ADVANCES IN THE IMMUNOLOGY OF HOST DEFENSE PEPTIDE: MECHANISMS AND APPLICATIONS OF ANTIMICROBIAL FUNCTIONS AND BEYOND

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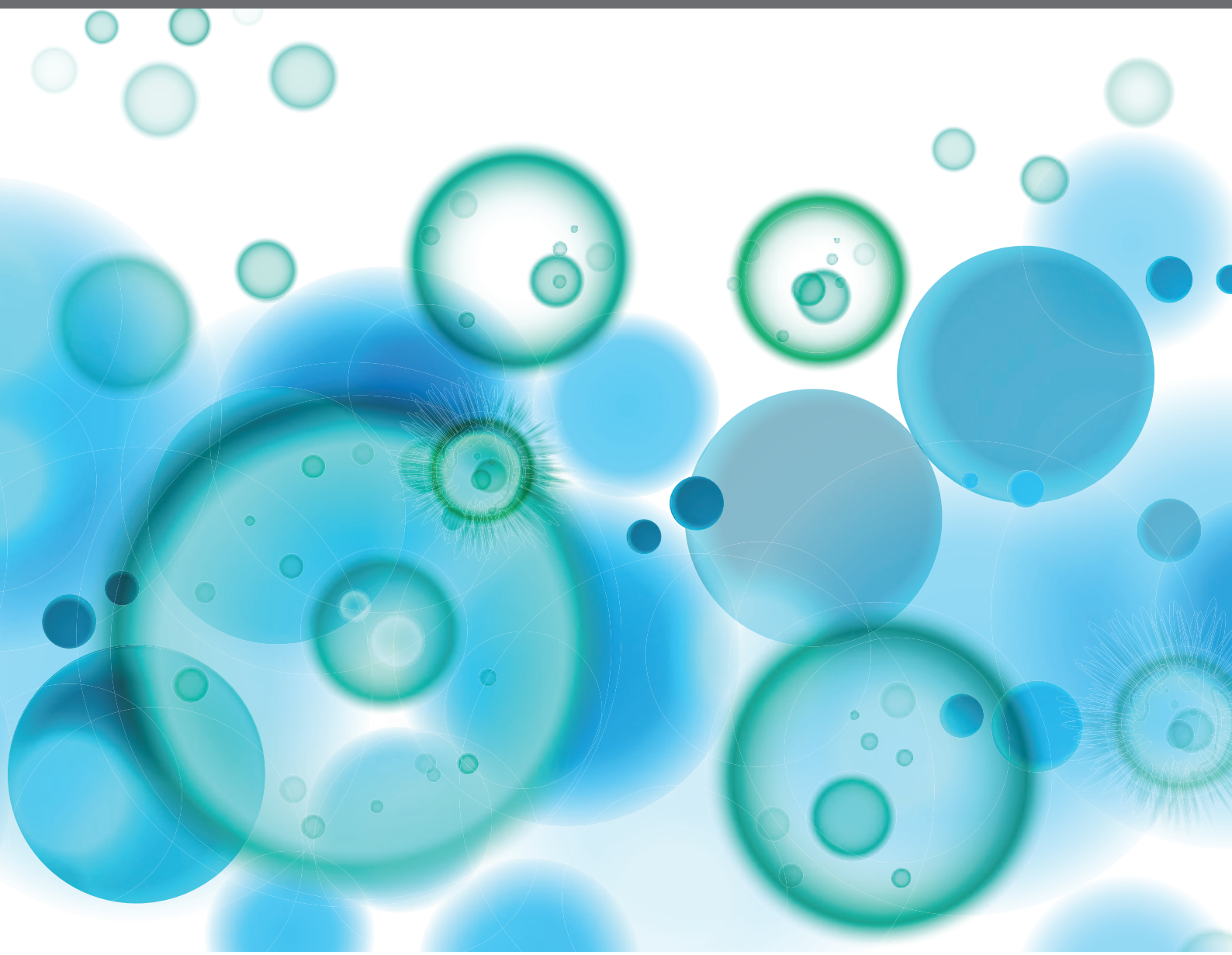

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Editorial: Advances in the Immunology of Host Defense Peptide: Mechanisms and Applications of Antimicrobial Functions and Beyond

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Editorial on the Research Topic

Advances in the Immunology of Host Defense Peptide: Mechanisms and Applications of Antimicrobial Functions and Beyond

INTRODUCTION

The term "antimicrobial peptides" (AMPs) classically refers to naturally occurring molecules that are made by most living organisms as key components of innate immunity that help shape the composition of colonizing micrbiota and provide a first line of defense against invading microbial pathogens. Although AMPS have diverse primary sequences, they are typically small in size, positively-charged, amphipathic, and contain α-helix and/or β-sheet rich structures often stabilized by disulfide bonds. AMPs can be constitutively expressed or induced by infectious or inflammatory stimuli, including proinflammatory cytokines and pathogen-associated molecular patterns. Since their discovery over a half century ago, research into AMPs has primarily focused on their direct antimicrobial activity, leading to them being touted as a promising molecular resource to tackle infectious diseases and antibiotic resistance. However, the direct microbicidal function of many AMPs may be limited under physiological and pathological conditions, due to a number of issues such as low local peptide concentration, suboptimal ion concentrations, physiological polyanions (e.g., mucins and glycosaminoglycans) and proteolytic degradation. More recently, a growing body of evidence suggests the biology and immunology of AMPs extends beyond the classical direct antimicrobial property. To highlight the multi-functionality and complexity of AMPs, in this Research Topic, we assembled a collection of original research and review articles that encompass recent advances in AMP research, with a particular emphasis on their alternative physiological and pathological functions. In doing so, we adopted a broader and more comprehensive term, "host defense peptides" (HDPs). New strategies to leverage HDPs for therapeutic uses, through exploitations of their multifaceted activities, are also highlighted.

IMMUNOMODULATORY FUNCTION OF HDPS

The ability of HDPs to mediate protection against microbial infection *in vivo*, under conditions that are often unfavorable for their direct antimicrobial actions, suggests that their immunomodulatory activities might be attributed to other functions. Indeed, HDPs have now been shown as potent immune effectors via multifunctional and multimodal orchestration of immune responses, including leukocyte chemotaxis, cytokine induction, angiogenesis and phagocytosis, modulation of leukocyte development and survival, as well as neutralization of pathogen-associated molecular patterns (PAMPs). HDPs also provide a link between innate and adaptive immunity by mediating chemotactic recruitment of antigen presenting cells and T lymphocytes, stimulating maturation of dendritic cell (iDC) maturation, and providing adjuvant-like functions. For example, proinflammatory HDPs, including iron-sequestering lipocalin 2 (Lcn2) can amplify antimicrobial responses to enhance pathogen killing and clearance. Lcn2-deficient mice, which result in reduced neutrophil recruitment and impaired cytokine production in macrophages, are more susceptible to *Escherichia coli* infections (Wang et al.). In contrast, Koeninger et al. demonstrated for the first time that systemically administered human β-defensin 2 (HBD-2, also know as DEFB2) suppresses inflammation induced by bacterial PAMPs including lipopolysaccharides (LPS), and in experimental colitis mouse models through chemokine receptor 2 (CCR2) targeting. The anti-inflammatory functions of HBD-2, and likely other HDPs, are required for controlling bacteria killing-associated inflammation and maintaining immune homeostasis, thus limiting unwanted inflammation-induced tissue damage. Similarly, cathelicidins attenuate LPS-induced Toll-like receptor-dependent inflammation through direct LPS-cathelicidin interaction. However, they can also bind to and stabilize bacterial DNAs and RNAs, promoting nucleic acid-sensing TLR activation. In their review, Scheenstra et al. provided a comprehensive discussion of these contrasting cathelicidin-mediated TLR modulations in reducing pathogen burdens during infection and resolving excessive inflammatory responses. From these articles, we can appreciate that it is not always straightforward to classify HDPs as either pro-inflammatory or anti-inflammatory. Instead, it may be appropriate to define them as inflammation regulatory molecules that balance host inflammatory responses and sustain immune homeostasis.

MICROBIOTA REGULATION AND TISSUE HOMEOSTASIS BY HDPS

Beyond their involvement in innate defense against pathogens, recent studies have uncovered the importance of HDPs in tissue homeostasis, dictated not only by their canonical antimicrobial function but also by their immunomodulatory properties. Notably, within protective epithelial barriers, it is clear that HDPs are critical, not only in defending against pathogenic pathogens, but also in regulating commensal microbiota as well. This microbiota balance within epithelial tissues is key for heathy epithelial homeostasis and immunity (Coates et al.). To this end, colonic HDPs [cathelicidins, β-defensins, regenerating islet-derived protein III (Reg3) and resistin-like molecules (Relm)] form an interactive network, as proposed by Blyth et al. and function synergistically and multimodally to prevent enteropathogenic colonization. Puértolas-Balint and Schroeder reviewed the complex interactions between diet, commensal microbiota and HDPs in gut homeostasis and diet-associated inflammatory diseases. They document that nutrient-sensing signaling mediators are involved in diet-associated modulation of HDPs, while immune mediators and effectors shape microbe-dependent HDP regulation. Furthermore, intestinal HDPs display unique mechanisms of action and spectrum of activity to regulate microbiota composition, evidently beyond direct antimicrobial activity. Of note, HDP-mediated control of gut microbiota is dependent on localization and the specific regulatory mechanisms of expression and activation. In support of this, in their original study, Nakamura et al. showed that Paneth cell-secreted α-defensins are topographically regulated from duodenum to ileum. Their findings of ex-germ free-dependent regulation of α defensins also suggest that the intestinal microbiota may partially affect HDP activity. Nevertheless, it remains to be determined precisely how microbes and certain dietary components can signal to the epithelium and mediate HDP function, as well as how HDPs mechanistically modulate gut microbiota through immunomodulation. Beyond human epithelial barrier, Le Bloa et al. discovered type IIa crustin, as a novel HDP in the deep-sea shrimp *Rimicaris exoculate*. This investigation uncovered an expression pattern of type IIa crustin that is spatio-temporally correlated with ectosymbiotic colonization through different life stages. These findings further signify the importance of HDPs in the establishment of vital and heathy host-symbiotic microbiota through their multifaceted functions.

HDPS IN DISEASES AND CHRONIC INFLAMMATORY CONDITIONS

As our understanding of molecular and cellular mechanisms underpinning HDP immunomodulation improves, the roles of HDPs in various diseases and chronic inflammatory conditions are drawing an important focus. This Research Topic features several reviews on the multitude of pathological outcomes associated with dysregulation of cathelicidins and defensins, including skin disorders (e.g., psoriasis, atopic dermatitis), inflammatory bowel diseases (e.g., ulcerative colitis, Crohn's disease), autoimmune diseases, lung disorders (e.g., cystic fibrosis, chronic obstructive pulmonary disease, asthma), and tumorigenesis (Alford et al., Liang and Diana, Shelley et al., Wehkamp and Stange, Xu and Lu). These review articles collectively provide a thorough overview of HDPs' functions in pathology and converge to highlight the "double-edge

sword" nature of HDP-mediated immunomodulatory responses. Of note, Lee et al. highlighted the unexpected similarities between Aβ amyloid aggregates and HDP protofibrils, hence posing new perspectives for HDP-associated pathogenesis. Intriguingly, by using ecogenetic analyses, Hanson et al. described an independent loss of immunologically costly and deleterious HDPs, such as diptericin, in true fly lineages in the absence of microbial challenges. The complexity of HDP-associated pathogenesis is further complicated by the heterogeneity of HDP (dys)regulation. In an original research contribution, Hemshekhar et al. showed that murine cathelicidin (CRAMP) and calprotectin (S100A8 and S100A9) are differentially regulated in the local tissues (joint and lung, respectively) in two immunopathologically-related mouse models of inflammatory arthritis and airway inflammation. Together, these reviews and research studies highlight that host immunity needs to strike a delicate balance in HDP regulation, to mediate an antimicrobial defense response on the one hand, whilst avoiding HDP-induced pathological perturbance on the other.

CLINICAL APPLICATIONS FOR HDPS: OPPORTUNITIES AND CHALLENGES

Insights into HDP mechanisms and functions have opened new and exciting therapeutic avenues, and have provided novel platforms for therapeutic design. In this Research Topic, potential clinical uses for HDPs are extensively discussed in various disease conditions, ranging from multidrug-resistant bacterial infection (Bergman et al.), fungal infection (Mercer and O'Neil), and cancer (Xu and Lu) to autoimmune disorders (Liang and Diana) and chronic inflammatory diseases (Alford et al., Koeninger et al., Shelley et al., Wehkamp and Stange). The broad-spectrum antimicrobial effects of HDPs have long-inspired the development of these peptides for treatment of infectious diseases. In addition, their actions on bacterial biofilms may benefit rational engineering and designs to tackle the biofilm-associated resistance against host immunity and antibiotics. For instance, Parducho et al. reported the antibiofilm activity of HBD-2 against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, is independent of biofilm regulatory pathways and instead mediated via induced alteration of biofilm integrity. It should also be noted that in the non-infection settings, the immunomodulatory functions of HDPs can also potentially be exploited to restore the balance to dysregulated immune responses that contribute to disease pathologies.

However, there are several challenging obstacles for HDP applications, notably their potential toxicity, proteolytic instability, salt intolerance and low *in vivo* efficacies. In an attempt to address the gap between bench-to-bedside translation of HDP-based therapies, Ting et al. highlighted different strategies in designing and translating the therapeutic potentials of HDPs, such as novel HDP synthesis, sequence optimization, cyclisation, N–/C–terminal modification and hybridization. Significantly, the authors also proposed a strategic pipeline for the developmental pathway of HDP-based therapeutics, from novel HDP prototyping to conducting preclinical studies to optimally inform further clinical trials. Consistent with these outlined strategies, Hernandez et al. demonstrated cyclic peptide [R_4W_4] with an improved antimycobacterial effect. Learning from the evolution of invertebrate big defensins toward β-defensin, Gerdol et al. also suggested to ameliorate HDPs' salt intolerance through N-terminal hydrophobicity. Ehmann et al. enhanced the potency of human neutrophil α-defensin four against multidrug resistant bacteria through truncation, N-terminal acetylation and C-terminal amidation. Additionally, Rodríguez-Rojas et al. found that *Staphylococcus aureus* is highly susceptible to toxic nucleoside analogs when exposed to pexiganan, and based upon this finding, proposed HDP-based combinatorial therapy for infectious diseases. Furthermore, Ting et al. provided a stimulating discussion on the use of artificial intelligence technology with machine learning algorithms as a tool to search for HDP sequences with desirable efficacy. Indeed, the BACIIα algorithm, a tensor search protocol, was used by Yount et al. to identify >700 putative new prokaryotic bacteriocins, which display potent *in vitro* antimicrobial effect against a wide range of human pathogens. Similarly, Cho et al. developed a digitized framework for *in silico* HDP identification and characterization, by which 19 novel cathelicidins from the gray short-tailed opossum were revealed and experimentally validated for their antibacterial and anti-West Nile virus activities. Undoubtedly, machine learning will help revolutionize future HDP discovery, which should not only shed light upon HDP biology in understudied organisms (such as plant HDPs as argued by Petre), but also expand the HDP arsenal for therapeutic exploitation.

CONCLUDING REMARKS

Together, the papers in this collection highlight and add new knowledge on the multifaceted function and importance of HDPs in contributing to the shape and complex nature of host immunity, as well as in regulating health and mediating disease progression. As our knowledge of HDP biology expands, the repertoire of HDP functions, particularly direct antimicrobial activity and immunomodulation, demonstratively opens the door for novel therapeutic development for treating infectious diseases, as well as inflammatory conditions. Nevertheless, while the classical microbicidal property of HDPs has been extensively studied, the research journey has only just begun for their non-antimicrobial properties. Forsaking the classical term "antimicrobial peptides" and embracing "host defense peptides" can not only properly acknowledge the multi-functionality and complexity of HDPs, but may also imprint the currently underexplored activity landscapes and enshrouded therapeutic potential of HDPs. We would like to take this opportunity to thank the authors for their invaluable contributions to this Research Topics, as well as all the reviewers for their time and constructive inputs.

AUTHOR CONTRIBUTIONS

TKP wrote the manuscript. CB and MH critically revised and approved the final version of the manuscript. All authors conceived the outline of the manuscript.

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Dynamic Evolution of Antimicrobial Peptides Underscores Trade-Offs Between Immunity and Ecological Fitness

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There is a developing interest in how immune genes may function in other physiological roles, and how traditionally non-immune peptides may, in fact, be active in immune contexts. In the absence of infection, the induction of the immune response is costly, and there are well-characterized trade-offs between immune defense and fitness. The agents behind these fitness costs are less understood. Here we implicate antimicrobial peptides (AMPs) as particularly costly effectors of immunity using an evolutionary framework. We describe the independent loss of AMPs in multiple lineages of Diptera (true flies), tying these observations back to life history. We then focus on the intriguing case of the glycine-rich AMP, *Diptericin,* and find several instances of loss, pseudogenization, and segregating null alleles. We suggest that *Diptericin* may be a particularly toxic component of the Dipteran immune response lost in flies either with reduced pathogen pressure or other environmental factors. As *Diptericins* have recently been described to have neurological roles, these findings parallel a developing interest in AMPs as potentially harmful neuropeptides, and AMPs in other roles beyond immunity.

Keywords: innate immunity, antimicrobial peptide (AMP), molecular evolution, population genetics, diptericin, drosophila, diptera, attacin

INTRODUCTION

The innate immune system plays a vital role in host defense against pathogens. This is particularly true in invertebrates, which lack an adaptive immune system. Antimicrobial peptides (AMPs) are one of the main effector molecules of innate immunity in many organisms and, as such, they represent the front lines in the coevolutionary struggle between host and pathogen. AMPs are often cationic, amphipathic peptides that defend their hosts against infection by disrupting the cell membranes of invading microbes (1). However, the dose makes the poison, and AMPs can also be toxic to eukaryotic host cells under certain conditions. This suggests that host immunity needs to strike a delicate balance: AMPs need to be potent enough to quickly inhibit pathogenic microbes, but not so potent that they upset the balance of the microbiota or damage host tissue.

Indeed, many pathologies in humans have been observed when this balance is perturbed. These include chronic inflammatory skin or bowel diseases (2–4), and pulmonary infections including cystic fibrosis wherein reduced levels of β-Defensins and the cathelicidin LL-37 are associated with increased risk of infection (5–9). The cathelicidin LL-37 is implicated in autoimmune reactions because it can be toxic to white blood cells (10), induce inflammation in the nervous system (11), or even damage host tissues during anti-cancer responses (12).

Recent studies have also suggested the Alzheimer's peptide Amyloid-beta is an AMP in the nervous system, and that Alzheimer's may in part be an infectious disease (13–15). These observations of AMPs as toxic agents are further supported by reduced lifespan in *Drosophila* fruit flies ubiquitously expressing AMPs in the brain (16), or systemically (17). During aging, *Drosophila* NF-κB signaling is also implicated in neurodegeneration with AMPs as prime suspects (18). Thus, AMP dysregulation can impose a significant threat to organismal health.

Insects, and particularly *Drosophila melanogaster*, have been integral to unraveling the innate immune response, including the regulation of AMPs by the Toll and Imd NF-κB signaling pathways (19). Thus, far seven AMP gene families have been described in *Drosophila*: *Defensin, Cecropin, Attacin, Diptericin, Drosocin, Metchnikowin*, and *Drosomycin*. Another class of AMP-like effectors called the *Bomanins* are also essential for Toll-mediated defense, however their antimicrobial properties await functional clarification (20, 21). *Drosophila* AMP evolution is shaped both by balancing and diversifying selection at the sequence level (22, 23). Following a duplication event and subsequent speciation, *Drosophila Diptericins* rapidly diverged into distinct *Diptericin* clades (24). In contrast, balancing selection seems to maintain a stable polymorphism amongst alleles that provide either moderate or poor protection against systemic infection with *Providencia rettgeri (P. rettgeri)* (25). Why selection should favor *Diptericin* alleles that result in loss of immune competence is unclear. One possibility is that the immune-poor *Diptericin* allele is selected for through trade-offs between poor immune defense when infected and higher fitness when uninfected. Indeed, rare *Diptericin* null alleles are observed in North American populations (25), and patterns of duplication and loss in *Diptericin* and other *Drosophila* AMPs have resulted in copy number variations amongst species (24, 26–28).

As AMP dysregulation can affect health, copy number variation may impose a significant challenge for the maintenance of optimal gene expression (29). Yet perhaps the most overt patterns in AMP evolution are duplication events affecting copy number, which is widespread in both humans and fruit flies (30–34). Therefore, conflict between maintenance of healthy expression levels and improved immune competence may drive patterns of AMP gain/loss or changes in expression patterns. In this model, we expect that AMPs are evolutionary liabilities in the absence of infection, and that host ecology and associated pathogen pressure will drive the evolution of AMP content both at the level of broad AMP gain/loss, and also of AMP expression: species with strong pathogen pressures would evolve to increase potential AMP production, while species whose ecologies involve less exposure to pathogens would be expected to reduce their AMP complement.

While characterizing pathogen pressure in different animal hosts is exceedingly difficult, we can use host ecology as a proxy for infectious pressures. The use of sterile food resources (such as plant sap) reduces the opportunities for microbes to inoculate hosts. There are several insects that spend large portions of their lives (larval, adult, or both) feeding on sterile or near sterile food resources—likely reducing the evolutionary benefits of AMPs and/or AMP induction. The pea aphid (which feeds on sterile plant phloem) is one such insect that has lost not just effectors, but also an entire NF-κB immune signaling pathway (35). Loss of immune signaling is also observed in plant-feeding *Tetranychus* mites (36, 37), as well as bed bugs (38) and body lice (39), suggesting blood-feeding may be a similarly clean feeding ecology. It should be noted that in some cases these hosts have intimate associations with endosymbionts, microbes that supplement nutrition or protect against infection. One argument to explain loss of immune signaling is that it is a direct consequence of endosymbiont presence to avoid negative consequences associated with chronic activation of the host immune response (40). However, cereal weevils live in sterile environments and have nutritional endosymbionts, but they instead utilize AMPs to regulate their symbiont populations (41). Thus, what factors of sterile lifestyles and/or endosymbionts promote immune gene loss remains poorly resolved.

As AMP copy number evolves rapidly, we suspected AMP evolution might respond to shifts in host ecology before entire immune pathways are affected. To test this, we surveyed Diptera (true flies) for AMP presence or absence and interpret this in the context of host ecology. Diptera are an extremely diverse lineage with equally diverse and unique ecologies and life histories, and boast numerous sequenced genomes and transcriptomes. We probed these diverse flies for classic AMP families described in *Drosophila* and other insects to better understand the forces driving AMP gain or loss. We further analyzed *Drosophila* copy number and sequence variation in conserved AMPs, tying these results back to life history. Globally, we describe a pattern suggesting AMPs are lost in Diptera lineages with more sterile life histories, with striking parallels to loss of immune signaling in other arthropods with sterile food resources. We also focus on *Diptericin*, which we suggest is a particularly costly AMP, describing distinct evolutionary patterns across ecologically diverse *Drosophila* and within *D. melanogaster*.

RESULTS

Some AMP Families Are Absent in Diptera Living in More Sterile Environments

Diptera diverged from other related holometabolous insects about 272 mya [timetree.org; Kumar et al. (42)] and diversified into extremely broad ecological habitats. We surveyed 31 Dipteran genomes as well as diverse *Drosophila* species for the presence of eight AMP/AMP-like families either described in *Drosophila* (*Bomanins, Drosocin, Drosomycin, Metchnikowin*) or characterized more broadly across Dipterans and other insects (*Diptericins, Cecropins, Attacins, Defensins*). We also annotated the feeding ecologies of these diverse flies to better understand which lineages may have reduced pathogen pressure owing to food resource use (**Supplementary Data File 1**). We performed an iterative reciprocal BLAST search using known AMPs against genomic or transcriptomic sequence. We found that *Drosocin, Metchnikowin, Bomanins,* and *Drosomycin* are

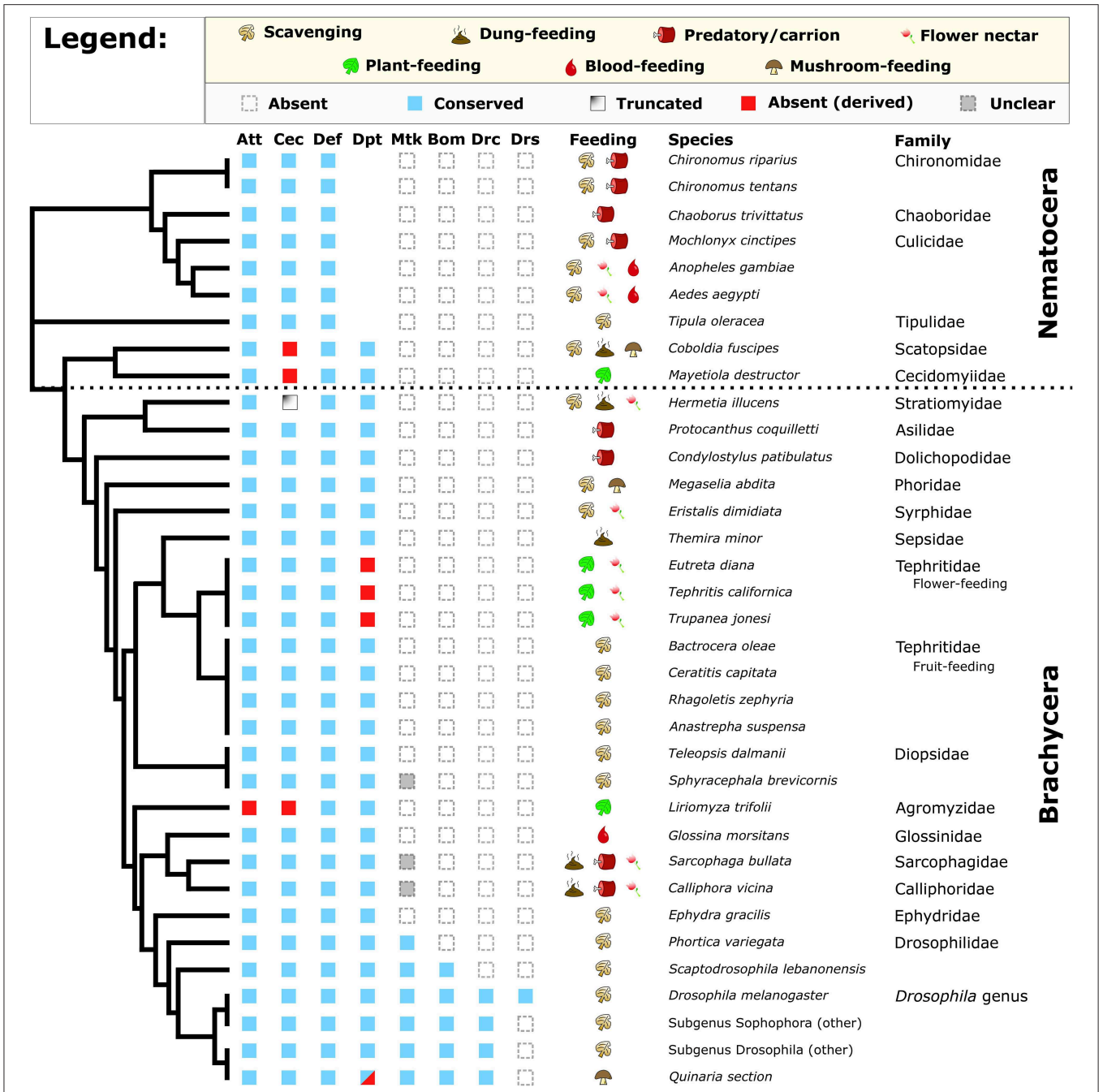

FIGURE 1 | Conservation of *Drosophila* AMP families in diverse Diptera. Broadly, *Attacin, Cecropin, Defensin, and Diptericin* are conserved in most flies. However, *Cecropins* are absent or truncated in select lineages, while flower-feeding Tephritids and *Liriomyza trifolii* lack subsets of AMPs. We also recover a pattern of *Diptericin* loss in members of the Quinaria section of *Drosophila* (shown later). Full annotation of larval and adult feeding ecologies is given in **Supplementary Data File 1**. Cladogram adapted from Vicoso and Bachtrog (43).

restricted to *Drosophila* and their close relatives (**Figure 1**). Using a lenient E-value threshold, we were able to recover *Metchnikowin* from diverse mushroom-feeding *Drosophila* and perhaps other flies, and confirmed their identities by alignment and reciprocal BLAST (**Figure S1**), improving on previous annotations of immune genes in this lineage (24). The other AMP families show a broader taxonomic distribution (**Figure 1**).

One striking pattern is the absence of *Cecropin* in two Nematocerans: the plant-feeding Hessian fly *Mayetiola destructor* and the oyster mushroom pest *Coboldia fuscipes*. The *Coboldia* genome is a small, well-assembled genome (~100 Mbp, scaffold

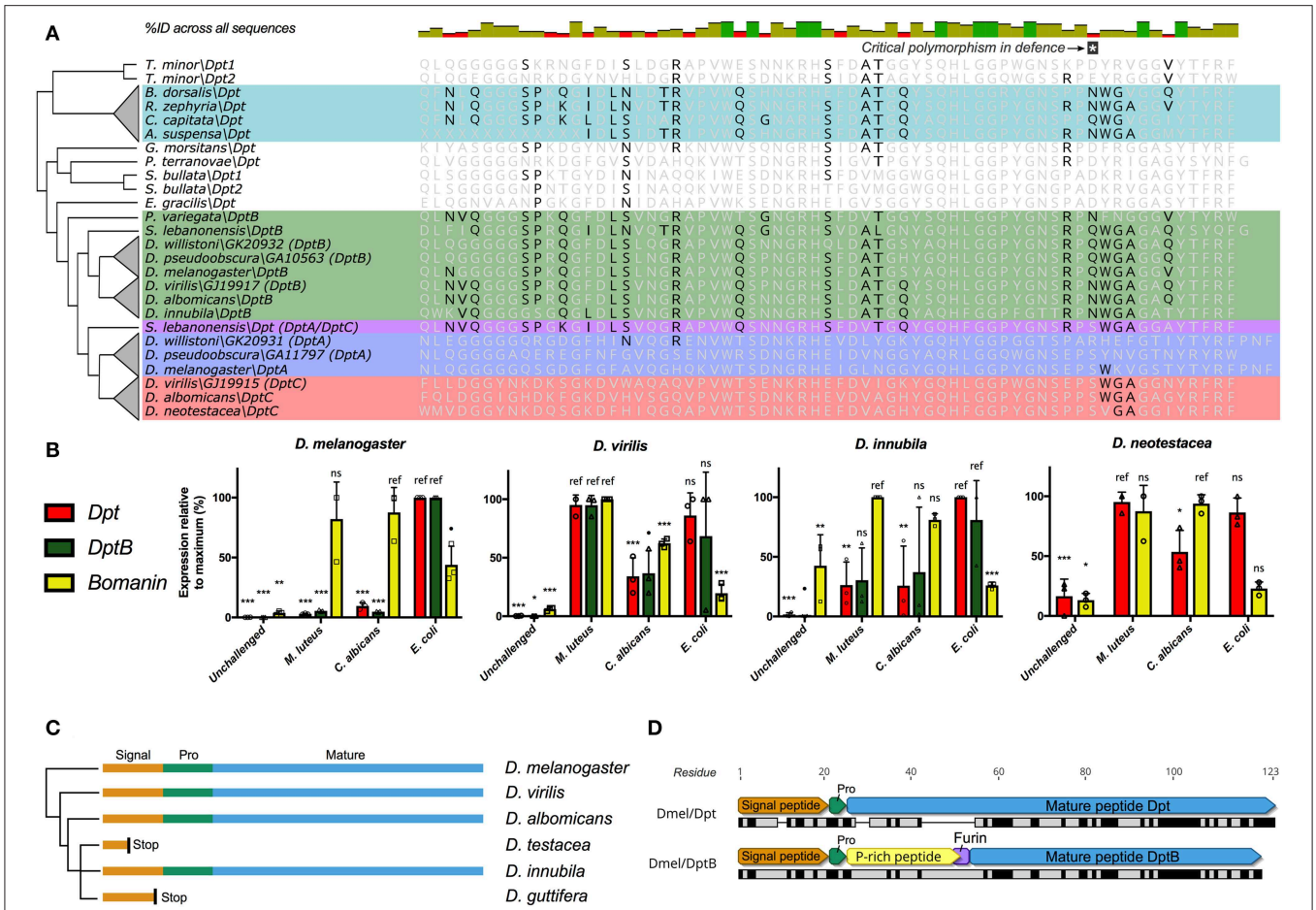

FIGURE 2 | Diptericin evolution in Tephritids and Drosophilids. **(A)** The Diptericin C-terminal sequence of fruit-feeding Tephritids has converged on *DptB*-like residues (also see **Figure S3**). Major *Diptericin* clades: Blue, *DptA*; Green, *DptB*; Red, *DptC*; Teal, Tephritid *Dpts*, and *S. lebanonensis Dpt* in purple is a direct outgroup to the *DptA* and *DptC* clades (collectively referred to as "*Dpt*" in **B**). The polymorphism critical for defense (25) is indicated, and Tephritid *Diptericins* have converged on the Q/N polymorphism found in the *Drosophila DptB* clade. **(B)** *Diptericin* expression in select lineages of *Drosophila*. Expression is normalized to the maximum for each gene within each species, and statistical comparisons are done with reference to the treatment that induced expression most (see Materials and Methods). **(C)** *DptB* has been pseudogenized in two independent lineages of mushroom-feeding *Drosophila*: *D. testacea* and *D. guttifera*. Signal, Signal peptide; Pro, propeptide; Mature, Mature peptide. **(D)** The mature peptides of *D. melanogaster* Dpt and DptB differ markedly in mature structure, including a critical furin cleavage site in DptB that likely leads to two mature peptides. At the protein sequence level Dpt has only 41.7% pairwise identity to DptB (black bars = identical sites).

N50 = 242 Kbp) (43, 44), and *Cecropins* throughout Diptera share similar motifs from the N terminus to C terminus. As such, we interpret this absence of *Cecropin* in both *M. destructor* (plant sap-feeding) and *C. fuscipes* (scavenger-feeding) as a likely true loss of *Cecropin* in this basal lineage. We also found a partial *Cecropin* sequence truncated by a premature stop codon in *Hermetia illucens* (scavenger-feeding). Finally, we did not recover *Cecropin* from the genome of the leafminer *Liriomyza trifolii*, an independent transcriptome of *L. trifolii* pupae (a life stage when AMPs are often highly upregulated), or from a sequence read archive (SRA) file (GenBank accession: DRX064600) of the related *Liriomyza chinensis*. We see no immediate pattern in feeding ecology or life history that predicts *Cecropin* loss, but we also failed to recover an *Attacin* from *Liriomyza*, suggesting *Liriomyza* has lost AMPs from two gene families (*Cecropins* and *Attacins*). Called "leafminers," *Liriomyza* larvae feed internally in the leaves of plants, an environment protected from external microbes by the immune system of the host plant; a more sterile food resource than most Dipterans. Moreover, we also failed to recover *Diptericin* in three flower-feeding Tephritid species. Like the leafminers, these flower-feeding flies live in a protected environment owing to larval development inside budding flower inflorescences (45).

Within the genus *Drosophila*, we observed two unique instances of AMP gain/loss we note separately. First, the genome of the cosmopolitan fly *Drosophila busckii* encodes no less than nine intact *Diptericin* genes, and we further recovered three pseudogenes in the *D. busckii Diptericin* gene region (**Figure S2**); for context, other *Drosophila* typically have only 2–3 *Diptericin* genes (24). *Drosophila busckii* is a cosmopolitan generalist species in common association with *D. melanogaster*, however *D. busckii* arrives later to rotting fruits and compost

relative to *D. melanogaster* (46, 47). To favor the retention of so many *Diptericin* copies suggests the *Diptericin* response is highly important *for D. busckii* ecology. Second, we found that one paralog of the *Attacin A/B* duplication event has been lost in *Drosophila sechellia*, a species closely-related to *D. melanogaster*. *Drosophila sechellia* is famous for its unique ecology, feeding on toxic morinda fruit that repels other flies (48). Beyond losing this *Attacin* paralog, *D. sechellia* also lacks the ability to encapsulate and kill invading parasitoid wasps, associated with loss of function in immune genes involved in the melanization and stress responses (49). It seems likely that the toxins in morinda fruit would repel parasites such as wasps, reducing infectious pressure on *D. sechellia*. Thus, this ecology—already associated with loss of immune genes—may have additionally promoted loss of an *Attacin* as well.

Overall, we observe numerous instances of AMP loss across the Diptera phylogeny. The loss of Cecropins in ecologically diverse lineages is puzzling. For the mushroom pest *C. fuscipes* and Hessian fly *M. destructor*, either scavenging (*C. fuscipes*) or sap-feeding (*M. destructor*) could reflect an ancestral ecology promoting *Cecropin* loss. These two last shared a common ancestor ∼250 mya [Timetree; (42)], and transitions from generalist to specialist ecologies, and back again, have been inferred in *Drosophila* (50). However, more strikingly we observe AMP gene family loss in all three strictly plant-feeding fly lineages assessed (the Hessian fly *M. destructor*, *Liriomyza* leafminers, and flower-feeding Tephritids), reminiscent of immune gene loss in sap-feeding Pea Aphids.

Parallel Loss of Diptericins in Lineages With Divergent Ecology

In our screen of AMP conservation in Diptera, we were intrigued by the loss of *Diptericin* in some Tephritid fruit flies and some *Drosophila*; *Diptericin* was previously shown to have rare null alleles segregating in a North American *D. melanogaster* population (25). While assembly quality was variable amongst the Tephritid genomes, the absence of *Diptericin* in three independent flower-feeding Tephritid species, but presence in all screened fruit-feeding Tephritid species suggests *Diptericin* is lost in the flower-feeding Tephritid lineage. *Diptericin* is an AMP that has attracted a great deal of attention as the canonical readout of Imd signaling in *D. melanogaster* (19), and for its highly specific interaction with *Providencia rettgeri* bacteria (25, 51). Interestingly, the *Diptericin* sequence retained in the fruit-feeding Tephritids has converged on a Drosophilid *DptB*-like sequence (**Figure 2A**). Furthermore, it was previously reported that *DptB* was pseudogenized in the mushroom-feeding *Drosophila* species *D. guttifera* and likely also in *Drosophila neotestacea (D. neotestacea)* (24). However, when we screened the recently-sequenced mushroom-feeding *Drosophila innubila* genome, we recovered intact coding sequence for *DptB*. It is possible that the intact *DptB* sequence in *D. innubila* could reflect that *DptB* in mushroom-feeding flies was initially pseudogenized not due to loss of coding sequence, but rather due to mutations affecting gene expression. We therefore performed qPCR following infection to determine the expression profile of *Diptericins* amongst mushroom-feeding flies and included outgroup *Drosophila* to inform our interpretations.

We used a Bomanin (*Bom791*) as a positive control for infections more specific to the Toll pathway in *D. melanogaster*. We further intended to use *Dpt* as a specific readout of Imd signaling, and as an independent control gene for assessing *DptB* expression. First, we found that *DptB* is strongly induced in *D. innubila*, suggesting it is not pseudogenized as in sister lineages. However, we found that *Dpt* expression is highly variable across *Drosophila* species (**Figure 2B**). *Dpt* is more specifically induced by Gram-negative bacterial challenge in *D. melanogaster*, and indeed we see this pattern in the outgroup flies *Drosophila pseudoobscura (D. pseudoobscura)* and *Drosophila immigrans (D. immigrans)* (**Supplementary Data File 1**), and also broadly in *D. innubila*. However, *Dpt* and *DptB* are similarly induced by either Gram-negative or Gram-positive bacterial challenge in *D. virilis*, and the same is true for *Dpt* in both *Drosophila testacea (D. testacea)* (not shown), and *D. neotestacea* (**Figure 2B**, and see Materials and methods). Using Sanger sequencing, we additionally confirmed that *DptB* is pseudogenized in *D. testacea* by a premature stop codon, supporting its absence in the *D. neotestacea* transcriptome (GenBank accession: MN311476). The mutation affecting *DptB* in the Testacea group is distinct from the mutation in the Quinaria group species *D. guttifera* (**Figure 2C**), suggesting independent loss events.

Thus, the pseudogenization of *DptB*-like genes in both flower-feeding Tephritids and two lineages of mushroom-feeding flies reflects that first there was convergent evolution toward *DptB*-like sequence in both Tephritids and Drosophilids. Thereafter, subsequent independent losses of *DptB*-like *Diptericins* occurred in lineages with ecologies that diverged from fruit-feeding. This pattern suggests *DptB* may be attuned to fruit-feeding ecology, but not as useful in other ecological niches.

We can only speculate on how evolution shapes patterns of *DptB* gain/loss: in *D. melanogaster*, functional characterization of *DptB* was long-ignored in favor of its more potently-induced paralog *Dpt*. However, recent studies have revealed that the two *Diptericins* have markedly different activities in immunity and host physiology. First, Unckless et al. (25) showed that a specific serine allele in *Dpt* confers defense against *P. rettgeri*, however no *DptB* gene in any fly encodes a serine at this site. Alternately, Barajas-Azpeleta et al. (52) found that *DptB*, but not *Dpt*, is required for long-term memory formation. There are a number of overt structure and sequence differences amongst *Dpt* and *DptB* (**Figure 2D**). First, *Dpt* encodes an 83-residue mature peptide with a proline-rich domain tailed by a glycine-rich Attacin-family domain. This 83-residue mature peptide is secreted following cleavage of the Dpt signal peptide and propeptide. On the other hand, *DptB* encodes a furin cleavage site (RVRR) between its proline-rich and glycine-rich domains, similar to other AMPs of the Attacin gene family (53); In Attacin C, this furin cleavage results in two secreted peptides, a proline-rich AMP (called MPAC) and a separate glycine-rich AMP (54). Furthermore, amongst the many sequence differences between *Dpt* and *DptB* (see **Figure 2A**) is the aforementioned serine residue of Dpt that confers defense against *P. rettgeri* bacteria. In *Dpt* genes, this residue is polymorphic (S/R/Q/N)

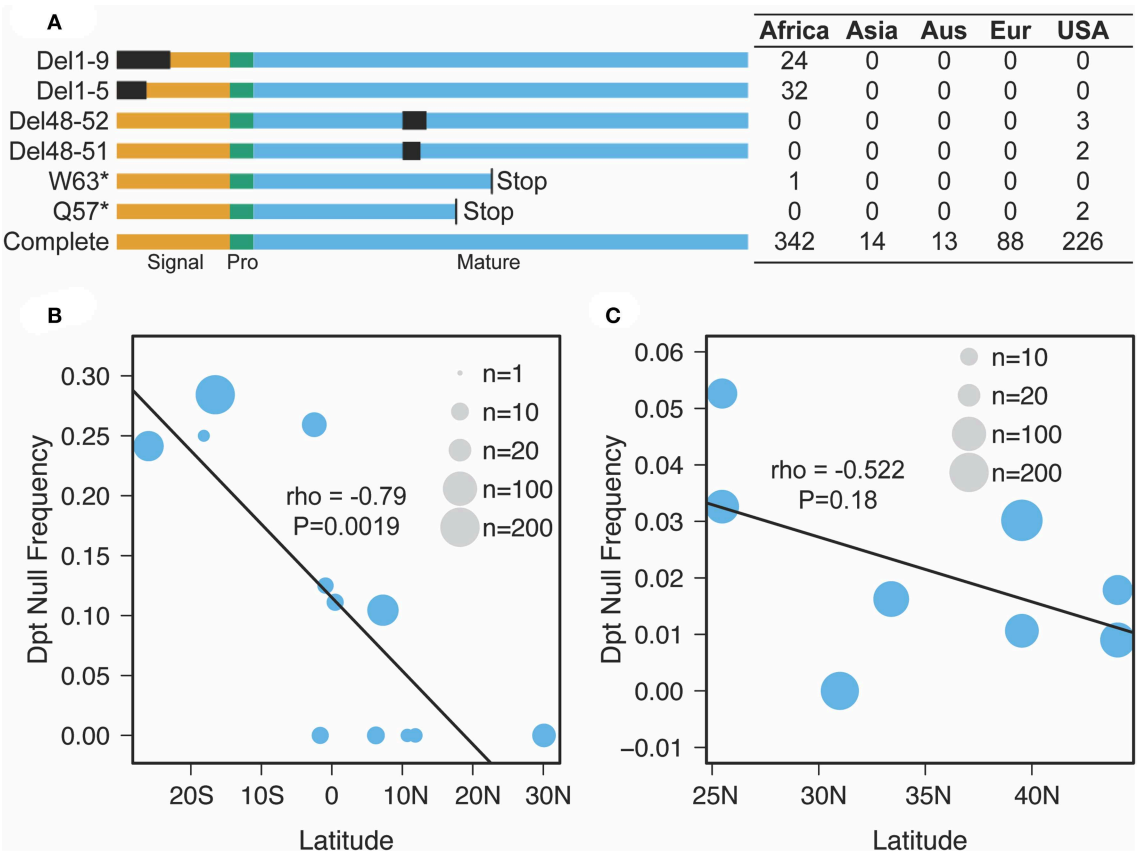

FIGURE 3 | Nature and geographic distribution of *Dpt* null alleles. **(A)** At least six unique null alleles segregate in *D. melanogaster Dpt* worldwide. Del1-9 and Del1-5 are deletions removing the first 9 or 5 amino acids, Del 48-52 and Del 48-51 are in frame deletions that remove amino acids in the mature peptide and W63* and Q57* are premature stop codons. Signal, Pro and Mature correspond to the signal peptide, propeptide and mature peptide of the protein. The box to the right denotes the counts of each allele in each population (Aus, Australia; Eur, Europe, USA, DGRP population only). **(B,C)** The correlation between latitude and null frequency in African **(B)** and North American **(C)** populations (data from pooled sequencing of populations along a cline). The size of the circle represents the number of individuals sequenced **(B)** or reads mapped **(C)** in each population.

in *D. melanogaster* and close relatives (25). However, DptB encodes a polymorphism for only Q/N at this site, including in convergent *DptB*-like *Diptericins* of Tephritid fruit flies. Globally, in *D. melanogaster*, *Dpt* appears to be key in mediating defense against *P. rettgeri* bacteria, while *DptB* is uniquely required for memory formation. Accordingly, these two *Diptericins* have overt differences in mature peptide products. Here we implicate host ecology as a likely determinant of *Diptericin* evolution, and suggest that these overt differences may have ecology-dependent effects on fitness leading to distinct patterns of gain/loss.

Null Alleles of Diptericin Are Segregating in Wild Populations of *D. melanogaster*

Our findings on *Diptericin* evolution coupled with recent descriptions of distinct *Diptericin* activities uniquely position this AMP family for providing insight into how conflicting roles in immunity and physiology can shape AMP evolution. Unckless et al. (25) reported the maintenance of two alternate alleles (serine/arginine) at residue 92 of the full length Dpt protein (residue 69 of the mature peptide) in wild populations of *D. melanogaster* and *D. simulans*. *Providencia rettgeri* is a natural pathogen of *D. melanogaster* (55), yet the *Dpt* arginine allele is maintained by balancing selection in the wild despite being associated with poor immune defense against *P. rettgeri* infection. Additionally, Unckless et al. (25) reported a rare null mutation in a *D. melanogaster* North American population (DGRP) (56) resulting in a premature stop codon affecting ∼1% of the reference strains. Surprisingly, when we investigated a set of African populations (DPGP) (57), we found multiple independent null mutations segregating in different sub-populations throughout the Africa sampling range (**Figure 3A**). Even more surprising, the prevalence of these null mutations reaches over 20% in some populations and appears to follow a latitudinal cline (**Figure 3B**). As such, selective pressure on *Dpt* may follow a clinal gradient in Africa, favoring *Dpt* loss in southern African populations. Note that the cline crosses the equator and so may not be driven by climate alone. We also recover a similar, though not significant, trend for null alleles segregating in North American collections (**Figure 3C**).

These data suggest that despite the described importance of *Dpt* in defense against the ecologically relevant pathogen *P. rettgeri*, null alleles associated with extremely poor immune defense are actively segregating in wild *D. melanogaster*. This suggests that the evolutionary forces behind *Diptericin* loss are not entirely passive. Taken together with the loss of *DptB* in other flies, instead this implicates active selection on *Diptericins* as peptides deleterious for fitness in alternate ecological conditions.

DISCUSSION

AMPs must maintain a fine balance: being potent enough that they can kill harmful pathogens but not so harmful that they damage host tissues directly or by damaging beneficial components of the host's microbiome. It stands to reason that host ecology drives pathogen pressure and therefore might indirectly shape the complement of AMPs in a given host. In our survey of AMPs across Diptera and within *Drosophila* presented above, we find some support for an adaptive loss of AMPs in hosts associated with more sterile habitats. There is increasing awareness that these classic immune molecules can play diverse physiological roles, and that evolution may be shaping AMP copy number and sequence due to selection on non-immune functions. When considering the internal plant parasites of the Tephritid family and the leaf miner, clear parallels can be drawn regarding sterile food resource use and other fluid-feeding arthropods that have similarly lost or re-organized their immunity genes, namely: aphids, some mites, bed bugs, and body lice (35–39). It may also be that these plant-parasitic flies have yet-uncharacterized bacterial endosymbionts that impose selection against certain AMPs, enabling their specialist lifestyle. We also describe multiple incidents of *Diptericin* loss in *Drosophila*: *DptB* in some mushroom-feeding flies, a lineage with a specialist ecology whose microbiota differs drastically from *D. melanogaster* (58), while *Dpt* null alleles are segregating in wild *D. melanogaster* populations.

The central question then becomes: why should immune-inducible AMPs, antimicrobial agents required for competent host defense, be lost so readily? We can think of two evolutionary scenarios that would lead to the loss of AMPs. First, when infection pressure is low, relaxed constraint on protein sequence and/or expression could lead to the accumulation of mutations that compromise protein function and eventually lead to pseudogenization and loss. This represents a neutral process where genetic drift is the force removing AMPs. The second evolutionary scenario is that AMPs are costly in the absence of infection, so when infection pressure is low, mutations that compromise function (indels, premature stop codons, cis-regulatory mutations) are actually selected for. If periods of low infection pressure persist long enough, those mutations can become fixed, and gene function is lost.

Several lines of evidence support the second, "selective loss" scenario. First, if relaxed constraint in the absence of infection drove AMP loss, we would expect the loss of AMPs to be somewhat random. However, we see convergent loss of *DptB-like* genes in independent lineages with divergent ecologies, and *Dpt* null alleles segregating in wild populations of *D. melanogaster*. Of course, if AMPs are specific to a small suite of pathogens (e.g., *Dpt* and *P. rettgeri*), perhaps those pathogens are relatively absent in some natural populations compared to others. This would also lead to increased loss of AMPs, but *via* neutral processes. In the case of *Dpt*, however, *Providencia rettgeri* is distributed worldwide including throughout Africa (59, 60). Therefore, it is unlikely to be completely absent from African populations of *D. melanogaster* where null alleles are common. Instead, the specific loss of *Diptericins* in *Drosophila* might reflect a deleterious consequence when dysregulated in non-immune tissues. For example, AMP expression increases dramatically in the head tissue of aging flies (18). This explanation seems more likely, as non-cell autonomous *DptB* is known to affect memory formation in *D. melanogaster* (52), evidence of direct *Diptericin* impact on brain function. A second line of support for the "selective loss" scenario is the null allele cline observed in African populations alongside a parallel (though not significant) cline in North America. Such parallel clines are often used as evidence for selection acting on alleles (61–63). While neutral processes could lead to clines in null alleles as well, with the null allele spreading from an initial source population, the likelihood of this happening in parallel on two continents is small. Finally, there is growing evidence that several AMPs may inflict damage on host tissues. For instance, the cathelicidin LL-37 is toxic to leukocytes (64), and constitutive expression of AMPs reduces lifespan in *Drosophila* (17). These direct observations of deleterious effects strongly undermine the idea that neutral processes are driving the loss of AMPs, instead suggesting these molecules impose a significant effect on host fitness.

One exciting explanation for AMP fitness costs is the idea that AMPs are dysregulated through aging, leading to chronic inflammatory responses and eventually cell death. Additionally, the idea that AMPs may be active in the nervous system is an attractive recent hypothesis that demands more consideration (65, 66), particularly to understand the roles these short peptides play in neuronal homeostasis (67). For instance, while implicated in Alzheimer's disease for decades, the specific nature of how Amyloid-beta contributes to dementia remains unclear (68). Understanding its role in immunity may shed light on the cause and progression of amyloid plaques (13), and reveal the true culprit(s) behind Alzheimer's progression; an interesting recent study found that Amyloid-beta binds to the human cathelicidin LL-37, forming heterodimers that reduce the toxicity of LL-37 to host cells (69). Alongside evidence that Alzheimer's may be an infectious disease (14), dysregulation of AMPs in the nervous system upon chronic infection could lead to host cell toxicity. Appreciating the role of AMPs in the nervous system, particularly during infection, may lead to breakthroughs in treating neurodegenerative disorders such as Alzheimer's disease or Parkinson's disease.

If indeed AMPs are deleterious in non-immune contexts, this may promote balancing selection in populations with dynamic immune pressures. Beyond AMPs, trade-offs between immunity and fitness are well-documented, implying that an immune system is advantageous only in the context of immune challenge, but otherwise is detrimental to reproductive fitness (70–73). As the front line of innate immunity, AMPs should be primary actors on this evolutionary stage, and selection for or against immunity genes should therefore act strongly

on context-dependent AMPs. Recent studies report that both balancing and diversifying selection has shaped the *Drosophila* AMP arsenal (22–25), revising how we view AMPs as actors in host-pathogen interactions (28). If balancing selection is driven by trade-offs between alleles that provide increased resistance during infection but are costly when hosts are uninfected, this could explain the dynamic patterns of AMP gain and loss described here. By characterizing e.g. *Diptericin* loss throughout Diptera, we provide the beginnings of an immunological fossil record with extinct (pseudogenes) and extant *Diptericin* gene copies in different lineages. The observations of other AMP gene losses throughout Diptera extend this fossil record back in time, describing lineages with different stages of loss stemming from an ancestral immune-competent fly to derived lineages lacking subsets of certain AMPs.

Globally, we highlight how host ecology predicts AMP loss, and follow the evolution of AMP lineages throughout Diptera. We describe that selection on the innate immune system can act swiftly and directly on AMPs, implicating some AMPs as deleterious molecules in the absence of microbial challenges. These results could relate to the newly discovered role of AMP-like peptides in neurodegenerative diseases and autoimmune disorders. If so, our findings offer evolutionary signatures supporting the notion that trade-offs between immunity and fitness are mediated by costs related to the maintenance of autotoxic host AMPs.

MATERIALS AND METHODS

Survey of AMP Families in Published Diptera Genomes and Transcriptomes

We first conducted a thorough literature review to annotate the life histories of diverse Diptera. We then searched for *Drosophila* AMP families present in other Diptera using an iterative step-wise tBLASTn approach followed by manual curation; of note, we used an extremely lenient E-value for the shortest peptides (e.g., E < 100 for Mtk, 26 residues long), followed by manual curation. In brief, we collected AMP genes from sequenced *Drosophila* and then BLASTed all available orthologs against outgroup genomes from Vicoso and Bachtrog (43). We collected all confirmed outgroup orthologs and re-performed this BLAST against any species where no match was found, until we ceased to recover new orthologs. To verify any patterns of loss we observed (e.g., *Dpt* loss in Tephritid species), we further searched for outgroup sequence data (genomes, transcriptomes, or raw SRAs) to include in our analyses as independent databases. For some orthologs, only a partial sequence was recovered on a scaffold assembled with many gaps (NNNs). If sequence similarity was highly conserved we annotated these AMPs as "present" but do not include them in phylogenetic analyses as their information content was poor. All sequence databases used in this study are included in **Supplementary Data File 1**. Sanger sequencing results are deposited in GenBank under accessions: MN311474-MN311476.

To investigate sequence similarity and validate curated orthologs, we aligned sequences using MAFFT and performed phylogenetic analysis using Neighbor-joining (1000 bootstraps) and/or Maximum likelihood (100 or 500 bootstraps) methods implemented in Geneious R10 and the PhyML webserver (74). For *Diptericin* evolution in **Figure S3**, sequences were also codon-aligned using MAFFT.

Fly Stocks and Strain Information

The following strains were used in this study for gene expression analysis and Sanger sequencing: *D. melanogaster* (DrosDel iso w^{1118}), *D. pseudoobscura, D. virilis, D. immigrans, D. innubila, D. testacea*, and *D. neotestacea*. *Drosophila pseudoobscura* and *D. immigrans* were generously provided by Ben Longdon and correspond to strains used in Duxbury et al. (75). *Drosophila innubila* used in this study is the same as the genome-sequenced strain from Hill et al. (76). *Drosophila virilis* was a gift from Richard Benton corresponding to Sackton et al. (28). Steve Perlman kindly provided Testacea group flies. The *D. testacea* strain used corresponds to the wild-type *D. testacea* described in Keais et al. (77) cleared of *Wolbachia* symbionts by the Perlman lab. The *D. neotestacea* strain is the same as used in Hanson et al. (24). *Drosophila melanogaster, D. pseudoobscura,* and *D. virilis* were reared on standard food medium for *D. melanogaster* and reared at 25°C. *Drosophila immigrans, D. innubila,* and *D. neotestacea* were reared on Nutri Fly^{TM} instant formulation supplemented with a piece of *Agaricus bisporus* mushroom, and reared at 22°C. All species were kept at 22°C during the course of infection. All flies used in this study were previously shown to be negative for *Wolbachia, a* common endosymbiont of *Drosophila.*

Gene Expression Analysis

Infections, RNA extraction, and cDNA synthesis were performed as previously described (51). Pooled samples of 6 flies (3 males and 3 females) were used for each replicate experiment, and three repeats were performed (18 total flies per treatment per species). Flies were frozen either 6 hpi (Unchallenged, *Escherichia coli (E. coli)*] or 24hpi (*M. luteus, C. albicans*) at −20°C in TRIzol. Quantitative PCR was performed on a LightCycler 480 (Roche) in 96-well plates using Applied Biosystems PowerUP Master Mix. Error bars represent one standard deviation from the mean. Statistical analysis was performed using One-way ANOVA with Holm's-Sidak *post-hoc* comparisons to the treatment that induced expression most in each species for each gene (marked as "ref"); e.g., the *E. coli* treatment was the point of comparison for *Dpt*, and *C. albicans* for Bomanin in *D. melanogaster*. *P*-values are reported as: not significant = ns, <0.1 = •, <0.05 = * <0.01 = ** and <0.001 = ***.

We note the pattern of *Dpt* induction we observed conflicts with a previous report that *Dpt* is not inducible in *D. neotestacea* (24), which is likely explained by measuring alternate *Dpt* isoforms. Primers used in this study, additional expression data for AMPs in different species, and *D. neotestacea Dpt* primer comparisons suggesting alternate *Dpt* isoforms can be found in **Supplementary Data File 1**.

Diptericin Evolution in Drosophila

We identified segregating putative null alleles in *Drosophila melanogaster* populations by visually inspecting alignments of

re-sequenced individual inbred lines (**Figures 3A,B**) or pool-seq alignments (North American populations in **Figure 3C**) (57, 78). We found three classes of putative null alleles: (a) premature stop codons, (b) deletions that disrupt core parts of the transcript (i.e., the start codon), and (c) deletions that are in frame but were associated with reduced immune defense against *P. rettgeri* in prior studies (79). Thus, while the counts in **Figure 3A** for the USA represent individual inbred lines, those in **Figure 3C** represent the proportion of reads at a given site carrying the particular null allele.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank database, accession numbers MN311474-MN311476.

AUTHOR CONTRIBUTIONS

MH performed live specimen experiments. MH and RU screened and analyzed data. MH, BL, and RU wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02620/full#supplementary-material

Figure S1 | Alignment of Metchnikowin (*Mtk*) and Mtk-like sequences. We recovered *Mtk* genes from mushroom-feeding flies (brown highlight) that retain the major portion of the *Mtk* mature peptide. We also recovered a clear *Mtk* ortholog in the outgroup Drosophilid *P. variegata* (Pvar\Mtk), which resembles Mtk-like sequence in the Brachyceran fly *Solenopsis brevicornis (S. brevicornis)* (Sbre\Mtk-like). Other Mtk-like sequences from Brachycerans are also shown, and the full open reading frame is shown for all sequences.

Figure S2 | The *Drosophila busckii Diptericin* gene region encodes 6 copies of the subgenus Drosophila-restricted *Dpt* (*DptC* clade, see **Figure S3**) and 3 copies of *DptB*. There are an additional 3 *Diptericin* pseudogenes apparent in the gene region.

Figure S3 | Maximum likelihood tree showing that Tephritid *Diptericins* paraphyletically cluster within the *Drosophila DptB* clade, though bootstraps for exact sorting are poor (as expected of paraphyletic clustering); 100 bootstraps, where bootstraps <30 are not shown. Major *Dpt* clades are highlighted as follows: Blue, *DptA*; Red, *DptC*; Green, *DptB*; Teal, Tephritid *Dpts*.

Supplementary Data File 1 | Annotated life histories: Annotations of feeding ecology in larvae and adults of Diptera in this study. Used in **Figure 1**. **Datasets used in this study:** Datasets and their accesions and quality scores for diverse Diptera used in this study. Used in **Figure 1**. Primers used in this study: Primers used in qPCR analysis or Sanger sequencing. qPCR reactions were run with a 60°C annealing and extension phase. Used in **Figure 2**. **qPCR data from alt. species:** qPCR data from initial explorations using Drosophila pseudoobscura, Drosophila immigrans, and Drosophila testacea (1 Experiment). Remarked as "not shown" in manuscript. **Dneo DptC primer comparison:** Comparison of Dneo\Dpt induction as measured by primers from Hamilton et al. (2014) and universal Testacea group Dpt primers from this study. Revises interpretation of Hamilton et al. (2014) and (24).

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Lipocalin 2 Protects Against *Escherichia coli* Infection by Modulating Neutrophil and Macrophage Function

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Lipocalin 2 (Lcn2) is an essential component of the antimicrobial innate immune system. It attenuates bacterial growth by binding and sequestering the iron-scavenging siderophores to prevent bacterial iron acquisition. Whereas, the ability of Lcn2 to sequester iron is well-described, the role of Lcn2 in regulating immune cells during bacterial infection remains unclear. In this study, we showed that upon infection with *Escherichia coli* (O157:H7), Lcn2-deficient ($Lcn2^{-/-}$) mice carried more bacteria in blood and liver, and the acute-phase sera lost their antibacterial activity *in vitro*. Neutrophils from $Lcn2^{-/-}$ mice were defective in homeostasis and morphological development. *E. coli* O157:H7 infection of $Lcn2^{-/-}$ mice resulted in a reduced neutrophil migration capacity, with 30% reduction of extravasated neutrophils, and impaired chemotaxis, as shown by a reduction in the secretion of chemoattractants, such as tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2, which are instrumental in eliciting a neutrophil response. We also found that some secreted cytokines [interleukin (IL)-6, IL-1β, and TNF-α] were decreased. Transcripts of inflammatory cytokines (IL-6, IL-1β, TNF-α, and IL-10), chemokines (MIP-2 and MCP-1), and iNOS production were all strongly repressed in $Lcn2^{-/-}$ macrophages. Furthermore, Lcn2 could induce the production of chemokines and promote the migration and phagocytosis of macrophages. Thus, Lcn2 deficiency could impair the migration and chemotaxis ability of neutrophils and disturb the normal secretion of inflammatory cytokines of macrophages. Therefore, the heightened sensitivity of $Lcn2^{-/-}$ mice to *E. coli* O157:H7 is not only due to the antibacterial function of Lcn2 but also a consequence of impaired functions of immune cells, including neutrophils and macrophages.

Keywords: lipocalin 2, Lcn2-deficient mice, bacterial challenge, neutrophil, macrophage

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INTRODUCTION

Iron is an essential micronutrient required for almost all aerobic organisms, with crucial functions in many critical metabolic processes, such as DNA synthesis, oxygen transport, redox reaction, and synthesis of hemoglobin (1). Both hosts and pathogens depend on and compete for iron for their proliferation and biologic functions. Therefore, iron always lies at the

center of an eons-long battle between hosts and their pathogens (2). In the struggle for iron, bacteria have evolved aggressive iron-acquiring mechanisms through the expression of siderophores to steal iron from host proteins, such as transferrin and ferritin (3). As a leading bacteria that cause diarrhea in humans and livestock animals, *Escherichia coli* can detect low iron signal as an environmental cue to trigger the synthesis of siderophore enterobactin, which has high affinity for iron (4, 5). In order to restrict bacteria from obtaining iron, the hosts have also adopted some "nutritional immunity" mechanisms for the competition of iron, including lipocalin 2 (Lcn2) (6). Lcn2 has higher affinity to enterobactin-Fe^{3+} than enterobactin receptor protein FepA of *E. coli*, so it can inhibit the iron uptake pathway of *E. coli* and disrupt bacterial iron acquisition (7).

Lcn2, also known as neutrophil gelatinase-associated lipocalin (NGAL), siderocalin, or 24p3, is a multipotent 25-kDa protein and mainly secreted by neutrophils. As a member of the lipocalin superfamily, Lcn2 forms a barrel-shaped tertiary structure with a hydrophobic calyx that binds several lipophilic molecules (8). It is a pleiotropic mediator of various biochemical processes, such as iron delivery (9), apoptosis (10), and cell migration and differentiation (11). Lcn2 also plays an important role as an early marker for kidney damage (12). Of all those functions, the best characterized one is that Lcn2 obstructs the siderophore iron-acquiring strategy of bacteria and thus inhibits bacterial growth. Indeed, Lcn2-deficient ($Lcn2^{-/-}$) mice were more sensitive to bacterial infection than wild-type (WT) mice and exhibit higher mortality rates after systemic administration of *E. coli* (13, 14). In this regard, Lcn2 plays an essential role in the innate immune response against bacterial infection.

Despite being named as a neutrophil protein and originally identified as a component of neutrophil granules, Lcn2 can also be expressed in other cell types, including macrophages, hepatocytes, epithelia, and adipocytes (13, 15, 16). Lcn2 has been reported to be an acute-phase protein based on elevated levels in serum, epithelium, urine, and feces of patients with active inflammatory disease (17–19). However, the precise role of Lcn2 in bacterial infection remains to be elucidated. Therefore, in this study, we investigated the role of Lcn2 in *E. coli* O157:H7 infection using gene-targeted $Lcn2^{-/-}$ mice. Our results present evidence to show that Lcn2 was dramatically upregulated and mainly induced in the liver in challenged mice. We showed that $Lcn2^{-/-}$ mice exhibited increased susceptibility to bacterial infections, in keeping with the proposed function of Lcn2 in iron sequestration. Moreover, we found that neutrophils derived from $Lcn2^{-/-}$ mice were defective in homeostasis, morphology, and migration. Additionally, Lcn2 was necessary for macrophages to induce inflammatory cytokines and phagocytose bacteria. Therefore, the observed sensitivity of $Lcn2^{-/-}$ mice to the pathogen *E. coli* O157:H7 is not only related to the antibacterial function of Lcn2 resulting from sequestration of iron but also a consequence of impaired immune cell function, such as neutrophils and macrophages.

MATERIALS AND METHODS

Mice and Cell Culture

C57BL/6 WT and C57BL/6 Lcn2-deficient ($Lcn2^{-/-}$) male mice (∼20 g) were obtained from Animal Center of Chinese Academy of Sciences (Shanghai, China) and Jackson Laboratory (USA), respectively. All mice were housed in specific pathogen-free cages and received food and water *ad libitum* in Zhejiang University with a 12-h dark-light cycle at 24°C. No mouse died during the experiment. Mouse studies were approved by the Animal Ethics Committee of Zhejiang University.

RAW264.7 macrophages were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), penicillin (KeyGen Biotech, China) (100 IU/ml), and streptomycin (KeyGen Biotech, China) (100 μg/ml) at 37°C in humidified air containing 5% CO_2. Cells were seeded in six-well dishes at 1×10^6 cells per well and grown overnight until 80% confluent. They were then digested by EDTA-trypsin (KeyGen Biotech, China) and used for a variety of experimental procedures.

E. coli in vitro Infection

E. coli O157:H7 (ATCC43889 strain) was obtained from China General Microbiological Culture Collection Center (Beijing, China) and grown in Luria-Bertani (LB). Prior to *in vitro* infection, cells were extensively washed with phosphate-buffered saline (PBS) (Genome Biotech, China) and incubated in complete RPMI-1640 without antibiotics for 2∼3 h until 90% confluent. The concentration of bacteria solution was determined by a standardized calibration curve of OD600/colony-forming units (CFU).

E. coli in vivo Infection

Each mouse (5–6 weeks) was infected by intragastric administration with 2×10^8 CFU of *E. coli* O157:H7 diluted in 200 μl PBS. Mouse behavior was carefully monitored every 12 h. For investigating the expression changes of Lcn2 after bacterial challenge, a total of 32 mice ($n = 4$ per time point) were euthanized by cervical dislocation at 1, 4, 8, 24, 32, 36, 48, and 60 h post infection (hpi). Liver, spleen, kidney, jejunum, colon, lung, and heart were collected for quantitative real-time PCR detection. For measuring the bacterial burden, heparinized blood samples and homogenized liver were collected at 32 hpi and plated on LB agar to determine CFU. Blood samples were also used for Wright-Giemsa staining (Phygene, China) and measurements of serum Lcn2 protein. Liver and jejunum tissues were fixed for paraffin sectioning and immunohistochemistry (frozen sections). Uninfected control group ($n = 4$) received 200 μl of sterile solution containing 10% (w/v) $NaHCO_3$ and 20% (w/v) sucrose.

For determining the bacteriostatic ability of endogenous Lcn2, WT and $Lcn2^{-/-}$ mice ($n = 6$ per group) were intraperitoneally injected with 2×10^8 CFU heat-killed *E. coli* O157:H7. Acute-phase serum was collected at 5 hpi. *E. coli* O157:H7 (10^3 CFU)

were then inoculated into RPMI-1640 with 20% acute-phase serum from WT and *Lcn2*$^{-/-}$ mice. The heat-killed *E. coli* O157:H7 solution was produced by heating in a water bath at 100°C for 30 min.

Determination of Neutrophils in Peripheral Blood and Peritoneal Exudates

Mice were intraperitoneally injected with 1 ml 2×10^8 CFU heat-killed *E. coli* O157: H7 to induce peritonitis ($n = 6$ per group). Mice were sacrificed at 5 hpi. The peripheral blood was drawn from retroorbital plexus, and the peritoneal exudates were extracted from the peritoneal cavity. Neutrophils from peripheral blood and peritoneal exudates were labeled by the phycoerythrin (PE)-conjugated rat anti-mouse Ly6G (Gr-1) mAb (clone 1A8) (BD Biosciences, USA). The percentage of neutrophils was determined by flow cytometry analysis.

Immunohistochemical Assay

Tissues sections from liver and jejunum were deparaffinized with xylene and rehydrated through a series of graded alcohol solutions to deactivate endogenous enzymes. Then, they were washed with PBS and immersed in 0.01M citric acid buffer at 98–100°C to reveal antigens. Cooled sections were stained using a goat anti-mouse Lcn2 (1:250; R&D, USA) and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000; Pierce, USA).

ELISA

Serum Lcn2 was quantified using Lcn2 Mouse ELISA kits (Boster, China) according to the manufacturer's instructions. Cytokine tumor necrosis factor α (TNF-α levels in serum and peritoneal lavages were measured by Duoset ELISA cytokine kits (Rapidbio, USA) according to the manufacturer's instructions. Interleukin (IL)-6, IL-1β, TNF-α, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 levels in the supernatants of the culture medium were quantified using Mouse ELISA kits (eBioscience, USA) according to the manufacturer's instructions.

Bone Marrow-Derived Macrophage (BMDM) Isolation and Culture

BMDMs were isolated from the cavity of femur and tibia of WT and *Lcn2*$^{-/-}$ mice ($n = 6$ per group) after removing the attached muscle tissues. They were then cultured in six-well plates with complete DMEM medium containing 20% fetal bovine serum (FBS), 1% penicillin and streptomycin, and 30% conditioned L929 media as a source of macrophage colony-stimulating factor (M-CSF). On day 7, BMDM cultures with nearly 100% confluence were stimulated with 5×10^6 CFU *E. coli* O157:H7 in DMEM for 24 h. Cytokines were analyzed in the supernatants.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from cells and animal tissues by the Trizol method (Sigma, China). For determining the tissue specificity of Lcn2 gene expression, kidney, pancreas, heart, lung, spleen, liver, jejunum, colon, adipose, bone, muscle, testis, and brain were collected for RNA extraction. Reverse transcription was performed on 2 μg of RNA using random hexamers and reverse transcriptase (Thermo-Fisher Scientific, USA). Quantitative real-time PCR was performed using the FastStart Universal SYBR Green Master fluorescence quantitative kit (Roche, Switzerland). All data were normalized to a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin measured in the same sample. The sequences of the specific primers are listed in **Table 1**. The fold change was calculated using ΔΔ threshold cycle method.

Analysis of Peripheral Blood Smears

A drop of peripheral blood was smeared onto a clean glass side and quickly air-dried for 30 min at room temperature. The smears were then stained with Diff-Quick staining kit (Dade-Behring) according to the manufacturer's recommended protocols. Representative images were shown.

Scratch Wound Healing Assay

Cell migration was examined using the scratch wound healing assay with RAW264.7 macrophages (20). RAW264.7 macrophages were seeded in six-well plates at a density of 2×10^3 cells/well and incubated overnight until cells were ∼70% confluent as a monolayer. The monolayer of cells was gently and slowly scratched linearly with a sterile 10-μl pipette tip to create a wound. Cells were washed twice with PBS to

TABLE 1 | Primer sequences for the real-time PCR amplification.

Gene	Primer (5′ → 3′)	Genebank no.
β-actin	F: CCACCATGTACCCAGGCATT	NM_007393.5
	R: AGGGTGTAAAACGCAGCTCA	
IL-1β	F: AGTTGACGGACCCCAAAAG	NM_008361.4
	R: TTTGAAGCTGGATGCTCTCAT	
IL-10	F: TGGGTTGCCAAGCCTTATCG	NM_010548.2
	R: TTCAGCTTCTCACCCAGGGA	
GAPDH	F: TGCGACTTCAACAGCAACTC	NM_008084.3
	R: GCCTCTCTTGCTCAGTGTCC	
MCP-1	F: GATGCAGTTAACGCCCCACT	NM_011333.3
	R: ACCCATTCCTTCTTGGGGTC	
TNF-α	F: GCTCTTCTGTCTACTGAACTTCGG	NM_013693.3
	R: ATGATCTGAGTGTGAGGGTCTGG	
MIP-2	F: CACTCTCAAGGGCGGTCAAA	NM_009140.2
	R: GGTTCTTCCGTTGAGGGACA	
IL-6	F: CCCCAATTTCCAATGCTCTCC	NM_031168.2
	R: CGCACTAGGTTTGCCGAGT	
GM-CSF	F: GCCATCAAAGAAGCCCTGAAC	NM_009969.4
	R: TCTTCAGGCGGGTCTGCAC	
iNOS	F: CTCACCTACTTCCTGGACATTAC	NM_010927.4
	R: CAATCTCTGCCTATCCGTCTC	
Lcn2	F: ACATTTGTTCCAAGCTCCAGGGC	NM_008491.1
	R: CATGGCGAACTGGTTGTAGTCCG	

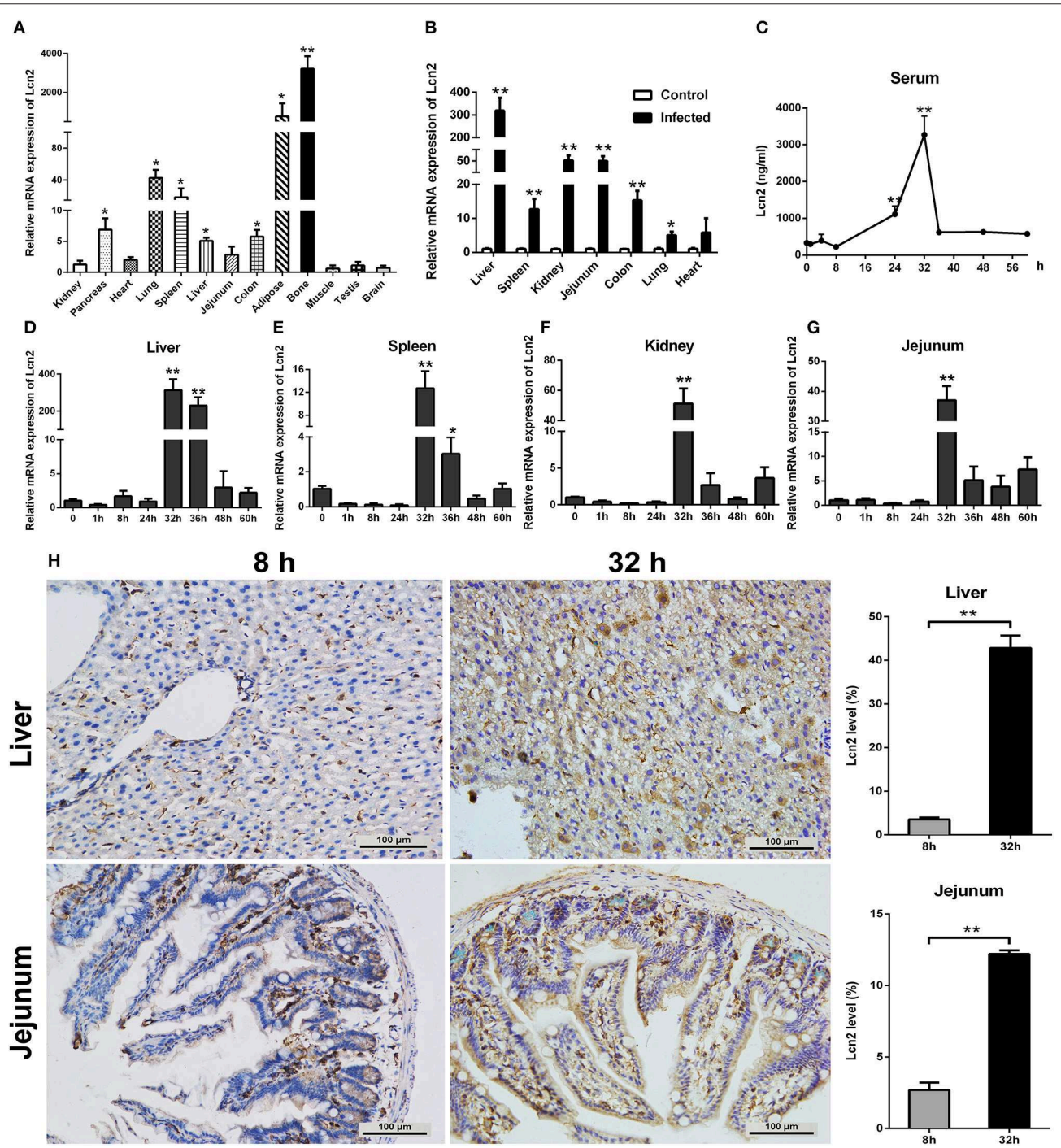

FIGURE 1 | Elevated lipocalin 2 (Lcn2) during *E. coli* O157:H7 infection. **(A)** Real-time PCR analysis of the levels of Lcn2 mRNA expression in indicated tissues of mice. **(B)** The mRNA levels of Lcn2 expression in indicated tissues of control and *E. coli* O157:H7-infected mice. **(C)** Serum levels of Lcn2 protein concentration in *E. coli* O157:H7-infected mice after challenge at different time points. **(D–G)** The mRNA expression levels of Lcn2 in indicated tissues from *E. coli* O157:H7-infected mice after challenge at different time points. **(H)** Protein levels of Lcn2 in mice detected on immunohistochemistry sections of liver and jejunum at 8 and 32 h after challenge with *E. coli* O157:H7. Original magnification was 200×. Values are average means of triplicate experiments. Error bars depict SEM ($n = 4$ per time point). Results are expressed as means ± SEM. $^{*}P < 0.05$, and $^{**}P < 0.01$.

remove floating cells and treated with 1 μg/ml recombined Lcn2 protein (Abcam, USA), while control groups were left untreated. The cells migrating from the leading edge were imaged at 0 (immediately after scratching) and 24 h. The migration distance was calculated by subtraction of the gap distance from the same point at 0 and 24 h.

Phagocytosis Analysis

After infection, the cells were incubated with serum-free medium containing 0.5 mg/ml fluorescein isothiocyanate (FITC)-dextran (4 kDa) (Sigma, America) for 2 h. Extracellular FITC-dextran was washed away with PBS. The cells were dissociated from the cell culture dishes (Corning, USA) with EDTA-trypsin solution (KeyGen Biotech, China), and the intracellular FITC fluorescence intensity was measured by flow cytometry.

Immunofluorescence Analysis for iNOS Determination

RAW264.7 macrophages seeded on glass-bottom dishes were fixed in 4% paraformaldehyde (KeyGen Biotech, China) and then permeabilized with ice-cold 0.5% TritonX-100. The cells were blocked in PBS containing 10% bovine serum albumin (KeyGen Biotech, China) for 30 min and then were incubated with rabbit monoclonal antibody inducible nitric oxide synthase (iNOS) (Bioss Antibodies, China) and rat monoclonal antibody F4/80 (Abcam, USA) overnight at 4°C. After washing with PBS, cells were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Abcam, USA) and Alexa Fluor 647 goat anti-rat IgG (Abcam, USA) for 1 h at 37°C. Finally, cells were counterstained with 50 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) (KeyGen Biotech, China) before capturing images with a confocal microscope (Zeiss, Germany).

Statistical Analysis

Statistical analysis of experimental data was performed by Student's *t*-test and one-way ANOVA in SPSS 20.0 software. The results are expressed as mean ± SEM. The test results were independently repeated three or six times. Levels of statistical significance were set at $P < 0.05$.

RESULTS

Lcn2 Increases Dramatically During *E. coli* O157:H7 Infection

In order to determine the tissue specificity of Lcn2 gene expression, Lcn2 mRNA expression was examined by qRT-PCR in most tissues of mice. Lcn2 mRNA was mainly expressed in bone marrow, adipose, lung, and spleen, while it was less expressed in muscle, testis, and brain (**Figure 1A**). The transcript of Lcn2 was highest in the bone marrow, which was more than 3,000 times of that in the kidney. To test the hypothesis that Lcn2 is one of the acute-phase proteins, we determined Lcn2 expression of challenged mice by intragastric administration with a sublethal dose of a clinical strain of *E. coli* O157:H7 (**Figure S1**). Thirty-two hours after challenge, Lcn2 mRNA levels were markedly ($P < 0.01$) increased in the liver, jejunum, kidney, colon, and spleen (**Figure 1B**). The ratio of Lcn2 mRNA in the liver of infected mice to that of control mice was about 300. Thus, it seems that Lcn2 expression is mainly induced in the liver during *E. coli* O157:H7 infection. To investigate the effect of bacterial infection on the level of Lcn2 in the bloodstream, sera were collected, and Lcn2 was detected by ELISA. The basal serum concentration of Lcn2 in uninfected mice was ∼300 ng/ml (**Figure 1C**). After intragastric infection, serum levels of Lcn2 increased to 1,110 ng/ml by 24 h, peaked at 3,270 ng/ml by 32 h, and then rapidly declined (**Figure 1C**). This expression profile of Lcn2 protein was in concordance with observations of Lcn2 transcripts in detected tissues, including the liver (**Figure 1D**), spleen (**Figure 1E**), kidney (**Figure 1F**), and jejunum (**Figure 1G**). Furthermore, immunohistochemical assay was employed to detect the Lcn2 protein in the liver and jejunum. *E. coli* O157:H7 infection induced a 12-fold increase of Lcn2 in the liver and a five-fold increase in the jejunum (**Figure 1H**). Thus, infection with *E. coli* O157:H7 elevated the levels of both Lcn2 mRNA and protein in the tissues and bloodstream of mice.

Lcn2 Is Involved in the Antibacterial Responses to *E. coli* O157:H7 Infection

Since *E. coli* O157:H7 challenge induced increased Lcn2 levels of transcription and protein in tissues and blood of mice, we speculated that Lcn2 might play a role in responses against *E. coli* O157:H7 infection. To test the role of Lcn2 in an acute lethal infection, we challenged $Lcn2^{-/-}$ or WT mice by intragastric administration with 2×10^8 CFU of *E. coli* O157:H7 and measured the bacterial burden. Compared with the titer in control WT mice, the bacteria recovered from $Lcn2^{-/-}$ mice were ∼20-fold higher in blood, where there are 2.7×10^6 CFU/mg in $Lcn2^{-/-}$ mice and 1.5×10^5 CFU/mg in WT mice (**Figure 2A**). In the liver, there were 1.2×10^8 CFU/mg in $Lcn2^{-/-}$ mice and 2.1×10^6 CFU/mg in WT mice. The bacteria

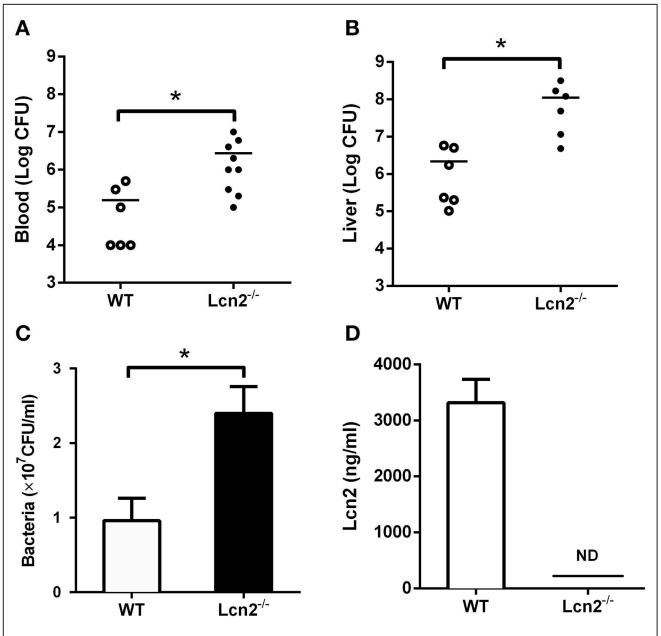

FIGURE 2 | The bacteriostatic characteristics of lipocalin 2 (Lcn2). **(A,B)** Bacterial loads in blood (CFU/ml) and livers (CFU/mg) of *E. coli* O157:H7-infected mice 32 hpi. **(C,D)** Serum levels of Lcn2 protein measured and growth of *E. coli* O157:H7 in RPMI with 20% acute-phase serum from wild-type (WT) or $Lcn2^{-/-}$ mice. Values are average means of triplicate experiments with two mice per genotype per experiment. Error bars depict SEM ($n = 6$ per group). Results are expressed as means ± SEM. $P < 0.05$ was considered statistically significant. *$P < 0.05$.

from *Lcn2*$^{-/-}$ mice were ∼60-fold higher (**Figure 2B**). In order to determine the direct bacteriostatic activity of Lcn2, sera from *Lcn2*$^{-/-}$ mice inoculated by heat-killed *E. coli* O157:H7 were collected 5 hpi and used for bacterial incubation. Compared with *Lcn2*$^{-/-}$ mice, the serum from WT mice exerted remarkable inhibition to *E. coli* O157:H7 ($P < 0.05$) (**Figure 2C**). Meanwhile, there were more than 3,000 ng/ml Lcn2 proteins in the serum of WT mice, but no Lcn2 was detected in serum of *Lcn2*$^{-/-}$ mice as expected (**Figure 2D**).

Lcn2 Deficiency Alters Neutrophil Homeostasis

The above results showed that Lcn2 deficiency could promote the growth of *E. coli* O157:H7, which indicated that Lcn2 might be involved in the innate immune response to bacterial infection. Previous studies showed that Lcn2 could limit bacterial growth by sequestering the iron-laden siderophore (9, 13). Lcn2 has also been shown to be dramatically upregulated in various inflammatory conditions and is considered as an acute-phase protein (12). Herein, we determined whether a deficiency of Lcn2 has consequences for neutrophil development. We first evaluated the hematological parameters of peripheral blood of *Lcn2*$^{-/-}$ and WT mice. The results showed that the numbers of white blood cells, monocytes, and eosinophils were significantly decreased in *Lcn2*$^{-/-}$ mice (**Figure 3A**). In contrast, lymphocyte and thrombocyte counts remained unchanged. Furthermore, a granulocyte-specific marker (Ly6G, clone 1A8) was detected using flow cytometry to confirm a decrease of neutrophils in the peripheral blood of *Lcn2*$^{-/-}$ mice (**Figure 3B**). In addition, Wright-Giemsa staining of peripheral blood smears showed that *Lcn2*$^{-/-}$ neutrophils had atypical bilobed nuclei (band cells), whereas neutrophils of WT mice bore all of the characteristics of mature cells, including ring-shaped segmented nuclei and pale abundant cytoplasm (**Figure 3C**). Furthermore, the number of circulating band cells in *Lcn2*$^{-/-}$ mice is around 3%, while WT mice had only 1% (**Figure 3D**).

Lcn2 Deficiency Reduces the Migration of Neutrophils

Since Lcn2 deficiency can affect the homeostasis of immune cells and the maturation of neutrophils, we speculated that Lcn2 might play an effect on other functions of neutrophils, such as chemotaxis and migration. Normally, the neutrophils from compartments of the bone marrow, peripheral blood, and extravascular space are in dynamic equilibrium (14). Under inflammatory conditions, neutrophils extravasate from the blood compartment to the sites of inflammation (21). We then analyzed neutrophil kinetics in peripheral blood and peritoneum by

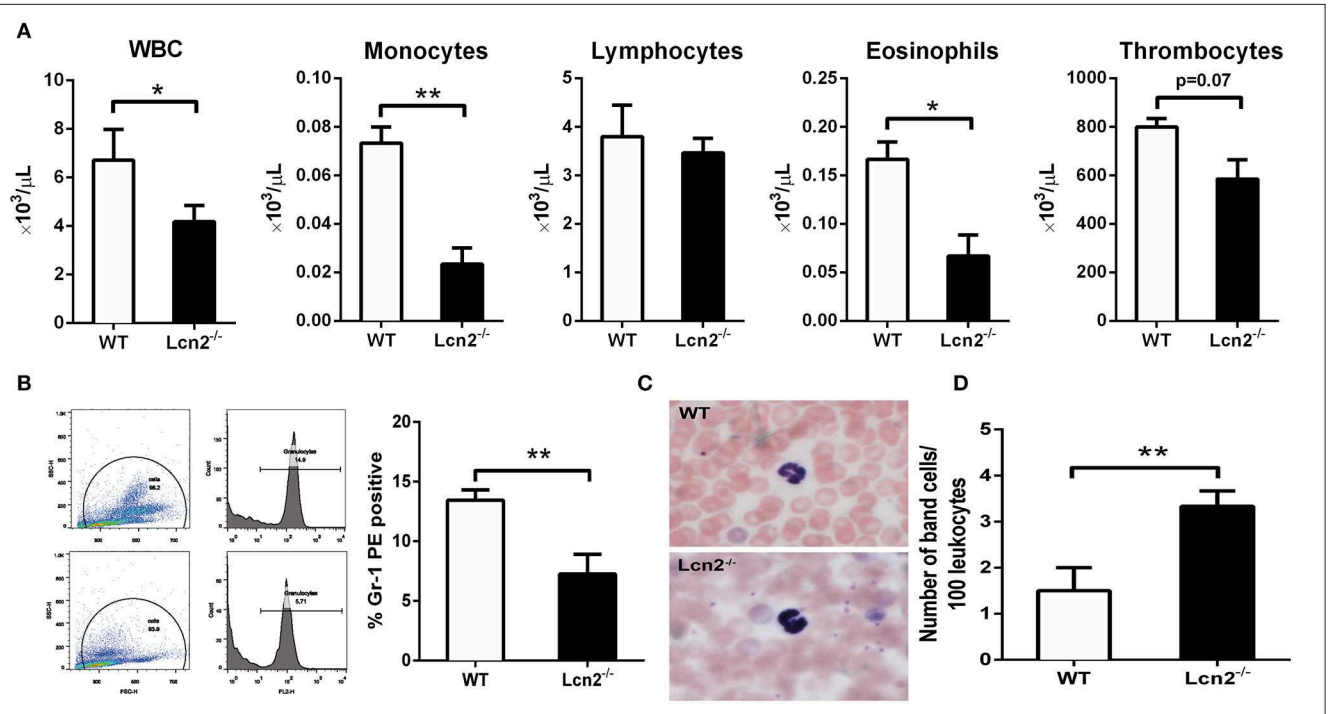

FIGURE 3 | Granulocyte abnormalities in *Lcn2*$^{-/-}$ mice. **(A)** Hematological parameters of peripheral blood from wild-type (WT) and *Lcn2*$^{-/-}$ mice. The data are presented as mean × 10^3 cells/μl. Error bars depict SEM ($n = 6$ per group). **(B)** Flow cytometry analysis of neutrophils in the peripheral blood after intragastric administration with 2 × 10^8 CFU of *E. coli* O157:H7. Cells were stained with indicated clones of Gr-1 Ab, and positive cells were determined by flow cytometry. Values are average means of triplicate experiments with two mice per genotype per experiment. Error bars depict SEM. **(C)** Wright-Giemsa staining of peripheral blood smears from *Lcn2*$^{-/-}$ mice identified atypical hyposegmented neutrophils in the peripheral blood. Original magnification ×63. In contrast, WT mice displayed normal neutrophil maturation. **(D)** Enumeration of the number of band neutrophils in the peripheral blood of *Lcn2*$^{-/-}$ mice. The data are presented as mean band cell numbers per 100 leukocytes. Error bars depict SEM. Results are expressed as means ± SEM. $P < 0.05$ was considered statistically significant. *$P < 0.05$, and **$P < 0.01$.

flow cytometry following Gr-1 staining. Leukocyte extravasation into the peritoneal cavity was studied in a mouse model of inflammation induced by intraperitoneal injection with heat-killed *E. coli* O157:H7 in WT and *Lcn2*$^{-/-}$ mice. Flow cytometry analysis of blood showed that the percentage of neutrophils in the peripheral blood had no significant difference ($P = 0.098$) between WT and *Lcn2*$^{-/-}$ mice (**Figure 4A**). In contrast, the percentage of extravasated neutrophils in challenged *Lcn2*$^{-/-}$ mice was 30% lower ($P < 0.01$) than that observed in challenged WT mice (**Figure 4B**). Thus, the results suggested that the migration of neutrophils from the blood into the peritoneum in *Lcn2*$^{-/-}$ mice was reduced. On the other hand, the release of chemokines and cytokines initiates the inflammatory response. Proinflammatory cytokines, such as TNF-α, and chemokines, such as MCP-1, are instrumental in eliciting a neutrophil response. The reduced migration of neutrophils may be due in part to the reduced secretion of any of these chemoattractants. To examine this possibility, we quantitated the levels of TNF-α proteins and transcripts of chemokines. After the inoculation with heat-killed *E. coli* O157:H7, TNF-α proteins of WT mice were increased sharply both in serum and in peritoneal lavage as expected (**Figures 4C,D**). However, the levels of TNF-α from *Lcn2*$^{-/-}$ mice were elevated mildly, which were significantly ($P < 0.01$) lower than those from WT mice. Moreover, the mRNA expressions of murine chemokines MCP-1 and MIP-2 were both decreased in livers of *Lcn2*$^{-/-}$ mice (**Figures 4E,F**). Thus, the above results showed that Lcn2 deficiency could impair the migration and chemotaxis ability of neutrophils and disturb the normal secretion of inflammatory cytokines under inflammatory conditions.

Lcn2 Deficiency Represses the Induction of Inflammatory Cytokines by Macrophages

Macrophages are the primary producers of cytokines. The observed reduction in cytokines in *Lcn2*$^{-/-}$ mice may also be explained that macrophages need Lcn2 to effectively recognize inflammatory stimuli and to mount an effective cytokine response. To directly test this assumption, we isolated BMDMs from mice treated with *E. coli* O157:H7 and determined the levels of various cytokines. There was no Lcn2 expression in macrophages from *Lcn2*$^{-/-}$ mice (**Figure 5A**). Secreted inflammatory cytokines including IL-6, IL-1β, and TNF-α were all significantly ($P < 0.05$) decreased in *Lcn2*$^{-/-}$ macrophages compared with WT macrophages (**Figures 5B–D**). Similarly, the transcripts of inflammatory cytokines, such as IL-6, IL-1β, TNF-α, and IL-10, were all strongly ($P < 0.01$) repressed in *Lcn2*$^{-/-}$ macrophages (**Figures 5E–H**). The transcript of granulocyte-macrophage colony-stimulating factor (GM-CSF) from *Lcn2*$^{-/-}$ macrophages was also significantly ($P < 0.01$)

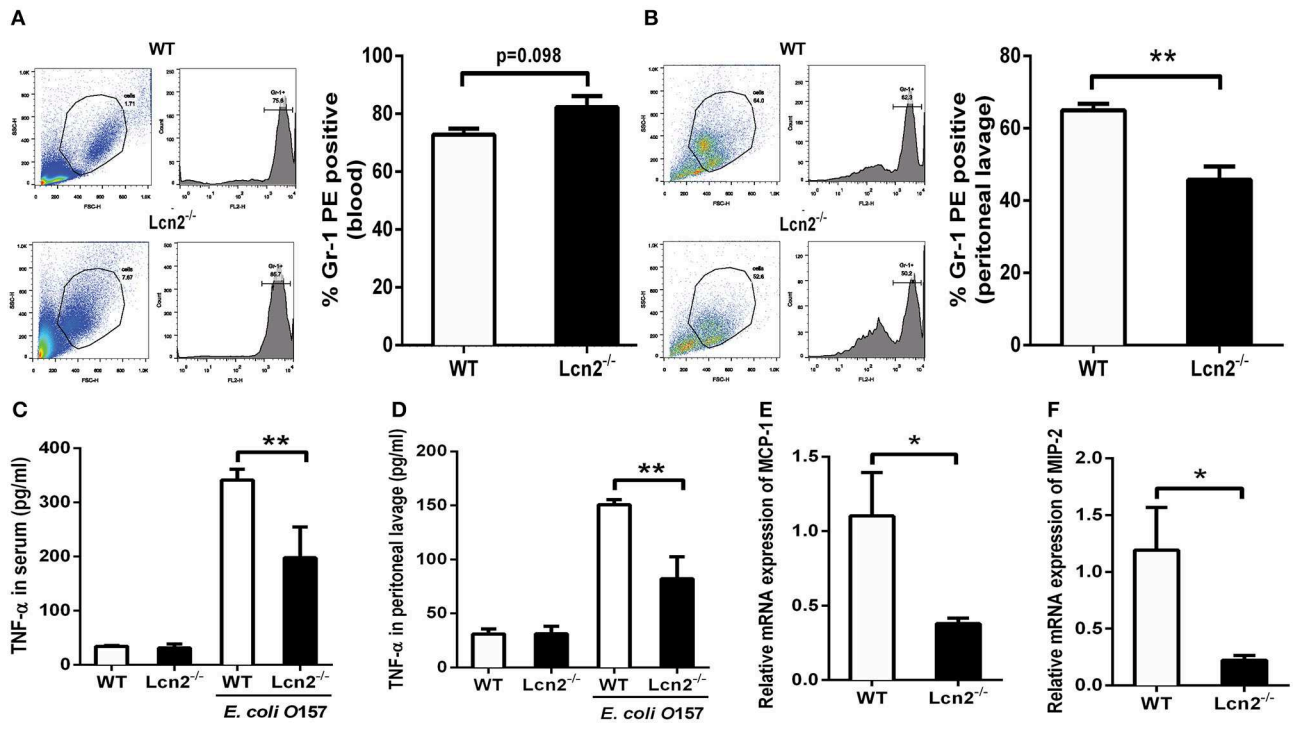

FIGURE 4 | Reduced migration of lipocalin 2-deficient (*Lcn2*$^{-/-}$) neutrophils. **(A,B)** Flow cytometry analysis of peripheral blood and peritoneal exudates of heat-killed *E. coli* O157:H7-challenged mice following staining with a Gr-1 PE Ab. **(C,D)** ELISA analysis of TNF-α in the serum and peritoneal exudates of heat-killed *E. coli* O157:H7-challenged mice. **(E,F)** Quantitative determination of chemokines MCP-1 and MIP-2 mRNA expression in the liver of heat-killed *E. coli* O157:H7-challenged mice. Values are average means of triplicate experiments with two mice per genotype per experiment. Error bars depict SEM. Results are expressed as means ± SEM. $P < 0.05$ was considered statistically significant. *$P < 0.05$ and **$P < 0.01$.

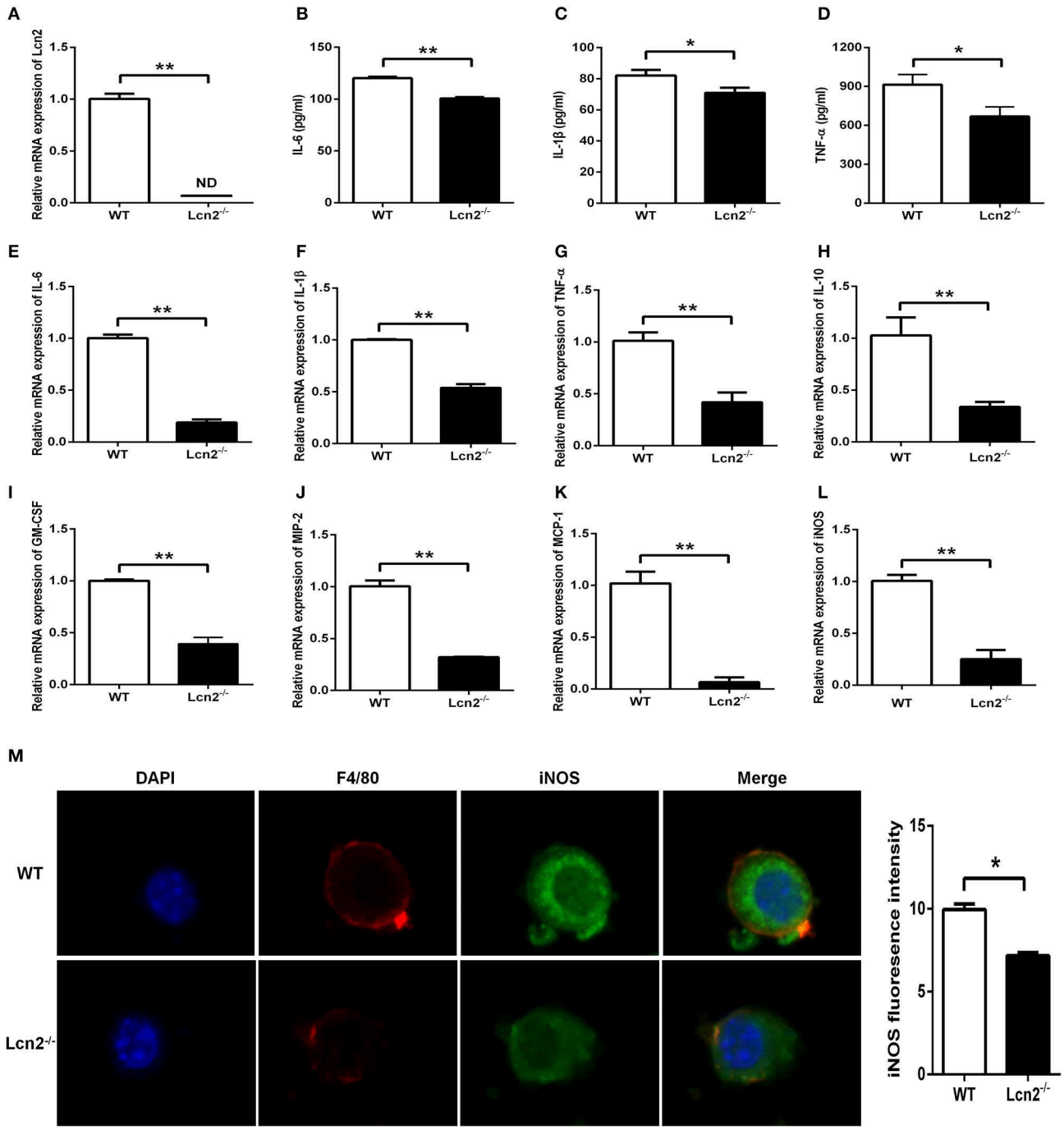

FIGURE 5 | Decreased expression of inflammatory cytokines produced by lipocalin 2-deficient (*Lcn2*$^{-/-}$) macrophages. **(A)** Real-time PCR analysis of Lcn2 mRNA expression levels in the *E. coli* O157:H7-infected primary bone marrow-derived macrophages (BMDMs) from wild-type (WT) and *Lcn2*$^{-/-}$ mice. **(B–D)** ELISA analysis of interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α levels in the culture medium of the *E. coli* O157:H7-infected BMDMs from WT and *Lcn2*$^{-/-}$ mice. **(E–L)** Real-time PCR analysis of cytokine mRNA expression levels in the *E. coli* O157:H7-infected pBMDMs from WT and *Lcn2*$^{-/-}$ mice. **(M)** The infected BMDMs of WT and *Lcn2*$^{-/-}$ mice were subjected to staining with rabbit monoclonal antibody iNOS, rat monoclonal antibody F4/80, Alexa Fluor 488 goat anti-rabbit IgG, and Alexa Fluor 647 goat anti-rat IgG in blocking buffer (1:200) and observed by fluorescence microscopy. Values are average means of triplicate experiments with two mice used for the isolation of BMDMs per genotype per experiment. Error bars depict SEM. Results are expressed as means ± SEM. $P < 0.05$ was considered statistically significant. *$P < 0.05$ and **$P < 0.01$.

decreased (**Figure 5I**), which might explain the reduction of proliferation and differentiation of monocytes. Chemokines are generated at local inflammatory milieu and play an important role in the local recruitment of immune cells. The mRNA levels of chemokines MIP-2 and MCP-1 were both significantly decreased in *Lcn2*$^{-/-}$ macrophages (**Figures 5J,K**). Macrophages

produce inducible nitric oxide synthase (iNOS) that enables the cell to kill pathogens through the production of NO. We further investigated whether deficiency of Lcn2 affected iNOS production. In line with the results of transcription, immunofluorescence analysis showed that iNOS expression was depressed ($P < 0.05$) in $Lcn2^{-/-}$ macrophages compared with that of WT macrophages (**Figures 5L,M**). Thus, the results indicated that Lcn2 played an important role in stimulating the production of these antimicrobial effectors by macrophages and maintained their balances.

Recombinant Lcn2 Promotes the Migration and Phagocytosis of Macrophages

Macrophages are professionally motile cells that carry out a variety of roles in immune surveillance. Transendothelial and interstitial motility is an essential aspect of their function as they must be able to move to specific sites upon demand. Since BMDMs from $Lcn2^{-/-}$ mice adhered to walls too firmly to move, we used RAW264.7 macrophages to detect the effect of Lcn2 on the migration of macrophages. Scratch wound healing assay showed that the migration distances of macrophages treated with recombinant Lcn2 were significantly ($P < 0.01$) longer than that of control cells (**Figures 6A,B**). In addition, chemoattractant cytokines MCP-1 and MIP-2 were both elevated significantly ($P < 0.05$) after Lcn2 treatment (**Figures 6C,D**), which might explain the increase of migration distance of Lcn2-treated macrophages. Moreover, because macrophages are also professional phagocytes and are highly specialized in the removal of dying or dead cells and cellular debris, we then determined the effect of Lcn2 on the phagocytosis of macrophages. Flow cytometry analysis exhibited that Lcn2 could promote the phagocytosis of FITC-Dextran by macrophages (**Figure 6E**). Above results indicated that Lcn2 was involved in the immunologic function of macrophages, such as migration and phagocytosis.

DISCUSSION

Lcn2 has been implicated in diverse physiological processes including apoptosis (22), iron trafficking (13), kidney development (12), and innate immunity (14, 23). We herein provide novel evidence that the absence of Lcn2 increased sensitivity to *E. coli* O157:H7 infection by altering neutrophil homeostasis, reducing the migration of neutrophils, and repressing the expression of inflammatory cytokines by macrophages. Additionally, Lcn2 can also promote the migration and phagocytosis of macrophages.

Lcn2 is considered to be the marker of many inflammatory diseases and involved in various inflammations, including intestinal inflammation, skin inflammation, and metabolic syndrome (24). It is an acute-phase protein known to be highly upregulated during inflammatory conditions (13, 17). In this study, we demonstrated that although bone marrow is the main site of Lcn2 expression normally, Lcn2 was highly induced in almost all detected tissues and mainly induced in the liver of mice in response to *E. coli* O157:H7 infection. This result was in line with that of a previous study (25). We also presented evidence to show that Lcn2 was induced in a parabolic pattern. After intragastric infection, the serum concentration of Lcn2 increased distinctly and then decreased fast. Both mRNA and protein expression showed that Lcn2 peaked by 32 h after challenge. These results are in concordance with observations that Lcn2 may be a marker for inflammation (13, 26). It suggested that strongly induced Lcn2 by *E. coli* O157:H7 infection might play an important role in innate defense against bacterial invasion.

E. coli O157:H7 has been a troublesome foodborne intestinal pathogen and involved in numerous human illness outbreaks (27), which requires iron to survive. Although humans or animals contain plenty of iron, the amount available to bacteria may be extremely limited because most iron is bound intracellularly by heme and ferritin, or extracellularly by transferrin and lactoferrin (28). Our previous studies showed that after the infection of *E. coli* K88, iron was inclined to be sequestered within cells and deposited more in tissues rather than serum, which was supposed to restrict iron available to the bacteria (29, 30). To remedy this difficulty, *E. coli* secretes siderophores to remove iron from host iron-binding proteins and transports it into the bacterial cell (29). However, Lcn2 from hosts can specifically bind to siderophores to prevent bacterial uptake of iron. As a result, Lcn2 is bacteriostatic. In this study, we found that there were more bacteria loaded in the blood and liver of $Lcn2^{-/-}$ mice, which indicated that $Lcn2^{-/-}$ mice succumb to bacterial infection more easily. Furthermore, in a murine model of inflammation with heat-killed *E. coli* O157:H7 infection, we also found that serum Lcn2 was protective against *E. coli* O157:H7. These results proved that elevated Lcn2 after *E. coli* O157:H7 challenge might mediate an innate immune response to inhibit bacterial infection based on iron sequestration.

Lcn2 is a neutrophil gelatinase-associated lipocalin, which is originally isolated from the specific granules of neutrophils (31). We were then wondering whether the knockout of *Lcn2* could affect the balance or development of neutrophils. First, hemocyte analyzer showed that the number of leukocytes, monocytes, and eosinophils in peripheral blood of $Lcn2^{-/-}$ mice was significantly lower than that of WT mice. It suggested that Lcn2 deficiency could disturb the normal homeostasis of immune cells in peripheral blood (32). Neutrophils account for a large proportion of leukocytes in the blood (50–70%) and lead the first wave of host defense to infection or tissue damage (33). They were derived from bone marrow hematopoietic stem cells and eventually developed into mature segmented cells through the following process: promyelocytes > myelocytes > meta-myelocytes > band cells > segmented cells (34). In this experiment, more immature band cells in peripheral blood of $Lcn2^{-/-}$ mice were observed, which indicated that maturation of neutrophils was impaired in $Lcn2^{-/-}$ mice. It is unclear how Lcn2 deficiency contributes to abnormal neutrophil development, but based on our observations, we propose that Lcn2 expression is required for normal neutrophil maturation. Normally, the majority of neutrophils are stored in the hematopoietic cords of bone marrow. Following infection, neutrophils start migrating from the bone marrow to the circulation. The circulating neutrophils

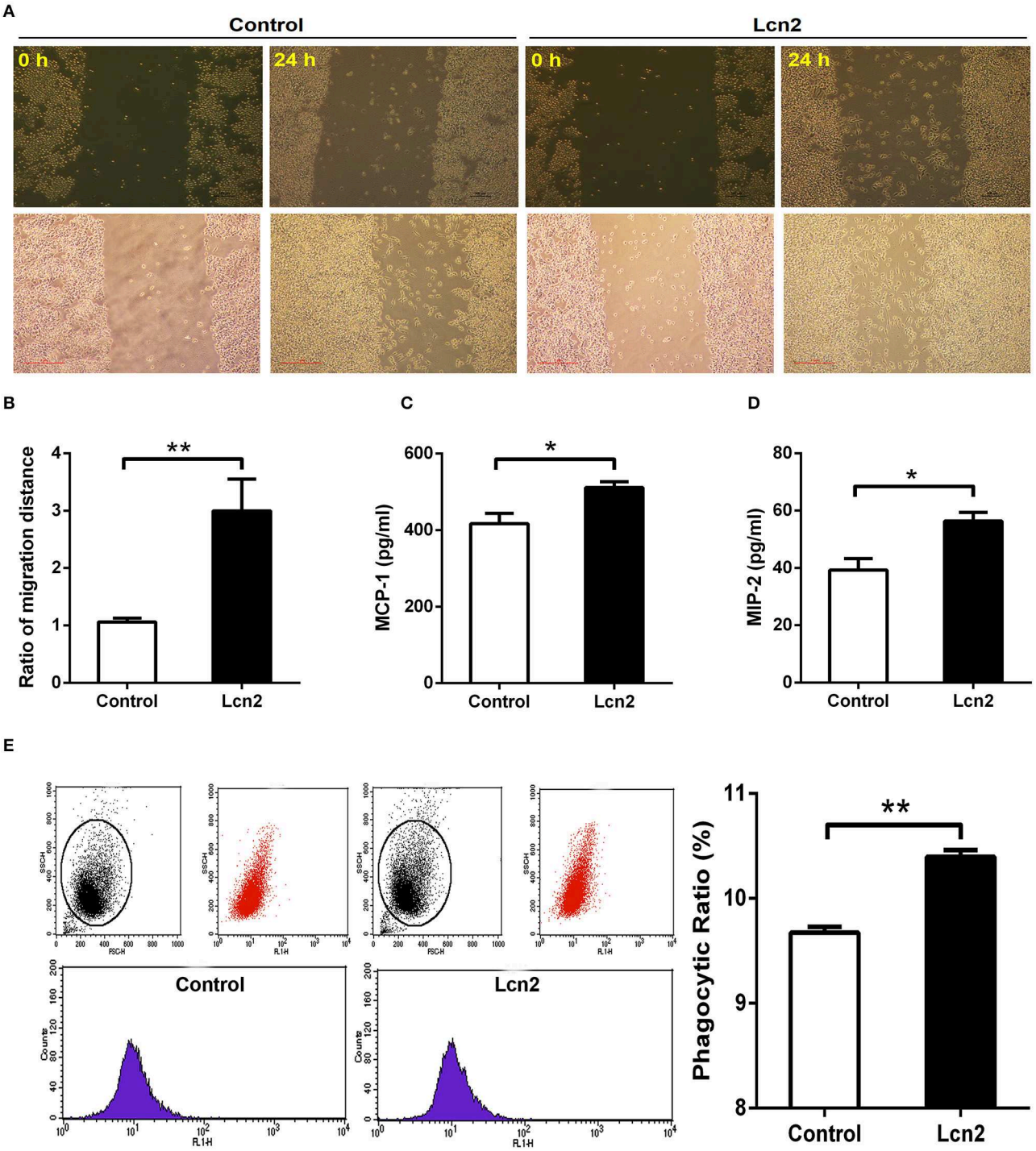

FIGURE 6 | Lipocalin 2 (Lcn2) can promote migration and phagocytosis of macrophages. **(A,B)** Scratch wound healing assay of mouse RAW264.7 macrophages and quantification of the fold change of average migrated distance of cells was measured with microscope ($n = 3$, mean ± SEM, scale bars, 100 μm). **(C,D)** ELISA analysis of monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-2 levels in the culture medium of Lcn2-treated macrophages. **(E)** Flow cytometry analysis of mouse RAW264.7 macrophages incubated with FITC-dextran. Values are average means of triplicate experiments with two repetitions per treatment per experiment. Error bars depict SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

then infiltrate from the bloodstream to the inflammatory sites through multiple processes that involve rolling, adhesion, and finally transmigration by passing through the endothelial cells (35). In this study, we also found that the percentage of neutrophils in the peritoneal fluid of challenged *Lcn2*$^{-/-}$ mice was significantly lower than that of WT mice, whereas there

was no significant difference in the ration of neutrophils in peripheral blood. This suggested that Lcn2 deficiency could reduce the migration of neutrophils from the blood into peritoneum. Chemoattractant-driven neutrophil migration to the sites of infection and inflammation is a well-coordinated and orderly process (36). In this study, reduced secretions of proinflammatory cytokine TNF-α were found in both serum and peritoneal lavage of *Lcn2*$^{-/-}$ mice. The mRNA expression of chemokines MCP-1 and MIP-2 were also both decreased in livers of *Lcn2*$^{-/-}$ mice. These results indicated that Lcn2 deficiency decelerated neutrophil migration by reducing the expression of some cytokines and chemokines. Lcn2 was reported to act as a central mediator to facilitate the crosstalk between neutrophils and hepatic macrophages via induction of the chemokine receptor CXCR2 (37). It was also found that the adhesion capacity of neutrophils was significantly decreased after Lcn2 deficiency (38). It suggested that the effect of Lcn2 on neutrophil chemotaxis might be related to the expression of adhesion protein and chemokine receptors. Further research is needed to clarify the mechanisms.

Both neutrophils and macrophages provide the first line of defense against invading pathogens. Our results showed that Lcn2 deficiency not only altered neutrophil homeostasis but also reduced their migration. We were then wondering whether Lcn2 deficiency would influence the function of macrophages. In this study, BMDMs were isolated from *Lcn2*$^{-/-}$ and WT mice and stimulated with *E. coli* O157:H7 to establish an inflammatory model. Macrophages monitor the invading pathogens, initiate an inflammatory response, and secrete a large amount of inflammatory factors. The results showed that Lcn2 deficiency resulted in the decreased production of inflammatory cytokines, such as IL-6, IL-1β, and TNF-α. It also downregulated the mRNA levels of all detected factors, including proinflammatory factors, such as IL-6, IL-1β, and TNF-α, anti-inflammatory factor IL-10, and chemokines MCP-1 and MIP-2. Similarly, both protein and mRNA levels of iNOS in BMDM of *Lcn2*$^{-/-}$ mice were also decreased significantly. These results suggested that Lcn2 deficiency could interfere with the normal expression of inflammatory factors secreted by macrophages after *E. coli* O157:H7 stimulation. This finding is similar to the reports that TNF-α is largely inhibited by Lcn2 deficiency in chronic inflammation caused by obesity (39), Lcn2 can induce both IL-6 and IL-10 cytokines during *Brucella abortus* infection (40). However, some studies have also shown that the deficiency of Lcn2 in murine inflammation model caused by lipopolysaccharide (LPS) can significantly increase the expression of proinflammatory factors (41). It was speculated that the difference was based on the way of causing inflammation. Our study focused more on the secretory function of macrophages from *Lcn2*$^{-/-}$ mice instead of the effects of Lcn2 on inflammatory responses. Macrophages can not only stimulate adaptive immunity by secreting cytokines but also kill pathogens and clear up cell debris through phagocytosis (42). Here, we also investigated the effect of Lcn2 on the migration and phagocytosis of RAW264.7 macrophages by treating with recombinant Lcn2. Exogenous Lcn2 could significantly increase the migration and promote the phagocytosis of macrophages, which is conducive to the rapid renovation of tissue damage when inflammation or trauma occurs.

In summary, upon encountering bacteria *E. coli* O157:H7, innate immune cells in most tissues produce and secrete Lcn2 immediately, which is induced in a parabolic pattern and, in turn, limits bacterial growth. Targeted disruption of Lcn2 gene has demonstrated its essential role in the early stages of the innate immune response to bacterial infection. An absence of Lcn2 can lead to an increased susceptibility of mice to *E. coli* O157:H7 infection. It was due to not only the loss of bacteriostatic action of Lcn2 by iron sequestration but also a deficiency of immunological functionality of neutrophils and macrophages. Lcn2 is required for animals to mount a proper host defense to bacterial infection by maintaining normal neutrophil maturation and modulating the function of macrophages.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request from the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Zhejiang University.

AUTHOR CONTRIBUTIONS

QW and HD designed the research. QW, SL, XT, and FW developed reagents and performed experiments. QW, SL, and HD analyzed the data. QW, SL, LL, and HD wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02594/full#supplementary-material

Figure S1 | Establishment of a mouse model of *E. coli* O157:H7 challenge. Effects of challenge on the morphology of liver, kidney, and jejunum of mice **(A)**, and transcription levels of inflammatory cytokines TNF-α, IL-1β, and IL-10 in the liver **(B)**. 18S rRNA was used as the housekeeping gene. The mRNA expression ratio was normalized to the mean value of control group of 1. Values are average means of triplicate experiments. Error bars depict SEM ($n = 4$). Results are expressed as means ± SEM. $^{**}P < 0.01$.

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REVIEW
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The Skin and Intestinal Microbiota and Their Specific Innate Immune Systems

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The skin and intestine are active organs of the immune system that are constantly exposed to the outside environment. They support diverse microbiota, both commensal and pathogenic, which encompass bacteria, viruses, fungi, and parasites. The skin and intestine must maintain homeostasis with the diversity of commensal organisms present on epithelial surfaces. Here we review the current literature pertaining to epithelial barrier formation, microbial composition, and the complex regulatory mechanisms governing the interaction between the innate immune system and microbiota in the skin and intestine. We also compare and contrast the skin and intestine—two different organ systems responsible creating a protective barrier against the external environment, each of which has unique mechanisms for interaction with commensal populations and host repair.

Keywords: skin, intestine, microbiome, innate immunity, AMPs

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INTRODUCTION

The skin and intestine both rely on multifaceted mechanisms to maintain homeostasis and protect against invading microbes. Components essential for proper homeostasis between the external environments and the skin or intestine include the physical barrier formed by epithelial cells, the chemical barrier, the presence of beneficial commensal microbiota, and finally the tissue-resident and infiltrating immune cells. The barrier surfaces of the skin and intestine are not only habitats for commensal microbiota, but they also represent potential entry sites for pathogens, including bacteria, viruses, fungi, and parasites. The direct interface between the epithelial tissue barrier and microbiota poses a challenge for the barrier-lining epithelial cells and resident immune cells to distinguish dangerous pathogens from commensals and respond accordingly. Therefore, complex regulatory mechanisms have evolved to allow for delicate coordination between host tissues and their resident microbes. In this review, we provide an overview of the epithelial anatomy of the skin and intestine and interactions between host and microbiota at these surfaces. We focus on the role of microbiota and the innate immune system at homeostasis, in protection against infections, and in tissue repair of the skin and intestine.

STRUCTURE OF THE PROTECTIVE BARRIER

The large surface areas of the skin and intestine—at least 30 m^2 of skin in adults and about 400 m^2 of intestinal epithelium—provides an expansive interface for interaction with the outside environment and increases the risk of invasion by pathogens (1–3). Given their extensive surface

areas, the skin and intestine not only harbor millions of commensal microbiota, but they also must rely on multiple protective strategies to prevent entry of pathogens. As a result, the skin and intestine have developed site-specific physical, chemical, microbial, and immunologic barriers to maintain health and eradicate pathogenic bacteria.

Physical Barrier

The physical barrier of the skin and intestine provides the first line of defense against external perturbation at these sites. The physical barrier of the skin is formed by numerous layers of epidermal and dermal keratinocytes (**Figure 1**). The outermost layer of the epidermis is the stratum corneum, composed of as many as 100 layers of keratinized cell envelopes (corneocytes) that form a protective barrier (5). Barrier lipids, derived from lamellar bodies form an occlusive matrix between corneocytes (6). Deeper epidermal layers, including the stratum granulosum and stratum spinosum, are major producers of keratin filaments, which form a structural support for the epidermis (5). Finally, the basal layer of the epidermis contains stem cells that proliferate in homeostatic conditions and in response to injury in order to reconstitute the physical epidermal barrier. Epidermal keratinocytes maintain tight physical contact through tight junctions and adherens junctions, which form protective layer that is nearly impermeable to microbes. In addition to providing physical protection at the skin barrier, tight junction proteins, such as zona occludins proteins, play roles in proliferation and differentiation of keratinocytes in the skin, allowing re-establishment of the barrier against microbes after breach of the skin from wounding (7).

In contrast to the stratified squamous epithelium of the skin, the intestinal barrier is composed of a single layer of columnar epithelial cells (**Figure 2**) (11). However, this single layer of intestinal epithelial cells (IECs) is made of diverse cell types with absorptive, secretory, and immune function (2). This includes not only the absorptive enterocytes, which encompass the majority of IECs, but also secretory goblet cells, Paneth cells, and enteroendocrine cells. All cells that make up the intestinal barrier are constantly renewed by intestinal epithelial stem cells located in the bases of mucosal crypts (**Figure 2**). As in the skin, IECs are connected via tight junctions, which form a strong physical barrier that impedes translocation of pathogenic microbes or toxins.

Chemical Barrier

The chemical barrier of the skin is formed by numerous secreted lipids and acids. As previously mentioned, the lipid layer secreted by lamellar bodies, is important for maintaining an occlusive matrix between cells and among layers of the stratum corneum (12, 13). Site-specific lipid content also influences the microbial composition of various cutaneous body sites (4, 14). In fact, microbial composition is relatively homogenous among multiple sebaceous sites but varies greatly between sebaceous and dry skin sites (4). Pathogenic microbes are also directly inhibited by some lipids or free fatty acids. For example, sapienic acid can efficiently inhibit pathogenic *Staphylococcus aureus* (*S. aureus*), but does not have sufficient activity against *Staphylococcus epidermidis* (*S. epidermidis*) (15). Overall, the chemical barrier formed by epidermal lipids and fatty acids is important for modulating microbial survival at the skin surface.

In addition, the stratum corneum of the epidermis maintains an acidic pH under homeostatic conditions. The term "acid mantle" has been used to describe the acidic condition of the stratum corneum (16). This acidic pH is important for skin barrier function and microbial defense by providing hostile environment for certain microorganisms (12). Furthermore, there are a number of pH-dependent enzymes that are critical for synthesis, production and maintenance of the lipid composition in the skin. Lipids, such as triglycerides and cholesterol, are hydrolyzed by skin-resident bacteria and yeasts into free fatty acids. Free fatty acids maintain a low pH that inhibits growth of pathogenic species such as *Staphylococcus aureus* (*S. aureus*), while allowing persistence of commensal bacteria such as coagulase negative *Staphylococcus* and *Corynebacterium* (1, 17).

The intestine relies on goblet cells to secrete a thick layer of jelly-like mucus made of glycoproteins to separate luminal bacteria from epithelial cells and create a distinct protected zone (**Figure 2**) (18). Mucins create both a chemical and a physical barrier between the intestinal lumen and EICs, and can even directly modulate expression of tolerogenic and inflammatory cytokines (19). In addition to providing physical protection, mucin layer is also rich in secretory IgA and antimicrobial proteins (AMPs) that provide a chemical immune defense against potential invading microorganisms (20, 21). Mucin synthesis is increased by short chain fatty acids (SCFAs), a fermentation product of bacterial metabolism (22). Furthermore, mucin production is decreased in germ-free mice, but production of mucin can be rescued by activation of microbe-sensing receptors, suggesting that commensal microbes enhance the intestinal barrier (2, 23). The composition of the mucin layer differs between the small and large intestine. The mucous layer of the small intestine is physically penetrable by bacteria, and epithelial cells are protected via secreted AMPs (24). In contrast, the large intestine contains both penetrable outer mucus layer and an impenetrable inner mucous layer (25).

DIVERSITY OF COMMENSAL MICROBIOTA

With the rise of new techniques such as 16S and whole genome metagenomic shotgun sequencing, we have begun to understand in greater detail the diversity and functions of microbiota that colonize the skin and intestine (14, 26). The skin and intestine support a tremendous diversity and number of microbiota. In both the skin and intestine, commensal microbiota are important for maintaining epithelial homeostasis and overall health of the tissue (4, 27).

Site-Specific Differential Composition of Microbiota

Although differing profoundly in taxonomic composition, the skin and intestine are similar in that the microbial composition varies among sites and niches. Recent sequencing studies have extensively mapped the species inhabiting various skin or

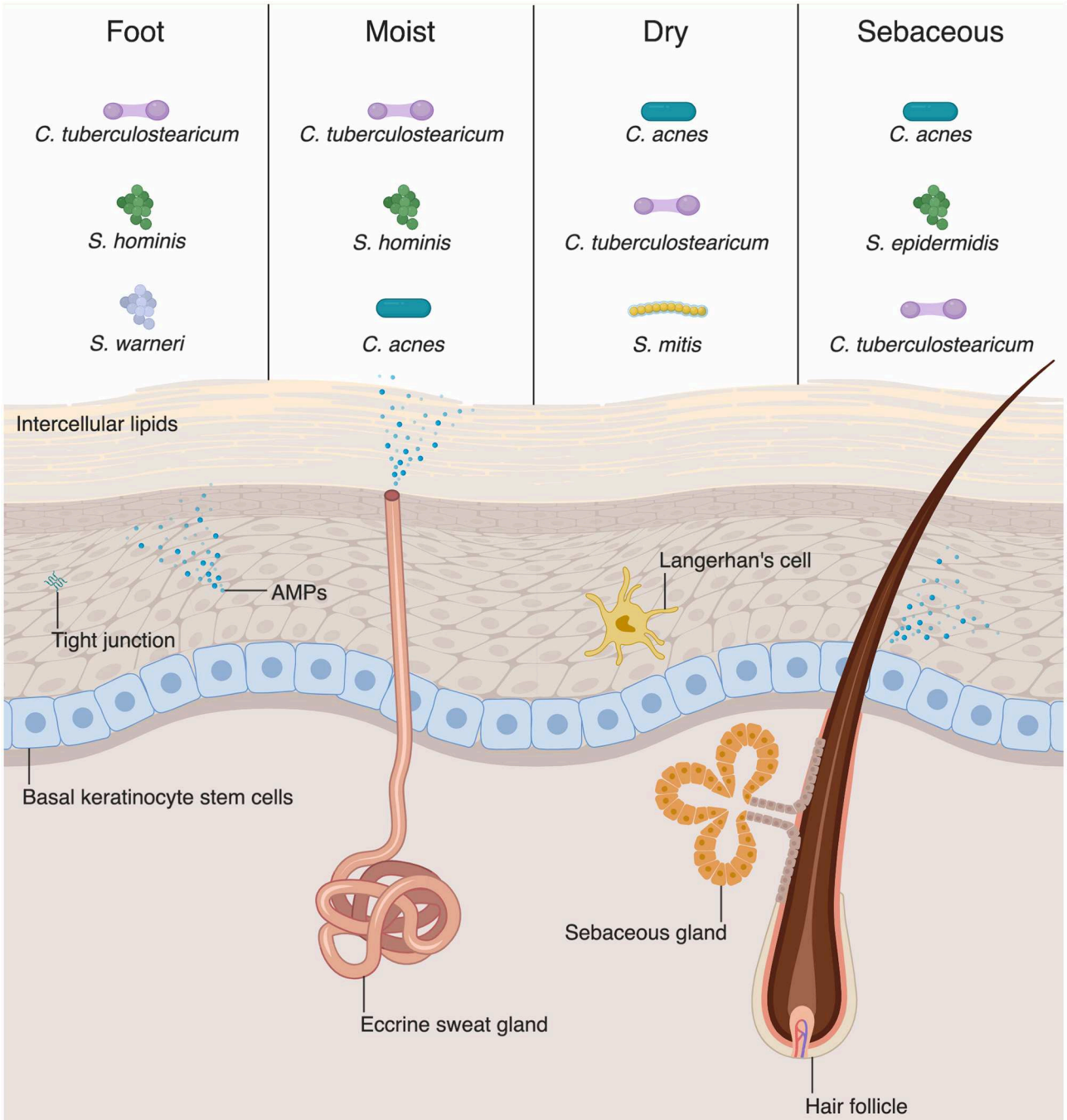

FIGURE 1 | Skin-microbial interactions promote innate immune function. The skin is an active immune organ whose function is augmented by the presence of commensal microbiota. The epidermis is made up of numerous keratinocytes. The stratum corneum is sealed via intracellular lipids, and other epidermal keratinocytes are connected via tight junctions. Dermal appendages include sweat glands, hair follicles, and sebaceous glands, all of which contribute to immune function. Keratinocytes and dermal appendages release antimicrobial peptides and proteins (AMPs), which provide defense against pathogenic microbes. A number of bacteria species are commensal colonizers of the skin surface. The top three bacterial species for each skin site are shown (4). Dry and sebaceous sites are colonized predominantly by *Cutibacterium acnes*, whereas moist sites and the foot are colonized chiefly by *Corynebacterium tuberculostearicum*.

body sites with different compositions, including wet, dry, and sebaceous sites (**Figure 1**) (14, 28). Distinct skin sites contain unique distribution of bacteria, in part governed by the lipid composition of a skin site (14). For example, sebaceous gland-rich areas, such as the glabella and back, are colonized most predominantly by *Cutibacterium* (formerly known as

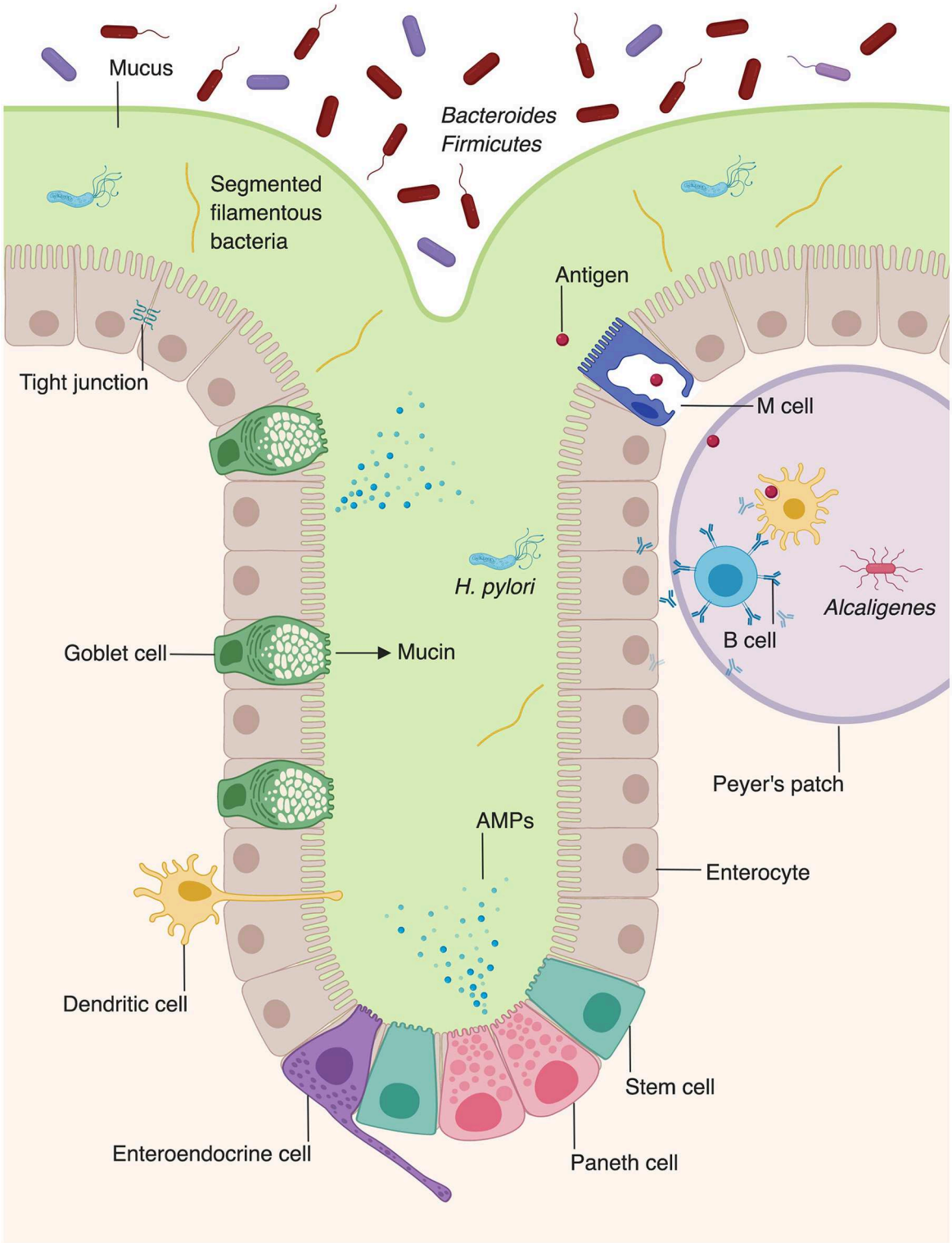

FIGURE 2 | Microbiota augment intestinal innate immunity. Intestinal epithelial cells, which make up the physical barrier of the intestine, secrete antimicrobial peptides and proteins (AMPs). Goblet cells secrete mucus which forms an additional layer of protection against pathogens. Dendritic cells present antigen to B cells within Peyer's patches, stimulating them to secrete IgA. The intestine provides unique niches in which commensal bacteria thrive. *Bacteroides* and *Firmicutes* species comprise the majority of luminal bacteria, whereas segmented filamentous bacteria and *Helicobacter pylori* can penetrate into the mucus layer of the intestine (8, 9). *Alcaligenes* species are able to inhabit Peyer's patches (10).

Propionibacterium) species, which are closely associated with the common condition acne vulgaris (14). Moist sites, such as the axilla and interdigital web spaces, are largely colonized by *Corynebacteria* and *Staphylococci* species (14).

In addition to bacteria, which are the most abundant kingdoms of organisms found on the skin, numerous fungi and viruses inhabit the skin (14). In contrast to bacteria, which are found in nearly all bodies sites and whose composition is governed by physiologic conditions, fungal distribution varies based on distinct body sites rather than physiologic conditions (29). The core body and arms have a relatively homogenous fungal composition and are predominantly colonized by *Malassezia* species, whereas the foot harbors a much greater fungal diversity (29). Viral composition, predominantly *Polyomaviridae* and *Papillomaviridae*, shows most diversity between individuals, rather than depending on body site or composition (28).

In contrast to the skin, which is inhabited by aerobic bacteria, aerotolerant anaerobes, or facultative anaerobes, the intestine is mostly colonized by anaerobes, such as bacteria of the phyla *Bacteroidetes* and *Firmicutes* (**Figure 2**) (8, 14). Whereas, the microbial composition of the skin is largely determined by environmental factors such as the presence or absence of sebum, the intestinal microbiota is dependent on location, niche, and external factors, such as diet (14, 30). The large intestine harbors a higher microbial diversity and density within individuals than the small intestine (31, 32). However, evidence suggests that the microbial composition of the small intestine is more dynamic than that of the large intestine, with large temporal fluctuations in ileal microbial constituents within a single day (33). Fewer studies have examined the microbial composition of the small intestine, compared to the large intestine. However, one study utilized 16s rRNA sequencing to examine the bacterial compositions of the jejunum, ileum, cecum, and recto-sigmoid colon (32). Facultative anaerobic bacteria were present in all four locations along the gastrointestinal tract. Lactobacilli, streptococci, and *Enterococcus* were detected at high frequencies in the jejunum and ileum. In addition to facultative anaerobes, which were the major operational taxonomic unit in both the small and large intestine, the large intestine was also found to contain obligate anaerobic bacteria (32).

Within the small or large intestine, the environmental niches can be luminal, mucus-associated, epithelial-associated, or lymphoid tissue-resident (30). Which phyla of bacteria inhabit a specific intestinal niche depends significantly on the characteristics of both the bacteria and the niche itself. Luminal bacteria are largely of the *Bacteroidetes* and *Firmicutes* phyla, and represent the largest percent of intestinal biomass (8). Recent studies have illuminated that the outer mucus layer of the large intestine forms a unique "mucus-associated" microbial niche with distinct bacterial communities (9). Specifically, bacteria of this niche are adapted to thrive in high levels of bioavailable iron and carbon, an ability conferred by their distinct genome-encoded metabolic and mucolytic activities. For example, *Helicobacter pylori* secretes urease to increase the pH of the mucin layer and disrupts the strong glycoprotein bonds, which allows it to burrow into the stomach mucosa (34).

The epithelial-associated bacteria make up a smaller proportion of intestine bacteria since fewer bacteria are able to infiltrate through the mucous layer (30). Epithelial-associated bacteria express distinct genes that allow them to colonize epithelial cells. For example, expression of commensal colonization factor (*Ccf*) genes allows *Bacillus fragilis* to metabolize carbohydrates present in the intestinal lumen and therefore promotes their colonization of intestinal epithelium, illustrating the importance of nutrient-specific factors in determining microbial composition (35). Furthermore, although *B. fragilis* is an anaerobic bacteria and thrives predominantly in the intestinal lumen, it also grows well in nanomolar oxygen concentrations, such as that found in intestinal crypts (36). Epithelial-associated bacteria are also important for proper function of the intestinal immune system. For example, segmented filamentous bacteria adhere tightly to EICs and induces a Th17 response, conferring protection against pathogenic mucosal bacteria (30). Intestine-associated lymphoid tissues, including Peyer's patches and lymphoid follicles, are colonized largely by *Alcaligenes* species (10). However, it should be noted that, under homeostatic conditions, these bacteria do not spread to the spleen or produce a systemic IgG response. Colonization of intestine-associated lymphoid tissues by these bacteria only results in the local production of *Alcaligenes*-specific IgA antibodies, highlighting the tolerogenic response to a lymphoid tissue-resident bacteria (10). Overall, the special distribution of intestinal bacteria is dependent on niche-specific factors, such as availability of nutrients or site specific microbial-host interactions.

Temporal Changes in the Commensal Microbiome

Commensal species, which can vary according to topography and anatomic environments, also undergo temporal changes as humans develop over time. It was previously thought that *in utero* fetuses were in a germ-free environment. However, data have shown that bacteria can be cultured from the umbilical cord and meconium of healthy full term babies (37, 38). 16S rRNA gene sequencing recently confirmed the presence of microbiota in newborn meconium and amniotic fluid (39). Meconium samples contained bacterial DNA, the majority of which mapped to *Pelomonas puraquae*. Conversely, amniotic fluid bacterial DNA mapped to skin commensal species such as *Cutibacterium acnes* and *Staphylococcus* species (39). The neonatal skin is first colonized by microbes present in the birth canal. Subsequently, an infant's microbiome is shaped by contact with the outside environment. Studies have shown that the skin flora of a baby is largely shaped by the mother's microbiome at birth and that there are notable differences in both skin and intestinal microbiota between infants born naturally or by C-section (40). The infant can also be exposed to viruses, such as herpes simplex virus type 2, present in the mother's vaginal tract (41). Over the course of the first year of life, the infant's skin microbiome is established and begins to resemble that of adults (42).

The intestine similarly has a temporal shift in its microbial flora as the baby transitions from an exclusively milk diet to

solid foods (42). An initial diet of breast milk results in high levels of facultative and obligate anaerobes, such as *Escherichia coli, Streptococcus*, and *Bifidobacterium* species (43). Breast milk provides a source of human milk oligosaccharides and milk glycoconjugates, which are consumed by *Bifidobacterium* species (44). *Bacteroides* and *Clostridia* species predominate as babies are weaned and ingest more complex carbohydrates (43). *Clostridia* species are particularly specialized in degrading plant polysaccharides and are therefore able to thrive in the intestine once complex carbohydrates are introduced into the infant diet (45).

Beyond the early years of life, both skin and intestinal microbiome become more stable, and within-individual variation in microbial communities over time is much less than between-individual variations (28, 46, 47). Despite the relative stability of the skin microbiome, it is less stable over time than the intestinal microbiome (48). Furthermore, the level of microbial stability over time is significantly different among individuals; some individuals have a very stable skin microbiome, whereas others do not. Skin sites that have extensive environmental contact, such as the palm, display the least stability in microbial composition. Interestingly, individuals with a more diverse intestinal microbiome (in terms of number of bacteria species) also have a more stable microbiome over time, whereas individuals with a more diverse skin microbiome have a less stable microbiome over time (48). Microbial diversity decreases in the elderly, coinciding with a decline in immunocompetence in older populations (49). The complex shifts in establishing a commensal population depending on skin and intestinal sites, physiologic conditions, and temporality highlight the importance of finely-tuned interactions between host and microbiota.

Environmental Influences on Microbiome Composition

In both the skin and intestine, microbial diversity is influenced by a plethora of exogenous factors, including diet, antibiotic use, and obesity (50–52). In the skin, treatment with topical or systemic antibiotics has been linked to shifts in the cutaneous microbiome. For example, use of topical antibiotics, such a bacitracin, neomycin, and polymyxin B (found in the commonly-used triple antibiotic ointment) lead to decreased commensal *Staphylococcus* strain in mice (53). Oral isotretinoin or tetracycline treatment leads to decreased abundance of *Cutibacterium* on the skin and the microbiome of sebaceous areas shifts to mimic that of dry sites, containing a greater proportion of *Staphylococcus* and *Streptococcus* species (54).

Diet is a strong driver of microbial composition in the intestine. An animal-based diet increases the abundance of bacteria that are bile-tolerant, such as *Alistipes*, *Bilophila*, and *Bacteroides* (50). In contrast, a vegan or vegetarian diet is associated with an increased prevalence of lactic acid bacteria, including *Ruminococcus, Eubacterium rectale, and Roseburia* (55). *Prevotella* species predominate in humans whose diets are high in carbohydrates and simple sugars (56). High fiber diets lead to a higher abundance of bacteria that ferment fiber into SCFAs, which have a broad range of beneficial effects, including immunomodulatory properties (57). Diet can even influence the circadian dynamics of intestinal microbiota: diet-induced obesity causes a dampening of diurnal variations in microbial composition (58).

MAINTAINING HOST-COMMENSAL HOMEOSTASIS VIA INNATE IMMUNITY

The skin and intestine have developed symbiotic relationships with commensal microbes and established a homeostasis that balances tolerating commensal microbes while defending against pathogens.

Commensal Microbiota Help Maintain Homeostasis in the Skin and Intestine

In the skin, the presence of commensal bacteria is crucial for maintenance of a healthy cutaneous environment. In development, skin immune tolerance begins developing in the post-natal period when T reg cells begin expressing the pathogen-specific FOXP3 transcription factor, coinciding with commensal colonization (59). Later in development, the continued presence of skin commensal bacteria modulates production of numerous cytokines and AMPs that help to protect the skin against pathogens. For example, commensal bacteria such as *S. epidermidis* can induce production of various cytokines by IL-$17^{+}CD8^{+}$ T cells (60). *S. epidermidis* can also produce ligands that suppress inappropriate immune activation by inhibiting production of tumor necrosis factor-α and IL-6 (61). Recent work demonstrated that germ-free mice have decreased expression of Toll-like receptors (TLRs), AMPs, complement cascades, and IL-1 cytokine signaling in the skin, when compared to specific pathogen free mice (62).

It is also well-established that bacterial colonization is essential for maturation of the intestinal innate immune system and, as in the skin, commensal microbiota work in tandem with the immune system to protect the host against pathogens (63, 64). For example, *Bacteroides fragilis* (*B. fragilis*) and commensal *Clostridium* cluster such as IV and XIVa can accumulate $Foxp3^{+}$ T_{reg} cells in mice and help build immune tolerance to the commensal microbiome (65, 66). In addition, the lamina propria harbors macrophages, whose function is phagocytosis of pathogens. However, lamina propria-associated macrophages do not express as strong proinflammatory phagocytic responses as macrophages at other sites (67). This suggests adaptation of the host in response to a large population of commensal microbiota, which minimizes unnecessary inflammation (34).

Innate Immune Responses Upon Barrier Injury Are Modulated by Commensal Microbiota

The physical and chemical barriers discussed above are crucial in preventing tissue penetration of the microbes by decreasing direct contact between them. However, pathogenic microbes may gain access to the tissue when there is a breach of these barriers. Disruption of the skin barrier may occur through physical cut or toxic chemical exposure. Barrier disruption is also associated with

chronic intestinal diseases such as inflammatory bowel disease, obesity, and diabetes, all of which can increase the intestinal permeability (68). In the following section, we discuss innate immune mechanisms upon barrier breach and how they are modulated by commensal bacteria.

Prompt recognition and eradication of pathogens are necessary to prevent infection. The innate immune system provides a first line of defense against pathogens upon barrier breach. Recent evidence has illuminated the role of commensal microbes in strengthening innate immune defense against pathogens (69). Pattern recognition receptors (PRRs) interact with microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) such as lipopolysaccharide (LPS) and peptidoglycan (PG) on bacteria and nucleic acids from bacteria (70). Upon activation of PRRs, downstream signaling leads to release of inflammatory cytokines or activation of immune cells. In the skin, PRRs are present on immune cells and keratinocytes. TLR stimulation can mediate direct antimicrobial action through the stimulation of macrophages to undergo phagocytosis and can also induce cytokines that mediate the differentiation of monocytes into macrophages and dendritic cells (70, 71). Commensal microbes secrete molecules which may act directly as TLR ligands; *S. epidermidis* secretes multiple small molecules that act as TLR2 and EGFR agonists, stimulating production of AMPs that have activity against group A *Streptococcus* and *S. aureus* (72–74).

AMPs play a critical role in innate immunity by acting under homeostatic conditions and destroying pathogenic microbes through various mechanisms (75). Keratinocytes, the major cell type in the skin, produce various AMPs (**Figure 1**) (49, 76). Human β-defensin-1 (hBD-1) is constitutively expressed by keratinocytes whereas hBD-2 and −3 are upregulated in response to inflammation (77–79). Human cathelicidin (hCAP-18) is cleaved and processed to the active form of antimicrobial peptide, LL-37 which then disrupts microbial membranes (80). Some specialized keratinocytes that make up appendages such as hair follicles, sweat glands, and sebaceous glands have various AMPs pertinent to their microenvironments (**Figure 1**). For example, dermcidin is traditionally thought to be a sweat-gland specific AMP, yet there are emerging evidences that it is also produced by sebaceous gland in humans and mice (81, 82). Sebaceous glands also produce cathelicidin and hBD-2 (83, 84). Commensal bacteria have been shown to secrete AMPs. *S. epidermidis* secretes phenol-soluble modulin γ and δ that have antibiotic effects on *S. aureus* (85). Commensal bacteria can also act on lipids secreted from sebaceous glands and hydrolyze them to free fatty acids (FFAs) (86). FFAs have intrinsic antibacterial effects against various Gram-positive bacteria; sapienic acid has activity against methicillin-resistant *S. aureus* (MRSA) (87). Furthermore, FFAs can induce sebocytes to upregulate expression of hBD-2 (88).

Just as external environmental effects are known to modulate skin microbial composition, environmental factors also regulate microbial recognition and AMP production in the skin. Ligand-dependent activation of the vitamin D receptor (VDR) is required for recruitment of macrophages to the injury site after wounding (89, 90). Genes coding for TLR2 are induced by the presence of 1,25-dihydroxy vitamin D3, which is regulated in part by exposure to UV light (83). Furthermore, vitamin D3-induced expression of TLR2 leads to cathelicidin production upon exposure to microbial components. Conversely, TLR2 activation can lead to increased expression of the VDR, which can be activated by vitamin D3 to produce cathelicidin (91). Vitamin D3-eluting wound nanodressings have even been shown to increased cathelicidin expression in human skin wounded explants (92).

Similarly to epidermal keratinocytes, IECs express PRRs, such as TLRS, NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) (93). PRR signaling is important both in homeostasis and in response to pathogenic bacteria, highlighting the diverse functions of innate immunity at steady-state and under disease conditions (94–96). PRRs also respond differently under homeostatic vs. inflammatory conditions, in part because of the presence of damage-associated molecular patterns (DAMPs), which are released by injured epithelial cells (97). Interestingly, steady-state activation of TLRs by commensal intestinal microbiota is also important for proper intestinal homeostasis. For example, mice deficient in TLRs, downstream signaling components of the TLR pathway, or normal commensal microbiota all displayed greater morbidity and mortality following intestinal epithelial disruption (95). Furthermore, TLR activation by commensal bacteria can enhance the protective function of tight junctions against pathogens by strengthening the zonula occludens-1 protein (94). The activated macrophages also signal repair pathway that promotes rapid enterocyte proliferation to repair the tissue defect by producing growth factors (34, 43). This highlights the importance of synergistic activity of commensal microbiota and host innate immunity in maintenance of a healthy epithelium.

In addition to producing barrier-protective mucins, EICs are also a rich source of AMPs (**Figure 2**) (2). Enterocytes produce AMPs including regenerating islet-derived protein III$_\gamma$ (REGIII$_\gamma$) and numerous β-defensins, which play diverse antimicrobial roles, including spatial segregation of bacteria (98, 99). Beyond their roles in barrier formation and production of AMPs, enterocytes also facilitate the translocation of secretory immunoglobulins, particularly IgA, across the intestinal wall (100). Paneth cells, present in intestinal crypts, produce additional AMPs, including α-defensins, lysozyme, and phospholipase A2 (98, 101, 102).

Intestinal commensals are able to induce AMP production in the intestine. *Lactobacillus* and probiotic *E. coli* strains are able to induce secretion of hBD-2 from enterocytes (103, 104). Commensal microbiota in the intestine are also capable of producing molecules that protect the host from chronic inflammatory diseases. For example, polysaccharide A, produced by *B. fragilis*, prevents inflammatory bowel disease (IBD) via an IL-10-producing CD4$^+$ T cell-dependent mechanism (105). SCFAs produced by commensals of the genuses *Bifidobacterium* and *Bacteroides* interact with the G-protein-coupled receptor 43 (GPCR43) (106). Mice deficient in GPCR43 have impaired resolution of inflammation in models of IBD, arthritis and asthma. Similarly to vitamin D3-dependent regulation of AMPs in the skin, butyrate regulates AMP production in the intestine. Butyrate is a SCFA that is produced by fermentation of

carbohydrates in the lumen by intestinal bacteria (107). Butyrate strongly induces cathelicidin production in colonic epithelial cells, and moderately induces h-BD1 and h-BD2 (108). Factors produced by commensal bacteria in the intestine may also prevent injury to IECs or facilitate intestinal repair. Numerous commensal bacteria produce compounds that prevent damage by noxious stimuli. Competence and sporulation factor (CSF) produced by *Bacillus subtilis* activates the mitogen-activated protein kinase (MAPK) pathway to protect epithelial cells from oxidative stress (109). Similarly, *Lactobacillus rhamnosus* produces two compounds, p75 and p40, which prevent cytokine-induced apoptosis of IECs through epidermal growth factor receptor (EGFR) signaling, which activates anti-apoptotic Akt/protein kinase B (110–112). Tight junction assembly is promoted by *Bifidobacterium* and butyrate from various bacteria, underscoring the ability of commensal bacteria to promote intestinal barrier function (113–115).

CONCLUSION

The skin and intestine are both active immune organs that are under constant environmental exposure. Therefore, complex regulatory mechanisms have evolved to maintain homeostasis. In addition to acting as physical barriers, epithelial cells of the skin and intestine produce AMPs, which act as endogenous antibiotics to protect against potential pathogens. Immune cells also constantly surveil both of these surfaces. More recently, it has been appreciated commensal microbiota may induce beneficial, tolerogenic immune responses under homeostasis or prime the immune system to fight against pathogens upon barrier breach. Some commensal bacteria may even produce AMPs on their own. An improved understanding of beneficial microbial-immune interactions has paved the way for new research involving exogenous supplementation of skin and intestinal microbial populations. For example, topical application of Gram-negative bacterial species obtained from healthy human volunteers improved atopic dermatitis in a mouse model (116). Manipulation of intestinal microbiota may be a promising therapeutic option for the treatment of numerous disease, including obesity, IBD, colorectal cancer, and liver disease (117). Although further studies will be needed to validate the safety and efficacy of a microbial-based therapeutic approach, it is clear that a healthy skin and intestinal microbiome is crucial for healthy epithelial homeostasis and immunity.

AUTHOR CONTRIBUTIONS

MC, ML, DN, and AM contributed to conception and design of the review. MC, ML, and DN performed initial literature search and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version. AM supervised all aspects of the review and manuscript writing and is the corresponding author.

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Human β-Defensin 2 Mediated Immune Modulation as Treatment for Experimental Colitis

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Defensins represents an integral part of the innate immune system serving to ward off potential pathogens and to protect the intestinal barrier from microbial encroachment. In addition to their antimicrobial activities, defensins in general, and human β-defensin 2 (hBD2) in particular, also exhibit immunomodulatory capabilities. In this report, we assessed the therapeutic efficacy of systemically administered recombinant hBD2 to ameliorate intestinal inflammation in three distinct animal models of inflammatory bowel disease; i.e., chemically induced mucosal injury (DSS), loss of mucosal tolerance (TNBS), and T-cell transfer into immunodeficient recipient mice. Treatment efficacy was confirmed in all tested models, where systemically administered hBD2 mitigated inflammation, improved disease activity index, and hindered colitis-induced body weight loss on par with anti-TNF-α and steroids. Treatment of lipopolysaccharide (LPS)-activated human peripheral blood mononuclear cells with rhBD2 confirmed the immunomodulatory capacity in the circulatory compartment. Subsequent analyzes revealed dendritic cells (DCs) as the main target population. Suppression of LPS-induced inflammation was dependent on chemokine receptor 2 (CCR2) expression. Mechanistically, hBD2 engaged with CCR2 on its DC target cell to decrease NF-κB, and increase CREB phosphorylation, hence curbing inflammation. To our knowledge, this is the first study showing *in vivo* efficacy of a systemically administered defensin in experimental disease.

Keywords: host defense peptides, antimicrobial peptides, β-defensins, IBD, innate immunity

INTRODUCTION

Inflammatory bowel diseases (IBD) are multifactorial disorders characterized by chronic relapsing inflammation of the intestine (1). They currently affect more than 4 million patients worldwide (2) and are classified in two major entities, Crohn's disease (CD) and ulcerative colitis (UC). While UC is mainly restricted to the colonic mucosa, CD can occur at any site of the gastrointestinal tract but predominantly in the terminal ileum and colon, and inflammation is typically transmural (3).

The etiology of the different forms of IBD is not fully understood. It has, however, been demonstrated that differential defects of the intestinal antimicrobial barrier play an important role in the pathogenesis of both CD and UC (4). Genetic analysis revealed that small intestinal vs. colonic CD are different disease entities and are characterized by distinct but overlapping genetic signatures (5). Moreover, in UC compromised mucus production, due to depletion of goblet cells, is a key triggering event in disease pathology, whereas CD is characterized by a defective intestinal barrier, which associates with complex defensin deficiencies based on a variety of mechanisms (6–10). The best described genetic links to small intestinal CD (11), i.e., NOD2, ATG16L1, XBPD1, are functionally involved in Paneth cell function (12–15). Paneth cells of the small intestine secrete different antimicrobial peptides into the intestinal lumen. Other mechanisms involving compromised α-defensin regulation of Paneth cells include the Wnt signaling pathway (16). A reduced monocyte derived Wnt ligand secretion in CD may further diminish Paneth cells and defensin expression (10). In the colon, we and others have shown an attenuated induction of the inducible human β-defensin 2 (hBD2) in CD patients, although the mechanisms remains elusive (7).

Defensins represent an ancient highly conserved part of the innate immune system. Most of these small endogenous peptides possess broad-spectrum antimicrobial activity as well as immunomodulatory functions. In humans, granulocytes as well as Paneth cells secrete different α-defensins whereas β-defensins are expressed by epithelial surfaces throughout the body (17). hBD2 was discovered using a functional antimicrobial readout by Harder and Schröder in the skin (18). As shown *in vitro* hBD2 has strong antimicrobial and immunomodulatory functions and is induced by inflammatory stimuli or exogenous microbial substances (19). hBD2 promotes intestinal wound healing (19) and angiogenesis (20) *in vitro* and can act as a chemoattractant for dendritic cells (DCs), monocytes and T-cells through interaction with the chemokine receptor 2 (CCR2) and 6 (CCR6) (21, 22). Thus, in addition to a lack of mucosal antibacterial activity (23) low defensin expression may also translate into a repressed anti-inflammatory activity. Together, these data provide evidence for an important role of defensins, including hBD2, in IBD disease pathogenesis and potential therapy, but its mode of action *in vivo* and their potential role as therapeutics remains to be described.

Standard therapy in IBD is based on immunosuppression with glucocorticosteroids and azathioprine as short and long term therapy, respectively. Antibodies that target tumor necrosis factor alpha (TNFα) attenuate disease-related inflammatory pathways rather than act as a general immunosuppressants, but 20–40% of patients are primary TNFα non-responders and up to 50% lose their effective response over time, termed secondary non-responders (24, 25). Despite successful development of other biologicals against specific targets like integrins or IL-12/23, the medical need for alternative therapeutic strategies targeting the molecular mechanisms underlying IBD is still high, providing a sound rationale for examining hBD2 as a potential biological therapy for the treatment of IBD and potentially other barrier function related inflammatory disorders. However, a major limitation for considering development of hBD2 was the difficulty to produce sufficient quantities of defensin peptides at industrial scale. We have therefore developed a cost efficient large-scale production method of recombinant hBD2 (26). In this study, we hypothesized that hBD2 could act as an anti-inflammatory peptide independently of its classical antimicrobial function. We found that recombinant hBD2 suppressed DC-mediated secretion of proinflammatory cytokines such as TNF-α, IL-12 and IL-1β. The mechanism was dependent on CCR2 signaling leading to a reduced NF-κB but increased CREB phosphorylation. Extending the *in vitro* findings, we next assessed the capability of hBD2 to suppress IBD in three different animal models of experimental colitis. We administered the therapeutic agent by subcutaneous injections to uncouple its classical antimicrobial actions from its immunomodulatory capabilities. hBD2 administration significantly improved the responding phenotype in both DSS-, TNBS-, and T-cell induced colitis, hence corroborating broad treatment efficacy in discrepant gastrointestinal disease pathologies. These data represent the first *in vivo* evidence that a human defensin, such as hBD2, offers a systemic, anti-inflammatory biologic agent, which could be used as a promising future therapeutic against human IBD.

MATERIALS AND METHODS

Human Blood Samples

In this study blood was obtained from healthy individuals (males and females in 1:1 ratio) that gave their written and informed consent after they were informed about the study purpose, sample procedure, and potential adjunctive risks. The study protocol was previously approved by the Ethical Committee of the University Hospital, Tübingen, Germany and the Ethical Committee of Region Capital, Denmark (Den Videnskabsetiske komite Region Hovedstaden).

Production and Purification of Recombinant hBD2

Recombinant hBD2 was expressed in *E. coli* as a his-tagged thioredoxin fusion protein with an enterokinase cleavage site and purified essentially as described in the patent (WO2010/007166 Treatment of inflammatory bowel diseases with human β-defensin 2). An additional reversed phase purification step was included to ensure removal of endotoxins. The processed and purified hBD2 was diluted in water for injection supplemented with 1% v/v formic acid and bound to a Daisogel SP-120-C18 column and eluted with 1% v/v formic acid in 30% v/v ethanol. The solvents were removed by evaporation in a speed-vac and the final product formulated in PBS before use. The proper folding and disulphide-bridge topology was verified using tryptic digestion coupled with LC-MS/MS and NMR spectroscopy. The purified hBD2 (endotoxin levels $<$ 0.05 EU/ml) were kept in its natural tertiary structure with purity $\geq$96%.

In vitro Toxicity Tests

Red Blood Cell Assay

Blood was collected using EDTA as anticoagulant and diluted in PBS to obtain an 8% red blood cell suspension. One part of the red blood cell suspension was added to three parts of test material (dissolved in PBS) in a poly propylene plate. One percent sodium dodecyl sulfate (SDS) was used as a positive control. Assay mixtures were incubated for 60 min at 37°C under constant agitation of the plate. Incubation was terminated by centrifugation at 2,000 rpm for 2–3 min. 50 µl of the supernatants were transferred to a microtiter plate and measured at 540 nm.

Murine Fibroblasts L929 Neutral Red Uptake

Cytotoxicity was measured by the neutral red uptake procedure of Borenfreund and Puerner, using mouse L929 fibroblasts (ATCC® CCL-1™) and a 24 h exposure to hBD2 (27). L929 fibroblasts were grown in EMEM supplemented with 10% fetal bovine serum (FBS). For testing 7.5×10^4 cells were seeded into 96 well plates and incubated for 24 h at 37°C to establish a near confluent monolayer. Cells were challenged with the indicated concentrations of hBD2, SDS was used as control.

In vivo Toxicity Test

In vivo toxicity of hBD2 was assessed in 6–8 weeks old female NMRI mice (Taconic Europe). All animal studies were performed according to Danish legislations for laboratory animals and approved by Novozymes science ethics committee. hBD2 was given subcutaneously (s.c.) in the intrascapular region using a 25G needle and a 1 ml syringe. Animals were dosed on day 0 with the indicated amount of hBD2, applied as 10 mg/kg according to the individual body weight. Clinical signs were recorded on day 0 and monitored until experimental endpoints. Body weights were recorded as a minimum on day 0, day 2, and day 4 prior to euthanasia. Necropsy was performed after euthanasia and kidneys, spleens and livers were weighed.

Pharmacokinetic Profile of hBD2

Two groups of female NMRI mice were weighed and injected s.c. with either 1 mg/kg ($n = 4$) or 10 mg/kg ($n = 3$) hBD2 in 300 µl. HBD2 was diluted in 10 mM sodium acetate in 0.9% NaCl. Blood samples were collected at different time points and stored at room temperature for a minimum of 20 min before centrifugation at 2,000 × g for 10 min. Serum was separated and stored at −20°C until analysis. Serum from the group that received 10 mg/kg was analyzed by LC-MS/MS, the serum from the other group was analyzed by HPLC.

Stimulation of Peripheral Blood Mononuclear Cells (PBMCs)

Heparinized blood was diluted 1/1 v/v with RPMI (Gibco) and was subjected to Ficoll-Paque Plus (GE healthcare) density centrifugation within 2 h of drawing. Plasma was collected from the top from individual donors and was kept on ice until it was used at 2% in the culture medium (autologous culture medium). Isolated PBMC's were re-suspended in autologous culture medium and seeded in 96-well culture plates with 115.500 cells per well (**Figure 1**) or 200.000 cells per well (**Figure 2**) in a total of 200 µl. PBMC's from the same donor were stimulated with 100, 10 or 1 µg/ml of hBD2 either alone or together with 20 pg/ml lipopolysaccharide (LPS) (*E. coli*, O111:B4, Sigma L4391) or with 0.3 µg/ml Pam3CSK4 (InvivoGen). The supernatants were collected after incubation at 37°C for 24 h, and stored at −80°C until cytokine measurement.

The experiment in **Figure 1** was performed with healthy volunteers, which were recruited under an approval from the Ethical Committee for Region Capital, Denmark. Interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α) (**Figure 1**) were quantified in supernatants by flow cytometry using a human inflammation cytometric bead array (CBA) according to manufacturer's instructions (BD) using a FACSarray flow

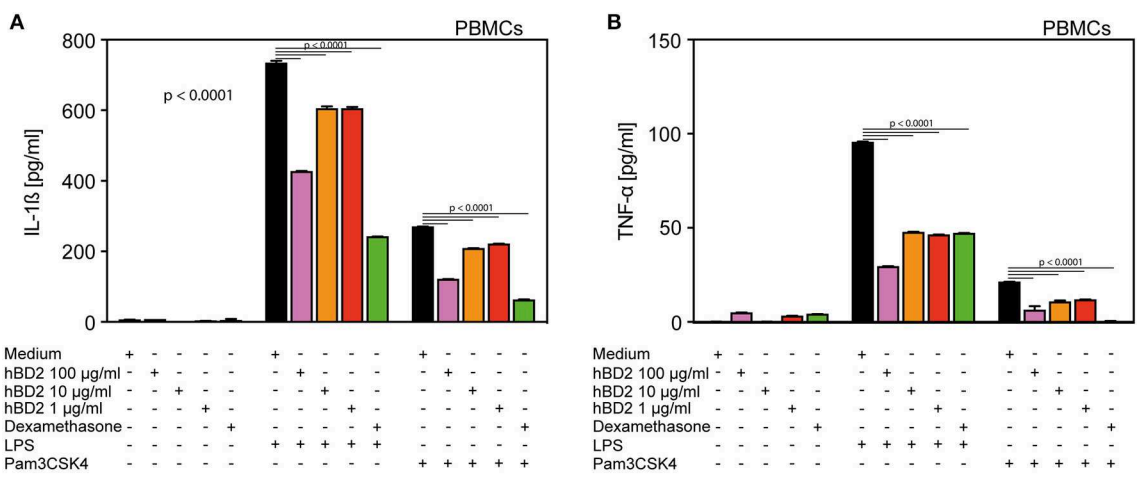

FIGURE 1 | hBD2 reduced the pro-inflammatory effect of LPS and Pam3CSK4 in human primary PBMC's. PBMC's were challenged with LPS (20 pg/ml) or Pam3CSK4 (0.3 µg/ml), respectively, and treated with various concentrations of hBD2 (100, 40, 10, or 1 µg/ml). Release of **(A)** IL-1β and **(B)** TNF-α. Results are presented as mean ± SEM, **(A)** and **(B)** $n = 150–194$. Statistical analysis was performed by one-way ANOVA with Bonferroni post-test.

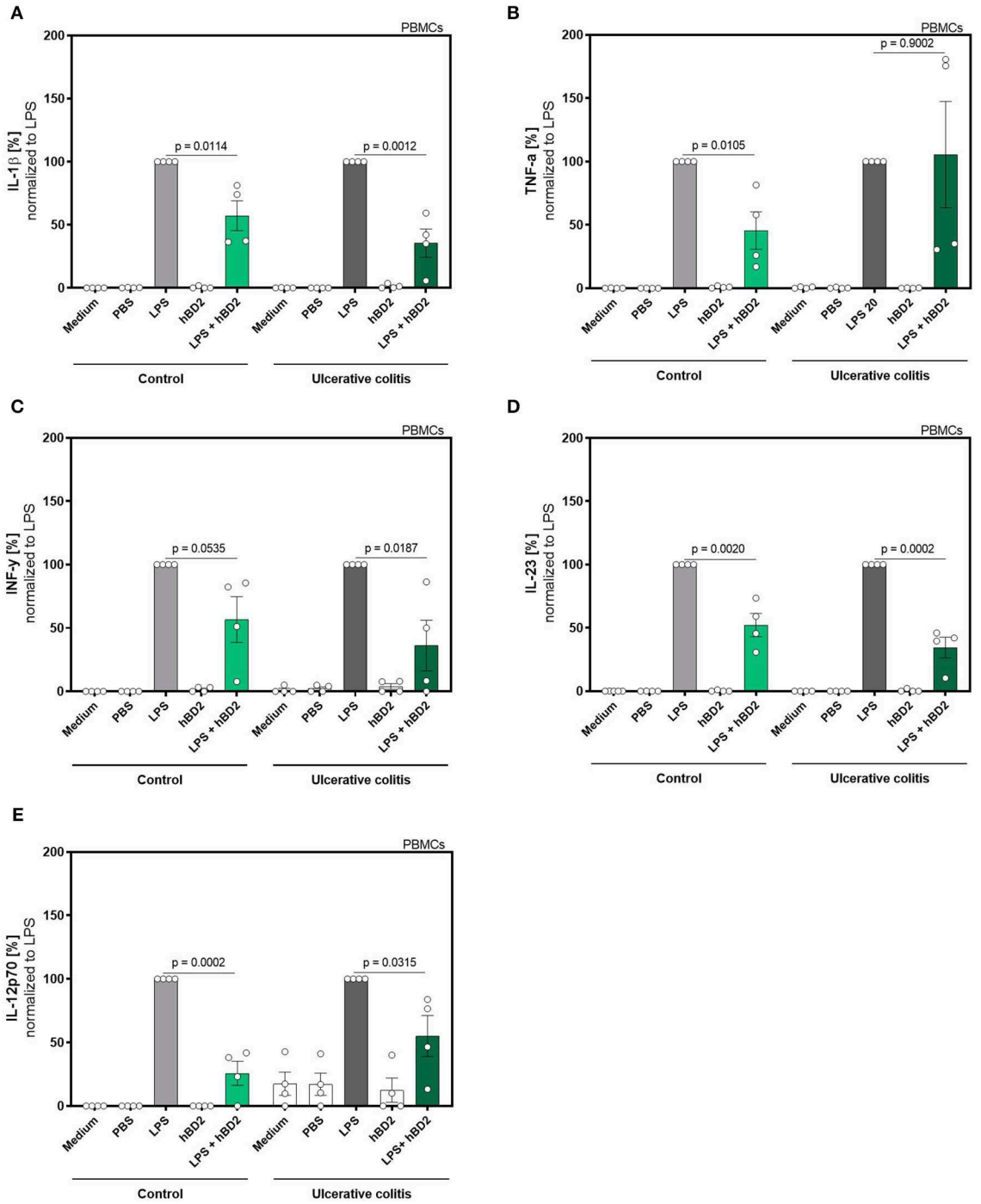

FIGURE 2 | hBD2 reduced the pro-inflammatory effect of LPS in human primary PBMC's of ulcerative colitis patients. PBMC's were challenged with LPS (20 pg/ml) and treated with 10 μg/mL hBD2. Release of of **(A)** IL-1β, **(B)** TNF-α, **(C)** INF-y, **(D)** IL-23, and **(E)** IL-12p70 is shown in % normalized to LPS. Data are presented as mean ± SEM ($n = 4$) and analyzed by unpaired *t*-test.

cytometer. IL-1β, TNF- α, Interferon gamma (INF-y), Interleukin 12p70 (IL-12), and Interleukin 23 (IL-23) were analyzed using a human inflammation cytometric bead array (LegendPlex Biolegends) according to manufacturer's instructions.

The experiment in **Figure 2** was performed with 8 subjects (4 controls and 4 colitis patients) to study the impact of disease status on treatment efficacy. Clinical status of these subjects were subtotal remission

(Simponi and Salofalk), remission (Vedolizumab), mild disease (Infliximab, Salofalk, and Prednisolon), and moderate disease (Vedolizumab). PBMCs from these subjects were analyzed for IL-1b, TNF-a, IFN-g, IL-12, and IL-23 expression using a human inflammation cytometric bead array (LegendPlex Biolegends) according to manufacturer's instructions.

Generation of Human Monocyte-Derived Dendritic Cells (Mo-DCs)

Peripheral blood was drawn from healthy donors and diluted one to one in PBS. The mixture was stacked in a falcon tube on Biocoll separation solution (Biochrome) with a ratio of three parts blood PBS mixture to two parts Biocoll. For a density gradient centrifugation the falcon tubes were centrifuged at 2,000 rpm for 30 min at RT. The PBMC's were removed and washed twice with PBS. 1.5×10^6 cells were seeded per well in a tissue culture-treated 6-well plate in RPMI-1640 medium (Merck) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich), 2 mM L-glutamine (Biochrome), 100 U/ml penicillin/streptomycin (Gibco), 50 μM 2-mercaptoethanol (Fluka), 1 mM sodium pyruvate (Biochrome), and 1x non-essential amino acids (Biochrome) and incubated for 1 h at 37°C and 5% CO_2 for monocyte adherence. Then the cells were washed with media and PBS to remove the non-adherent cells and were cultivated for 6 days with media additional supplemented with 50 ng IL-4 and 100 ng GM-CSF (Miltenyi). Cytokines were added a second and third time on day 2 and 4 whereas the cells were harvested at day 6.

Generation of Bone Marrow-Derived Dendritic Cells (BM-DCs)

BM-DC's were generated using granulocyte-macrophage colony-stimulating factor (GM-CSF) and RPMI-1640 medium (Merck) supplemented with 10% fetal calf serum (FBS; Life Technologies), 2 mM glutamine (Thermo Fisher), 100 U/ml penicillin/streptomycin (Gibco), 50 μM 2-mercaptoethanol (Roth), 1 mM sodium pyruvate (Biochrome), and 1x non-essential amino acids (Biochrome) as previously described (28). Shortly, 2×10^6 bone marrow cells flushed from the tibias and femurs of C57BL/6 mice were seeded in dishes containing 200 U/ml GM-CSF. After 3 days extra medium containing GM-CSF was added to the cells and on day 6 half of the medium was replaced by fresh serum containing GM-CSF. After 7 or 8 days slightly attached cells were harvested. Female C57BL/6JolaHsd mice were purchased from Janvier (St. Berthevin Cedex, France). Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocol was approved by the Regierungspräsidium Tübingen (Anzeige 09.01.2014).

Cytokine Production by Human Mo-DC's and Murine BM-DC's

2×10^5 murine BM-DC's or human Mo-DC's were seeded in 96-well round bottom plates. First they were pretreated with 100 ng/ml pertussis toxin (Sigma-Aldrich) or 5 μM of the CCR2 inhibitor RS 504393 (Tocris) and subsequently stimulated with 100 ng/ml LPS or 0.2 mg/ml TNF-α, 0.2 mg/ml IL-6 and 0.2 mg/ml IL-1β and 10 μg/ml or 100 μg/ml hBD2. 24 h later supernatants were collected and TNF-α [Biolegend (BM-DC's); R&D (Mo-DC's)] was analyzed according to the manufacturer's instructions. IL-10, IL-12, and IL-1β (LEGENDplex Biolegend) was as well-analyzed according to the manufacturer's instructions.

Flow Cytometry

2×10^5 murine BM-DC's were seeded in 96-well round bottom plates and treated as described above. Cells were removed from the plate using Accutase (Sigma-Aldrich) and stained for 20 min at room temperature with Zombie Aqua (Biolegend) to exclude dead cells and extracellular antibodies against CD11c-APC (N418) (Miltenyi), MHCII-FITC (M5/114.15.2) (Miltenyi) and CD86-BV421 (GL-1) (Biolegend). For p-CREB staining cells were fixed and permeabilized with Foxp3 Staining Buffer Set (eBioscience) and stained with primary antibody phosphor-CREB mAb (Ser133; clone 87G3) (Cell Signaling) for 30 min in the dark at room temperature followed by secondary goat anti-rabbit IgG-DyLight™649 (Jackson ImmunoResearch) for 15 min at 4°C. To detect intracellular p-NF-κB BM-DCs were fixed with 2% paraformaldehyde (VWR) in PBS, permeabilized with 90% freezing methanol (Applichem) and stained with the primary antibodies to phosphor-NF-κB p65 (93H1) (Cell Signaling) for 60 min in the dark at room temperature followed by goat anti-rabbit IgG-PE-Cy7 (Santa Cruz Biotechnology) for 15 min at 4°C. PBS with 0,5% bovine serum albumin (Biomol) was used for all incubations and washing steps. At least 50,000 cells were acquired using a Canto-II flow cytometer (BD nces) with DIVA software (BD Biosciences) and were further analyzed using FlowJo 10.5 software (Tree Star).

DSS Colitis Model

The DSS colitis study was performed by Farma-Cros Ibérica according to directive 86/609 EEC and approved by Novozymes science ethics committee. 7–8 weeks old male C57BL/6 mice were used (Charles River) and each group consisted of 10 animals. Animal allocation to all experimental groups was done in a randomized manner. Colitis was induced by supplementing the drinking water with 2% dextran sodium sulfate (DSS, 30–50 kDA, MP Biomedicals) for 7 days. On day 1 all mice were weighed and the drinking bottle was filled with the DSS solution, this solution was replaced on day 3 and 5. On day 8 the remaining solution was discarded and replaced with autoclaved water. Mice were divided into 3 groups. One group received PBS as sham treatment intravenously. One group received on day 1, 4, and 8, 300 μg/mouse of a mouse anti-TNF-α antibody (αTNF, Ramcon) intraperitoneally. The animals in the other group were dosed s.c. with 0.1 mg/kg hBD2 once a day, starting at day 1 until day 10. Animals were euthenized on day 10. Daily clinical assessment was

carried out to calculate a validated clinical disease activity index (DAI) ranging from 0 to 4 according to the following parameters: body weight loss, presence or absence of rectal bleeding, stool consistency. One mouse in the DSS control group had to be euthanized before the end of the study.

TNBS Colitis Model

The TNBS colitis study was performed by Farma-Cros Ibérica according to directive 86/609 EEC and approved by Novozymes science ethics committee. Male BALB/cByJ mice were used (Janvier) and each group consisted of 15 animals. Animal allocation to all experimental groups was done in a randomized manner. Colitis was induced on day 0 by intracolonic (distal) administration of trinitrobenzene sulfonic acid (TNBS), 1 mg/mouse in 50% ethanol under mild anesthesia (ketamine/xylazine). Treatment of the animals started on day 0 after induction of colitis. All compounds were applied s.c. Mice received PBS (TNBS control), Prednisolone (10 mg/kg), or hBD2 0.1 mg/kg), respectively. In the TNBS control group as well as in the Prednisolone group, 2 animals had to be sacrificed before the end of the experiment. All other animals were sacrificed on day 10. Daily clinical assessment was carried out to calculate a validated clinical DAI ranging from 0 to 4 according to the following parameters: body weight loss, presence or absence of rectal bleeding, stool consistency.

T Cell Colitis Model

This study was performed by the Department of Biomedical Science at the University of Catania according to directive 86/609 EEC and approved by Novozymes science ethics committee. 8 weeks old female BALB/c and C.B-17 female SCID mice were purchased from Harlan (Italy). Colitis was induced in severe combined immunodeficiency (SCID) mice by transplantation of $CD4^+/CD25^-$ T cells from the BALB/c mice. Briefly, lymphomonocytes isolated from spleen or lymph nodes from BALB/c mice were subjected to negative selection of $CD4^+$ T cells. Afterwards, $CD4^+/CD25^+$ cells were positively isolated by binding to the beads from the $CD4^+$ T cell suspensions and the $CD4^+/CD25^-$ were collected from supernatant. Cell preparation was considered successful if the analysis of purified cells by flow cytometry (FACSCalibur, BD Bioscience, Heidelberg, Germany) using CellQuest software showed that >95% of the cells were viable (based on forward and side-scatter characteristics and/or 7- actynomycin-D staining) and CD4-positive (using a FITC-conjugated anti-mouse CD4-antibody, BD, Heidelberg, Germany), as well as more than 98% depleted of CD25 (using a APC-conjugated anti-mouse CD25-antibody, BD, Heidelberg, Germany). $CD4^+/CD25^-$ cells were intraperitoneally injected to SCID mice at a concentration of 500,000 cells in a final volume of 0.2 ml RPMI 1640. Sham treated animals ($n = 6$) received 0.2 ml pure RPMI 1640. Diseased mice were randomized divided into 4 groups ($n = 11$) and treated once daily by s.c. application of PBS (vehicle), hBD2 (0.1 and 1 mg/kg, respectively) or 0.3 mg/kg Dexamethasone (Dexa., applied intraperitoneally). Treatments started 7 days post T cell transfer and continued daily for 86 days. Animals were weighed three times a week and monitored twice a week starting from day 42 for the clinical status, summarized as DAI. The DAI included body weight loss, stool consistency, and the presence of blood at the rectum. One animal in the vehicle and one in the Dexamethasone treated groups had to be sacrificed before the end of the study. At the end of the study, animals were sacrificed and the colon was removed and carefully cleaned for further analysis. First, the colon was weighed then a section from the middle was removed and fixed for pathologic survey. The rest of the colon (5 cm) was used to quantify the activity of myeloperoxidase in the tissue.

Myeloperoxidase Activity Assay

Myeloperoxidase activity was performed on 5 cm of colon previously frozen at −80°C. Colon was homogenized in 0.5% of HETAB dissolved in 10 mM of Phosphate-Citrate Buffer (pH 7.0) to enable the release of MPO enzyme from the neutrophil granules. Homogenated samples after three freeze-thaw cycles were spun at 3,000 × g at 4°C for 30 min. Pellets were resuspended in 0.5% HETAB in 10 mM Phosphate-Citrate Buffer and spun again at 3,000 × g at 4°C for 30 min. 500 ul of supernatant were delivered into a vial along with 500 ul of TMB in Phosphate-Citrate Buffer containing Perborate Sodium. Changes in absorbance at 620 and 450 nm were read by a spectrophotometer (IRIS). Peroxidase enzyme diluted in 0.5% HETAB in 10 mM Phosphate-Citrate Buffer and H2O was used as standard. Two-fold dilutions of standard were prepared at the highest concentration of 50 pg/ml. Reagents and samples were kept refrigerated. Substrate solution was prepared just before the assay. MPO activity expresses the amount of enzyme that is able to degrade 1 μM of peroxide/min and it is expressed as U/g tissue weight.

Preparation of Histological Samples

Sections of the colon were taken from each animal and preserved in neutral buffered formaline for subsequent histological analysis. Sections were stained using haematoxylin and eosin for further scoring.

Statistical Analysis

Release of cytokines from PBMC's was compared using two-way ANOVA and Tukey post-test or unpaired student's *t*-test as appropriate. Weight change, colon weight and MPO activity were compared using one-way-ANOVA and Bonferroni post-test, while clinical and pathological scores were analyzed by a Kruskal-Wallis-test for non-parametric data with a Dunn's post-test. All statistical analyses and graphs were done using GraphPad Prism 8 (GraphPad Software, USA).

RESULTS

hBD2 Modulated the Effects of Toll-Like Receptor Ligands in PBMC's

We selected human PBMCs challenged with LPS as an *ex vivo* read out model to assess the anti-inflammatory potential of hBD2. While LPS-challenged PBMCs secreted ample amount of interleukin 1-β (IL-1β) (**Figure 1A**) and TNF-α (**Figure 1B**), co-treatment with increasing doses of hBD2 consistently mitigated release of both cytokines. hBD2 is a

positively charged peptide and could potentially bind to the negatively charged LPS, hence mediate the observed effect indirectly without interfering with the pathway. To investigate this possibility, we included an alternative toll like receptor (TLR) ligand, Pam3CSK4; a neutral/negative charged synthetic peptide. As shown in **Figure 1**, hBD2 significantly reduced the Pam3CSK4 induced release of IL-1β and TNF-α, corroborating a strong anti-inflammatory effect of hBD2 on human PBMCs *ex vivo*.

We next evaluated if the immune modulating capabilities of hBD2 could be extended to colitis patients. This patient group is known to exhibit an altered response to immunomodulatory stimuli compared to healthy controls. We therefore expanded the proinflammatory panel to also include interferon (INF)-**γ**, IL-12p70, and IL-23. Despite the low n-size in this proof-of-concept sub-experiment, hBD2 treatment successfully attenuated the proinflammatory immune response in LPS-stimulated PBMCs (**Figure 2**).

Cytokine Production of DC's Was Affected by hBD2 in a TLR- and CCR2-Dependent Manner

Since PBMCs comprise a variety of cell types we aimed to characterize the responsive cell type, and furthermore the receptor that was targeted by hBD2. DCs upregulate cytokine production when their TLRs engage their cognate ligands (29). Thus, we hypothesized that DC mediated cytokine secretion was affected by hBD2. We generated human mo-DCs and murine BM-DCs *in vitro,* and subsequently challenged them with LPS with or without two different concentrations of hBD2. Similar to the previously reported PBMC response (**Figures 1**, **2**), hBD2 treatment dose-dependently curbed TNF-α production in LPS-challenged DCs (**Figures 3A,B**).

To test whether the hBD2-mediated cytokine modulation was restricted to TLR signaling, we next investigated the cytokine production of BM-DCs stimulated with a TLR-independent cytokine cocktail. While hBD2 treatment alleviated LPS-induced TNF-α, IL-12p70, and IL-1β secretion concomitant with a substantial induction of the anti-inflammatory cytokine, IL-10, same treatment failed to modulate TLR-independent activation of BM-DCs (**Figures 3C,D** and **Figures S1B,C**).

Since hBD2 is able to bind to G protein-coupled receptors expressed on monocytes and DCs (22), we pretreated BM-DCs with pertussis toxin prior to the stimulation with LPS and hBD2. Inhibition of G protein-coupled receptor signaling prevented the observed immunomodulatory capacity of hBD2. Thus, BM-DCs pre-treated with pertussis toxin and stimulated with LPS and hBD2 showed similar TNF-α, IL-1β, IL-10, and IL-12p70 secretion compared to BM-DCs treated solely with LPS (**Figures 3E,F** and **Figures S1D,E**).

To specify the G protein-coupled receptor interaction of hBD2 we pre-treated BM-DCs with the CCR2 specific inhibitor, RS504393, and subsequently stimulated with LPS and hBD2. Pre-treatment with RS504393 prevented the anti-inflammatory effect of hBD2 (**Figures 3G,H** and **Figures S1F,G**). Together these data demonstrate a central involvement of CCR2 in hBD2-mediated DC cytokine modulation; a trait that was shared between human (**Figure S1A**) and mouse DCs.

hBD2 Modulates NF- κB and CREB Phosphorylation

NF-κB represents a key signaling pathway triggered by TLRs. This pathway can also be activated by the proinflammatory cytokines TNF-α and IL-1β (30). NF-κB activity is mediated by direct interaction with the CREB coactivator CBP. However, phosphorylated CREB, that can be induced by G protein-coupled receptors, needs in addition CBP to compete with NF-κB and thereby limiting the NF-κB activity (31). We analyzed the NF-κB and CREB phosphorylation of hBD2 treated BM-DCs. Stimulation with proinflammatory cytokines showed an increase in NF-κB phosphorylation although to a lower extent than what was observed during LPS stimulation. Co-incubation of both stimuli with hBD2 showed only a reduced NF-κB phosphorylation when BM-DCs were treated with the TLR ligand LPS in combination with hBD2 (**Figures 4A,I**). However, pre-treatment with the CCR2 inhibitor RS504393 prior to stimulation showed comparable levels of NF-κB phosphorylation irrespective of hBD2 treatment (**Figures 4B,I**). CCR2 inhibition therefore prevented the hBD2-mediated reductions in NF-κB activation. Although the mechanism behind this observation remains elusive, LPS-challenged BM-DCs showed reduced maturation status when stimulated with hBD2, corroborated by lower levels of their key activation markers, MHCII and CD86 (**Figures 4C,E**). Again, pre-treatment with the CCR2 inhibitor prevented this lower expression (**Figures 4D,F**). We hypothesized that the CREB signaling pathway could counter NF-κB phosphorylation. We therefore analyzed the CREB phosphorylation status in a next step of experiments. We demonstrated that triggering G protein-coupled receptors led to an increase in CREB phosphorylation (**Figures 4G,I**) and simultaneously to a reduced NF-κB phosphorylation (**Figure 4A**), suggesting that phosphorylated CREB competes with NF-κB for their mutual cofactor, CBP. In contrast, pre-treatment with the CCR2 inhibitor prevented the increase in CREB phosphorylation (**Figures 4H,I**).

Recombinant hBD2 Showed Good Tolerability and Rapidly Entered the Bloodstream After Subcutaneous Administration, Hence Allowing Systemic Immunomodulatory Actions

To identify possible toxicity of recombinant expressed hBD2 we first addressed the hemolytic potential of hBD2 on human red blood cells. As shown in **Figure S2A** we could not detect any hemolytic effect of $\leq$300 μg/ml hBD2. We next tested the effect on the viability of murine fibroblasts. No negative effect of hBD2 up to a concentration of 1750 μg/ml could be identified (**Figure S2B**). Combined, these data corroborates high *in vitro* tolerability. To assess hBD2 toxicity *in vivo*, NMRI mice were challenged s.c. with different doses of hBD2 and monitored for 4 days for the development of clinical symptoms. On day 4, mice were euthanized for necropsy. **Table 1** records the clinical

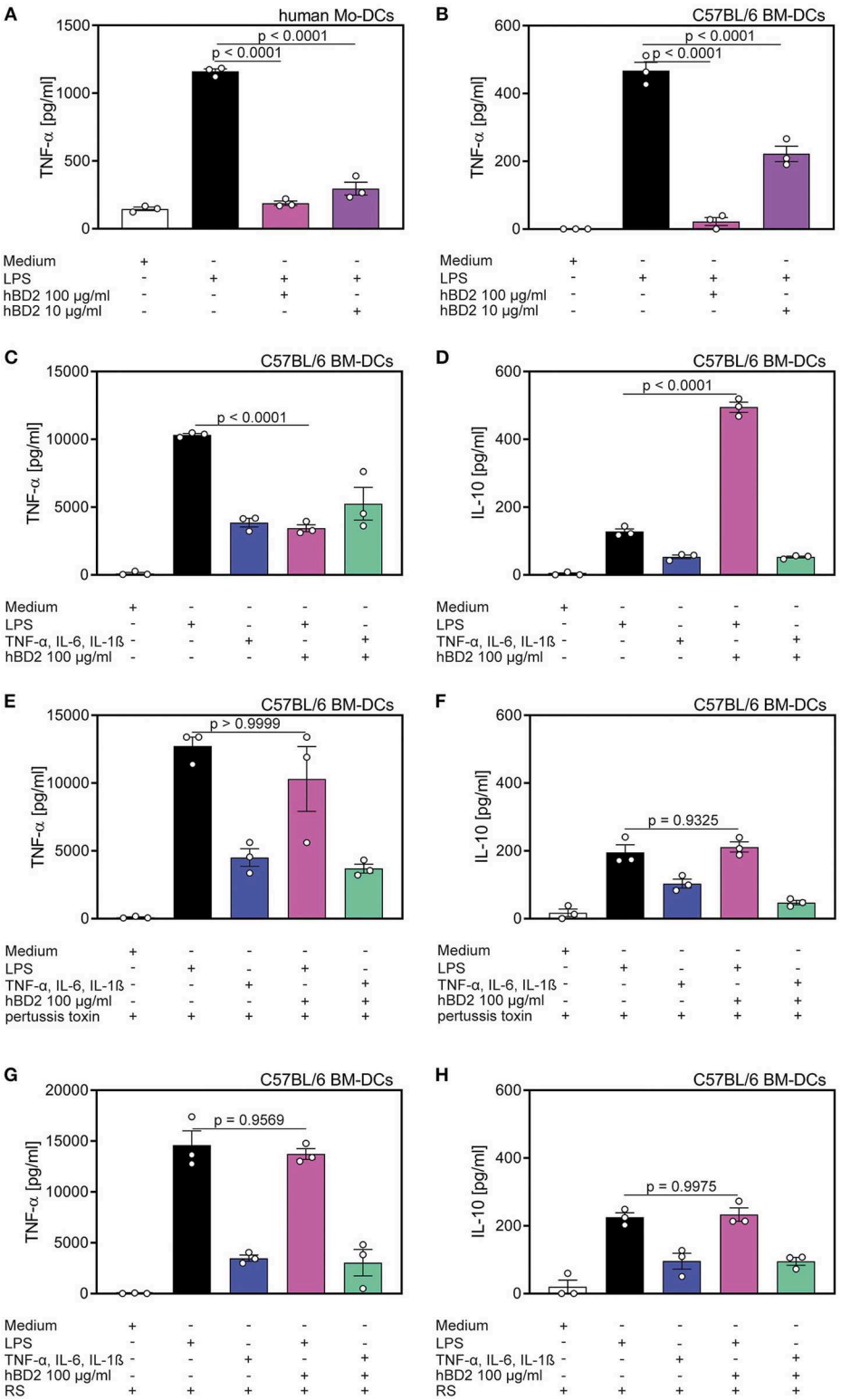

FIGURE 3 | Cytokine production of DC's was affected by hBD2 in a TLR- and CCR2-dependent manner. Human mo-DC's and murine BM-DC's were treated with LPS (10 µg/ml) alone or co-incubated with various concentrations of hBD2 (100 µg/ml or 10 µg/ml). Murine BM-DCs were additionally treated with pertussis toxin or the CCR2 inhibitor RS prior to stimulation with LPS or a cytokine cocktail containing TNF-α (0.2 mg/ml), IL-6 (0.2 mg/ml), and IL-1β (0.2 mg/ml). Release of TNF-α in **(A)** human Mo-DC's and in **(B)** murine BM-DC's was quantified by ELISA. Release of TNF-α **(C,E,G)** and IL-10 **(D,F,H)** in murine BM-DCs was quantified by LEGENDplex. Results are presented as mean ± SEM, $n = 3$. Statistical test used is one-way ANOVA with Bonferroni post-test.

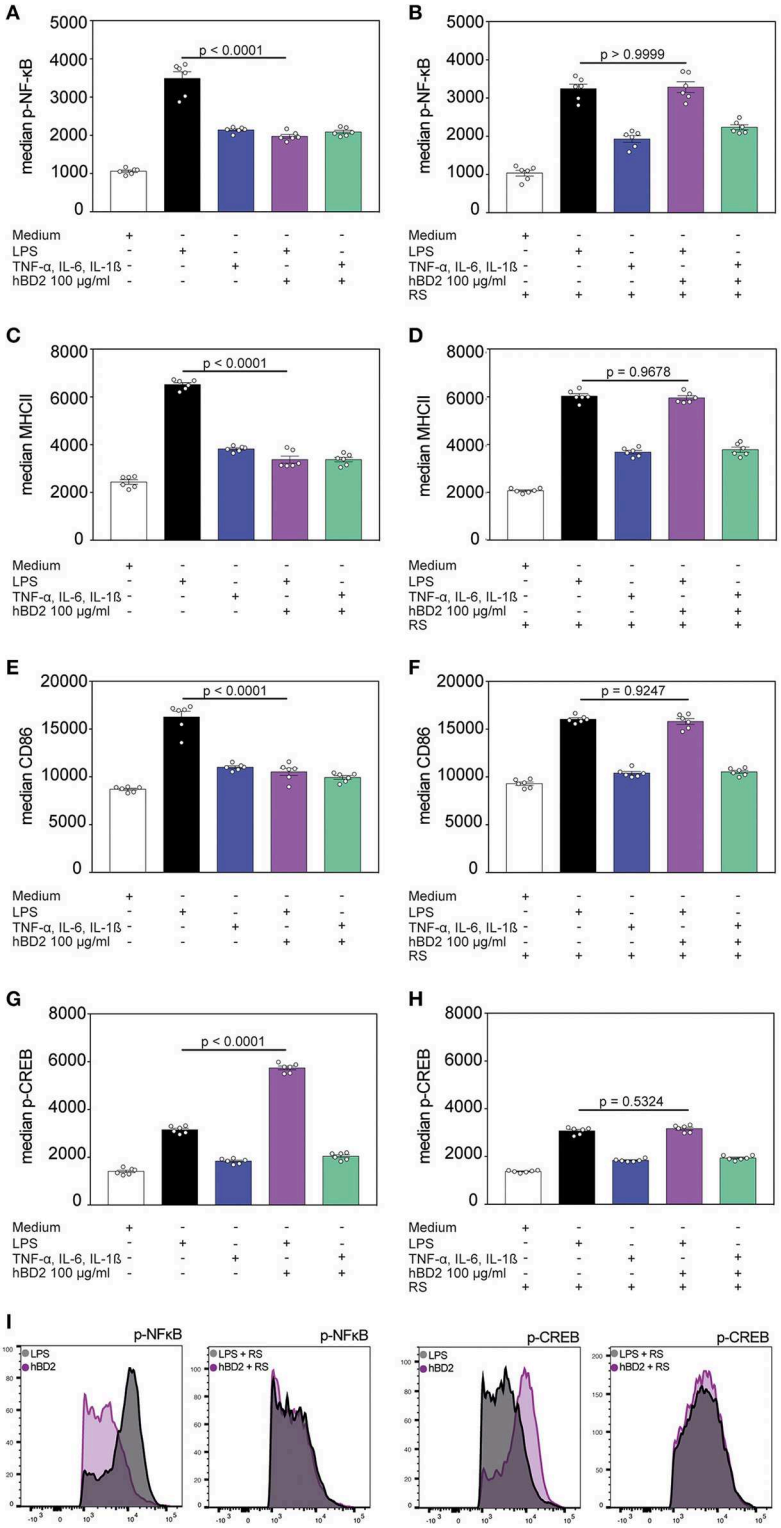

FIGURE 4 | hBD2 modulates NF-κB and CREB phosphorylation. BM-DC's were incubated for 60 min with LPS (10 µg/ml) or a cytokine cocktail containing TNF-α (0.2 mg/ml), IL-6 (0.2 mg/ml), and IL-1β (0.2 mg/ml) alone or in combination with hBD2 (100 µg/ml). BM-DCs were additionally pretreated with pertussis toxin or the CCR2 inhibitor RS prior to stimulation. The cells were stained with CD11c, MHCII, and CD86 antibodies followed by intracellular staining against p-NF-κB or p-CREB and analysis by flow cytometry. Statistical analysis of **(A,B)** p-NF-κB, **(C,D)** MHCII, **(E,F)** CD86, and **(G,H)** p-CREB staining. **(I)** Shows histograms of FACS analysis. Results are presented as mean ± SEM, $n = 6$. Statistical test used is one-way ANOVA with Bonferroni post-test.

TABLE 1 | Clinical symptoms after subcutaneous administration of hBD2.

Test compound	Dose level mg/kg	Clinical signs on the day of dosing				Necropsy
		No clinical signs	Pruritus	Clinical signs	Clinical signs at the end of the day of dosing	Macroscopic findings
Vehicle	0	3/3	0/3	0/3	0/3	0/3
HBD2	0.5	3/3	0/3	0/3	0/3	0/3
	10	0/3	3/3	3/3	0/3	0/3
	40	0/3	3/3	3(severe)/3	0/3	0/3

symptoms observed. Although minor acute effects were observed (minutes), no adverse effects were observed at necropsy. Shortly after challenge (10–15 min) the mice receiving 10 or 40 mg/kg hBD2 showed mild clinical signs such as decreased locomotor activity, proneness, ptosis, piloerection, pruritus, bradypnoea, reddish discoloration around the eyes and swelling around the eyes and snout. All clinical signs were transient and only mice dosed with 40 mg/kg hBD2 were still affected at 60 min post dosing. Of note, 40 mg/kg is 40 times higher than the highest dose used in our *in vivo* experiments. The lowest dose group, 0.5 mg/kg hBD2 and the vehicle group did not develop any clinical signs after systemic challenge. No further clinical signs were observed during the subsequent 4 day observation period nor could we find any abnormalities during necropsy. Additionally, body weight gain and organ weight were recorded. Mice did not show any weight loss nor did the organ weight differ significantly between the groups (**Figures S2C,D**). In summary, these results indicate that hBD2 is well-tolerated *in vivo*.

We next analyzed whether s.c. administered hBD2 would enter the blood stream. For that purpose we injected mice s.c. with 1 or 10 mg/kg hBD2 and quantified the amount of hBD2 in serum at different time points after injection. As shown in **Figure S2E**, hBD2 entered the blood stream rapidly after s.c. injection and remained detectable for more than 2 h. These findings indicate that s.c. applied hBD2 might not only act locally but could also have systemic effects *in vivo*.

hBD2 Ameliorated the Outcome of DSS Colitis *in vivo*

The above described results prompted us to investigate the clinical potential of hBD2 using murine models of experimental colitis with different disease pathologies. First, we assessed the anti-inflammatory and protective effect of hBD2 in DSS colitis. DSS causes a chemical injury to the intestinal mucosa. This results in the exposure of the lamina propria and submucosal compartments to luminal antigens and enteric bacteria which results in inflammation and ulcer formation (32). Treatment of DSS colitis with s.c. administered hBD2 (0.1 mg/kg) resulted in a significant improvement of colitis; the therapeutic effect was superior to anti-TNF-α treatment (**Figure 5**). hBD2 prevented excessive weight loss (**Figure 5A**) and improved the DAI (**Figure 4B**). Furthermore, scoring of the colonic mucosa for damage revealed a significantly reduced mucosal damage in mice treated with hBD2 (**Figure 5C**). Histological assessment of colon (**Figure 5D**) showed strong mucosal damage caused by DSS, characterized by a massive loss of the crypt architecture. In contrast, treatment with hBD2 prevented this loss of crypts and maintained a normal mucosa, comparable to the naïve mice.

hBD2 Significantly Improved TNBS Colitis *in vivo*

We next tested the efficacy of hBD2 in TNBS induced colitis. TNBS reacts with proteins in the colon (haptenation), thus making them immunogenic. TNBS is dissolved in ethanol, which permeabilizes the colonic epithelium. The immunogenic proteins then cause a predominantly Th1 type response restricted to the colon (33). In contrast to DSS colitis, TNBS colitis did not result in weight loss, but rather prevented weight-gain in mice during the time of our experiment. Weight changes were similar between groups, although hBD2 treated mice trended toward increased weight gain from day 1–7 (**Figure 6A**). Yet, s.c. treatment with 0.1 mg/kg hBD2 significantly reduced colon weight and a similar tendency was also observed for prednisolone (**Figure 6B**), indicating a reduced infiltration of inflammatory cells. Finally, s.c. treatment with hBD2 significantly improved the macroscopic (**Figure 6C**) as well as the microscopic score of mouse colons (**Figures 6D,E**) comparable with the effect of prednisolone. Microscopic analysis of the colon showed a loss of crypts in diseased mice, while mice receiving 0.1 mg/kg hBD2 showed a nearly normal mucosa comparable to prednisolone treated mice.

Protective Effect of hBD2 in T Cell Transfer Colitis

Finally, we tested hBD2 in a model of T cell transfer colitis. In this model $CD4^+$ T cells from immunocompetent mice are adoptively transferred into severe combined immunodeficiency (SCID) mice, lacking T cells. The transferred T cells respond to enteric bacteria with the release of IL-2 and INF-γ (34). The inflammation is restricted to the colon, and extends diffusely from the cecum to the rectum. Besides affecting the lamina propria, the pathogenesis can also be transmural (35). Importantly, in addition to the different disease pathology, this model also results in chronic inflammation as oppose to the acute models (DSS and TNBS) previously examined. Colitis was induced in SCID mice by transferring $CD4^+/CD25^-$ T cells from WT mice. One group of SCID mice did not receive a T cell transfer (naive). Mice that

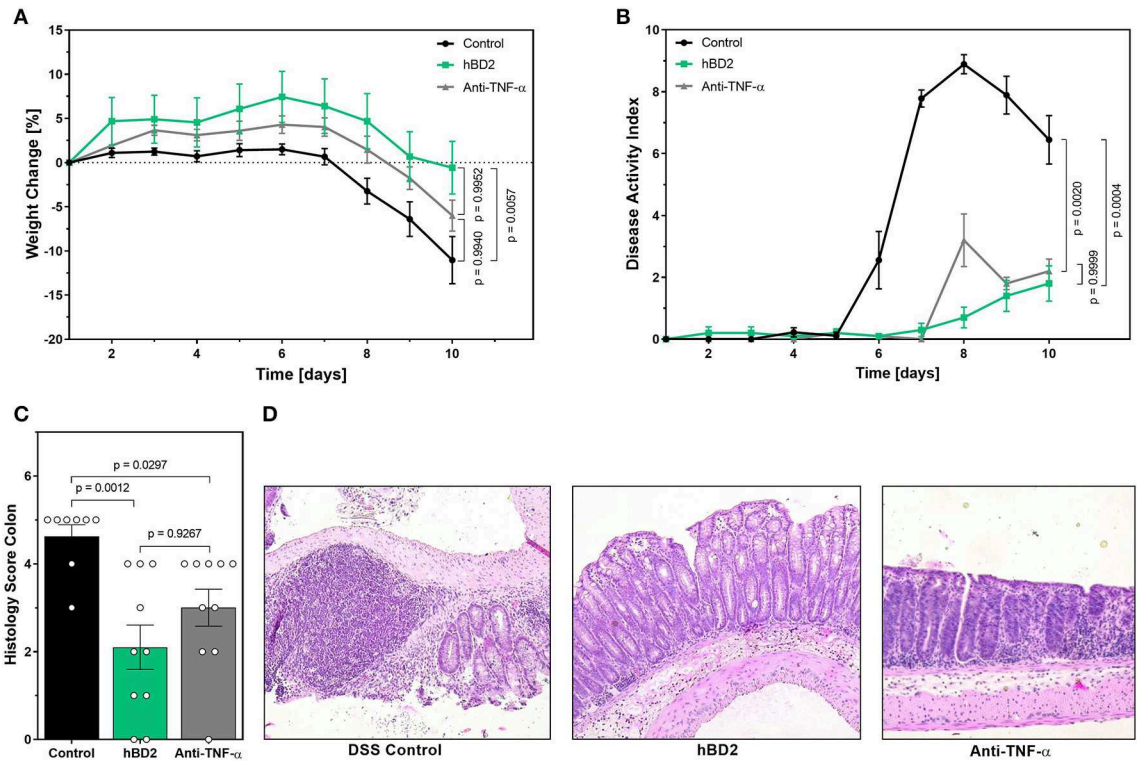

FIGURE 5 | hBD2 ameliorated the outcome of DSS colitis *in vivo*. Colitis was induced by adding 2% DSS into the drinking water. On day 8 DSS was removed from the drinking water. Mice were treated either once a day s.c. with 0.1 mg/kg hBD2 or intraperitoneally with an anti-TNFα antibody (300 μg/mouse) on day 1, 4, and 8. **(A)** Weight change of mice during the experiment, **(B)** development of DAI, **(C)** histology score from the colon of the mice at the end of the experiment, and **(D)** representative images from the colon of differentially treated mice. Results are presented as mean ± SEM, control group $n = 9$ and treated groups $n = 10$. Appropriate statistical comparison are shown within the graph by a Kruskal-Wallis-test for non-parametric data with a Dunn's post-test.

received a T cell transfer gained significantly less weight than naive mice. Based on the chronic nature of this model and the involvement of numerous cell type of the immunological arsenal, we applied two different doses of hBD2 to increase the therapeutic window. Colitis mice treated with 1 mg/kg hBD2 s.c. showed less weight loss (**Figure 7A**) and demonstrated an improved DAI (**Figure 7B**) in comparison to the T cell colitis control group. Same trait was observed for the stool score (**Figure 7C**). The increased scores in the untreated colitis group appeared earlier than in the hBD2 treated group indicating a protective effect of hBD2. Furthermore, treatment with 1 mg/kg hBD2 significantly reduced the colon weight (**Figure 7D**) supporting the conclusion of mitigated inflammation. Only a minor effect of hBD2 on colonic myeloperoxidase activity could be observed (**Figure 7E**). Histological analysis (**Figure 7F**) showed pronounced inflammation in colitis mice without treatment, while mice treated with 1 mg/kg hBD2 showed less signs of inflammation and tissue disruption. Less signs of inflammation were also observed in the colon of dexamethasone treated mice. Furthermore, dexamethasone also improved DAI, stool score and colon weight significantly and was therefore superior to hBD2 in the T cell transfer model. The high dose of hBD2 (one log higher than previous experiments) seemed essential as 0.1 mg/kg hBD2 did not improve the outcome of T cell mediated colitis (**Figures 7A–F**).

DISCUSSION

Herein we report that hBD2 can be used as a systemically administered anti-inflammatory biological. hBD2 is well-tolerated, both *in vitro*, and *in vivo*. These observations are in line with published *in vitro* studies testing hBD2 with human mesenchymal stem cells, osteoblasts, keratinocytes, and HeLa cells without observing cytotoxic effects (36). Otte et al. (19) additionally found that hBD2 was well-tolerated by intestinal epithelial cells. Of note and in contrast to hBD2, high concentrations of hBD3 demonstrated cytotoxic effects in human dendritic cells and keratinocytes (37). After subcutaneous injection we observed a dose dependent pruritus of short duration which only occurred at doses magnitudes higher than the later identified therapeutic doses. At all doses (up to 40 mg/kg), body weight and organ weights of liver, spleen and kidney were unaffected.

Peak hBD2 drug serum concentrations were also dose dependent and in the range of 2–10 μg/ml after single doses of 1 vs. 10 mg/kg. Using medium concentrations in a similar range we confirmed that hBD2 attenuates inflammatory responses

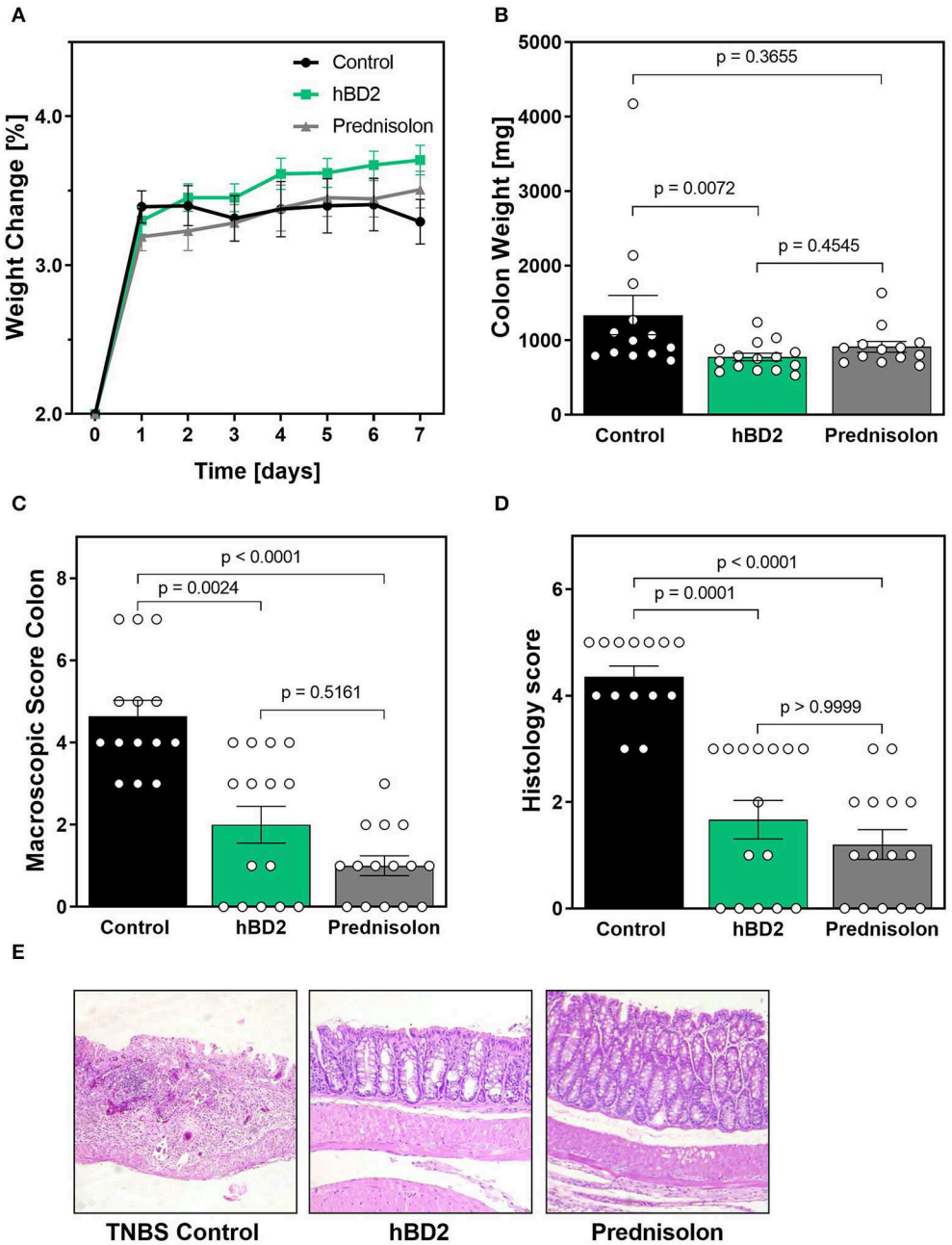

FIGURE 6 | hBD2 significantly improved TNBS colitis *in vivo*. Colitis was induced by a single dose of TNBS into the colon. Mice were then treated s.c. with different doses of hBD2 (0.1 mg/kg) or Prednisolone (10 mg/kg) once a day and monitored for 7 days. **(A)** Development of mouse weight during the experiment. After euthanization, the colon of the mice was examined for **(B)** colon weight, **(C)** macroscopic abnormalities, and **(D)** microscopic evidence of inflammation. **(E)** Representative images from the colon of differentially treated mice are shown. Results are presented as mean ± SEM, control group $n = 14$, prednisolone group $n = 14$, and hBD2 group $n = 15$. Appropriate statistical comparisons are shown within the graph by a Kruskal-Wallis-test for non-parametric data with a Dunn's post-test.

of human PBMC's *in vitro*. TNF-α, IL-1β, and IL-12p70 were consistently reduced after hBD2 treatment, whereas the anti-inflammatory cytokine, IL-10, was significantly increased. TNF-α is a well-known key inflammatory mediator of IBD and a successful target of modern biologicals in the treatment of IBD and other inflammatory diseases (38). In addition, IL-1β has recently been described to mediate intestinal inflammation in IBD patients with IL-10 receptor deficiency, and is thus proposed as a potential therapeutic target (39). In a follow-up pilot study using 4 colitis patients and 4 healthy controls, we confirmed the

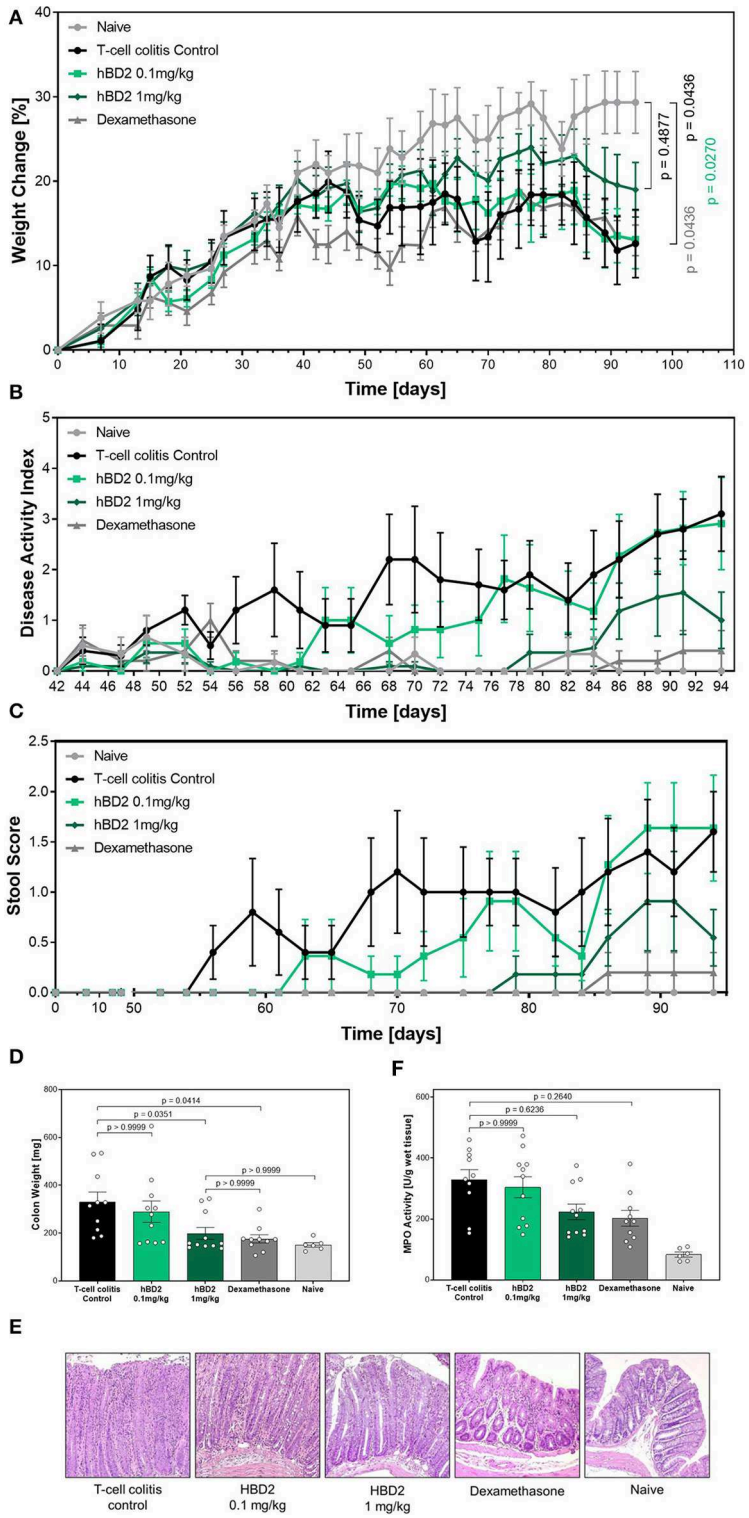

FIGURE 7 | Protective effect of hBD2 in T cell transfer colitis. Colitis was induced by transferring $CD4^+/CD25^-$ T cells from WT mice to SCID mice. Development of colitis was observed for 94 days. Daily treatment with hBD2 (0.1 mg/kg or 1 mg/kg, s.c.) and Dexamethasone (0.3 mg/kg, i.p.) started 7 days after the transfer. **(A)** Weight change of mice during the experiment was monitored as well as **(B)** development of clinical symptoms as DAI. **(C)** Alteration of stool consistency was assessed as stool score. At the end of the experiment **(D)** colon weight was measured and **(E)** activity of myeloperoxidase (MPO) was quantified. **(F)** Representative images from the colon of differentially treated mice are shown. Results are presented as mean ± SEM, $n = 6$ (sham treated animals), $n = 10$ (vehicle and dexamethasone mice) and $n = 11$ (hBD2 mice). Appropriate statistical comparisons are shown within the graph by a Kruskal-Wallis-test for non-parametric data with a Dunn's post-test.

immunomodulatory capabilities of hBD2 in both groups, lending further credence to the hypothesis that hBD2 might be used as a novel biological to either treat colitis patients or alternatively to keep such patients in remission.

In a next step, we identified DCs as one cell population amongst PBMCs whose cytokine secretion is modulated by hBD2. Because it is already known that hBD2 is able to induce chemotaxis by binding to the CCR2 receptor (22), we investigated whether the observed hBD2 mediated downregulation of inflammation might depend on CCR2. In line with this hypothesis, the effects of hBD2 in human mo-DC's as well as in murine BM-DC's were completely blocked by a CCR2 inhibitor. We found a potential interaction between the downstream signaling molecules of the TLR and CCR signaling pathways. One possibility is that the signaling molecules NF-κB and CREB compete for the coactivator CBP. But also other signaling molecules such as the extracellular signal-regulated kinase (ERK) that plays an important role in TLR signaling (40) as well as CCR signaling, by inducing e.g., expression of CCR1 and CCR2 in human monocytic cells may play important roles (41, 42). Unfortunately, it is not possible to test the CCR2 dependency of the hBD2 effect *in vivo* because $CCR2^{-/-}$ mice are protected from experimental induced colitis (43).

Based on the feasibility of recombinant hBD2 production (26), negligible toxicity, and strong anti-inflammatory CCR2 dependent modulation of DCs, we hypothesized that hBD2 could be used as a systemic anti-inflammatory biological. This strategy uncouples the classical intra-intestinal functions of hBD2 from their potent immunomodulatory capacity, and thus represents a new paradigm in therapeutic use of antimicrobial peptides. Indeed, subcutaneously administered hBD2 improved the outcome of colitis in three different *in vivo* models of IBD, namely DSS-, TNBS-, and T-cell transfer-mediated colitis. This is in line with our observation of hBD2 to rapidly enter the blood stream after s.c. administration and is likely mediated by its anti-inflammatory activity on several blood cell populations, and especially the DC fraction, as described above. We therefore tested and found that hBD2 administered s.c. could act as an immune-modulator, attenuating inflammation, that characterizes IBD. This finding is consistent with the observation of Aden et al. (44) who studied the development of colitis in IL-23 receptor deficient mice ($Il23R^{\Delta IEC}$) and found these to display decreased levels of leukocyte derived IL-22 and of Reg3b, a C-type lectin with antimicrobial activity (45). Systemic administration of Reg3b significantly improved DSS-colitis in $Il23R^{\Delta IEC}$ mice by recruiting IL-22 secreting neutrophils supporting a protective role for Reg3b in colitis (44). In addition, the hBD2-induced wound healing in intestinal epithelial cells *in vitro* (19) may add another mechanism of action. Finally, rectally applied porcine β-defensin 2 (pBD2) has been used for the treatment of DSS colitis in mice by Han et al. (46). They found pBD2 to be protective against mucosal injury and disruption of the epithelial barrier associated with DSS colitis. Furthermore, they reported decreased inflammatory infiltrates and expression of inflammatory mediators upon treatment with pBD2 as well as an increase in intestinal tight junction structure and function compared to untreated DSS control mice. In contrast to the local administration employed by Han et al., we provide evidence for a distinct anti-inflammatory effect with systemic application.

Despite ongoing development of therapeutic approaches, new treatment strategies for the management of IBD are still urgently needed. Corticosteroids remain the standard therapeutic options for active CD and UC. However, their beneficial effects are associated with severe side effects such as osteoporosis, moon face, mood disturbances, glaucoma, and hypertension (47). In recent years, several new therapeutics targeting the molecular mechanisms of intestinal and systemic inflammation have been developed. Among these, anti-TNFα antibodies have been the most successful and most commonly used biological (48). However, primary and secondary lack of response as well as serious side effects, limits their use. More recently, antibodies targeting IL-17 in CD and IL-13 in UC have been proposed as IBD management. Yet, despite promising preclinical data, both antibodies failed to improve the outcome of CD or UC (49) in patients. Secukinumab, an anti-IL-17 antibody, even worsened the disease in a clinical trial in CD patients (50) and two anti-IL-13 antibodies also failed to produce positive results in clinical trials for UC (51, 52). This negative effect of IL-17 blockade may in part be explained by blocking the hBD2 pathway, which is also mediated by IL-17.

In conclusion, the results presented here constitute the first *in vivo* proof of therapeutic efficacy of a systemically administered human defensin. It is, however, important to stress, that in all tested models, hBD2 were administered before the onset of clinical inflammation. We therefore provide strong evidence for the potential of hBD2 treatment to keep patients in remission, but studies are warranted to examine if hBD2 also exhibits treatment efficacy of acute and relapsed inflammation. The use of natural host defense peptides could provide a new chapter of effective, minimal-side-effect treatment strategies of IBD.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the University Hospital Tübingen. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Novozymes science ethics committee.

AUTHOR CONTRIBUTIONS

LK, NA, KB, SK, BA, PN, BJ, and JW conceived the study, designed the experiments, and analyzed the data. LK, NA, KB, BA, and CL performed the experiments. SA, DS, and NM contributed reagents, materials, analysis tools, and participated in scientific discussion. LK, NA, ES, PN, BJ, and JW wrote the paper.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: KB, SK, and BA was employed by Novozymes. PN was employed by Defensin Therapeutics. PN and JW hold shares of Defensin Therapeutics. Defensin Therapeutics holds patents on treatment with defensins.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Opossum Cathelicidins Exhibit Antimicrobial Activity Against a Broad Spectrum of Pathogens Including West Nile Virus

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This study aimed to characterize cathelicidins from the gray short-tailed opossum *in silico* and experimentally validate their antimicrobial effects against various pathogenic bacteria and West Nile virus (WNV). Genome-wide *in silico* analysis against the current genome assembly of the gray short-tailed opossum yielded 56 classical antimicrobial peptides (AMPs) from eight different families, among which 19 cathelicidins, namely ModoCath1 – 19, were analyzed *in silico* to predict their antimicrobial domains and three of which, ModoCath1, -5, and -6, were further experimentally evaluated for their antimicrobial activity, and were found to exhibit a wide spectrum of antimicroial effects against a panel of gram-positive and gram-negative bacterial strains. In addition, these peptides displayed low-to-moderate cytotoxicity in mammalian cells as well as stability in serum and various salt and pH conditions. Circular dichroism analysis of the spectra resulting from interactions between ModoCaths and lipopolysaccharides (LPS) showed formation of a helical structure, while a dual-dye membrane disruption assay and scanning electron microscopy analysis revealed that ModoCaths exerted bactericidal effects by causing membrane damage. Furthermore, ModoCath5 displayed potent antiviral activity against WNV by inhibiting viral replication, suggesting that opossum cathelicidins may serve as potentially novel antimicrobial endogenous substances of mammalian origin, considering their large number. Moreover, analysis of publicly available RNA-seq data revealed the expression of eight ModoCaths from five different tissues, suggesting that gray short-tailed opossums may be an interesting source of cathelicidins with diverse characteristics.

Keywords: antimicrobial peptides, antiviral function, West Nile virus, host defense peptides, cathelicidins, *Monodelphis domestica*, gray short-tailed opossum, circular dichroism

INTRODUCTION

Antimicrobial peptides (AMPs), known as host defense peptides (HDPs), serve as alternatives to antibiotics owing to their antimicrobial properties and immunomodulatory responses, along with the rare possibility of acquiring bacterial resistance (1). Cathelicidins are a family of AMPs that have been identified in most vertebrates as innate immune defense peptides; they contain

characteristic well-conserved cathelin-like domains (CLDs) comprising four Cys residues and a mature bioactive peptide at the C-terminal end (2). Cathelicidin propeptides undergo post-translational modification through proteolytic cleavage by specific enzymes, releasing the active antimicrobial domain (3). The number of cathelicidin genes differs among species, from one in humans to at least twelve in the opossum (4).

The identification and characterization of endogenous AMPs has been limited to only a few species. However, the availability of genome sequences from diverse species along with AMP databases and bioinformatic tools facilitates the identification of novel AMPs (5, 6).

Marsupials differ from eutherian mammals, particularly in their reproductive and developmental traits (7, 8). For instance, marsupials are exposed to harsh environments containing pathogenic bacteria during early development within a brood pouch or burrow containing an abundant and diverse microbial flora (9, 10). Cathelicidins in milk and the brood pouch lining immunologically protect naïve joeys from harmful bacteria in the environment (11, 12). Therefore, such biological characteristics may have contributed to the expansion and diversification of AMPs during evolution in marsupials (4, 13). Cathelicidins of *Monodelphis domestica* have been studied previously; however, the genes were not completely identified and characterized (14).

Although several studies have reported that LL-37 exerts antiviral activity against human immunodeficiency virus (HIV), herpes simplex virus type 1 (HSV-1), influenza A virus, and Zika virus, the list of AMPs exhibiting antiviral effects and their characterization remain limited (15–19).

West Nile virus (WNV) is an arthropod-borne virus of genus *Flavivirus*, similar to the Dengue, Zika, or yellow fever viruses. WNV has recently emerged in different regions worldwide and poses a major threat to public health (20). This mosquito-borne virus causes infections in humans and is considered the primary cause of viral encephalitis worldwide (21).

At the initial site of viral inoculation, the skin serves as a first-line host defense against flaviviruses and leads to the initiation of the early innate immune response (22). Keratinocytes, the most abundant epidermal cells, are permissive to WNV and express inflammatory and antiviral proteins upon infection (23). Thus far, no antiviral agent to combat WNV infections or vaccines are available. Therefore, the characterization of antimicrobial peptides provides insights into new antiviral and antibacterial therapies.

MATERIALS AND METHODS

In silico Identification of AMP-Like Sequences From the Genome of *Monodelphis domestica*

The sequences of 1,173 non-redundant AMPs of mammalian, avian, and fish origins were downloaded from UniProtKB/Swiss-Prot[1], using the query "antimicrobial peptide AND reviewed: yes." Consequently, 420 sequences corresponding to eight major AMP families including alpha-defensin, apolipoprotein A2, beta-defensins, BPI/LBP superfamily, calycins, cathelicidins, hepcidin, and LEAP-2 were identified (**Supplementary Table S1**). Thereafter, BLASTp and tBLASTn analyses were carried out[2] against the reference genome of the opossum (GCF_000002295.2 MonDom5). Furthermore, keyword searches were carried in the NCBI and Immunome Database for Marsupials and Monotremes (IDMM) (24) with the query, "cathelicidin AND *Monodelphis domestica*."

In silico Functional Characterization and Nomenclature of Cathelicidin-Like Sequences in the *Monodelphis domestica* Genome

Exons/introns were predicted using Splign Transcript to Genomic Alignment Tool[3] (25). Signal peptides and CLDs were determined using SignalP 4.1 server[4] (26) and HMMER[5] (27), respectively. DBAASP[6] (28) and Antimicrobial Sequence Scanning System (AMPA[7]) (29) were used to predict potential antimicrobial activity using the default threshold. Protein secondary structures were analyzed using the PSIPRED protein sequence analysis workbench[8]. Proteolytic cleavage sites were predicted to define the mature peptide region, using ExPasy PeptideCutter[9] and PROtease Specificity Prediction servER (PROSPER[10]) (30), respectively. The mature peptide regions in the predicted cathelicidin sequences of *M. domestica* were named ModoCath 1 to 19, concurrent with the previous annotation in IDMM, where "Modo" and "Cath" stand for *M. domestica* and cathelicidin, respectively. Hydrophobicity, net charges, molecular weight and sequence similarities of ModoCaths were analyzed using APD3[11] (31) and Protparam tool[12].

Bioinformatic Analysis of ModoCath Expression Using RNA-Seq Data

We downloaded 74 RNA-seq runs of *Monodelphis domestica* from NCBI SRA database[13] (**Supplementary Table S2**). Expression levels of cathelicidins were determined relative to *EMC7* (accession, XM_001380762.4). Downloaded fastq sequences were mapped to the full-length coding sequences of 19 ModoCaths and *EMC7*, using BWA aligner (version 0.7.17) (32). Sorted bam files with uniquely mapped reads were obtained using samtools (version 1.9) (33). The average depth and coverage of expressed transcripts was calculated using bedtools (version 2.25.0) (34) and R software (version 3.6.0) (35).

[1]http://www.uniprot.org/uniprot/

[2]http://blast.ncbi.nlm.nih.gov

[3]https://www.ncbi.nlm.nih.gov/sutils/splign

[4]http://www.cbs.dtu.dk/services/SignalP/

[5]https://www.ebi.ac.uk/Tools/hmmer/

[6]https://dbaasp.org/prediction

[7]http://tcoffee.crg.cat/apps/ampa/do

[8]http://bioinf.cs.ucl.ac.uk/psipred/

[9]http://web.expasy.org/peptide_cutter/

[10]https://prosper.erc.monash.edu.au

[11]http://aps.unmc.edu/AP/prediction/prediction_main.php

[12]https://web.expasy.org/protparam/

[13]https://www.ncbi.nlm.nih.gov/sra

Peptide Synthesis and Evaluation of Antibacterial Activity

Peptides corresponding to the predicted antimicrobial regions of ModoCath1 (ΔModoCath1, N-VKRTKRGARRGL TKVLKKIFGSIVKKAVSKGV-C), ModoCath5 (ΔModoCath5, N-WYQLIRTFGNLIHQKYRKLLEAYRKLRD-C), ModoCath6 (ΔModoCath6, N-VRRSKRGIKVPSFVKKVLKDVVSESIS-C) and PMAP36 (N-GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSI PLGCG-C) were synthesized via solid-phase peptide synthesis and purified via high-performance liquid chromatography using a commercial service (GenScript, Piscataway Township, NJ, United States). The MIC of synthesized peptides was determined against a panel of bacteria comprising 3 gram-positive strains, *Staphylococcus aureus* ATCC 6538 (ATCC, Manassas, VA, United States), *Bacillus cereus* ATCC 10876, and *Enterococcus faecalis* ATCC 29212, and 3 gram-negative strains, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella typhimurium* ATCC 14028. Ampicillin (Sigma Aldrich, St. Louis, MO, United States) and gentamicin sulfate (Sigma Aldrich) were used as controls for antimicrobial activity. The MIC was determined using a colorimetric method specified by the Microbial Viability Assay Kit-WST (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's protocol and the Clinical and Laboratory Standards Institute (CLSI) guidelines (2018). Briefly, four colonies of each bacterium were inoculated into 5 mL Luria-Bertani (LB) broth medium at 37°C for 4 to 6 h. The cells were washed with sterile saline (0.9% NaCl) twice and seeded in a single well of a 96-well plate at the cell density of 10^5 CFU/well. Subsequently, 180 μL/well of fresh Mueller-Hinton broth (MHB) was added to the plate. Different concentrations of each peptide and reference antibiotics were serially diluted in 10 μL of MHB and added to each well. The plate was incubated at 37°C for 6 h. Cation-adjusted MH broth (CAMHB) was used to culture *E. faecalis*. Subsequently, 10 μL of the coloring reagent was added, and cells were incubated at 37°C for 2 h. UV absorbance was measured for each well at 450 nm, using a microplate spectrophotometer (xMark spectrophotometer; Bio-Rad, Hercules, CA, United States). MIC values were determined when the difference in the optical density (OD) between treatments and blanks (media and coloring reagent only) decreased to < 0.05. Experiments were conducted in triplicate. The MIC of ΔModoCath1 and 5 in different physiological salts (150 mM NaCl, 1 mM $MgCl_2$, 4.5 mM KCl and 2.5 mM $CaCl_2$) and pH conditions (pH 5, 6, and 7) was also determined against *E. coli* (ATCC 25922). The pH conditions were achieved by using acetic acid (Sigma Aldrich).

In vitro Analysis of Serum Stability of the AMPs

The Ethics Committee of the Konkuk University Hospital approved the use of human serum samples for research studies, and human serum was obtained from Konkuk University Medical Center (KUMC) Biobank.

Antimicrobial peptides were dissolved in 25% (v/v) pooled human serum from five individuals, and incubated at 37°C. Aliquots were extracted in triplicate after 0, 60, and 120 min incubation, and their antimicrobial activity against *E. coli* (ATCC 25922) was assessed using the incubated samples, as described above.

Determination of *in vitro* Mammalian Cell Cytotoxicity

Two mammalian cell lines, including human embryonic kidney cells (HEK293T) and human breast cancer cells (MCF7) were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone^{TM}, Logan, UT, United States) supplemented with 10% FBS (Hyclone^{TM}) and 1% penicillin/streptomycin (Hyclone^{TM}) and incubated at 37°C and 5% CO_2 up to 80% confluence. Cellular adherence to the substratum was disrupted using Accutase (Innovative Cell Technologies, San Diego, CA, United States). In total, 1×10^4 to 4×10^4 cells in each well of a 96-well plate containing 8, 16, 32, and 64 μg/mL of ModoCath peptides were incubated for 24 h at 37°C and 5% CO_2. Additionally, HEK293T cells were incubated in the FBS-free medium. Triton X 100 (Sigma Aldrich) was used as a positive control for complete cell lysis, and untreated cells were used as the negative control. After incubation, the medium was removed from the wells, and 10 μL of coloring solution (Cell Proliferation Reagent WST-1^{TM}; Sigma Aldrich) and 100 μL of DMEM (Hyclone^{TM}) were added to the wells in accordance with the manufacturer's protocol. Absorbance was measured for each well at 440 nm (peptide-treated and control) and 650 nm (background) and recorded as the OD, using a microplate reader (xMark^{TM} spectrophotometer; Bio-Rad). Cell viability was calculated using the following equation:

$$Cell\ Viability(\%) = 100 \times \frac{(OD\ peptide\ -\ OD\ background)}{(OD\ negative\ -\ OD\ background)} \quad (1)$$

All experiments were performed in triplicate.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra signals were recorded at 25°C using a Jasco J-810 spectropolarimeter (Jasco, MD, United States) at an emission range of 195 – 260 nm, scanning speed of 50 nm/min, 1 nm bandwidth, 4 s response time, and four accumulations using a rectangular quartz cell (0.1 cm path length). All peptides were scanned at a concentration of 25 μM dissolved in 10 mM sodium phosphate buffer, pH 7.0. Lipopolysaccharide (LPS, Sigma Aldrich) titrations were carried out with increasing concentrations from 0 – 0.16 mg/mL to 25 μM peptide in 10 mM sodium phosphate buffer pH 7.0. LPS was prepared via temperature cycling between 4 and 70°C, and vortexed for 10 min. LPS was stored at 4°C overnight before use. The CD spectrum signal for the peptides was obtained after subtracting LPS respective spectrum from that of LPS and peptide mixtures. All experiments were triplicated.

Dual-Dye Membrane Disruption Assay

Four colonies of *B. cereus* (ATCC 10876), *E. coli* (ATCC 25922), and *S. aureus* (ATCC 6538) were inoculated into 5 mL LB broth and incubated at 37°C for 5 h. Cells suspensions were

centrifuged at 3000 × g and 25°C, washed and resuspended in phosphate buffered saline (PBS) + (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 supplemented with 10 mM glucose and 0.5 mM $MgCl_2$; pH 7.4) to an OD_{600} of 0.1. Concurrently, a blank PBS+ sample without cells and cells treated with Nisin (Sigma Aldrich) and vancomycin (Sigma Aldrich) were used as controls (36). TO-PRO-3 iodide (Sigma Aldrich) and $DiOC_2(3)$ (Sigma Aldrich) dyes were then added into the sample and controls to a final concentration of 625 nM and 10 μM, respectively. The plates were incubated at 25°C in the dark for 5 min, and the cells were then treated with ModoCath peptides to final concentrations of 0.1×, 0.2×, and 1× MIC, respectively. Thereafter, the absorbance spectra for TO-PRO-3 iodide and $DiOC_2$ were determined (**Supplementary Figure S1**). The absorbance of the plates was read at λ_{ex} 640 nm and λ_{em} 700 nm for TO-PRO-3 iodide and λ_{ex} 480 nm and λ_{em} 530 nm for $DiOC_2(3)$ using a fluorescence microplate reader (Gemini EM, Molecular Devices, Sunnyvale, CA, United States), where λ_{ex} and λ_{em} indicate wavelengths for excitation (λ_{ex}) and emission, respectively.

Field Emission Scanning Electron Microscopy

Escherichia coli (ATCC 25922) cells at an OD_{600} value of 0.2 were inoculated in LB medium with 1.5 μg/mL ΔModoCath1, 10 μg/mL ΔModoCath5 or 4 μg/mL PMAP36 followed by incubation for 2 and 4 h, respectively, at 37°C. The bacterial cells were harvested by centrifugation at 4,500 rpm, after which the pellets were washed twice with PBS and fixed with 2.5% glutaraldehyde (Sigma Aldrich) in PBS for overnight at 4°C. Cells were then washed thrice with PBS and dehydrated using graded ethanol at 50, 70, and 90% for 10 min each, and 100% for 15 min. Subsequently, samples were dried with hexamethyldisilazane (Daejung Chemicals and Metals Co. Ltd., Siheung, South Korea) for 15 min. For observation, prepared samples were sputter-coated with platinum using Ion Sputter MC1000 (Hitachi High-Technologies, Tokyo, Japan) prior to imaging with a Hitachi HR FE-SEM SU8010 (Hitachi High-Technologies).

Isolation and Culturing of Normal Human Epidermal Keratinocytes From Skin Samples

The Ethics Committee of the Poitiers Hospital approved the use of human skin samples for research studies. All subjects provided written informed consent in accordance with the tenets of the Declaration of Helsinki. Normal abdominal or breast skin samples were obtained from patients undergoing plastic surgery and thoroughly washed with PBS free of calcium and magnesium (PBS; Gibco, Thermo Fisher Scientific, Waltham, MA, United States) after fat removal. The skin samples were minced into fragments of approximately 125 mm^2, using scalpel blades. Skin samples were incubated overnight at 4°C in a dispase solution (25 U/mL; Life Technologies, Carlsbad, CA, United States). Epidermal sheets were removed from the dermis, and keratinocytes were dissociated via trypsin digestion (trypsin-EDTA; Gibco) for 15 min at 37°C. The cell suspension was then filtered through a 280-μm sterile filter. DMEM (Gibco) supplemented with 10% (vol/vol) of FBS (Gibco) was added, and the suspension was centrifuged at 300 × *g* and 25°C for 10 min. Keratinocytes were seeded at a density of 10^7 cells in 75-cm^2 tissue culture flask in keratinocyte-serum free medium (K-SFM; Invitrogen, Carlsbad, CA, United States) supplemented with bovine pituitary extract (25 μg/mL; Invitrogen) and recombinant epidermal growth factor (EGF) (0.25 ng/mL; Invitrogen). The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 until 80% confluence and then stored frozen in liquid nitrogen until use. Finally, keratinocytes were seeded in sterile 24-well culture plates at 10^5 cells/well in K-SFM supplemented with bovine pituitary extract and EGF and cultured to 80% confluence. Cells were then starved overnight in K-SFM alone before stimulation.

Assessment of the Viability of Keratinocytes

Primary keratinocytes were cultured in 96-well plates at 4 × 10^4 cells per well in 0.1 mL K-SFM (Invitrogen) up to 80% confluence before being treated with various concentrations of ModoCath peptides for 24 h. Cell viability was assessed using the cell proliferation kit II (XTT; Roche, Basel, Switzerland) in accordance with the manufacturer's protocol. The XTT labeling mixture was added after 24 h of incubation in the absence or presence of peptides at the indicated concentrations.

WNV Strain Production

A lineage WNV clinical strain, isolated from a human brain during an epidemic occurring in Tunisia in 1997, was provided by Dr. I. Leparc Goffart (French National Reference Center for Arboviruses, Marseille, France). The viral stock was produced on the *Aedes albopictus* clone C6/36 cells (ATCC® CRL-1660™). Cells were cultivated in Leibovitz's L-15 medium (Gibco) supplemented with 2% of tryptose-phosphate (Gibco) and 5% of FBS (Gibco) in a 75-cm^2 tissue culture flask at 28°C until 50% confluence and then infected at a multiplicity of infection (MOI) of 0.01 for 72 h. Cell supernatants of infected cells and uninfected C6/36 cells, used as the control, were clarified via centrifugation in 50-mL tubes for 15 min at 1500 × *g*. Thereafter, the viral suspension and the supernatant from the uninfected C6/36 suspension were ultrafiltered using Amicon ultra-4 centrifugal filter units 100 kDa (Dominique Dutscher, Brumath, France) for 5 min at 3,000 × *g*. The viral suspension and the supernatant from the uninfected C6/36 suspension were frozen at −80°C in cryotubes containing 500 μL of Leibovitz's L-15 medium supplemented with 0.5 M sucrose and 50 mM HEPES. The final viral titer was $10^{7.97}$ TCID50 (50% tissue culture infective dose) per milliliter as determined using 10-fold serial dilutions of the virus sample on Vero cell monolayers (described below).

Viral Quantification via the End-Point Dilution Assay

Vero cells were seeded in 96-well plates the day before titration at 2 × 10^3 cells/well in DMEM (Gibco) supplemented with 2% SVF. The suspension was successively diluted from a dilution of

10^{-1} to 10^{-9} in DMEM medium supplemented with 2% SVF. Thereafter, 100 μL of each dilution was deposited in a row of six wells. Initial data were obtained after 120 h of incubation at 37°C in 5% CO_2. The wells containing cells with cytopathic effects were considered positive for viral infection. The titer of the viral suspension was then determined using the Kärber's method for assessing the TCID50.

Viral Infection

Human primary keratinocyte cultures (60–80% confluence) from three different patients were infected at a MOI of 0.1 and incubated for 24 h at 37°C in 5% CO_2 in K-SFM (Invitrogen) medium. Cell culture supernatants and cell monolayers were harvested for viral quantification via RT-qPCR and transcriptomic analysis of inflammatory markers, as described below.

Antiviral Assays

The antiviral properties of ModoCath peptides were first assessed by evaluating their impact on growth kinetics of the virus inoculated on primary human keratinocytes. Keratinocyte cultures from three different patients were incubated with one of the three peptides at a final concentration of 16 μg/mL for 1 h before addition of WNV at a MOI of 0.1 TCID50 per cell. Uninfected cultures with or without the peptides were used as the control. After 24 h of incubation at 37°C in 5% CO_2 in K-SFM (Invitrogen) medium, cell culture supernatants and cell monolayers were harvested for viral quantification via RT-qPCR and transcriptomic analysis of inflammatory markers. The virucidal properties of ModoCath peptides directly on the virus were assessed by pre-incubating 0.1 mL of the virus stock (described above) with peptides for 1 h at 37°C before titration via the end-point dilution assay using Vero cells, as described above. The viral titer thus determined was compared to that of similarly assayed untreated viral suspensions.

RNA Extraction

For viral RNA quantification in cell supernatants, 200 μL of total DNA/RNA from keratinocyte supernatants was extracted using a NucliSENS easyMAG® automated system (bioMérieux, Marcy-l'Étoile, France) in accordance with the manufacturer's protocol. For intracellular viral RNA quantification and evaluation of the host inflammatory response, total RNA was extracted from the keratinocyte monolayer using the Nucleo-Spin RNA extraction kit in accordance with the manufacturer's instructions (Macherey-Nagel, Düren, Germany). RNA concentrations and purity were determined using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

Viral Quantification via RT-qPCR

Viral quantification in cell supernatants and keratinocytes was performed using a previously described one-step real time RT-PCR assay (23). Total RNA (5 μL) was added to the reaction mixture containing 12.5 μL of Master Mix (Invitrogen), 0.5 μL (0.2 μM) of forward (5′-GTGCGGTCTACGATCAGTTT-3′) and reverse primers (5′-CACTAAGGTCCACACCATTCTC-3′), 0.25 μL (0.1 μM) of 5′FAM and 3′Dark Quencher probe (5′-AATGTGGGAAGCAGTGAAGGACGA-3′), 0.5 μL of SuperScript III reverse transcriptase (Invitrogen) and DNA polymerase platinum Taq (Invitrogen), 0.5 μL of RNase out (Invitrogen), and 5.25 μL of water. The calibration range was determined using a transcript produced using a plasmid containing the WNV genome without the genes encoding structural proteins. Transcripts were diluted to obtain a calibration range allowing for the quantification of viral load from 10^2 to 10^7 RNA copies/mL.

Transcriptomic Analysis of the Innate Antiviral Immune Response in Keratinocytes

Total RNA (1 μg) was reverse-transcribed using SuperScript II kit (Invitrogen). Quantitative real time PCR was performed in 96-well plates with a LightCycler 480 system (Roche). A reaction mixture comprised 5 μL of AceQ SYBR Green qPCR Master Mix (Vazyme Biotech, Nanjing, China), 1 μM forward and reverse primers designed using Primer 3 software, and 12.5 ng of cDNA template in a total volume of 10 μL. PCR conditions were as follows: 5 min at 95°C, 40 amplification cycles for 20 s at 95°C, 15 s at 64°C, and 20 s at 72°C. Relative mRNA expression of target genes was normalized to that of two independent control housekeeping genes (GAPDH and 28S rRNA gene) and reported using the ΔΔCT method as fold-changes in RNA: $2\Delta\Delta CT = 2^{\Delta CTsample - \Delta CTreference}$.

Quantification of Type III Interferon Secretion

Keratinocyte secretion of active type III IFNs (IL-28A, IL-28B, and IL-29) was quantified using HEK-Blue™ IFN-λ reporter cells expressing an inducible secreted embryonic alkaline phosphatase (InvivoGen, San Diego, CA, United States) according to the manufacturer's instructions. The activity of the secreted alkaline phosphatase was measured as a colorimetric reaction at 630 nm using the Quanti-Blue reagent (InvivoGen).

RESULTS

Identification of 56 AMP Genes From *in silico* Analysis of the *Monodelphis domestica* Genome

The strategy for the *in silico* identification of AMPs from the *Monodelphis domestica* genome is described in **Supplementary Figure S2**. We identified a total of 56 putative AMP genes in *M. domestica*, including one alpha-defensin, one apolipoprotein A2, 3 beta-defensins, 21 BPI/LBP superfamily members, 7 calycins, 21 cathelicidin-like, one hepcidin, and one LEAP-2 (*e*-value < 0.001; **Supplementary Tables S3, S4**). Among the 21 cathelicidins, 7 were previously undescribed, and their putative names were assigned as ModoCath 13 to 19 after excluding

secreted phosphoprotein 24 and cathelicidin-related peptide Oh-Cath-like isoform X2, which failed to meet the characteristics of functional cathelicidins (**Supplementary Tables S5, S6**). The conservation of the CLD and cysteine motif among the 20 opossum cathelicidins is shown in **Supplementary Figure S3**. Basic proline-rich protein-like isoform X1 was excluded because it had a longer sequence than that of others.

In silico Prediction of Eight *Monodelphis domestica* Cathelicidins With Antimicrobial Activity

The 21 cathelicidin-like sequences were analyzed *in silico* to predict protein secondary structures and the antimicrobial activity core region using AMPA, DBAASP and PSIPRED protein sequence analysis workbench databases. The analysis identified eight sequences, including ΔModoCath1, 2, 4 to 7, 12, and 19, which were strongly predicted to possess antimicrobial activity-related structures (**Supplementary Figure S4** and **Supplementary Table S6**). Biochemical features of the core sequences from the eight cathelicidins, deduced using APD3 and Protparam, showed the antimicrobial activity-conferring regions to be 27 to 41 amino acids long and 3.04–4.85 kDa in molecular weight (**Table 1**). The ratios of hydrophobic residues and net charges for the core regions were 25 to 41% and +4 to +12, respectively. Their sequence similarities to known AMPs were less than 50%, indicating that they were novel. Interestingly, ΔModoCath12 and ΔModoCath19 shared the same core sequence (**Supplementary Figure S4** and **Table 1**).

Difference in Antibacterial Specificity of ΔModoCath1, 5, and 6

Among the eight ModoCath peptides with predicted antimicrobial activity, we chemically synthesized three peptides, ΔModoCath1, 5, and 6, based on the uniqueness of their sequences which are not based on statistical evaluation (**Supplementary Figures S4, S5**). The antimicrobial activity of the peptides was evaluated against our bacterial panel, comprising 3 gram-negative strains, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*, and 3 gram-positive strains, *Staphylococcus aureus*, *Bacillus cereus*, and *Enterococcus faecalis*. All three peptides showed strong antibacterial activities with differences in bacterial strain specificity (**Table 2**). ΔModoCath1 showed the strongest and broadest activity against both gram-positive and gram-negative bacteria, with MICs of 0.75 to 3 μg/mL, except for *B. cereus* (30 μg/mL). ΔModoCath5 showed antibacterial activity toward gram-positive strains with MICs of 1.5 to 6 μg/mL. ΔModoCath6 showed bactericidal activity only against *E. coli* in our panel.

Stability of the Bactericidal Activity of ΔModoCath1 and 5 in Serum and Various Salt and pH Conditions

For the pharmaceutical application of AMPs, their stability in serum as well as the physiological condition in which they are placed are important factors to consider. We evaluated the effect of serum on the antimicrobial activity of ΔModoCath1 and 5 at different concentrations. The peptides were incubated in 25% human serum for 0–120 min, and the antimicrobial activity of the peptides against *E. coli* was assessed over time (**Supplementary Figure S6**). Results show that the antimicrobial activity of ΔModoCath1 and 5 was affected to varying degrees at different concentrations and incubation times. However, the activity was unaffected at $> \sim 2\times$ MIC after 60 min incubation, and decreased following 120 min incubation with human serum. Therefore, our results showed that the two cathelicidins, ΔModoCath1 and 5, with broad-spectrum antimicrobial activity do not exhibit significant susceptibility to inhibitory substances within human serum.

We also evaluated the antimicrobial activity of ΔModoCath1 and 5 in various salt and pH conditions (**Supplementary Table S7**). No inhibitory effect was observed in their activity at 150 mM NaCl, 1 mM $MgCl_2$, 4.5 mM KCl or 2.5 mM $CaCl_2$, all of which correspond to various physiological conditions (37). Interestingly, the MIC of ΔModoCath1 was found to decrease slightly from 0.75 to 0.5 μg/mL, potentiating the activity in the physiological salt conditions than in bacterial culture media. Regarding the varying pH conditions, a minimum of 2-fold increases were observed in the activity of ΔModoCath1 and 5 in acidic conditions (pH 5 and 6) compared to neutral pH.

Low-to-Moderate Level Cytotoxicity of ΔModoCath1, 5, and 6 to Mammalian Cells

Cathelicidins with strong antimicrobial activity could negatively affect mammalian cells (38, 39). Therefore, the degree of cellular damage caused by ModoCaths to HEK293T and MCF7 cells and human primary keratinocytes was assessed by evaluating the viability of cells exposed to various concentrations (8–64 μg/mL) of ΔModoCath1, 5, and 6 (**Table 3**). Cell survivability was $>\sim$90% at concentrations below 16 μg/mL for all tested cells for ΔModoCath1 and 6, indicating that the cells were minimally affected at that concentration. However, ΔModoCath5 showed variation in cell viability from 67 to 96% at the same concentration, showing slightly higher cytotoxicity than the other ModoCaths. Moreover, the viability of MCF7, a human breast cancer cell line, was not significantly affected by the three ModoCaths, indicating the lack of direct antitumorigenic activity (**Table 3**). In addition, the cytotoxicity of ModoCaths to HEK293T cells in the absence and presence of serum was not different significantly (**Supplementary Table S8**).

Secondary Structure Formation of ΔModoCath1 and 5 After Interaction With *E. coli* LPS

Next, we analyzed the peptide conformation of ΔModoCath1 and 5 using circular dichroism (CD) analysis. LPS was titrated against each peptide to evaluate the secondary structure formation upon interactions. Results reveal gradual formation of the α-helical structure upon increasing concentration of *E. coli* LPS (**Figure 1**), demonstrating that peptide binding to LPS triggers formation of ΔModoCath1 and 5 secondary structure. Further, ΔModoCath1 contained a randomly coiled

TABLE 1 | Characteristics of the antimicrobial activity domain for the eight cathelicidins of *Monodelphis domestica* selected for their antimicrobial activity.

Name in this study	Core sequences with antimicrobial activity	Length	<H>[a]	z^b (+)	Molecular weight (Da)	Similarity (%)[c]
ΔModoCath1	VKRTKRGARRGLTKVLKKIFGSIVKKAVSKGV	32	37	12	3510.37	41.66
ΔModoCath2	VKRTKRGIKKGISKVLKKFFSSMIKKAVSK	30	36	12	3422.31	39.47
ΔModoCath4	GIRGFWNGFRGR	12	33	3	1422.61	46.15
ΔModoCath5	WYQLIRTFGNLIHQKYRKLLEAYRKLRD	28	35	5	3623.27	35.71
ΔModoCath6	VRRSKRGIKVPSFVKKVLKDVVSESIS	27	37	6	3042.66	36.66
ΔModoCath7	IVRRSKRGIKVPGFVKKFLKDVVSETI	27	40	6	3100.79	37.50
ΔModoCath12[d]	VKRTKREISKILEEIFSTVIKIFIPKGFYKGIQLVNEIIKE	41	41	4	4849.87	38.63
ΔModoCath19[d]	VKRTKREISKILEEIFSTVIKIFIPKGFYKGIQLVNEIIKE	41	41	4	4849.87	38.63

[a] *The ratio of hydrophobic residues.* [b] *The charge.* [c] *Similarity to known AMPs.* [d] *The sequence of the predicted antimicrobial activity domain is identical.*

TABLE 2 | Antimicrobial activity of three opossum cathelicidins comparing to conventional antibiotics against standard bacterial strains.

Strains	MIC (μg/mL, μM)						
	ΔModoCath1	ΔModoCath5	ΔModoCath6	Ampicillin[a]	Gentamycin[a]	Nisin[b]	Vancomycin[b]
Gram-negative bacteria							
E. coli ATCC 25922	0.75 (0.21)	5 (1.38)	5 (1.64)	1 (2.86)	1 (2.09)	>32 (9.54)	32 (22.08)
P. aeruginosa ATCC 27853	2 (0.57)	>32 (8.83)	>32 (10.51)	>128 (366.33)	1 (2.09)	ND	ND
S. typhimurium ATCC 14028	2 (0.57)	>32 (8.83)	>32 (10.51)	1 (2.86)	1 (2.09)	ND	ND
Gram-positive bacteria							
S. aureus ATCC 6538	3 (0.85)	1.5 (0.41)	>32 (10.51)	1 (2.86)	1 (2.09)	15 (4.47)	5 (3.45)
B. cereus ATCC 10876	30 (8.5)	6 (1.66)	>32 (10.51)	16 (45.79)	1 (2.09)	32 (9.54)	4 (2.76)
E. faecalis ATCC 29212	2 (0.57)	2 (0.55)	>32 (10.51)	2 (5.72)	5 (10.45)	ND	ND

[a] *Antibiotics for control according to the CLSI standard.* [b] *Antimicrobials for control used in the membrane activity assay. ND, Not determined.*

TABLE 3 | Viability of human cells treated with varying concentrations of three opossum cathelicidins.

Peptide	Concentration (μg / mL)	Cell viability ± SD (%)		
		HEK293T	Human primary keratinocytes	MCF7
ΔModoCath1	8	108.1 ± 9.9	97.1 ± 2.9	91.2 ± 2.5
	16	105.5 ± 6.9	91.4 ± 1.6	89.2 ± 0.6
	32	66.9 ± 5.0	56.2 ± 8.4	79.1 ± 1.8
	64	55.7 ± 6.2	5.8 ± 3.7	42.4 ± 2.1
ΔModoCath5	8	93.7 ± 8.3	98.1 ± 0.6	98.4 ± 4.2
	16	67.4 ± 3.1	96.2 ± 3.5	84.6 ± 0.6
	32	47.8 ± 2.7	79.1 ± 13.0	28.4 ± 0.7
	64	35.0 ± 0.8	48.9 ± 15.5	10.3 ± 2.8
ΔModoCath6	8	103.8 ± 5.0	98.0 ± 1.6	89.2 ± 1.4
	16	95.6 ± 8.4	100.3 ± 1.3	93.2 ± 0.9
	32	89.0 ± 13.1	101.0 ± 2.6	92.8 ± 1.0
	64	88.8 ± 8.1	89.2 ± 7.2	95.2 ± 1.0
Triton X-100[a]		11.6 ± 2.5	8.2 ± 0.4	11.4 ± 0.4

[a] *The control for cell lysis. The experiment was triplicated.*

structure in LPS-free aqueous solution with a negative peak at 198 nm (**Figure 1A**). Surprisingly, ΔModoCath5 showed a helical structure even in aqueous solution, with a negative peak at 208 nm (**Figure 1B**). Although both ΔModoCath1 and 5 demonstrated increased mean residual ellipticity at 208 and 222 nm with increasing LPS concentrations and increased helicity (40), the spectra for ΔModoCath5 contained double minima at 208 and 222 nm, indicating higher helicity compared to that of ΔModoCath1. Comparatively, PMAP36, a well-characterized cathelidicin with a helical structure (41), also showed a similar CD spectral signature to ΔModoCath1 with different concentrations of LPS (**Figure 1C**).

Disruption of Bacterial Membrane Permeability by ModoCaths

Fluorescent dyes have been used to determine the integrity of membranes (36). The increased fluorescence intensity of TO-PRO-3 iodide and $DiOC_2(3)$ in cells indicates the penetration of dyes to the cytoplasm due to the damaged membrane and disruption of membrane potential. The treatment of *B. cereus*, *E. coli*, and *S. aureus* with ΔModoCath1, 5, and 6 resulted in an increase in fluorescence from the cells cultured with either dyes regardless of cell type (**Figure 2**). This result is identical to that obtained on treatment with Nisin, a lantibiotic, known to create a pore that disrupts membrane permeability. The lower effect of Nisin treatment on the membrane permeability of *E. coli* than that of *S. aureus* and *B. cereus* was also consistent with the activity preference of Nisin on gram-positive bacteria rather than gram-negative bacteria (42).

The disruption of membrane potential, as indicated by $DiOC_2(3)$ fluorescence after peptide treatment, differed among

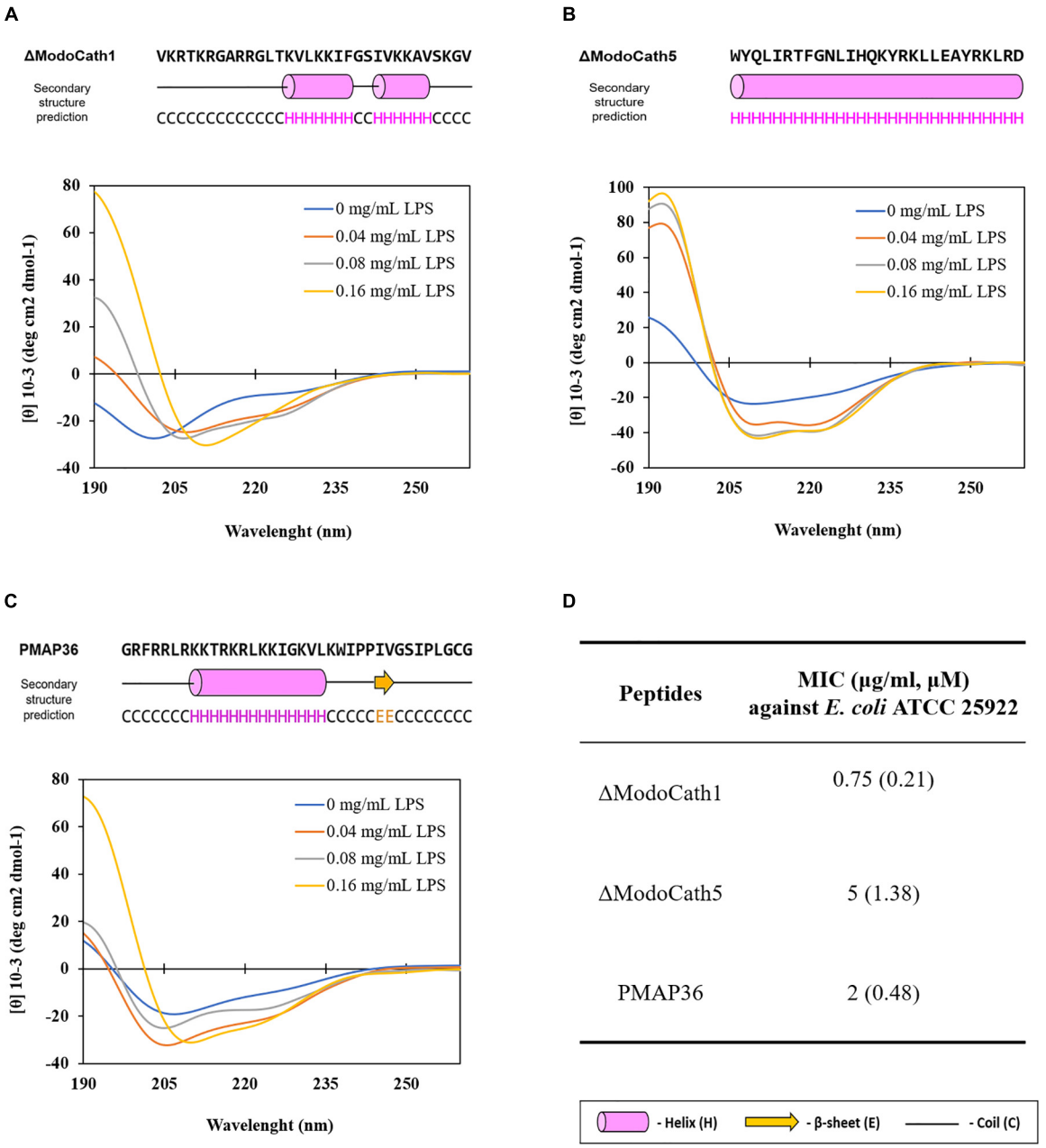

FIGURE 1 | Changes in the structural conformation of ΔModoCath1 and 5 peptides upon interaction with LPS. The CD spectra were recorded for ΔModoCath1 **(A)**, Δ ModoCath5 **(B)**, and PMAP36 **(C)** following incubation with increasing concentration of LPS. The CD spectra of LPS were subtracted from their respective spectra in combination with the peptide mixtures. The predicted secondary structures of these peptides using PSIPRED are presented together with amino acid sequences. The MICs of ΔModoCath1, 5 and PMAP36 against *E. coli* are also presented **(D)**. Representative data from one set of experiments are shown.

bacterial strains. However, the magnitude of changes was consistent with the strength of the bactericidal activity of ModoCaths (**Table 2**). In contrast, vancomycin, which does not disrupt the cell membrane, showed no changes in the fluorescence output of either dye. Therefore, we concluded that the antibacterial activity of the three ModoCaths validated in this study was mediated by the increase in membrane permeability owing to membrane disruption.

Damage to Bacterial Cell Envelop and Leakage of Cell Contents Following Exposure to ModoCaths

To visualize the morphological changes occurring in bacteria following treatment with ΔModoCath1 and 5, high resolution electron microcopy was performed. The synthesized PMAP36 peptide, which is a representative of cathelicidins with membrane disrupting activity, was used as a comparison (**Figure 3A**). Intact

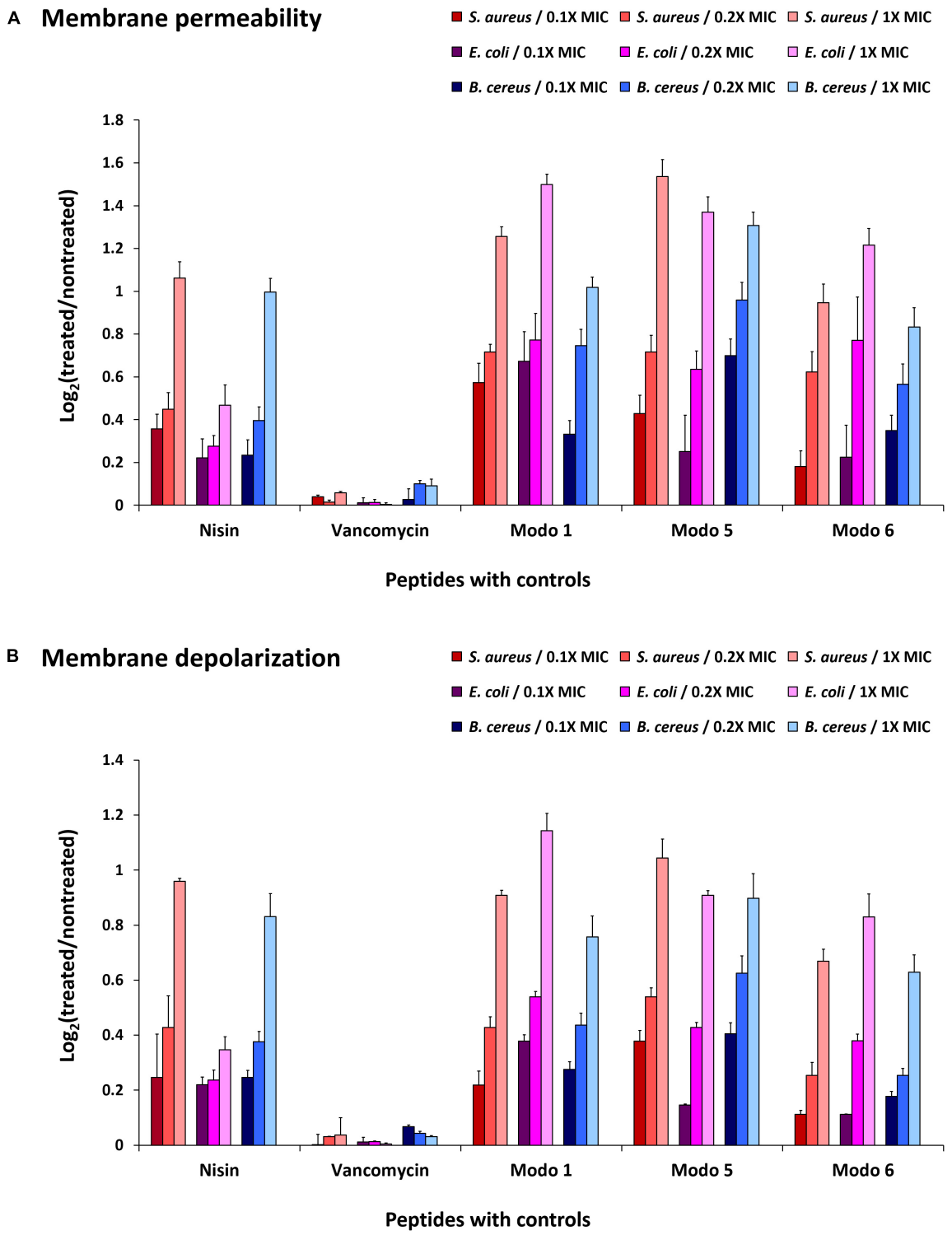

FIGURE 2 | Disruption of bacterial membrane permeability by ΔModoCath peptides. The fluorescence of TO-PRO-3 iodide and $DiOC_2(3)$ was detected from *B. cereus* (ATCC 10876), *E. coli* (ATCC 25922), and *S. aureus* (ATCC 6538) treated with ΔModoCath1, 5, and 6 at the concentration of 0.1×, 0.2×, and 1× MIC. Nisin and vancomycin were used as controls. **(A)** The fluorescence of TO-PRO-3 iodide was measured for membrane permeability at λ_{ex} 640 nm and λ_{em} 700 nm. **(B)** The fluorescence of $DiOC_2(3)$ was detected at λ_{ex} 480 nm and λ_{em} 530 nm for cell polarity. The log2 ratio of detected signals between treated and non-treated wells is expressed on the *Y*-axis. Error bars represent the standard deviation from three replicated experiments. Modo 1, 5, and 6 indicate ΔModoCath1, 5, and 6, respectively.

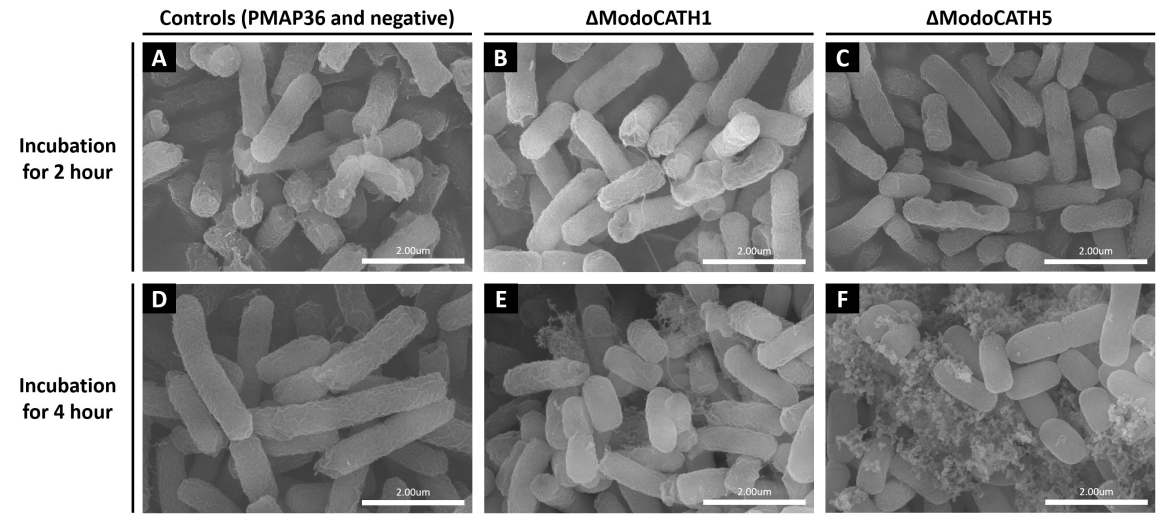

FIGURE 3 | Scanning electron microscopy images of bacterial cells treated with ΔModoCath1, 5 and PMAP36. Electron micrographs showing *E. coli* cells treated with 2× MIC of ΔModoCath1 (1.5 μg/mL, **B,E**), ΔModoCath5 (10 μg/mL, **C,F**), and PMAP36 (4 μg/mL, **A**) for 2 or 4 h. Panel **D** represents the untreated control. PMAP36 was used as a representative control for membrane disrupting cathelicidins. Scale bar, 2 μm.

E. coli without any treatment was used as negative control (**Figure 3D**). Field emission scanning electron microscopy (FE-SEM) images of *E. coli* cells following 2 or 4h of peptide treatments showed complete or partial disruption of bacterial membranes with subsequent outflow of cytoplasm (**Figures 3B,C,E,F**). The images show that both ΔModoCath1 and 5 induce formation of pores (**Figures 3B,C**) and blisters (**Figures 3E,F**) on the bacterial surface, leading to leakage of cytoplasmic materials (**Figures 3E,F**) in peptide-exposed cells, which ultimately becomes so severe as to cause formation of coral reef-like structures among bacterial cells, most notably in *E. coli* cells treated for 4 h with ΔModoCath5 (**Figure 3F**).

Strong Inhibition of West Nile Virus Replication by ΔModoCath5

Before studying the ability of ModoCaths to inhibit West Nile virus replication, their cytotoxic effects on human primary keratinocytes were evaluated (**Table 3**). The concentration of 16 μg/mL, resulting in cell viability greater than 90% for the three peptides, was chosen to test the antiviral activity of ΔModoCath1, 5, and 6. Results showed a potent inhibitory effect of ΔModoCath5 on WNV replication in primary keratinocytes; ModoCath1 and 6 exhibited no antiviral effects (**Figure 4**). The concentration of viral RNA was significantly reduced in supernatants of cells treated with ΔModoCath5, resulting in approximately 500-fold decrease in virus production. In the cell monolayer, ΔModoCath5 treatment also resulted in a 1-log (93%) decrease in WNV viral loads.

In the second step, the cell innate immune response to WNV infection was characterized in absence and presence of the peptide, in order to evaluate the potential immunomodulatory effects of ΔModoCath5 on cellular antiviral responses. The expression profile of players involved in the cellular antiviral response was studied by transcriptomic analyses. mRNA expression levels of molecules, such as the type III interferon interleukin 28A (IL28A), the chemokine CXCL10, as well as three interferon-stimulated genes (ISGs; IFIT2, ISG20, and viperin), which are known for their antiviral activities, were monitored (**Supplementary Figure S7A**). ΔModoCath5 treatment in keratinocytes during WNV infection did not significantly modulate the cellular antiviral response compared to that in untreated infected cells. The presence of ΔModoCath5 resulted in lower CXCL10, IFIT2, and IL28A mRNA levels, which can be related to the reduced viral replication in keratinocytes treated with this peptide. While viperin expression was not modified, ΔModoCath5 tended to increase ISG20 expression in WNV-infected keratinocytes. However, at the protein level, type III interferon secretion in response to WNV infection was not modulated by ΔModoCath5 treatment (**Supplementary Figure S7B**).

In addition, the virucidal properties of ΔModoCath1, 5, and 6 were assessed by measuring WNV infectious titers following pre-incubation in the presence of these peptides at a final concentration of 16 μg/mL for 1 h at 37°C. The infectious titer measured in the presence of the peptides was compared to that of the untreated virus suspension. No direct virucidal effect of the ModoCath peptides was observed (**Supplementary Figure S8**). Taken together, our results show that ΔModoCath5 strongly inhibits WNV replication in primary human keratinocytes by mechanism of action that remains to be defined.

DISCUSSION

Endogenous AMPs are natural substances encoded by a diverse group of genes in vertebrates to eliminate pathogens or control microbiota of the host. Although many AMPs are being discovered from diverse species, detailed analysis of their

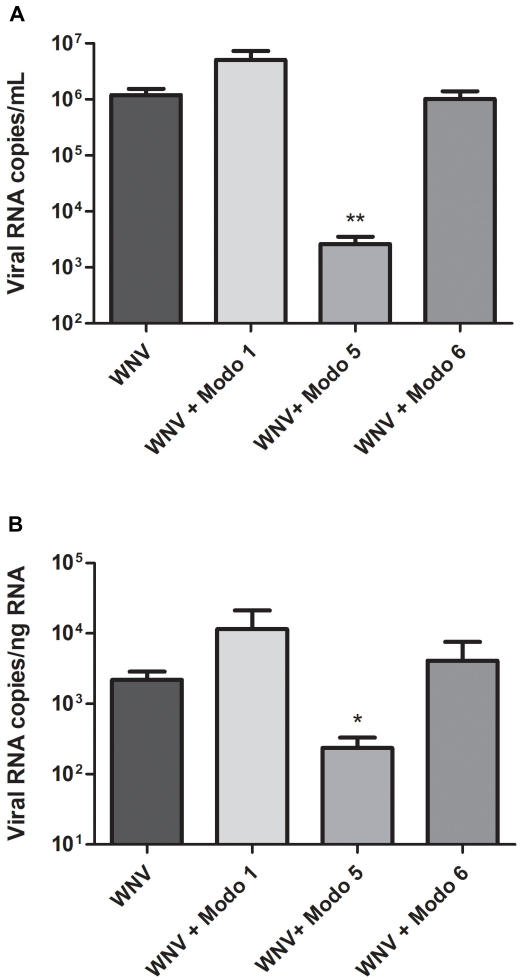

FIGURE 4 | West Nile virus replication in human primary keratinocytes treated with ΔModoCath1, 5, and 6. Primary keratinocytes were treated with the peptides at a final concentration of 16 μg/mL before being infected with WNV at a MOI of 0.1 for 24 h. Viral loads were determined in the cell supernatant (**A**, in log10 viral RNA copies/mL) and in cell lysates (**B**, in log10 viral RNA copies/ng total RNA) from infected keratinocytes. Data are represented as mean ± SEM of three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$, compared with the infected control without peptides. Modo 1, 5, and 6 indicate ΔModoCath1, 5, and 6, respectively.

biological activity, which is critical for the development of possible clinical applications, is still lacking. Here, we sought to address this shortfall by characterizing the AMP repertoire of the gray short-tailed opossum by *in silico* analyses, particularly for cathelicidins, which are greatly expanded in the species in comparison to eutherian species (4, 13). We determined the core sequences of eight opossum cathelicidins predicted to have strong antimicrobial activity. Three selected peptides, ΔModoCath1, 5, and 6, were demonstrated to possess potent antimicrobial activity with different antimicrobial spectra, including potent antiviral activity against WNV.

The antimicrobial activity of a single opossum cathelicidin, ModoCath4, has been previously demonstrated (14). Determination of the active core sequence for cathelicidin propeptides depends on the predicted enzyme cleavage site, secondary structures, cationic charges, and hydrophobicity (3, 28, 29). In this study, we slightly revised the core sequence of three opossum cathelicidins previously thought to have no antimicrobial activity after adjusting the neutrophil elastase cleavage site (14). Interestingly, we were able to detect antimicrobial activity against pathogenic bacteria for all three tested peptides, suggesting that the precise determination of the active domain sequence is critical to assess the activity of cathelicidins. These results suggest that the five untested sequences listed in **Table 1** may also show antimicrobial activities. Together with ModoCath4 from a previous study (14), antimicrobial activity has so far been confirmed for four opossum cathelicidins.

With the exception of certain marine organism AMPs that have been found to tolerate salt concentrations up to 450 mM, higher salt concentrations often interfere with AMP activity (43). Salt sensitivity is also observed in various antimicrobial peptides such as magainins, indolicidins, gramicidins, bactenecins (44). Notably, defensins have been reported to become inactivated in the presence of high salt concentrations (45, 46). In fact, studies have shown that lung infection by *P. aeruginosa* in cystic fibrosis patients is often related to inactivity of AMPs at the higher salt concentrations found in the lungs of these patients (46). For this reason, increasing the salt tolerance of AMPs to a minimum of 150 mM NaCl has been a goal of numerous studies (43, 47–49). Interestingly, the activity of ΔModoCath1, which had the highest net charge among the peptides used in this study, was potentiated in various salts at physiologic concentrations, while the bactericidal activity of ΔModoCath5 was negatively affected (**Supplementary Table S7**). Hence, the charge distribution and conformational stability appear to be important factors contributing to salt insensitivity (43, 47–49). Alternatively, the salt tolerance exhibited by ΔModoCath1 may have been attributed to the extreme positive net charge (+12) and structural stabilization by salts.

The antimicrobial properties of ΔModoCath1 and 5 were improved by a minimum 2-fold at solutions with a lower pH (**Supplementary Table S7**). This result is consistent with previous studies which reported that the bactericidal activity of AMPs is equal to or superior at lower pH than at a neutral pH (50). Particularly, His-rich peptides, such as clavanins which contain a number of histidines in place of the more common lysine or arginine residues, were potentiated at an acidic pH, reducing their microbicidal concentrations and shortening their killing times (51, 52). These enhanced bactericidal activities in acidic conditions is attributed to an increase in the positive charge of AMPs facilitating electrostatic interactions of peptides with anionic microbial surfaces. Our results suggest that ModoCaths, or their derivatives, may prove effective as treatment options for infections occurring in areas of the body with a physiologically acidic pH.

Therefore, the high level of antimicrobial activity elicited by ΔModoCath1 and 5 was due to the high net positive charge and amphiphilic α-helical conformation observed in CD spectroscopy (**Figure 1**). Although ΔModoCath1 and PMAP36

share only 42% sequence homology, they showed similar activity against *E. coli* (**Figure 1D**) which is likely due to the similar CD spectra for these two peptides upon LPS interactions, as well as their net charges (**Table 1**).

Further, ModoCath peptides induced blebs on bacterial surfaces similar to that observed following treatment with PMAP36 (**Figures 3A,E,F**). This phenomenon has been previously described for other AMPs with membrane-perturbing activity, such as magainin 2, temporin L and SMAP-29 (53–55). Additionally, the appearance of blebs has been reported as indicative of a given peptide's ability to destabilize the outer membrane of gram-negative bacteria following displacement of divalent cations that function to bridge and neutralize LPS (56). The results from SEM analysis are consistent with those of the membrane disruption assay (**Figure 2**).

Comparison of AMP gene numbers among eutherian and marsupial species indicated the extensive expansion of cathelicidins in marsupials during evolution (**Supplementary Table S9**). Our analysis identified 19, 7, 11, and 11 cathelicidin genes from the gray short-tailed opossum (*Monodelphis domestica),* tammar wallaby (*Macropus eugenii*), Tasmanian devil (*Sarcophilus harrisii*), and koala (*Phascolarctos cinereus*), respectively. This, in contrast to the presence of a single gene in humans and mice, suggests the importance of cathelicidins to marsupials, for instance to compensate for the lack of an adaptive immune system in neonate marsupials (8).

Using publicly available RNA-seq data (**Supplementary Table S2**), we detected the expression of eight cathelicidins–ModoCath1, 2, 4, 5, 7, 8, 16, and 18–from 5 tissues: placenta, lung, spleen, kidney, and Meckel's cartilage and anterior malleus (**Supplementary Table S10**). ModoCath4 and 8 in particular were expressed at higher levels than the others. The diversity of cathelicidin expression was highest in the spleen.

Although antiviral properties of cathelicidins have been less intensively studied than their bactericidal effects, several cathelicidins including LL-37, protegrin-1, SMAP-29, BMAP-27, and frog temporin have shown antiviral activity against several pathogenic human viruses (15–18, 57). To our knowledge, our study is the first to report that ΔModoCath5 possesses strong antiviral activity against WNV. Although we only tested its antiviral activity on WNV, ΔModoCath5 may also exhibit broad antiviral activity against other flaviviruses and, potentially, other enveloped viruses. The antimicrobial spectrum of ModoCath5 seems to be similar to that of LL-37 showing both antibacterial and antiviral activities. LL-37 acts either through direct inactivation of the viral particles or upregulation of the cellular antiviral response (15, 16, 58, 59). However, by contrast with LL-37, ΔModoCath5 displayed no direct virucidal effect at the tested concentrations (**Supplementary Figure S8**). Moreover, ΔModoCath5 did not modulate inflammatory mediator production in WNV-infected human primary keratinocytes (**Supplementary Figure S7**). Therefore, further research is still required to determine the mechanism of antiviral activity of ΔModoCath5 assessed during keratinocyte infection.

Despite great interests in the therapeutic use of endogenous AMPs and current progresses on characterization of new AMPs, including research on its potency and specificity toward pathogens, action mechanisms, and cytotoxicity to mammalian cells, our knowledge is still limited. Animals such as opossums harboring a large-sized cathelicidin repertoire could become an interesting model to study the systematic effect of cathelicidins. Considering the broad-spectrum bactericidal and antiviral activity, ΔModoCath5 could be an interesting candidate for therapeutic exploitation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank database under the accession numbers: XM_001381586.3, XM_007476942.1, XM_007474477.1, XM_007499675.2, XM_007499676.2, XM_007499719.2, XM_007499718.2, XM_003341720.3, XM_007499716.1, XM_007500054.2, XM_007500053.2, XM_007500052.2, XM_007499717.2, XM_007499678.1, XM_007505482.2, XR_001623701.1, XM_007499715.1, XM_007499714.1, XM_007499677.1, XM_007491824.2, XM_001372004.3, XP_003339699.1, XP_007485040.1, XP_001381623.1, XP_007477004.1, XP_007474539.1, XP_007474439.1, XP_007474434.1, XP_007474435.1, XP_007474436.1, XP_007474534.1, XP_007474533.1, XP_007474532.1, XP_007474437.1, XP_016283821.1, XP_007503439.1, XP_016281316.1, XP_007474984.1, XP_016281317.1, XP_016284649.1, XP_001381804.1, XP_007474950.1, XP_007474951.1, XP_007474952.1, XP_001381797.1, XP_007474444.1, XP_007474443.1, XP_007475462.1, XP_007475432.1, XP_016287018.1, XP_001374266.1, XP_016287021.1, XP_007475429.1, XP_016288248.1, XP_001372041.1, XP_007491886.1, XP_007499778.1, XP_007499740.1, XP_007499737.1, XP_007499738.1, XP_007499781.1, XP_003341768.1, XP_007499780.1, XP_007500114.1, XP_007500115.1, XP_007500116.1, XP_007499779.1, XP_007505544.1, XP_007499777.1, XP_007499739.1, XP_007499776.1, and XP_007505545.1. The raw data supporting the conclusions of this article will be available in **Supplementary Data** by the authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committees of Poitiers Hospital and Konkuk University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HC, CP, and CB designed and coordinated the study. HC, JY, AL, NL, QL, NS, and HJ performed all experiments to characterize ModoCaths. HC, CB, and CP analyzed the data and interpreted the results. JY and BA carried out bioinformatic analysis. HC, JY, CB, and CP wrote the manuscript. KH and J-HK provided administrative, technical, or material support. The final version of the manuscript was reviewed by all the authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00347/full#supplementary-material

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REVIEW
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An Update Review on the Paneth Cell as Key to Ileal Crohn's Disease

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The Paneth cells reside in the small intestine at the bottom of the crypts of Lieberkühn, intermingled with stem cells, and provide a niche for their neighbors by secreting growth and Wnt-factors as well as different antimicrobial peptides including defensins, lysozyme and others. The most abundant are the human Paneth cell α-defensin 5 and 6 that keep the crypt sterile and control the local microbiome. In ileal Crohn's disease various mechanisms including established genetic risk factors contribute to defects in the production and ordered secretion of these peptides. In addition, life-style risk factors for Crohn's disease like tobacco smoking also impact on Paneth cell function. Taken together, current evidence suggest that defective Paneth cells may play the key role in initiating inflammation in ileal, and maybe ileocecal, Crohn's disease by allowing bacterial attachment and invasion.

Keywords: Paneth cell, Crohn's disease, ileum, bacterial recognition, autophagy, endosomal stress, necroptosis

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INTRODUCTION

Crohn's disease was originally described and finally established (1) as a chronic ileal inflammation leading to strictures and finally resection of the involved segment. Over time it became evident that there was also a form of colonic Crohn's disease (2) and actually the disease may involve all parts of the gastrointestinal tract from mouth to anus. The respective localization is remarkably stable in a given patient whereas the disease behavior may advance from a mere inflammatory process to strictures as well as fistulas penetrating the gut wall (3). This categorization into ileal, colonic, and combined, usually ileocecal or ileocolonic localization also has a genetic background (4). Originally thought to be an autoimmune type of disease, the current view is that the immune response is directed against and induced by the intestinal microbiome and the gut inflammation is at least in part a collateral damage of this interaction (5). The separate localization types imply that if indeed Crohn's disease was characterized by a defective barrier toward intestinal microbes (6), the cellular and molecular basis of this defect was likely to be local and differ between ileal and colonic Crohn's disease.

One possible explanation may be provided by the Paneth cell which resides predominantly in the small intestine, although it may also be induced by inflammation as a metaplastic cell in other parts of the intestine, such as in the colon. The history of this cell (7) dates back to 1872 when it was first observed by Schwalbe in Freiburg but described in more detail in 1888 by Josef Paneth in Vienna (who actually quoted Schwalbe and showed one of his pictures). It took nearly a century to elucidate the function of Paneth cells: in an exhaustive study on the Paneth cell in gastrointestinal disease published in 1969 it was still speculated that the granules contained a kind of zymogen, possibly a peptidase and was therefore involved in digestion (8). Finally, lysozyme was detected in Paneth cells of the small intestine (9), compatible with their now established role in bacterial killing. However,

quantitatively and biologically the most important Paneth cell products are the antibacterial α-defensins, i.e., human defensin 5 (HD5) (10) and, to a lesser degree, human defensin 6 (HD6) (11). Apparently, the antibiotic peptides secreted form a chemical barrier preventing bacterial invasion and any defect in Paneth cell function may therefore compromise mucosal integrity. We therefore provocatively (and tongue in cheek) renamed this entity of ileal Crohn's disease as "Paneth's disease" (7) and ten years after it seems appropriate to look at the current state of the Paneth cell in Crohn's disease. Paneth cells are Janus-faced: they were given the title "maestros of the crypt" (12) but they may also be the culprits in Crohn's disease, hiding backstage behind the *T*-cells.

PANETH CELL AND DEFENSIN PHYSIOLOGY

Located at the base of the crypts of Lieberkühn, the Paneth cells appear to serve a dual function: support of the surrounding LGR5 positive stem cells and antibacterial secretion. The first and quite essential role is based on the local secretion of trophic factors supporting the stem cell niche of neighboring crypt base columnar cells, from which all other small intestinal cell lineages originate (13). The trophic factors include epidermal growth factor, transforming growth factor α and Wnt3. Coculture of Paneth cells with stem cells is 10 times more efficient in the formation of organoids than single type stem cell cultures. This suggests an important role of this interaction also *in vivo*.

The limited population of about 5–15 Paneth cells per crypt is under strict control by a complex net of differentiation factors, the most important being the Wnt-factor TCF4 (also known as TCF7L2) (14). TCF4 drives both a stem cell/progenitor gene program and a Paneth cell maturation program. Indian hedgehog is another important mediator, that is secreted by mature Paneth cells and forms a feedback loop down-regulating differentiation from Paneth cell precursors (15). Finally, during mouse Paneth cell development colony stimulating factor-1 is important (16) as well as other downstream mediators of Wnt (17).

Following differentiation, Paneth cell granule secretion into the crypt lumen is governed by cholinergic and bacterial factors (18), probably mediated by NOD2 (19) and TLR9 (20). TLR (toll like receptor) signals are transferred through MyD88, limiting microbial adherence and invasion through Paneth cell direct sensing and antibacterial secretions (21). Interestingly, as shown in organoids only the apical and not the basolateral surface of Paneth cells was responsive to lipopolysaccharide or live bacteria (22). However, even simple molecules like butyric acid or leucine may induce Paneth cell α-defensin secretion (23). Another factor involved, especially in maintaining the α-defensin precursor activating enzyme MMP7 in the starving mouse is mTOR (24). However, regulation may also be independent of microbiota such as lymphocyte derived interleukins which trigger Paneth cells to secrete antibacterial peptide, in this case angiogenin 4 (25). In addition, it was recently shown that also monocytes may induce Paneth cell defensins, probably via Wnt-factors (26). Others emphasized the key role of interferon signaling in Paneth cell function (27), thereby affecting microbial ecology (28). It is conspicuous that the Paneth cell also seems to be the main source of IL17 (29) as well as TNF, a major inflammatory cytokine and therapeutic target in the intestine (30).

Notably, Paneth cells produce a whole array of antibacterial peptides in addition to the α-defensins and angiogenin, including lysozyme as mentioned above but also lectins like RegIIIα in man or RegIIIγ in the mouse as well as type II secretory phospholipase A2 (12). Nevertheless, the key antibacterials are the two α-defensins (31), with different main modes of action. HD5 is a direct antibacterial and, if the human gene is "knocked into" a mouse, this will then change its commensal microbiome composition (32) and the mouse becomes resistant to Salmonella infection (33): thus the host defensins select its commensal microbiota but also protect against invaders. HD5 peptide in the intestine is unstable, however, and may be degraded by proteases into up to 8, 000 new antimicrobial peptide combinations which dramatically increase the host's ability to control pathogens and commensals (34). In contrast, HD6 is rather stable and predominantly acts by forming peptide nanonets inhibiting bacterial movement (35) rather than direct killing. Killing is only observed upon chemical reduction of the peptide (36), similar to HBD1 (37). It should be noted that these α-defensins are not only observed in the crypts and lumen of the small intestine but in the mouse are also transported intact from the small intestine to the colonic lumen, suggesting an impact also on the colonic microbiome (38). In this species α-defensins are called cryptdins also exhibiting strong bactericidal activity (39). However, their primary function likely is the prevention of bacterial migration through the ileocecal valve from the colon into and up the small intestine, resulting in about 1000-fold lower bacterial counts in the terminal ileum compared to the colon.

PANETH CELL FUNCTION IN CROHN'S DISEASE

In a first series of ileal Crohn's disease patients from Germany both ileal HD5 and HD6 were diminished compared to controls (40) whereas those with unaffected ileum and colonic disease exhibited a normal expression. In the colon enhanced expression of both α-defensins reflected Paneth cell metaplasia. In a second series of American and German patients combined low HD5-expression and protein in the affected Crohn's ileum was confirmed, and this finding was shown to be independent of the degree of tissue inflammation, whereas IL-8 was directly related to inflammation (41). Concomitantly antimicrobial activity of ileal mucosa was compromised and all other non-defensin antimicrobial peptides measured including lysozyme or phospholipase A2 were in the normal range. This suggested that the relative defensin deficiency was the key to defective antibacterial activity. However, other antibacterials like angiogenin (42) may also have important roles.

In further investigations this diminished Paneth cell defensin expression was linked to the Wnt system, in particular TCF4 (43), LRP6, and TCF 1 (44). As mentioned above, monocytes may activate Paneth cells, probably through Wnt factors but this mechanism was shown to be defective in monocytes from

Crohn's disease (26). Thus, there is a direct link between bone marrow derived and Paneth cells controlling the microbiome.

In a study from Australia low HD5 expression was confirmed but not independent of inflammation (45). The authors explained their findings by a loss of surface epithelium during inflammation, i.e., in ulcerated areas inflammation may indeed also affect the Paneth cell area. Avoiding problems of varying biopsy sites an English study quantitated HD5 in ileal effluents and found these to be reduced in Crohn's patients. This occurred without apparent inflammation compared to controls, but levels were particularly low if there was active disease (46). Moreover, HD5 in Crohn's disease gut lumen persisted in a complex of trypsin and chymotrypsin as well as in an immature precursor form, probably compromising its antibacterial activity. It is conceivable that the multiple proteolytic imbalances described in Crohn's disease affect the intraluminal degradation of HD5 mentioned above (33). In uninvolved Crohn's jejunum HD6 expression was diminished but not HD5 (47). More recently it was demonstrated that the HD5-gene showed a higher methylation status in Crohn's disease, regardless of inflammation, although the number of HD5 positive Paneth cells was normal (48). Thus, this apparently permanent gene methylation may be important in silencing the HD5 gene.

In an initial pediatric cohort both HD5 and TCF4 were low and correlated (49), whereas in another study of children with Crohn's disease only ileal TCF4 was diminished but not HD5 (50). Interestingly, in a very recent large study looking at a global pattern of ileal gene expression low HD5 expression was observed specifically in older children of 10 years age and above while younger children did not exhibit this decrease (51). Therefore, the authors suggested that this defensin deficiency may explain the rapid rise of IBD during puberty. Finally, also in pediatric patients, and independent of the genetic associations with Paneth cell defects discussed below, a phenomenon related to autophagy induced crinophagy was described specifically in ileal Crohn's disease (52). This was independent of inflammation and resulted in a significant decrease in the number of secretory granules. Taken together, despite some inconsistencies and remaining controversy, the current evidence, independent of the genetic studies discussed below, relates compromised Paneth cell function and even morphology to ileal Crohn's disease both in (older) pediatric and adult populations. However, to prove a primary role of such a defect, the genetic basis has to be clarified.

In addition to the changes in α-defensins, interesting observations suggest that HBD-3 peptide expression (but not mRNA) is actually increased in ileal Crohn's disease and it is relocated from the luminal surface and Paneth cell granules to the basolateral surface and the lamina propria (53).

THE PANETH CELL AND GENETIC LINKS TO CROHN'S DISEASE

NOD2 (nucleotide binding oligomerization domain 2) came into the focus since the revolutionary observation that single nucleotide polymorphisms in various genes are related to the risk of Crohn's disease, in particular ileal Crohn's disease (54, 55). This first and relevant link is an intracellular receptor for bacterial derived muramyldipeptide (MDP)and is expressed in several cell types including monocytes and, notably, the Paneth cell (19). After binding to MDP, NOD2 oligomerizes and binds to the serine-threonine kinase RIP2 and finally the complex mediates the signal to the IKK complex which then activates NFκB. Expression of NOD2 and the NOD2/RIP2 complex is enhanced in Crohn's disease (19, 56) and, somewhat paradoxically, NOD2 may actually suppress HD5 and HD6 formation in cultured Caco2 cells differentiated to Paneth like cells through action of FGF9 (57). On the other hand, MDP-NOD2 stimulation induced the defensin HNP-1 (human neutrophil peptide 1) in Caco-2 cells (58) and hBD2 (human ß-defensin-2) in several epithelial cells (59). In the latter study induction with a mutated NOD2 failed to induce HBD2. This fits the concept that the NOD2 mutations in Crohn's disease share a signaling defect, the most pronounced occurs in the frameshift mutation 1007*fs*. Quite strikingly, NOD2 is also a directly active antibiotic and this action is also compromised by these mutations (60). However, the relevance of this mechanism *in vivo* is unclear.

When ileal α-defensins were related to the NOD2 status of the patients, in a first study (40) their expression was particularly low in those with mutations. In a second study these results were confirmed in a different cohort and the most pronounced effect was noted in the patients with the frame shift mutation (41). This was not observed in an Australian study (45) and also not in the pediatric study comparing the older and younger children (47). On the other hand, in the ileostomy patients (46) HD5 levels in the effluent of NOD2 homozygotes and compound heterozygotes were the lowest observed in the cohort. Looking at Paneth cell morphology, Crohn's patients carrying at least two NOD2 mutations exhibited an increased number of abnormal granules in Paneth cells (61). Finally, following small bowel transplantation, with 35% of the patients possessing NOD2 polymorphisms, rejection was characterized by decreased expression of Paneth cell antimicrobial peptides in the NOD2 mutant recipients, prior to the onset of inflammation (62). Finally, it has been repeatedly demonstrated that the NOD2 genotype impacts on the ileal microbiome in Crohn's disease (63). It seems likely but is unproven that this alteration is mediated by defensins. Unfortunately, in experimental animals the findings are similarly controversial (64, 65) and NOD2$^{-/-}$ mouse organoids were not impaired in α-defensin expression (66). In contrast, in NOD2 deficient mice helicobacter hepaticus induced ileal granulomatous inflammation and this was reversed by transgenic expression of α-defensins in Paneth cells (67). Thus, NOD2 may well be important for Paneth cell defensin expression or secretion in mouse and man but the issue is not yet resolved.

Another risk gene identified in genome wide association studies is ATG16L1 (68) and this moved autophagy into the limelight. Autophagy is a process of degradation and recycling of cellular components, reducing cellular stress, but also of degrading bacterial components upon entry into the cell. It operates through the encapsulation of organelles and cytoplasm as well as bacteria within a membrane-bound organelle, termed the autophagosome (69). In a similar sequence of events to NOD2, next it was demonstrated that ATG16L1 and ATG5,

another autophagy protein, play key roles in intestinal Paneth cells (70). ATG16L1 and ATG5 deficient or defective Paneth cells in both mouse and man exhibited striking abnormalities in the granule exocytosis pathway. During an infection lysozyme may be rerouted via secretory autophagy as an alternative secretory pathway and this is also affected in the ATG16L1 mutated mouse (71). At the same time some injury signals like acute phase reactants and adipocytokines were enhanced. When combined with a murine norovirus there was enhanced pathology following administration of toxic dextran sodium sulfate (72). Finally, the group succeeded in introducing the defective human Atg16L1 T300A variant gene into the mouse and again observed abnormalities in Paneth, but also in goblet cells (73). In human epithelial cells the variant impaired autophagy of *S. typhimurium* (74). Most importantly, however, it was demonstrated in Crohn's disease patients that genetic variants synthesize to produce Paneth cell phenotypes of Crohn's disease: i.e., the granule defects were more pronounced it the patient carried multiple NOD2 and ATG16L1 risk genes (61). Moreover, high proportions of abnormal Paneth cells were associated with shorter time to disease recurrence after surgery. The additive action of these genes is not surprising because NOD2 recruits ATG16L1 to the plasma membrane at the bacterial entry site and mutant NOD2 fails in this regard (75). Quite surprisingly, in Japanese patients there was a similar number of defective Paneth cells as in American patients, but this phenomenon was related to LRRK2 rather than ATG16L1 polymorphisms (76). LRRK2 is known to help sort lysozyme in cooperation with NOD2 and is also suppressor of autophagy: both processes may affect Paneth cell morphology (77).

Next, the focus turned from autophagy in Paneth cells to endosomal stress. This leads to accumulation of unfolded proteins within the endoplasmic reticulum (ER) lumen and a response directed by the receptor inositol-requiring enzyme 1 (IRE-1) which double-cleaves mRNA for XBP-1 (X-box binding protein 1) synthesis (78). This splicing activates XBP-1 to induce the unfolded protein response and, if it fails, cellular apoptosis is induced. For example, ischemia/reperfusion or obesity may lead to ER stress but there is also evidence that inflammatory bowel disease mucosa is "ER-stressed" (79). Moreover, the group reported that XBP-1 knockout mice exhibit loss of Paneth and goblet cells, reduced antibacterial activity and spontaneous enteritis. To complete the picture, and similar to NOD2 and ATG16L1, there was a clear-cut genetic link of hypomorphic XBP-1 polymorphisms to IBD. In elegant studies with single or double ATG and/or XBP-1 knockout mice it was demonstrated that *both* pathways affect and partly compensate each other. The combination of these genetic defects in the single mouse at last established the Paneth cell as a site of origin for intestinal inflammation (80). Further evidence that both pathways are interlinked is based on the observation that defective ATG16L1-mediated removal of IRE1α drives Crohn's disease like ileitis in the mouse (81). Finally, both pathways are involved in interleukin-22 signaling (82), a classical epithelial-protective cytokine. Novel findings now suggest that IL-22 actually orchestrates a pathological endoplasmic reticulum stress response and may also have deleterious facets (83).

Other genetic links of relevant Paneth cell genes to Crohn's disease are KCNN4 (84) and the Wnt factors TCF4 (85) and LRP6 (86). In addition, an unbiased genetic screen may well unravel further links as demonstrated recently (76).

THE PANETH CELL AND NON-GENETIC LINKS TO CROHN'S DISEASE

Another important role in Paneth cell survival is played by caspase-8 which, if knocked out, induces TNFα-induced epithelial necroptosis and terminal ileitis. Its knock-out is also associated with lack of Paneth cells and reduced numbers of goblet cells (87). Accordingly, caspase-8 is essential to maintain intestinal barrier function and restrict pathogen colonization during *S. typhimurium* infection (88). Interferon lambda was recently shown to promote Paneth cell death in mice and is increased in inflamed ileal tissue in patients with Crohn's disease (89). Interestingly, glucocorticoids and tofacitinib, in current use in IBD, prevented Paneth cell death. Recently, it was described that also patients with inherited caspase-8 deficiency may develop intestinal inflammation but the role of caspase 8-genetics in Crohn's disease is not fully established (90).

An overarching factor affecting inflammatory response, amino acid metabolism, autophagy and also endoplasmic reticulum stress is ATF4 (activating transcription factor 4). Its levels were significantly decreased in inflamed mucosa of IBD patients and its deletion in mice was associated with diminished Paneth cell defensins (91). It should be emphasized, however, that although non-genetically deleted animal-models of terminal ileitis like the SAMP1/YitFc mouse also exhibit Paneth cell alterations (92), not all Paneth cell defects lead to spontaneous inflammation. In some models of ileitis the defective antibacterial system may be secondary to dysbiosis (93). In a very recent report, it was elucidated elegantly that even the Paneth cell specific knockout of prohibitin 1 triggers Paneth cell defects and ileitis in the mouse (94). Prohibitin 1 is not genetically linked to IBD but mitochondrial dysfunction and low levels of this mitochondrial protein have been observed. Interestingly, some species like the pig, not the cleanliest animal on earth, appear to perform quite well without Paneth cells.

THE PANETH CELL AND ENVIRONMENTAL RISK

In a recent review of metaanalyses several environmental risk factors for Crohn's disease were reevaluated and confirmed including smoking, antibiotic exposure, and vitamin D deficiency (95). All of these three factors impact on Paneth cell function and this link may represent a plausible mechanism of risk increase. For example, exposing mice to intragastric smoke condensate leads to alterations of ileal Paneth cell granules, antimicrobial peptide production and a reduction of bactericidal capacity (96). In Crohn's disease patients the combination of tobacco smoking and the ATG16L1 polymorphism combine to trigger Paneth cell defects and apoptosis (97).

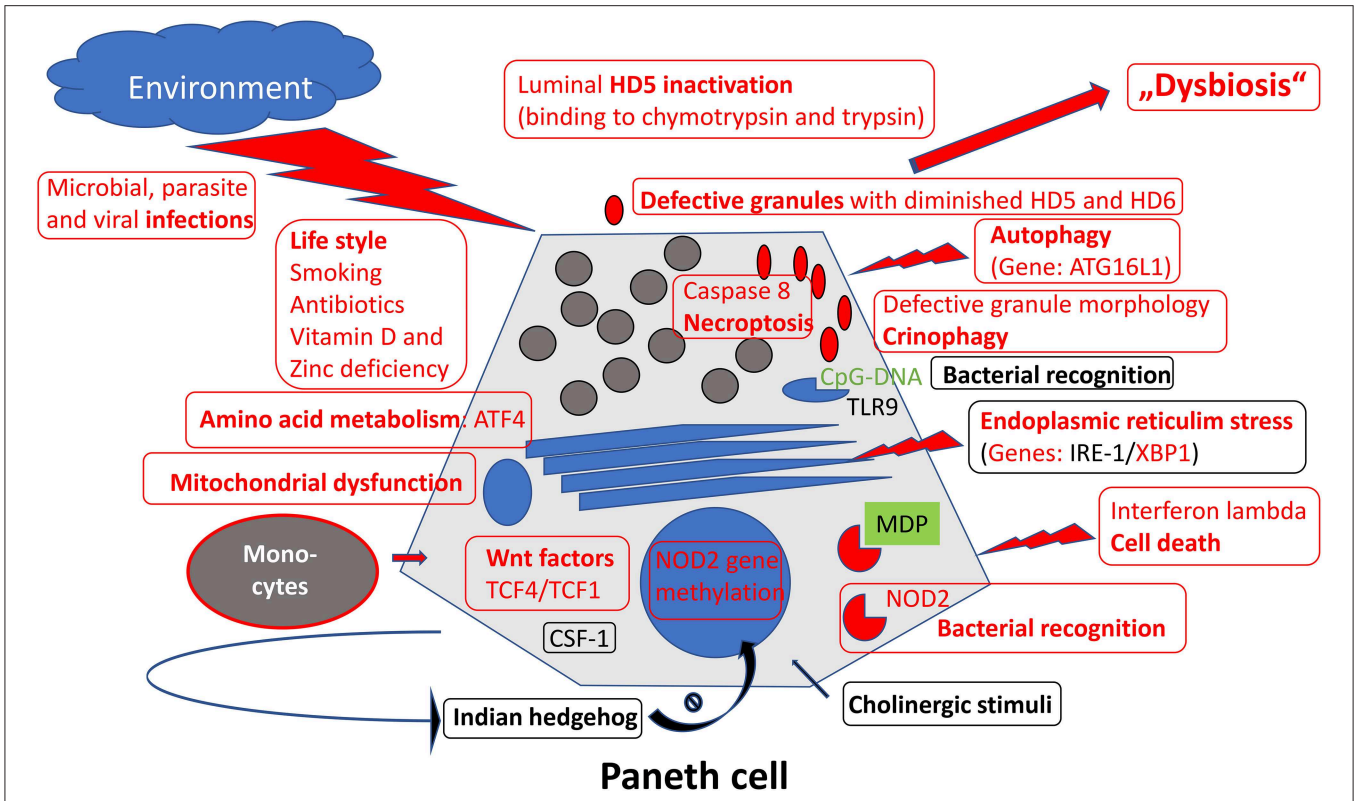

FIGURE 1 | Overview of mechanisms regulating Paneth cell function and morphology. Those defective in ileal Crohn's disease were labeled in red.

Acute antibiotic treatment is known to decrease the protein level of lysozyme and of RegIIIγ as well as the mRNA level of α-defensin 5 (98). However, the long-term effects of "earlier in life" antibiotic treatment are unknown and therefore the analogy to patients with antibiotics in childhood and later Crohn's disease is speculative. Also, in animal models vitamin D deficiency together with high-fat feeding reduces α-defensin 5 and its activator MMP 7, similar to vitamin D receptor knockouts (99). Obese individuals exhibit decreased jejunal levels of HD5 and lysozyme, whereas Paneth cell numbers were unchanged (100). Thus, Paneth cell problems are not necessarily specific for Crohn's disease. Finally, chronic ethanol feeding also reduced α-defensin 5 in the mouse intestine (101) and possibly zinc deficiency plays a negative role in this context (102). Remarkably, in some of these circumstances (99, 101) oral administration of HD5 reversed the pathological changes. However, at least alcohol consumption is not an established risk factor for Crohn's disease, whereas zinc and vitamin D deficiency may well occur.

Finally, the microbiome may play a major role because bacteria (103), Listeria and Salmonella in particular (104, 105), as well as parasites like toxoplasma (106) and even viruses (107) all interact with Paneth cell physiology. It is common that patients report on an episode of gastrointestinal infection prior to developing IBD but this is not, to the best of our knowledge, an established link. Innate host defense, of course, is opposed to these infections but also "sculpts" the local commensal microbiome (30): as a consequence, Paneth cell defects may induce dysbiosis (108–110). However, it still remains an open question whether this dysbiosis is the hen or the egg, or both, with respect to the inflammatory process (6, 111). A detailed discussion of these host vs. microbiome issues is beyond the scope of this review but it is quite conspicuous that adherent-invasive *E. coli* associated with Crohn's disease are resistant to both α- and ß-defensins (112).

CONCLUSION

After the first hints of a Paneth cell role in ileal Crohn's disease (19, 40), the Paneth cell as the key cell of defensin production in the small intestine proved to be an exciting focus of IBD-research, in recent years and in many respects. The various defects of this specialized cell in ileal Crohn's disease (**Figure 1**), in particular the (necessarily primary) genetic defects, have convinced many in the field that deficient defensins may represent one of the key events in triggering the disease (7, 113). The microbiome directed immune response and the stable localization over time is unlikely to be explained by a mere T-cell overresponse and, therefore, unlikely to represent an autoimmune disease (107). Future studies on the regulatory network of Paneth cells, maybe like those reported recently, using transcriptomics approaches may delineate additional complexity in these already remarkably versatile cells (114). Finally, and this is what counts for the patients: if the chance to substitute for mucosal defensins by systemic or oral administration, as

mentioned above, really works out, this originally unlikely hypothesis may lead to a promising new therapy both in Crohn's disease (115, 116) as well as in intestinal graft vs. host disease (117).

AUTHOR CONTRIBUTIONS

Both authors have made substantial, direct and intellectual contribution to the work and approved it for publication.

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Conflict of Interest: JW holds different patents on defensin treatments in different diseases including inflammatory bowel disease, asthma, and metabolic syndrome. ES declares that he serves as a consultant to Curevac, Tübingen, Germany.

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REVIEW
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Functional Insights From the Evolutionary Diversification of Big Defensins

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Big defensins are antimicrobial polypeptides believed to be the ancestors of β-defensins, the most evolutionary conserved family of host defense peptides (HDPs) in vertebrates. Nevertheless, big defensins underwent several independent gene loss events during animal evolution, being only retained in a limited number of phylogenetically distant invertebrates. Here, we explore the evolutionary history of this fascinating HDP family and investigate its patchy distribution in extant metazoans. We highlight the presence of big defensins in various classes of lophotrochozoans, as well as in a few arthropods and basal chordates (amphioxus), mostly adapted to life in marine environments. Bivalve mollusks often display an expanded repertoire of big defensin sequences, which appear to be the product of independent lineage-specific gene tandem duplications, followed by a rapid molecular diversification of newly acquired gene copies. This ongoing evolutionary process could underpin the simultaneous presence of canonical big defensins and non-canonical (β-defensin-like) sequences in some species. The big defensin genes of mussels and oysters, two species target of in-depth studies, are subjected to gene presence/absence variation (PAV), i.e., they can be present or absent in the genomes of different individuals. Moreover, big defensins follow different patterns of gene expression within a given species and respond differently to microbial challenges, suggesting functional divergence. Consistently, current structural data show that big defensin sequence diversity affects the 3D structure and biophysical properties of these polypeptides. We discuss here the role of the N-terminal hydrophobic domain, lost during evolution toward β-defensins, in the big defensin stability to high salt concentrations and its mechanism of action. Finally, we discuss the potential of big defensins as markers for animal health and for the nature-based design of novel therapeutics active at high salt concentrations.

Keywords: antimicrobial peptide, defensin, evolution, nanonet, host defense (antimicrobial) peptides

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INTRODUCTION

Host defense peptides (HDPs) comprise bioactive molecules produced by virtually all life forms. Initially characterized for their antimicrobial properties and accordingly named antimicrobial peptides (AMPs), they were described as peptides, usually cationic, which selectively target essential

microbial components (1). More than natural antibiotics, HDPs perform a wide range of both immune and non-immune functions (2). Although every species has typically its own repertoire of HDPs, molecular evolution has led to the convergence on a few highly successful structural scaffolds widely distributed in multicellular organisms. Defensins probably represent the most striking example of this process, as they are found in nearly all multicellular Eukaryotes, from fungi and spermatophyte plants to animals (both Protostomia and Deuterostomia) (3).

Defensins are gene-encoded disulfide-rich antimicrobial peptides (4). They are produced by various tissues according to species, and can be constitutively expressed or induced in response to different stimuli (infection, injury, inflammatory factors, etc.). Recent phylogenetic studies have classified defensins into two analogous superfamilies, namely *cis*-defensins and *trans*-defensins, that arose from different origins, but that underwent convergent evolution in terms of structure and function (5) (**Figure 1**). This classification is based on the spacing and pairing of the cysteine residues and the orientation of the peptide secondary structure. *Cis*-defensins contain two parallel disulfide bonds that stabilize the final β-strand to an α-helix (6). This folding is a key element in a 3D structure known as cysteine-stabilized α-helix/β-sheet (CSαβ) motif, which is shared by all *cis*-defensins as well as by plant trypsin inhibitors and scorpion neurotoxins (7). In *trans*-defensins, two disulfide bonds point in opposite directions from the final β-strand and stabilize different secondary structure elements (6). All *trans*-defensins share a conformational structure consisting of three anti-parallel β-strands stabilized by three disulfide bonds (8) but they adopt a diversity of 3D structures that do not systematically include α-helices (**Figure 1**). CSαβ-containing peptides from the *cis*-defensin superfamily may have six, eight or ten cysteines whereas *trans*-defensins contain six cysteine residues.

Cis-defensins are widely distributed across the fungal, plant and animal kingdoms. In contrast, *trans*-defensins have arisen and evolved exclusively in animals. Based on the disulfide bond arrangement of their six conserved cysteine residues and 3D structures, *trans*-defensins are subdivided into different families: α-defensins, β-defensins and big defensins (**Figure 1**). Aside from these families, a defensin with a cyclic peptide backbone was named θ-defensin; it is related to α-defensins and exists only in some non-human primates (9). α-defensins are peptides stabilized by the cysteine pairing $Cys_{1-6}Cys_{2-4}Cys_{3-5}$ (4), they were the first group of defensins to be described. Originally isolated from rabbit granulocytes in 1984 (10), they have only been identified in a few mammalians. β-defensins are peptides holding three intramolecular disulfide bonds paired as $Cys_{1-5}Cys_{2-4}Cys_{3-6}$. They occur from teleost fish to mammals and are considered as the oldest type of vertebrate defensin (11).

The last family of *trans*-defensins known as big defensins was isolated from the hemocytes, i.e., circulating immune cells, of the horseshoe crab *Tachypleus tridentatus*, an ancient marine chelicerate (Merostomata) (12). Big defensins are composed of a C-terminal β-defensin-like domain combined with a hydrophobic globular N-terminal domain (**Figure 2**). The *T. tridentatus* big defensin (*Tt*-BigDef) is stored in hemocyte granules (13) and displays antimicrobial activities and LPS-binding properties (12). Homologs of *Tt*-BigDef have been identified in bivalve mollusks (Bivalvia) and amphioxus (Cephalochordata) by molecular approaches (14–17).

With few exceptions, big defensin precursors are synthesized as prepropeptides in which a prodomain is located downstream of the signal sequence (15) (**Figure 2A**). This prodomain, whose function remains unknown, ends with a conserved dibasic site (either Lys-Arg or Arg-Arg), which is likely recognized by a furin-like peptidase during big defensin maturation, like other invertebrate AMPs (18, 19) (**Figure 2A**). Additional post-translational modifications (e.g., oxidation of disulfide bonds, C-termination amidation) give rise to mature big defensins (15).

Mature big defensins harbor a N-terminal hydrophobic region and a C-terminal region that contains six cysteines (**Figure 2A**). To date the horseshoe crab *Tt*-BigDef and the Pacific oyster *Cg*-BigDef1 are the only two big defensins for which a three-dimensional structure has been obtained (20, 21) (**Figure 2B**). Both molecules are highly soluble in solution. They are composed of two distinct globular domains connected by a flexible linker. Their hydrophobic N-terminal domain adopts a β1-α1-α2-β2 fold while their cationic C-terminal domain shows the cysteine pairing of β-defensins ($Cys_{1-5}Cys_{2-4}Cys_{3-6}$). The flexible linker is longer in *Cg*-BigDef1 than in *Tt*-BigDef, which determines a different orientation of the N- and C-terminal domains in each molecule (**Figure 2B**). Basic or dibasic sites (Arg-Arg or Lys-Arg) are found between the two structural domains of big defensins. The proteolytic cleavage of the native *Tt*-BigDef at this dibasic site, experimentally achieved (12), generated two fragments with distinct antimicrobial activities, as also observed for the two synthetic domains of *Cg*-BigDef1 (21). The covalent association of *Cg*-BigDef1 domains is synergistic and essential for salt-stable antimicrobial activity (21).

The discovery of big defensins has rekindled the discussion about the evolutionary history of *trans*-defensins (22). Both structural and phylogenetic studies have provided compelling evidence that big defensins could be the missing link in vertebrate defensin evolution, as an invertebrate big defensin gene has been hypothesized as the most probable ancestor of present-day β-defensins (5, 22). It is noteworthy that the N-terminal hydrophobic region is the hallmark of big defensins, a trait that was lost during the transition from basal chordates to their vertebrate relatives (22). In the subsequent sections we explore the taxonomic distribution and extraordinary diversification of the big defensin family in terms of sequence, tissue expression, gene regulation and mechanism of action. We discuss the functional meaning of the N-terminal domain conservation and translational insights that can be gained from a functional perspective.

PREAMBLE

In this review, we discuss the molecular diversity and biochemical properties of big defensin sequences subject of previous studies and deposited in publicly available repositories. However, to provide a more comprehensive overview of the taxonomic distribution of these HDPs, we extend our investigation to several large, but still unexplored phyla, for which genomic or taxonomic

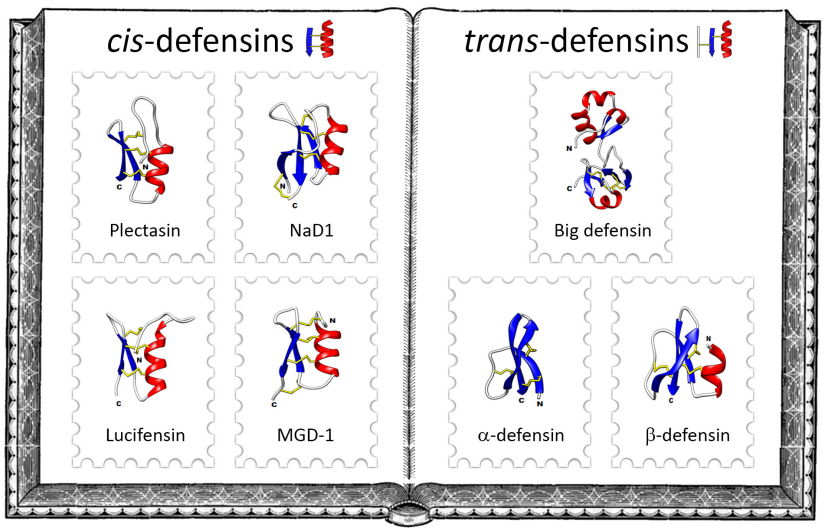

FIGURE 1 | The family album of defensins. Left-hand side of the album illustrates some classical *cis*-defensins: the fungal defensin plectasin from the ebony cup mushroom *Pseudoplectania nigrella* (PDB: 1ZFU), the plant defensin NaD1from the flowering tobacco *Nicotiana alata* (PDB: 1MR4), the insect defensin lucifensin from the green bottle fly *Lucilia sericata* (PDB: 2LLD) and the mollusk defensin MGD-1 from the Mediterranean mussel *Mytilus galloprovincialis* (PDB: 1FJN). Right-hand side of the album exemplifies some members of the main families of *trans*-defensins: the big defensin *Cg*-BigDef1 from the Pacific oyster *Crassostrea gigas* (PDB: 6QBL), the α-defensin HD5 (PDB: 2LXZ) and the β-defensin hBD-1 (PDB: 1IJV) from humans. Protein Data Bank (PDB) numbers are indicated in parentheses, α-helices in red, β-strands in blue and disulfide bonds in yellow.

resources are available. Consequently, while all the big defensin sequences described in this review derive from the screening of previously published sequence data, most of them had not been formally identified or described before. The big defensin sequences described in this article, with IDs and references, are reported in **Supplementary Table S1**.

Our approach was based on *in silico* data mining and exploited the conserved phylogenetic signal shared by all big defensins. In brief, known big defensin sequences were retrieved from the NCBI nr protein database and the redundancy of the dataset was reduced with CD-HIT v4.6.8 (23), based on a pairwise sequence identity threshold of 60%. The multiple sequences alignment obtained with MUSCLE (24) was used to generate a Hidden Markov Model profile for HMMER v3.3 (25). This profile was used to screen the genomes and transcriptomes of the species mentioned in the following sections based on an *e*-value threshold of $1E^{-3}$. In detail, gene annotations, whenever available, were used to obtain protein predictions from genomes, and TransDecoder v5.5.0 was used to virtually translate transcriptomes. tBLASTn[1] was used as a complementary tool for the identification of unannotated genes, using an *e*-value threshold of $1E^{-3}$. All retrieved hits were manually curated and the approach was re-iterated, by regenerating the HMM profile, until no new hits could be found.

The results here presented are largely dependent on the availability of -omic resources for the screening, on the completeness of the transcriptomes that we analyzed and on the quality of the genome assemblies and annotations. Therefore, our inference about the presence or absence of big defensins in a given taxa, as well as the estimates of the number of paralogous genes per species are subject to future update and revision.

[1]https://blast.ncbi.nlm.nih.gov/Blast.cgi

THE BROAD BUT DISCONTINUOUS TAXONOMIC DISTRIBUTION OF BIG DEFENSINS

Ecdysozoa (A Large Monophyletic Group of Invertebrate Animals Belonging to Protostomia, Which Undergo Molting, e.g., Arthropods, Nematodes, and Other Minor Phyla)

Although 25 years have passed since the initial discovery of big defensins in *T. tridentatus* (12, 13), horseshoe crabs (class Merostomata) still remain the only clade of arthropods where these HDPs have been formally described. Indeed, while orthologous sequences are expressed in the transcriptomes of the two other extant genera of horseshoe crabs, i.e., *Limulus* and *Carcinoscorpius*, no trace of big defensins has ever been found in insects, arachnids and crustaceans, in spite of the high amount of -omic data available. Based on the analysis of fully sequenced genomes, this consideration can be further extended to the Tardigrada, Nematoda and Priapulida, which points out a very narrow taxonomic distribution of big defensins within Ecdysozoa (**Figure 3**), the largest group of animals, with over 4.5 million estimated extant species (26).

The only other known case of peptides bearing a β-defensin-like cysteine array in Ecdysozoa is that of panusins, a family of

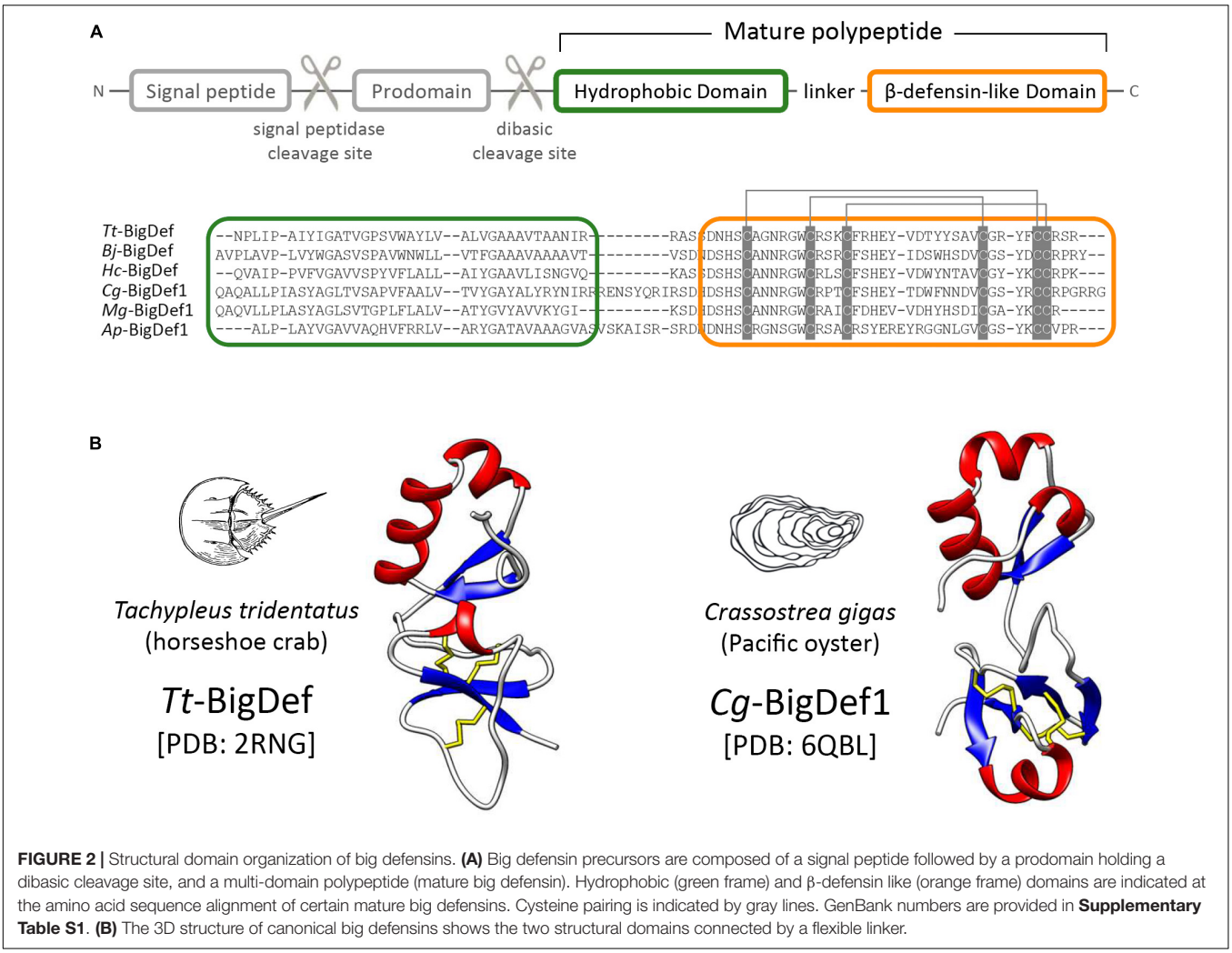

FIGURE 2 | Structural domain organization of big defensins. **(A)** Big defensin precursors are composed of a signal peptide followed by a prodomain holding a dibasic cleavage site, and a multi-domain polypeptide (mature big defensin). Hydrophobic (green frame) and β-defensin like (orange frame) domains are indicated at the amino acid sequence alignment of certain mature big defensins. Cysteine pairing is indicated by gray lines. GenBank numbers are provided in **Supplementary Table S1**. **(B)** The 3D structure of canonical big defensins shows the two structural domains connected by a flexible linker.

HDPs which have been first identified in a crustacean, the spiny lobster *Panulirus* spp. (27, 28). In spite of a significant primary sequence homology with the C-terminal domain of big defensins, panusins are completely devoid of the N-terminal region and more closely resemble the architecture of vertebrate β-defensins. A similar sequence was recently identified in another decapod crustacean, the lobster *Homarus americanus* (29).

Lophotrochozoa (A Large Monophyletic Group of Invertebrate Animals Belonging to Protostomia That Share the Lophophore Feeding Structure and the Trochophore Developmental Stage, e.g., Mollusks, Annelids, and Many Other Minor Phyla)

In stark contrast with the scarce number of reports in Ecdysozoa, big defensins have been found on multiple occasions in Lophotrochozoa. They have been described in nearly all lineages of Bivalvia (Mollusca), mostly including marine species of mussels (16), scallops (14, 30, 31), oysters (15), clams (32, 33) and ark shells (34), but also in a freshwater species belonging to the family Unionidae (35). While no big defensin has been formally reported in the other molluscan classes, the results of our screening suggest that the phylogenetic spread of these HDPs in Mollusca extends far beyond bivalves (**Figure 3**).

In spite of the relevant amount of -omic resources available for Gastropoda (which include over 80, 000 classified species of snails and slugs) (36), we could identify big defensins only in abalones and in a few snails, which suggests that these HDPs are likely to be present only in some (but not all) species. The existence of big defensins in cephalopods (e.g., octopuses and squids) is supported by both genomic and transcriptomic evidence: while the only sequence deposited in public databases is a mRNA expressed in the photophore of the squid *Pterygioteuthis hoylei*, we could identify unannotated big defensin orthologs in the genomes of *Octopus* spp. and *Architeuthis dux*. Moreover, a big defensin transcript was also detected in *Chiton olivaceus*, a species belonging to a minor molluscan class (Polyplacophora).

Very fragmentary information is available for the other lophotrochozoan phyla, most likely due to the limited -omic resources available and to the lack of efforts specifically focused

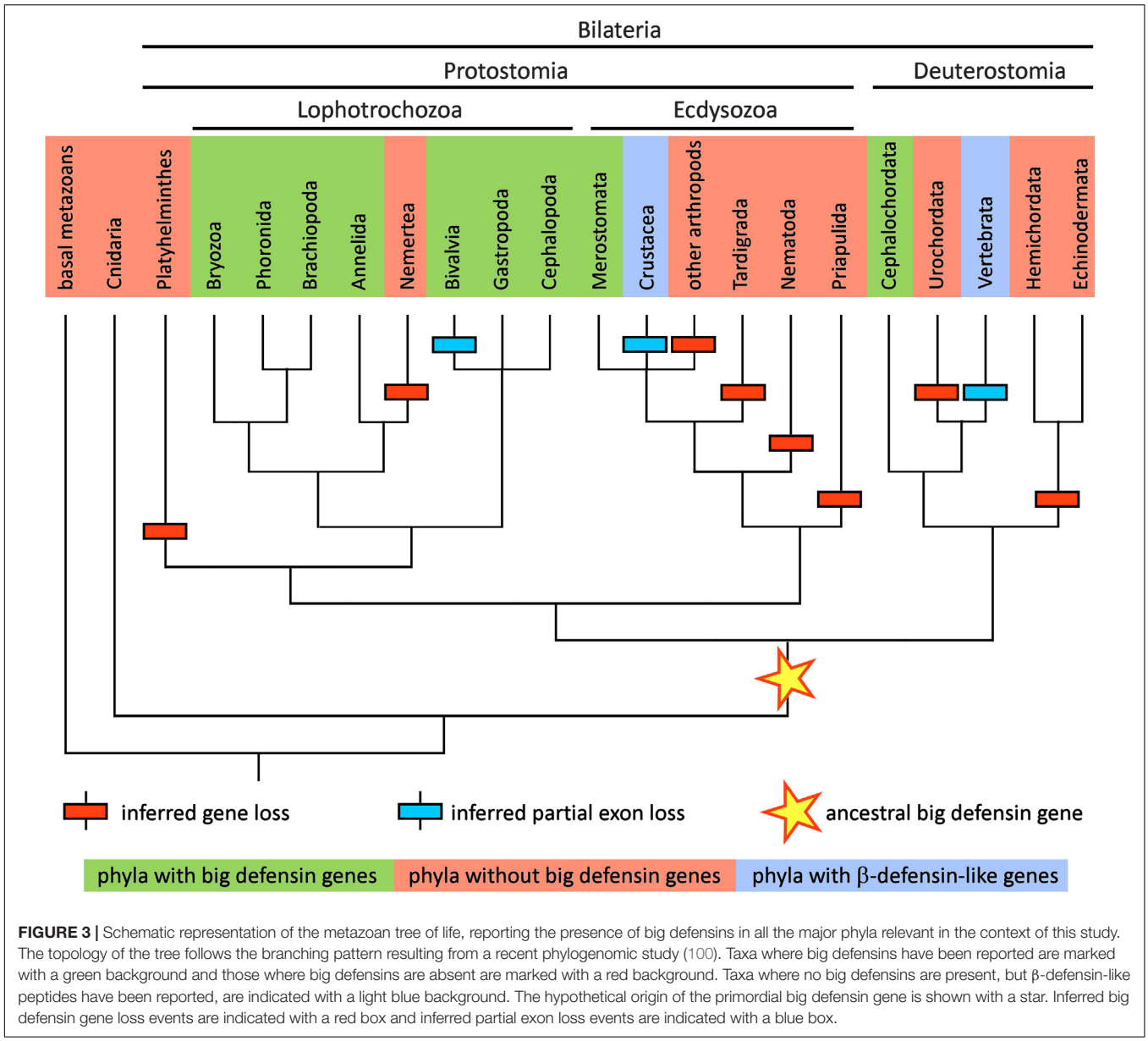

FIGURE 3 | Schematic representation of the metazoan tree of life, reporting the presence of big defensins in all the major phyla relevant in the context of this study. The topology of the tree follows the branching pattern resulting from a recent phylogenomic study (100). Taxa where big defensins have been reported are marked with a green background and those where big defensins are absent are marked with a red background. Taxa where no big defensins are present, but β-defensin-like peptides have been reported, are indicated with a light blue background. The hypothetical origin of the primordial big defensin gene is shown with a star. Inferred big defensin gene loss events are indicated with a red box and inferred partial exon loss events are indicated with a blue box.

on the study of AMPs in these organisms. Big defensins have been previously evidenced in Rhynchonelliformea, one of the three subphyla of the phylum Brachiopoda (37). Here we can also report the presence of big defensins in the transcriptomes of several other distantly related lophotrochozoan species, which include the bryozoan *Flustra foliacea*, two species of sabellid polychaetes (Annelida), and two congeneric species of phoronids. On the other hand, the genomes of many other lophotrochozoans, such as the annelids *Capitella teleta* and *Helobdella robusta*, or the ribbon worm *Notospermius geniculatus*, as well as the genomes of the early branching spiralian groups (e.g., Platyhelminthes, Rotifera and Gastrotricha) are completely devoid of big defensin genes, confirming the scattered distribution of these HDPs in metazoans (**Figure 3**).

Deuterostomia (i.e., the Sister Group of Protostomia, Characterized by a Different Embryonic Development. This Group Includes, Among the Others, Echinoderms, Tunicates, Amphioxi, and Vertebrates)

Among deuterostomes, big defensins have been only found in Cephalochordata (amphioxi, or lancelets). The cloning of a big defensin cDNA from *Branchiostoma japonicum* (17) finds full support in the presence of orthologous sequences in the genomes of the other cephalochordate species *Branchiostoma belcheri*, *Branchiostoma floridae* and *Asymmetron lucayanum*. On the other hand, big defensins are apparently present neither in Ambulacraria (Hemichordata + Echinodermata) nor in

Urochordata (**Figure 3**). Even though vertebrates do not display big defensins, they possess a related family of *trans*-defensins named β-defensins (38). These defense peptides underwent a remarkable diversification in vertebrates in which they spread from teleosts to mammals (39). Although they share an identical pairing of cysteines, they entirely lack the N-terminal region typical of big defensins.

BIG DEFENSINS, β-DEFENSINS AND PANUSINS: A SHARED EVOLUTIONARY ORIGIN?

The phylogenetic distribution of big defensins suggests that these sequences are monophyletic and derive from a primordial big defensin gene already present in the latest common ancestor of all bilaterian animals, before the speciation process that gave rise to protostomes and deuterostomes (**Figure 3**). However, the timing of the appearance of the first big defensin gene is presently unclear, since no big defensin or any other *trans*-defensin-encoding genes have ever been described in extant representatives of early branching metazoan phyla (e.g., Porifera, Cnidaria, Ctenophora, etc.). Nevertheless, the scattered distribution of these molecules in the animal phylogeny may seem counterintuitive and requires some explanation. Over long evolutionary timescales, gene death occurs with high frequency (40), contributing to animal genetic and phenotypic variation (41). The multiple independent rounds of lineage-specific gene contraction/loss events documented along metazoan evolution (42, 43) may be fully consistent with the discontinuous taxonomic distribution of big defensins (**Figure 3**).

A key question that remains to be answered is whether big defensins are evolutionarily related with vertebrate β-defensins and crustacean panusins, or the similarity in the disulfide array of these peptides is rather the product of convergent evolution. Zhou and Gao provided compelling evidence in support of a shared evolutionary origin for vertebrate β-defensins and invertebrate big defensins (22). Both gene types share a phase I intron (i.e., with the splicing site placed between the first and the second position of a codon) in a highly conserved position, at the 5′ end of the region encoding the C-terminal cysteine-rich region. The conservation of gene structure and intron phase are both considered important indicators of shared ancestry among distantly related genes (44). This is further supported by the recent release of horseshoe crab (*Limulus polyphemus*) and gastropod (*Pomacea canaliculata*) genomes in which big defensin genes share the very same highly conserved phase I intron (**Figure 4A**).

Most big defensin genes are characterized by the presence of three exons, with the coding region being split between the second and the third exon, as in the case of most bivalves and amphioxus (**Figure 4A**). However, several exceptions to this general and likely ancestral gene architecture exist. For example, the position of the initial ATG codon slid back into exon 1 in gastropods, leading to the creation of a phase 0 intron (**Figure 4A**). Moreover, the big defensin genes of horseshoe crabs and scallops display an additional intron (found in phase 0 and phase 2, respectively), which splits exon 2 in two smaller exons (**Figure 4A**).

Different genetic mechanisms may explain the divergent structure of the precursor peptides encoded by invertebrate big defensins and vertebrate β-defensins. Zhou and Gao (22) proposed two equally plausible alternative hypotheses to explain the loss of the N-terminal region in the vertebrate lineage: (i) partial intronization of exon 2; (ii) exon shuffling and combination of the 3′ exon, encoding the cysteine-rich module, with diverse upstream leader regions.

The genomes of two bivalve mollusks, the Manila clam *Ruditapes philippinarum* (45) and the zebra mussel *Dreissena rostriformis* (46), may represent cornerstones for understanding the molecular mechanisms behind the generation of genes encoding β-defensin-like peptides from a canonical big defensin gene. Indeed, both species display the simultaneous presence of canonical big defensins (carrying the N-terminal hydrophobic domain typical of this peptide family) and shorter non-canonical peptides (lacking this domain), which are characterized by large indels (∼40 amino acids) and resemble vertebrate β-defensins and crustacean panusins (**Figure 4B**) (33). As suggested by phylogenetic inference (see the following section), these two types of sequences are likely encoded by paralogous genes, ruling out the possibility of their origin by exon shuffling. The genetic mechanisms that led to the loss of the N-terminal region in *Ruditapes* and *Dreissena* are, however, largely different. In fact, the presence of a phase 1 intron and the contemporary presence of a short exon 2 in the Manila clam would be fully consistent with the intronization hypotheses proposed by Zhou and Gao (22) (**Figure 4A**). On the other hand, the non-canonical big defensin zebra mussel genes entirely lack intron 2 and therefore only display a single uninterrupted open reading frame, which is entirely embedded in the second exon (**Figure 4A**). This observation strongly suggests that the loss of the N-terminal region in *Dreissena* was not driven by intronization, but rather by the deletion of the genomic region comprising the 3′ end of exon 2 along with the entire intron 2, paired with the in-frame rejoining between the remnant part of exon 2 and exon 3. Unlike panusins in decapods and β-defensins in vertebrates, this evolutionary process acted on paralogous gene copies, maintaining the original canonical big defensin genes intact.

Altogether, these observations highlight that different genetic mechanisms may have independently originated β-defensin-like molecules using canonical big defensin genes as templates in vertebrates, crustaceans (i.e., panusins), bivalves and possibly other unexplored taxa.

INTER- AND INTRA-SPECIFIC SEQUENCE DIVERSITY: BIVALVES AS A CASE STUDY

Due to the abundant literature on big defensins and the good number of fully sequenced genomes available, bivalves represent an excellent case study for investigating the processes behind the remarkable primary sequence diversity observed, both between and within species (**Figure 5**).

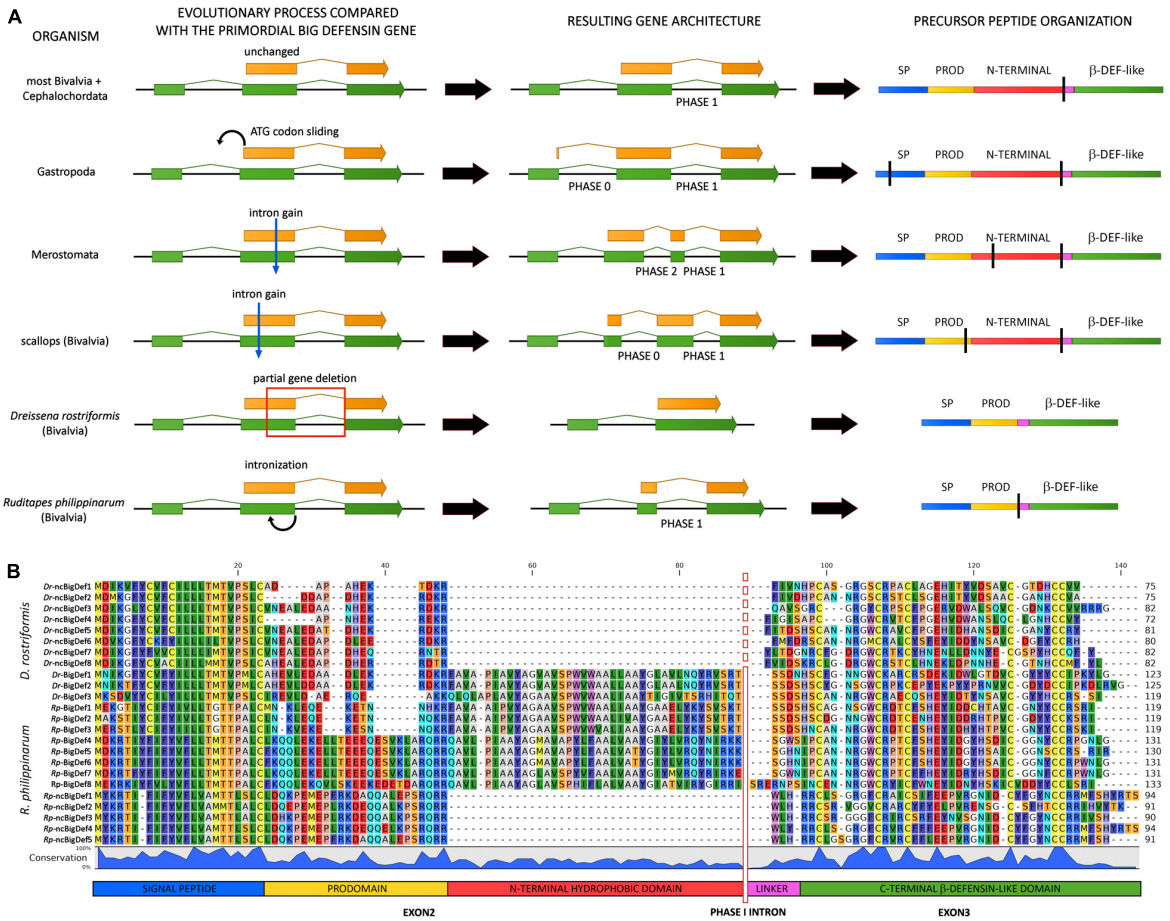

FIGURE 4 | Inferred evolutionary processes that may have led to the big defensin gene architecture observed in extant metazoan taxa. **(A)** mRNAs are indicated by green arrows and protein-coding regions are indicated with orange arrows. On the right-hand side, a schematic organization of the encoded precursor peptides is shown, including the signal peptide (SP), prodomain (PROD), N-terminal hydrophobic domain, linker and C-terminal β-defensin-like domain. Vertical black bars highlight the positioning of introns. **(B)** Multiple sequence alignment of the canonical (BigDefs) and non-canonical (ncBigDefs) big defensin peptides identified in the genomes and transcriptomes of *Dreissena rostriformis* and *Ruditapes philippinarum*. The organization of the main regions of the precursor peptides is shown at the bottom of the figure. The location of the phase I intron is indicated by a vertical bar (dashed in *Dr*-ncBigDefs, where it was lost). Note the large deletion of the N-terminal region which characterizes the non-canonical genes of both species.

Quite surprisingly, bivalve genome data support the presence of a highly variable number of big defensin genes per species, ranging from zero to several copies. In line with the hypothesis of multiple independent rounds of lineage-specific gene loss, a few bivalve species are completely devoid of big defensins (e.g., *Modiolus philippinarum*, *Sinonovacula constricta,* and *Lutraria rhynchaena*), or only show relict pseudogenes with in-frame stop codons (e.g., the pearl oyster *Pinctada fucata*). Other bivalve species, such different scallops, the deep-sea hydrothermal vent mussel *Bathymodiolus platifrons* and the ark shell *Scapharca broughtonii* only carry a single functional big defensin gene. In contrast, many bivalve species retain two or more potentially functional big defensin genes, such as the freshwater mussel *Venustaconcha ellipsiformis*, with two paralogous gene copies, and *R. philippinarum*, with four (two canonical and two non-canonical big defensin genes, respectively).

A particularly complex situation can be observed in oysters whose genomes usually bear multiple big defensin genes. Nine out of the ten genes found in the reference genome of the Eastern oyster (*Crassostrea virginica*) are found in two distinct clusters of tandemly duplicated genes located on chromosome 2, containing 5 and 4 genes each. In a similar fashion, the genome of the Sidney rock oyster *Saccostrea glomerata* is characterized by the presence of six tandemly duplicated big defensin gene models, organized in a single cluster. In both oyster species, the precursor peptides encoded by these gene clusters display a highly variable level of pairwise sequence identity, which ranges from over 90% to as low as ∼25%, suggesting very different timings for the underlying gene duplication events. While at least three different big defensin genomic sequences have been described in the Pacific oyster *Crassostrea gigas* (15), the recent release of novel genomic data suggests that the big defensin gene repertoire of this species may be even larger (47).

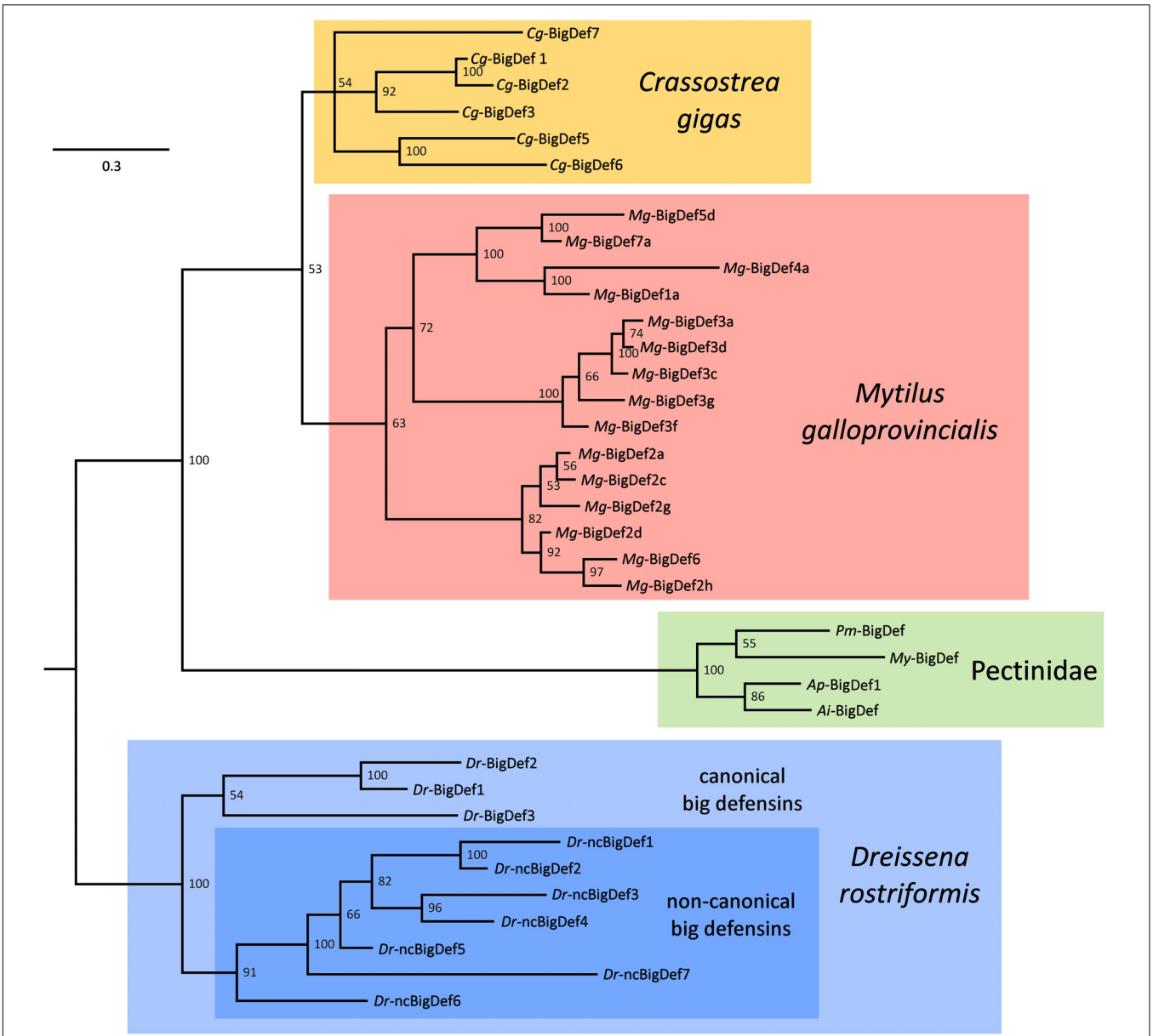

FIGURE 5 | Simplified phylogeny of bivalve big defensins, exemplified by variants found in the genomes and transcriptomes of the oyster *Crassostrea gigas* (*Cg*-BigDefs), the mussel *Mytilus galloprovincialis* (*Mg*-BigDefs), the clam *Dreissena rostriformis* (*Dr*-BigDefs and (*Dr*-ncBigDefs) and the four scallop species *Pecten maximus* (*Pm*-BigDef), *Mizuhopecten yessoensis* (*My*-BigDef), *Argopecten purpuratus* (*Ap*-BigDef1) and *Argopecten irradians* (*Ai*-BigDef). The tree was obtained through Bayesian inference using MrBayes v3.2.1, with two parallel MCMC analyses run for 500, 000 generations each, based on a VT + G model of molecular evolution. For simplicity's sake, variants sharing >90% pairwise identity have been removed. The tree was rooted on *D. rostriformis*, as the only member of the superorder Imparidentia. Posterior probability support values are shown for each node.

Another example of a bivalve species bearing multiple big defensin genes is the zebra mussel *D. rostriformis*, which only shows a single gene encoding a canonical big defensin (a second copy is a pseudogene), and four tandemly duplicated non-canonical genes. Finally, the genome of the Mediterranean mussel *Mytilus galloprovincialis* contains six paralogous big defensin genes, which are mostly scattered in different genomic locations and encode proteins with different levels of pairwise similarity (ranging from ∼45 to over 90%) (48).

Although this has not been established yet, recurrent gene conversion among recently duplicated paralogs may explain, at least to some extent, the high level of intraspecific sequence variation of big defensins, mirroring the case of some insect AMPs, like attacins and diptericins (49, 50). The intricate evolutionary scenario of bivalve big defensin genes can be only in part disentangled with the aid of phylogenetic inference. Here we present a highly simplified overview of the relationships between the sequences identified in the mussel *M. galloprovincialis*, in

the oyster *C. gigas*, in the freshwater mussel *D. rostriformis* and in four scallop species (**Figure 5**). Although some uncertainties remain due to the presence of some poorly supported nodes, the topology of the tree enables to assert that:

(i) All the different variants found in the same species appear to be monophyletic, suggesting an origin by independent species-specific gene family expansion events, driven by tandem gene duplication and, possibly, gene conversion among paralogs;
(ii) Gene duplication has often been followed by a fast process of molecular diversification, as evidenced by the high diversity of the variants found in *C. gigas*, *M. galloprovincialis* and *D. rostriformis*;
(iii) The magnitude of intraspecific big defensin sequence diversity often exceeds interspecific diversity, as highlighted by the comparison between the three aforementioned species and the four scallop orthologs;
(iv) The *D. rostriformis* canonical and non-canonical big defensin genes are monophyletic, which reinforces the hypothesis concerning the shared evolutionary origins of these HDPs;
(v) Altogether, these observations suggest that all bivalve big defensins have originated from a single ancestral gene, which was maintained in a single copy with little variation is some taxa (e.g., Pectinidae) or underwent repeated duplications and fast diversification in others.

The gene presence/absence variability (PAV) phenomenon (which indicates the presence of a gene in some, but not all the individuals belonging to the same species), adds a further layer of complexity to the highly dynamic genomic context outlined above. A growing body of evidence indicates that PAV is pervasive in some bivalve species, such as mussels, where it often targets HDP-encoding gene families (48). PAV most certainly shapes the individual repertoire of big defensins in *C. gigas*, as revealed by the patterns of presence/absence documented by PCR in 163 specimens (51) (**Supplementary Figure S1**). Although this situation would be potentially compatible with the presence of a single big defensin gene characterized by three highly polymorphic alleles (*Cg*-BigDef1, *Cg*-BigDef2, and *Cg*-BigDef3), the release of two complete genome assemblies (47, 52) revealed that the Pacific oyster, like the congeneric species *C. virginica*, most certainly holds multiple big defensin gene copies.

The data recently collected from the analysis of the *M. galloprovincialis* genome provide further data in support of the relevance of PAV in the context of big defensin intraspecific sequence diversity. Overall, a total of 33 unique variants, belonging to six sequence clusters (*Mg*-BigDef1, *Mg*-BigDef2/6, *Mg*-BigDef3, *Mg*-BigDef4, *Mg*-BigDef5, and *Mg*-BigDef7), were identified in 15 resequenced individuals. Although this categorization did not always allow to discriminate between paralogous genes and allelic variants encoded by the same genomic locus (e.g., up to four variants per cluster were found in some mussels), it allowed to ascertain that mussel big defensin genes are frequently subject to PAV (**Supplementary Figure S1**).

POLYMORPHISM OF BIG DEFENSIN EXPRESSION

Big defensins display highly different patterns of expression in terms of tissues and inherent array of genes in one species. According to species, big defensins are expressed in hemocytes or epithelia, tissues which play important roles in immunity. The expression of big defensins is specific to hemocytes in oysters (15) and horseshoe crabs (12). In contrast, in mussels (16, 35), scallops (30, 31) and clams (34), big defensins are mainly expressed in epithelial tissues (**Figure 6**); their expression in hemocytes is either undetectable or lower than in other tissues. Tissue-specific expression of big defensin genes is sometimes observed. For instance, in the Mediterranean mussel *M. galloprovincialis*, *Mg*-BigDef1, *Mg*-BigDef3, and *Mg*-BigDef6 are constitutively expressed in the digestive gland, gills and mantle, respectively (16). Therefore, it can be speculated that mussel big defensin genes carry distinct biological functions and control the host-microbiota homeostasis at the main epithelial surfaces. While the reasons behind the marked differences in tissue specificity observed among different species is still unknown, one possible explanation may be sought in the functional replacement with

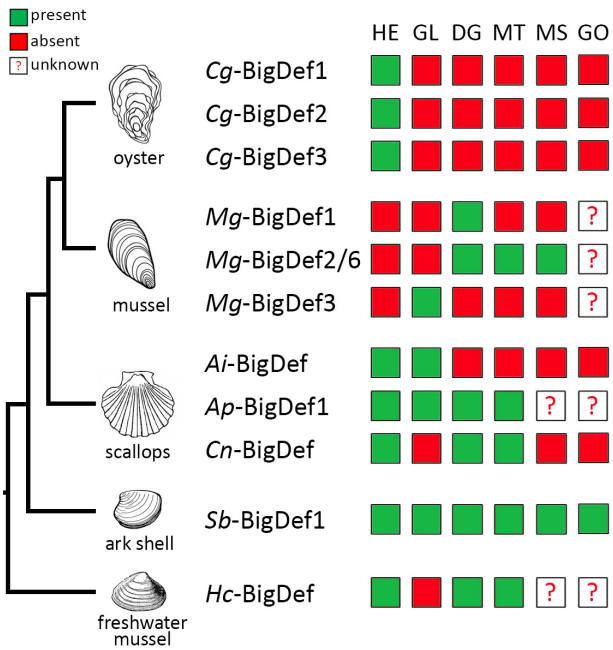

FIGURE 6 | Expression profiles of big defensin genes among different tissues from bivalve mollusks. Big defensin expression in hemocytes (HE), gills (GI), digestive gland (DG), mantle (MT), muscle (MS), and gonad (GO) tissues from the oyster *Crassostrea gigas*; the mussel *Mytilus galloprovincialis*; the scallops *Argopecten irradians, A. purpuratus,* and *Chlamys nobilis*; the ark shell *Scapharca broughtonii* and the freshwater mussel *Hyriopsis cumingii*, is represented in a color code schema. Green squares indicate detection of big defensin transcripts; red squares represent that the expression is undetected. Question mark indicates that the expression in those tissues has not been analyzed.

other AMPs, such as mytilins, myticins and invertebrate-type defensins in mussel hemocytes.

In addition, big defensin genes respond differently to environmental stimuli. For instance, in healthy *C. gigas* oysters, *Cg*-BigDef1 and *Cg*-BigDef2 are expressed at very low basal levels whereas *Cg*-BigDef3 is constitutively expressed, and only *Cg*-BigDef1 and *Cg*-BigDef2 are induced in response to bacterial challenge (15) (**Figure 6**), mirroring the case of other human β-defensins (53). While *Cg*-BigDef3 does not respond to bacterial challenge (15) its expression is repressed by OsHV-1 (Ostreid herpesvirus type 1) viral infection (54). These different regulatory patterns strongly suggest different roles in oyster immunity. In the ark shell *S. broughtonii* and the freshwater mussel *Hyriopsis cumingii*, *Sb*-BigDef1 and *Hc*-BigDef are expressed constitutively in different tissues and overexpressed in some of them after challenge (34, 35). In scallops, a single big defensin gene is expressed at low levels in hemocytes compared with other tissues such as mantle or gills, but it is overexpressed in hemocytes and epithelia after *Vibrio* challenge (30). A feature common to all big defensins is the lack of response to damage-associated molecular patterns, triggered by wounding or injection of sterile seawater.

Further supporting an important role in immunity, recent studies have shown that big defensins are regulated by the NF-κB/Rel pathway, as revealed by transcriptional knockdown of genes implicated in the pathway. Specifically, the silencing of *Cg*Rel expression in the oyster *C. gigas* (55) and the inhibitor of NF-kB transcription factor in the scallop *Argopecten purpuratus* (56) indicates the participation of NF-κB/Rel pathway in the regulation of big defensin expression.

In mussels and oysters, a third and important degree of variability in big defensin expression is introduced by PAV (see section above), with an extreme inter-individual variability in the expression of big defensin isoforms encoded by different genes and/or alleles (51).

Whether and how tissue-expression, gene-regulation and PAV affecting big defensins may impact the biology and ecology of mollusks remains to be determined. To better understand the functions of big defensins more research is now needed at a protein level. Until now this has been hampered by limitations in producing big defensins and, as a consequence, specific antibodies. Recently, in the scallop *A. purpuratus*, *Ap*-BigDef1 was localized not only inside hemocytes but also in the digestive gland, mantle and gill tissues from challenged scallops (30). With the recent developments to produce big defensins in large amounts (21), new perspectives are now open for understanding the biology of these HDPs, from tissue distribution to function across multiple species.

FUNCTIONAL CONSEQUENCES OF BIG DEFENSIN MOLECULAR EVOLUTION

An amazing feature of big defensin molecular evolution discussed in this review is their loss, pseudogenization and molecular evolution toward novel forms, including β-defensins, in diverse phyla (Ecdysozoa, Deuterostomia) (**Figure 3**), as opposed to their conservation and major diversification in Lophotrochozoa, particularly in mollusks.

Potential Trade-Offs Between Immune Function and Host Fitness

In species harboring the canonical big defensin structure (horseshoe crab, amphioxus, scallop and oyster), big defensins play immune functions: actually, they have antimicrobial activities at the physiological salt concentrations of their marine host, indicating they likely contribute to the defense against infections (12, 14, 17, 21). The oyster *Cg*-BigDef1 remains the only big defensin produced in sufficient amounts to establish a large activity spectrum (21). It shows a broad range of bactericidal activities against reference, environmental, and clinical strains, including strains multiresistant to antibiotics. Supporting further a role in controlling infections, *Cg*-BigDef1 is one of the few AMPs of *C. gigas* having significant antimicrobial activities against *Vibrio* species pathogenic for oysters (21, 57).

The loss of big defensins in several classes of Ecdysozoa and Deuterostomia suggests that the maintenance of these HDPs could be highly costly for their hosts. AMP gene loss and pseudogenization can result from a high fitness cost either because AMPs can damage host tissues or kill beneficial components of the host microbiota (58). As a consequence, for species exposed to low infection pressures, AMPs can be readily lost or accumulate mutations that compromise their function (58). This trade-off hypothesis is supported by the presence of a high number of pseudogenes in bivalves. In species where only β-defensin-like peptides but no big defensins are found, a likely hypothesis is an evolution toward other functions. In deuterostomes, this is exemplified by human β-defensins, which lack direct antimicrobial activity at physiological salt concentrations but act as key immunomodulators controlling infections (59). The ability of AMPs to carry multiple functions beyond antimicrobial is illustrated by myticins, another family of cysteine-rich peptides found in mussels (60, 61), or macins, CSαβ-containing peptides found in a number of invertebrates (62). The coexistence of both canonical big defensins and non-canonical peptides lacking the N-terminal region in some bivalve mollusks (*R. philippinarum* and *D. rostriformis*) may be indicative of an ongoing process of neofunctionalization.

We have earlier hypothesized that strong selection pressures imposed by marine environments may explain the scattered distribution of big defensins across animal species, mostly in marine species (21). Indeed, while most β-defensins are salt-sensitive (63), big defensins retain antimicrobial activity at high salt concentration and this property was assigned to the hydrophobic N-terminal domain lost during evolution toward big defensins (21). However, this view is partly questionned by our identification of canonical big defensins in different freshwater bivalve and gastropod species and by the observation that big defensins are absent in many large phyla of marine organisms (e.g., echinoderms and tunicates). As discussed in the previous sections, the current discontinuous distribution of big defensins may appear consistent with the massive genome reduction events that have led to the loss of several thousand

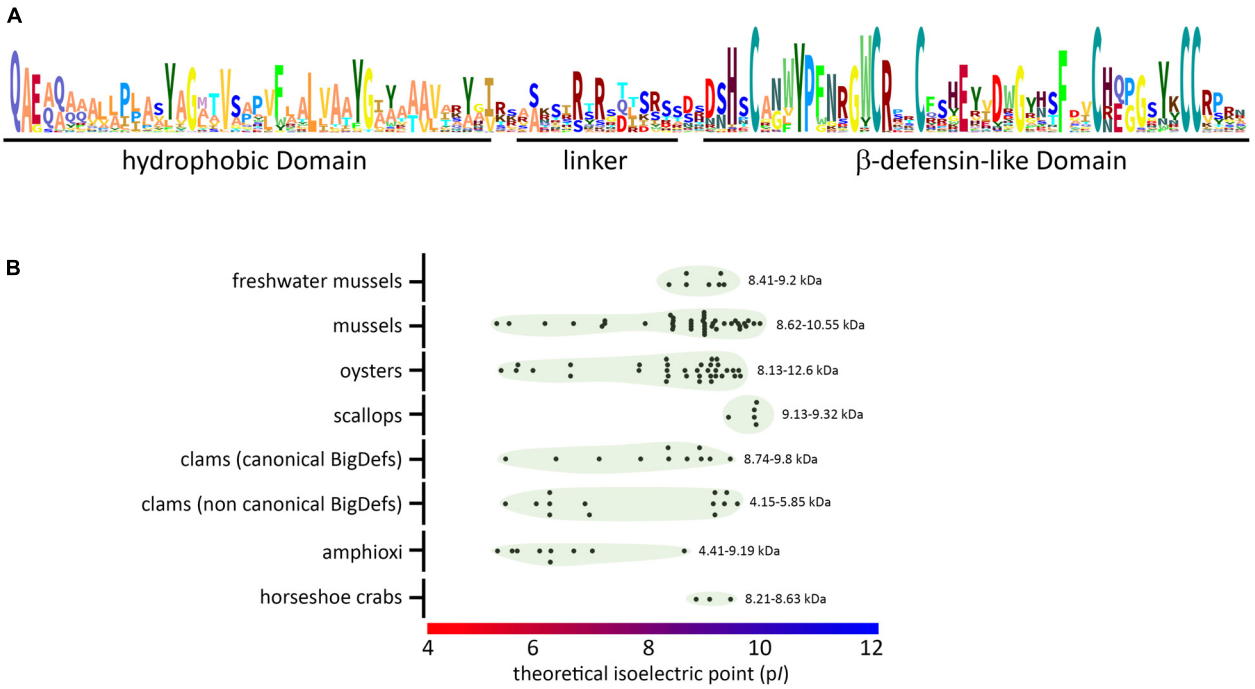

FIGURE 7 | Conserved molecular patterns and diverse biophysical properties of big defensins. **(A)** Schematic view (sequence logo) of the conserved amino acids found in big defensins. **(B)** Variability of isoelectric point (p*I*) and molecular weight (kDa) among big defensins from different taxa. Black dots represent single big defensin sequences from each taxon, and green circles indicate the dispersion of the p*I* values (indicated on the *X* axis). At the right side of each group, the range of deduced molecular weight is presented (kDa).

genes in multiple metazoan lineages (64, 65). At the same time, it is certainly noteworthy that, to date, no big defensin has ever been identified in terrestrial species. Therefore, while marine habitats cannot be considered as the only drivers of the retention of big defensins, it is likely that the evolutionary scenario we have highlighted in this review is the product of a combination between multiple ecological and evolutionary factors whose relative weight could be only addressed in the future though advanced phylogenomic profiling studies.

Conserved and Diversified Molecular Patterns in Canonical Big Defensins

The ancestral N-terminal domain preserved in canonical big defensins does not present any homology with known sequences outside this AMP family, questioning its role in big defensin mechanism of action. Remarkably, it has a well conserved sequence that retains hydrophobic properties (**Figure 7**), suggesting a similar function across big defensins. It has been suggested that the insertion of the N-terminal domain into membranes is involved in the antimicrobial activity of the horseshoe crab *Tt*-BigDef (20). However, such a membrane activity was shown to be uncoupled to the activity of oyster *Cg*-BigDef1. Instead, *Cg*-BigDef1 N-terminal domain drives bacteria-triggered peptide assembly into nanonets that entrap and kill *Staphylococcus aureus* (**Figure 8**). The hydrophobicity of this domain would be essential to nanonet formation and salt-stable antimicrobial activity (21). Such nanonets were earlier identified

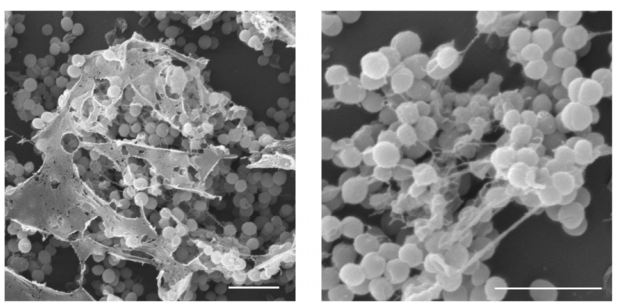

FIGURE 8 | Bacterially triggered assembly of *Cg*-BigDef1 into nanonets. Large and branched fibers entrapping *Staphylococcus aureus* are observed by scanning electron microscopy when *S. aureus* SG4511 is exposed to *Cg*-BigDef1. The same observations are made with *S. aureus* exposed to the N-terminal domain alone (21). Bars represent 3 μm.

for human α-defensin 6 (HD6) (66) and human β-defensin 1 (hDB1) (67). They were recently observed for the scallop *Ap*-BigDef1 (68). This indicates that this property is shared by different *trans*-defensins and among them distinct big defensins regardless of sequence diversity. It suggests a key role in their mechanism of action.

In canonical big defensins, sequence diversification is observed at different levels: (i) amino acid composition and (ii) length of the flexible linker that connects the two domains. Changes in amino acid composition strongly affect

the charge of the big defensins, which can vary from anionic to cationic (**Figure 7** and **Supplementary Table S1**), with potential consequences on interactions with microorganisms. However, up to now, there is no evidence that electrostatic interactions are involved in the mechanism of action of big defensins. More unexpectedly, surface properties of big defensins were shown to drastically vary with the length of the flexible linker that connects the two domains. Indeed, the linker length induces a different orientation of the N- and C-terminal domains (**Figure 2**). As a consequence, *Tt*-BigDef is amphipathic whereas *Cg*-BigDef1 is hydrophobic (21). This likely has major consequences on the interaction of big defensins with prokaryotic and eukaryotic cells. Linker length varies also significantly between big defensins at an intraspecific level (**Supplementary Figure S2**). The role of linkers in big defensin 3D structures and activities remains an important aspect to be explored.

It was hypothesized that antimicrobial activity of big defensins could be carried by their β-defensin-like domains, while the N-terminal domains would promote close contact with bacteria (21). This raises another important unsolved question. Are the two domains cleaved apart upon interaction with bacteria? Although big defensin cleavage has only been evidenced *in vitro* (12), it is likely that the basic and di-basic sites often present on big defensin flexible linkers (**Figure 4**) are accessible to bacterial proteases, which can trigger the release of the β-defensin like-domains at the close vicinity of the microbes. If so, the N-terminal domains of big defensins could serve as a cargo to release active and concentrated peptides on microorganisms, the specificity of which could be carried by the β-defensin-like domains and vary with the sequence diversity of these domains.

BIG DEFENSINS AT THE HOST-MICROBIOTA INTERFACE

As a compromise between effective defense responses and optimal host fitness, the AMP/HDP repertoire of a given animal species must inhibit or kill pathogenic microbes without seriously unbalancing the host-associated microbiota nor damaging host tissues (58). The evolutionary diversification of AMP/HDP genes and families disclosed so far in bivalve mollusks and available functional data indicate unexpected individual differences (44), multiple action modes (20, 53) and even the possible maintenance of isoforms or alleles coding poorly effective antimicrobials (69). In essence, healthy bivalves can be regarded as a dynamic assemblage of host-microbe interactions in which the host maintains tolerable amounts of symbionts or commensals and even opportunists or parasites (70). Environmental factors such as temperature and salinity changes, microorganism blooms and inappropriate farming practices can break such homeostasis, leading to diseases and death (71, 72).

The unique gene landscapes and expression profiles of big defensins and other AMPs support a tight control of microbiota in healthy oysters and mussels (3, 48). However, the impact of big defensins on the host microbiota has been poorly studied until now. Recently, the Ostreid herpesvirus type 1 (OsHV-1) was shown to suppress the expression of AMPs, in particular big defensins (both the inducible *Cg*-BigDef1/2 and the constitutively expressed *Cg*-BigDef3). This led to a fatal dysbiosis characterizing the Pacific oyster mortality syndrome (POMS) (54, 73). This is certainly the best indication that big defensins could be key players in the interaction with the host microbiota. In agreement, important microbiota changes were observed upon induction of big defensin in scallops (74), which further suggests a role for big defensins in host-microbiota homeostasis.

This view of big defensin peptides as key defense effectors in mollusks is consistent with data reported for other invertebrate AMPs that select species-specific microbiota (75), control the hemolymph microbiota (76) or control pathogenic infections (77).

TRANSLATIONAL INSIGHTS FROM BIG DEFENSINS

Big Defensins in Marker-Assisted Selection of Bivalve Broodstock

Either in the whole or as an archetypal gene family, species-specific AMP repertoires were shown to determine host aptitude for pathogen-resistance. This was recently illustrated in *Drosophila melanogaster* through experimental knockout of ten known AMP genes (78). Therefore, AMPs could serve as a proxy for the immune system competence in a marker-assisted selection of bivalve broodstock.

Individual phenotypes are shaped by complex gene-environment interactions and the host immune response is a metric (not dichotomous) phenotypic trait resulting from the action of several genes, each one subjected to multiple regulation levels, with specific alleles generating additive or non-additive genetic effects, such as dominance or epistasis. Following the hypothesis of polygenic and mostly additive genetic effects (79), a low heritability of the "response to infection" provided by a single defense molecule would not be surprising. Breeding programs in *C. gigas* produced oyster families with different (stable) levels of resistance to POMS (80, 81), triggered by the OsHV-1 μvar virus. Resistance was identified as heritable (80, 82) with some candidate markers having a role in distinct antiviral pathways (79, 83). Big defensins and other AMPs were not identified as associated with resistance to POMS, probably because the bacteremia comes as a secondary infection in this virus-induced immunosuppressive disease. Due to their potent activities against vibrios, it can be speculated that AMPs will rather arise as good resistance markers in disease where bacteria (e.g., *Vibrio aestuarianus*) are the primary infectious agents (84).

The mounting demand of support to fish and shellfish aquaculture requires knowledge-based solutions and oriented research work. Sequence diversity, salt-stable antimicrobial activity and gene presence/absence variation indicate bivalve

big defensins as key candidates to be considered among other AMPs/HDPs, in the assessment of host immune-competence for the genetic improvement of farmed stocks.

Big Defensin-Inspired Nanonets

Antimicrobial resistance is a major concern for public health worldwide (85). Antibiotics have been extensively used for decades, generating strong selective pressures on microorganisms. This has selected resistant genotypes that currently threaten the sustainability of the "modern medicine" (86, 87). Even though AMPs are often seen as possible alternatives to antibiotics and huge research efforts are made to isolate new AMPs (88), resistance and tolerance to AMPs, i.e., their capacity to survive a transient exposure to AMPs (89) are already well described phenomena (90–93). This highlights the need to develop new anti-infectives less prone to induce resistance (94).

The diversity of AMP structures and the multiple functions they harbor as HDPs offer a platform to design new drugs (95). A few examples in the literature have shown that HDPs from eukaryotic organisms show considerable advantage over antibiotics as they induce only limited resistance (low increase in mutation rate and horizontal gene transfer) (96). Over the past years, the immunomodulatory functions of AMPs/HDPs have been successfully exploited to develop effective anti-infectives with very limited risk to induce resistance (97).

With the recent discovery of nanonet formation as an indirect way by which AMPs/HDPs control infections by entrapping bacteria without necessarily killing them (21, 66), a new field is open to design new drugs. The present review illustrates that the hydrophobic N-terminal domain of big defensins, which carries the capacity to form nanonets upon contact with bacteria, has hydrophobic and nanonet-forming properties. Engineering of nanonet-forming AMPs, in which the N-terminal domain of big defensins is used as a cargo to deliver AMPs with different activities in close contact with microorganisms, is promising in many regards. Such multi-domain antimicrobials should provide an important advantage in terms of AMP resistance as (i) their activity depends on the nanonet formation rather the only interaction with a receptor, which can be easily mutated, and (ii) combination of peptides (cocktails) with multiple bacterial targets have already proved to limit resistance (98). It is also important as salt-stable AMPs are currently needed to treat infections associated to cystic fibrosis, a disease in which salt-treatment is used to control infections. It can be argued that nanonets (peptide aggregates) present a risk of having toxic effects, as known for amyloid fibers involved in ageing (Alzheimer's disease) (99). The current literature shows that nanonets are an evolutionary-conserved defense strategy in AMP families from highly divergent phyla (21, 66). It is likely that, if toxic, this defense mechanism would have been counter-selected. Moreover, no toxicity was observed for *Cg*-BigDef1 on human cell lines (21). For all these reasons, we believe that novel translational insights can be gained through the design of novel antimicrobials inspired by big defensins.

CONCLUSION

Big defensins have a complex and fascinating evolutionary history in the animal kingdom, with gene losses in many species of Ecdysozoa and Deuterostomia as opposed to major diversification in Lophotrochozoa. With two species where the canonical big defensins co-exist with shorter β-defensin-like peptides (non-canonical big defensins), this review highlights an ongoing evolutionary process and supports the hypothesis that β-defensins derived from big defensins. The massive expansion and diversification of β-defensins in Deuterostomia echoes to big defensin molecular evolution in Lophotrochozoa, which appears to be the product of independent lineage-specific gene tandem duplications followed by a rapid molecular diversification, with an additional layer of complexity provided by the PAV phenomenon. Like β-defensins, big defensins have therefore likely acquired novel functions, which remain to be uncovered. Key features conserved in canonical big defensins highlight the importance of the hydrophobic N-terminal domain, which drives the formation of nanonets and plays an important role in maintaining big defensin antimicrobial activity at high salt concentrations, an additional reason to consider them as animal health markers. Having maintained broad and salt-stable antimicrobial activities, and being active against multi drug resistant bacteria, the ancestral (canonical) structure of big defensins also inspires the nature-based design of novel therapeutics.

AUTHOR CONTRIBUTIONS

All authors have contributed to the writing of the review article, they agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, and they provided approval for publication of the content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00758/full#supplementary-material

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Defensins: A Double-Edged Sword in Host Immunity

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Defensins are a major family of host defense peptides expressed predominantly in neutrophils and epithelial cells. Their broad antimicrobial activities and multifaceted immunomodulatory functions have been extensively studied, cementing their role in innate immunity as a core host-protective component against bacterial, viral and fungal infections. More recent studies, however, paint defensins in a bad light such that they are "alleged" to promote viral and bacterial infections in certain biological settings. This mini review summarizes the latest findings on the potential pathogenic properties of defensins against the backdrop of their protective roles in antiviral and antibacterial immunity. Further, a succinct description of both tumor-proliferative and -suppressive activities of defensins is also given to highlight their functional and mechanistic complexity in antitumor immunity. We posit that given an enabling environment defensins, widely heralded as the "Swiss army knife," can function as a "double−edged sword" in host immunity.

Keywords: antimicrobial peptide, host defense peptide, defensin, innate immunity, *Shigella*, host–pathogen interaction

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INTRODUCTION TO HUMAN DEFENSINS

Defensins are a family of small (2–5 kDa), cationic host defense peptides with a β-sheet core structure stabilized by three conserved intramolecular disulfide bonds. The first mammalian defensin, also termed microbicidal cationic protein, was isolated in 1980 by Lehrer and colleagues from rabbit lung macrophages (1, 2). It was not until 1985 when the same lab discovered homologous peptides in human neutrophils did Lehrer coin the term defensin (3, 4) to describe disulfide-stabilized cationic peptides of mammalian origins with broad antimicrobial activity against bacteria, viruses and fungi. Based on disulfide topology, mammalian defensins are classified into three subfamilies, α, β, and θ-defensins (5–8). In humans, there exist only α and β-defensins. θ-defensins, with a unique circular structure stabilized by three parallel disulfide bonds in a ladder pattern, are only found in leukocytes of rhesus macaques (9). Although RNA transcripts homologous to the rhesus θ-defensin gene are found in humans, they contain a premature stop codon in the upstream signal sequence that abolishes their subsequent translation (10).

To date, six human α-defensins have been identified, which are further divided into two major classes according to their expression patterns and gene structures: myeloid defensins or human neutrophil peptides (HNPs) 1 to 4 and human (enteric) defensins (HDs) 5 and 6 (11–13). HNPs are stored in the azurophilic granules of human neutrophils, of which HNPs 1–3 and their much less abundant fourth cousin HNP4 account, collectively, for 5–7% of the total

neutrophil protein (4, 14, 15). HNPs-containing granules normally undergo restricted secretion and are commonly directed for fusion with phagolysosomes, where high concentrations of HNPs directly kill phagocytosed microbes (16, 17). Upon holocrine secretion and neutrophil infiltration during inflammation, HNPs are released into the extracellular milieu through degranulation of activated neutrophils (17–19). HD5 and HD6 are constitutively expressed in and secreted by Paneth cells at the bottom of the small intestinal crypt (12, 13, 20, 21). While the concentration of HD5 at the luminal surface of the small intestine is estimated to be as high as 50–250 μg/ml, it is significantly lower at the colonic mucosal surface due to the distance from secretion (21). HD5 ranging from 1 to 50 μg/ml is also found in vaginal fluid from healthy women (22) and induced in the male and female reproductive tract in response to sexually transmitted infections (STIs) (23–25). Although more than 30 β-defensin genes exist in the human genome, only a few have been extensively characterized at the genomic and functional levels (26). Unlike α-defensin expression, which is commonly regulated at the level of secretion, β-defensin expression is transcriptionally regulated and restricted to keratinocytes of the skin and epithelial cells. For instance, while human β-defensin 1 (HBD1) is constitutively expressed, HBD2 and HBD3 are induced by microbial insults and pro-inflammatory cytokines in various epithelial and mucosal tissues (27, 28).

Since their first discovery in the early 1980s, defensins have been intensively investigated for their broad antimicrobial activities and multifaceted immunomodulatory functions under both physiological and pathogenic conditions. Many excellent reviews have shed light on a multitude of sophisticated molecular and cellular mechanisms by which defensins act against bacteria, viruses and fungi and function as pleiotropic immune effectors in inflammation, development and cancer (5, 11, 26, 29–34). By and large, defensins are heralded as the "Swiss army knife" in innate immunity against microbial pathogens. Nevertheless, accumulating recent evidence has unveiled a potential pathogenic role defensins play in host-pathogen interactions and tumorigenesis, indicating that the mechanisms of action of defensins are far more complex than previously thought. The growing recognition that defensins can be both advantageous and detrimental, depending on their spatial-temporal settings, gives us the impetus to review the recent literature on their protective and pathogenic roles in health and disease.

DEFENSINS IN VIRAL INFECTION

Defensins directly inactivate and inhibit the replication of a variety of viruses, and their multifaceted mechanisms of action have been elucidated (30, 31); the underlying mechanisms of the role of defensins in host-virus interactions are more complex as evidenced with HIV-1 (**Figure 1**). Early studies demonstrated that defensins are able to target multiple steps of host-virus interactions to reduce the infectivity of both enveloped and non-enveloped viruses. HNP1–3, HD5 and retrocyclins 1 and 3 deduced from human θ-defensin pseudogenes effectively block adhesion of enveloped herpes simplex virus 2 (HSV-2) to host cells by preventing HSV-2 gB interactions with its receptor HSPGs (35–37). Defensins also inhibit fusion of virions of several enveloped viruses with their host cells. Retrocyclin 2 and HBD3 interfere with viral fusion mediated by influenza virus hemagglutinin (HA) and other viral proteins such as baculovirus gp64 and Sindbis virus E1 protein (38). While HNP1 is well recognized for its direct anti-HIV activity (39, 40), it also restrains HIV-1 uptake by inhibiting Env-mediated viral fusion and downregulating host cell surface expression of CD4 and coreceptor CXCR4 (41), a controversial mechanism for HBD2 and HBD3 inhibition of HIV-1 infection (42–44).

Post-entry inhibition of viral infection by defensins has been observed with several families of non-enveloped viruses, notably HPV (45). Without affecting the binding and entry steps, α-defensins effectively block intracellular uncoating of HPV and its escape from cytoplasmic vesicles by stabilizing its viral capsid structure to prevent interactions of viral proteins and genome with host factors essential for productive infection (45–49). This general inhibitory mechanism has been verified for other non-enveloped viruses such as human adenovirus (HAdV) and JC polyomavirus where α-defensins stabilize viral capsid proteins, thus diminishing subsequent intracellular infection (50–54). Of note, post-entry inhibition of enveloped viruses such as HIV-1 and influenza by HNP1 is mediated through interfering with cell signaling pathways such as PKC that are required for viral replication (39, 55).

More recent studies, mostly by the same research groups who demonstrated the beneficial role of defensins in controlling viral infection, unveil infection-promoting effects of defensins in HIV-1 and certain serotypes of HAdV infections (25, 51, 56–60). Chang and colleagues reported that HD5 and HD6, induced by *Neisseria gonorrhoeae* infection in a cervicovaginal tissue culture system, increase HIV infectivity in a CD4- and HIV coreceptor-independent manner (25). HD5 and HD6 promote HIV infection by acting on the virion to enhance viral attachment to its target cells (57). These defensins antagonize anti-HIV activity of polyanion microbicide candidates that block HIV entry (56). HNP1, the prototypic α-defensin extensively studied for its multifaceted anti-HIV activity, is also capable of disrupting epithelial integrity to promote HIV traversal across epithelial barriers, thus facilitating viral infection and dissemination (60). These findings by the Chang group are of particular interest since increased HNP1 and HD5 expression in the genitourinary tract upon STIs could potentially generate sufficiently high concentrations of defensins to enhance HIV-1 infection under physiological conditions. Other examples regarding the enhancing effect of defensins on enveloped virus infection have been reported. For example, cryptdin 3, one of several mouse α-defensins expressed in the small intestine (61) also enhances HIV infection *in vitro* presumably by facilitating viral entry (58). A recent study shows that an alphaherpesvirus, equine herpesvirus type 1, is resistant to equine β-defensins 2–3, which inhibit bacteria and viruses, and exploits these defensins to invade the host for viral spread (62).

HNP1- and HD5-promoted viral infection has also been observed with certain serotypes of HAdV as reported by

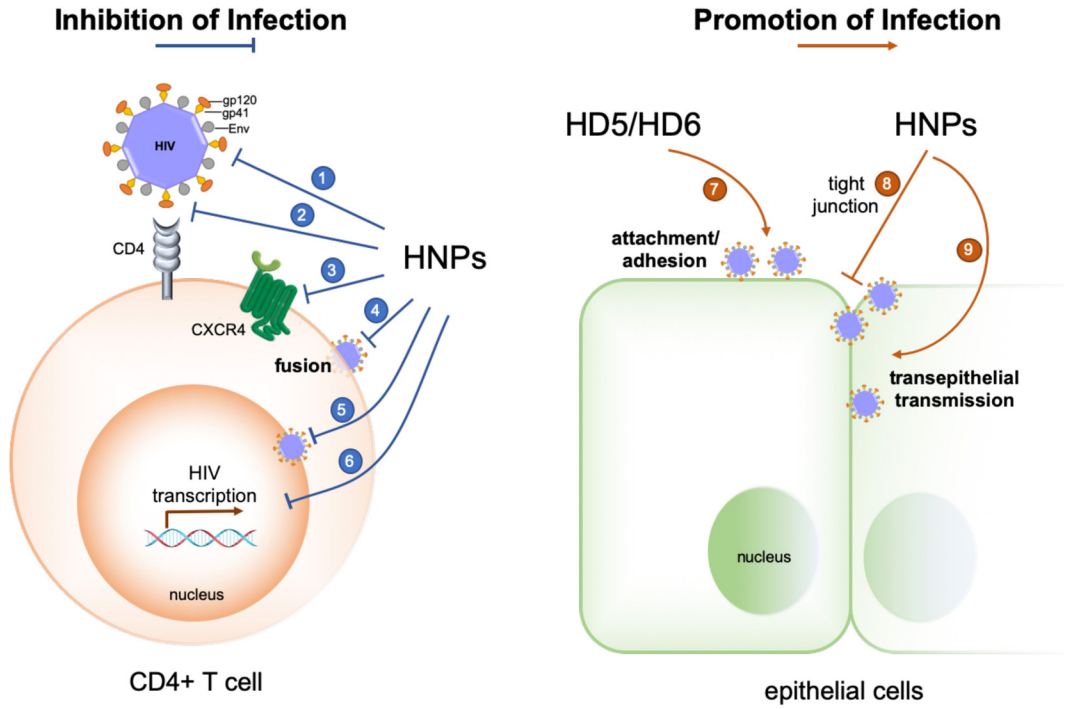

FIGURE 1 | Opposing effects of human α-defensins on HIV-1 infection. Infection-inhibiting mechanisms include: (1) direct inactivation of the virus (39, 40), (2) blockade of gp120-CD4 interactions (164, 165), (3) coreceptor downregulation (41, 166), (4) inhibition of gp41- and Env-mediated viral fusion (41), (5) inhibition of nuclear import of viral RNA (30, 39), and (6) suppression of HIV transcription (30, 39, 167). Infection-promoting mechanisms include: (7) enhancing viral adhesion/attachment (25, 56, 57, 59), and (8, 9) disrupting tight junction to promote trans-epithelial transmission of HIV (60).

the Smith group (51), who previously deciphered the capsid-stabilizing mechanism of defensins against HPV and HAdV and delineated their structural determinants of antiviral activity (46–54, 63). As is the case with HIV-1, HNP1- and HD5-dependent enhancement in infection by HAdV-D and -F correlates with increased viral attachment to target cells independently of receptor binding (51). To address the physiological relevance of defensin-enhanced adenovirus infection, Smith and colleagues utilized a murine enteric organoid (enteroid) to examine the impact of naturally secreted cryptdins on the infectivity of an enteric mouse pathogen, mouse adenovirus 2 (MAdV-2). MAdV-2 infection increases in the enteroids expressing mouse α-defensins but not in the ones devoid of them (64). This *ex vivo* study demonstrates that α-defensin-enhanced viral infection occurs not only in traditional cell cultures, but also under physiologic conditions.

DEFENSINS IN BACTERIAL INFECTION

Defensins are capable of killing bacteria or inhibiting bacterial growth through a multiplicity of antimicrobial mechanisms such as direct membrane disruption (11, 65, 66) and inhibition of bacterial cell wall synthesis (67–69). Defensins can also reduce bacterial infection by neutralizing secreted toxins (70–73). In general, human α-defensins are less cationic but more hydrophobic than β-defensins, and they can differ mechanistically in the killing of bacteria (11). While HBD1 and HBD2 are active preferably against Gram-negative bacteria (74), their significantly more cationic counterpart HBD3 is potently bactericidal against both Gram-positive and -negative strains (75). Due to its heavily cationic nature, HBD3 broadly kills bacteria in a structure-independent manner (76, 77). Notably, disulfide reduction of the weakly bactericidal HBD1 turns it into a potent antimicrobial peptide against opportunistic pathogenic fungi and Gram-positive commensal bacteria (78). Excellent reviews on the antifungal activity of defensins are also available (79, 80). Our review focuses on the role of human α-defensins in host-bacteria interactions to contrast their protective and pathogenic functions.

Bevins and colleagues demonstrated that HD5-transgenic mice are markedly resistant to oral challenge with virulent *Salmonella typhimurium*, consistent with the antibacterial activity of HD5 *in vitro*, whereas wild-type mice are susceptible to infection (81). An *in vivo* protective role against *Salmonella* infection is also illustrated for mouse intestinal α-defensins or cryptdins (82). Of note, enteric HD6, while exhibiting little bactericidal and membranolytic activity *in vitro*, protects mice from *Salmonella* infection by entrapping bacteria with a unique self-assembled "nanonets" structure to preclude the pathogen's direct contact with the intestinal epithelium (83).

HNP1–3 secreted by infiltrating neutrophils in *Staphylococcus aureus* infection induce TNF-α and IFN-γ release from macrophages, which, in turn, increase phagocytosis of pathogens

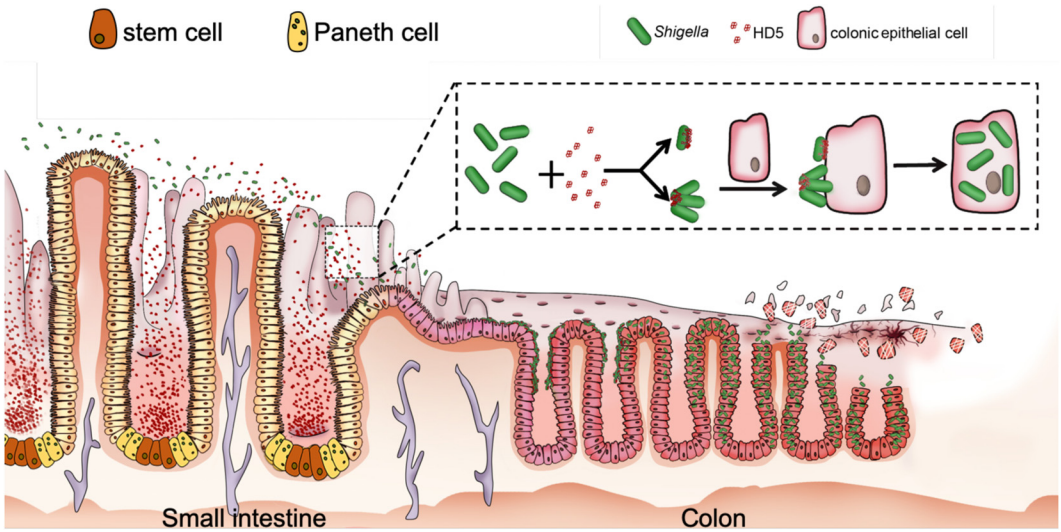

FIGURE 2 | Proposed model for HD5-promoted *Shigella* infection of the colonic epithelium. HD5 in the lumen of the small intestine encounters poorly adhesive *Shigella* in transit, binds to *Shigella* surface, and promotes *Shigella* adhesion to the colonic epithelium by bridging single bacterium and host cell and/or clustering multiple bacteria for multivalent attachment to host cell, leading to increased bacterial infection from the apical surface (89, 91).

TABLE 1 | Suppressive and proliferative properties of human defensins in tumorigenesis.

Beneficial (tumor-suppressing)				Detrimental (tumor-promoting)			
Defensins	**Cancers**	**Mechanisms**	**Refs**	**Defensins**	**Cancers**	**Mechanisms**	**Refs**
HBD1	Bladder	Inhibiting growth	(110, 119)	HBD3	Oral	Stimulating growth	(122, 123, 126–128)
	Renal	promoting apoptosis	(110)		neck and head	promoting migration	
	prostate	inhibiting migration	(120)		cervical	trafficking TAM	(127, 128)
	oral					resisting apoptosis	(127)
HBD2	*Oral*	*Inhibiting growth and invasion*	(136)	HBD2	Esophageal	Stimulating growth	(131, 132, 139)
					Lung	promoting angiogenesis	(137)
					Cervical		
HNP1–3	Colorectal	Direct cytolysis (high concentration)	(157)	HNP1–3	Renal	Stimulating growth (low concentration)	(150)
	lung	inducing apoptosis	(158–160)		bladder	promoting invasiveness	(152, 153)
	bladder	inhibiting angiogenesis	(160–162)		oral		
	renal	reversing immune alteration	(163)				
	neck and head						
	oral						

by macrophages – an essential step in bacterial clearance (84, 85). HNP1 also inhibits phagosomal escape and intracellular multiplication of *Listeria monocytogenes* and *Mycobacterium tuberculosis* in macrophages (86, 87), suggesting that the defensin, although not being expressed by macrophages, contributes to their antimicrobial function. Notably, HNP1 acts in the aftermath of *Salmonella* infection as a "molecular brake" on macrophage-driven inflammation by preventing protein translation to ensure both pathogen clearance and the resolution of inflammation with minimal bystander tissue damage (88).

While the protective roles of defensins in bacterial infection are widely reported in the field, we have made a surprising recent discovery that α-defensins can contribute to the pathogenicity of *Shigella* (89–92). Unlike other enteropathogenic bacteria, *Shigella* lacks general adhesion machinery such as fimbriae due presumably to pervasive genome reduction during the course of adaptation to the intracellular environment (93–95). As a result, *Shigella* is much less adhesive and invasive *in vitro* than other fimbriated enteropathogenic bacteria despite its extraordinary infectivity in humans. Further, although highly infectious in humans, *Shigella* hardly infects any other animals including mice with abundant enteric α-defensins (cryptdins) (96, 97). This seemingly paradoxical phenomenon or conundrum in *Shigella* pathogenesis has remained largely obscure mechanistically at the molecular and cellular levels (97–99). We found that the

lack of fimbriae in *Shigella* affords the pathogen a unique bacterial surface, onto which HD5 forms multimeric structures to mediate *Shigella* adhesion to host epithelium; enhanced bacterial adhesion in turn strongly promotes *Shigella* invasion of host cells, ensuing dramatically augmented infection *in vivo* and *ex vivo* (**Figure 2**). These studies support the premise that *Shigella* exploits HD5 for virulence (89, 91), thereby explaining not only its extraordinary pathogenicity but also its restricted host selectivity.

HNP1 is also active in promoting *Shigella* infection of epithelial cells (90), consistent with an earlier finding that human neutrophil granular proteins (containing HNPs) enhance *Shigella* adhesion *in vitro* at sub-lethal concentrations (100). Although HNP1 is weaker than HD5 with respect to their ability to promote *Shigella* adhesion, its strong activity in disrupting the epithelial barrier contributes additionally to *Shigella* infection (90). It is worth noting that HD5 exacerbates the pathogenicity of *Shigella* in macrophages. Despite that HD5 boosts phagocytosis of *Shigella* by macrophages, an antimicrobial event generally unfavorable to invading pathogens, it fails to prevent subsequent phagosomal escape and intracellular multiplication of *Shigella*, resulting in necrosis of infected macrophages induced by multiplying *Shigella* and massive release of intracellular bacteria (92).

For human α-defensins, their hydrophobicity and selective cationicity segregated on a dimeric structure stabilized by intramolecular disulfides are critical for antimicrobial activity (11). Several mutational studies have identified the functional determinants of α-defensins in promoting viral and bacterial infections (25, 56, 57, 59, 60, 90–92). Briefly, disulfide bonding in defensins is absolutely required for their ability to enhance HIV-1 infection (25, 60) and to promote *Shigella* adhesion and invasion (91, 92); hydrophobic residues in α-defensins, i.e., Trp26 and Phe28 in HNP1, Leu16, Leu26, Tyr27 and Leu29 in HD5, and Phe2 and Phe29 in HD6, play a pivotal functional role (59, 90, 91); dimerization and/or oligomerization of α-defensins are functionally indispensable (59, 83, 91, 101, 102); selective cationicity, as exemplified by Arg28 in HD5, can be critical for promoting HIV and *Shigella* infection (59, 91, 92). Obviously, although α-defensins are highly variable in amino acid sequence, their functional determinants are rather conserved, irrespective of their pathogenic and protective roles in host immunity.

DEFENSINS IN TUMORIGENESIS

Most cancers develop from epithelial cells and tissues (carcinomas) where β-defensins are expressed for mucosal surface protection against microbial infection (26, 27, 103, 104). Since β-defensins are differentially expressed in normal tissues and tumors, their role in tumor development and progression has attracted considerable interest (32, 105–107). HBD1 is downregulated in most carcinomas (108–118), and the stimuli of this downregulation are yet to be identified. Growing evidence suggests that HBD1 functions as a tumor suppressor in most carcinomas (110, 119, 120). By contrast, HBD3 is frequently overexpressed in various carcinomas (121–124), and its upregulation has been ascribed to LPS-stimulated EGFR activation (121) or HPV co-infection-induced p53 degradation (125), among others. Importantly, HBD3 stimulates tumor growth and migration (122, 123, 126), confers resistance of tumor cells to apoptosis (127), and helps the recruitment of tumor-associated macrophages that promote tumor progression (127, 128). Consistent with the oncogenic role of upregulated HBD3, mouse β-defensin 14, the ortholog of HBD3, acts as a chemoattractant to enhance angiogenesis and tumor development *in vivo* (129). The regulation of HBD2 and its influence in tumorigenesis vary from cancer to cancer (106) and can be controversial at times (130, 131). HBD2 is upregulated in esophageal, lung and skin cancers (108, 109, 118, 132), but downregulated in oral and colon cancers (112, 114, 133). While the mechanisms of HBD2 regulation are only partially understood (132, 134, 135), HBD2 appears to play a suppressive role in tumor development and progression when it is downregulated (136), but a proliferative role when upregulated (131, 132, 137–139), in agreement with HBD1 and HBD3. The suppressive and proliferative properties of defensins in tumorigenesis are tabulated in **Table 1**.

The role of α-defensins in tumorigenesis has also been extensively examined (140, 141). Elevated levels of myeloid α-defensins, HNP1–3, are frequently detected in many different types of tumor tissues and in biological fluids from cancer patients (142–155). While tumor-infiltrating immune cells, and neutrophils in particular, are likely a major contributor to increased HNP1–3 in tumors (151), several studies also suggest that tumor cells themselves may produce HNP1–3 through a yet-to-be-identified mechanism (142, 150). HNP1–3 have been shown to promote tumor cell proliferation (150, 156), contributing to tumor progression and invasiveness (152, 153). Due to their membranolytic activity toward bacteria and limited sites of expression, much of the early studies of α-defensins have focused on their ability to lyse tumor cells at high concentrations (157). More recent work, however, has shed light on the mechanistic complexity of the antitumor activity of HNP1–3, including inducing apoptosis (158–160), inhibiting angiogenesis (160–162), and altering immune milieu in HPV-associated neoplasia by recruiting immature dendritic cells (163).

CONCLUDING REMARKS

Long recognized as a class of host defense peptides and immunomodulators important for innate immune responses to viral, bacterial and fungal infections, human defensins are widely thought to be host protective. Growing recent evidence suggests, however, that they can also be pathogenic under certain biological conditions by promoting viral and bacterial infections. The interchangeable roles between a "Swiss army knife" and a "double-edged sword" played by human α-defensins in host immunity are under-appreciated in the field, despite the well-recognized fact that defensins can be both suppressors and promotors in tumorigenesis, depending on which defensin and cancer type are studied. While the mechanisms of host protection by human defensins are

well-understood, much remain obscure with respect to the molecular and cellular events dictating defensins' pro-infective activity. A better understanding of how human defensins promote infection may ultimately lead to new therapeutic interventions of infectious diseases.

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DX and WL wrote the manuscript.

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The Antimicrobial Peptide Human Beta-Defensin 2 Inhibits Biofilm Production of *Pseudomonas aeruginosa* Without Compromising Metabolic Activity

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Biofilm production is a key virulence factor that facilitates bacterial colonization on host surfaces and is regulated by complex pathways, including quorum sensing, that also control pigment production, among others. To limit colonization, epithelial cells, as part of the first line of defense, utilize a variety of antimicrobial peptides (AMPs) including defensins. Pore formation is the best investigated mechanism for the bactericidal activity of AMPs. Considering the induction of human beta-defensin 2 (HBD2) secretion to the epithelial surface in response to bacteria and the importance of biofilm in microbial infection, we hypothesized that HBD2 has biofilm inhibitory activity. We assessed the viability and biofilm formation of a pyorubin-producing *Pseudomonas aeruginosa* strain in the presence and absence of HBD2 in comparison to the highly bactericidal HBD3. At nanomolar concentrations, HBD2 – independent of its chiral state – significantly reduced biofilm formation but not metabolic activity, unlike HBD3, which reduced biofilm and metabolic activity to the same degree. A similar discrepancy between biofilm inhibition and maintenance of metabolic activity was also observed in HBD2 treated *Acinetobacter baumannii*, another Gram-negative bacterium. There was no evidence for HBD2 interference with the regulation of biofilm production. The expression of biofilm-related genes and the extracellular accumulation of pyorubin pigment, another quorum sensing controlled product, did not differ significantly between HBD2 treated and control bacteria, and *in silico* modeling did not support direct binding of HBD2 to quorum sensing molecules. However, alterations in the outer membrane protein profile accompanied by surface topology changes, documented by atomic force microscopy, was observed after HBD2 treatment. This suggests that HBD2 induces structural changes that interfere with the transport of biofilm precursors into the extracellular space. Taken together, these data support a novel mechanism of biofilm inhibition by nanomolar concentrations of HBD2 that is independent of biofilm regulatory pathways.

Keywords: airways, antimicrobial peptides, biofilm, cystic fibrosis, epithelial cells, innate immunity, mucosa, *Pseudomonas aeruginosa*

INTRODUCTION

Biofilms are composed of microbial communities encased in a protective layer of self-produced, extracellular polymers. Biofilms are formed on both abiotic and biotic surfaces and play a significant role in a variety of settings such as aquaculture (1), the food industry, and the clinical field as a factor for antimicrobial drug resistance. Biofilms can colonize body surfaces and mechanisms regarding how our bodies prevent biofilm formation are under extensive investigation (2). In part, biofilms provide tolerance to host immune factors and antibiotics through impeding their diffusion. Furthermore, biofilms enhance bacterial resistance to these factors by altering bacterial metabolism resulting from the decreased oxygen levels in the center of the biofilm mass as well as the acidification of the local microenvironment (2–5). The biofilm matrix is primarily composed of exopolysaccharide, proteins, and extracellular DNA and has been particularly well studied in *Pseudomonas aeruginosa*, a ubiquitous, opportunistic, Gram-negative bacterium. The major structural polysaccharides of *P. aeruginosa* biofilms are Pel, which is composed of positively charged amino sugars, and Psl, which is a polymer of glucose, rhamnose, and mannose; and in certain strains, alginate – an anionic polysaccharide (6–8). Proteinaceous components of biofilm include type 4 pili and cup fimbriae serving attachment and various proteins that connect matrix components adding strength to the biofilm (9). Extracellular DNA (eDNA), which is released via cell lysis (10), plays an important role in priming surfaces for the initial adhesion of the bacteria as well as in maintaining the structural integrity of the polysaccharide fibers (3, 6, 11–14).

Multiple regulatory networks govern the complex process of biofilm formation (15), which progresses from initial attachment mediated by the flagella and the production of pili, to downregulation of flagellar genes, upregulation of the production and secretion of matrix components, maturation, and eventual reappearance of flagella and dispersion. For *P. aeruginosa*, biofilm regulation has been well studied and several regulatory systems have been identified including the Las, Rhl, and quinolone quorum sensing systems, the GacA/GacS two-component system, and c-di-GMP controlled pathways. Key quorum sensing molecules for Las, Rhl, and quinolone systems are N-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL), N-butanoyl-homoserine lactone (C4-HSL), and 2-heptyl-3-hydroxy-4-quinolone (known as Pseudomonas Quorum Sensing molecule or PQS), respectively (16, 17). These overlapping regulatory systems not only control the production of biofilm but also the production of pigment and various other virulence factors (17, 18). Genes whose expression is modulated during biofilm formation include *flgF*, which encodes for the basal rod in bacterial flagellin, and *pslA*, which is the first gene in the polysaccharide synthesis locus (19, 20).

In addition to being able to produce biofilm, *P. aeruginosa* possesses potent virulence factors such as: a type III secretion system, which allows it to directly deliver exotoxins to host cells (21); rhamnolipids, which enable *P. aeruginosa* to disrupt the tight junctions of respiratory epithelia (22); and pigments with diverse functions in metal-chelation, competitive inhibition of other bacteria, and resistance to oxidative stress (23–25). All of these virulence factors and resistance mechanisms contribute to *P. aeruginosa* being one of the leading isolates in healthcare-associated pneumonia in intensive care units and chronic lung infection in patients with cystic fibrosis, a genetic disorder characterized by impaired anion transport and increased mucous viscosity (26). Yet, despite its ubiquity in nature and its prevalence in healthcare-associated infections, *P. aeruginosa* is not known to cause lung infection in healthy adults, suggesting that humans possess effective innate defense mechanisms in the airways against this organism.

Antimicrobial peptides (AMPs) are small, highly conserved effector molecules that play a key role in innate immunity (27, 28). Present in plants, insects, and mammals, most AMPs are between 2 and 5 kDa in size and are cationic with varying degrees of hydrophobicity. Upon the detection of microbial components via pattern recognition receptors, AMPs can be synthesized by epithelial cells and myeloid cells as part of the first line of defense against microbes (29–33). A wealth of research has been performed on the ability of AMPs to displace cations bound to bacterial membranes, which are rich in either negatively charged lipopolysaccharides or lipoteichoic acids in addition to anionic phospholipids (34). After binding to bacterial membranes, AMPs can perturb the membrane structure and form pores mediated by hydrophobic and electrostatic forces. In addition to the charge of the membrane, phospholipid species and the presence or absence of cholesterol, which is absent in bacterial membranes, also affect the binding and orientation of AMPs and hence, their pore-forming capabilities (35–40). While pore-formation has been a widely studied mechanism of action, an increasing body of research suggests that the antimicrobial activity of AMPs may also depend on other mechanisms – disruption of cell wall synthesis, metabolic activity, ATP and nucleic acid synthesis, and amino acid uptake (33, 41). Furthermore, certain AMPs interact with the eukaryotic host cells and have immunoregulatory functions in addition to their antimicrobial activity. A notable example is that LL-37 can also: act as a chemotactic agent to recruit other immune cells and modulate cytokine and chemokine expression in host cells, bind bacterial lipopolysaccharide, and dysregulate the expression of genes involved in biofilm formation (42–46). Other AMPs have also shown multi-functional capabilities, in particular human beta-defensin 2 (HBD2) and 3 (HBD3), which have been proven to possess mechanisms of action that are more complex than simple pore formation and membrane perturbation (47–49). In fact, HBD2 was the first human beta defensin to demonstrate chemotactic activity (50). Beta-defensins are characterized by three, antiparallel β-strands stabilized by three conserved disulfide linkages preceded by an α-helical domain near the N-terminus (51–53). Although HBD2 and HBD3 share amino acid sequence and some structural similarities, their overall net charge, hydrophobicity, and charge distribution differ significantly (**Table 1**) and may play a role in their unique and distinct mechanisms of action. Expression of HBD2 and HBD3 is low or absent during steady state but both peptides are induced in airway epithelial tissues during infection or inflammation (31, 32, 48, 54).

TABLE 1 | Human beta defensins-2 and -3 physicochemical properties.

Peptide	Amino acid sequence[a]	MW (Da)	Net Charge	Hydrophobicity index	
				Kyte-Doolittle[b]	Wimley-White[c]
HBD2	GIG*D*PVTC^{1}L**K**SGAIC^{2}HPVFC^{3}P**RR**Y**K**QIGTC^{2}GLPGT**K**C^{1}C^3**KK**P	4,328.22	6	−0.1	6.16
Linear HBD2	GIG*D*PVTAL**K**SGAIAHPVFAP**RR**Y**K**QIGTAGLPGT**K**AA**KK**P	4,141.88	6	−0.21	8.62
HBD3	GIINTLQ**K**YYC^1**R**V**R**GG**R**C^{2}AVLSC^{3}LP**K***EE*QIG**K**C^{2}ST**R**G**RK**C^{1}C^3**RRKK**	5,155.19	11	−0.7	12.65

[a]*Amino acid sequences are given in one-letter code starting from the N and ending with the C terminus. Underlined residues denote mutation sites for the linearized HBD2. Cationic residues are in boldface. Anionic residues are italicized.* [b]*Values were calculated based on the Kyte–Doolittle hydrophobicity scale (120) using the grand average of hydropathy (GRAVY) program. Higher values represent an increase in hydrophobicity.* [c]*Values were calculated based on the Wimley–White whole residue hydrophobicity interface scale (121) using the APD3 antimicrobial peptide calculator and predictor. Lower values represent an increase in hydrophobicity.* $^{1-3}$ *Numbers denote disulfide bond connectivity.*

Due to their lasting potency for millions of years and the feasibility of modifying AMP structures, AMPs continue to be in the spotlight as potential antimicrobial agents (33). The importance of biofilm in the infection process and in their resistance to antimicrobial agents has been recognized, yet there is a lack of drugs that interfere with biofilm. Therefore, knowledge on the structure-function relationships of AMPs, and the effects of AMPs on bacterial biofilm formation may benefit rational engineering and design of novel AMP variants and therapeutic regimens that are effective against microbial biofilms (55). Considering the induction of HBD2 and HBD3 and their secretion to the epithelial surface in response to bacteria and their products, we hypothesized that HBD2 and HBD3 have biofilm inhibitory activity. We discovered that biofilm and metabolic inhibition are proportionally reduced by HBD3 but not by HBD2. At low concentrations, HBD2 inhibits biofilm production, but not metabolic activity. We undertook multiple approaches to delineate the underlying mechanism for the selective biofilm inhibitory effects of HBD2. This research may lead to the identification of novel targets for the engineering of antimicrobials, which, in the era of increasing multi-drug resistance, is of great importance.

MATERIALS AND METHODS

Antimicrobial Peptides

Chemical synthesis and purification of human beta-defensin 2 (HBD2/L-HBD2), its D- form (D-HBD2) comprised entirely of D-amino acids, its linearized mutant (Linear HBD2 with alanine replacing all cysteine residues), and human beta-defensin 3 (HBD3, in L-form) have been described previously (56, 57). **Table 1** summarizes their physicochemical properties. Stock solutions (500 μM) were prepared in 0.01% acetic acid and stored at −20°C. For experiments, peptides were used as 10-fold concentration in 0.01% acetic acid.

Bacterial Culture

For this study, a pyorubin-producing *P. aeruginosa* strain (a cystic fibrosis isolate previously obtained from Dr. Michael J. Welsh, University of Iowa, Iowa City) and *Acinetobacter baumannii* ATCC 19606 were used. For each experiment, snap-frozen 18 h cultures in Tryptic Soy Broth (TSB) (Oxoid) were quickly thawed, subcultured into prewarmed TSB (750 μL into 50 mL), and brought to mid-log growth phase (3 h at 37°C, 200 rpm). Bacterial cells were then sedimented and washed with 140 mM NaCl by centrifugation for 10 min at 805 × *g* in a precooled centrifuge (4°C), and resuspended in 500 μL 140 mM NaCl. For gene expression analysis, the suspended bacteria were used directly. For all other assays, the concentration of bacteria was first adjusted to 5×10^7 CFU/mL in 140 mM NaCl, and then further diluted as needed.

Biofilm Quantification

In a round bottom 96-well polystyrene microtiter plate (Costar #3795), 90 μL mid-logarithmic growth phase bacteria were added to 10 μL of 10-fold concentrated defensin or 0.01% acetic acid as solvent control to yield the following final assay conditions: 1×10^6 CFU/mL, 10% Mueller-Hinton broth (Oxoid, without cations), and 140 mM NaCl. Samples were incubated for 18 h at 37°C and biofilms were quantified according to Merritt et al. (58). Briefly, the content of sample wells containing non-adherent bacteria (planktonic and/or dead) was carefully discarded without disturbing the biofilm, and the well walls were rinsed three times with dH_2O (200 μL/well) followed by addition of 125 μL of 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, United States). After 10 min incubation at RT, the crystal violet solution was removed, wells were rinsed three times with dH_2O (200 μL/well) and air dried for at least 30 min. To solubilize crystal violet bound to biofilm, 200 μL of 30% acetic acid was added to each well and after 15 min incubation at RT 125 μL was transferred to optically clear flat-bottom 96-well polystyrene microtiter plates (Perkin Elmer from Waltham, MA, United States). Absorbance was read at 570 nm using a Victor X3 Plate Reader (Perkin Elmer). Wells containing only 125 μL of 30% acetic acid were used to subtract baseline absorbance values from samples for analysis.

Metabolic Activity Measurement

Resazurin reduction was employed as a measure of bacterial metabolic activity (59, 60). Metabolites accumulating during bacterial growth reduce the weakly fluorescent resazurin to the highly fluorescent resorufin. Samples were prepared as described above but with resazurin (Sigma) added to the assay buffer to obtain a final concentration of 0.01% resazurin (w/v). Relative fluorescent units (RFU) were measured every 3 h with a preheated

Victor X3 Plate Reader (Perkin Elmer) at 530 nm excitation and 616 nm emission wavelength and a top read.

ATP Quantification

ATP concentrations of non-adherent bacteria were determined using the BacTiterGlo kit (Promega), with ATP standard curves prepared according to the manufacturer's instructions. Bacteria were prepared and incubated with defensins for 18 h as described for the biofilm assay. Then, the entire well contents were transferred to a new 96 well plate, thoroughly resuspended, and of this 75 μL from each well was transferred to a black 96-well half area plate (Perkin-Elmer). After addition of 75 μL ATP substrate solution to each well and 5 min mixing on an orbital shaker, luminescence was quantified with a Victor X3 plate reader. Seventy-five μL aliquots of serially diluted ATP standard were treated in the same way.

Pyorubin Quantification

Pyorubin is a collection of pigments produced by certain *P. aeruginosa* strains including our test strain. Although its full chemical composition is unknown, it consists of at least two, water-soluble, red-colored pigments (61). Pyorubin quantification was based on Hosseinidoust et al. (23). Briefly, bacteria were grown for 18 h in 10% Mueller-Hinton and 140 mM NaCl in the presence of 0.125–1 μM of HBD2 or solvent control in final assay volumes of 1 mL in 12-well microtiter plate (non-tissue culture treated, Costar). After 18 h incubation, well contents were collected and centrifuged at 5,000 × *g* for 10 min at 4°C to remove non-adherent bacteria. Equivolume mixtures of cell free supernatant (900 μL) and chloroform (900 μL) were mixed and centrifuged at 12,000 × *g* for 15 min at 4°C to separate the aqueous and organic phases and remove cell debris and other molecules. The aqueous phase containing pyorubin was lyophilized, dissolved in 125 μL volume of dH_2O. From this, 100 μL were transferred to a 96-well flat bottom plate (Perkin Elmer) followed by an absorbance reading at 535 nm using a Victor X3 Plate Reader (Perkin Elmer).

In silico Molecular Docking Studies

The *in silico* modeling of binding between QS molecules and HBD2 was performed using Autodock Vina (The Scripps Research Institute) through the UCSF Chimera program[1]. LasR receptor (RSCB 3IX3) and HBD2 (RSCB 1FQQ) were considered as rigid receptors and were docked with *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C12-HSL), *N*-butanoyl homoserine lactone (C4-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS) as ligands. Phosphorylcolamine (NEtP) was used as a negative control. Free energy of binding was used to calculate dissociation constants using Eq. (1) with R = 0.00198 kcal/(mol K) and T = 37°C = 310.15 K (62).

$$K_{D,pred} = e^{(([\Delta G_{bind}]/[(R/1000)\times T]))} \quad (1)$$

[1]https://www.cgl.ucsf.edu/chimera/

Gene Expression Analysis

Mid-logarithmic growth phase bacteria were prepared and washed as described above. The assay was up-scaled using 12-well polystyrene flat bottom plates with non-reversible lids with condensation rings (Genesee Scientific, San Diego, CA, United States). Twenty μL of the washed bacteria was added to HBD2 or solvent (100 μL of 10-fold concentrated defensin in 0.01% acetic acid or 0.01% acetic acid, respectively, diluted in 900 μL 10% Mueller Hinton/140 mM NaCl) yielding about 1×10^8 CFU/mL. After incubation at 37°C for the specified time points, biofilm and planktonic phase bacteria were homogenized by 10 min vortexing with 1 mm glass beads and tightly secured lids (Sigma-Aldrich, St. Louis, MO, United States). RNA extraction was performed on the homogenized samples using an RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's enzymatic lysis and mechanical disruption protocol with acid-washed 425–600 μm glass beads (Sigma-Aldrich). Residual genomic DNA was removed with in-solution TurboDNase treatment (2 U/μL, Invitrogen, Carlsbad, CA, United States) according to the manufacturer's recommendations followed by purification and concentration of RNA samples with RNA Clean and Concentrator-5 kit (Zymo Research, Irvin, CA, United States). Purity of RNA was confirmed by lack of amplification in SsoAdvanced™ Universal SYBR® Green (Bio-Rad, Hercules, CA, United States) real-time PCR using the RNA samples as template and primers for the housekeeping gene *gapA* (see **Table 2**). Confirmed pure RNA samples were reverse transcribed with iScript Reverse Transcription Supermix (Bio-Rad) and resulting cDNA was diluted to 25 ng/μL in nuclease free water. SsoAdvanced™ Universal SYBR® Green real-time PCR was performed with target primers for *pslA* and *flgF* and housekeeping gene *gapA* as reference gene (see **Table 2**, used at 0.75 μM final concentrations) in 10 μL reaction volumes and 12.5 ng cDNA input. Primers (Integrated DNA Technology's, IDT, Coralville, IA, United States) were designed using IDT's primerQuest Tool. Quantitative PCR (qPCR) and subsequent melt curve was performed using BIO-RAD's CFX96 Real Time Thermocycler following standard conditions with annealing/extension at 60°C. CT values and relative gene expression were determined with BIO-RAD's CFX Maestro Version 1.1. Amplified products were verified through size determination via standard agarose gel electrophoresis and melt curve analysis. Each time point was assessed in three independent experiments conducted in duplicates for a total *n* of 6. Initially, *16S rRNA* was considered as a second housekeeping

TABLE 2 | Primers used in this study.

Gene target		5′–3′ sequence	T_M (°C)	Product size (bp)	Product melt peak (°C)
pslA	F	CGTTCTGCCTGCTGTTGTTC	56.9	160	88.5
	R	TACATGCCGCGTTTCATCCA	57.3		
gapA	F	CCATCGGATCGTCTCGAA	61.0	130	88.0
	R	GTTCTGGTCGTTGGTGTAG	60.0		
flgF	F	ACAACCTGGCGAACATCTC	62.0	137	89.0
	R	GCCATGGCTGAAATCGGTA	62.0		

gene. However, its CT values (around 5) were substantially earlier than the CT values for the target genes and *gapA* (at or above 20) and thus, *16S rRNA* gene expression was not further evaluated in this study.

Outer Membrane Protein Profile Analysis

Pseudomonas aeruginosa outer membranes were harvested after incubation with HBD2 or solvent control according to Park et al. (63) with minor modifications. Briefly, bacteria were prepared as above and then grown for 18 h in 10% Mueller-Hinton and 140 mM NaCl in the presence of 0.125 to 1 μM of HBD2 or solvent control in final assay volumes of 1 mL in 12-well microtiter plate (Costar® not treated, Corning). After 18 h incubation, the well contents were resuspended, transferred into microfuge tubes, and centrifuged at 5,000 × *g* for 10 min at 4°C to pellet the bacterial cells. Cells were then resuspended in 80 μL of 0.2 M Tris–HCl, pH 8.0. Then, 120 μL lysis buffer was added to the resuspended cells (final conditions were 200 μg/mL hen egg white lysozyme (Sigma-Aldrich), 20 mM sucrose and 0.2 mM EDTA in 0.2 M Tris-HCl, pH 8.0). After a 10 min incubation at RT, 2 μL of Protease Inhibitor Cocktail (Sigma Aldrich P8340) was added followed by 202 μL of extraction buffer (10 μg/mL DNAse I [Sigma-Aldrich DN25] in 50 mM Tris–HCl/10 mM $MgCl_2$/2% Triton X-100). After 1.5 h incubation on a rocker at 4°C, samples were centrifuged at 1500 × *g* at 4°C for 5 min. The resulting supernatants from triplicate samples, which contain the outer membranes, were pooled and placed into 4 mL ultrafiltration tubes with 5 kDa cut off molecular weight (Amicon Ultracel, 5k, Millipore). PBS was added to yield a volume of 4 mL, and then the tubes were centrifuged at 2400 × *g* until about 500 μL residual volume was obtained. The outer membranes in this residual were then washed by suspending in 3.5 mL PBS and then centrifuging at 2400 × *g* for 25 min at RT, yielding a residual volume of approximately 200 μL. Of this, 4 μL were subjected to standard SDS-PAGE using Bio-Rad 16.5% Mini-Protean Tris-Tricine gels followed by silver stain. Images were acquired with Versadoc (Bio-Rad) and analyzed with Image Lab version 6.01 software from Bio-Rad Laboratories.

Atomic Force Microscopy

P. aeruginosa (1 × 10^6 CFU/mL inoculum) was incubated in 10% Mueller Hinton broth/140 mM NaCl/12.5 mM sodium phosphate pH 7.0 with and without HBD2 (0.25 μM), on glass coverslips (Borosilicate glass square coverslips, Thermo Fisher Scientific) in 6-well plates (Corning) for 18 h at 37°C. As negative controls for HBD2 the peptide solvent 0.01% acetic acid was included, respectively. Coverslips were then transferred into wells of a fresh six-well plate and adherent bacteria were fixed with 2.5% glutaraldehyde (Ted Pella, CA; 25%, electron microscopy grade) diluted in PBS for 20 min at 4°C followed by washing with deionized water according to Chao and Zhang, 2011 (64), and stored at 4°C until imaging by atomic force microscopy (AFM).

All AFM tests (65) were carried out with a NX12 AFM system (Park System) using an aluminum coated PPP NCHR (Park systems) cantilever with a spring constant of 42 N/m, a resonance frequency of 330 kHz, and a nominal tip radius of <10 nm. At least five images were acquired per sample in air with non-contact mode (NCM) with settings of 256 pixels/line and 0.75 Hz scan rate and continuous monitoring of the tip integrity. The images were first order flattened and the roughness and height of all bacteria were measured using XEI software (Park Systems). Specifically, roughness of each bacterium was calculated from the root mean square value (RMS, i.e., standard deviation of the distribution of height over the whole bacterium surface).

Data and Statistical Analysis

Data graphs were generated using Microsoft Excel® 2016 or GraphPad Prism 7.04 Software. Statistical analyses were performed using IBM SPSS version 24 or GraphPad Prism 7.04 Software. A p-value < 0.05 was considered statistically significant.

RESULTS

At Low Concentrations, HBD2 Does Not Reduce Metabolic Activity but Inhibits Biofilm Production by *P. aeruginosa*, Unlike HBD3

To compare the antimicrobial activities of HBD2 and HBD3, *P. aeruginosa* was exposed to either peptide at various concentrations over a period of 18 h. Viability was assessed by measuring metabolic activity every 3 h via quantification of resazurin reduction to the highly fluorescent resorufin by bacterial metabolites. Biofilm was assessed at 18 h post-incubation via quantification of crystal violet staining through absorbance readings. The resazurin reduction assay showed that both HBD2 and HBD3 reduced metabolic activity in a dose-dependent manner, with HBD3 being more effective on a per molar basis, producing around a 30% reduction at 0.5 μM compared to the 4 μM needed by HBD2 at 18 h for the same effect (**Figure 1**). However, when comparing the effect on biofilm production between the two peptides, a notable difference was observed. At concentrations of 0.25 and 0.5 μM, HBD2 reduced *P. aeruginosa* biofilm to ∼75% of the control without significantly reducing the metabolic activity (**Figure 2A**). In contrast, at these concentrations, HBD3 reduced the formation of *P. aeruginosa* biofilm in a dose dependent manner that was directly proportional to the cumulative effect on metabolic activity and further reduced both biofilm and resorufin production to nearly undetectable levels at a concentration of 1 μM (**Figure 2B**) consistent with direct microbicidal activity. ATP concentrations measured at the end of the 18 h incubation period corroborated the resazurin data (**Figure 3**), showing maintained ATP levels in HBD2 treated bacteria but a significant reduction of ATP levels in HBD3 treated *P. aeruginosa* (at 2 μM defensin, 17.65 ± 5.31 nM ATP compared to 3.6 ± 2.88 nM ATP, respectively, p = 0.011). These data suggest a differential mechanism for the antimicrobial activity between HBD2 and HBD3, and that HBD2 selectively inhibits biofilm formation at low concentrations.

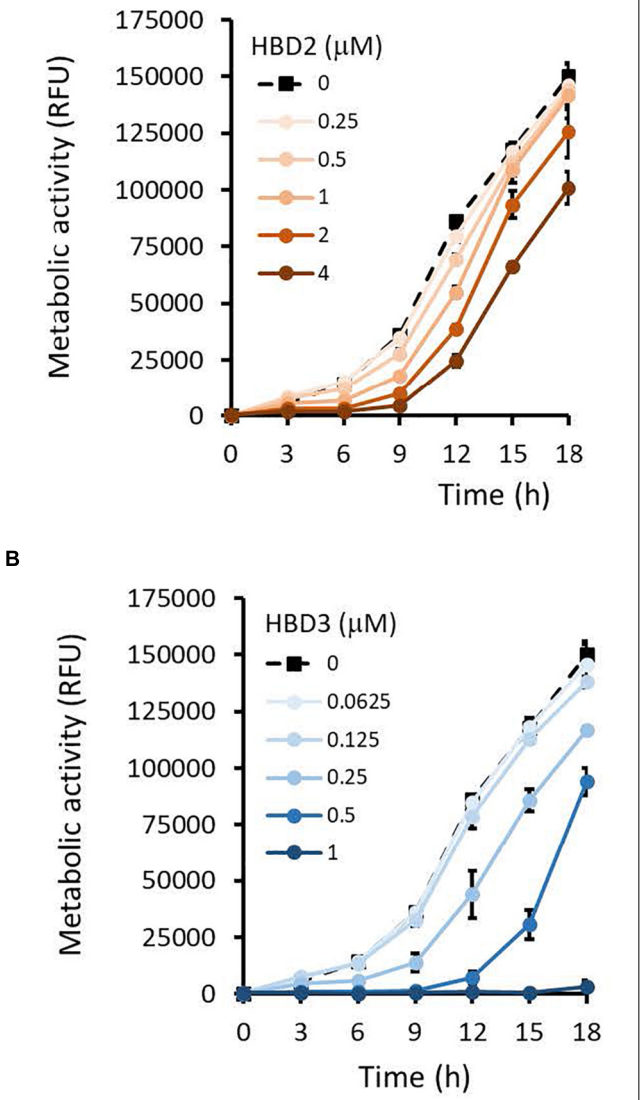

FIGURE 1 | Metabolic activity of *P. aeruginosa* in the presence and absence of HBD2 and HBD3 over 18 h. Bacteria were incubated in 10% Mueller-Hinton/140 mM NaCl supplemented with 0.01% resazurin and fluorescence emitted by resorufin reflecting the production of reducing metabolites was measured every 3 h (530 nm_{ex}, 616 nm_{em}). Shown are the means ± SD of three independent experiments conducted in duplicates. RFU: relative fluorescence units. $p < 0.001$ for HBD2 **(A)** at 1, 2, and 4 μM and for HBD3 **(B)** at 0.25, 0.5, and 1 μM compared to the solvent control in univariate ANOVA with Bonferrroni *post hoc* analysis. All other concentrations were not significantly different from the solvent controls.

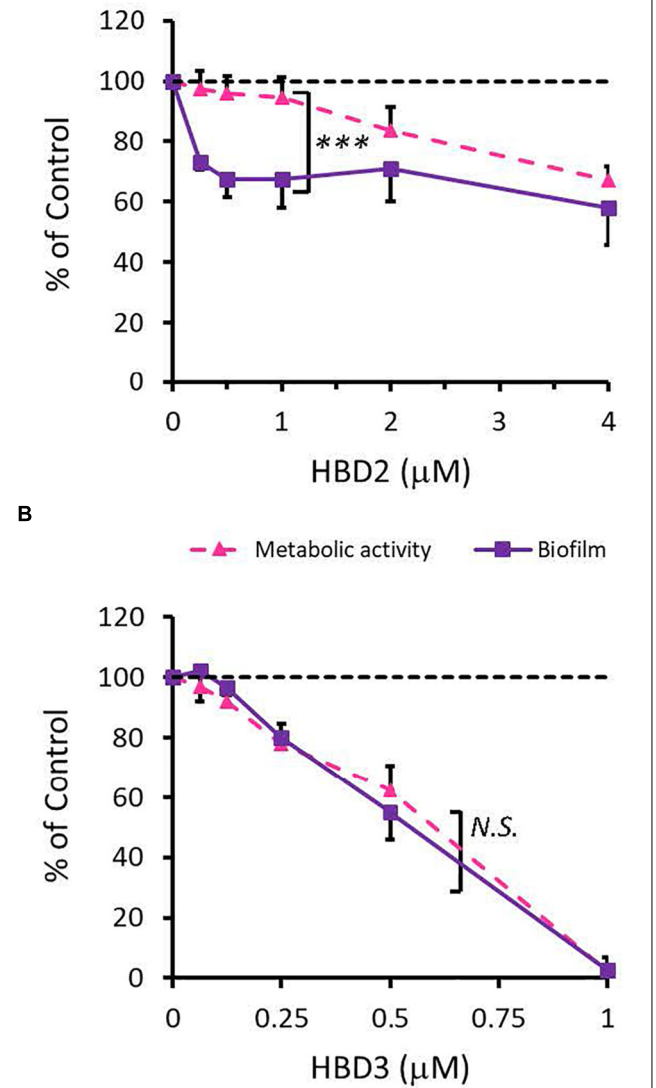

FIGURE 2 | Comparative effects of HBD2 and HBD3 on *P. aeruginosa* biofilm and metabolic activity. Shown are biofilm formation and accumulated resorufin fluorescence after 18 h of incubation with HBD2 **(A)** and HBD3 **(B)** at the concentrations given. Data are expressed relative to the control and represent means ± SD of three independent experiments conducted in triplicates. ***$p = 0.0004$ in Two-way ANOVA. N.S: not significant ($p = 0.7721$).

HBD2 Similarly Inhibits Biofilm Production by *A. baumannii* Without Reducing Metabolic Activity at Lower Concentrations

To rule out that the observed differential biofilm reducing activity of HBD2 activity was strain-specific and restricted to *P. aeruginosa*, we also subjected *A. baumannii*-another opportunistic Gram-negative rod of clinical relevance – to varying doses of HBD2 and determined resazurin reduction and biofilm production after 18 h incubation. As shown in **Figure 4**, at low concentrations, HBD2 similarly inhibited biofilm formation while not reducing metabolic activity of *A. baumannii*. For example, at 1 μM, HBD2 effected a significant reduction of biofilm to 51.77 ± 2.93% of the control ($p < 0.001$) while resazurin reduction was still at 115 ± 0.67% ($p = 1.0$) of the control (means ± SD, $n = 3$). At higher concentrations though, HBD2 appeared to have greater effects on *A. baumannii*

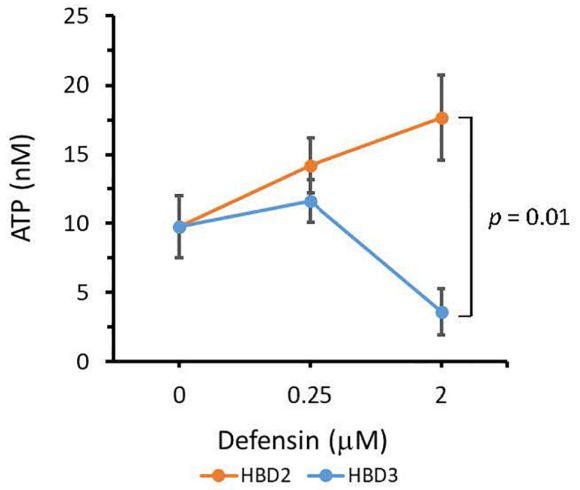

FIGURE 3 | ATP quantification in *P. aeruginosa* after 18 h incubation in the presence or absence of HBD2 and HBD3 at the concentrations given. ATP concentrations are in nM and were calculated based on a standard curve. Shown are means ± SD of three independent experiments conducted in duplicates. $p = 0.01$ in One way ANOVA with Bonferroni *post hoc* analysis for 2 μM HBD2 compared to 2 μM HBD3.

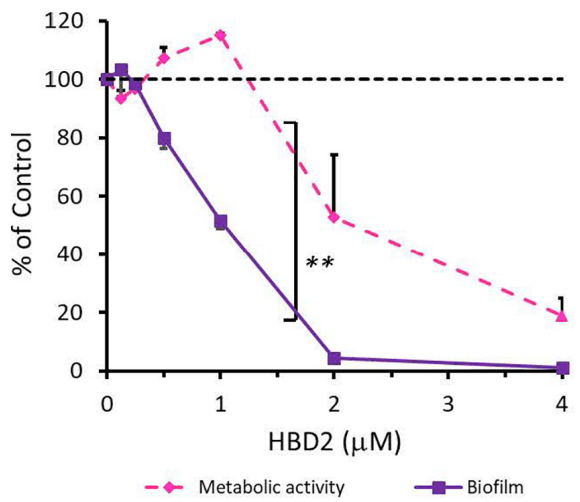

FIGURE 4 | Effects of HBD2 on *A. baumannii* biofilm formation and metabolic activity. Shown are crystal violet absorbance and accumulated resorufin fluorescence expressed as % of the control after 18 h of incubation with HBD2 at the concentrations given. Data represent means ± SD of three independent experiments conducted in triplicates. $^{**}p = 0.004$ for biofilm reduction versus reduction of metabolic activity in two tailed Paired Samples Test. In Oneway ANOVA with Bonferroni *post hoc* analysis, $p = 0.001$ for resazurin reduction at 4 μM HBD2, and $p < 0.001$ for biofilm reduction at 1, 2, and 4 μM HBD2, compared to the solvent control. All other data points were not significantly different from the control.

compared to *P. aeruginosa* as both biofilm and metabolic activity were reduced to less than 2 and 20% of the control at 4 μM HBD2, respectively (1.23 ± 0.48 and 18.71 ± 10.43%, means ± SD, $n = 3$).

HBD2 Biofilm Inhibitory Activity Does Not Depend on Chirality but on Folding State

Since HBD2 appeared to selectively reduce biofilm formation and it has been known to bind to chemokine receptors on eukaryotic cells (66, 67), it was possible that the effects of HBD2 were due to binding to receptors involved in the biofilm regulatory pathway such as the GacA/GacS system. To test this, we assessed the activity of the D-form of HBD2, which, due to mismatched chirality, does not bind to proteinaceous receptors of L-HBD2. Like L-HBD2, D-HBD2 effected a significant reduction of biofilm production by *P. aeruginosa* without reducing metabolic activity (**Figure 5A**). Thus, this suggests that the observed HBD2 effect on *P. aeruginosa* biofilm production was not due to binding to receptors important for biofilm regulatory pathways.

Upon proper folding, defensins form three intramolecular disulfide bridges, which stabilize an amphipathic structure where cationic and hydrophobic amino acid residues are spatially segregated. To assess the importance of the structure and thus, charge distribution of HBD2 for its observed activity, a comparison was made between wildtype HBD2 and a linearized HBD2 mutant (Linear HBD2) with cysteine residues replaced by alanine residues. Loss of the cysteine residues prevents the formation of stabilizing disulfide bonds, drastically limits proper folding, and disrupts the organization of charged domains thought to be critical for AMP activity (68–70). As shown in **Figure 5B**, linearization of HBD2 resulted in a pronounced loss of activity.

Taken together, these data provided evidence for a receptor-independent activity that requires proper sequestration of charged and hydrophobic residues. We next asked whether HBD2 disrupts regulatory pathways of biofilm production through QS molecule binding. To answer this question, we took a three-pronged approach and performed *in silico* docking studies with known QS molecules involved in biofilm regulation, employed qPCR probing for genes differentially expressed during biofilm formation, and quantified pyorubin, a pigment regulated by the pathways that also affect biofilm production.

HBD2 Binding to QS Molecules Is Unlikely Based on Autodock Vina Prediction

QS molecules are small and flexible molecules with a potential for hydrogen bonding and hydrophobic interactions. Thus, they may bind to and be sequestered by HBD2. To explore this further, Autodock Vina was used (**Figure 6**) to predict HBD2 binding to known *P. aeruginosa* QS molecules representing three different QS systems, namely 3-oxo-C12-HSL – as the major QS molecule for *P. aeruginosa* utilized by the Las system, C4-HSL primarily utilized by the Rhl system, and PQS a key sensing molecule in the 4-quinolone system (71). As a positive control, Autodock Vina was also used to match the known binding pocket of the QS molecule 3-oxo-C12-HSL to its receptor LasR that has been previously assessed by X-ray diffraction (RSCB 3IX3) (72). Phosphorylcolamine (NEtP), which is not expected to bind to either LasR receptor or HBD2, was used as a negative control. Using the same methodology that confirmed binding

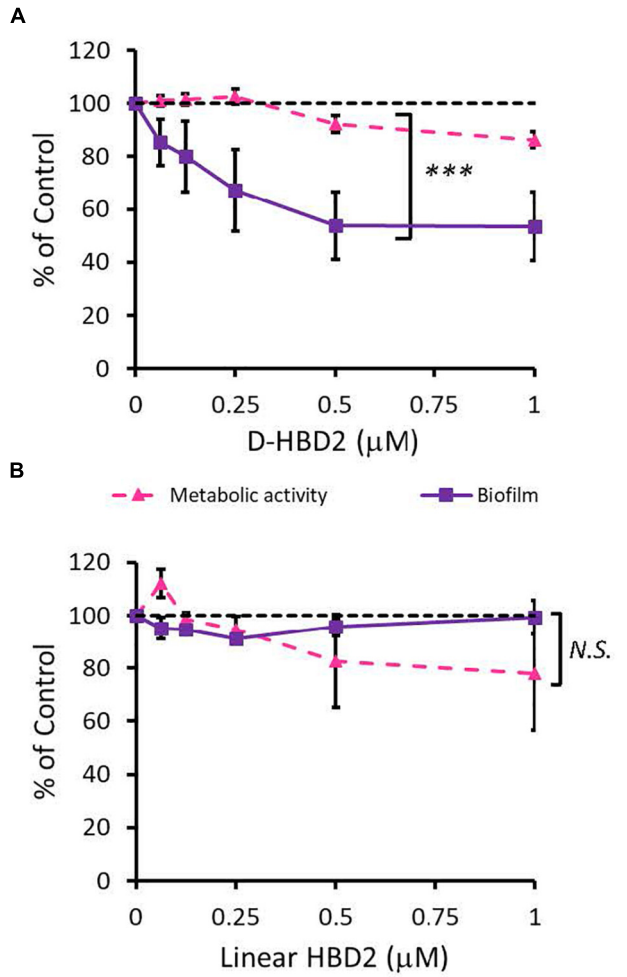

FIGURE 5 | Comparative effects of D-HBD2 and linear HBD2 on *P. aeruginosa* biofilm and metabolic activity. Shown are biofilm formation and accumulated resorufin fluorescence expressed as % of the control after 18 h of incubation with all D-HBD2 **(A)** and linear HBD2 **(B)** at the concentrations given. Data represent means ± SD of three independent experiments conducted in triplicates. In paired *T* test comparing biofilm reduction and reduction of metabolic activity, ***$p < 0.001$ for D-HBD2 **(A)** and not significant (N.S.) for linear HBD2 **(B)**. In Oneway ANOVA with Bonferroni *post hoc* analysis, biofilm formation ($p = 0.033$) but not metabolic activity ($p = 0.473$) is significantly reduced by D-HBD2. For linear HBD2, none of the data is significantly different from the solvent control.

of 3-oxo-C12-HSL to LasR here (**Figure 6A**) no binding of 3-oxo-C12-HSL to HBD2 was found (**Figure 6B**). Furthermore, we calculated the free energy of binding and found for LasR values corresponding to those reported in the literature (62, 73). Employing a −6 kcal/mol threshold for likely binding between ligand and receptor, binding between LasR and 3-oxo-C12-HSL, C4-HSL, and PQS was much more favorable (**Figure 6C**) than binding between HBD2 and these sensing molecules (**Figure 6D**).

Using Eq. (1), the dissociation constants (K_D) for the most favorable binding pair between either LasR or HBD2 with each QS molecule was calculated (**Table 3**). This method predicted the K_D of 3-oxo-C12-HSL and LasR (1.15 μM) near that of previously reported values (∼5.5 μM) (74). Furthermore, K_D values for LasR binding with all three *P. aeruginosa* QS molecules were consistently two to three orders of magnitude lower than those of HBD2 binding with any of these QS molecules. This suggests that it is unlikely for HBD2 at physiological concentrations (75–77) to significantly bind these QS molecules.

Gene Expression of *flgF* and *pslA* Is Not Affected by HBD2

During biofilm formation, motility and production of exopolysaccharide are reciprocally regulated with reduction of the expression of flagella-related genes and increase in the expression of genes contributing to polysaccharide synthesis including Psl polysaccharide. Thus, we compared the expression of *flgF* (**Figure 7A**) and *pslA* (**Figure 7B**) in *P. aeruginosa* treated with 0.25 μM HBD2 or solvent at various timepoints for up to 12 h. For solvent treated control bacteria, as expected, *flgF* gene expression decreased within 2 h reaching statistical significance after 6 h and the expression of *pslA* was signficantly increased after 2 h compared to the later time points ($p < 0.01$ and $p < 0.05$ in multivariate ANOVA with Bonferroni *post hoc* analysis). As observed for control bacteria, *flgF* gene expression decreased over time and was significantly reduced in HBD2 treated bacteria ($p < 0.05$) though changes in *pslA* gene expression did not reach statistical significance. However, there was overall no statistical significant difference between solvent and HBD2 treated bacteria. Thus, expression analysis of genes altered early in the biofilm production process does not support that HBD2 interference with biofilm production occurs at the transcriptional level.

Pyorubin Accumulation Is Not Reduced in Media Collected From HBD2 Treated *P. aeruginosa*

Pigment production in *P. aeruginosa* has been shown to be also regulated by QS (24, 61). To further corroborate that HBD2 does not interfere with QS, we quantified pyorubin released into culture supernatants in the presence and absence of HBD2. At 0.125 and 0.25 μM HBD2 there was no difference in pyorubin accumulation compared to the control (data not shown). In the presence of 0.5 and 1 μM HBD2, there was a slight increase of pyorubin (109.5 ± 4.9 and 109.9 ± 5.8% of the control, respectively, $p < 0.01$ in univariate ANOVA with Bonferroni *post hoc* adjustment). This finding further supports that HBD2 does not inhibit quorum sensing and next, we explored whether HBD2 may induce structural changes in the outer membrane that could interfere with the transport of biofilm precursors to the extracellular space.

HBD2 Alters the Outer Membrane Protein Profile of *P. aeruginosa*

Outer membrane proteins participate in the process of biofilm formation (78). Hence, we probed whether incubation with HBD2 leads to changes in the outer membrane protein profile of *P. aeruginosa* (**Figure 8**). A representative image of outer membrane preparations resolved by silver stained SDS-PAGE is depicted in **Figure 8A**. Numerous bands are detected ranging

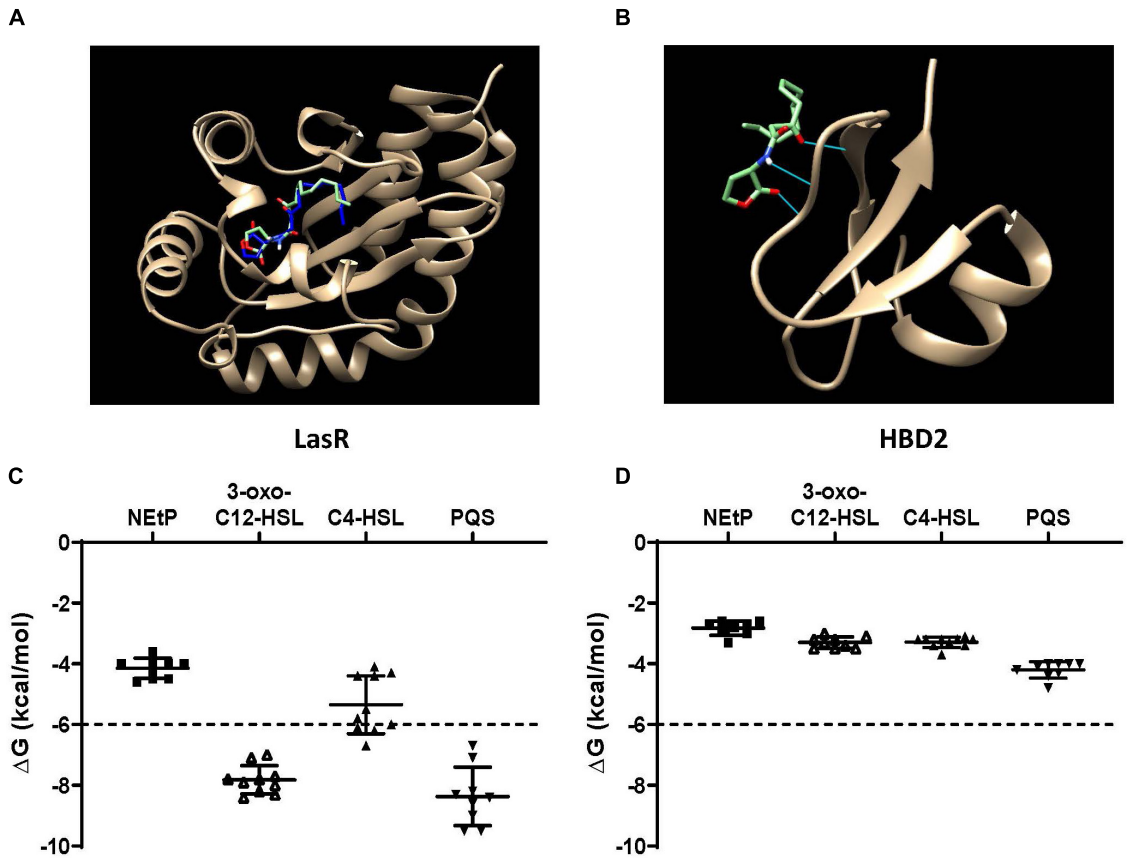

FIGURE 6 | *In silico* docking and binding energies (ΔG) of various QS molecules calculated for LasR and HBD2. AutoDock Vina was used to predict binding sites and potential hits for HBD2 and quorum sensing molecules in comparison to LasR. **(A)** Test *N*-(3-oxohexanoyl) homoserine lactone (3-oxo-C12-HSL, green) lies inside the LasR binding pocket in the same region as co-crystallized 3-oxo-C12-HSL (blue) with LasR (RSCB 3IX3). **(B)** HBD2 does not contain a binding pocket for test 3-oxo-C12-HSL (green). Free energy of binding (ΔG) for various hits were determined for phosphorylcolamine (NEtP), 3-oxo-C12-HSL, *N*-butyryl homoserine lactone (C4-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS) as ligands with either LasR **(C)** or HBD2 **(D)** as rigid receptors. Dashed lines indicate the –6 kcal/mol threshold for actively bound molecules.

TABLE 3 | Dissociation constants for quorum sensing molecules calculated using AutoDock Vina measurements.

Receptor	NEtP	3-oxo-C12-HSL	C4-HSL	PQS
LasR	558 μM	1.15 μM	18.3 μM	191 nM
HBD2	4.637 mM	3.348 mM	2.417 mM	403 μM

The best binding energies (predicted by AutoDock Vina) for each ligand-receptor pair were used to manually calculate dissociation constants (K_D) using Eq. (1). NEtP: phosphorylcolamine; 3-oxo-C12-HSL: N-(3-oxododecanoyl) homoserine lactone; C4-HSL: N-butanoyl homoserine lactone; PQS: 2-heptyl-3-hydroxy-4-quinolone.

from about 10 kDa to over 200 kDa with the most dominant bands appearing above 25 kDa, in particular a band around 35 kDa similar to the molecular weights of previously reported *P. aeruginosa* outer membrane proteins (79). Two weaker bands around 10 kDa are consistently visible only in the outer membrane preparations from control bacteria. Overall, the outer membranes from HBD2 treated bacteria appear to contain less proteins between 35 and 75 kDa. A prominent band between 10 and 15 kDa is detected in all samples, including the medium control, consistent with the molecular weight of the lysozyme (14 kDa) added during the extraction procedure. **Figure 8B** summarizes the protein profiles of the outer membrane preparations from control bacteria and HBD2 treated bacteria. To control for variations during the ultrafiltration process and gel loading, the band intensities of the various proteins were normalized with the presumptive lysozyme band intensity. HBD2 appears to affect a decrease in outer membrane proteins in particular at about 22, 34, 40, 45, and 50 kDa, with the changes noticeable at all concentrations tested.

Atomic Force Microscopy Reveals Ultrastructural Changes in HBD2 Treated Bacteria Reflected in Increased Surface Roughness

We also assessed whether the changes at the outer membrane induced by HBD2 resulted in topographical changes and employed atomic force microscopy to measure bacterial height and roughness after incubation for 18 h in the presence or

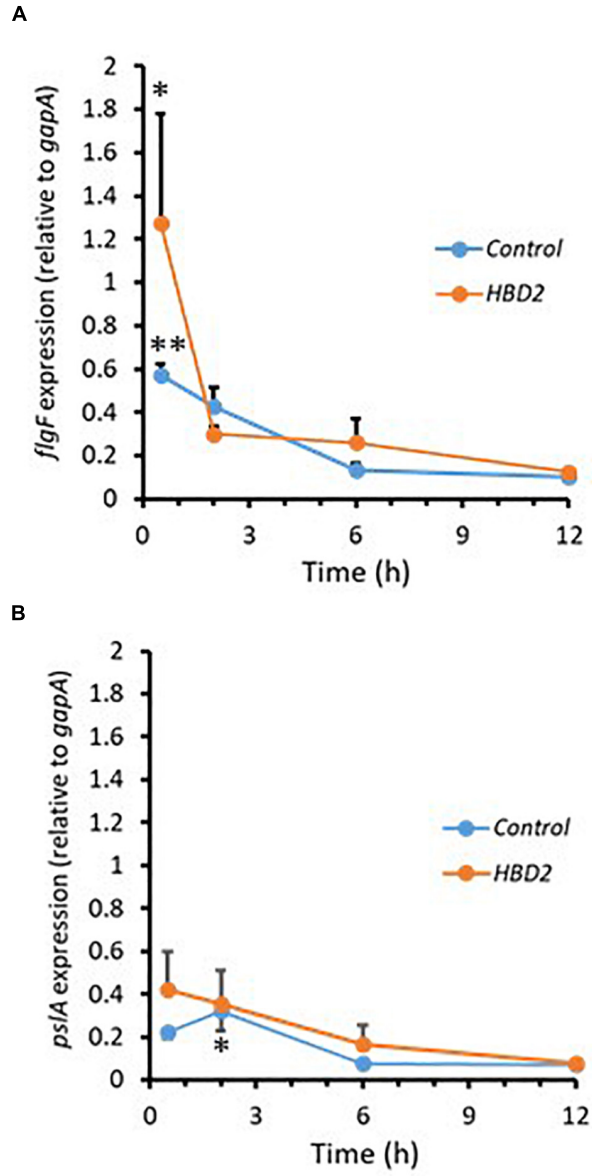

FIGURE 7 | Relative gene expression of *flgF* and *pslA* in the presence and absence of 0.25 μM HBD2 as determined by qPCR. Gene expression of *flgF* **(A)** and *pslA* **(B)** in *P. aeruginosa* was calculated relative to the reference gene *gapA* after incubation in the presence or absence of HBD2 for up to 12 h. Shown are means ± SEM, $n = 6$. In multivariate ANOVA with Bonferroni *post hoc* analysis (*$p < 0.05$ and **$p < 0.01$), gene expression of *flgF* and *pslA* changed over time (Control: $p < 0.01$ for *flgF* 0.5 h versus 6 h and 12 h, and $p < 0.05$ for *pslA* 2 h versus 6 h and 12 h; HBD2: $p < 0.05$ for *flgF* 0.5 h versus 12 h) but there was no significant difference between the control and HBD2 treated bacteria.

absence of 0.25 μM HBD2 (**Figure 9**). Representative images of control and HBD2 treated bacteria are shown in **Figure 9A**. The surface of control bacteria appears smoother compared to the surface of HBD2 treated bacteria, the latter showing irregular dents. While the overall bacterial height is not significantly different in HBD2 treated bacteria compared to solvent only exposed bacteria (215.22 ± 3.96 nm versus 220.24 ± 3.23 nm, means ± SEM, $n = 85$ and $n = 69$, respectively, $p = 0.343$), there is a significant increase in roughness in HBD2 treated samples (**Figure 9B**) consistent with a structurally altered surface (43.39 ± 1.52 versus 51.86 ± 1.5 nm, means ± SEM, $n = 85$ and $n = 69$, $p < 0.001$ in independent samples *T* test).

Taken together, our data demonstrate that at low concentrations L- and D-forms of HBD2 inhibit biofilm formation while not reducing metabolic activity in Gram-negative bacteria of two different genera, *Pseudomonas* and *Acinetobacter*. Furthermore, this activity appears to be receptor-independent and not mediated by interference with quorum sensing or other regulatory pathways of biofilm production at the transcriptional level. Instead, our data are consistent with structural changes induced by HBD2 that interfere with the transport of biofilm precursors into the extracellular space suggesting a novel mechanism of action for the antimicrobial peptide HBD2.

DISCUSSION

In this study, we demonstrate that, HBD2, at nanomolar concentrations, and independent of its chiral state, significantly reduced biofilm formation of *P. aeruginosa* without affecting metabolic activity. This was unlike HBD3, which equally reduced biofilm and metabolic activity at nanomolar concentrations. HBD2 similarly affected *A. baumannii*, another Gram-negative bacterium, at low concentrations. *In silico* modeling did not support direct binding of HBD2 to QS molecules, the release of a QS regulated pigment was not inhibited, and the expression of biofilm-related genes was not influenced by HBD2. However, the outer membrane protein profile was altered in HBD2 treated bacteria with reduced representation of several proteins, which was accompanied by increased roughness of the bacterial surface. Taken together, these data support a novel mechanism of biofilm inhibition by HBD2 at low concentrations that is independent of biofilm regulatory pathways but involves structural changes induced by HBD2 that may interfere with the transport of biofilm precursors into the extracellular space.

HBD2 has been previously reported to reduce bacterial survival in existing biofilm cultures of *Lactobacillus* ssp., Gram-positive bacteria, at higher, micromolar concentrations (80). However, inhibition of biofilm formation by HBD2 has not been reported previously to the best of our knowledge. Considering the rapid induction of HBD2 in epithelial cells' response to proinflammatory cytokines and bacterial challenge (81), the ability to interfere with biofilm formation at low concentrations adds importance to the role of HBD2 in innate host defense during the early interaction between host and pathogen. Bacteria are more susceptible to host-derived and exogenous antimicrobial agents while they are metabolically active in the planktonic state prior to biofilm production. Thus, HBD2 may amplify host defenses early in the attempted infection process and could improve the action of antibiotics in a clinical setting (82). Synergism studies will be able to address this experimentally in the future.

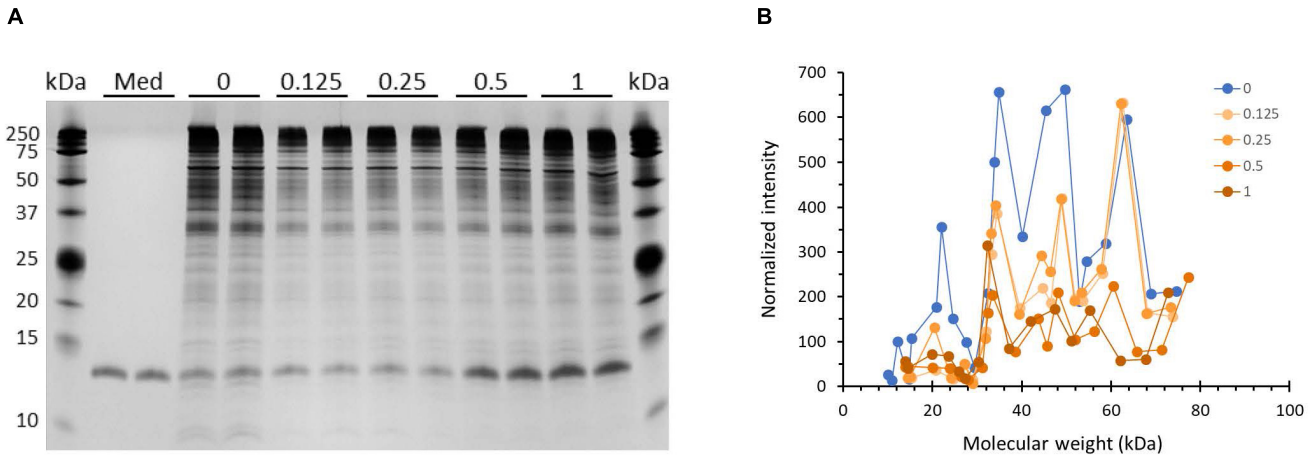

FIGURE 8 | Outer membrane protein profile of *P. aeruginosa* after 18 h incubation in the presence and absence of HBD2. **(A)** Four μL of concentrated outer membrane preparations from HBD2 treated (0.125–1 μM) or solvent control exposed bacteria (0) were resolved by SDS Tris-Tricine PAGE and visualized by silver stain. (Med) indicates medium only processed like bacteria-containing samples. The band migrating between 10 and 15 kDa in all samples is consistent with the expected molecular weight of lysozyme (14 kDa) that was added to the extraction buffer. **(B)** Approximate molecular weight and intensities of bands were quantified with Image Lab software and band intensities detected in both replicates were normalized to the intensity of the presumptive lysozyme band. Each data point represents the average of replicates. Each line represents the protein profile for the indicated HBD2 concentration (in μM).

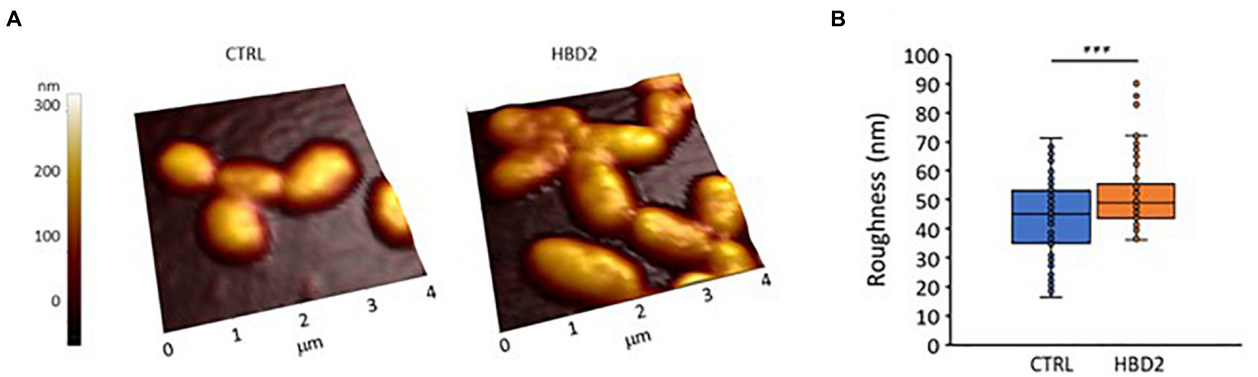

FIGURE 9 | Atomic force microscopy of *P. aeruginosa* after 18 h incubation in the presence and absence of 0.25 μM HBD2. Bacteria were incubated on glass slides and fixed with 2.5% glutaraldehyde prior to imaging. Images taken with the atomic force microscope were first order flattened before extracting measurement for bacterial roughness. **(A)** Representative images. CTRL: solvent control exposed bacteria. **(B)** Box and whisker chart (with inner points and outliers) of roughness measurements from multiple images of solvent exposed control bacteria (CTRL, $n = 85$) and 0.25 μM HBD2 treated bacteria ($n = 69$). ***$p < 0.001$ in independent samples T test.

Anti-biofilm activity of HBD2 in the absence of inhibition of metabolic activity of *P. aeruginosa* occurred only at low concentrations. A concentration dependent mechanism of action has been well documented for the lantibiotic nisin, which, at nanomolar concentrations, preferentially binds to lipid II disrupting cell wall synthesis and, at micromolar concentrations, embeds into the bacterial membrane causing pore formation (83–87). More recently, the alpha-defensin human neutrophil peptide 1 (HNP1) has been added to the list of AMPs that initially interact with lipid II, and when concentrations increase, with the bacterial cell membrane (88). Binding of HBD3 to lipid II has also been described, albeit at higher concentrations, in the micromolar range (47). It is conceivable that HBD2 could similarly interfere with membrane-embedded proteins responsible for the transport of biofilm components (17) at low concentrations followed by membrane perturbation at higher concentrations.

The differential effect of HBD2 on biofilm production and metabolic activity of *P. aeruginosa* was not observed in the related beta-defensin HBD3, which was active at lower concentrations than HBD2 and equally reduced biofilm and metabolic activity reflecting a strong bactericidal activity. These differences in their activity could be at least in part attributed to the differences in their physicochemical properties with respect to net charge, surface charge distribution, hydrophobicity index, and behavior in solution (51, 89).

Biofilm is a complex matrix with numerous components that can be affected in different ways by HBD2 and HBD3. For example, alginate has been shown to affect antimicrobial peptide conformation inducing alpha-helices contigent on the hydrophobicity (90), and HBD2 and HBD3 substantially differ in their hydrophobicity with HBD2 being more hydrophobic than HBD3.

HBD2, at low concentrations, similarly inhibited biofilm production in *A. baumannii* without reducing metabolic activity suggesting the observed effects are not strain specific. However, at higher HBD2 concentrations differences between the effects on *P. aeruginosa* and *A. baumannii* emerged as reflected in a near complete inhibition of biofilm production of *A. baumannii* contrasting the stalled biofilm inhibition of *P. aeruginosa*. The lesser susceptibility of *P. aeruginosa* to HBD2 may be due to a greater outer membrane vesicle production in *P. aeruginosa* that may sequester HBD2 before it reaches the bacterial cell (91).

Like other defensins, HBD2 forms three intramolecular disulfide bridges and linearization of the peptide can reveal the importance of its structure for its antimicrobial activity (92). Here, linearization of full length HBD2 led to a pronounced loss of both its antimicrobial and biofilm inhibitory activity. This contrasts reports for other defensins including HBD3 and could be attributed to a lack of accumulation of positively charged amino acid residues at the C-terminus of HBD2 compared to HBD3. Chandrababu et al. (93) have shown that positively charged residues cluster in the C-terminal segment of a linearized form of HBD3 allowing them to interact with the negatively charged phospholipids of micelles. The inherent antimicrobial activity of this patch of cationic residues is also reflected in studies with HBD3 analogs truncated to the C-terminal region (94). The here observed loss of activity after linearization could indicate that HBD2 functions through a receptor (56). However, D- and L forms of HBD2 did not differ in their activity and thus, we interrogated the possibility that HBD2 interferes with regulatory networks of biofilm production.

QS molecules are key to the regulation of virulence factor production including biofilm and pigment in *P. aeruginosa*. They are small hydrophobic molecules (95) and thus, we interrogated possible binding of HBD2 to QS molecules *in silico*. We found favorable binding of LasR to not only its cognate ligand 3-oxo-C12-HSL but also to C4-HSL and PQS. This is in line with a recent study describing LasR as promiscuis for binding a variety of QS molecules (96). The unfavorable binding energies derived for HBD2 suggest that interference of QS-dependent processes through direct HBD2 binding to individual QS molecules is unlikely. Another type of QS molecule, (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (S-THMF-borate), has been shown to increase biofilm formation in *P. aeruginosa* (97, 98). However, although S-THMF-borate – a molecule with a distinct structure from major Gram-negative QS molecules – has been identified in some Gram-positive and Gram-negative bacteria (99), *P. aeruginosa* does not encode the *luxS* gene required for its synthesis (100) and binding to this S-THMF-borate should not be further considered as an underlying mechanism for the observed biofilm inhibition.

In agreement with the *in silico* data, HBD2 did not affect the expression of *flgF* and *pslA*. Thus, interference of HBD2 with regulatory networks at the transcriptional level is not likely to account for its biofilm inhibitory activity. However, we cannot rule out that HBD2 has posttranscriptional effects through interference with the two component signal transduction system GacS/GacA (71, 101). GacS is a transmembrane sensor kinase phosphorylating GacA, which in turn induces the expression of small RNA molecules that antagonize the protein RsmA, a translational repressor interfering with *psl* translation and known to normally block exopolysaccharide production (102). It is conceivable that HBD2 could interfere with GacS upon inserting into the bacterial membrane. Finally, HBD2 might bind to the secondary messenger molecule c-di-GMP, which regulates biofilm formation in *P. aeruginosa* at multiple levels (103). Previously, de la Fuente-Nunez et al. (104) demonstrated that peptide 1018, derived from the antimicrobial peptide bovine Bac2a (105), inhibited biofilm formation in *P. aeruginosa* while not affecting planktonic growth by binding to the second messenger p(pp)Gpp and promoting its degradation. A similar mode of action could apply to HBD2.

Further supporting that HBD2 does not act through interference with regulatory networks is our finding that pyorubin accumulation in the extracellular fluid was not diminished after incubation with HBD2. Pyorubin is composed of several pigments including aeruginosin A, which is a phenazine, like the much better studied *P. aeruginosa* pigment pyocyanin (106). Phenazines typically traverse the bacterial membrane freely and their production is under the same controls that govern biofilm production (107, 108).

Considering the lack of evidence for interference with regulatory networks and the stereoisometry independent activity of HBD2, we conceived that the observed HBD2 mediated inhibition of biofilm production is most likely due to embedding in the bacterial membrane and disruption of transport of biofilm precursor molecules across the membrane. An increasing amount of research suggests that AMPs can target discrete loci in bacterial membranes and thereby disrupt biological processes (109). For example, AMPs are known to impair the assembly of multicomponent enzyme complexes in the bacterial cell membrane (110) or disrupt periplasmic protein-protein interaction interfering with molecular transport (111). In 2013, Kandaswamy et al. showed that HBD2 localizes to the mid-cell region of the Gram-positive bacterium *E. faecalis* (112). The authors determined that this mid-cell region is rich in anionic phospholipids and that HBD2 delocalized the spatial organization of protein translocase SecA and sortases, both of which are important for pilus biogenesis (112, 113). It is possible that HBD2 targets similar machinery in *P. aeruginosa* to impair biofilm formation. SecA also plays a role in the transport of outer membrane proteins in Gram-negative bacteria (114) and outer membrane proteins have been shown to participate in biofilm formation, including the 11 kDa LecB protein and the 38 kDa OprF (115, 116). Consistent with this we found an

altered outer membrane protein profile in HBD2 treated bacteria with a paucity of proteins around 10 kDa and proteins around the molecular weights of previously reported outer membrane proteins. This may indicate structural changes of the outer membrane, which was further supported by our atomic force microscopy data demonstrating an increased roughness of the bacterial surface after HBD2 treatment. It is important to note, however, that increased roughness could also represent changes in the LPS profile. Atomic force microscopy has been previously employed elsewhere to demonstrate outer membrane damages in *P. aeruginosa* (117). Resolving the outer membrane proteins by 2D gel electrophoresis could further delineate the observed changes in future experiments, which should also revisit the action of the D-form of HBD2 and effects on the outer membrane of *A. baumannii*. Finally, outer membrane vesicles have been recognized to take part in the formation of biofilm by interacting with extracellular DNA and HBD2 interference with proper outer membrane formation may disrupt this process (118).

CONCLUSION

This study reveals distinct activity of two epithelial beta-defensins, HBD2 and HBD3, and provides evidence for a novel antibacterial action of HBD2. At low concentrations in the nanomolar range, HBD2 reduced biofilm formation without reducing the metabolic activity of *P. aeruginosa*. Biofilm production of *A. baumannii* was similarly affected, indicating that the observed HBD2 activity is not strain specific. This activity is unlikely mediated through a receptor-dependent interference with regulatory networks but contingent on preservation of HBD2 structure. Our findings are consistent with a membrane-targeted action of HBD2 that affects proper function of membrane-associated proteins involved in biofilm precursor transport into the extracellular environment. Future studies dissecting the molecular basis for the described HBD2 activity may inform the development of new methods for the manipulation of biofilms in aquaculture, in the food industry, and in the healthcare setting, which is in particular of interest for the latter considering the rise of multidrug resistance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

KP, BB, TY, MB, EE, AT, AC, and YW: acquisition of the data. KP, BB, TY, MB, EE, AT, AC, HP, YW, WL, and EP: analysis and interpretation of the data. KP, MM, and CA: method development. KP and EP: statistical analysis. KP: molecular docking. KP, HP, YW, WL, and EP: conceptual and experimental design. KP, HP, and EP: drafted the manuscript. KP, BB, TY, MB, EE, AT, AC, MM, CA, HP, YW, WL, and EP: critical revision of the manuscript for important intellectual content. All authors approved the final manuscript submission.

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REVIEW
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The Network of Colonic Host Defense Peptides as an Innate Immune Defense Against Enteropathogenic Bacteria

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Host defense peptides, abundantly secreted by colonic epithelial cells and leukocytes, are proposed to be critical components of an innate immune response in the colon against enteropathogenic bacteria, including *Shigella* spp., *Salmonella* spp., *Clostridium difficile*, and attaching and effacing *Escherichia coli* and *Citrobacter rodentium*. These short cationic peptides are bactericidal against both Gram-positive and -negative enteric pathogens, but may also exert killing effects on intestinal luminal microbiota. Simultaneously, these peptides modulate numerous cellular responses crucial for gut defenses, including leukocyte chemotaxis and migration, wound healing, cytokine production, cell proliferation, and pathogen sensing. This review discusses recent advances in our understanding of expression, mechanisms of action and microbicidal and immunomodulatory functions of major colonic host defense peptides, namely cathelicidins, β-defensins, and members of the Regenerating islet-derived protein III (RegIII) and Resistin-like molecule (RELM) families. In a theoretical framework where these peptides work synergistically, aspects of pathogenesis of infectious colitis reviewed herein uncover roles of host defense peptides aimed to promote epithelial defenses and prevent pathogen colonization, mediated through a combination of direct antimicrobial function and fine-tuning of host immune response and inflammation. This interactive host defense peptide network may decode how the intestinal immune system functions to quickly clear infections, restore homeostasis and avoid damaging inflammation associated with pathogen persistence during infectious colitis. This information is of interest in development of host defense peptides (either alone or in combination with reduced doses of antibiotics) as antimicrobial and immunomodulatory therapeutics for controlling infectious colitis.

Keywords: colonic epithelium, host defense peptides, cathelicidin, defensin, colitis

INNATE IMMUNITY IN INFECTIOUS COLITIS AND THE PRESENCE OF HOST DEFENSE PEPTIDES

Infectious diarrhea causes inflammation of the gastrointestinal tract, clinically manifested by diarrhea, dehydration and, in severe cases, death. Infectious diarrhea is a major cause of morbidity and mortality worldwide, particularly in developing countries (1). Diarrhea is regarded as the 8th leading cause of death, with children (<5 year of age) being responsible for over a quarter of deaths (2). Of the >1 million diarrheal deaths attributed to infectious agents, bacterial pathogens were collectively responsible for ∼57% (2). Likewise, of the >2 billion global cases of diarrhea due to foodborne illnesses in 2010, 32% were due to bacteria (3). In addition, bacterial diarrhea is a main cause of illness in travelers seeking medical care after returning from developing nations (4).

The main genera of bacteria that cause infectious colitis are *Escherichia*, *Salmonella*, *Campylobacter*, *Vibrio*, *Listeria,* and *Shigella*. Increased emergence of antibiotic resistant bacterial strains have limited our ability to treat important enteric pathogens including *Escherichia* and *Salmonella*, and raises the possibility of increased prevalence and mortality due to intestinal bacterial infections (5–7). Indeed, antibiotic resistance is predicted to be a major future public health problem, with antibiotic resistant bacteria expected to cause > 10 million deaths globally by 2050 (8). Development and commercialization of new antibiotics is minimal and there are predictions that without substantial changes, bacterial resistance will continue to increase. Therefore, understanding innate immune mechanisms that aid in pathogen clearance and resolution are critical to understanding pathophysiology of infectious colitis and developing novel antimicrobial and immunomodulatory therapeutics.

The gastrointestinal tract has metabolic functions of digestion and nutrient absorption and also provides a barrier against large numbers of commensal or pathogenic microbes in the lumen. The colon employs multiple innate mechanisms to prevent and clear bacterial infections. For example, MUC2 mucin glycoprotein secreted by goblet cells and host defense peptides (HDPs) secreted by intestinal epithelial cells into

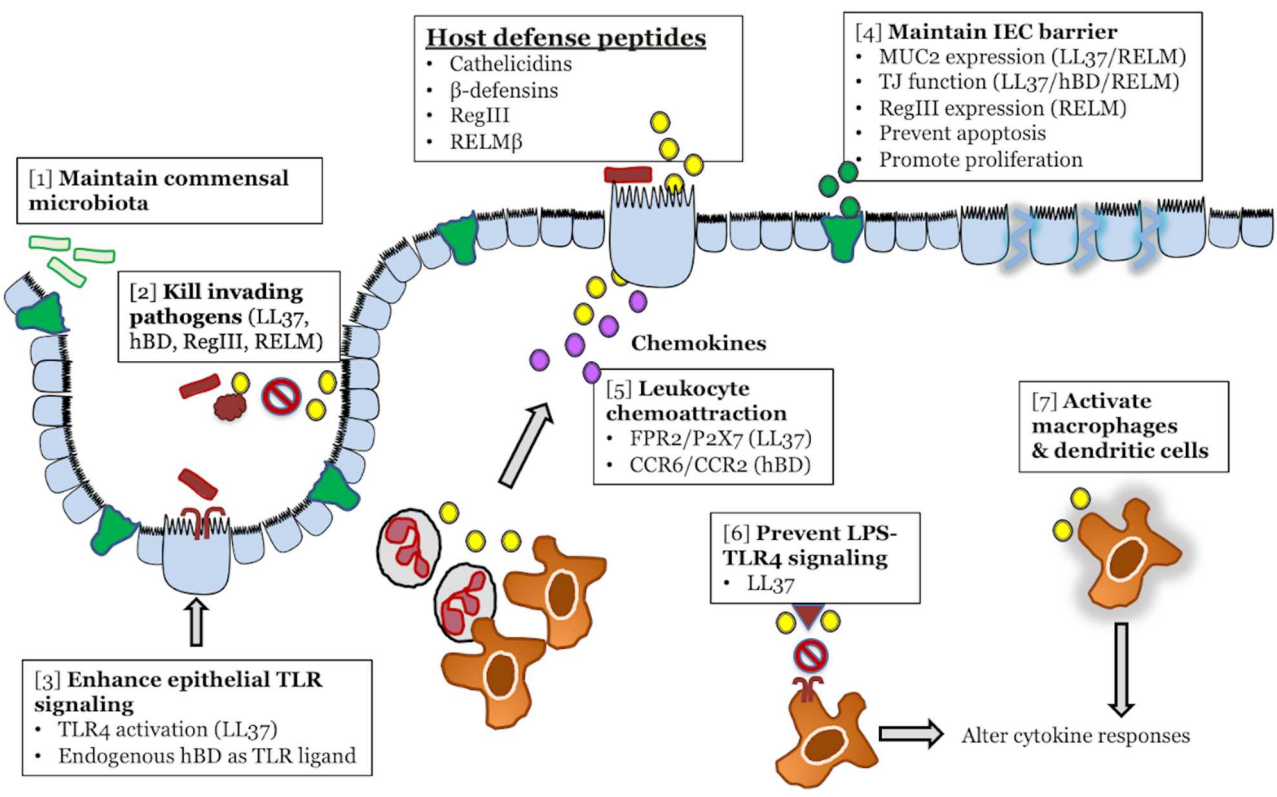

FIGURE 1 | Colonic host defense peptides (HDPs) contribute to intestinal homeostasis and innate immune defense during infectious colitis through multiple overlapping mechanisms. HDPs secreted from intestinal epithelial cells (yellow) exert direct antibacterial effects on both the intestinal microbiota [**1**] and invading bacterial pathogens [**2**]. In terms of immunomodulatory functions, HDPs can enhance the immune signaling capacity of intestinal epithelial cells by forming complexes with LPS/TLR4 (LL-37), or by directly activating TLRs (β-defensins) [**3**]. HDPs might maintain intestinal epithelial cell barrier and prevention of pathogen invasion by increasing MUC2 secretion (green) and tight junction protein expression [**4**]. In the lamina propria, HPDs (yellow) secreted by epithelial cells or infiltrating leukocytes can directly chemoattract leukocytes from the blood (neutrophils, macrophages), through activation of FPR2, P2X7, CCR2, and CCR6, or promote the secretion of chemoattractant (CXCL8/IL-8) by epithelial cells (purple) [**5**]. In tissue resident macrophages, HDPs (yellow) can either promote anti-inflammatory responses by blocking LPS-TLR4 interaction [**6**] or activate macrophages and dendritic cells [**7**] to alter cytokine responses.

the luminal environment compose the mucus layer, an acellular first line of defense. Intestinal epithelial cells, held together by apical junctional complexes, form a second line of defense to prevent penetration of bacteria into the lamina propria. If pathogenic bacteria are able to penetrate mucus and epithelial barriers, underlying leukocytes can protect the host by initiating inflammatory responses to clear invading pathogens.

Among innate effectors in the colon, HDPs are short cationic peptides abundantly secreted into the lumen by leukocytes and the intestinal epithelium, with key functions in maintenance of gut homeostasis (**Figure 1**). In the colon, HDPs are mostly represented by cathelicidin, β-defensins, and members of the Regenerating islet-derived protein III (RegIII) and Resistin-like molecule (RELM) families. Known as broad-spectrum antimicrobials, their contribution to innate gut defenses is expected to extend beyond direct lytic effects on bacteria to include immune functions reported *in vitro* (e.g., recruitment of immune cells, wound healing, and cytokine production; **Table 1**) (9, 42, 43). These immunomodulatory functions have been mostly described for myeloid-derived immune cells (10, 44). A key question is the extent to which immunomodulatory effects of HDPs occur in the gut and their relevance in infectious diseases. There are indications that secreted colonic HDPs are not merely antimicrobial, but also contribute to orchestrated immune responses. Understanding these aspects of HDP function is necessary for identifying novel anti-inflammatory and anti-infective targets as alternatives to conventional antimicrobials. Herein, we review recent advances in our understanding of HDPs in gut innate immune defenses and their role in pathogenesis of infectious colitis.

TABLE 1 | Immunomodulatory cellular functions attributed to colonic host defense peptides.

Host defense peptide	Immune function	References
Cathelicidin	• Alter chemokine responses • Inhibit TLR4 activation (leukocytes) • Enhance TLR4 signaling (enterocytes) • Chemoattractant • Increase MUC2 expression • Induce NET formation	(9–23)
β-defensin	• Chemoattractant • Activate dendritic cells and monocytes • Stimulate cytokine release • Increase epithelial barrier function • Induce epithelial cell migration • Induce MUC2 expression	(24–32)
REGIII	• Promote epithelial cell proliferation • Prevent apoptosis	(33, 34)
RELM-β	• Increase epithelial cell proliferation • Chemoattract T-cells • Regulate REGIII expression • Regulate epithelial cell barrier function • Increase mucus secretion • Promote fibrosis	(35–41)

CATHELICIDINS

Cathelicidins are small, cationic, amphipathic peptides produced by epithelial cells, macrophages, and polymorphonuclear leukocytes (44, 45). These peptides are synthesized as pro-peptide precursors with a highly conserved N-terminal region (cathelin domain) and a highly variable antimicrobial cathelicidin peptide domain in their C-termini. Cleavage of the C-terminal domain from the holoprotein (e.g., by serine proteases) is required for antimicrobial activity. Humans have a single cathelicidin gene (*cathelicidin antimicrobial peptid*e, *CAMP*), which yields a 37 amino acid peptide (leucine-leucine, LL-37) generated by extracellular cleavage of the C-terminus (46). The murine counterpart is cathelicidin-related-antimicrobial-peptide (CRAMP), encoded by the gene *Camp* (formerly *Cnlp*) (47).

In the colon, cathelicidins are mostly secreted by neutrophils and epithelial cells (42, 48). Differentiated colonic epithelial cells at the peak of crypts constitutively secrete cathelicidins (42, 48, 49), which are normally present in intestinal mucus of healthy individuals (50). Protective roles of cathelicidin in infectious colitis have been demonstrated in mice homozygous for null mutations in *Camp* ($Camp^{-/-}$). These mice had exacerbated diarrhea, destructive colitis, and increased pathogen burden after challenge by chemical (43) or infectious (*Clostridium difficile*) (51) agents. Accordingly, $Camp^{-/-}$ mice were more susceptible to infection with attaching/effacing bacteria including *C. rodentium* (42) and *E. coli* O157:H7 (11). Consistent with these findings, upregulation of endogenous cathelicidin ameliorated colitis caused by enteropathogenic *E. coli* in rabbits (52).

Signaling pathways that regulate cathelicidin synthesis in the colon respond to both bacterial and endogenous stimuli. Regarding bacterial stimuli, colonic epithelium produced cathelicidins in response to bacterial by-products, such as short chain fatty acids (e.g., butyrate) (48, 53) via MEK-ERK signaling (49, 54). Bacterial DNA also stimulated cathelicidin in colonic lamina propria macrophages through TLR9 (43). This mechanism was observed *in vivo* when intracolonic exposure to *E. coli* genomic DNA upregulated cathelicidin expression in mice via TLR9 signaling (43). Similar to bacterial DNA, double-stranded RNA mimic poly(I:C) induced cathelicidin expression from intestinal epithelial cells via PI3-kinase-PKCζ-Sp1 signaling independent of MAPK pathways (55). MAPK signaling was also required for cathelicidin expression from colonic epithelial cells exposed to a combination of IL-1β and purified MUC2 (56).

Direct antibacterial activity was the first function identified for cathelicidins (57) with most studies focusing on the role of LL-37 against *E. coli*. Whereas, multiple antibacterial mechanisms may occur simultaneously, a principal bactericidal action of cathelicidins is membrane pore formation followed by direct bacterial death. LL-37 recognizes negatively charged lipids, a major component of Gram-negative bacterial membranes (58, 59). Binding of LL-37 to the bacterial surface leads to formation of transmembrane pores that induce bacterial cell lysis (60, 61). This pore formation depends on the alpha-helical amphipathic structure of LL-37, which shape its interactions with negatively-charged and hydrophobic targets on bacterial membranes. Because the structure of LL-37 is highly dependent

on environmental factors (e.g., pH and anion concentration), the ability of cathelicidins to kill *E. coli* by transmembrane pore formation may be affected in physiological conditions (61).

Other antimicrobial mechanisms of cathelicidins include binding LPS to cross bacterial outer membranes into the periplasmic space, where LL-37 binds and immobilizes peptidoglycan to impede cell wall biogenesis and growth (62). Additionally, there is a large influx of LL-37 into the bacterial cell after permeabilization of outer and cytoplasmic membranes that rigidifies the cytoplasm and halts motion of chromosomal DNA and ribosomes, thereby arresting *E. coli* growth (63). The polycationic nature of LL-37 allows it to form a network of electrostatic bonds with polyanionic DNA and ribosomes, preventing proper diffusion of cellular components (63). However, some of these antibacterial effects may be simply bacteriostatic and may not be effective at the population level. In high-density *E. coli* cultures exposed to LL-37, a sub-population of non-growing bacterial cells absorb massive amounts of LL-37 to deplete it from the surrounding environment, enabling a second sub-population to continue growing (64). Unlike LL-37, which interacts directly with microbial cell surfaces [e.g., *E. histolytica* (56)], other cathelicidins seem to internalize within bacterial cells and trigger non-lytic mechanisms. For example, porcine proline rich cathelicidin (PR-39), abundant in myeloid cells in pigs, crosses the cell membrane and likely kills pathogens by blocking bacterial DNA and peptide synthesis (65).

In an attempt to establish infection, intestinal pathogens may actively dampen cathelicidin defenses by multiple strategies. One strategy is to decrease cathelicidin expression in the colon during bacterial colonization. Cathelicidin production was decreased in colonic epithelium and leukocytes of shigellosis patients during early infection, where both live *Shigella* and bacterial plasmids blocked transcription of cathelicidin mRNA (66). Cathelicidin was also transcriptionally suppressed in colonic epithelial cells by exotoxins of *Vibrio cholera* and *E. coli* (cholera toxin and labile toxin, respectively) (67). Thus, cathelicidin silencing is likely a key virulence mechanism used by bacterial pathogens to facilitate intestinal colonization. Another evasion strategy is to repel direct killing by cathelicidins. While cathelicidins displayed *in vitro* killing activity against multiple strains of *E. coli* (11, 68), *Salmonella enterica* serovar Typhimurium resisted killing by cathelicidin through modulation of its outer membrane properties (69). Of note, despite these findings, the real relevance of cathelicidin antimicrobial activity in the gut is still controversial. Cathelicidins showed broad *in vitro* antibacterial activity (either bactericidal or bacteriostatic) against both Gram-positive and -negative bacteria (12, 70–73) (**Table 2**). However, this antibacterial activity is often abolished under physiological conditions, including presence of high salt concentrations (68, 85), serum (86), plasma alipoprotein-A1 (87), and sugars (88). Antibacterial activity of certain cathelicidins [synthetic cathelicidin (C18G), protegrin, magainin-like peptide] could be further inhibited by bacterial surface modifications, e.g., lipid A acylation by *Salmonella spp.* (89). Therefore, it is still questionable if cathelicidins undertake extensive antimicrobial activities in the colonic lumen. It is possible that microbicidal activities are restricted to certain conditions or niches (e.g., deeper in intestinal crypts, within the inner mucus layer) where cathelicidins can reach high concentrations and/or overcome inhibitory physiological effects.

TABLE 2 | Direct *in vitro* antimicrobial functions of colonic host defense peptides.

Host defense peptide		Antibacterial activity		References
		Gram-negative	Gram-positive	
Cathelicidin		*E. coli* *C. rodentium* *S. enterica* *S. enteritidis* *K. pneumoniae*	*L. monocytogenes* *S. aureus* *E. faecalis*	(12, 42, 70–73)
β-defensin	-1	*E. coli* *S. enteritidis*	*Bifidobacterium* spp. *Lactobacillus* spp. *B. subtilis* *S. aureus*	(29, 74)
	-2	*E. coli* *P. aeruginosa* *H. pylori*	*S. aureus* *S. pyogenes*	(75–78)
	-3	*S. enterica* *E. coli* *P. aeruginosa*	*L. monocytogenes* *S. aureus* *E. faecalis*	(78)
	-4	*E. coli* *P. aeruginosa*	*S. aureus*	(79)
RELM-β		*P. aeruginosa* *C. rodentium*	*L. monocytogenes* *E. faecalis*	(80)
REGIII	-β	*E. coli* *Bacteroides* spp.		(81, 82)
	-γ		*L. monocytogenes* *L. innocua* *E. faecalis*	(83, 84)

On the other hand, there is growing evidence that immunomodulation is a critical function of cathelicidins in gut homeostasis. Such immunomodulation can be achieved by signaling through both colonic epithelial cells and immune cells, often at concentrations lower than is required for antimicrobial activity (**Figure 1**, **Table 1**). A first role of cathelicidins in gut innate immuninty could be enhancement of Toll-like receptor (TLR) sensing and prevention of pathogen invasion into colonic epithelial cells. For instance, human adenocarcinoma colonic epithelial (HT-29) cells exposed to a combination of synthetic LL-37 and LPS had increased TLR4 gene and protein expression (13). Such TLR4 activation is expected to increase production of pro-inflammatory cytokines, since LL-37 was required for CXCL8 and IL-1β production from colonocytes exposed to bacterial stimuli (13, 14). Moreover, the combination of cathelicidin and LPS prevented invasion of *Salmonella enterica* serovar Typhimurium into HT-29 cells (14). Although the synergistic effect between cathelicidins and LPS has not be tested *in vivo*, HT-29 cells served to examine colonic epithelial cell responses, as they constitutively express TLR4 and secrete CXCL8 in response to LPS as do primary intestinal epithelial cells (90). In support of this presumptive role in the colonic mucosa, LL-37 primed inflammatory responses in airway epithelial cells during *Pseudomonas aeruginosa* infection, promoting IL-1β

and IL-18 secretion in an NLRP3 and caspase-1 dependent fashion (91).

Other immunomodulatory roles of cathelicidins in the gut include regulation of the intestinal epithelial barrier. This is important because the epithelial barrier plays a critical role in colonic histopathological changes and diarrhea that characterize infectious colitis. The epithelial paracellular barrier is largely maintained by tight junctions (TJs), and is critical for water absorption and restricting invasion of enteric luminal bacteria. TJs are disrupted in the colon exposed to enteric pathogens (92), but cathelicidins induced TJ gene expression in mammalian enterocytes and porcine intestines (9, 93). In addition, LL-37 prevented disruption of the TJ protein ZO-1 during *S. enterica* serovar Typhimurium infection in colonic epithelial (T84) cells (14). Effects of cathelicidins are not restricted to gut epithelium, as LL-37 induces upregulation of tight junction proteins and increases epithelial barrier function in keratinocytes (94). Although significance of cathelicidins in maintaining the gut barrier is incompletely understood, these functions might contribute to the increased pathogen burden and histopathological damage in $Camp^{-/-}$ mice infected with *C. rodentium* (42).

The colonic epithelial barrier is also maintained by the mucus layer, mainly composed of MUC2 mucin derived from intestinal goblet cells (56). MUC2 mucin limited *in vitro* colonization by pathogenic *E. coli* (95) and mechanically expelled pathogens from the gut to prevent *C. rodentium* propagation (96). The colonic mucus barrier is comprised of a firmly attached inner layer devoid of bacteria and a more loosely attached outer layer which contains large numbers of commensal bacteria (97). $Camp^{-/-}$ mice had a thinner colonic mucus layer and were more easily penetrated by *E. coli* O157:H7 (11), demonstrating the importance of cathelicidin for forming an effective mucus barrier. Moreover, stimulation of HT-29 cells with LL-37 induced gene expression of mucins *MUC1* and *MUC2* via the P2X purinoceptor 7 (P2X7) and MAP kinase pathway (9, 15). This function has been demonstrated in other epithelia, where stimulation of airway epithelial (NCI-H292) cells with LL-37 resulted in MUC5AC production through EGFR activation (98). Thus, it has been postulated that cathelicidins and mucin coexist as first line defenders in the intestinal lumen. Moreover, the more compact inner mucus layer could retain cationic peptides due to its overall negative charge and provides a gradient of antimicrobial HDPs that separates commensal microbiota from the epithelium (50). Indeed, cathelicidins are implicated in maintaining the colonic microbiota. $Camp^{-/-}$ mice display a different colonic microbiota in comparison to $Camp^{+/+}$ mice, mostly associated with increased populations of *Odoribacter lanues*, *Desulfovibrio piger,* and *Desulfomicrobium orale* in $Camp^{-/-}$ mice (99).

It is known cathelicidins can act as direct chemoattractants to promote leukocyte influx to the site of infection; a role that could be critical in infectious colitis (100, 101). In leukocytes, cathelicidins signal through a range of receptors, including P2X7 and Formyl Peptide Receptor 2 (FPR2) that recognize extracellular ATP and N-formyl peptides, respectively. Accordingly, LL-37 inhibited neutrophil apoptosis by signaling through P2X7 and PI3K pathway (102), and chemoattracted FPR2-expressing peripheral blood monocytes, neutrophils and $CD4^+$ T cells (16). The inhibition of apoptosis in neutrophils was abrogated by blocking P2X7 and the PI3K pathway (102), while the chemoattractant function of cathelicidin was inhibited by both a specific FPR2 inhibitor and the G-protein coupled receptor inhibitor pertussis toxin (16). Moreover, LL-37 directly activated CD11b/CD18 to increase monocyte migration (103) and phagocytosis of LL-37 coated bacteria (104), indicating a role of cathelicidin in leukocyte phagocytosis and migration through CD11b/CD18. Indirect activation of CD11b/CD18 by LL-37 and CRAMP on monocytes can also occur through activation of FPR2 (105). Cathelicidins can promote additional antimicrobial functions in neutrophils, such as induction of neutrophil extracellular trap (NET) formation. Stimulation of human neutrophils with both LL-37 and CRAMP resulted in an increase in NET formation (17, 18). Further research is needed to define the importance of these chemoattractant and pro-phagoctytic effects of cathelicidins in gut physiology and defenses in infectious colitis.

One intriguing aspect of cathelicidins is their pleiotropic nature, exerting either pro-inflammatory effects or attenuating inflammation depending on the environment. Cathelicidins inhibit LPS-induced pro-inflammatory responses in leukocytes. LL-37 inhibited the LPS-induced secretion of TNF-α from phagocytic THP-1 cells (10) by blocking binding of LPS to CD14 (19). This LPS neutralization was also important for preventing LPS-induced apoptosis in endothelial cells (106) and LPS/ATP-induced macrophage pyroptosis (20). Likewise, LL-37 lowered *TNF-α, Cxcl-1,* and *IL-1β* expression in both cultured murine macrophages and mammary epithelial cells exposed to the pathogenic algae *Prototheca bovis* (107). On the other hand, cathelicidins seem to promote inflammatory responses in intestinal epithelium (13, 14). Cathelicidin exhibited pro-inflammatory functions in intestinal epithelial cells exposed to LPS or *S. enterica*, including increased TLR4 expression, increased CXCL8 expression, and increased IL-1β (13, 14). It is likely that pro- or anti-inflammatory immunomodulatory function of cathelicidin is cell-type specific, and depends on the receptors expressed by either leukocytes or epithelial cells, infection status, the class of infecting pathogen, and the surrounding cytokine milieu.

The inter-species activity of cathelicidins is of interest for understanding the ontogeny of these ancenstral defenses and the development of therapeutics. Although all cathelicidins share a highly conserved N-terminal cathelin domain, the C-terminal antimicrobial domain is highly variable, both in sequence identity and secondary structure (45). For example, LL-37 and CRAMP share <70% sequence identity, however, they are still considered homologous. This is because both LL-37 and CRAMP share similar structure (α-helical and net charge of +6), antimicrobial capabilities (12, 108), and show interspecies functional capacity (109, 110). Both peptides comparably regulated chemokine expression and TLR 4 activity in myeloid cells (12, 21, 22) and neutrophil recruitment via FPR2 (16, 23). Moreover, mice infected with H1N1 influenza A virus had enhanced survival and reduced viral titer upon treatment with nebulized LL-37

(109). Likewise, intranasal administration of LL-37 increased inflammatory responses in sinonasal tissue of mice (110), while CRAMP was chemoattractant for human monocytes, neutrophils, and macrophages (23). However, an analysis of the interspecies functionality of 12 cathelicidins from 6 different species showed varying immunomodulatory activity results. While cathelicidins from all species demonstrated antimicrobial and LPS neutralizing function, there was large variability in the peptides' abilities to induce cytokine secretion from RAW264.7 macrophages (12). Thus, although they share common aspects, each cathelicidin should be studied as a unique peptide with specific activities in each host.

Given the antimicrobial and immunomodulatory characteristics of endogenous cathelicidins, the use of exogenous cathelicidin peptides (or their derivatives) as therapeutics for infectious colitis is appealing. Systemically administered exogenous cathelicidins were shown to attenuate colitis and reduce *Salmonella* burden in mice (111), while intracolonic CRAMP administration attenuated murine *C. difficile* colitis (51). Furthermore, intraperitoneal injection of porcine cathelicidin PR-39 in EHEC-infected mice improved survival, attenuated inflammatory cell infiltration and pro-inflammatory cytokine production (IL-1β, TNF-α, and IL-6) in the colon, and restored jejunal tight junction formation (112). Treatment of EHEC-infected mice with a cathelicidin derived from the snake *Bungarus fascia* (cathelicidin-WA) was similarly effective as the antibiotic enrofloxacin for increasing survival, reducing histopathological colonic damage and attenuating inflammatory colonic IL-6 production (113). Moreover, cathelicidin-WA was more effective than enrofloxacin for restoring jejunal mucus thickness and goblet cell number in EHEC-infected mice (93). A CRAMP-vancomycin conjugate demonstrated increased antibacterial activity against Gram-negative bacteria when compared to vancomycin or CRAMP alone, or to a 1:1 mixture of vancomycin and CRAMP (114). Synthetic HDPs derived from bovine cathelicidin peptide sequences with direct bactericidal and immunomodulatory functions (named immune defense regulator peptides, IDRs) have been developed for the treatment of diverse bacterial infections (115). IDR-HH2,−1002, and−1018 stimulated neutrophil functions including chemokine secretion, expression of adhesion molecules and release of antimicrobial HDPs, resulting in increased neutrophil killing of *E. coli* (116). IDR-1002 showed anti-inflammatory functions in a murine ear sterile inflammation model, decreasing IL-6, MCP-1, and KC production in PMA inflamed ears (117). Treatment of *P. aeruginosa* infected mice with IDR-1002 showed decreased bacterial burden and associated inflammation, including decreased IL-6 and MCP-1 in bronchoalveolar lavage fluid (118). Additionally, RAW 264.7 macrophages pre-treated with IDR-1002 and then stimulated with LPS showed reduced TNF-α and COX-2 expression (119). IDRs could also be combined with conventional antibiotics; IDR-1018 demonstrated anti-biofilm activity against *P. aeruginosa* and synergistic capabilities with antibiotics to kill biofilms of *P. aeruginosa*, *E. coli*, *Acinetobacter baumannii*, and *S. enteria*, among others (24). Thus, cathelicidins show promise as a potential future therapeutic against infectious colitis to reduce or replace antibiotics.

β-DEFENSINS

β-defensins are small cationic HDPs characterized by their cysteine-rich nature and disulphide bridges. There are at least 48 human β-defensin (hBD) genes (120), including hBD-1, -2, -3, and -4 that are highly expressed in the colon (25, 121). In mice, murine β-defensin (mBD)-1, -4, and -14 have been proposed as orthologous to hBD-1, -2, and -3, respectively (120). In terms of gut regulation, hBD-1 is constitutively expressed in colonic epithelium but does not appear to be upregulated by inflammatory signals (26), whereas hBD-2, -3, and -4 are minimally expressed in healthy colonic epithelium but are induced during inflammation (27–29).

Specific pro-inflammatory cytokines regulate colonic β-defensins. For example, IL-1α/β, and TNF-α enhanced expression of hBD-2 in intestinal epithelial cells without affecting hBD-1 expression (26). Such β-defensin induction occurred mostly through NF-κB (26). Likewise, activation of TLR2 and TLR4 directly activated hBD-2 expression in colonic epithelial cells through NF-κB and AP-1 (30), as well as activation of Nucleotide-binding Oligomerization Domain-like Receptor 2 (NOD2) (31). NF-κB-independent mechanisms have also been involved in β-defensin synthesis. Corticosteroids increased β-defensin expression independent of NF-κB in intestinal epithelial (Caco-2) cells (32). Additionally, hBD-3 was upregulated independently of NF-κB in human colonic epithelial cells exposed to extracts from medicinal plants (andrographolide, oridonin, and isoliquiritigen) (122). This upregulation of β-defensin increased bactericidal activity against *Listeria monocytogenes* and, bacteriostatic activity against *S. enterica* in supernatants from human colonic epithelial cells (122). The colonic mucus layer is also important in hBD-2 regulation. The major colonic secretory mucin, MUC2, upregulated hBD-2 in HT-29 cells in response to IL-1β (75). Moreover, mice genetically deficient in Muc2 ($Muc2^{-/-}$) had decreased expression of mBD-4 and mBD-14 (75). Interestingly, fully glycosylated mature MUC2 reduced antibacterial activity of hBD-2 against pathogenic (EPEC) and commensal *E. coli*, indicating mucin may protect enteric bacteria from killing by β-defensins (75). These results are particularly important for ulcerative colitis patients, who commonly have diminished or disrupted intestinal mucus layers, suffer from dysbiosis, and are more prone to *Clostridium difficile* infection (123, 124).

Direct antibacterial functions of β-defensins are attributed to a disruption of microbial membranes by pore formation, causing release of intracellular contents and death (125). β-defensin homologs have a broad range of antibacterial activity (**Table 2**). For instance, hBD-2 has bactericidal activity against Gram-negative bacteria (i.e., *E. coli, P. aeruginosa, Helicobacter pylori*) and fungicidal activity against yeast (*Candida albicans*), but is merely bacteriostatic against the Gram-positive bacterium *Staphylococcus aureus* (76, 77). Conversely, hBD-3 is directly bactericidal against *S. aureus* and vancomycin-resistant *Enterococcus faecium* (VRE) (78). hBD-4 has bactericidal activity against both Gram-negative *E. coli* and *P. aeruginosa* in addition to Gram-positive *S. aureus* (79). Interestingly, reduction of disulphide-bridges in hBD-1 increases its bactericidal activity

against *C. albicans* and Gram-positive commensal bacteria (29). Noteworthy, hBD-1 may have additional antibacterial functions beyond direct bacterial lysis; hBD-1 forms an entrapping net that abolished bacterial translocation across polycarbonate membranes and would prevent bacterial invasion (74).

β-defensins have chemoattractant function, although these roles in colitis are poorly defined. Both human (hBD-1, -2, and -3) and murine (mBD-4 and -14) β-defensins induce chemotaxis in leukocytes in a CCR6 dependent fashion (126–129). Moreover, hBD-2 and -3, and the orthologous mBD-4 and -14 induce migration of monocytes, macrophages and neutrophils through interactions with CCR2 (25). β-defensin immunomodulatory function also includes maturation and activation of leukocytes. mBD-2 activates immature dendritic cells, functioning as a TLR4 ligand and upregulating co-stimulatory molecules toward to a TH1 polarized response (130). In addition, hBD-3 activates monocytes in a TLR1/2 dependent fashion (131), whereas hBD-2 and -3 increase pro-inflammatory cytokine release from TLR-stimulated macrophages by ATP release and P2X7 activation (132). Interestingly, hBD-1, -2, and -3 all stimulate cytokine release from human peripheral blood mononuclear cells, with each β-defensin stimulating a unique array of cytokines (133). The presence of hBD-1 in human plasma (134), and expression of hBD-1 and -2 by human monocytes indicates systemic functions of β-defensins (135).

At the gut mucosa, β-defensins regulate epithelial cell responses including proliferation and migration, which are critical for resolution of injury, infection, and inflammation. hBD-2 signals through CCR6 on colonic epithelial (Caco-2 and T84) cells to induce actin cytoskeleton rearrangements and promote cell migration (136). Likewise, hBD-2 increased cell migration, induced *MUC2* transcription and protected against apoptosis in Caco-2 and HT-29 cells (137). Studies in other epithelia infer hBDs may additionally have a role in regulation of intestinal epithelial permeability. hBD-3 improved keratinocyte barrier function through upregulation of tight junction proteins (138). Overall, protective mechanisms of β-defensins during intestinal infection include direct bacterial killing and regulatory functions on immune and intestinal epithelial cells.

REGENERATING ISLET-DERIVED PROTEIN (REG) III

The Regenerating islet-derived protein III (RegIII) proteins are C-type lectins, ∼16–17 kD (139), with the capacity of binding bacterial carbohydrate motifs, independent of calcium, to mediate pore formation in bacterial membranes (140). The *Reg* gene family was originally identified from the *Reg* gene expressed in rat pancreatic regenerating islets (141). A large *Reg* gene family was later characterized and separated into 4 subgroups (I-IV), based on DNA and protein sequence similarity (142, 143). In humans, the *Reg* family consists of 5 genes [*REGIα*, *REGIβ*, Hepatocarcinomal-Intestine-Pancreas/Pancreatitis-Associated Protein (*HIP/PAP*), *REGIIIγ*, and *REGIV*], whereas 7 *Reg* genes are present in mice (*RegI*, *RegII*, *RegIIIα*, *RegIIIβ*, *RegIIIγ*, *RegIIIδ*, and *RegIV*) (144). In intestines, *RegIII* genes are the most prevalent *Reg*, with higher expression in the small intestine (83, 145, 146). Mice express 4 *RegIII* family members (*RegIIIα, -β*, *-γ*, and *-δ)*, whereas humans express 2 *RegIII* genes (*HIP/PAP* (*REGIIIα)* and *REGIIIγ)* with certain homologies (147, 148). Human HIP/PAP and murine RegIIIγ are orthologous and share 67% homology, whereas human REGIIIγ shares 68% homology with murine RegIIIβ (149).

RegIII gene expression is increased during intestinal inflammation, as observed in IBD patients and mice afflicted with DSS-induced colitis (*HIP/PAP* and *RegIIIγ*) (145). *RegIII* expression in non-hematopoietic cells is mainly induced by activation of pattern recognition receptors and MyD88 signaling. Such innate upregulation of *RegIIIγ* in intestinal epithelium conferred protection against *L. monocytogenes* infection (150). Likewise, oral LPS upregulated *RegIIIγ* through TLR4 in antibiotic-treated mice, providing increased resistance to vancomycin-resistant *Enterococcus* (VRE) (151). Specialized intracellular nucleotide-binding oligomerization domain-like receptors (NOD-like receptors) also regulate *RegIII.* Mice deficient in Nod ($Nod1^{-/-}$/$Nod2^{-/-}$) had decreased expression of colonic *RegIIIγ*, associated with increased susceptibility to DSS-induced colitis (152).

Expression of *RegIIIγ* and *RegIIIβ* in colonic epithelial cells can also be regulated by IL-22 via STAT3 (153, 154). Indeed, mice genetically deficient in STAT3 have delayed wound healing during DSS-colitis, associated with decreased *RegIIIγ* and *RegIIIβ* expression in intestinal epithelial cells (155). Likewise, enteric infections could regulate *RegIII* via IL-22. *RegIIIγ* is upregulated during *C. rodentium* infection in response to IL-22 (154), whereas the TLR5 ligand flagellin increased expression of *RegIIIγ* in intestinal epithelial cells through IL-22 induction and protected against VRE infections (156). A main source of IL-22 is Th17 cells that regulate *RegIIIγ* in human and murine colonic epithelial cells (154, 157). Therefore, upregulation of intestinal *RegIII* can be mediated through cytokines, such as IL-22, or potentially through recognition of pathogen associated molecular patterns, e.g., LPS or flagellin. Collectively, these functions could work to drive pathogen clearance and restore homeostasis.

Antimicrobial functions of RegIII in colonic defense were recently demonstrated (**Table 2**). *In vitro,* HIP/PAP kills Gram-positive bacteria (*L. monocytogenes* and *E. faecalis*) through formation of an oligomeric pore in the bacterial membrane, although HIP/PAP failed to kill Gram-negative bacteria (83, 84). Unlike other C-type lectins, HIP/PAP binds to bacterial peptidoglycan in a calcium-independent fashion, and bacterial killing requires a conserved EPN motif (140). Accordingly, $RegIII\gamma^{-/-}$ mice have increased abundance of Gram-positive mucosa-associated commensal bacteria (158). Interestingly, whereas RegIIIγ disrupts Gram-positive bacterial membranes, RegIIIβ preferentially kills Gram-negative bacteria (81, 82, 159). The ability of RegIIIβ to specifically target Gram-negative bacteria is due to its ability to bind to carbohydrate motifs of lipid A, a main component of LPS (159). The antibacterial function of RegIIIγ and RegIIIβ to preferentially target Gram-positive and Gram-negative bacteria, respectively, offers an interesting mechanism to for these two related HDPs to jointly protect

against different types of bacterial pathogens. The antibacterial activity of RegIIIγ and HIP/PAP is activated through proteolytic removal of an N-terminal pro-segment by trypsin; the protein is inactive until it is secreted into the intestinal lumen and proteolytically processed to generate an active peptide (160). This fine control of RegIIIγ activity is similar to other regulatory mechanisms described for small intestinal α-defensins that require activation by the metalloproteinase matrilysin or trypsin (161, 162).

Roles of RegIII proteins in gut homeostasis may extend beyond direct bactericidal functions (**Table 1**). In the skin of psoriasis patients, HIP/PAP is highly expressed via IL-17 and promotes keratinocyte proliferation through engagement of exostosin-like 3 (EXTL3) and activation of the PI3K-AKT signaling pathway (33). Some of these immunomodulatory roles for RegIII proteins could apply to the gut, including preventing apoptosis. Treatment of HT-29 cells with recombinant HIP/PAP protected against apoptosis (34). Moreover, IL-22 protected mice from intestinal stem cell apoptosis during graft-vs.-host disease (GVDH) through upregulation of *RegIIIγ* (34).

Intestinal microbiota is an important regulator of RegIII. *RegIIIγ* genes were upregulated in the colon of germ-free mice upon bacterial colonization (83), and specific commensal bacteria induce *RegIIIγ* genes in the colon. Monocolonization of germ-free mice with Gram-positive *Bifidobacterium breve*, but not Gram-negative *E. coli* JM83, increased *RegIIIγ* production (149). Similarly, specific pathogen free (SPF) Nod1$^{-/-}$/Nod2$^{-/-}$ mice showed decreased *RegIIIγ* expression, which was restored with altered Schaedler flora (ASF) (in gnotobiotic Nod1$^{-/-}$/Nod2$^{-/-}$ mice) or *B. breve* (in SPF Nod1$^{-/-}$/Nod2$^{-/-}$ mice) (152). In addition, monocolonization of germ-free SCID mice with segmented filamentous bacteria (SFB), strong inducers of Th17 cells and mucosal IL-22, increased *RegIIIγ* expression in intestinal epithelial cells (163). Likewise, monocolonization of germ-free mice with Gram-negative *Bacteroides thetaiotaomicron* resulted in a modest upregulation of *RegIIIγ* (2.5-fold), whereas monocolonization with Gram-positive *Listeria innocua* had no effect on *RegIIIγ* expression (83). Interestingly, monocolonization of Rag-1$^{-/-}$ germ-free mice with *B. thetaiotaomicron* and *L. innocua* resulted in a large (40 to 50-fold) increase in *RegIIIγ* expression, respectively (83). This phenomenon is hypothesized to be due to the absence of luminal IgA, which normally sequesters commensal bacteria away from intestinal epithelial cells, suggesting contact between bacteria and intestinal epithelial cells is a major driver of *RegIIIγ* expression (83).

It has been proposed that RegIIIγ antimicrobial activity is critical to separate commensal bacteria from underlying intestinal epithelial cells (158), or modulate the intestinal microbiota population when stimulated by intestinal infection or inflammation. In fact, RegIII-mediated alteration of the intestinal microbiota can have implications on outcomes of infectious colitis. *RegIIIβ* expression resulted in prolonged and worsened disease in a streptomycin murine model of *Salmonella*-induced colitis, corresponding with decreased presence and re-establishment of commensal *Bacteroides* spp. (81). Similarly, the regulation of RegIII by the microbiota has implication on infections, as depletion of the intestinal microbiota by antibiotic treatment decreased intestinal *RegIIIγ* expression and increased VRE burden (151). Production of RegIIIγ varies across the gut, with lower *RegIIIγ* expression in colon compared to small intestine (146); this difference may account for differential microbiome-host interactions. Whereas, *RegIIIγ*$^{-/-}$ mice did not display increased commensal bacteria/epithelial cell contact in the colon, they had more intimate bacterial contact in the terminal ileum associated with increased production of inflammatory IL-22 and myeloperoxidase (164). The antibacterial function of RegIIIγ is thought to be of particular importance due to its presence within the mucus layer, preventing penetration of both commensal and pathogenic bacteria. The mucus layer contains RegIIIγ (164) and its larger size compared with other HDPs (e.g., defensins or cathelicidins) could favor closer interactions with mucus glycoproteins thereby preventing RegIIIγ diffusion from the mucus layer into the lumen (158). Thus, RegIII may contribute to gut homeostasis via direct antibacterial functions against intestinal bacterial pathogens, microbiome regulation and immunomodulation (**Figure 1**).

RESISTIN-LIKE MOLECULES (RELM)

Resistin-like molecules (RELMs) are a family of secreted proteins characterized by a conserved cysteine-rich C-terminus (165). Previously named Found in Inflammatory Zone (FIZZ) and Hypoxia-Induced Mitogenic Factor (HIMF) proteins (166), the RELM protein family has been studied in a wide range of diseases, including asthma, diabetes, and bacterial and parasitic infections. RELM proteins range in size from 105 to 114 amino acids, with 3 conserved domains: a signal sequence, a variable middle domain, and a C-terminal domain (165). The latter is particularly rich in cysteine residues, invariantly spaced following the sequence C-X_{11}-C-X_8-C-X-C-X_3-C-X_{10}-C-X-C-X-C-X_9-CC-X_{3-6} (165). Mice have 4 Relm proteins (RELMα, -β, -γ, and resistin) encoded by *Retlna*, *Retlnb*, *Retlng*, and *Retn* genes, respectively, whereas humans possess only RELMβ and Resistin, encoded by *RETLNB* and *RETN* genes (165, 167). Murine RELMα is mostly restricted to airway epithelial cells (168) and immune cells (i.e., macrophages and dendritic cells) (169, 170), while murine RELMγ is expressed in bone marrow, spleen, and lungs (171). Human Resistin is expressed in immune cells (172), whereas murine Resistin is restricted to adipocytes (173). Resistin and RELMβ form large hexamers consisting of 2 trimers, linked by disulphide bonds present in their N-terminal coiled-coiled domains (174), although RELMβ may also exist as secreted dimers (175).

RELMβ is the most abundantly expressed RELM in the large intestine of both humans and mice (176), mostly in cecum and distal colon (176). The peptide is produced as a 111-amino acid protein with an N-terminal 11-amino acid signal sequence, and is predominantly restricted to secretory granules of mucus-secreting goblet cells (176). Unsurprisingly, RELMβ expression is regulated by factors that influence goblet cell functions, e.g., IL-18 and IL-22 (177). Expression of RELMβ is dependent

on the microbiota, being transcriptionally induced in germ-free mice upon colonization with a conventional microbiota (176). Moreover, oral antibiotic treatment of mice decreased *Firmicutes,* with persistence of *Bacteroidetes* and *Proteobateria*, and decreased RELMβ production, along with decreased IFNγ and IL-17 production from CD4+ T-cells (178).

RELMβ is particularly reactive to helminth intestinal infections, including *Trichinella muris*, *Trichuris spiralis*, and *Nippostrongylus brasiliensis* as a part of a TH2-driven immune response, mainly mediated through IL-4 and IL-13 (179). Accordingly, RELMβ-deficient mice were more susceptible to *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* infections (180). Detection of RELMβ in feces of mice with gastrointestinal nematode infections has also been demonstrated as a non-invasive tool to assess intestinal changes in response to intestinal infections (181). Mechanistically, RELMβ is necessary for IL-4-mediated intestinal worm expulsion, impairing the ability of worms to feed on host tissues and generate ATP (180). In addition, RELMβ can directly bind chemosensory components of parasitic nematodes to block their sensory functions (179).

Colonic RELMβ expression is increased during bacterial infection (e.g., *C. rodentium*), with secreted RELMβ present in feces (35). Indeed, RELMβ deficient mice are more susceptible to *C. rodentium* infection, with decreased survival and increased bacterial colonization deep within colonic crypts (35). Direct microbial killing may be a key role of RELMβ in this gut defense. RELMβ causes pore formation and bacterial death in both Gram-positive and Gram-negative bacteria, including *L. monocytogenes*, *E. faecails*, *C. rodentium,* and *P. aeruginosa* (80) (**Table 2**). Although it is broad-spectrum bactericidal, RELMβ preferentially kills Gram-negative bacteria, with antibacterial functions observed for both monomeric and dimeric forms of the protein (80). Additionally, colonic RELMβ may protect against *C. rodentium* indirectly, by promoting intestinal epithelial cell (IEC) proliferation and chemoattracting T-cells. RELMβ$^{-/-}$ mice infected with *C. rodentium* had increased mortality, with reduced CD4+ T-cell infiltration, reduced IL-22 production, and impaired IEC proliferation in colons (35). Interestingly, RELMβ$^{-/-}$ mice displayed decreased expression of RegIII C-type lectins (36, 37). Thus, decreased CD4+ T-cell infiltration and IL-22 production in the colons of RELMβ$^{-/-}$ mice may be major drivers of decreased *RegIIIγ* gene expression, as IL-22 is a strong inducer of RegIIIγ production (153, 154).

RELMβ expression is induced in mice during DSS colitis, with increased expression requiring IL-13, as IL-13$^{-/-}$ mice were unable to induce RELMβ (36). Some pro-inflammatory roles for RELMβ are postulated based on decreased colitis in RELMβ$^{-/-}$ mice exposed to DSS (e.g., decreased weight loss, colonic shortening, and mortality) (36). However, in the same study, RELMβ$^{-/-}$ mice were more susceptible to TNBS-induced colitis (36). These apparent conflicting results are likely due to the underlying inflammatory mechanisms behind these 2 models of colitis. While DSS colitis causes inflammation driven by direct erosion of the epithelial barrier, TNBS-induced colitis is mediated by potent TH1 immune responses (182). In addition, RELMβ$^{-/-}$ mice responses to DSS and TNBS-induced colitis could be regulated by RegIIIβ expression, which showed to reduce TNF-α induced immune responses in monocytes and epithelial cells (36, 183). Thus, RELMβ could undertake either pro- or anti-inflammatory roles depending on the inflammatory stimuli or surrounding immune activation.

RELMs may enhance gut mucosal barrier defenses against pathogenic bacteria by promoting colonic mucin. RELMβ upregulated MUC2 and increased secretion of MUC5AC in mucin-producing colonic epithelial (HT29-Cl.16E) cells, signaling through calcium and PKC (38). Moreover, mice pre-treated with synthetic RELMβ experienced increased mucus production and attenuated TNBS colitis (38). However, RELMβ could still exert pro-inflammatory roles beyond a mucin secretagogue effect. Mice genetically deficient in Muc2 (*Muc2*$^{-/-}$) developed spontaneous colitis, an effect that was diminished in mice double knock out for Muc2 and RELMβ (*Muc2*$^{-/-}$/RELMβ$^{-/-}$) (37). Interestingly, RELMβ expression in *Muc2*$^{-/-}$ mice induced expression of both RegIIIγ and RegIIIβ, which corresponded to a decrease in *Lactobacilli* spp. (37). It is likely that spontaneous colitis in *Muc2*$^{-/-}$ mice responds to replenishment of *Lactobacilli* spp. and a pro inflammatory role of RELMβ affecting healthy intestinal microbiota. Furthermore, the presence of RELMβ in *Muc2*$^{-/-}$ mice could contribute to increased RegIII expression, resulting in microbial dysbiosis and more severe colitis in comparison to *Muc2*$^{-/-}$/RELMβ$^{-/-}$ mice (37). Effects of RELMβ on gut permeability could also impact intestinal homeostasis. RELMβ$^{-/-}$ mice have decreased trans-epithelial electrical resistance (TEER) and increased permeability to (4-kDa) dextran in whole intestinal mounts (36). RELMβ stimulation of rat jejunal tissue promoted glucose transport mediated by alteration of glucose transporter proteins (diminished SGLT-1 and increased GLUT2 expression) and activation of PKC and AMPK signaling (184). Thus, the function of RELMβ in colitis is, at least in part, due to its ability to induce expression of RegIII proteins, modulate the intestinal microbiota, and influence epithelial permeability.

Other RELMs, including RELMα, usually restricted to immune cells (e.g., macrophages and dendritic cells) (169, 170), are expressed by epithelial cells, macrophages, and eosinophils during *C. rodentium* infection (185). However, RELMα could promote gut inflammation without microbicidal activities. RELMα$^{-/-}$ mice infected with *C. rodentium* did not show higher pathogen burden, but decreased colitis with decreased CD4+ T cell expression of pro-inflammatory IL-17A (185). Additionally, intraperitoneal injection of mice with recombinant RELMα increased *C. rodentium*-induced colitis, including increased IL-17, whereas IL-17A$^{-/-}$ mice did not display increased colitis in response to RELMα treatment (185). These data demonstrate a pro-inflammatory role of RELMα during *C. rodentium* infection, working mainly through the induction of IL-17. Similarly, RELMα$^{-/-}$ mice have decreased inflammation during DSS colitis (186, 187). Thus, influence of RELMs on intestinal inflammation and infection is complex and involves

direct antimicrobial activity, regulation of intestinal RegIII C-type lectins, modulation of the microbiota, and potential direct immunomodulatory effects.

CONCLUSIONS

Aspects of major HDPs (i.e., cathelicidins, β-defensins, and members of RegIII and RELM families) in the colon and their relevance in pathogenesis of infectious colitis reviewed herein aid to uncover roles of these peptides in promoting epithelial defenses beyond direct microbial elimination (**Figure 1**). The presence of HDPs abundantly secreted into the intestinal lumen by epithelial cells and leukocytes during inflammation must be critical components of the innate immune response against enteropathogenic bacteria. They are bactericidal against enteric pathogens as well as the microbiota, while simultaneously modulating numerous cellular responses including leukocyte chemotaxis and migration, wound healing, cytokine production and pathogen sensing (**Tables 1**, **2**). In fact, crude colonic mucus isolates from uninfected mice had no direct antibacterial activity against *C. rodentium* (96). Thus, there is increasing interest to decipher major mechanisms of HDPs in innate defense, which seem to be largely attributed to immunomodulatory functions.

To date, our understanding of HDP function in the colon is mostly limited to studies using mice genetically deficient in a single HDP, or via stimulation of mice with a specific exogenous peptide (commonly a synthetic peptide derived from an endogenous HDP). We propose a theoretical framework of how these peptides may work together in the gut. Host defense peptides would form an interactive peptide network capable of preventing colonization of enteropathogens by: (1) direct bacterial killing and (2) fine-tuning of the host immune response in the colon (i.e., modulation of epithelial cell responses and migration of leukocyte populations to the site of infection). In these activities, HPDs likely have overlapping and potentially complementary roles. Cathelicidins from different species displayed synergistic antibacterial activity against *P. aeruginosa*, *E. coli*, *E. faecalis,* and MRSA (188). Additionally, cathelicidins demonstrated synergistic antibacterial ability with human lysozyme (188). Thus, in the real scenario of numerous HDPs co-existing with other innate factors (e.g., MUC2 mucin, lysozyme), antibacterial activity against specific pathogenic species is likely enhanced.

The synergistic effects of multiple HDPs could apply to cytokine and chemokine signaling in the gut. Combined hBD-3 and LL-37 showed a synergistic reduction in secretion of proinflammatory factors (GRO-α, G-CSF, MCP-1, and IL-6) in gingival fibroblast-epithelial cells exposed to LPS, although they displayed only additive effects reducing IL-8 secretion (189). Other synergies can be predicted to occur during infectious colitis based on the effects of single HDPs. RELMβ regulates T-cell migration, IL-22 production and RegIIIγ in the colon (35–37), while cathelicidins, β-defensins, and RELMβ have all been demonstrated to regulate both mucus production and epithelial permeability (9, 11, 14, 15, 36, 38, 137, 138). This HDP network may decode how the intestinal innate immune system functions to quickly restore gut homeostasis and avoid damaging inflammation associated with pathogen colonization. Exploring how these peptides act synergistically in innate immune defenses and the complex signaling networks they activate during infectious colitis should lead to identification of therapeutic anti-infectious targets, or development of synthetic HDPs that work in combination to resolve intestinal infections. Such synthetic HDPs could be used either alone or in combination with reduced doses of antibiotics. These strategies of infectious disease control would be greatly beneficial, as emergence of antimicrobial resistance is rendering conventional antibiotics use unsustainable.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Strategies in Translating the Therapeutic Potentials of Host Defense Peptides

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The golden era of antibiotics, heralded by the discovery of penicillin, has long been challenged by the emergence of antimicrobial resistance (AMR). Host defense peptides (HDPs), previously known as antimicrobial peptides, are emerging as a group of promising antimicrobial candidates for combatting AMR due to their rapid and unique antimicrobial action. Decades of research have advanced our understanding of the relationship between the physicochemical properties of HDPs and their underlying antimicrobial and non-antimicrobial functions, including immunomodulatory, anti-biofilm, and wound healing properties. However, the mission of translating novel HDP-derived molecules from bench to bedside has yet to be fully accomplished, primarily attributed to their intricate structure-activity relationship, toxicity, instability in host and microbial environment, lack of correlation between *in vitro* and *in vivo* efficacies, and dwindling interest from large pharmaceutical companies. Based on our previous experience and the expanding knowledge gleaned from the literature, this review aims to summarize the novel strategies that have been employed to enhance the antimicrobial efficacy, proteolytic stability, and cell selectivity, which are all crucial factors for bench-to-bedside translation of HDP-based treatment. Strategies such as residues substitution with natural and/or unnatural amino acids, hybridization, L-to-D heterochiral isomerization, C- and N-terminal modification, cyclization, incorporation with nanoparticles, and "smart design" using artificial intelligence technology, will be discussed. We also provide an overview of HDP-based treatment that are currently in the development pipeline.

Keywords: antibiotic, antimicrobial peptide, antimicrobial resistance, artificial intelligence, host defense peptide, nanoparticle, peptide

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INTRODUCTION

Antimicrobial resistance (AMR) currently represents a major global health threat of the twenty-first century and is estimated to cost 10 million deaths per year by 2050 (1, 2). Tackling AMR requires a multi-faceted and synergistic approach, including understanding of the main mechanisms and drivers of AMR at the microorganism, individual and population levels, antimicrobial stewardship within the healthcare and agriculture sectors, in tandem with discovery and development of new classes of antimicrobial therapy (3, 4).

Host defense peptides (HDPs), also known as antimicrobial peptides, are a group of evolutionary conserved molecules that play critical roles in the innate immune system (5–7). These molecules are usually small in size (around 12–50 amino acid residues), cationic (with a net charge of +2 to +13), and amphiphilic geometry (5, 6). So far, more than 3,000 naturally occurring and synthetic HDPs have been discovered across six life kingdoms (8, 9), underlining their essential roles during the adaptation and evolutionary process. HDPs have been shown to exhibit broad-spectrum antimicrobial activities against a plethora of microorganisms, including drug-resistant bacteria, fungi, parasites, and viruses (5, 10). In contrast to conventional antibiotics (which usually target a particular extracellular or intracellular binding site), cationic HDPs bind to the anionic surfaces of bacteria causing lytic cell death through varied mechanisms. Additionally, HDPs could also kill bacteria via inhibition of macromolecules that are involved in the biosynthesis of the surface membrane, and components of metabolic organelles (11, 12). Such unique mechanism of action accounts for the rapid and broad-spectrum antimicrobial action and the low risk of developing AMR as modification of the entire microorganisms' anionic membrane incurs a substantially higher fitness cost when compared to alteration of a particular binding site targeted by the conventional antibiotics (e.g., alteration in the 30S subunit inhibits the action of aminoglycosides) (13).

In addition to the antimicrobial activity, there has been emerging evidence highlighting the non-antimicrobial roles of HDPs, including antibiofilm (14), immunomodulatory (15), wound healing (16), anti-viral, and anti-cancer properties (17), rendering them an attractive and novel class of clinical therapeutics (18). For instance, corneal infection or infectious keratitis is a sight-threatening ocular disease that can be caused by a wide range of microorganisms, including bacteria, fungi, parasites, and viruses (19, 20). In addition, corneal infection may be caused by polymicrobial infection which can present significant therapeutic challenges to the clinicians (21). The dual broad-spectrum antimicrobial and wound healing properties of HDPs would not only provide a comprehensive antimicrobial coverage for the infection but also expedite the healing process of corneal ulceration, limiting the damage to the cornea and ultimately preserving the vision. The complete repertoire of antimicrobial and non-antimicrobial functions of HDPs has been recently summarized in a seminal review by Mookherjee et al. (22).

Despite decades of research efforts have been invested in the field of HDP, the mission of translating novel HDP-derived molecules from bench to beside has yet to be fully accomplished. The sluggishness in the HDP-derived drug development pipeline is primarily attributed to their complex structure-activity relationship (SAR), undesired toxicity to host tissues, instability in host and microbial environment, lack of correlation between *in vitro* and *in vivo* efficacies, and dwindling interest from large pharmaceutical companies (23–26). A list of synthetic derivatives of HDPs that are currently under clinical trials are summarized in **Table 1** for readers' convenience. Detailed overview on the mechanisms of actions and clinical applications of HDPs have also been provided elsewhere in published review articles (27–29). Based on the literature and our experience, this review article aims to highlight the strategies that have been attempted to enhance the antimicrobial efficacy, proteolytic stability, and cell selectivity (for microbial cells instead of host cells; i.e., improved safety) of HDPs, which are all important factors to be considered during the translation of these molecules.

STRATEGIES FOR ENHANCING ANTIMICROBIAL EFFICACY AND SAFETY OF HDPs

Structurally, HDPs are mainly characterized by the presence of key charged residues (e.g., Arg and Lys), with a high proportion of hydrophobic residues (constituting 50% or more) and amphipathicity (7, 30). Optimization of HDP sequences to improve antimicrobial efficacy has been previously detailed (30, 31). Here, we aim to provide key strategies to improve the selectivity of HDPs toward microbial membranes through reported examples of model HDPs that were enhanced through rational design approaches (**Table 1**).

Residue Substitution With Natural Amino Acids (AAs)

HDPs should fulfill key functional requirements to qualify for clinical use, including low toxicity, high antimicrobial activity, and good *in vivo* stability (24). These requirements are closely linked to their biochemical selectivity toward anionic and zwitterionic surfaces (24). The antimicrobial activity is attributed to a fine balance of hydrophobicity, cationic residues, amphipathicity, and structural conformation (e.g., α-helical, β-sheet, and cyclized) (32). On the other hand, the hydrophobic interaction between specific residues of HDPs (e.g., Leu, Ile, Val, Phe, Tyr, Trp) and zwitterionic phospholipids on host cell surfaces is responsible for its toxicity. For example, peptide derivatives of mastoparan (a key constituent of wasp venom) that were designed based on fixed five rules utilizing the quantitative structure-activity relationship (QSAR) approach showed potent antimicrobial efficacy against *Bacillus subtilis* (33). It was shown that the potency of these derivatives was mainly dependent on the presence of Trp, Lys, and His (33). Lata et al. (34) analyzed 486 HDPs from the antimicrobial peptide database (APD) for AA frequency in these sequences using the bioinformatic tools. Residues such as Gly, Arg, Lys, and Leu were shown to be commonly found in HDPs, whilst AAs such as Ser, Pro, Glu, and Asp were least common at both N- and C-terminus. A recent study from Hilpert group has demonstrated through *in silico* designed library of 3,000 *de novo* short peptides (9-mer in length) that the specific design characteristics of HDPs did not apply to short peptides (35). The peptide sequences that were grouped as "super active" based on their activity toward *Pseudomonas aeruginosa* were mainly composed of Lys, Arg, Trp, and Val/Ile/Leu (35). However, the activity of these super peptides toward host cells and *in vivo* stability was not yet reported.

Melittin, a key constituent of honey-bee venom, is a potent HDP with strong antimicrobial activity (36). However, its clinical use is largely limited by the high hemolytic activity (36). Blondelle

TABLE 1 | Derivatives of host defense peptides in clinical trials.

Drug/peptide name	Peptide derivative	Progress	Application	References/Trial registry (Status; Last update posted)
Omiganan (CLS001/MBI226)	Indolicidin	Phase II/III	Facial seborrheic dermatitis; Genital warts; Rosacae; Vulvar neoplasia; Atopic dermatitis; Acne vulgaris; Skin antisepsis; Prevention of catheter infections	NCT03688971 (recruiting; May 2019) NCT02576847 (completed; Jul 2018) NCT02547441 (completed; Jul 2018) NCT02576860 (completed; Mar 2018) NCT03091426 (completed; Dec 2017) NCT02596074 (completed; Aug 2017) NCT03071679 (completed; May 2017) NCT02571998 (completed; Mar 2017) NCT02849262 (completed; Mar 2017) NCT02456480 (completed; Jul 2016) NCT02066545 (completed; Sept 2015) NCT01784133 (completed; May 2015) NCT02028286 (completed; Apr 2014) NCT00608959 (completed; Jan 2010) NCT00231153 (completed; Aug 2009) NCT00027248 (completed; Sept 2005)
Pexiganan (MSI-78)	Magainin	Phase III	Diabetic foot ulcer (topical)	NCT01594762 (completed; Jun 2017) NCT01590758 (completed; Jun 2017) NCT00563433 (completed; Nov 2007) NCT00563394 (completed; Nov 2007)
Iseganan (IB-367)	Protegrin	Phase II/III	Prevention of radiation-induced oral mucositis; Ventilator-associated Pneumonia	NCT00022373 (unknown; Oct 2014) NCT00118781 (terminated; Jul 2005) (failed in Phase III; https://www.thepharmaletter.com/article/intrabiotics-lead-drug-hits-snag-in-phase-iii)
C16G2	Novispirin G10	Phase II	Dental cavities	NCT02594254 (completed; Aug 2019) NCT02509845 (completed; Aug 2019) NCT03196219 (completed; Aug 2019) NCT02254993 (completed; Aug 2019) NCT03052842 (completed; Aug 2019) NCT03004365 (completed; Aug 2019) NCT02044081 (completed; Aug 2019)
Brilacidin (PMX-30063)	Defensin mimetic	Phase I/II	Prevention of radiation-induced oral mucositis, treatment of gram-positive bacterial skin infections	NCT04240223 (completed; Feb 2020) NCT02324335 (completed; Jan 2019) NCT02052388 (completed; Sep 2018) NCT01211470 (completed; May 2012)
Novexatin (NP213)	Polyarginine cyclic HDP	Phase II	Onychomycosis (topical)	www.novabiotics.co.uk (no registered trial)—PMID 32232410
PAC-113	Histatin-3	Phase IIb	Oral candidiasis (mouth rinse)	NCT00659971 (completed; Jun 2008)
hLF_{1-11}	Human lactoferrin	Phase II	Bacteraemia; Candidemia; anti-infectives for haematopoietic stem cell transplant recipients	NCT00430469 (withdrawn; Jun 2015) NCT00509834 (withdrawn; Jun 2015) NCT00509847 (withdrawn; May 2014) NCT00509938 (completed; Oct 2008)
OP-145 (AMP60.4Ac)	Human LL-37	Phase II	Chronic suppurative otitis media	ISRCTN12149720 (completed; Feb 2019) ISRCTN84220089 (completed; Jan 2008)
Glutoxim (NOV-002)	Synthetic hexapeptide	Phase II/III	Tuberculosis; myelodysplastic syndromes; ovarian cancer; non-small cell lung cancer; breast cancer;	NCT00960726 (withdrawn; Jul 2012) NCT00499122 (completed; Jan 2018) NCT00347412 (completed; Nov 2011) NCT00345540 (completed; Mar 2015)
Lytixar (LTX-109)	Synthetic peptidomimetic	Phase I/IIa	Impetigo; drug-resistant gram-positive nasal and skin infections (topical); atopic dermatitis	NCT01158235 (completed; Jun 2011) NCT01223222 (completed; Feb 2011) NCT01803035 (completed; Apr 2014)
Opebacan (rBPI-21)	Bactericidal/permeability increasing protein (BPI)	Phase I/II	Prevention of endotoxemia following myeloablative allogeneic stem cell transplantation; anti-sepsis in patients with burn injury	NCT00454155 (terminated; Jul 2012) NCT00462904 (terminated; Nov 2019)

et al. (37) studied the function of Trp in melittin activity through serial Trp substitution starting from N- to C-terminus. Substitution of Leu→ Trp at 9th position was shown to decrease the hemolytic activity whereas substitution of Pro→ Trp at 14th position improved the alpha-helical conformation and reduced the hemolytic effects compared to parent melittin peptide.

HDPs with Pro residues are widely known to display a disrupted helix conformation, which eventually affects their surface retention time and penetration into microbe cytoplasm (38, 39). A recent study using peptide analog (Anal 3, 19-AA long) from N-terminus of *Helicobacter pylori* ribosomal protein L1 has demonstrated that an insertion of Pro-hinge into Anal 3 (via Glu→ Pro substitution at 9th position) significantly improves the peptide selectivity toward microbes with no effect on host cells (40, 41). This was attributed to the helix-hinge-helix conformation of Anal 3-Pro analog at the surface of bacteria allowing peptide penetration and DNA binding in the cytoplasm. This study suggested that rational insertion of Pro residue through SAR analysis could improve the biological membrane selectivity of microbicidal peptides. Proline-rich designed HDPs such as ARV-1502 (42), oncocin (43), and Bac-5 (44, 45) have shown significant efficacy against Gram-negative pathogens but not host cells membranes. Unlike cationic HDPs, proline-rich peptides kill bacteria through inhibition of protein synthesis (12, 45–47). Histidine-rich (48, 49), alanine-rich (50), and tryptophan-rich (51) short HDPs have also been developed. These were shown to be highly effective at acidic pH against a range of Gram-negative and Gram-positive bacteria (52).

Magainin-2 is 23 residues long antimicrobial peptide (AMP) isolated from frog skin, *Xenopus laevis* (53). Due to its non-hemolytic and broad-spectrum antimicrobial properties, magainin-2 was widely studied as a model peptide to understand the SAR of naturally occurring AMPs (54–58). Chen et al. (59) have demonstrated that the alpha-helical conformation of magainin-2 could be stabilized through Gly→ Ala substitution (at both 13th and 18th position) and C-terminal amidation. This was shown to increase the antibacterial activity by 2-fold against a range of bacteria without modulating its safety against erythrocytes (59). Numerous groups have made attempts to improve the activity of magainin-2 against Gram-negative bacteria through residue substitution. It was demonstrated that the substitution of Phe→ Trp in magainin-2 (F12W mutant) increased its activity against Gram-negative bacteria; however, this increased its selectivity toward erythrocytes, causing significant hemolysis (60). This could be attributed to the bulkiness of Trp compared to Phe and the presence of NH-group in Trp that is capable of forming hydrogen bonds with zwitterionic phospholipids (39, 61). Further modification through reduction of net charge of F12W mutant (Lys→ Glu substitution at 10th position) was shown to reduce the hemolytic effect. However, this made the mutant magainin-2 less effective against Gram-negative bacteria (60). Extensive SAR studies from Zasloff's laboratory led to the development of MSI-78 (also known as pexiganan), a derivative of magainin-2, which showed improved safety/efficacy profile compared to parent magainin-2 sequence (62, 63). However, it failed in the phase III clinical trial for the treatment of infective diabetic foot ulcers. Further details on pexiganan clinical journey can be reviewed elsewhere (64–66).

Typically, natural HDPs display a net positive charge between +2 and +13. It has been widely demonstrated that modification of total net charge of synthetic HDPs through cationic residue substitution enhances electrostatic interaction between HDPs and lipopolysaccharide (LPS) (30, 60). However, this approach has shown to increase the toxicity of certain HDPs. For example, magainin-2 analogs with positive charge above +5 were shown to display hemolytic effects (rank order +6 > +5 > +4 > +3) (67). To overcome the inherent issues associated with peptide optimization, Mishra and Wang (68) adopted an *ab initio* design approach which involved utilization of novel database-filtering technology (DFT). This led to the development of a 13-AA long, leucine-rich, anti-MRSA peptide template—termed "DFTamP1" (68). A subsequent study demonstrated that DFT503, an optimized variant of DFTamP1, was shown to be safe and effective in *in vivo* killing of MRSA in a neutropenic mouse model. This anti-MRSA activity was attributed to its eight Leu residues and a single Lys at position 11 (net charge +1) (69). These studies suggested that lower cationic charge and high hydrophobicity is preferred for anti-MRSA synthetic peptides. This strategy could form the basis for the development of species-specific peptide-based therapy against multidrug resistant (MDR) pathogens.

LL-37 is a lone member of the cathelicidin family of HDPs reported in humans (70–72). It was widely studied due to its multi-functional abilities, including microbicidal (73), anti-cancer, immunomodulatory (74), chemotactic (75), and wound-healing properties (76). Numerous groups have exploited the structure of LL-37 to design a range of synthetic antimicrobial analogs through residue substitution (77, 78). FK-13 (residues 17-29 of LL-37) was identified as a core antimicrobial and anti-cancer domain using nuclear magnetic resonance (NMR) technique (79). Subsequently, the deletion of Phe at 17th position led to the development of KR-12, which showed potent antimicrobial efficacy equivalent to LL-37 and FK-13 against *Escherichia coli*, but devoid of toxic activity against host cells (80). KR-12 and KE-18 analogs were recently shown to possess anti-*Candida* and anti-*Staphylococcal* properties (81). Specifically, KE-18 showed anti-biofilm activity even at sub-killing concentration against yeast and bacteria (81). Further variants of KR-12 were also reported and the less cationic analogs, a5 and a6, were shown to possess potent immunomodulatory, antibiofilm, antimicrobial, and osteogenic properties (82–85). Variants of LL-23, corresponding to 23 N-terminal residues of LL-37, were generated through substitution of Ser→ Ala and Ser→ Val at the 9th position. LL-23V9 peptide was shown to display increased antimicrobial and immunosuppressive activities compared to LL-23 and parent LL-37 (86). Wang et al. (77), Mishra and Wang (87) have recently demonstrated that titanium surface immobilized FK-16 (a short variant of LL-37) is highly antimicrobial against ESKAPE pathogens. Our group has recently demonstrated that FK-16 could be used for repurposing conventional antibiotics such as vancomycin as a strategy to counter antimicrobial resistance (88). Further improvement of FK-16 by Narayana et al. (89) have also led to the development of GF-17, 17BIPHE2, and other related variants of superior efficacy and safety compared to LL-37. Nell et al. (90) designed a range of short peptides through residue substitution based on the LL-37 sequence for neutralization of lipopolysaccharide (LPS) and lipoteichoic acid (LTA). P60.4, a 24-AA derivative, was shown to possess similar LPS/LTA neutralization ability and antimicrobial effects compared to LL-37, but with negligible *in vivo* toxicity toward

audible canal, skin, and eyes. This peptide was subsequently termed as OP-145 and was proven to be safe and efficacious in the treatment of chronic otitis media in phase I/II clinical trials (91). However, the activity of OP-145 was recently shown to be reduced in human plasma (92). Subsequent modification led to the development of synthetic antimicrobial and antibiofilm peptides (SAAPs) such as SAAP-145, −148, and −276, which showed potent anti-biofilm activity against a range of MDR pathogens (92).

Residue Substitution With Unnatural AAs

HDPs are essentially a group of small bioactive molecules made of different combinations of 20 naturally occurring AAs. The nearly infinite chemical space (20^n) and varying physicochemical properties account for the vast structural and functional diversities of naturally occurring HDPs (8, 9). However, susceptibility to host cell interaction (e.g., human erythrocytes, albumin, etc.) (93, 94) and proteolytic degradation from the host and bacterial proteases (e.g., human proteases in serum, staphylococcus aureolysin, pseudomonas elastase, etc.) (95–98) remains one of the key impediments in translating HDP-based treatment to clinical therapeutics. For instance, the anti-staphylococcal activity of cathelicidin (LL-37)—one of the most widely studied human HDPs—was shown to be inhibited by the proteases produced by *Staphylococcus aureus*, namely the aureolysin (a metalloproteinase) and V8 protease (glutamylendopeptidase), via cleavage and hydrolysis of the intramolecular peptide bonds (96).

To overcome this barrier, incorporation of unnatural or non-proteinogenic AAs has been employed to increase the proteolytic stability and/or antimicrobial efficacy of HDPs. It is known that the antimicrobial efficacy of HDPs is greatly influenced by the cationicity (99). To preserve the cationicity and thence efficacy, researchers have attempted to optimize the HDPs by replacing the cationic residues (e.g., lysine) with its analogs such as ornithine, 2,4-diamino-butyric acid (DAB), and 2,3-diamino-propionic acid (DAP), which have three, two, and one methylene (CH_2) groups in the side chain, respectively (100, 101). Using Trp-rich peptides as the design template, Arias et al. (101) reported a 4-fold length-dependent increase in the antibacterial activity against *E. coli* when the side chain of lysine was shortened from 4-carbon (lysine) to 1-carbon (DAP). Such effect was likely attributed to an increase in membrane permeabilization based on calcein leakage study. In addition, a substantial improvement in the stability against trypsin was observed when the side chain of arginine or lysine was shortened (101). Oliva et al. (102) investigated the potential role of integrating unnatural AAs within the 9-residue synthetic HDPs and discovered that unnatural AAs such as 2-naphthyl-L-alanine (an aromatic residue) and S-tert-butylthio-L-cysteine residues enhanced the antimicrobial efficacy and proteolytic stability in 10% serum for 1 and 16 h (to a lesser extent). In addition, incorporation of unnatural AAs dipeptides (tetrahydroisoquionolinecarboxylic acid-octahydroindolecarboxylic acid; or Tic-Oic) within magainin analogs has been shown to induce an amphipathic and loose alpha-helical structure with enhanced antimicrobial potency and selectivity against Gram-positive, Gram-negative and mycobacterium (103).

Unnatural AAs have been successfully utilized for improving the efficacy and stability of various peptidomimetics (104). For example, Saralasin, a partial angiotensin II receptor agonist, was developed by incorporation of sarcosine (an unnatural AA) at a key position in angiotensin II molecule (105). This provided resistance against aminopeptidases and improved bioactivity. Carbetocin, a cyclic 8-AA derivative of oxytocin is currently used for the treatment of post-partum hemorrhage, was developed through incorporation of unnatural AAs such as methyltyrosine which improved its metabolic stability and overall therapeutic benefits (106).

Hybridization

It is widely known that a cocktail of HDPs are produced at the tissue sites in response to infection (107, 108). This natural synergism between HDPs was shown to be beneficial to the host, providing the first line of defense against pathogens. This was very well-exploited through *in vitro* and *in vivo* studies, which proved that the combination of two HDPs produces strong activity against bacteria (107, 108). However, this was not deemed as a cost-effective approach and the issue of host toxicity remains unresolved. Hybridization strategy was shown to circumvent these known issues, which involves the combination of key residues from two to three HDPs of different mechanisms of actions into a single sequence (109–111). In 1989, Boman et al. (112) elegantly showed that a hybrid of cecropin-A (1-13) and melittin (1-13) was highly bactericidal and less toxic toward host cells compared to parent cecropin-A and melittin. Subsequent modifications led to the development of numerous short hybrids of cecropin-A and melittin (15–18 residues in length), which showed similar activity as the first-generation hybrids (113, 114). Chimeras of cecropin-A (CA) and magainin-2 (MA) were also developed that exhibited potent antibacterial and antitumor activities. Insertion of hydrophobic residues through residue substitution in the hinge region (at 16th position) of CA(1-8)-MA(1-12) hybrid was shown to improve its antibacterial and antitumor activity with no hemolytic effects (115). A recent study has demonstrated that substitution of key residues in CA(1-8)-MA(1-12), specifically Phe5Lys, Lys7His, Phe13His, Leu14Phe, and His17Leu, could stabilize the alpha-helical conformation, resulting in improved LPS binding affinity, increased bactericidal activity against clinical Gram-negative isolates, and low cytotoxicity (116). Hybrids of human-derived and animal-derived HDPs were also developed to comprise the membrane-lytic and immunomodulatory properties of cationic HDPs. For example, hybrids of cecropin-A (1-8)-LL37(17-20) (117), melittin(1-13)-LL37(17-30) (118), and BMAP27(9-20)-LL37(17-29) (119) were shown to be highly bactericidal and improved the efficacy of conventional antibiotics against a variety of bacteria. Another study involving "triple hybrid" of cecropin-A, melittin, and LL-37 showed that this approach could significantly enhance the bactericidal against a range of Gram-negative and Gram-positive organisms (120). Similarly, Dutta et al. (121) reported good *in vitro* and *in vivo* efficacies of an antimicrobial contact lens coated with melimine (derived

from melittin and protamine) for treating infectious keratitis in a rabbit model. These studies have clearly indicated that an optimized rational design approach could enable development of chimeras with improved biological selectivity.

In addition to overcoming the issue of host toxicity (as described above), the hybrid strategy has also led to the development of numerous species-specific and targeted bactericidal peptides to prevent damage to useful microbiome (122–124). Kim et al. (122) have developed a targeted chimeric peptide for the treatment of *P. aeruginosa* infection. Through phage-display library screening, they identified an outer-membrane porin F (OprF) binding peptide motif, termed PA2. Hybridization of this tag sequence to a membrane-lytic short peptide, GNU-7, was shown to improve the antimicrobial efficacy of parent GNU-7 by 16-fold toward *P. aeruginosa* both in *in vitro* and *in vivo* model systems. LPS-targeting GNU-7 variants were also developed through hybridization with lactoferrin (28-34), BPI (84-99), and *de novo* sequence (125). Chimeric bactericidal peptides targeted to *E. faecalis* (123) and *S. mutans* (124) were also developed based on the species-specific pheromones. This approach was proven to be highly targeted and would prevent the damage to commensal microbes.

L-to-D Heterochiral Isomerization

Depending on the geometric arrangement, all naturally occurring AAs (except for glycine) can exist as stereoisomers, either in L- or D-form, albeit only the L-configuration can be utilized by cells (126). That said, there is emerging evidence showing that most organisms are able to produce D-AAs, primarily through spontaneous racemization of L-AAs or post-translational enzymatic modification (127). In addition, D-AAs such as D-alanine and D-glutamic acid are found in peptidoglycan, which is a key component of the cell wall of Gram-positive bacteria. These D-AAs have been shown to increase resistance to host proteases that usually cleave the peptide bonds between L-AAs, thereby maintaining their virulence (126).

Capitalizing on the evolutionarily advantageous strategy equipped by microbes, L-to-D isomerization has been utilized to enhance the proteolytic stability of HDPs against a range of host and microbes' proteases (128–133). L-to-D isomerization can be utilized to either modify specifically one or several L-AAs (131, 134), or the entire sequence of a L-form HDP (130–132). Carmona et al. (130) demonstrated that L-to-D isomerization of Panidin-2 (D-Pin2) improved the cell selectivity (i.e., reduced hemolysis) and proteolytic stability in human serum, elastase, and trypsin, while maintaining the antimicrobial activity against a range of Gram-positive and Gram-negative bacteria. In a similar vein, Jia et al. (131) reported an improved stability of D-AA derivative of polybia-CP (which was originally derived from the venom of social wasp *Polybia paulista*), in chymotrypsin and trypsin for 1 and 6 h and reduced hemolytic activity (D-lysine derivative). In addition to the beneficial effect of proteolytic stability and/or cell selectivity, the D-form AAs may enhance the antimicrobial efficacy of HDPs. For example, the D-form KLKLLLLLKLK-NH2 (derived from sapesin B) was shown to exhibit increased antimicrobial efficacy against *S. aureus* (due to increased interaction with the peptidoglycan), *E. coli* and *Candida albicans* when compared to the L-form (132). However, the enhanced antimicrobial efficacy was not observed in other tested D-forms of HDPs such as mastoparan M and temporin A, suggesting that the D-isomerization effect is sequence-dependent (132).

L-to-D isomerization has also been shown to confer unique changes to the peptide-folding and secondary structure of HDPs (128, 131, 133). Based on circular dichroism analysis, the D-form derivatives of naturally occurring alpha-helical HDPs typically exhibit a left-hand alpha-helical spectrum (instead of a right-hand spectrum) whereas partial D-isomerization of HDPs may result in some degree of loss of alpha-helicity, depending on the position and number of D-AAs being introduced (128, 131, 133). Such changes in the secondary structure are likely accountable for the reduced host toxicity and improved cell selectivity in some HDPs (131).

This strategy was found to be successful in the development of daptomycin, an antibacterial cyclic lipopeptide, which was approved by the US Food and Drug Administration (FDA) in 2003 for the treatment of skin and systemic Gram-positive infections (104, 135). Structurally, daptomycin is comprised of 13 residues including D-alanine and D-serine (134). In addition, it also contains non-canonical amino acids such as ornithine, L-kynurenine, and L-3-methylglutamic acid (134).

C- and N-Terminal Modification

A range of N- and C-terminal modification strategies have been proposed to enhance the antimicrobial efficacy and/or cell selectivity of natural and synthetic HDPs (102, 136, 137). Amongst all, N-terminal acetylation (CH_3CO-) and C-terminal amidation (-NH_2) are the two most commonly attempted strategies (102). N-acetylation is a common protein modification observed in eukaryotic and prokaryotic cells (138). By neutralizing the positive charge (NH_3^+) at the N-terminal, N-acetylation can result in a range of irreversible changes to the protein properties, including the folding, stability, and protein-protein interactions (138). Saikia et al. (139) examined the antimicrobial efficacy and salt sensitivity of *E. coli*-derived MreB (a bacterial cytoskeleton protein found in non-spherical cells) and its N-acetylated analogs and found that N-acetylated W-$MreB_{1-9}$ demonstrated a higher antimicrobial efficacy (in salt) compared to W-$MreB_{1-9}$. However, N-acetylation may result in a decrease in antimicrobial efficacy of certain synthetic HDPs due to a reduction in the overall cationicity (139, 140), suggesting that the benefit of this modification strategy is only selective for certain HDPs.

On the other hand, C-amidation is a common post-translational modification that is widely observed in nature, including the natural synthesis of HDPs (137). C-amidation has been shown to improve the antimicrobial efficacy of certain HDPs, including aurein (141), melittin (142), modelin-5 (143), anoplin (144), and esculentin-1 (145), amongst others. The enhanced antimicrobial efficacy of these HDPs is likely ascribed to the increased alpha-helix stability at the peptide-membrane interfaces, enabling a greater membrane disruption and pore formation (141–144). In addition, Oliva et al. (102) demonstrated that simultaneous N-acetylation and C-amidation enhanced the proteolytic stability of HDP derived from human apolipoprotein B by more than 4-fold when exposed to fetal bovine serum 10%

for 1 h. Similarly, the proteolytic stability of tachyplesin I (a beta-hairpin HDP from the horseshoe crab, *Tachypleus tridentatus*) in fresh human serum was significantly enhanced using the similar N-acetylation and C-amidation strategy (146).

Other N- or C-terminal modification strategies have also been described in the literature, including N-methylation of certain cyclic HDPs to enhance the antimicrobial efficacy (136), introduction of 6-aminocaproic acid at the N- and C-terminals to protect HDPs from the action of exopeptidases (102, 147), and pegylation of the C-terminus of M33, a branched peptide, to increase the resistance against *P. aeruginosa* elastase (148).

Cyclization

Cyclization is a common phenomenon observed in natural HDPs that can exist in three main forms: (a) sidechain to sidechain; (b) backbone to backbone; and (c) sidechain to backbone (137). It has been shown to demonstrate several favorable biological properties, including enhanced antimicrobial efficacy, stability against proteases (due to conformational rigidity), enhanced cell selectivity, and reduced host toxicity (137, 149), rendering it an attractive strategy for translating HDPs from bench to bedside. Some of the notable examples of cyclic glyco- or lipopeptides that are already in clinical use include vancomycin, daptomycin, and colistin/polymyxin, which are commonly used as last resorts for combatting MDR bacteria, albeit their widespread use are hindered by the inherent toxicity and emergence of AMR (149–151).

In view of the structural stability, Dathe et al. (152) were able to create a series of short cyclic hexapeptides (based on AcRRWWRF-NH_2) with enhanced antimicrobial efficacy (up to >16-fold increase) against *Bacillus subtilis* and *E. coli* compared to the linear form, though the hemolytic activity was increased by 3-fold (152). It was also found that the antimicrobial activities of those small Arg/Trp-rich cyclic peptides were influenced by the self-assembling behavior of peptides at the bacterial membrane instead of their hydrophobic surface area, amphiphilicity, and ring size (153). In addition, a number of small cyclic D,L-alpha-peptides (with six or eight alternating D- and L-form residues) have also demonstrated strong antimicrobial efficacy against Gram-positive and/or Gram-negative bacteria via self-assembly on the bacterial membranes as organic nanotubules, which could increase membrane permeability and disrupt transmembrane ion potentials with resultant cell lysis (154, 155). Furthermore, molecular dynamic simulations and biophysical assays have provided further supportive evidence that cyclic peptides are able to bind to negatively charged membrane more strongly than the linear peptides and adopt a beta-sheet structure at the membrane surface (156).

Another form of cyclization that is found abundantly in natural HDPs, mainly in defensins, is the disulfide intramolecular cross-link between cysteine residues, which has been shown to enhance proteolytic stability (157–160). Inspired by the nature, Scudiero et al. (160) engineered a 17-residue cyclic synthetic hybrid HDP, based on the internal hydrophobic domain of human-beta defensin (HBD)-1 and positively charged C-terminal of HBD-3 (RRKK residues), and demonstrated good antimicrobial efficacy against Gram-positive and Gram-negative bacteria and herpes simplex virus, with low toxicity and good proteolytic stability. Similarly, Mwangi et al. (161) successfully developed a cyclic HDP-based molecule called ZY4 by introducing a disulfide bond to a derivative of cathelicidin-BF, which is an antimicrobial peptide derived from the snake venom of *Bungarus fasciatus*. This molecule was shown to exhibit significant *in vivo* antimicrobial efficacy against MDR *P. aeruginosa* and *A. baumannii* with high stability in mice lung infection and septicemia models (161).

Incorporation With Nanoparticles (NPs)

Nanotechnology is a rapidly growing field in biotechnology that involves characterization, manipulation and synthesis of materials that are in nanoscales (or one billionth of meter; 10^{-9} m) (162). NPs, with sizes ranging from 1 to 100 nm, can exist in many forms, including lipid-based, metal-based, carbon-based, ceramics, semiconductor, and polymeric NPs (163). It has increasingly been applied in the field of antimicrobials, either employed as antimicrobial agents or nano-carriers for drug/peptide delivery in view of their enhanced protection against extracellular degradation, improved bioavailability, and cell selectivity (164–167). Recently, Biswaro et al. (166) have provided an excellent review on the role of nanotechnology in delivering HDPs. To avoid any significant overlap, this section aims to only recapitulate the fundamental principles of NPs and provide some notable examples regarding the potential values of incorporating NPs with HDPs.

In principle, there are two types of nano-delivery systems: (a) passive delivery where the intended drugs/peptides are encapsulated within the nanocarriers through hydrophobic interaction without any surface modification; and (b) active delivery where the drugs/peptides are directly conjugated with the nanocarriers with surface modification with ligands or other moieties to facilitate delivery to the targeted site (168). Among all, LL-37 and its mimics are some of the most commonly explored HDPs that have been incorporated with different types of NPs, including polymeric NPs [e.g., poly lactic-co-glycolic acid (PLGA)] (169), gold NP (170, 171), and magnetic NPs (172).

PLGA is a FDA-approved biodegradable and biocompatible polymer that has demonstrated promising potential as a drug delivery carrier (173). Chereddy et al. (169) described using PLGA as a nanoparticle carrier for delivering a sustained release of LL-37 treatment. Compared to PLGA or LL-37 alone, PLGA-LL37 nanoparticles were reported to expedite the wound healing process with significantly higher collagen deposition, re-epithelialization and neovascularization (169). It also demonstrated better antimicrobial activity against *E. coli* compared to PGLA alone, though the efficacy was lower than LL-37 alone (169). Cruz et al. (174) similarly reported that the encapsulated form of GIBIM-P5S9K peptide within PLGA or polylactic acid exhibited around 20 times stronger antimicrobial efficacy against methicillin-resistant *S. aureus* (MRSA), *P.* aeruginosa, and *E. coli* when compared to the free peptide.

In addition, gold NPs have been increasingly applied in the field of HDPs (175). Comune et al. (171) demonstrated that

LL-37 conjugated with gold NPs (via an additional cysteine residue at the C-terminus of LL-37) demonstrated superior *in vitro* and *in vivo* wound healing properties compared to LL-37 alone. This was attributed to the prolonged phosphorylation of epidermal growth factor receptor (EGFR) and extracellular-signal-regulated kinase (ERK)1/2, which increased the migration of keratinocytes. Gold NPs have also been used as nanocarriers for other HDPs such as Esc(1-21), a derivative of a frog skin HDP called esculentin-1a (176). Compared to Esc(1-21), it was found that the conjugated form of Esc(1-21) with gold NPs via a poly-ethylene glycol linker improved the antimicrobial efficacy against *P. aeruginosa* by around 15-fold, with increased resistance to proteolytic degradation (176). Certain peptides have also demonstrated self-assembling ability as a nanocarrier for drug delivery (166), though this is beyond the scope of our review.

Smart Design Using Artificial Intelligence (AI) Technology

AI serves as one of the major breakthroughs in the mankind's history. Long been deployed in the automobile and technology industries, AI has only started gaining traction in the field of science and medicine, owing to the advancement in computer power, availability of big data, publicly available neural networks, and improvement in AI algorithms using machine learning and deep learning (177–180). In view of the infinite chemical space and complex SAR of natural and synthetic HDPs, AI serves as an attractive solution to identify and predict novel peptide sequences with potentially good antimicrobial efficacy (181–184).

To date, a number of machine learning algorithms such as artificial neural network (ANN) (34, 182), support vector machine (SVM)-based classifier (34, 183, 184), quantitative matrices (34), and fuzzy K-nearest neighbor (FKNN) (185) have been developed to search for the ideal synthetic HDPs. Cherkasov et al. (182) trained an atomic-based QSAR model using ANN and inductive chemical descriptors based on two large 9-mer peptide libraries. The model was then tested against 200 peptides that were chosen from a virtual library of 100,000 random 9-mer peptides. The model not only successfully predicted the antimicrobial efficacy of the synthetic peptides but also identified potent peptide candidates (HHC-10 and HHC-36) which were highly active against a range of Gram-positive and Gram-negative superbugs, with low risk of toxicity (182).

On the other hand, Lee et al. (183) developed a SVM-based classifier coupled with Pareto-optimization (186) to deduce the functional and structural similarities of alpha-helical HDPs. By employing antimicrobial assays and small-angle X-ray scattering, it was found that the SVM distance to hyperplane σ correlated strongly with the ability of HDP in generating a negative Gaussian curvature (NGC), which is commonly responsible for the membrane disruption mechanism of HDP (183). Subsequently, Yount et al. (184) were able to identify a unifying physicochemical characteristic of alpha-helical HDPs in a 3-dimensional space, termed the alpha-core signature, using knowledge-based annotation and pattern recognition analysis of bioinformatics databases. The antimicrobial efficacy of this alpha-core signature (i.e., the ability to induce NGC) was further validated with the previously developed SVM-based classifier (184).

DISCUSSION AND FUTURE DIRECTIONS

Since the serendipitous discovery of HDPs in nature during early 1980s, immense research effort have been invested in realizing the therapeutic potentials of HDPs in clinic (7). In this review, we provided a comprehensive overview on eight key strategies (with examples) in improving and translating the therapeutic potentials of HDP-based treatment from bench to bedside. Moreover, we summarized HDP derivatives that are currently in the development pipeline.

Lessons From Previous Experience

So far, a number of HDP-based treatment have entered advanced (phase II/III) clinical trials (**Table 1**) but none had reached the market due to regulatory hurdles (27, 187). Nevertheless, many lessons have been learnt from past experience. One of the notable examples (as described above) is MSI-78 or pexiganan, a magainin-derived HDP, which did not obtain the FDA approval after failing to demonstrate any superiority to the normal standard wound care with oral ofloxacin for infected diabetic foot ulcers in two phase 3 trials (188). Although the discouraging results have painted a gloomy outlook for HDP-based treatment at that time, a closer look at the development pathway of MSI-78 has shed light on the plausible reasons accounting for the failure. First, although the molecule demonstrated a broad-spectrum antimicrobial activity against 3,109 clinical isolates (with an average MIC_{90} of 32 μg/ml or less) (63), the activity remained considerably weaker than the conventional antibiotics (62). Second, peptide-based treatment including MSI-78 are more susceptible to proteolytic degradation when compared to the conventional small molecule antibiotics. That means HDP-based treatment need to be administered in a higher concentration to achieve the intended *in vivo* efficacy, which could inevitably lead to increased host toxicity. In addition, MSI-78 exhibits several favorable properties over conventional antibiotics, including low risk of developing AMR and good activity against MDR isolates (63), but the phase 3 clinical trials of MSI-78 were conducted for mild infective diabetic foot ulcers which did not fully capitalize on these strengths. This highlights the importance of setting the right research question during the development of HDP-based treatment.

Learning from the previous (unsuccessful) experience, a plethora of strategies have been proposed and attempted to overcome the inherent limitations of HDP-based treatment, with enhanced antimicrobial efficacy, proteolytic stability, and cell selectivity (for microbial cells). Although the design of ideal HDPs is not governed by a single overarching rule (7), it is apparent from the literature that peptide design guided by the fundamental principles and systematic SAR analysis is able to yield potential efficacious peptide candidates with desired properties (189). In fact, *de novo* designed synthetic peptides were successfully developed purely based on Arg and Trp with 50% hydrophobicity and demonstrated significantly antimicrobial and anti-biofilm efficacies against MDR staphylococci (190).

Proposed Strategy in Designing and Developing HDP-Based Treatment

Based on the literature and our experience, we propose a potential strategy in streamlining the drug discovery and development pathway of HDP-based treatment, starting from designing new HDP treatment to conducting well-designed pre-clinical studies (**Figure 1**). So far, more than 3,000 naturally occurring and synthetic HDPs (with reported antimicrobial and/or non-antimicrobial functions) have been discovered (8, 9); therefore, it would be a good strategy to use an existing template with proven effect as a starting point for designing a new HDP-based treatment. Alternatively, employing artificial intelligence technology in predicting potentially efficacious molecules could be utilized. Once a starting template is identified (either a linear peptide or a cyclic peptide), systematic SAR analysis of the sequence via rational substitution of specific residues is required to optimize the antimicrobial efficacy and cell selectivity toward microbial cells. If hybridization strategy is used, functioning sequence of each single peptide should be first determined before being hybridized. This is then followed by further SAR analysis to determine the optimal sequence.

Once the efficacy and safety are optimized, the next hurdle is to overcome the issue of proteolytic degradation, which could be achieved through the strategies (either singularly or in combination) mentioned in **Table 2** and **Figure 1**. However, it is noteworthy to mention that the beneficial effects of these modifications may be unique to specific HDPs. In addition, antimicrobial efficacy and/or microbial cell selectivity of the HDPs may also be affected during the modification. Subsequently, the potential lead compound should be validated in *in vitro* conditions mimicking the physiological or host disease environment. For instance, when designing HDP-based treatment for corneal infection, the designed HDPs should be tested in tear fluid or in salt of physiological concentration, which are known to affect the efficacy and stability of HDPs. The findings enable a better prediction of the *in vivo* results and help minimize the unnecessary use of animals (191). Finally, well-designed pre-clinical studies need to be performed with appropriate sample size calculation and positive/negative controls, which will increase the success rate of clinical trials. For example, efficacy of the designed HDP needs to be compared with antibiotic treatment that reflects the current clinical practice for the disease of interest. Otherwise, subsequent clinical trials are likely to fail and necessary regulatory approval will not be obtained.

Ideally, it is best to optimize the previous steps before progressing to the next step. For instance, validating the potential lead compound in *in vitro* conditions mimicking physiological environment is crucial before proceeding to pre-clinical studies. Modification strategies proposed in each step may also be applicable to other steps. For example, introduction of unnatural AAs primarily improves the proteolytic stability but may also enhance the antimicrobial efficacy (101), and incorporation of HDPs with nanoparticles may reduce host toxicity as well as improve bioavailability. In addition, several strategies may be employed in combination to achieve the intended therapeutic effect and stability.

There are also increasing reports examining and exploiting the strategy of using peptide-antibiotic combination to counter AMR, increase the lifespan of conventional antibiotics and HDPs, as well as to reduce the undesired toxicity to host tissues (88, 192–194). The synergistic effect of peptide-antibiotic combination treatment is likely attributed to the different underlying mechanism of action whereby the membrane perturbation effect of peptides facilitates the passive diffusion of conventional antibiotics into the cells for intracellular targeting action (192).

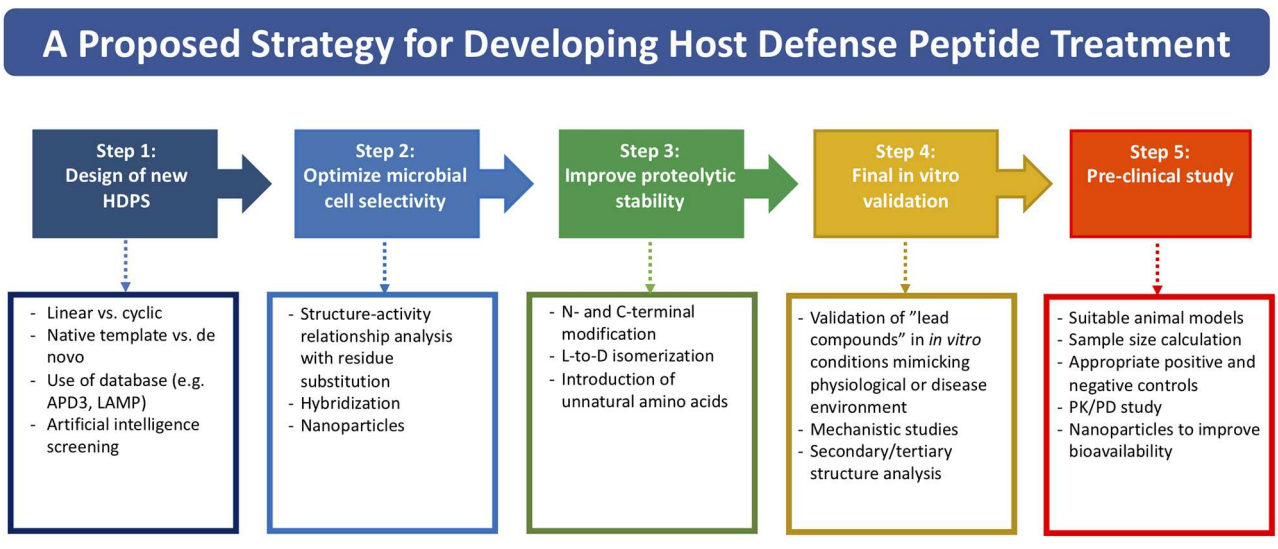

FIGURE 1 | A potential strategy for streamlining the drug discovery and developmental pathway of HDP-based treatment, covering from designing of new HDP treatment to conducting well-designed pre-clinical studies.

TABLE 2 | Summary of different strategies in translating the therapeutic potentials of host defense peptides (HDPs).

Methods	HDPs template	Strategies	Biological effects
Residue substitution			
Lee et al. (40)	HP ribosomal protein 1	Pro substitution	Increased antimicrobial efficacy
Wang et al. (86)	LL-37	Ala/Val substitution	Increased antimicrobial efficacy
Blondelle et al. (37)	Melittin	Trp substitution	Reduced host tissue toxicity
Hybridization			
Wei et al. (117)	Cecropin and LL-37	Hybridization	Increased antimicrobial efficacy and reduced host tissue toxicity
Wu et al. (118)	Melittin and LL-37	Hybridization	Increased antimicrobial efficacy and reduced host tissue toxicity
Boman et al. (112)	Cecropin and melittin	Hybridization	Improved antimicrobial efficacy and reduced host tissue toxicity
Unnatural AA			
Arias et al. (101)	Indolicidin	Ornithine, DAB, DAP, Agb, and hArg	Improved antimicrobial activity against GN and proteolytic stability
Clemens et al. (100)	Cecropin and magainin	Ornithine	Good antimicrobial and anti-biofilm efficacies against GP and GN
Hicks et al. (103)	Magainin	Tic-Oic	Increased antimicrobial activity against GP, GN and mycobacterium and reduced host tissue toxicity
L-to-D isomerization			
Jia et al. (131)	Polybia-CP	LDI	Improved proteolytic stability and reduced host tissue toxicity
Manabe et al. (132)	Sapesin B	LDI	Improved antimicrobial efficacy against GP, GN and fungi
Carmona et al. (130)	Pandinin 2	LDI	Reduced host tissue toxicity
C- and N- terminal modifications			
Saikia et al. (139)	MreB	N-acetylation	Improved antimicrobial efficacy in salt
Falciani et al. (148)	M33	C-pegylation	Increased proteolytic stability
Dennison and Phoenix (143)	Modelin-5	C-amidation	Improved stabilization of alpha-helix and antimicrobial efficacy
Cyclization			
Mwangi et al. (161)	Cathelicidin-BF	Cyclization	Increased antimicrobial and antibiofilm efficacies against MDR-GN and good proteolytic stability
Scudiero et al. (160)	HBD-1 and−3	Cyclization	Increased proteolytic stability
Fernandez-Lopez et al. (154)	*De novo*	Cyclization of D,L-alpha peptides	Increased antimicrobial efficacy
Incorporation with nanoparticles			
Comune et al. (171)	LL-37	Gold NP	Improved wound healing
Casciaro et al. (176)	Esculentin-1a	Gold NP	Improved antimicrobial efficacy, wound healing, and proteolytic stability
Chereddy et al. (169)	LL-37	PLGA NP	Improved wound healing
Smart design with artificial intelligence technology			
Yount et al. (184)	5,200 12-mer peptide sequence	SVM-based classifier	Identification of a unifying alpha-core signature of peptide with good correlation with ability to generate NGC
Lee et al. (183)	572 alpha-helical peptides	SVM-based classifier	Accurate prediction of peptide ability to generate NGC
Cherkasov et al. (182)	Random 9-mer peptide database	QSAR model using ANN	Generation of highly active synthetic peptides against MDR GP and GN, with low toxicity

Three representative examples are provided for each strategy, in order of chronology.
HP, Helicobacter pylori; GP, Gram-positive bacteria; GN, Gram-negative bacteria; DAB, 2,4-diamino-butyric acid; DAP, 2,3-diamino-propionic acid (DAP); Agb, (S)-2-amino-4-guanidinobutyric acid; hArg, homo-arginine; Tic-Oic, tetrahydroisoquionolinecarboxylic acid-octahydroindolecarboxylic acid dipeptide; HBD, Human-beta-defensin; PLGA, Poly lactic-co-glycolic acid; SVM, support vector machine; NGC, Negative Gaussian curvature; ANN, Artificial neural network; MDR, Multidrug resistant.

Risk of AMR Related to HDP

Although HDP-based treatment has long been envisioned as a novel solution to tackle AMR, emerging evidence are suggesting that HDPs could also develop AMR, albeit with a lower risk than the conventional antibiotics (7, 195). Spohn et al. (196) have highlighted the influence of physicochemical characteristics of HDPs, including the proportion of polar AAs, cationicity, and hydrophobicity, on the risk of developing HDP-related AMR, thereby providing invaluable insights into the design of future HDPs. Reassuringly, cross resistance between HDPs was found to be limited to those with similar modes of action, underscoring the importance

and necessity of having HDPs with different antimicrobial mechanisms within the therapeutic armamentarium of antimicrobials (197).

With the advancement in peptide design strategy, synthesis techniques and AI technology, it is hopeful that clinical deployment of HDP-based treatment for a range of diseases will soon become a reality. However, further studies will need to be conducted to decipher the mechanism of HDP-related AMR in order to prepare for the potentially self-perpetuating vicious cycle of AMR in the future.

METHOD OF LITERATURE SEARCH

Electronic databases, including MEDLINE (January 1950–March 2020) and EMBASE (January 1980–March 2020), were searched for relevant articles related to host defense peptides. Keywords such as "host defense peptide," "antimicrobial peptide," "hybrid," "cyclization," "unnatural amino acid," "D-amino acid," "nanotechnology," "nanoparticles," "artificial intelligence," and "machine learning" were used. Only articles published in English were included. Bibliographies of included articles were manually screened to identify further relevant studies. The final search was updated on 31 March 2020.

AUTHOR CONTRIBUTIONS

DT and IM: conception and design of work, data collection and interpretation, drafting the manuscript, and final approval of the work. RB and HD: data interpretation, critical revision, and final approval of the work. RL: conception and design of work, data interpretation, critical revision, and final approval of the work.

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Fragmentation of Human Neutrophil α-Defensin 4 to Combat Multidrug Resistant Bacteria

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The occurrence and spread of multidrug-resistant bacteria is a prominent health concern. To curb this urgent threat, new innovative strategies pursuing novel antimicrobial agents are of the utmost importance. Here, we unleashed the antimicrobial activity of human neutrophil peptide-4 (HNP-4) by tryptic digestion. We identified a single 11 amino acid long fragment (HNP-4_{1-11}) with remarkable antimicrobial potential, exceeding that of the full length peptide on both mass and molar levels. Importantly, HNP-4_{1-11} was equally bactericidal against multidrug-resistant and non-resistant strains; a potency that was further enhanced by N- and C-terminus modifications (acetylation and amidation, respectively). These observations, combined with negligible cytotoxicity not exceeding that of the full length peptide, presents proteolytic digestion of innate host-defense-peptides as a novel strategy to overcome the current health crisis related to antibiotic-resistant bacteria.

Keywords: host defense peptides, α-defensins, proteolytic digestion, multidrug resistance, HNP-4

INTRODUCTION

The spread and occurrence of new multidrug-resistant bacteria represents a prominent and emerging health care threat on a global scale. At large, the pharmaceutical industry and governments alike have failed to develop new antibiotics which has urged World Health Organization (WHO) to call out for new cost-effective strategies to fight these devastating pathogens (Tacconelli et al., 2018). A major challenge is the divergent motivation from society vs. companies, where novel strategies are welcomed yet shelved by regulatory authorities. The rationale behind such decisions, mitigating the risk of multidrug resistance while ensuring these novel therapies in case of an outbreak, is justified, yet jeopardizes the costly development of novel antibiotics. Thus, new cost-effective strategies more resilient to multidrug resistance are urgently needed (Sukkar, 2013; Falagas et al., 2016). Host-defense-peptides (HDP) – previously known as antimicrobial peptides (AMPs) – possess a broad range of antimicrobial properties, which could be useful to develop new antimicrobials in the fight against resistant pathogens (Zasloff, 2002). Defensins are the most prominent class of HDPs in humans. These small cationic molecules share as a common motive six conserved cysteines, which from three disulfide bonds classifies them into α- and β-defensins (White et al., 1995; Ganz, 2003; Selsted and Ouellette, 2005). Four of the six human α-defensins are expressed by immune cells, namely human neutrophil peptides 1–4

(HNPs), whereas the remaining two, human α-defensin 5 and 6 (HD-5 and HD-6) are expressed by Paneth cells in the small intestine (Lehrer and Lu, 2012). All HNPs are processed from propeptide to mature form during their trafficking activated by proteolytic digestion in polymorphonuclear neutrophils azurophilic granules (Valore and Ganz, 1992). These granules fuse with the lysosome after phagocytosis of pathogens allowing for context specific bactericidal activity (Ganz et al., 1985; Selsted et al., 1985). Based on the biological control of these processes it is hypothesized that synthetic production of said peptides could be used as an antibiotic tool against extracellular pathogens. Yet, large-scale expression of accurately folded defensins is a major cost-challenge. Inspired by our recent observation that duodenal fluid degrades full length HD-5 to multiple biological active fragments with different antimicrobial properties including potency, efficacy and bacterial spectrum (Ehmann et al., 2019), we hypothesized that enzymatic digestion of mature HDPs could unleash their antimicrobial capacity and concomitantly solve the production-cost challenge of full length peptides. To this end, the least expressed HNP, HNP-4 (Harwig et al., 1992; Hu et al., 2019), is more bactericidal against Gram-negative bacteria than any of HNP-1-3 (Ericksen et al., 2005). While HNP-1-3 only differs internally in the first amino acid sequence, HNP-4 is more divergent combined with an increased negative charge ultimately enhancing antimicrobial activity (Lehrer and Lu, 2012). We used HNP-4 as precursor to identify new therapeutic agents. To this end, tryptic digestion of the linearized full length peptide liberated its antimicrobial potential. We identified a single fragment with a remarkable bactericidal potency, exceeding the MIC of the full length peptide on molar level. Surprisingly, we observed the antimicrobial efficacy of said peptide to be equally efficient against multidrug-resistant and non-resistant strains, hence presenting HDP fragmentation (Latendorf et al., 2019) as an innovative and cost-effective strategy to aid curbing the emerging threat of antibiotic resistance.

MATERIALS AND METHODS

Bacterial Strains

B. adolescentis Ni3,29c and *B. breve* were provided by Ardeypharm GmbH (Herdecke, Germany). *L. rhamnosus* GG was obtained from InfectoPharm Arzneimittel and Consilium GmbH (Heppenheim, Germany). *A. baumannii* DSM30007, *B. vulgatus* DSM1447, *E. coli* MC1000 DSM6214, *E. coli* DSM8695 (EPEC), *E. coli* DSM10729 (UPEC), *E. faecalis* DSM20478, *E. faecium* DSM20477, *K. pneumoniae* DSM30104, and *S. epidermidis* DSM20044 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkultur GmbH (Braunschweig, Germany). *A. baumannii* 4-MRGN, *B. longum*, *E. coli* ATCC25922, *E. faecium*, *E. faecalis ATCC29212*, *K. pneumoniae* 3-MRGN, *L. fermentum*, *L. salivarius*, *P. aeruginosa* ATCC27853, *P. aeruginosa* 4-MRGN, *S. enterica* serovar Enteritidis, *S. aureus* ATCC25923 *and S. salivarius* were obtained as clinical isolates from the Robert-Bosch-Hospital Stuttgart, Germany. *B. subtilis* (trpC2), *E. coli* JM83, *P. aeruginosa* PAO1, *P. aeruginosa* XPAT1, *P. aeruginosa* XPAT2, *S. aureus* USA300 and *Y. enterocolitica* were provided by the Interfaculty Institute for Microbiology and Infection Medicine, Tübingen, Germany.

Peptides

HNP-4 (Purity ≥ 99%) was obtained from PeptaNova GmbH (Sandhausen, Germany). All peptide fragments, HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$ were chemically synthesized by EMC Microcollections GmbH (Tübingen, Germany) and purified by precipitation. EMC Microcollections guarantees a purity >> 90% by HPLC analysis (**Supplementary Figure S3**). All peptides were dissolved in 0.01% acetic acid.

Screening for Fragments of HNP-4 Using LC/MS

As previously described (Ehmann et al., 2019), 2.5 μg of HNP-4 were incubated in 50 mM NH_4HCO_3 buffer (pH 8.0; Fluka) with 2 mM *tris* (2-carboxyethyl) phosphine for 15 min at 37°C. Afterward, 0.05 μg trypsin [1:50 (w/w)] was added and incubated for additional 30 min at 37°C. Lastly, formic acid and acetonitrile in a final concentration of 0.5 and 10% were added, respectively, and the samples analyzed by mass spectrometry. Mass spectrometry was performed as a LC/MS system using an Agilent 1200 series HPLC with an Agilent Advanced Bio Peptide Map (2.1 × 150 mm, 2.7 μm) column with a flow of 0.4 ml/min at 55°C column temperature and a 6540 UHD Q-TOF LC/MS system (Agilent) for mass analysis. The samples were separated by a gradient of acetonitrile in 0.1% formic acid. The gradient started at 2% acetonitrile for 4 min and then increases during 35 min to 45%. Mass spectrometric analyses were performed in single MS mode from 100 to 3400 m/z with positive ion polarity and were analyzed by Agilent MassHunter Quantitative Analysis B 06.00 software.

Screening for Potential Dimers of HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$ Using HPLC-MS

To analyze possible inter-/intramolecular dimer formation HPLC-MS were performed by EMC Microcollections GmbH Tübingen. HPLC-MS was performed using a Chromolith Fast Gradient RP18e, 50 × 2 mm column (Merck) with detection at a wavelength of 214 nm, followed by an ESI-MS analysis. The samples were separated by a gradient of MeCN (acetonitrile) containing 0.1% FA (monofluoroacetic acid) from 0 to 100% in 30 min.

Radial Diffusion Assay

Antimicrobial activity of all peptides was assessed with a modified version of the radial diffusion assay as described earlier (Schroeder et al., 2011b). Briefly, bacteria were cultivated (anaerobic bacteria in anaerobic jars with AnaeroGen, Oxoid, United Kingdom) for up to 18 h in liquid TSB medium. Log-phase bacteria were washed with 10 mM sodium phosphate buffer; pH 7.4 and diluted to 4×10^6 CFU/ml in 10 ml agar (10 mM sodium phosphate buffer, pH 7.4 with 0.3 mg/ml TSB powder and 1% (w/v) low EEO-agarose (AppliChem).

Bacteria were incubated under aerobic or anaerobic conditions, respectively, with 2 μg HNP-4 or 4 μg of each fragment for 3 h at 37°C. Afterward, plates were covered with 10 ml of an overlay-gel containing 6% (w/v) TSB powder, 1% (w/v) agar and 10 mM sodium phosphate buffer and incubated for 24 h. The diameter of the inhibition zones corresponds to the antimicrobial activity, when subtracting the diameter of 2.5 mm corresponding to the diameter of the punched well. Experiments were repeated at least three times.

Turbidity Broth Assay

Log-phase bacteria were washed twice with 10 mM sodium phosphate buffer containing 1% (w/v) TSB. Approximately 4×10^5 CFU/ml bacteria were incubated with serial peptide concentrations (1.56–100 μM) in a final volume of 100 μl in 10 mM sodium phosphate buffer containing 1% (w/v) TSB for 2 h at 37°C. Afterward, 100 μl of 6% TSB (w/v) were added and absorbance was measured at 600 nm (Tecan, Switzerland) and monitored for 12 h. Experiments were carried out at least three independent times.

Time-Kill Assay

Log-phase bacteria (5×10^5 CFU/ml) were incubated with 6.25 μM of HPN-4_{fl}, HNP-4_{1-11}, HNP-$4_{1-11mod}$ or 0.01% acetic acid as a control in 10 mM sodium phosphate buffer containing 1% (w/v) TSB. After incubation at 37°C and 150 rpm for 0 to 120 min, a sample was taken from the suspension and added to a 0.05% (v/v) sodium polyanethole sulfonate (Sigma-Aldrich) solution, which neutralizes remaining peptide activity, and plated on LB agar to determine the number of viable bacteria. Experiments were carried out at least three independent times.

Reduction Assay

The amino acid sequences of HNP-4_{1-11} and HNP-$4_{1-11mod}$ contain cysteines which might form disulfide bonds with another fragment. As reducing agent Dithiothreitol (DTT) was used. Both peptides, HNP-4_{1-11} and HNP-$4_{1-11mod}$ were pre-incubated with either 0.1 mM or 1 mM DTT for 1 h at room temperature followed by a turbidity broth assay with ˜4×10^5 CFU/ml bacteria as described above. The MIC of HNP-4_{1-11} and HNP-$4_{1-11mod}$ was determined against different bacteria strains. Experiments were carried out at least three independent times.

Protease Inhibitor Assay

Log-phase bacteria were cultivated for up to 18 h in TSB containing different concentrations (0.01 or 0.1) of Bacterial ProteaseArrest™ (G-Biosciences) and 0.5 M EDTA. Bacteria were washed with twice with 10 mM sodium phosphate buffer containing 1% (w/v) TSB and the optical density at 600 nm was adjusted to 0.1. Approximately 5×10^5 CFU/ml bacteria were incubated with serial peptide concentrations (1.56–12.5 μM) in a final volume of 100 μl in 10 mM sodium phosphate buffer containing 1% (w/v) TSB and (0.01 or 0.1) of Bacterial ProteaseArrest™ and 0.5 M EDTA for 2 h at 37°C. After incubation, 100 μl of 6% TSB (w/v) were added and absorbance was measured at 600 nm (Tecan, Switzerland) and monitored for 12 h. Experiments were carried out at least three independent times.

Cell Toxicity Assay

Experiments were conducted with the human colonic epithelial adenocarcinoma cell line CaCo2 subclone TC7 which was obtained from the Robert-Bosch-Hospital Stuttgart, Germany. HT29 MTX cells subclone E12 (Merck, Germany) were used as an additional colorectal carcinoma cell line. Cells were used at an internal early passage of about 25–40. For experiments, 1500 cells/well were seeded in a 96-well plate in 90 μl media.

Cells were treated with serial peptide concentrations (1.56–100 μM) in a final volume of 100 μl and incubated for 96 h. Afterward, the CellTiter-Glo® 2.0 Cell Viability Assay (Promega, United States) was performed based on the company's protocol. Experiments were carried out at least three independent times.

Hemolytic Activity of HNP4 Fragments

Hemolytic activity assay was performed as described earlier (Oddo and Hansen, 2017). Briefly, 1 ml O neg whole blood was washed twice with PBS, centrifuged and 1% (v/v) erythrocytes suspension prepared. Erythrocytes were incubated with serial peptide concentrations (1.56–100 μM) for 1 h at 37°C. Then, samples were centrifuged, supernatant collected and optical density measured at 414 nm. Toxicity against erythrocytes was relative determined to the hemolytic activity of 0.1% Triton X-100. Experiments were carried out in duplicates and performed twice.

Ethics Statement

The study protocol was previously approved by the Ethical Committee of the University Hospital Tübingen, Germany. Patients and controls who were included in this study all gave their written and informed consent after the study purpose, samples procedure, and potential adjunctive risks were explained. All experiments were conducted in accordance with the relevant guidelines and regulations.

RESULTS

Identification of a Novel HNP-4 Fragment After Tryptic Digestions

To generate possible fragments out of HNP-4 we used trypsin as a serine protease. It is known from previous work that folded defensins seemed to be stable against proteolytic digestion (Schroeder et al., 2011a). We incubated HNP-4 with 2 mM TCEP (tris(2-carboxyethyl)phosphine; Sigma-Aldrich) to open the disulfide bonds leading to a more linear structure susceptible to proteolytic digest. We analyzed the trypsin-incubated reduced HNP-4 via LC/MS methods and were able to detect several fragments according to the observed ions and their mass to charge ratio (**Figure 1A**).

Identified fragments were mostly located in the N-terminal region based on the cleaving sites of trypsin (**Figure 1B**). As it is commonly accepted that the net charge of AMPs could play an important role to their antimicrobial activity, we focused on HNP-4_{1-11} with a positive net charge of +3 **Figure 1B**, marked in red.

Antimicrobial Efficacy of HNP-4_{1-11} and HNP-$4_{1-11mod}$

The natural *in vivo* stability of short linear peptides is generally weak; we therefore used an additional modified form of HNP-4_{1-11} (HNP-$4_{1-11mod}$). Here we exchanged the L-amino acids with D-amino acids and modified the N-terminus (acetylation) and C-terminus (amidation). Both modifications should result in a gain of stability (Brinckerhoff et al., 1999; Hong et al., 1999), hence potentially leading to a stronger antimicrobial activity. To analyze the antimicrobial activity of HNP-4_{fl}, HNP-4_{1-11}, and HNP-$4_{1-11mod}$ we used RDAs against a subset of different commensal and pathogenic bacteria (**Supplementary Figures S1**, **S2**). All of our tested peptides showed an antimicrobial activity against tested bacteria (**Figure 2**). While the RDA is the suitable assay to determine a general antimicrobial activity of different peptides, a comparison between different peptides is not possible according to their different abilities (like diffusion) in an agarose gel. We therefore next used a turbidity broth assay to determine the minimal inhibitory concentration (MIC) of HNP-4_{fl}, HNP-4_{1-11}, and HNP-$4_{1-11mod}$ against pathogenic (some multidrug-resistant) Gram negative and positive bacteria (**Figure 3A**). While all peptides displayed antimicrobial activity against tested bacteria (sole exception: HNP-4_{fl} against *K. pneumoniae* DSM30104), HNP-4_{1-11} was surprisingly equimolar to HNP-4_{fl}, indicating that the antimicrobial potency of the natural complex-to-produce HNP-4_{fl} is chiefly driven by the first 11 amino acids (HNP-4_{1-11}), at least in its linear form. To this end, *Hu* and colleagues recently observed some dependency of specific residues post position 11 in the fully folded native peptide (Hu et al., 2019). Pointing further toward enhanced bactericidal efficacy of this linear fragment, HNP-$4_{1-11mod}$, which is expected to exhibit increased stability over the non-modified version, was superior to both HNP-4_{fl} and HNP-4_{1-11} with a MIC several fold lower than the one observed for the natural occurring full length peptide. Additionally, we performed a time-kill assay to investigate the efficacy of HNP-4_{1-11} and HNP-$4_{1-11mod}$ compared to the HNP-4_{fl}. Although we observed a higher potency of HNP-4_{1-11}, the efficacy was similar to HNP-4. In contrast, HNP-$4_{1-11mod}$ was superior in both aspects (**Figure 3B**).

In vitro Stability of HNP-4_{1-11} and HNP-$4_{1-11mod}$

We modified the turbidity broth assay to determine the stability and potential resistance against proteolysis and/or natural degradation. To this end, we determined the antimicrobial activity of HNP-4_{1-11} and HNP-$4_{1-11mod}$ against *E. coli* ATCC25922 in presence a protease inhibitor cocktail (**Figure 4A**). Increasing amounts of protease inhibitors did not improve the bactericidal potential of any of the tested fragments, indicating bacterial proteases do not further degrade mentioned fragments, hence corroborating their stability. Instead, the data points toward a potential fragment:protease interaction, as high concentrations of protease inhibitors reduced the bactericidal efficacy of both fragments.

Enhanced prevalence of cysteine residues on most HDPs led to the current models of multimer formation, combined with a high net charge, as a mechanism to interact with the surface of microorganisms (Brogden, 2005; Mukherjee and Hooper, 2015). To address if multimers were essential for bactericidal efficacy, we determined the MIC of HNP-4_{1-11} and HNP-$4_{1-11mod}$ against *E. coli* ATCC25922 in the presence of increasing levels of the reducing agent, DTT (**Figure 4B**). Elevated DTT concentrations did not affect antimicrobial activity of neither HNP-4_{1-11} nor HNP-$4_{1-11mod}$, suggesting that monomeric peptides were sufficient to kill *E. coli* ATCC25922. To further substantiate these observations, we next performed a HPLC-MS analysis to determine possible inter-/intramolecular dimer formation (**Figures 4C,D**). In line with the results from our reduction assay, we did not detect any formation of oligomeric or polymeric peptide fragments.

Cytotoxic and Hemolytic Effects of HNP-4_{1-11} and HNP-$4_{1-11mod}$

To determine the potential of HNP-4_{1-11} and HNP-$4_{1-11mod}$ for *in vivo* applications as therapeutic agents, we used two different cell lines to investigate their cytotoxic abilities.

While we only observed minor cytotoxic effects on CaCo2/TC7 cells at higher peptide concentration (**Figure 5A**), HT29 MTX E29 cells were more susceptible to both peptide-derivates (**Figure 5B**). Importantly, at lower concentrations (e.g., 12.5 μM, where HNP-$4_{1-11mod}$ has a strong antibacterial effect), the fragments exhibited only modest cytotoxicity. We additionally examined the hemolytic activity of said peptides (**Figure 5C**).

While HNP-$4_{1-11mod}$ has a 20% hemolytic effect at 150 μM (by far exceeding the highest concentration needed for bactericidal efficacy) there was negligible toxicity at ≤18.75 μM, i.e., the highest biological relevant concentration. Thus, compared to the honey bee toxin, Melittin, which showed an 80% hemolytic effect at 1.25 μM both HNP-4_{1-11} and HNP-$4_{1-11mod}$ appeared with low hemolytic activity. In conclusion, the cytotoxic concentrations identified were magnitudes higher than the corresponding bactericidal concentration.

DISCUSSION

Loss of antibiotic efficacy causes increased number of hospitalizations, treatment failures and spread of drug-resistant pathogens (Martens and Demain, 2017). WHO called out to develop new strategies against Gram-negative bacteria in general, and in particularly those from the WHO priority list (Tacconelli et al., 2018). To meet this request, alternatives to

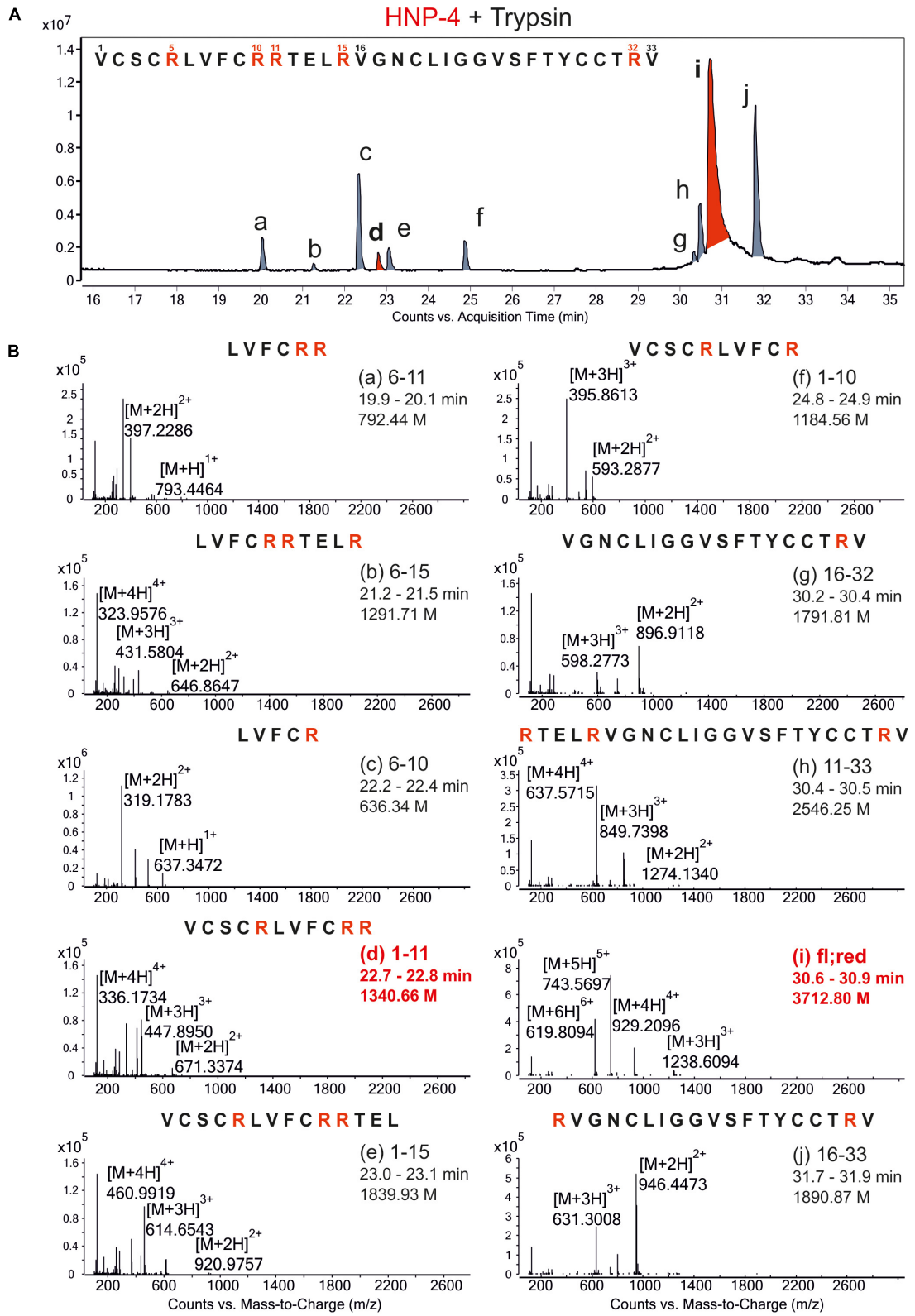

FIGURE 1 | Proteolytic digestion of reduced HNP-4 by trypsin produced different fragments. **(A)** Displays an overview of the chromatogram from an incubation of reduced HNP-4 with trypsin after reduction with 2 mM TCEP. All detectable fragments were marked in red or gray (a–j) and listed due to their retention time. Panel **(B)** show the mass-to-charge (m/z) graphs of all detected fragments. In all mass-to-charge graphs we pointed out the neutral mass based on the detected ions. All peptides marked in red were chose for synthesis and further investigations.

Commensal bacteria	HNP-4		
	fl	1-11	1-11mod
B. subtilis 168trpC			
B. vulgatus			
B. adolescentis Ni3,29c			
B. breve			
B. longum			
E. coli MC1000			
L. fermentum			
L. rhamnosus			
L. salivarius			
S. salivarius ssp.*salivarius*			

Pathogenic bacteria	HNP-4		
	fl	1-11	1-11mod
A. baumannii 4-MRGN			
A. baumannii DSM30007			
E. faecalis DSM20478			
E. faecium DSM20477			
E. coli (EPEC) DSM8695			
E. coli (UPEC) DSM10729			
K. pneumoniae 3-MRGN			
K. pneumoniae DSM30104			
P. aeruginosa 4-MRGN			
P. aeruginosa ATCC27853			
S. Enteritidis			
S. aureus ATCC25923			
S. aureus USA300			
S. epidermidis DSM20044			

High activity | Low activity | No activity

FIGURE 2 | HNP4-derivates display a high antimicrobial activity against commensal and pathogenic bacteria. We analyzed the antimicrobial potential of the identified fragment and its modified version against commensal and pathogenic bacteria. In this heat map, we listed all bacteria and the activity of the fragments in RDA against them. We used 2 μg of the full-length peptide and 4 μg of each fragment. An inhibition zone greater than 8 mm was determined as highly active, between 2.5 and 8 mm as low active, while a diameter of 2.5 mm (diameter of the punched well) was marked as no activity. The heat map is based on three independent experiments.

A

MIC after 12 hours		HNP-4$_{fl}$		HNP-4$_{1-11}$		HNP-4$_{1-11mod}$	
		μM	μg/ml	μM	μg/ml	μM	μg/ml
Gram negative	*A. baumanii* 4-MRGN	12.5	46.4	25	33.5	6.25	8.6
	A. baumanii DSM30007	3.125	11.6	6.25	8.4	6.25	8.6
	E. coli JM83	12.5	46.4	6.25	8.4	6.25	8.6
	K. pneumoniae 3-MRGN	>>>	>>>	25	33.5	6.25	8.6
	K. pneumoniae DSM30104	100	371.5	25	33.5	6.25	8.6
	P. aeruginosa 4-MRGN	50	185.8	50	67.1	6.25	8.6
	P. aeruginosa ATCC27853	6.25	23.2	12.5	16.8	6.25	8.6
	P. aeruginosa PAO1	12.5	46.4	12.5	16.8	12.5	17.3
	P. aeruginosa XPAT1	12.5	46.4	12.5	16.8	12.5	17.3
	P. aeruginosa XPAT2	12.5	46.4	12.5	16.8	12.5	17.3
	S. Enteritidis	100	371.5	25	33.5	12.5	17.3
	Y. enterocolitica	12.5	46.4	12.5	16.8	6.25	8.6
Gram positive	*E. faecium* 475747	3.125	11.6	3.125	4.2	3.125	4.3
	E. faecium DSM20477	6.25	23.2	12.5	16.8	6.25	8.6
	S. aureus ATCC25923	6.25	23.2	12.5	16.8	3.125	4.3
	S. aureus USA300	6.25	23.2	50	67.1	12.5	17.3

B

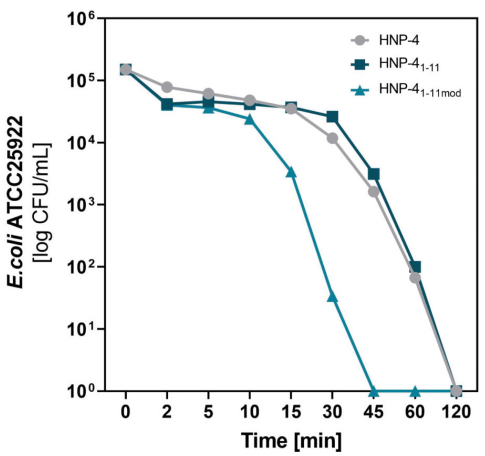

FIGURE 3 | Comparison of the potency (MIC) and efficacy (killing rate) of HNP-4$_{fl}$, HNP-4$_{1-11}$ and HNP-4$_{1-11mod}$. **(A)** The minimal inhibitory concentration (MIC) in μM and μg/ml as a concentration without any bacterial growth. Peptides were incubated with tested bacteria and changes in optical density (OD_{600}) were measured after 12 h at 37°C. If we were able to observe an antimicrobial effect but did not detect a total inhibition of bacterial growth we marked it with ">>>."Each experiment was carried out three independent times. **(B)** Killing of *E. coli* ATCC25922 after 0–120 min exposure to 6.25 μM (1× MIC) HNP-4$_{fl}$, HNP-4$_{1-11}$ and HNP-4$_{1-11mod}$. Results are expressed as the number of viable bacteria (in log10 CFU) per milliliter. Values are means of three independent experiments.

conventional antibiotics are urgently needed (Ghosh et al., 2019; Theuretzbacher et al., 2019, 2020). Thus, new strategies, including those of antimicrobial peptide-derivates must, be developed in the battle against multi-drug resistant bacteria (Fosgerau and Hoffmann, 2015; Breij et al., 2018). To this end proteolysis of HD-5 generated various antimicrobial active peptides with selectivity to certain bacteria (Ehmann et al., 2019). These fragments possess abilities to shift microbiota composition without decreasing diversity. Moreover, mice treated with HD-5_{1-9}, the most potent fragment identified, harbored an increased amount of *Akkermansia* sp. (Ehmann et al., 2019). The same could be shown for the human β-defensin

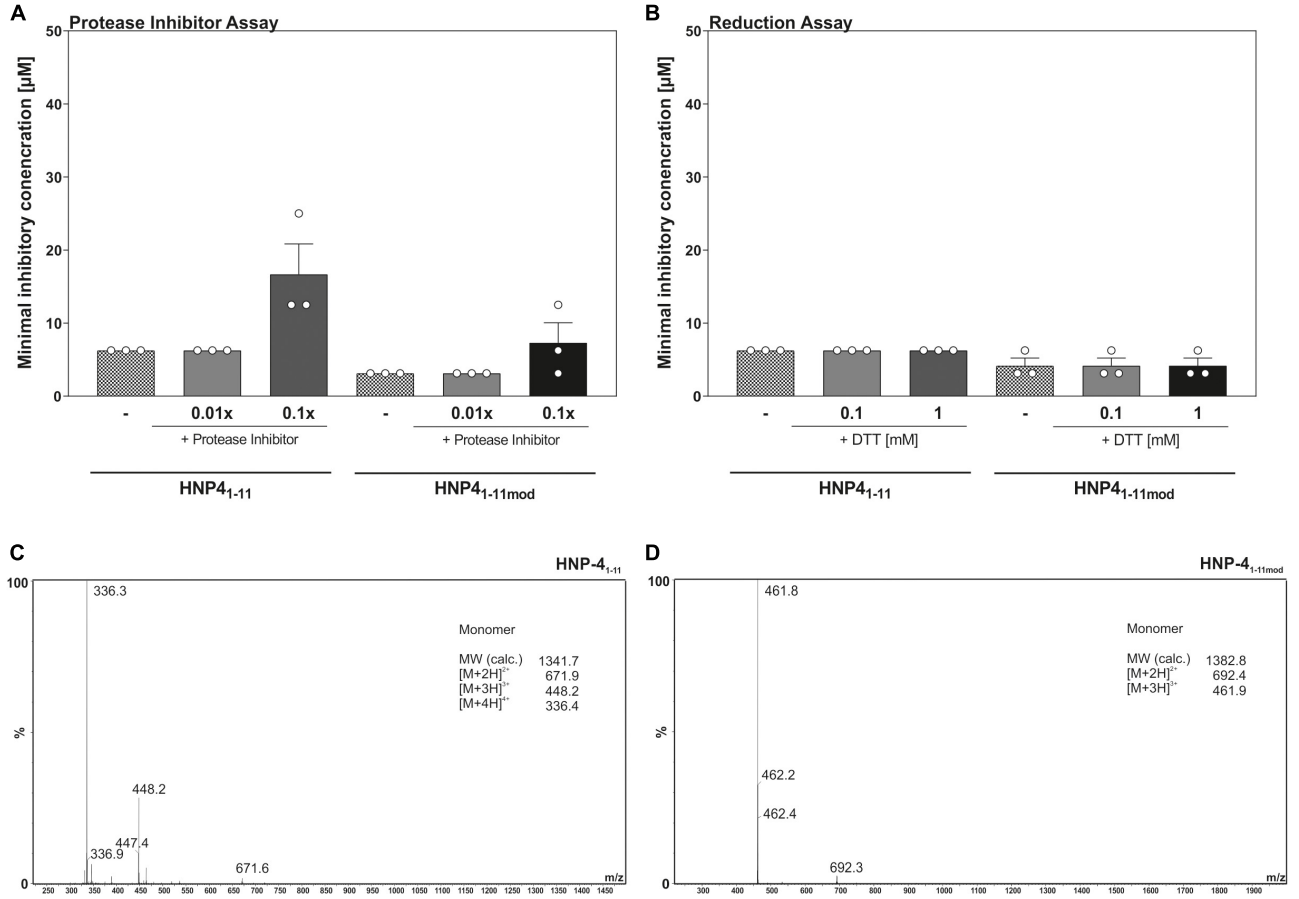

FIGURE 4 | Reduction as well as proteolysis of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$ have no influence on the antimicrobial activity. **(A)** Changes in the antimicrobial activity against *E. coli* ATCC25923 were analyzed in the presence of a protease inhibitor cocktail. **(B)** The minimal inhibitory concentration of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$ was determined against *E. coli* ATCC25922 under reducing conditions due to the optical density after 12 h. Results from three independent experiments with ±SEM are represented. **(C)** ESI-MS analysis of HNP-$4_{1\text{-}11}$ to detect potential dimer's after peptide dilution. **(D)** Analysis of HNP-$4_{1\text{-}11\text{mod}}$ using ESI-MS to detect potential dimer's after peptide dilution.

1, where digestion also led to a diverse set of biological active antimicrobial fragments (Wendler et al., 2019). This study complements our earlier reports with the discovery that proteolytic digestion of HNP-4 led to a highly active easy-to-produce 11 amino acids short fragment (HNP-4_{1-11}) with a broad antimicrobial spectrum against Gram negative and Gram positive bacteria. We hypothesize that this interesting phenomenon represents a general feature of HDPs rather than being specific to HNP-4, in part based on the observation that also the N-terminal part of HNP-1 is antimicrobial active (Varkey and Nagaraj, 2005). It is thus possible that this method of tryptic digestion of HNP-4 may be used as a general technique to unleash the antimicrobial potential of endogenous expressed HDPs to aid curbing the antibiotic resistance crises.

Interestingly, HNP-4_{1-11} possesses equal or better antimicrobial activity against bacteria than the full-length peptide on molar level. A modified version of this fragment further improved both potency and efficacy. Remarkably, HNP-$4_{1-11\text{mod}}$ was highly effective *in vitro* against various multidrug-resistant bacteria including *A. baumannii* 4-MRGN, *K. pneumoniae* 3-MRGN and *P. aeruginosa* 4-MRGN; all top "members" of the WHO priority and Centers for Disease Control and Prevention lists (Tacconelli et al., 2018; CDCP, 2019). Lending credence to the hypothesis of modified HDPs representing an underexplored plethora of drug candidates against multi-drug-resistant bacteria, a recent study elegantly corroborated that this exact class of bacteria are more susceptible to HDPs (Lázár et al., 2018), hence stressing their potential as new therapeutic agents. While we were able to show that HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$ displayed a broad spectrum antimicrobial activity pattern, we did not focus on their antimicrobial mechanisms, but the capacity to induce rapid killing of Gram-negative bacteria indicates membrane interactions as part of the mode(s) of action. From a general point of view cysteines and charged amino acids are often relevant for antimicrobial activity (Jiang et al., 2008). Importance of those amino acids led to the current models of HDP mechanism forming multimers as well as the need of charged amino acids to interact with the surface of microorganisms (Brogden, 2005; Mukherjee and Hooper, 2015).

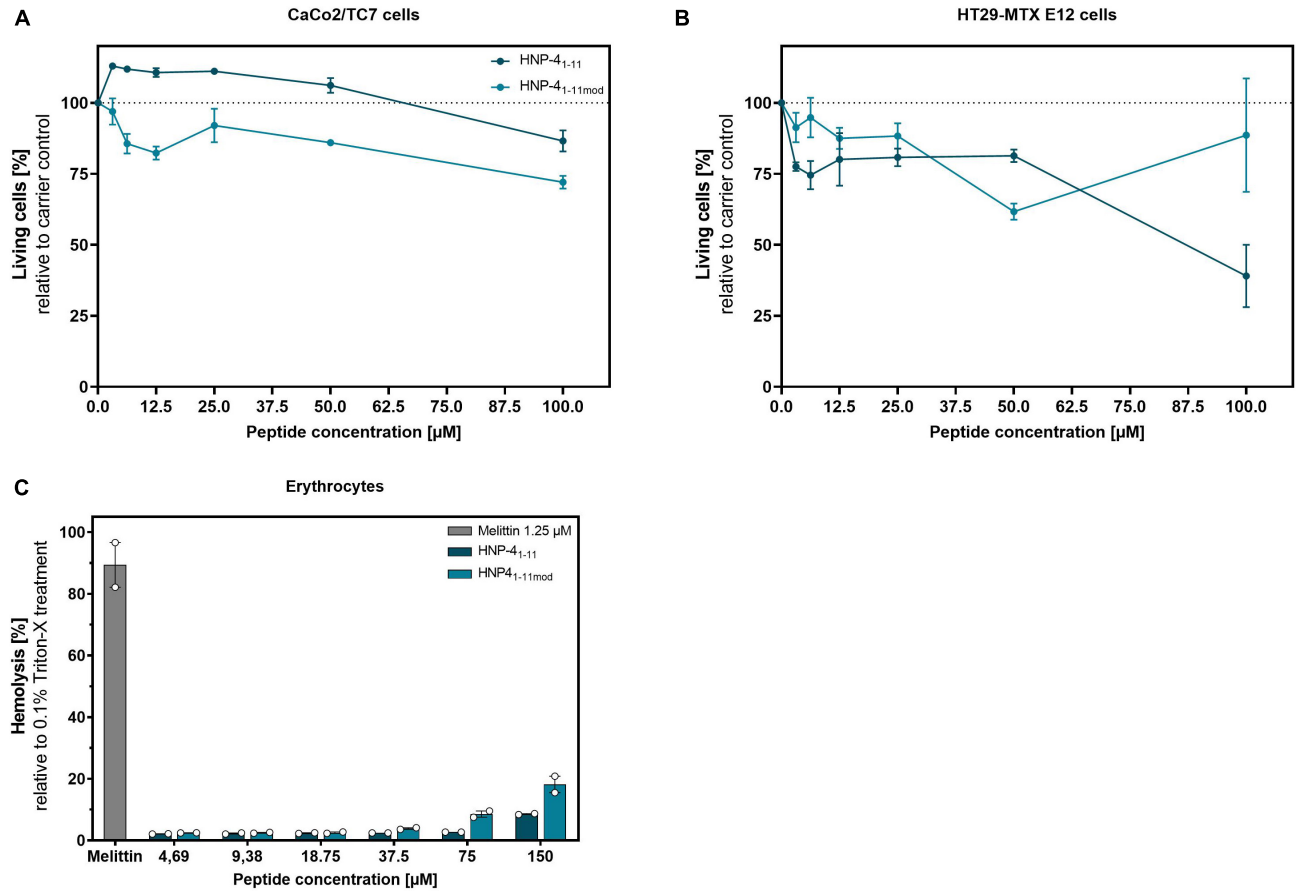

FIGURE 5 | HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$ show only minor cytotoxic and hemolytic activity at high concentrations. We investigated the cytotoxic activity of HNP-$4_{1\text{-}11}$ and HNP-$4_{1\text{-}11\text{mod}}$ against **(A)** CaCo2/TC7 or **(B)** HT29 MTX E 29 cells. We seeded 1500 cells per well and treated them after 24 h with different peptide concentrations. Living cells were determined after 96 h treatment using a CellTiter Glo2.0 assay. **(C)** Hemolytic activity on human erythrocytes of the peptides compared to 0.1% Triton-X treatment. **(A,B)** Results from three independent experiments with ±SEM are shown, and **(C)** Results from two independent experiments with ±SEM are represented.

Due to these observations, we initially assumed that also the antimicrobial activity of the here presented fragments depended on dimerization. Yet, our reducing assays followed by HPLC-MC analysis illustrated that monomeric formation was sufficient for the observed bactericidal activity, pointing toward a different mode of action of these hallmark peptide fragments, disputing the current dogma in the field.

Although covalent dimers are absent, non-covalent oligomeric forms of both peptides cannot be entirely excluded. Additional analyses are necessary to determine the importance of supramolecular peptide forms for antimicrobial activity, as non-covalent oligomerization can be relevant for antimicrobial activity of several and in particular amyloid-forming peptides (Latendorf et al., 2019).

A challenge with HDPs in therapeutic contexts is their susceptibility to proteolysis by bacterial proteolytic enzymes (Reijmar et al., 2007), in particular in reduced environments (Schroeder et al., 2011a), as exemplified by the outer membrane protease of *Salmonella enterica* which degrades and thereby inactivates HDPs, thus supporting an essential role of bacterial proteases in bacterial resistance to HDPs (Guina et al., 2000). The conceptual advancement of utilizing protease-degraded biologically active fragments, as showcased here by trypsin digest is therefore intriguing. Such fragments should, by nature, be resistant to further degradation and may prove valuable to aid fight multi-drug resistant pathogens. In keeping with this notion, our analysis revealed that HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$ activity was not further boosted by protease inhibitors, suggesting that proteases *per se* do not hamper their function. Instead, high levels of protease inhibitors appeared to limit the bactericidal efficacy of both HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$ suggesting that these fragments conversely interact with proteases, rather than being annulled by them, to induce bacterial killing. Future studies are warranted to elucidate the extent of such potential fragment:protease interaction.

For potential therapeutic application, we assessed toxicity of HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$. Both peptides showed cell-type dependent cytotoxicity and hemolytic activity at higher concentrations. To this end, HNP-$4_{1-11\text{mod}}$ exerted a greater

impact on CaCo-2 cells, whereas HNP-4_{1-11} possessed higher cytotoxicity against HT29-MTX E12 cells, but for both tested cell types the cytotoxic concentration range were magnitudes higher than the concentrations needed for antimicrobial activity.

In summary, although future *in vivo* experiments are warranted to determine the full potential of HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$, our results demonstrate promising efficacy of HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$ against multidrug-resistant bacteria. From this point of view, proteolytic digestion of HDPs could be used to generate new biologically active fragments to overcome the antibiotic-resistance crisis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study protocol was previously approved by the Ethical Committee of the University Hospital Tübingen, Germany. Patients and controls who were included in this study all gave their written and informed consent after the study purpose, samples procedure, and potential adjunctive risks were explained. All experiments were conducted in accordance with the relevant guidelines and regulations.

AUTHOR CONTRIBUTIONS

DE, LK, and JaW designed the study. DE, LK, and JuW performed the experiments. DE, LK, BJ, and JaW analyzed the data. DE, LK, JaW, and BJ wrote the manuscript. JuW and NM assisted with data interpretation and manuscript editing. JaW and BJ supervised all parts of the study. All authors were involved in data discussion and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01147/full#supplementary-material

FIGURE S1 | RDA with the HNP-4 fragments against commensal bacteria. Here we show the detailed results of the RDA experiments. Data are presented as mean ± SEM. Experiments were carried out three independent times.

FIGURE S2 | RDA with the HNP-4 fragments against pathogenic bacteria. Here we show the detailed results of the RDA experiments. Data are presented as mean ± SEM. Experiments were carried out three independent times.

FIGURE S3 | Analytical data sheet of HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$. Here we show the detailed analysis of purity of HNP-4_{1-11} and HNP-$4_{1-11\text{mod}}$.

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frontiers in Immunology

REVIEW
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Does an Apple a Day Also Keep the Microbes Away? The Interplay Between Diet, Microbiota, and Host Defense Peptides at the Intestinal Mucosal Barrier

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A crucial mechanism of intestinal defense includes the production and secretion of host defense peptides (HDPs). HDPs control pathogens and commensals at the intestinal interface by direct killing, by sequestering vital ions, or by causing bacterial cells to aggregate in the mucus layer. Accordingly, the combined activity of various HDPs neutralizes gut bacteria before reaching the mucosa and thus helps to maintain the homeostatic balance between the host and its microbes at the mucosal barrier. Defects in the mucosal barrier have been associated with various diseases that are on the rise in the Western world. These include metabolic diseases, such as obesity and type 2 diabetes, and inflammatory intestinal disorders, including ulcerative colitis and Crohn's disease, the two major entities of inflammatory bowel disease. While the etiology of these diseases is multifactorial, highly processed Western-style diet (WSD) that is rich in carbohydrates and fat and low in dietary fiber content, is considered to be a contributing lifestyle factor. As such, WSD does not only profoundly affect the resident microbes in the intestine, but can also directly alter HDP function, thereby potentially contributing to intestinal mucosal barrier dysfunction. In this review we aim to decipher the complex interaction between diet, microbiota, and HDPs. We discuss how HDP expression can be modulated by specific microbes and their metabolites as well as by dietary factors, including fibers, lipids, polyphenols and vitamins. We identify several dietary compounds that lead to reduced HDP function, but also factors that stimulate HDP production in the intestine. Furthermore, we argue that the effect of HDPs against commensal bacteria has been understudied when compared to pathogens, and that local environmental conditions also need to be considered. In addition, we discuss the known molecular mechanisms behind HDP modulation. We believe that a better understanding of the diet-microbiota-HDP interdependence will provide insights into factors underlying modern diseases and will help to identify potential dietary interventions or probiotic supplementation that can promote HDP-mediated intestinal barrier function in the Western gut.

Keywords: antimicrobial peptides, defensins, microbiota, diet, prebiotics and probiotics, high-fat diet, intestinal barrier function, gut bacteria

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INTRODUCTION

The human gut is the interface between the body and the environment and is colonized by a community of trillions of microorganisms, including bacteria, fungi, and archaea. While the small intestine is responsible for nutrient absorption, the large intestine can rather be considered as a bioreactor in which gut bacteria carry out different biological functions, such as processing of dietary fibers (1, 2), maturation and regulation of the immune system (3, 4), and production of metabolites that exhibit various metabolic and neurological effects (5–7). At the intestinal interface, the immune system has the challenging task of maintaining a stable microbiota by keeping beneficial commensal bacteria at bay and by recognizing and eliminating disease-causing microbes. When this equilibrium is lost, the microbiota composition enters a state termed "dysbiosis," which has been associated with a wide array of diseases.

Several environmental factors are known to directly alter or disturb the microbial composition, including diet and medicine use (8), but also intrinsic host factors such as host defense peptides (HDPs) (9), and host genetics (10). Therefore, strict regulation of immune signals in response to intrinsic and extrinsic stimuli is prompted at the intestinal interface to maintain homeostasis.

The intestinal defense system is composed of the gut associated lymphoid tissue (GALT), formed by a single layer of intestinal epithelial cells that are arranged in crypts and villi, and the underlying mesenteric lymph nodes and lamina propria. Goblet cells are dispersed over the epithelial layer and secrete mucus that functions as a physical barrier to maintain microorganisms at a safe distance from the intestinal epithelium. Immunoglobulin A (IgA)-secreting B-cells contribute to controlling local microbial communities (11). Paneth cells are specialized small intestinal cells at the bottom of the crypts of Lieberkühn that specialize in the production of HDPs, and together with enterocytes, which produce HDPs in the small and large intestine, they represent the primary source of HDPs in the gut.

HDPs are mostly small cationic peptides with unique mechanisms of action and different specificity against Gram-positive and Gram-negative bacteria (12). These antimicrobial molecules are the effector molecules of the intestinal immunity with potent bactericidal activity that has mostly been tested against intestinal pathogens (13). On the contrary, much less is known about how HDPs affect commensal bacteria, and several studies suggest that antimicrobial activity against the resident microbiota is comparably low or even absent (14–16). Yet, two independent studies demonstrated that transgenic intestinal expression or oral application of human alpha-defensin 5 (HD5) in mice could shape the intestinal microbiota composition *in vivo* (9, 17). It is therefore possible that previous activity testings of HDPs in *in vitro* assays did not appropriately reflect the *in vivo* conditions, as already demonstrated for human beta-defensins 1 (HBD1) and the Paneth cell-derived human alpha defensin 6 (HD6), which gained activity under adjusted conditions that reflected the intestinal microenvironment (18, 19). However, we are only about to begin to understand how HDPs affect commensal microbes and how the functionality of this defense system can influence the way the host copes with its inner microbial world in the intestine.

The secretion of Paneth cell HDPs can occur in response to bacterial stimuli and is largely regulated by signals from the transcription factor 4 (TCF-4)/Wnt signaling pathway in Paneth cells, while epithelial-derived HDPs rather seem to be controlled through IL-22, derived from immune cells (12, 20, 21). Microbial ligands are recognized through pattern recognition receptors (PPRs) present in intestinal epithelial cells or immune cells. Upon recognition and activation, immune cells of the GALT send signals to Paneth cells, goblet cells and enterocytes to coordinate their function and maintain the epithelial barrier function (22). In addition, the presence of microbes and their metabolites seems to be implicated in the control of the antimicrobial programming at the intestine, as germ-free (GF) mice have reduced HDP expression (23) and since probiotic supplementation or microbial-metabolite enrichment stimulates HDP production (24–27).

Members of the gut microbiota can influence HDP expression, and diet is considered one of the most influencing factors determining gut microbiota composition. Accordingly, diet composition, and whether it is of animal or plant-based origin, has profound implications in defining the gut microbial composition. For example, a diet rich in plant-derived fiber is associated with increased diversity in microbial communities, and more specifically, with an increase in *Bifidobacterium* abundance, which has been shown to be a positive regulator of intestinal barrier function (28, 29). As for proteins, animal-derived proteins were shown to decrease the abundance of Firmicutes, a phylum that has been associated with obesity and high body mass index (30), whereas plant-derived proteins were shown to promote the growth of beneficial *Bifidobacterium* and *Lactobacillus* genera and reduce the abundance of pathogenic bacteria (31). A Western-style diet (WSD), characterized by its low dietary fiber but high-fat and high carbohydrate content, markedly changes the microbiota composition in humans and mice (29, 32–35). Moreover, a WSD promotes a pro-inflammatory response through different dietary components (e.g., cholesterol, saturated and non-saturated fatty acids) and can cause microbiota-induced mucus defects as a result of the reduced fiber content (29, 36–38). Importantly, various studies indicate that the increased consumption of WSD in our modern societies, often accompanied by food additives such as artificial sweeteners and emulsifiers, is likely one of the drivers for the worldwide increase in non-communicable diseases, including metabolic syndrome and inflammatory bowel disease (IBD) (3, 39–42).

In this review, we summarize recent findings linking the effect of microbiota, diet, and food availability on the HDP-mediated intestinal defense function during intestinal homeostasis. Moreover, a defective HDP function has been linked to modern diseases associated with a Western-lifestyle, including IBD and metabolic disease. In particular, reduced levels of human defensins have been described in ileal Crohn's disease (43–45). Therefore, we also aim to decipher possible interactions along the diet-HDP-microbiota axis (**Figure 1**), that could be relevant

in Western diseases, in which gut microbiota can gain access to the host epithelium due to an impaired barrier function. In that context, modulation of the gut microbiota through diet has been much discussed as a therapeutic alternative to protect the intestinal epithelium. Thus, we pose the question whether an apple a day keeps the microbes away, and we chose this fruit for several reasons: an apple is an easy-accessible everyday product that does not only contain fibers and polyphenols to potentially support the growth of HDP-stimulating bacteria, as we discuss below, but it was also recently shown that apples carry thousands of bacteria (46). Thus, it is theoretically possible that this fruit could serve as a natural pre- and pro-biotic to strengthen antimicrobial HDP function in the gut.

MICROBIOTA IN HEALTH AND DISEASE

The human microbiota is generally dominated by the Bacteroidetes (*Bacteroides*, *Parabacteroides*, *Prevotella*, *Alistipes* genera) and Firmicutes (*Clostridium*, *Eubacterium*, *Blautia*, *Roseburia*, *Lactobacillus*, *Faecalibacterium*, *Ruminococcus*, *Streptococcus* genera) phyla. Other phyla, such as Proteobacteria *(Escherichia* genus), Actinobacteria (*Bifidobacterium* genus), and Verrucomicrobia (*Akkermansia* genus), are less represented and ratios of these phyla vary highly between individuals (47). A classification into "enterotype" groups was previously proposed, based on the function and relative abundance of the *Bacteroides*, *Prevotella,* and *Ruminococcus* genera within an individual, but the authors also stressed the fact that non-abundant species can exert high-abundant functions (e.g., methanogens), and that high-abundant microbes should thus not be regarded as solely responsible for the entire functionality of the human intestinal microbiota (48). Consequently, enterotypes do not seem to be as discrete as previously suggested, as they can be confounded by environmental variables, the clustering model used and stability over time (49).

Numerous studies have collectively attempted to define what constitutes a healthy microbiota, as the gut microbiota of healthy and diseased individuals differs in its composition. For example, the microbiota has been implicated in several disorders, such as IBD (50–52), obesity (53–56), diabetes (57–59), allergic diseases (60, 61), Parkinson's disease (62), autism spectrum disorder (63), and atherosclerosis (64), among others. Although there is in most cases no solid evidence that changes in the microbiota may cause these diseases, these microbial associations have encouraged the effort of finding strategies to modulate the microbial community composition through dietary intervention to improve the symptoms accompanying these disorders. Yet, the establishment of a healthy "ideal" microbiota is complex, as many factors are known to influence its composition (65). Here, we will focus on two key factors that are continuously affecting the intestinal microbiota, namely diet, and HDPs.

Dietary Influence on Gut Microbiota Composition

The impact of different diets on the intestinal microbiota has been extensively reviewed in recent years (28, 30, 31, 66). The composition of the diet (defined by macronutrient ratio—carbohydrates, fats and proteins), the origin of these components (plant or animal-based) and the availability of different dietary factors are recognized as determinants of gut microbial metabolism and composition, with the potential to influence human health (67, 68) (**Figure 2**). The three major macronutrients carbohydrates, fats and proteins, can reach the colon after escaping the primary digestion in the small intestine when the intake surpasses the rate of digestion, or due to the biomolecules' intrinsic structural complexity (69–71). Therefore, the proportion of macronutrients present in, for example, a Western-style, protein-rich, vegan, vegetarian, or fiber-rich diet will have different effects on the colonic microbiota.

Fiber

The dietary compound that has been found to be the strongest contributor to gut microbial community structure is dietary fiber. Dietary fiber is almost exclusively of plant-based origin and can be found as soluble or insoluble carbohydrate polymers that are inaccessible to the human body due to the limited number (ca. 17) of carbohydrate-active enzymes (CAZymes) (2). In contrast, it is estimated that the gut microbiota is equipped with 11,000 CAZymes that carry out the hydrolysis of different sets of soluble fibers (72, 73), also known as microbiota-accessible carbohydrates (MACs) (2). As a result of bacterial fiber fermentation, short chain fatty acids (SCFAs), including acetate, propionate, and butyrate, and gases such as H_2 and CO_2, are produced by different gut bacteria in a complex cross-feeding network. Enterocytes utilize SCFAs as an energy substrate, and these metabolites have also been shown to improve the intestinal barrier integrity, regulate glucose homeostasis and lipid metabolism, and induce both anti-inflammatory and tolerogenic immune reactions (74). Conversely, insoluble fibers are not fermented by the microbiota and do not convey the aforementioned benefits.

Microbial fermentation is determined by the origin, chemical composition and physicochemical properties of the fibers present in food (28). As a rare example of animal-derived carbohydrate, honey includes a diverse mixture of mono- and disaccharides as well as complex carbohydrates. Although the effect will depend on the specific type, honey was shown to promote the growth of *Bifidobacterium* and *Lactobacillus* (75). Fibers that originate from plants—derived from either cereals, grains, vegetables, legumes or nuts—have unique chemical compositions and physicochemical properties (28). Therefore, the variety of fibers present in plant-based diets can support more diverse gut microbial communities (76, 77).

Fruits are another common source of plant-derived fibers. For example, complex pectins found in apples and wine can be degraded by *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) (78), and kiwifruit supplements increased the abundance of *Faecalibacterium prausnitzii* (*F. prausnitzii*) in patients with constipation (79), while a crude extract of kiwi was shown to support the growth of *Bifidobacterium* and *Bacteroides in vitro* (80).

Clinical studies assessing the impact of different types of fibers on the microbiota report that *Bifidobacterium spp.* are

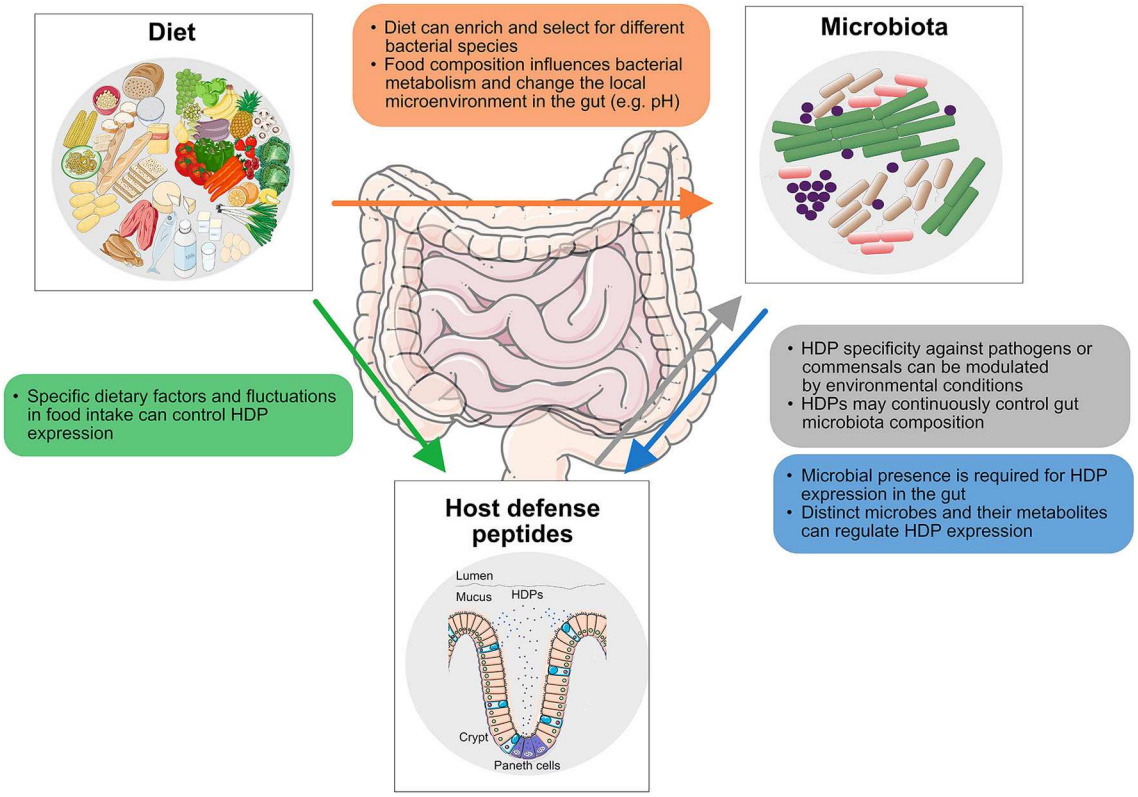

FIGURE 1 | Interactions along the diet-HDP-microbiota axis discussed in this study. The impact of diet on gut microbiota composition (orange), the effect of diet on host defense peptide (HDP) expression (green), the activity of HDPs against gut microbes (gray), and the influence of bacteria and associated metabolites in HDP expression (blue) are displayed.

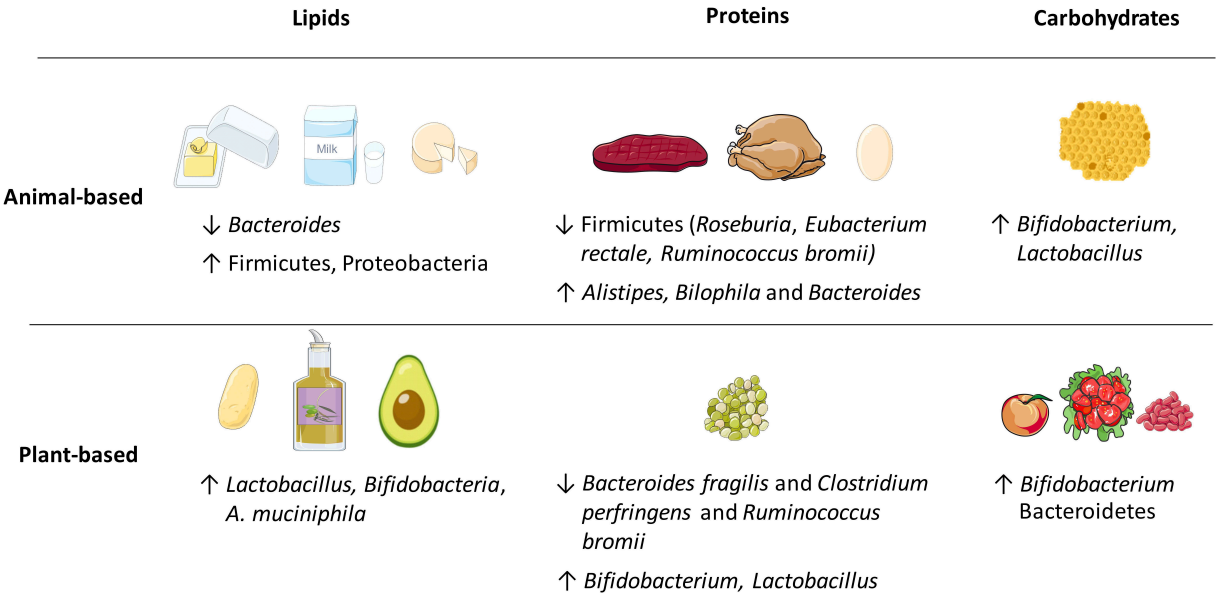

FIGURE 2 | Examples of energy-delivering macronutrients, including lipids, proteins, and carbohydrates that produce various changes in the relative abundance of gut microbiota. The animal or plant-based origin dramatically influences the outcomes.

enriched following consumption of diets with certain fibers, including galacto-oligosaccharides (GOS), inulin-type fructans, xylo-oligosaccharides, and arabinoxylan-oligosaccharides, and that microbes in the Bacteroidetes and Firmicutes phyla are differentially stimulated by soluble fibers from corn or polydextrose (28, 81). In addition, studies comparing the low-fiber diet of Westernized populations with the high-fiber diet of unindustrialized communities show dramatic differences in the microbiota composition between both populations (34, 35), including that the Westernized societies having decreased diversity and apparent loss of certain microbes that are present in the unindustrialized communities (82).

Lipids

A high-fat WSD, mainly containing saturated or trans-fat, is associated with a decrease in *Bacteroides* and an increase in Firmicutes and Proteobacteria relative abundance (34, 83–85). Conversely, mono- and polyunsaturated fat present in low levels in vegan/vegetarian diets increase the levels of lactic acid bacteria, *Bifidobacteria,* and *Akkermansia muciniphila* (*A. muciniphila*) (30, 31). In mice, both lard-based and palm-oil based HFDs increased the relative abundance of the *Clostridiales* and *Bacteroidales* classes in specific pathogen free (SPF) mice (86). However, no significant differences in microbiota composition were observed between both diets that mainly differ in their cholesterol content, where a lard-based diet contains 10 times more cholesterol than the palm-oil based HFD (86).

Agans et al. demonstrated in an *in vitro* multi-vessel analysis that distinct gut microbiota can utilize dietary fatty acids as a sole carbon source through β-oxidation and anaerobic respiration pathways (87). Thus, bacteria that possess fatty acid oxidation enzymes, for example *Alistipes spp.* and members of the Proteobacteria phylum (*Bilophila*, *Escherichia/Shigella*, *Citrobacter,* and *Enterobacter spp.*) were enriched in a medium containing only capric acid, palmitic acid, stearic acid, oleic acid, and linoleic acid (87). Interestingly, however, in the small intestine, where most of the macronutrient digestion and absorption occurs, the intestinal microbiota was also shown to be capable of regulating host dietary fat digestion and absorption in mice (88). In that study, consumption of a HFD increased the relative abundance of the *Clostridiaceae* family at the mucosa, most markedly in the jejunum and ileum, and one member of this family was shown to secrete an unknown metabolite capable of mediating lipid absorption (88). Thus, the mucosa-associated microbiota can be highly sensitive to dietary lipid changes and can play an important role in nutrient absorption.

Proteins

In addition to fiber fermentation, protein metabolism by bacteria can produce a small fraction of SCFAs too, but also more detrimental metabolites originating from animal diets (eggs, beef, pork). For example, the food-derived microbial metabolite trimethylamine N-oxide (TMAO) is linked to cardiovascular disease and atherosclerosis (89, 90). Moreover, a diet rich in animal protein is associated with a decrease in members of Firmicutes phylum that are known to metabolize plant polysaccharides (e.g., *Roseburia*, *Eubacterium rectale,* and *Ruminococcus bromii*) and with an increase in the levels of bile-tolerant bacteria (*Alistipes*, *Bilophila,* and *Bacteroides*) (67, 91). However, individuals consuming pea protein—a plant-based alternative for meat—displayed increased intestinal SCFA levels and a bloom in beneficial *Bifidobacterium* and *Lactobacillus*, while pathogenic *Bacteroides fragilis* and *Clostridium perfringens* levels were reduced (31). Furthermore, observations in protein supplementation studies showed an increase in the total amount of bacteria, as determined by absolute-abundance (92). This was proposed to be linked to the increased availability of nitrogen, an otherwise limited nutrient in the gut, as a result of the higher protein intake (92).

Other: Micronutrients and Food Additives

Besides the discussed macronutrients, micronutrients are increasingly acknowledged to influence the gut microbiota. Some of these compounds transit the small intestine, where a large number of digestible nutrients are already absorbed, and reach the colon intact, where they concentrate and interact with the microbiota (66, 93). Examples of micronutrients include polyphenols—naturally occurring plant metabolites—(e.g., lignans, isoflavones, stilbenes), trace elements and vitamins. Polyphenols in plant-based diets are generally considered to have a prebiotic effect, i.e., supporting the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* (94–96), can be antimicrobial against different bacterial pathogens and can have anti-inflammatory effects (31, 97). Trace-elements, such as iron and zinc, have a low abundance in the gut and are thus competed for amongst pathogens and commensals, thereby also affecting gut microbial composition and/or favoring pathogen colonization (98–100). Other micronutrients such as vitamins B6 and B12 serve as cofactors for microbial enzymes and consequently, gut microbial species compete with the host for these diet-derived vitamins in the small intestine (101).

Remarkably, food additives that are often present in modern diets (e.g., non-caloric artificial sweeteners (NAS) such as sucralose, saccharin and aspartame) and emulsifiers [e.g., carboxymethyl cellulose (CMC) and polysorbate-80 (P80)], induce significant dysbiosis. The microbiota of NAS-consuming mice provoked an overgrowth of *Bacteroides spp.* and reduced levels of *A. muciniphila* (39). Emulsifier-treated mice had a reduction in the *Bacteroidales* population and an increase of the mucolytic bacterium *Ruminococcus gnavus*, which was accompanied by decreased SCFA production and the development of metabolic syndrome (40).

In summary, individual macronutrients and micronutrients can have distinct effects on gut microbiota composition, which in turn can have subsequent effects on human health. However, caution is prompted when linking a phylum to a specific diet, given the dynamic nature of the microbiome and due to the challenging task of disentangling which dietary effector in the complex composition of the diet is driving the observed changes (66). For example, the changes in gut microbial composition observed in HFDs could be biased by the low dietary fiber content and may not be a direct consequence of the fat content or composition. Indeed, Morrison et al. showed that switching from a regular chow diet to a refined low-fat/low soluble fiber

diet was accountable for the change in the fecal community structure in mice (102). In contrast, a switch from the low-fat/low soluble fiber diet to a low soluble fiber/HFD kept the initial observed changes without further alteration. Remarkably, the authors observed expansion of *Clostridia* and Proteobacteria and a reduction of Bacteroidetes when switching from a chow diet to a diet low in fat and lacking soluble fibers; these alterations are typical of HFD interventions (102).

INTESTINAL HDPs AS KEY EFFECTORS OF MUCOSAL BARRIER FUNCTION

Besides the nutrients derived from the ingested food, the host also plays an active role in shaping the gut microbial community. While the production of IgA and mucus as modulators of gut microbiota composition have been discussed elsewhere (103, 104), we will here focus on the release of HDPs, which due to their positive charge are retained in the intestinal mucus layer (105–107). Intestinal HDPs are a diverse group of proteins that possess unique mechanisms of action and spectrum of activity against microbes. In part, these mechanisms depend on the HDP localization in the intestine (**Figure 3**) and the specific regulatory mechanisms of expression and activation (12).

Location

In the intestine, different epithelial cell subsets produce distinctive HDPs. Enterocytes produce the regenerating islet-derived protein 3-gamma (Reg3γ) and β-defensins in both the small and large intestine, and the Ly6/PLAUR domain containing 8 protein (Lypd8) exclusively in the large intestine (108). While the primary function of goblet cells relies on the production and secretion of MUC2, they also secrete resistin-like molecule beta (RELMβ), an HDP that is active predominantly in the colon (109, 110). However, the vast majority of HDPs are produced by small-intestinal Paneth cells and include lysozyme, α- and β-defensins (α-defensins are alternatively called cryptdins in mice), angiogenin-4 (Ang4), secretory phospholipase A2 group IIA (sPLA2), and Reg3γ. Although mature Paneth cell HDPs have been isolated from the large intestine, they probably also originate from small-intestinal Paneth cells (111).

Immune cells also contribute to the HDP repertoire with secretion of lipocalin-2 (Lcn2) by neutrophils and lysozyme by macrophages (112). Also, neutrophils produce human neutrophil peptides (HNPs), a class of α-defensins, which are only produced by humans and not by mice (113). To compensate for this, however, mice seemingly evolved and acquired an additional set of peptides closely related to α-defensins, called cryptdin-related sequence (CRS) peptides, that are also produced by Paneth cells (114).

Antimicrobial Activity of HDPs

Defensins possess a broad spectrum of antimicrobial activity, as *in vitro* studies demonstrated that they are bactericidal against Gram-positive and Gram-negative bacteria, fungi, viruses, and unicellular parasites (115). These small secreted peptides (30–40 amino acids long, 3 to 5 kDa) are characterized by six conserved cysteine residues that form three disulfide bridges (116). The in-sequence linkage of the cysteines distinguishes α- and β-defensins, the two largest defensin families (113). The cationic nature of these peptides advantageously attracts them to the negatively charged outer envelope of bacteria, produced by the presence of phospholipids in Gram-negatives and of teichoic acid in Gram-positives. In its majority, defensins are thought to act by disrupting the bacterial membrane integrity or via inhibition of the cell wall synthesis by interacting with lipid II (112, 113, 117). Interestingly, due to this cationic property, HDPs have recently been attributed different tumor killing capabilities, as the membrane of tumor cells have increased expression of negatively charged cell surface glycoproteins (118).

Mice possess a wide array of α-defensin and CRS-peptides in the intestine, both with many gene paralogs and high sequence similarity between different laboratory mouse strains, which complicates research on these molecules (119, 120). In addition, expression levels of mice defensins vary along the small intestine (23). α-defensins, also called cryptdins in mice, exhibit a variable spectrum of activity depending on their oxidation status (discussed below); for example displayed reduced α-defensin 4 a greater antimicrobial activity against 7 different commensal bacteria than the oxidized form (15). Furthermore, the antimicrobial activity spectrum of CRS-peptides relies on their characteristic ability to form covalent disulfide-bridged homo- and heterodimers, conferring different killing capabilities against commensal *Enterococcus faecalis (E. faecalis)* and *Lactobacillus fermentum* and the pathogens *Streptococcus pyogenes*, *Listeria monocytogenes, Escherichia coli* (*E. coli*) and *Salmonella typhimuriu*m (*S. typhimurium*) (114).

In humans, the most abundant intestinal HDPs are HD5 and HD6 (121). The potent antimicrobial activity of HD5 was shown against *Staphylococcus aureus* (*S. aureus*) and *E. coli*, which was comparable to the activity of HNP2 and HNP4, respectively (122). HD6 was shown to self-assemble and acquire a nanonet formation, capable of entrapping bacteria (123), and of disrupting the cell envelope of bacteria in a reducing environment (19). Other intestinal HDPs in humans include the β-defensins HBD1, HBD2, and HBD3. Interestingly, HBD1 seems to be constitutively expressed whereas HBD2 and HBD3 can be induced by microbial products (116, 124). HBD1 and HBD3 were found in human rectal-mucus extracts and their activity was not affected by binding to mucus (107). HBD3 possesses a broad spectrum of activity against facultative anaerobic commensal and pathogenic bacteria (19). Of note, HBD1 can exist as an oxidized, disulfide-bridged form and a reduced, linear peptide, which differ in their antimicrobial activity spectrum: reduced HBD1 exhibits a higher antimicrobial effect against the opportunistic pathogen *Candida albicans* (*C. albicans*) and different commensal *Bifidobacterium* and *Lactobacillus* strains when compared to its oxidized form (18). Recently, it was shown that even an HBD1-derived octapeptide fragment that was generated through digestion by gastrointestinal proteases caused cell wall and membrane defects as well as the disintegration of cytosolic structures of *E. coli* and *C. albicans* (125).

Further HDP classes include Reg proteins, angiogenins, and Lcn2. Reg3γ in mice (or the human homolog Reg3A,

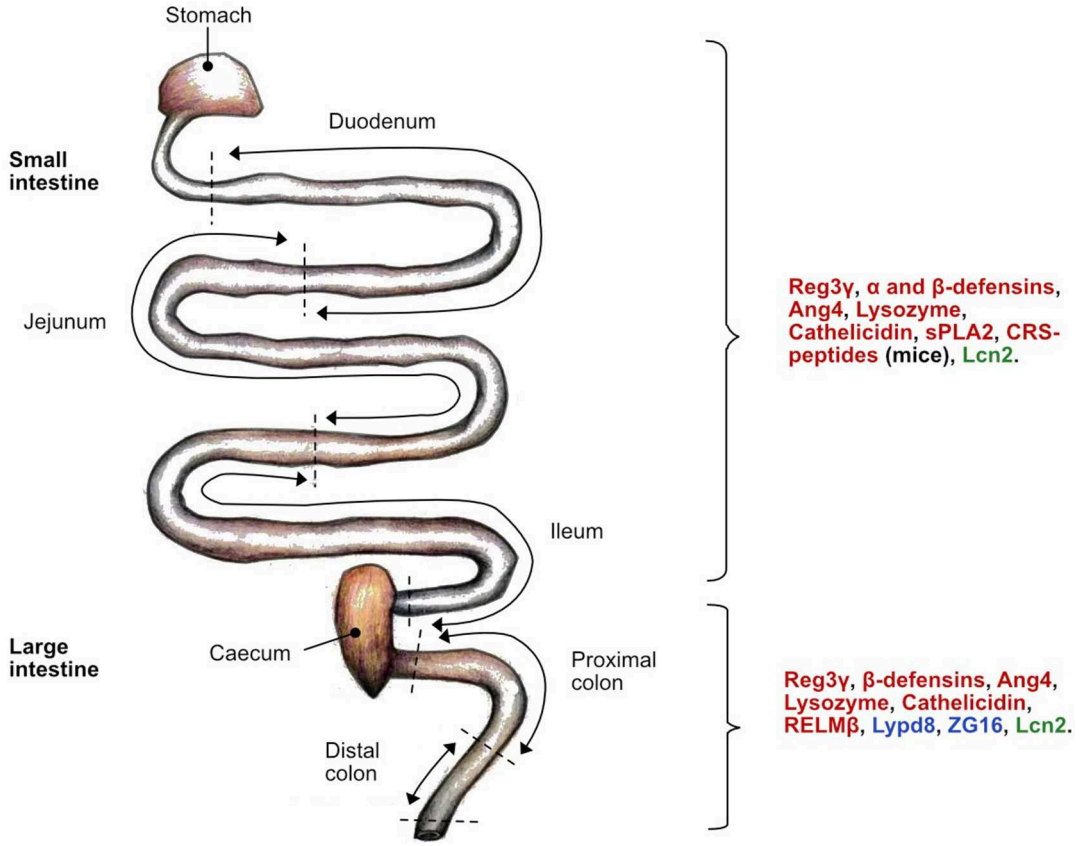

FIGURE 3 | Location of host defense peptide (HDP) expression along the mice intestine under normal physiological conditions. The antimicrobial activity is specific to each HDP class: bactericidal (red), bacteriostatic (green) and aggregation (blue). α-defensins can also be referred to as Defas in mice. C-type lectin regenerating islet-derived protein 3 gamma (Reg3γ); Angiogenin 4 (Ang4); secretory phospholipase A2 group IIA (sPLA2); cryptdin-related sequence peptides (CRS-peptide)s; Lipocalin-2 (Lcn2); Ly6/PLAUR domain containing 8 protein (Lypd8); zymogen granule protein 16 (ZG16); resistin-like molecule beta (RELMβ).

previously known as human HIP/PAP), present antimicrobial activity against Gram-positive bacteria via formation of an hexameric pore structure in the bacterial membranes (126). Interestingly, angiogenins have been attributed to different functions, including tumorigenesis, cell growth, and apoptosis, and Ang4 was shown to be bactericidal against Gram-positive *E. faecalis* and *L. monocytogenes* through a yet unknown mechanism of action (127, 128). Lcn2 is mainly secreted by neutrophils and prevents bacterial growth by sequestering iron-scavenging siderophores (129). Additionally, in the absence of Lcn2, mice were more susceptible to bacterial colonization, not only owing to the reduction of the bactericidal effect but also by altering the migration of neutrophils and reducing the expression of cytokines by macrophages (130).

ZG16, Lypd8, and RELMβ have been described as peptides that maintain the spatial segregation between the gut microbiota and the hosts' epithelial cell surface in the colonic mucus layer (108, 110, 131). ZG16 is a highly abundant protein in the colon that intertwines with the mucin polymeric network and contributes to space separation by binding to Gram-positive bacteria (131). Lypd8, a highly glycosylated glycosylphosphatidylinositol-anchored protein, binds flagellated bacteria and was recently shown to inhibit the attachment of *Citrobacter rodentium* to epithelial cells through competitive binding of bacterial intimin, thereby interrupting its interaction with the translocated intimin receptor (Tir) in the host (108, 132). Lypd8 thus specializes in inhibiting the colonization of intestinal pathogens. RELMβ controls the levels of Gram-negative Proteobacteria in the inner colonic mucus layer of mice by forming pores into the bacterial cell membrane (110). Of note, the human homolog (hRETN) was shown to be specifically bactericidal against Gram-negative pathogens but lacked activity against commensal bacteria such as *E. faecalis* and *B. thetaiotaomicron* (110). Additionally, RELMβ is inducible by the microbiota and was shown to be elevated during intestinal inflammation and potentially also aging, as observed in 104 weeks old mice (133, 134).

Altogether, there is reason to believe that HDPs in the small intestine are mainly aiming to "kill" bacteria, whereas those in the large intestine have a more spatial segregation function and are not necessarily bactericidal.

Transcriptional Regulation of HDPs

Key to the antimicrobial effect of HDPs are the regulatory cues behind their function, which can occur at the level of granule-release, expression, and activation. For example,

defensin exocytosis from Paneth cell granules is mediated by the ATG16L1 and ATG5 proteins of the autophagy pathway (135), and can also occur in response to bacterial stimuli (21), which will be discussed later. In the crypts of the small intestine, stem cells differentiate into Paneth cells via the Wnt/β-catenin signaling pathway under the activity of the transcription factor TCF-4 (20, 136). Studies performed in mice show that the Paneth cell maturation process is accompanied by the appearance of α-defensins and CRS-peptides during the weaning period (23, 137). Moreover, TCF-4 can control the expression of α-defensins and CRS-peptides in mice and humans—as the promotor region of these genes has binding sites for TCF-4, suggesting their baseline release levels occur in parallel to Paneth cell differentiation (20, 119). Recently, the pro-inflammatory cytokine interferon gamma (IFN-γ) was also identified as a potent inducer of Paneth cell degranulation and goblet cell mucus production in a model of murine primary organoid culture (138).

Although several studies report that Paneth cells and intestinal epithelial cells can directly respond to bacterial stimuli, immune cell-derived signals are the main effectors triggering HDP expression (138). Upon recognition of microbe-associated molecular patterns (MAMPs) by Toll-like receptors (TLRs) present in innate lymphoid cells (ILCs) and dendritic cells, downstream signaling orchestrates an inflammatory response and integrates different signals oriented to control the expression of different HDPs. Activation of the NLRP6 inflammasome signaling by the microbiota—as shown in GF vs. SPF mice—directed the release of IL-18, which induced the HDPs intelectin 1a (ITLN1), RELMβ, and Ang4, implicating IL-18 as a regulator of the antimicrobial program of the colonic mucosa (27). Likewise, IL-25, a Th2 cytokine mainly known for its anti-helminth function, was shown to also induce the expression of *Ang4* in an IL-23 dependent manner (139). Furthermore, IL-22 has been demonstrated to induce *Reg3β*, *Reg3γ*, β-defensins, *Lcn2*, and *Ang4* (12, 12, 140–144), as well as mucin production (145). The cellular sources of IL-22 include type 3 innate lymphoid (ILC3), natural killer (NK), Th17 and Th22 cells as well as dendritic cells (146–148).

Whereas, Reg3γ is induced by the microbiota-dependent inflammatory signals of the TLR-MyD88-IL-22 axis (24, 120, 143), evidence for a role of MyD88 in α-defensin regulation is conflicting. Castillo *et al.* reported that MyD88 is not involved in defensin regulation, as the total defensin copy number in the small intestine of *Myd88*$^{-/-}$ mice, was not different from the *Myd88*+/+ control group (120). Conversely, Menendez *et al.* showed a drop in ileal defensin (*Defa*) expression in *MyD88*$^{-/-}$ mice, using a relative expression approach (149). Likewise, Liang *et al.* showed that *MyD88*$^{-/-}$ mice had reduced gene expression of *IL-22*, and of *Mmp7*, the coding gene for the matrix metalloproteinase-7 (Mmp7) which is a key enzyme that is required to activate mouse α-defensins (discussed below), and diminished mature α-defensins under normal conditions (150). These last observations suggest that MyD88 regulates *Mmp7* expression, and therefore the post-transcriptional activation of α-defensins (150). Thus, MyD88 may not regulate transcription of *Defa* genes directly, but affect α-defensins rather indirectly through regulation of Mmp7. However, this explanation does not provide reasoning to the transcriptional downregulation of *Defa* observed by Menendez *et al.* (149). Finally, most recently the TIR domain-containing adaptor molecule 1 (TRIF or TICAM1) was described as a key homeostatic regulator of epithelial barrier function by controlling the expression and protein levels of Mmp7, Reg3γ, and Defa1 (151). In this study, *MyD88*$^{-/-}$ mice again had no influence on *Defa1* expression and showed only a slight reduction in *Mmp7* expression (151).

Post-transcriptional Regulation of HDPs

On the activation level, environmental conditions and the presence of different proteases can determine the activity of defensins. HDPs with membrane-lysing capacities can be toxic to eukaryotic cells. Because of this, defensins are secreted as pro-peptides that are processed and activated in the gut lumen by Mmp7, also known as matrilysin, in mice and by trypsin in humans (152, 153). Cutting off the pro-region will activate their antimicrobial function, and once in its mature form, some peptides can resist proteolysis, as was shown for α-defensin 4 (111). In the gut, HDPs can be further processed by other proteases such as gelatinase (*GelE*) or serine protease E (*SprE*), secreted by *E. faecalis,* or chymotrypsin and neutrophil elastase, produced by the host (111).

Another form of regulation of HDP activity relies on the local microenvironment in the intestine. Defensins form disulfide bridges that can modulate the antimicrobial effect, depending on whether they are in their reduced (linear/open) or oxidized (closed) forms, as exemplified for α-defensin 4 (15). Redox-potential and pH differ between the bottom of the intestinal crypts (where most HDPs are secreted) and the gut lumen and can thus shape the tertiary structure of α-defensins. Furthermore, the enzymatic thioredoxin system in the gut is a host-dependent mechanism to control redox reactions, and this system mediated the reduction of HBD1, thereby revealing its potent antimicrobial activity against common anaerobic gut bacteria (18). Of note, antimicrobial activity of α- and β-defensins was differently modulated when conditions were adjusted for pH and redox-potential, and was independent of bacterial genus, cell wall composition, or defensin class (19). In this manner, a reducing environment exposed a bactericidal effect of HD6, while the nanonet conformation was maintained under these conditions (19). Importantly, in the gut the reduced or oxidized conformations will be subject to protease activity to either activate or deactivate them (125, 154). While oxidized HBD1 and HD5 were resistant to protein digestion, $HBD1_{red}$ was readily digested *in vitro* and generated a C-terminal octapeptide (125). This octapeptide gradually lost its activity in acidic conditions, further highlighting the influence of environmental regulation on HDP activity (125). Similarly, $HD5_{red}$ was efficiently degraded by host proteases and produced ten new fragments, some of which exhibited an antimicrobial effect against commensal bacteria, and thus greatly increased the known spectrum of activity of this peptide (154). In contrast, $HD6_{red}$ was unaffected by host proteases, mainly due to its characteristic nanonet formation (154).

In summary, the antimicrobial activity of intestinal HDPs is controlled by a complex interplay between transcriptional

and post-transcriptional signals that are central to maintaining homeostasis in the gut (12). Some of these regulatory factors are under the influence of the host, but other factors tightly depend on the presence and the composition of the gut microbiota. While HDPs have historically been considered to protect the host against pathogens, many studies preferentially included pathogenic bacteria and fungi in their antibiotic activity tests. It is thus possible that the activity against anaerobic, commensal bacteria is underestimated, due to a study bias and due to the selection of simple testing conditions that did not resemble the conditions in the gut. In fact, only by modulating some of the environmental parameters in the activity tests, the antimicrobial effect of several HDPs against commensal bacteria could be revealed (15, 18, 19, 154). It is thus required to keep these factors in mind in order to increase our understanding of the function of these peptides in shaping microbial communities in the gut.

IMPACT OF HDPs ON INTESTINAL MICROBIOTA

Intestinal HDPs protect the host against microbial intruders in the gut and have the potential to shape the intestinal microbiota (155) (**Table 1**). Specifically, the HDP family of defensins has been shown to exert noticeable effects on gut microbiota composition. Transgenic mice expressing HD5 on top of their indigenous HDP repertoire were shown to have an expansion of the Bacteroidetes and a reduction of the Firmicutes phyla in the small intestine (9). Interestingly, a fragment produced after proteolysis of HD5 ($HD5_{1-9}$), shifted the fecal microbiota composition and influenced the microbial diversity in the small intestine, specifically increasing *Akkermansia* and a member of the Ruminococcaceae family and decreasing *Intestimonas* from the Clostridiaceae family (154). Furthermore, administration of HD5 in a murine model of diet-induced obesity reversed dyslipidemia and improved the overall glucose regulation (17). The latter was partly attributed to HD5-induced changes in the fecal microbiota, namely an increase in *Bifidobacterium* and *Alloprevotella* abundance, that correlated with improved metabolic parameters (17). However, the effect on microbiota composition in the small intestine was less marked. Thus, both the transgenic expression and administration of defensins can shape the microbial communities in the mouse intestine.

The impact of mice defensins on gut microbial communities was also studied in mice lacking Mmp7 (152). *Mmp7*$^{-/-}$ mice have an increase in the levels of Firmicutes (mainly *Clostridia*) and a significantly lower proportion and abundance of *Bacteroides* in the small intestinal microbiota, which is contrary to the result observed in mice expressing HD5 (9). As both the HD5 transgenic and the *Mmp7*$^{-/-}$ mice models showed no effect on the total bacterial numbers, the defensin function seems likely restricted to shaping the composition of the microbiota rather than controlling its abundance in the small intestinal lumen. Interestingly, and in contrast to the small intestine, the caecal and colonic microbiota of *Mmp7*$^{-/-}$ mice were not different from their wild-type controls (111). These findings were attributed to the existence of other host and microbial proteases present in the large intestine that could convert inactive defensin precursors into active peptides, thereby explaining the lack of an effect on the microbiota composition at this intestinal site (111).

TABLE 1 | Influence of host defense peptides (HDP) on microbiota composition in *in vivo* studies.

Host defense peptide treatment	Effect on SI microbiota (relative abundance)	Effect on fecal/colonic microbiota (relative abundance)	References
Transgenic expression of HD5	↑ Bacteroidetes ↓ Firmicutes	NA	(9)
Mmp7$^{-/-}$	↑ Firmicutes ↓ Bacteroidetes	NA	(9)
	NA	No difference when compared to wild-type	(111)
$HD5_{1-9}$	↑ *Akkermansia* and members of the Ruminococcaceae family ↓ *Intestimonas* from the Clostridiaceae family	↓*Bacteroides* and *Lactobacillus* genera ↑ *Akkermansia spp.* and *Parasutterella*	(154)
Administration of HD5	↑*Bifidobacterium*	↑*Bifidobacterium* and *Alloprevotella* ↓ *Parabacteroides*	(17)

NA, not available, was not investigated in the study. SI, small intestine.

However, as discussed above, the overall impact of HDPs on gut microbiota community structure is expected to be reflected by the potential antimicrobial effect against gut commensals. And indeed, administration of HD5 increased *Bifidobacterium* relative abundance (17), while transgenic expression of HD5 on top of the own mouse antimicrobial arsenal led to increased relative abundance of Bacteroidetes (9). This suggests that defensins can promote the growth of selected microbial taxa by specifically eliminating other microbes. While some HDPs, such as hRETN, human LL37 or mouse cathelicidin related antimicrobial peptide (CRAMP) show no antimicrobial effect against commensal microbes (16, 110), defensins have anti-commensal activity that was dependent on an environment that resembled the conditions in the gut (15, 18, 19). Regardless of this, most *in vitro* studies have investigated the antimicrobial effect against isolated pathogens and not against complex communities of commensals. Accordingly, these findings have contributed to build up the notion that HDPs do not affect commensals. Therefore, more work is required to determine the effect of several HDP classes with adjusted microenvironmental conditions and to truly understand the impact of HDPs on the gut microbiota community.

Altered HDP Expression in Microbiota-Associated Diseases

An indication that HDP modulation of gut microbiota composition might also be true in humans is based on the fact that gut-associated inflammatory disorders are commonly

accompanied by dysbiotic communities, and that ileal Crohn's disease, a form of IBD, can be linked to Paneth cell dysfunction (156–158). Accordingly, in two German cohorts patients with ileal Crohn's disease had reduced levels of HD5 and HD6, which was even more pronounced in patients carrying a mutation in the intracellular nucleotide binding oligomerization domain 2 (NOD2) receptor (43, 44). However, in another cohort in Australia, reduced levels of HD5 were associated with inflammation and not with the NOD2 genetic status (45). Yet, despite that Paneth cells are the predominant cell types expressing NOD2 at the intestinal mucosa, it is unclear whether mutations in NOD2 are indeed causing the altered expression of the human defensins in ileal Crohn's disease (159).

In addition to NOD2, other genetic risk factors that may affect Paneth cell function in Crohn's disease include mutations in ATG16L1 (160), which is part of the autophagy pathway and implicated in Paneth cell degranulation, or in the transcription factor X-box binding protein-1 (Xbp1), a key regulator of the endoplasmic reticulum (ER) stress response (161). Mutations in Xbp1 can result in ER stress, defects in Paneth cell granule morphology and reduced lysozyme levels, leading to intestinal inflammation (162).

Besides patients with ileal Crohn's disease, patients with obesity (BMI>35) evidenced defective Paneth cell secretions, which was linked to the activation of the unfolded protein response (UPR) during ER stress (163). Even though the morphology and number of Paneth cells were normal, these individuals had reduced HD5 and lysozyme protein levels, despite having an increased gene expression of these HDPs, suggesting the presence of transcriptional arrest (163). Similarly in mice, an obesogenic diet was associated with Paneth- and goblet cell abnormalities, a worsened colonic inflammation and expansion of *Atopobium spp.* and Proteobacteria in the fecal microbiota (164). Taken together, a defective antimicrobial defense that cannot sufficiently control microbial communities in the gut may likely be a contributing factor in the pathogenesis of ileal Crohn's disease and obesity. However, it is still not fully clear if defects in defensin expression or secretion precede or follow the onset of disease.

DIRECT EFFECT OF DIET ON HDP EXPRESSION

We have described the implications of different dietary compounds on the microbial communities in the gut. To add to this function, evidence of a direct effect of diet (i.e., the presence of certain dietary components or the impact of complex diets) and nutritional status (i.e., fasting or starvation condition) on HDP function, has been accumulating over the past decade. Takakuwa *et al.* tested 20 amino acids in mice-derived enteroids to investigate their defensin-inducing capacity *in vitro*. The release of Defa1 was strongly induced by leucine, and to a lesser extent, by tryptophan (165). Their observations thus suggest a direct role of distinct amino acids in the induction of defensin expression by intestinal cells. Another study explored how diets supplemented with kidney bean flour, which is rich in fiber and phenolic compounds, influenced colonic barrier function in a mouse model of colitis. When compared with a basal control diet, the dietary flour intervention increased SCFAs (acetate, butyrate, and propionate) and up-regulated MUC1 and RELMβ in unchallenged mice (166). This effect was more pronounced after the induction of colitis and at the same time, the bean flour treatment decreased the expression of pro-inflammatory cytokines and improved colitis symptoms (166). Moreover, Bentley-Hewitt *et al.* demonstrated that *in vitro* fermented kiwifruit products significantly increased the production of HBD1 and HBD2 by intestinal epithelial cells. This effect, which was mainly mediated by the production of SCFAs and was not observed after treatment with the digested kiwifruit lacking fermentation products, suggests that the HDP modulating effect was exerted by fermentation products and not directly by the digested kiwifruit (167).

Food Availability

In addition to individual components of the diet (i.e., food quality), food availability (i.e., food quantity) has also been linked to HDP function. Mice deprived of food for 48 h had reduced expression of the Paneth cell antimicrobial peptides lysozyme, defensins, and *Reg3γ*, which was confirmed on the protein level for the precursor of Reg3γ and lysozyme (168). Although the numbers of Paneth cells were not changed, the physiology of their granules was altered. Furthermore, a 2-fold increase in bacterial translocation to the mesenteric lymph nodes was observed in the starved mice (168). Interestingly, the same authors showed opposite results in a second study, in which they evaluated the impact of total parenteral nutrition on Paneth cell function and regulation of intestinal homeostasis in rats. Here, the absence of enteric food caused an up-regulation of lysozyme and rat α-defensins 5 and 8 (169). Remarkably, the authors noted an inverse correlation between lysozyme expression and Firmicutes abundance in the small intestine, implying a link between HDP function and the small intestinal microbiota (169). Regarding the contrasting results, the authors speculated that the main difference between both studies was that the rats fed with parenteral nutrition could still respond to changes in the microbiota, as the Paneth cells were still functional, as opposed to the altered granule physiology observed in starved mice (169). Indeed, Liang *et al.* expanded these observations in a longer mouse starvation study of 72 h and identified that the starved group showed a drop in *Reg3β*, *Reg3γ* and *Mmp7* gene expression after the first 48 h, and later an increased expression of *Mmp7* at 72 h (150). The authors investigated the V-shaped pattern of expression of *Mmp7* and were able to demonstrate that an initial drop in the microbial population caused the decreased expression 48 h post-starvation through Myd88-IL-22 signaling, and that the recovered expression 72 h post-starvation was regulated by the transcriptional repressor Hes1, controlled by the mTOR nutrient-sensing transcriptional regulator in response to the nutrient fluctuations (150). They additionally demonstrate that mTOR controlled Hes1 translation by sensing amino acids and glucose (150). Recently, intestinal neurons secreting vasoactive intestinal peptide (VIP) in response to food consumption were also implicated in the ILC3-mediated

regulation of *Reg3γ* expression (170). Although the molecular food-sensing mechanism causing a VIPergic response remains unknown, this study evidences a link between neural-immune regulation of HDP expression and food intake.

Taken together, these studies suggest that microbiota as well as nutritional status play a role in HDP-modulation. Accordingly, these observations highlight the importance of the presence of food for the correct functioning of the antimicrobial program in the small intestine, as starvation has been associated with increased risk of bacterial translocation in patients receiving parenteral nutrition (171).

The Effect of High Fat Diet (HFD) on HDP Expression

HFD is a general term for a diet with increased fat content. However, the amount and composition of fat content, as well as other dietary compounds can vary between studies, as can the length of the intervention. Such variations have the potential to change HDP expression pattern in the small intestine to varying degrees. **Table 2** summarizes various mice and rat studies that investigated how different HFD treatments affect the expression of selected HDPs. Diets with 20–60% fat content substantially modify HDP expression patterns in the small intestine, whereas short term treatments (between 2 and 20 weeks) lowered HDP expression, which was in some cases also confirmed on protein levels. At the same time, longer treatments (>20 weeks) seemed to shift this pattern toward higher HDP expression levels.

HFDs, which also include the high fat/high carbohydrate WSDs, contain components that can directly (e.g., cholesterol or saturated fatty acids) or indirectly (e.g., TMAO) trigger inflammation (37). Specifically, saturated fatty acids present in WSDs, such as palmitic or stearic acid, induce ER stress, an insult that is sensed by macrophages (180). Also, a recent study demonstrated that HFD feeding caused a 50% increase in Lgr5+ intestinal stem cell numbers but at the same time a 23% reduction in Paneth cell numbers (181). Thus, we hypothesize that the initial reduction in HDP expression upon HFD feeding can be caused by the reduced numbers of Paneth cells (**Table 2**) (176, 179), and in addition by further detrimental effects, such as ER stress, which was previously linked to obesity and HDP malfunction (163). After prolonged treatment—for example, 5-month HFD intervention—a chronic inflammatory response is initiated in the gut that is reflected by the increased HDP expression.

Different pathways were suggested to control the dietary regulation of HDP expression. Tomas *et al.* focused on the pathological effects of a HFD in the small intestine, a region with key nutritional functions, and which seemed to have been understudied in the context of obesity, diabetes and metabolic syndrome (**Table 2**) (172). Among different detrimental effects, the authors identified that HFD treatment led to a downregulation of HDPs and the cystic fibrosis transmembrane conductance regulator (Cftr), as well as the peroxisome proliferator-activated receptor (Ppar)-γ, a nuclear receptor involved in lipid sensing and mucosal defense regulation (172, 182, 183). In addition, mice fed the HFD had increased numbers of bacteria in the intervillous zone in the small intestine—an otherwise sterile area. This aberrant colonization of bacteria in the villi was associated with the defective antimicrobial response in the ileum, and indeed, the administration of rosiglitazone, a Ppar-γ agonist, restored HDP expression levels; thus revealing the role of this receptor in controlling the antimicrobial function in the intestine (172).

While research on high-fat diets naturally focuses on the role of fat, these dietary compounds can also interact with other dietary factors. Su *et al.* demonstrated that both dietary fat and vitamin D can modulate *Defa* and *Mmp7* expression in mice. Vitamin D is a dietary component that supports mucosal barrier function when supplied in adequate amounts (184, 185). HFD treatment reduced the expression of *Defa5*, *Defa1*, *Defb1,* and the protein levels of Defa1 and Mmp7, and the reduction in HDP expression was even stronger in mice fed a diet low in vitamin D (**Table 2**) (173). Remarkably, the combination of a high-fat and vitamin D-deficient diet (called "the double hit model") caused hepatic steatosis and insulin resistance. This effect was attributed to a state of dysbiosis in the small intestine, as a consequence of the low antimicrobial response. Accordingly, this hypothesis was confirmed by the administration of synthetic HD5 in the double hit model, which corrected the expression of HDPs, resolved the systemic inflammation and improved the observed insulin resistance (173).

Importantly, dietary supplements in the context of HFDs revealed additional factors with the potential of modulating mucosal barrier function. These factors included the polyphenol rutin and the prebiotics inulin and oligofructose (**Table 2**) (175, 178). While a 20 week HFD treatment elevated the expression of HDPs, both the administration of rutin or rutin and inulin reduced their transcription back to base expression levels (178). In addition, the administration of oligofructose significantly increased the expression level of *Reg3γ* (>50-fold), which was otherwise reduced by a HFD (175). These examples implicate polyphenols and prebiotics as keepers of intestinal homeostasis by correcting the aberrant expression of HDPs. As prebiotic interventions will promote the growth of beneficial bacteria, their HDP-modulatory effect might operate via modulation of microbial communities, which will be discussed in detail below.

Finally, a more indirect effect of HFD on HDP expression has been described recently. Upon consumption of a (high-fat) meal the host secretes bile acids to emulsify the fatty acids and facilitate their absorption. To test the effect of bile acids in mice, dietary supplementation with the primary bile-acid chenodeoxycholic acid (CDCA) was shown to induce the transcript levels of *Defa20*, *Reg3β*, and *Reg3γ*, and stimulate the production of Reg3β, and Reg3γ in different cell types along the villi in the ileum (186). While the effect on the microbiota was minimal and only increased relative abundance of Bacteroidetes, the increase in antimicrobial defenses protected the host from enteric *Salmonella* and *Citrobacter* infection, two microbes that are otherwise bile-resistant (186). Although the mechanism behind the induction could not be identified, this implicates bile acids as indirect effector molecules from the host that can regulate HDP expression and microbiota composition.

TABLE 2 | High fat diet (HFD)-modulation of host defense peptide expression in different studies.

Duration of HFD treatment	Analyzed variables (% of fat content)	Changes in HDP expression compared with control group after HFD treatment	Intestinal area	Observations	References
4 weeks	HFD (40%)	↓*Reg3γ* in all regions (except ileum), and ↓*Mmp7*, *Ang4*, *Lyz1*, *Defa3*, *Defa5*, *Defa20*, *Pla2g4a* in the ileum	Duodenum, jejunum and ileum	Increased bacterial colonization of the intervillous space and retention of Muc2 in the ileum after HFD treatment The HDP defects were corrected after administration of rosiglitazone, a Ppar-γ agonist, and after switching back to a standard diet	(172)
18–20 weeks	HFD (60%) and vitamin D deficiency	↓*Defa5*, *Defa1* and *Defb1*, and ↓*Mmp7* protein level	Ileum	Both the HFD and vitamin D deficiency induced insulin resistance and fatty liver	(173)
8 weeks + 19 days gestational period	Undernutrition (UN) HFD (60%) in the mother. Measured effects on the fetus's intestinal barrier function	Mothers UN ↓*Lyz2* and *Reg3γ* HFD ↓*Reg3γ*, *Muc2* and ↑ *Lyz2* (also shown in a protein level) Fetus UN ↓*Muc2* HFD ↑*Lyz1* and *Reg3γ*	Mothers jejunum Fetal gut	Maternal UN was associated with reduced gut barrier function and integrity, fetal gut development and mucus production Maternal HFD was associated with increased barrier function and lysozyme production and with improved fetal gut barrier function and integrity	(174)
8 weeks	HFD (60%) Prebiotic treatment	↓*Reg3γ*, *Pla2g2, Lyz1* and *Ang4*	Jejunum	When compared to HFD, HFD-prebiotic treated mice had increased *Reg3γ* expression and epithelial cell turnover, decreased Firmicutes/Bacteroides ratio (evidencing an opposite effect in taxonomic shifts observed in gut microbiota), heightened SCFA production and reduced levels of plasma leptin	(175)
22–26 weeks	HFD (60%) Villin-Cre (VC) recombinase-mediated intestinal epithelial cell specific insulin receptor deletion (VC-IR knockout)	↓*Muc2* and ↑*Lyz1* and *Defa1a*. These last 2 HDP were not increased in VC-IR knockout	Jejunum	Deletion of the intestinal epithelial insulin receptor diminished the HFD-induced elevations in cholesterol and expression of Paneth cell peptides	(176)
8, 12, and 16 weeks	HFD (60%) different time points	↓*Lyz1*, *Reg3γ*, and *Ang4* at either 8, 12 and 16 weeks of HFD feeding	Small intestine (Ileum)	A HFD may stimulate intestinal inflammation via altering gut microbiota, which can occur prior to the increase of circulating inflammatory cytokines	(177)
20 weeks	HFD (60%) Supplementation with the polyphenol rutin or with rutin and polysaccharide inulin	↑*Defa5*, *Lyz1*, *Ang4*. No effect observed for Reg3γ. Lower protein levels of lysozyme	Small intestine (Ileum)	Rutin supplementation alleviated the increase of plasma triglycerides or leptin, attenuated the inflammatory response and improved ER stress caused by HFD. There was a positive correlation between increased expression of HDP with plasma LPS and inflammatory mediators, suggesting a link between Paneth cell HDPs and obesity-associated inflammation	(178)
2 weeks + 48 h induction with sodium taurocholate	HFD (20% saturated animal fat) and induction of acute necrotizing pancreatitis	↓*Lyz1* and *Defa5*	Ileum	Intestinal dysbiosis may contribute to the pathogenesis of intestinal barrier dysfunction in the context of acute necrotizing pancreatitis and hypertriglyceridemia	(179)*
14 weeks	HFD (60%) and dextran sodium sulfate (DSS) treatment	↓*Defa1*, *Defa4*, *CRS1C*, Lyz1 protein level	Ileum	The HFD treatment increased the susceptibility to DSS-induced colitis which was transferable through fecal transplantation and abolished after antibiotic treatment	(164)

*Unless specified, all studies were performed in mice. *Study done in rats. Lyz1 (Paneth cell) and Lyz2 (myeloid specific).*

Altogether, food availability and specific dietary factors have the potential to significantly influence the intestinal antimicrobial defense system, an effect that is only starting to be understood. While most studies observed HDPs at the expression level, protein levels are expected to reveal more information about this function. Also, most of these studies were performed in mice and the effect of these factors in human trials remains to be demonstrated.

EFFECT OF MICROBES AND THEIR METABOLITES ON HDP EXPRESSION

Dietary factors elicit different responses that control microbial communities in the gut. Nevertheless, both the presence or absence of the microbiota as well as specific individual microbes have been linked to modulation of HDP expression. It is well-known that germ-free (GF) mice, which are completely devoid of a microbiota, undergo an incomplete development of the immune system (187, 188). As such, early studies of GF mice intestines revealed a decrease in expression of *Reg3γ* and *CRS4C*, as determined by total transcript copy number, when compared to conventional mice (23). Likewise, conventional mice had higher expression of *Reg3γ*, *Reg3β*, *RELMβ*, and *CRS4C4* when compared to GF mice or to mice in which the microbiota had been depleted by an aggressive antibiotic regime (14). Similarly, observations in antibiotic depleted mice further corroborated the decreased expression of *Mmp7* and *Reg3β* in the ileum and of *Ang4*, *Pla2g2a*, *RELMβ*, *Reg3γ*, and *Reg3β* in the colon (150, 189). Altogether, these observations seem to be a result of immature immune development in the absence of HDP-stimulating microbes.

Microbes That Stimulate HDP Expression

Different studies have linked the enrichment of a particular microbe or the administration of a probiotic bacterium to increased expression of HDPs. For example, *B. thetaiotaomicron* has been shown to stimulate the production of Reg3γ and Ang4 in the small intestine and CRAMP in the colon (24, 127, 190). Moreover, *B. thetaiotaomicron* mediated the colonization resistance against *C. albicans* via induction of HIF-1α (190), a transcription factor involved in the activation of innate immune effectors that also regulates CRAMP in the murine gut and of HBD1 in humans (191, 192). Interestingly, this microbe has been described to be resistant to HDPs and could therefore survive the antimicrobial stimulation (16, 24). Similar to *B. thetaiotaomicron,* supplementation with *A. muciniphila* increased the expression of *Reg3γ* in the mouse colon when mice were fed a control diet, but not during treatment with a HFD, in which ileal *Pla2g2a*, *Defa,* and *Lyz1* were not stimulated by *A. muciniphila* (193). Also, monocolonization of mice with the probiotic *Bifidobacterium breve* NCC2950 induced *in vivo* and *in vitro* expression of *Reg3γ*, an effect that was mediated by the MyD88-Ticam axis of the TLR signaling pathway (194). Furthermore, Cazorla *et al.* isolated intestinal fluid from mice treated with the probiotic strains *L. casei* CRL431 and *L. paracasei* CNCM-I and showed that the extracted fluid had enhanced antimicrobial activity against *S. typhimurium* and *S. aureus* (195). Although this study did not measure HDP expression, the probiotic treatment increased the Paneth cell numbers in the crypts, hence suggesting an overall increase in the antimicrobial peptide function in the gut (195). In the same line, the probiotic strain *E. coli* Nissle 1917 evidenced a strong induction of HBD2 in *in vitro* studies (196), an effect that could be confirmed *in vivo* in a small human study, in which administration of an *E. coli* probiotic preparation (Symbioflor® 2) for 2–3 weeks increased the levels of this peptide in the feces (25). While the *in vitro* HBD2-inducing effect was caused by flagellin of *E. coli* Nissle 1917 (197), the *E.coli* strains in the probiotic preparation did not produce flagellin, thus suggesting that multiple mechanisms can mediate this effect and that they differ between individual strains (25).

Microbial-Derived Components and Molecules Implicated in HDP Control

Specific microbial-derived molecules and metabolites have been associated with regulation of HDP function by stimulating their expression or by promoting their release. Early studies demonstrated that Paneth cells can respond directly to bacterial stimuli, either live or dead bacteria, lipopolysaccharide (LPS) or membrane components (21). This observation was performed in isolated crypt cells and shed light on defensin secretion capabilities. However, since a mucus layer covers the small intestinal epithelium, the probability of microbial derivates reaching the bottom of the crypts is expected to be low. Yet, the described Paneth cell response is likely a back-up response that could take place in the presence of severe mucosal damage or during abnormal microbial growth at this site. Still, isolated crypts may contain exposed basolateral receptors and are perhaps more responsive to bacterial stimuli. Accordingly, using mouse-derived small intestinal organoids, Farin *et al.* showed that Paneth cell degranulation did not occur after stimulation with bacterial agents, but rather after direct induction with IFN-γ or a supernatant derived from stimulated iNKT cull culture (138). Similarly, the SCFA butyrate was shown to directly enhance the production of Defa1 in isolated crypts from the small intestine (165), but it is unclear whether Paneth cells could be stimulated by this fermentation product in the small intestine *in vivo*, where the concentration of butyrate is relatively low (165, 198). Furthermore, by using a human-derived reporter cancer cell line, Sugi *et al.* investigated the transcription of *Defa5* after challenge with the bacterial ligands LPS, the synthetic lipopeptide P3CSK4 or with the bacterial metabolites acetate, lactate, butyrate, and propionate. Among these molecules, lactate strongly suppressed the transcription of *Defa5* while propionate and butyrate were suppressive only at a high concentration (9 mM) (198). However, this intestinal cell line represents absorptive epithelial cells, and their defensin expression capacities are lower when compared to Paneth cells. Nevertheless, lactate was found in high concentrations in the small intestine, suggesting it could suppress the transcription of *Defa5 in vivo* and thereby prevent the release of pyrogenic molecules and the probable aberrant activation of inflammation (198).

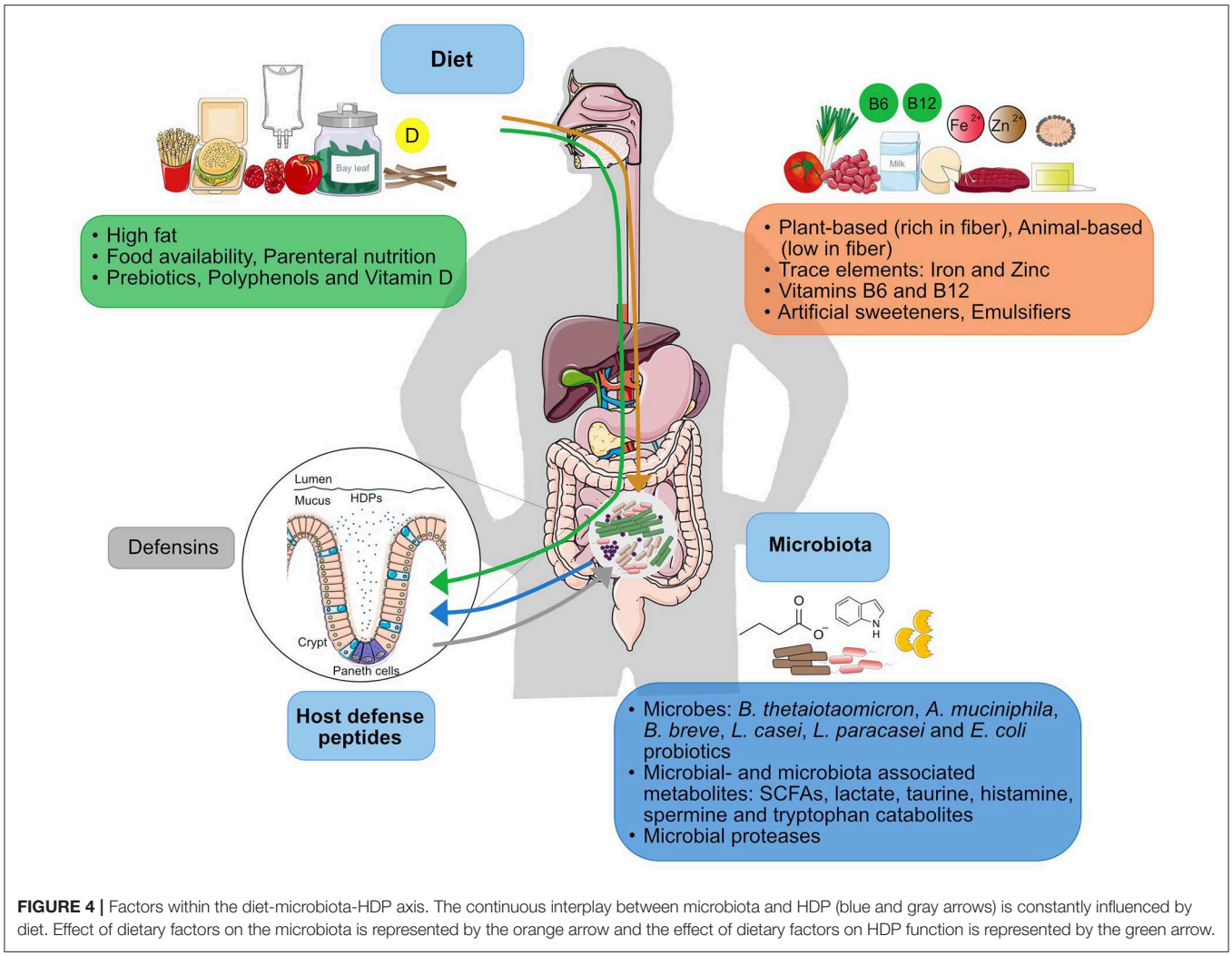

FIGURE 4 | Factors within the diet-microbiota-HDP axis. The continuous interplay between microbiota and HDP (blue and gray arrows) is constantly influenced by diet. Effect of dietary factors on the microbiota is represented by the orange arrow and the effect of dietary factors on HDP function is represented by the green arrow.

In addition to SCFA-mediated HDP regulation, microbial-derived tryptophan catabolites (e.g., indole or indole-3-aldehyde) can bind the aryl hydrocarbon receptor (AhR) and activate IL-22 secretion (199). The AhR is a transcriptional factor expressed by several immune cell types, including RORgt+ ILC3s, that is crucial for the control of intestinal homeostasis in a ligand dependent manner (200). Binding of microbial AhR ligands induced IL-22 secretion by ILCs and stimulated the expression of the HDPs *Lcn*-2, *S100A8*, and *S100A9*, which was shown to be protective against *C. albicans* infection (26).

As described in section 3, Levy *et al.* demonstrated the IL-18 mediated induction of colonic *ITLN1*, *RELMβ* and angiogenins (27). By using colonic explants and colonic spheroids, the authors identified the microbiota-associated metabolite taurine, a bile acid conjugate, as the most potent activator of IL-18, which stimulated HDP expression via the activation of the NLRP6 inflammasome. Conversely, histamine and spermine (polyamine) featured the strongest suppression of IL-18. Furthermore, when taurine was administered to mice with dextran sodium sulfate (DSS)-induced colitis, this metabolite greatly improved colitis severity and weight loss, stressing its *in vivo* importance in the context of disease (27).

Consequently, the members of the microbiota play an essential role in the innate immune development and regulation of HDP function against pathogen infection. This effect may be mediated by the presence of beneficial microbes or by their associated metabolites. Identifying microbes or metabolites that have high potential to influence HDPs will aid in the selection of potential new probiotic strains. Furthermore, the use of such microbial metabolites could be exploited as a "postbiotic" treatment to control the interactions between host and microbiota (27).

DIET-MICROBIOTA-HOST DEFENSE INTERACTION

Diet composition can define microbial communities in the intestine and the antimicrobial programming in the intestinal epithelium. Given the close interactions between microbes and HDPs, there is undoubtedly a close interrelationship of diet,

HDPs, and the intestinal microbiota (**Figure 4**), in which it is difficult to define the extent to which each component is regulating each other.

The microbiota is a dynamic community and different types of diets change the microbiota composition. As we discussed, plant-based components seem to enrich beneficial microbes with the potential of improving immune function, whereas HFDs tend to enrich pro-inflammatory Proteobacteria communities. In both cases, however, HDP expression is also altered, but it is unclear if diet-dependent alterations of the microbiota composition can at least in part be caused by modulation of HDP expression. Most HFDs studies discussed here report that both the changes in HDP expression (**Table 2**) and microbiota alterations take place at the same time. However, by looking at different time-points, Guo *et al.* found that HFD-mediated alterations of the microbiota preceded changes in the levels of circulating inflammatory cytokines (**Table 2**) (177). Thus, HFD may presumably first change microbiota composition which in turn influences the immune response. In addition, diet composition and microbial metabolism will likely affect environmental conditions such as redox-potential and pH that can further mediate defensin function (18, 19). As a result of microbial fermentation, the pH in the gut becomes more acidic (from 6.5 to 5.5 as determined *in-vitro*), which can directly influence the activity of HDPs and favor the growth of butyrate-producing Firmicutes, such as *Roseburia spp.*, while reducing the proliferation of the acid-sensitive *Bacteroides spp.* (201, 202).

Microbes can regulate HDP function directly through their structural components, through proteolytic activation/deactivation, and via their metabolites. However, defining the causative contribution of complex microbial communities on HDP expression is more challenging. Yet, a dysbiotic microbiota obtained from the caecum of SPF mice with ileitis was shown to transmit the inflammatory phenotype to genetically susceptible GF mice, which also led to a reduced expression of lysozyme, but not *Defa2* (203). The transferred dysbiotic microbiota was characterized by an increase in the relative abundance of *Clostridiales* and decreased abundance of *Porphyromonadacaeae* (order of Bacteroidales), suggesting a pro-inflammatory potential of these taxa (203). Remarkably, it was the caecal microbiota that could induce the defect in the small intestine, which is unexpected, as the small intestine possess its own characteristic microbial community.

When attempting to understand the role of microbes in controlling HDP function, the mucosa-associated microbiota is expected to have an even stronger effect on the host as the luminal microbiota, since it is in closer interaction with the epithelium. Furthermore, in the case of defensin function, the ileal microbiota is expected to have a stronger influence than the colonic community. Indeed, Su *et al.* observed in their HFD experiment a reduced HDP expression (**Table 2**) that was accompanied by changes in microbiota composition in the ileum (173). They observed a pronounced increase in Proteobacteria with members *Campylobacterales* and *Helicobacteraceae* (including the hepatotoxic *Helicobacter hepaticus*), a mild increase in Firmicutes and a reduction in Bacteroidetes. Thus, a closer examination of the missing microbial communities mentioned in these studies could help in identifying microbes with distinct HDP modulating function.

CONCLUDING REMARKS

We are only beginning to understand how certain dietary components and microbes can signal to the epithelium and mediate HDP function. We discussed that the nutrient-sensing signaling mediators Ppar-γ, AhR, VDR, mTOR, and VIP-producing neurons are involved in diet-associated HDP control, while immune mediators in the TLR-Myd88/TRIF signaling pathway as well as the cytokines IFN-γ, IL-22, and IL-18 shape the microbe-dependent HDP regulation. However, more research is required to define the individual contribution of these different mediators to the complex regulation of intestinal HDP expression, and we believe that identifying strategies to fine-tune diet-HDP-microbiota interplay is a promising approach to strengthen the innate defenses in HDP-related disorders. In addition, further studies are required to understand whether HDPs can modulate and kill commensal bacteria in the gut or whether their activity is indeed limited to kill pathogenic microorganisms.

So, does an apple a day also keep the microbes away? Unfortunately, our current knowledge does not allow us to answer this question, but instead prompts us to ask other questions: Do we in fact want the microbes away from the intestinal mucosa, and thus eliminate beneficial microbes that stimulate HDPs? Or can we specifically fine-tune our defenses to target only pathogens and not commensals? Thus, rather than just relying on an apple, a diverse diet with different proportions of macronutrients and micronutrients is likely the food of choice to maintain a stable microbiota community that is separated from the host through a balanced HDP-microbiota homeostasis.

AUTHOR CONTRIBUTIONS

The manuscript was written and edited by FP-B and BS. FP-B generated the figures included in this study using templates from Servier Medical Art by Servier licensed under a Creative Commons Attribution 3.0 Unported License.

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REVIEW
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Cathelicidins Modulate TLR-Activation and Inflammation

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Cathelicidins are short cationic peptides that are part of the innate immune system. At first, these peptides were studied mostly for their direct antimicrobial killing capacity, but nowadays they are more and more appreciated for their immunomodulatory functions. In this review, we will provide a comprehensive overview of the various effects cathelicidins have on the detection of damage- and microbe-associated molecular patterns, with a special focus on their effects on Toll-like receptor (TLR) activation. We review the available literature based on TLR ligand types, which can roughly be divided into lipidic ligands, such as LPS and lipoproteins, and nucleic-acid ligands, such as RNA and DNA. For both ligand types, we describe how direct cathelicidin-ligand interactions influence TLR activation, by for instance altering ligand stability, cellular uptake and receptor interaction. In addition, we will review the more indirect mechanisms by which cathelicidins affect downstream TLR-signaling. To place all this information in a broader context, we discuss how these cathelicidin-mediated effects can have an impact on how the host responds to infectious organisms as well as how these effects play a role in the exacerbation of inflammation in auto-immune diseases. Finally, we discuss how these immunomodulatory activities can be exploited in vaccine development and cancer therapies.

Keywords: macrophages, dendritic cells, antimicrobial peptides, cathelicidins, LL-37, Toll-like receptors, MAMPs, DAMPs

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INTRODUCTION

The discovery of penicillin in 1928 by Alexander Fleming was a major breakthrough in medicine. Ever since, the use of antibiotics saved millions of lives around the world, curing infections that were previously life threatening (1). However, due to the continuous expansion of antibiotic resistance among clinically relevant bacterial species, novel antimicrobials are urgently required to counter infections by these pathogens (2). One promising alternative to conventional antibiotics is the use of host defense peptides (HDPs), which refers to a large family of peptides with varying functions, including direct antimicrobial activity against a wide variety of bacterial, fungal and viral pathogens. Of special interest is the more recent description of the immunomodulatory functions of these peptides, which provides additional opportunities for potential clinical applications (3).

One group of HDPs that has been extensively studied in the context of their immunomodulatory activity is the cathelicidin family. This peptide family can be found in nearly all vertebrate species and has been shown to have a major impact on host responses toward various highly conserved

microbe-associated molecular patterns (MAMPs). MAMPs activate the innate immune system through pattern recognition receptors (PRRs), of which Toll-like receptors (TLRs) are the most well-known receptor family. TLRs can be roughly divided into two subgroups; extracellular TLRs and intracellular TLRs, that recognize microbial membrane components and extracellular proteins or nucleic acids, respectively (4).

In this review, we aim to summarize the current knowledge on the mechanisms by which cathelicidins affect TLR activation and downstream signaling as well as how this impacts immune responses during both infections and sterile inflammation, including auto-immune responses.

CATHELICIDINS

Cathelicidins belong to the family of HDPs with each cathelicidin being encoded by a single gene, consisting of four exons. Cathelicidins are translated as a pre-pro-peptide, consisting of a signal peptide on the N-terminus that directs the peptide to secretory granules, followed by the highly conserved cathelin domain and ending in the active mature peptide at the C-terminus (5). Cathelicidins, such as the human cathelicidin LL-37, are commonly secreted by neutrophils in their biologically inactive pro-peptide form and require cleavage by extracellular enzymes such as elastase or proteinase-3 to release the biologically active C-terminal peptide (6). In the human skin, proteases such as kallikrein-5 have also been shown to cleave the LL-37 pro-peptide (hCAP18) once it is secreted by epithelial cells and keratinocytes. This leads not only to release of the active LL-37 peptide, but also to many different smaller fragments, such as LL-23, LL-29, and KS-27 (7). The mature cathelicidin peptides are highly variable in both amino-acid sequence and size, which leads to considerable differences in their 3D structure. They can contain to α-helices, β-hairpins, extended structures or form cyclic peptides. Some cathelicidins are rich in specific tryptophan, proline or arginine residues, while others are arranged in short tandem repeats (8–10). Since the mature peptides are highly diverse, not all cathelicidins will have similar activities which is important to keep in mind when studying these peptides. Importantly, despite these highly diverse structures, most peptides have a characteristic amphipathic nature and a net positive charge (8, 11).

Cathelicidins are expressed in nearly all vertebrates. In some species, only one cathelicidin has been identified, like human (LL-37), mouse (CRAMP) and dog (K9CATH). Other species, like chicken, horse, pig and cattle, express multiple cathelicidins (10). The main source of LL-37 in humans are neutrophils, which store the inactive pro-peptide in their secretory granules (12) and secrete them upon activation (13, 14). However, other cell types, including lymphocytes, macrophages, epithelial cells and keratinocytes, can also produce cathelicidins (15–17). Under homeostatic conditions, cathelicidins reach *in vivo* concentrations of around 0.2–0.5 μM in the plasma (12, 18), 0.2–2.0 μM in the lung mucosa (18), 0.01–1.1 μM in sweat (19), 0–4.4 μM in ascites fluid and 4–6 μM in saliva (18). Many cathelicidins are strongly upregulated during infection due to TLR activation by MAMPs, such as LPS, LTA and flagellin (20, 21). In addition, cathelicidins can be upregulated when tissues are damaged or by exposure to specific compounds, such as vitamin D3, butyrate and PGE2 (22–25). Under extreme conditions, for example in psoriatic lesions, more than 300 μM cathelicidin can be detected (26).

While best known for their direct antimicrobial activity against a broad spectrum of bacteria (27–29), viruses (30–32), fungi (33, 34), and parasites (35, 36), it is now well-established that these peptides also have the potential to modulate immune responses in various ways. This includes regulation of neutrophil and monocyte chemotaxis (37–39), induction of chemokine expression (27, 40), skewing of macrophage polarization (41), influencing phagocytosis (27, 42–44), and regulation of both extracellular and intracellular TLR activation (27, 40, 45–49). Due to this plethora of effects, it is perhaps not surprising that the reduced expression or total lack of cathelicidins is correlated with increased risk of infection (50, 51) but also has an impact on the development of autoimmune diseases (52–55).

CATHELICIDINS INHIBIT THE ACTIVATION OF LIPID-SENSING TLRs

Lipid-Sensing TLRs

Extracellular TLRs are important in the detection of bacteria-derived lipid-containing molecules. Detection of such lipids is often the first step in the initiation of an immune response against many bacterial pathogens. Bacterial lipid-containing molecules that can activate TLRs include lipopolysaccharides (LPS) from the Gram-negative bacterial outer membrane (TLR4), lipoteichoic acids (LTA) from the Gram-positive bacterial cell wall and diverse di- and tri-acylated bacterial lipoproteins (TLR1/2/6). During activation, TLRs form homo- or heterodimers that are the basis of the TLR receptor complex. However, various co-receptors, such as MD-2 and CD14 have been shown to improve ligand detection by TLRs. Upon stimulation, TLR4 forms a receptor complex consisting of a TLR4 homodimer and two MD-2 proteins (4, 56, 57). The expression of the CD14 co-receptor can further enhance LPS detection and cellular responses. The soluble LPS-binding protein (LBP) can further act as a chaperone by extracting LPS from the bacterial membrane or bacterial-derived outer membrane vesicles and delivering it to the TLR4 receptor complex. TLR2 on the other hand forms heterodimers with either TLR1 or TLR6 (58, 59). These TLR2 heterodimers are responsible for the recognition of a variety of MAMPs, including LTA, di- and tri-acylated bacterial lipoproteins such as the highly common Braun lipoprotein in *E. coli*, lipoarabinomannan from mycobacteria, zymosan from fungi and hemagglutinin from measles viruses. In addition, synthetic lipoproteins based on these natural ligands, such as the di-acylated Pam2CSK4 (TLR2/6) and tri-acylated Pam3CSK4 (TLR1/2), are commonly used as TLR2 ligands for *in vitro* studies. Similar to TLR4 activation, expression of CD14

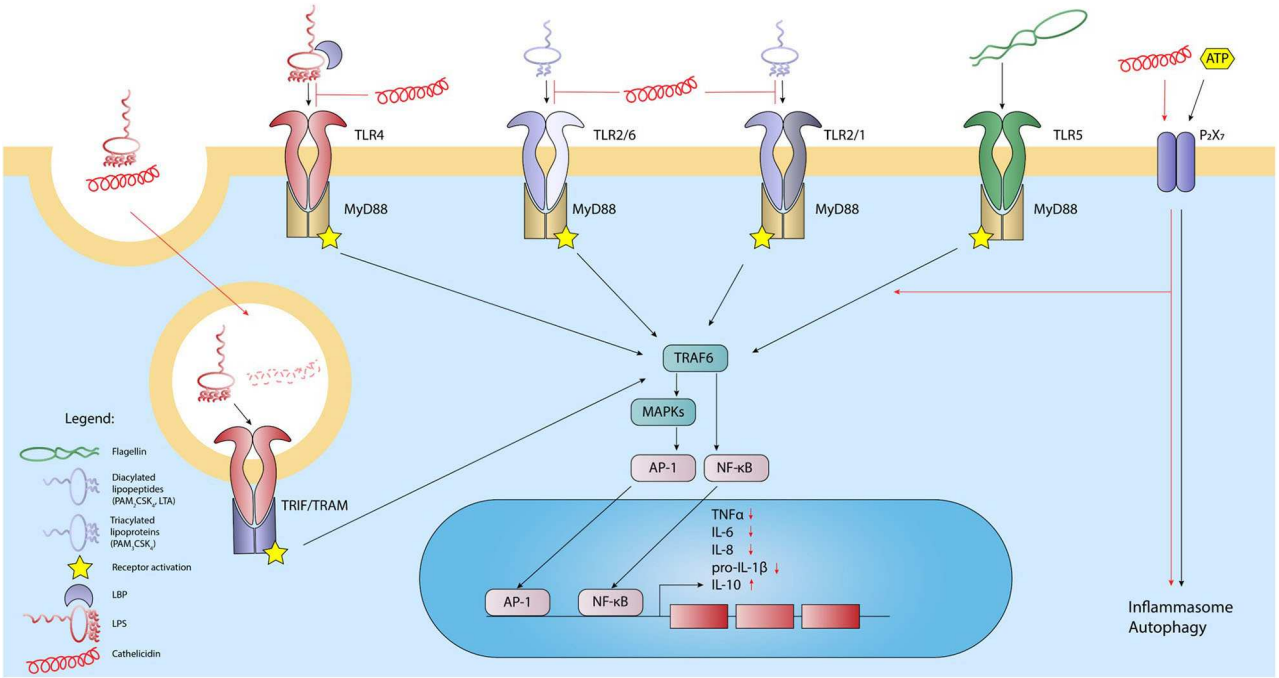

FIGURE 1 | Cathelicidins inhibit the activation of lipid-sensing TLRs and modulate the response of other extracellular receptors. Activation of TLR4 by LPS, TLR1/2, or-2/6 heterodimers by LTA, and TLR5 by bacterial flagellin leads to activation of the intracellular domain. Through the adaptor MyD88 and subsequent downstream signaling through MAPKs, NFkB, and AP-1 are released and translocated to the nucleus, leading to transcription of inflammatory cytokines. LPS can also activate TLR4 located in endosomal compartments, which leads to activation through adaptors TRIF and TRAM. Additionally, P_2X_7 receptor responds to extracellular ATP, which leads to inflammasome formation. In general, cathelicidins inhibit lipid-induced TLR-activation and thereby the downstream inflammatory response by neutralizing lipid MAMPs. LPS-cathelicidin complexes can be taken up, after which the cathelicidins are degraded to allow LPS-induced TLR4 stimulation. Finally, cathelicidins can signal through the P_2X_7 receptor by which the peptides can stimulate inflammasome formation and autophagy. *Black lines: normal signaling; red lines: effects exerted by cathelicidins*.

further increases the detection efficiency of TLR1/2/6 receptor complexes (56). Both TLR4 and TLR1/2/6 signal via the MyD88-dependent pathway, which ultimately leads to activation of NF-κB and AP-1 and thereby to the secretion of pro-inflammatory cytokines (56, 60). Importantly, TLR4 can also be present in endosomal compartments where activation can lead to TRIF-mediated signaling pathways, leading to the production of anti-inflammatory cytokines like IL-10 and type I interferons, predominantly IFN-β (61) (**Figure 1**).

Cathelicidins Inhibit LPS-Induced TLR4 Activation

Due to the fact that cathelicidins were initially described for their antimicrobial and membrane disruptive activity, many studies have focused on elucidating how cathelicidins interact with bacterial membranes or specific membrane components, such as LPS and LTA. Through these studies, it has become clear that cathelicidins are attracted to the bacterial membrane via electrostatic interactions between the negatively charged lipids in the bacterial outer membrane and the positively charged peptide (62). Indeed, loss of negatively charged phosphate-groups in the LPS core-region, for example due to mutations in LPS-controlling genes such as *PhoP/PhoQ* or *PmrB/PmrA*, reduces the susceptibility of Gram-negative bacteria to host defence peptides, like cathelicidins (63). While LPS-cathelicidin interaction may be important for eliciting antimicrobial activity, it was first shown in 1994 by Hirata et al. (64), that the 18 kDa rabbit cationic protein (CAP18) also exerts LPS-neutralizing activity, which drastically inhibits the inflammatory responses toward LPS both *in vitro* and *in vivo* (65–67). Later studies showed that this LPS-cathelicidin interaction resulted in reduced TLR4 activation and was not limited to hexa-acylated *E. coli* LPS (68–72), but was also observed in the context of penta-acylated *P. aeruginosa* LPS and the tetra-acylated *P. gingivalis* LPS (68, 70, 73). For several cathelicidins, including the human LL-37, it has now been shown that direct complex formation between LPS and cathelicidins plays an important role in preventing the binding of LPS to the TLR4 receptor complex, thereby reducing immune activation (66, 74–79). More detailed studies on LL-37 showed that binding of *E. coli* LPS occurs in a two-step process, with strong ionic interactions being followed by lower affinity interactions that are more dependent on entropic forces, such as interaction between hydrophobic regions of LPS and LL-37 (79, 80). That LPS neutralization is at least partially dependent on this ionic interaction is underlined by the fact that citrullinated LL-37 loses its ability to reduce the

LPS-induced activation of macrophages (81, 82). Importantly, cathelicidins can influence LPS-induced TLR4 activation at different stages. Chicken cathelicidin-2 (CATH-2) and LL-37 have been shown to directly penetrate the bacterial membrane and bind to membrane lipids during the bacterial killing process (79, 80). Furthermore, several cathelicidins are able to bind LPS which was already bound to LBP or are capable of reducing the LPS concentration on the host cell surface (67, 76, 83, 84), suggesting competition with cell surface receptors (**Figure 1**). Important to note is that the mature cathelicidin peptides are highly diverse, which explains why some cathelicidins exert a strong inhibitory effect, while others do not seem to affect TLR4 activation by LPS at all (27). Similarly, cleavage of cathelicidins, which can alter the overall peptide charge or structure, can influence the LPS binding as well. For example, LL-23, a cleaved biological variant of LL-37 containing 23 amino acids, is unable to neutralize LPS (85), whereas the 31 amino acid long LL-31 is still able to inhibit the LPS-induced TLR4 activation (68).

While the interaction between cathelicidins and LPS is important for the regulation of TLR4 activation, several studies have suggested more indirect TLR4 regulation by cathelicidins. LL-37 pre-incubation, for instance, still leads to a reduction of the LPS-induced immune response *in vitro*, albeit to a lesser extent compared to LPS-LL-37 co-incubation (71). Furthermore, in human monocytes, LPS-mediated p50/p105 as well as TNF induced protein 2 expression were strongly inhibited by LL-37, in contrast to the much milder effects of LL-37 on for instance RELB, CCL4 and CXCL1 (71, 72). Similarly, LPS stimulation of bone marrow-derived macrophages from CRAMP knockout mice results in enhanced IL-10 production compared to stimulation of wildtype macrophages. However, no difference in TNF or MIP-2 production was observed between wildtype and CRAMP knockout cells (86). This selective influence on TLR4 activation could be the result of regulating the expression or functions of signaling molecules downstream of TLR4. The murine cathelicidin CRAMP for instance, reduced MyD88 synthesis and impaired the interaction between MyD88 and IRAK in murine macrophages upon LPS stimulation. In addition, CRAMP inhibited the phosphorylation of p38 and ERK downstream in this cascade, leading to a strong reduction of TNF production (86). Similarly, LL-37 was shown to inhibit the LPS-induced translocation of the NF-κB subunits p50 and p65, also resulting in a strong reduction of TNF (71, 87) and reduces the LPS-induced upregulation of TREM-1 by MyD88 (88). However, as these studies co-incubated cells with LPS and cathelicidins, it is difficult to assess to what extent these effects are just the result of reduced TLR4 activation due to the blocking of receptor activation. Nevertheless, it has been suggested that LL-37 can interact with intracellular GAPDH, an important enzyme in the glycolysis pathway, which subsequently promotes MAPK activation and chemokine expression (71). Such effects on MAPK activation can also have an impact on LPS-induced signaling pathways, which also use MAPK as intermediate signaling molecules. However, more research is needed to clarify to what extent these indirect regulatory effects of cathelicidins influence activation of TLR4 as well as other TLRs (**Figure 1**).

While most studies have clearly shown an inhibitory effect of cathelicidins on LPS-induced immune activation, there are also indications that in specific cases the interaction between LPS and cathelicidins can lead to enhanced cellular activation. This effect was first shown by Shaykhiev et al. (89), where LL-37-LPS complexes were shown to be taken up more efficiently *in vitro* by human bronchial epithelial cells, which subsequently led to enhanced intracellular TLR4 activation and increased IL-6 production (89). Similarly, a human adenocarcinoma colonic epithelial cell line also responded with an enhanced inflammatory response toward LPS-LL-37 complexes compared to LPS by itself (24) (**Figure 1**). Nevertheless, increasing the cellular uptake of LPS does not always lead to an enhanced response. For instance, cathelicidin-mediated uptake of LPS was also observed in cultured human liver sinusoidal endothelial cells; however, this did not lead to an altered immune activation in these cells (90).

Cathelicidins Inhibit LTA-Induced Activation of TLR1/2/6

Whereas the effects of cathelicidins on LPS-induced TLR4 activation are very well-studied, the influence of cathelicidins on lipid-induced TLR1/2/6 activation is less well-known. Nevertheless, several cathelicidins were shown to inhibit LTA- or Pam3CSK4-induced TLR1/2 activation and Pam2CSK4-induced TLR2/6 activation. This includes LL-37-mediated inhibition of TNF and IL-6 release in LTA-stimulated PBMCs and dendritic cells, as well as inhibition of LTA-induced TNF release in macrophages by several cathelicidins from different species (27, 72, 91). However, similar to the inhibition of LPS-induced activation, not all cathelicidins are able to reduce TLR1/2/6-activation or might be less effective (27, 68, 72, 75). Like LPS-neutralization, the mechanism by which cathelicidins inhibit TLR2 has also been associated with direct interaction between cathelicidins and the TLR2 ligands. Using isothermal titration calorimetry as well as competition assays, both chicken CATH-2 and human LL-37 were shown to bind LTA and Pam3CSK4 directly (77, 79, 92), albeit with a lower affinity compared to their binding to LPS (77, 78). In addition, citrullinated LL-37 loses its ability to inhibit LTA-induced activation of macrophages (81), indicating that interaction with LTA, similar as with LPS, is dependent on ionic interactions. Despite the observed interaction between LL-37 and LTA, LL-37 is not able to inhibit TLR2 activation on all cell types. In human primary bronchial cells, co-incubation of LL-37 enhanced the Pam3CSK4-induced expression of IL-8 and IL-6, while no effect of LL-37 was observed in relation to LTA-induced keratinocyte activation (93). This indicates that the function of cathelicidins might differ in the context of different cell types. In addition, as TLR2 and TLR4 signaling pathways share many downstream signaling molecules, it is likely that the indirect effects of cathelicidins on downstream TLR signaling pathways related to TLR4 activation will also play

a role in the cathelicidin-mediated regulation of TLR2 activation, although no proof has been provided for this yet (**Figure 1**).

CATHELICIDINS ENHANCE THE ACTIVATION OF NUCLEIC ACID-SENSING TLRs

Nucleic Acid-Sensing TLRs

Foreign nucleic acids from invading viruses, as well as several bacteria, are sensed by several intracellular PRRs. These include several DNA- and RNA-receptors in the cytoplasm, as well as specific TLRs (TLR3, -7, -8, and-9) expressed in endolysosomal compartments (94). Depending on the localization of a pathogen as well as the stage of infection, receptors at these different cellular compartments can be activated. For instance, viruses are obligate intracellular parasites, meaning they rely completely on host cells for their replication and survival and replicate their genome within the host cytoplasm. Alternatively, viral nucleic acids can be found extracellularly as well in apoptotic particles, which can be engulfed by host cells and thereby end up in TLR-containing endolysosomes (95). In contrast to extracellular TLRs, which can rapidly respond to ligands in the extracellular environment, several barriers exist for the activation of intracellular TLRs by nucleic acids. Firstly, due to the many nucleases present in the extracellular environment, most free extracellular nucleic-acids are degraded before cells have the opportunity to respond (96, 97). Secondly, because the nucleic acid sensing TLRs are located intracellularly, cells have to actively endocytose the DNA or RNA for it to reach its complementary TLR receptor (98, 99).

TLR9 recognizes unmethylated CpG-containing DNA motifs in bacterial genomic DNA and viral double stranded DNA (dsDNA). The unmethylated CpG-containing DNA motifs in bacterial DNA are mimicked by synthetic CpG oligodeoxynucleotides (ODN), which are widely used in experimental systems (94, 100, 101). TLR21 is the avian equivalent of TLR9, which also recognizes CpG-ODN (102). TLR7 and -8 are highly homologous to each other due to gene duplication and recognize viral single stranded RNA (ssRNA), RNA from bacteria such as group B Streptococci and possibly siRNAs as well. The synthetic agonists for TLR7 and -8 are antiviral nucleoside analogs such as R848 and imiquimod. TLR3 recognizes viral double-stranded RNA (dsRNA), which is mimicked by the synthetic analog polyinosinic-polycytidylic acid [poly(I:C)]. TLR3 might also respond to some ssRNA viruses, most likely during the replication phase when they copy their RNA (56, 94, 100, 101, 103).

Like most extracellular TLRs, TLR7, -8, and -9 signal through the MyD88-dependent signaling pathway, resulting in the secretion of proinflammatory cytokines. In addition, activation of the highly expressed TLR7 and -9 in plasmacytoid DCs (pDCs) results in high levels of type-I interferons, like IFN-α, which is important during anti-viral responses. TLR3 on the other hand activates the TRIF-TRAF pathway in a MyD88 independent manner, which leads to IFN-β secretion (94, 101, 103).

Cathelicidins Promote Nucleic Acid Stability and Endocytosis

Cathelicidins have been found to play an important role in improving the detection of nucleic acids by cells. First of all, the positively charged cathelicidins can directly interact with the negatively charged DNA or RNA through ionic interactions, which protects it from degradation by DNAses and RNAses that are abundantly present in the extracellular environment (48, 49, 98, 104, 105). Through this interaction, cathelicidins can stabilize DNA and RNA released from damaged or dying cells as well as DNA released by bacteria, for instance during the process of biofilm formation. Once nucleic acids are bound by cathelicidins and protected from degradation, cathelicidins can assist in improving the uptake of DNA by different cell types, such as macrophages, dendritic cells and B cells (48, 49, 106, 107). While this increase in uptake could theoretically be the result of increased DNA stability, increased uptake has also been observed in the context of short DNA oligos with a phosphorothioate backbone, which are resistant to DNAse degradation due to a sulfur group replacing an oxygen group on the DNA-backbone (48, 107). Furthermore, it has been shown that the positively charged peptide can act as a bridge between the nucleic acids and proteoglycans on the cell surface, which appears to be involved in the lipid-raft-mediated uptake of these cathelicidin-nucleic acid complexes (105) (**Figure 2A**).

The ability of cathelicidins to increase DNA stability and enhance DNA internalization is also important during NETosis, the process by which neutrophils undergo cell death by expelling their DNA into a neutrophil extracellular trap (NET) to trap invading microbes. LL-37-DNA complexes for instance, are formed within NETs produced by neutrophils that are infected by mycobacteria. These LL-37-covered NETs, still containing mycobacteria, are more efficiently internalized by macrophages, which allows the macrophages to kill the mycobacteria in lysosomal compartments in an LL-37-dependent manner (108). Cathelicidins are also commonly found within NETs under other circumstances (104, 108, 109), where they can both contribute to the antimicrobial activity of the NET, as well as prevent the NETs from being degraded by bacterial nucleases (104).

Cathelicidins Influence TLR Responses To Nucleic Acids Depending on the Cell Type

Plasmacytoid Dendritic Cells (pDCs)

pDCs play an important role in many inflammatory processes, which include wound healing, antiviral and antibacterial responses, but also autoinflammatory responses (49, 110–113). Within all these processes, their main role is the production of high quantities of IFN-α, which is produced upon activation of the endosomally located TLR7 by ssRNA or TLR9 by DNA (113). pDCs were the first cell type in which LL-37 was shown to enhance DNA-induced IFN-α responses in a TLR9-dependent manner (49). Shortly after, a similar finding was done in the context of ssRNA-LL-37-complexes, which enhanced IFN-α production in pDCs in a TLR7-dependent manner (98). While both these processes depend on the stabilization of nucleic

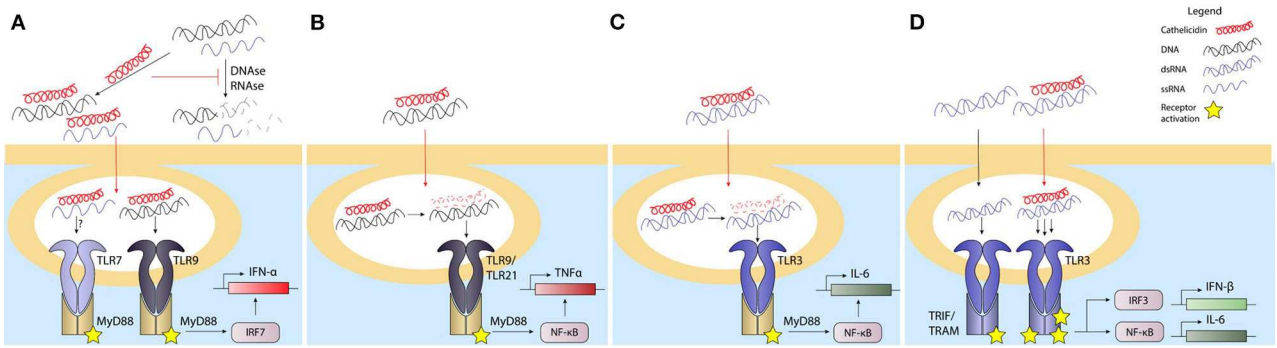

FIGURE 2 | Cathelicidins influence the response to nucleic acids depending on the cell type. Extracellular nucleotides are an inflammatory signal through various pathways. Phagocytosis or endocytosis of DNA and/or RNA is required for their delivery to endosomal compartments leads to activation of TLR3 by dsRNA, TLR7/8 by ssRNA, and TLR9 by bacterial or self-DNA. Activation of the TLR response leads to activation of the NFkB and/or IRF transcription factors, an subsequently to inflammatory cytokine production. Extracellular DNAses and RNAses degrade the nucleotides, which prevents uptake by phagocytic cells and reduces TLR activation in the endosomal compartements. Cathelicidins can bind to extracellular DNA/RNA to stabilize it and prevent it from degradation by DNAses or RNAses. The uptake of DNA/RNA-cathelicidin complexes is enhanced and this complex formation enhances TLR9 and possibly TLR7 activation in pDCs **(A)**. The uptake of DNA-cathelicidin complexes by macrophages **(B)** or RNA-cathelicidin-complexes by bronchial epithelial cells **(C)** is enhanced as well, but the cathelicidin is degraded in order to DNA or RNA to activate their respective TLRs. In keratinocytes, the dsRNA-cathelicidin complex is transported to the endosome and enhances the TLR3 stimulation **(D)**. *Black lines: normal signaling or route; red lines: effects exerted by cathelicidins*.

acids by LL-37 to allow endocytosis, it was recently shown that the structural organization of DNA-LL-37-complexes is another important step in TLR9 activation. Schmidt et al. showed that DNA, when bound to LL-37, forms columnar complexes where the spacing between the DNA strands is related to the structure of the LL-37 molecules. This spacing created by LL-37 is crucial, as it approximates the width of the TLR9 ectodomain (114, 115). This ensures that the TLR9 molecules bound to the DNA do not interfere with each other, as the LL-37-mediated spacing leaves enough room for binding of other TLR9 molecules to parallel DNA strands. Furthermore, it was shown that the outside of the TLR9 ectodomain, which is not in contact with the DNA bound in the binding pocket, can interact with an adjacent DNA strand, which improves the binding affinity of the whole DNA-complex (114). Overall, the compactness of the DNA-induced by the binding with LL-37 provides an optimal spatial arrangement for the DNA to bind a high number of TLR9 receptors, which boosts the downstream signaling and IFN-α release (**Figure 2B**). However, similar to the indirect effects of cathelicidins on TLR4 activation, LL-37 could also play a more indirect role in TLR activation in pDCs. In these cells, autophagy is needed to deliver viral TLR7 ligands to compartments containing TLRs in order to induce an antiviral response (116). While LL-37 has not yet been shown to directly activate autophagy in pDCs, it has been shown to boost autophagy in other phagocytic cell types. Vitamin D3 for instance, a potent LL-37 inducer, triggered autophagy in human macrophages in an LL-37-dependent manner by downregulation of the PI3K/Akt/mTOR pathway (117–119), which improved the intracellular killing of *Mycobacterium tuberculosis* (119).

Macrophages, Conventional Dendritic Cells and B Cells

Besides pDCs, other cell types, such as macrophages (120, 121), conventional DCs (cDCs) (122, 123) and B cells (124–126), express nucleic acid recognizing TLRs. However, in contrast to pDCs, these cells do not produce IFN-α upon nucleic acid sensing, but mostly signal through MyD88-dependent proinflammatory signaling pathways, mainly involving MAPK and NF-κB (126, 127). B cells show an increased TLR9 activation upon stimulation with DNA-LL-37-complexes, which results in enhanced surface expression of activation markers CD40 and CD86, as well as increased production of IL-6 and IgG (107, 128). In human cDCs, which express TLR8 but not TLR9, ssRNA-LL-37 complexes increase the surface expression of CD80 and CD86 activation markers as well as production of IL-6 and TNF. Interestingly, IFN-α, derived from pDCs activated by ssRNA-LL-37-complexes, can further enhance the activation of cDCs by these same complexes (98).

Similar to B cells, macrophages express TLR9 in endosomal compartments and induce a proinflammatory response that includes TNF production upon DNA detection. However, where LL-37 was shown to improve responses in B cells, it has a very limited ability to enhance activation of macrophages toward DNA when tested in murine RAW264.7 macrophages. Alternatively, cathelicidins from several other species, including equine CATH-2, chicken CATH-2 and porcine PR-39, but not murine CRAMP, were able to enhance TNF responses in these cells (27). Importantly, while TLR9 activation in pDCs was shown to be dependent on the sustained complex formation between DNA and LL-37 (114), chicken macrophage activation by chicken CATH-2-DNA complexes depends on the actual release of cathelicidin from the DNA within the endosomal compartment (48). This release was the result of peptide degradation due to endosomal proteases and was a requirement for TLR21 activation. Interestingly, while exogenous CRAMP appears unable to enhance DNA-induced macrophage responses, endogenous CRAMP expression improves DNA-induced macrophage activation by upregulating TNF, IL-6 and

IL-12p40 production, likely due to direct interaction between DNA, CRAMP and the endosomal TLR9 (129) (**Figure 2C**). While macrophage responses toward RNA-LL-37 complexes are less well-studied, some studies show that stimulation of RAW264.7 cells or alveolar macrophages with RNA-LL-37 complexes results in reduced IL-6 expression, which indicates that this complex formation actually has an inhibitory effect on the activation of TLR3 in these cells (130–132).

Keratinocytes and Epithelial Cells

Keratinocytes are crucial in the protection against skin infections and are a major source of LL-37 during both infections as well as wound-healing processes (133–136). The secretion of LL-37 by these cells strongly contributes to direct antimicrobial activity in the skin and enhances bacterial internalization by the keratinocytes (133). Besides acting as an antimicrobial factor, LL-37 can also influence nucleic acid detection by keratinocytes in several ways. First of all, whereas keratinocytes normally only express low levels of TLR9, LL-37 strongly induces the expression of this TLR in a dose-dependent manner, thereby increasing the capacity of keratinocytes to respond to endocytosed DNA (137–139). Secondly, expression of LL-37 during infection or skin damage gives LL-37 the opportunity to interact with host-DNA or -RNA released from the damaged tissue and influence the cellular uptake of these nucleic acids. Interestingly, while keratinocytes have an increased DNA uptake when DNA is complexed with LL-37, this DNA does not end up in endosomes, but in the cytoplasm. This alternative localization could be the reason why TLR9 cannot be activated by LL-37-DNA-complexes in keratinocytes, while consecutive stimulation with LL-37 and DNA does lead to a strong type I IFN response, possibly due to the increased expression of TLR9 induced by LL-37 (139). Nevertheless, while LL-37 promotes cytoplasmic uptake of DNA and many cytoplasmic nucleic acid receptors exist, such as the inflammasome-activating DNA receptor AIM2, the cytoplasmic localization of LL-37-DNA-complexes does not lead to activation of AIM2 nor does it activate the inflammasome-mediated release of IL-1β. The lack of AIM2 activation could potentially be caused by steric hindrance by LL-37, which might prevent the binding of self-DNA to AIM2 (140, 141) and thereby could play a role in the prevention of autoimmunity. While the studies mentioned here have mostly focused on the interaction between LL-37 and host-DNA, the upregulation of TLR9 expression could of course also influence the detection of bacterial DNA released passively by dying bacteria or actively during the programmed cell death during bacterial biofilm formation (142, 143). In contrast to LL-37-DNA complexes, complexes of LL-37 with dsRNA are in fact capable of reaching endosomal compartments in both human epidermal keratinocytes and human bronchial epithelial cells, which results in the activation of TLR3 (130, 131, 144, 145). TLR3 activation by RNA-LL-37-complexes depends on different processes in both cell types. In keratinocytes, complex formation between LL-37 and the dsRNA provides an RNA structure where the intercalating LL-37 provides optimal spacing between RNA molecules to bind a higher number of TLR3 molecules per RNA molecule, enhancing IFN-β and IL-6 production, with a similar mechanism as LL-37-DNA-mediated activation of TLR9 in pDCs. Human bronchial epithelial cells on the other hand require the dissociation of LL-37 from the LL-37-RNA-complex to activate TLR3, which is caused by a decrease in pH and protease activation in endolysosomal compartments. Degradation of LL-37 then allows the RNA to bind to the TLR3 receptor, which is reminiscent of the mechanism by which TLR21 is activated by CATH-2-DNA complexes in chicken macrophages (130, 131) (**Figure 2D**). Together, all these studies demonstrate both the complexity by which cathelicidins can influence nucleic acid-sensing as well as the different requirements that TLR activation by RNA/DNA-cathelicidin-complexes has depending on the cell type and species investigated.

LIMITED EFFECTS OF CATHELICIDINS ON TLR5 ACTIVATION

While the effects of cathelicidins on the previously described TLRs are well-studied, the influence of cathelicidins on TLR5 activation remains less well-understood. TLR5 detects the conserved flagellin protein present in the flagella of Gram-negative bacteria and its activation leads to pro-inflammatory cytokine production via MyD88- and NF-κB-signaling pathways (56). Some studies have shown that LL-37 enhances the flagellin-induced IL-8 secretion by adult human keratinocytes (93, 146), which was depended on P_2X_7 receptor signaling and Scr/Akt pathway activation (146). In human bronchial epithelial cells, co-incubation of LL-37 and flagellin resulted in an increased IL-8 and IL-6 secretion (93), regulated via the PI3K/p38 pathway (147). On the other hand, LL-37 had no or a slightly inhibitory effect on the flagellin activation in human dendritic cells (91), macrophages (81), PBMCs (68, 87), or TLR5-tranfected HEK cells (77) (**Figure 1**). This shows that the influence of cathelicidins on TLR5 is highly dependent on the cell type, although more research is required to understand the mechanisms underlying these differences.

CATHELICIDINS ACTIVATE THE INFLAMMASOME VIA P_2X_7

Another innate immune mechanism involved in sensing microbe- or damage-related signals involves the inflammasome. The formation of the inflammasome complex can be triggered by a diverse set of environmental stimuli, including ATP, cytokines and TLR ligands, and might be affected by cathelicidins as well. Inflammasome activation and signaling results in the conversion of pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18. ATP is one of most common ligands studied in this respect, triggering the inflammasome formation via the P_2X_7 receptor, leading to the activation of caspase-1 and thereby the cleavage of pro-IL-1β to active IL-1β (148). However, this P_2X_7-mediated inflammasome activation can also be induced by other ligands, such as LL-37. This has for instance been shown by LL-37 treatment of LPS-activated monocytes or stimulation of macrophages with both LL-37 and *P. aeruginosa*, which in both cases leads to P_2X_7-dependent IL-1β release (149, 150). In addition, NET-associated LL-37 has been found to activate caspase-1 in a P_2X_7 receptor

dependent fashion in macrophages, leading to IL-1β and IL-18 release (151), which provides yet another function for LL-37 in NETs.

THE INFLUENCE OF CATHELICIDINS ON TLR ACTIVATION IN HEALTH AND DISEASE

Cathelicidin-Mediated TLR Regulation Balances the Inflammatory Response to Bacterial Infection

Cathelicidin-Mediated TLR Regulation *in vitro*

Cathelicidins are capable of reducing the inflammatory response of the immune system by inhibiting LPS- or LTA-induced TLR activation. This reduction is dependent on the direct interaction between cathelicidins and these lipid-containing molecules. Until recently, these effects were mostly studied in the context of purified TLR ligands and little was known about how cathelicidins affect TLR signaling in the context of a complete bacterium. However, some recent studies are now shedding some light on how cathelicidins balance inflammation in the context of whole bacterial cells. For instance, human adenocarcinoma colonic epithelial cells produce higher amounts of LL-37 upon activation, which prevents internalization of the enteric pathogenic Gram-negative bacterium *S. typhimurium*. Alternatively, knockdown of LL-37 increases *Salmonella* invasion in enterocytes and allows for more efficient immune evasion by these bacteria due to lower TLR4 expression and a reduced IL-1β response (152). While reducing the invasion and internalization of *Salmonella* in colonic epithelial cells, LL-37 enhances the clearance of *Mycobacterium avium subsp. paratuberculosis* (MAP), a bacterium causing chronic diarrheic intestinal infections in domestic and wild ruminants, by increasing bacterial uptake in murine macrophages. In addition, macrophage treatment with LL-37 suppresses TLR2 upregulation and thereby the production of tissue-damaging inflammatory cytokines released during MAP infection (153), as well as during *A. fumigatus* infection (154).

Besides the ability of cathelicidins to regulate TLR expression they can also influence bacterial-induced TLR activation through direct interaction with these bacteria. Importantly, the ability of cathelicidins to regulate bacterial-induced TLR activation directly is highly dependent on bacterial viability. For instance, when cathelicidins such as human LL-37, chicken CATH-2 or porcine PMAP-36 are co-incubated with heat-inactivated *E. coli* or *P. aeruginosa*, they strongly reduce macrophage responses against these bacteria by blocking TLR2 and TLR4 activation through direct interaction with the lipoproteins and LPS normally activating these TLRs. However, when these cathelicidins are co-incubated with live *E. coli* or *P. aeruginosa*, no inhibition is observed as long as these peptides remain below bactericidal concentrations. Importantly, when instead bactericidal concentrations are used, inhibition of macrophage activation can be observed again. Alternatively, using cathelicidins that lack antimicrobial activity, but possess LPS-neutralizing activity, such as the canine K9CATH, it was shown that these peptides can in fact reduce macrophage activation in the context of killed bacteria, but not in the context of viable bacteria (73, 77, 92). This viability-dependent regulation of TLR activation provides an elegant way for the host to respond to bacterial molecules only when needed. At the start of an infection, activation of the immune system leads to the production and release of cathelicidins and cytokines from both macrophages and neutrophils at the site of infection. These cathelicidins will target and fight the bacteria to reduce the bacterial burden at the site of infection. During this phase, cathelicidins will only be able to neutralize the LPS- and lipoprotein-induced inflammatory responses against already killed bacteria, while still allowing a response against any remaining viable bacteria. This leads to a balancing act, where a reduction or increase of viable bacteria, i.e., a reduced or increased threat, also leads to a corresponding reduced or enhanced inflammatory response. Therefore, this cathelicidin-mediated regulation based on bacterial viability could be an important factor in maintaining a proportional inflammatory response based on the present bacterial threat and thereby limiting excessive inflammation which can lead to unwanted tissue damage (**Figure 3**).

Cathelicidin-Mediated TLR Modulation *in vivo*

While the strong antimicrobial potential of cathelicidins has driven the *in vivo* testing of these peptides, many studies have also investigated how these peptides influence immune activation during infection. A common tool for testing the effects of cathelicidins on infection and inflammation *in vivo* is the *clnp*-null mouse model, which lacks the expression of the only murine cathelicidin CRAMP. Using this model, it has been shown that mice lacking cathelicidin expression were more prone to necrotic skin infection caused by Group A *Streptococcus* (155), *P. aeruginosa* infection of the cornea (50), *S. aureus*-induced endophthalmitis (156), cecal-ligation and puncture-induced sepsis (157), dextran sulfate sodium (DSS)-induced colitis (158), *K. pneumoniae*-induced lung infection (159), caerulein-induced experimental acute pancreatitis (160) and meningitis-induced by intracerebral injection *S. pneumoniae* (161, 162). In general, these CRAMP-deficient mice suffered from a higher bacterial load, increased proinflammatory cytokine production and increased tissue damage. In addition, two studies also reported an increased influx of neutrophils in *clnp*-null mice (50, 162). These findings indicate the importance of cathelicidins not only in reducing the pathogenic burden during infections, but also their importance in limiting inflammation. Similarly, alternative models relying on exogenous treatment with cathelicidins, or transgenic overexpression of LL-37, show comparable results. For instance, intravenous administration of LL-37 in a cecal-ligation and puncture-induced sepsis mouse model improved the survival of these mice and reduced the bacterial load in the blood and peritoneum. In addition, LL-37 reduced the levels of several proinflammatory cytokines in the peritoneal fluids as well as in the serum, despite causing an increase in NET formation by neutrophils (163, 164). In a similar fashion, intratracheal treatment of *A. fumigatus*-instilled mice

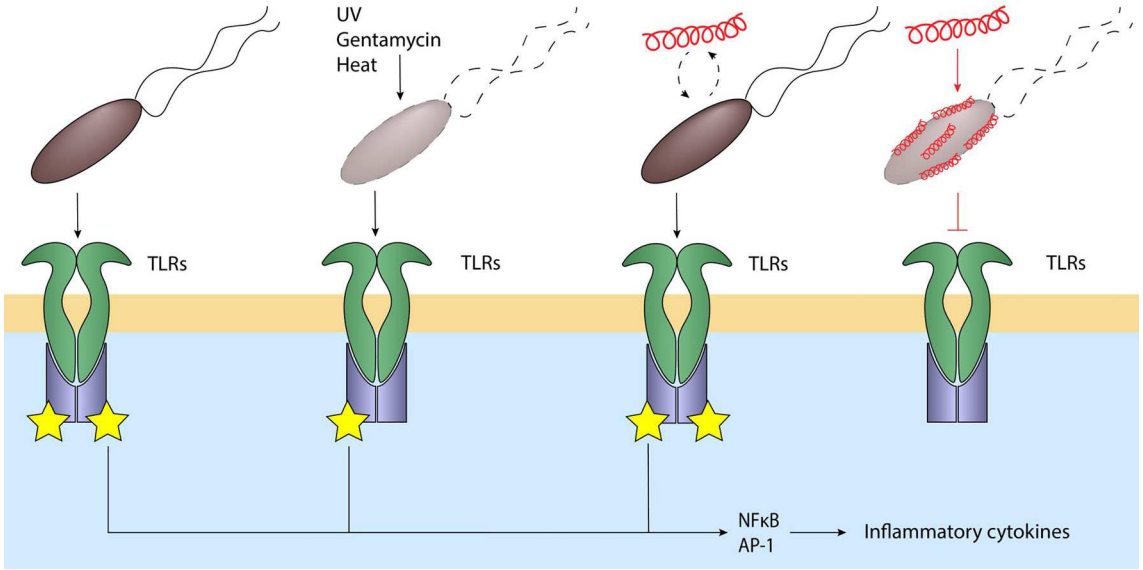

FIGURE 3 | Cathelicidins balance the immune response to bacteria. Live bacteria activate various TLRs through their MAMPs, leading to NFkB and AP-1 activation and inflammatory signaling. Bacteria killed by for example UV, heat or gentamycin still activate these TLRs. Addition of cathelicidins to viable bacteria does not inhibit TLR activation; however, cathelicidin-killed bacteria or addition of cathelicidins to non-viable bacteria strongly inhibits TLR activation. This silent killing reduces the inflammatory response and thereby the subsequent inflammatory tissue damage when the bacteria are already killed and are no longer a threat. *Black lines: normal signaling; red lines: effects exerted by cathelicidins*.

with exogenous LL-37, as well as transgenic overexpression of LL-37, causes enhanced fungal clearance, reduced lung damage and less proinflammatory cytokine production (154). Taken together, all these studies show a potent antimicrobial and anti-inflammatory role for cathelicidins during both bacterial and fungal infections. However, despite the cathelicidin-related anti-inflammatory effect seen in many of these studies, it remains difficult to distinguish if these anti-inflammatory effects can be partially caused by direct TLR inhibition or by alternative immunomodulation or that the effects can be explained by a lower bacterial burden due to the antimicrobial activity of these cathelicidins.

In an effort to separate these effects, several studies have now used different types of inactivated bacteria to investigate the direct anti-inflammatory role of cathelicidins *in vivo*. For instance, inflammatory responses induced by intratracheal administration of heat-inactivated *P. aeruginosa* or *S. aureus* isolates in mice can be inhibited by co-administration or post-administration of chicken CATH-2 (73, 165). In addition, intratracheal administration of chicken CATH-2-killed *P. aeruginosa* induces less neutrophil influx and inflammatory cytokine production compared to either administration of heat-killed or gentamicin-killed *P. aeruginosa* (73). These findings indicate that it is likely that the reduction in inflammatory markers observed upon treatment of a bacterial infection with cathelicidins, is not only the result of a reduced bacterial burden due to antimicrobial activity but is indeed also affected by direct inhibition of immune activation by cathelicidins.

Nucleic Acid-Cathelicidin Complexes Can Lead to Autoimmune Disease

Cathelicidins have been extensively described for various protective functions that are beneficial for host survival. These functions include their direct antimicrobial activity against Gram-positive and Gram-negative bacteria (27, 74, 166), inhibition of viral replication (132) or direct antiviral activity (167), promotion of wound healing (110) as well as their ability to modulate immune responses (15, 37, 71), which has been shown to protect against excessive inflammation (77). However, their ability to improve nucleic acid detection may also lead to the onset of various autoimmune diseases.

The pathways leading to excessive inflammation in autoimmune diseases are often complex and involve numerous cell types. For SLE, psoriasis and diabetes, these processes most likely start with some type of tissue damage that initiates TLR signaling and autoimmune inflammation (49, 54, 168). In diabetes, cell death of the insulin producing β-cells of the pancreas initiates a cascade that leads to more cell death and subsequently more inflammation (54). In SLE and psoriasis, anti-DNA and -RNA antibodies can be found that most likely are the result of DNA released upon tissue damage and play an important role in the exacerbation of these diseases (168, 169). In 2007, Lande et al. were first to link these processes to cathelicidins by describing how LL-37 enhances DNA-induced inflammation in psoriatic skin lesions (49). In these lesions, the release of DNA and RNA from damaged tissue binds LL-37 (49, 98), which is expressed at extremely high concentrations (up to 300 μM) by either keratinocytes or neutrophils under these conditions (26).

These complexes then stimulate pDC-derived IFN-α production that subsequently drives the activation of cDCs and T cells, which in turn exacerbate the tissue damage (98). In addition, enhanced activation of B cells by DNA-LL-37 complexes also increases the production of anti-DNA antibodies (128). In diabetes, the complex formation of these anti-DNA antibodies with DNA and CRAMP triggers pDC activation, which again leads to high IFN-α production. These high levels of IFN-α in turn increase T cell activation and thereby autoreactivity against pancreatic β-cells (54). Other complexes that can increase auto-inflammation include RNA-LL-37 complexes. These trigger TLR8-mediated cytokine production and can induce neutrophil NETosis in psoriatic skin (170). Similarly, anti-RNA immunocomplexes were shown to activate neutrophil NETosis in SLE, which results in the release of additional NET-derived DNA into the extracellular environment (168). Importantly, as these NETs are coated with both LL-37 and anti-DNA antibodies, they can serve as new ligands for pDC activation and thus IFN-α production, which leads to a further exacerbation of the inflammatory response (169).

Cathelicidins as Adjuvant for Vaccination

Vaccination strategies aim to induce a modest immune response against one or more specific pathogens, in order for a host to be able to respond with a humoral response toward such pathogens when they are encountered later in life. To induce such a specific immune response, vaccination therapies require one or more antigens in the form of whole live, inactivated or attenuated viruses or bacteria, or alternatively, specific microbial components, such as outer membrane vesicles or specific viral or bacterial proteins. However, as not all antigens can stimulate the immune system sufficiently to build an immune memory, adjuvants, including several TLR agonists, are commonly used to improve the strength of the immune response during vaccination. Since cathelicidins can modulate these TLR responses, their possible role as adjuvant during vaccinations has been investigated in various studies. For instance, intranasal vaccination with attenuated pseudorabies virus (PRV), complexed with CpG-DNA and a bactenecin-derived innate defense regulator (IDR) peptide, resulted in an enhanced protection of piglets (171). Similarly, the combination of an IDR peptide and CpG-DNA as adjuvants for a pertussis toxoid vaccine improved *in vitro* DC maturation, cytokine production and expression of surface activation markers, while also enhancing *in vivo* antigen presentation and specific IgG1 and IgG2a antibody titers (172, 173). While the mechanisms behind these improved responses are hard to discern, improvement of DNA-induced TLR9 responses by these peptides could very well play a role in this. On the other hand, cathelicidins have also been shown to improve vaccination responses toward various viral pathogens in the absence of CpG-DNA as an adjuvant. Intramuscular or intranasal administration of piglets with inactivated porcine reproductive and respiratory syndrome virus (PRRSV) microparticles complexed with an IDR peptide or LL-37 enhanced the response toward the antigens *in vitro*; however, *in vivo* only little improvement in vaccination efficacy was observed (132). In addition, intranasal vaccination of mice using a nanoparticle-based vaccine for an H1N1-ovalbumin influenza virus, also benefits from IDR peptides as adjuvant, which together with c-di-AMP, induced a strong humoral and cellular immune response (174). Furthermore, subcutaneous vaccination of mice with the HPV E7 epitope of human papillomavirus (HPV) using CRAMP as adjuvant, reduced HPV-induced tumor growth (175). Finally, besides their effect on anti-viral vaccinations, IDR peptides were also shown to improve vaccination efficiency against other types of pathogens. This includes intravenous administration of an IDR peptide as an adjunctive therapy for an oral administered anti-malarial therapy, which strongly enhanced the protection against late-stage malarial infection in mice (176). Alternatively, using an IDR peptide as adjuvant resulted in a balanced increase in IgG1 and IgG2a antibody titers upon subcutaneous vaccination of beef calves, using a mix of *Mycoplasma bovis* subunits and IDR peptide. However, in this last study it is unclear whether this is due to the addition of the IDR peptide, since a control without IDR peptide is missing (177). Together, these results indicate the potential usefulness of cathelicidins and other similar host defense peptides in vaccination therapies; however, more detailed studies will be required to discriminate the contribution of direct immunomodulatory activities from the TLR-modulatory activities of these peptides in such vaccination therapies.

Cathelicidins as Anticancer Therapy

Besides their role in inflammation, both cathelicidins and TLRs play an important role in the development and progression of cancer. TLR2 activation for instance, has been suggested as a possible therapeutic target, with local administration of a TLR2/6 agonist resulting in reduced tumor growth and prolonged survival in a pancreatic carcinoma mouse model (178). Alternatively, TLR9 activation by CpG-ODN has been shown to reduce metastasis and improve survival in pancreatic cancer (179) and neuroblastoma mouse models (180). As LL-37 has been shown to improve DNA-induced TLR9 activation, it might not be surprising that co-administration of CpG-ODN and LL-37 enhanced survival in a mouse ovarian-tumor model compared to CpG-ODN alone and that the LL-37-CpG-ODN combination enhanced the activation and proliferation of NK-cells, but not of T cells or macrophages, in the peritoneal cavity (181). On the other hand, inhibition of LPS-induced TLR4 activation reduces the migration and invasion capacity of the SW480 cancer cell line (182) and reduces pancreatic tumorigenesis in mice (183). While not specifically tested with cathelicidins, peptides such as LL-37 could also provide efficient inhibition of TLR4 and could thereby be of therapeutic value in tumors which have strongly enhanced TLR4 expression, for example numerous ovarian epithelial cancers (184). Furthermore, cathelicidins can also have beneficial anti-cancer effects in the absence of specific TLR ligands. In gastric or colon tumors, where the expression of LL-37 is reduced (185), treatment with LL-37 activates caspase-independent apoptosis and reduces tumor progression (186). Additionally, the application of CRAMP as adjuvant for HPV vaccination reduces tumor growth, albeit no direct anti-tumor effects of CRAMP were observed when used to treat HPV-induced tumors (175).

Nevertheless, overexpression of LL-37 has also been linked to increased tumor growth, enhanced invasiveness and bad prognosis in malignant melanomas and ovarian, lung, prostate and breast cancers, by stimulating the growth receptors of the EGFR and ERB-family (187). In addition, increased TLR expression in ovarian and pancreatic cancers is associated with poor clinical outcome (188, 189). Together, this shows that cathelicidins, either directly or through modulation of TLR activation, can be useful in the development of novel anti-cancer therapies, but that the potential negative effects of these peptides should not be overlooked. In this respect, modification of synthetic cathelicidin-like peptides could provide a solution to these issues, by structurally improving the peptide to increase desirable effects while at the same time limiting unwanted side effects.

CONCLUSION AND OUTLOOK

The fact that cathelicidins are a highly conserved part of the innate immune system in vertebrates, together with their apparent multifunctional nature, has led to a large interest in these peptides across various disciplines, including e.g., microbiology, immunology, oncology and dermatology. This multidisciplinary approach has uncovered a wide variety of effects that these peptides can exert. However, the potency of these different effects can vary strongly. Among their immunoregulatory functions, the regulation of TLR activation can be counted among their more potent functions, with cathelicidins being able to strongly reduce LPS- or LTA- induced TLR activation and almost completely inhibiting TLR-mediated inflammatory responses when they kill bacteria. On the other hand, DNA can be transformed from a quickly degradable extracellular factor into a very potent inflammatory signal in the context of various auto-immune diseases. While direct interaction between cathelicidins and TLR ligands in most of these cases plays an important role in regulating TLR activation, more research is required to uncover the more complex direct effects of cathelicidins on signaling pathways such as the induction of autophagy. Autophagy can play a role in an enormous set of different intracellular pathways. Activation of autophagy by cathelicidins could also have a major impact on TLR signaling besides those effects mentioned in this review. Importantly, while cathelicidins are conserved among vertebrates, the amino acid composition and 3D structure of the mature peptides vary a lot. This means that one should be careful to extrapolate findings between different cathelicidins without supporting laboratory evidence.

Finally, the literature discussed in this review shows that cathelicidins could have strong prophylactic and therapeutic value aside from their antimicrobial activities. This includes dampening inflammation when treating infections to prevent sepsis as well as improving vaccine responses through an improved immune response against target antigens. In addition, it has been shown using structurally modified cathelicidins that certain functions can be enhanced or, alternatively, be limited. Thus, cathelicidin derivatives can be designed with specific therapeutic properties while limiting any unwanted side effects. Overall, it may be concluded that, despite the fact that cathelicidins have been discovered nearly 30 years ago, the elucidation of new properties and functions in recent years continues to provide more insight in the physiological roles and potential applications of cathelicidins.

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MS and MC have written the review. RH designed the graphics. HH, EV, and RH, carefully read and corrected the text and wrote paragraphs of their expertise.

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REVIEW
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Host Directed Therapy Against Infection by Boosting Innate Immunity

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The innate immune system constitutes the first line of defense against invading pathogens, regulating the normal microbiota and contributes to homeostasis. Today we have obtained detailed knowledge on receptors, signaling pathways, and effector molecules of innate immunity. Our research constellation has focused on ways to induce the expression of antimicrobial peptides (AMPs), the production of oxygen species (ROS and NO), and to activate autophagy, during the last two decades. These innate effectors, with different mechanisms of action, constitute a powerful defense armament in phagocytes and in epithelial cells. Innate immunity does not only protect the host from invading pathogens, but also regulates the composition of the microbiota, which is an area of intense research. Notably, some virulent bacteria have the capacity to downregulate innate defenses and can thereby cause invasive disease. Understanding the detailed mechanisms behind pathogen-mediated suppression of innate effectors are currently in progress. This information can be of importance for the development of novel treatments based on counteraction of the downregulation; we have designated this type of treatment as host directed therapy (HDT). The concept to boost innate immunity may be particularly relevant as many pathogens are developing resistance against classical antibiotics. Many pathogens that are resistant to antibiotics are sensitive to the endogenous effectors included in early host defenses, which contain multiple effectors working in cooperation to control infections. Here, we review recent data related to downregulation of AMPs by pathogenic bacteria, induction of innate effector mechanisms, including cytokine-mediated effects, repurposed drugs and the role of antibiotics as direct modulators of host responses. These findings can form a platform for the development of novel treatment strategies against infection and/or inflammation.

Keywords: phagocytes, gene expression, antimicrobial peptides (AMPs), antibiotic, epithelia

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INTRODUCTION

After Alexander Fleming first discovered penicillin, several generations of different types of classical antibiotic drugs were developed. Most of these antibiotics target a specific molecule or an essential mechanism needed for survival of the bacterium. However, the targets of antibiotics can be altered by mutations, rendering them ineffective in controlling the infection. Bacterial enzymes

can also degrade active antibiotics, and membrane proteins can pump the antibiotic drug out of the bacterial cell and thus prevent it from reaching the intended target. Genes encoding the degrading enzymes and the membrane pumps can be located on plasmids that are horizontally transferred between bacterial strains (1, 2). Excessive antibiotic usage has exerted a selection pressure on the bacterial population, which has increased the spread of antibiotic resistant strains. Although novel antibiotics have been developed the selection of resistance continues, and in recent years, antibiotic resistance has increasingly resulted in prominent problems for healthcare worldwide. Excessive use and abuse of antibiotics are major drivers of antimicrobial resistance (AMR). Livestock and agriculture industries are, for example, major contributors to AMR, due to the application of medically important antibiotics in livestock production and in the food/agriculture industry (3, 4). A critical scenario may emerge with the spread of resistant bacterial strains that no classical antibiotics exhibit activity against, i.e., pan-drug-resistant (PDR) pathogens. Consequently, we are entering an era similar to the pre-antibiotic time, where common infections would be serious or even lethal. Thus, there is an urgent need to find novel strategies to prevent and control infection.

Various alternative strategies have been suggested, including blockage of host microbe interactions, inhibition of virulence factors, and the development of bacterial phage therapy (5). All these approaches have potential but have not yet resulted in therapies that can be utilized in a clinical setting. Furthermore, any single target strategy would theoretically select for resistant bacteria, since novel resistance mechanisms are likely to occur upon application of a new treatment. The consequences of resistance to treatments could be disastrous and thus, the application of new or adapted treatments should be critically studied before introduction into clinical settings.

We have developed a strategy based on direct stimulation of host cells by activation of endogenous defense mechanisms, such as antimicrobial peptides (AMPs), reactive oxygen species (ROS and NO) and autophagy, in both epithelial cells and macrophages. These are early immune mechanisms aiming to limit the spread of pathogens by killing them [(6–8); **Figure 1**]. Pathogens have developed different mechanisms to evade the first line of defense and are often not metabolically adapted to a life in the external environment like the lumen of the gut, but instead they need to invade and exploit the host tissues (9). However, counteracting the pathogen-mediated downregulation on host cells by recapturing the expression of innate antimicrobial effectors can lead to elimination of the pathogenic intruder (**Figure 1**). Interestingly, by this strategy, it is possible to induce multiple genes, generating a powerful response of innate effectors in cells. The genes encoding response factors are co-regulated in an orchestrated fashion to create effective responses and are selected for even in early eukaryotic lifeforms. The simultaneous induction of multiple effectors is the key for this strategy, since the combination should limit the selection of resistant pathogenic strains.

Alternative strategies based on direct application of AMPs have been in development. First, topical treatment using modified peptides on skin infections, relying on direct antimicrobial activity have been presented (10). However, the use of a single peptide would be expected to select for resistant strains. Furthermore, modified AMPs have also been used as injections for immune modulation of the peptides, in order to modify innate responses (11). This second strategy has been reviewed elsewhere (12).

Our approach of using induction of innate immunity with emphasis on the expression of AMPs has resulted in inducers that stimulate a range of innate antimicrobial effectors with limited inflammation. The initial inducers we have studied are butyrate and phenylbutyrate (PBA), which are effective in animal models (6, 13). In human trials, PBA has been used together with vitamin D3 as adjunct therapy to boost immunity against *Mycobacterium tuberculosis* (Mtb) with a beneficial outcome (14). An additional clinical trial utilizing butyrate as adjunct therapy to treat shigellosis showed early reduction of local inflammation (15). Our aim is to develop inducers with optimal properties to activate innate effector mechanisms as host directed therapies (HDTs) to combat infection in the absence of the selection of resistant strains. In addition, a combination of HDTs and classical antibiotics might enhance the treatment efficacy and shorten the duration of antibiotic usage. Indeed, cooperative action between classical antibiotics and innate antimicrobial

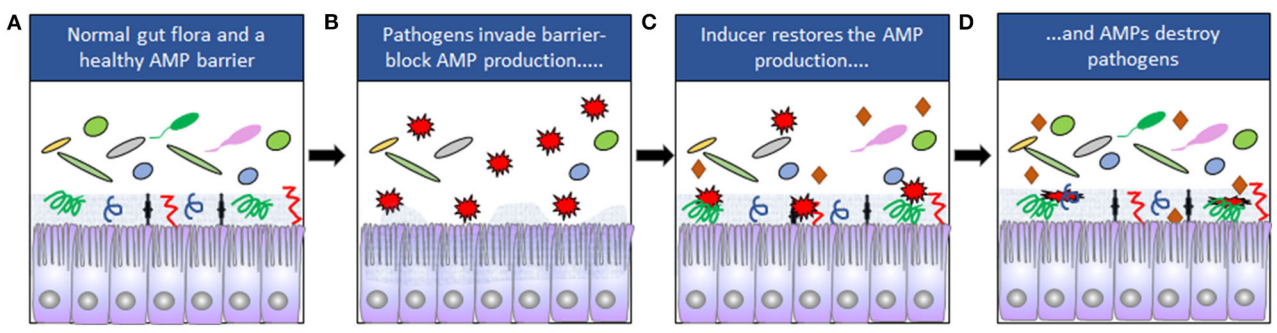

FIGURE 1 | (A) The composition of the normal gut microbiota is dependent on expression of AMPs. Mucins are indicated by the gray zone at the apical site of the cells and AMPs by symbols ( ). **(B)** Some pathogens ( ) can downregulate the expression of AMPs and disrupt the epithelial barriers. **(C)** Downregulation can be counteracted by inducers ( ). **(D)** The pathogens are eliminated when AMP expression is restored.

effectors has been confirmed *in vitro* (16). These combination therapies could retrieve usage of early generations of classical antibiotics and would be in line with improved stewardship, aiming to limit the spread of resistant strains.

Our focus in this review will be induction of antimicrobial effector mechanisms in mucosal epithelial cells and phagocytes of the macrophage lineage.

INNATE IMMUNITY AND FRONT LINE DEFENSES

Innate immunity constitutes the first line of defense and includes specific cells that produce various effector molecules to activate mechanisms resulting in the elimination of pathogens. Different cells of hematopoietic origin constitute the effector cells of innate immunity, such as NK cells and dendritic cells, as well as the professional phagocytic cells monocytes/macrophages and granulocytes. Furthermore, the epithelial cells, while of non-hematopoietic origin, are of fundamental importance, making up a vital surface layer and working as a continuous defense barrier. In our models, the target cells of innate induction have been epithelial cells and macrophages (6, 14). Epithelial cells are sewed and tilted together with tight junctions and adherent junctions, respectively. These linkages between epithelial cells have organ specific adaptations depending on the function of the tissues, such as uptake of nutrients in the small intestine, gas exchange in the lung, and filtration of the blood in the kidney. Moreover, controlled para-cellular transport can also occur. In innate immunity, epithelial cells are functional players and constitutively secrete innate antimicrobial effectors, keeping microbes at bay. Epithelial cells signal to internal sites, secrete specific cytokines, and contribute to defense in the local environment. If microbes pass the epithelial barrier, macrophages, and dendritic cells in the underlying tissue serve as defense mediators with links to adaptive immunity. Further recruitment of neutrophils and monocytes represent another wave of active antimicrobial defenses, originating from the circulation. Innate lymphoid cells (ILCs) are essential in this context, especially in the gut. They sense immunological mediators that are released from epithelial cells and secrete the specific cytokines IL-22 and IL-17 that in turn enhance the secretion of epithelial antimicrobial peptides (17). Indeed, the ILCs have been indicted as important local orchestrators of environmental signals to an immune response for the maintenance of homeostasis. This underlines that the initial defenses rely on a complex network of cell communication (18).

AMPs and reactive radicals, such as nitric oxide (NO) and oxygen species (ROS) with the capacity to eliminate invading pathogens are some of the effector molecules produced by epithelial cells and phagocytes. The combination of these effector systems characterized best in phagocytes, and a similar system operates on epithelial surfaces. Together these effectors work in cooperation in either an additive or a synergistic manner as an efficient armament against microbes. Notably, these effector molecules are evolutionary ancient and have co-evolved with the natural microbiota and have done so without selecting for invasive resistant bacterial strains. This is in contrast to the situation with classical antibiotics that have been only been used for several decades and their usage has selected multiple resistant strains.

Innate effectors, such as AMPs, are not only included in the armament for killing pathogenic intruders but work also as modulators of cell activity. In this respect, AMPs resemble cytokines and chemokines by activating and recruiting different immune cells. Interestingly, this dual activity has been described for several AMPs, indicating a common character. Some AMPs constitute links to adaptive immunity for specific responses and memory functions. As an example, the human cathelicidin LL-37 is chemotactic to human T cells (19), and human beta-defensins can recruit immature dendritic cells and memory T cells (20). Molecules of the adaptive arm of immunity are also included in front line defenses, such as IgA antibodies, that are secreted into the mucosa and make up an efficient barrier together with innate effector molecules. This underlines the cooperation between adaptive and innate immunity at epithelial mucosal surfaces, including multiple effectors for efficient protection of the tissues.

It is of importance to consider the turnover and differentiation of epithelial cells and phagocytes, since the cells have to reach maturity for full activity. Epithelial cells are derived from basal cells in the lungs (21) and from crypt stem cells in the small intestine (22). Upon maturation, epithelial cells use tight, and adherent junction proteins for linking together adjacent cells and seal off the interior tissues from the outside, allowing only small molecules to pass in specific situations. Therefore, tight junctions define a physical character of the epithelial barrier that is important for innate defenses. Microbes do not pass the para-cellular space, with the exception of certain pathogens that actively break the barrier to enter (20, 23, 24). Epithelial cells are exposed to friction and stress and hence are shed continuously and must be renewed. The timing of this is dependent on the specific epithelia. In the small intestine, epithelial cells have a lifespan of ∼4 days and in the lung, they last for over 4 weeks (25, 26). The removal of infected cells and renewal of the epithelial cells will maintain active innate defenses. The high turnover of epithelial cells is thus vital for an efficient barrier protection.

Neutrophils are potent phagocytes loaded with a dazzling array of antimicrobial effector molecules. These cells, of hematopoietic origin, differentiate from the bone marrow and enter the circulation as mature phagocytes. The lifetime of the neutrophils is short in the circulation. If they get alarmed through chemotaxis by bacterial compounds, such as N-formyl-MetLeuPhe (fMLF), they will transmigrate from the blood to the site of infection loaded with effectors and exert their functions by phagocytosis of microbes or by release of antimicrobial molecules. It should be mentioned that mouse neutrophilic granulocytes do not produce alpha-defensins, thus questioning results from mouse models as responses in human with reference to studies of these AMPs (27).

As indicated above, mature epithelial cells and phagocytes are constantly alert and ready to kill microbes by constitutive expression of genes encoding antimicrobial effectors. However, both epithelial cells and phagocytes must be activated to enhance the expression of innate immune effectors. Several pathogens

suppress these immune effectors as part of their virulence in order to invade the body.

DOWNREGULATION OF AMP EXPRESSION

The gut microbiota plays a major role in the maintenance of the overall health of the host. AMPs and additional innate effector molecules are generally produced by epithelial cells as well as by circulating inflammatory cells. The epithelial-derived AMP-based defense system has co-evolved with diverse microorganisms and has been shown to regulate the composition of the commensal microbiota (28, 29). Expression of AMPs in the gastrointestinal tract is considered constitutive and has a symbiotic relation to the microbiota. It appears that the natural microbiota is partially spared from the lethal action of AMPs and certain bacteria have adapted resistance. One potential mechanism by which the commensal microbiota reduce their susceptibility to AMPs is by membrane modification, reducing the net negative charge of the bacterial surface, thus decreasing the interactions with AMPs (30). The unique microbiome composition of an individual host is influenced by multiple factors, including the use of specific drugs and antibiotics. Interestingly, activation of endogenous AMPs can eliminate pathogenic bacteria and promote a balanced microbiota in the gut, while conventional antibiotics non-selectively decrease bacterial numbers and diversity in the gut (31).

Different bacterial species thrive in the luminal milieu of the intestinal tract and the microbiota of the gut has evolved by metabolic adaptations. The microbial ecosystems produce a spectrum of metabolites within the human host with diverse functions. Many of these metabolites with specific functions have been characterized and have the ability to limit the growth of surrounding bacteria. The natural microbiota lacks virulence factors needed to break through the epithelium (9). In addition, there is interspecies competition and regulation of the composition between the different bacteria of the natural microbiota. Interestingly, the expression of surface defense molecules is regulated by the microbiota that secrete metabolic products, such as butyrate and lithocholic acid (LCA) (32). Bacterial products modulate the expression of defense molecules that in turn regulate the composition of the microbiota. Butyrate, a short chain fatty acid (SCFA), is produced by fermentation of starches, dietary fiber, sugars, and glycosylated proteins by the intestinal microbiota.

Pathogens exhibit different metabolic adaptation compared to the natural microbiota, and several pathogens have evolved strategies, making them resistant to AMPs [reviewed in Duperthuy (33)]. These strategies include modifications of lipopolysaccharide, modifications of phospholipids, efflux pumps and proteolytic degradation of AMPs. Interestingly, some pathogens in contact with AMPs at low concentration can induce resistant mechanisms and major virulence factors, which would promote their invasive potential.

Furthermore, many pathogenic bacteria have evolved mechanisms to invade the tissue by sophisticated mechanisms to break through the mucosal epithelial barrier in order to avoid or circumvent the defense pathways. We first reported down-regulation of LL-37 and human beta-defensin-1 (HBD-1) expression in human biopsies of patients with early stage of *Shigella* infection (34). Plasmid DNA appeared to be responsible for turning off the expression of these AMPs in the epithelial surface as a virulence strategy to escape the immune surveillance. Others also confirmed the downregulation of AMPs, and secreted proteins were claimed to be the mediators responsible for this downregulation (35). As a proof-of-principle, using an animal model of shigellosis, we were able to demonstrate that *Shigella* spp. down-regulated CAP-18 (rabbit cathelicidin) in the large intestine, at the site of infection (13). Interestingly, downregulation of CAP-18 was also observed in remote epithelial lining of lungs and trachea, where no infection occurred (6). Later, it was shown that *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) suppress LL-37 and HBD-1 expression in intestinal epithelial cells through the actions of cholera toxin (CT) and labile toxin (LT), respectively (36). A similar scenario was observed in patients with cholera and ETEC diarrhea during early infection (37). Furthermore, our group showed that pathogenic strains of *Neisseria gonorrhoeae* downregulated the expression of LL-37 in a human cervical epithelial cell line, while non-pathogenic or heat killed pathogenic strains of *Neisseria* did not have such an effect. This result suggested a survival strategy of the pathogen during invasion of the genital tract (38). In experimental studies, we further demonstrated that *Vibrio cholera* or enteropathogenic *E. coli* (EPEC) also can downregulate CAP-18 in the small intestinal epithelia (39, 40).

These studies led to the conclusion that counteracting or blocking pathogen-mediated down-regulation of endogenous AMPs could be used for treatment of infections. Various small molecular compounds are known to induce endogenous AMPs, where immunomodulatory properties could be harnessed to treat infections. Host directed therapy (HDT) against pathogens by boosting the expression of endogenous AMPs is thus considered as a novel alternative for treatment of infectious diseases (**Figure 1**).

INDUCTION OF INNATE EFFECTORS IN PHAGOCYTES AND EPITHELIUM

AMPs are potent antibacterial substances with activity comparable to classical antibiotics. Initially it was suggested that the peptides could be utilized in therapy as direct antimicrobial substances. The disadvantage with this approach is that the peptides are enzymatically degraded in the gastrointestinal tract, and when injected they might evoke production of antibodies, limiting the usage to topical administration for skin infections and treatment of wounds. Furthermore, monotherapy with single synthetic peptides could lead to selection of resistant strains (41). Therefore, our logical approach was to stimulate AMP production by activating regulatory pathways in the cells, without stimulating inflammatory responses through NFκB. This would result in induction of several genes encoding active peptides, mimicking the true response in our front-line defenses

(42). Accordingly, small molecules that can be administrated orally have been used as inducers of AMP-expression both *in vitro* and *in vivo*. By this strategy multiple peptides with different killing mechanisms together with NO and ROS would be engaged, making selection of resistant bacterial strains unlikely. These small inducing molecules can enter the blood stream and have systemic effects, as well as also boosting innate defenses at mucosal surfaces and in phagocytic cells (6, 43–46).

The idea to improve mucosal defenses was proposed by Fehlbaum et al. (47) where they showed that isoleucine could induce transcription of human beta defensin 2 in bovine epithelial cells. Shortly after this, we identified butyrate as a major inducer of AMP-expression (48, 49). Butyrate is a product of fiber fermentation by the natural microbiota and the carbon energy source for colonic epithelial cells. Butyrate is known to be an HDAC inhibitor and has earlier been used in a *Shigella* infection model with beneficial effects (50). However, the mechanisms underlying these beneficial effects remained elusive at that time (51).

Today several of the inducers of AMP expression have been identified as histone deacetylase inhibitors (HDACi) (52). The activity relates to histone modifications, where histone acetyltransferase (HAT) mediates acetylation of lysine residues, resulting in reduced ionic interactions between the basic histones and the acidic DNA with more open chromatin structure. In contrast, HDAC reverses this reaction, and if inhibited the histones are maintained in the acetylated form. Therefore, HDAC inhibition affect the chromatin structure from tightly packed toward open with enhanced access of different transcription factors and machinery for transcription, including the RNA polymerase II. Typically, the responses to HDACi are complex and involve many genes, and some pathways of HDACi are physiologically relevant. Butyrate is considered an important part of the normal human physiology together with other short chain fatty acids (SCFA), enhancing the expression of mucins and AMPs in intestinal epithelial cells. SCFAs strengthen defenses and enhance epithelial functions by increasing tight junctions and thus maintaining barrier integrity. Various bacterial species produce butyrate in the human colon, most of them belong to the Firmicutes phylum, in particular the clostridial clusters IV and XIVa, which have been associated with a healthy gut homeostasis (53). Altered composition of the microbiota with reduced growth of butyrogenic *Clostridium* species has been linked to susceptibility to infections and inflammation (54).

Butyrate also affects various immune cells in the gut with beneficial effects of the host, balancing inflammatory reactions to homeostasis of host microbe interactions in the gut (55). The specific receptors of butyrate GPR41 (FFAR3) and GPR43 (FFA2) are G-protein coupled receptors that have been characterized on several immune cells (56). The anti-inflammatory properties of butyrate have been shown to be dependent on the expression of GPR43 in regulatory T-cells (57). Butyrate can also enter cells for example via the SLC5A8 transporter (58), which is needed for the HDACi activity. In summary, both the HDACi and receptor activation by butyrate are needed for imparting the beneficial effects of butyrate, leading to enhanced barrier integrity (59).

The concentration of butyrate in the gut is 2.3–26.1 mmol/kg, while it is 1–64 μM (60) in the blood stream. Accordingly, butyrate can be absorbed from the colon, and affect remote organs, which we have shown for the pancreas and the lungs (6, 44).

Butyrate is a foul-smelling compound and is not possible to use it as an oral drug. Therefore, we searched for alternative compounds and identified phenyl-butyrate (PBA), a drug already approved for treatment of urea cycle disorders by virtue of the ammonium scavenging capacity (61). Importantly, PBA is available in tablet form and using animal models, we have confirmed that it has a similar induction profile of AMPs as butyrate (6). It is also an HDACi as well as a chemical chaperone (62). Since PBA is a registered drug for clinical use, all toxicity, and regulatory studies have already been performed and thus, it could enter the clinic in a fast track for additional indications (Phase IIB-trials).

The most recent research on butyrate further underlines its important role. In a mouse model, butyrate was found to imprint antimicrobial programs in macrophages (63). Butyrate was demonstrated to increase antimicrobial activity, reduce mTOR kinase activity, increase LC3-associated host defense, as well as enhancing AMP expression without increasing the production of inflammatory cytokines. The antimicrobial response was dependent on HDAC3-expression, which was shown by blocking specific transcription using siRNA (63). In line with this result, butyrate was shown to protect intestinal epithelial cells from damage caused by *Clostridium difficile* in a mouse model (64). This effect was mediated by stabilization of the transcription factor HIF-1α, resulting in attenuated inflammation and improved intestinal barrier. Thus, butyrate is a key player in host defense against inflammation and infections (64). The pathway involving HIF1α has also been shown to be important in the inhibition of *Candida albicans* by commensal bacteria, where cathelicidin was highlighted as an important contributor (65).

HDAC inhibition has long been known and, as the name indicates, relates originally to the effect on histones and chromatin structure. In recent years, acetylation has been shown to be much broader, comprising many non-histone proteins. These proteins are involved in key cellular events including signal transduction, autophagy, metabolism, protein folding, and cell division (66). Accordingly, the acetylation was renamed to lysine acetyltransferase (KAT) and lysine deacetylase (KDAC) to underline broader targets. These epigenetic mechanisms have recently been thoroughly reviewed in relation to innate immunity and infections (67). Clearly, the regulatory circuits of HDACi, or rather KDACi, in relation to induction of AMPs are complex and many specific details are still to be clarified. Therapeutic interventions enhancing defenses by affecting these regulatory pathways to prevent or fight infections are thus worth pursuing. Adverse effects have been of concern for HDACi and must be considered for each inducing compounds. However, HDACi is a part of our physiology exemplified by the production and release of butyrate in the gut.

We have shown co-operative inducing activity between butyrate/PBA and vitamin D3 on the expression of the *CAMP*

gene encoding LL-37 in lung and gut epithelium (68). The cooperativity with respect to the *CAMP* gene relates most likely to effects on different regions of the promoter. Interestingly, in lung epithelial cells with the VDR expression knocked-down by siRNA, the induction of the *CAMP* gene by PBA and butyrate was reduced (69). Butyrate and PBA likely altered chromatin structure and thus increased the access of VDR to the *CAMP* gene promoter. PBA and butyrate are not cognate ligands to VDR, but acetylation of components in the signal transduction pathway of VDR is the most likely explanation for the observed cooperative effect.

Vitamin D3, a nutritional component, has been shown to be a potent AMP inducer. This activity of vitamin D3 was originally found in a general screen of vitamin D3 induced genes. The induction of the *CAMP* gene expression was outstanding compared to other affected genes identified (70). Subsequently, the effects of vitamin D3 on AMP-expression in relation to tuberculosis (TB) and leprosy were demonstrated both *in vitro* and *in vivo* (71), and the anti-mycobacterial activity was dependent on the *CAMP* gene (72). Binding of the vitamin D receptor (VDR) to the promoter of the *CAMP* gene, with subsequent production of the human cathelicidin LL-37, was shown by Gombart et al. (73) and more specifically in the skin by Weber et al. (74). Importantly, the vitamin D3 effect was specific for cathelicidin-expression in primates and was dependent on a transposable element that entered the gene late in evolution (75). Rodents do not have this insertion element and hence the mouse has not been a suitable *in vivo* model for vitamin D-mediated responses against infections. However, a mouse model of the vitamin D-induced human cathelicidin was recently generated. This transgenic mouse has some of the features seen in humans after vitamin D3 induction, with enhanced killing of *Staphylococcus aureus,* as well as induced *CAMP* gene expression following topical induction in the skin (76).

Notably, vitamin D3 has been known to induce the differentiation of monocytes into macrophages. The presence of vitamin D during differentiation promotes the expression of cathelicidin and intracellular control of mycobacterial growth (77, 78).

The observation of the synergy between PBA and vitamin D, together with the potent effect of *CAMP* expression in the lung epithelial cell-line VA10, and against Mtb prompted us to continue with clinical studies. Previous clinical trials using vitamin D as an adjunctive therapy together with standard TB-treatment have failed to show positive effects on pre-specified clinically relevant endpoints (79). However, a significant effect on time to sputum culture conversion was observed when a polymorphic variant (TT genotype) of the VDR receptor variant TaqI was present (80).

Interestingly, in our study using PBA and vitamin D as adjunct therapy together with the four classical TB drugs Isoniazid, Rifampicin, Ethambutol, and Pyrazinamide, enhanced the antibiotic activity. Furthermore, the outcome was significant for sputum clearance together with a decline in clinical score. These results indicate that host directed therapy is effective against difficult to treat infections (14).

Additional inducers would be of interest to broaden our concept for host directed therapy. We therefore set up a strategy to screen for novel inducers that included the development of a reporter-system cell line containing the *CAMP* gene fused to luciferase (52). The initial screen was with a panel of HDAC inhibitors followed by a Prestwick library of 1,200 compounds of FDA approved drugs. Several compounds were identified as *CAMP* gene inducers, but the HDACi Entinostat gave the most prominently induced response at a low concentration (2.5 μM). Subsequently, we showed Entinostat to be effective in animal models for *Shigella* and *Vibrio cholera* infections (8, 40). Detailed analyses of the mechanism for induction of Entinostat was dependent on the transcription factors STAT3 and HIF1α. HIF1α was found bound to the *CAMP* gene promoter, while STAT3 activates HIF1α (81), but the initial steps of the activations are still not clarified. Since Entinostat is toxic at high concentrations and has been developed as a cancer drug candidate, we therefore made several variants of Entinostat called aroylated phenylenediamines (APDs), with the aim to improve water solubility and to reduce toxicity (8). Several of these non-toxic lead compounds are active in inducing several AMP-genes in colon and lung epithelial cells (8, 82). We are currently in the process of developing these compounds further as drugs for host directed therapy against infection. The approach is to utilize these novel inducers alone or in combination with conventional antibiotics.

The effects of vitamin D on the *CAMP* gene are well-documented and relevant for *Mycobacterium* infections, both leprosy and tuberculosis (71, 78). The vitamin D receptor (VDR) binds to DNA as a heterodimer in complex with the retinoid X receptor (RXR). The role of the RXR partner in this context has not been defined in detail. Partners of class II nuclear receptors, other than VDR, are farnesoid X receptor (FXR) and the retinoic acid receptor (RAR). Interestingly, these partners are also linked to AMP induction and innate immunity.

Bile acids are ligands to FXR that can induce *CAMP* gene expression. The *CAMP* gene induction by FXR was initially linked to biliary duct sterility (83). In mouse models, utilizing knockout mice of the *CYP27* gene with reduced bile synthesis or knockout mice for the *FXR* gene (NR1H4 gene), a broader effect of FXR was seen to regulate innate immunity in small intestinal epithelial cells (84). FXR and bile acids regulated the expression of several antimicrobial components and affected the occludin protein of the tight junctions (84). Pronounced effects of deleted FXR or reduced bile synthesis on antimicrobial activity and bacterial translocation were observed. FXR has also been highlighted in inflammation as counteracting TLR-4 mediated response in myeloid cells (85).

Furthermore, the synthesis of retinoic acid, the ligand for RAR/RXR heterodimer, has been linked to epithelial transcription programs of defense. Commensal *Clostridia* species can modulate retinoic acid availability by affecting the synthesis and thereby the IL-22-dependent antimicrobial responses. Bacterial regulation of retinoic acid synthesis was shown to be important for the balance of the microbiota (86), indicating a role for RAR/RXR in the homeostasis of the natural microbiota.

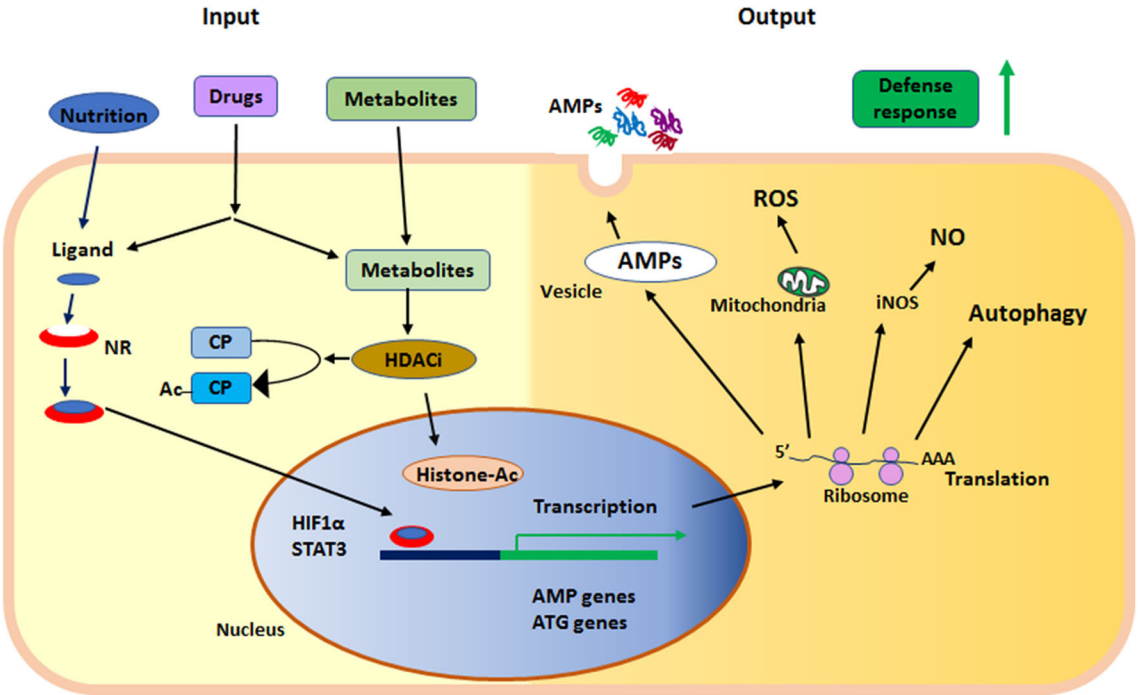

FIGURE 2 | Summary of the main events in early defense by innate effector mechanisms described in the text. NR, nuclear receptor; HDACi, histone deacetylase inhibition; AMPs, anti-microbial peptides; ROS, reactive oxygen species; NO, nitric oxide; iNOS, inducible nitric oxide synthase; ATG, autophagy related gene; HIF1α, hypoxia-inducible factor 1-alpha; STAT3, signal transducer and activator of transcription 3; CP, cytoplasmic protein; Ac, acetylation; Histone-Ac, acetylated histone and open chromatin.

Other orphan members of nuclear receptors with unknown natural ligands have emerged as crucial regulators of inflammation and immune responses (87), but are not directly linked to induced expression of AMPs.

In summary, nuclear receptors and KDACs are sensors of metabolites and nutritional components, regulating the status of innate immunity. Together these proteins serve as sensors in the epithelium at the interphase of the outside environment and the tissue, where microbial exposure occurs. Both nuclear receptors and KDACs can be affected by synthetic ligands and are thus motives for enhancing defenses to prevent or fight infections (**Figure 2**).

ACTIVATION OF AUTOPHAGY TO FIGHT BACTERIAL INFECTION

Autophagy is a conserved mechanism to maintain homeostasis and is tightly linked to cellular metabolism. It is also involved in the cellular defense mechanisms against a range of intracellular microbes. Several important primary pathogens, including Mtb, *Salmonella,* and *Legionella*, have evolved mechanisms to avoid or block the autophagic process (88). Thus, efforts to restore autophagy by using small molecular drugs could be used to fight infections against these bacteria. Here we will review recent reports and results, including drugs that have been used for this particular purpose.

Autophagy is a general concept that can be further subdivided into macro-autophagy, mitophagy, xenophagy, LC3-associated phagocytosis, and chaperone-mediated autophagy, with specific but also overlapping functions (89). Here we will mainly discuss xenophagy, which specifically targets bacteria (90).

Mtb specifically targets autophagy and blocks the fusion between the phagosome and the lysosome (91, 92). This mechanism facilitates the intracellular survival of Mtb in human macrophages and is essential for the virulence of Mtb. Thus, restoration of autophagy has been selected as a target for host-directed therapies against Mtb. Several FDA-approved drugs with the potential to restore autophagic function during Mtb-infection have been identified. Vitamin D, for example, has been known to have beneficial effects against Mtb since the pre-antibiotic time, but the mechanisms have not been well-described. However, Liu et al. (71) showed that intracellular control of Mtb growth is regulated by vitamin D via the induction of the AMP LL-37. LL-37 has direct anti-mycobacterial effects *in vitro*, but other mechanisms may also be involved. One intriguing possibility is that LL-37 acts as a mediator of autophagy activation (93), which is a claim that has been further substantiated by our results, showing that vitamin D activates autophagy via a paracrine loop and activation of the surface associated P2X7-receptor (7). In fact, the autophagy-related genes Atg5 and Beclin-1 are induced by vitamin D in human macrophages (7, 93). It was recently shown in a *Helicobacter pylori*-model that vitamin D can restore lysosomal degradation by activation of the protein

disulfide isomerase family A, member 3 (PDIA3) receptor via upregulation of Ca^{++} channels, resulting in a normalized lysosomal acidification (94).

Notably, the autophagy-activating effect of vitamin D is further enhanced by the addition of PBA and the combined effect of these compounds depends on LL-37 in order to activate autophagy. The mechanism behind this effect involves VDR-activation but most likely also chromatin remodeling caused by the histone deacetylase effects exerted by PBA (7). In addition, PBA alone has a direct growth inhibitory effect on Mtb (95). Due to the promising effects of vitamin D on autophagy and inhibition of Mtb growth in human macrophages, several clinical trials of vitamin D supplementation as adjunctive treatment to patients with pulmonary TB have been performed (79). However, the clinical outcome in these trials have been limited in relation to the primary endpoint (sputum culture conversion). The combination of vitamin D with PBA resulted in a faster conversion of sputum culture and better clinical TB-score in two clinical trials (14, 96). Furthermore, some positive effects of vitamin D3 have been observed with regard to pre-selected secondary endpoints, including pro-inflammatory cytokines (97).

The discrepancy between the *in vitro* results showing beneficial effects of vitamin D3 and the mostly negative results from clinical trials is intriguing. One reason for the lack of effect of vitamin D3 in clinical TB-trials is that the standard treatment is usually very effective, and any beneficial effect of adjunctive vitamin D3 treatment would require a very large study cohort to detect a statistically significant difference. Based on these assumptions it has been proposed that the true benefit of an adjunctive protocol with any immunomodulatory drug would be found in trials on MDR TB-patients, where the standard drug regimens are ineffective. In fact, a recent meta-analysis comprising 1,850 participants in 8 interventional trials found that vitamin D3 accelerated sputum culture conversion in patients infected with MDR Mtb, but not in those patients with susceptible Mtb isolates (79). Another important factor may be the daily dose vs. bolus dose of vitamin D3 given over a period of weeks or months, which may not be suitable to impart beneficial impacts on the immune system to fight TB disease (14, 96). Finally, vitamin D3 supplementation has been shown to exhibit the best effect against respiratory tract infections in individuals with prominent vitamin D3 deficiency (<25 nmol/L). However, the beneficial effect was evident also in those individuals with serum levels of 25 OH-vitamin D3 up to 75 nmol/L (98).

On the other hand, it has been shown that the common TB-drugs isoniazid and pyrazinamide activate autophagy (99). The beneficial effect of this *in vitro* finding remains to be shown in a clinical context, but adds another layer of complexity to the story.

In addition to vitamin D3, several other drug-like compounds have been identified in different screens for autophagy inducing agents. For example, the anti-parasitic drug nitazoxanide, the anti-depressive drug fluoxetine and the EGFR-inhibitor gefitinib, have all been shown to activate autophagy. In addition, the anti-epileptic drugs carbamazepine and valproic acid also activate autophagy and impair Mtb growth in human macrophages (100).

Autophagy is also important for host defense against other bacteria, not classically considered to have an intracellular lifestyle. One example is *Klebsiella pneumoniae*, where downregulated or impaired autophagy leads to increased bacterial growth and increased mortality in mice (101). However, it should be noted that for some additional extracellular bacteria, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, autophagy appears to be exploited by the pathogens to cause disease. Consequently, impaired autophagy has been shown to lead to a beneficial outcome in infectious models (102–104), which represent a reverse situation in comparison to Mtb and other strictly intracellular bacteria.

To conclude, activation of autophagy clearly seems to have beneficial effects during infection with Mtb, but for other bacteria the effects might be different. Thus, it is important to delineate the role of autophagy for specific bacteria since modulation of autophagy could potentially lead to beneficial or detrimental effects, depending on the context.

ANTIBIOTICS AND EFFECTS ON HOST IMMUNITY

Antibiotic treatment is the natural first choice of treatment against bacterial infections. The traditional paradigm considers that antibiotic drugs specifically target bacteria. However, several antibiotic drugs also affect innate immunity via direct effects on host signaling pathways. The most potent antibiotic drug classes in this respect are the quinolones, the macrolides and drugs used for treatment of tuberculosis, mostly studied for the first-line drug rifampicin. In this section, we include examples of antibiotics that have been shown to have direct effects on host immunity.

Ciprofloxacin, one of the best-studied antibiotics of the quinolone class, was developed as a potent inhibitor of topoisomerase 2 in bacteria. It is active against both gram-positive and gram-negative bacteria. It is widely used by virtue of its good and broad intracellular penetration effects and low adverse event profile. However, resistance against ciprofloxacin has emerged, and consequently the recommendations regarding empirical use of this drug have been changed. In addition, potentially serious adverse side effects are also increasingly acknowledged. These side effects include cardiac arrhythmias, tendon ruptures, and the selection for *Clostridium difficile*-associated diarrhea (CDAD).

Ciprofloxacin has a direct bactericidal effect against bacteria via the SOS-response, which releases large amounts of ROS, causing DNA-damage, and bacterial death (105). Consequently, scavengers of ROS inhibit the effects of ciprofloxacin, which provide evidence for a ROS-dependent bacterial killing. It is well-known that ciprofloxacin has profound effects on the intestinal microbiome. In fact, an altered microbiota was evident up to 1 year after treatment of ciprofloxacin (106, 107). Clinical use of ciprofloxacin is associated with a higher risk of developing CDAD, which could possibly be mediated via direct effects on the microbiota (108). However, ciprofloxacin has also been shown to downregulate colonic expression of

AMPs (109). Recently, it was also shown that ciprofloxacin caused a loss of the mucosal barrier and immune-function in the intestine (110). Thus, it is becoming increasingly clear that ciprofloxacin has potent and direct effects on innate immunity. For example, ciprofloxacin has been shown to upregulate IL1-β and TNF-α expression in human macrophages (111). In a clinical study, patients with gram-negative sepsis that were treated with ciprofloxacin exhibited lower levels of pro-inflammatory cytokines, compared to those patients treated with a beta-lactam antibiotic (112). To conclude, ciprofloxacin is widely used and it has a profound impact on the microbiota. However, ciprofloxacin also exerts immunomodulatory functions, which may be beneficial (anti-inflammatory) or detrimental (down-regulation of AMP-expression) depending on the circumstances.

Azithromycin (AZM) is a macrolide antibiotic drug targeting the 50S subunit of the bacterial ribosome. It is widely used against respiratory tract infections, since it is also active against atypical bacteria, including *Mycoplasma* spp., atypical mycobacteria and *Legionella* spp. It also has been extensively used as a prophylactic drug in patients with asthma and bronchiectasis. The prophylactic use of AZM was further substantiated recently in a randomized and placebo-controlled trial, where the number of asthma exacerbations was reduced, and the quality of life was increased (113). A Cochrane review has also concluded that there are beneficial effects of AZM in patients with bronchiectasis (114). The drawbacks are the potential risk of the development of resistance against AZM and adverse events, such as arrhythmias, diarrhea, and arthralgia (115).

In addition to its bacterial target, AZM has potent immuno-modulatory effects. These effects are considered to contribute to its efficacy as a preventive agent in patients with respiratory diseases. For example, it was recently shown that AZM protects against *Pseudomonas* infections in the lung via direct inhibition of the inflammasome (116). In addition, AZM polarizes macrophages toward an M2 phenotype via inhibition of STAT1 and NFκB (117). AZM also contributes to the strengthening of the barrier in the airways by induction of tight junction proteins (118). Further, AZM induces epidermal differentiation, which potentially can protect the lungs during infection (119). Another layer of recently added complexity is that AZM was shown to alter the microbiome and metabolome of the lung, promoting bacterial metabolites with anti-inflammatory effects of the airways. Finally, AZM may protect against virus infections, as shown in the context of zika-virus, where AZM induced protective type 1 and type 3 interferon responses (120). The antiviral effects of AZM could also explain the beneficial effects in asthmatic patients, where rhinovirus often triggers exacerbations (121). One way to reduce the problems with antibiotic resistance is to use a macrolide analog without antimicrobial properties but with retained anti-inflammatory effects. One such non-antibiotic macrolide drug candidate was recently shown to reduce inflammation in an allergy mouse model (122).

A third group of antibiotics with potent interactions with the immune system is the TB-drugs. This has been best studied for the first-line drug rifampicin (RIF), which has an impact on the immune responses during TB-infection at several levels. One key mechanism appears to involve direct interactions between RIF and nuclear receptors. For example, it has been shown that RIF activates pregnane X receptor (PXR) that can promote TB growth in macrophages via increased efflux of TB-drugs out of the cell (123). This leads to sustained bacterial growth in the presence of the drug, which may accelerate the development of resistance. Notably, *M. tuberculosis* with a single nucleotide polymorphism in the rpoB-gene bypasses the protective IL1-β response and instead promotes an interferon-β response. This causes metabolic reprogramming of macrophages, ultimately promoting bacterial growth (124). A recent study links the aryl hydrocarbon receptor (AhR) to TB-treatment. AhR is a member of the family of basic helix-loop-helix transcription factors and can be activated by tryptophan metabolites. RIF, and the related drug rifabutin, are sensed by the AhR, leading to impaired phagocytosis of Mtb by macrophages. The application of a small molecule inhibitor of AhR caused decreased phagocytosis and improved killing of Mtb (125). These findings collectively demonstrate that TB-drugs are sensed by the innate immune system, which activate changes in host cells that may be detrimental for the control of intracellular bacterial growth. Nevertheless, the clinical implications of innate immune sensing of TB-drugs remain to be shown. It should be noted that rifampicin has been the most essential drug for TB-treatment for decades. However, it is possible that drug concentrations below the MIC-value will impair endogenous defenses, as suggested by Puyskens et al. (125), which could play a role in the resolution of infection.

CONCLUSION

Initial innate defenses at epithelial surfaces are complex and include AMPs as important effectors. Many of the surface effectors including ROS and NO are also present in phagocytes, the second line of early defenses. The expression of AMPs is included in the differentiation program of innate immune cells but is also influenced by signals from the environment. Over the last decade, it has emerged that metabolites from bacteria and nutritional components are influential environmental mediators for the expression of the innate immune effectors. These innate components constitute an important barrier against invading pathogenic microbes, preventing them from entering the internal host environment. The strength of the barrier is decisive for keeping microbes at bay and the environmental signals are important for a tight barrier. Several pathogens suppress the expression of barrier components and thereby manage to open an entrance into the body, leading to infections. Details behind the interactions between bacteria and host cells must be resolved for each pathogen and much work remains to be done. However, powerful analytical models based on gene deletions in bacteria and host cells from different organisms are developing fast and open up for novel treatment approaches. A detailed knowledge on pathogen-mediated suppression of innate immunity and the discovery of counteracting compounds is the focus of our research. For example, inducers of innate effector mechanisms may be used alone or in combination with antibiotics. Such approaches would likely reduce the spread of resistance strains. Future research efforts on the interaction between multidrug-resistant bacteria and the host is warranted. The use of host directed therapy

holds the promise to promote health and reduce the spread of antibiotic-resistant strains.

AUTHOR CONTRIBUTIONS

PB, RR, BA, and GG planned the outline, wrote, and edited the manuscript. RSR edited and made the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Dichotomous Responses Driven by β-Defensins

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Defensins are short, rapidly evolving, cationic antimicrobial host defence peptides with a repertoire of functions, still incompletely realised, that extends beyond direct microbial killing. They are released or secreted at epithelial surfaces, and in some cases, from immune cells in response to infection and inflammation. Defensins have been described as endogenous alarmins, alerting the body to danger and responding to inflammatory signals by promoting both local innate and adaptive systemic immune responses. However, there is now increasing evidence that they exert variable control on the response to danger; creating a dichotomous response that can suppress inflammation in some circumstances but exacerbate the response to danger and damage in others and, at higher levels, lead to a cytotoxic effect. Focussing in this review on human β-defensins, we discuss the evidence for their functions as proinflammatory, immune activators amplifying the response to infection or damage signals and/or as mediators of resolution of damage, contributing to a return to homeostasis. Finally, we consider their involvement in the development of autoimmune diseases.

Keywords: beta defensin, psoriasis, atopic dermatitis, autoimmunity, immunomodulation, AMP

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INTRODUCTION

Defensins and defensin-like peptides are found throughout multicellular organisms including plants, insects and fungi, as well as vertebrates. They were first described as antimicrobial peptides (AMP), with the ability to rapidly penetrate and disrupt the outer membrane of bacteria, viruses and fungi to varying degrees and subsequently disrupt metabolic processes within (1). It is appealing to consider that this innate, protective mechanism is so fundamental that the defensin genes have been evolutionarily conserved for this purpose. In fact, the value of the structure of these disulphide-stabilised, cysteine containing, positively charged loop peptides has resulted in two evolutionarily distinct defensin families that have arisen separately by convergent evolution (2). The cis-defensin superfamily (present in insects, fungi and plants), has the central beta-strand stabilised by disulphide bridges, connected to the same alpha-helix in the "cis" orientation. This is in contrast to the vertebrate (and some invertebrate) defensins, in which the central beta strand has disulphide bridges that stabilise structures in non-cis or "trans" orientations (3, 4). Both cis and trans families have undergone rapid expansion and evolutionary change to reveal a repertoire of diverse functions that are only recently becoming clear (5).

Here we focus on the human, trans-defensins—specifically β-defensins. We discuss their role(s) in host defence other than by direct microbial killing. We consider whether the function of these molecules is purely as an acute "alarmin"-type response to danger/damage (alerting the body and promoting both local innate, and also local and systemic adaptive immune responses), or if they are also instrumental in controlling inflammation (limiting the damage response and mediating resolution)—thus speeding a return to homeostasis.

BETA-DEFENSIN BACKGROUND

The defensin family is a large, multigene family that is rapidly changing and evolving. In humans there are two functional subfamilies of defensins (α and β) which differ in their cysteine connections but retain the central structure of a trans-defensin cysteine knot. Both α- and β- defensins are generally encoded by two (sometimes three) exons, with the first exon containing the hydrophobic, anionic leader sequence and the second exon encoding the mature, cationic peptide. The α-defensins are stored in this inactive form in the granules of either neutrophils or intestinal Paneth cells, while β-defensins are expressed predominantly in epithelial cells and believed to be cleaved by signal peptidase as they are secreted (6).

β-defensins are an ancient family, from which the α-defensins have evolved. The amino acid sequence of human β-defensins is highly divergent, and has been subject to complex positive and negative selection (7). During this process, other than the cysteines, only a core glycine and aspartic acid are well-conserved. β-Defensins have an identifiable consensus sequence of $X_{2-10}CX_{5-7}(G/A)XCX_{3-4}CX_{9-13}CX_{4-7}CCX_n$ and the three disulphide connections following oxidation are assumed to be the same for all β-defensins (C_I—C_V, C_{II}—C_{IV}, and C_{III}—C_{VI}). The highly variable residues in the mature peptide are rich in the positively charged amino acids lysine and arginine to varying degrees (7). Some of the peptides have extended peptide tails with clusters of lysines and residues for additional potential glycosylation sites (8). In the human genome there are five β-defensin clusters located over three chromosomes, with around 33 genes, of which only a few have known function (9, 10) and seven of which (in humans) are hyper copy number variable (CNV) (5). The many gene duplications in the defensin family result in gene "birth and death" and as the gene number and sequence changes, some genes become specialised for a new function; while at the species level, there are increased numbers or complete loss of gene clades. Mice, for example, have different numbers of cryptdins (intestinal α-defensins) even between different strains of *Mus domesticus* (11) and no longer have neutrophil expressed α-defensin genes. The sequence diversity and gene number variation in the defensin genes is not surprising as strain specific diverse regions (SSDR) between mouse strains are highly enriched for genes involved in immunity, infection and reproduction functions, all of which are associated with defensins (12).

Gene duplication and sequence change, followed by selection for advantageous changes, allows functional change of paralogues. The structure of some off-shoots of the main β-defensin tree has been so advantageous that there are examples of both reptiles (snakes and lizards) and mammals (egg laying platypus) independently giving rise to venom toxins, with a variety of actions that include antimicrobial function (13) and potassium channel blocking ability (14). Additionally, Kudryashova et al. (15) showed that both α and β-human defensins could target, destabilise and inactivate bacterial protein toxins (16). This work implies that defensins may have protective abilities that are not limited to microbe destruction. Intriguingly, and perhaps indicative of roles of immunological modulation/damage, the human defensin HBD2 has been shown to bind to the outer pore domain of potassium channel Kv1.3 and efficiently inhibit channel currents and suppress IL-2 production in both human primary T cells and peripheral mononuclear cells (17).

At this point, the expression pattern of β-defensins in humans is worthy of mention [*see useful reviews on this here* (18, 19)]. All the many β-defensin members are strongly expressed in various segments of the epididymis post puberty (20, 21) and a major function of β-defensins is in sperm maturation. A β-defensin mutation in human *DEFB126* was found to reduce sperm motility and fertility in Chinese men (22). In addition, mice deleted for several β-defensins (in pairs or more) are infertile and this demonstrates their synergistic function in sperm maturation, movement and protection against premature acrosome reaction (23, 24). Sperm are rich in β-defensin in the glycocalyx of the head and this may protect the sperm from inappropriate activation. However, mice with transgenic over-expression of an epididymal specific β-defensin (orthologous to human β-defensin SPAG11), while being resistant to *E. coli* infection, simultaneously show reduced expression of inflammatory cytokines IL-1α and IL-1β, indicating multiple functions and implying immunomodulatory properties.

Expression of β-defensins is not just evident in the male genitourinary system, as these peptides are also widely expressed in other tissues. In this review we are focussing primarily on HBD1–4, as these genes are the most studied in human. Their peptide sequence, gene name and charge are given in **Table 1**. HBD1–3 are found in the female reproductive tract in endometrium, vagina and cervix, while HBD1 is found in fallopian tubes (26). These defensins are increased in expression at a number of sites in the body, including the tracheal epithelium, gingival mucosa, respiratory epithelium, gastrointestinal epithelium, genitourinary tract epithelium and skin (27–30). In addition, HBD1 is produced constitutively in a range of other epithelial tissues, including the small intestine, pancreas, and kidney. Expression of HBD1 may also be increased in various cell types following viral stimulation (31) and both HBD2 and HBD3 are inducible proteins, with expression occurring in various cell types in response to infection (32, 33), proinflammatory cytokines (including IL-1β, IL-17,TNFα, and IL-22) (34–36) and injury. The response to these inducers is not the same for every gene or for every condition. For example, plasma levels of β-defensins are variable in individuals with asthmatic vs. normal airways, where HBD3 is elevated by HBD1 and 2 are reduced (37). HBD3 and HBD4 are significantly increased, but HBD2 is decreased. The level of mouse DEFB14 was also increased in asthmatic animals. Expression may also be varied by genomic copy number of *DEFB103, DEFB4, DEFB104* but *DEFB1* does not show copy number variation (CNV). Both copy number and promoter sequence variation has been shown to contribute to expression of *DEFB4* and *DEFB103* (38, 39), but inflammatory stimuli can override these.

This widespread pattern of expression, and inducibility in infection and inflammation, raises the question of what is the principle function of these peptides? A number of studies have been conducted addressing whether β-defensins act as

TABLE 1 | Mature Peptide sequence of the four human β-defensins described most commonly in the literature.

PEPTIDE	GENE	Mature peptide sequence	Charge
HBD1	*DEFB1*	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK	4
HBD2	*DEFB4*	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP	6
HBD3	*DEFB103*	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK	11
HBD4	*DEFB104*	ELDRICGYGTARCRKKCRSQEYRIGRCPNTYACCLRKWDESLLNRTKP	7

Sequences are in single letter code and conserved cysteines are highlighted. DEFB4, DEFB103, and DEFB104 are on the hyper-copy number variable gene cluster in the human genome chr 8p and termed A or B in ensemble to distinguish their independent location in the genome (25). Please note we use here the common peptides names rather than the official designated peptide names (e.g., HBD1 instead of DEFB1etc.). Net charge at pH 7 calculated using https://pepcalc.com/.

immune or inflammatory modulators, but it is important to bear in mind that synthetic preparation and oxidation of defensins is not trivial. Correct cysteine disulphide bonding and oligomerisation may have an important effect on function as has been shown for the chemoattractive role of defensins (40). In some cases, recombinant peptides have been used, which poses some concern regarding contamination with Lipopolysaccharide (LPS). Some β-defensins are highly charged molecules and their structure *in vivo* can be monomeric or dimeric, oxidised or reduced, depending on the tissue, with known effects on both antimicrobial and other function(s) (40–43). In addition, apart from the reproductive tract, where expression is strong and constitutive, β-defensins are generally expressed at very low levels until induced by inflammatory mediators. The concentration of peptide used in *in vitro* experiments is therefore likely crucial to determine the true *in vivo* effect. Thus, studies using peptides *in vitro* are important, but may not always reflect physiological functional relevance. With those consideration in mind, we discuss below the evidence for β-defensins as host defence peptides, able to modulate the immune system in various ways (see **Figure 1**).

BETA-DEFENSINS AS ALARMINS

Alarmin is a term first coined by Yang and Oppenheim, for endogenous molecules that act as signals for tissue and cell damage (45). They are characterised by a number of central principles, which include the ability to recruit and activate innate immune cells, and bridge to and/or promote adaptive immune responses, whether through direct or indirect mechanisms (46, 47). Increasingly, β-defensins are shown to be involved in pathways of this type, acting as both chemokines for adaptive immune cells and as innate immune stimuli (detailed below). This is suggestive of an alarmin role for these peptides.

Chemokines

Both β and α-defensins can act as chemoattractants for immune cells (see **Figure 1**). Some years ago the similarity of defensin structure to chemokines was noted, alongside recognition that many chemokines can have antimicrobial activity under similar experimental conditions to those under which defensins were studied (48, 49). In addition, similarly to chemokines, defensins bind glycosaminoglycans (GAG) and oligomerise (50). Various human β-defensins can attract immune cells including immature dendritic cells, memory $CD4^+$ T cells, monocytes, and activated neutrophils at low (∼10–100 ng/ml) concentrations, similar to known chemokines (∼0.02 μM) (40, 51, 52). When this chemoattractant ability was first described, it was a very exciting observation, revealing defensins as a bridge between the innate response and adaptive immune cell recruitment. CCR6 (receptor for CCL20) was identified as a receptor through which defensins could mediate chemotaxis of lymphocytes and neutrophils, with structural similarities to CCL20 being detected (51–53). However, it was also shown that an as yet unidentified receptor, independent of CCR6, could mediate chemoattraction of CD4+ T cells and dendritic cells by a murine β-defensin (54). In addition, monocytes were shown to be attracted by HBD3 and this activity was shown to be dependent on the cysteine stabilised structure, whereas antibacterial activity was not (40). Interestingly, restoration of a single cysteine (cysV) was sufficient to enable human monocyte chemoattractant activity for HBD3 and its mouse orthologue Defb14 (55). Of physiological relevance, *in vivo* studies only found evidence for the HBD2-mediated attraction of macrophages (and not dendritic cells) following intraperitoneal injection of mice with the peptide (54, 56). Subsequently, CCR2 was shown to be a macrophage receptor through which HBD3 (and Defb14) could induce monocyte/macrophage cell movement (57). Indeed, HBD3 expression has been suggested to result in tumour associated macrophage attraction *in vivo* through CCR2 (58). In addition, HBD1, 2, and 4 can all have their expression increased by ΔNp63 in normal and squamous cell carcinomas and exert a chemotactic activity for (lymphatic) endothelial cells in a CCR6-dependent manner (59).

In addition to these direct chemoattractant properties, defensins can also function indirectly by inducing chemokine expression. Human keratinocytes exposed to a high concentration (30 μg/ml; ∼6 μM) of HBD-2, -3, or -4, increase the gene expression and protein production of IL-6, IL-10, IP-10, CCL2, CCL20, and RANTES. The treated cells displayed enhanced Ca^{2+} mobilization, chemoattraction, proliferation and phosphorylation of epidermal growth factor receptor (EGFR); signal transducer and activator of transcription (STAT)1, and STAT3 (60). This pro-inflammatory response was markedly suppressed by G protein coupled receptor inhibitors.

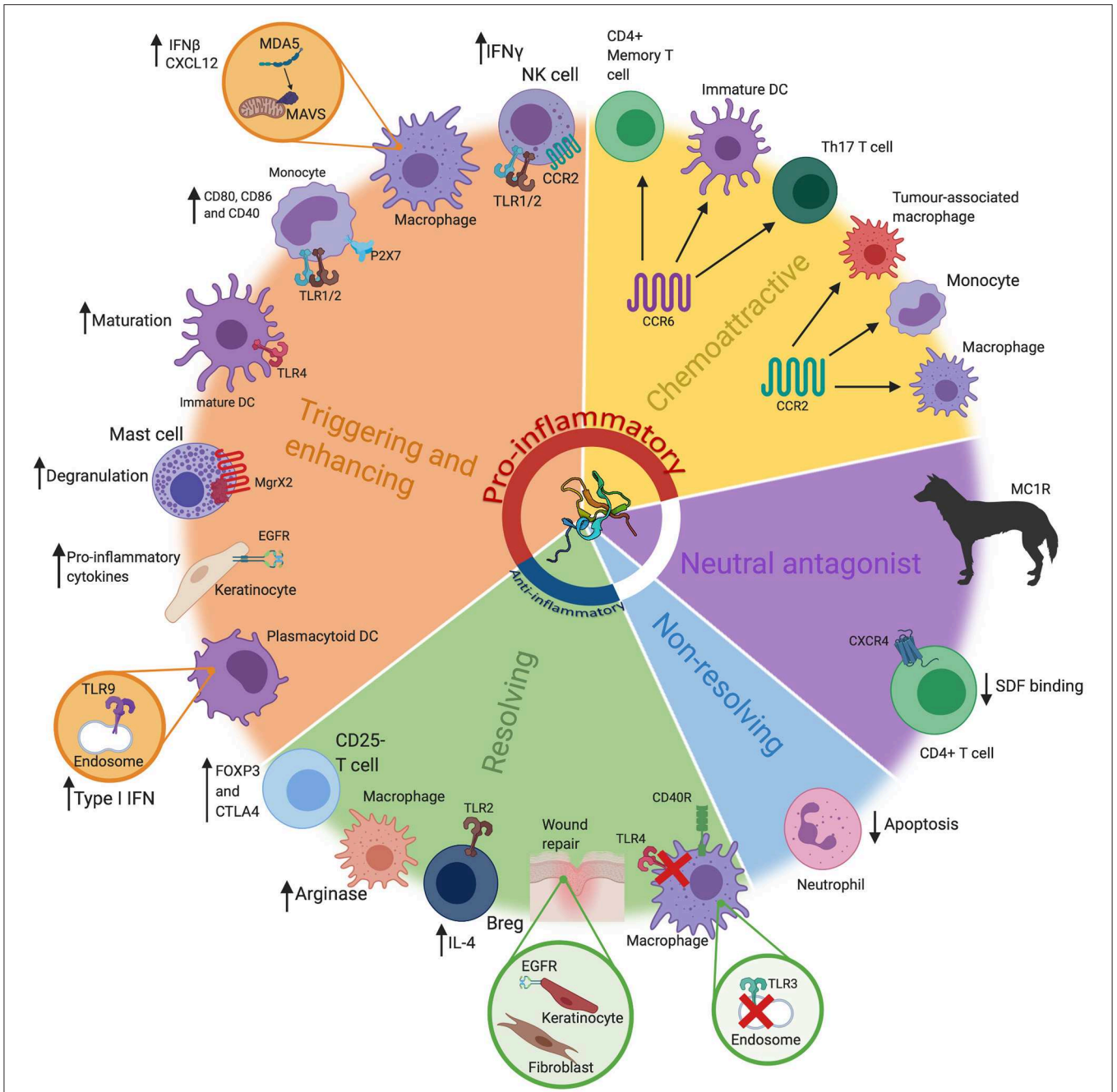

FIGURE 1 | The many roles of β-defensins. β-defensins have been shown to have a wide range of roles, that go far beyond basic antimicrobial activity. These can loosely be grouped into five key groups; triggering and enhancing, chemoattraction (chemoattractive), neutral antagonist, non-resolving and resolving. These functions are represented here, alongside the most prominent cell types/tissues/organisms associated with that particular role. Particular receptors that are known be involved in these pathways have also been highlighted, alongside the consequence of the β-defensin stimulus. These have also been grouped into pro-inflammatory or anti-inflammatory (or neither). Abbreviations: interferon (IFN), toll like receptor (TLR), Dendritic Cell (DC), interleukin (IL), T helper (Th), regulatory B cell (Breg). *β-defensin structure taken from PBD reference 1kj6 (44).*

Innate Triggers

In addition to acting to promote chemotaxis of a range of immune cells, the β-defensins have a range of other modulatory functions that expand their repertoire beyond simplistic microbicidal activity (see **Figure 1**). A proinflammatory response to HBD3 was observed in monocytes, when a robust concentration of 3.8 μM (20 μg/ml) was used to induce an increase in co-stimulatory molecules CD80, CD86m and CD40 and proinflammatory cytokines in a TLR1/2 dependent manner (61). However, unlike TLR2 ligands, HBD3 did not increase

levels of IL-10 and did not reduce co-stimulatory molecule expression (62). At 1 μM (5 μg/ml) HBD3, we see no evidence of proinflammatory responses in primary macrophages. At 5 μM and above, HBD3 can cause membrane damage in monocytes (but not B and T cells) through interaction with the negatively charged phospholipids (63), thus care is required to consider the concentrations at which cellular stress responses to supraphysiological conditions might occur. HBD3-mediated CD86 expression (but not CD80) was shown to be induced via the ATP-gated channel P2X7 (64). Similarly, recombinant mouse β-defensin 2 (*Defb2* peptide) was shown to induce maturation of dendritic cells via TLR4, proposing it as a potential adjuvant, although this was only observed with a fusion protein incorporating this peptide, and not with peptide alone (65).

Innate Enhancement

In addition to these stimulatory effects of antigen presenting cells, defensins have been shown to alter cellular processing, and inflammatory responses to DNA and RNA. In plasmacytoid dendritic cells (pDC), enhanced intracellular uptake of CpG or self-DNA was observed when the DNA was associated with either HBD3 or HBD2 at a 1:2 μM ratio, thus promoting TLR9-dependent IFN-α production in both human and mouse (66, 67). This was also observed with bacterial DNA in human and mouse pDC, but a response to self-DNA was only seen in the human cells (68). It is likely that these observed increases in ligand uptake and TLR9 signalling are due to the ability of these cell-penetrating peptides to increase the transport of the DNA into the cells (69). However, HBD3 is also able to oligomerise and may increase the ability of the DNA to interact with TLR9 effectively. This has been shown for HBD3 and another cationic host defence peptide cathelicidin, LL-37, as well as other cationic peptides. Schmidt et al. (70) elegantly showed that the peptides can form columnar nanocrystalline complexes with dsDNA and that the distance between the DNA columns influence a stronger or weaker interaction with the TLR9 receptor, which signals to produce type I interferon (71). Importantly these effects have also been observed *in vivo*, with intravenous injection of mice with CpG DNA:HBD3 complexes generating increased IL-6, IFN-γ, IL-12p70, IL-10, and IFN-α in the serum 24 h later when compared to CpG DNA alone, and an increase in antigen presenting cells in the spleen (66).

In addition, primary mouse macrophages, when pre-stimulated for 4 h with a fusion protein of IgG1 and the mouse orthologue of HBD3, Defb14, then subsequently stimulated for 24 h with endosomal (TLR3 and TLR9) or heterodimer (TLR1/2) ligands, showed an increase in proinflammatory cytokines and chemokine CXCL12, independent of the presence of CCR2 or CCR6 (72). The Defb14 fusion did not induce a cytokine signal on its own. These studies reveal a complex interplay with other factors, via which these defensins may contribute to enhanced adaptive responses.

Our lab has shown that the presence of HBD3 alters innate signalling to double stranded RNA poly I:C, increasing the Interferon-β (IFNβ) response and decreasing CXCL10 (IP10) production *in vivo* and *in vitro* in both mouse and human primary macrophages (73). PolyI:C is a synthetic double stranded RNA (dsRNA) and consequently acts as a mimic of virus or product of damaged cells. It is recognised by endosomally located TLR3 and also by cytoplasmic RIG-I-like receptors (RLRs). High molecular weight (HMW) poly I:C preferentially signals through the RLR MDA5 (Melanoma Differentiation-Associated protein 5), also known as IFIH1 (interferon induced with helicase C domain 1) and produces Interferon β (IFNβ). We showed that 0.1 μM HBD3 enhanced poly I:C-mediated MAVS (IPS-1) and MDA5 signalling, increasing IFNβ, but decreased TLR3 stimulation and CXCL10 signalling (72) in primary murine macrophages. The peptide rapidly entered the macrophages (within 10 min), decreased the endosomal localisation of the HMW PolyI:C and increased cytoplasmic localisation. This contrasted with the effect of the cationic lipid lipofectamine on HMW PolyI:C, which increased endosomal signalling through TLR3. LL-37, a cathelicidin cationic AMP with some similar immunomodulatory actions to HBD3 (74), can also increase dsRNA induced signalling through MAVS and TLR3 to increase production of proinflammatory cytokines and IFNβ in keratinocytes (75). For TLR3 this is partially due to the alpha helical LL-37 peptide forming crystalline structures with dsRNA which matches the steric size of TLR3, allowing recruitment and engagement of multiple TLR3 receptors and an increased cytokine signalling response (76) in a similar way to peptide-induced DNA association with TLR9. The increased signalling by MDA5 in the presence of HBD3 and HMW polyI:C might also be structurally dependent. Of note, linear HBD3 peptide does not increase IFNβ production and MDA5 normally forms filaments around dsRNA for oligomerization; we therefore speculate that this may be optimised in the presence of correctly-folded HBD3 (77).

The properties of other immune cells can also be modified by exposure to defensins, to promote host defence mechanisms. In the presence of HBD3, human NK cells increase CD69 C-Type lectin protein expression and secrete IFNγ, killing the NK sensitive myeloid cell line K562. In addition, HBD3 can function through the Mas related gene X2 to activate and initiate degranulation of mast cells (78, 79). Other cationic amphipathic peptides, such as LL-37, have also been shown to have this capacity.

Finally, defensins may modulate cell death, with possible consequences for inflammation. β-defensins have been shown to downregulate the pro-apoptotic truncated protein Bid and upregulate the anti-apoptotic Bcl-xL, leading to inhibition of mitochondrial membrane potential change and decreased caspase 3 activity and apoptosis (80). HBD3 is the most potent of the human β-defensins in this regard. Neutrophil apoptosis is important in resolution of tissue damage, thus limiting apoptosis may also be pro-inflammatory. In contrast, in human airway smooth muscle cells, the addition of HBD3 (at high concentrations of 5 or 10 μM) has been shown to induce CCR6-dependent production of IL-8 and cell apoptosis. This apoptotic effect appeared to be induced by ERK1/2 MAPK and ROS-induction (37). This may be important context for scenarios in which higher concentrations of the peptide are seen to be inflammatory and leading to cytotoxic effects. Cytotoxicity has been seen for high concentrations of HBD3 (over 20 μM) in

a wide variety of cells in culture, including DC, normal and immortalised keratinocytes and primary oral gingival epithelial cells (81).

Receptor Neutral Antagonism

There are several examples of defensins acting as promiscuous ligands for receptors (see **Figure 1**) helping to explain the pleiotropic properties observed. This may be due to complementary electrostatic interaction between the cationic peptide and receptors with anionic regions. HBD3, the most highly charged β-defensin (charge of +11) has been demonstrated to be a neutral antagonist, through charge based interaction with melanocortin receptor 1 and 4 (82). In dogs, a three base pair deletion in the canine orthologue of HBD3 results in an increase in the level of expression, which then allows this peptide to promiscuously bind the melanocortin receptor 1 (MC1R)—resulting in dogs with black, rather than agouti, fur (83). When the mutant or wildtype dog genes are expressed ubiquitously in transgenic mice, under a powerful promoter, their coat colour is also black (despite being genetically agouti). This demonstrates that an inappropriately high, level of β-defensin can result in promiscuous receptor binding *in vivo*. A further example of promiscuous receptor binding and neutral antagonist behaviour is the ability of recombinant HBD3 (at 5, 10, 20, and 40 μg/ml) to compete with stromal-derived factor 1 (SDF-1), in a structural and charge dependent manner, for cellular binding to CXCR4, without increasing calcium mobilization or chemotaxis (84, 85). CXCR4, also known as fusin, is used for HIV entry into CD4+ T cells. However, copy number increase of the HBD3 gene does not associate with protection against HIV (86).

BETA-DEFENSINS AS RESOLVERS

In contrast to alarmin activity (see section Beta-Defensins as Alarmins) we use the term "resolvers" here to describe the anti-inflammatory pro-resolving activity of β-defensins.

Innate Suppression

As discussed above, in the presence of defensins, some pattern recognition receptors increase the response to stimulation. However, exposure to TLR4 ligands (such as LPS) or CD40 activation in the presence of HBD3 (1 μM) results in a decrease in cytokine responses in primary macrophages (87, 88). This anti-inflammatory effect was also observed *in vivo*, where serum from mice displayed a decrease in proinflammatory cytokines following injection of LPS and HBD3 peptide compared to LPS alone (87). This suppression was independent of defensin binding to TLR4 or LPS and could be observed even if the peptide was added up to an hour after the LPS. HBD3 suppressed cytokine and type I interferon production through the MyD88 and TICAM1 pathways, respectively (89). Exposure to HBD3 and LPS compared to LPS alone showed reduced transcription of many genes associated with TLR4 activation, while others were increased, including TLR2—demonstrating that this was not simply an inhibition of all signalling downstream of the receptor. HBD3 alone had no effect on macrophage transcription. Further pathway analysis, using InnateDB, showed that many LPS-induced proinflammatory signalling pathways were downregulated when HBD3 was also present but that metabolism, classical complement activation and FcγR-dependent phagocytosis were upregulated (74). The anti-inflammatory effect of HBD3 on macrophages was also seen in the acute inflammatory cytokine response to *Porphyromonas gingivalis in vitro* and *in vivo* (88). Indeed, mice with an exaggerated response to *P. gingivalis* LPS (*ApoE* –/–) showed an increase in CCL2, TNF-α, IL-6, and NO levels at 2 h—but HBD3 (10 μg/mouse) could suppress this. The authors also report an increase in *Arginase 1*, a key marker of mouse alternatively activated macrophages (AMM or termed M2), possibly indicating a change in cellular polarisation as a consequence of defensin exposure.

A similar inflammation suppressive effect has also been recently observed with HBD2, which reduced TNFα and IL-1β secretion from dendritic cells in human peripheral blood mononuclear cells exposed to LPS. The effect was lost in the presence of a CCR2 inhibitor. When HBD2 was delivered systemically to a variety of mouse models of inflammatory bowel disease, the colitis was reduced to a level comparable to steroids and anti-TNFα (90). In addition, in the infected cornea of mice, silencing of the murine orthologues of HBD2 and 3 resulted in increased production of proinflammatory cytokines, with a simultaneous increase in bacterial load (91). The effect on bacterial load is postulated to be due to the defensins [at low concentration of 1 μg/ml (0.2 μM)] inhibiting macrophage autophagy and in this way increasing phagocytic receptor expression leading to intracellular killing of the *Pseudomonas aeruginosa* (92). All these studies indicate that, under specific infections scenarios, defensins are capable of contributing to anti-inflammatory response, or at least a rebalancing of the nature of the cellular response.

Adaptive Suppression

In addition to effects on innate responses to infectious and inflammatory stimuli, defensins have also been shown to have suppressive effects on adaptive immunity. UVB radiation induces Defb14 production in keratinocytes while DEFB14 peptide injection into mice suppressed contact hypersensitivity, but this was shown to involve the induction of antigen-specific regulatory T cells (Tregs), rather than the UV suppression pathway (93). The HBD3 peptide [at 10 μg/ml (2 μM)] has a demonstrated capacity to alter CD4+ CD25- T cells, from a non-regulatory phenotype towards a regulatory phenotype with expression of both the characteristic regulatory T cell (Treg) transcription factor (*FoxP3*) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4), a protein which downregulates immune responses (94). Treatment of CD4+CD25– cells with DEFB14 resulted in reduction in methylation of the *Foxp3* promoter compared to cells without DEFB14 (and closer to the level seen in Tregs) which correlated with an increase in FoxP3 expression. Additionally, treatment with DEFB14 before, or after, the induction of experimental autoimmune encephalomyelitis (mouse model of multiple sclerosis), was found to ameliorate the disease, with less

central nervous system inflammation and decreased levels of proinflammatory cytokines and cytotoxic T cells (94). The beneficial effect was lost upon depletion of regulatory T cells. These observations were attributable to an increase in suppressive CD4+ T cells, possibly through a change in cellular polarisation but the mechanism underpinning defensin-induced modulation of CD4+ T cells to a regulatory phenotype requires further investigation.

Further evidence of immune suppression by HBD3 (or DEFB14) arises from studies of diabetes. β-defensins were shown to be expressed in endocrine cells in both the human and mouse pancreas (95). DEFB14 treatment of non-obese diabetic mice was found to dampen the autoimmune response and to reduce subsequent diabetes development. This disease limitation was shown to be due to DEFB14 increasing proliferation of pancreatic B cells, expressing the regulatory cytokine IL-4 and the repair cytokine active TGFβ, which enabled polarisation of alternatively activated macrophages and a subsequent increase in Treg cells. This immune modulating pathway was believed to account for the reduction in autoimmune inflammation, with DEFB14 playing an integral role possibly through induction of TLR2.

Wound Healing Resolution

In addition to roles in innate and adaptive immune responses, β-defensins have been found to play important roles in resolution of damage pathways, via effects on wound healing. Characterising chronic wounds, β-defensin expression is found to be decreased in diabetic ulcers (96). This is thought to contribute to increased infection and also to a lack of wound healing, through mechanisms such as stimulating the migration of fibroblasts, as well as the proliferation of keratinocytes (97, 98). HBD2 also is reported to promote wound healing of intestinal cells *in vitro* (99) and *in vivo* by stimulating keratinocyte migration and proliferation in rats (100). The physiological significance of these findings are demonstrated in mice with *Defb14* deletion, which display delayed wound healing *in vivo,* with significantly increased wound area, delayed epithelialisation and an altered wound microbiota (97). In addition, there is an observed increase in classically activated macrophages in these wound sites and a trend towards decreased alternatively activated macrophages, together with an increased bacterial load in the skin (97). This implies that DEFB14 is important in wound repair and that insufficient peptide expression may reduce wound healing as a consequence of inappropriate macrophage polarisation (section Innate Suppression) and/or alteration in the ratio of local cellular populations. Macrophages are key in wound repair and can be central in the process by promoting a resolution of inflammation leading to tissue repair. CCL2, the major macrophage chemoattractant, can reverse the impaired wound healing in diabetic mice (101) and HBD3 can chemoattract macrophages through CCR2 and also modulate pattern recognition receptors relevant to wound repair (102, 103). As with all resolution milieu, successful wound healing will be multifactorial, but these data suggest that β-defensins are likely to contribute.

HUMAN DISEASE ASSOCIATION

Given the range of roles that β-defensins display, it is not surprising that their expression and influence are demonstrably intertwined into various disease states. For the sake of examining β-defensins in human non-infectious disease, however, discussion will be based on the main expression sites, epithelial cells, predominantly localised to the gut and skin. We will not be addressing their influence on cancer, although the involvement of β-defensins in cancer also demonstrates dichotomous behaviour. For example, their expression can be increased or decreased in tumours, their influence can be to promote or suppress, and these effects can be dependent upon the specific defensin peptide, the cancer type and the cells involved [for an excellent, recent review of the literature see (104)].

In addition to the complex, localised environmental influences that dictate β-defensin function, there is the issue of copy number variation, as mentioned previously. Six β-defensin genes (*DEFB4, DEFB103, DEFB104, DEFB105, DEFB106, DEFB107*), and the β-defensin related gene *SPAG11*, are present at chromosome 8p23.1, at two loci 5 Mb apart and are hyper CNV, changing through unequal crossing over at the rate of ∼0.7% per gamete (25). Worldwide, the average copy number of this unit is four, although copy numbers range from two to twelve (this does not occur in all species, with mice being an example of no copy number variation). The variation in these genes, combined with the alteration in expression based on localised stimulation, gives a large range of expression for these peptides, with overall inflammation in disease considered a stronger influence on expression than copy number (5, 105). The link between the level of expression of β-defensins and disease is discussed below.

Psoriasis

Indication that β-defensin copy number associates with disease development is evident in psoriasis. Psoriasis is a disease principally characterized by skin plaques, commonly found on the elbows, knees and trunk. Psoriatic lesions are described as sites of chronic skin inflammation with thickened, hyperplastic epidermis, increased vascularity and immune cell invasion. Lesions display overexpression of several inflammatory peptides and cytokines. The overexpression of these local cytokines (such as TNFα, IFNγ and IL-1) leads to increased expression of β-defensins within the lesions (106, 107), to a degree that allowed both HBD2 and HBD3 to be first isolated from psoriatic scales. In addition, there is a significant, replicated association between more than four copy numbers of the β-defensin seven gene repeat unit and psoriasis occurrence (108, 109). This goes beyond localised disease region expression, however, as serum levels of HBD2 correlate with copy number, both in normal individuals and disease state (where increase in serum HBD2 also correlates with psoriasis severity) (110) and HBD3 expression is increased in both normal and lesional skin of psoriasis patients, possibly adding to the reduced bacterial burdens in lesions compared with those in atopic dermatitis (see below) (111). It is not clear how increased β-defensin expression contributes to disease aetiology, but, as mentioned above, β-defensins HBD3 and 2 have

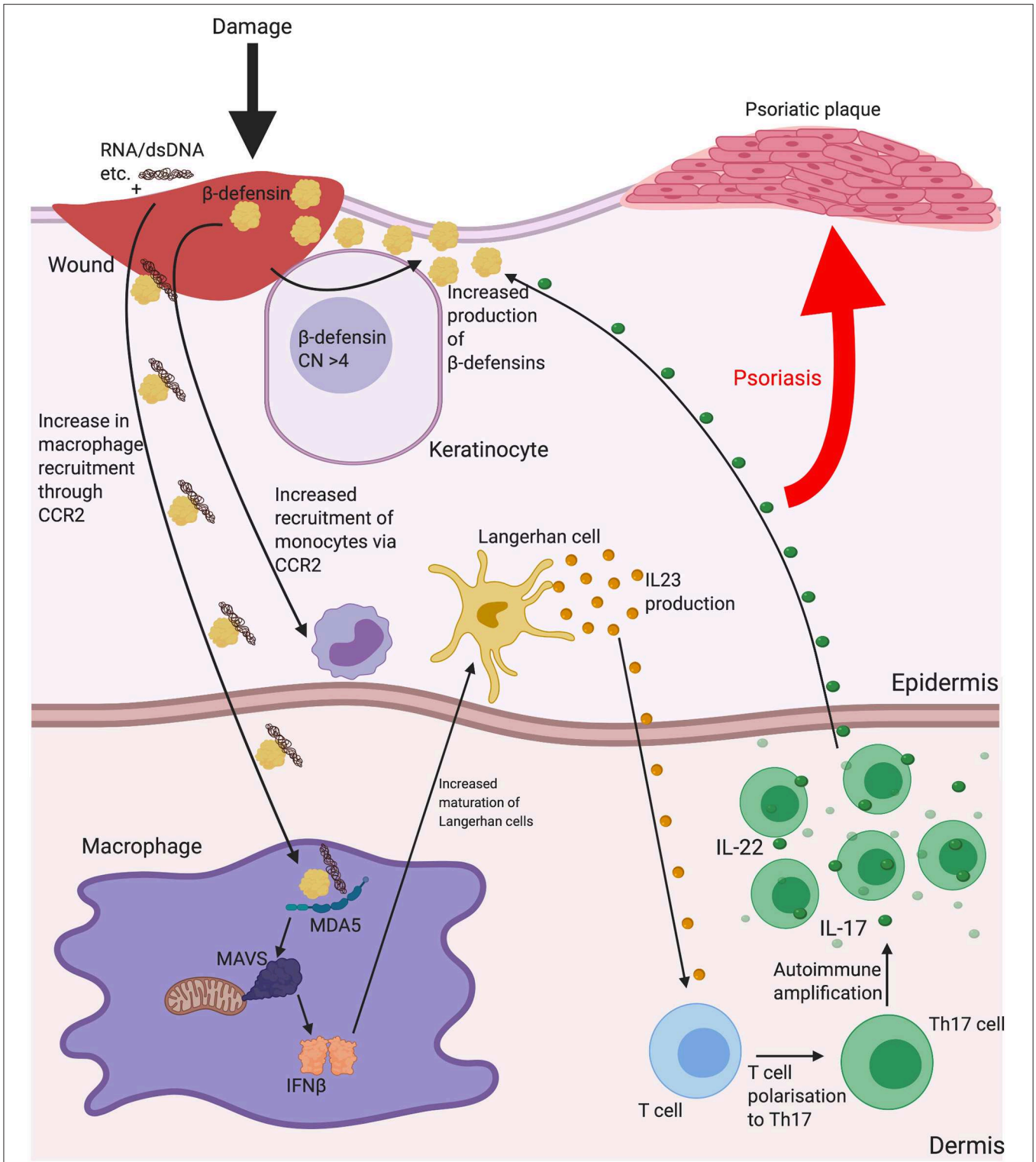

FIGURE 2 | The possible implications for β-defensins in psoriasis. Psoriasis is a disease characterised by scaly lesions, hyperplastic epidermal thickening, immune cell accumulation and is triggered by some sort of insult to the skin. HBD2 and 3 may contribute to the disease process as a consequence of increased gene copy number increasing the level of the peptide response to inflammation and enhancing monocyte/macrophage recruitment and increasing uptake of nucleic acids released from dying cells or microbes at the site of damage. Shown here is increased dsRNA entering macrophages with β-defensin and enhancing IFNβ secretion leading to Langerhan cell maturation and interleukin (IL)-23 release to influence mature T cell polarisation to T helper (Th)17. IL-22 production from Th17 may then further stimulate β-defensin production and amplify the process. Abbreviations: Copy number (CN), C-C chemokine receptor type 2 (CCR2), interleukin (IL), melanoma differentiation-associated protein 5 (MDA5), T helper (Th).

been shown to increase the Interferon-α response to DNA via TLR9 and the Interferon-β response to RNA through stimulation of the MDA5/MAVS pathway (see section Innate Triggers). HBD2 has been shown to be evident in the same dermal compartment as pDC in psoriatic skin, leading to a hypothesis that this peptide may be instrumental in breaking tolerance to self-DNA following infection or damage (67). Injection of CpG DNA:HBD3-defensin complexes subcutaneously in mice, increased epidermal hyperplasia and both neutrophil and lymphocyte recruitment at 24 h (66), supporting this hypothesis. Psoriasis is a Th17 predominated disease and effective treatment with UV irradiation is linked to suppression of type I Interferon and Th17 cells (112). In addition, psoriasis can be induced in multiple sclerosis patients using IFNβ therapy (113). The current effective treatments for psoriasis are biologics against IL-17 production or IL-12p40 (subunit common to both Il-12 and IL-23), to limit Th17 cell production and action. Of note, IL-22 is expressed by Th17 cells, which triggers β-defensin expression (95). Interestingly, individuals with missense variants in Human MDA5 gene (*IFIH1*) are protected from psoriasis (114) and gain of function MDA5 mutations have related type I interferonopathy with musculoskeletal disease that includes psoriasis (115). These lines of evidence strongly support the involvement of Interferon β in psoriasis and the genetic link between increased β-defensin CNV and psoriasis may be due to an increase in β-defensin expression having a functional consequence in the responses to dsRNA released from wounds, via MDA5/MAVS signalling and production of IFNβ (see **Figure 2**).

Atopic Dermatitis

Another skin disease associated with β-defensin expression is atopic dermatitis (AD) [for a more extensive review of the associations between β-defensin and AD see Chieosilapatham et al. (116)]. AD is another chronic inflammatory skin disease, characterised by itchy, inflamed lesions across a range of different body sites (117). In comparison with psoriatic plaques, AD lesions have a decrease in expression of these β-defensins (118) with induction of peptide levels found to be impaired for the level of inflammation. This has not been found to be related to copy number variation, however, and is instead due to the local Th2-skewed cytokine milieu and thus focused inhibition of β-defensin expression (119). Despite these lesional differences, HBD2, but not HBD3, is found to be increased in AD serum (120). Reduced defensins at the sites of disease may contribute to the pathology of AD in a number of ways, including the increase in lesional skin infections that are characteristic of the condition (121) (see **Figure 3**). In addition to the direct bactericidal properties of some of these peptides, it has been shown that HBD3 can increase expression of tight junction components in keratinocytes and improve barrier function (122). Further, we recently demonstrated that some β-defensins, such as HBD2, are able to inhibit the barrier-damaging effects of bacterial proteases, such as from the common AD lesional pathogen *Staphylococcus aureus*, which can contribute to this disease, in which loss of barrier integrity is critical (123). The mechanism of this is not yet fully elucidated and is subject to further investigation.

Inflammatory Bowel Disease

In addition to inflammatory disorders of the skin, β-defensin expression has been shown to be altered in chronic inflammatory disorders of the gut. Unlike the skin, however, β-defensins are not the key AMP type in the gastrointestinal tract. Instead, the most abundantly expressed AMP group is the human α-defensins, including human defensin 5 (HD5) and human defensin 6 (HD6), which are constitutively expressed by Paneth cells located at the base of the crypts of Luberkühn (unique to the small intestine) (124, 125). As well as being known to have antibacterial (HD5) and antiviral (HD6) activities (126), these peptides are known to be chemoattractive for naïve and memory CD4+ T lymphocytes, as well as macrophages and mast cells (127). Similarly to their β-defensin cousins, they are also linked chronic inflammatory disorders, with decreased levels of both HD5 and HD6 being demonstrated in ileal Crohn's disease (affecting the upper parts of the intestine) (128). It is thought that this lack of expression allows for increased pathogenic bacteria and therefore worsening of pathology (129).

While α-defensin are present in Paneth cell granules in the upper parts of the intestine, β-defensin expression is conducted by enterocytes, which are the most abundant epithelial cell lineage in both the small and large intestine (130). Enterocytes of the colon express HBD1 constitutively, with HBD2 being induced by TLR stimulation (131). HBDs can also be induced in the gastric mucosa, when faced with bacterial challenge (132) and expression of β-defensins is shown to be altered in chronic inflammatory bowel diseases (IBD) of the gut. Comparably to the relationship between β-defensin levels and different chronic inflammatory disorders of the skin, there appears to be a discrepancy in activity in different IBD disorders of the gut. Patients who suffer from Crohn's disease present a decrease in HBD2 β-defensin levels, and a concomitant decrease in the gene copy repeating unit suggested as a factor for predisposition to the disease (133, 134). HBD2 has recently been delivered subcutaneously to mice with induced models of intestinal bowel disease and successfully reduced the level of inflammation (90). In opposition to Crohn's disease, patients with ulcerative colitis (a disease of the colon) have a highly increased expression of HBD2, although not HBD1 (135). This has been argued to be due to changes in localised cytokine milieu, rather than variations in copy number. Aldhous et al. demonstrate that *DEFB4* mRNA and HBD2 protein levels varied upon stimulation with inflammatory cytokines in samples from IBD patients, independent of variations in HBD2 copy number (105). In this case, the influence of copy number variation is overridden by the impact of the local inflammatory environment. However, this is in the context of the high level of variation in defensin expression from one region of the gut to another, which is also in combination with differences between biopsy location and inflamed vs. non-inflamed areas of the bowel (105). This requires further study and the influence of the microbiota on defensin

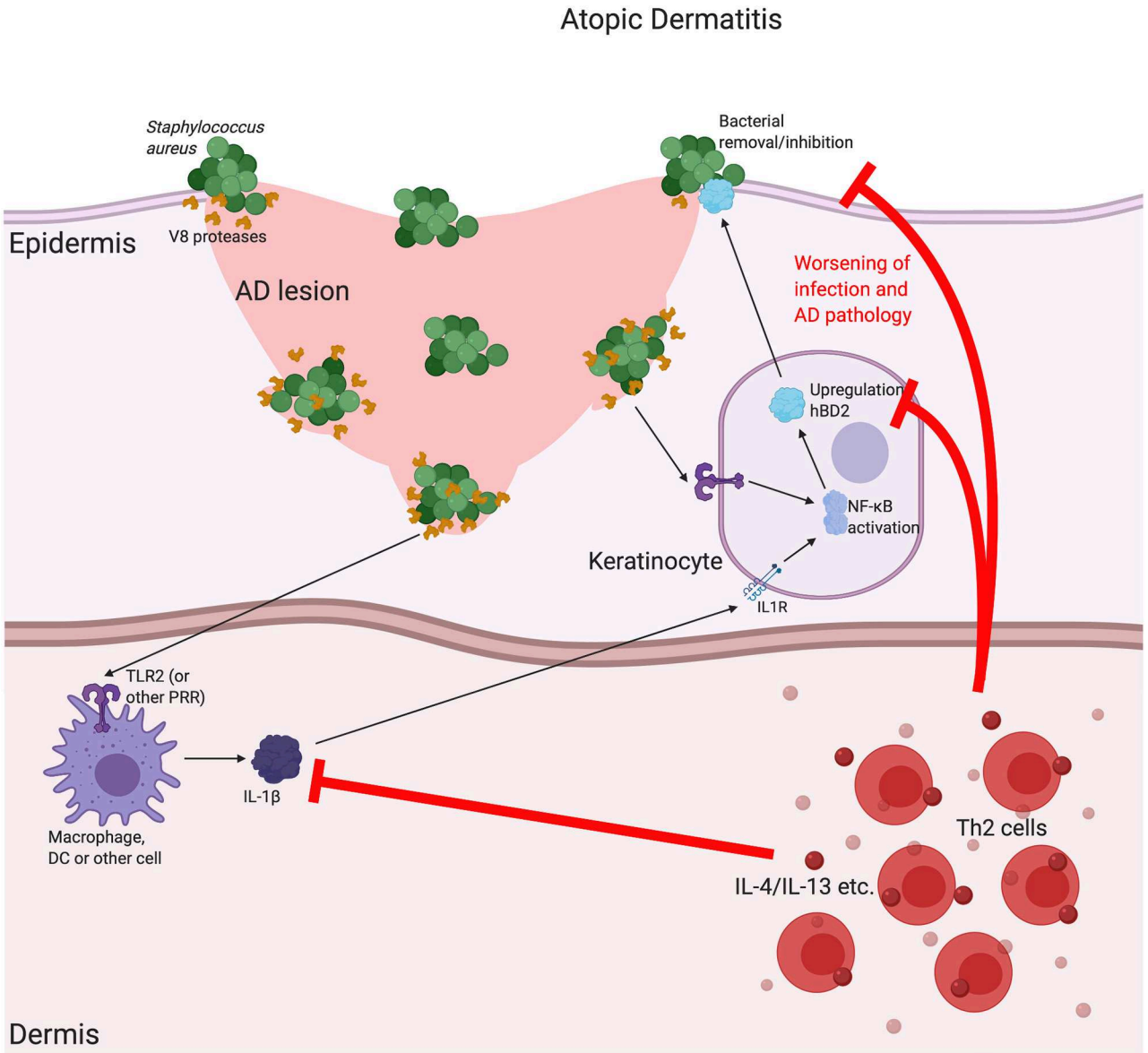

FIGURE 3 | The possible implications for β-defensins in atopic dermatitis. Atopic dermatitis (AD) is a chronic, relapsing disease associated with itchy lesions on the skin, across a large proportion of the body. These lesions are characterised by a breakdown in the barrier function of the uppermost regions of the skin (the epidermis). This allows for an increase in bacterial infection, which is made worse by bacterial production of proteases that further breakdown the junctions between cells, as is the case for V8 (SspA) production by *Staphylococcus aureus*. In AD, there is a downregulation in a number of Th1-associated cytokines, such as Tumour Necrosis Factor (TNF)α and interleukin (IL)-1β, as well as antimicrobial peptides, such as the β-defensins (including HBD2). It is thought that the AD-associated, localised cytokine milieu, which has a T helper (Th)2-skewed phenotype, is responsible for this reduction. Inhibition of the induction of β-defensins prevents proper bacterial removal/inhibition, worsening infection and AD pathology. Abbreviations: interleukin (IL), Toll Like Receptor (TLR), pathogen recognition receptor (PRR).

expression and defensin expression on microbiota composition needs clarification.

CONCLUSION

It is clear that β-defensins are not only AMPs, and their ability to change the behaviour of eukaryotic (particularly immune) cells at similar concentrations as those required to kill pathogens is intriguing. Here we have described the increasing body of research that has revealed the ability of β-defensins to behave in a dichotomous way with respect to inflammation. Under certain conditions they behave as alarmins and yet under other conditions they are suppressors of inflammation. The difference in effect does not seem to be due to the levels of peptide. As the Dorin lab has shown, the same peptide preparation

on human and mouse primary macrophages can suppress or increase inflammatory signalling, dependent on which PRR ligand is used. The charged nature of β-defensins is likely to be important in how it interacts with a variety of molecules and may explain why HBD3, with its high charge, consistently gives the most potent responses. Blocking receptors, binding to nucleic acids to enhance receptor engagement, and inducing chemoattraction, are all likely to be driven by the cationic and amphipathic nature of the peptides. At higher levels (above 2 μM) β-defensins certainly have a cytotoxic effect, but this may be supraphysiological. During infection, rapid killing, detection and innate response are essential; therefore, in this regard, high HBD3 copy number and potentiation of PRR may be beneficial. However, an undesirable effect of increased copy number of the defensin cluster (and concomitant increase in expression of defensin peptides) may be over stimulation of PRRs leading to exuberant production of type I interferons. This double-edged sword may provide protection against pathogens in the short term, but in the longer term contribute to the development of psoriasis in individuals with an increased copy number of the β-defensin cluster.

In vivo experiments are the most compelling to attribute function, because other cationic host defence peptides will also play a part, as some have synergistic actions and come from recruited, as well as resident cells at the site of injury. The *in vivo* evidence that DEFB14 or HBD3 can increase the inflammatory state of mouse skin but increase wound healing and suppress development of autoimmune diabetes are clear demonstrations of the dichotomy of the influence of β-defensins on mammalian cells. The influence of increased β-defensin expression in psoriasis and reduced expression in atopic dermatitis may reflect the different disease environments; in this case increased copy number in psoriasis may be the causative factor. This is an exciting area of research and further clarification of the factors that give rise to the type of response β-defensins encourage is important for therapeutic strategies.

AUTHOR'S NOTE

We apologize to our colleagues whose work we were unable to cite due to space limitation.

AUTHOR CONTRIBUTIONS

Manuscript conceived, researched and written by JS and JD, with additional interpretation, writing and editing by DD.

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Antimicrobial Peptides and Ectosymbiotic Relationships: Involvement of a Novel Type IIa Crustin in the Life Cycle of a Deep-Sea Vent Shrimp

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The symbiotic shrimp *Rimicaris exoculata* dominates the macrofauna inhabiting the active smokers of the deep-sea mid Atlantic ridge vent fields. We investigated the nature of the host mechanisms controlling the vital and highly specialized ectosymbiotic community confined into its cephalothoracic cavity. *R. exoculata* belongs to the Pleocyemata, crustacean brooding eggs, usually producing Type I crustins. Unexpectedly, a novel anti-Gram-positive type II crustin was molecularly identified in *R. exoculata.* Re-crustin is mainly produced by the appendages and the inner surfaces of the cephalothoracic cavity, embedding target epibionts. Symbiosis acquisition and regulating mechanisms are still poorly understood. Yet, symbiotic communities were identified at different steps of the life cycle such as brooding stage, juvenile recruitment and molt cycle, all of which may be crucial for symbiotic acquisition and control. Here, we show a spatio-temporal correlation between the production of Re-crustin and the main ectosymbiosis-related life-cycle events. Overall, our results highlight (i) a novel and unusual AMP sequence from an extremophile organism and (ii) the potential role of AMPs in the establishment of vital ectosymbiosis along the life cycle of deep-sea invertebrates.

Keywords: extreme, hydrothermal, symbiosis, host-microbe interaction, invertebrate immunity, crustacean

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INTRODUCTION

In marine habitats such as in deep-sea hydrothermal ecosystems, bacterial associations with invertebrates are well-described (1). The important animal biomass observed around hydrothermal vents is based on the existence of dense chemosynthetic prokaryotic communities (2, 3). Among these communities, a large number forms highly specialized symbiotic associations with metazoan hosts. These relationships are now quite well-studied, e.g., as in the case of the bacterial community inhabiting the cephalothoracic cavity of the shrimp *Rimicaris exoculata* (4–7). By

contrast, understanding the mechanisms by which hosts selectively recruit bacteria for long-term (core) or short-term (flexible) specific relationships is still a considerable challenge (8). To date, most of the existing literature has focused on the role of immune receptors (lectins, PGRPs, FREPs, TLR, SRCR) in marine symbiotic associations (9–11). A given immune receptor recognizes some families of microbes (bacteria, fungi, viruses) on the basis of motifs of recognition called Microbe Associated Molecular Patterns (MAMPs) commonly exposed on the membrane of friend and foe microorganisms (12). However, other selective and specific host processes are required to discriminate between pathogenic or mutualistic microbes in order to selectively kill or tolerate them. Amongst the few other immune substances known to be involved in host-symbiont associations, host defense antimicrobial peptides (AMPs) represent promising actors (13–18). AMPs are chemical components that take part in both the internal and external immune defenses (i.e., they can be secreted in the outer parts of the body), thus playing functions in the control/establishment of ectosymbiosis as described for the hydrothermal worm *Alvinella pompejana* (19–21). From an evolutionary perspective, the adaptive diversification of AMPs at the interspecific and intraspecific levels makes them of particular interest to decipher the immune mechanisms driving bacteria-specific and environment-dependent symbioses (22–24).

The Pleocyemata shrimp *R. exoculata* dominates the fauna at several hydrothermal vent sites of the Mid-Atlantic Ridge (MAR) (25, 26). This deep-sea crustacean thrives in such hostile habitats through an association with two distinct ectosymbiotic microbial communities. One housed in its gut (27–29) and the other in its enlarged cephalothoracic cavity (4, 5, 7, 30–34). Previous studies have suggested and then demonstrated the chemotrophic role of the symbionts that colonize the cephalothoracic cavity (6, 35–37). This specialized ectosymbiosis composed of few specific bacterial lineages, mainly proteobacteria and *Campylobacterota* (previously *Epsilonproteobacteria*) (38) is confined to the internal faces of the lateral carapace (branchiostegites) and the mouthparts (scaphognathites) of the cephalothorax cavity, but not of the gills (5, 31, 32). While the gut symbiotic community harbors proteobacteria and *Campylobacterota*, other symbionts have been found in the digestive system (stomach and digestive tracts, respectively), such as Mollicutes or Deferribacteres (27–29) evoking an organ-dependent mode of selection of the symbionts by the host. Recently, ectosymbionts have also been described on eggs along their development (P. Methou, personal communication). Interestingly, every 10 days, the microbial community of the cephalothoracic cavity, but not of the gut, is eliminated during the molt of the adult and ectosymbionts rapidly re-colonize the host cephalothoracic cavity (39, 40). This re-colonization process is strictly similar for each individual and strictly located on the same area of mouthparts and branchiostegites, suggesting a tight selection of the bacteria by the host (5, 39). However, the immune mechanisms involved in this association remain mostly unknown. Only the recent work by Liu and his colleagues, has characterized the potential immune role of a C-type lectin highly expressed in the scaphognathites, which has a broad nonself-recognition spectra and could agglutinate some of the cephalothoracic symbionts (10, 11).

The immune system of crustaceans is based on cellular and humoral responses involving, among other substances, the production of AMPs (41). Several classes of both gene-encoded and non-ribosomally synthetized AMPs have been identified and characterized in major commercial species of decapod crustaceans (42, 43). To our knowledge, despite a particularly well-described role of these molecules in the immune response of crustaceans against pathogens (41), no studies have ever been conducted on their involvement in mutualistic symbiosis.

Crustins form a diverse and multigenic family of AMPs found in virtually all crustacean groups and in some hymenopteran insects (44). They are mainly active against Gram-positive bacteria, but their unique feature is the presence of a C-terminal whey acidic protein (WAP) domain, a conserved cysteine-rich motif (four-disulfide core or 4DSC) that exhibits antiprotease activities (45). Crustins are divided into four groups (Types I to IV) according to the presence/absence of two N-terminal structural domains: the glycine-rich and the cysteine-rich regions (43). Type I crustins contain an N-terminal cysteine-rich region (with four conserved cysteine residues) followed by the typical C-terminal WAP domain. In addition to the cysteine-rich region, Type II crustins also harbor a highly hydrophobic glycine-rich region at the N-terminus. Comparatively, crustin members from Types III and IV are composed of one and two WAP domains, respectively, and are devoid of any other domains. Interestingly, while Type I crustins are widely distributed across decapod crustaceans (Pleocyemata and Dendrobranchiata), Type II crustins (Sub-Types IIa and IIb) are mainly present in penaeid shrimps (Dendrobranchiata) (46).

In this study, we explored the sequence conservation of a novel glycine-rich crustin member, Re-crustin, produced by the extremophile Pleocyemata shrimp, *R. exoculata*. Then, we investigated expression patterns in different host tissues and throughout its life cycle in order to identify possible correlations with the main symbiosis related events taking place at different life stages of this vent shrimp. These events include embryonic development (5, 47), juvenile settlement into adult habitats (P. Methou, personal communication), and through the molt cycle involving re-establishment of the symbiotic community after each molting event (39).

MATERIALS AND METHODS

Specimen Collection

Rimicaris exoculata were collected at two MAR hydrothermal vent fields, TAG (26°08' N; −3,640 m) and Snake Pit (23°23' N; −3,480 m), with the Research Vessel (R/V) *Pourquoi pas?* using the suction sampler of the remotely operated vehicle (ROV) Victor 6,000 and the human operated submersible Nautile during the oceanographic cruises BICOSE2014 (https://doi.org/10.17600/14000100) and BICOSE2 2018 (http://doi.org/10.17600/18000004) (**Figures 1A–C**). The isobaric collection device PERISCOP (49) was used to collect shrimps at different life stages (several females with early or late eggs, recruited juveniles collected within adults' aggregates (**Figure 1D**), and adults at

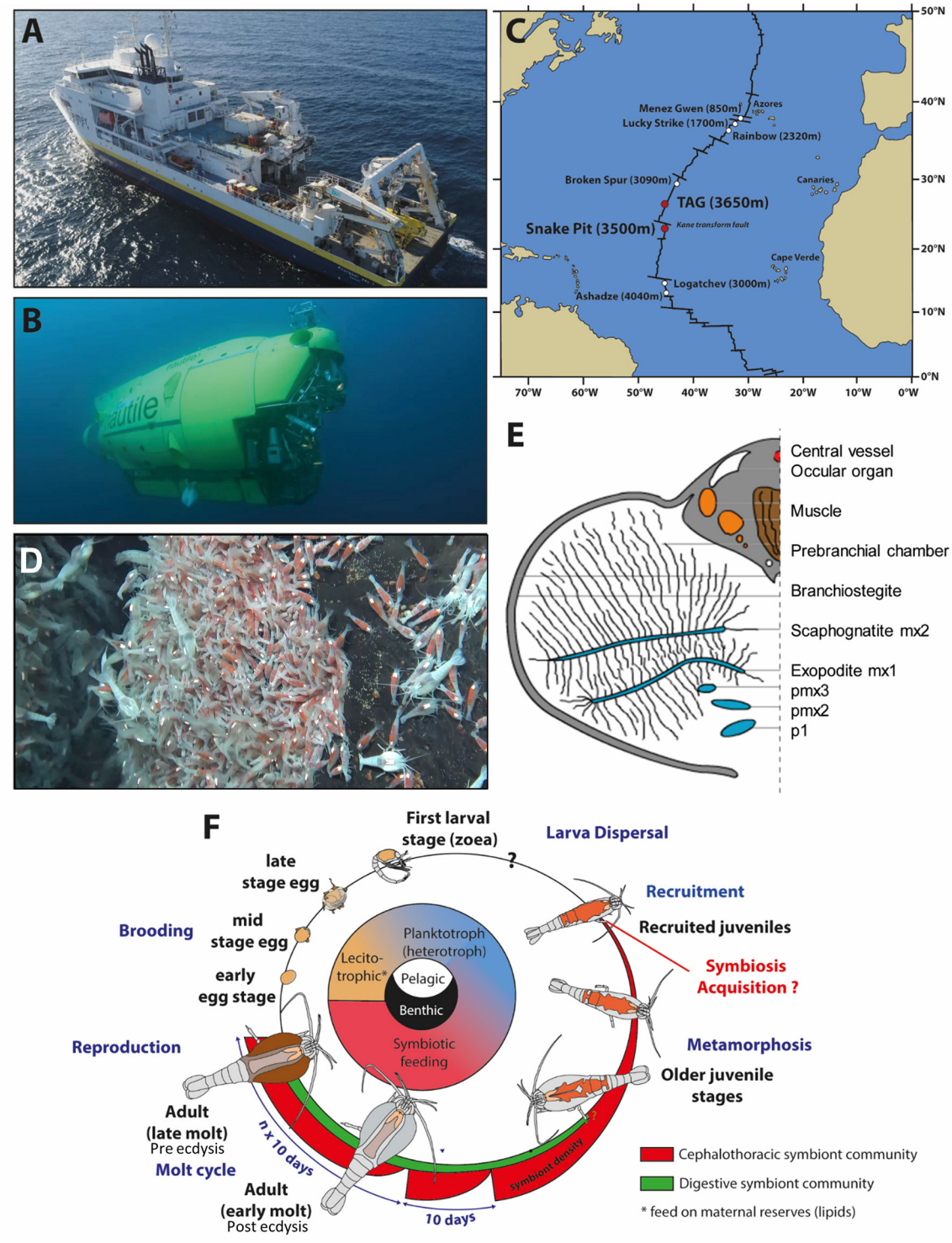

FIGURE 1 | *Rimicaris exoculata* sampling and summary of its symbiotic relationships through its life cycle **(A)**. Research vessel (R/V) "*Pourquoi pas*?" and **(B)** HOV Nautile used for sampling. **(C)** Location of the two hydrothermal vent sites presently studied along the Mid-Atlantic Ridge. **(D)** Suction sampling of the shrimps at Snake Pit with the human operated submersible Nautile during the oceanographic cruise BICOSE2018. **(E)** *Rimicaris exoculata* cephalothoracic chamber [modified from Segonzac et al. (26)]. **(F)** Life cycle of *Rimicaris exoculata*. Inspired from the figure of Laming et al. (48) copyright BICOSE2-Nautile@Ifremer.

different molting stages). They were dissected aboard, and pieces were either flash frozen in liquid nitrogen before being kept at −80°C (with or without Trizol ReagentTM, Invitrogen) or were kept straight after sampling at 4°C (in 4% Paraformaldehyde) until further use at the laboratory (**Figures 1E,F**).

Molecular Identification of Re-crustin

The nucleotide sequence of the Re-crustin was obtained by RT-PCR using 2 μg of template cDNA, with the forward primer 5′- GACAAACACCTCCTCCTCCTCCA−3′ designed from the incomplete 5′ coding sequence of a crustin sequence available in GenBank (accessing number FJ573157) and the oligo (dT)18 primer.

cDNA Synthesis

Whole animals were ground in Trizol ReagentTM using the Ultra-Turrax T25® (IKA). RNA was extracted according to the manufacturer's instructions. The concentration of extracted RNA was estimated with the Qubit® 3.0 Fluorometer (ThermoFisher Scientific). RNA extracts were treated by RQ1 RNase-free DNase (Promega) and used for cDNA synthesis with the RevertAid M-MuLV RT kit (ThermoFisher Scientific) according to the manufacturer's protocol. Reaction mixtures for PCR amplifications contained 0.1 μM of each primer, 0.25 mM of each desoxynucleotide triphosphate, 5 × Go Taq G2 Flexi buffer (Promega), and 5 U of GoTaq G2 Flexi DNA polymerase (Promega). The PCR program involved an initial denaturation step at 95°C for 3 min, followed by 39 cycles of 95°C for 1.5 min, 55°C for 1.5 min, and 72°C for 1.5 min, with a final elongation step at 72°C for 5 min.

Molecular Cloning and Sequence Analysis

PCR products from each replicate were pooled and then purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Purified PCR products were cloned using the TOPO-TA kit (Invitrogen, Carlsbad, CA, USA). Clones were sequenced according to the Sanger method (50) on a 310 ABI prism (Applied Biosystems). Sequences were imported into Geneious© version 8.1 software (Biomatters, available from http://www.geneious.com/). Prediction of signal peptide was performed with the SignalP 4.1 program (51) and the presence of conserved domains was tested using the SMART 7.0 protein analysis tool (52). Homology searches were performed using BLAST from NCBI. Multiple alignments of the deduced amino acid sequences (Type I, Type IIa, and Type IIb crustins) were generated using the MAFFT software (scoring matrix BLOSUM62) (53). Maximum likelihood phylogenetic analyses were generated in MEGA X (54) using best-fit WAG model assuming gamma distribution with invariant sites (G+I) for substitution rates. Gaps and missing data were included in data subset as relevant phylogenetic sites. Trees were resampled 1,000 times.

Determination of the Level and Site of Re-crustin Gene Expression by RT-qPCR

Tissues from branchiostegite, scaphognathite, gills, abdomen, stomach, hepatopancreas and eggs dissected aboard as wells as whole adults and juveniles were ground in Trizol ReagentTM using FastPrep-24® 5G (MP Biomedicals). Total RNA extraction and RT were performed as described in the previous section. The primers used for the quantitative PCR were designed with the Primer3 Input software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/primer3/; www.cgi).

- crustin primers: forward: 5′-ACTGCTGTGAGAACGGGAAC-3′; reverse: 5′-AACATGTTTGAGGGGGTCCT-3′
- Rpl8 primers: forward: 5′-GAAGCTCCCATCAGGTGCCAAGAA-3′; reverse 5′-TTGTTACCACCACCGTGAGGATGC-3′.

The Rpl8 gene was used as the reference gene (55). Real-time quantitative PCR reactions (RT-qPCR) were conducted on a LightCycler® 480 system (Roche) using a hot start enzyme. RT-qPCR assays were submitted to an initial denaturation step of 10 min at 94°C followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 59°C for 1 min and extension at 72°C for 30 s. Reference and target were amplified in separated wells. After amplifications, a melting curve analysis was performed in order to confirm the specificity of the PCR products. Re-crustin primers generated a single and discrete peak in the dissociation curve (data not shown). A negative control and a 5-fold dilution series protocol of pooled cDNAs were included in each run. The 5-fold dilution series were used to construct a relative standard curve to determine the PCR efficiencies and for further quantification analysis. In all experiments, all primer pairs gave amplification efficiencies of 90–100%. Each reaction was run in triplicates. Analysis of relative gene expression data was performed using the ΔΔCq method (56). For each couple of primers, a plot of the log cDNA dilution vs. ΔCq was generated to validate the RT-qPCR experiments (data not shown).

Immuno-Location of Re-crustin Protein by Western Blot and by Immunohistochemistry

Polyclonal Antiserum

The chemically synthesized region of Re-crustin (PTRFGGPPQTCSSDSSCTNNYTDK) was coupled to ovalbumin and used for the immunization procedure of two New Zealand White rabbits (Saprophyte Pathogen free) according to the protocol of Covalab™ (France).

Protein Extraction and Electrophoresis

Total proteins were isolated from the samples used for RNA extraction according to the manufacturer's instructions (Trizol ReagentTM, Invitrogen). The white band (interphase) containing the proteins was washed with a solution of 0.3 M guanidine hydrochloride in 95% ethanol, and then resuspended in 9.5 M urea and 2% CHAPS. The protein concentration was determined by the Bradford method using BSA as a standard (57). Proteins were separated by a denaturing SDS-PAGE electrophoresis. The running gel was composed of 12% acrylamide (12% acrylamide; Tris-HCL 1.5 M, pH 8.8; 0.1% SDS; 0.1% ammonium persulfate; 0.01% TEMED) and the stacking gel was composed of 4% acrylamide (4% acrylamide; Tris-HCL 0.5 M pH 6.8; 0.1% SDS; 0.1% ammonium persulfate; 0.01% TEMED). A total of 22 μg of protein was loaded in Laemmli buffer (Tris 125 mM pH 6.8; 20%

glycerol; 4% SDS and 5% β-mercaptoethanol). Gels were run at 70 V for 15 min and then at 180 V for 20 min.

Immunoblot

The proteins of the SDS-PAGE gel were transferred to a nitrocellulose membrane 0.2 μm (BIO RAD) by semi-dry electro blotting (0.8–1.2 mA/cm²). After transfer, the gel was stained by Coomassie Brilliant Blue R-250 (BioRad). The membrane was blocked for 1 h in PBS at 0.1 M containing 0.05% Tween 20 and 5% casein and was then probed with the rabbit polyclonal anti-Re-crustin antibody (1:300 dilution) in the blocking solution (PBS at 0.1 M with 5% w/v non-fat dry milk) overnight at 4°C. After three washes with PBS/0.05%-Tween 20, the membrane was incubated for 1 h in the blocking solution at room temperature with the peroxidase-conjugated anti-rabbit secondary antibody Abcam (1:5000 in PBS at 0.1 M containing 0.05% Tween 20; at 1 h). A Clarity™ Western ECL Substrate (Bio Rad) was used for the chemoluminescence visualization of the immunolabeling with a Kodak Bio Max light film.

Immunocytochemistry and Immunohistochemistry

Eggs, juveniles and tissues were fixed aboard in 4% paraformaldehyde. Later, immunohistochemistry was performed on paraffin sections of eggs (thickness of 4 μm), juveniles and adult tissues (thickness of 7 μm). Consecutive paraffin sections were made with a LEICA RM 2255 microtome. Immunocytochemistry and immunohistochemistry were performed with the rabbit anti-Re-crustin (1:400) and the FITC-conjugated anti-rabbit secondary antibody (1:100; Jackson Immunoresearch Laboratories). Samples were examined using a confocal microscope (Zeiss LSM LSM780) and the Fluorescence microscope (Zeiss Axio Imager 2).

Determination of Antibacterial Activities

Bacteria

One Gram-positive *Micrococcus luteus* and one Gram-negative *Vibrio diabolicus* were chosen for being easily carriable and cultivable onboard a ship. *M. luteus* routinely used in laboratory, is found in soil, dust, water and air and *V. diabolicus* was isolated from deep sea hydrothermal vents (58).

Samples

Branchiostergites and scaphognathites were crushed with the rotor CoolPrep, MP system (3 times 20 s at 60 rpm) in 0.1 M PBS at 4°C. 10 μL of samples were incubated without (control) or with 0.5 μl of the anti-Re-crustin antibody (dilution 1:400) at 4°C for 20 min.

Radial Diffusion Assay

10 μL of each sample were spotted onto LB-agar (Luria-Bertani) plates containing alive *M. luteus* or alive *V. diabolicus* (1×10^5 Colony Forming Unit (CFU)/mL of LB agar). After an overnight incubation at 37°C, the activity was quantified by measuring the diameter of the bacterial growth inhibition.

Experiments were performed in triplicate, once aboard the *Pourquoi pas?* R/V during the BICOSE2 2018 cruise with freshly dissected tissues and twice back to the laboratory in Lille with tissues frozen during the same cruise.

RESULTS

Re-crustin, a Novel Member of Type IIa Crustins and a Novel AMP From an Extremophile Organism

The complete nucleotide sequence of Re-crustin was obtained by 5′-RACE RT-PCR from total RNA extracted from the entire shrimp *R. exoculata* (GenBank accession number: MT102281). Only one sequence of crustin was identified from our molecular subcloning and sequencing. The complete cDNA sequence encodes a precursor of 190 amino acid residues, which includes a 15-residue signal peptide (**Figure 2**). The mature polypeptide is predicted to consist of 175 residues with a calculated molecular weight of 17.82 kDa and a theoretical isoelectric point (pI) of about 8.5. The mature Re-crustin is composed by a hydrophobic glycine-rich region followed by a C-terminus containing a cysteine-rich region (with 4 conserved cysteine residues) and a single WAP domain (**Figure 2B**). The glycine-rich region of Re-crustin possesses ten sequential repeats of the heptapeptide Gly-Gly-(Gly/Val)-Phe-Pro-Gly-Gln [GG(G/V)FPGQ].

Besides the presence of an N-terminal glycine-rich region, multiple sequence alignment analysis confirmed that Re-crustin is an authentic Type II member from the Sub-Type IIa (**Figure 3**). The Re-crustin sequence showed highest homology to Type IIa crustins from other decapods from the Pleocyemata suborder, including the red cherry shrimp *Neocaridina heteropoda* (NhCrustin, 67% amino acid identity), the morotoge shrimp *Pandalopsis japonica* (Paj-CrusIIc, 64% amino acid identity), the Japanese spiny lobster *Panulirus japonicus* (PJC1-4, 59–66% amino acid identity) and the Chinese mitten crab *Eriocheir sinensis* (Escrustin-1, 63% amino acid identity). Within Type II crustins from penaeid shrimp (Dendrobranchiata), Re-crustin was 52–59% identical to Type IIa crustins and 44–53% identical to Type IIb crustins. On the other hand, the mature Re-crustin displayed 36–44% identity to Type I crustins from decapods from both Pleocyemata and Dendrobranchiata suborders. Less than 30% amino acid identity was observed between Re-crustin and Type III (single WAP domain-containing proteins or SWD) and Type IV crustins (double WAP domain-containing proteins or DWD). Western blot analysis from total protein extracts showed a band at the predicted molecular weight confirming (i) the specificity of the antibody designed to recognize the WAP domain of Re-crustin and (ii) the translation of the Re-crustin transcripts (**Figure 4B**).

In Adults, Re-crustin Is Produced by Tissues on Which Ectosymbionts Develop

RT-qPCR and Western blot analyses were performed on exactly the same tissues from the same individuals (**Figure 4**). Results showed the presence of both transcripts (**Figure 4A**) and proteins (**Figure 4B**) in the pieces of the cephalothoracic cavity. Neither the transcripts nor the proteins were detected in the gut, the hepatopancreas and the stomach. The major Re-crustin producing tissues were clearly those on which the ectosymbiotic community of the cephalothoracic cavity develops, i.e., the branchiostegites and scaphognathites. Cellular localization of

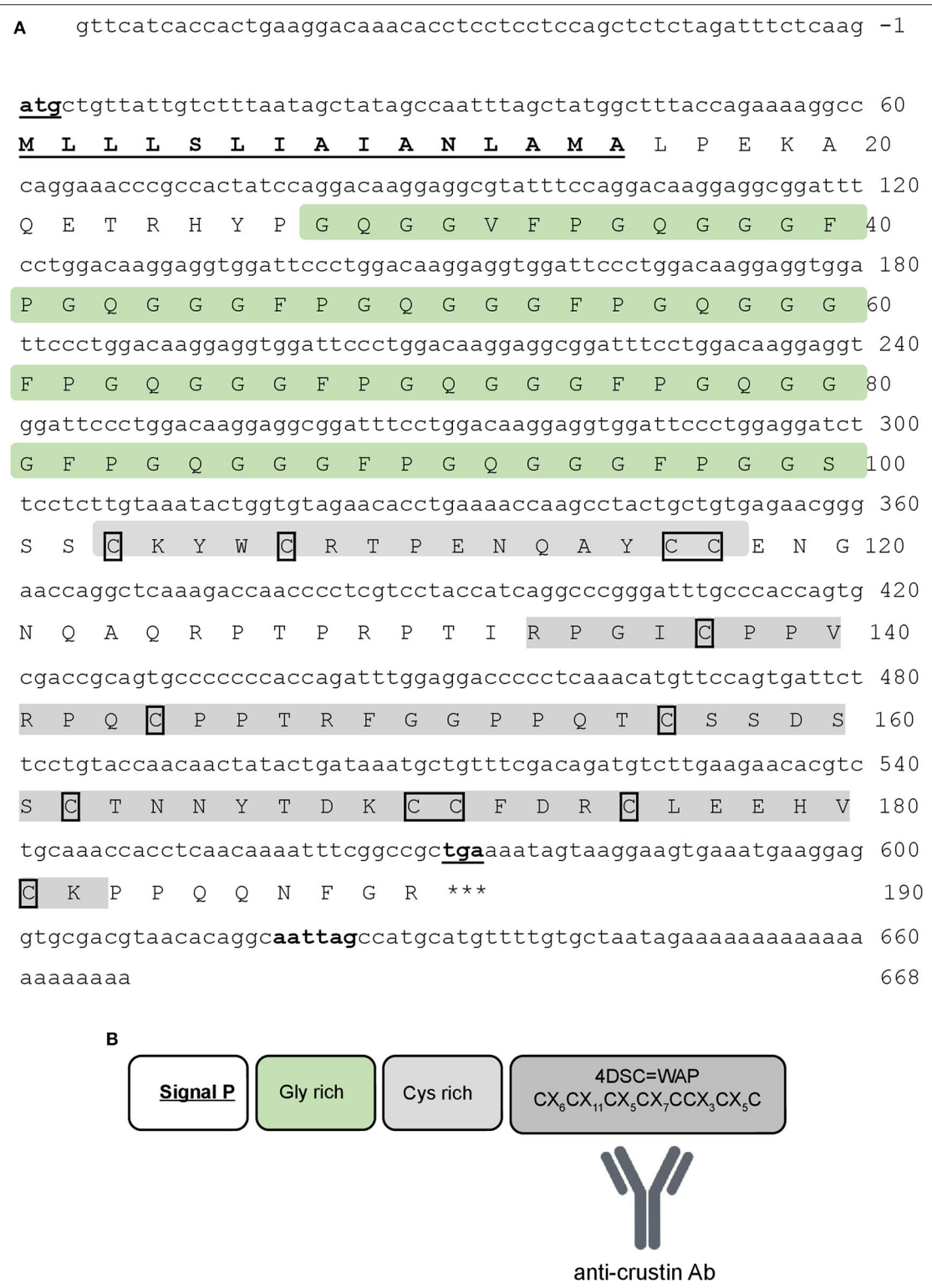

FIGURE 2 | Re-crustin sequence. **(A)** The full-length nucleotide (above) and predicted amino acid (below) sequences of Re-crustin cDNA from *Rimicaris exoculata*. The start and stop codons and the putative polyadenylation site are in bold and underlined. The signal peptide is underlined. The 12 conserved cysteine residues are framed. **(B)** The predicted organization of WAP domain is shown in the dark gray box.

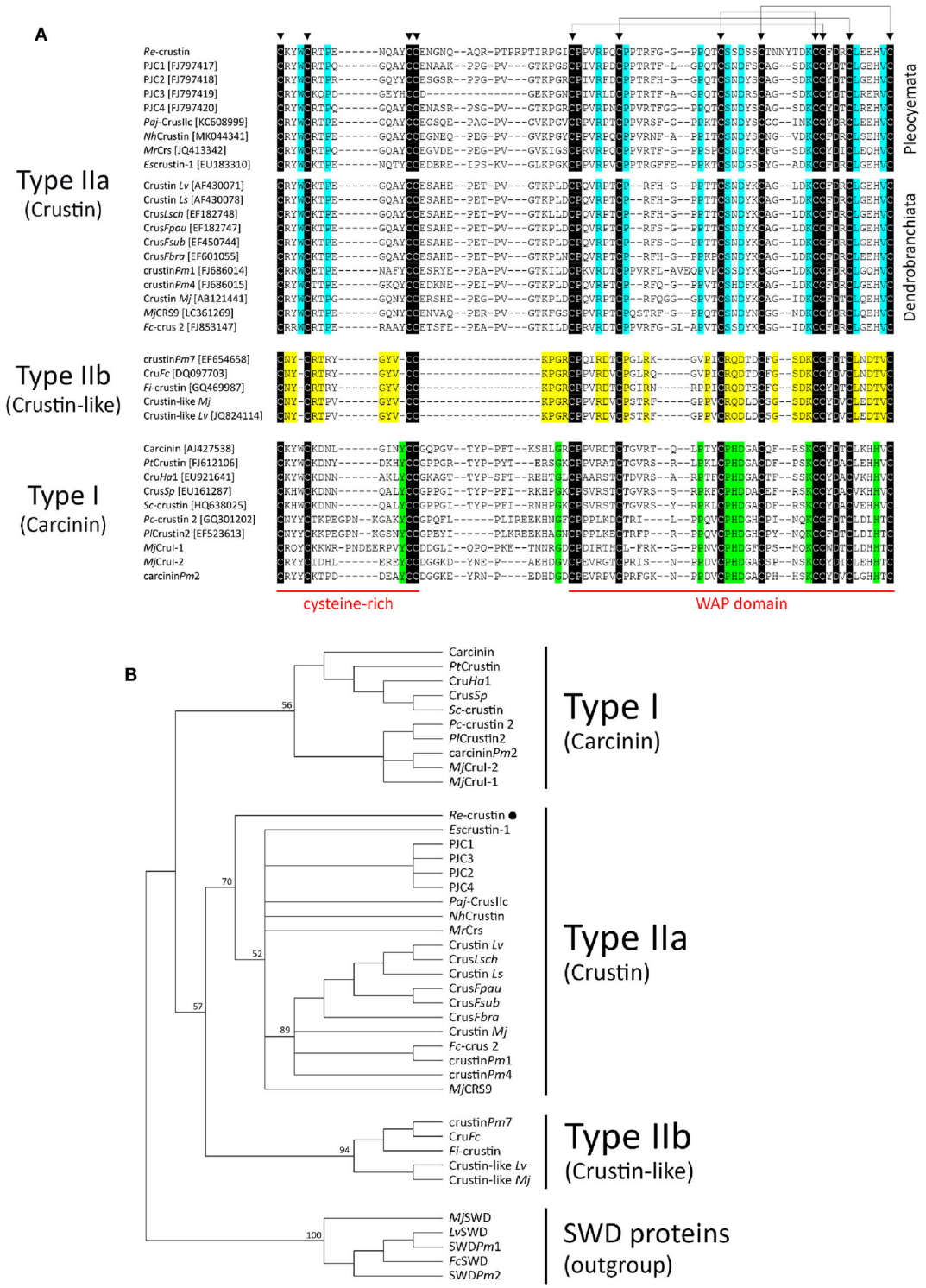

FIGURE 3 | Comparison of Re-crustin with other Type I and Type II crustins from decapod crustaceans. **(A)** Amino acid sequence alignments of the cysteine-rich region and the WAP domain of crustins. Identical amino acid residues are highlighted in black while specific amino acid residues found in Type IIa ("Crustin"), Type IIb ("Crustin-like"), and Type I ("Carcinin") peptides are highlighted in blue, yellow and green, respectively. Triangles (▼) indicate the 12 conserved cysteine residues. **(B)** Phylogenetic analysis of Type I and Type II crustins. The tree was constructed using the Maximum Likelihood method with bootstrap values calculated from 1,000 trees.

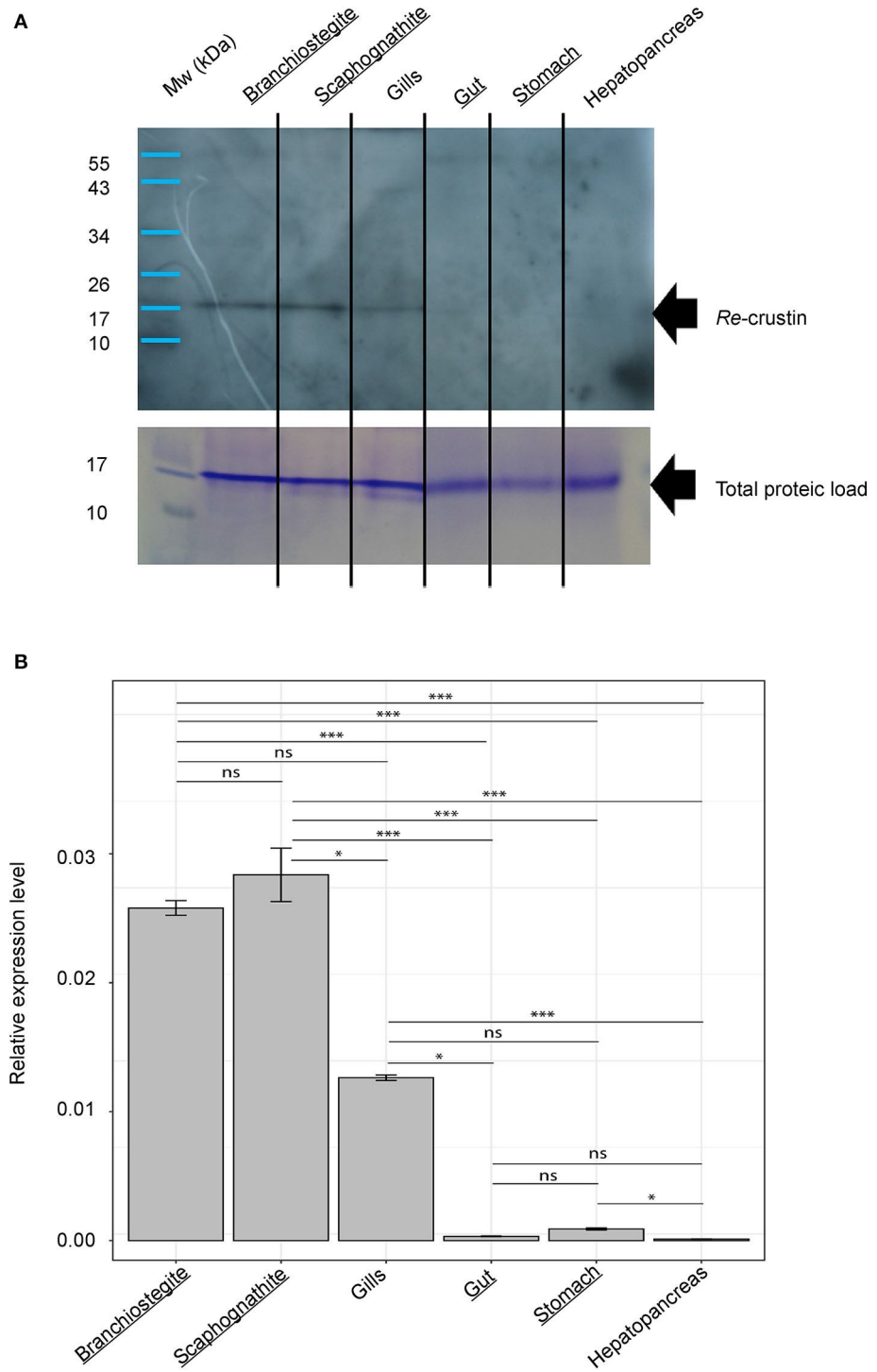

FIGURE 4 | Re-crustin distribution in tissues of adults of *Rimicaris exoculata*. **(A)** Western blot analysis was performed using total protein extracts (22 μg) from branchiostegite; scaphognathite; gills; gut; stomach and hepatopancreas dissected from adults. Immunostaining with the anti-Re-crustin antibody revealed one band of approximately 17 kDa corresponding to Re-crustin mass prediction. Mw, molecular weight markers. Equivalent well-loading was assessed by a generic protein coloration of the gel (Coomassie Brilliant Blue R-250). **(B)** Quantification of the levels of expression of Re-crustin in the cephalothoracic cavity (branchiostegite; scaphognathite; gills) and in the digestive tract (gut; stomach; hepatopancreas) by RT-qPCR analysis using the $\Delta\Delta$Cq method. Known symbiotic tissues are underlined. The graphs show the mean ± SEM for each organ ($n = 10$ in all cases) and significance level for each intergroup comparisons (ns, $p > 0.05$; *$p < 0.05$; ***$p < 0.001$; Dunn tests). Kruskal–Wallis: $\chi 2 = 55.812$; $p < 0.001$. Reference (Rpl8) and target were amplified in separated wells ($n > 10$ in all cases). A technical triplicate was applied for each sample.

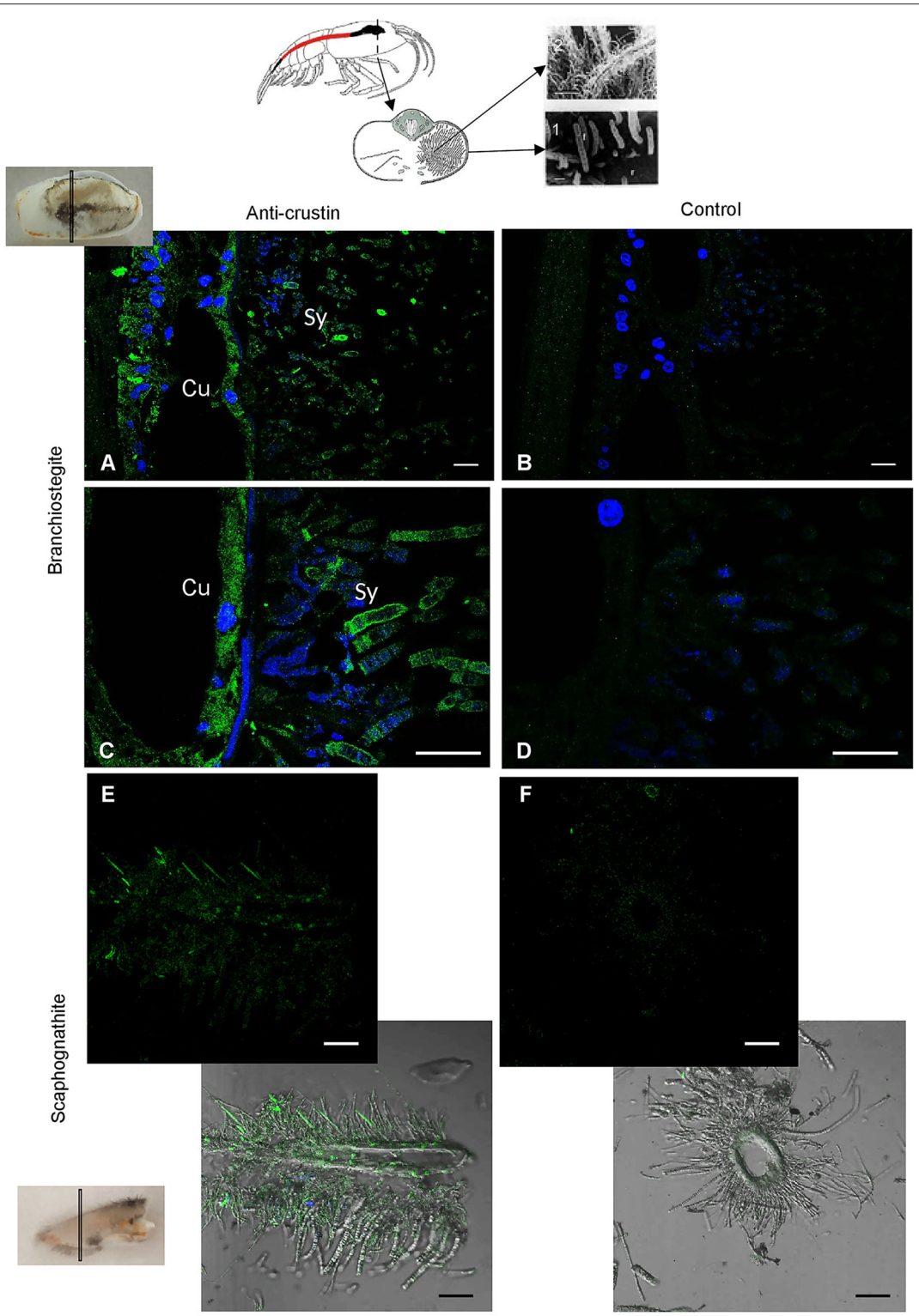

FIGURE 5 | Immunolabeling on paraffin sections with a thickness of 7 μm of Re-crustin on pieces of carapace colonized by bacteria. Schematic *Rimicaris exoculata* cephalothoracic chamber illustrated by two photos in electron microscopy (1, 2) the filamentous *Campylobacteria* epibionts. **(A–D)** Branchiostegite and **(E,F)** scaphognathite. **(A–C)** The anti-crustin antibody specifically labels in green the tissue that lines the inside of the cephalothoracic cavity and also covers the surface of some attached bacteria. **(E)** Some bacteria attached to the scaphognathite are also labeled. **(B–F)** No labeling appears with the pre-immune control. Nucleic acids are labeled in blue (DAPI). The white field is superimposed in E and F to see the shadows of the structures. The observations were made using the confocal microscope, Zeiss LSM 780. Scale bars correspond to 20 μm. Sy, symbiont; Cu, Cuticle.

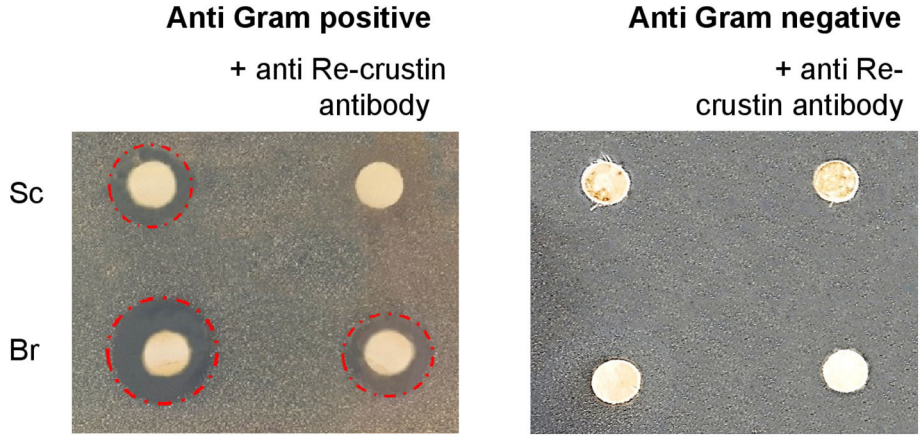

FIGURE 6 | Antimicrobial activities of crude extracts of branchiostegites (Br) and scaphognathites (Sc) against Gram-positive and Gram-negative bacteria. The antibody added to the extracts acts as a blocking agent of the endogenous Re-crustin. The red circles underline the antimicrobial activities.

Re-crustin was then investigated in these pieces of carapace colonized by symbionts using immunohistochemistry and confocal microscopy analyses (**Figure 5**). Immunolabeling with the anti-crustin antibody provided evidence for the synthesis of Re-crustin by the epithelium cells beneath the cuticle of the branchiostegites (**Figure 5A–D**) and scaphognathites (**Figures 5E,F**) from adults and its accumulation on the cuticle that delimits the cephalothoracic cavity. Interestingly, Re-crustin covered some of intact ectosymbiotic bacteria anchored to these mouth pieces (**Figures 5C,E**).

Re-crustin Produced by Branchiostegites and Scaphognathites Displays Antibacterial Activities

Antibacterial assays performed in triplicate from crude extracts of branchiostegites and scaphognathites showed antibacterial activities against the Gram-positive *M. luteus* but not against the Gram-negative *Vibrio diabolicus* (**Figure 6**). Part of this antibacterial effect is significantly reduced when the endogenous Re-crustin is blocked by adding the specific Re-crustin antibody to the extract, confirming the production and the antibacterial activity of Re-crustin in both scaphognathites and branchiostegites. Since the anti-Re-crustin antibody does not fully inhibit the antibacterial activities, active substances others than Re-crustin are presumably produced by the scaphognathites and the branchiostegites.

The Production Site of Re-crustin Along the Life Cycle of the Shrimp Is Correlated With the Acquisition of Ectosymbionts

The transcriptomic and protein levels of Re-crustin were investigated at different stages of the shrimp life-cycle for which the colonization states by the ectosymbionts were already described (**Figure 7**) (5, 47). In late eggs, when they are covered by large amount of symbionts (47), the gene is slightly expressed (**Figure 7A**), but the protein is not detected in the western blot analysis (**Figure 7B**). Using immunohistochemistry, which is a more sensitive method, we detect a small amount of the protein in the membrane of freshly spawned eggs (early eggs, almost deprived of symbionts, Methou et al. (47) (**Figures 8A,B**). In late stages, Re-crustin was immunodetected into vesicles beneath the cell membrane (**Figures 8CA,D**) and on the bacteria that form the biofilm surrounding the eggs (**Figures 8CB,D**). Juvenile specimens are young shrimps recruited close to adults aggregates where they have been sampled, and start their development toward adult symbiotic life (Methou et al., submitted). RT-qPCR data using total RNAs extracted from these whole juveniles combined with western blot show that Re-crustin transcripts and proteins are highly abundant at this transition stage (**Figure 7A**). Re-crustin was immuno-localized on the cuticle that delimits the cephalothoracic cavity, in the gills and also in the nervous system (**Figure 9**).

Because the life of adults is punctuated by molt cycles, the same protocol was applied to "white" adults at the beginning of their molt cycle, where almost all epibiont have been eliminated, in comparison with "red" adults at the end of their molt cycle, highly colonized but where epibionts are encrusted in minerals impairing their activities (39) (**Figure 10**). In both cases, Re-crustin transcripts were not detected by RT-qPCR using RNA extracted from whole animals, probably because of an over dilution of the transcripts (**Figure 7A**). By contrast, the protein was abundantly present in red adults at their pre-ecdysial stage while it was undetectable in adults that have just molted and are starting a new cycle (**Figure 7B**). Immunohistochemistry showed an accumulation of Re-crustin in the epidermis beneath the cuticle colonized by epibionts of red animals only (**Figure 10**).

DISCUSSION

We open hypothesis for a novel biological role for gene-encoded antimicrobial host defense peptides (AMPs) in crustacean-microbe interactions. Our results revealed that the expression of a new member of the classic crustin AMP family (Re-crustin) is spatio-temporally correlated with the

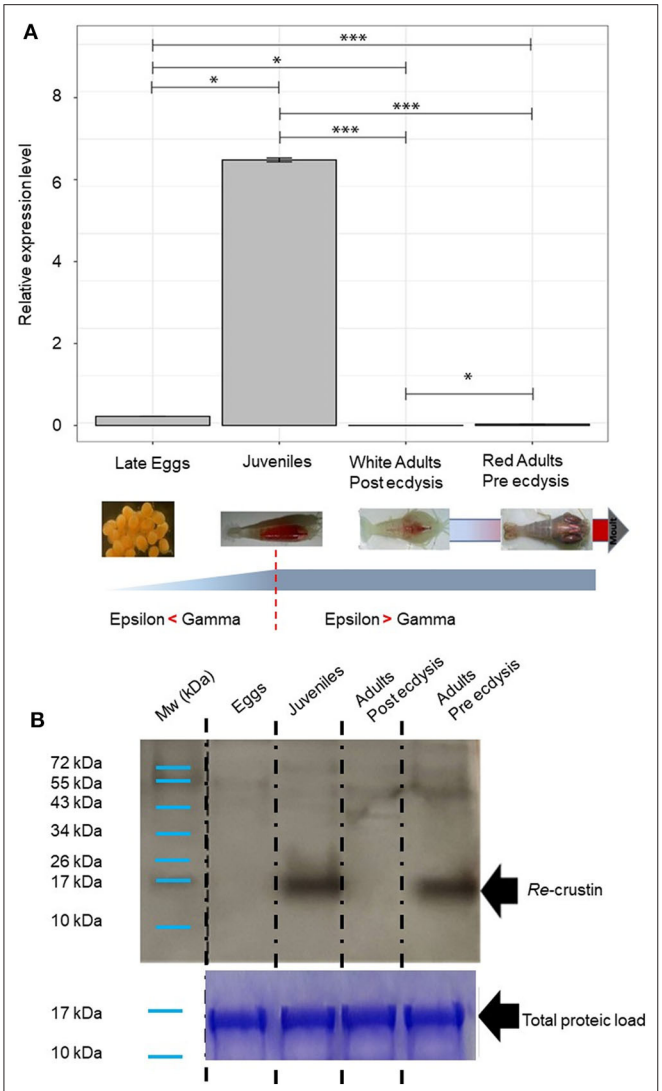

FIGURE 7 | Re-crustin distribution along the *Rimicaris exoculata* life cycle and molt stages. As represented, a shift occurs in symbiotic population between Gammaproteobacteria and Campylobacteria along the host life stage with the first one dominating at early stages while the second dominating at adult stages (5, 47) for early stages and (4, 33) for adults. **(A)** Gene expression analysis by RT-qPCR. Reference (Rpl8) and targets were amplified in separated wells ($n > 10$ in all cases). The graph shows the mean + SEM for each life stages and molt stages ($n = 10$ in all cases) as well as significance level for each intergroup comparison (ns, $p > 0.05$; *$p < 0.05$; ***$p < 0.001$; Dunn tests). Kruskal–Wallis: $\chi 2 = 36.596$; $p < 0.001$. Reference (Rpl8) and targets were amplified in separated wells. A technical triplicate was applied for each sample. **(B)** Western blot analysis was performed using total protein extracts (22 μg) from eggs, juveniles, adults (beginning of molt cycle), and adults (end of molt cycle). Immunostaining with the anti-Re-crustin antibody revealed one band of approximately 17 kDa corresponding to Re-crustin mass prediction. Mw, molecular weight markers. Equivalent well-loading was assessed by a generic protein staining of the gel (Coomassie Brilliant Blue R-250).

establishment of the ectosymbiotic microbial communities inhabiting the cephalothoracic cavity of the extremophile deep-sea shrimp *R. exoculata*.

Different from its Pleocyemata counterparts that mainly produce Type I crustins, *R. exoculata* expresses in its tissues a N-terminal glycine-rich crustin that belongs to the Sub-Type IIa. Indeed, the Type II comprises a distinct group of crustins usually found in penaeid shrimp (Dendrobranchiata suborder) that is subdivided into two Sub-Types: Type IIa ("Crustins") and Type IIb ("Crustin-like") (44). Although unusual, the presence of Type IIa crustins has been reported for other decapod crustaceans from the Pleocyemata suborder (59, 60). On the other hand, Type IIb crustins were only described in penaeid shrimp species (46).

The presence of a signal peptide in the Re-crustin precursor, together with the results obtained from the western blot analysis, provides evidence of its processing prior to the release of the Re-crustin into the extracellular compartment where it exerts its biological properties. The accumulation of Re-crustin on the surface of some ectosymbionts, as evidenced by immunohistochemistry, also supports the extracellular secretion and clearly shows an interaction of Re-crustin with the ectosymbiotic community of the cephalothoracic cavity *in vivo*. This interspecific interaction appears as an important function of Re-crustin in *R. exoculata*. Our multiple approaches all demonstrate that Re-crustin is essentially produced by the mouthparts in the cephalothoracic cavity, which is hugely colonized by the ectosymbionts (4, 5, 30, 32). Re-crustin was slightly detected in the digestive tract, which is colonized by a microbial community different from the one of the cephalothoracic cavity (28, 29). This suggests a low contribution of Re-crustin in this organ and underlines the importance of the molecule in the cephalothoracic cavity of *Rimicaris*. When tested for their production of antibiotics, the scaphognathites and branchiostegites showed an antibacterial activity that was partially, but not only, due to Re-crustin, thus suggesting the synthesis of other still undiscovered antimicrobial substances by these appendages or their associated bacteria. No activity was observed against the tested proteobacteria, confirming Re-crustin as belonging to the Type IIa crustins which are known to be mainly active against Gram-positives (61) while the Type IIb crustin from *Penaeus monodon* (crustinPm7/Crus-likePm) showed antibacterial activity against both Gram-positive and Gram-negative bacteria (62, 63). Because they are also uncultivable, the ectosymbionts could not be used for *in vitro* antimicrobial assays. However, immunodetection with the anti-Re-crustin antibody showed that the AMP covers the surface of the filamentous ectosymbionts (mainly composed of Gram-negative Proteobacteria/Campylobacterota) without killing them. A control of the bacterial growth via bacteriostatic activities of Re-crustin as observed in the endosymbiostasis of beetles cannot be excluded (64). One could also hypothesize that when embedding the ectosymbionts, Re-crustin contributes to their success by acting as an anti-competitive agent against other environmental bacteria and by favoring indirectly or directly their growth. A chemoattractant effect of Re-crustin favoring the recruitment of target symbionts and their subsequent attachment to the host might also be proposed as an explanation to the accumulation of Re-crustin at the surface of some ectosymbionts. The production of the recombinant molecule is planned to enlarge the spectrum of antibacterial activities and to decipher the other putative biological functions of Re-crustin.

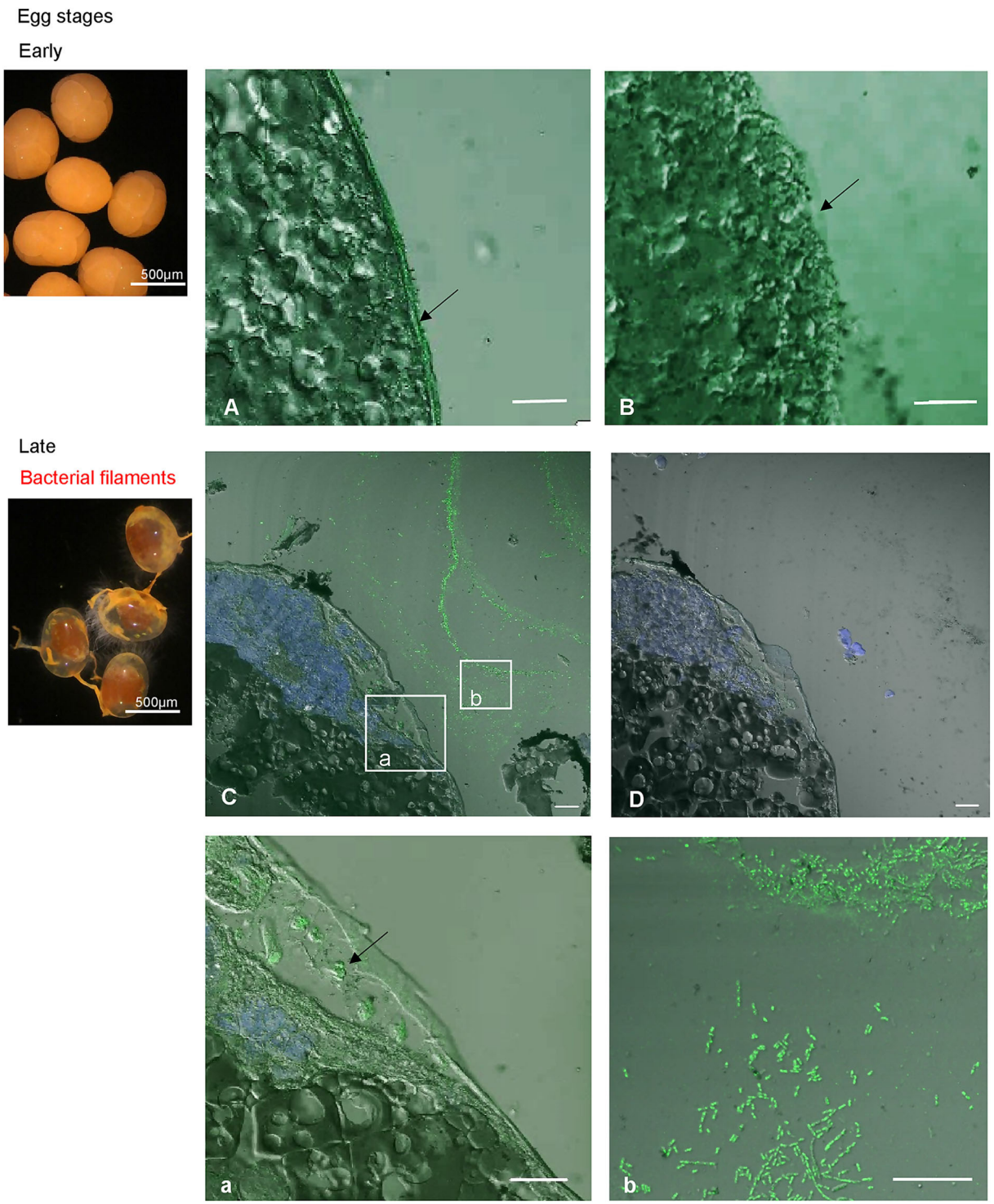

FIGURE 8 | Immunolabeling on paraffin sections with a thickness of 4 µm of Re-crustin on eggs of early stages (**A,B**, no biofilm) and of late stages (**C,D**, a,b, orange biofilm). A slight labeling is visible in the panels corresponding to eggs at young stage, with the specific antibody **(A)** and is absent with the pre-immune control **(B)**. On late stages, the biofilm that covers the eggs is clearly labeled (**C**, b for a closer view), and some vesicles containing Re-crustin are visible beneath the membrane of the egg (**C**; a; for a closer view). **(D)** No labeling is visible with the pre-immune control. Nucleic acids are labeled in blue (DAPI). The white field is superimposed to see the shadows of the structures. The observations were made using the confocal microscope, Zeiss LSM 780. Scale bars correspond to 20 µm.

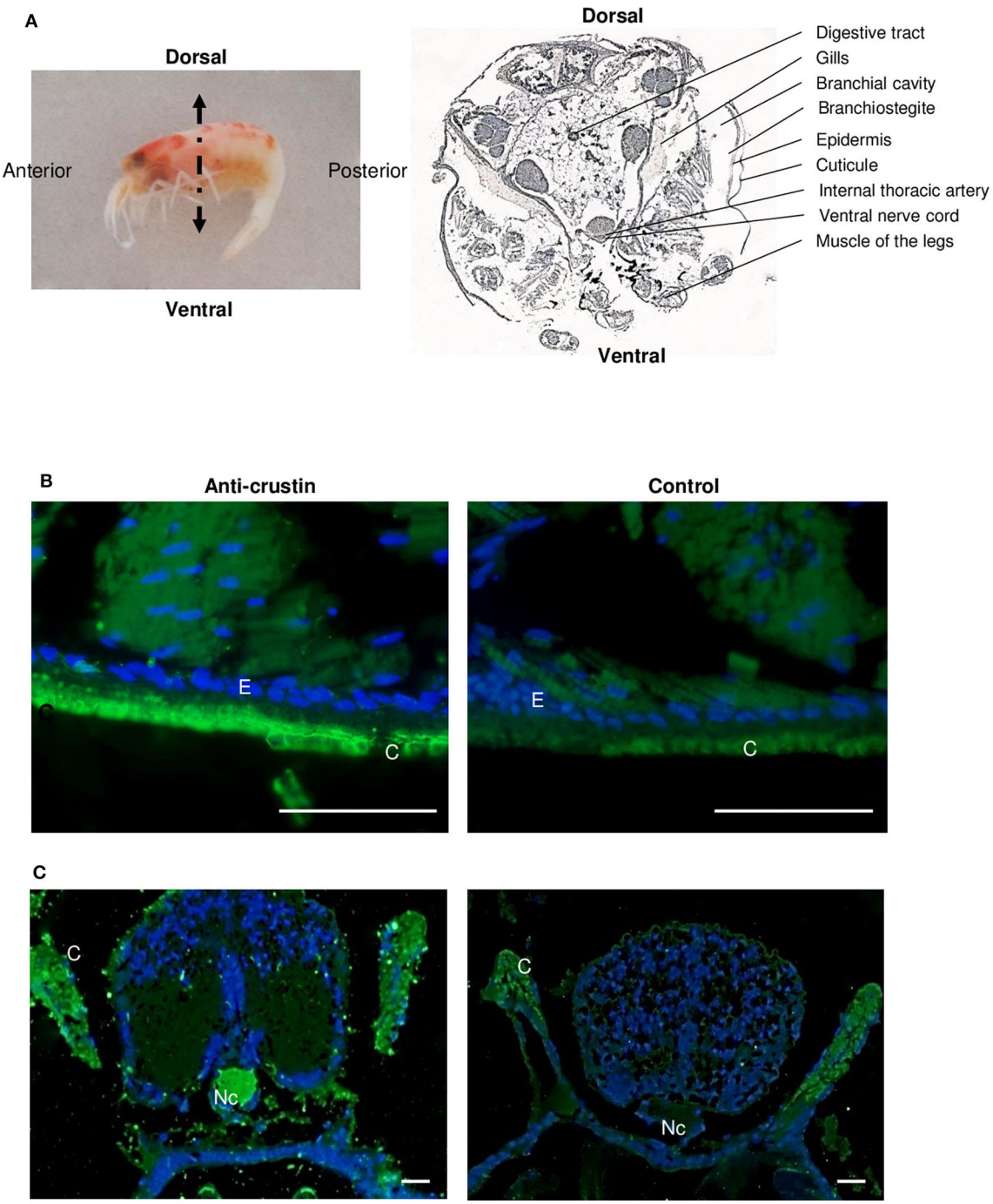

FIGURE 9 | Immunolabeling on paraffin sections with a thickness of 7 μm of Re-crustin on juveniles. **(A)** Image of a juvenile fixed in 4% paraformaldehyde to illustrate the detailed section shown on the right. The anti-crustin antibody specifically labels in green the cuticle **(B)**, gills and nerve cord **(C)** in juveniles. No labeling is visible in the control panels. Nucleic acids are labeled in blue (DAPI). The observations were made using the fluorescence microscope, Zeiss Axio Imager 2. Scale bars correspond to 20 μm. Abbreviations used: C, cuticle; E, Epidermis; G, Gills; Nc, nerve cord.

Re-crustin is probably part of a cocktail of AMPs and immune receptors, such as the recently characterized type C lectin (10), acting synergistically to shape the symbiont community and to prevent the colonization of the gills by pathogens and/or competitors such as Gram-positive bacteria and also Archaea. Further investigations will have to be performed to identify the other antibiotics involved in the emblematic ectosymbiotic association of *Rimicaris*.

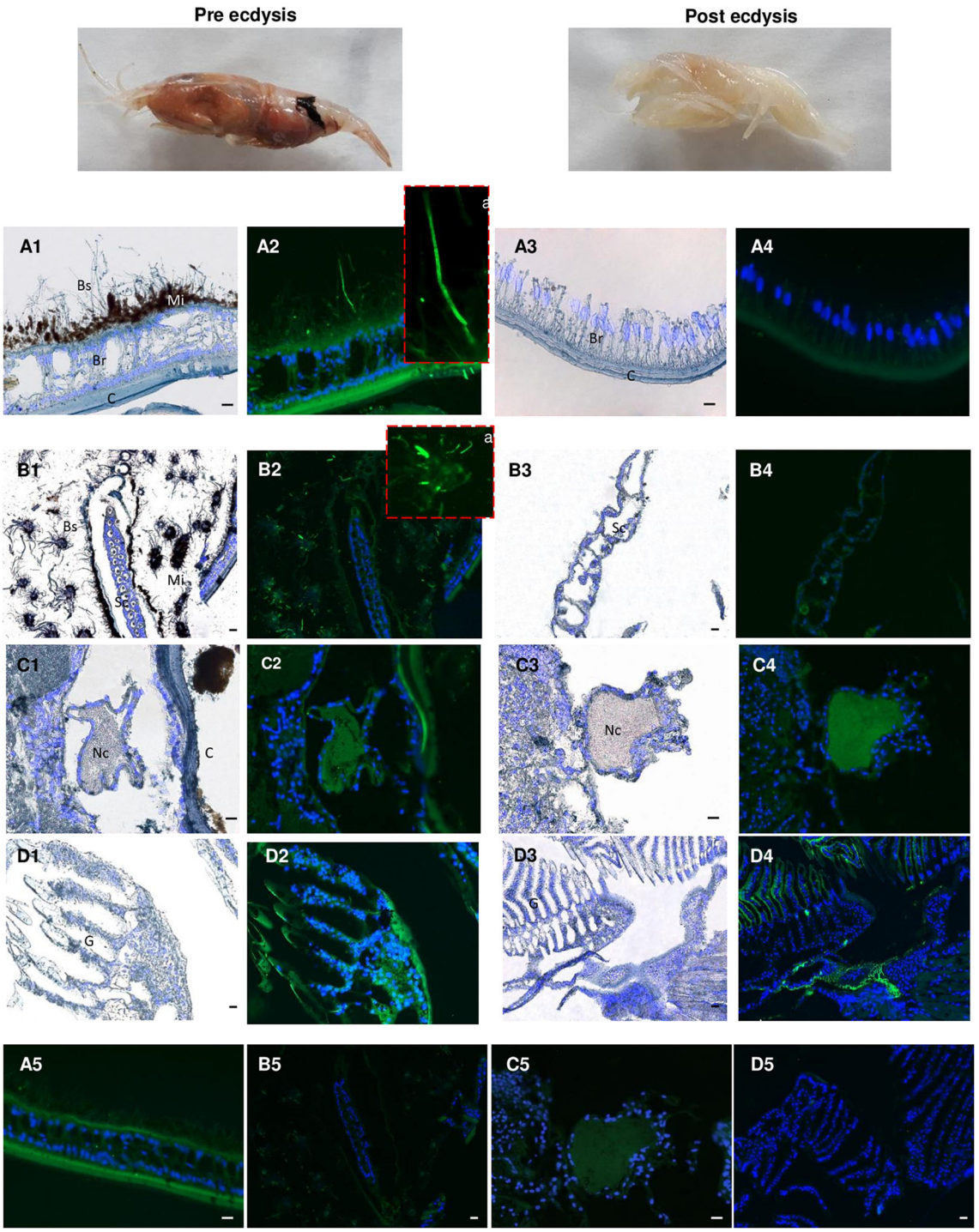

FIGURE 10 | Immunolabeling on paraffin sections with a thickness of 7 μm of Re-crustin in adult shrimps before and after molting. Two images present an adult shrimp at the end of the molting cycle (Pre ecdysis) and after molting (Post ecdysis). **(A2-a)** The anti-crustin antibody specifically labels in green the tissue that lines the inside of the cephalothoracic cavity and also covers the surface of some attached bacteria at the Pre ecdysis but not at the Post-ecdysis stage **(A4)** which is devoid of symbionts. **(B2-a)** Some bacteria attached to the scaphognathites are labeled at Pre ecdysis. No labeling is observed in the scaphognathites free of ectosymbionts of postecdysial adults **(B4)**. An immune-staining of the Re-crustin is observed in the Nerve cord **(C2-C4)** and in the gills **(D2-D4)** of adults at both the pre and post ecdysis stages. Nucleic acids are labeled in blue (DAPI). **(A5-B5-C5,D5)** No labeling appears with the pre-immune control. The white field is shown in **(A1, A3, B1, B3, C1, C3, D1, D3)** to see the structures. The observations were made using the fluorescence microscope, Zeiss Axio Imager 2. The 20 μm scale bars are placed on the white fields and on the pre-immune controls. Abbreviations used: Br, branchiostegites; C, cuticle; Bs, Bacterial symbionts; G, gills; Mi, mineral deposits; Sc, scaphognathite; Nc, nerve cord.

Re-crustin was also immunodetected in the central nervous system (CNS). Further investigations should be performed to determine whether the expression sites are the neurons themselves or the epithelial cells infiltrated into the nervous system as documented for the crustin named PET-15 in the spiny lobster (65). A multifunction of Re-crustin appears also as a potential hypothesis to explore, following e.g., results on the neuronal growth activities of AMPs produced by the CNS of other invertebrates (66).

To go further into the role of Re-crustin in the ectosymbiostasis of *Rimicaris*, we explored the spatio-temporal correlations between the levels of production of Re-crustin and the ectosymbiotic acquisition/loss/re-colonization events that punctuate the life cycle of the hydrothermal shrimp (**Figure 1**). Our data show that Re-crustin, is already present at the surface of just spawned eggs. The synthesis is enhanced in late eggs still attached to the abdomen of the mother, as they are being colonized by a dense mat of bacteria. Interestingly, Re-crustin secreted by late eggs covers the bacteria forming the biofilm. The biofilm formation appears concomitant to Re-crustin synthesis/secretion in eggs during their embryonic stages and thus may contribute to the development of the immune system. Re-crustin may also serve as an anti-competitive and/or a growth factor for the symbionts. During their embryonic development, *R. exoculata* egg envelopes are colonized by bacterial communities that partly differ from adult's symbiotic communities for late stage eggs (5, 47). After hatching, larvae undergo several molting events, still uncharacterized, to the juvenile stage A, while dispersing in the water column before their recruitment to a new hydrothermal vent field (5, 67). Although we cannot rule out the possibility that some of the bacteria present on eggs during embryonic development are carried along during post-hatching larval life until recruitment, recruited juveniles appear to be mostly colonized anew shortly after settlement by an ectosymbiotic community i.e., the one of the cephalothoracic cavity identified in surrounding adults (40). In late eggs and even much more pronounced in juveniles recruited to the sites inhabited by adults, the peak of synthesis of Re-crustin likely corresponds to the development of a novel bacterial community corresponding to the adult one. Re-crustin would then appear as a developmental, metamorphic signal induced by the symbiotic community associated to the eggs and to the juveniles starting their development toward adult symbiotic forms.

Immunohistochemistry combined with RT-qPCR and western blot data also show a spatial correlation between the AMP and the symbionts, with an abundant presence of Re-crustin in eggs and in the epidermis cells beneath the cuticle of the branchiostegites and scaphognathites carrying the ectosymbionts, in recruited juveniles and in adults.

As far as we know, only few studies have been devoted to the investigation of crustin expression during the early stages of crustacean's life and none were correlated with the associated bacterial community (43, 45, 68, 69). Hauton and colleagues have shown that Type I crustin expression levels are similar in lobster *Homarus gammarus* postlarvae stages IV and VI (68). Larvae of the shrimp *P. monodon* have been reported to express a Type IIb crustin transcript at high levels at all stages of development from nauplii IV through to juveniles (46). In all reported cases, an immune function of crustins in larvae was proposed but their involvement in the symbiostasis was not investigated.

Like most crustaceans and contrary to metabole insects, *Rimicaris* still molt during their adult stage. Molting results in the complete renewal of the cuticle (including mouth appendages) together with the loss of the attached ectosymbiotic community, notably on scaphognathites and branchiostegites in our case. Interestingly, the production of Re-crustin by the scaphognathites and branchiostegites reaches a peak when the re-colonization occurs, suggesting a role of Re-crustin in the control of the symbiosis acquisition and presumably in the selection and success of the appropriate bacteria from the habitat. Surprisingly, the protein load is increased in the pre ecdysis stage while the mRNA expression of Re-crustin is not. In addition to the technical explanation (see results), a neosynthesis might also occur in between the two stages (i.e., in between the post and the pre ecdysis) and should be investigated. An increased gene expression starting after the post ecdysis would lead to an accumulation along the molt cycle of the protein (embedding the symbionts that have colonized the gill chamber) which quantity would reach a maximal at the pre-ecdysis stage whereas the mRNA transcription would have already stopped. Since *Rimicaris* forms large and dense colonies constituted by individuals desynchronized in their molting and development, the symbiotic bacteria remain always available within the population, such as on shed exuvia, thus allowing the horizontal transfer of the symbionts. Because Re-crustin accumulates on the surface of both the symbionts and the cuticle of the appendages, the molecule may also serve as a communication pathway (such as a chemoattractant effect see before) in between the host and the bacteria thus favoring the recruitment and the horizontal transfer of the free-living symbionts.

During the extremely short molt cycle (10 days), the symbiont growth is accompanied by a progressive mineral accumulation, caused in part by the symbiont activities (39, 70). In pre-ecdysial advanced stage, the mineral crust completely surrounds the symbionts and some lysis forms can even be observed. The recovery induced by molting could "stifle" the symbionts that would no longer be able to properly feed their host. The host might thus trigger its molt to recycle its ectosymbionts. Therefore, the *R. exoculata* molt cycle could be compared to the concept of symbiotic trade-off retrieved in insects (64) whereby insects offer "board and lodging" to the endosymbionts as long as they can rely on their metabolic supply, and recycle them by autophagy and apoptosis when the cost of symbiont maintenance overcomes the provided benefits.

Our results overall highlight a novel AMP sequence from an extremophile organism and suggest the role of AMPs in the establishment of vital ectosymbioses that take place along the life cycle of marine organisms. *R. exoculata* enlarged cephalothoracic cavity offers a mechanic but also, an immune protective and growth environment for the symbionts against the harsh hydrothermal vent conditions and their microbial competitors while the symbionts provide nutrients *via* a transcuticular transfer (6) and maybe an immunity to the shrimp. The

developmental and metamorphic role of the ectosymbiosis *via* the ıproduction of Re-crustin would have to be investigated to decipher the correlated production of the AMP with the symbiotic colonization and the transition stages of the host.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Re crustin, GenBank accession number: MT102281.

AUTHOR CONTRIBUTIONS

AT, M-AC-B, and FP designed the research. SL, CB-W, VC-G, LD, and PM developed reagents and performed experiments. SL, CB-W, RR, VC-H, M-AC-B, and AT analyzed the data. SL, CB-W, RR, VC-H, PM, FP, M-AC-B, and AT wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Functional Reciprocity of Amyloids and Antimicrobial Peptides: Rethinking the Role of Supramolecular Assembly in Host Defense, Immune Activation, and Inflammation

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Pathological self-assembly is a concept that is classically associated with amyloids, such as amyloid-β (Aβ) in Alzheimer's disease and α-synuclein in Parkinson's disease. In prokaryotic organisms, amyloids are assembled extracellularly in a similar fashion to human amyloids. Pathogenicity of amyloids is attributed to their ability to transform into several distinct structural states that reflect their downstream biological consequences. While the oligomeric forms of amyloids are thought to be responsible for their cytotoxicity via membrane permeation, their fibrillar conformations are known to interact with the innate immune system to induce inflammation. Furthermore, both eukaryotic and prokaryotic amyloids can self-assemble into molecular chaperones to bind nucleic acids, enabling amplification of Toll-like receptor (TLR) signaling. Recent work has shown that antimicrobial peptides (AMPs) follow a strikingly similar paradigm. Previously, AMPs were thought of as peptides with the primary function of permeating microbial membranes. Consistent with this, many AMPs are facially amphiphilic and can facilitate membrane remodeling processes such as pore formation and fusion. We show that various AMPs and chemokines can also chaperone and organize immune ligands into amyloid-like ordered supramolecular structures that are geometrically optimized for binding to TLRs, thereby amplifying immune signaling. The ability of amphiphilic AMPs to self-assemble cooperatively into superhelical protofibrils that form structural scaffolds for the ordered presentation of immune ligands like DNA and dsRNA is central to inflammation. It is interesting to explore the notion that the assembly of AMP protofibrils may be analogous to that of amyloid aggregates. Coming full circle, recent work has suggested that Aβ and other amyloids also have AMP-like antimicrobial functions. The emerging perspective is one in which assembly affords a more finely calibrated system of recognition and response: the detection of single immune ligands, immune ligands bound to AMPs,

and immune ligands spatially organized to varying degrees by AMPs, result in different immunologic outcomes. In this framework, not all ordered structures generated during multi-stepped AMP (or amyloid) assembly are pathological in origin. Supramolecular structures formed during this process serve as signatures to the innate immune system to orchestrate immune amplification in a proportional, situation-dependent manner.

Keywords: antimicrobial peptides, amyloids, self-assembly, Toll-like receptors, innate immunity, autoimmune diseases, neurodegenerative diseases

INTRODUCTION

Amyloids and antimicrobial peptides (AMPs) are two classes of proteins that have fascinating biophysical and structural properties. Until recently, they were thought to be distinct entities with vastly different functions. Amyloids were strictly pathologic and accumulation in tissues invariably led to diseases (1). In comparison, AMPs are considered essential components of the innate immune system, defending against invasive microbial infections and sounding the alarm to activate cellular-mediated immune responses (2, 3). Within the last 5–10 years, emerging work from collaborations between bioengineers, amyloid biologists, and immunologists has dramatically blurred the lines between amyloids and AMPs. AMPs and amyloids have strikingly similar structural and biophysical properties that enable them to self-assemble with immune ligands like DNA to amplify immune responses (4–6). Surprisingly, many amyloids possess hidden antimicrobial activity in addition to their known cytotoxic properties, suggesting a potential endogenous role in host defense (7, 8). AMPs and bacterial amyloids have also been implicated in the pathogenesis of autoimmune diseases like lupus and psoriasis (5, 9–13), parallel to the proinflammatory role of amyloids in neurodegeneration (14). Disentangling the molecular basis for the homeostatic and pathologic functions of both amyloids and AMPs has proven challenging (15).

The goal of this review is to highlight fundamental studies that showcase the unexpected similarities between amyloid and AMP self-assembly and discuss how these findings can transform our understanding of their functional roles in host defense, inflammation, and disease. While some effort has been made in the literature to compare and contrast amyloids and AMPs, it has been difficult to identify common themes due to the sheer diversity of sequences and structures in both classes of molecules (**Figure 1**). Here, we begin by first providing a short overview of AMPs and their known antimicrobial and immunomodulatory functions. We focus on recent work from our group that outlines a novel emerging paradigm for understanding how AMPs talk to the innate immune system. We find that AMPs self-assemble into amyloid-like protofibrils that act as molecular templates to scaffold canonical immune ligands into spatially periodic nanocomplexes, which amplify immune responses via pattern-recognition receptors (PRRs) such as the Toll-like receptors (TLRs) (**Figure 2**). We demonstrate how this paradigm is general to other immune proteins beyond AMPs such as chemokines as well as other TLRs. We then discuss implications for the synergistic role of AMPs in normal host defense as well as in autoimmunity. In the second part of the review, we compare AMP self-assembly to amyloid self-assembly in the contexts of antimicrobial and membrane-remodeling activity (**Figure 3**). Lastly, we summarize how the functional similarities between AMPs and amyloids extends to bacterial amyloids as well in the realm of immunomodulation. By borrowing lessons and tools from the AMP literature, we find that amyloids potentially have endogenous functions beyond their pathologic consequences. We conclude by suggesting future research directions that can integrate our knowledge of AMP and amyloid biology to uncover mechanisms of disease and develop new targeted therapies.

AMPs ORGANIZE IMMUNE LIGANDS INTO SPATIALLY PERIODIC NANOCOMPLEXES TO AMPLIFY TLR ACTIVATION

AMPs are part of an ancient arm of the innate immune system that represents the first line of defense against microbial infections (2). AMPs are found in almost all living organisms including vertebrates, invertebrates, and plants (16–18), and can be broadly categorized by their secondary structures: the α-helical AMPs, β-sheet AMPs, AMPs with cross α-β structures, and extended linear peptides with specific enriched amino acids (19–21) (**Figure 1**). The prototypical human AMP is cathelicidin (LL37), which is an α-helical AMP with essential anti-infective and immunomodulatory functions (28, 29). Prototypical human β-sheet AMPs are the defensins. The mechanisms underpinning the antimicrobial activity of AMPs are thoroughly reviewed elsewhere but we briefly discuss it here (3, 30–32). In general, AMPs are cationic (+2 to +9) and amphiphilic with segregated groups of hydrophobic and polar/charged residues (2). These properties enable AMPs to electrostatically bind to negatively charged bacterial membranes and embed themselves into the membrane via hydrophobic interactions. Several models have been proposed for membrane permeation, including the "barrel-stave" model, "carpet" model, and "toroidal-pore" model (32). In the "barrel-stave" and "toroidal-pore" models, AMPs self-assemble into bundles that cylindrically insert into bacterial membranes to form aqueous pores, whereas in the "carpet" model, AMPs disintegrate the membrane via micellization (33). We have shown that AMP antimicrobial function correlates with its ability to induce negative Gaussian curvature (NGC) in bacterial membranes, a topological criterion for pore formation and membrane permeation (34). However, antimicrobial peptides are not only

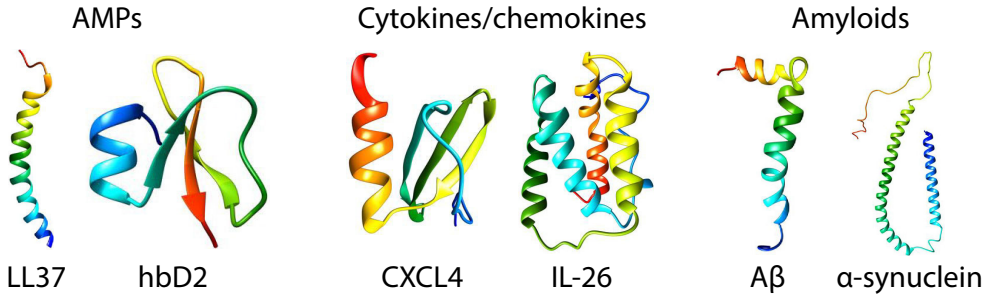

FIGURE 1 | Structures of prototypical antimicrobial peptides, cytokines/chemokines, and amyloids. LL37 (22) and human β-defensin 2 (23) are canonical α-helical and β-sheet AMPs, respectively. CXCL4 (24) and IL-26 [homology model shown based on IL-19 (25)] are representative immune signaling molecules that also have known direct antimicrobial properties. Amyloid β (26) and α-synuclein (27) are the amyloids implicated in Alzheimer's disease and Parkinson's disease. The monomeric structures were taken from the Protein Data Bank (PDB) and visualized in Chimera (UCSF).

limited to membrane permeation. AMPs can also kill bacteria and fungi by disrupting metabolic gradients, inhibiting ribosomes, and binding to intracellular nucleic acids (35). However, the most underappreciated aspect of AMP function is their ability to amplify immune responses by autocrine signaling via PRRs such as TLRs. AMPs can signal through PRRs via direct binding. LL37 has been shown to be a chemoattractant for leukocytes by binding to the formyl peptide receptor-like 1 (FPRL1) (36). Furthermore, β-defensins are known to be chemotactic for monocytes and macrophages by binding to the CCR6 receptor (37), and β-defensin 2 is a known ligand for Toll-like receptor 4 (TLR4) (38). Despite this work, it was not known until recently whether AMPs could signal to PRRs without being direct ligands, or whether they could serve as chaperones by binding to immune ligands such as nucleic acids.

In a series of groundbreaking studies, Lande et al. showed that LL37 can break immune tolerance to self-DNA in diseases like lupus and psoriasis by forming insoluble complexes that are phagocytosed by immune cells. In these diseases, LL37 is overexpressed in the skin and blood and are predominantly produced by neutrophils and keratinocytes (39–41). LL37-DNA complexes are formed extracellularly and are internalized into the endosomes of plasmacytoid dendritic cells (pDCs), amplifying type I interferon (IFN-α) production by binding to Toll-like receptor 9 (TLR9). They also showed that other cationic AMPs in the skin possess a similar property, including the β-defensins and lysozyme (42). To understand the molecular basis for how LL37 and other AMPs signal through TLR9, we characterized the structures of numerous AMP-DNA complexes using X-ray scattering and correlated them with their ability to activate pDCs via TLR9 (43). We found that LL37 and β-defensins electrostatically self-assemble with DNA into spatially periodic grill-like nanostructures with well-defined inter-DNA spacings, and that the inter-DNA spacing within these complexes correlated directly with the quantitative degree of cytokine production (**Figures 2A–D**). The biophysics of the hierarchical electrostatic self-assembly of rigid polyelectrolytes like DNA has been well-described in the literature and is thoroughly discussed elsewhere (45–47). AMP-DNA complexes with spacings well-matched with the steric size of TLR9 enabled multivalent binding to clustered TLR9 on the endosomal membrane and IFN-α production orders of magnitude higher than expected from individual ligands (45). Surprisingly, this phenomenon was independent of the degree of endosomal uptake, suggesting that this differential response was solely due to differences in the nanostructures of the complexes. This conceptual transformation suggested that a much broader range of molecules could be predicted to activate TLR9 if they had the right physicochemical properties to organize and present DNA at optimal periodic positions that promote multivalent interactions with an ensemble of TLR9.

Inspired by this, we set out to discover general rules for how α-helical AMPs like LL37 can self-assemble into molecular templates for DNA binding and amplify immune responses. Previous work has shown that artificial patchy amphiphiles can be designed to self-assemble into various unique structures (48, 49). By combining computer simulations with X-ray structural characterization, we found that LL37 oligomerizes into a superhelical amyloid-like protofibril in the presence of DNA, with hydrophobic residues buried in the interior and outward-facing cationic residues (4) (**Figures 2A,B**). The LL37 protofibril cross-links DNA into a 4-fold coordinated lattice with inter-DNA spacings commensurate with the size of TLR9. We conducted experiments with other α-helical AMPs with different charge densities and hydrophobicities such as melittin (50) and buforin (51). We discovered that formation of this amyloid-like protofibril requires sufficient hydrophobicity to enable polymerization into a superhelix and cationic charge density well-matched to the high anionic charge density of DNA. Remarkably, we discovered that although melittin was able to form optimized complexes with DNA for TLR9 activation, its cytotoxicity to immune cells prevented cytokine production. By attenuating its cytolytic activity while retaining its ability to self-assemble into 4-fold coordinated nanocomplexes with DNA at the optimal inter-DNA spacing, we rescued its ability to activate TLR9 (4). This highlighted that there exist natural tradeoffs in antimicrobial and immunomodulatory functions of AMPs, and that we can deterministically modulate them by altering the AMP's physicochemical properties.

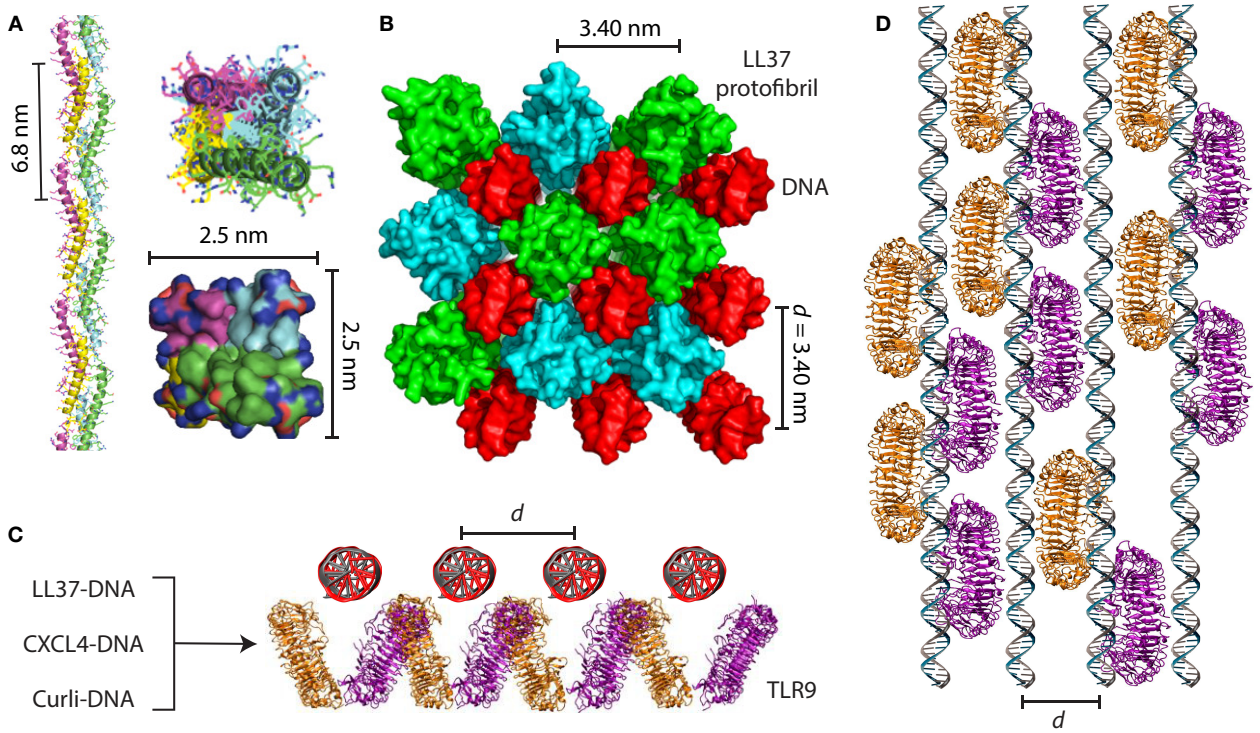

FIGURE 2 | AMPs and amyloids organize immune ligands into spatially periodic nanocomplexes to amplify TLR activation. **(A)** LL37 self-assembles into a 4-fold amyloid-like superhelical protofibril in the presence of DNA. Hydrophobic residues are buried in the interior of the protofibril while cationic residues are exposed at the perimeter. **(B)** Structure of the LL37-DNA complex showing cross linking of spatially periodic DNA strands by LL37 protofibrils at an inter-DNA spacing of 3.40 nm, which is optimal for TLR9 binding and amplification of cytokine production. **(C)** End-on view and **(D)** top-down view of geometrically organized DNA immune complexes binding to clustered TLR9 in the endosomal membrane. In addition to the LL37-DNA complex, CXCL4-DNA complexes formed in scleroderma and curli-DNA complexes from *Salmonella* biofilms also demonstrate similar structural properties that enable amplification of TLR9 in immune cells and type I interferon production. **(A,B)** are adapted with permission from (4). **(C,D)** are adapted with permission from (44) and (10).

The next natural question to ask is whether this phenomenon is general to other immune ligands and innate immune receptors. Gallo and colleagues have previously shown that LL37 can break immune tolerance to double-stranded RNA (dsRNA) released from keratinocytes in psoriasis and other cutaneous diseases (52–55). Given the structural homology of TLR9 to Toll-like receptor 3 (TLR3) and DNA to dsRNA, respectively, we decided to map out the structural rules for immune activation of TLR3 by dsRNA complexes (56). We characterized the structures of numerous AMP-dsRNA complexes (LL37 and various truncated variants) and tested their ability to induce IL-6 production from psoriatic keratinocytes via TLR3. Cognate to LL37-DNA complexes, we found that LL37-dsRNA complexes formed nanocrystalline structures with well-defined inter-dsRNA spacings, and that complexes that maximally activated TLR3 had spacings perfectly matched with the steric size of TLR3. A mathematical model and computer simulation of TLR3 binding to spatially periodic AMP-dsRNA complexes recapitulated the experimental data and showed that both the inter-dsRNA spacing and the number of repeat units within the complexes were primary determinants of immune activation (56). This validated the idea that innate immune receptors like TLR9 and TLR3 can recognize both single ligands, as well as the crystallinity of spatially periodic, geometrically patterned ligands templated by molecular chaperones like AMPs.

As it turns out, this phenomenon is not limited to AMPs, but is rather general to other immune signaling proteins. Chemokines are a well-studied class of immune signaling molecules that are known to exert their biological activities by binding to G-protein coupled receptors (GPCRs) on the surface of immune cells. We discovered an unexpected signaling pathway for chemokine (C-X-C motif) ligand 4 (CXCL4)/platelet factor 4 (PF4) and its role in the pathogenesis of scleroderma. Interestingly, CXCL4 naturally self-assembles into an oligomeric homotetramer and has a cationic, amphipathic cross α-β structure that is homologous to that of defensin antimicrobial peptides (57) (**Figure 1**). It has also previously been shown to exert antimicrobial activity (58–62). CXCL4 is typically highly expressed in platelets and plays a key role in hemostasis and wound healing (63). CXCL4 is known to bind to anionic heparin, particularly in the context of heparin-induced thrombocytopenia (64–66), but its causal role in inflammatory diseases was unclear. We discovered that

like LL37 and other AMPs, CXCL4 can self-assemble with microbial and self-DNA to form nanocomplexes to amplify IFN-α production via TLR9 within skin pDCs (**Figures 2C,D**). We identified CXCL4-DNA complexes in the blood and skin of scleroderma patients, and levels of these complexes correlated directly with the type I interferon signature (44). Surprisingly, this activity was independent of the canonical CXCL4 receptor, CXCR3. We predict that many other chemokines likely possess similar properties, since they share a structural backbone and have close physicochemical similarity, including the ability to self-assemble into oligomers. Taken together, our findings are consistent with a robust emerging conceptual framework where diverse classes of molecules can signal to the innate immune system by scaffolding endogenous immune ligands into spatially periodic nanocomplexes, rather than being direct agonists.

SYNERGY BETWEEN THE ANTIMICROBIAL AND IMMUNOMODULATORY PROPERTIES OF AMPs AND CHEMOKINES

Thus far, we have demonstrated that AMPs and chemokines are multifunctional, and can exert direct antimicrobial activity and modulate immune responses via PRRs. Due to their cationicity and amphipathicity, AMPs are capable of directly killing microbes through membrane permeation, inhibition of metabolic machinery, and disruption of electrostatic gradients. However, the same physicochemical features allow them to also self-assemble into ordered nanocrystalline complexes with immune ligands such as DNA and dsRNA by functioning as structural scaffolds. These complexes can potently induce inflammation by amplifying Toll-like receptor activation via receptor clustering, and the crystallinity of these complexes can determine the degree of immune amplification (43). What are the consequences of this multifunctionality for host defense?

Synergy between the dual antimicrobial and immunomodulatory functions of many AMPs and chemokines enables them to play an important role in protection against infections and in mediating autoimmune disease and inflammation. Certain AMPs and chemokines are capable of lysing and killing bacteria and presenting fragments of bacteria such as DNA to innate immune receptors. For instance, Meller et al. demonstrated that interleukin 26 (IL-26), a cytokine secreted by human interleukin-17 producing helper T cells (T_H17), both kills bacteria and promotes immune sensing of bacterial and host cell death, driving the potent antimicrobial and proinflammatory function of T_H17 cells (67) (**Figure 1**). IL-26 is a highly cationic and amphipathic protein that possess broad-spectrum antimicrobial activity against several gram-negative bacterial strains including *P. aeruginosa, E. coli,* and *K. pneumoniae*, and gram-positive bacteria *S. aureus* (67, 68). IL-26, like AMPs, can oligomerize into multimers and lyse bacteria by forming pores in their membranes. The antimicrobial properties of T_H17 cell-derived IL-26 helps explain why patients defective in T_H17 cells are highly susceptible to *S. aureus* infections (69), and why depletion of T_H17 cells during infection by simian immunodeficiency virus results in the dispersal of gut bacteria (70).

Upon bacterial killing, T_H17 cell-derived IL-26 triggers potent immune activation. IL-26 forms nanocrystalline complexes with bacterial DNA released during the antimicrobial response. These complexes are internalized into the endosomal compartments of pDCs and induce an amplified production of IFN-α via recruitment and super-selective binding of TLR9 receptors. Type I interferons are responsible for driving many proinflammatory responses, including CD8+ T cell activation (71, 72), T_H1 cell differentiation (72), NK cell activation, dendritic cell maturation (73, 74), and promotion of antibody-secreting plasma cells (75). Consequently, their production has been shown to be beneficial in the context of extracellular bacterial infections, including the resolution and control of infections caused by *P. aeruginosa*, *S. pneumoniae*, and *E. coli* (76, 77), and reducing inflammation in mouse models of bacterial sepsis (78). In addition to serving as a direct antimicrobial, IL-26 has evolved the ability to amplify and regulate innate and adaptive responses to extracellular bacteria. Its dual functionality allows our immune system to more effectively clear bacterial infections. Modulating the endogenous activity of IL-26 may offer promising strategies to enhance our natural host defense against microbes.

IL-26 and CXCL4 are likely several of many examples of multifunctional molecules that play a synergistic role in host defense against microbes via direct killing and immunomodulation, in addition to their other homeostatic functions. Recently, other interferons like IFN-β (79) and IFN-γ (80) were shown to exhibit direct antimicrobial properties in addition to their known immunomodulatory functions. These findings suggest that the nature has evolved a way to bioconjugate multiple distinct functions into the same amino acid sequence (81), and that understanding how the immune system works requires us to examine these hidden functions.

COMPARISON OF AMP AND AMYLOID SELF-ASSEMBLY

Here, we draw comparisons between the self-assembly of AMPs and the classical self-assembly of amyloids. Amyloids constitute a broad class of proteins that have the unique ability to aggregate into fibrils with characteristic secondary structures. The structural, physicochemical, and biological properties of AMPs are similar to those of many amyloid proteins. The majority of amyloids have a β-sheet secondary structure, but recently a subset of α-helical amyloids was identified (82, 83). Amyloids can be broadly categorized into those of eukaryotic and prokaryotic origins. Human endogenous amyloids are associated with over 50 distinct disease processes, the most famous of which is amyloid β-peptide (Aβ) in Alzheimer's disease (AD) (**Figure 1**). More and more proteins are being discovered to have amyloidogenic properties. Whether amyloids play a causal role in disease or are merely a consequence of disease is hotly debated. However, amyloids have unequivocally been shown to exhibit direct cytotoxic activity against human cells. The best data is

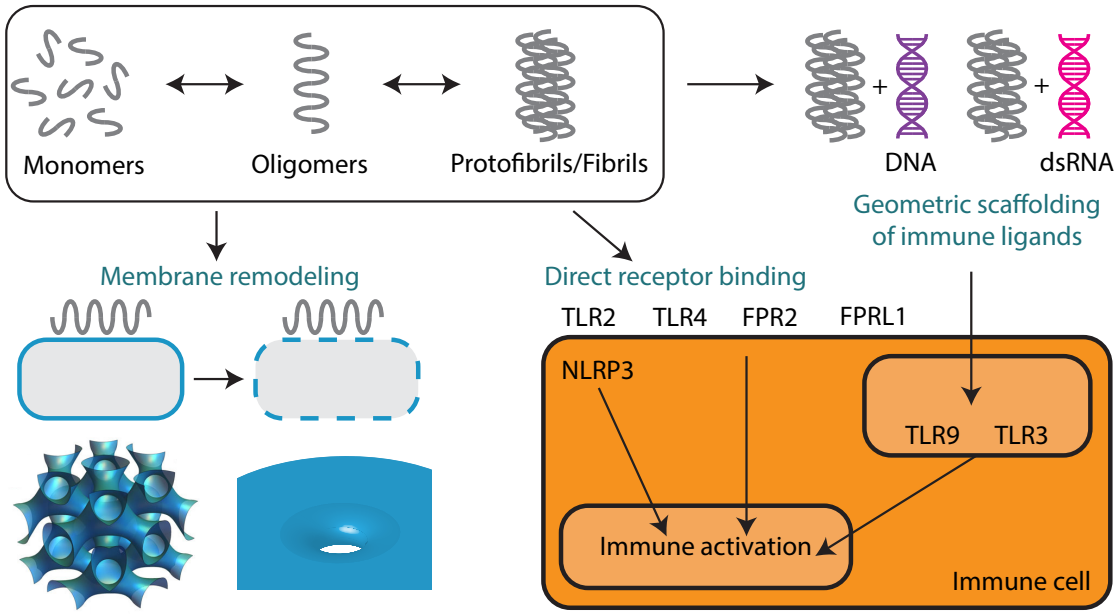

FIGURE 3 | Supramolecular self-assembly of AMPs and amyloids enables membrane remodeling activity and immunomodulation. Monomers of AMPs and amyloids sequentially self-assemble into oligomers and protofibrils or fibrils. Oligomeric forms are predominantly responsible for mediating membrane permeation, including pore formation and membrane fusion leading to direct antimicrobial activity and cytotoxicity. Protofibrils and fibrils can signal to the innate immune system either by direct receptor binding or by the geometric scaffolding of immune ligands such as DNA and dsRNA. Both AMPs and amyloids can engage a broad range of immune receptors including TLR2, TLR3, TLR4, TLR9, FPR2, FPRL1, and NLRP3.

available for Aβ, but many other amyloids have been shown to self-assemble into structures that can disrupt membranes (84) and signal to the immune system (**Figure 3**).

Aβ is the main component of amyloid plaques found within neurons in AD brains and is thought to induce cytotoxicity leading to neuronal cell death (85) via multiple mechanisms (86–89). Traditionally, Aβ has been characterized as a functional catabolic byproduct of amyloid precursor protein (APP) without much evidence for a possible endogenous homeostatic function (90). However, recent *in vitro* studies have shown that Aβ can exhibit AMP-like direct antimicrobial activity by disrupting membranes (91) and may play a role as an effector molecule of innate immunity, exhibiting broad-spectrum activity against several common and clinically relevant organisms (92) (**Figure 3**). In a directly related study, Kumar et al. highlighted the potent antimicrobial activity of Aβ and demonstrated its biological relevance in host defense through *in vivo* models of infection. Aβ expression is associated with increased host survival in both nematode and mouse models of bacterial (93) and viral infection (94). Low Aβ expression resulted in greater death of APP-KO mice after infection. The protective role of Aβ can be attributed to classic AMP mechanisms characterized by reduced microbial adhesion, bacterial membrane disruption, and entrapment of microbes by Aβ fibrils (93). Alternatively, low levels of fibrillar Aβ may signal to the immune system and elicit inflammation to keep the immune system or the infection in check. Low levels of Aβ can get cleared without amyloid deposition. Nonetheless, these data imply that Aβ possesses a normally protective role in host defense that, when dysregulated, can lead to neurodegenerative disease. Aβ may normally function as an endogenous inducible AMP that is cleared upon resolution of inflammation. However, when dysregulated in the right of genetic or environmental context, Aβ instead forms toxic amyloid oligomers leading to neuronal cell death and eventually deposits leading to chronic inflammation (95).

It is important to note that genetic factors may also be involved in the dysregulation of Aβ production in addition to environmental factors like bacterial and viral infections (96, 97). Overexpression of APP on chromosome 21 is associated with AD, and individuals with Down syndrome (Trisomy 21) are at a higher risk of AD relative to the population (96). In addition, Aβ from individuals with the "Arctic" mutation (E693G 669 on APP) tends to self-assemble into protofibrils at a much higher rate than the wild type protein (98). A larger number of additional genetic polymorphisms have been identified which affect Aβ cytotoxicity (99), but their consequences on Aβ in host defense is currently unknown. It is also possible that genetic polymorphisms in other immune and inflammatory genes can alter Aβ production and contribute to AD. For example, the apolipoprotein gene ApoE4 is another major genetic risk factor for AD (100, 101), and deficient clearance of Aβ is associated with disease (102). Further work will be required to elucidate how these genetic changes affect the function of Aβ in host defense and inflammation.

Similarly, while AMPs are typically protective, dysregulation of AMP expression can lead to host cell toxicity, degenerative pathologies, and chronic inflammation and autoimmunity as

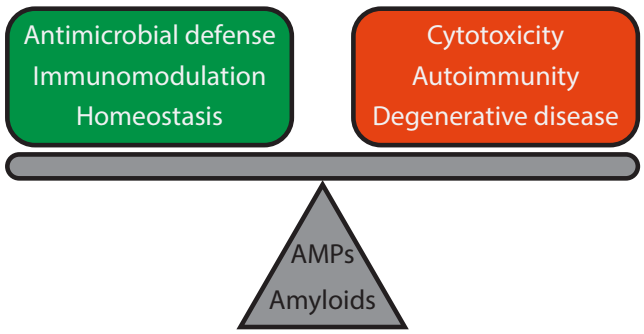

FIGURE 4 | Functional reciprocity of AMPs and amyloids and consequences for host defense and immune signaling. Both AMPs and amyloids are involved in antimicrobial defense, immunomodulation, and homeostasis, but dysregulation can lead to adverse outcomes such as cytotoxicity, chronic inflammation, and autoimmune diseases like lupus, and degenerative diseases like Alzheimer's disease and Parkinson's disease. Further work will be required to elucidate the mechanisms of how such a delicate balance is attained.

described above (103–105) (**Figure 4**). For example, LL37 is a human cathelicidin AMP essential for normal immune function and protection against lethal infections (106). However, at elevated physiological concentrations, it is cytotoxic to host smooth muscle cells (107) and implicated in the pathogenesis of late-stage diseases including atherosclerosis, rheumatoid arthritis, and systemic lupus erythematosus (29). Interestingly, certain AMPs are deposited as amyloids in common human amyloidopathies including isolated atrial and senile seminal vesicle amyloidosis (7, 92, 108). In fact, a large number of naturally occurring AMPs including LL37 (4, 109), lysozyme (110), protegrin-1 (111), plant defensins (112), temporins (113, 114), histatin 5 (115), HAL-2 (116), uperin 3.5 (117), dermaseptin S9 (118), Cn-AMP2 (119), and longipin (120) and apolipoprotein A-I (121) from invertebrates form amyloids or amyloid-like fibrils *in vitro* and *in vivo*. A number of synthetic amyloid-inspired peptides have been designed primarily as novel broad-spectrum antibiotics (83, 122), and many AMPs are known to oligomerize before or upon membrane binding and pore formation (123, 124).

The potential protective effects of host-generated amyloids have only recently emerged (7, 8, 125) despite recognition of the association between chronic bacterial infections and amyloidosis for nearly a century (1). Findings related to the role Aβ plays in neuronal innate immune defense may extend to proteins associated with amyloidopathies other than AD, several of which have been shown to exhibit antimicrobial activity (18, 108, 126–129). Pathways that regulate innate immunity in AD and other amyloidopathies may serve as novel targets for therapeutic intervention. Parkinson's disease (PD)-associated α-synuclein has been long-studied as a model system for amyloid-mediated cytotoxicity (130–134) due to its propensity for membrane interactions (135, 136) via its N-terminal helix (137, 138) (**Figure 1**). Recently, it was shown to be antimicrobial against a variety of bacteria and fungi (139). Unexpectedly, it was found to be also involved in the chemoattraction of immune cells, suggesting a potential endogenous role in host defense (140). In human patients with chronic gut inflammation, α-synuclein was found to be upregulated in enteric neurons (141), a fascinating finding given that PD often begins in the gut as constipation before neurologic symptoms appear (142, 143). Disruption of the ability of α-synuclein to self-assemble into oligomers on neuronal membranes appears to be a potential therapeutic strategy in a nematode model of PD (144). Beyond Aβ and α-synuclein, several other amyloids or their fragments have been shown to have antimicrobial or membrane-lytic properties, including tau (145), islet amyloid polypeptide (IAPP) (146–148), human prion protein (128), superoxide dismutase (127), and endostatin (149). The functional bacterial amyloid curli, which is a key stromal component of *Salmonella* biofilms (150), was also shown to form cytotoxic oligomeric intermediates (151).

Interestingly, a recent machine learning tool originally trained to identify antimicrobial activity in α-helical AMPs identified a subset of naturally occurring amyloid peptides that possess predicted membrane-permeating activity (33, 152–154), among numerous other classes of molecules (155, 156). This demonstrates that data-driven approaches may be helpful in further identifying amyloids that are involved in host defense, but it is clear that much more work needs to be done to validate the extent and relevance of that function.

IMMUNOMODULATORY ASPECTS OF AMYLOIDS AND SIMILARITY TO AMPs

The functional similarities between AMPs and Aβ amyloids extend to bacterial amyloids as well. In bacterial biofilms, bacterial amyloids form the building blocks of the biofilm extracellular matrix alongside extracellular DNA (eDNA) (157). In a series of landmark papers, Tükel and colleagues showed that the biofilm amyloid curli from *Salmonella* and *E. coli* activated TLR2 (158–160) (**Figure 3**). Subsequent studies have shown that TLR2/TLR1 heterocomplex recognized the fibrillar structure of amyloids from both prokaryotic and eukaryotic origin including curli, Aβ and serum amyloid A (SAA) (158, 160–162). In the case of curli, the adaptor molecule CD14 further enhanced the recognition of curli via the TLR2/TLR1 heterocomplex (163). These data instigated further studies investigating whether the conserved fibrillar structure of amyloids serve as a pathological molecular signature for the innate immune system. Consistent with this idea, fibrillar curli (164), Aβ (14), serum amyloid A (165), and IAPP (166) elicited IL-1β cytokine production by directly activating the NLRP3 inflammasome in macrophages. This process impacts the innate immune system in multiple ways: (1) TLR2 activation initiated the pre-IL-1β production and amyloid internalization, (2) NLRP3 inflammasome activation by cytosolic fibrils activated caspase1 and cleaved the pre-IL-1β into mature IL-1β (164). In addition to TLR2, possible activation of TLR4 and TLR6 by Aβ was also reported (167, 168). However, it is not known whether the observed activation of TLR4 and TLR6 was due to the generation of additional Aβ structural conformations during *in vitro* fibrillization or any other contaminating factors. In invertebrates, amyloid formation

is key to activation of the innate immune system and host defense. SAA from marine bivalves resembling SAA from vertebrates is a potent acute phase protein and are induced upon bacteria infection (169). In insects such as *Heliothis virescens*, the functional amyloid P102 is synthesized and released to protect against pathogens such as bacteria and parasites. This can occur in response to lipopolysaccharide stimulation (170). The secreted amyloid layer acts as a molecular scaffold to promote localized melanin synthesis and immune cell adhesion to foreign invaders (171). However, it is unknown whether they play a role in direct receptor binding.

Previously, we showed that AMPs like LL37 can self-assemble into an amyloid-like superhelical protofibril to present spatially ordered DNA to TLR9, and that AMP self-assembly with immune ligands can enable signaling through a broad range of PRRs without being direct agonists. Interestingly, nucleic acids have previously been shown to accelerate amyloid fibrillation and serve as molecular templates for self-assembly (172, 173). AD amyloids like Aβ in particular have a propensity to bind to DNA (174) and co-localize within nuclei of affected cells (175, 176). Autoimmune responses to Aβ-containing amyloid structures have been described in AD patients (177). PD-associated α-synuclein fibrils have the ability to self-assemble with DNA (178). Surprisingly, another endogenous amyloid serum amyloid P component (SAP) was shown to be protective against lupus by binding to DNA to prevent formation of anti-DNA antibodies (179, 180), suggesting that perhaps different amyloids are involved in regulating inflammation and recognition of immune ligands. Previously, we showed how structural scaffolding of immune ligands like DNA by AMPs and amyloids dramatically affects immune outcomes (10, 43, 56). AMP-DNA complexes with inter-DNA spacings well-matched with the size of TLR9 amplifies cytokine production, but those with spacings that are much smaller or larger can actually inhibit TLR9 activation and inflammation (4, 43, 45). SAP may potentially regulate inflammation by out-competing binding of proinflammatory amyloids to DNA. This challenges the notion that amyloid assembly is strictly proinflammatory or pathologic.

The ability of amyloids to act as a carrier for nucleic acids to promote endosomal TLR signaling was only recently discovered. Di Domizio et al. showed that artificially formed amyloid fibrils bound to DNA to form amyloid-DNA complexes (181). When administered systemically, these amyloid-DNA promoted systemic autoimmunity, autoantibody production, and lupus-like syndromes in mice by amplifying TLR9 activation in pDCs (6) (**Figure 3**). A similar observation was made with curli proteins and eDNA found at close proximity in the extracellular matrix of the biofilm. Curli and eDNA formed irreversible complexes together. Similar to what was observed with human amyloids, DNA accelerated the self-assembly process of bacterial amyloid curli (182). Incorporation of DNA into curli rendered DNA resistant to enzymatic degradation. Systemic administration of curli-DNA complexes induced autoantibody production and type I interferon production (12) suggesting that complexes of curli-like bacterial amyloids with DNA may promote inflammatory disorders (183). These findings are fascinating in the setting of our previous work showing that LL37 self-assembles into amyloid-like protofibrils to amplify TLR9 activation. We set out to examine the structures of curli-DNA complexes and found that, similar to LL37 and other AMPs and chemokines, curli was able to organize DNA into geometrically optimal nanostructures to amplify TLR9 activation (**Figures 2C,D**). Immune activation occurred via a two-step process—curli-DNA complexes were first internalized into immune cells via binding to TLR2 (158–160) and then activated TLR9 once inside the endosome leading to the generation of type I interferons (5). Engagement of TLR2 and TLR9 also contributed to the autoantibody production through unknown mechanisms.

For the longest time, it has been known that infections initiate and/or exacerbate autoimmune diseases. However, the mechanisms of how infections trigger autoimmunity remained a mystery. Besides curli producing enteric bacteria, many important human pathogens such as *Borrelia burgdorferi* (184), *Mycobacterium tuberculosis* (185), *Pseudomonas aeruginosa* (186, 187), and *Staphylococcus aureus* (188) also produce amyloids. Individuals infected with these pathogens develop some form of autoimmune sequelae such as inflammatory arthritis (13). Phenol soluble modulins (PSMs) from *Staphylococcus* biofilms (189–191) and Fap amyloids from *Pseudomonas* biofilms (186) have been studied concisely, but at present, the mechanisms of DNA binding by other functional amyloids remain unclear, and it remains to be seen whether this has consequences for immune signaling and inflammation. Nevertheless, extracellular DNA is known to facilitate the formation of functional amyloids in *Staphylococcus* biofilms (192), and PSMs are known to bind directly to human formyl peptide receptor 2 (FPR2) (193). Together, these studies strongly suggests a link between chronic bacterial infections, biofilms, and autoimmune diseases (13, 194) (**Figure 4**). By therapeutically targeting curli amyloid fibers (195), disruption could potentially eradicate bacterial biofilms and secondary autoimmunity.

Formation of amyloid deposits by subunits of different amyloid fibrils is termed as cross-seeding. The co-existence of combinations of α-synuclein, tau, prion protein, and Aβ have all been observed in amyloid deposits in humans (144). In the past several years, few studies also investigated cross-seeding events and a possible link between neurodegenerative diseases and bacterial amyloids. Cross-seeding between SAA and curli was reported in a mouse model of secondary amyloidosis (147). Recent studies have shown that curli can also seed the self-assembly of human α-synuclein (6, 196–198). Colonization of α-synuclein-overexpressing mice with curli-producing *E. coli* exacerbates motor impairment and GI dysfunction, and promotes α-synuclein deposition in the brain (199). However, the spatial interactions between bacterial and host amyloids that would allow for cross-seeding and how these interactions could be influenced by binding to nucleic acids to induce inflammation still remains unknown. We feel that this is an area that should attract and reward attention.

CONCLUSIONS AND OUTLOOK

In this review, we discussed the unique functional reciprocity of amyloids and antimicrobial peptides, and how supramolecular self-assembly changes our understanding of their respective roles in host defense and immune activation. We outlined recent work highlighting novel molecular mechanisms for AMP-mediated immunomodulation via TLRs, and implications for antimicrobial responses and inflammatory diseases. We then compared AMP and amyloid self-assembly in the contexts of antimicrobial and membrane-remodeling activity, cytotoxicity, and immune signaling using LL37, Aβ, and curli as fundamental examples.

By critically examining the AMP and amyloid literature together, we discover several convergent themes. First, the amphiphilic properties unique to AMPs and amyloids enable them to cooperatively self-assemble into supramolecular nanostructures to modulate the innate immune system and defend against microbial infections. AMPs, which were thought of as only having antimicrobial function, are now known to modulate innate immune receptors by forming amyloid-like protofibrils and scaffolding canonical immune ligands like DNA and RNA into geometrically organized patterns (**Figure 2**). Recognition of these complexes by the immune system drives autoimmunity in diseases like lupus, psoriasis, and scleroderma. In a parallel direction, functional bacterial amyloids such as curli from *Salmonella* has shown how these stromal biofilm proteins organize eDNA into cognate spatially ordered complexes to induce autoimmunity in diseases like lupus. Further studies will be required to map out the immune activation landscape of both eukaryotic and prokaryotic amyloids and their distinct mechanisms (**Figure 3**). For example, exploring how amyloids bind to other immune ligands and identifying the structural rules for immune activation would be incredibly fascinating, analogous to our work with AMP self-assembly. Can we adapt this paradigm to explain autoimmune sequelae of other bacterial infections? We imagine that lessons learned from work on the α-helical AMPs can inform new research directions for α-helical amyloids such as the *Staphylococcus* PSMs, and vice versa. Similarly, our strong understanding of the self-assembly of β-sheet amyloids may inform a better understanding about how β-sheet rich AMPs and AMP-like molecules such as chemokines oligomerize.

Second, the revolutionary work demonstrating that Aβ, which has no known primary function, is an AMP that protects the nervous system against bacterial and fungal infections fundamentally challenges our view of endogenous human amyloids as solely pathologic. This model of Aβ activity suggests that excessive β amyloid deposition in AD and pathogenesis may not necessarily arise from an intrinsic abnormal propensity for Aβ to aggregate, but rather as a consequence of dysregulation of the brain's normal host defense system against invasive infections, similar to how dysregulation of AMP expression and production in tissues can adversely lead to autoimmune diseases (**Figure 4**). The discovery that α-synuclein, which also has no previous known primary function, is a chemoattractant and is induced to alert the immune system during gut infections opens up incredible opportunities for discovery. Are there other amyloids with hidden antimicrobial activity with potential roles in host defense? What are the primary roles of other endogenous amyloids?

We are just beginning to elucidate the role of supramolecular assembly in immune recognition and modulation. Recent studies have shown that innate immune receptor adaptor proteins like melanoma differentiation-associated protein 5 (MDA5), which senses cytosolic dsRNA, can self-assemble into amyloid-like helical filaments in the presence of dsRNA (200, 201). Helical filament assemblies can also be observed in the signaling pathways of the RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), and mitochondrial antiviral-signaling protein (MAVS) (202–204). Given that we know how AMPs and amyloids self-assemble with nucleic acids to talk to TLRs, further work will be required to illuminate how they interact with filamentous assemblies of cytoplasmic immune receptors. In summary, we hope that this review will serve to highlight the advances, opportunities, and outlook for the AMP and amyloid communities, and stimulate collaborations between AMP and amyloid biologists, immunologists, as well as bioengineers.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Type III Secretion Protein, PcrV, Impairs *Pseudomonas aeruginosa* Biofilm Formation by Increasing M1 Macrophage-Mediated Anti-bacterial Activities

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Pseudomonas aeruginosa biofilms employ a variety of strategies to hijack the host immune defense system to achieve chronic infection. However, the bacterial components that are involved in this process are not yet fully understood. PcrV, a needle tip protein of the *P. aeruginosa* type III secretion system (T3SS), was downregulated during *P. aeruginosa* biofilm infection. The impaired expression of the *P. aeruginosa pcrV* gene is associated with attenuated immune activation and an increased percentage of M2 macrophages following *P. aeruginosa* biofilm infection. Treatment with exogenous PcrV produced from *Escherichia coli* elevated tissue inflammation and the percentage of M1 macrophages, resulting in reduction in the biofilm burden. Further analyses demonstrated that the potential of PcrV to induce classically activated M1 macrophages as evidenced by the increased production of proinflammatory cytokines and anti-bacterial mediators, including inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS), as well as increased phagocytosis of bacteria. Mechanistically, PcrV-mediated promotion of macrophage M1 polarization and phagocytosis occurs through the activation of mitogen-activated protein kinases (MAPKs) and NF-κB signaling pathways. Collectively, these findings reveal a potential role of PcrV in skewing host immune defense to promote *P. aeruginosa* biofilm infection and provide new insights into the therapeutic strategies for *P. aeruginosa* biofilm infection.

Keywords: ***Pseudomonas aeruginosa*, biofilm, PcrV, immune evasion, macrophage polarization, biofilm eradication**

INTRODUCTION

Pseudomonas aeruginosa-mediated chronic infections are commonly associated with biofilm formation on host tissues and indwelling medical devices. Bacterial biofilm communities are encased in a self-produced extracellular matrix which precludes antibiotic penetration, which impedes eradication of biofilm bacteria by conventional antibiotic treatment. To combat biofilm infections, various strategies have been investigated, including utilization of antimicrobial peptides/lipids (Verderosa et al., 2019), quorum sensing inhibitors (Chang et al., 2019), and bacteriophages

(Geredew Kifelew et al., 2019). In addition to the methods that directly target bacterial components, promising new strategies to control biofilm infection rely on improving host anti-bacterial immune responses, such as the induction of proinflammatory cytokines and antibacterial mediators, for instance, nitric oxide (NO) and reactive oxygen species (ROS), and the enhancement of phagocytosis activity (Campoccia and Mirzaei, 2019).

Macrophages act as the first line of defense against bacterial infection by secreting proinflammatory cytokines and bactericides, as well as by increasing phagocytotic activity against bacteria (Keewan and Naser, 2020). However, differences in the polarization of macrophage phenotypes determine the diverse efficacy in the eradication of bacteria. The classically activated M1 macrophages release large amounts of proinflammatory cytokines, such as tumor necrosis factor (TNFα), interleukin 6 (IL6), and IL12, and antibacterial mediators, such as NO, ROS, and reactive nitrogen species (RNS). As such, M1 macrophages mediate efficient phagocytosis and are actively involved in microbicidal action (Qian and Pollard, 2010; Shapouri-Moghaddam et al., 2018). Conversely, the alternatively activated M2 macrophages are characterized by enhanced production of anti-inflammatory cytokines, IL4 and IL10 and arginase. As such, M2 macrophages are involved in attenuating microbicidal activity (Panagi et al., 2019). Although M1 macrophages play critical roles in eliminating planktonic bacteria, studies have demonstrated that macrophages display an M2 phenotype following activation by *Staphylococcus aureus* biofilms (Thurlow et al., 2011), suggesting that biofilms play a role in inducing an anti-inflammatory activation of macrophages that benefits biofilm persistence. Conversely, treatment of biofilms with M1-activated macrophages indicates the potential importance for controlling biofilm infections. However, the issues regarding the bacterial elements that affect biofilm persistence following *P. aeruginosa* biofilm infection, as well as the efficacy of M1 macrophages against *P. aeruginosa* biofilm infections, have not yet been reported.

PcrV, which is a critical needle tip protein of the type III secretion system (T3SS) of *P. aeruginosa*, is an indispensable factor that allows the translocator protein PopB/D to form pores on the host cell membrane through which effector proteins translocate into host cells. In addition to this biological function, PcrV has been reported to possess a possible proinflammatory function (Wangdi et al., 2010). As a V-antigen, a PcrV-originated vaccine elicited a multifactorial immune response and conferred broad protection in an acute *P. aeruginosa* pneumonia model (Hamaoka et al., 2017; Wan et al., 2019), indicating the potential of PcrV for the treatment of bacterial biofilm infection through activation of the host immune response. Nevertheless, the role of PcrV in modulating macrophage polarization and improving the efficacy of *P. aeruginosa* biofilm eradication remains to be elucidated.

In this study, we demonstrated that *pcrV* gene expression is reduced during *P. aeruginosa* biofilm infection. Injection of PcrV into the milieu surrounding the biofilm-infected tissues induced a proinflammatory response with increased infiltration of M1-polarized macrophages, decreasing the biofilm burden. Further analyses demonstrated that PcrV promotes biofilm eradication, macrophage M1 polarization, and phagocytosis *via* the mitogen-activated protein kinases (MAPKs) and NF-κB signaling pathways. Taken together, these results suggest that decreased expression of PcrV during *P. aeruginosa* biofilm infection promotes biofilm persistence and provide novel clues into the therapeutic strategies against *P. aeruginosa* biofilm infection.

MATERIALS AND METHODS

Mice and Ethics Statement

Male mice on a C57BL/6 background were purchased from biocytogen CO., Ltd. (Beijing, China). Animal experiments were conducted according to the experimental animal guidelines of the Army Medical University of China.

Expression and Purification of PcrV Protein

Pseudonomas aeruginosa PcrV gene was cloned into pQE31 (Qiagen, Germany), which introduces an N-terminal fusion of the protein to a His_6 tag. *Escherichia coli* JM109 strain carrying expression plasmids was propagated in LB medium containing 100 μg/ml ampicillin until the OD_{600} nm reached 0.5 and was induced using 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 4 h. Cells were harvested and resuspended in phosphate buffered saline (PBS) and lysed by sonication. The fusion protein was purified by affinity chromatography using His-Trap HP (GE healthcare, Germany). Endotoxin was removed from PcrV by using Detoxi-Gel endotoxin removing gel (Thermo fisher, USA) following the manufacturer's instructions.

Isolation and Induction of BMDMs

Tibias and femurs from the euthanized C57BL/6 mice were excised, and marrow cells were washed out with a 25-gauge needle attached to a 5 ml-syringe. Cells were cultured in 10% FBS/dulbecco's modified eagle medium (DMEM), penicillin, streptomycin, and 50 ng/ml recombinant murine macrophage colony stimulating factor (M-CSF) at 37°C and 5% CO_2 for 3 days. At day 4, the medium was replaced, and cells were cultured at same condition for an additional 3 days.

Mouse Model of *P. aeruginosa* Catheter-Associated Biofilm Infection

A mouse model of catheter-associated biofilm infection was established as described previously with some modifications (Thurlow et al., 2011; Hanke et al., 2013). Briefly, a sterile 1 cm intravenous catheter was implanted subcutaneously into the flank of mice under pentobarbital sodium anesthesia. A suspension (20 μl) of log-phase PAO1 (1×10^5 colony forming unit, CFU) was injected through the skin into the catheter lumen. Biofilm formation was monitored throughout the course of infection, and mice were sacrificed on days 2, 5, and 8 post-infection. For scanning electron microscopy (SEM) analysis, biofilms on catheters were fixed and dehydrated according to a standard SEM protocol and were observed under a Crossbeam 340 SEM (Carl Zeiss, Germany). Tissues surrounding infected catheters were homogenized and weighed after freezing in liquid nitrogen. The bacterial burdens of catheters and surrounding tissues were enumerated using *P. aeruginosa* isolation agar (PIA) plates.

Macrophage Administration Into Biofilm Infections *in vivo*

To determine the efficacy of biofilm clearance by differentially polarized macrophages, 10^6 non-activated or PcrV-activated bone marrow-derived macrophages (BMDMs) were subcutaneously injected at the sites surrounding infected catheters on days 5, 6, and 7 post-infection. The infected catheters and surrounding tissues were harvested on day 8 for subsequent analysis.

Generation of *P. aeruginosa* Static Biofilms *in vitro*

Static biofilms were generated as previously described (Thurlow et al., 2011) with minor modifications. Briefly, sterile 24-well cell culture plates were treated with 20% human plasma in sterile carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C to facilitate bacterial attachment. The PAO1 strain was cultured overnight at 37°C with shaking in DMEM supplemented with 10% FBS. The bacterial culture was adjusted to an initial OD_{600} of 0.05 and then incubated in the plasma pre-coated plate at 37°C under static aerobic condition for 3 days. Medium was carefully replenished every 24 h to prevent disruption of the biofilm structure.

Phagocytosis Assay

The phagocytic efficacy of macrophages against planktonic bacteria or biofilms was evaluated according to a previously described method with modifications (Thurlow et al., 2011). Briefly, planktonic bacteria or biofilms were co-cultured for 30 min with RAW264.7 cells at a ratio of 1:10 at 37°C under 5% CO_2. Cells were then treated with gentamicin (final concentration 400 μg/ml) for 2 h at 37°C under 5% CO_2 to remove extracellular bacteria. For immunofluorescence staining, the cytoskeleton and bacteria were visualized by phalloidin (red) and anti-PAO1 antibody (green), respectively. For evaluation of the intracellular bacteria, cells were lyzed with 0.5% TritonX-100/PBS and enumerated on PIA plates.

Western Blot

Cell pellets were lysed using RIPA buffer (Beyotime, China) supplemented with protease inhibitor cocktail (Roche, USA). Equal amounts of proteins were separated on 10% SDS-PAGE and then transferred electrophoretically to PVDF membranes (Millipore, USA). The membrane was blocked using 5% skim milk in TBST at real time (RT) for 1 h. Then, the membrane was incubated with the appropriate first antibody at 4°C overnight and horseradish peroxidase-conjugated secondary antibody at RT for 1 h.

Real-Time Quantitative PCR

Total RNA extraction and reverse transcription to cDNA were performed according to the manufacturer's instructions. Quantitative PCR was performed using an ABI 7500 RT PCR system (Applied Biosystems, Germany). For macrophages, the relative gene expression levels of *cd11c*, *inos*, *ptgs2*, *cd206*, and *ppary* were normalized to GAPDH. For PAO1 strain, the relative gene expression level of *ndk* was normalized to *gyrB*, *rpoD*, or *rplU* gene. The primers used in this study were provided in **Supplementary Table S1**.

Enzyme-Linked Immunosorbent Assay

Supernatants of stimulated macrophages or biofilm infected tissues were assessed for their levels of TNFα, IL 12 p40/p70, and IL6 by using the sandwich enzyme-linked immunosorbent assay (ELISA) kits (BD biosciences, USA) according to the manufacturer's instructions.

Measurement of Intracellular ROS

Macrophages were harvested and incubated with 2'7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Santa Cruz, USA) dye in DMEM medium at a final concentration of 5 μM for 30 min at RT. Intracellular ROS were measured by flow cytometry.

Statistical Analysis

Data were expressed as means ± standard errors of the means (SEM). The statistical analysis was carried out with GraphPad (GraphPad Software Inc., San Diego, CA). Data were analyzed by unpaired *t* test when comparing two groups and one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test for multiple groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

P. aeruginosa Biofilm Persistence Is Associated With Attenuated Activation of Host Proinflammtory Responses and Decreased Bacterial Eradication Ability

To evaluate the condition of the host immunity and bacterial killing efficacy in response to a *P. aeruginosa* biofilm, we used a mouse model of catheter-associated biofilms to mimic bacterial biofilm formation on medical devices in humans (Hanke et al., 2013). Bacterial biofilm formation involves a transformation from an immature to a mature structure involving bacterial surface attachment and bacterial colony formation in which bacteria are surrounded by matrix materials. In this study, we primarily observed the different stages of *P. aeruginosa* biofilm formation on catheters by SEM. Examination of the catheter lumen by SEM revealed that an immature biofilm was formed with few extracellular matrix-encompassed bacteria within 4 days of the initial infection (**Figure 1A**). With the extension of time post-infection (7 days), a mature biofilm was formed with a contiguous bacterial layer in which extracellular matrix and interior holes were found (**Figure 1A**), suggesting the successful establishment of a mature *P. aeruginosa* biofilm.

To compare the host immune responses and bacterial eliminating efficacy between *P. aeruginosa* biofilms and planktonic bacteria-associated infections, we analyzed the production of inflammation-related cytokines, arginase, and inducible nitric oxide synthase (iNOS), as well as the bacterial burdens in the tissues surrounding

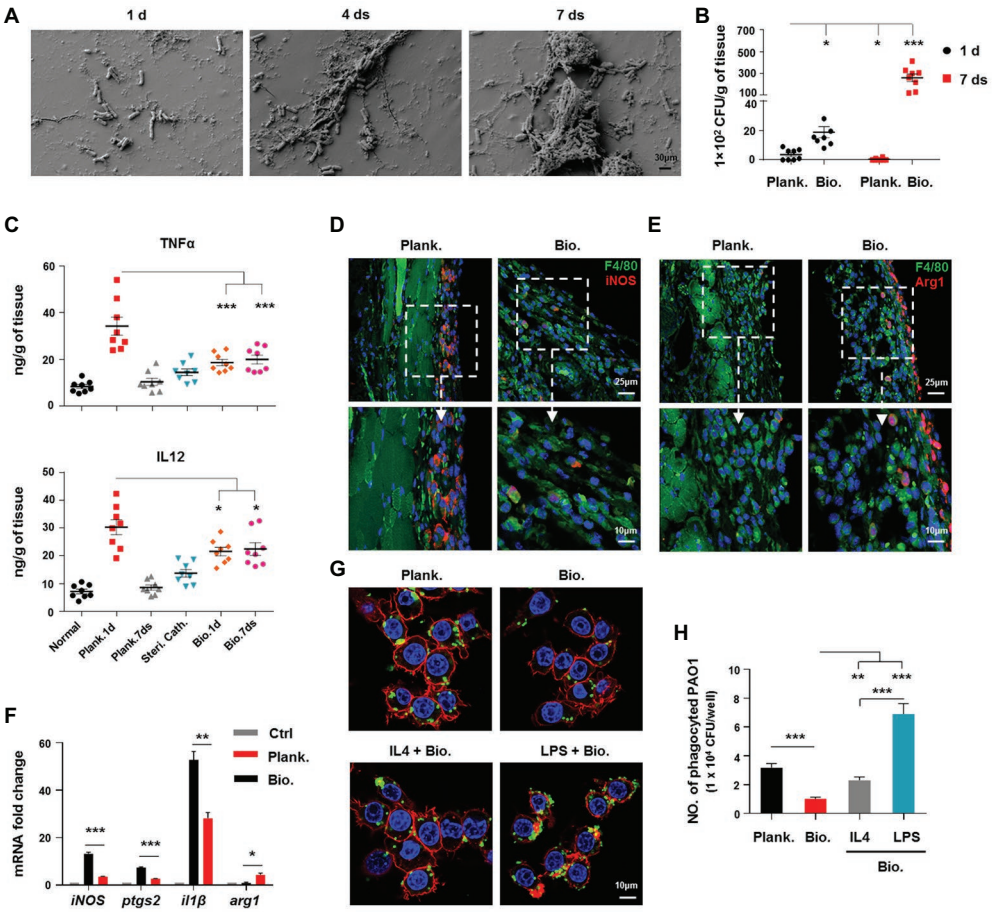

FIGURE 1 | *Pseudomonas aeruginosa* biofilm persistence is associated with attenuated activation of host proinflammtory responses and decreased bacterial eradication ability, whereas increased programming of macrophages toward M2 phenotype. **(A)** *P. aeruginosa* biofilm formation on catheters was observed by scanning electron microscopy (SEM; 5000X magnification). Bacterial loads **(B)**, production of tumor necrosis factor (TNFα) and IL12 p40/70 **(C)**, and F4/80$^+$/iNOS$^+$ and F4/80$^+$/Arg1$^+$ macrophages **(D,E)** in planktonic *P. aeruginosa* (1 day)- or catheter biofilm (7 days)-infected tissues were determined by colony forming unit (CFU) enumeration, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence staining, respectively. For immunofluorescence staining, M1 macrophages were counterstained with fluorescein isothiocyanate (FITC)-conjugated anti-F4/80 and AF647-conjugated anti-inducible nitric oxide synthase (iNOS) antibodies. M2 macrophages were counterstained with FITC-conjugated anti-F4/80 and AF647-conjugated anti-Arg1 antibodies; cellular nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The static *P. aeruginosa* biofilms were co-cultured with Raw264.7 at 37°C, with 5% CO_2 for 3 h (MOI = 10), and relative gene expression levels were analyzed by real-time quantitative PCR (RT-qPCR) using mouse GAPDH as a reference gene **(F)**. To generate M1 and M2-polarized macrophages, Raw264.7 cells were pretreated with 100 ng/ml lipopolysaccharide (LPS) + 50 ng/ml IFNγ (named as LPS group) or 20 ng/ml IL4 for 6 h, respectively. The cells were then co-cultured with static *P. aeruginosa* biofilms at a ratio of 1:10 at 37°C, 5% CO_2 for 30 min. Phagocytosis was detected by immunofluorescence staining **(G)** and CFU enumeration **(H)**. Cytoskeleton was labeled with phalloidin (red); PAO1 was visualized by FITC anti-PAO1 antibody (green); cellular nuclei were stained with DAPI (blue). Error bars indicate the means ± standard errors of the means (SEM). An unpaired Student's *t* test was used for statistical analysis **(B,C,F,H)**. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Planktonic (Plank.), biofilm (Bio.), sterile catheter (steri cath).

the site of the infection. At 1 day post-infection, bacterial loads in planktonic bacteria-infected tissues were lower than biofilm bacteria-infected group (**Figure 1B**; $p < 0.05$). At 7 days post-infection, the planktonic bacteria were almost eradicated, while the bacterial loads in the biofilm remained high (**Figure 1B**). In contrast to the decreased bacterial loads, the levels of the proinflammatory cytokines, TNFα (**Figure 1C**; $p < 0.001$) and IL12 p40/70 (**Figure 1C**; $p < 0.05$), and the percentages of iNOS$^+$ cells were higher in planktonic bacteria-infected tissues (1 day post-infection) than those in biofilm-infected tissues (**Supplementary Figure S1**). Arg1, which is involved in activating anti-inflammatory responses, was also higher in biofilm-infected tissues than in planktonic bacteria-infected tissues (**Supplementary Figure S1**), suggesting a lower ability of *P. aeruginosa* biofilms to activate proinflammatory responses to infection.

Phagocytosis by phagocytes, such as macrophages, accelerates the clearance of bacteria following infection; therefore, we compared the phagocytotic ability of macrophages against *P. aeruginosa* in the planktonic and biofilm forms *in vitro*. The results showed that the ability of macrophages to phagocytose biofilm bacteria was inferior to that observed for planktonic bacteria (**Figures 1G,H**; $p < 0.001$). Collectively, these results indicated that *P. aeruginosa* biofilms enhance bacterial chronic infection by circumventing the host immune response and anti-bacterial activities.

P. aeruginosa Biofilm-Mediated Programming of Macrophages Toward an M2 Phenotype Attenuates Immune Activation, and Promotes Biofilm Persistence

Based on the above results, we further investigated the association of the impaired activation of host immune responses to *P. aeruginosa* biofilm infection with alternatively activated M2 macrophages. Result revealed that a higher percentage of F4/80$^+$/Arg1$^+$ macrophages and a lower percentage of F4/80$^+$/iNOS$^+$ macrophages in biofilm-infected tissues (7 days post-infection) than those in planktonic bacteria-infected tissues (1 day post-infection; **Figures 1D,E**), reflecting a less M1-like phenotype of macrophages under a biofilm-associated infection. To further verify this phenomenon, we analyzed the gene expression profiles of Raw264.7 cells infected with *P. aeruginosa* planktonic bacteria or biofilms *in vitro*. In accordance with our *in vivo* findings, macrophages associated with the biofilm infection exhibited decreased expression of proinflammation-related genes, including *inos*, *il1β*, and *ptgs2*, compared with those associated with planktonic bacterial infection (**Figure 1F**), whereas the expression of *arg1* was increased (**Figure 1F**; $p < 0.05$).

The decreased phagocytic ability of M2 macrophages skewed by bacterial biofilms is also responsible for the delayed bacterial clearance and biofilm persistence. To this end, we analyzed the phagocytic ability of differentially polarized macrophages against *P. aeruginosa* biofilms. Although, lipopolysaccharide (LPS)- or IL4-primed M1 or M2 macrophages both displayed higher phagocytic ability than non-activated macrophages (**Figures 1G,H**), the M1 macrophages exhibited a superior ability for phagocytosis of biofilm bacteria than M2 macrophages (**Figures 1G,H**; $p < 0.001$). Collectively, these results suggested that *P. aeruginosa* biofilm-mediated skewing of macrophages toward the M2 phenotype enhances biofilm persistence.

PcrV Production Is Attenuated in Chronic *P. aeruginosa* Biofilm Infection, and the Addition of Exogenous PcrV Accelerates Macrophage-Mediated *P. aeruginosa* Clearance in Biofilms

The reduced activation of host proinflammatory responses that allows *P. aeruginosa* biofilms to persist may be due to the inactivation of the T3SS, whereby components of the T3SS could be immunostimulatory. Previous research has shown that components of the *P. aeruginosa* needle tip complex, including PcrV, are involved in enhancing proinflammatory responses in mouse lung tissues following infection (Wangdi et al., 2010). Therefore, we speculated that *pcrV* gene expression is downregulated during *P. aeruginosa* biofilm infection, and the decrease in PcrV production in the milieu surrounding the infection accounts for the impaired activation of immune responses mediated by M2 macrophages following biofilm infection, which ultimately results in biofilm persistence. To verify this hypothesis, we first analyzed *pcrV* gene expression in *P. aeruginosa* biofilm-infected catheters *in vivo* by real-time quantitative PCR (RT-qPCR) analysis of three different *P. aeruginosa* reference genes. Compared to the immature biofilm bacteria (1 day post-infection), *pcrV* gene transcription was decreased both in the incompletely matured (4 days post-infection) and mature biofilm bacteria (7 days post-infection; **Figure 2A**). A similar trend was also observed in the *in vitro* static biofilm system (**Figure 2B**; $p < 0.001$). To further investigate the correlation between PcrV production and biofilm eradication, PcrV protein was subcutaneously injected into the tissues surrounding the biofilm-infected catheters. Bacterial burdens in PcrV-treated catheters (**Figure 2C**; $p < 0.001$) and surrounding tissues (**Figure 2C**; $p < 0.05$) were lower than those in the PBS-treated control group, whereas the levels of TNFα (**Figure 2D**; $p < 0.01$) and IL12 p40/70 (**Figure 2D**; $p < 0.001$) were elevated.

To explore the involvement of macrophages in PcrV-mediated elimination of biofilm bacteria, M1 and M2 polarization was analyzed in the macrophages associated with *P. aeruginosa* biofilm catheters-infected tissues following treatment with or without PcrV. The percentages of M1 macrophages (F4/80$^+$/iNOS$^+$) in the PcrV-treated groups were higher than those in the PBS-treated groups (**Figure 2E**), whereas the opposite trend was observed in M2 macrophages (F4/80$^+$/Arg1$^+$; **Figure 2F**). These results suggested that PcrV promotes the elimination of biofilm bacteria through polarization of macrophages toward an M1 phenotype.

PcrV Is Involved in Skewing Macrophages Toward an M1 Phenotype

Given that subcutaneous injection of PcrV promoted biofilm clearance and increased the percentages of M1-polarized macrophages around the infected catheter, we investigated the ability of PcrV to directly drive macrophage differentiation toward an M1 phenotype. To this end, we first evaluated the extensive inflammatory modulation effects of PcrV on BMDMs by gene and protein chip analysis. Gene chip analysis showed that PcrV treatment extensively upregulated macrophage M1 activation-related genes including proinflammtory cytokines (e.g., *tnf*, *il1β*, *il12*, and *il6*), chemokines (e.g., *cxcl3*, *cxcl9*, and *cxcl11*), bacterial killing molecules (*inos*), antigen presentation (e.g., MHCI, MHCII, and CD86), and others (e.g., *cd11c*, *ptgs2*, *egln3*, and *inhba*), whereas M2 activation-related genes, such as *cd206*, *pparγ*, *cd83*, and *egr2*, were downregulated (**Figure 3A**). Protein chip assays revealed that PcrV-primed BMDMs displayed increased production of macrophage M1 polarization-related cytokines (e.g., GM-CSF, TNFα, IFNγ, IL12 p40/70, and IL1α/β), chemokines (e.g., CXCL1 and CCL4/6), and IL2, which is responsible for T cell activation (**Figure 3B**). Despite the extensive upregulation of M1 markers following PcrV treatment, and decreased expression of M2 markers in BMDMs, some of the M2 molecules, such as *ccl2*, *ccl22*, IL4, and IL10 were also elevated in PcrV-primed BMDMs (**Figures 3A,B**), suggesting a balance of immune responses following PcrV treatment.

To observe the dose-dependent effects of PcrV on macrophages, BMDMs and the RAW264.7 cells were treated with different concentrations of PcrV. Similar to LPS-primed M1 macrophages, PcrV-pulsed BMDMs and RAW264.7 cells showed upregulated M1-related gene expression (*inos*, *ptgs2*, and *cd11c*; **Figure 3C**; **Supplementary Figure S2A**), increased production of iNOS (**Figure 3E**) and NO (**Figure 3F**), TNFα, IL12 p40/70, IL6 (**Figure 3D**; **Supplementary Figure S2A**), and ROS (**Figures 3G,H**),

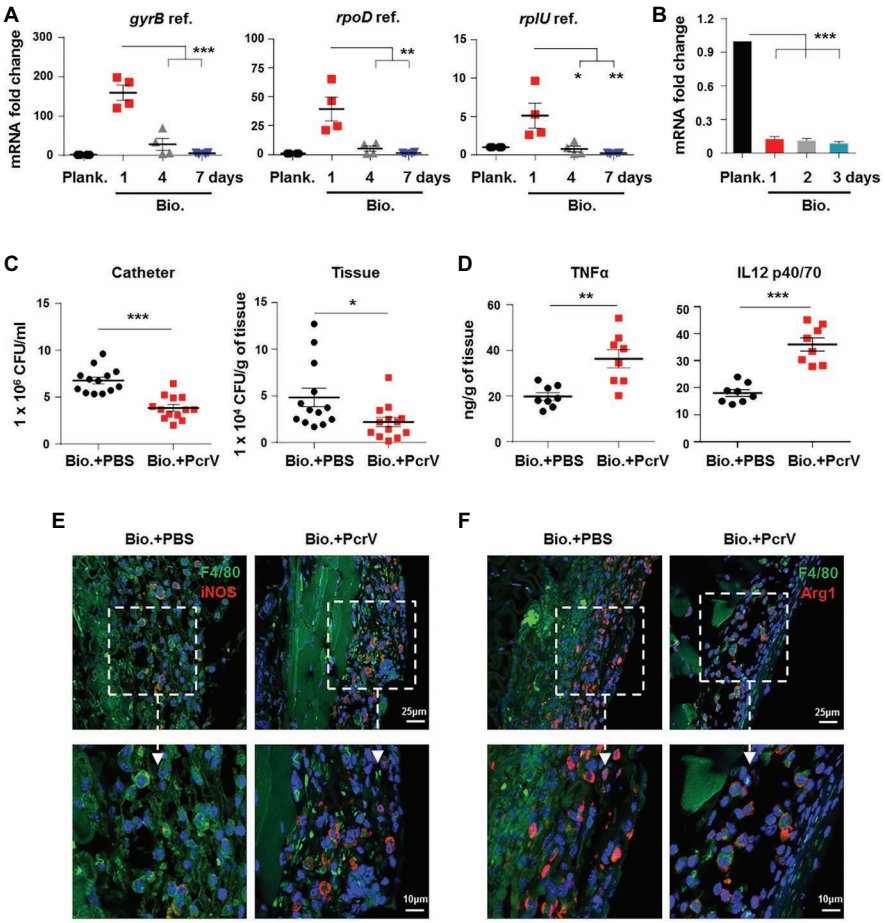

FIGURE 2 | Decreased expression of *P. aeruginosa* PcrV gene following biofilm infection promotes biofilm persistence. **(A)** *P. aeruginosa*-infected catheters were subcutaneously implanted into C57BL/6 mice, and catheters were harvested after infection for 1, 4, and 7 days. The *pcrV* gene expression in biofilm catheter or planktonic bacteria harvested before infection was analyzed by RT-qPCR. *P. aeruginosa gyrB*, *rpoD*, and *rplU* genes were used as reference genes (abbreviated as ref.). **(B)** The *pcrV* gene expression in *P. aeruginosa* static biofilms or planktonic bacteria was analyzed by RT-qPCR using *rplU* genes as reference gene. After infection for 4 days, 10 μg of PcrV was daily injected into the tissues surrounding the biofilm-infected catheters for three times. Infected tissues and catheters were harvested at day 8. Bacterial loads in infected tissues or catheters were determined by counting CFU **(C)** and production of TNFα and IL12 p40/70 was detected by ELISA assay **(D)**. F4/80$^+$/iNOS$^+$ and F4/80$^+$/Arg1$^+$ macrophages in tissues surrounding the biofilm-infected catheters were detected by immunofluorescence staining **(E,F)**. For immunofluorescence staining, M1 macrophages were counterstained with FITC-conjugated anti-F4/80 and AF647-conjugated anti-iNOS antibodies. M2 macrophages were counterstained with FITC-conjugated anti-F4/80 and AF647-conjugated anti-Arg1 antibodies; cellular nuclei were stained with DAPI. One-way ANOVA (Tukey's *post hoc*, **A,B**) or an unpaired Student's *t* test **(C,D)** was used for statistical analysis. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

whereas expression of M2-related genes, including *cd206*, *ppary* (**Figure 3C**; **Supplementary Figure S2B**), and *arg1* (**Supplementary Figure S2B**) was downregulated in a dose-dependent manner compared with the untreated- or hydrolyzed PcrV (PK)-treated groups. Taken together, these results suggested that PcrV plays a role in skewing macrophage differentiation toward an M1 phenotype.

PcrV Repolarizes Macrophages From M2 to M1 Phenotype

To evaluate the ability of PcrV to promote a macrophage switch from the M2 to M1 phenotype, BMDMs were pretreated with the M2 inducer IL4, before PcrV treatment. IL4 significantly increased the expression of M2-specific genes, including *cd206*, *ppary*, and *arg1* in BMDMs (**Figure 4**); however, the IL4-mediated upregulation of these genes was abolished by PcrV treatment (**Figure 4**). Despite the anti-inflammatory conditions of macrophages pretreated with IL4, PcrV treatment significantly elevated the expression of proinflammatory M1-related genes in BMDMs, such as *inos*, *ptgs2*, and *cd11c* (**Figure 4**), demonstrating that PcrV plays a role in inducing transformation from the M2 to M1 phenotype.

The Proinflammtory M1 Macrophages Polarized by PcrV Are Involved in Biofilm Elimination

Considering that PcrV is involved in skewing macrophage toward the M1 phenotype, we further investigated the efficacy of PcrV-pulsed M1 macrophages in biofilm elimination both *in vitro* and *in vivo*. *In vitro* studies revealed that PcrV treatment significantly increased the phagocytosis ability of macrophages against biofilm

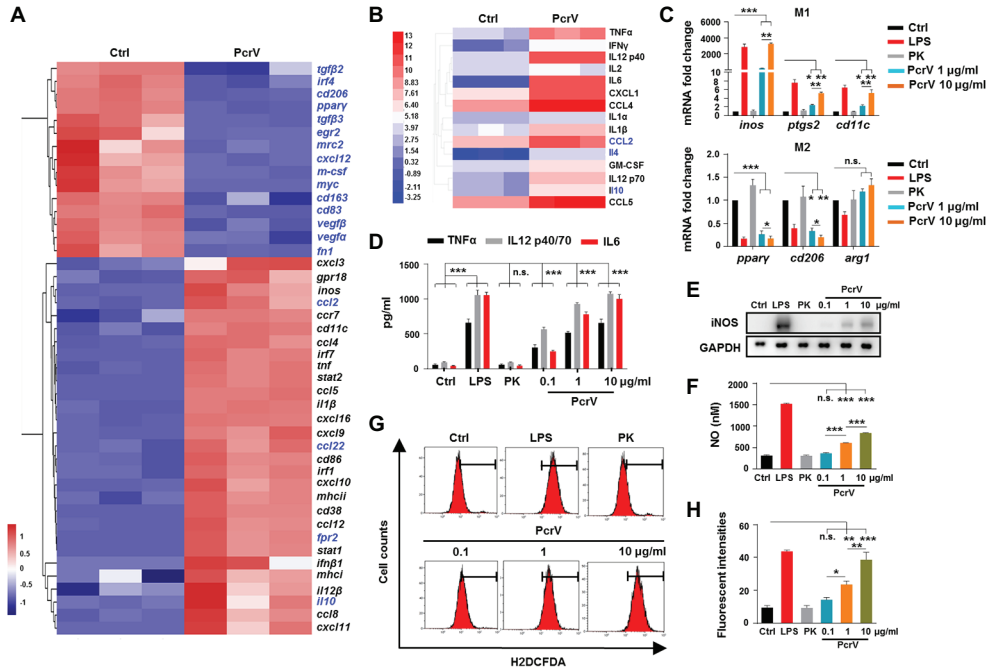

FIGURE 3 | PcrV promotes macrophage M1 polarization. **(A)** Gene expression and **(B)** cytokine production in bone marrow-derived macrophages (BMDMs) treated with or without PcrV (10 μg/ml) for 24 h were analyzed by gene chip and protein chip, respectively. M1-related genes were marked in blank; M2-related genes were marked in blue. **(C)** Gene expression in BMDMs treated with LPS + IFNγ, hydrolyzed PcrV (PK), and PcrV (10 μg/ml) for 24 h was verified by RT-qPCR. **(D)** Levels of TNFα, IL12 p40/70, and interleukin 6 (IL6) in the culture supernatants of the treated BMDMs were assayed by ELISA assay. The production of iNOS **(E)** and reactive oxygen species (ROS; **G,H**) in Raw264.7 treated with LPS + IFNγ, PK, and PcrV for 6 h were detected by western blot and flow cytometry, respectively. **(F)** The concentration of nitric oxide (NO) in the culture supernatant of Raw264.7 treated with the indicated compound for 24 h was detected by NO detection kit. One-way ANOVA (Tukey's *post hoc*, **C,D,F,G**) was used for statistical analysis. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s. indicates no significance.

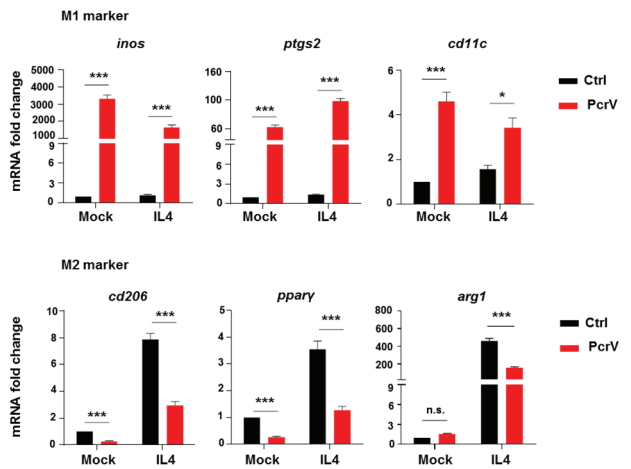

FIGURE 4 | PcrV repolarizes macrophages from M2 to M1 phenotype. BMDMs pretreated with 20 ng/ml of IL4 for 12 h were primed by PcrV (10 μg/ml) for another 24 h. Macrophage M1 and M2-related genes were analyzed by RT-qPCR. An unpaired Student's *t* test was used for statistical analysis. $^{*}p < 0.05$; $^{***}p < 0.001$; n.s. indicates no significance.

bacteria in a time-dependent manner (**Figures 5A,B**; $p < 0.001$). Following injection of BMDMs pretreated with PcrV for 24 h into the tissues surrounding biofilm catheters *in vivo*, the percentage of F4/80^{+}iNOS^{+} macrophages was higher in PcrV/BMDMs treated mice than that in mice treated with non-activated BMDMs (**Figure 5C**), suggesting that functional M1 macrophages were present at the site of infection. Analyses of bacterial burdens and tissue inflammation status revealed that PcrV-primed M1 macrophages reduced bacterial loads in catheters and infected tissues (**Figure 5D**; $p < 0.01$), whereas the production of TNFα and IL12 p40/70 was augmented (**Figure 5E**; $p < 0.001$). Collectively, these results suggested that PcrV-primed M1 macrophages are effective in accelerating biofilm clearance.

MAPKs and NF-κB Signaling Pathways Play Dominant Roles in Promoting PcrV-Mediated Macrophage M1 Activation and Phagocytosis

MAPK and NF-κB signaling pathways are among the most extensively reported pathways that are involved in activating anti-bacterial immune responses, involving macrophage M1 polarization (Akhtar et al., 2019; Justino et al., 2020). Our results demonstrated that PcrV promoted the phosphorylation of extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPKs and IKBα (an indicator of NF-κB pathway activation) in RAW264.7 cells in a dose- and time-dependent manner (**Supplementary Figures S3A,B**). Further utilization of the corresponding signal pathway inhibitors U0126,

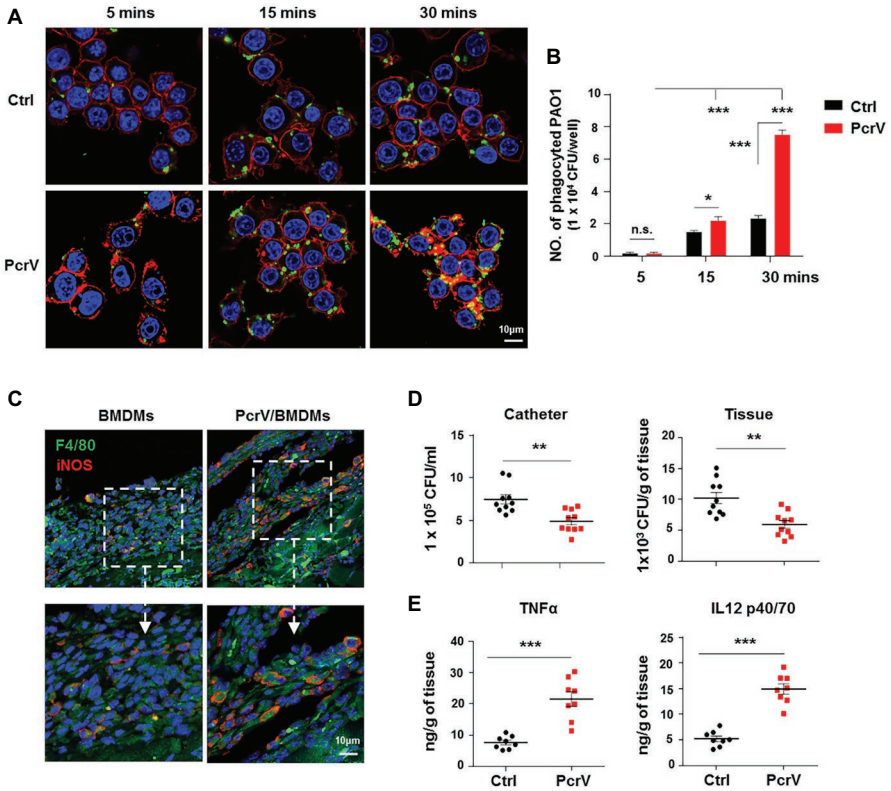

FIGURE 5 | PcrV-primed macrophages display increased phagocytosis, bacterial killing efficacy, and induction of proinflammtory cytokines. Raw264.7 cells pretreated with or without PcrV (10 μg/ml) for 6 h were co-cultured with *P. aeruginosa* biofilms (MOI = 10) for the indicated time point. The phagocyted bacteria were detected by immunofluorescence staining **(A)** and CFU enumeration **(B)**. Cytoskeleton was labeled with phalloidin (red); PAO1 was visualized by FITC anti-PAO1 antibody (green); cellular nuclei were stained with DAPI (blue). BMDMs primed with or without PcrV (10 μg/ml) for 24 h were daily injected into the tissues surrounding biofilm-infected catheters after biofilm infection for 4 days. The infected tissues and catheters were harvested after the injection of PcrV for 3 days. $F4/80^+/iNOS^+$ macrophages in infected tissues **(C)**, bacterial burdens **(D)**, and production of TNFα and IL12 p40/70 **(E)** were analyzed by immunofluorescence staining, CFU enumeration, and ELISA, respectively. For immunofluorescence staining, M1 macrophages were counterstained with FITC-conjugated anti-F4/80 and AF647-conjugated anti-iNOS antibodies. M2 macrophages were counterstained with FITC-conjugated anti-F4/80 and AF647-conjugated anti-Arg1 antibodies; cellular nuclei were stained with DAPI. One-way ANOVA (Tukey's *post hoc*, **B**) or unpaired Student's *t* test **(D,E)** was used for statistical analysis. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

SP600125, SB203580 for ERK, JNK, and p38 MAPKs, respectively, revealed that PcrV-mediated activation of the three pathways was successfully inhibited (**Figure 6A**). Since the nuclear translocation of NF-κB p65 results in the activation of NF-κB signaling pathway, we used JSH23, an inhibitor of NF-κB p65 nuclear translocation and transcriptional activity, to treat the cells. Given NF-κB p65 might regulate the transcriptional level of itself, the JSH23 treatment also lead to a massive reduction of the expression of NF-κB p65 in the JSH23 treated groups (**Figure 6B**). In spite of that, LPS or PcrV-mediated promotion of nuclear translocation of NF-κB p65 was impaired by JSH23 (**Figure 6B**). Analyses of the downstream cytokines, gene expression, and phagocytosis showed that the levels of TNFα, IL6 (**Supplementary Figure S3C**; $p < 0.001$), and ROS (**Figures 6C,D**), expression of the M1-related genes, *cd11c* and *ptgs2* (**Supplementary Figure S3D**), and phagocytosis (**Figures 6G,H**) were reduced in PcrV-treated RAW264.7 in the presence of both MAPK and NF-κB inhibitors; however, ERK inhibition elevated iNOS (**Figure 6E**; **Supplementary Figure S3D**) and NO (**Figure 6F**; $p < 0.001$), while JNK and p38 inhibition did not alter NO production (**Figure 6F**). Meanwhile, MAPK inhibition did not elevate *pparγ* expression in RAW264.7 cells primed with PcrV (**Supplementary Figure S3D**). In contrast, JSH23 treatment reduced the levels of iNOS (**Figure 6E**; **Supplementary Figure S3D**; $p < 0.001$) and NO (**Figure 6F**; $p < 0.001$) and reversed the decrease in *pparγ* expression in PcrV-stimulated RAW264.7 cells (**Supplementary Figure S3D**; $p < 0.05$). Collectively, these results indicated that the MAPK and NF-κB signaling pathways are involved in PcrV-mediated regulation of macrophage M1 polarization and phagocytosis.

DISCUSSION

Bacterial biofilm formation on human tissues and implanted/indwelling devices provides a basis for persistent infections. The remodeling of host immune responses by biofilms following infection is one of the most important factors that benefit bacterial survival and chronicity of infection (Gonzalez et al., 2018; Campoccia and Mirzaei, 2019). Previous studies have revealed

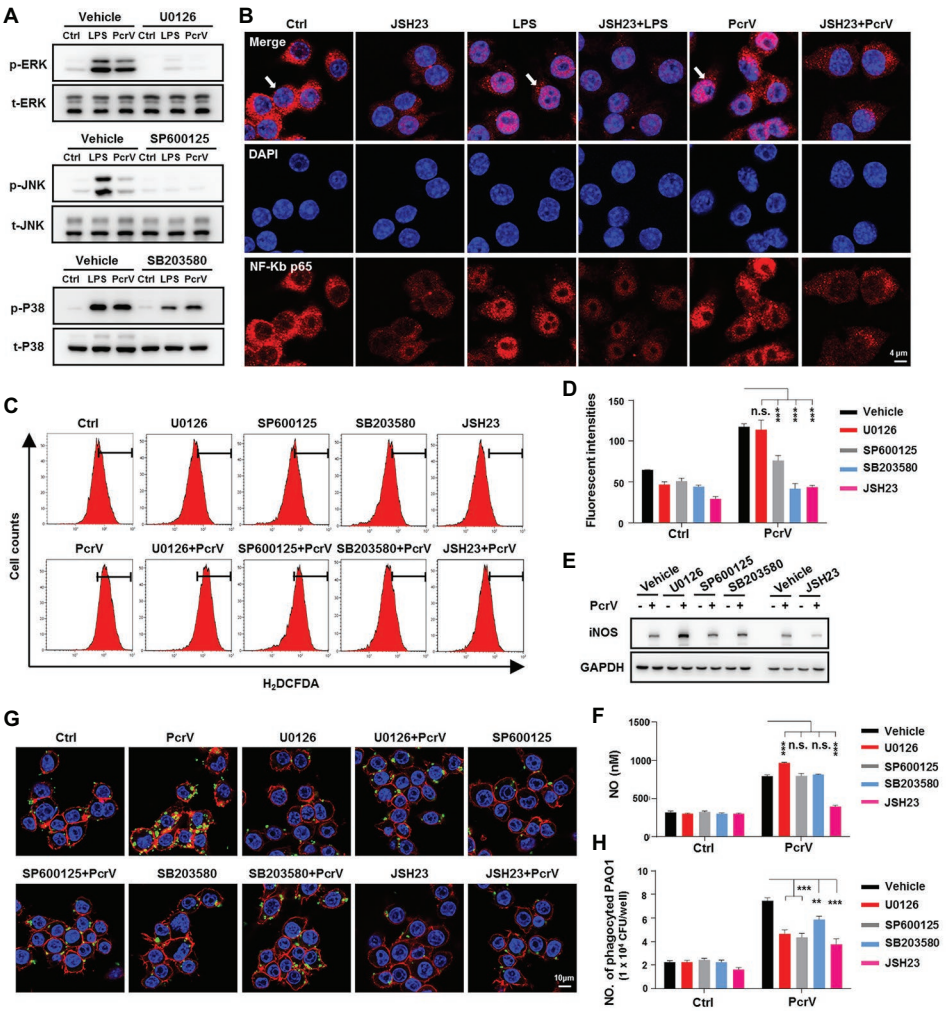

FIGURE 6 | Mitogen-activated protein kinases (MAPKs) and NF-κB signaling pathways are involved in PcrV-mediated activation of M1 macrophages and increasing of phagocytosis. Raw264.7 cells pretreated with the corresponding inhibitors U0126 (5 μM), SP600125 (10 μM), SB203580 (5 μM), and JSH23 (15 μM) for extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), p38 MAPKs, and NF-κB, respectively, were primed by LPS + IFNγ (named as LPS group) or PcrV (10 μg/ml) for 6 h. The total and phosphorylation levels of JNK, ERK, and p38 MAPKs were analyzed by western blot **(A)**. The cellular translocation of NF-κB was visualized by immunofluorescence staining **(B)**. NF-κB was labeled with AF647-conjugated anti-NF-κB p65 antibody (red); cellular nuclei were stained with DAPI (blue). ROS **(C,D)** and iNOS **(E)** production were analyzed by flow cytometry and western blot, respectively. **(F)** The concentration of NO in the culture supernatant of Raw264.7 treated with the indicated compound for 24 h was detected by NO detection kit. Raw264.7 pretreated with the corresponding inhibitors was primed by PcrV (10 μg/ml) for 6 h. The cells were then co-cultured with static PAO1 biofilms (MOI = 10) for 30 min. Phagocytosis was detected by immunofluorescence staining **(G)** and CFU enumeration **(H)**. Cytoskeleton was labeled with phalloidin (red); PAO1 was visualized by FITC anti-PAO1 antibody (green); cellular nuclei were stained with DAPI (blue). Unpaired Student's *t* test was used for statistical analysis **(D,F,H)**. $^{**}p < 0.01$; $^{***}p < 0.001$; n.s. indicates no significance.

that *S. aureus* biofilms attenuated the production of proinflammatory cytokines/chemokines, including IL1β, TNFα, and CXCL2, as well as iNOS, and exacerbated bacterial biofilm infection (Thurlow et al., 2011). Chronic *P. aeruginosa* biofilm infections in cystic fibrosis patients are dominated by a Th2 response with increased and decreased levels of IL4 and IFNγ, respectively (Moser et al., 2000; Hartl et al., 2006), suggesting that *P. aeruginosa* biofilms persist by reducing host proinflammatory responses to infection. *In vitro* co-culture of *P. aeruginosa* biofilm matrix exopolysaccharides (EPS) and extracellular DNA (eDNA) with RAW264.7 cells induced a lower-grade inflammatory response than that induced by planktonic bacteria- or LPS-treated cells (Ramirez et al., 2019). In accordance with these reports, we have demonstrated that compared to planktonic bacteria, *P. aeruginosa* biofilms impaired the production of proinflammatory cytokines, as well as iNOS, while promoting the expression of the anti-inflammatory enzyme, Arg1, both *in vitro* and *in vivo*. Thus, our findings further confirm that *P. aeruginosa* biofilm-associated infection reduces proinflammatory responses to benefit biofilm bacterial survival.

Accumulating evidence demonstrates that the biofilm-mediated hijacking of host immune defense relies on several processes, such as interference in the release of antimicrobial peptides (AMPs), enzymes, ROS, RNS, and NO from leukocytes; impaired phagocytosis; and the recruitment of immunosuppressive cells,

such as MDSCs (Campoccia and Mirzaei, 2019). In addition, studies have demonstrated that the re-education of M1 macrophages to an anti-inflammatory M2 phenotype is also involved in biofilm-mediated immune suppression (Thurlow et al., 2011; Hanke et al., 2013). In accordance with this phenomenon observed in *S. aureus*, we demonstrated that *P. aeruginosa* biofilms obstructed the host immune response by activation of M2 macrophages. Mechanistically, it has been shown that the cyclic di-AMPs, alpha-toxin (Hla), and leukocidin AB (LukAB; Scherr et al., 2015), released from *S. aureus* biofilms promote biofilm persistence by enhancing macrophage anti-inflammatory polarization or inhibiting phagocytosis (Gries et al., 2016). In *P. aeruginosa*, the biofilm formation-related exopolysaccharide alginates (Leid et al., 2005) and rhamnolipids (Alhede et al., 2014) have been implicated in the protection of biofilm bacteria against macrophage-mediated phagocytosis or have been shown to exert direct cytotoxic effect against macrophages. However, the mechanisms by which *P. aeruginosa* biofilms skew macrophage phenotypes to favor their survival following infection still remain obscure. Although excessive activation of *P. aeruginosa* T3SS during acute infection might aggravate inflammation-mediated tissue damage and immune dysfunction, activation of the host immune response by T3SS might also accelerate bacterial recognition and eradication by host immune cells (Galle et al., 2012; Klockgether and Tummler, 2017). However, during a chronic infection, bacterial T3SS is inactivated (Jain et al., 2008), which enhances the ability of bacteria to evade host immune recognition and clearance, ultimately resulting in biofilm persistence. Due to the reverse correlation between T3SS and biofilms (Kuchma et al., 2005), it is highly possible that regulatory elements that control T3SS inhibition/activation might also regulate biofilm persistence. In this study, we found that the T3SS protein PcrV, which is involved in enhancing proinflammatory polarization and phagocytosis of macrophages, is downregulated during *P. aeruginosa* biofilm formation *in vitro* and *in vivo*. The addition of exogenous PcrV or PcrV-pulsed M1 macrophages reversed M2 macrophage-mediated immune inhibition and increased biofilm bacterial elimination, indicating that the decreased expression of PcrV during biofilm formation might impair the M1 macrophage-mediated proinflammatory response and bacterial clearance, ultimately promoting biofilm persistence. Studies have demonstrated that *P. aeruginosa* T3SS genes, including *pcrV*, are negatively regulated in bacteria by the intracellular second messenger cyclic di-GMPs (Romling and Galperin, 2017) and the three-component SadARS regulatory system (Kuchma et al., 2005). Given that cyclic di-GMPs and the SadARS system are also involved in positively regulating *P. aeruginosa* biofilm formation (Kuchma et al., 2005; Ha and O'Toole, 2015; Sharp and Rietsch, 2019), it is likely that these factors are involved in modulation of PcrV-mediated regulation of biofilm persistence during infection *in vivo*. Considering that PcrV, which is involved in inducing macrophage M1 polarization and enhancing phagocytosis, is downregulated during biofilm infection, the addition of exogenous PcrV shows promise as a therapeutic strategy in patients with biofilm infections or chronic immune suppression.

Macrophage polarization is influenced by a variety of factors, such as different types of inflammatory cytokines/chemokines and infiltrated immune cells, as well as cell membrane and intracellular molecule-related mechanisms (Zhou et al., 2014). In this study, we demonstrated that PcrV-mediated polarization of M1 macrophages is through the activation of MAPK and NF-κB signal pathways. Similar to our findings, the bacterial pathogenicity associated molecular patterns (PAMPs) derived from Gram-negative bacteria, such as *Yersinia enterocolitica* LcrV (Sing et al., 2002), *Brucella abortus* cell-surface protein 31 (BCSP31) protein (Li et al., 2014), and *Vibrio cholerae* porin OmpU (Khan et al., 2015), have also been demonstrated to induce macrophage M1 polarization *via* MAPK and NF-κB signaling pathways. Peroxisome proliferator activated receptor (PPARγ), which is mainly expressed in adipose tissue and immune cells, is a member of the nuclear receptor superfamily of ligand-inducible transcription factors that regulate a variety of biological activities, including adipogenesis, lipid metabolism, and insulin sensitization, as well as inflammation (Janani and Ranjitha Kumari, 2015). PPARγ-mediated regulation of inflammation often leads to an inhibitory effect on the activation of immune cells, as well as the production of inflammatory factors through the suppression of signaling pathways, such as NF-κB and JNK/p38 signal pathways (Wang et al., 2018). In this study, we found that PcrV treatment significantly decreased the expression of *pparγ* gene in macrophages, suggesting that the impaired *pparγ* expression might exacerbate NF-κB and MAPK pathway-mediated inflammation in PcrV-treated macrophages.

In summary, this study reveals a role for PcrV in altering biofilm persistence of *P. aeruginosa*, and subsequently the potential therapeutic effects of treating *P. aeruginosa* biofilm infections with PcrV to reverse biofilm-mediated immune suppression.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by ethics committee of Army Medical University.

AUTHOR CONTRIBUTIONS

HY and KZ conceived and designed the experiments. HY, JX, JQ, XH, HS, QD, DL, RX, LJ, QL, QC, JP, and MW performed the experiments. HY, XR, and KZ analyzed the data. HY, XR, and KZ wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01971/full#supplementary-material.

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Cyclic Peptide [R_4W_4] in Improving the Ability of First-Line Antibiotics to Inhibit *Mycobacterium tuberculosis* Inside *in vitro* Human Granulomas

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Tuberculosis (TB) is currently one of the leading causes of global mortality. Medical non-compliance due to the length of the treatment and antibiotic side effects has led to the emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* (*M. tb*) that are difficult to treat. A current therapeutic strategy attempting to circumvent this issue aims to enhance drug delivery to reduce the duration of the antibiotic regimen or dosage of first-line antibiotics. One such agent that may help is cyclic peptide [R_4W_4], as it has been shown to have antibacterial properties (in combination with tetracycline) against methicillin-resistant *Staphylococcus aureus* (MRSA) in the past. The objective of this study is to test cyclic peptide [R_4W_4] both alone and in combination with current first-line antibiotics (either isoniazid or pyrazinamide) to study the effects of inhibition of *M. tb* inside *in vitro* human granulomas. Results from our studies indicate that [R_4W_4] is efficacious in controlling *M. tb* infection in the granulomas and has enhanced inhibitory effects in the presence of first-line antibiotics.

Keywords: cyclic peptide, tuberculosis, host–bacteria interaction, cytokine, antimicrobial peptides

INTRODUCTION

Mycobacterium tuberculosis (*M. tb*) is the etiological agent that is responsible for causing tuberculosis (TB). According to the World Health Organization (WHO), it is estimated that around 9 million people are suffering from active TB disease with an approximate global mortality of 1.43 million people annually (1, 2). Furthermore, about a quarter of the world's population is affected with latent TB (1, 2).

Current treatment for drug-sensitive TB includes first-line antibiotics, which are administered for approximately 6–9 months (3). Although treatment may be effective, problems with drug toxicity can lead to severe side effects, interfering with patients' compliance with medical treatment. Non-compliance to treatment often leads to the emergence of multidrug-resistant (MDR) strains of *M. tb* and the development of MDR-TB, which is difficult to treat, highlighting the need for new therapeutic strategies.

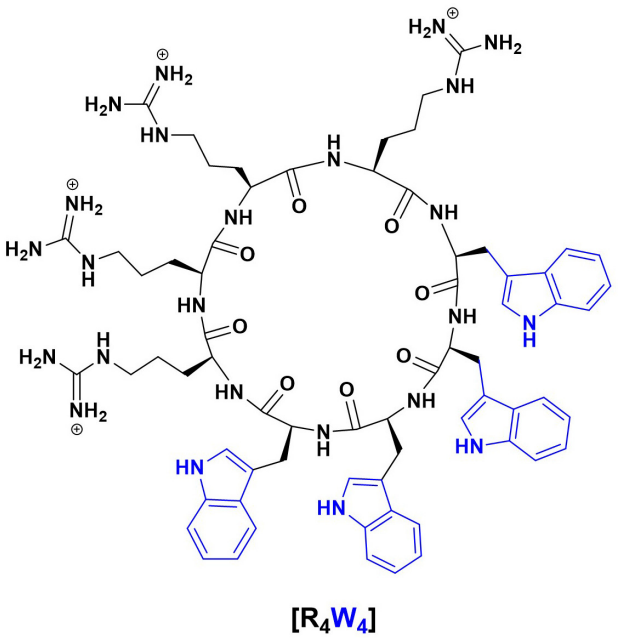

FIGURE 1 | Chemical structure of cyclic peptide [R_4W_4].

Novel therapeutic approaches that can achieve complete cure along with reduced duration of treatment and dosage of first-line anti-TB drugs are highly warranted. These strategies may be crucial in alleviating drug side effects and in addressing patient non-compliance.

Antimicrobial peptides (AMPs) are one such alternative therapeutics against antibiotic-resistant pathogens since they may act as effectors and regulators of the immune system as well as inhibitors of bacterial cell growth (4). Additionally, there are cell-penetrating peptides (CPPs) that share amphiphilicity and cationic structural properties with antimicrobial peptides. Cell-penetrating peptides are particularly interesting since they may help deliver other drugs intracellularly due to their ability to move across the eukaryotic cell membrane. In the grand scheme, it is thought that the correlation between antimicrobial activity and cell-penetrating property may be due to the interaction between positively charged amphiphilic peptides and bacterial membranes that have negatively charged components (4). One such antimicrobial peptide is cyclic peptide [R_4W_4], which has a cyclic structure consisting of four arginine and four tryptophan residues (**Figure 1**), enabling it to interact with cell membranes and deliver cargo. We, therefore, tested the effects of [R_4W_4] both alone and in combination with the first-line antibiotics in restricting the growth of *M. tb* inside the *in vitro* generated human granulomas.

Our study findings indicate that [R_4W_4] causes a significant decrease in the viability of *M. tb* and works effectively with first-line antibiotics such as isoniazid (INH) and pyrazinamide (PZA) by modulating the levels of cytokine release, oxidative stress, and autophagy.

MATERIALS AND METHODS

Human Subjects and Whole Blood Collection

In this study, we recruited eight healthy human subjects aged between 18 and 65 years irrespective of other demographic characteristics. Subjects were excluded if they self-reported ever having a positive purified protein derivative (PPD) skin test, a *Bacille Calmette-Guerin* (BCG) vaccination, a history of excessive alcohol intake, and/or chronic Hepatitis B or C infection(s). Informed consent was obtained from each subject prior to the initiation of any research procedures. After obtaining written informed consent, ∼35 ml of whole blood was obtained from each subject using Acid Citrate Dextrose tubes by research nursing staff at the WesternU Health Patient Care Center. All studies were approved by the Institutional Review Board (IRB) and the Institutional Biosafety Committee (IBC) of the Western University of Health Sciences. All study participants were above the legal age of consent at the time of participation, and written informed consent was obtained from all volunteers prior to participation in the study.

Isolation of Peripheral Blood Mononuclear Cells

Whole blood samples were distributed between two 50-ml conical tubes containing Ficoll-Histopaque (Sigma, St. Louis, MO, USA) at a 1:1 ratio. Following centrifugation (1,800 rpm for 30 min), peripheral blood mononuclear cells (PBMCs) were collected and washed twice with sterilized 1× phosphate buffer saline (PBS) (Sigma, St. Louis, MO, USA). PBMCs were suspended in RPMI with 5% human AB serum (Sigma, St. Louis, MO, USA). Cell counts were achieved using Trypan Blue exclusion staining and a hemocytometer.

Infection, Treatment, and Termination of PBMC-Derived Granulomas

PBMCs (6×10^5) with verified counts were infected with *M. tb* (Erdman strain) using a multiplicity of infection ratio of 1:10 (*M. tb*: PBMCs) and distributed into sterile cell culture 24-well plates (Corning, Corning, NY, USA). Two wells per category contained cover glasses that were used to collect *in vitro* granulomas for staining procedures. Treatments were applied one time in quadruplicate using the following categories: Control (sham treatment), INH, PZA, [R_4W_4] (4 μg/ml), [R_4W_4] (8 μg/ml), INH and [R_4W_4] (4 μg/ml), INH and [R_4W_4] (8 μg/ml), PZA and [R_4W_4] (4 μg/ml), and PZA and [R_4W_4] (8 μg/ml). All treatments that included INH and PZA used minimum inhibitory concentrations (0.125 μg/ml and 50 μg/ml, respectively). Treated infected PBMCs were cultured for 8 days at 37°C in the presence of 5% CO_2 for granuloma formation. At the conclusion of the 8-day incubation, each well was terminated. Supernatants of each well were collected and aliquoted into sterile Eppendorf tubes by treatment categories. Sterile cold 1 × PBS was added into each experimental well without cover glasses to lyse the *in vitro* granulomas. To further dislodge the *in vitro* granulomas, each well was scraped using a sterile micropipette. Lysates from each category were then collected and aliquoted into

sterile Eppendorf tubes by treatment category. Wells with cover glasses were treated with 4% paraformaldehyde (PFA) for 1 h at room temperature to affix *in vitro* granulomas to their respective cover glasses for imaging analyses. PFA-treated wells were also washed three times with PBS to remove any cellular debris that could impede imaging analyses.

Quantification of Intracellular *M. tb* Survival

In order to quantify the intracellular survival of *M. tb* in these treated *in vitro* granulomas, plates containing Middlebrook 7H11 agar media (Hi Media, Santa Maria, CA, USA) supplemented with albumin-dextrose-catalase (ADC) (GEMINI, Calabasas, CA) were inoculated with previously collected supernatants and lysates. Plates were then incubated at 37°C for a minimum of 4 weeks. Plates were then read, and colony-forming units (CFUs) were counted.

Cytokine Measurements

Cytokine analyses were conducted to quantify the produced levels of TNF-α, IFN-γ, and IL-10 in collected supernatant samples. The sandwich enzyme-linked immunosorbent assay (ELISA) was used. Assays and analyses were conducted per the assay manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

Imaging of *in vitro* Granulomas

Cover glasses with fixed *in vitro* granulomas were permeabilized with Triton-X for 2 min and stained overnight with FITC-conjugated CellROX and with antibodies against LC3B conjugated with PE. Cover glasses were washed with PBS and mounted on clean glass slides with mounting media containing 4′,6-diamidino-2-phenylindole (DAPI). Slides were observed under the fluorescent microscope. Fluorescent images were captured, and the fluorescent intensity was quantified using the ImageJ software.

Statistical Analysis

All data analyses for this study were conducted using GraphPad Prism 8.0. (version 8, GraphPad, San Diego, CA, USA). A one-way ANOVA (analysis of variance) with Tukey corrections was performed for datasets greater than two groups. Reported values are in means with each respective category. Data represent ±SE from experiments performed in eight different individuals. Analyses with a $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Antimicrobial peptides are promising candidates that can be used as adjunctive therapy for TB. The structural and functional qualities of the synthetic AMP, $[R_4W_4]$, led us to test its efficacy against *M. tb*., which has a thick peptidoglycan layer along with other lipid layers on the cell wall. $[R_4W_4]$ has already been shown to have potent antibacterial activity against pathogenic gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) with an inhibitory concentration (MIC) of 2.67 μg/ml (1.95 μM). In addition, a follow-up 24-h study showed bactericidal activity against MRSA with the combination of two times the MIC of $[R_4W_4]$ and four times the MIC of tetracycline (0.5 μg/ml) (4). Cytotoxicity studies of $[R_4W_4]$ have been reported using MTS proliferation assay against two cancer cell lines (human ovarian adenocarcinoma SK-OV-3 and human leukemia CCRF-CEM) and one normal human embryonic kidney HEK 293T cell line at 24-h incubation. The cyclic peptide $[R_4W_4]$ demonstrated more than 84% cell viability at a concentration of 20.5 μg/ml in both cancer and normal cell lines (4). Furthermore, $[R_4W_4]$ showed minimal hemolytic activity by showing <10% hemolysis of normal red blood cells at the concentration of 128 μg/ml. Therefore, these studies demonstrate negligible or minimum cytotoxicity of $[R_4W_4]$ up to 20.5 μg/ml to the normal and cancerous cell lines. A fluorescent-labeled $[R_4W_4]$ peptide was synthesized and reported to disperse into the nucleus and cytoplasm. This demonstrated the transporter property of the $[R_4W_4]$ peptide. Furthermore, it has been reported that cyclic peptides have higher stability against proteases and found resistant toward proteolytic degradation as compared to linear peptides (5). Cyclic peptides have demonstrated stability under disease condition, and some of them are successfully used as drugs, including vancomycin, daptomycin, polymyxin B, colistin, caspofungin, and cyclosporine (6–8). A series of cyclic WR peptides has been reported to be stable against serum and found to have molecular transporter properties (9, 10). Therefore, $[R_4W_4]$ will be stable against proteases due to very similar amino acid composition and cyclic nature. As $[R_4W_4]$ has shown bactericidal activity (alongside tetracycline) against MRSA (4), it is an attractive candidate that can be tested along with first-line anti-TB drugs against *M. tb*.

Two of the first-line antibiotics used to treat active TB are INH and PZA, both of which have MICs of 0.125 and 50 μg/ml, respectively, against *M. tb*. In our past studies, one-time treatment of *in vitro M tb*-infected granulomas (or immune cell clusters) with lone INH and PZA at MIC did not result in complete clearance of *M. tb*. (11). We, therefore, tested the effects of $[R_4W_4]$ both alone and in combination with either INH or PZA against *M. tb* in the *in vitro* granulomas generated from PBMCs isolated from healthy subjects.

We first tested the direct antimycobacterial effects of $[R_4W_4]$ added at 4 and 8 μg/ml concentrations to static cultures of Erdman strain of *M. tb*. Static cultures of *M. tb* were grown in 7H9 media in the presence and absence of $[R_4W_4]$ (added at 4 μg/ml and 8 μg/ml) and the growth of *M. tb* was monitored for 8 days (**Figure 2A**). The selection of the two concentrations (4 μg/ml and 8 μg/ml) of $[R_4W_4]$ was based on the rationale of reported MIC values of peptide $[R_4W_4]$ against MRSA (4, 12, 13). The bacterial suspension was plated on 7H11 media and incubated for 4 weeks for colony formation. Compared to the untreated control group, *M. tb* survival (demonstrated by CFU counts) in 7H9 was significantly reduced by 2.2-fold in the group treated with $[R_4W_4]$ at 4 μg/ml concentration ($p = 0.0223$) (**Figure 2A**). There was also reduced *M. tb* survival in the group treated with $[R_4W_4]$ at 8 μg/ml, but this was not a statistically significant finding ($p = 0.1709$) (**Figure 2A**).

We then determined the viability of *M. tb* inside the *in vitro* granulomas treated with $[R_4W_4]$ at 4 and 8 μg/ml. The sham control group of human *M. tb*-infected granulomas was

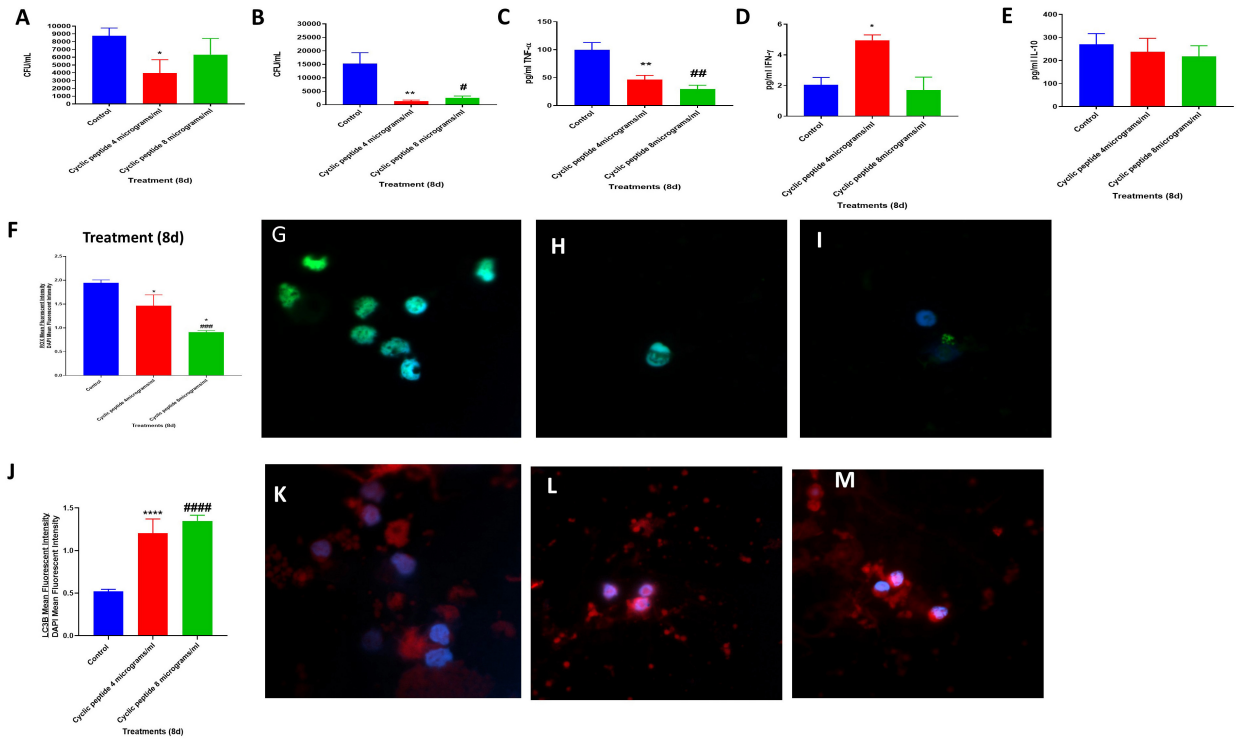

FIGURE 2 | Efficacy of [R_4W_4] against *M. tb*. Sensitivity Erdmann strain of *M. tb* to the cyclic peptide [R_4W_4] added at 4 and 8 μg/ml concentrations. *$P < 0.05$ when comparing CFUs at the 8-day time point of 4 μg/ml [R_4W_4]-treated Erdman strain to untreated control category **(A)**. Effects of [R_4W_4] in altering the viability in *M. tb* in the *in-vitro* granulomas **(B)**. Effects of [R_4W_4] in altering the production of TNF-α **(C)**. Effects of [R_4W_4] in altering the levels of IFN-γ **(D)**. Effects of [R_4W_4] in altering the production of IL-10 **(E)**. Effects of [R_4W_4] on reactive oxygen species production **(F)**. Mean fluorescent intensity of CellROX-stained (green fluorescent) control granulomas **(G)**, [R_4W_4] (4 μg/ml)-treated **(H)**, and [R_4W_4] (8 μg/ml)-treated granulomas **(I)**. Effects of [R_4W_4] on LC3B expression **(J)**. Mean fluorescent intensities of LC3B-stained (PEt)control granulomas **(K)**, [R_4W_4] (4 μg/ml)-treated **(L)**, and [R_4W_4] (8 μg/ml)-treated granulomas **(M)**. Anti-LC3B conjugated with PE and CellROX conjugated with FITC were applied on the same specimen. Fluorescent images were captured for each marker either from the same field or from a different representative field. Reported values are in means with each respective category. Data represent ± SE from experiments performed from eight different individuals. Analysis of figures utilized a one-way ANOVA with Tukey test. The *p* value above 0.05 is not considered significant, one symbol (* or #) denotes significant difference below 0.05, two symbols (** or ##) denote significant difference below 0.005, three symbols (*** or ###) denote significant difference below 0.0005, and four symbols (**** or ####) denote significant difference below 0.0001. An asterisk (*) indicates comparison to the direct previous category. A hash mark (#) indicates comparison between one category and a previous category that is one column before it. A dollar sign ($) indicates comparison between one category and a previous category that is two columns before it.

compared against *in vitro* granulomas treated with [R_4W_4] at 4 and 8 μg/ml, respectively. Compared to the control group, intracellular *M. tb* survival was significantly reduced in both groups treated with [R_4W_4] at 4 μg/ml ($p = 0.0348$) and 8 μg/ml concentrations ($p = 0.0423$) (**Figure 2B**). In comparison to the untreated granulomas, the fold reduction in the CFUs was 10.86 and 5.95 for granulomas treated with [R_4W_4] at 4 and 8 μg/ml, respectively. However, there was no significant difference in the intracellular *M. tb* survival between the groups treated with 4 and 8 μg/ml ($p = 0.2719$) (**Figure 2B**). These findings indicate that the treatment of *in vitro* granulomas with R_4W_4 caused a reduction in the *M. tb* burden (**Figure 2B**).

To understand the effects of [R_4W_4] in altering the underlying immune effector mechanisms against *M. tb* infection, we first measured the levels of several related cytokines that cause the activation (TNF-α and IFN-γ) and inhibition (IL-10) of immune effector mechanisms. TNF-α expression was significantly downregulated by [R_4W_4] at both 4 μg/ml ($p = 0.036$) and 8 μg/ml concentrations ($p = 0.0003$) compared to sham-treated control categories (**Figure 2C**). In comparison to the untreated granulomas, the fold reduction in the levels of TNF-α was 2 and 3.3 for granulomas treated with [R_4W_4] at 4 and 8 μg/ml, respectively. TNF-α, a pro-inflammatory cytokine, activates the effector immune functions and causes recruitment of immune cells to form a solid and stable granuloma to contain *M. tb* infection (14–18). Excess TNF-α can cause cell death by necrosis, leading to inflammation (19, 20). Our findings indicate a positive correlation between diminished numbers of *M. tb* in [R_4W_4]-treated granulomas with a corresponding decrease in the levels of TNF-α explaining the immunomodulatory effects of [R_4W_4].

IFN-γ is crucial in both innate and adaptive immunity, acting as a macrophage activation factor and mediating MHC molecule expression (21). There was a 2.5-fold increase in IFN-γ production in the supernatants from *in vitro* granulomas treated with 4 μg/ml concentration of [R_4W_4] ($p = 0.0161$) compared

to sham control (**Figure 2D**). In our previously published study (18), we demonstrated that IFN-γ increase was associated with enhanced phagosome acidification and improved killing of *M. tb* in the *in vitro* granulomas. Therefore, higher amounts of the IFN-γ release may therefore serve as an additional effector mechanism by which *in vitro* granulomas treated with 4 μg/ml of [R_4W_4] control *M. tb* infection.

IL-10 is an immune-suppressive cytokine that can inhibit phagosome–lysosome fusion and dampen other effector responses against *M. tb* infection (11, 22–24). We observed a notable decrease in the levels of IL-10 in [R_4W_4]-treated granulomas at both concentrations (4 and 8 μg/ml). These findings indicate that [R_4W_4] supplementation augments immune responses against *M. tb* infection by downregulating the levels of IL-10 (**Figure 2E**) and by increasing the levels of IFN-γ (**Figure 2D**).

It is well-known that oxidative stress generated during *M. tb* infection can contribute to both protection and pathogenesis (25). *M. tb* infection triggers the release of pro-inflammatory cytokines and reactive oxygen species (ROS) leading to oxidative stress. It is important to point out that the survival of *M. tb* is dependent on the extent of ROS produced by the host immune cells. Exacerbation in the levels of ROS can damage the immune cells causing diminishment in the effector responses against *M. tb* infection (25, 26). CellROX staining was performed to determine the extent of oxidative stress in sham control and [R_4W_4]-treated granulomas (**Figure 2F**). In comparison to the control group, there was a significant decrease in the intensity of CellROX staining in the [R_4W_4]-treated granulomas (**Figures 2F–I**). In comparison to the untreated granulomas, the fold reduction in the intensity of CellROX staining was 1.3 and 2.1 for granulomas treated with [R_4W_4] at 4 and 8 μg/ml, respectively. This significant reduction in the mean fluorescence intensity implies attenuation of oxidative stress levels. These data further illustrate that reducing the oxidative stress restores favorable immune responses against *M. tb* infection. TNF-α plays a central role in triggering the inflammatory response via ROS production (18, 27, 28). Additionally, ROS can induce TNF-α production (18, 27). Our study findings therefore confirm this direct link between TNF-α levels and oxidative stress and the effects of [R_4W_4] in downregulating the levels of both.

Autophagy is a self-degradative process, which plays a critical role in promoting cellular senescence, antigen presentation on the cell surface, and eliminating intracellular waste aggregates and damaged organelles, thereby preventing necrosis (29–31). It has been reported that autophagy functionally benefits some diseases such as cancer, cardiomyopathy, diabetes, liver disease, and infections (29–31). LC3B is a mammalian homolog of the yeast ATG8 protein, which is a ubiquitin-like protein related to autophagosomal membranes (32, 33). Therefore, LC3B is an important and direct marker used for detecting autophagy levels (32, 33). The LC3B protein in the granulomas were stained by an anti-LC3B antibody tagged with PE, a red fluorescent dye. Increased LC3B expression is indicative of increased autophagy. We observed that the mean fluorescence intensity of LC3B was significantly elevated in the [R_4W_4]-treated granulomas at both concentrations compared to the untreated granulomas (**Figures 2J–M**). In comparison to the untreated granulomas, the fold increase in the expression levels of LC3B was 2.4 and 2.6 for granulomas treated with [R_4W_4] at 4 and 8 μg/ml, respectively. Our results specify that autophagy may be one of the immune effector mechanisms by which [R_4W_4]-treated *in vitro* granulomas control *M. tb* infection.

We also tested the effects of [R_4W_4] and first-line antibiotics (INH or PZA) in reducing the burden of *M. tb* in the *in vitro* granulomas. Infected PBMCs were treated with [R_4W_4] at 4 and 8 μg/ml concentrations and cultured in the presence and absence of INH or PZA at their respective MIC. Consistent with our previous observations, there was a significant and 18-fold reduction in viability of *M. tb* in INH-treated granulomas ($p = 0.0314$). Treatment with INH in conjunction with [R_4W_4] at either 4 μg/ml ($p = 0.0287$) or 8 μg/ml ($p = 0.0292$) concentration resulted in a further reduction in the viability of *M. tb* when compared with the control category (**Figure 3A**). In comparison to the untreated granulomas, the fold reduction in the viability of *M. tb* was 104 and 57 in granulomas treated with INH + [R_4W_4] at 4 μg/ml and INH + [R_4W_4] 8 μg/ml, respectively. Although these findings were statistically significant, there were no significant statistical findings when comparing the INH category with INH treatment in conjunction with [R_4W_4] at 4 μg/ml ($p = 0.2787$) and 8 μg/ml ($p = 0.3803$) concentrations (**Figure 3A**).

The reduction in the viability of *M. tb* in INH + [R_4W_4]-treated granulomas was accompanied by a significant diminishment in the levels of TNF-α ($p = 0.0113$) along with a notable decrease in the levels of IL-10 ($p = 0.1336$) in the granuloma supernatants from INH + [R_4W_4] treatment categories when compared to untreated and INH-alone treated groups (**Figures 3B,D**). INH + [R_4W_4] treatment did not result in any significant changes in the production of IFN-γ (**Figure 3C**). Furthermore, treatment with INH + [R_4W_4] resulted in a significant decrease in the intensity of CellROX when compared to INH alone and control categories (**Figures 3E–I**). INH + [R_4W_4] treatment also resulted in a significant decrease in the intensity of LC3B staining when compared to the INH-alone treatment group. However, when compared to the control category, INH + [R_4W_4] treatment resulted in a statistically significant 2-fold increase in the intensity of LC3B staining (**Figures 3J–N**). Our results demonstrate that INH, when given in conjunction with [R_4W_4], decreases *M. tb* burden and downregulates oxidative stress and production of TNF-α and IL-10, when compared to lone INH treatment and control. Although the combination of INH + [R_4W_4] did not increase the expression of LC3B when compared to treatment with INH alone, INH + [R_4W_4] treatment downregulated the intensity of CellROX staining and diminished the production of TNF-α and IL-10. Although TNF-α plays a central role in the formation and maintenance of granulomas, overexpression of TNF-α has also been linked to many inflammatory and autoimmune diseases (14–20). IL-10, an immunosuppressive cytokine, can

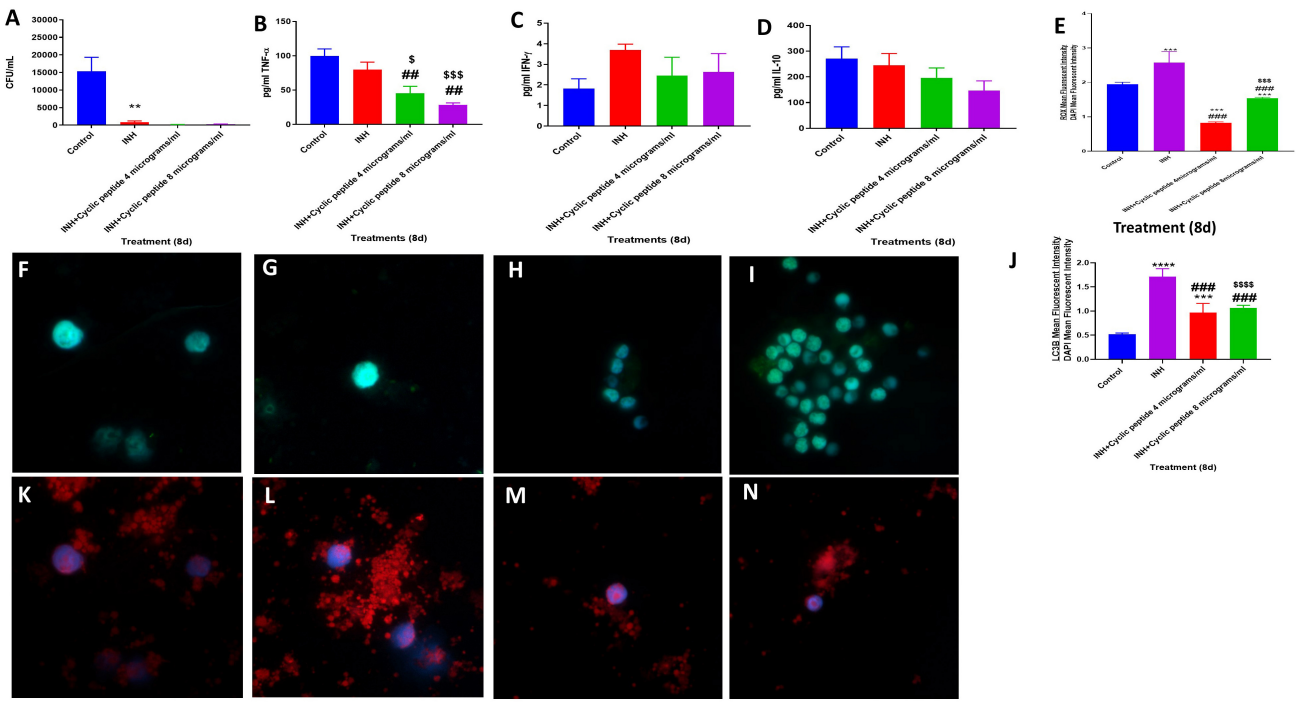

FIGURE 3 | Efficacy of INH and INH + $[R_4W_4]$ against *M. tb*. Effects of INH and INH + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) in altering the viability in *M. tb* in the *in vitro* granulomas **(A)**. Effects of INH and INH + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) in interfering with the production of TNF-α **(B)**. Effects of INH and INH + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) in interfering with the production of IFN-γ **(C)**. Effects of INH and INH + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) in interfering with the production of IL-10 **(D)**. Effects of INH and INH + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) on reactive oxygen species production **(E)**. Mean fluorescent intensity of CellROX-stained (green fluorescent) control granulomas **(F)**, INH-treated **(G)**, INH + $[R_4W_4]$ (4 μg/ml)-treated **(H)**, and INH + $[R_4W_4]$ (8 μg/ml)-treated granulomas **(I)**. Effects of INH and INH+ $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) on LC3B expression **(J)**. Mean fluorescent intensities of LC3B-stained (red fluorescent) control granulomas **(K)**, INH-treated **(L)**, INH + $[R_4W_4]$ (4 μg/ml)-treated **(M)**, and INH + $[R_4W_4]$ (8 μg/ml)-treated granulomas **(N)**. Anti-LC3B conjugated with PE (red) and CellROX conjugated with FITC (green) were applied on the same specimen. Fluorescent images were captured for each marker either from the same field or from a different representative field. Reported values are in means with each respective category. Data represents ± SE from experiments performed from 8 different individuals. Analysis of figures utilized a one-way ANOVA with Tukey test. The *p* value above 0.05 is not considered significant, one symbol (*, #, or $) denotes significant difference below 0.05, two symbols (**, ##, or $$) denote significant difference below 0.005, three symbols (***, ###, or $$$) denote significant difference below 0.0005, and four symbols (****, ####, or $$$$) denote significant difference below 0.0001. An asterisk (*) indicates comparison to the direct previous category. A hash mark (#) indicates comparison between one category and a previous category that is one column before it. A dollar sign ($) indicates comparison between one category and a previous category that is two columns before it.

downregulate the effector functions of macrophage against *M. tb* infection (11, 22–24, 34). Therefore, combination of $[R_4W_4]$ with INH can downregulate oxidative stress and production of TNF-α and IL-10, which in turn can favor improved control of *M. tb* infection when compared to treatment with INH alone.

When compared to the untreated control category, PZA treatment also resulted in a statistically significant and 30-fold reduction in the viability of *M. tb* in the granulomas (**Figure 4A**) ($p = 0.0302$). A further decrease in the viability of *M. tb* was observed with PZA + $[R_4W_4]$ treatments. The fold reduction in the viability of *M. tb* was 511 and 136 in granulomas treated with PZA + $[R_4W_4]$ at 4 μg/ml and PZA + $[R_4W_4]$ 8 μg/ml, respectively, when compared to untreated granuloma. The levels of TNF-α, IL-10, and IFN-γ were measured in the granuloma supernatants from control, PZA-treated, and PZA + $[R_4W_4]$-treated groups (**Figures 4B–D**). There was a statistically significant and 3.3-fold reduction in the levels of TNF-α in the PZA-treated group when compared to the control (**Figure 4B**). PZA + $[R_4W_4]$ treatment resulted in a further significant decrease in the production of TNF-α when compared to the PZA-alone category and control groups. PZA + $[R_4W_4]$ treatment also resulted in a significant decrease in the production of IL-10 (**Figure 4D**).

Although not significant, PZA + $[R_4W_4]$ (4 μg/ml) treatment resulted in an increase in the levels of IFN-γ (**Figure 4C**). PZA treatment resulted in attenuation in the fluorescence intensity of CellROX. However, the granulomas treated with PZA + $[R_4W_4]$ showed a significant increase in the intensity of CellROX staining compared to the PZA-alone group (**Figures 4E–I**). There was increased intensity of LC3B staining in PZA-treated granulomas compared to the untreated control group. PZA + $[R_4W_4]$ treatment caused a significant increase in the intensity of LC3B staining compared to the control and PZA-alone group. In comparison to the untreated granulomas, the fold increase in the intensity of LC3B staining was 2 and 2.4 in granulomas

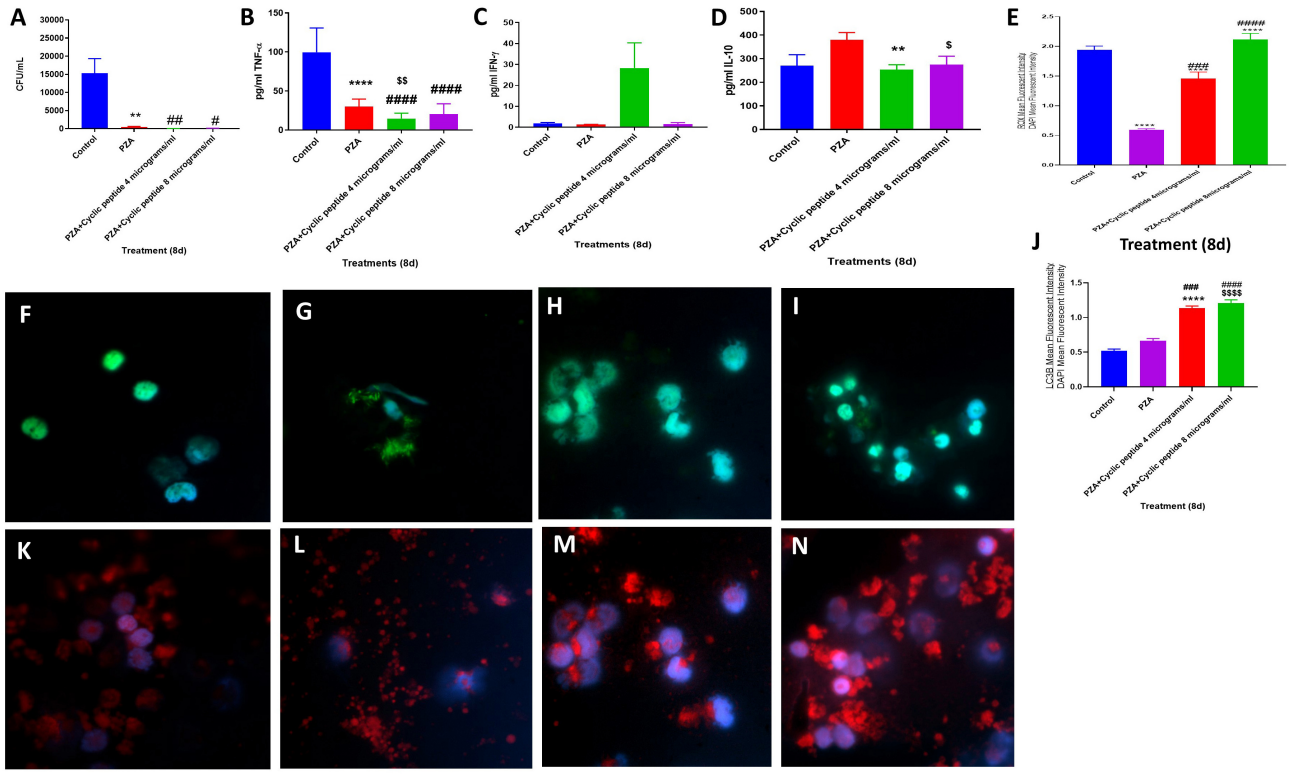

FIGURE 4 | Efficacy of PZA and PZA + $[R_4W_4]$ against *M. tb*. Effects of PZA and PZA + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) in altering the viability in *M. tb* in the *in vitro* granulomas **(A)**. Effects of PZA and PZA + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) in interfering with the production of TNF-α **(B)**. Effects of PZA and PZA + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) in interfering with the production of IFN-γ **(C)**. Effects of PZA and PZA + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) in interfering with the production of IL-10 **(D)**. Effects of PZA and PZA + $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) on reactive oxygen species production **(E)**. Mean fluorescent intensity of CellROX-stained (green fluorescent) control granulomas **(F)**, PZA-treated **(G)**, PZA + $[R_4W_4]$ (4 μg/ml)-treated **(H)**, and PZA + $[R_4W_4]$ (8 μg/ml)-treated granulomas **(I)**. Effects of PZA and PZA+ $[R_4W_4]$ (added at 4 and 8 μg/ml concentrations) on LC3B expression **(J)**. Mean fluorescent intensities of LC3B-stained (red fluorescent) control granulomas **(K)**, PZA-treated **(L)**, PZA + $[R_4W_4]$ (4 μg/ml)-treated **(M)**, and PZA + $[R_4W_4]$ (8 μg/ml)-treated granulomas **(N)**. Anti-LC3B conjugated with PE (red) and CellROX conjugated with FITC (green) were applied on the same specimen. Fluorescent images were captured for each marker either from the same field or from a different representative field. Reported values are in means with each respective category. Data represents ± SE from experiments performed from 8 different individuals. Analysis of figures utilized a one-way ANOVA with Tukey test. The *p* value above 0.05 is not considered significant, one symbol (*, #, or $) denotes significant difference below 0.05, two symbols (**, ##, or $$) denote significant difference below 0.005, three symbols (***, ###, or $$$) denote significant difference below 0.0005, and four symbols (****, ####, or $$$$) denote significant difference below 0.0001. An asterisk (*) indicates comparison to the direct previous category. A hash mark (#) indicates comparison between one category and a previous category that is one column before it. A dollar sign ($) indicates comparison between one category and a previous category that is two columns before it.

treated with PZA + $[R_4W_4]$ at 4 μg/ml and PZA + $[R_4W_4]$ at 8 μg/ml, respectively (**Figures 4J–N**). Our results indicate that when compared to the PZA-alone category, treatment with PZA + $[R_4W_4]$ caused increased production of IFN-γ, decreased levels of IL-10, increased autophagy, and improved control of *M. tb* infection. These findings further support the previous findings that decreased IL-10 along with increased IFN-γ will favor effective control of *M. tb* infection (21).

Our study findings demonstrate that $[R_4W_4]$ elicits direct antimycobacterial ability against virulent Erdman strain of *M. tb* at 4 μg/ml. Treatment of *in vitro* granulomas with $[R_4W_4]$ both alone and in combination with first-line antibiotics such as INH and PZA at MIC concentration resulted in a further decrease in the viability of *M. tb*. The addition of $[R_4W_4]$ to granulomas both in the presence and absence of PZA led to a reduction in the levels of TNF-α and IL-10, and elevation in the levels of autophagy. Our study findings, therefore, indicate that $[R_4W_4]$ causes a significant decrease in the viability of *M. tb* and works in conjunction with first-line antibiotics by modulating the levels of cytokine release and autophagy. In conclusion, we believe that $[R_4W_4]$, along with anti-TB treatment, would not only attenuate the side effects of antibiotics but can also enhance the immune responses to eliminate the active *M. tb* infection.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Western University of Health Sciences. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AA synthesized cyclic peptide. RK and VV conceived the study, developed study design, analyzed the data, and prepared the manuscript. JH, DA, and RC conducted the studies and drafted the manuscript. AY, KT, RA, and TN provided technical assistance. All authors contributed to the article and approved the submitted version.

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Cathelicidin and Calprotectin Are Disparately Altered in Murine Models of Inflammatory Arthritis and Airway Inflammation

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Cationic host defense peptides (CHDP) are immunomodulatory molecules that control infections and contribute to immune homeostasis. CHDP such as cathelicidin and calprotectin expression is altered in the arthritic synovium, and in the lungs of asthma and COPD patients. Recent studies suggest a link between airway inflammation and the immunopathology of arthritis. Therefore, in this study we compared the abundance of mouse cathelicidin (CRAMP), defensins, and calprotectin subunits (S100A8 and S100A9) in murine models of collagen-induced arthritis (CIA) and allergen house dust mite (HDM)-challenged airway inflammation. CRAMP, S100A8, and S100A9 abundance were significantly elevated in the joint tissues of CIA mice, whereas these were decreased in the lung tissues of HDM-challenged mice, compared to naïve. We further compared the effects of administration of two different synthetic immunomodulatory peptides, IG-19 and IDR-1002, on cathelicidin and calprotectin abundance in the two models. Administration of IG-19, which controls disease progression and inflammation in CIA mice, significantly decreased CRAMP, S100A8, and S100A9 levels to baseline in the joints of the CIA mice, which correlated with the decrease in cellular influx in the joints. However, administration of IDR-1002, which suppresses HDM-induced airway inflammation, did not prevent the decrease in the levels of cathelicidin and calprotectin in the lungs of HDM-challenged mice. Cathelicidin and calprotectin levels did not correlate with leukocyte accumulation in the lungs of the HDM-challenged mice. Results of this study suggest that endogenous cathelicidin and calprotectin abundance are disparately altered, and may be differentially regulated, within local tissues in airway inflammation compared to arthritis.

Keywords: inflammation, cathelicidin, calprotectin, host defence peptides, antimicrobial peptides, arthritis, asthma, airway

INTRODUCTION

Cationic host defense peptides (CHDP), also known as antimicrobial peptides, are endogenous molecules that orchestrate host immune responses to infection and inflammation (1, 2). Immunity-related functions of CHDP include enhancing leukocyte migration and innate immune responses to resolve infections, regulation of endotoxin- and/or specific cytokine-induced inflammatory responses, promotion of the maturation and differentiation of leukocytes, induction of anti-inflammatory cytokines, contributing to tissue remodeling or wound healing, and overall maintenance of immune homeostasis (1–3). CHDP bridge innate and adaptive immune responses and play a role in promoting initiation, polarization, and amplification of adaptive immunity (1). These peptides are expressed by both immune and structural cells, and expression levels of some CHDP are enhanced in presence of infection or inflammatory challenge. Several studies have demonstrated altered levels of CHDP cathelicidin and calprotectin (a complex of subunits S100A8 and S100A9) in various chronic inflammatory diseases including rheumatoid arthritis (RA), chronic obstructive pulmonary disease (COPD), asthma, inflammatory bowel disease (IBD) and atherosclerosis (3–12). However, alteration of abundance of specific CHDP in chronic inflammatory diseases has not been completely defined.

Recent evidence suggests the involvement of airway inflammation to the onset and the immunopathology of RA (13, 14). Environmental factors such as smoking and air pollution that lead to the exacerbation of airway inflammation are also critical risk factors for the development of RA (13). Thus, although airway inflammation and inflammatory arthritis have distinct phenotype, these exhibit overlapping molecular mechanisms of immune dysregulation. A mechanism linking airway inflammation to RA is the production of antibodies directed against citrullinated peptide targets (anti-CCP). These antibodies are found in the lungs during airway inflammation and are defined as autoantibodies in RA (15, 16). Interestingly, CHDP such as LL-37 can get citrullinated in airway inflammation, which alters the functions of the peptide (17). Moreover, the abundance of circulating LL-37 is associated with anti-CCP in early RA (18). However, the role of CHDP in respiratory inflammatory disease progression and in the immunopathology of RA remains elusive. We have previously shown that inhaled air pollution, which is a critical environmental risk factor for both airway inflammation and the development of RA, alters the expression profile of CHDP in the human lungs (19). We have also shown that cytokines that are elevated during inflammation in both the lungs and joints, such as IL-17 and TNF, can significantly change the expression profile of CHDP in epithelial cells (20). Therefore, in this study we examined *in vivo* the expression of a panel of CHDP selected from these previous studies, in tissues isolated from murine models of airway inflammation and arthritis. The objective was to examine if specific endogenous CHDP are altered similarly within the local tissues in these two interconnected disease processes.

It is unknown whether change in the abundance of endogenous CHDP during inflammation is solely related to enhanced accumulation of inflammatory leukocytes within the local tissues. Synthetic peptides based on CHDP sequence have been previously shown to mitigate local inflammation in chronic inflammatory disease models (2). For example, the human cathelicidin LL-37-derived peptide IG-19, which represents the minimum region of LL-37 required for immunomodulatory functions (21), can suppress pro-inflammatory cytokine secretion (22, 23), and reduce leukocyte accumulation and control inflammatory mediators in the joints in a collagen-induced arthritis (CIA) mouse model (24). Similarly, a bovine cathelicidin-derived peptide IDR-1002 mitigates airway inflammation, controls leukocyte accumulation in the lungs, and improves airway hyper-responsiveness (AHR) in an allergen house dust mite (HDM)-challenged mouse model (25). Therefore, in this study we used the synthetic immunomodulatory peptides IG-19 and IDR-1002 as probes to examine whether the change in the abundance of endogenous CHDP is associated with enhanced inflammation within the local tissues. The hypothesis being that if CHDP abundance is altered solely due to enhanced inflammation such as that mediated by the influx of inflammatory leukocytes within the local tissues, then the administration of the specific anti-inflammatory synthetic peptides would result in the altered CHDP levels being reversed and/or restored to baseline, in both models.

In this study, we investigated the abundance of the mouse cathelicidin CRAMP, α - and β-defensins, and calprotectin subunits S100A8 and S100A9, in the two different murine models of chronic inflammation; a CIA model of inflammatory arthritis and allergen HDM-challenged model of airway inflammation (25–27). We showed that CRAMP and calprotectin subunits S100A8, and S100A9 are disparately altered in these mouse models, being significantly increased in the joint tissues of CIA mice, but decreased in the HDM-challenged lungs. Overall, the results in this study suggest that endogenous CHDP cathelicidin and calprotectin abundance are altered disparately, and may be differentially regulated within local tissues in airway inflammation compared to inflammatory arthritis.

METHODS

Collagen-Induced Arthritis (CIA) Murine Model

The protocol used for the CIA murine model was based on our previous study (26) and approved by the University of Manitoba Animal Research Ethics Board (protocol no 16-009). ARRIVE guidelines were followed in designing and reporting animal data (28). Briefly, highly susceptible DBA/1 male mice (∼6 weeks old) were obtained from Jackson laboratories, sorted into 4 mice per cage by animal care staff and allowed to acclimatize to the facility for 2-weeks at the central animal care facility at University of Manitoba. Mice were anesthetized using isoflurane (4%) and challenged with a tail injection (s.c) of 100 μg bovine collagen type II (CII) emulsified in complete Freund's adjuvant. A boost of CII emulsified (50 μg) in incomplete adjuvant was administered

on day 21 after the initial CII challenge. On day 25 after the first CII challenge, mice were injected intra-peritoneally (i.p.) with LPS from *E. coli* 0111:B4 (20 μg per mouse). Administration of low dose of LPS synchronizes the disease incidence in the CIA model, and therefore allows for accurate comparative evaluation of outcomes between different groups (26, 29, 30). Collagen and LPS challenge were performed between 10:00 am and noon. All reagents for the CIA challenge were obtained from Chondrex Inc. (Redmond, WA, USA). Mice with saline injections were used as the control group. Mice were monitored for change in body weight every alternate day, and were visually monitored for grooming and activity levels every day. Joint thickness was monitored daily using a digital caliper from day 22 onwards to assess disease progression as described previously (24, 26). Disease severity was assessed in a blinded manner using a standardized clinical score based on joint thickness / swelling data, as previously described by us (24, 26). Briefly, clinical score assessment was as follows: Score 0 = normal joint; 1 = paw swelling only; 2 = one joint of one limb along with paw swelling; 3 = multiple joints on a limb involved; and 4 = all joints involved or limb fusion. Therefore, a total clinical score ranging from 0 to 16 was assigned to each mouse by combining the scores of each paw (24, 26). On day 29 after the first CII challenge, mice were anesthetized with isoflurane (4%) and euthanized by cardiac puncture, blood collected and used to obtain serum samples. Serum obtained was aliquoted and stored in −20°C until use. Mice joints were collected, cleaned to remove skin/tissues and homogenized to prepare protein lysates.

HDM-Induced Allergic Asthma Mice Model

The protocol used for the HDM murine model was based on our previous study (25, 27) and approved by the University of Manitoba Animal Research Ethics Board (protocol no 16-040). ARRIVE guidelines were followed in designing and reporting animal data (28). Briefly, female BALB/c mice (6–7 weeks) were obtained from the Genetic Modeling of Disease Center at the University of Manitoba. Mice were sorted into 4 mice ($n = 4$) per cage by animal care staff and housed at the central animal care facility at University of Manitoba. Animals were acclimatized for a minimum of 1 week. Mice were sedated using isoflurane and challenged with intranasal (i.n) administrations of 35 μL of HDM extract (0.7 μg/mL of saline) or saline, 5 times a week for 5 weeks. HDM extracts used in this study were obtained from Greer laboratories (Wilmington, MA, USA), with low endotoxin levels between 600 and 800 EU/vial (which is 115 and 260 EU/mg of protein weight). HDM challenge was performed in the morning between 10:00 a.m. and noon. Mice were visually monitored for grooming and activity levels every day. Mice were sacrificed 24 h after the last HDM-challenge based on our previous study (25), and right lower lobe lung tissues collected for protein analyses.

Administration of Cathelicidin-Derived Synthetic Peptides in Mice

Peptides IG-19 and IDR-1002 were obtained from CPC Scientific (CA, USA) (21, 23, 25, 31). IG-19 (IGKEFKRIVQRIKDFLRNL-NH_2) is derived from the human cathelicidin LL-37 (amino acids 13-31) (21, 23). Innate Defense Regulator (IDR) peptide, IDR-1002 (VQRWLIVWRIRK-NH2), is derived from bovine cathelicidin Bac-2A (25, 31). These synthetic peptides were re-suspended in sterile saline and administered by s.c. injections (6 mg/kg body weight) in both models. The dose and mode of administration of these peptides were based on our previous studies in the CIA model and HDM-challenge model (24–27). In the CIA model, the peptides were administered starting from day 20 [1 day before boost, after the initial arthritis induction phase (32)] and subsequently every 48 h until the end of the study. As the anti-inflammatory effect of the peptide IG-19 was previously demonstrated in the CIA model without LPS boost (24), we also tested the peptide IDR-1002 in the CIA model without LPS boost. In the HDM-challenge model, the peptides were administered 3 days per week starting from day 1, for 2 weeks. Peptide treatments were performed between 10:00 a.m. and noon.

Tissue Sample Preparation

After the mice were euthanized, joint tissues were collected from mice in the CIA model, and lung tissues were obtained from mice in the HDM-challenge model, as described above. The tissues were flash frozen in liquid nitrogen and stored in −80°C until further use. The flash frozen tissues were homogenized on ice using a tissue homogenizer (Omni International, USA), in protein extraction buffer T-PER (Thermo Scientific, USA) containing protease inhibitor cocktail (Cell Signaling Technology, Denver, USA). The homogenates were centrifuged at 10,000 × g, at 4°C for 10 min. The supernatants were collected, aliquoted and stored in −20°C until use. Total protein amount was estimated in the supernatants using micro-Bicinchoninic acid (BCA) assay (Thermo Scientific, USA) according to the manufacturer's instructions.

Immunoblotting

The joint and lung tissue lysates (20 μg per sample) were resolved on NuPage 4–12% Bis-Tris protein gels (Invitrogen) and transferred onto nitrocellulose membranes. The membranes were blocked overnight with 5% milk powder (w/v) and probed with antibodies for murine CRAMP (rabbit polyclonal, Abcam, USA, catalog number ab93357), S100A8 (rat monoclonal [clone ABM4A69], Abcam, catalog number ab220174), S100A9 (rat monoclonal [clone 2B10], Abcam, catalog number ab105472), α-defensin 1 (goat polyclonal, Abcam, catalog number ab122884), β-defenisn 2 (rabbit polyclonal, MyBioSource, USA, catalog number MBS2005685) and β-defensin 14 (rabbit polyclonal, MyBioSource, catalog number MBS1490249). Antibody to β-actin (Cell Signaling Technologies) was used to normalize for protein loading. Affinity-purified horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling, USA) along with Amersham ECL Prime (GE Healthcare) was used for detection. The blots were imaged using AmershamTM Imager 680 blot and gel imager. Densitometry assessment of band intensity was determined using AmershamTM Imager 680 analysis software version 2.0. The relative band intensity was assessed after normalization with the band intensity for β-actin.

Evaluation of Anti-collagen Type II (CII) Antibodies in Serum

Circulating levels of mouse anti-collagen antibodies (auto-antibodies) and bovine anti-collagen antibodies was assessed by ELISA in serum collected from mice in the CIA model. A mouse anti-mouse type II collagen IgG antibody assay kit and mouse anti-bovine type II collagen IgG antibody assay kit was used, according to the manufacture's protocol (Chondrex Inc. Redmond, WA, USA). The antibody concentrations in the samples were calculated by comparison with the optical density (OD) values of standard anti-CII antibody (units/ml) provided in each of the assay kits as previously described (24, 26).

Cell Differential Assessment in Bronchoalveolar Lavage Fluid (BALF)

Mice in the HDM-challenged model were anesthetized with sodium pentobarbital (90 mg/kg, i.p), tracheotomised, and lungs were washed with 1 mL of cold saline twice for a total of 2 mL. BALF obtained was centrifuged at 150 × g for 10 min at room temperature and cell differentials were assessed using a modified Wright-Giemsa staining (Hema 3® Stat Pack, Fisher Scientific, Hampton, NH, USA) using a Carl Zeiss Axio Lab A1 (Carl Zeiss, Oberkochen, Germany) microscope, as previously described (25, 27). Cell differentials were counted blinded by two different personnel in 5 image frames at 20X magnification per slide, as previously described by us (25).

Lung Function Measurements

Mice from the HDM-challenged model were anesthetized with sodium pentobarbital (90 mg/kg, i.p.) and tracheostomized. Prior to lung function testing, mice received additional 45 mg/kg pentobarbital to maintain a stable anesthetized state. Lung function measurements was performed using a *flexi*Vent™ small animal ventilator (SCIREQ Inc, Montreal, QC, Canada) as previously described (33, 34). Briefly, high frequency forced oscillation with positive end-expiratory pressure of 3 cmH_2O was used to assess Newtonian resistance (Rn) to monitor central airway constriction, tissue damping (G) as an index of alveolar tissue restriction, and tissue elastance (H) to determine alveolar tissue stiffness. A muscle paralytic agent was not used in this method. Data was collected using flexiWare Software and transferred to Microsoft Excel and GraphPad Prism software for further analysis. Changes in Rn, G and H were monitored in response to nebulized saline (baseline measures), followed by increasing concentrations of nebulized methacholine (3–50 mg/mL), using Quick Prime-3 and Snapshot perturbations (25).

Histological Assessment of Joints for Cellular Infiltration

The ankle joints of mice were collected on the day of sacrifice and fixed in 10% buffered formalin for 48 h. The joints were decalcified using 10% EDTA for 14 days followed by dehydration in increasing concentrations of ethanol. The tissues were embedded in paraffin and serial sagittal sections (5 μm) were obtained. The sections were stained with hematoxylin and eosin (H&E) to assess influx of leukocytes in the joints. Sections were imaged and processed with a Zeiss imager M2 (Germany) using the Zen 2011 software. The stained sections were scored as previously described (24, 26) in a blinded manner by three independent personnel. Briefly, a histology score to assess the leukocyte infiltration and integrity of the joints was as follows: A score of 0 = normal synovium, 1 = synovial membrane hypertrophy and cell infiltration, 2 = pannus formation and cell infiltration, 3 = joint degeneration and robust cell infiltration, and 4 = loss of joint integrity along with robust cellular infiltration (24, 26).

Statistical Analysis

GraphPad Prism 7.05 software was used for data analyses. Statistical significance was determined using Kruskal–Wallis One-way analysis of variance (ANOVA) followed by Dunn's *post-hoc* test when comparing three or more groups. Mann–Whitney *U*-test was used to determine the *p*-values between any two groups as presented in **Table 1**. Pearson's correlation analysis was performed to examine the correlation between CHDP abundance with joint histology score in the CIA model, or total cell count in the BALF in the HDM model. A p-value of < 0.05 was considered to be statistically significant.

RESULTS

Endogenous CRAMP and Calprotectin Abundance Are Altered in CIA and HDM-Challenged Mice

The abundance of CRAMP, S100A8, S100A9, α-defensin 1, β-defensin 2 and β-defensin 14 were examined by western blots in the joint tissue lysates obtained from CIA and saline-treated control mice, and in the lung tissue lysates obtained from HDM-challenged and allergen-naïve mice. Defensins were not detected in measureable abundance in the joint or lung tissue lysates. CRAMP, S100A8, and S100A9 were not detected in the joint tissue lysates of saline-treated control mice, whereas the abundance of these CHDP was robust in joint tissue lysates of CIA mice (**Supplementary Figure 1**). Densitometry analyses demonstrated that the levels of CRAMP, S100A8, and S100A9 peptides were significantly ($p \leq 0.002$) increased between 70 and 500-fold in the joint tissues of CIA mice, compared to saline-treated mice (**Figure 1**). In contrast, the levels of CRAMP, S100A8, and S100A9 peptides were significantly ($p \leq 0.001$) decreased by 65 ± 6.5%, 60 ± 6%, 62 ± 6.7%, respectively in the lung tissues of HDM-challenged mice, compared to allergen-naïve mice (**Figure 2**).

Administration of LL-37-Derived Synthetic Peptide IG-19 Alters the Abundance of CRAMP and Calprotectin in the Joints of CIA Mice

We have previously shown that administration (s.c.) of the human cathelicidin LL-37-derived peptide IG-19, from day 20 after initial CII challenge, prevents arthritic symptoms and suppresses anti-collagen antibodies, in CIA mice using a protocol without LPS boost (24). The CIA mouse model

TABLE 1 | Change in the relative abundance of CRAMP and calprotectin in murine models of CIA and HDM-challenged airway inflammation.

	Relative fold change in joint tissues compared to saline (densitometry)				Relative fold change in lung tissues compared to saline (densitometry)			
	CIA	CIA +IG-19	% change	*p*-value	HDM	HDM+ IDR1002	% change	*p*-value
CRAMP	75 ± 27	8 ± 1.3	89 ± 1.28%	0.028	0.36 ± 0.1	0.49 ± 0.16	22.6 ± 7.1%	0.48 ns
S100A8	688 ± 252	95 ± 34	86 ± 1.82%	0.028	0.29 ± 0.08	0.48 ± 0.12	31.4 ± 16%	0.34 ns
S100A9	593 ± 350	58 ± 25	90.7 ± 6.2%	0.028	0.30 ± 0.1	0.31 ± 0.11	1.3 ± 9%	0.99 ns

ns, non-significant.

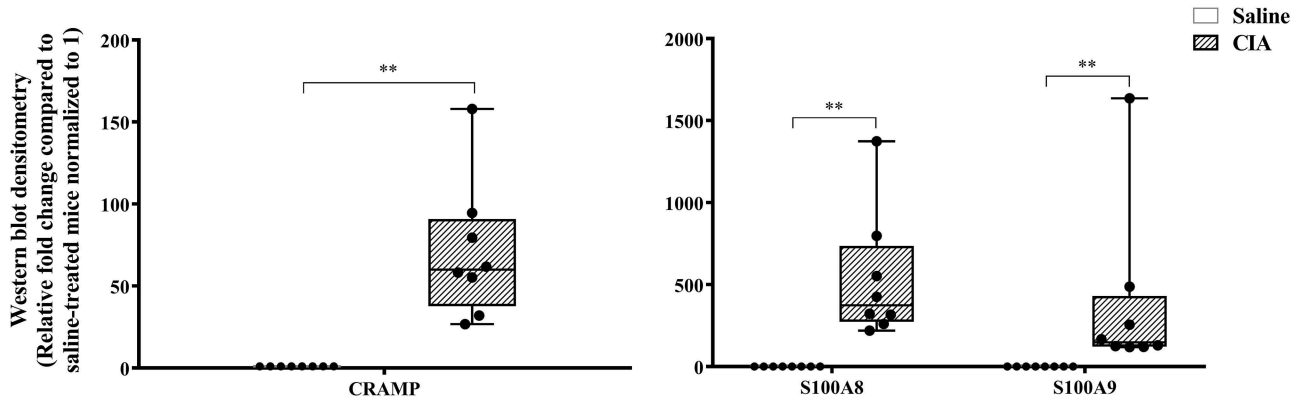

FIGURE 1 | CRAMP and Calprotectin levels are elevated in murine model of CIA. Flash frozen tissues obtained from joints of the CIA model ($n = 8$) and saline control ($n = 8$) were homogenized and protein concentration was determined using miroBCA. The samples (20 μg protein each) were resolved on NuPage 4–12% Bis-Tris protein gels and transferred onto nitrocellulose membranes. The membranes were probed with antibodies for mouse CRAMP, S100A8, S100A9 and β-actin (loading control). Densitometry for band intensity was determined using AmershamTM Imager 680 analysis software version 2.0. The relative band intensity was determined by normalizing to the β-actin band intensity for each sample. The represented values are the relative fold change compared to saline-treated mice normalized to 1. GraphPad Prism 7.05 software was used for statistical analyses. Mann–Whitney *U*-test was used to determine the *p*-values. A *p*-value of < 0.05 was considered to be statistically significant (**$p \leq 0.005$).

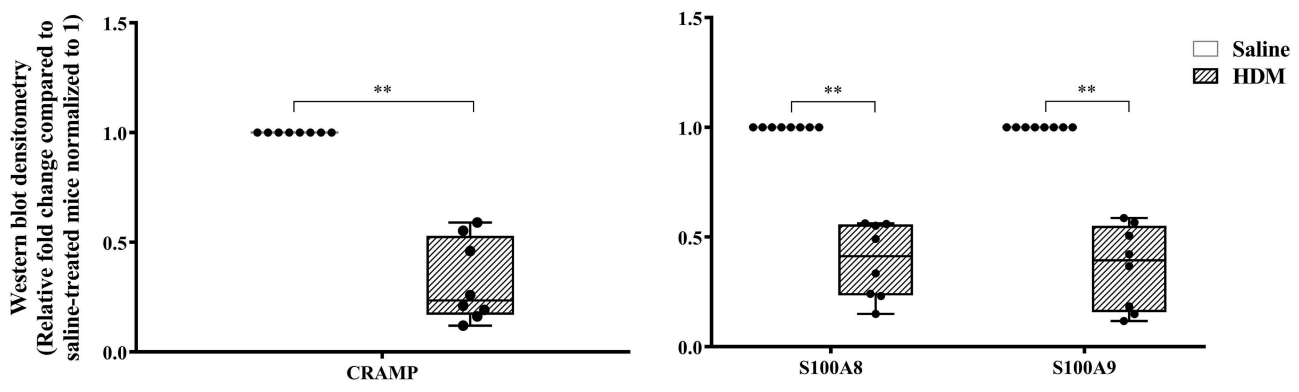

FIGURE 2 | CRAMP and Calprotectin levels are decreased in murine model of HDM-challenge-induced airway inflammation. Flash frozen tissues obtained from lungs of HDM-challenge model ($n = 8$), and saline control ($n = 8$) were homogenized and protein concentration was determined using miroBCA. The samples (20 μg protein each) were resolved on NuPage 4–12% Bis-Tris protein gels and transferred onto nitrocellulose membranes. The membranes were probed with antibodies for mouse CRAMP, S100A8, S100A9 and β-actin (loading control). Densitometry for band intensity was determined using AmershamTM Imager 680 analysis software version 2.0. The relative band intensity was determined by normalizing to the β-actin band intensity for each sample. The represented values are the relative fold change compared to saline-treated mice normalized to 1. GraphPad Prism 7.05 software was used for statistical analyses. Mann-Whitney U test was used to determine the *p*-values. A *p*-value of < 0.05 was considered to be statistically significant (**$p \leq 0.005$).

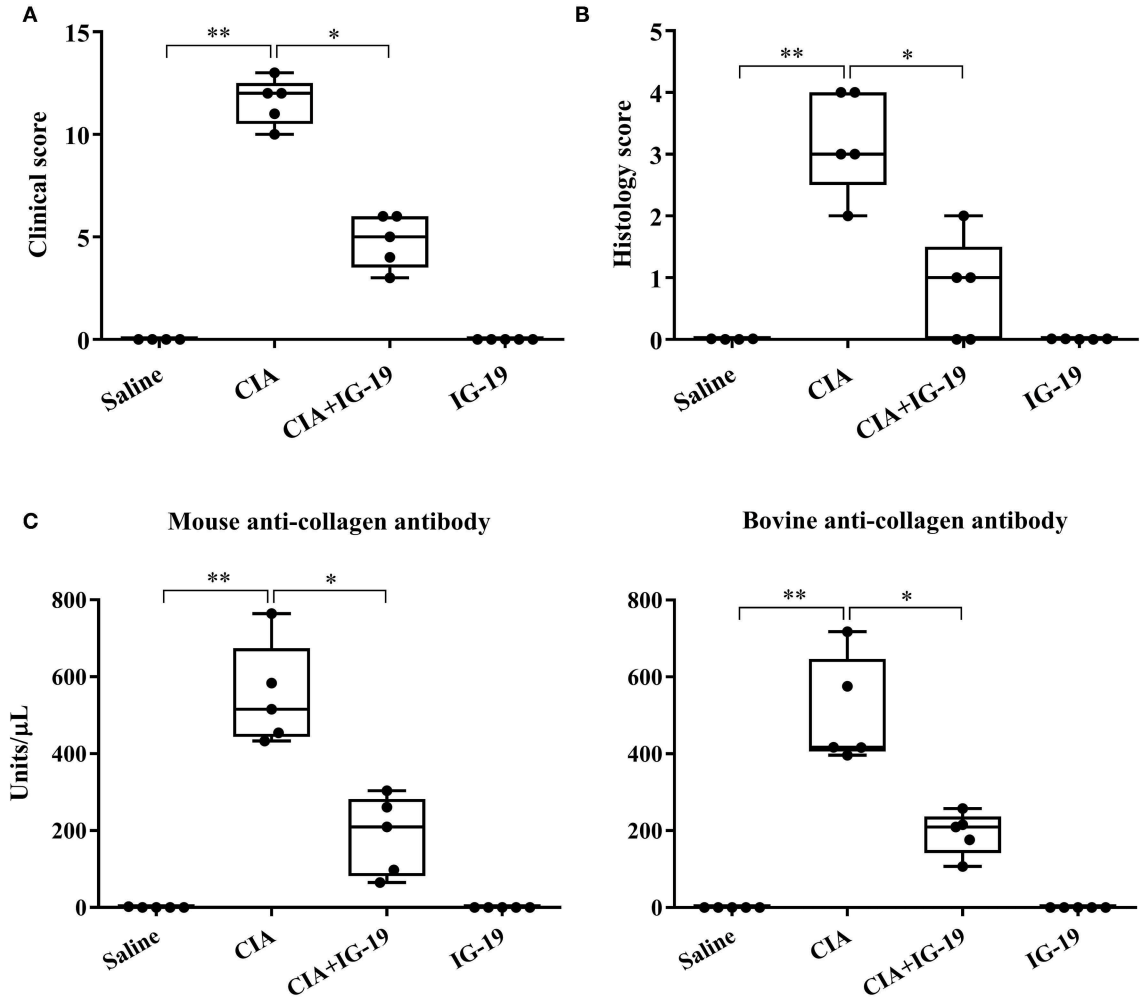

FIGURE 3 | Administration of IG-19 reduces clinical scores and anti-collagen II antibody levels in CIA mice. DBA/1 male mice (8 weeks) were challenged (s.c.) with bovine CII ($n = 4$), followed by a booster dose of CII (s.c) on day 21 and a LPS boost (i.p.) on day 25 after the initial CII challenge. Peptide IG-19 was administered (s.c) every 48 h from day 20 after the first CII challenge (one day before boost). Mice were monitored for disease severity and assigned clinical scores from day of CII boost (day 21 after the first CII challenge), every alternate day. Mice were euthanized by cardiac puncture under anesthesia on day 29 after the first CII challenge and blood collected for serum. **(A)** Clinical scores, **(B)** histology scores, and **(C)** serum concentration of anti-mouse collagen II autoantibodies and anti-bovine collagen II antibodies (to the immunizing antigen), assessed on the day of sacrifice. GraphPad Prism 7.05 software was used for statistical analyses. Kruskal–Wallis One-way ANOVA followed by Dunn's multiple comparison test was used to determine the significance (*$p \leq 0.05$, **$p \leq 0.01$).

protocol as detailed in this study includes a low dose LPS boost which has been shown to synchronize the disease onset and progression in the CIA model (26). Low dose of LPS in the CIA model synchronizes the arthritic clinical symptoms without compromising the severity and other characteristic features of the disease, which allows for an accurate comparative evaluation of outcomes across different groups (26, 29, 30). In this study, we tested the effects of s.c. administration of IG-19 in the CIA model synchronized with LPS boost as detailed in the methods of this study, as well as in the HDM-challenged mouse model of airway inflammation. Administration of IG-19 significantly decreased the clinical and histology scores by >50%, and suppressed both anti-bovine collagen antibody (antibodies to immunizing antigen) and anti-mouse autoantibodies by > 60% in the CIA mice (**Figure 3**; **Supplementary Figure 2**). These results were consistent with our previous study using the non-synchronized (without LPS) CIA mouse model (24). Taken together, these results indicated that s.c. administration of IG-19 from 1 day before CII boost prevents the development of the disease, suppresses anti-collagen antibodies, and suppresses accumulation of leukocytes in the joints, in CIA mice (24, 26). In contrast, administration of IG-19 did not improve HDM-induced AHR, and did not reduce leukocyte accumulation in the lungs of HDM-challenged mice (**Supplementary Figure 3**). Therefore, we further evaluated the effects of administration of IG-19 on the abundance of CRAMP and calprotectin in joint tissues in the CIA model in this study. Administration (s.c.) of IG-19 significantly suppressed elevated levels of CRAMP, S100A8, and S100A9 by 89

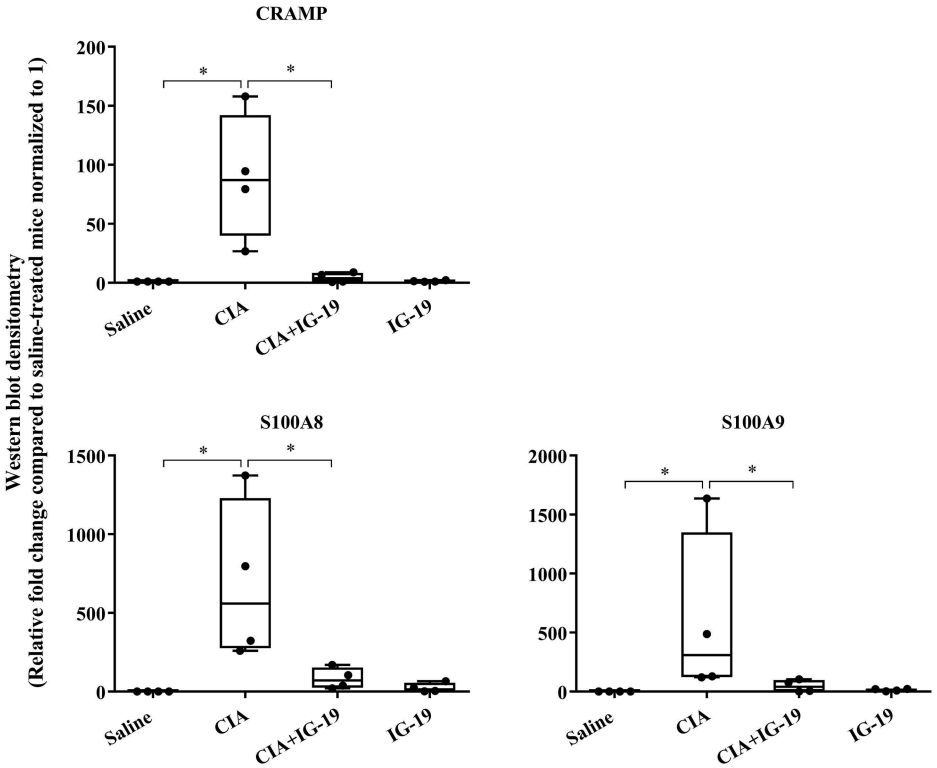

FIGURE 4 | Administration of peptide IG-19 alleviates elevated levels of CRAMP and Calprotectin in joint tissues of CIA mice. Flash frozen tissues obtained from joints of saline treated ($n = 4$), CIA ($n = 4$), CIA mice administered with either peptide IG-19 ($n = 5$) or peptide alone ($n = 5$ each), were homogenized and protein concentration was determined using miroBCA. The samples (20 μg protein each) were resolved on NuPage 4–12% Bis-Tris protein gels and transferred onto nitrocellulose membranes. The membranes were probed with antibodies for mouse CRAMP, S100A8, S100A9 and β-actin (loading control). Densitometry for band intensity was determined using AmershamTM Imager 680 analysis software version 2.0. The relative band intensity was determined by normalizing to the β-actin band intensity for each sample. The represented values are the relative fold change compared to saline-treated mice normalized to 1. GraphPad Prism 7.05 software was used for statistical analyses. Kruskal–Wallis One-way analysis of variance (ANOVA) followed by Dunn's *post-hoc* test was used to determine the significance. A *p*-value of < 0.05 was considered to be statistically significant ($^*p < 0.05$).

± 1.28%, 86 ± 1.82%, and 90 ± 6.2%, respectively in CIA mice, effectively restoring the elevated abundance of these CHDP to baseline levels (**Figure 4**, **Supplementary Figure 4**, **Table 1**).

Administration Synthetic Peptide IDR-1002 Does Not Alters the Abundance of Endogenous CRAMP and Calprotectin

We have previously shown that administration (s.c) of the bovine cathelicidin-derived synthetic peptide IDR-1002 alleviates airway inflammation and improves AHR in HDM-challenged mice (25). In contrast, administration of IDR-1002 did not prevent disease progression, and did not suppress anti-collagen antibodies in the CIA mice (**Supplementary Figure 5**). Therefore, we examined effect of administration (s.c.) of IDR-1002 on CRAMP and calprotectin abundance in lung tissues obtained from HDM-challenged mice. Administration of IDR-1002 peptide did not prevent the decrease of CRAMP, S100A8, or S100A9 in the lung tissues of HDM-challenged mice (**Figure 5**, **Supplementary Figure 4**, **Table 1**).

Correlation of CRAMP and Calprotectin Abundance With Leukocyte Accumulation

In this study, we have shown that abundance of endogenous CRAMP and calprotectin subunits are altered in the joints of CIA mice and in the lungs of HDM-challenged mice (**Figures 1**, **2**, respectively). Administration of immunomodulatory peptides IG-19 and IDR-1002, which controls inflammation in CIA mice and HDM-challenged mice, respectively (24, 25), decreased the enhanced levels of CHDP back to baseline in the CIA mice (**Figure 4**), but did not alter CHDP levels in the HDM-challenged mice (**Figure 5**). To determine whether change in the abundance of CRAMP and calprotectin is associated with inflammatory status within the local tissues, we performed correlation analyses between the abundance of CRAMP, S100A8, and S100A9 with histology score in the CIA model, and with total leukocyte count in BALF in the HDM model. Abundance of CRAMP, S100A8, and S100A9 in the joint tissues showed a significant linear correlation with histology scores in the CIA model (**Figure 6A**). In contrast, there was no significant correlation between the levels of CRAMP and calprotectin with total leukocytes in BALF in the HDM-challenged murine model (**Figure 6B**).

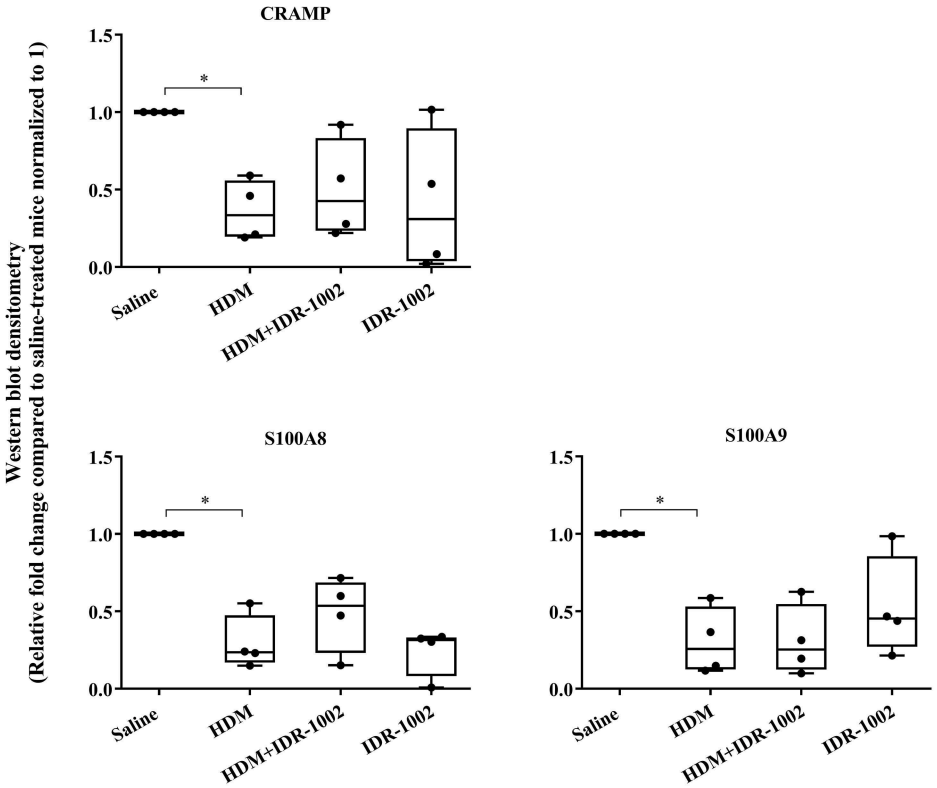

FIGURE 5 | Administration of peptide IDR-1002 does not alter CRAMP and Calprotectin in lung tissues of HDM-challenged mice. Flash frozen tissues obtained from lungs of saline treated mice, HDM-challenged mice, HDM-challenged mice administered with peptide IDR-1002 or peptide alone ($n = 4$ mice per group), were homogenized and protein concentration was determined using miroBCA. The samples (20 μg protein each) were resolved on NuPage 4–12% Bis-Tris protein gels and transferred onto nitrocellulose membranes. The membranes were probed with antibodies for mouse CRAMP, S100A8, S100A9 and β-actin (loading control). Densitometry for band intensity was determined using AmershamTM Imager 680 analysis software version 2.0. The relative band intensity was determined by normalizing to the β-actin band intensity for each sample. The represented values are the relative fold change compared to saline-treated mice normalized to 1. GraphPad Prism 7.05 software was used for statistical analyses. Kruskal–Wallis One-way analysis of variance (ANOVA) followed by Dunn's *post-hoc* test was used to determine the significance. A p-value of < 0.05 was considered to be statistically significant (*$p < 0.05$).

DISCUSSION

In this study, we demonstrated that the levels of mouse cathelicidin (CRAMP) and calprotectin (S100A8 and S100A9) were significantly increased in the joint tissues of CIA mice, a model that is used for preclinical studies of inflammatory arthritis (24, 26, 35). In contrast, CRAMP, S100A8, and S100A9 were significantly decreased in the lung tissues of allergen HDM-challenged mice, a model of airway inflammation that is used for preclinical studies of asthma (25, 27, 36). We further showed that administration of an immunomodulatory LL-37-derived synthetic peptide IG-19, which prevents disease progression and controls inflammation in CIA mice (24), significantly suppressed the elevated levels of CRAMP and calprotectin, restoring these to baseline levels in the joints of the CIA mice. However, administration of a bovine cathelicidin-derived synthetic peptide IDR-1002, which is known to alleviate HDM-induced airway inflammation and AHR (17), did not prevent the decrease of the levels of CRAMP or calprotectin from baseline in the lungs of HDM-challenged mice. Overall, the results in this study indicate that endogenous levels of CHDP cathelicidin and calprotectin are altered in local tissues in chronic inflammatory disease. However, whether these levels are enhanced or reduced depends on the disease type. Our results also suggest that changes in the abundance of endogenous cathelicidin and calprotectin may be directly related to the local inflammatory status in inflammatory arthritis, but not in airway inflammation.

Functions of cathelicidins and calprotectin in immunity and inflammation are well-documented (1–3, 37–41). For example, cathelicidins human LL-37 and mouse CRAMP are multifunctional immunomodulatory peptides that can act both as effector and regulator of inflammation (1, 3, 4, 42–44). These peptides mediate pro-inflammatory responses such as chemotaxis of immune cells, production of chemokines, and polarization and maturation of dendritic cells and T-lymphocytes (1, 3, 42). Cathelicidins can also suppress the inflammatory process by intervening in endotoxin or pro-inflammatory cytokine-induced signaling cascade, and by mediating anti-inflammatory responses such as activation of the dual phosphatase MKP-1 and production of anti-inflammatory

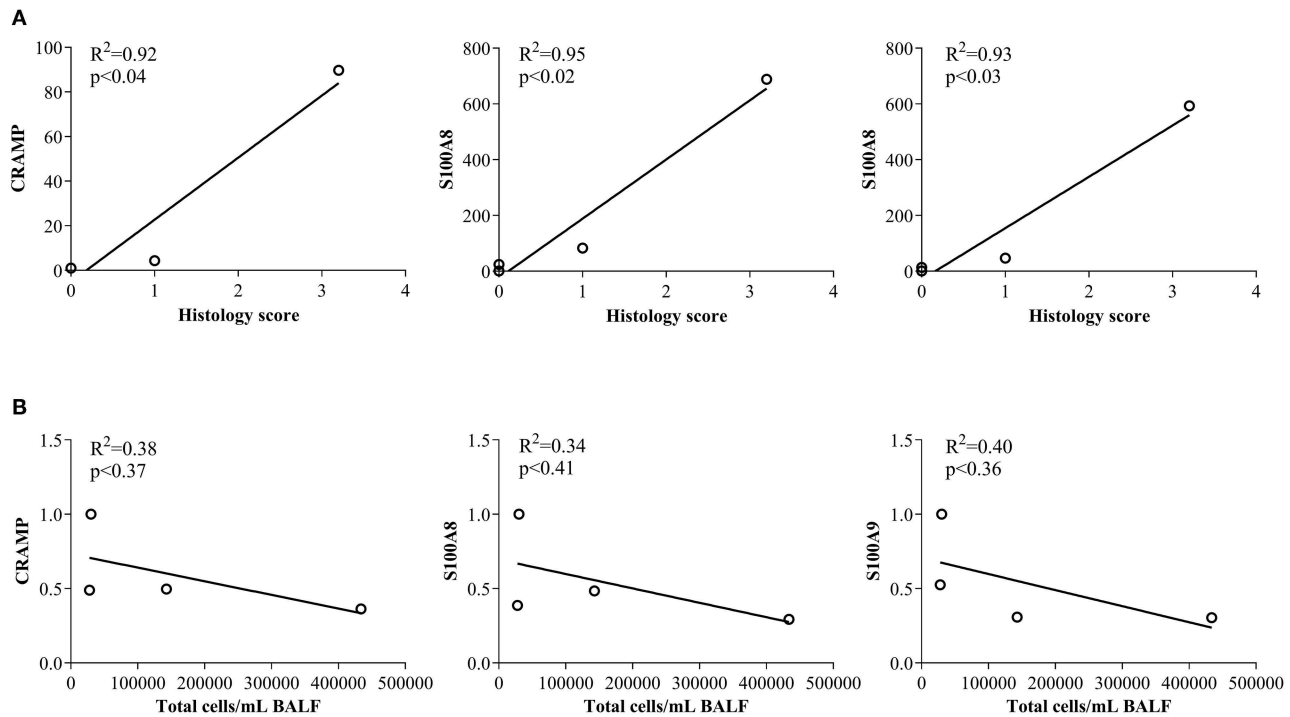

FIGURE 6 | Correlation analyses of abundance of CRAMP and Calprotectin with cellular accumulation in local tissues. **(A)** Flash frozen tissues obtained from joints of saline treated ($n = 4$), CIA ($n = 4$), CIA mice administered with either peptide IG-19 ($n = 5$) or peptide alone ($n = 5$ each), were homogenized and used for western blots to examine the abundance of CRAMP, S100A8 and S100A9, using β-actin (loading control). Pearson's correlation analysis was performed to determine the correlation between the abundance of each peptide in the joints with histology score. **(B)** Flash frozen tissues obtained from lungs of saline treated mice, HDM-challenged mice, HDM-challenged mice administered with peptide IDR-1002 or peptide alone ($n = 4$ mice per group), were homogenized and used in western blots to examine the abundance of CRAMP, S100A8, S100A9, using β-actin (loading control). Pearson's correlation analysis was performed to determine the correlation between the abundance of each peptide in the lung tissues with total leukocyte count in the BALF. A p-value of < 0.05 was considered to be statistically significant.

cytokines IL-10 and IL-1RA (23, 43–47). Similarly, calprotectin is a calcium-binding protein, a heterodimer of subunits S100A8 and S100A9, that exhibits chemokine- and cytokine-like activity, initiate pro-inflammatory responses such as chemotaxis (37, 39, 41, 48), and also exhibit anti-inflammatory functions by acting as oxidant scavengers (48, 49). The wide repertoire of pro- and anti-inflammatory functions of cathelicidins and calprotectin has propelled interest in examining their role in chronic inflammatory diseases.

Several studies have noted that circulatory levels of LL-37 and calprotectin are altered in variety of chronic inflammatory diseases such as RA, COPD, asthma, cystic fibrosis, IBD, systemic lupus erythematosus and psoriasis (4, 37, 39, 41, 50, 51). We show in this study that cathelicidin and calprotectin are significantly enhanced in the joints in the CIA mice. Elevated levels of both LL-37 and calprotectin have been demonstrated in the serum and synovium of RA patients (6, 11, 52–54). Previous studies in pristane-induced models of arthritis have shown increase of CRAMP expressing cells in mouse (55), and enhanced levels of rat cathelicidin rCRAMP in the joints, blood and secondary lymphoid organs (11). Expressions of cathelicicidin and calprotectin subunit encoding genes were also shown to be elevated in CIA mouse model without low dose LPS boost (56). Taken together, these studies indicate that the enhanced abundance of calprotectin and cathelicidin in the joints reported here is not due to the low dose LPS injection in the CIA model, rather related to the inflammatory phenotype in the joints. Consistent with this, we showed that administration of an immunomodulatory synthetic peptide that reduces clinical scores and suppresses inflammation in the CIA mice, also decreased the elevated levels of cathelicidin and calprotectin restoring these to baseline levels in the joints of CIA mice. Moreover, there was a significant correlation between the abundance of cathelicidin and calprotectin with that of histology scores in the CIA mouse model. Therefore, the increase in the endogenous cathelicidin and calprotectin abundance in the joints is likely directly associated with the enhanced inflammatory phenotype in the joints in arthritis. However, how the enhanced CHDP levels relate to arthritis disease activity remains unclear. A study by Vogl et al. used optical imaging to demonstrate a significant correlation between increase in calprotectin subunit S100A9 and disease activity in the joints of CIA mice (57). Various studies have suggested a critical role of S100A8 and S100A9 in synovial activation and cartilage degradation during inflammatory arthritis (58–61). These evidences suggest that calprotectin may be involved in the activation and tissue

degradation in arthritis. However, despite increase in cathelicidin being correlated with pathological changes in arthritis (11, 52, 53), cathelicidin-deficiency does not seem to affect the disease process in inflammatory arthritis (55). Thus, there is a lack of conclusive evidence for pathogenic role of cathelicidins in inflammatory arthritis. Nevertheless, results in our study corroborate previous findings that abundance of cathelicidin and calprotectin (S100A8 and S100A9) are significantly increased in the joint tissues, and that this is associated with increase in cellular influx indicative of increased inflammatory mediators in local tissues during the disease process in inflammatory arthritis. Contrary to this paradigm, we demonstrate in this study that endogenous levels of cathelicidin and calprotectin are decreased in the lung tissues of allergen HDM-challenged mice, which is a model of airway inflammation.

The HDM-challenged model results in increase in airway inflammation and AHR, and enhanced levels of various inflammatory proteins in the lungs (25, 27). Despite increase in airway inflammation in response to HDM challenge (27), here we show that CRAMP, S100A8, and S100A9 are significantly decreased in the lungs. We also showed that administration of the peptide IDR-1002, which has anti-inflammatory effects in the HDM-challenged mice, did not alter the abundance of the endogenous CHDP. Furthermore, there was no correlation between the levels of either cathelicidin or calprotectin with leukocyte accumulation in the BALF in the HDM-challenged mouse model. These results suggest there is no direct correlation with increase in inflammation and the change in endogenous CHDP cathelicidin and calprotectin levels in the lungs of HDM-challenged mice. Note that changes in cathelicidin and calprotectin levels reported from studies in diseases characterized by airway inflammation show considerable variability. A study showed an increase in LL-37 in BALF following segmental allergen challenge in adult allergic patients (62). In animal models of allergen challenge, mouse cathelicidin CRAMP was shown to be increased in the lungs in ovalbumin-challenged mice (63). In contrast, CRAMP was notably absent in the BALF of ovalbumin-sensitized mice infected with *Pseudomas aeruginosa,* despite significant increase in inflammatory cytokines (64). Such variability in cathelicidin levels is also observed in other diseases characterized with airway inflammation such as COPD. Plasma level of cathelicidin LL-37 is reduced in severe COPD patients (65, 66), but shown to be increased in sputum and blood in stable and in acute exacerbations of COPD patients (5, 65). A cross-sectional study by Golec et al. showed that concentration of LL-37 varied in BALF depending on the stage of the disease in COPD, significantly higher in early stages and lower in advanced disease stage, compared to healthy individuals (67). Similarly, studies examining calprotectin in airway inflammation show considerable variability. S100A8 gene expression is increased in blood samples obtained from children with asthma (68), and S100A9 is increased in the sputum of patients with asthma and COPD (69–71). These studies have suggested that the role of calprotectin is to amplify inflammation in respiratory disease. In contrast, other studies have suggested a protective role of calprotectin in airway inflammation, using exogenous administration of S100A8 and S100A9 in animal models of allergen-induced airway inflammation (72, 73). Therefore, how endogenous cathelicidin and calprotectin levels are altered, and their role in diseases characterized by airway inflammation remains ambiguous. The variability reported across studies may be due to the heterogeneity of processes related to immune dysregulation in airway inflammatory diseases. Results reported in this study demonstrating that endogenous levels of cathelicidin and calprotectin are differently altered in airway inflammation compared to inflammatory arthritis, may also be due to the heterogeneity in immune dysregulation within local tissues in these different diseases. Our results suggest that CHDP may be disparately regulated in airway inflammation compared to inflammatory arthritis. Although the immunopathology of airway inflammation (such as in asthma) and arthritis have been shown to be interconnected (13, 14), our results indicate that cathelicidin and calprotectin expression and related functions may not be one of the overlapping molecular mechanisms of immune dysregulation in these two disease processes. The mechanisms related to the differential regulation of CHDP in airway inflammation compared to arthritis warrants further investigation.

In summary, in this study we demonstrate that abundance of mouse cathelicidin (CRAMP) and calprotectin (S100A8 and S100A9) are inversely regulated in CIA and allergen HDM-challenged mice, being elevated in the joints but decreased in the lungs, respectively. We show that a synthetic immunomodulatory peptide that controls inflammation in CIA mice can also restore the elevated levels of cathelicidin and calprotectin to baseline in the joints. We also demonstrate that changes in these CHDP show a linear correlation with leukocyte accumulation in the joints in the CIA murine model. These results indicate that the enhancement of cathelicidin and calprotectin levels may be directly related to the inflammatory phenotype in the joints in arthritis. In contrast, we show that administration of an immunomodulatory peptide that controls airway inflammation cannot prevent the decrease in cathelicidin and calprotectin level in the lungs, and that the changes in these endogenous CHDP do not correlate with leukocyte influx in the lungs, in the HDM-challenged model. The results of this study indicate that the expression and/or regulation of CHDP within the local tissues in airway inflammation (e.g. in asthma) and arthritis are not similar, although the immunopathology of these diseases are known to be interconnected. Thus, the CHDP-mediated functions may not be among the common mechanisms of immune dysregulation in these two interconnected disease processes. Overall, the results of this study suggest that cathelicidin and calprotectin are disparately regulated in the local tissues in airway inflammation compared to inflammatory arthritis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by The University of Manitoba Animal Research Ethics Board.

AUTHOR CONTRIBUTIONS

MH, HP, and NM conceived the study and designed the experiments. MH, HP, DM, and LC performed the experiments and analyzed the data. NM directly supervised the study and obtained funding for reagents, materials, and analysis tools. MH wrote the manuscript. NM and AH extensively edited the manuscript. All authors reviewed the manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01932/full#supplementary-material

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Toward the Discovery of Host-Defense Peptides in Plants

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Defense peptides protect multicellular eukaryotes from infections. In biomedical sciences, a dominant conceptual framework refers to defense peptides as host-defense peptides (HDPs), which are bifunctional peptides with both direct antimicrobial and immunomodulatory activities. No HDP has been reported in plants so far, and the very concept of HDP has not been captured yet by the plant science community. Plant science thus lacks the conceptual framework that would coordinate research efforts aimed at discovering plant HDPs. In this perspective article, I used bibliometric and literature survey approaches to raise awareness about the HDP concept among plant scientists, and to encourage research efforts aimed at discovering plant HDPs. Such discovery would enrich our comprehension of the function and evolution of the plant immune system, and provide us with novel molecular tools to develop innovative strategies to control crop diseases.

Keywords: antimicrobial peptides, peptide elicitors, concept transfer, plant immunity, defensins, thaumatin-like proteins

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INTRODUCTION

Defense peptides protect multicellular eukaryotes against pathogens such as microbes, and represent key tools to develop innovative disease control strategies in medicine and agriculture (1, 2). In biomedical sciences, defense peptides are often bifunctional, simultaneously able to directly kill microbes and to modulate host immunity. In 2006, Hancock and Sahl proposed to refer to these peptides as Host-Defense Peptides (HDPs) (**Box 1**) (3). The HDP concept has been rapidly captured by the biomedical research community, and has provided researchers with a robust conceptual framework to further discover and characterize HDPs (**Figure 1A**, left hand side) (4–6). In the plant science literature, no HDP (i.e., a peptide simultaneously able to kill pathogens and modulate host immune responses) has been convincingly reported so far (7, 8). The very concept of HDP is absent from the literature, and does not seem to have been captured by the research community.

Plant science literature currently categorizes defense peptides into two groups: antimicrobial peptides (AMPs) and peptide elicitors (PEs) (**Figure 1A**, right hand side). Plant AMPs are secreted proteins that interact with microbes and directly kill them (9, 10). Noteworthy, plant AMPs can be promiscuous and exhibit additional biological activities (8, 11–13), although the activities reported so far are unrelated to the modulation of immune responses; so that no plant AMP with immunomodulatory activity (i.e., an HDP) has been convincingly described to date. Plant PEs are small peptides that derive from larger precursor proteins and that function as ligands of cell-surface immune receptors to modulate plant immunity (14, 15). Thus, the current conceptual framework in plant science does not consider defense peptides as being able to simultaneously kill microbes and modulate plant immunity, therefore hindering efforts that could lead to the discovery of plant HDPs.

BOX 1 | Host-Defense Peptides - more than promiscuous AMPs.

In this study, the term "Host-Defense Peptides" (HDPs) refers to defense peptides that exhibit two well-defined activities within the host immune system: an antimicrobial (or more broadly a biocidal) activity (i.e., direct killing of an invading organism) and an immunomodulatory activity (i.e., modulation of immune responses), as originally proposed (3). According to that definition, HDPs perform two functions that directly pertain to host immunity; and which have probably been selected throughout evolution. Understanding such HDPs is therefore key to better comprehend host immunity. Noteworthy, this article does not consider promiscuous plant AMPs (i.e., AMPs that display additional activities unrelated to the modulation of host immune responses) as HDPs.

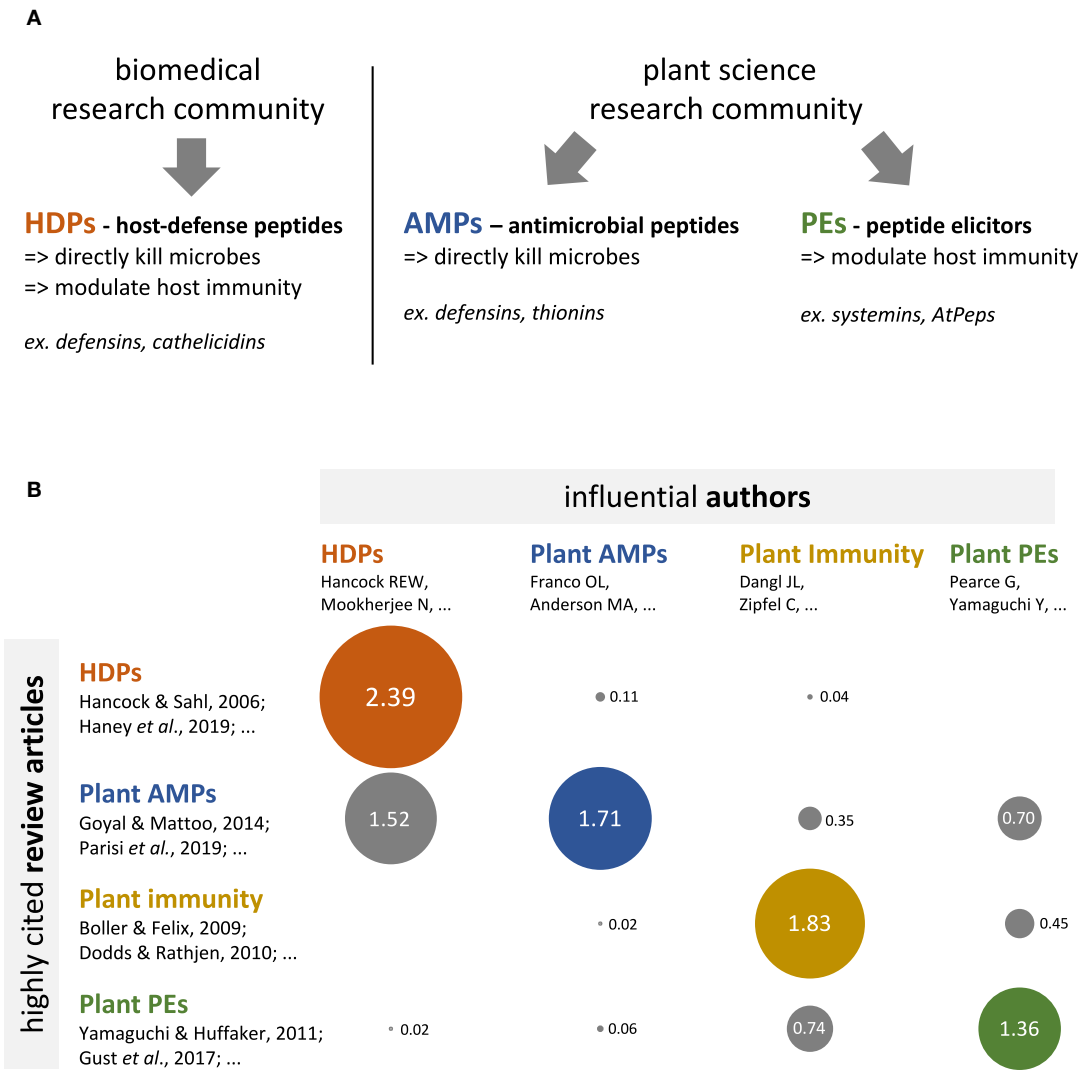

FIGURE 1 | Biomedical and plant science fields have different conceptual frameworks regarding defense peptides, and limitedly cite each other's. **(A)** Conceptual frameworks pertaining to defense peptides in the biomedical and plant science research communities. The biomedical literature (left hand side panel) conceptualizes defense peptides as multifunctional molecules, with both antimicrobial and immunomodulatory activities, which are referred to as Host-Defense Peptides (HDPs). The plant science literature (right hand side panel) conceptualizes defense peptides as specialized molecules, which display either antimicrobial activities (antimicrobial peptides or AMPs) or immunomodulatory activities (peptide elicitors or PEs). **(B)** Bubble table chart depicting the results of a bibliometric analysis regarding the cross-referencing between HDP (red), plant AMP (blue), plant immunity (yellow), and plant PE (green) research communities. Numbers indicate the citation score for each field intersection (e.g., HDP vs. plant AMPs); that is expression of the average number of citations per author per article for a given field intersection (see **Supplementary Methods** for details). The diameter of the bubbles directly correlates with the indicated values. The analysis reveals that research communities are strongly compartmentalized, though some articles and 'transversal' authors evolve at the interface of the various research communities (see **Supplementary Table 1** for details).

Conceptual frameworks guide research investigations and structure research communities. Indeed, I surmise that powerful concepts or models, often shared via influential review articles, shape the way researchers think and organize themselves. For example, the "zig-zag model" in plant immunity, proposed by Jones and Dangl in 2006, has cemented a robust research community and provided it with a strong conceptual framework to coordinate efforts and further investigate plant immunity (16). I claim here that the lack of awareness of the HDP concept within the plant science research community hinders the discovery of plant HDPs, as researchers lack the conceptual framework that would coordinate and encourage them to look for HDPs. Considering the innovative potential of HDPs, this ultimately deprives modern agriculture from the innovations it requires to be sustainable and efficient.

This perspective article has two goals: raise awareness of the HDP concept among plant scientists and encourage the search for HDPs in plants. To reach the first goal, I have used a bibliometric approach to identify articles and authors that may bridge biomedical and plant science communities and thus assist concept transfer. To reach the second goal, I have performed a literature survey to identify and list promising HDP candidates (i.e., AMPs with suspected immunomodulatory activities or PEs with suspected antimicrobial activities).

A BIBLIOMETRIC ANALYSIS REVEALS THE ABSENCE OF THE HDP CONCEPT IN PLANT SCIENCE, AND IDENTIFIES OPPORTUNITIES TO TRANSFER THE CONCEPT FROM THE BIOMEDICAL FIELD

To evaluate the status of the HDP concept in plant science, I performed a bibliometric analysis. I have first identified a set of 30 influential (i.e., highly cited) review articles published between 2009 and 2019, which focus on plant immunity, plant AMPs, or plant PEs (10 article for each category) (**Supplementary Table 1**). These 30 review articles have been collectively cited 6 813 times, and have probably shaped the dominant conceptual frameworks in their sub-fields. Secondly, I screened the main text of these articles for the term "HDP" or "host-defense peptides" using the Zotero key word search tool. I found zero occurrence of these terms. I further scrutinized the articles, and found no explicit reference to the HDP concept within them, although one article implicitly referred to the HDP concept (17). I conclude from that analysis that the most influential literature in plant science and plant immunity does not refer to the HDP concept, suggesting that plant science research community as a whole has not integrated this concept.

To identify influential articles and authors at the interface of the biomedical and the plant science communities (i.e., contact points) that could assist the HDP concept transfer, I have analyzed citation patterns between the plant science and the HDP literature. To this end, I have first identified a set of 10 influential (i.e., highly cited) review articles published between 2009 and 2019 that pertain to the HDP concept (**Supplementary Table 1**). These articles have been collectively cited 4 251 times, and are currently the most visible source of information about HDP in the academic literature. I have then identified the corresponding authors of all the review articles from **Supplementary Table 1** (40 articles in total) and quantified how often they were cited in each of the review article, using a citation score that disregarded self-citations (**Figure 1B**; see **Supplementary Methods** for details). The analysis first shows strong 'intra-community' citation, as the articles from one particular sub-field (HDP for instance) cite predominantly the authors from the same community (average citation score of 1.83). In contrast, "cross-community" citation (i.e., citation between different research communities) is lower (average citation score of 0.33), with even null values at the intersection of HDP articles *vs.* plant PEs authors and plant immunity articles vs. HDP authors; this altogether suggests no—or seldom—cross-community information flow. Finally, and most interestingly, the analysis shows that the plant AMP article set cites authors from the three other communities (average citation score of 0.86). A further detailed investigation of the citation pattern revealed a handful of specific articles and authors that cite—and are cited—beyond the communities boundaries (**Supplementary Table 1**) (10, 17–21). Such "transversal" articles and authors are probably and simultaneously knowledgeable about the HDP concept and visible within the plant science research community; they are therefore in a good position to assist cross-community concept transfer.

CANDIDATE HDPS IN PLANTS: DEFENSINS, THAUMATIN-LIKE PROTEINS, AND OTHERS

To encourage investigations aimed at discovering HDPs in plants, I identified and listed what are in my view the most promising candidate HDPs. To do so, I screened the literature for reports of AMPs that exhibit an additional activity that could be related to modulation of the plant immune system, or for PEs (or their precursors) that might exhibit antimicrobial activities. In total, I found six such peptides; two that belong to the defensin superfamily (alfalfa MsDef1 and tomato DEF2), two that belong to—or derive from—the thaumatin-like protein (TLP) superfamily (sweet potato IbACP and european plum PdPR5-1), and two that do not belong to large conserved multigene families (poplar RISP and pepper CaAMP1) (**Table 1**).

Among the six peptides listed in **Table 1**, four (MsDef1, DEF2, CaAMP1, and RISP) were reported as AMPs that directly inhibit microbe growth (7, 22, 24, 27). In contrast, two peptides (IbACP and PdPR5-1) have been shown to function as PEs, and their direct antimicrobial activity was not tested (25, 26). However, both PdPR5-1 and the probable precursor of IbACP are members of the TLP superfamily, which is a well-characterized AMPs family in plants (29). I therefore consider likely that PdPR5-1 and IbACP precursor both display a direct antimicrobial activity.

The six above-mentioned peptides alter plant physiology in a way that suggests a potential role as immunomodulators. On the one hand, purified IbACP and RISP both trigger rapid plant cell culture alkalinization, while purified MsDef1 inhibits plant root growth (7, 23, 25). Both cell culture alkalinization and

TABLE 1 | Plant HDP candidates.

Peptide name	Peptide information	Peptide origin	Antimicrobial activity	Immunomodulation-related activity	References
MsDef1	MsDef1 (*Medicago sativa* Defensin 1) belongs to the defensin family	Alfalfa (*Medicago sativa*)	Purified MsDef1 inhibits fungal growth	Purified MsDef1 inhibits root growth (suggesting immune response activation)	(22, 23)
DEF2	DEF2 belongs to the defensin family	Tomato (*Solanum lycopersicum*)	DEF2-containing foliar extracts inhibit fungal growth	Ectopically-expressed DEF2 perturbs tomato plant development and reduces seed production (trade-off between growth and defense)	(24)
IbACP	IbACP (*Ipomea batatas* anti-cancer peptide) probably derives from the N-terminus of a PR-5/TLP precursor	Sweet potato (*Ipomea batatas*)	IbACP precursor belongs to the TLP family, whose members are well-established AMPs	Purified IbACP induces tomato cell culture alkalinisation (suggesting immune response activation)	(25)
PdPR5-1	PdPR5-1 (*Prunus domestica* Pathogenesis-related Protein 5 1) belongs to the Thaumatin-Like Protein (TLP) family	European plum (*Prunus domestica*)	PdPR5-1 belongs to the TLP family, whose members are well-established AMPs	Ectopically-expressed PdPR5-1 induces the expression of plant defense pathways (suggesting immune response activation)	(26)
CaAMP1	CaAMP1 (*Capsicum annuum* antimicrobial protein 1) is a 186-amino-acid antimicrobial protein from pepper	Pepper (*Capsicum annuum*)	Purified CaAMP1 inhibits the growth of bacteria, fungi, and oomycetes	Ectopically-expressed CaAMP1 modulates the expression of plant defense-related proteins (suggesting immune response modulation)	(27, 28)
RISP	RISP (Rust-Induced Secreted Protein) is a 82-amino-acid peptide that belongs to a Salicaceae-specific family of Cysteine-rich, cationic, secreted peptides	Poplar (*Populus trichocarpa*)	Purified RISP inhibits fungal growth	Purified RISP triggers poplar cell culture alkalinisation (suggestive of immune response activation)	(7)

root growth inhibition are hallmarks of—and are commonly used as a readout for—the activation of plant immunity (30, 31). This suggests that these three peptides can directly control immunity, potentially working as a ligand to an immune receptor, as hypothesized for RISP (32). On the other hand, ectopically-expressed DEF2, PdPR5-1, and CaAMP1 affect plant physiology. PdPR5-1 and CaAMP1 modulate the expression of defense-related genes, or genes that participate in defense pathways, suggesting that the expression of the peptides in the plant stimulates immune responses (26, 28). DEF2 alters plant growth, and notably reduces seed production (24). Considering the trade-off that exists between growth and defense in plants (33), this alteration could result from an alteration of the growth/defense balance.

Altogether, this set of observations suggests that the six above-mentioned peptides represent priority candidates in the search for plant HDPs. It also indicates that HDP candidates can be detected in well-characterized and conserved plant AMP gene families (such as TLPs or defensins), which would facilitate further functional investigations.

DISCUSSION

In this perspective article, I have combined a bibliometric analysis with a literature survey to evaluate the status of the HDP concept in plant science and to encourage research efforts aimed at discovering plant HDPs. The bibliometric analyses showed that the HDP concept has not been captured by the plant science community, but also revealed interfacial research communities that could assist concept transfer. The literature survey identified a list of six defense peptides that I propose to consider as priority HDP candidates.

To bolster the effort aimed at discovering HDPs in plants, I see two obvious perspectives. Firstly, we could test known AMPs for additional immunomodulatory activities. In most cases, this task would take advantage of available purified peptides (usually used to demonstrate the antimicrobial activity) that could be directly used in assays that rely on exogenous peptide application (e.g., cell culture alkalinization or root-growth inhibition assays). Secondly, we could test known PEs, as well as their precursor proteins, for direct antimicrobial activities. Priority could be given to PE precursors that are predicted to be secreted out of the cells (e.g., Hydroxyproline-rich systemins; HypSys) and/or that are organized into well-characterized multigene families with known immunomodulatory roles (e.g., Rapid Alkalinisation Factors; RALF). Completing such tasks may rapidly help us determine whether defense peptide with HDP-like properties evolved in plants. An alternative to these two approaches consist in searching for synthetic peptides with HDP-like properties (34–36). Although such an approach would not inform much about the evolution and the function of the plant immune system, it would still provide us with valuable tools to develop phytosanitary products, such as peptide-based biopesticides for instance, to protect crops from diseases.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

BP is the only author of the manuscript, he prepared the manuscript himself.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01825/full#supplementary-material

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REVIEW
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Cathelicidin Host Defense Peptides and Inflammatory Signaling: Striking a Balance

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Host-defense peptides (HDPs) are vital components of innate immunity in all vertebrates. While their antibacterial activity toward bacterial cells was the original focus for research, their ability to modulate immune and inflammatory processes has emerged as one of their major functions in the host and as a promising approach from which to develop novel therapeutics targeting inflammation and innate immunity. In this review, with particular emphasis on the cathelicidin family of peptides, the roles of natural HDPs are examined in managing immune activation, cellular recruitment, cytokine responses, and inflammation in response to infection, as well as their contribution(s) to various inflammatory disorders and autoimmune diseases. Furthermore, we discuss current efforts to develop synthetic HDPs as therapeutics aimed at restoring balance to immune responses that are dysregulated and contribute to disease pathologies.

Keywords: host-defense peptide, innate immunity, homeostasis, toll-like receptor, self-antigen

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INTRODUCTION

Host defense peptides (HDPs) have evolved across all species of animals and are recognized as vital components of innate immune processes (Haney et al., 2019a; Mookherjee et al., 2020). HDPs are short, gene-encoded polypeptides (10–50 residues in length) that are broadly characterized by a net positive charge and a high proportion of hydrophobic amino acids (Fjell et al., 2012). They can exhibit potent bactericidal activity in buffer, which is why they are often referred to as antimicrobial peptides (AMPs), although this activity is often abrogated by host physiological conditions including (especially divalent) cation concentrations and the presence of polyanions such as glycosaminoglycans (Bowdish et al., 2005a; Hancock et al., 2016). Conversely, under host-like physiological conditions and in animal models, many natural AMPs are able to modulate the host innate immune response. Indeed, the immunomodulatory activity of these molecules might be more representative of their natural functions and potential for development as therapeutic agents. Numerous studies have focused on unraveling the mechanisms that underlie the various immunomodulatory functions of HDPs in diverse scenarios (Davidson et al., 2004; Chen Y. et al., 2017; Chen S. et al., 2018). While no general mechanism has been described for all HDPs, several features of the immunomodulatory response to HDPs have been described for a variety of cell types

and animal models, including cellular recruitment, anti-inflammatory activity, and wound healing, among others (Hancock et al., 2016).

Current knowledge about the activities of HDPs has been largely derived from the study of naturally-occurring peptides from vertebrates (Van Dijk et al., 2018). Some HDPs are expressed constitutively by immune cells, whereas the local concentration of others can be upregulated in response to a particular stimuli and/or secreted into the local environment or released from phagocytes by degranulation (Elloumi and Holland, 2008). Several HDPs are also expressed by epithelial, cells of the skin, gastrointestinal, genital, and respiratory tracts as well as a variety of other cell types (Lee et al., 2016). The most abundant and best characterized HDPs in mammals are those classified as cathelicidins and defensins (Fruitwala et al., 2019). Numerous cathelicidins have been described in mammals as well as other phyla including birds, reptiles, amphibians, and fish (Uzzell et al., 2003; Xiao et al., 2006; Van Harten et al., 2018).

Here we focus on the features of cathelicidins that contribute to their immunomodulatory properties and highlight the potential for developing synthetic HDP derivatives as novel therapies for various inflammatory conditions. An overview of the structure, function, and expression of naturally-occurring cathelicidins across vertebrates is provided with a particular emphasis on their ability to maintain homeostasis by influencing immune signaling and mitigating damaging inflammatory responses (Mookherjee et al., 2006). In addition, we discuss disorders that are made more severe by cathelicidins acting as self-antigens, and describe various diseases associated with dysregulated expression of cathelicidins. Several examples of synthetic peptides that have been designed to harness the beneficial effects of natural peptides are highlighted, particularly for their capacity to modulate innate immune processes (Hilchie et al., 2013). In addition, we examine an emerging role for cathelicidins and synthetic HDP derivatives in the management of dysregulated immunity present in sepsis (Martin et al., 2015). Finally, we highlight several ongoing clinical trials aimed at exploiting the immunomodulatory functions of HDPs and discuss emerging peptide formulation strategies and studies in animal models that bridge the gap between pre-clinical and clinical development of novel peptide therapies.

EVOLUTIONARY PERSPECTIVES OF CATHELICIDINS ACROSS VERTEBRATE SPECIES

The cathelicidin family of HDPs exhibits a broad diversity in structure and function across all vertebrates. The number of genes encoding cathelicidin analogs can vary by species. For instance, only a single cathelicidin gene is encoded in humans, mice, and dogs, while 2–11 cathelicidin-coding genes have been identified in certain species of fish, amphibians, reptiles, birds, and most other mammals (Ramanathan et al., 2002; Masso-Silva and Diamond, 2014; Kim et al., 2017; Qi et al., 2019). The organization of the coding sequence seems to be well conserved among vertebrates and is comprised of four exons that collectively encode the precursor peptide consisting of a signal peptide sequence, the cathelin pro-domain, and the mature cathelicidin sequence (Ramanathan et al., 2002; Dalla Valle et al., 2013). Although there is high amino acid sequence identity for the cathelin domain between species, the mature form of the cathelicidin peptide is remarkably diverse in length, composition, net charge, and structure (**Figure 1**).

Mature cathelicidin peptides can be loosely grouped into four structural classes: α-helical or linear peptides that can adopt helical conformations under physiological conditions or in the presence of biological membranes; linear peptides that are disproportionately high in particular amino acids such as glycine, serine, proline or tryptophan; and two classes stabilized by disulfide bridges, namely β-structured and cyclic peptides (Zanetti, 2005). The α-helical peptides are the most widely distributed and present in all vertebrate groups, but other structural classes are observed across species (Masso-Silva and Diamond, 2014; Chen Y. et al., 2017 see **Supplementary Material**).

It has been suggested that HDPs found in multicellular organisms arose as a protective mechanism against microbes, particularly against bacteria (Boman, 2003; Lazzaro et al., 2020). In such a scenario, it is assumed that host-microbial interactions and direct antimicrobial activity drove the evolution of HDP sequences to optimize them collectively for anti-bacterial potency. However, as mentioned above, the antimicrobial potency of most HDPs remains rather modest in host-like environments. A recent study of mammalian homologs of LL-37 proposed that the driving force behind the evolution of cathelicidins might be their interaction with host receptors (Zhu and Gao, 2017), which is consistent with the concept that immune response elements are one of the most highly evolving groups of proteins across mammalian species (Hahn et al., 2007; Kosiol et al., 2008).

An earlier study suggested that the disordered C-terminus of LL-37 interacts with the N-formyl peptide receptor-like (FPR) family of proteins (Singh et al., 2014), as part of the process to mediate chemotaxis. Sequence analysis of the human FPR2 receptor indicated high variability in the ligand-binding extracellular loop domain, while the C-terminus of mammalian LL-37 homologs was disordered; thus statistical analysis revealed a possible co-evolution of this peptide as a cognate binding partner for FPR2 was proposed (Zhu and Gao, 2017). Furthermore, the elimination of the disordered N- and C-terminal regions in the rabbit LL-37-homolog (CAP18-FV) or their replacement with disordered regions from the dolphin (ttLL-37) or human homologs had no impact on the anti-bacterial activity. Unfortunately, since the immunomodulatory properties of the resulting species-hybrid mutants of LL-37 were not evaluated, the direct influence of this proposed interaction was not confirmed. Regardless, several other host receptors with immune functions have been proposed to interact with cathelicidins, including purinergic receptors P2Y11 and P2 × 7,

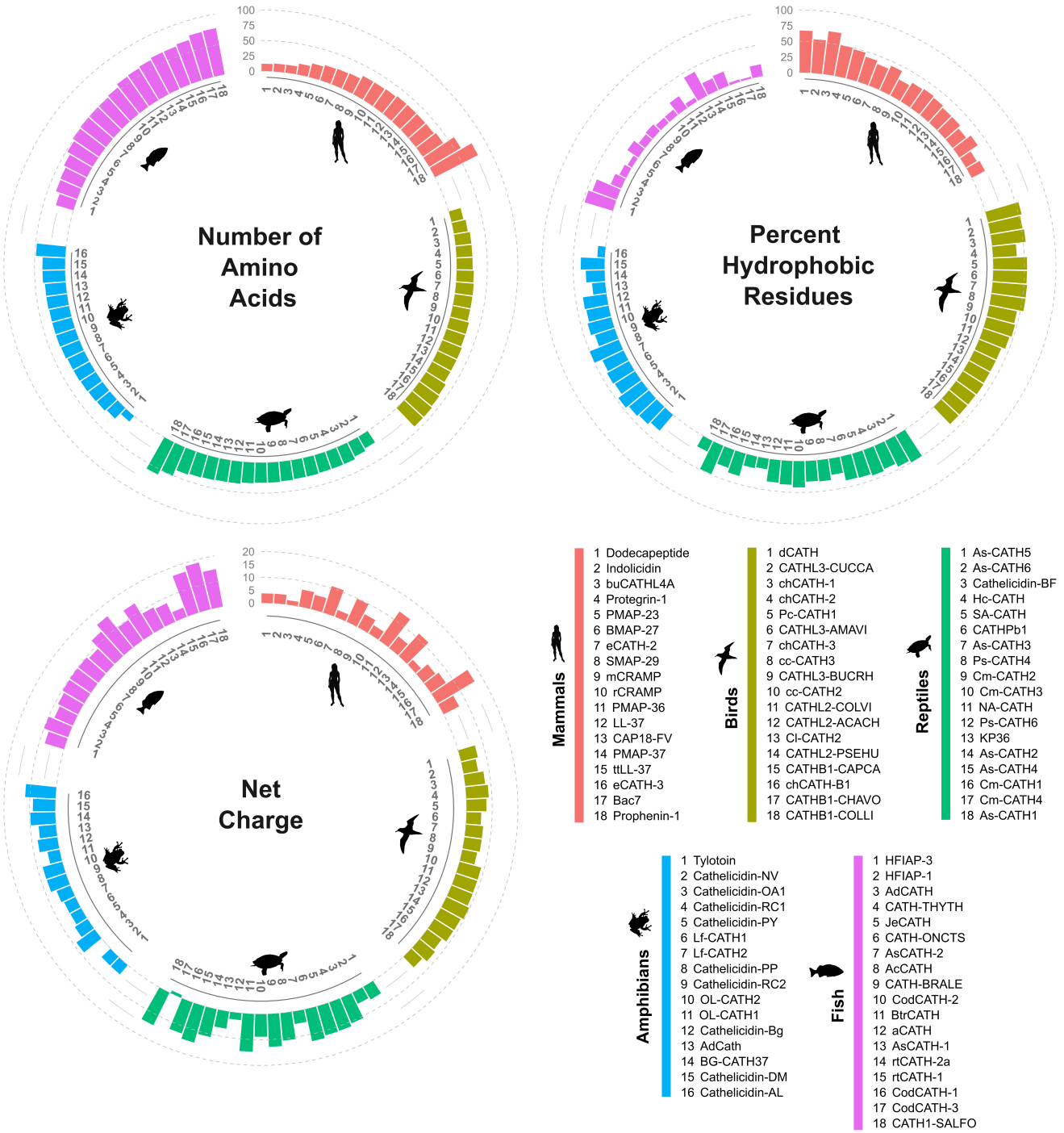

FIGURE 1 | Diversity of cathelicidin peptides among vertebrate groups. Circular bar plots show the distribution of length, charge and proportion of hydrophobic amino acids among representative vertebrate cathelicidin peptides (see **Supplementary Table S1**). The order of sequences in each group is sorted by peptide length. Physico-chemical properties were computed using the *Peptides* package v2.4.2 (Osorio et al., 2015) in R v4.0.0 (R Core Team, 2020). Net charge was predicted using the Bjellqvist's pK scale implemented in the *Peptides* package. Animal silhouettes were created by: NASA (mammals; human), Juan Carlos Jerí (birds; shearwater), uncredited (reptiles; turtle), Will Booker (amphibians; tree frog) and Felix Vaux (fish), and downloaded from http://phylopic.org/.

the CXC chemokine receptor type 2, Mas-related gene X2 (MrgX2), GAPDH, and others (Verjans et al., 2016). This provides strong evidence that receptor binding directly impacts the biological functions of cathelicidins. Curiously, a similar evolutionary analysis did not identify highly variable residues in avian cathelicidins, suggesting that this putative co-evolutionary

relationship might be specific to mammalian LL-37 homologs (Cheng et al., 2015).

REGULATION OF CATHELICIDIN EXPRESSION

Since the repertoire and cell/tissue distribution of cathelicidins varies by species, we focus below on discussing the expression and activity of the human cathelicidin antimicrobial peptide (*CAMP*) gene found on chromosome 3p21 (Elloumi and Holland, 2008). The *CAMP* gene encodes the 18 kDa precursor human cationic antimicrobial protein, hCAP18, which is cleaved by proteases to generate the active peptide known as LL-37. It is expressed in a variety of tissues and cell types, including epithelial cells and many cells of the immune system (Hancock et al., 2016). Expression of hCAP18 is highest in the bone marrow in healthy individuals (Fagerberg et al., 2014), although expression can be detected in many organs and tissues. Secretory glands enhance basal expression at mucosal surfaces, with hCAP18 secreted in the semen, saliva, and sweat (Andersson et al., 2002). Most studies of the regulation of *CAMP* expression in various tissues reflect recognition of inflammatory stimuli by neutrophils and monocytes, since these cell types produce more hCAP18/LL-37 than other immune cells. In addition, neutrophils store the inactive hCAP18 precursor in specific (azurophilic) granules for rapid deployment during immune responses (Kai-Larsen and Agerberth, 2008). Recognition of inflammatory signals leads to cascading activation of immune cells and an increase in *CAMP* expression, particularly in leukocytes, as well as LL-37 secretion due to neutrophil degranulation. Increased expression of *CAMP* has been attributed to endoplasmic reticulum (ER) stress which is in part associated with NF-κB activation and concomitant downstream signaling events (Park et al., 2011), although other factors aside from ER stress might contribute to enhanced *CAMP* expression following inflammatory stimulus. Consistent with this, LL-37 production is induced by a variety of inflammatory disorders that are not associated with infection (Kahlenberg and Kaplan, 2013), and exogenous host defense metabolites, such as short chain fatty acids and butyrate, which strongly induce *CAMP*/LL37 expression (Chen and Vitetta, 2020). During secretion, proteinase 3 or kallikreins, produced by monocytes or cells at the skin surface, respectively, cleave the precursor hCAP18 protein to generate the active LL-37 peptide as well as truncated forms with varying biological activities (Murakami et al., 2004; Yamasaki et al., 2006).

Beyond the enhanced production of hCAP18 in response to inflammation and pathogen exposure, a growing body of research is addressing *CAMP* expression following exposure to physiologically-important metabolites (Coorens et al., 2017). The *CAMP* promoter is directly targeted by the cognate vitamin D receptor (VDR) found in various tissues, and thus vitamin D_3 and its metabolites can induce widespread *CAMP* expression, especially in myeloid cells (Wang et al., 2004). For example, the hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3, upregulates the expression of *CAMP* in immortalized human keratinocytes, acute myeloid leukemia, and colon cancer cell lines as well as in primary bone marrow derived macrophages (Gombart et al., 2005). Combining exogenous vitamin D_3 with cytokines, such as IL-13, that favor T_h2 polarization of $CD4^+$ T cells, further enhances this VDR-mediated *CAMP* expression (Schrumpf et al., 2012).

BIOLOGICAL ROLE OF CATHELICIDINS

LL-37 and mCRAMP

HDPs, in general, exert an incredible array of immunomodulatory functions and many of these features are shared by members of the cathelicidin family of peptides (**Figure 2**), although individual peptides tend to favor a subset of these properties (reviewed in ref 4). Of the many biological functions of HDPs, their antimicrobial functions have undoubtedly been the most widely researched in part due to the simple assays involved. While many studies have emphasized the important role of cathelicidins as antimicrobials at epithelial surfaces, particularly the skin (Travis et al., 2000), such conclusions must be qualified due to the conditions under which such activities were assessed, often in very dilute salts. For instance, phosphate buffer, in which many of these studies were undertaken, is decidedly not physiological since *in vivo* conditions involve high concentrations of divalent and monovalent cations and polyanionic sugars that can inhibit antimicrobial activity (Bowdish et al., 2005b).

Admittedly, studies in mice deficient in the cathelin-related antimicrobial peptide (mCRAMP), the murine ortholog of LL-37, have demonstrated an enhanced susceptibility to a variety of infections including necrotizing skin infections caused by Group A *Streptococcus* (Nizet et al., 2001), keratitis produced by *P. aeruginosa* (Huang et al., 2007), and meningitis induced by *Streptococcus pneumoniae* (Merres et al., 2014). However, it is possible that such studies could reflect the immunomodulatory effects of cathelicidins that enables protection against infections (Bowdish et al., 2005b; Hancock et al., 2016). In the following section, we discuss studies demonstrating that cathelicidins, such as LL-37, have a primary role in modulating the (innate) immune response which is robust, complex, and occurs under physiological conditions both *in vitro* and in animal models.

One outstanding feature of LL-37 is its ability to suppress pro-inflammatory signaling. This likely involves a complex series of both direct and indirect mechanisms (Koo and Seo, 2019). Regarding direct mechanisms, cathelicidins bind to and neutralize the bacterial toll-like receptor (TLR) ligands, such as lipopolysaccharide (LPS) or lipoteichoic acid (LTA) (Kandler et al., 2006), which would otherwise engage TLRs and trigger inflammatory processes associated with cascading activation of immune cells (Horibe et al., 2013). In addition, LL-37 can substantially attenuate LPS-mediated TNF-α production from peripheral blood mononuclear cells (PBMCs) when added either before or after the LPS stimulus, consistent with the notion of a variety of indirect mechanisms that reflect LL-37 uptake into cells and modulation of various intracellular signaling events

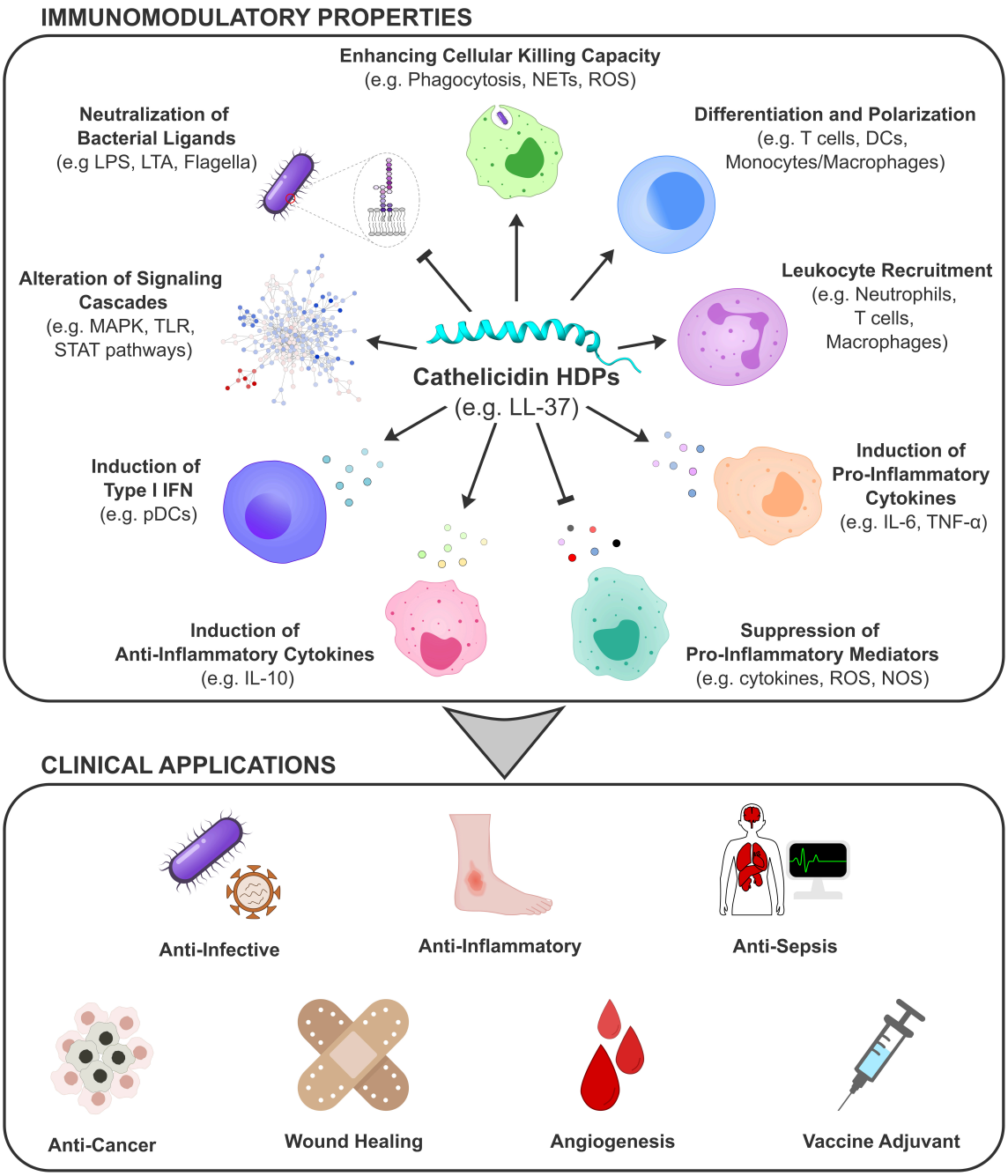

FIGURE 2 | The immunomodulatory features of cathelicidins. Immunomodulatory functions of cathelicidin HDPs include, but are not limited to: enhancing cellular killing capacity, differentiation and polarization of immune cells, leukocyte recruitment, induction or suppression of pro-inflammatory mediators, induction of anti-inflammatory cytokines, induction of type I IFN, alteration of signaling cascades and neutralization of bacterial ligands. These functions can be harnessed for anti-infective, anti-inflammatory, anti-sepsis and anti-cancer applications or contribute to wound healing, angiogenesis and improved vaccine effectiveness. DCs, dendritic cells; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; LTA, lipotechoic acid; MAPKs, mitogen-activated protein kinases; NOS, nitrous oxide species; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; TLRs, toll-like receptors; TNF, tumor necrosis factor.

(Hilchie et al., 2013). Indeed, in PBMCs challenged with TLR-2, TLR-4, or TLR-9 agonists, LL-37 generally suppresses the production of the pro-inflammatory cytokines TNF-α and IL-1β and alters expression of IL-6 and IL-8 (Mookherjee et al., 2006; Hilchie et al., 2013). Moreover, based on microarray analysis, more than 160 genes up-regulated in LPS-stimulated THP-1 monocytic cells were suppressed in LL-37 treated cells (Mookherjee et al., 2006). Thus, LL-37 neutralizes bacterial signature molecules (TLR agonists) that normally induce pro-inflammatory immune responses.

Several lines of evidence support the host-cell-directed activity of cathelicidins that is independent of bacterial ligand binding. For example, LL-37 modulates more than a dozen signaling pathways including the p38, Erk1/2, JNK MAP-kinases, NFκB, PI3K/Akt, Src family kinase, TRIF-IRF, TREM, Wnt/β-Catenin, JAK-STAT, and autophagy signaling pathways independent of LPS, LTA and/or flagellin stimulation (Scott et al., 2002; Mookherjee et al., 2009; Hancock et al., 2016). Furthermore, systems biology and biochemical studies on human $CD14^+$ monocytes treated with LL-37 showed nearly 500 genes changing expression, reflecting the involvement of many of these pathways in chemokine induction in response to LL-37 (Mookherjee et al., 2009). Activation of Wnt/β-Catenin and PI3K/Akt signaling cascades elicited by LL-37 was also demonstrated in samples from non-small cell lung carcinoma patients (Ji et al., 2019) with a positive correlation between LL-37 concentration in tissues and relevant gene expression. Similarly, in the A549 pneumocyte cell line *in vitro*, LL-37 stimulated growth through a β-Catenin-dependent but TLR-independent manner (Ji et al., 2019). Overall, such studies provide evidence that LL-37 has multiple surface and intra-cellular targets (Hilchie et al., 2013; Levast et al., 2019) that contribute to the biological functions of this HDP *in vivo*, resulting in diverse outcomes mediated by a broad array of signaling events (**Figure 2**).

A typical outcome is the induction of certain chemokines in a variety of cell types, including phagocytes and epithelial cells. Although the resting concentrations of LL-37 at mucosal surfaces can be quite low, local LL-37 concentrations at sites of infection or during acute inflammation can be much higher due to degranulation of phagocytes releasing LL-37 into the vicinity. The high local concentration promotes the well-studied ability of cathelicidins to enhance the recruitment of immune cells, especially phagocytes (monocytes, macrophages, and neutrophils), to the sites of infections in mice (Hilchie et al., 2013). For example, the release of LL-37 by human keratinocytes activate the Src family kinases and enhance TLR-5 activation upon flagellin stimulation, thereby inducing the release of chemokines (Nijnik et al., 2012). LL-37 and other HDPs can also act as chemokines to directly attract immune cells. Thus, binding of the FPR2 receptor by LL-37 enables the recruitment of neutrophils, monocytes, T cells, and mast cells (Yang et al., 2000) although, generally speaking, this occurs at higher peptide concentrations than other chemokine inducing activities, likely due to low ligand binding affinity.

However, not all of the functions of LL-37 are beneficial. For example, LL-37 promotes histamine release from mast cells, which promotes loosening of blood vessel walls to enable enhanced uptake of immune cells, but this process is also allergenic (Niyonsaba et al., 2002). Mast cell degranulation (and histamine release) is mediated by LL-37-induced activation of the MrgX2 receptor that triggers a range of signaling pathways such as PI3K/Akt, Erk1/2, and JNK (Yu et al., 2017). LL-37 also promotes microbially-induced apoptosis of epithelial cells while extending the lifetime of neutrophils (Barlow et al., 2010), which could contribute to damage associated with respiratory infections.

TLRs play a crucial role in innate immunity and govern pathogen recognition by and activation of important sentinel cells, such as macrophages, neutrophils, dendritic cells (DCs), and epithelial cells (Beutler, 2009). In addition to their ability to suppress proinflammatory signaling through TLRs, cathelicidins can also influence the expression of TLRs in a variety of cell types including mast cells, monocytes, neutrophils, renal cells, epithelia lining the colon, and other mucosal surfaces (Agier et al., 2018). Interestingly, the influence of LL-37 on TLR expression is both tissue- and time- dependent. For example, protein levels of TLR-2, TLR-4, TLR-5, and TLR-9 increased in Wistar rat mast cells in the presence of LL-37 in a time-dependent manner and peaked 3 hours following stimulation *in vitro* (Agier et al., 2018). Furthermore, LL-37 downregulated TLR and co-receptor expression induced by LPS in human gingival fibroblasts, but did not influence expression in untreated cells (Inomata et al., 2019), although the relevance of this is unknown since, as mentioned above, LL-37 strongly reduces LPS/LTA-mediated proinflammatory cytokine expression (Kandler et al., 2006).

Cathelicidins can alter the host immune response prior to, during, and even after infection. By directly influencing lymphocytes while also altering the chemokine profile associated with T and B cells, as well as affecting innate immune responses (e.g., cytokines, etc.) that prime and activate lymphocytes, cathelicidins link the innate and adaptive immune systems (Mookherjee et al., 2009; Nicholls et al., 2010). LL-37 and its derivatives also have the ability to induce differentiation and maturation of DCs as well as activate plasmacytoid DCs (pDC) and macrophages that prime and activate adaptive immunity (Davidson et al., 2004; Kandler et al., 2006). Overall, the features of LL-37 that promote modulation of the immune system, rather than direct microbicidal effects, seem to assist the resolution of an infection while also regulating harmful inflammation. The immunomodulatory effects of cathelicidins include selectively enhancing and diminishing inflammation by direct and/or indirect chemotaxis, pro-inflammatory and anti-inflammatory cytokine production, blocking TLR activation and downstream signaling pathways, and promoting activation of adaptive immunity.

Cathelicidins From Other Vertebrates

Although human LL-37, and to a lesser extent mCRAMP, have been the most highly studied HDPs (Van Harten et al., 2018), cathelicidins are ubiquitous in vertebrates. To determine similarities between the biological activities of natural cathelicidins, Coorens et al. (2017) compared the activity of 12 cathelicidins from chickens and five different mammalian species (human, mouse, dog, horse, and pig). They found that some functions of cathelicidins, such as suppression of bacterial ligand-induced TNF-α secretion and modest antimicrobial activity, were largely conserved. Therefore, while they speculated that these might represent the "core" biological activities of cathelicidins across vertebrate species, the above-mentioned caveat about limited antimicrobial activity under physiological conditions is worth taking into account. Critically, studies comparing the various biological activities of cathelicidins from different vertebrate groups under the same experimental conditions are

lacking. We contend that the massive sequence and structural variability of cathelicidins (and more generally HDPs), is the result of a complex evolutionary process where different peptides have evolved toward similar functions. It is expected that the individual host-specific activities of HDPs will differ, especially since each species produces its own repertoire of HDPs for which expression varies depending on tissue distribution and immune stimulus. Thus, the overall function of HDPs would be the important evolutionary driver and one might not expect any given peptide to have a single optimized purpose.

The immunomodulatory activities of non-human and non-murine cathelicidins are usually tested against human- or mouse-derived cell lines, which may obscure host-specific responses and functions of the peptides. However, functional insights into the biological roles played by these cathelicidins in their native hosts can still be gleaned by these types of studies. In the following section, we highlight some of the research related to the lesser studied cathelicidin peptides from other vertebrate groups and highlight several conserved activities that contribute to their likely biological function and therapeutic potential.

In amphibians, the skin plays a major role in water regulation, respiration, and defense. However, it also constitutes a permissive environment for potential pathogens, being rich in water, nutrients, and oxygen to enable growth (Clarke, 1997). To protect themselves, amphibians secrete a wide assortment of bioactive peptides, including HDPs, with diverse functions (Xu and Lai, 2015). In this regard, several amphibian cathelicidin peptides have been identified, and many of these have potent immunomodulatory functions (Mu et al., 2014; Cao et al., 2018; Wu et al., 2018; Shi et al., 2020). For example, cathelicidin-PP is an inducible β-structured cathelicidin from the skin secretions of the tree frog *Polypedates puerensis* that can significantly inhibit the production of nitric oxide (NO) and pro-inflammatory cytokines [TNF-α, interleukins (IL)-1β and IL-6] by LPS-stimulated mouse peritoneal macrophages (Mu et al., 2017). The suppression of pro-inflammatory mediators was proposed to be mediated through direct binding of LPS, but also led to decreased phosphorylation of MAP kinases and inhibition of NF-κB signaling pathways (Mu et al., 2017).

Several cathelicidin peptides have been found in reptiles, often exerting broad antimicrobial activities toward bacteria and fungi (Dalla Valle et al., 2013; Chen Y. et al., 2017; De Barros et al., 2019; Qiao et al., 2019). Recent studies have also demonstrated that they inhibit biofilm formation and can eradicate pre-formed biofilms of various species of bacteria (Chen Y. et al., 2017; De Barros et al., 2019; Qiao et al., 2019). In addition, reptilian cathelicidins typically possess low hemolytic and cytotoxic activity against mammalian cells and can inhibit production of LPS-induced NO and pro-inflammatory cytokines by mammalian macrophages *in vitro*. These inhibitory activities are usually ascribed to direct binding of the peptide to LPS and to the TLR-4/MD2 complex, which prevents receptor activation and inhibits MAP kinase and NF-κB signaling (Chen Y. et al., 2017; Qiao et al., 2019), although direct inhibition of signaling responses is frequently not examined. On the other hand, the induction of chemokines like MCP-1 and anti-inflammatory cytokines like IL-10 has been observed for several cathelicidins across reptilian species (Cai et al., 2018; Shi et al., 2019).

Reptilian cathelicidins can also influence immune processes without directly modulating cytokine signaling. For example, the snake cathelicidin-WA/-BF (CWA) promoted M2-like polarization of *E. coli*-stimulated murine macrophage RAW264.7 cells (Chen S. et al., 2018). When co-administered with live bacteria *in vitro*, CWA indirectly suppressed the production of pro-inflammatory mediators through phosphorylation of STAT-6, while inhibiting STAT-1 and NF-κB pathways. The resulting M2-like $CD206^{+}$ macrophages displayed higher expression of M2 anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β, but other crucial cellular functions such as phagocytosis were unchanged. CWA was also evaluated as a treatment for diarrhea in weaned piglets (Yi et al., 2016). Diarrheal diseases are a leading cause of death in young pigs and are usually associated with the proliferation of enterotoxigenic *E. coli* and heightened intestinal inflammation (Rhouma et al., 2017). CWA administration attenuated diarrhea and reduced levels of systemic and jejunal inflammation in piglets, similar to that observed using the fluoroquinolone antibiotic, enrofloxacin. However, only CWA treatment improved intestinal morphology, integrity and barrier functions, as well as microbial composition and metabolism (Yi et al., 2016).

Recently, Chen Y. et al. (2017) characterized the activities of six novel alligator cathelicidin peptides (As-CATH1-6). In general, As-CATH1-6 differed in their *in vitro* activity profiles and *in vivo* activities in murine peritonitis models of *E. coli* and *S. aureus* infection. Interestingly, the efficacy of these peptides in the murine models was related to their ability to recruit immune cells to the site of infection rather than assessed *in vitro* activities. Thus, peptides As-CATH4-6 displayed good antimicrobial, antibiofilm, and LPS-neutralization activities, while also suppressing production of pro-inflammatory mediators (NO, IL-6, IL-1β, and TNF-α) from LPS-stimulated murine peritoneal macrophages in contrast to the limited *in vitro* activities of As-CATH1-3. Nevertheless, when evaluated *in vivo*, As-CATH2-6 were all able to recruit neutrophils, monocytes, and macrophages to the infection site and protected mice against bacterial infection to varying degrees. Conversely, As-CATH-1 only moderately recruited macrophages and showed no protective effect *in vivo* (Chen Y. et al., 2017), suggesting a minor role for this HDP in preventing bacterial infections in alligators. Although the underlying reasons for these differences in efficacies among As-CATH2-6 were not further explored, these results highlight the importance of immune modulation in promoting the *in vivo* anti-infective activity of crocodylian cathelicidins.

In general, cathelicidins from a range of vertebrate species have displayed strong immunomodulatory effects including induction of chemokines and suppression of pro-inflammatory mediators that are induced in response to microbial signature molecules. Secondary to their role as immune modulators, cathelicidins reduce bacterial load in clinically relevant infection models. Cathelicidins also promote wound healing, cell recruitment and differentiation, and production of anti-inflammatory cytokines depending on their amino acid sequence. This functional plasticity likely contributes

to the overall *in vivo* efficacy of these peptides. Thus, improving our understanding of the amino acid sequence requirements that control these biological functions is critical to decipher their role in preventing infections and maintaining overall health.

CATHELICIDINS IN AUTOIMMUNITY

Beyond their involvement in innate immunity, recent research has unveiled additional pathological roles for cathelicidins in auto-immune diseases and, in some instances, they have even been implicated as self-antigens. A common feature of immune-mediated chronic inflammation is an imbalance of cytokine and chemokine levels (Chen X. et al., 2018) and an imbalance in host levels of cathelicidin and other HDPs can potentially influence the concentrations of these inflammatory mediators. As our understanding of cellular and molecular mechanisms underlying HDP activities improves, their roles in various disease conditions are beginning to emerge.

Psoriasis is a common skin condition that afflicts an estimated 7.4 million adults in the United States alone (Rachakonda et al., 2014). Incidence rates vary from 1 to 3% based on age and geographical location and known risk factors include environmental stressors like smoking and a family history of psoriasis (Parisi et al., 2013). As an autoimmune condition, psoriasis is characterized by an excessive differentiation and maturation of T cells and DCs. Persistent epidermal inflammation and keratinocyte expansion coupled with reduced rates of cellular apoptosis results in scaly plaque formation on the skin surface. While the exact cause of psoriasis is unknown, this condition is associated with increased levels of LL-37 in the epidermis, which triggers the rapid recruitment and activation of neutrophils and T lymphocytes, likely contributing to a chronic inflammatory response encompassing innate and adaptive immunity (Schön, 2019). It was originally suggested that LL-37 protects RNA against degradation by forming complexes that induce translocation and activation of the TLR-7/8 and TLR-9 receptors (Ganguly et al., 2009). This was proposed to result in the enhancement of IFN-α and IFN-β release by pDCs, spurring the chronic inflammation typically associated with psoriatic epidermis.

Recent reports challenge this model, instead suggesting a role for neutrophils and neutrophil extracellular traps (NETs), rather than DCs, in psoriasis disease progression (Herster et al., 2020). NETs are DNA structures released from neutrophils with embedded cellular proteins and peptides, including HDPs, that are proposed to have various functions such as trapping of pathogens. Neutrophil RNA:LL-37, but not DNA:LL-37 complexes, activate signaling through human TLR-8 and murine TLR-13, to propagate the release of cytokines (e.g., TNF-α, IL-6, IL-8, IL-1β) and chemoattractants (e.g., IL-16, MIP-1β). The RNA:LL-37 complex further promotes inflammation by the enhanced activation of neutrophil-derived NETs that, in turn, propagate additional rounds of immune stimulation. Furthermore, NET-associated RNA can potentially trigger immune activation which might also play a part in other NET-associated diseases such as systemic lupus erythematosus (SLE).

The pathogenesis of SLE is poorly understood due to its heterogeneous nature, unexplained higher incidence rates in females, and unknown etiology (Rees et al., 2017). A common feature in SLE patients is that several innate immune functions are altered including functional disruptions of neutrophils, monocytes, macrophages, and DCs along with severe organ impairment. Interestingly, skin biopsies from SLE patients have reported slightly (1.3- to 1.5-fold) increased levels of LL-37, along with IFN-α and pDCs (Sun et al., 2011). Additionally, SLE-derived neutrophils exhibit an enhanced capacity to release self-DNA and peptides to form NET structures (Lande et al., 2011; Kahlenberg and Kaplan, 2013). NETs are stabilized and protected from degradation by LL-37 and activate signaling through TLR-9 in pDCs to produce type I IFNs. The NET-associated LL-37 complex leads to sustained inflammatory responses, therefore implicating LL-37 in SLE pathogenesis. Interestingly, auto-antibodies to LL-37 are also able to directly induce NET formation which, in turn, promotes higher levels of IFN-α and further propagates the chronic inflammation seen in SLE (Lande et al., 2011).

Rheumatoid arthritis (RA), is another autoimmune chronic inflammatory disease that afflicts more than a million people in the United States (Hunter et al., 2017). RA affects the synovial membrane in joints resulting in decay, disability and increased mortality (Aletaha and Smolen, 2018). Within the synovial membrane, human RA patients express higher LL-37 levels (assessed by immunohistochemistry) in both CD66b granulocytes and CD68 macrophages when compared to healthy donors (Hoffmann et al., 2013). The pristane-induced arthritis model in rats has been used to mimic human RA and reveals that rCRAMP (rat CRAMP) as well as defensin expression is enhanced in the synovial fluid compared to healthy controls (Hoffmann et al., 2013). These augmented levels (3- to 9-fold) of rat HDPs were found in the joints, blood, and lymphoid organs of pristane-treated rats and were associated with higher levels of IFN-γ and autoantibodies against rCRAMP, further strengthening the link between cathelicidins/HDPs and RA progression. Conversely, analogs of LL-37 have protective effects in another RA model, the murine collagen-induced arthritis model (Chow et al., 2014). Subcutaneous administration of IG-19, a 19 amino acid peptide derived from the internal sequence of LL-37, suppressed cellular infiltration of the collagen injection site and reduced inflammation markers when compared to untreated mice. IG-19 treatment was also found to improve the clinical score and reduce disease severity by alleviating the harmful inflammation associated with collagen-induced murine arthritis. Possibly increased expression of the parent peptide, LL-37, might represent a natural defense mechanism against RA.

LL-37 has also been implicated in atherosclerosis (Kahlenberg and Kaplan, 2013). Atherosclerosis is an inflammatory condition characterized by the deposition and accumulation of fatty and/or fibrous plaques on the artery walls. Cardiovascular diseases associated with atherosclerosis are common around the world and are a leading cause of heart attacks and strokes (Libby et al., 2019). LL-37 in atherosclerotic lesions, generated mainly by macrophages, was reported to enhance host immunity by promoting the secretion of cell surface adhesion molecules and

chemokines (Edfeldt et al., 2006). Alternatively, it has been suggested that complexes of mitochondrial DNA and LL-37 escape autophagic degradation to amplify the inflammatory cascade and are key mediators of atherosclerotic plaque development (Zhang et al., 2015). Thus, there is evidence that abnormal levels of HDPs, like cathelicidins, might contribute to (or reflect) the development and pathogenesis of a range of immune-associated disorders. In the following section, we examine what is arguably the most serious case of immune system dysregulation, sepsis, and how HDP activities might combat this grave medical condition.

APPLICATIONS OF CATHELICIDINS IN SEPSIS

Sepsis is a life-threatening condition caused by an abnormal and dysfunctional immune response to infection (Rittirsch et al., 2008). A recent global estimate reported 48.9 million cases of sepsis in 2017 and sepsis-associated mortality was found to be high, accounting for nearly 11 million (or 19.7% of all) deaths worldwide (Rudd et al., 2020). Despite its high global burden, a complete understanding of sepsis pathogenesis remains a daunting challenge in medicine. Sepsis was initially defined as blood poisoning, referring to the presence of bacteria in the blood, that led to an initial amplified inflammatory response, dubbed a "cytokine storm" (Chousterman et al., 2017), which a few days later was followed by a hypo-inflammatory phase (Lyle et al., 2014). A clinical consensus on the definition of sepsis was established in 1992 focusing on the initial uncontrolled inflammation and requiring a diagnosis of systemic inflammatory response syndrome. However, this failed to address the subsequent immunosuppressive characteristics of this syndrome that further complicated diagnosis and treatment, and that was associated with the greatest mortality (Lyle et al., 2014).

Transcriptomic studies have identified gene expression signatures associated with the progression of sepsis in patients (Pena et al., 2014). Whole blood, PBMCs, and even specific immune cells like neutrophils and monocytes have showcased both inflammation and immunosuppression/cellular reprogramming at early stages of sepsis in the clinic. Cellular reprogramming in early sepsis patients is related to repeated exposure of the immune system to bacterial endotoxin, thereby reducing the host response to microbial ligands, resulting in a situation dubbed "immune amnesia." This type of immune dysfunction is implicated in high rates of ICU admission plus increased rates of organ failure that correlate with worse sepsis patient outcomes (Pena et al., 2014).

The majority of therapeutic candidates evaluated to date have sought to directly or indirectly dampen the initial pro-inflammatory immune response, but do not address the heterogeneity of sepsis pathogenesis, thereby resulting in ineffective therapies (Marshall, 2014). This high failure rate has led many to regard the development of sepsis treatment options as the "graveyard of biotech" (Riedemann et al., 2003). Since hyper-inflammation and immunosuppression appear to be acting in tandem, but manifest in a heterogeneous manner within the population, stratification of patients into phenotypically and mechanistically similar groups (endotypes) may well allow more targeted beneficial treatments and aid in future drug development (Van der Poll et al., 2017).

Perhaps unsurprisingly, the ability of natural HDPs to modulate immune responses has led researchers to examine whether these polypeptides play a role in sepsis progression and/or resolution. Interestingly, sepsis patients have been found to have ∼15-fold higher levels of defensins and lactoferrin HDPs, but not LL-37, relative to surgical controls (Berkestedt et al., 2010), suggesting an association of sepsis with an imbalance in natural HDP levels. Early work examining HDPs as treatments for sepsis used a murine model of endotoxemia where mice were given intravenous *E. coli* LPS. Exogenous application of human LL-37 protected mice from lethal sepsis by neutralizing endotoxin and suppressing the production of proinflammatory cytokines (Gough et al., 1996). LL-37 also protected against *S. aureus* septicemia (Bowdish et al., 2005b). Intriguingly, intratracheal instillation of LL-37 resulted in a reduction of TNF-α levels and significantly increased levels of chemoattractant MCP-1 in bronchoalveolar lavage fluid, suggesting that LL-37 specifically influenced the production of cytokines and chemokines (Scott et al., 2002). Using microarray studies, Scott et al. (2002) treated the murine macrophage cell line RAW 264.7 with LL-37 and found that upregulated genes fell into two broad categories associated with receptor binding and cellular communication. Both of these pathways promoted immune surveillance and the migration of immune cells, consistent with LL-37 having a protective role in sepsis progression. LL-37 and IB-367, a short analog of a the pig cathelicidin protegrin-1, were also evaluated in 3 rat models of sepsis: intraperitoneal delivery of LPS, peritonitis induced by live *E. coli,* and cecal ligation puncture (CLP) (Giacometti et al., 2003; Cirioni et al., 2006). Both LL-37 and IB-367 treatment in all three of these models dampened TLR signaling and reduced TNF-α secretion, thereby improving sepsis-associated inflammation. The CLP model in particular mimicked a multispecies intra-abdominal bacterial infection and has been found to best mirror the immune dysfunction in sepsis patients (Parker and Watkins, 2001). Lethality in the CLP model decreased from 100% in no treatment controls to 33.3% after treatment with LL-37 as well as the antibiotics polymyxin B and imipenem (Cirioni et al., 2006). Similarly, IB-367 led to 75% survival, as did piperacillin, when compared to the 100% lethality observed in the CLP control group (Giacometti et al., 2003). CLP lethality was completely overcome by the combination treatment of IB-367 and antibiotic, implying a combined effect of neutralizing endotoxin and limiting bacterial growth. *In vitro* and *in vivo* models have implicated certain mechanisms for LL-37 reduction of sepsis-induced injury, including decreased neutrophil infiltration, suppression of endothelial cell apoptosis, subdued inflammatory signal, and enhanced wound healing (Suzuki et al., 2011; Koziel et al., 2014; Qin et al., 2019).

The application of HDPs *in vitro* and *in vivo* has unveiled a variety of beneficial immunomodulatory effects that impact systemic inflammation in the context of sepsis (Martin et al., 2015). However, clinical applications of HDPs and related immunomodulatory proteins to treat sepsis are very limited,

and results have been mixed. One of the more extensively studied proteins related to innate immunity is that of 80 kDa iron-binding protein human lactoferrin (LF) and various peptide fragments derived therefrom (Sinha et al., 2013). Bovine LF (bLF; 77% homologous to human LF) supplementation was evaluated in a randomized, placebo-controlled trial and led to a reduced incidence of sepsis in very low-birth-weight (VLBW) neonates compared to the placebo group, while oral bLF prevented necrotizing colitis of VLBW human infants by increasing regulatory T-cell counts (Akin et al., 2014). Inspired by the restoration of the immune imbalance by LF in human neonatal sepsis, a large clinical trial of 2,203 participants across 37 neonatal units was initiated to evaluate the effect of enteral feedings of bLF on sepsis prevention; unfortunately, this failed to improve neonatal sepsis rates (Griffiths et al., 2019). Similarly, LF derivatives like talactoferrin (Guntupalli et al., 2013) and the N-terminal 11 amino acids of LF, hLF1-11 (Martin et al., 2015), exhibited promising efficacy in animal models of sepsis, but failed to progress as successful sepsis therapeutics.

Alternative therapies are needed to improve sepsis patient outcomes and there is some evidence that the immunomodulatory features of HDPs and cathelicidins might prove beneficial in this regard. Monocytes and macrophages from septic patients exert immunosuppressive characteristics (Venet and Monneret, 2018) and share many commonalities with M2 macrophages (Pena et al., 2011). A novel method to restore immune balance has been proposed to promote an intermediate (M1-M2) macrophage phenotype using a modified synthetic bovine cathelicidin bactenecin known as innate defense regulator (IDR)-1018 (Pena et al., 2013). Specifically, maturation of primary blood monocytes in the presence of IDR-1018 altered the release of certain M1 cytokines (e.g., TNF-α, COX2, IP-10) while also enhancing the anti-inflammatory features of M2 macrophages (e.g., IL-10, CCL-22 but not TGF-β) to promote a return to a balanced immune response. Harnessing this type of macrophage reprogramming, in conjunction with the other immunomodulatory properties of synthetic HDPs, may allow beneficial manipulation of inflammatory responses in conditions such as sepsis.

SYNTHETIC HDPs AND CLINICAL APPLICATIONS

To demonstrate that small synthetic peptides with desirable activity profiles can be derived from natural HDPs, one needs to look no further than several LL-37 fragments that are naturally generated through the action of proteases found throughout the body (**Table 1**). LL-37 is released from the hCAP18 precursor protein through the action of proteinase 3 in the blood (Sørensen et al., 2001) or kallikrein 5 in the skin (Yamasaki et al., 2006). Interestingly, further proteolytic processing at the skin surface by serine proteases generates LL-37 fragments with enhanced antibacterial activity in dilute medium toward various skin pathogens such as *S. aureus* and *C. albicans* (Murakami et al., 2004; Yamasaki et al., 2006). Several studies have characterized various fragments of LL-37 primarily for their antimicrobial activity (Kanthawong et al., 2012) but also for their antibiofilm, antiviral, spermicidal, and immunomodulatory functions (Tripathi et al., 2015). These examples of synthetic cathelicidin-derived peptides do not even begin to scratch the surface of the many studies that manipulate the amino acid sequence of LL-37 and various peptide fragments to rationally design derivatives with enhanced biological activities (e.g., Chen et al., 2019).

The majority of studies aimed at optimizing synthetic HDPs have focused on improving their direct antibacterial effects. As such, efficacy has been determined using endpoints such as recovered bacterial counts in dilute medium to measure success. Likely because factors present within the host milieu confound the direct antimicrobial activity of many HDPs, most of the subsequent trials have failed to advance therapeutic peptides beyond the pre-clinical stage (Mansour et al., 2014). Interestingly, even though the LL-37 fragments generated by the action of the kallikrein proteases have increased antibacterial potency, their ability to stimulate IL-8 release from keratinocytes is lost, suggesting that the peptide sequence requirements that underlie antibacterial activity are independent of those promoting immunomodulatory activity (Murakami et al., 2004). This may be another reason why many synthetic HDPs in clinical trials have failed to demonstrate success, since peptides with optimal antimicrobial activity *in vitro* may lack the immunomodulatory effects that are important or essential for their *in vivo* efficacy (Haney et al., 2019a). Considering that the immunomodulatory roles of HDPs are increasingly appreciated as their major natural function *in vivo* (Hancock et al., 2016), specifically selecting for peptides with enhanced immunomodulatory activities may prove more fruitful for advancing synthetic HDPs toward clinical applications.

Currently, 36 peptides are in clinical (27 peptides) and preclinical (9 peptides) trials for their potential applications in the context of infectious disease and immune modulation (Koo and Seo, 2019). Among HDPs in clinical trials is LL-37 itself, that was evaluated as a potential treatment of venous leg ulcers (VLUs). VLUs are the most prevalent form of chronic wounds caused by venous insufficiency or arterial disease (i.e., malfunctioning circulatory valves) that lead to significant intravenous pressure, and inflammation (Chi and Raffetto, 2015). Acute skin wounding of normal healthy skin induces the expression of human hCAP18 by epidermal keratinocytes at the wound margin. However, hCAP18 mRNA is rapidly degraded and functional peptide is reduced in chronic skin wounds, such as those associated with VLUs (Heilborn et al., 2003). Reduced LL-37 levels correspondingly reduce the activation of leukocytes and inhibit inflammatory cascades that are important for clearing pathogens and promoting angiogenesis, thereby preventing venous leg ulcers from healing appropriately.

Topical administration of LL-37 at low doses was assessed in combination with compression therapy for treatment of venous leg ulcers and comorbid bacterial infections in a randomized, placebo-controlled, double-blind Phase IIa clinical trial (Grönberg et al., 2014). A significant improvement in wound healing was observed in patients treated with 0.5 or 1.6 mg/ml LL-37 compared to patients treated with placebo, whereas no

TABLE 1 | Naturally produced LL-37 fragments and their processing enzymes.

Peptide	Sequence	Processing enzyme[a]	References
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	PR3, K5	Sørensen et al., 2001; Yamasaki et al., 2006
RK-31	RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	SSP, K7	Murakami et al., 2004; Yamasaki et al., 2006
KS-30	KSKEKIGKEFKRIVQRIKDFLRNLVPRTES	SSP, K5	Murakami et al., 2004; Yamasaki et al., 2006
KR-20	KRIVQRIKDFLRNLVPRTES	SSP, K7	Murakami et al., 2004; Yamasaki et al., 2006
KS-22	KSKEKIGKEFKRIVQRIKDFLR	K5	Yamasaki et al., 2006
LL-29	LLGDFFRKSKEKIGKEFKRIVQRIKDFLR	K5	Yamasaki et al., 2006

[a]*PR3, proteinase 3 from neutrophils; K5, Kallikrein 5; K7, Kallikrein 7; SSP, Sweat serine protease.*

improvement was seen for patients treated with 3.2 mg/ml LL-37. Indeed, patients treated with the highest concentration of LL-37 demonstrated increased inflammation at the wound site although only one of these cases was classified as severe. An ongoing Phase IIb clinical trial has been initiated to advance the peptide through the drug development pipeline (Rivas-Santiago et al., 2012), but the results of that clinical trial have not been published to date.

Although vitamin D_3 and 1,25-dihydroxyvitamin D upregulate the expression of LL-37 and enhance wound healing in a keratinocyte model of diabetic foot ulcers *ex vivo* (Gonzalez-Curiel et al., 2014), endogenous levels of vitamin D_3 do not correlate with healing of VLUs *in vivo* (Krejner et al., 2017). These contrasting results highlight the discrepant influence of vitamin D_3 on LL-37 activity *in vitro* vs. *in vivo* and demonstrate the importance of considering the effects of physiological solutes in cell-culture based mechanistic characterization of HDPs. Furthermore, the rapid degradation of vitamin D_3 might explain the failure of oral supplementation to improve diabetic wound healing in a randomized, placebo-controlled clinical trial (NCT03813927). In contrast, a recent randomized, double-blind, placebo-controlled trial in Bangladesh examined the effect of supplementation with vitamin D3 and 4-phenyl butyrate, both of which are potent inducers of LL-37, on recovery of adult tuberculosis patients aged 18–24. After 4 weeks of treatment, 71% of tuberculosis patients given both supplements ($p = 0.001$) and 61.3% of vitamin D3 supplemented patients ($p = 0.032$) were culture negative, compared to only 42.2% in the placebo-group (Mily et al., 2015). LL-37 levels were correspondingly increased and intracellular growth of *Mycobacterium tuberculosis* decreased, consistent with published animal model studies (Rivas-Santiago et al., 2013). Thus the induction of LL-37 represents a novel and exciting new strategy for the treatment of tuberculosis utilizing rather inexpensive inducers.

P60.4Ac is a 24-residue C-terminal truncated derivative of human LL-37 that was originally designed to inhibit inflammation associated with chronic sinusitis and other diseases of the upper respiratory tract (Nell et al., 2006). In a bronchial epithelial model of respiratory mucosa *in vitro*, P60.4Ac reduced LPS-induced TLR signaling and downstream activation of extracellular signal-related kinase Erk1/2, which in the context of upper respiratory infections is known to trigger damaging inflammatory responses (Nell et al., 2006). Excitingly, when administered as ear drops to patients suffering from chronic otitis media in a randomized, placebo-controlled Phase IIb clinical trial, P60.4Ac demonstrated efficacy in reducing bacterial load and neutrophil infiltration (Peek et al., 2009). In 2010, plans for a Phase III clinical trial were announced for this peptide, renamed as OP-145[1] but the company making this announcement was sold and there is no indication the trial has proceeded. Nevertheless, formulated OP-145 continues to be studied preclinically for potential applications in the context of biomaterial-associated infections (De Breij et al., 2016).

Omiganan is a 12-residue derivative of the bovine cathelicidin, indolicidin, that has been in development as a topical antimicrobial compound for several years (Rubinchik et al., 2009). When applied as an antimicrobial, omiganan was unsuccessful in a Phase IIIb clinical trial for the treatment of catheter-related urinary tract infections due to failure to reach its primary endpoint (significant decrease in physician-determined infections) although it showed efficacy in its secondary endpoints of significantly decreased microbiologically confirmed infections and decreased tunnel infections, demonstrating its potential (Koo and Seo, 2019). More recently, formulation of omiganan (also known as CLS-001, MBI-226, and/or MX-226 across studies) as a topical gel revealed clinical promise as an immunomodulatory/anti-inflammatory treatment of rosacea, acne, vulvar intraepithelial neoplasia, and atopic dermatitis, according to preliminary results from Phase IIIa clinical trials[2]. Interestingly, little mechanistic data has been reported to elucidate its immunomodulatory influence on host cells. *In vitro*, omiganan has broad spectrum antimicrobial and antifungal activity against numerous resistant species including *S. aureus* and *Candida albicans*, respectively; however, proteases endogenous to the skin can cause deactivation of the peptide *in situ* (Ng et al., 2017). In contrast, the D-enantiomer of omiganan is metabolically stable toward skin proteases and is comparable to the L-enantiomer in terms of antibacterial and antifungal potency. Although further studies are needed to determine suitability of the D-enantiomeric peptide for *in vivo* use, preliminary studies suggest that the stereoisomer of omiganan remains a promising candidate for future clinical applications (Zapotoczna et al., 2017).

The possibility of extrinsically manipulating endogenous expression of *CAMP* for systemic and localized therapeutic benefit, as mentioned above for LL-37 in the case of tuberculosis, has also attracted significant attention in recent years (Brandwein et al., 2017). Several epidemiological studies have linked

[1]http://www.octoplus.nl

[2]http://www.mallinckrodt.com/

serum and/or tissue levels of LL-37 to clinical outcomes in seemingly unrelated diseases such as bacterial meningitis, rosacea, respiratory syncytial virus (RSV) bronchiolitis, and type II diabetes mellitus. In the context of bacterial meningitis, a substantial bacterial load in the membranes that surround the spinal cord and brain is associated with poor outcomes and cerebrospinal fluid with high levels of LL-37 was linked to reduced neurological damage and audiological sequelae, but not improved survival in children (Savonius et al., 2018). Rosacea is characterized as an erythematous pustular rash on the face with overexpressed LL-37 levels in tissues and serum of patients (Park et al., 2018). Interestingly, LL-37 expression in the skin of rosacea patients is abnormally high (Yamasaki et al., 2007) and it has been proposed that this leads to an overactive innate immune response that contributes to disease pathogenesis. Regarding viral infections, higher levels of serum LL-37 in human infants were associated with lower rates of RSV bronchiolitis, but the causality of LL-37 levels and disease severity lacks elucidation (Mansbach et al., 2017). Lastly, in the case of type II diabetes mellitus, decreased LL-37 levels are associated with lowered high-density lipoprotein (HDL) cholesterol, which in turn diminishes the buildup of atherosclerotic plaque load and therefore, cardiovascular damage (Meguro et al., 2014). Unfortunately, attempts to modulate *CAMP* levels at the transcriptional and protein levels have demonstrated variable success in the clinic (Mily et al., 2013; Mily et al., 2015). This may be related to several issues including how inducers are delivered, endogenous levels of LL-37 in patients and/or proteolytic degradation *in situ*.

NOVEL FORMULATIONS FOR PEPTIDE DELIVERY

To date, the inability to translate animal model data into clinical applications for many peptide-based drugs might be due to a range of factors including a short half-life, a tendency for peptides to aggregate, non-specific toxicity at high concentrations as well as confounding physiological factors *in vivo*. These factors need to be considered when advancing a novel peptide through clinical trials and many of them can be addressed through the use of drug formulation strategies (Eckert, 2011), or circumvented by incorporating active peptides directly into biomedical devices (Riool et al., 2017). For example, peptides can be immobilized into fibrous scaffolds, called electrospun nanofibers, to create functional wound dressings (Amariei et al., 2018). Another strategy involving the synthetic antimicrobial peptide, HHC-36, incorporated this peptide into titanium coatings to control the release of peptide from orthopedic implants and prevent the development of bacterial biofilms on implant surfaces (Kazemzadeh-Narbat et al., 2013). A similar approach sought to prevent bacterial attachment on implant surfaces by coating medical devices with a polymer brush decorated with covalently-attached synthetic AMPs while maintaining their bactericidal effects against Gram-positive and Gram-negative bacteria (Yu et al., 2015). Although some peptide delivery methods have been evaluated in the clinical trials described above, many are still being assessed in preclinical studies. Oftentimes, *in vitro* efficacy testing of nascent and formulated peptides hinges on isolation of primary cells from human volunteers or working with immortalized cell lines. From there, testing in animal models allows efficacy to be determined under physiological conditions. In these models, peptides can be delivered locally to a site of infection or inflammation in a variety of ways (e.g., via implanted catheters or adsorption to other implanted biomedical plastics) but most have been best studied following topical cream formulation (Easton et al., 2009). The application of novel formulations in animal models that best mimic these scenarios is crucial to bridging the gap between pre-clinical and clinical development of cathelicidins.

The incorporation of synthetic HDPs with potent immunomodulatory properties into vaccine adjuvants is an attractive area of research with the goal of enhancing the adaptive immune response elicited by existing, clinically-approved therapies to garner greater immune protection (Nicholls et al., 2010). The feasibility of such an approach was demonstrated with a polyphosphazene microparticle formulation incorporating IDR-1002 with the TLR-9 agonist, CpG oligodeoxynucleotide, leading to enhanced protection in a murine model of whooping cough (Polewicz et al., 2013). Compared to microparticles alone, the peptide formulated adjuvant stimulated enhanced immune activity against *Bordetella pertussis* by upregulating the secretion of numerous chemokines and cytokines including MCP-1 and TNF-α as well as dampening the secretion of anti-inflammatory IL-10.

Several other peptide formulation strategies have focused on prolonging the release of active compound to provide a sustained therapeutic effect, or to prevent some of the negative effects of administering high concentrations of peptides (e.g., aggregation and/or toxicity). Cathelicidin-BF is a peptide isolated from the venom of the banded krait snake, *Bungarus fasciatus,* that has proven amenable to microparticle formulation. Formulation of this peptide in poly(D,L-lactide-co-glycolide) (PLGA) microspheres allowed for the slow release of cathelicidin-BF *in vitro* for more than 15 days. Importantly, the microspheres prevented peptide degradation while still maintaining antimicrobial efficacy against *E. coli*, *Shigella dysenteriae,* and *Salmonella typhi* (Bao et al., 2018). In addition, using a murine model of *P. aeruginosa* pneumonia, intravenous pre-treatment of formulated cathelicidin-BF activated the immune response to improve antibacterial functions while enhancing macrophage clearance and dampening inflammation via obstruction of the NF-κB signaling cascade (Liu et al., 2018). Cathelicidin-BF has also been conjugated to PLGA polymers, resulting in even slower release (up to 30 days) while exhibiting minimal toxicity toward eukaryotic cells as well as low hemolysis (Schlosser et al., 2008). The slow release of co-encapsulated PLGA microparticles resulted in enhanced DC uptake and activation of cytotoxic T cells and furthermore, protected against vaccinia virus *in vivo*. Lastly, IDR-1018 peptide formulated with derivatized hyperbranched polyglycerols (dHPG) provided a novel strategy to minimize peptide aggregation and toxicity (Haney et al., 2019b). Administration of IDR-1018 formulated with dHPG sustained *in vitro* immunomodulatory features toward human PBMCs stimulated with LPS and a human bronchial epithelial cell

line stimulated with polyinosinic:polycytidylic acid, modulating TLR-4 or TLR-3 signaling, respectively.

Liposomal/micellar formulations serve to stabilize peptides in *in vitro* and *in vivo* models. While cellular toxicity of peptides is often diminished when formulating with lipids, activity is often preserved as seen in studies examining methicillin resistant *S. aureus* skin infections (Kumar et al., 2019), herpes simplex virus 1 infection (Ron-Doitch et al., 2016), and has even shown effectiveness as a tumor delivery system (Zhang et al., 2016). Formulation of a synthetic antibiofilm peptide, DJK-5, in nanogels composed of octenyl succinic anhydride-modified hyaluronic acid decreased cutaneous toxicity 4-fold when compared to DJK-5 alone (Kłodzińska et al., 2019). Importantly, DJK-5 nanogels maintained their efficacy against *P. aeruginosa* LESB58-generated murine abscesses. Thus, formulation strategies that promote peptide stability while also mitigating potentially negative effects related to peptide toxicity and aggregation hold tremendous promise to aid in clearing the final hurdle to advancing synthetic HDPs to the clinic.

CONCLUSION AND FUTURE DIRECTIONS

HDPs are distributed across a broad range of vertebrate and invertebrate species. The cathelicidin subgroup in vertebrates in particular contributes to the complex signaling mechanisms that are associated with the innate immune response as well as various inflammatory processes. Since HDPs are able to bind to and modulate signaling through TLRs and other extracellular and intracellular receptors, they massively alter gene expression in cells and can influence downstream effects in the cell to modulate the immune response. This leads to the alteration of cytokine and chemokine levels, depending on other underlying immune stimuli, promoting chemotaxis and cellular proliferation, suppressing inflammation, and providing a link between the innate and adaptive immune systems. Rather than complete suppression of cellular responses and signaling, HDPs, and cathelicidins in particular, enforce a more balanced immune response. Unsurprisingly, atypical cathelicidin expression has been shown to exhibit contradictory and harmful effects as evidenced by reports of cathelicidins acting as self-antigens as well as being implicated in the pathogenesis of complex autoimmune diseases. In the context of persistent dysfunctional inflammation in sepsis, the ability of HDPs to modulate cellular differentiation and alter inflammatory signaling pathways may prove to be beneficial for disease resolution. To harness the useful characteristics of peptides under physiological conditions, the choice of an appropriate formulation for synthetic HDPs is of great importance. Formulation lends itself to improved peptide stability and thus sustained functionalities, enabling HDPs to impact on their diverse signaling pathways in the context of inflammatory and infectious diseases. As we seek new therapies based on natural HDPs, a strategy that seeks to maximize the host immune response is potentially the best path forward toward advancing synthetic HDPs toward clinical applications.

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All authors were involved in writing and editing the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: RH and EH have developed peptides, related to the ones discussed here, for commercial application, assigned these to the employer the University of British Columbia and licensed these to ABT therapeutics Inc., a Victoria company owned in part by RH with EH as a minor shareholder.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Dual Role of Antimicrobial Peptides in Autoimmunity

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Autoimmune diseases (AiDs) are characterized by the destruction of host tissues by the host immune system. The etiology of AiDs is complex, with the implication of multiple genetic defects and various environmental factors (pathogens, antibiotic use, pollutants, stress, and diet). The interaction between these two compartments results in the rupture of tolerance against self-antigens and the unwanted activation of the immune system. Thanks to animal models, the immunopathology of many AiDs is well described, with the implication of both the innate and adaptive immune systems. This progress toward the understanding of AiDs led to several therapies tested in patients. However, the results from these clinical trials have not been satisfactory, from reversing the course of AiDs to preventing them. The need for a cure has prompted many investigators to explore alternative aspects in the immunopathology of these diseases. Among these new aspects, the role of antimicrobial host defense peptides (AMPs) is growing. Indeed, beyond their antimicrobial activity, AMPs are potent immunomodulatory molecules and consequently are implicated in the development of numerous AiDs. Importantly, according to the disease considered, AMPs appear to play a dual role in autoimmunity with either anti- or pro-inflammatory abilities. Here, we aimed to summarize the current knowledge about the role of AMPs in the development of AiDs and attempt to provide some hypotheses explaining their dual role. Definitely, a complete understanding of this aspect is mandatory before the design of AMP-based therapies against AiDs.

Keywords: autoimmune diseases, antimicrobial host defense peptides, innate immunity, defensin, cathelicidin

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INTRODUCTION

Autoimmune diseases (AiDs) have been originally characterized by the destruction of a specific host cell type by autoantigen-specific T and B cells. A higher frequency of autoreactive T cells circulates in the body of autoimmune-prone individuals due to a defect in the thymic selection. Additionally, a defect in the peripheral tolerance allows the activation of the autoreactive lymphocytes and the subsequent destruction of the host cells. This terminal step of the immunopathology of AiDs is well documented, and this knowledge led to several clinical trials based on the modulation of the adaptive autoimmune response. However, for most AiDs, these clinical trials were unsatisfactory, with limited success and few definitive cures (1, 2). In parallel, during the last decades, the role of innate immunity in the immunopathology of AiDs has emerged, and it is tempting to speculate that targeting this innate part of the immune system may be a promising therapeutic approach against AiDs (3). Among the numerous molecules produced by the innate immune system, the antimicrobial peptides (AMPs) have recently been identified as important factors in

the development of AiDs. Importantly, the role of AMPs in the autoimmune process appears to be complex, with both a deleterious and a protective role. Originally discovered in silk moth 40 years ago, it is now recognized that AMPs, also known as host defense peptides, represent a major component of the innate immune system of every living organism (4). AMPs are a large group of small cationic polypeptide molecules largely produced at the epithelial surfaces, but not exclusively, and their first described function is to protect against the continuous exposure to environmental microorganisms (5); more recently, their antimicrobial function extends to the maintenance of the host microbiota (6). AMPs encompass representatives of several distinct molecules including cathelicidins, alpha-defensins (human neutrophil peptides, HNPs), beta-defensins (BDs), regenerating islet-derived protein, ribonucleases, or S100 proteins. These polypeptides exert their antimicrobial activity by directly disrupting the membrane of microorganisms or by the sequestration of metals essential for microorganism growth, for example (7). Importantly, high concentrations of AMPs may be toxic for eukaryotic host cells which support their use as anticancer drugs (8). More recently, AMPs were shown to directly modulate the function of non-immune cells; for example, AMPs regulate the intestinal barrier integrity by stimulating tight junction protein synthesis by enterocytes (9) or promoting insulin secretion by pancreatic beta cells (10). Importantly, AMPs have been rediscovered in the last decades as an important player in the regulation of the immune responses, which supports their use as potential therapeutic molecules against immune-related diseases (8, 11). Here, we will discuss the role of AMPs in several AiDs and attempt to propose some hypothesis regarding their contrasting role in autoimmunity. The increasing knowledge about the role of AMPs in autoimmunity may open new therapeutic opportunity to prevent or cure AiDs.

ANTIMICROBIAL PEPTIDES AS IMMUNOMODULATORY MOLECULES

During the last decades, the ability of AMPs to act as modulators of the immune response has been extensively studied, and their role in innate and adaptive immunity has become increasingly appreciated (8, 12). The immunomodulatory roles of cathelicidin and defensins have been extensively investigated as these AMPs are expressed in various cell types, including epithelial cells and cells of the immune system (13). First of all, it is important to mention that the impact of AMPs on the immune system is widespread and complex, with both pro- and anti-inflammatory effects, likely reflecting the necessity of a tight control of any immune responses. Due to their ability to bind chemokine receptors, cathelicidins and defensins are potent chemoattractants for several immune cell types, including monocytes *via* CCR2, neutrophils *via* formyl-peptide receptors, dendritic cells (DCs), and T cells *via* G protein-coupled receptors (14–16). However, the immune cells recruited by AMPs can potentially have either inflammatory or regulatory functions, and the chemotactic activity of AMPs cannot be necessarily associated with inflammation. The presence of AMPs during the differentiation of macrophages and DCs can bias their polarization toward a pro-inflammatory phenotype (17–20). However, one study also demonstrated the role of HNP1–3 on human monocyte-derived DCs showing that, depending on the dose of HNPs, they can either promote at a low dose or prevent at a high dose the differentiation and maturation of DCs (21). AMPs also modulate the activation of macrophages and DCs through their capacity to bind Toll-like receptor (TLR) ligands. By sequestrating TLR ligands or perturbating intracellular signaling pathways, AMPs inhibit the activation of macrophages and DCs. After being endocytosed in monocytes, LL-37 binds to GAPDH, and the resulting complex interacts with p38 MAPK and other signaling molecules to prevent excessive inflammation (12, 22). However, AMPs also have an adjuvant role by enhancing the pro-inflammatory response to TLR ligands such as viral RNA *via* TLR3 in epithelial cells, flagellin *via* TLR5 in keratinocytes, and CpG *via* TLR9 in B cells and plasmacytoid DCs (pDCs) (23–25). Finally, AMPs regulate the apoptosis of innate immune cell types as neutrophils prolonging their life after activation (26, 27). The above-mentioned effects of AMPs on innate immune cells, and particularly on antigen-presenting cells, impact the adaptive immune response by modulating Th1, Th17, or regulatory T (Treg) cell responses (18, 28, 29). In summary, AMPs occupy a central place not only in the innate immune defense against invading pathogens but also in the modulation of the adaptive immune response. AMPs may be required to initiate a fast immune response and then to efficiently terminate the response and prevent immune-induced tissue damage. Consequently, a dysregulated expression of AMPs in a specific tissue may participate in the development of the autoimmune response, as described in the following sections. Besides, research during the last decade has revealed the significant role of the microbiota in the regulation of autoimmune diseases (30). Consequently, thanks to the ability of AMPs to regulate microbiota composition (6), they likely also modulate the autoimmune response in this way.

THE ROLE OF AMPs IN AUTOIMMUNE DISEASE

From primary observations that the expression of AMPs is dysregulated in many tissues affected by autoimmune or autoinflammatory diseases, their involvement in the pathophysiology of these diseases is now established or suspected, as in systemic lupus erythematosus (SLE), psoriasis, rheumatoid arthritis (RA), type 1 diabetes (T1D), Sjögren's disease (SjS), and multiple sclerosis (MS). The most documented aspect is that an aberrant production of AMPs produced by neutrophils or epithelial cells promotes inflammation, favoring the autoimmune response (31). Activated neutrophils in the tissue produce neutrophil extracellular traps (NETs) that are made of self-nucleic acids from the nucleus bound to granular cytoplasmic proteins rich in AMPs (32). These NETs are normally produced in infectious context to immobilize and kill pathogens (33, 34). Aberrant production of NETs in sterile condition and impaired clearance of these NETs would stimulate

pDCs *via* TLR7 and TLR9 to produce type I interferons (IFNs), which are important contributors to autoimmune diseases by activating antigen presentation by DCs and the production of autoantibodies by B cells (35–39). On the other hand, recent studies have shown that AMPs produced by specific non-immune cells carry immunoregulatory properties on various innate and adaptive immune cell types, leading to the induction of Treg cells, preventing the development of autoimmune disease (40). In the present review, we discuss the present knowledge about the role of AMPs in autoimmune diseases.

Systemic Lupus Erythematosus

Systemic lupus erythematosus is a systemic autoimmune disease that results from defects of the immune system that can occur at different levels of the immune response, explaining the vast heterogeneity of the clinical presentation of the disease. Affected tissues include the central nervous system (CNS), kidney, blood, skin, and joints (41). SLE is a disease caused by an inappropriate reaction of the innate and adaptive immune systems and is characterized by the presence of autoantibodies to nuclear antigens forming immune complexes with DNA or RNA. SLE is also characterized by a type I IFN signature that results from the sterile activation of pDCs by the immune complexes (42). Both HNPs and cathelicidin have been implicated in the physiopathology of SLE (43). Increased levels of HNP1–3 expressed by activated neutrophils have been detected by enzyme-linked immunosorbent assay (ELISA) in the blood of SLE patients (44–46). These HNPs harbor chemotactic and pro-inflammatory activity for immune cells such as DCs and T cells (47). More importantly, autoantibodies against HNPs are detected in the sera of SLE patients, and the HNP level correlates with disease activity (48). Using antibody suspension bead array, Idborg et al. determined that the level of S100 calcium-binding protein A12 was increased in the serum of patients compared with healthy individuals (49). Higher levels of cathelicidin have been observed by *in situ* hybridization in the skin of SLE patients compared with healthy individuals (50). However, the serum level of LL-37 measured by ELISA did not increase in patients vs. healthy individuals and did not correlate with disease activity in patients (51). Gilliet's group described the pathogenic behavior of cathelicidin in SLE. The pathogenic role of cathelicidin in SLE originates from its presence in NETs and its ability to form and stabilize immune complexes with DNA and autoantibodies. As described above, these complexes promote type I IFN secretion by pDCs and autoantibody production by B cells (52, 53). Recently, another aspect of the role of cathelicidin in SLE has been identified. The authors show that cathelicidin-specific T cells circulate in patients and support the production of cathelicidin-specific pathogenic autoantibodies by B cells (54). Animal models of lupus also demonstrated the role of AMPs in the physiopathology of the disease. In the New Zealand mixed (NZM) model, the accumulation of NETs and autoantibodies against the NET component including cathelicidin have been reported (55). However, using a model of pristane-induced lupus, cathelicidin-related antimicrobial peptide (CRAMP)-deficient mice were not protected against the disease, minimizing the causative role of cathelicidin in lupus (56).

Psoriasis

Psoriasis is an autoimmune disease affecting mainly the skin with the presence of inflammatory plaques for the most common form. The immune pathogenesis of psoriasis implicates dysfunction of the innate and adaptive immunity with the recruitment of inflammatory macrophages and type I IFN-producing pDCs and the generation of an uncontrolled Th17 response (57). By reverse transcription PCR (RT-PCR) and immunohistochemistry, cathelicidin and human beta-defensins 2 and 3 (hBD2/3) have been shown to be highly expressed in the psoriatic skin of patients (58–63). Gilliet's group elegantly deciphers the pathogenic role of cathelicidin in psoriasis. As described above, cathelicidin binds to self-DNA/RNA released from keratinocytes to form immunogenic complexes that activate type I IFN-secreting pDCs through TLR9/TLR7 (25, 64). Moreover, cathelicidin-immune complexes activate 6-sulfo LacNAc (slan) DCs *via* TLR7/8 that, in response, secrete inflammatory cytokines [interleukin (IL)-6, IL-12, and IL-23], inducing Th1/Th17 responses (65). A recent study also demonstrates the role of cathelicidin from infiltrating neutrophils in the disease. Complexes of cathelicidin with RNA that are rich in psoriatic skin trigger *via* TLR8/TLR13 inflammatory cytokine production by neutrophils and the formation of NETs perpetuating chronic inflammation in psoriasis (66). In addition to activating the innate immune system, cathelicidin was identified as an autoantigen with the presence of cathelicidin-specific T cells that produce IFN-gamma in the skin of patients with psoriasis (67). Also, circulating autoantibodies to cathelicidin and its citrullinated or carbamylated derivatives were found in psoriasis patients. However, their role in the pathogenesis of the disease remains to be determined (68, 69).

Rheumatoid Arthritis

Rheumatoid arthritis is a chronic inflammatory disease of the joints resulting in cartilage and bone damage (70). The synovial fluid of RA patients is infiltrated by innate immune cells (e.g., monocytes, DCs, mast cells, and innate lymphoid cells) and adaptive immune cells (e.g., Th1 and Th17 cells and B cells). While RA is pathologically heterogeneous, more severe symptoms are associated with the presence of autoantibodies against posttranslationally modified self-peptides, especially from proteins that have been citrullinated or carbamylated (71). Different AMPs are expressed constitutively or are inducible in articular joints such as hBD1–3 and cathelicidin (72). Proteomic analysis and ELISA revealed that, in patients, HNP1–3 expressions are also increased in the synovia of patients with an observed correlation between joint erosion and the HNP levels (73, 74). The presence of pro-inflammatory cytokines in the diseased joints may likely explain the increased expressions of some AMPs, such as hBD2/3 (72, 75). hBD3 may participate in the physiopathology of RA since this AMP stimulates the production of metalloproteinases by chondrocytes, degrading the extracellular matrix of cartilage (72, 75). Besides, hBDs are also known as potent chemotactic agents for human monocytes, dendritic cells, and T cells (47). Increased expressions of hBD2/3 may also contribute to recruiting immune cells and amplifying

inflammatory response in the joint. Increased expression of cathelicidin has been described in the synovia of RA patients, with macrophages and neutrophils as cell sources identified by flow cytometry (76, 77), and cathelicidin induces the apoptosis of osteoblasts, indirectly contributing to altered bone formation in arthritic joints (78). Finally, using the pristane-induced arthritis model in rats, Hoffmann et al. have demonstrated that cathelicidin is produced by neutrophils in the synovial fluids of diseased rats and that the transfer of pristane-primed neutrophils induced arthritis, whereas type I IFNs or autoantibody responses in control rats did not (77). Altogether, exaggerated cathelicidin expression in the joints may participate in the development of RA; however, the exact pathogenic mechanism remains unclear. It could be hypothesized that cathelicidin from neutrophils may prime pDCs to secrete type I IFNs, as demonstrated for other autoimmune diseases (38). Interesting in the context of psoriatic arthritis, posttranslationally modified cathelicidin from neutrophils represents a source of self-antigens, supporting that autoantibodies against cathelicidin participate in inflammation and the autoimmune process (79).

Type 1 Diabetes

Type 1 diabetes is an autoimmune disease ultimately resulting from the destruction of the insulin-producing β-cells of the pancreas by autoreactive T cells. However, many different innate and adaptive immune cell types are implicated in the long diabetogenic process. Due to the inability to produce insulin, T1D patients are unable to control their glycemia, and even with replacement therapy, i.e., insulin injection, they can develop diabetes-associated complications in multiple organs (80). Few studies have examined the expressions of AMPs in T1D patients. By ELISA, Brauner et al. described reduced levels of cathelicidin and hBD1 in the serum of T1D patients compared with type 2 diabetic patients or healthy individuals (81). Besides, Nemeth et al. showed by ELISA and reverse transcription quantitative PCR (RT-qPCR) increased levels of HNP1–3 in the plasma of T1D patients; however, similar increases were observed in type 2 diabetes (T2D) patients, suggesting that hyperglycemia may be responsible for such increases and, consequently, may only be a consequence of the disease and not a cause (82). Indeed, hyperglycemia was demonstrated in a diabetic rat model to promote NET formation (83). Importantly, these studies measured the circulating levels of AMPs in patients that may not reflect the levels in the pancreas. Moreover, the highest concentrations of HNPs were detected in T1D patients with complications including diabetic kidney disease (82). The explanation might be that the elevations in the plasma HNP1–3 levels are the consequence of the decreased renal degradation of the peptides in patients with advanced nephropathy (84). The role of cathelicidin in T1D development has been well demonstrated in a non-obese diabetic (NOD) mouse model. A first study from our group demonstrated that cathelicidin participates in the initiation of the disease in young NOD mice (85). Around the age of weaning, netting neutrophils transiently infiltrate the pancreas and produce cathelicidin in complex with self-DNA and anti-DNA immunoglobulin G (IgG). These complexes activate pDCs *via* TLR9, inducing the production of type I IFNs that promotes the progression of T1D (86). Importantly, a similar mechanism may be at play in human since aberrant neutrophil activation in the blood and the presence of NETs in the pancreatic section have been identified in pre-diabetic and diabetic patients (87, 88). In addition, in a follow-up study, we have demonstrated the protective role of cathelicidin against the disease. Indeed, we identified that cathelicidin is normally produced by pancreatic β-cells in adult non-autoimmune mice, but not in NOD mice. Conversely, treatment of pre-diabetic adult NOD mice with recombinant cathelicidin induces regulatory macrophages and T cells in the pancreas, preventing the development of the disease. We demonstrated that the gut microbiota-derived metabolites short-chain fatty acids (SCFAs) promote the pancreatic production of cathelicidin, and the alteration in the gut microbiota explains the defective production of cathelicidin in NOD mice (89). The protective effect of SCFAs against T1D has been demonstrated by others in mouse models (90) and in patients (91). How the same AMP, cathelicidin, has apparent opposite effects in T1D is under investigation by our group. Finally, we recently demonstrated that the pancreatic β-cells also produce mouse β-defensin 14 (mBD14) under the control of the gut microbiota. This expression of mBD14 in the pancreas is defective in the NOD mice compared with the non-autoimmune mouse strains, and treatment of pre-diabetic NOD mice with recombinant mBD14 prevents diabetes development by the induction of regulatory B cells in the pancreas (92). Overall, the pancreatic β-cells harbor the capacity to produce different immunoregulatory AMPs targeting different immune cell types, ensuring the maintenance of the immune tolerance in the pancreas. Defective AMP expression by the pancreatic β-cells allows the inflammation to develop in the pancreas, favoring the diabetogenic autoimmune adaptive response. However, cathelicidin aberrantly expressed by neutrophils infiltrating the pancreas in a diabetes-prone genetic background participates in the initiation of the disease *via* a classical mechanism described for other autoimmune diseases.

Sjögren's Syndrome

Sjögren's syndrome is a chronic autoimmune disease affecting primarily the exocrine glands; hallmarks of the disease associate with dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). Moreover, multiple organs can be affected, including the lung, kidney, liver, joint, skin, and so on. The impairment of the salivary and lacrimal glands (SGs and LGs, respectively) is caused by the infiltration of various immune cell types, including T and B cells, macrophages, and DCs (93). In addition, SjS diagnosis relies on the presence of autoantibodies against ribonucleoproteins, type I IFN production by infiltrating pDCs, and actually many features of SjS are indeed in common with other systemic autoimmune diseases (94–96). The literature regarding the potential role of AMPs in SjS is not abundant; however, some studies suggest that cathelicidin and defensins may have a role in the physiopathology of the disease. Cathelicidin expression is detected by RT-qPCR and immunohistochemistry in both mouse and human SGs at steady state, and cathelicidin expression is upregulated with inflammation of the oral cavity (97, 98). Svensson et al. also reported that cathelicidin of the parotid and

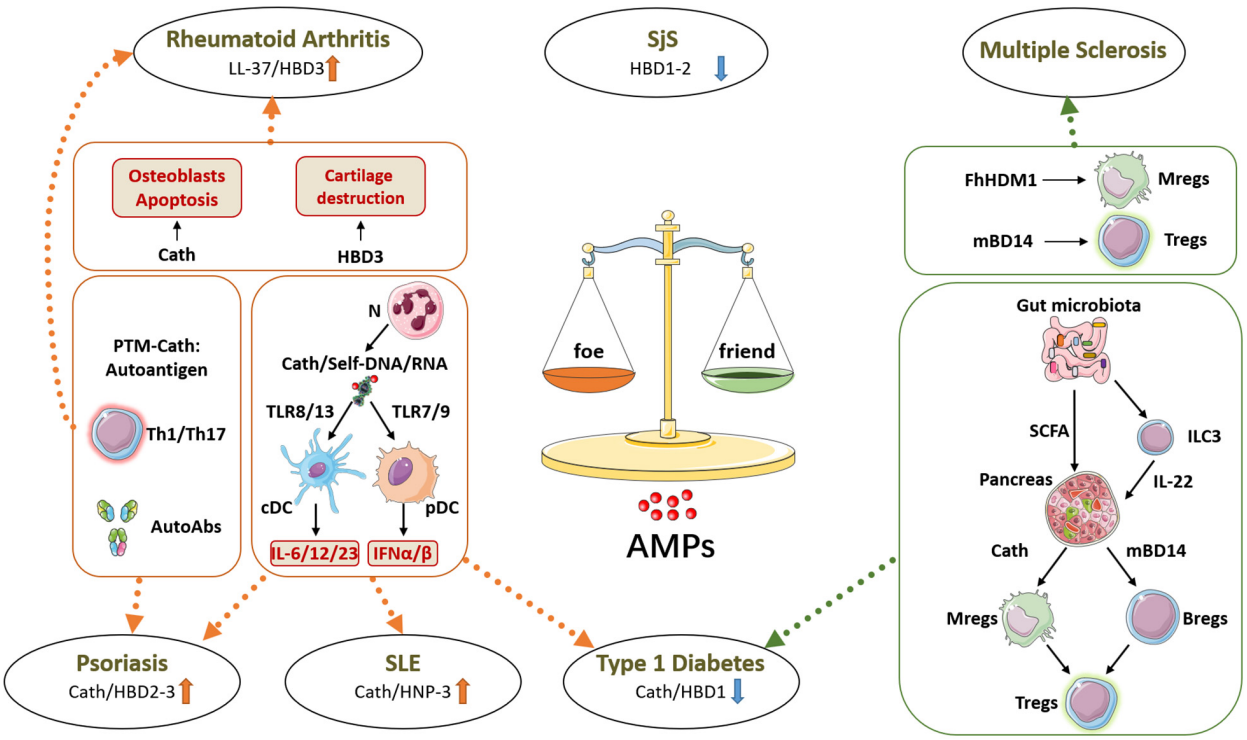

FIGURE 1 | Friend or foe: roles of antimicrobial peptides (AMPs) in autoimmune diseases. AMPs have a dysregulated expression in many tissues affected by autoimmune diseases, where they can promote or prevent the autoimmune response. On one side, as shown in psoriasis, systemic lupus erythematosus (SLE), and type 1 diabetes (T1D), cathelicidin (Cath) produced by neutrophils (N) forms immune complexes with self-nucleic acids, activating conventional and plasmacytoid dendritic cells (cDCs and pDCs, respectively) to release inflammatory cytokines which boost the autoimmune response. Besides, in psoriasis and in rheumatoid arthritis, posttranslational modifications of AMPs can generate neo-self-antigens recognized by effector T cells (Th1/Th17) and produce autoantibodies (AutoAbs). Moreover, in rheumatoid arthritis, AMPs directly support cartilage destruction by acting on osteoblasts and osteoclasts. On the other side, AMPs can also prevent autoimmune diseases, as shown in T1D. The gut microbiota-derived metabolites short-chain fatty acids (SCFAs) can induce AMP expression in pancreatic islets, and these AMPs directly or indirectly induce protective and regulatory immune cells including macrophages (Mregs), B cells (Bregs), and T cells (Tregs). In multiple sclerosis, mBD14 and *Fasciola hepatica* helminth defense molecule 1 (FhHDM1) show similar immunoregulatory functions and protective effects in a mouse model of the disease. h, human; m, mouse, BD, beta-defensin; HNP, alpha-defensin; SjS, Sjögren syndrome.

submandibular/sublingual saliva originates from the glandular blood vessel neutrophils (99). Besides, patients with morbus Kostmann have congenital neutropenia; neutrophils from these patients were deficient in LL-37, and no cathelicidin is detected by mass spectrometry and Western blot in the plasma and the whole saliva of these patients, suggesting that salivary LL-37 is indeed derived from neutrophils (100). The reports above support that chronic inflammation may be responsible for the increase of cathelicidin expression observed in SGs in the context of SjS. hBD1–3 mRNAs have been detected in SGs, including the parotid, submandibular, and minor glands, as well as the oral epithelium (101, 102). One study confirmed the expressions of hBD1–3 in SGs and showed by immunohistochemistry that the expressions of hBD1/2 were decreased in minor SGs from SjS patients compared with healthy subjects (103). By proteomic analysis, HNP1 expression was found specifically upregulated in the SGs of SjS patients, together with other inflammatory genes (104, 105). Unlike cathelicidin, hBD1/2 in SGs may largely derive from ductal epithelial cells, which may explain the decreased hBD levels as a consequence of the destruction of ductal epithelial cells during inflammation. Our laboratory is currently investigating how these AMPs may participate in the development of SjS. Regarding the above literature, it is tempting to speculate that cathelicidin or HNP-forming immune complexes may trigger type I IFN production by pDCs infiltrating the SGs; however, we could also hypothesize that cathelicidin or BDs are able to maintain immune tolerance in the SGs by inducing immunoregulatory immune cells.

Multiple Sclerosis

Multiple sclerosis is a chronic inflammatory disorder of the CNS. Hallmarks of the disease associate with multifocal demyelination, axonal loss, activation of glial cells, infiltration by innate and adaptive immune cells, and the presence of autoantibodies, together initiating the demyelination of axons (106, 107). AMPs appear to be part of the CNS immune system as defensins and cathelicidins are produced by a variety of cell types in the brain such as astrocytes and microglia (108, 109). Using RT-qPCR and Western blot, AMPs have been detected in the CNS of rodents and humans at steady state and in inflammatory conditions (109–113). Accumulating evidences support that infiltrating neutrophils may play an important role in the diseases affecting

the CNS (114), including MS (115); however, whether these neutrophils express cathelicidin remains unknown. Using the experimental autoimmune encephalomyelitis mouse model of MS, one study showed that recombinant mBD14 has a protective and even a therapeutic effect against the disease by directly stimulating Treg cells (116). Lastly, a study has shown that a parasitic cathelicidin-like peptide is protective against both T1D and MS in mouse models (117). Although more studies are required to support the role of AMPs in MS, AMPs may represent interesting therapeutic tools against MS.

CONCLUSION

Since their discovery 50 years ago as microbicidal molecules, AMPs appear today as key molecules in the regulation of the immune responses, and not surprisingly, the dysregulation of their expression participates in the development of various autoimmune diseases. However, the precise role of AMPs in autoimmunity seems complex, with both detrimental and protective effects even considering the same AMP and the same disease, such as cathelicidin in T1D (**Figure 1**). Understanding the opposite role of AMPs in autoimmune diseases is a crucial step before the development of new therapeutic strategies based on AMPs for resolving the progression of these diseases. AMPs are chemoattractants for various immune cells; however, the phenotype of these cells can be either inflammatory or regulatory. Overall, cathelicidin produced by neutrophils appears to be a potent inducer of type 1 IFNs and inflammatory cytokines favoring the development of the autoimmune responses. Recent studies also support that cathelicidin and HNPs from neutrophils are a source of autoantigens. On the other side, secretion of cathelicidin and BDs by the cells targeted by the autoimmune attack may represent a mechanism of protection *via* the induction of regulatory immune cells. One attractive hypothesis to explain the dual role of AMPs in AiDs is that the immune function of AMPs is related to posttranslational modifications of peptides, such as citrullination or carbamylation. Indeed, such modifications of cathelicidin reduce its positive charge, increase its chemotactic activity, and alter its ability to bind nucleic acids, thereby reducing their pro-inflammatory potential (118, 119). Also, modified AMPs may represent a source of autoantigens, but not their native forms. Importantly, modifications of susceptible proteins that occur in inflammatory conditions such as in activated neutrophils may represent a general mechanism of control of the inflammatory response. Whether this mechanism of innate immune tolerance is defective in the autoimmune context remains to be determined. Considering the growing knowledge about the role of AMPs in AiDs, it is tempting to suggest their use as therapeutic targets or agents to prevent or treat AiDs (120). However, due to their conflicting and pleiotropic immunomodulatory roles, the use of AMPs should be considered with care. Unexpectedly, a safer and efficient AMP-based therapy against AiDs may take advantage of their ability to shape the microbiota. Using this skill, AMPs may correct the pathological microbiota prevailing in autoimmune-prone individuals, hence preventing the development of AiDs.

AUTHOR CONTRIBUTIONS

WL and JD reviewed the literature, wrote the manuscript, and designed the figure.

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Discovery of Novel Type II Bacteriocins Using a New High-Dimensional Bioinformatic Algorithm

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Antimicrobial compounds first arose in prokaryotes by necessity for competitive self-defense. In this light, prokaryotes invented the first host defense peptides. Among the most well-characterized of these peptides are class II bacteriocins, ribosomally-synthesized polypeptides produced chiefly by Gram-positive bacteria. In the current study, a tensor search protocol—the BACIIα algorithm—was created to identify and classify bacteriocin sequences with high fidelity. The BACIIα algorithm integrates a consensus signature sequence, physicochemical and genomic pattern elements within a high-dimensional query tool to select for bacteriocin-like peptides. It accurately retrieved and distinguished virtually all families of known class II bacteriocins, with an 86% specificity. Further, the algorithm retrieved a large set of unforeseen, putative bacteriocin peptide sequences. A recently-developed machine-learning classifier predicted the vast majority of retrieved sequences to induce negative Gaussian curvature in target membranes, a hallmark of antimicrobial activity. Prototypic bacteriocin candidate sequences were synthesized and demonstrated potent antimicrobial efficacy *in vitro* against a broad spectrum of human pathogens. Therefore, the BACIIα algorithm expands the scope of prokaryotic host defense bacteriocins and enables an innovative bioinformatics discovery strategy. Understanding how prokaryotes have protected themselves against microbial threats over eons of time holds promise to discover novel anti-infective strategies to meet the challenge of modern antibiotic resistance.

Keywords: bacteriocin, host-defence, anti-infecive agents, computational biology, antimicrobial

INTRODUCTION

One of the most urgent threats facing medicine and society today is the emergence of multi-drug resistant (MDR) pathogens. Estimates from the World Health Organization and like agencies suggest deaths due to MDR infections will outpace nearly all other causes by the year 2050 (1, 2). Compounding this issue is reduced pharmaceutical investment in anti-infective drug discovery, yielding a dearth of mechanistically novel anti-infectives in the drug development pipeline.

Virtually all modern anti-infectives identified to date were originally derived from microbial sources. Among these, bacteriocins are the earliest host defense peptides (HDPs), derived from bacteria to protect against microbial competitors. Although they originated in prokaryotes, HDPs have been retained throughout evolution and have been identified in virtually all organisms from which they have been sought. Such HDPs are typically small, cationic and amphipathic, and structurally categorized as predominantly α-helical, β-sheet or more complex secondary structure architecture, such as the cysteine-stabilized-αβ peptides. Mechanistically, a body of experimental data indicated that cationicity and amphipathicity as distributed in 3-dimensional space are essential for antimicrobial functions of HDPs. For example, cationicity is likely important for their propensity to target electronegative microbial membranes, while amphipathicity is likely essential for subsequent membrane perturbing events.

Bacteriocins are represented by a number of highly diverse families created through ribosomal or non-ribosomal synthesis (3–6). Of those generated by ribosomal synthesis, perhaps the best characterized are the Class II bacteriocins produced mainly by Gram-positive bacteria (7, 8). Class II bacteriocins are typically small (<60 amino acids) and heat-stable, and often synthesized as pre-bacteriocins containing an N-terminal signal sequence that is cleaved during secretion (4, 7, 8). This family of bioactive peptides can be further subclassified: Class IIa (pediocin-like); Class IIb (dimeric); Class IIc (cyclic) (4, 8). Hallmark of the Class IIa bacteriocins is an N-terminal consensus sequence (KYYGNG[L/V]XCXKXXCXVDW) comprised of an anti-parallel β-sheet stabilized by a disulfide bridge that is integral to antimicrobial activity (4, 7)

Previous investigations seeking to find novel bacteriocin sequences largely used computational screens for a conserved signal peptide motif (9, 10). However, in many of these investigations, this signal term has been class-specific, such that genomic screens that do not account for degeneracy, codon-use biases or open-reading-frame limitations are negatively restricted. Hence, while highly specific, such scans have missed large groups of bacteriocin sequences (10). In the present investigation, a novel and high-dimensional bioinformatics strategy—the BACIIα algorithm—was developed to overcome the above limitations. It incorporates a relaxed signal peptide motif that is inclusive of consensus bacteriocin leader sequences, along with key physicochemical and genomic pattern recognition to selectively identify putative bacteriocins from published sequence databases. Furthermore, this algorithm targets the α-helical core element of bacteriocins as a means to power specificity and sensitivity. Application of the novel BACIIα protocol retrieved all families of known Class IIa and IIb bacteriocin peptides, validating its inclusive scope. Moreover, it discovered >700 putative new bacteriocin sequences, many from prokaryotes for which no bacteriocin had been characterized to date. The retrieved sequences were predicted by a validated machine-learning classifier (11–14) with high probability to induce negative Gaussian curvature (NGC) in target membrane structures, which is a hallmark of antimicrobial activity. As proof-of-principle, prototype bacteriocin candidates were synthesized and found to have potent microbicidal activity against a panel of medically-relevant and drug resistant pathogens. Together, these data suggest the BACIIα algorithm is a rapid and efficient tool to identify novel bacteriocins which have retained efficacy against MDR pathogens over an evolutionary timespan. In this light, a greater understanding of host defense peptides that prokaryotes use to protect themselves against microbial competitors holds promise for discovery and development of innovative anti-infectives to meet the burgeoning threat of multi-drug resistant infections.

METHODS

Generation of the Type II Bacteriocin Consensus Formula (BACIIα)

To identify a consensus formula inclusive of known class II bacteriocins, multiple sequence alignments integrating prototypic representatives of this family were carried out using CLUSTALW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) and refined using MEGA 6 (15). Sites of potential conservation were scored for residue or physicochemical identity to generate a 12-residue core consensus formula. In some cases, positions in the formula are degenerate for inclusivity, based on sequence or biochemical (polar residues) properties conserved at these positions. Initial sequence alignments were generated using CLUSTALW2, followed by manual adjustment to align the double glycine motif using MEGA 6 (15).

Screen for Amphipathic α-Helices Within Retrieved Dataset

This consensus formula, termed BACIIα, was then used with ScanProsite (https://prosite.expasy.org/scan-prosite/) to conduct computational pattern searches of the UniProtKB Swiss-Prot and TrEMBL databases (https://www.uniprot.org/). Search results were further filtered for: (1) protein size (<80 residues); (2) bacterial source; and (3) localized to the first 25 residues of the query protein with a "<X(0.25)" logical operator. Results were submitted as a sequence database against which additional pattern searches could be carried out using Prosite. This database was queried using a systematic degenerate amphipathic sequence formula strategy [(11); https://prosite.expasy.org/scan-prosite/] to scan for α-helical domains within the retrieved protein dataset. The formula was advanced sequentially one position at a time through 18 iterations to encompass an entire 18-residue helical wheel span. Iteration one of this query sequence is listed below:

X-[VILMCFWYAG]-[KRHEDNQSTAG]-
[KRHEDNQSTAG]-[VILMCFWYAG]-[VILMCFWYAG]-
[KRHEDNQSTAG]-[KRHEDNQSTAG]-[VILMCFWYAG]-
X-[KRHEDNQSTAG]-[VILMCFWYAG]

As mature bacteriocins are typically located near C-termini of holoproteins, search parameters included a "X(0.30)>" logical operator to restrict results to the final 30 residues of target proteins.

Physicochemical Parameter Determination

Retrieved datasets were subjected to batch analysis to compute physicochemical parameters. The isoelectric point (pI) of individual sequences was determined using ExPASy (https://web.expasy.org/compute_pi/), while the hydrophobic moment, mean hydrophobicity, net charge (K and R [+1], H [+0.5], D and E [−1]) and K and R residue frequency (N_K/N_K+N_R) were determined using Python programs coded for this purpose. Residue frequency analysis was carried out using the Sequence Manipulation Suite in Protein Stats (https://www.bioinformatics.org/sms2/).

Genomic Operon Characterization

To probe for unforeseen or novel bacteriocins, genomic regions surrounding uncharacterized hits were analyzed. A total of 20,000 base pairs (10,000 each upstream and downstream) from search hit sequences were scored for the presence of typical bacteriocin operon genes (e.g., ABC transporters, immunity proteins, pheromones). Sequences consistent with bacteriocin-operon genomics signatures were prioritized for further study.

Design of the BACIIα Algorithm

Multiple structural elements may impact antimicrobial activity of host defense peptides, including biochemical features such as sequence motifs and electrostatic charge. However, of key importance to overall antimicrobial function is how these physicochemical properties are distributed in 3-dimensional space. To improve specificity and probe for membrane-active amphipathic α-helical structures which are important for membrane permeabilization and antibacterial activity, sequences retrieved from 1° searches were subjected to further computational screens collectively comprising the BACIIα algorithm:

Amphipathic Helix Motif

The BACIIα sequence formula returns hits based on their sequence alignment. To assess 3-dimensional patterns, hit sequences were assessed using a recently-identified tool that identifies core signatures of α-helical antimicrobial peptides [termed the α-core; (11)]. This analysis enabled spatial patterns of residues encompassed in the helical domains of retrieved proteins.

Physicochemical Profile

Proteins were also scored for intrinsic physicochemical parameters including: electrostatic charge [Q]; hydrophobic moment [μH]; mean hydrophobicity [H]; isoelectric point [pI]; and lysine-to-arginine ratio (N_K/N_K+N_R). These analyses were performed using Python algorithms specifically created for this study. Hydrophobicity values were derived using the Fauchère and Pliska octanol-water interface scale (16). PI was calculated using the ExPASy Compute pI/MW tool https://web.expasy.org/compute_pi/).

Machine-Learning Validation

To further characterize the datasets retrieved by the BACIIα formula, a previously developed support vector machine (SVM)-based classifier (12–14) was used to screen the obtained sequences for antimicrobial activity. Briefly, the SVM classifier was trained to optimally partition known α-helical sequences present in the Antimicrobial Peptide Database [APD, http://aps.unmc.edu/AP/main.php; (17)] from decoy peptides with no reported antimicrobial activity. The SVM generated 12 descriptors from the peptide sequence and output a score σ specifying the distance of the peptide from the 11-dimensional hyperplane separating antimicrobial and non-antimicrobial sequences. Using small-angle X-ray scattering (SAXS) experiments, the σ scores were found to correlate with the ability to generate NGC by α-helical test sequences (12). Thus, a large, positive σ score correlates with the ability to induce NGC in membranes, whereas a negative σ score indicates a lack of NGC activity. This membrane curvature feature is characteristic of antimicrobial peptides that have cell membrane-permeating functions (12–14). Sequences retrieved from the α-core search tool were screened using this algorithm, and σ scores calculated. Spearman correlations were quantified between σ and α-core metrics using Mathematica software (https://www.wolfram.com/mathematica/online/).

Synthesis of Prototypic Bacteriocin Candidates

Select putative bacteriocin sequences were commercially synthesized (BioMatik, https://www.biomatik.com/) at a 100 mg scale. All sequences were authenticated for mass and amino acid composition and purified using RP-HPLC to >98% purity. Lyophilized peptides were reconstituted with double-distilled and deionized water (ddIH20) and stored in aliquots at −20°C.

Antimicrobial Assay

Antimicrobial assays of putative bacteriocins were performed using a well-established radial diffusion method at pH 5.5 (a surrogate for contexts of serum or acidic phagolysosomes) or 7.5 [a surrogate for bloodstream context; (18)]. These peptides were assayed for antimicrobial activity against a panel of human pathogens paired for susceptibility (S) or resistance (R) phenotypes: Gram-positive *Staphylococcus aureus* [ISP479C [S], ISP479R [R]; (19)]; Gram-negative *Salmonella typhimurium* [MS5996s [S], MS14028; (20)], *Pseudomonas aeruginosa* (PA01 [R]), *Acinetobacter baumanni* (17,928; [R]) and the fungus *Candida albicans* [36082S [S] or 36082R [R]; (21)]. In brief, logarithmic-phase organisms were inoculated (10^6 CFU/ml) into buffered agarose, and poured into plates. Peptides (10 μg) were introduced into wells in the seeded matrix, and incubated for 3 h at 37 °C. Nutrient overlay medium was

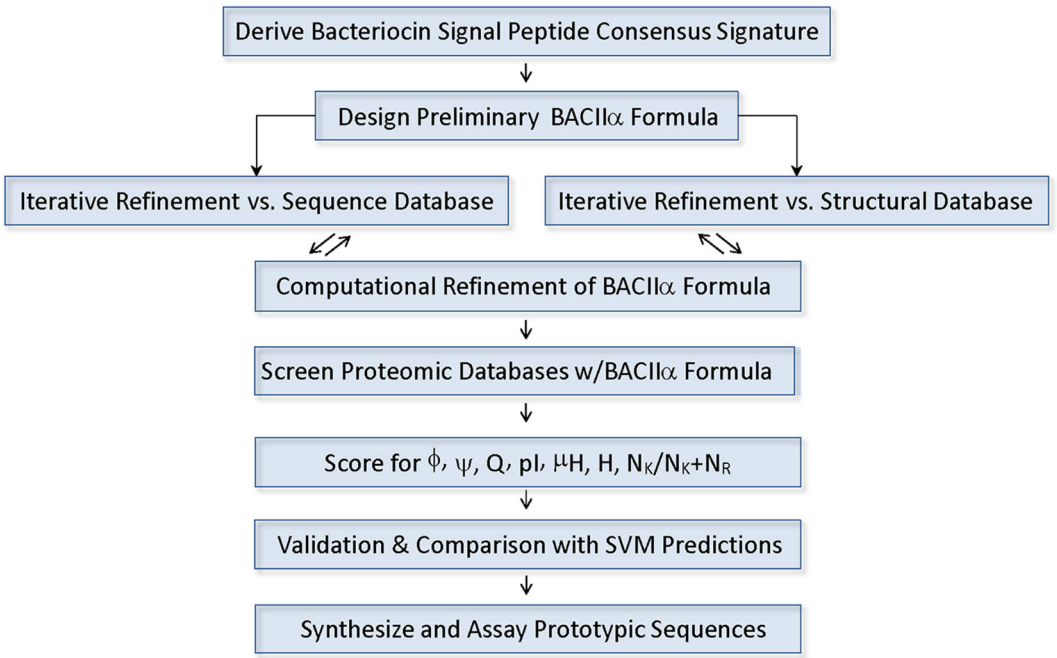

FIGURE 1 | Components and Process of the BACIIα Algorithm.

applied and assays incubated at 37 or 30°C for bacteria or fungi, respectively. Zones of inhibition were quantified after 24 h incubation. Independent experiments were repeated a minimum of three times, and assessed by parametric analysis for statistical significance (22).

RESULTS

Defining the BACIIα Probe Sequence Formula

A consensus formula consistent with the vast majority of known Class II (a-d) bacteriocins was identified and used to probe protein databased (**Figure 1**). Conserved residues in the signal peptide domain were used to generate a 12 residue consensus element comprising the formula:

−12	−11	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1
[LI]	[KREDNQSTYH]	X	[KREDNQSTYH]	X	[MLV]	X	X	[IVLT]	X	G	G

Notably, several positions within this formula were conserved predominantly at the level of physicochemical properties (positions −9 and −11). These positions are represented by degenerate search terms reflecting the propensity for a polar residue at these positions. Using this BACIIα probe sequence formula, a primary computational pattern search of the UniProtKB/Swiss-Prot and TrEMBL databases yielded a total of 3,050 sequences. Of the characterized sequences (706), the following bacteriocin-related classes were represented: bacteriocins (53%); competence enhancing peptides (18%); autoinducing peptides (2%); and pheromones (1%) (**Table 1**).

TABLE 1 | Retrieved sequences using BACIIα algorithm by stage of study.

Group	Signal peptide search	%	Amphipathic pattern search	%
Characterized sequences				
Bacteriocins	376	53	308	82
Competence enhancing peptides	129	18	6	2
Pheromones	7	1	1	0.3
Autoinducing peptides	12	2	8	2
Other	182	26	52	14
Total characterized Sequences	706	–	375	–

Collectively, 74% of known characterized sequences were bacteriocin or bacteriocin-related sequences.

Application of the BACIIα Algorithm

Applying the BACIIα algorithm, the total number of high-priority sequences was 1,563. Among the characterized sequences (375), 82% were bacteriocins and 4% included other bacteriocin-related sequences (**Table 1**). Hence, application of the BACIIα algorithm increased specificity for bacteriocins from 53 to 82%.

TABLE 2 | Bacteriocin peptides retrieved by multi-component formula search.

Class		Peptide	Organism
IIa	Acidocin	8912, LF221B, M	*Lactobacillus acidophilus*
	Avicin	A	*Enterococcus avium (Streptococcus avium)*
	Carnobacteriocin	A, B2, BM1	*Carnobacterium maltaromaticum*
	Curvacin	A	*Lactobacillus curvatus*
	Divergicin	750	*Carnobacterium divergens (Lactobacillus divergens)*
	Enterocin	B, 1071A/1B, CRL35, C2, NKR-5-3	*Enterococcus faecium*
	Leucocin	A, A-Qu 15, B, C, K, N, Q	*Leuconostoc gelidum, Leconostoc carnosum*,
	Mundticin	KS, L	*Enterococcus pallens ATCC BAA-351*
	PapA		*Listeria aquatica FSL S10-1188*
	Pediocin	PA-1, AcH	*Pediococcus acidilactici*
	Piscicolin	126	*Carnobacterium maltaromaticum*
	Plantaricin	A, F, J, 1.25 beta, NC8, c81F, S	*Lactobacillus plantarum*
	Sakacin	A, D98c, P, T, X	*Lactobacillus sakei*
IIb	Amylovorin	L alpha, L beta, L471	*Lactobacillus amylovorus*
	Bacteriocin	GatX, BacSJ2-8	*Streptococcus pneumoniae*
	Brevicin	925A T A/7B	*Lactobacillus brevis*
	Gassericin	705 alpha, 705 beta	*Lactobacillus gasseri*
	Lactobin/Cerein	LafA, LafX	*Streptococcus australis ATCC 700641*
	Lactocin		*Lactobacillus curvatus*
	Lactacin F		*Lactobacillus johnsonii*
IId	Lactococcin	A, A1, G beta, Q beta	*Clostridium perfringens (strain SM101 / Type A)*
	Mesentericin	B105, Y105	*Leuconostoc mesenteroides*
	Weissellicin	L	*Weissella hellenica*

Inclusion of bacteriocin-related peptides increased specificity to 86% within the subset of proteins having known functions. The resulting dataset included members from nearly all Class IIa and IIb bacteriocin families within the UniProtKb database (**Table 2**). In particular, the formula identified representatives from ∼90% of Class IIa families and 88% of Class IIb families. Class IId (other) structural class bacteriocins were less predominant (13%). As expected, representatives from the cyclic, Class IIc bacteriocin group, which do not contain a helical element, were not retrieved with this search. For many bacteriocins more than one representative of each family was retrieved; and in some cases a large number of family members were returned, such as for the Class IIb Lactobin family where more than 70 members were identified.

Origin Species Classification

The majority of sequences (bacteriocins and related) retrieved with the BACIIα formula originated from Gram-positive Firmicutes (74% [50% *Lactobacillus* spp.; 14% other *Bacillus* spp.; 10% *Clostridium* spp.]) and other Gram-positive organisms (Actinobacteria [2%]). Sequences were also retrieved from a number of Gram-negative organisms (**Table 3**). Additionally, a number of putative bacteriocins were retrieved from organisms for which bacteriocins have yet to be characterized.

TABLE 3 | Source organisms of retrieved dataset proteins.

Organism	Phylum	% Class	Class	% Subclass
Gram-Positive				
	Firmicutes	74		
			Lactobacilli	50
			Other bacilli	14
			Clostridiae	10
	Other	2		
			Actinobacteria	2
Gram-Negative	*Proteobacteria*	24		
			Bacteroides	13
			Proteobacteria	9
			Cyanobacteria	2
			Chlamydiae	<1.0
			Planctomycetia	<1.0
Non-gram staining		<1.0		
			Mollicutes	<1.0
Unclassified		<1.0		<1.0

Physicochemical Properties of Known Bacteriocins

Known bacteriocins retrieved using the BACIIα formula were analyzed for multiple physicochemical properties. The amphipathic spans of the 308 identified bacteriocins had the following average values: charge (Q), +1.1; hydrophobic moment (μH), 0.33; and mean hydrophobicity (H), 0.46 (**Table 4**). The lysine to arginine ratio (N_K/N_K+N_R) indicated lysine was preferred over arginine at an ∼2:1 ratio, particularly at positions 1, 8 and 15, nearer the termini of helices. Moreover, as the N_K/N_K+N_R ratio increased over amphipathic spans, so did net hydrophobicity (**Figure 2**). This finding suggests that lysine propensity is compensated by increasing hydrophobicity in bacteriocins or other HDPs (11, 23).

TABLE 4 | Biophysical properties of retrieved dataset proteins.

Group	*n*	%	μH	Q	N_K/N_K+N_R	H	PI
Known bacteriocins	308	22	0.33 (±0.2)	1.1 (±1.5)	0.71 (±0.3)	0.46 (±0.1)	6.8 (±2.3)
Bacteriocin-related*	15	1	0.51 (±0.1)	1.9 (±1.9)	0.85 (±0.9)	0.37 (±0.2)	8.5 (±2.3)
Non-bacteriocin	52	4	0.40 (±0.1)	0.4 (±1.5)	0.42 (±0.4)	0.43 (±0.2)	7.1 (±2.4)
Uncharacterized	1038	73	0.39 (±0.2)	0.1 (±1.5)	0.68 (±0.4)	0.41 (±0.4)	6.4 (±2.1)

**Includes pheromones, competence-inducing peptides and others.*
μH, hydrophobic moment; Q, charge; NK/NK+NR relative percentage of lysine vs. arginine; H, hydrophobicity; PI, isoelectric point. Values are presented ± standard deviation.

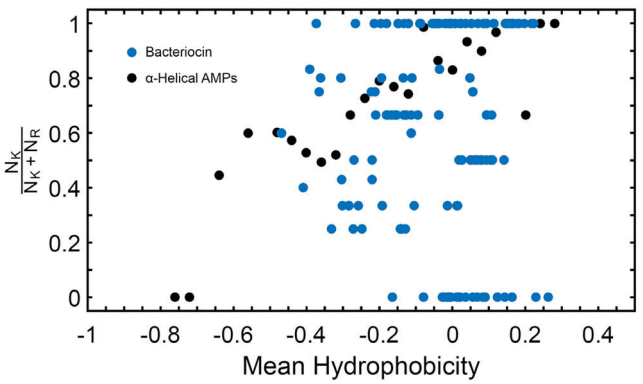

FIGURE 2 | N_K/N_K+N_R Ratio and Mean Hydrophobicity in Study Molecules. Percentage of lysine (N_K) relative to arginine (N_R) expressed as (N_K/N_K+N_R) vs. hydrophobicity (H) in study αHDPs Preference of lysine as compared to arginine is reflected in an increased value of H for peptides capable of generating NGC in membranes as predicted by the saddle-splay rule.

Global Residue Frequencies

Residue frequency analyses of known bacteriocins revealed an enrichment in certain residues. In particular, residues glycine and alanine collectively represented more than one third (35%) of all amino acids (**Figure 3A**). Of the charged residues, the basic amino acid lysine (5%) was the most abundant. Other cationic (R) and anionic (D, E) residues were represented at a lower frequency overall (∼3%). The aliphatic (non-polar) hydrophobes, leucine, isoleucine or valine had equivalent frequencies (6–7%), and occurred nearly twice as often as the aromatic hydrophobes phenylalanine, tryptophan or tyrosine (2.4–3.5%).

Positional and Spatial Residue Frequencies

The BACIIα formula identifies hits based on alignment to its sequence formula. Three-dimensional assessment is also informative regarding positional and spatial localization of residues along the identified amphipathic spans (**Figure 3B**). Glycine and alanine, the most abundant residues, were distributed across the amphipathic spans and found on both hydrophobic and hydrophilic facets with a similar frequency. On the polar facet, the next most abundant residues were the cationic residue lysine and neutral hydrophilic residues threonine and serine. On the non-polar facet, the most abundant residues were the aliphatic hydrophobes, valine, leucine and isoleucine.

Analysis of Uncharacterized Sequences

Beyond retrieving known bacteriocins, the BACIIα algorithm identified a large number (1,038) of as yet uncharacterized sequences. To assess this sequence dataset based on physicochemial properties of known bacteriocins, we applied a mathematical scoring system of factors inherent to membrane permeabilizing, microbicidal sequences (11). Hydrophobic moment (μH) and net charge (Q), represented by a combinatorial index μH*Q (HMQ), were quantified. These data were binned and values representing the top 25th and 50th highest HMQ quartiles (HMQ_{25} and HMQ_{50}) were derived. Application of these thresholds revealed a significant portion of the uncharacterized dataset ($n = 208$, HMQ_{25}; $n = 319$, HMQ_{50}) are likely to have antimicrobial properties (**Table 5**). Therefore, more than 700 (>74%) of the uncharacterized molecules retrieved by the BACIIα algorithm are putative novel bacteriocins.

Membrane Active Propensity

Search hits were assessed for membrane active propensity characteristic of antimicrobial peptides (**Table 5**). The sequence dataset was evaluated using a validated SVM machine-learning classifier for sequences capable of generating negative Gaussian curvature in model membranes (12–14). The SVM algorithm integrates specific physicochemical parameters such as amphipathicity (μH), charge (Q), and sequence-order. The output score, σ, quantifies confidence of this classification; high positive σ values have high probability of NGC which is characteristic of membrane permeabilizing, antimicrobial properties. The known bacteriocins retrieved were predicted to be membrane active, with average σ scores of 0.80 (HMQ_{25}) and 0.58 (HMQ_{50}). Likewise, a high percentage of the dataset encompassing unknown proteins was also predicted to have membrane permeabilizing activities with σ scores of 0.80 (HMQ_{25}) and 0.65 (HMQ_{50}). To test the accuracy of the BACIIα retrieved datasets relative to the SVM classifier, Spearman correlations were performed to assess monotonic ranking. This assessment revealed highly significant correlations ($R = 0.46$–0.74; range, $P = 2.5 \times 10^{-9}$ to 6.0×10^{-44}) between datasets generated by the two methods (**Figure 4**). This strong congruence suggests the BACIIα algorithm accurately detects unforeseen antimicrobial sequences (e.g., novel bacteriocins) and converges with the SVM on attributes conferring microbicidal properties.

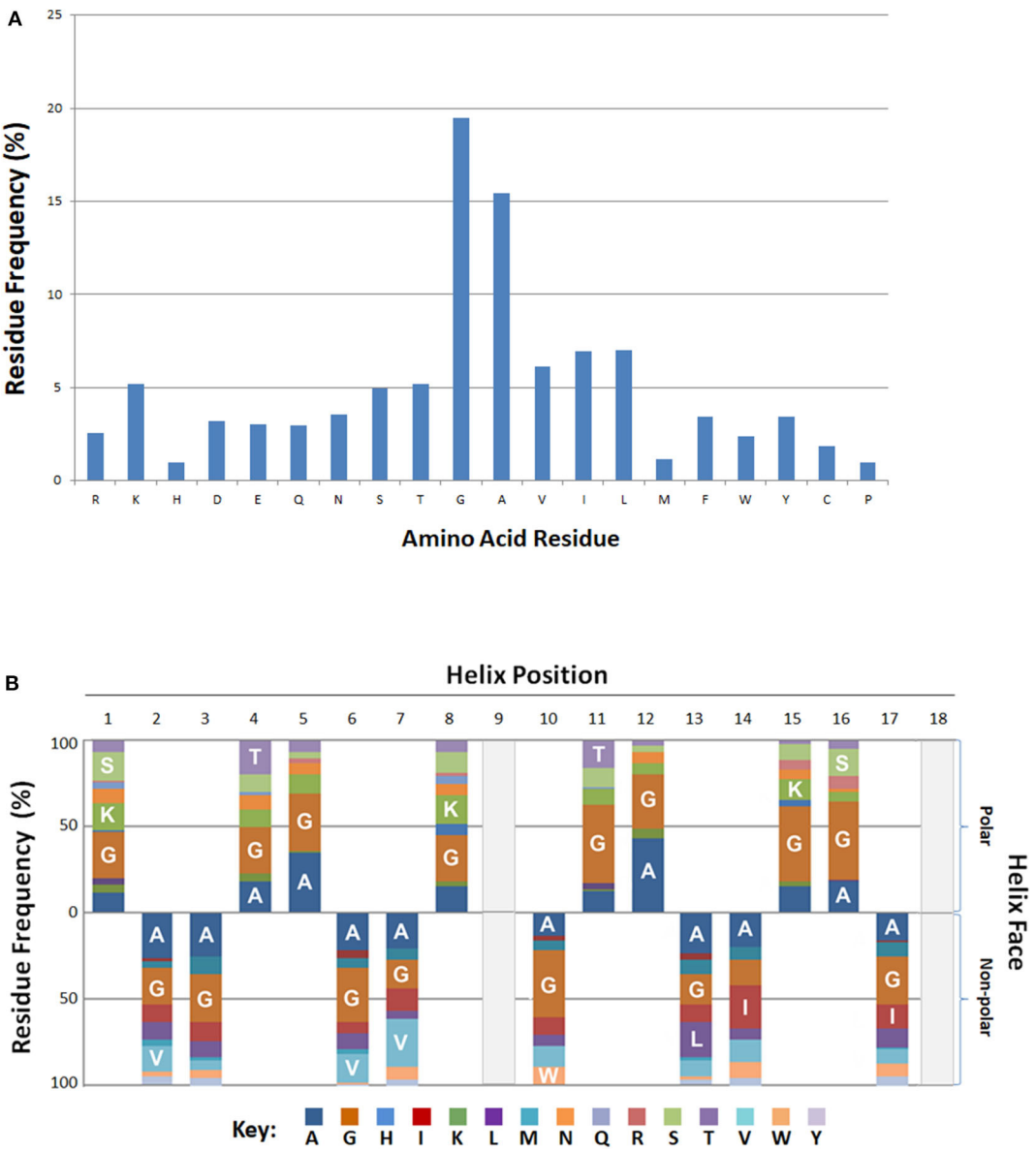

FIGURE 3 | Positional and Spatial Amphipathic Residue Frequency. **(A)** Relative amino acid percentages are displayed for bacteriocins. **(B)** Percentages of individual residues associated with either the polar or non-polar search term group are represented as various color blocks. Residues above the x-axis are associated with the polar residue group and residues below the axis are found on the.

Selection of Bacteriocin Candidates

Uncharacterized sequences representing putative novel bacteriocins were selected based on high BACIIα algorithm scores and genomic analyses. Among these, sequences from phylogenetically distinct organisms were chosen to assess correlates of source and target organisms: (SwissProt accession [*species*; study name]): A0RKV8 (*Bacillus thuringiensis*; peptide-1); D6E338 (*Eubacterium rectale*; peptide-2); B3ZXE9 (*Bacillus cereus*; peptide-3); R2S6C2 (*Enterococcus pallens*; peptide-4). At a genome level, peptides 1–4 localized to bacteriocin-like operons containing bacteriocin-associated genes (**Figure 5**). All were localized within 20 kb of an ABC transporter protein and ABC transporter accessory genes, such as C39 peptidases and ATP binding proteins. Candidate bacteriocins also localized within gene loci characteristic of known bacteriocin sequences and/or pheromones. In some cases, prototypic bacteriocin immunity peptides also localized to putative bacteriocin operons.

Antimicrobial Activity of Bacteriocin Candidates

Selected peptides 1–4 (**Figure 6**) were assessed for antimicrobial activity against a panel of human pathogens (**Figures 7A,B**). All

TABLE 5 | Quartile analysis of dataset protein properties vs. SVM scoring.

	Original *n*	Subset *n*	%	μH	Q	N_K/N_K+N_R	H	PI	σ
Category	Total				μH*Q > 1.0				SVM
Known bacteriocins	308	43	14	0.52	3.2	0.68	0.38	8.68	0.90
Bacteriocin-related	15	9	60	0.56	3.4	0.91	0.29	9.98	0.64
Non-bacteriocin	52	10	19	0.52	2.7	0.27	0.34	8.18	0.72
Uncharacterized	1,038	85	8	0.53	3.2	0.56	0.33	8.67	0.91
Category	Total				μH*Q > 0.50				SVM
Known bacteriocins	308	79	26	0.46	2.6	0.69	0.42	7.9	0.80
Bacteriocin-related	15	10	66	0.57	3.2	0.92	0.32	9.6	0.63
Non-bacteriocin	52	15	29	0.50	2.4	0.38	0.35	8.1	0.65
Uncharacterized	1,038	208	20	0.49	2.3	0.63	0.36	7.6	0.80
Category	Total				μH*Q > 0.25				SVM
Bacteriocins	308	161	52	0.36	2.1	0.75	0.42	7.2	0.58
Bacteriocin-related	15	12	80	0.52	2.8	0.76	0.35	9.1	0.54
Non-bacteriocin	52	16	31	0.50	2.3	0.4	0.35	7.9	0.67
Uncharacterized	1,038	319	31	0.44	1.9	0.65	0.38	7.2	0.65

*The μH*Q values represent different percentile cutoffs for peptide groups (dark orange, >1.0; middle orange; >0.50; and light orange, >0.25). Definition legend: μH–hydrophobic moment; Q–charge; N_K/N_K+N_R relative percentage of lysine vs. arginine; H–hydrophobicity; PI–isoelectric point.*

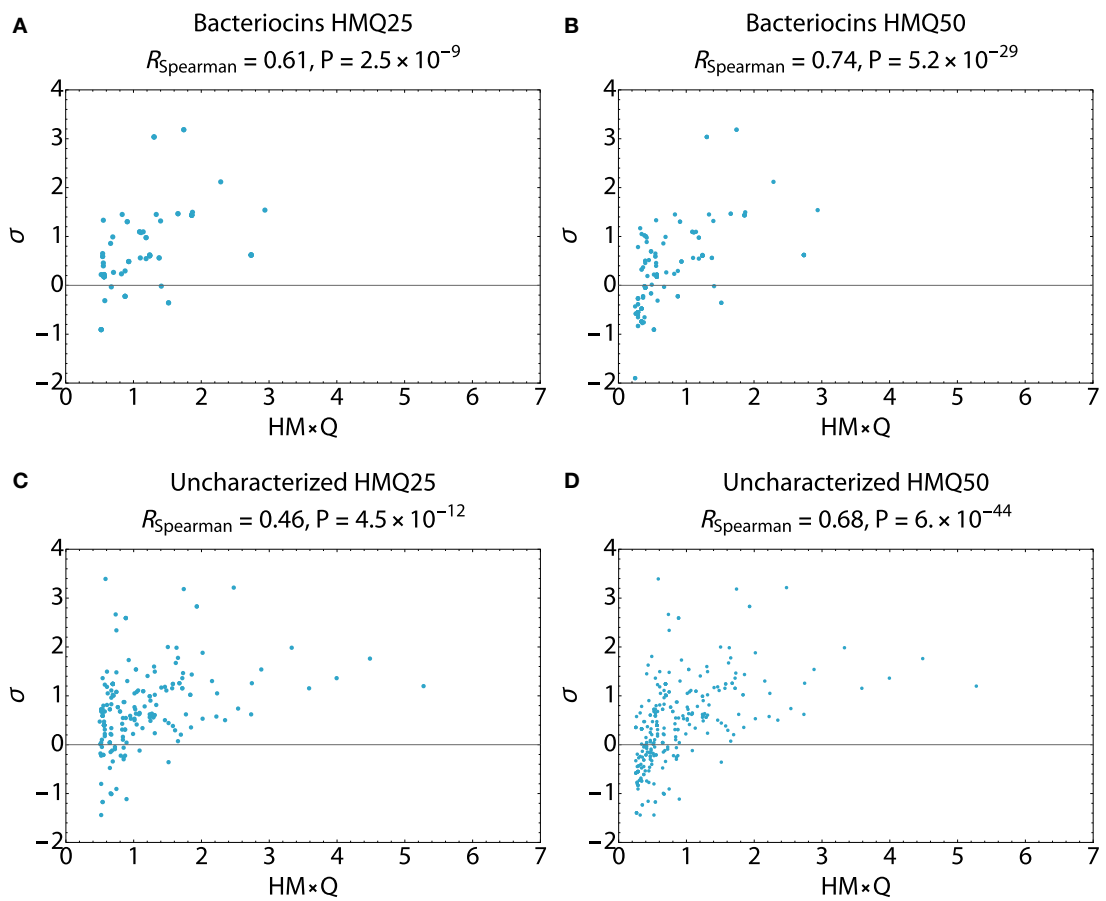

FIGURE 4 | Spearman Correlations Multi-Component BACIIα Formula and ML Classifier. Correlations were carried out to assess the predictive accuracy and monotonic ranking between the BACIα algorithm and the SVM classifier scored peptide sequences. Plots compare HMQ (BACIIα predictive) vs. sigma (classifier probability) scores for study peptides in the top 25th (HMQ25) and 50th (HMQ50) percentiles. The bacteriocin groups **(A,B)** display scores for identified bacteriocins. The uncharacterized groups **(C,D)** reflect those peptides which are also predicted to be membrane permeabilizing by the two protocols. All comparisons were found to be significant given a cutoff value of $P \leq 0.05$. Correlations were carried out using Mathematica (Wolfram).

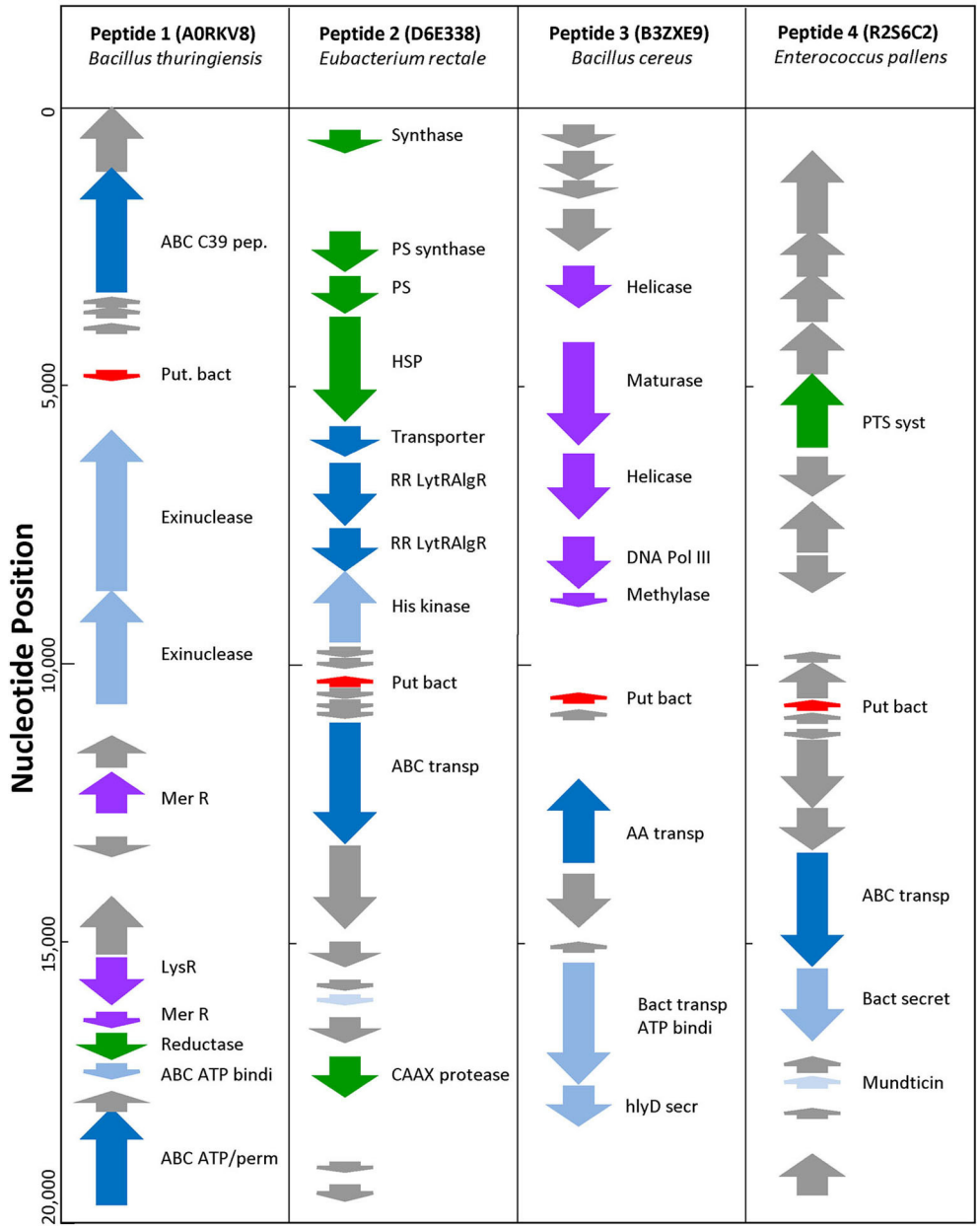

FIGURE 5 | Genomic Environment Surrounding Putative Bacteriocins. Analysis of 20 kb region surrounding putative bacteriocin genes. Red—putative bacteriocin; gray—hypothetical proteins; dark blue—C39 bacteriocin processing peptidase; medium blue—exinuclease ABC subunit; light blue—ABC transporter, ATP binding protein; green other enzyme; purple—polymerase related protein.

four putative bacteriocins possessed microbicidal activity against Gram-positive (*S. aureus*), Gram-negative (*S. typhimurium, P. aeruginosa, A. baumanni*) and a fungus (*C. albicans*). While active against all organisms tested, peptides 1–4 had generally greater activity vs. Gram-negative pathogens. The relative activity of peptides 1–4 was greater at pH 7.5 than at pH 5.5. Notably, peptide three lost nearly all activity against the Gram-positive pathogen *S. aureus* at pH 5.5. Beyond individual efficacy, cluster analyses reveal patterns of peptide efficacy against organism groups and as influenced by pH. For example, at pH 7.5, peptide one was relatively less active than the other peptides against all organisms except *Ps. aeruginosa* (**Figures 7C,D**).

DISCUSSION

Class II bacteriocins are typically small, cationic peptides of bacterial origin that often contain a conserved signal sequence important for downstream processing of the mature

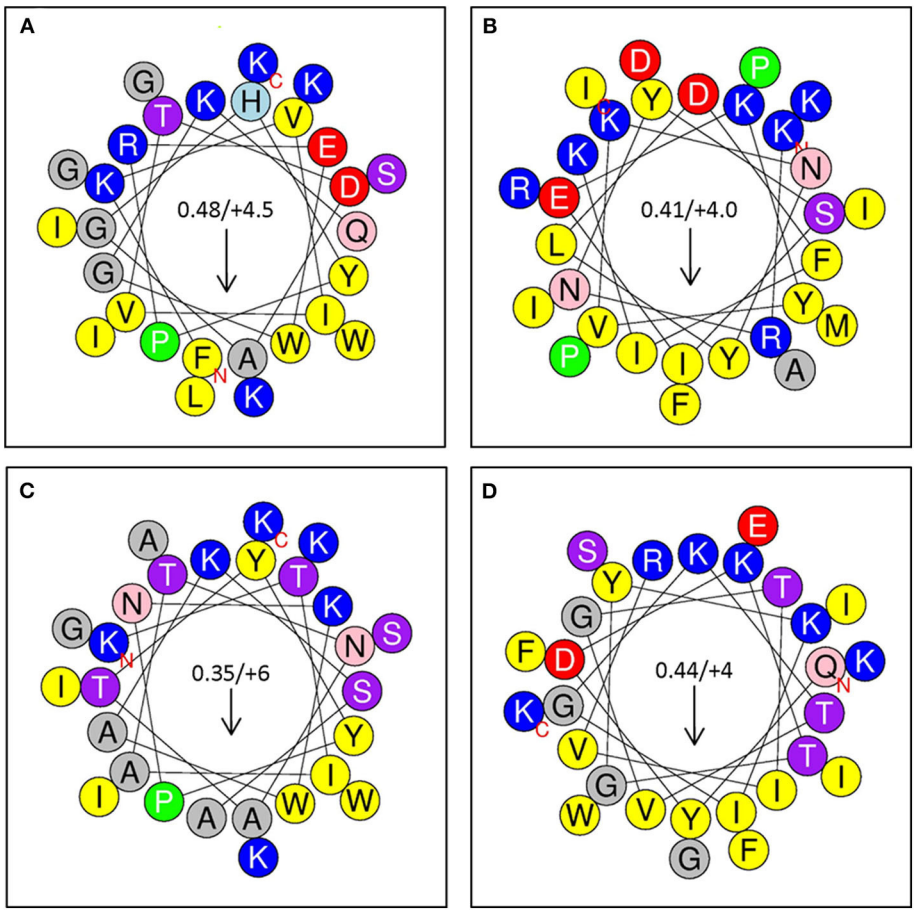

FIGURE 6 | (A) Sequence Analysis and Antimicrobial Activity of Putative Bacteriocins. Putative bacteriocins synthesized for assessment of antimicrobial activity. Arrows indicate hydrophobic moment and direction. **(A)** Peptide 1: A0RKV8 (+4.5), PI−10.7; *Bacillus thuringiensis* (G+); FKVIVTDAGHYPREWGKQLGKWIGSKIK (24); **(B)** Peptide 2: D6E338 (+4), PI 10.3; *Eubacterium rectale;* KRNYSIEKYVKNYIDFIKKAIDIFRPMPI (25); **(C)**; Peptide 3: B3ZXE9 (+6), PI−10.9; *Bacillus cereus*; KTIATNATYYPNKWAKSAGKWIASKIK (26). **(D)** Peptide 4: R2S6C2 (+4), PI−10.5; *Enterococcus pallens*, QYDKTGYKIGKTVGTIVRKGFEIWSIFK (24).

peptide. This leader domain is characterized as having a highly conserved double-glycine motif essential for proper cleavage of the bacteriocin precursor and maturation of the active mature peptide (4, 6, 27). Prior reports have made use of the signal peptide consensus to search for unidentified bacteriocin sequences in published genomic or proteomic sequence databases (28). However, these studies largely employed a very strict formulae [e.g., LSX_2ELX_2IXGG; (29)], often selecting only the most abundant residue at a position as a component of their search term. Hence, results conveyed a high degree of specificity, but had very limited sensitivity to identify novel bacteriocin molecules or classes within emerging proteomic databases.

In the present study, an alignment of more than 200 prototypic class II bacteriocins was carried out to generate an inclusive consensus formula. A primary component of this BACIIα formula was a convergent signal sequence. In addition to the C-terminal double glycine motif in this signal domain, the consensus formula included a strategic design to account for specific residues in key positions. For example, it allowed for inclusion of any polar residue at positions −9 and −11 of the signal peptide backbone. Further, a specific set of hydrophobic residues was allowed at positions −4 and −7. These features encompass the class II bacteriocin leader consensus originally identified by Nes and colleagues (6). The resulting consensus signature formula, BACIIα, represents an innovative probe for unforeseen bacteriocins. This formula retrieved members from nearly all known classes of type II bacteriocins, and the vast majority (∼90%) of Class IIa and IIb linear bacteriocins.

The BACIIα formula was used as the first step in the multifactorial BACIIα search algorithm designed to discover novel bacteriocins. To improve specificity for membrane-active sequences characteristic of antimicrobial activity, the BACIIα algorithm integrated a strategy to probe for α-helical domains in retrieved peptides (11). The current results are in concordance with Class IIa and IIb bacteriocin propensity to adopt α-helical conformation in membrane

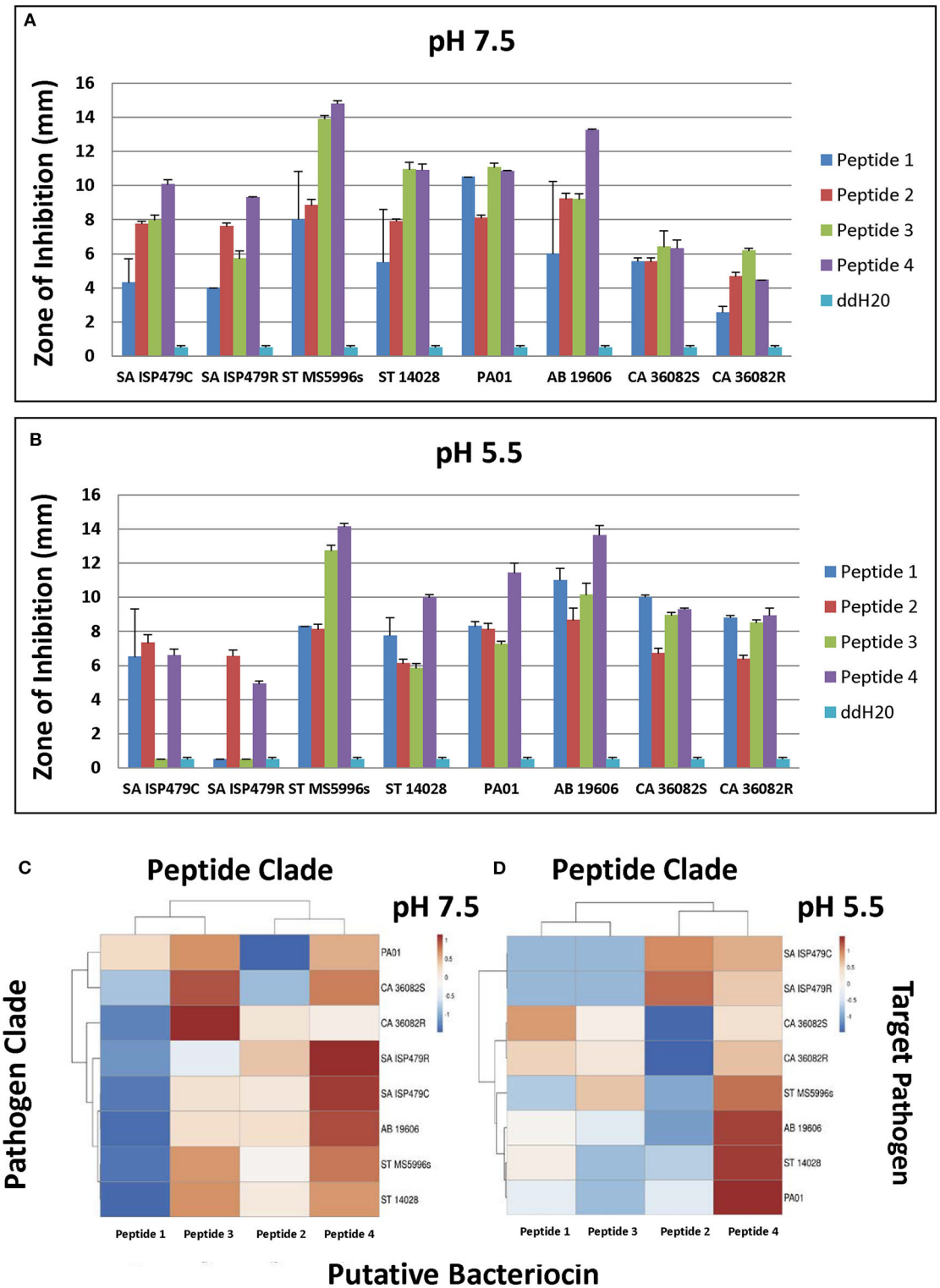

FIGURE 7 | Microbicidal activity of study test peptides vs. a panel of prototypic gram-positive (*S. aureus*), gram-negative (*S. typhimurium, P. aeruginosa, A. baumannii*) and fungal (*C. albicans*) pathogens at two pH representing: **(A)**–bloodstream (pH 7.5); or **(B)**–phagolysosomal/abscess (pH 5.5). Data represent experiments independently performed a minimum of $n = 3$ times. Error bars represent the standard error of the mean. All study peptides were found to have statistically significantly greater activity ($P < 0.01$) than the dilution vehicle (ddH_20) in at least one pH condition. Note the differential pH dependent efficacy of Peptide 3 against *S. aureus*. The relative efficacies of study peptides against representative organisms at pH 7.5 or pH 5.5 are shown in the cluster analyses in panels **(C,D)**, respectively (red, relatively greater efficacy; blue, relatively lesser efficacy).

mimetic environments (4). One notable exception was for the Class IIa pediocins, which were retrieved by the BACIIα formula, but not with the α-helix screen. This result would be expected, as many members of the pediocin-like bacteriocin group form a hairpin-like structure at the C-terminus (26). Given the high efficiency and specificity with which it captures bacteriocins, the BACIIα sequence formula and ensuing BACIIα algorithm provide a comprehensive strategy to reveal previously unrecognized bacteriocins. For example, the BACIIα search algorithm discovered putative bacteriocin sequences that were not returned using existing bacteriocin identification tools (e.g., BAGEL3; data not shown). As BAGEL3 employs an internal ORF calling component, its limits may reflect a difficulty of identifying the very small ORFs ($\leq$ 0.5 kb) that are typical of bacteriocins (24).

To support results of the BACIIα algorithm, retrieved bacteriocin candidates were analyzed using a validated SVM-learning classifier to score membrane-active propensity (12–14). The SVM analyses confirmed that the vast majority of proteins prioritized by the BACIIα algorithm were likely to have a propensity for generating NGC in membrane environments and be antimicrobial in nature. This congruence was supported by regression analyses that yielded robust statistical significance. Thus, the BACIIα and SVM protocols, which derive from highly divergent knowledge-based and machine-learning strategies, converge on the same set of bacteriocin candidates. As the SVM was previously shown to generate high σ values for eukaryotic HDPs, the current findings further suggest that core features integral to antimicrobial activity are conserved in HDPs from eukaryotic and prokaryotic hosts.

Residue frequency analysis of the BACIIα dataset revealed that alanine and glycine are strongly preferred among amphipathic spans in bacteriocins (>33% of residues). These residues are distributed to both the polar and non-polar facets in these proteins. Such findings lend support to a new hypothesis regarding the mechanism by which α-helical HDPs may limit self-toxicity. Specifically, an abundance of small, sterically-unrestrained residues with a high degree of rotational freedom (e.g., glycine and alanine) may serve to keep α-helical antimicrobial peptides in an unstructured and thus non-toxic conformation in aqueous environments. Only when adopting their amphipathic structure in context of the hydrophobic milieu of a target membrane do they become cytotoxic. The fact that HDPs typically have higher affinity for prokaryotic vs. eukaryotic membrane constituents enhances this antimicrobial specificity. Support for this hypothesis is provided by: (1) the abundance of glycine, and to a lesser extent alanine, in α-helical HDPs of many organisms (11); (2) structural studies (25, 30–32) finding that α-helical HDPs are often unstructured in aqueous solutions, and only adopt α-helical conformation in membrane environments; and (3) propensity for α-helical HDPs to target cardiolipin or phosphatidylglycerol moieties common to prokaryotic membranes, with less affinity for phospholipids or sterols more common to eukaryotic membranes. In the current study, the abundance of glycine and alanine in retrieved sequences suggests these peptides may also utilize a similar mechanism to limit self-toxicity. Prokaryotes also express other safeguards to protect themselves from the very bacteriocins they produce. For example, organisms which make bacteriocins also produce immunity proteins, encoded within the bacteriocin-producing operon, which help to minimize self-toxicity (4, 8). In this respect, bacteriocins made by one bacterium can preferentially kill other competitive or pathogenic bacteria or fungi. Therefore, bacteriocins have a plausible role in host defense against infection, be it the bacterium producing the bacteriocin, or the host in which it resides. These concepts form a fundamental tenet for the protective roles of the beneficial human microbiome (33, 34).

It was also of interest that neutral serine and threonine residues were more highly represented than many other uncharged (Q, N) and/or charged (R, H, D, E) polar residues. This finding reflects prior observations of a similar evolutionary preference for these small uncharged residues in eukaryotic HDPs (35). While the reason for this propensity is unknown, such residues may act as neutral "spacers" to aid incorporation of more biochemically reactive polar and charged residues within amphipathic HDPs. Also, given the availability of their hydroxyl moiety for H-bonding, serine and threonine residues may facilitate miscibility in aqueous vs. lipid environments (35, 36).

The current study also provided information regarding the global biophysical properties found within amphipathic bacteriocins. As similar studies have been carried out in eukaryotes (11), we were interested in whether the bacteriocin amphipathic domains differed substantively from those found in higher organisms with phylogenetically advanced immune systems, or whether key physicochemical parameters are essentially immutable (37–39). The bacteriocin sequences identified in the current study exhibited a net cationic charge, reflecting a property that is nearly universal in microbicidal HDPs of eukaryotes. Cationicity is thought to be important mechanism of selective HDP affinity for anionic membrane lipids (e.g., phosphatidylserine, cardiolipin and phosphatidylglycerol), which are enriched in prokaryotes, and inward rectifying net electronegative potential of many bacterial membranes (40–42). The bacteriocin sequences were moderately cationic with an average net charge of +1.1 (n = 308). By comparison, a parallel study using the same amphipathic search tool identified a somewhat higher net charge in eukaryotic HDPs (Q = +2.0; n = 907; 11). This difference in net charge was also reflected in the relative percentage of cationic residues within bacteriocin amphipathic spans (K+R = 8%) vs. those in eukaryotic HDPs (K+R = 16%). The biological reasons for the slightly lower charge density in bacteriocins are not known, but ostensibly could reflect the potential for a greater degree of compartmentalization of HDPs in eukaryotic cells, such that charged and potentially toxic microbicidal sequences are safely stored until targeted release.

Similarly, charge composition analyses revealed that of the cationic residues, lysine was preferred over arginine in the amphipathic spans of prokaryotic bacteriocins in the current study (K:R = 2:1), and in eukaryotes (K:R = 5:1) (11). Importantly, lysine and arginine residues interact with

membrane phospholipid head groups in fundamentally different ways. The single ε-amino group of lysine can only form a monovalent hydrogen bond with one membrane phospholipid headgroup at a time. In contrast, the guanidinium amino moiety of arginine can form multiple hydrogen bonds with phospholipid headgroups simultaneously. These differences lead to alternate membrane perturbation events, with arginine generating negative Gaussian curvature (NGC) oriented to achieve both positive and negative curvature along two perpendicular directions, whereas lysine generates only negative curvature. These biophysical constraints are supported by studies that have found that lysine is less efficient at generating negative Gaussian curvature (NGC), and pore-like structures, than arginine (12–14, 23). Notably, many lysine-rich HDPs have a net hydrophobic propensity, a feature that may compensate for this reduced permeabilizing efficiency, in a phenomenon known as the "saddle-splay" rule (23).

The observed preference for lysine over arginine common in the amphipathic spans of HDPs of prokaryotic and eukaryotic organisms suggest a crucial biophysical constraint within α-helical HDPs enabling membrane permeabilization. Several concepts support this hypothesis, including: (1) lysine-rich domains may be more energetically favorable for the transition from random coil to α-helical structures, as is common among these peptides; (2) reduced arginine frequency may make amphipathic helices less toxic toward "self" [relative to prokaryote (e.g., bacteriocin) or eukaryote (e.g., defensin) host] membranes; (3) a specific K/R ratio may facilitate a interaction with a cognate receptor or lipid II/LPS, and avoid off-target effects on ion channels; and (4) this ratio may confer some alternate evolutionary advantage.

Lastly, the BACIIα formula and algorithm retrieved a large number of sequences it classified as bacteriocins, but are as yet uncharacterized. As a proof-of-concept, several prototypes of these unknown sequences prioritized based on logical selection criteria were synthesized and assessed for antimicrobial activity. Notably, each of these peptides exerted activity against a broad spectrum of human pathogens, with generally greater activity vs. Gram-negative pathogens. In addition, each of the peptides demonstrated differential activity in pH conditions simulation bloodstream vs. abscess / phagolysosomal contexts. Historically, bacteriocins have been generally viewed as having relatively narrow spectrum activity, and greatest potency against closely-related Gram-positive organisms. However, more recent studies show that bacteriocins have broad spectra, with microbicidal activity against Gram-negative and fungal organisms as well (43, 44). It is interesting that HDPs from a variety of prokaryotes and eukaryotes can be active against fungi. There are at least two plausible targets of HDPs in fungi: (1) fungal envelope and/or cell membrane; and (2) mitochondria, which in effect are considered ancestral prokaryotic endosymbionts. With respect to the former, mechanisms for HDP targeting of fungi are believed to be related to unique components such as sphingolipids, glycolipids, phosphatidic acid and ceramides (45, 46). Considerable data suggest HDPs may target specific proteins integral to the fungal surface (47, 48). With respect to mitochondria, it is known that certain eukaryotic HPDs such as Histatin-5 target energized fungal mitochondria (49). Moreover, our previous work has demonstrated that HDPs can induce regulated cell death mechanisms leading to fungal cell death (50). These latter reports are in alignment with our current findings.

In summary, development of the BACIIα search formula and algorithm allowed for high-dimensional and rapid screening of proteomic databases to discover putative new bacteriocin species. Moreover, this process enabled characterization of essential features of prokaryotic bacteriocins, revealing fundamental similarities and differences with respect to analogous eukaryotic HDPs. These results offer key insights into essential, immutable features, as well as plasticity of evolution of HDPs from prokaryotes and eukaryotes. In this regard, such knowledge should improve our understanding of host defense against infection, and provide important templates for development of innovative anti-infectives.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NY conceived of studies, performed computational analyses, and wrote the manuscript. DW wrote programs for data analysis. JA performed computational analyses and assisted with writing the manuscript. EL performed computational analyses for the manuscript. ML provided input and assisted with writing the manuscript. GW conceived of studies and wrote the manuscript. MY conceived of studies and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Innate Inspiration: Antifungal Peptides and Other Immunotherapeutics From the Host Immune Response

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The purpose of this review is to describe antifungal therapeutic candidates in preclinical and clinical development derived from, or directly influenced by, the immune system, with a specific focus on antimicrobial peptides (AMP). Although the focus of this review is AMP with direct antimicrobial effects on fungi, we will also discuss compounds with direct antifungal activity, including monoclonal antibodies (mAb), as well as immunomodulatory molecules that can enhance the immune response to fungal infection, including immunomodulatory AMP, vaccines, checkpoint inhibitors, interferon and colony stimulating factors as well as immune cell therapies. The focus of this manuscript will be a non-exhaustive review of antifungal compounds in preclinical and clinical development that are based on the principles of immunology and the authors acknowledge the incredible amount of *in vitro* and *in vivo* work that has been conducted to develop such therapeutic candidates.

Keywords: antimicrobial peptide, host defence peptide, innate immunity, antifungal, immunotherapeutics

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INTRODUCTION

Medical and technological advances, improvements in hygiene and availability of vaccines to important life-threatening diseases means that since 1900 global average life expectancy has more than doubled and is now more than 70 years (1). Despite this, the prevalence of both life-threatening and superficial fungal infections has increased and has largely coincided with progress in the treatment of other diseases (2). Systemic fungal infections are significant causes of morbidity and mortality, responsible for the deaths of more than 1.6 million people per annum (3); comparable to tuberculosis and more than 3-fold higher than malaria. All fungal infections have risen in prevalence over recent decades, including allergic bronchopulmonary aspergillosis (ABPA) and superficial fungal infections, with the increased use of immunosuppressive medications for cancer and transplantation and patients with HIV/AIDS and other immunodeficiences (including genetic disorders), as well as indiscriminate antibiotic use, parenteral nutrition and permanent indwelling catheters. Climate change, pollution and environmental disruption are also considered likely to contribute to the increased incidence of fungal infection and fungal antigenicity (4–7). Defects in innate immune responses, including neutropenia, alveolar macrophage dysfunction, and mutations in STAT3 (resulting in autosomal dominant hyper IgE syndrome) and impaired NAPDH oxidase activity facilitate the development of pulmonary, and in some cases invasive, aspergillosis (8), whereas mutations in the gene for CARD9 (signaling adaptor protein for the C-type lectin receptor) results in increased susceptibility to many types of fungal infection,

including dermatophytosis (9, 10). The reasons for the increased incidence of fungal infections over recent decades are beyond the scope of this manuscript and readers are directed to several excellent reviews on the subject (3, 4, 7, 11–15). Fungi are ubiquitous throughout nature and we are constantly exposed to these microbes from the environment via inhalation, ingestion or on epithelial surfaces including the skin and mucosae (16–21). Most fungi are not pathogenic to humans, and most of those that are do not cause life-threatening infections in immunocompetent individuals and such infections are relatively rare. Of the fungi that are able to colonise the human body, many co-exist (commensalism) without causing infection under normal circumstances, e. g. *Candida* spp. (22, 23). *Candida* spp., (∼750,000 cases of invasive candidiasis/year) *Cryptococcus* spp. (∼225,000 cases per annum in AIDS patients/year) and *Aspergillus* spp. (∼3.75 million cases of chronic pulmonary or invasive aspergillosis/year) are responsible for a significant number of life-threatening fungal infections, whereas other fungi are responsible for substantial levels of systemic infection, including *Pneumocystis* spp. (∼500,000 cases/year), *Histoplasma* spp. (∼500,000 cases/year), *Coccidioides* spp. (∼25,000 cases/year) and mucorales (>10,000 cases/year) (4, 11). Fungi cause superficial infections of the skin, hair, nails and mucosal membranes, including dermatophytes, *Candida* spp. and *Malassezia* spp. that are normally readily treatable. There are at least 1 billion cases of superficial fungal infection each year and this is both under-reported and increasing in incidence (3, 24). Dermatophytes are the main cause of superficial fungal infections and each year 20–25% of humans and animals suffer dermatophyte infections (25). Fungal exposure is also thought to contribute to allergies and worsening of asthma symptoms (e.g., ABPA), affecting millions of individuals worldwide (8, 26, 27). Difficulties in diagnosis, the limited antifungal armamentarium, the lack of any fungal vaccines and our limited understanding of the immune response to fungal infection all contribute to this disappointingly high level of morbidity and mortality (**Table 1**).

There is a limited armamentarium of antifungal drugs for the treatment of fungal infection and significantly, drug resistant fungal infections are emerging as important clinical challenges (46–51). Currently available antifungals fall into a limited number of classes; polyenes (e.g., amphotericin B and nystatin), azoles (e.g., fluconazole, itraconazole, voriconazole, isavuconazole, efinaconazole and posaconazole), echinocandins (e.g., caspofungin, anidulafungin and micafungin), allylamines (e.g., terbinafine and naftifine) and other lesser used or topical therapies including flucytosine, ciclopirox olamine, tavaborole, amorolfine, butenafine, griseofulvin, tolnaftate and natamycin. Most serious fungal infections are treated with drugs from only 3 classes; azoles, echinocandins and the polyene, amphotericin B (46, 52) and therefore, resistance to one class of antifungal limits treatment options to a significant degree (51). Whilst resistance rates are low compared to those, for example, of the bacterial ESKAPE pathogens, ∼3% of *A. fumigatus* are resistant to more than one azole, whereas 1.0–1.5% of *Candida* spp. are resistant to echinocandins and rates of resistance are increasing (47, 48, 51). Analogous to antibiotic resistance, antifungal resistance may be caused by acquired resistance mechanisms as well as primary resistance (also referred to as inherent resistance). For example, azole antifungals inhibit the ergosterol biosynthesis pathway (an essential component of the fungal cell membrane) by targeting lanosterol 14-α-demethylase, encoded by Erg11 in yeasts and Cyp51A/Cyp51B in filamentous fungi. Resistance to azole antifungals can be as a result of over-expression of the target gene (*ERG11*), loss of function of other enzymes involved in ergosterol biosynthesis (e.g., Δ-5,6-desaturase enzyme Erg3), up-regulation of multidrug transporters (e.g., Cdr1, Cdr2 and Mdr1 in *Candida* spp.), genome plasticity causing chromosomal duplications (aneuploidy) and the inherent resistance of *C. auris* to fluconazole (48). The recent emergence of *C. auris*, a predominantly nosocomial pathogen first isolated from a patient in 2009, is associated with high rates of mortality and antifungal resistance. In the US ∼90% of *C. auris* isolates are fluconazole resistant, 30% are amphotericin B resistant, although <5% of isolates are resistant to echinocandins. Additionally, multi-drug resistance of *C. auris* has commonly been reported, as has its ability to persist following disinfection of surfaces (49, 50, 53).

Clearly, new therapeutic options for the treatment of fungal infections are urgently needed (54). The global antifungal drug market was valued at US $11.92 Bn in 2018 and is expected to grow to US $13.87 Bn by 2026 (fiormarkets.com, 2020)[1]. Understanding the immune responses to fungal infection is essential for the rational design of more effective therapies and therefore improved patient outcomes in the future. Depending on the site and type of infection, the immune response can mount fungus-specific and/or site-specific antifungal responses. The development of antifungal drug candidates that replace or correct defective elements or dysregulation in appropriate immune responses to fungal infection and/or enhance the host immune response appear to be logical starting points for the development of new antifungal therapies. Despite the prevalence of fungal infection, its significant morbidity and mortality and the increasing problem of antifungal resistance, antifungal drug development has been under-represented in the development of antimicrobials. The design and development of antifungal therapeutics is, arguably, more complex than the design of antibacterial drugs, as both humans and fungi are eukaryotes and therefore share many common cellular features (55). One of the most obvious differences between fungal and mammalian cells is the cell surface (cell membrane and wall in the case of fungi) and it is perhaps no surprise that the most successful antifungal drugs available today target fungal cell walls (echinocandins) or membranes (azoles, amphotericin B). If we are to design future generations of antifungal drugs, we should look to the immune system as this can readily distinguish between fungi and self and to target fungi for eradication. AMP are one such example of this and are ripe for exploitation as antifungal therapeutic candidates as we discuss in this review (56–59).

[1]Antifungal Drugs Market by Drug Class (Azoles, Echinocandins, Polyenes, Allyamines, Others), Indication, Dosage Form, Regions, Global Industry Analysis, Market Size, Share, Growth, Trends, and Forecast 2019 to 2026. Available online at: https://www.fiormarkets.com/report/antifungal-drugs-market-by-drug-class-azoles-echinocandins-407129.html

TABLE 1 | Human fungal infections, incidence and treatment options [adapted from (3)].

Infection	Fungus	Infection type	Infection site	Incidence (cases per annum/global burden)	Therapeutic options	Reference/s
ABPA[1]	*Aspergillus* spp.	Allergic	Lung	~5 M (GB[2])	Glucocorticoids ± itraconazole	(28)
Pulmonary aspergillosis[3]		Severe	Lung	~3 M (GB)	Voriconazole, itraconazole	(29)
Invasive aspergillosis		Severe	Disseminated	>300 K	Voriconazole	(30)
Oropharyngeal candidiasis	*Candida* spp.	Mucosal	Mouth	~3.3 M	Oral nystatin, miconazole or clotrimazole[4]	(31)
Vulvovaginal candidiasis		Mucosal	Genitourinary tract	~134 M (GB)	Topical antifungal, fluconazole	
Invasive candidiasis		Severe	Disseminated	~750 K	Echinocandin, fluconazole	
Cryptococcosis	*Cryptococcus* spp.	Severe	Lung, CNS[5], disseminated	~225 K	Fluconazole, amphotericin B + flucytosine	(32)
Tinea	Dermatophytes (e.g., *Trichophyton rubrum*)	Superficial	Skin, hair, nails	>1,000 M (GB)	Terbinafine, itraconazole	(33)
Severe dermatophytosis		Invasive	Disseminated	Very rare	Terbinafine, itraconazole, posaconazole	(34)
Mucormycosis	Mucorales (e.g., *Rhizopus oryzae*)	Severe	Rhinocerebral, lung, skin, disseminated	>10 K	Amphotericin B, posaconazole, isavuconazole	(35)
Chromoblastomycosis	Chaetothyriales (e.g., *Exophiala dermatitidis*)	Severe	Skin	>10 K (GB)	Itraconazole, terbinafine, posaconazole	(36)
Coccidioidomycosis	*Coccidioides* spp.	Severe	Lung, skin	~25 K (GB)	Fluconazole	(37)
Paracoccidioidomycosis	*Paracoccidioides* spp.	Severe	Lung	~4 K (GB)	Itraconazole, amphotericin B	(38)
Histoplasmosis	*Histoplasma* spp.	Severe	Lung	~600 K	Itraconazole	(39)
Sporotrichosis	*Sporothrix* spp.	Severe	Skin, lung, disseminated	>40 K	Itraconazole, amphotericin B	(40)
Pneumocystis jirovecii pneumonia	*Pneumocystis jirovecii*	Severe	Lung	~500 K	Trimethoprim/ sulfamethoxazole	(41)
Eumycetoma	Fungi (e.g., *Scedosporium* spp.)	Severe	Skin	~9 K (GB)	Itraconazole	(42)
Fungal Keratitis	Fungi (e.g., *Fusarium* spp.)	Superficial	Eye	~1 M (GB)	Voriconazole	(43)
Fungal rhinosinusitis	Fungal antigens	Allergic	Lung	~12 M (GB)	Corticosteroids	(44)
Talaromycosis	*Talaromyces marneffei*	Severe	Skin, lung, liver, disseminated	~8 K	Amphotericin B, itraconazole, voriconazole	(45)

[1] *Allergic bronchopulmonary aspergillosis.*
[2] *Global burden.*
[3] *Includes aspergilloma.*
[4] *For more severe cases oral or intravenous fluconazole can be administered.*
[5] *Central nervous system.*

INNATE IMMUNITY AND HUMAN FUNGAL INFECTIONS

In immunocompetent individuals, innate immunity is the first-line of defence against invasive fungal infection. Host defence peptides (HDP), also termed antimicrobial peptides (AMP), form a key part of the innate immune response to infection and inflammation (60–62). HDP have been found at most sites in the human body, including the oral cavity, skin (including sweat and wound fluid), lungs, blood, tears, gastrointestinal tract, urinary tract & reproductive organs, breast milk and cerebrospinal fluid (63). A number of HDP are produced constitutively by epithelia and this basal level of HDP production can provide a first line of protection against fungal infection. Continuous interactions between fungal pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) and host pattern recognition receptors (PRR) initiate low levels of NF-κB activation that drives amplified expression of HDP-encoding genes (64–66). Upon greater levels of colonisation, inflammation and/or epithelial damage, expression of HDP genes, and concomitant HDP production, increases significantly (67, 68). For example, human β-defensins, cathelicidin and other HDP are considered integral to the innate immune response to fungal infection in the skin (69), whereas histatins are considered key effectors in the oral cavity (70). In addition to direct antimicrobial activity, HDP can act as immune modulators, for example, by promoting migration of neutrophils and monocytes to the site of infection, by upregulating tumour necrosis factor alpha (TNF-α) production and by chemoattraction of immature dendritic and T cells to modify the adaptive immune response (61, 62, 71). Perhaps unsurprisingly, most studies on the antimicrobial activities of AMP have focused on their antibacterial properties. Most research on antifungal AMP has been directed against *Candida* spp, especially *C. albicans*, with a smaller number of studies assessing activity against *A. fumigatus*, *Cryptococcus neoformans* and the questionably relevant *S. cerevisiae*. Thus, the direct antifungal activity of HDP, and most other AMP, may be significantly under-realised. In this review we will focus on the direct antifungal activity of AMP and anti-biofilm properties where relevant, but the immunomodulatory properties of HDP/AMP are largely beyond the scope of this manuscript and readers are directed to several excellent reviews on this subject (61, 62, 72–74).

Histatins

Histatins (Hst) are small histidine-rich HDP with an α-helical conformation in membranes. Histatins, and derivatives, have been investigated for their potential to treat localised infections, including vulvovaginal candidiasis, skin infections, cystic fibrosis lung infections, mucositis and gingivitis/periodontitis (75, 76). First isolated from human parotid saliva, Hst are also found in the saliva of other higher primates. Histatins are secreted by the parotid and submandibular salivary glands. Histatins comprise 12 structurally related members of which Hst-1 and Hst-3 are full-length proteins encoded by two genes, HTN1 (encoding Hst-1) and HTN3 (encoding Hst-3). The smaller proteins, Hst-2 (derived from Hst-1) and Hst-4 to−12 (derived from Hst-3), are generated by proteolytic cleavage of the parent Hst by salivary proteases during secretion (59, 77, 78).

Histatins comprise 3 main HDP (Hst -1, -3 and -5), of which Hst-5 (**Figure 1A**) has the most potent antifungal activity and can be found at concentrations of 15–30 μM in whole saliva (80). Fungicidal activity of Hst has been demonstrated against *Candida* spp. (albeit with little or no activity against *C. glabrata*), *Cryptococcus neoformans* and *A. fumigatus* (70, 81–83). In a study on the efficacy of Hst-5 on *Candida* spp. biofilms, Hst-5 was not effective against planktonic *C. glabrata* (2 isolates; $IC_{50} > 100$ μM). However, Hst-5 was effective against preformed biofilms of *C. albicans* and *C. glabrata* on poly(methyl methacrylate) discs, resulting in a 50% reduction in biofilm metabolic activity at concentrations of 1.7–62.5 μM (83, 84), albeit less effective than 0.12% chlorhexidine gluconate (84). Hst-1, -3 and -5 can also inhibit germination of *C. albicans* spores, leading to reduced virulence and ability to cause infection (85, 86).

Unlike the membrane-active defensins and cathelicidin, Hst act at multiple levels by mechanisms of action conserved across the Hst family of AMPs. Histatins bind metal ions, including copper, and the presence of Cu improved the antifungal activity of Hst-5 against *C. albicans* (87). In *C. albicans*, Hst-5 binds to fungal cell wall glycans, predominantly β-1,3-glucan (88), and cell wall proteins Ssa1 & Ssa2. Hst-5 is transported into the cell via the fungal polyamine transporters Dur3 and Dur31 in an energy-dependent process (76), and it is the lack of these transporters that forms the basis of the lack of sensitivity of *C. glabrata* to Hst-5 (89). Hst-5 can also be internalized by endocytosis (76) and by direct uptake via interaction with the plasma membrane (90). Hst-5 causes release of K^+, via the ion transporter Trk1, which causes osmotic imbalance and a consequent loss of cell volume and viability (76, 91). Hst also induce formation of reactive oxygen species (ROS), ATP efflux, inhibition of oxidative phosphorylation and metal ion chelation and these properties could contribute to the fungicidal activity of Hst-5 (76, 92–94). Human saliva also contains other non-immune proteins with antifungal properties, including lactoperoxidase, lactoferrin and lysozyme (95, 96). Interestingly, the antifungal caspofungin (inhibitor β-1,3-glucan biosynthesis) causes a loss of β-1,3-glucans in the *Candida* spp. cell wall, resulting in reduced susceptibility to Hst-5 (88).

Additionally, antibacterial properties of Hst-5 against ESKAPE pathogens have been demonstrated, including anti-biofilm properties (97). Hst may also exert their antimicrobial activities by inhibiting host and microbial proteases and may attenuate tissue damage and microbial propagation during the onset of disease (63). Hst have other functions in the oral cavity, including acceleration of wound healing, tooth enamel mineral homeostasis and pellicle formation (78, 98). Hst-1, -2 and -3, but not Hst-5, can promote re-epithelialization and angiogenesis during wound healing (78) and can prevent the translocation of bacteria across cell layers (99).

Defensins

There are three distinct families of defensins, α-, β- and θ-defensins which are cationic AMP characterised as three-stranded β-sheet folds stabilised by three conserved and

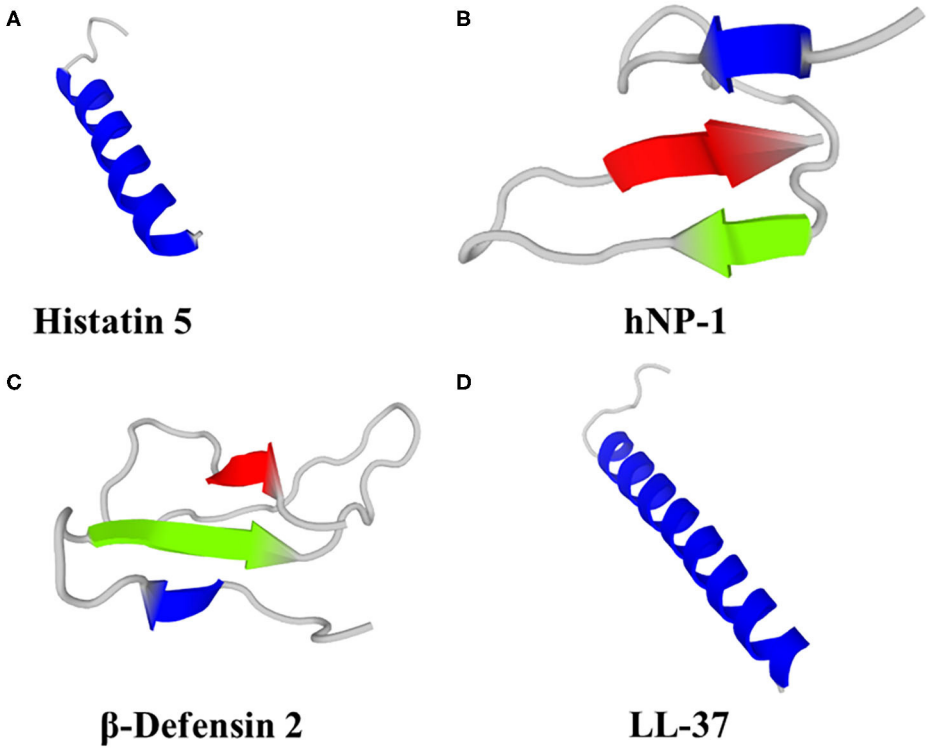

FIGURE 1 | Predicted 3D structures of human HDP; **(A)** Histatin 5, **(B)** Neutrophil Peptide 1 (α-defensin), **(C)** β-Defensin 2 and **(D)** LL-37. Models were generated using PEP-FOLD 3 (79).

regiospecific disulphide bridges. Humans produce only α- and β-defensins. In addition to their antimicrobial activity, roles in immunomodulation, fertility, development and wound healing have also been indicated (67, 100–102). The immunomodulatory activities of β-defensins include pro-inflammatory responses via recruitment (chemoattraction) of monocytes, macrophages, immature dendritic cells (DC) and T cells to sites of infection/inflammation, thereby providing a link between the innate and adaptive immune system (101, 103–106).

Humans produce six α-defensins; 4 are produced by neutrophils and some other myeloid cells [Human Neutrophil Peptides (HNP) 1–4] and a further two α-defensins (HD-5 and HD-6) are produced by the Paneth cells of the small intestine and some epithelial cells in the reproductive tract (HD-5 only) (101, 107). HNP-1 (**Figure 1B**) can kill *C. albicans* by depleting intracellular ATP (108) and was fungicidal against *A. fumigatus* (109). HNP-3 demonstrated limited activity against *Cryptococcus neoformans* planktonic cells and biofilms; 72 and 80% survival, respectively, after exposure to 8 μM HNP-3 for 30 min (110). HD-6 prevented adhesion of *C. albicans* to human intestinal epithelial cells, thereby preventing biofilm formation and cell invasion, but not hyphal transition. HD-6 functionality against *C. albicans* is dependent on the self-assembly properties of HD-6 and is non-lethal. HD-6 self-assembles into oligomers, termed nanonets, that entrap pathogens, including *C. albicans*, and prevent them from entering host cells (111).

Humans produce 4 β-defensins (hBD-1 - 4), primarily from epithelial cells that form biological barriers to pathogens at internal-external interfaces of the skin, gastrointestinal tract, respiratory tract and urogenital tract. Computational and bioinformatic approaches suggest at least 28 human β-defensin genes (112). Human β-defensins have direct antimicrobial activity, including via membrane permeabilization, against bacteria, fungi, viruses and unicellular parasites, as well as roles in immunomodulation, reproduction and pigmentation. Human β-defensin 1 is constitutively expressed, whereas hBD-2 (**Figure 1C**), 3 and -4 are induced in response to various stimuli, including inflammation and infection (101). hBD-1, hBD-2 and hBD-3 killed *C. albicans* by membrane permeabilization (113), hBD-2 was fungicidal against *A. fumigatus* (109) and hBD-3 was fungicidal against *C. glabrata* (114). hBD-1 in reduced form (i.e., lacking disulphide bridges) demonstrated activity against *C. albicans*, unlike the oxidised form, and is found in human colonic mucosa, small intestine crypts and skin epidermis (115). hBD-2 and hBD-3 reduced *C. albicans* adhesion by mediating elevation of Xog1 activity (116). hBD-2 and hBD-9 gene expression was induced by *A. fumigatus* and hBD-2 peptide co-localised with *A. fumigatus* conidia that had been phagocytosed by A549 cells (human alveolar basal epithelial adenocarcinoma cells), but not hyphae (117). Antifungal properties of hBD-1, hBD-2 and hBD-3 have been demonstrated against *C. albicans* (113), including antibiofilm properties of a 15 amino acid fragment from the C-terminus of hBD-3 (118). hBD-1 and hBD-3 were active against

Cryptococcus neoformans planktonic cells and biofilms, albeit less effective against biofilms (110).

Cathelicidin

Cathelicidins are cationic HDP containing 12–80 aa (predominantly 23–37 aa) and adopt either α-helix or β-sheet secondary structures in amphipathic helices and include the single human cathelicidin, LL-37 (**Figure 1D**). The classification of cathelicidins as one family is due to the large evolutionary conserved N-terminal cathelin sequence. However, the highly variable C-terminal region is responsible for most of the broad-spectrum antimicrobial and immunomodulatory activities. Cathelicidin knockout mice were more susceptible to bacterial and viral infection, resulting in a higher morbidity and mortality (119–121). The myriad other properties of cathelicidin have been the subject of several recent reviews (68, 74, 122–124) and are beyond the scope of this manuscript.

The candidacidal activity of LL-37 has been demonstrated in a number of *in vitro* studies (125–129), but activity against other fungi has been demonstrated in a limited number of reports. Antifungal activity of LL-37 was demonstrated against *T. rubrum* ($n = 2$) and *T. mentagrophytes* ($n = 2$) with an MIC/MFC = 12.5–25 μM and was fungicidal against *Malassezia furfur* (25 μM) (130). LL-37 demonstrated antifungal activity (MIC <64 μM) against selected vaginal *Candida* spp. isolates (*C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis*), albeit the majority of isolates tested had MIC >64 μM, and was ineffective against preformed *C. albicans* biofilms at ≤32 μM. LL-37 (64 μM) was able to inhibit adhesion of *C. albicans* SC5314 to polystyrene and silicone surfaces, thereby preventing biofilm formation (128). LL-37 associated with the cell wall and/or membrane of *C. albicans* and caused membrane lysis, generation of ROS and release of ATP and other molecules (≤40 kDa) (131). Murine cathelicidin was fungicidal against *Pneumocystis murina* in a dose-dependent manner (10–50 mg/L) (132). The *C. albicans* cell wall β-1,3-exoglucanase, Xog1, interacts directly with LL-37 leading to elevated enzyme activity and subsequent cell wall remodelling and reduced adhesion of *C. albicans* to plastic surfaces (116), oral epidermoid OECM-1 cells and murine urinary bladder at concentrations that were not fungicidal (133). *C. albicans* that did not adhere were aggregated when LL-37 was bound to the cell surface, mediated by preferential binding to cell wall mannans and to a lesser extent chitin and cell wall glucans (133). Secreted aspartyl proteases (SAP1 – 4, 8 & 9) of *C. albicans* were able to hydrolyse LL-37 into smaller peptides *in vitro* and this correlated with a reduction in antifungal and immunomodulatory activity and may facilitate survival of *C. albicans* at sites where LL-37 is produced (134). Interestingly, the *in vitro* growth of *A. fumigatus* and *A. flavus* was stimulated by physiological concentrations of LL-37 (0.97–31.25 mg/L) found in the lung, whereas a scrambled analogue of LL-37 had no such effect (135).

Other Human Antifungal AMP/HDP

A number of other human AMP/HDP possess documented antifungal activity, including RNases, psoriasin, dermcidin, lactoferricin, antileukprotease/secretory leukocyte protease inhibitor (SLPI), calprotectin, trappin-2/pre-elafin, granulysin, thrombocidins, hepcidins, α-melanocyte stimulating hormone, the chemokine CCL20, substance P, calcitonin gene-related peptide, neuropeptide Y, amyloid β-peptide and vasostatin-1 (136–152).

RNase 3 and RNase 7 demonstrated activity against *C. albicans* (MFC 2.5–5.0 μmol/L) (151), whereas dermcidin demonstrated pH-dependent activity against *C. albicans* with optimal activity at pH 5.5–6.5 (143). SLPI was active against *A. fumigatus*, including spores (137) and *C. albicans* (153). Hepcidins, Hepc20 and Hepc25, inhibited sporulation of *A. fumigatus* and *A. niger* and Hepc20 was fungicidal at 40 μM, whereas both Hepc20 and Hepc25 were only moderately antifungal against *C. albicans* at 30 μM (∼1 log kill) (142). Hepc20 was fungicidal against a panel of *C. glabrata* (MIC 60–100 μM), which was enhanced in acidic conditions, whereas Hepc25 was not fungicidal (150, 154). The neuropeptides Substance P, Calcitonin gene-related peptide and Neuropeptide Y demonstrated activity against *C. albicans* (MIC 8.1, 63.1, and 46.5 mg/L, respectively) (146). Lactoferrin and peptides derived from it demonstrated broad-spectrum antifungal activity, including against important pathogenic moulds (e.g., *Aspergillus* spp., *Alternaria* spp., *Fusarium* spp., *Absidia* spp. and dermatophytes) and yeasts (e.g., *Candida* spp., *Cryptococcus* spp. and *Exophiala* spp.) (152). RNase 7, hBD-2 and psoriasin demonstrated activity against dermatophytes, including *T. rubrum*, *T. mentagrophytes* and *Epidermophyton floccosum*, albeit only psoriasin demonstrated significant activity against *Microsporum canis* (148). Psoriasin demonstrated broad-spectrum antifungal activity with a 90% MIC of ∼2 μM against *A. fumigatus*, *Malassezia furfur*, *M. canis*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *T. rubrum* and *T. mentagrophytes*, but was not active against *C. albicans* at concentrations up to 20 μM (155).

NOVEL ANTIFUNGAL PEPTIDES IN CLINICAL AND PRECLINICAL DEVELOPMENT

A number of synthetic AMP have been investigated as antifungal therapies (156, 157). AMP with antifungal activity show the same structural diversity as other AMP and include linear and cyclic peptides, lipopeptides and depsipeptides. Over 1100 putative endogenous AMP with antifungal activity have been described (The Antimicrobial Peptide Database; http://aps.unmc.edu/AP/main.php). Antifungal peptides may form α-helices, β-sheets or mixtures thereof and may be cysteine-stabilised. Some are rich in specific amino acids, contain non-natural amino acids or contain non-protein modifications including lipid and carbohydrate moieties. Therapeutic candidate antifungal peptides mostly have a membrane-lytic mechanism of action, but peptides with alternative and even multiple mechanisms of action have been investigated (57–59, 158–161). The structure/composition of fungal cellular membranes vary between species and between yeast and hyphal forms, but in general are more negatively charged than mammalian cell membranes and this may account for the specificity of membrane-active antifungal peptides (58). There have been a number of mechanisms of action

both proposed and proven for the interactions of AMP with membranes including the formation of toroidal pores, barrel-stave pores (162), disordered toroidal pores (163), aggregate pores (164), the carpet model (peptide interaction with phospholipid head groups leading to membrane solubilisation) (165). Other less documented mechanisms of action include peptide-induced membrane curvature, induction of cubic lipid phases (166), membrane-thinning/thickening (167), membrane domain formation (168), membrane flip-flop (169), lipid clustering (170) and disruption of membrane potential (171).

NP213 (Novexatin®)

NP213 is a novel, first-in-class, synthetic AMP therapeutic candidate derived from HDP that was designed specifically as a topical therapy for the treatment of onychomycosis (fungal nail infection) by NovaBiotics Ltd. NP213 is a backbone-cyclised homopolymer of 7 L-arginine residues with a net charge of +7. NP213 is rapidly fungicidal against dermatophytes and other fungi causative of onychomycosis and is more active in the presence of human nail and keratin than in conventional antifungal susceptibility testing (RPMI-1640 liquid medium). NP213 was equally effective against dermatophyte spores and hyphae, unlike terbinafine, which demonstrated limited activity against spores, and demonstrated a 3 log kill within 3–4 h, compared to >24 h for terbinafine. NP213 is membranolytic and dependent on its positive charge for activity. NP213 was efficacious in *ex vivo* models of fungal nail infection, eradicating different *Trichophyton rubrum* isolates after only 28 d application, unlike ciclopirox and amorolfine (172). Preclinical and clinical safety and toxicity testing revealed no systemic exposure following topical application to the skin of mini-pigs or humans (including a maximum exposure study) with no NP213 detectable in plasma. In clinical trials, NP213 was safe and well tolerated. In two randomized, double-blind, placebo-controlled Phase IIa efficacy studies, daily application of NP213 for 28 d demonstrated clearance of infection in 43.3% (after 180 d; trial 1) and 56.5% (after 360 d; trial 2) of patients with mild-to-moderate onychomycosis (determined by culture) (173). NP213 has also been the subject of a Phase IIb study and further clinical studies are planned.

HXP124

HXP124 is an investigational novel AMP drug candidate in clinical development for the topical treatment of onychomycosis by Hexima Ltd. HXP124 is a novel plant defensin with a cysteine-stabilised αβ-motif structure. HXP124 demonstrated broad-spectrum fungicidal activity against clinically important human pathogens, including *Candida* spp., *Cryptococcus* spp., dermatophytes and other moulds. HXP penetrated human nails and was active in an *ex vivo* model of nail infection. Additionally, HXP124 demonstrated a favourable safety profile in preclinical testing (174). HXP124 has been the subject of a first in human Phase I/IIa trial to evaluate the safety, tolerability and efficacy of daily topical application for 6 weeks in otherwise healthy patients with mild-to-moderate toenail onychomycosis (Australian Clinical Trials ID: ACTRN12618000131257). HXP124 was safe and well-tolerated and substantially reduced the area of infection (>40%) in 15 of 41 patients (37%) analysed after 12 weeks, compared to only 3 of 17 patients (18%) in the vehicle-only group (6 weeks post-treatment) (https://hexima.com.au/).

CZEN-002

CZEN-002 is a synthetic octapeptide, $(CKPV)_2$, derived from α-melanocyte stimulating hormone (α-MSH). α-MSH had previously demonstrated antifungal activity against *C. albicans* (175). CZEN-220 contains the C-terminal tripeptide (KPV) of α-MSH with a Cys-Cys linker to create an octapeptide. CZEN-002 was candidacidal against *C. albicans*, *C. krusei* and *C. glabrata* at sub-mM concentrations. CZEN-002 is not membranolytic (176). In a rat vaginitis model of *C. albicans* infection, CZEN-002 dose-dependently reduced the number of surviving *C. albicans* over 18 d. CZEN-002 inhibited *C. albicans* phagocytosis by macrophages and inhibited the production of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6, while increasing arginase activity and the secretion of the anti-inflammatory cytokine IL-10, indicating anti-inflammatory properties (177). $(CKPV)_2$ exhibited anti-inflammatory effects against human neutrophils (178) and inhibited TNF-α release form endotoxin-stimulated peripheral blood mononuclear cells *in vitro* and *in vivo* (179).

Zengen Inc., developed CZEN-002 for the topical treatment of vulvovaginal candidiasis as an intravaginal gel (56, 180). A phase I/IIa clinical trial reported 88.2% and 87.5% cure (KOH test and culture, respectively) in a total of 18 female VVC patients with VVC that completed the trial in 2004 (https://www.eurekalert.org/pub_releases/2004-05/z-zrp052404.php). A larger dose-ranging Phase IIb clinical trial was planned for 2005 in Canada & EU. The development status of CZEN-002 is not currently known.

P113

P113 (also known as PAC-113, PAC113 and P-113) is a synthetic amphipathic, α-helical 12 amino acid histatin 5 derivative (AKRHHGYKRKFH) with membrane-permeabilising activity against *Candida* spp. (181) and bacteria (182–185). P113 progressed through clinical development as a topical treatment for oral candidiasis. Complexation with zinc confers greater mechanical stability to the peptide (186). P113 represents the smallest fragment of histatin 5 that retains activity against *Candida* spp. that was comparable to the parent compound. An analogue of P113 containing D-amino acids, P113D, was equally active against *C. albicans*. Substitution of the 3 His residues with Phe or Tyr had little effect on activity against *C. albicans* (MIC 2.2–2.5 mg/L, but substitution of the 2 Arg and 2 Lys residues with Gln abrogated activity (MIC >80 mg/L) (181). P113 was candidacidal against *Candida* spp. (*C.* albicans, *C. tropicalis*, *C. famata*) in a time- and dose-dependent manner. A series of P113 derivatives have been designed, including a dimer and trimer. P113, P113 dimer and P113 trimer demonstrated limited cytotoxicity against human gingival epithelial cells (LD_{50} > 400 mg/L). The P113 dimer and trimer were more efficacious than P113 against *C. albicans* and *C. krusei* and similarly active against *C. tropicalis*, *C. dubliniensis* and *C. parapsilosis*, whereas *C. glabrata* was insensitive to all 3 peptides. The P113 trimer retained activity at high sodium acetate concentrations

(31.25–93.75 mM), unlike P113 (187). P113, the dimer and the trimer, increased ROS generation and inhibited cellular respiration in *C. albicans* by targeting mitochondrial complex I. This activity was predominantly caused by inhibition of the NADH dehydrogenase in mitochondrial complex I. The P113 dimer and trimer were also able to target an alternative NADH dehydrogenase not present in mitochondrial complex I. The rapid killing by P113, dimer and trimer mostly occurs via ROS generation, rather than depletion of energy (188). In another study, *Candida glabrata* was not sensitive to P113 or other histatins and derivatives (189). As well as evidence for P113 causing membranolysis, similar to the histatins from which it is derived, P113 is rapidly taken up into the cytosol of *Candida* spp. after initial binding to the cell wall, and this process is facilitated by Ssa2p (Heat shock protein 70 (HSP70) chaperone) that can transfer cell wall-bound peptides to membrane permeases to specifically transport peptides into the cytosol. Thus, the antimicrobial activity of P-113 acts through binding to and destabilization of the microbial membrane and through a specific protein receptor on the microbial cell surface (190).

When the His residues at positions 4, 5, and 12 were replaced with the bulky, non-natural amino acids β-naphthylalanine (Nal-P113), salt sensitivity was less pronounced and activity against *Candida* spp. was retained. Such amino acid substitutions may improve activity under physiological salt concentrations (191). P113 was subject to proteolysis by *C. albicans* intracellular enzymes at Ala4 and Lys11, whereas P113D was not (192). Based on studies with Hst-5, the Lys residue at position 8 would be subject to cleavage by *Candida* spp. secreted aspartyl proteases, Sap2 and Sap9 (193). Additionally, histatins (and potentially P113) can form complexes with salivary proteins, e.g., salivary amylase, that can inhibit antifungal activity (194). A possible solution to improve the antifungal efficacy of P113 and other AMP in saliva is to formulate the peptides in delivery systems such as liposomes that facilitate gradual release and limit proteolysis (195). Interestingly, in a rat oral mucosal ulcer model, Nal-P113 increased expression of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) and decreased the expression of transforming growth factor-β1 (TGF-β1), whereas in an *in vitro* wound healing assay, Nal-P113 promoted migration of human immortalized oral epithelial cells, indicating that application of Nal-P113 might be an effective therapeutic approach for recurrent aphthous stomatitis (196).

General Biologicals Corporation (GBC) currently market P113-containing compounds as part of their over-the-counter antibacterial "oh-care" range Whilst apparently continuing development of P113 for the treatment of oral candidiasis. A Phase I/IIa clinical trial demonstrated that P113 as an oral mouthrinse was generally safe and well-tolerated and similarly efficacious in curing oral candidiasis as the gold standard, as 37% of PAC-113 patients were assessed as clinically cured at day 14 compared to 36% of Nystatin patients (56, 180). A randomized, examiner-blinded, positive-controlled, parallel design Phase IIb clinical trial of PAC113 oral mouth rinse was carried out in 2008 in 223 HIV seropositive individuals with oral candidiasis and included 3 different concentrations of PAC113 (0.15, 0.075, and 0.0375%) compared to Nystatin oral suspension to determine whether there was elimination or a reduction in clinical signs and symptoms of oral candidiasis. Unfortunately, no results were posted for this trial (ClinicalTrials.gov Identifier: NCT00659971). In a double-blinded, randomized, controlled clinical trial to evaluate the safety and toxicity of three histatin (P-113) concentrations in gel formulations, and to assess potential clinical benefit on the development of gingivitis, 106 healthy subjects without gingivitis were enrolled. All formulations were safe and well-tolerated and efficacy data revealed that P113 gels applied twice daily may reduce experimental gingivitis in humans (197). In another phase 2 multi-centre clinical study, a P113 mouth rinse was safe and well-tolerated and reduced the development of gingivitis in 294 healthy subjects using the formulation twice daily in place normal oral hygiene procedures (198). In a double-blinded, randomized clinical study, 37 patients with moderate or severe chronic periodontitis were treated on one tooth with 20 mg/L Nal-P113 or placebo on days 0 and 3 and on day 7 teeth were sampled. Treatment with Nal-P113 improved periodontal clinical status, reduced plaque/biofilm formation compared to controls (199).

Omiganan

Omiganan (MX-226 or MBI-226) is a synthetic AMP (ILRWPWWPWRRK-amide) derived from indolicidin, originally isolated from bovine neutrophils, with antifungal (200, 201), antibacterial (200, 202), anti-biofilm (203, 204), antiviral (205) and immunomodulatory properties (206). Omiganan was active against *Candida* spp.; *C. albicans* (MIC 32–>512 mg/L; $n = 104$), *C. glabrata* (MIC 128–>512 mg/L; $n = 27$), *C. krusei* (MIC 16–256 mg/L; $n = 26$), *C. parapsilosis* (MIC 32–256 mg/L; $n = 30$) and *C. tropicalis* (MIC 8–64 mg/L; $n = 27$) (200) and moulds, including *Aspergillus* spp. (MIC 16–1,024 mg/L; $n = 10$), *Curvularia* spp., *Fusarium* spp., *Paecilomyces variotii* and *Penicillium* spp. (MIC 1–256 mg/L; $n = 10$) (201). 100 mg/L omiganan caused a 1–2 log kill against *C. albicans* ($n = 3$) within 1 h exposure (129). Interestingly, an omiganan analogue with the sequence reversed (KRRWPWWPWRLI-NH_2) was more active against *C. albicans* (Forward MIC 128 mg/L; Reverse MIC 32–64 mg/L) and both were equally effective against *A. niger* ATCC16404 (MIC 64 mg/L) (207). An all D-enantiomer analogue of omiganan demonstrated the same antimicrobial activity as L-omiganan, but was less susceptible to skin proteases (t1/2 >120 min and t1/2 = 10 min, respectively) (208). In an *ex vivo* pig skin infection model, ≥0.1% (w/w) omiganan (in an aqueous gel) was active against *C. albicans* ATCC14053, causing a 2–3 log kill after 24 h, whereas in an *in vivo* guinea pig skin infection model 1% (w/w) omiganan cased a 2 log kill after 24 h (209).

Omiganan has been the subject of 16 clinical trials in the US and 10 in Europe (ClinicalTrials.gov clinicaltrialsregister.eu), probably making it the most studied AMP in humans, albeit all trials were for topical application, including acne vulgaris, rosacea and sebhorrhoeic dermatitis. Unfortunately, none of these clinical trials investigated the antifungal properties of omiganan, although one trial into the use of omiganan for the prevention of central venous catheter-related bloodstream infections described that they would test for

fungaemia, bacteraemia and sepsis (ClinicalTrials.gov Identifier: NCT00027248). Unfortunately, no results for this study, sponsored by BioWest Therapeutics Inc, have been posted. In a later Phase III study of 1,859 hospitalised patients, omiganan 1% gel was compared to 10% povidone-iodine for the prevention of catheter infection/colonisation in patients with central venous catheters, but results were disappointing and the trial failed to achieve its primary efficacy end-point of reducing local catheter site infections (ClinicalTrials.gov Identifier: NCT00231153) (56, 180, 209). In two recent Phase II clinical trials sponsored by Cutanea Life Sciences (EudraCT Number: 2015-002724-16 & 2015-005553-13), the safety and efficacy of omiganan in the treatment of human papillomavirus-induced genital lesions ($n = 12$) or external ano-genital warts ($n = 24$) was assessed. Omiganan was safe and well tolerated by all patients. Human papillomavirus load significantly reduced after 12 weeks of treatment with omiganan compared to placebo, but only in the external ano-genital warts patients (205). Whilst clinical development of omiganan appears to be ongoing, omiganan has been proven to be safe and generally well-tolerated as a topical antimicrobial. Its efficacy has yet to be proven in the clinic. Carefully designed trials with appropriate efficacy/outcome measures and application of the peptide in appropriate formulations will be critical to ensure success and potential translation of this compound's promising *in vitro* antifungal data.

hLF1-11

The AMP hLF1-11 (GRRRRSVQWCA) comprises the first 11 amino acids of human lactoferrin and is a multi-functional peptide with antibacterial activity (185, 210, 211), antifungal activity (212) and immunomodulatory properties (213, 214). hLF1-11 demonstrated antifungal activity *in vitro* against *C. albicans* (MIC 22–44 mg/L; $n = 11$), including oral and vaginal isolates (212, 215). Pre-treatment of fluconazole-resistant *C. albicans* with non-candidacidal concentrations of hLF1-11 (4–8 μM) was synergistic with fluconazole, rendering this strain fluconazole sensitive. The combination of hLF1-11 and fluconazole was also effective against *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* (216). hLF1-11 caused mitochondrial calcium uptake which stimulated an increase in mitochondrial membrane potential and permeability, resulting in the synthesis and secretion of ATP and ROS production, leading to *C. albicans* cell death (217). hLF1-11 was also active against *A. fumigatus* hyphae (EC_{50} 29 ± 5 μM) and spores (MIC 5 ± 4 μM) (218).

hLF1-11 (88–176 mg/L) prevented *C. albicans* biofilm formation with almost complete inhibition of metabolic activity, a 2 log reduction in cell viability (176 mg/L) and decreased expression of selected biofilm-associated genes. However, hLF1-11 demonstrated poor activity against pre-formed biofilms (215). hLF1-11 (88–176 mg/L) prevented *C. parapsilosis* ($n = 3$) biofilm formation and 55 mg/L hLF1-11 significantly reduced the amount of biofilm formed. When *C. parapsilosis* CP7 was allowed to adhere to the surface of 96-well plates or peripheral Teflon catheter pieces for 1.5 or 3 h, hLF1-11 (≥44 mg/L) significantly reduced the amount of biofilm formed and metabolic activity, whereas after being allowed to adhere for 6 h, 44 mg/L hLF1-11 was ineffective at preventing adhered cells developing into biofilms and both 44 and 88 mg/L hLF1-11 were ineffective when *C. parapsilosis* had been allowed to adhere for 24 h. Incubation of *C. parapsilosis* CP7 with 44 mg/L hLF1-11 led to reduced expression of the adhesin gene CpALS7, the biofilm formation-associated gene *CpACE2* and the β-glucan synthase catalytic sub-unit 1 gene *CpFSK1* (219). Coating of hLF1-11 onto titanium surfaces by atom transfer radical polymerization reduced adhesion of *Streptococcus sanguinis*, *Lactobacillus salivarius* and a mixed microflora derived from human dental plaque (220), whereas attachment of hLF1-11 to chitosan films via the cysteine residue increased the adhesion of *Staphylococcus aureus* ATCC33591 to the film, albeit with some reduction in viability (221). Thus, hLF1-11 may have application in prevention of infection of implanted medical devices provided careful consideration is given to the manner of surface attachment. hLF1-11 was not haemolytic at concentrations up to 200 mg/L and caused no significant loss of viability of murine osteoblast MC3T3-e1 cells at a concentration of 400 mg/L (222).

hLF1-11 demonstrated synergistic inhibition of *C. albicans* SC5314 in combination with caspofungin *in vitro*. When tested in the *Galleria mellonella* (wax moth) larva model of infection hLF1-11 was not toxic (≤100 mg/kg), but these concentrations were not effective at improving survival in larvae infected with *C. albicans* ($2.8–3.0 \times 10^5$ cfu inoculum) and in this model, the combination of 25 mg/kg hLF-11 and 0.5 mg/kg caspofungin also resulted in no enhanced survival (223). In a neutropenic murine model of systemic candidiasis (established for 24 h) with a fluconazole-resistant *C. albicans* isolate 0.4 μg/kg hLF1-11 caused a ∼1.5 log reduction in *C. albicans* kidney burden after 18 h and mice treated with up to 40 μg/kg hLF1-11 had smaller and fewer infectious foci in their kidneys and grew predominantly as yeast, unlike the hyphal growth observed in the kidneys of untreated mice. hLF1-11 was also able to inhibit the yeast-hyphal transition *in vitro* (217).

Exposure of monocytes to hLF1-11 during GM-CSF-driven differentiation is sufficient to direct differentiation of monocytes toward a macrophage subset characterized by both pro- and anti-inflammatory cytokine production (IL-10 and TNF-α) when subsequently exposed to heat-killed *C. albicans* and these macrophages also demonstrated increased responsiveness to bacterial lipopolysaccharide (LPS), lipoteichoic acid (LTA) and heat-killed *C. albicans* (213). Following intracellular uptake by monocytes, hLF1-11 bound to myeloperoxidase (MPO) and inhibited the chlorination and peroxidation activity of MPO (224). hLF1-11 also facilitated differentiation of human monocytes to dendritic cells (DC) with increased expression of HLA class II antigens and dectin-1 (a *C. albicans* PRR) and increased phagocytosis of *C. albicans*, but not *Staphylococcus aureus*. Upon stimulation with *C. albicans*, hLF1-11-differentiated DC produced increased amounts of ROS and the cytokines IL-6 and IL-10, but not IL-12p40 or TNF-α. Supernatants from hLF1–11-differentiated DCs caused CD4+ T cells to produce increased concentrations

of IL-17, but reduced IFN-γ, following stimulation with *C. albicans* (214).

hLF1-11 has been the subject of 4 proposed human trials, sponsored by AM-Pharma, and registered on ClinicalTrials.gov although only one was completed. The completed trial was to determine the safety of a single intravenous dose of hLF1-11 (5 mg, single dose IV) in 8 autologous haematopoietic stem cell transplant recipients (HSCT) (ClinicalTrials.gov Identifier: NCT00509938). The safety and tolerability of hLF1-11 had to be established in HSCT recipients as they are at risk of developing, but have not yet developed, infectious complications due to invasive fungal disease. An earlier study in 48 healthy volunteers (36 hLF1-11 and 12 placebo) had established that single ascending intravenous doses (0.005–5 mg, single dose IV) and multiple intravenous doses (0.5 & 5 mg, single dose IV) were safe and well tolerated. HSCT patients differ from healthy volunteers as they have received myeloablative treatment which arrests haematopoiesis, resulting in neutropenia, but also causes mucosal barrier injury. Both of these predispose HSCT patients to fungal infections which typically occur during the week after transplant. It was therefore essential to know that hLF 1-11 is safe when given during neutropenia and mucosal barrier injury before infections ensue. A single 5 mg (single dose IV) dose was well-tolerated in patients with a side effect of elevated liver enzymes (alanine aminotransferase and aspartate aminotransferase) that was reversible and may have been related to treatment (225). A further study to determine the effect of multiple doses of hLF1-11 in HSCT patients (ClinicalTrials.gov Identifier: NCT00430469) was withdrawn by the sponsor prior to patient recruitment. Another of the withdrawn studies, one was a phase IIa, double-blind, randomized study to determine the tolerability and efficacy of hLF1-11 in patients with proven candidemia with concomitant fluconazole treatment (ClinicalTrials.gov Identifier: NCT00509834), but unfortunately the target patient population was not available. It is clear that hLF1-11 is generally safe and well-tolerated in healthy subjects and HSCT patients at the dose ranges tested thus far and that this peptide has *in vitro* and preclinical efficacy in models of fungal infection. It remains to be seen how effective this peptide can be in clinical use.

Iseganan (IB-367)

Iseganan (IB-367) is a synthetic AMP containing 17 amino acid residues derived from protegrin I, part of the cathelicidin family of AMP, that has been in clinical development for the treatment of oral mucositis (226–228) and ventilator-associated pneumonia (229). Iseganan was selected as the most promising candidate for the prevention of oral mucositis based on a study of structure–activity relationships of synthetic protegrin analogues (230). Iseganan demonstrated antibacterial activity (231, 232), antifungal activity (233, 234), anti-parasitic (235), anti-biofilm activity (236) and both antibacterial and anti-endotoxin activity in rat models of septic shock (237). Iseganan was fungicidal against dermatophytes (MIC 8–16 mg/L ($n = 20$; MFC 16–32 mg/L ($n = 20$) (234). and *C. albicans* (MIC 4–8 mg/L; MFC 4–32 mg/L ($n = 5$), *C. glabrata* (MIC 2–16 mg/L; MFC 2–16 mg/L ($n = 5$), *C. parapsilosis* (MIC 8–32 mg/L; MFC 16–>128 mg/L ($n = 5$), *C. krusei* (MIC 4–16 mg/L; MFC 4–64 mg/L ($n = 5$) and *C. tropicalis* (MIC 2–4 mg/L; MFC 2–4 mg/L ($n = 5$) (233), although activity against *A. fumigatus* ATCC16404 was poor (MIC/MFC = 256 mg/L) (238). Local application of Iseganan (IB-367) reduced mucositis severity in a hamster model of oral mucositis which correlated with a >100-fold reduction in oral microflora densities in a dose-dependent manner (239).

A multi-centre double-blind, placebo-controlled Phase III trial to determine the efficacy of Iseganan HCl rinse in reducing the severity of oral mucositis in 323 patients (163 iseganan and 160 placebo) receiving stomatotoxic chemotherapy (PROMPT-CT trial). Iseganan (9 mg in 3 ml) was administered as a swish and swallow solution, six times daily for 21–28 d and was safe and well-tolerated. In this study, 43 and 33% of Iseganan and placebo patients, respectively, did not develop ulcerative oral mucositis. Iseganan patients experienced less mouth pain, throat pain and difficulty swallowing compared to placebo patients and experienced lower stomatitis scores (226). However, other studies failed to demonstrate a benefit of Iseganan in causing reduction in oral mucositis (227, 228). Stomatotoxic chemotherapy can induce changes in the oral microflora that may cause oral and systemic infections in myelosuppressed cancer patients and studies suggest that reduction of the microbial load in the oral cavity has some clinical benefit. A sub-analysis of the first trial was conducted to assess the antimicrobial activity of Iseganan in this patient population. Microbial cultures were generated before and after the daily Iseganan mouth rinse. Iseganan significantly reduced total microbial load in the oral cavity, mainly due to decreased numbers of streptococci and yeasts. This antifungal activity is of interest as oropharyngeal candidiasis is common in immunocompromised patients and some elderly populations (240). A multinational, double-blind, randomized, placebo-controlled trial of Iseganan (371 patients) applied topically to the oral cavity vs. placebo (354 patients) in intubated patients receiving mechanical ventilation for up to 14 d was conducted to determine the occurrence of microbiologically confirmed ventilator-associated bacterial pneumonia (VAP) measured among survivors up through Day 14 (ClinicalTrials.gov Identifier: NCT00118781). The peptide was deemed to be safe and well tolerated but the study's primary efficacy end-points were not met [no significant differences in the rate of VAP among survivors between patients treated with Iseganan (16%) and those treated with placebo (20%; $p = 0.145$) (229)]. The design of the study was potentially flawed due to the short exposure time of Iseganan to potential pathogens (241). Thus, as a proven safe and well tolerated candidate when applied topically in very sick patients with preclinical antifungal activity, Iseganan has the potential to be developed as an AMP for the treatment of oropharyngeal candidiasis and for topical application for the treatment of other fungal infections.

LTX-109

LTX-109 (LTX109, Lytixar, LTX 109) is an AMP peptidomimetic (Arg-Tbt-Arg-NH-EtPh) that was in clinical development by Lytix Biopharma AS. LTX-109 contains 2 arginine residues, a central modified tryptophan residue (2,5,7-tri(*tert*-butyl)tryptophan) and an ethylphenyl group at the C-terminus (242) with antibacterial (243, 244) and antifungal activity

(245). LTX-109 was fungicidal against *S. cerevisiae* (MIC 8 mg/L), causing a 3 log kill within 60 min, and was also active against pre-formed *S. cerevisiae* biofilms. LTX-109 disrupted *S. cerevisiae* membrane integrity by a sphingolipid-dependent mechanism (245).

Topical LTX-109 has been the subject of 3 clinical trials in Gram-positive bacterial infections; nasal decolonisation of *Staphylococcus aureus* (Clinical trials identifier: NCT01158235), a role in non-bullous impetigo (Clinical trials identifier: NCT01803035) and Gram positive skin infections including patients with mild eczema/dermatoses such as atopic dermatitis (Clinical trials identifier: NCT01223222). The study for nasal decolonisation of *Staphylococcus aureus* was a randomized, double-blind, dose escalation phase I/IIa study conducted at a single centre to compare the efficacy, safety, tolerability, bioavailability and efficacy of 3 days nasal treatment with LTX-109 (TID) applied directly to the anterior nares vs. vehicle in persistent nasal carriers of *Staphylococcus aureus*. LTX-109 was safe and well-tolerated and treatment with LTX-109 resulted in a reduction in *Staphylococcus aureus* counts after only 1 day of application. A significant reduction of the number of CFU below the detection limit compared to the vehicle group was demonstrated in subjects treated with 2 and 5% LTX-109 after 2 days of treatment. The most frequently reported AEs related to the application site were itching, burning, pain, and redness ($n =$ 26) and the subjects in the 2 and 5% LTX-109 treatment groups reported more of these symptoms than did the 1% or vehicle groups (246). Unfortunately, no results are available for the other 2 LTX-109 clinical trials. Given the positive clinical safety and tolerability data following topical application over multiple days in bacterial infection, together with promising antifungal activity *in vitro*, LTX-109 could be a promising candidate for the treatment of fungal infection.

ANTIBIOFILM PEPTIDES

The ability of fungi to form biofilms have been associated with high rates of morbidity and mortality, yet compared to bacterial biofilms and bacterial anti-biofilm compounds, the field of fungal biofilm research remains in its infancy. Fungal biofilms consist of adherent cells (on biotic or abiotic surfaces) surrounded by an extracellular matrix which can reduce antifungal efficacy and impair immune responses (247, 248). In addition to direct antifungal activity some AMP/HDP, *in vitro*, can prevent biofilm formation and/or eradicate preformed biofilms via mechanisms associated with fungal adhesion, cell wall perturbation, generation of ROS and gene regulation (59, 249). Although not yet in clinical use, the search for AMP with "druggable" antibiofilm properties remains ongoing (56, 250). For example, in the case of *Cryptococcus neoformans* biofilms, formation is dependent on the production of the polysaccharide capsule (251). hBD-1 and hBD-3 were active against *Cryptococcus neoformans* planktonic cells and biofilms, albeit less effective against biofilms (110), whereas lactoferrin was not effective against *Cryptococcus neoformans* biofilms (251). Hst-5 was effective against planktonic *C. albicans* (IC_{50} 2.6–4.8 μM; $n = 3$), but not *C. glabrata* ($IC_{50} > 100$ μM; $n = 2$). However, Hst-5 was active against preformed biofilms of *C. albicans* and *C. glabrata* on poly(methyl methacrylate) discs, resulting in a 50% reduction in biofilm metabolic activity at concentrations of 1.7–6.9 μM (*C. albicans*; $n = 3$) and 31.2–62.5 μM (*C. glabrata*; $n = 2$) (83). LL-37 was able to prevent *C. albicans* biofilm formation on silicone elastomer discs (used in the manufacture of medical devices) at sub-MIC concentrations without a concomitant reduction in *C. albicans* viability, whereas LL-37 had no effect on pre-formed *C. albicans* biofilms (128). Thus, AMP have promise as anti-biofilm agents against fungi as well as bacteria.

FUTURE DIRECTIONS FOR ANTIFUNGAL PEPTIDE DESIGN AND DEVELOPMENT

Antifungal Peptides in Preclinical Development

A limited number of AMP are in preclinical development for the treatment of fungal infections and have been extensively reviewed (156, 157, 252–254). In this section we will provide a non-exhaustive review of some of the later stage preclinical antifungal AMP candidates likely to be closer to clinical testing.

NP339 is a preclinical drug candidate being developed as an intravenous therapy for life threatening invasive fungal disease (bloodstream and deep tissue fungal infections) including those caused by yeasts and moulds that are resistant to existing antifungal therapies. An inhaled form of NP339 is also under development for direct delivery into the airways in patients with, or at risk of respiratory fungal infections, including Allergic Bronchial Pulmonary Aspergillosis (ABPA) and pulmonary fungal infections in cystic fibrosis patients. NP339 is a synthetic 2 kDa cationic linear AMP that has been engineered from β-defensins.

NP339 targets the fungal membrane and kills fungi by membrane disruption and cell lysis. This mechanism of action is specific to fungal cells and NP339 is not cytotoxic at significantly higher concentrations than are required to achieve antifungal activity. NP339 kills more rapidly than conventional classes of antifungals, including against metabolically active and inactive fungi and is also sporicidal. NP339 is active against a broad range of clinically relevant fungal pathogens, including *Aspergillus* spp., *Candida* spp. and *Cryptococcus* spp., as well as emerging fungal pathogens including Mucorales, *Scedosporium* spp. and *Exophiala* spp. (255). Nebulised NP339 as a monotherapy, or in combination with amphotericin B, elicited a reduction in lung burden relative to vehicle in murine models of invasive pulmonary aspergillosis (256).

In addition to P113 (see Section P113), Demegen had a second AMP, D2A21, in pre-clinical development (257). D2A21 was a synthetic peptide derived from cecropin (258) being investigated for a number of antimicrobial indications and was formulated as a topical gel (Demegel). D2A21 demonstrated *in vitro* antifungal activity against *C. albicans*, *A. niger*, *Mucor* spp. and *T. mentagrophytes*, as well as antibacterial, antiparasitic and potential anti-tumorigenic activity (257). Potential antimicrobial indications include fungal infections,

sexually-transmitted infections caused by *Chlamydia trachomatis* (259), and *Trichomonas vaginalis* (260) (for which *in vitro* activity was demonstrated) and burn wound infections (261, 262), In an *in vivo* infected burn wound model in Wistar rats, D2A21 demonstrated significant antibacterial activity against *P. aeruginosa* infection, sterilized burn eschar and decreased the bacterial load in subeschar, leading to significantly improved survival (261, 262).

ETD151 is a preclinical AMP drug candidate derived from ARD1 (a heliomycin peptide), a naturally occurring AMP from the lepidopteran *Heliothis virescens* (tobacco budworm). ETD151, developed by EntoMed SA, is a 44 aa AMP intended for the treatment of serious invasive fungal infections affecting immunocompromised patients (263). ETD151 was derived from ARD1 by site-directed mutagenesis, following recombinant expression in *Saccharomyces cerevisiae* to create a peptide with increased cationicity (264). ETD151 demonstrated promising antifungal activity *in vitro* (MIC_{50} 0.1–6.25 mg/L against *C. albicans*, *Cryptococcus neoformans*, *A. fumigatus*, *F. solani* and *Scedosporium prolificans*) (264). In murine models of systemic *C. albicans* or *A. fumigatus* infection, EDT-151 was effective when compared to amphotericin B and azoles and was non-toxic following intravenous administration (263). ETD151 has yet to enter clinical trials to the knowledge of the authors, however, most recently, the antifungal activity of ETD151 has been assessed against *Botrytis cinerea*, a necrotrophic plant pathogen responsible for gray mold disease, for use as a fungicide in crop protection (265).

TABLE 2 | Factors influencing preclinical antimicrobial activity testing of AMP.

In vitro	*Ex vivo*
pH & ionic strength	Biological matrices (e.g., blood)
Temperature	Mammalian cells
Medium type/composition	Intracellular pathogens
Nutrient concentrations	
Buffer	
Bicarbonate	
Metal ions	
Salt (NaCl)	
Polysorbate-80	
Synergy/Antagonism with other antimicrobials	
Inoculum size	
Growth Phase (e.g., biofilms, persisters, spores, small colony variants and other phenotypic variants)	
Charge effects	
Solubility	
Laboratory materials	
Proteolysis	
Biological macromolecules (e.g., protein, DNA)	
Oxygen (hyper-, norm- & hypoxia)	
Mono/Polymicrobial interactions	

Preclinical Activity Testing

Antimicrobial peptides, whether antifungal, antibacterial, antiparasitic or antiviral, cannot be developed through the same preclinical and clinical pathways as small molecule drugs. We cannot assume or expect that methods for determining antimicrobial activity that are employed in the development of antibiotics and other "small" molecule antimicrobials will be appropriate for the development of AMP as drug candidates. We say "small" as the authors acknowledge that many clinically used antimicrobials do not obey the traditional definition of small, i.e., <500 Da, from Lipinski's rule of five (266), but are nevertheless generally smaller than most AMP. Evaluation of AMP as antimicrobial drug candidates begins with *in vitro* antimicrobial susceptibility testing in which a number of key parameters need to be taken into consideration, including media composition, growth phase, oxygen, temperature and other biological matrices (**Table 2**) (267, 268). This also applies to *in vitro* cytotoxicity testing (269, 270), formulation and delivery considerations (see Section Formulation and Delivery) and the choice of models for *in vivo* testing (271). It is probable that with adequate consideration given to the factors outlined above and also appropriately designed clinical trials there would be significantly more AMP in preclinical and clinical development and the importance of this is described in detail in a new review of the subject (268).

Rational Drug Design

As stated above, most manuscripts describing AMP &/or peptidomimetics focus on antibacterial properties, but when considering the AMP themselves, and not their target, most reports focus on the isolation of AMP from increasingly unusual organisms (272–274), library screening (275–277) and attempting to identify or modify AMP to have the highest possible level of antimicrobial activity (i.e., lowest MIC). This is perhaps reflected by the fact that The Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) now contains over 3100 entries. Despite this, no AMP has achieved approval by the regulatory authorities as an antimicrobial therapeutic in clinical practice.

Whilst our understanding of the biology and function of AMP remains incomplete, especially how peptides behave in complex biological systems, we are gaining sufficient insight that researchers are increasingly making use of this biological knowledge and even computational approaches to design novel, synthetic AMP (278–282). Novel, informed drug-design approaches to identify AMP is aided by the vast sequence space available (78, 283). Other approaches have taken known host defence peptides and attempted to optimise them using a variety of approaches (281, 284–286).

At a less complex level, rational drug design principles can be applied to designing AMP to target specific pathogens at specific anatomical sites. As described above, NP213 has completed Phase II clinical trials for the treatment of onychomycosis (173). NP213 was designed at the outset as an antifungal peptide, but one that also needed to have specific physicochemical properties that would facilitate penetration into human nail (172). Human nail is a highly effective biological barrier and delivery of therapeutics to the nail and nail bed is challenging (287, 288). Additionally,

keratin, the major constituent of the nail, binds to and inactivates many of the existing small molecule antifungal classes, thus compromising therapy (289). AMP/HDP are expressed and produced in the nail (290–293) and several HDP/AMP are antifungal against dermatophytes, including LL-37 (130), hBD-2, RNase7 and Psoriasin (148). AMP therefore constituted a logical starting point for the design of a novel therapeutic for the treatment of onychomycosis. NP213 is highly hydrophilic and positively charged (net charge +7), properties that should facilitate nail penetration as the nail is a negatively-charged, concentrated hydrogel under physiological conditions (294). Additionally, NP213 is small compared to most AMP/HDP (7 aa vs. ∼12–>50 aa) that are already known to penetrate nail (292, 293) and this low molecular weight should also facilitate nail penetration (295). One of the known drawbacks of peptide/protein therapeutic candidates is susceptibility to hydrolysis, especially proteolysis (296), which is of especial concern with respect to dermatophytes as they are known to produce multiple classes of proteases/peptidases that enable them to hydrolyse keratin (297, 298). NP213 is a cyclic peptide and therefore not prone to hydrolysis by exoproteases and the limited sequence diversity within NP213 limits the classes of endoproteases that could hydrolyse NP213 (https://www.ebi.ac.uk/merops). Therefore, even prior to peptide synthesis, NP213 had been designed to function as an antifungal at this unique site of infection.

Formulation and Delivery

In comparison to the considerable body of research focusing on the discovery of AMP and the optimisation of their activity, considerably less effort has been given to delivery systems, formulation or routes of administration for AMP. Formulation and delivery of AMP will play key roles in efficacy outcomes including reducing degradation of protease-susceptible AMP, limiting binding to plasma and other proteins and macromolecules, controlling dose-exposure parameters and even potentially targeting pathogens directly (e.g., intracellular pathogens or pathogens in biofilms). This topic merits a separate manuscript and several excellent reviews have already been written to that end (299–303).

As has been published widely, an issue for the development of certain peptide therapeutics is the potential for proteolysis, whether by proteases of host or microbial origin (304, 305). Infected tissue is often characterised by high levels of proteases, both microbially- and host-derived (306). Possible solutions to the problem of proteolysis include formulation of the peptide to afford protection from proteases, including liposomal formulations, as used for other drugs (307), use of non-natural or D-enantiomer amino acids (308, 309), design and development of peptidomimetics (310, 311) and multivalent peptides (312).

When considering formulation of AMP, the characteristics of both the AMP and the carrier require consideration. AMP charge (and its type and distribution), size, solubility, hydrophobicity and structure can affect loading and activity, as can the properties of the carrier including charge, pH, ionic strength, pore/mesh size, conjugation method (where appropriate). Formulation and delivery approaches that have been tested for AMP include the use of hydrogels (313–315), liposomal formulations (195, 316), carbon nanotubes (317), PEGylation (270, 299) and nanoparticles (318, 319). Appropriate formulation and delivery strategies may also allow us to resurrect and re-investigate some of the candidate AMP therapies that have previously been abandoned because *in vivo* and/or clinical efficacy was significantly diminished vs. *in vitro* data.

OTHER ANTIFUNGAL IMMUNOTHERAPEUTICS

The antifungal properties of endogenous HDP are such that these peptides are obvious templates for the design and development of synthetic therapeutic antifungal AMP. As described in preceding sections of this review, AMP have shown early promise as therapeutic candidates. The optimal clinical pathway (trial design, endpoints, formulation etc.) to demonstrate translation of their therapeutic potential into clinical use may not have been carved out as yet however, to the detriment of a number of molecules no longer in development as a result. There are, however, other potential immunotherapeutics that could be deployed alongside antifungal AMP and even existing classes of antifungal therapy; in each case to further enhance infection resolution and eradication. In particular, invasive (systemic) fungal infections predominantly affect immunocompromised patients and there are potential benefits in strengthening those aspects of the immune response that remain functional in these individuals in order to combat systemic fungal infection (30, 320, 321). In cases of invasive aspergillosis or systemic candidiasis, clinical practice guidelines recommend reduction or reversal of immune suppression (31, 322, 323), but in many cases this is simply not feasible due to the initial pathology in cases of stem cell malignancy. In some cases, the reversal of immune suppression can result in immune reconstitution inflammatory syndrome (IRIS), causing increased morbidity and mortality due to "cytokine storm" and an exaggerated host inflammatory response (324, 325). Identifying patients, therefore, for whom particular antifungal immunotherapies are appropriate is critical. It is essential to avoid overtly "boosting" any aspect of the host response in patients who are not entirely immunodeficient in order to mitigate potential immunotoxicity or hyperinflammation. Directly acting antifungal AMP with no host cell pharmacology are potentially the class of immunotherapy with broadest cross-patient applicability for fungal disease in this context. The development of biomarkers to predict responses to antifungal immunotherapy may be beneficial for broader, future adoption of fungal immunotherapy (326) and clinical trial design for these treatments will also require careful consideration as potential patient pools are likely to be limited compared to oncology trials where immunotherapeutics are more commonly used.

Adjunct immunotherapy strategies include the adoptive transfer of activated immune cells with antifungal activity, the

administration of immune-activating cytokines in combination with antifungal therapy or the use of antibody therapy. Other approaches being studied include transfusion of leukocytes pre-loaded with antifungals, modulated T cells (e.g., stimulated *ex vivo* and re-infused) and investigation of potential vaccine strategies (321, 327–332). Some of these approaches will be described in subsequent sections.

Immunostimulatory Molecules

Interferon-γ

A number of clinical studies have demonstrated beneficial effects of recombinant interferon-γ (IFN-γ) administration in combination with antifungal therapy in immunocompromised patients with systemic fungal infections, including *Candida* spp. and *Aspergillus* spp. infection ($n = 8$ patients) (333), chronic granulomatous disease (CGD) ($n = 130$) (334–336), HIV infection ($n = 173$) (337–339), leukaemia ($n = 5$) (340, 341), and transplant patients ($n = 7$) (342), in a single patient with *S. aureus* liver abscess and invasive *C. albicans* infection (343), in a single patient with intracerebral aspergillosis (344), in two patients with progressive chronic pulmonary aspergillosis (345), and in two patients with idiopathic CD4 lymphopenia and cryptococcal meningitis (346). In the study of Delsing and co-workers, rIFN-γ administration partially restored immune function as evidenced by increased production of proinflammatory cytokines involved in antifungal defence by leukocytes (IL-1β, TNFα, IL-17, and IL-22) and increased human leukocyte antigen DR (HLA-DR) positive monocyte production in patients where levels were low (333). IFN-γ is FDA-approved for the treatment Chronic Granulomatous Disease patients at risk of invasive fungal and other infections in combination with antifungal therapy and Granulocyte-macrophage colony-stimulating factor (GM-CSF) (347).

Colony Stimulating Factors

In cancer patients with chemotherapy-associated neutropenia, the prophylactic use Granulocyte Colony-Stimulating Factor (G-CSF; e.g., filgrastim) is FDA-approved and results in a decrease in rates of infection and infection-related morbidity (all causes) in patients receiving cancer therapy or undergoing stem-cell transplantation, although the effect on infection-related mortality was moderate (348). In a clinical study of patients with haematological malignancy and suspected or proven systemic fungal infection, nearly twice as many responded to amphotericin B therapy with concomitant G-CSF compared to those receiving amphotericin B alone (349). Another small study (8 patients with leukaemia ($n = 7$) or breast cancer ($n = 1$) demonstrated that adjuvant therapy with G-CSF in addition to amphotericin B resulted in cure ($n = 4$), partial response ($n = 2$) or failure ($n = 2$), indicating potential utility of G-CSF in resolving fungal infection in patients with malignancy (350). In another study, G-CSF in combination with fluconazole resulted in faster infection resolution in non-neutropenic patients with invasive candidiasis/candidemia (324, 351). Treatment with G-CSF before chemotherapy resulted in a dose-dependent increase in the number of neutrophils and treatment after chemotherapy initiation reduced the number of days on which the neutrophil count was ≤1,000/μl, the number of days on which antibiotics were used to treat fever and the incidence and severity of mucositis was decreased (352). G-CSF also enhanced the respiratory burst response of human phagocytes *in vitro* to fungal conidia or yeast cells, but not hyphae (353).

Granulocyte-macrophage colony-stimulating factor (GM-CSF; e.g., sargramostim) promotes neutrophil, monocyte, macrophage and lymphocyte production, maturation, activation and migration (as well as progenitor cells), whereas G-CSF primarily affects myeloblasts and neutrophils and M-CSF primarily affects only monocytes. GM-CSF is licensed for the treatment of chemotherapy-associated neutropenia and stem cell transplantation (354, 355) and is likely to have advantages over G-CSF therapy due to its wider effects on fungi and the immune system (324). In a randomized trial of patients receiving allogenic haematopoietic stem cell transplantation HSCT, 100-day cumulative mortality and 100-day transplantation-related mortality were lower in patients receiving GM-CSF than receiving G-CSF and after 600 days of follow-up infection-related mortality and invasive fungal disease-related mortality was lower in the GM-CSF group compared to the G-CSF group (355). In other studies of acute myeloid leukaemia patients, administration of GM-CSF led to recovery of neutrophil counts and was associated with a more rapid clearance of infection when compared with a historical control group that did not receive GM-CSF (356), including fungal infections (357). In a small study of neutropenic patients with fungal infection, eight patients received amphotericin B and GM-CSF. Six patients responded to treatment, with four undergoing complete recovery, whereas the remaining two patients died of fungal infection. Although this study did not have controls, the survival rate is higher than would be infected from antifungal treatment alone (358). In a study of 11 AIDS patients with fluconazole-refractory oropharyngeal candidiasis that received GM-CSF and fluconazole, a mycological response was seen in seven patients and three patients were cured (359). Three patients with rhinocerebral zygomycosis were successfully treated with adjunctive GM-CSF when added to antifungal therapy (amphotericin B) and surgery (360). However, in a study of acute myelogenous leukaemia in elderly patients (55–75 years), GM-CSF therapy (114 patients) did not improve complete remission rates when compared to patients receiving placebo (126 patients), but did prolong disease-free survival and overall survival. The number of patients with infections, including serious fungal infections, was not different between the GM-CSF and placebo groups (361).

Macrophage Colony-Stimulating Factor (M-CSF) can rapidly increase myeloid differentiation of haematopoietic stem cells. In a study of bone marrow transplant patients that developed invasive fungal infection and that received recombinant human M-CSF (rhM-CSF), survival was greater than historical patients not receiving rhM-CSF with *Candida* spp. infection, but not in patients with *Aspergillus* spp. infection or in any patients with Karnofsky scores of <20% (362, 363). Exogenous M-CSF was protective in murine models of *Aspergillus* spp. and *Pseudomonas aeruginosa* infection following haematopoietic stem cell or

progenitor cell transplantation and was more efficacious than G-CSF (364). Synergy of M-CSF with fluconazole was observed in human monocyte-derived macrophages infected with *Cryptococcus neoformans*. M-CSF alone also reduced counts of *Cryptococcus neoformans* in this model (365) and in a murine model of *Cryptococcus neoformans* infection (366). In a rat model of acute candidiasis, administration of ≥0.1 mg/kg M-CSF with 0.3 mg/kg fluconazole enhanced survival (>30 d) compared with fluconazole alone (5 d) and similarly reduced *C. albicans* kidney burden in a chronic model of candidiasis (367). Conversely, another study of mice infected intravenously with *C. albicans* demonstrated that treatment with M-CSF exacerbated disease and led to significantly earlier death (368). Clearly, M-CSF has potential in the treatment of invasive fungal infection, either alone, or in combination with antifungal therapy, but more research is clearly required and it is possible that the effect may be dependent on the infecting pathogen. Thus, whilst showing clear promise the use of colony-stimulating factor therapy should be the subject of appropriately controlled clinical studies in patients with accurately diagnosed fungal infections and comparable antifungal therapeutic regimens.

Immune Checkpoint Inhibitors

Immune checkpoints are important regulators of immune homeostasis. Immune checkpoints consist of both stimulatory and inhibitory pathways that are important for maintaining self-tolerance and regulating the type, magnitude, and duration of the immune response (369, 370). Immune checkpoint therapies in oncology target regulatory pathways in T cells to enhance anti-tumour responses (370–373) and are used for the treatment of squamous-cell carcinoma and advanced melanoma. The checkpoint programmed cell death 1 (PD1) (a member of the B7-CD28 superfamily) is expressed on monocytes, natural killer cells, T- and B-lymphocytes. Binding of PD1 to the ligand PD1-L1 on myeloid cells impairs T-cell functions including cytokine production and cytotoxic activity, whereas blocking binding of PD1 to its ligand with an anti-PD1 antibody can restore immune function. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is another immune checkpoint that can impair T-cell function and Ipilimumab (an anti-CTLA-4 antibody) was the first immune checkpoint inhibitor approved for the treatment of cancer (374). The PD1 and CTLA-4 pathways have roles to play in antifungal defences (374), as demonstrated *in vitro* in a murine model of *Histoplasma capsulatum* infection (PD1) (375) and in blood from patients with paracoccidioidomycosis (CTLA-4) (376). In a murine model of *C. albicans* sepsis, antibodies to PD1 and PD-L1 were effective at improving survival, as was an antibody to CTLA-4 in this model (377) and in a murine model of *Cryptococcus neoformans* infection (378). Nivolumab, an antibody drug that blocks PD1, was used successfully in combination with IFN-γ and antifungal therapy (liposomal amphotericin B and posaconazole) in a case of invasive mucormycosis following unsuccessful antifungal therapy for 28 days (379). The use of Nivolumab for immune checkpoint inhibition in sepsis (documented or suspected infection) has been the subject of a recent Phase 1b clinical trial (NCT02960854) (380).

Vaccines

It is estimated that vaccination prevented at least 10 million deaths globally between 2010 and 2015 (381). No fungal vaccine has yet been approved for use in humans although clinical trials of fungal vaccines have been reported and a number are in preclinical and clinical development (382, 383). Our ever-improving knowledge of the immune system ought to increase the likelihood of developing fungal vaccines, but a number of challenges exist and for a number of infectious diseases, treatment rather than vaccination remains the optimal strategy. Eliciting a protective response to immunisation in immunocompromised individuals who have developed/are at risk of invasive fungal infection is unlikely, particularly without risk of aggravating underlying disease and/or development of the fungal infection due to attenuated vaccine administration (382–384). Additionally, developing a vaccine against commensal microorganisms, e.g., *Candida* spp. could represent an additional problem (385). The high costs associated with vaccine development are a challenge considering that revenue will only be obtained from vaccinating only populations at risk of developing fungal infection, or in the case of endemic mycosis, only a limited patient population cannot attract sufficient investment (386).

A vaccine (NDV-3A) containing the N-terminal portion of the agglutinin-like sequence 3 (Als3) protein of *C. albicans*, is in development by NovaDigm Therapeutics for the prevention of recurrent vulvovaginal candidiasis (VVC). Als3 is a hyphal-specific virulence factor that mediates adherence to and invasion of human epithelial and vascular endothelial cells. In a Phase II randomized, double-blind, placebo-controlled clinical trial, NDV-3A demonstrated a statistically significant increase in the percentage of symptom-free patients at 12 months after vaccination and a doubling in the median time to first symptomatic episode for a subset of patients aged <40 years (ClinicalTrials.gov Identifiers: NCT01926028 and NCT02996448) (382, 387). Another vaccine, PEV7, has been the subject of a successful Phase I clinical trial (ClinicalTrials.gov Identifier: NCT01067131) for the prevention of recurrent VVC. PEV7 was developed by Pevion Biotech (rights subsequently acquired by NovaDigm Therapeutics) and contains recombinant secreted aspartyl protease 2 (rSAP-2) incorporated into influenza virisomes. Trial results demonstrated the generation of specific and functional B cell memory in 100% of the vaccinated women and a favourable safety profile (388). Earlier reports of an oral vaccine, D.651, for the prevention of VVC recurrence was prepared using ribosomes of *C. albicans* serotypes a and b. A Phase II clinical trial reported a good safety profile and efficacy, in which 13 of 20 patients taking the vaccine did not experience recurrence of VVC during the 6 months taking the vaccine (389). The current status of this vaccine is not known. In another study, a vaccine consisting of formaldehyde-killed spherules of *Coccidioides immitis* was tested in humans, but a statistically significant reduction of the incidence of infection was not observed in those vaccinated (390). A number of other fungal vaccines have been tested in animal models and are beyond the scope of this manuscript, but have been the subject of several recent reviews (328, 383, 388, 391–394). The vaccines described

TABLE 3 | Selected immunology-based approaches for the treatment of fungal infection.

Antifungal therapy	Target fungal infection	Developmental therapeutic	Target pathogen/s	Development stage as antifungal	Reference/ ClinicalTrials.gov identifier
AMP					
Antifungal	Onychomycosis	NP213	Dermatophytes	Phase IIb	(173)
	Onychomycosis	HXP124	Dermatophytes	Phase I/IIa	(174)
	VVC[1]	CZEN-002	*Candida* spp.	Phase I/IIa	(56)
	Oral candidiasis	P113	*Candida* spp.	Phase IIb	NCT00659971
	Dermal infection	Omiganan	*Candida* spp.	*In vivo* (porcine)	(209)
	Prophylaxis in HSCT[2] patients	hLF1-11	Not Specified	Phase I	(225)
	Oral mucositis	Iseganan	Yeasts	Phase III	(240)
	Not specified	LTX-109	*S. cerevisiae*	*In vitro*	(245)
	Aspergillosis & Candidiasis	NP339	*Aspergillus* spp., *Candida* spp., mucorales	*In vitro*	(290)
	Fungal infection	D2A21	*Mucor* spp., *T. mentagrophytes*	*In vitro*	(259)
	Systemin infection	ETD151	*C. albicans*, *A. fumigatus*	*In vivo* (murine)	(263)
Anti-biofilm	Not specified	Histatin-5	*C. albicans*	*In vitro*	(83)
	Not specified	LL-37	*C. albicans*	*In vitro*	(128)
	Not specified	hLF1-11	*C. albicans*	*In vitro*	(215)
	Not specified	LTX-109	*S. cerevisiae*	*In vitro*	(245)
Immunostimulatory molecules				Clinical	
Interferon-γ	Systemic infection	IFN- γ	*Aspergillus* spp., *Candida* spp.		(347)
Colony stimulating factors	Prophylaxis	G-CSF[3]	Fungal	Clinical	(348)
	Prophylaxis	GM-CSF[4]	Fungal	Clinical	(355)
	Prophylaxis	M-CSF[5]	*Candida* spp.	Phase I/II	(363)
Antibodies					
Prophylaxis	Cryptococcosis	18B7	*Cryptococcus neoformans*	Phase I	(408)
	Candidiasis	mAb 3D9.3	*C. albicans*	*In vitro*	(404)
	Fungal	mAb C7	*Candida* spp., *Cryptococcus* spp., *A. fumigatus*, *Scedosporium prolificans*	*In vitro*	(405)
Therapeutic	Disseminated candidiasis	Ab119 & Ab120	*Candida* spp.	*In vivo* (murine)	(409)
Vaccines	VVC[1]	NDV-3A	*Candida* spp.	Phase II	(387)
	VVC[1]	PEV7	*Candida* spp.	Phase I	(388)
	VVC[1]	D.651	*Candida* spp.	Phase II	(389)
Immune checkpoint inhibitors	Mucormycosis	Nivolumab	Mucorales	Case study (1 patient)	(379)
Cell-based therapies					
Antifungal-loaded leukocytes	Pulmonary aspergillosis	Posaconazole-loaded leukocytes	*Aspergillus* spp.	*In vivo* (murine)	(414)
CAR-T	Murine lung infection	D-CAR$^+$ T cells[6]	*Aspergillus* spp.	*In vivo* (murine)	(415)

[1] *Vulvovaginal candidiasis.*
[2] *Haematopoietic stem cell transplantation.*
[3] *Granulocyte Colony-Stimulating Factor.*
[4] *Granulocyte-Macrophage Colony-Stimulating Factor.*
[5] *Macrophage Colony-Stimulating Factor.*
[6] *Dectin-Chimeric Antigen Receptor Positive T-cells.*

above represent the only ones to reach clinical trials to the best of the authors knowledge.

On a cautionary note, in some cases, live, attenuated fungi (*Blastomyces dermatitidis* and *Histoplasma capsulatum*) have demonstrated the induction of protective immunity in mice (395). Naturally, caution would be required before testing live attenuated fungi in immunocompromised individuals although live, attenuated vaccines are arguably much more appropriate candidates for vaccination against endemic fungal infections, such as histoplasmosis and sporotrichosis, in otherwise immunocompetent, healthy subjects.

Interestingly, heat-killed *Saccharomyces cerevisiae* administered as a vaccine was protective against systemic aspergillosis, candidiasis, cryptococcosis and coccidioidomycosis in mouse models (396), but to the best of our knowledge has not yet been tested in humans.

Antifungal Monoclonal Antibodies

Monoclonal antibodies (mAb) represent some of the world's best-selling therapeutics, of which more than 80 have received marketing approval and more than 100 are in development. In 2018 alone, twelve new mAb were approved by the FDA, representing 20% of the total number of approved drugs and sales of mAb were forecast to reach US $125 Bn by 2020. Most therapeutic monoclonal antibodies are used for the treatment of cancer or immunological disorders (397, 398). The development of monoclonal antibodies for the prevention and treatment of infectious diseases lags somewhat behind their development for other therapeutic areas, e.g., cancer and autoimmune diseases (399), and only three monoclonal antibodies have received approval for infectious disease prophylaxis or treatment; palivizumab for prevention of respiratory syncytial virus in high-risk infants (400); and obiltoxaximab (401) and raxibacumab (402) for prophylaxis and treatment of anthrax. The lack of development of mAb for infectious diseases may be because consensus on clinical end-points and definitions on conditions of use are lacking, as well as high costs associated with their development and lack of a cle arly defined market for these products (399). Fungal-specific mAb can mediate protection from fungal infection by direct action on fungal cells or via promotion of phagocytosis and complement activation. However, some mAb to fungi can be disease-enhancing or have no effect (403). Protective mAb against human fungal pathogens are currently in preclinical development (382), including examples with narrow spectrum reactivity [e.g., mAb 3D9.3 (anti-Als3) that specifically recognises *C. albicans* (404)] and broad-spectrum reactivity with a number of fungal pathogens (e.g., mAb C7 (anti-Als3) which inactivates germ tubes and spores of *Candida* spp., *Cryptococcus neoformans*, *A. fumigatus* and *Scedosporium prolificans* (405).

A murine mAb, 18B7, was raised against *Cryptococcus neoformans* and bound to capsular glucuronoxylomannan in infected mouse tissues (406). 18B7 was protective in a murine intraperitoneal model of *Cryptococcus neoformans* infection (407). In a human Phase I dose escalation study of human immunodeficiency virus (HIV)-infected patients who had been successfully treated for cryptococcal meningitis, the maximum tolerated dose was established as 1.0 mg/kg and serum cryptococcal antigen titres declined by a median of 3-fold at 2 weeks post-infusion. However, titres subsequently returned toward the baseline values by week 12, 3 of 4 subjects in the 1.0-mg/kg dosing cohort had a 0.5 log10 increase in HIV load and 18B7 was not detected in cerebrospinal fluid (408).

Interestingly, Rudkin et al. generated the first set of fully human anti-*Candida* spp. mAb isolated from B cells of patients suffering from candidiasis and that demonstrated morphology-specific, high avidity binding to the cell wall, including mAb specific for the *C. albicans* hyphal cell wall protein Hyr1. Cell wall mAb demonstrated cross-reactivity with other *Candida* spp., whereas anti-Hyr1 mAb were cross-reactive with only *C. albicans*. Importantly, tested mAb promoted phagocytosis of *C. albicans* by macrophages and reduced fungal burden in therapeutic or prophylactic murine models of disseminated candidiasis (409), but these have yet to be tested in humans. Efungumab (Mycograb) is a recombinant human mAb against fungal HSP90 with activity against *C. albicans*, *C. krusei*, *C. tropicalis*, *C. glabrata,* and *C. parapsilosis* (410, 411). *In vitro* studies revealed synergy with fluconazole, amphotericin B (AmB) and caspofungin, and in a murine model of systemic candidiasis, efungumab improved the killing of *Candida* spp. (*C. albicans*, *C. krusei,* and *C. glabrata*) in combination with AmB (412). However, the combination effect of efungumab and AmB was later revealed to be a nonspecific protein effect, as addition of efungumab or other unrelated proteins, including human serum, resulted in similar decreases in the MIC of AmB (413). Although clinical trials of this product were conducted, they were unsuccessful and development of this drug candidate has been abandoned.

Therefore, the potential for the use of mAb for treatment or prophylaxis against fungal infection remains a possibility, but large-scale clinical trials will be required to bring this promise to fruition.

CONCLUSIONS

Antimicrobial peptides are promising candidates as therapeutics for the treatment of fungal infection and are much needed in clinical practice due to the limited array of treatment options and increasing resistance to existing antifungals. Unfortunately, we are not seeing enough drug candidates making it through the drug development pipeline, as *in vitro* and *in vivo* testing approaches are not always appropriate and/or optimised for AMP (268). The same is true in part for clinical efficacy trials which must be appropriate for AMP (end points in particular). These factors are undoubtedly part of the reason behind there not being more AMP progressing through the drug development cycle and/or AMP candidates are confined to topical therapy status as delivery systems, formulation, routes of administration and duration of therapy for AMP have not been adequately optimised. The time is now coming for greater exploitation of AMP and other immunotherapeutics as antifungal drug candidates as we gain a greater understanding of how best to

test these drug candidates *in vitro* and how regulatory pathways and clinical studies can be more accommodating for peptides (**Table 3**). As the global AMR crisis worsens and emerging fungal diseases increase, the potential of these drug candidates must be fulfilled sooner rather than later.

AUTHOR CONTRIBUTIONS

DM and DO'N contributed to the writing and editing of this manuscript. All authors contributed to the article and approved the submitted version.

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Antimicrobial Peptide Induced-Stress Renders *Staphylococcus aureus* Susceptible to Toxic Nucleoside Analogs

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Cationic antimicrobial peptides (AMPs) are active immune effectors of multicellular organisms and are also considered as new antimicrobial drug candidates. One of the problems encountered when developing AMPs as drugs is the difficulty of reaching sufficient killing concentrations under physiological conditions. Here, using pexiganan, a cationic peptide derived from a host defense peptide of the African clawed frog and the first AMP developed into an antibacterial drug, we studied whether sub-lethal effects of AMPs can be harnessed to devise treatment combinations. We studied the pexiganan stress response of *Staphylococcus aureus* at sub-lethal concentrations using quantitative proteomics. Several proteins involved in nucleotide metabolism were elevated, suggesting a metabolic demand. We then show that *Staphylococcus aureus* is highly susceptible to antimetabolite nucleoside analogs when exposed to pexiganan, even at sub-inhibitory concentrations. These findings could be used to enhance pexiganan potency while decreasing the risk of resistance emergence, and our findings can likely be extended to other antimicrobial peptides.

Keywords: pexiganan, antibiotic resistance, antimetabolites, antimicrobial peptides, antibiotics, nucleoside analogs

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INTRODUCTION

Antimicrobial peptides (AMPs, we use AMPs here as synonymous with host defense peptides) are immune effector molecules used by multicellular organisms to control infections (1–3). These peptides are usually active against a broad spectrum of bacterial pathogens and some display activity against antibiotic-resistant bacteria. Thus, antimicrobial peptides are considered a promising source of new antibacterial drugs (4, 5) to tackle the current antibiotic crisis (6).

Some of the factors that make AMPs attractive are their high diversity across the tree of life (7) and the finding that, although drug resistance also evolves against AMPs (8–12), it evolves at a much lower probability in comparison to conventional antibiotics (3, 13, 14). One common problem with the development of AMPs as drugs is that, under physiological conditions, their antimicrobial activity cannot be easily recaptured and the required dosage is extremely high (15). This dosage issue can be addressed by making use of synergistic combinations of AMPs (16), a property common in natural defense cocktails (17, 18).

While the mode of action on bacterial membranes has been worked out for some AMPs (19), the consequence of AMP-induced stress on bacterial physiology is less studied. The first goal of this study, therefore, is to understand how the pathogen *Staphylococcus aureus* reacts to different doses of pexiganan at the minimum inhibitory concentration (MIC). Pexiganan is a drug that was mostly developed against this bacterium (20). This molecule is a 22-amino-acid peptide (Gly-Ile-Gly-Lys-Phe-Leu-Lys-Lys-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Lys-Ile-Leu-Lys-Lys-NH2); molecular weight, 2478 Da [free peptide base] and has cationic nature. It is a derivative analog of the magainin II peptide isolated from the skin of the African clawed frog *Xenopus laevis*. Pexiganan exhibited broad-spectrum antibacterial activity *in vitro* when tested against 3,109 clinical isolates of gram-positive and gram-negative, anaerobic and aerobic bacteria (20). Pexiganan shows a barrel-stave type mechanism of membrane disruption (or channel formation). The consensus is that Pexiganan exerts its antibacterial effect by forming toroidal pores in the bacterial membranes. Pexiganan effectively induced the uptake and leakage of small compounds from both bacterial membranes and *in vitro* assembled lipid vesicles (21).

Using pexiganan as an example, we found that different concentrations induce the upregulation of several genes depending on nucleotides or related to nucleotide metabolism. Based on these results, we hypothesized that this would lead us to identify phenotypic collateral sensitivity. We hypothesized that the response to pexiganan sensitizes *S. aureus* against certain nucleoside antimetabolites or toxic nucleoside analogs. Interestingly, these analogs have been proposed as an alternative to antibiotics as a consequence of resistance emergence (22). Nucleoside analogs have the advantage of being clinically approved for cancer therapies, but also as antiviral and antifungal treatments (22). Pyrimidine and purine analogs, as we use here, showed potent antimicrobial activity against *S. aureus* in the past (22–25).

In this study, we show how proteomic changes in *S. aureus* in response to low-dose pexiganan uncover cellular soft spots that help to identify intervention opportunities. In addition, our findings contribute to the understanding of the early stages of resistance evolution to antimicrobial peptides. Here, we first study the global proteomic response of *S. aureus* to the cationic antimicrobial peptide pexiganan at concentrations similar to and below MIC. This helps us to detect the possible metabolic changes that open the path to collateral sensitivity to nucleoside analogs. We then confirm that these treatments sensitize *S. aureus* to antimetabolite purine and pyrimidines analogs.

MATERIALS AND METHODS

Bacteria and Growth Conditions

We used *S. aureus* SH1000 (26) for all experiments. Bacteria were cultured in non-cation-adjusted (unsupplemented) Mueller–Hinton broth (MHB) as recommended for antimicrobial peptides susceptibility testing (27).

Global Proteomics by LC-Mass Spectrometry

Staphylococcus aureus strain SH1000 was grown in non-cation-adjusted MHB to the mid-exponential-phase (OD_{600} 0.5) at 37°C with vigorous shaking. The cultures were diluted 100 times in fresh MHB in a separate tube to a final volume of 5 ml. Pexiganan was added to tubes for a final concentration of 0.5, 1, 2 and 4 μg/ml (1/8,1/4, 1/2, 1x MIC, respectively) in a final culture volume of 10 ml per tube. Non-treated samples were used as controls. After the addition of pexiganan, all tubes were incubated for 30 min with moderate shaking at 37°C. The pellets were collected by centrifugation at 10,000 × g for 5 min and the supernatant was removed by aspiration using a sterile vacuum line. Fifty microlitre of denaturation urea buffer (6 M urea/2 M thiourea/10 mM HEPES, pH 8.0) were then added to each pellet. The resulting suspensions were transferred to new 1.5 ml Eppendorf tubes and exposed to 5 freeze-thawing cycles alternating between liquid nitrogen and a 37°C water bath. The tubes were centrifuged at 20,000 × g for 10 min and the resulting supernatants were transferred to fresh tubes and used as starting protein material for digestion. Each experimental condition had six independent biological replicates. Approximately 50 μg proteins were processed per sample and were in-solution digested as described elsewhere (28). Denaturation buffer-containing protein solutions were reduced by adding 1 μl of 10 mM DTT (final concentration) and incubated for 30 min. The reactions were then alkylated by adding 1 μl of 55 mM iodoacetamide and incubated for 20 min in the dark. Lysyl endopeptidase (LysC, Wako, Japan) resuspended in 50 mM ABC was added to the digestion reaction in a proportion of 1 μg per 50 μg of total sample protein and incubated for 3 h. The samples were diluted with four volumes of 50 mM ammonium bicarbonate (ABC) and digested overnight with 1 μg of sequencing grade modified trypsin (Promega, USA). All digestion steps were performed at room temperature. The next day, the digestions were stopped by adding final concentrations of 5% acetonitrile and 0.3% trifluoroacetic acid (TFA). The samples were desalted using the Stage-tip protocol as described previously (28), and the eluates were vacuum-dried. Peptides were reconstituted in 10 μl of 0.05% TFA, 2% acetonitrile, and 6.4 μl were analyzed by a reversed-phase capillary nano liquid chromatography system (Ultimate 3000, Thermo Scientific) connected to an Orbitrap Velos mass spectrometer (Thermo Scientific). Samples were injected and concentrated on a trap column (PepMap100 C18, 3 μm, 100 Å, 75 μm i.d. × 2 cm, Thermo Scientific) equilibrated with 0.05% TFA, 2% acetonitrile in water. After switching the trap column inline, LC separations were performed on a capillary column (Acclaim PepMap100 C18, 2 μm, 100 Å, 75 μm i.d. × 25 cm, Thermo Scientific) at an eluent flow rate of 300 nl/min. Mobile phase A contained 0.1% formic acid in water, and mobile phase B contained 0.1% formic acid in acetonitrile. The column was pre-equilibrated with 3% mobile phase B followed by an increase of 3–50% mobile phase B in 50 min. Mass spectra were acquired in a data-dependent mode utilizing a single MS survey scan (m/z 350–1,500) with a resolution of 60,000 in the Orbitrap, and MS/MS scans of the 20 most intense precursor ions in the

linear trap quadrupole. The dynamic exclusion time was set to 60 s and automatic gain control was set to 1×10^6 and 5,000 for Orbitrap-MS and LTQ-MS/MS scans, respectively.

MS and MS/MS raw data were analyzed using the MaxQuant software package (version 1.6.4.0) with an implemented Andromeda peptide search engine (29). Data were searched against the reference proteome of *S. aureus* strain NCTC 8352 downloaded from Uniprot (2,889 proteins, taxonomy 93061, last modified September 2017) using label-free quantification and the match between runs option was enabled. Filtering and statistical analysis was carried out using the software Perseus (30). Only proteins with intensity values from at least 3 out of 6 replicates were used for downstream analysis. Missing values were replaced from normal distribution (imputation) using the default settings (width 0.3, down shift 1.8). Student's *T*-tests were performed using permutation-based FDR of 0.05.

Antimetabolite Nucleosides

In this study, we used four nucleoside analogs. We used the pyrimidine analogs 6-azauracil, gemcitabine, 5-fluorouracil and the purine analog 6-thioguanine. All drugs were purchased from Sigma Aldrich (Germany). 6-azauracil is used as a growth inhibitor of various microorganisms via depletion of intracellular GTP and UTP nucleotide pools (31). Gemcitabine is a chemotherapy medication used to treat different types of cancer. Gemcitabine is a synthetic pyrimidine nucleoside analog in which the hydrogen atoms on the 2′ carbon of deoxycytidine are replaced by fluorine atoms and competitively takes part and disrupts several pathways where pyrimidines are needed (24). 5-Fluorouracil is also used as an anticancer treatment and it works by inhibiting cell metabolism by blocking many pathways, but its major action is the inhibition of the thymidylate synthase. By doing so, the synthesis of the pyrimidine thymidine is stalled, which is an essential nucleoside required for DNA replication (32). 5-Fluorouracil causes a drop on dTMP, causing cells to undergo cell death via thymineless death (32, 33).

Pexiganan and Antimetabolite Nucleosides Susceptibility Testing

Minimal inhibitory concentration (MIC) was determined by broth micro-dilution method modified for cationic antimicrobial peptides (34). Briefly, 2 μl of the mid-exponential phase culture diluted 1:100 (around 10^5 bacteria) were inoculated into each well of a polypropylene V-bottom 96-well plates with anti-evaporation ring lids (Greiner Bio-One GmbH, Germany). Prior to inoculation, pexiganan and the analogs (a kind gift from Dr. Michael A. Zasloff, Georgetown University) were 2-fold serially diluted in a final volume of 100 μl MHB per well using 32 μg/ml as starting concentration. Each assay was performed with eight replications and plates were incubated at 37°C in a humid chamber. The MIC was defined as the lowest concentration where no visible bacterial growth was observed after 24 h.

Isobologram Assay

The combined activity and interactions between peptides, pexiganan, purine and pyrimidine analogs against *S. aureus* in MHB were determined using isobolographic combinations, also called the checkerboard assay method, (8 × 8 matrix of concentrations combinations) (35). In a 96-well plate, 50 μl of pexiganan at 4x MIC concentration was 2-fold serially diluted ranging from 32 to 0.25 μg/ml in the direction of the columns from 1 to 8. In another 96-well plate, 100 μl of nucleoside analogs at 8x MIC concentrations were prepared identically to the previous plate, but diluted in the direction of the rows from A to H. Half of the content (50 μl) of each well from the analog drug plate was transferred to the corresponding well of the plate containing pexiganan in an equal 1:1 mix fashion, halving the concentration of both compounds. In the same plate, the columns 9 and 10 were used to serially dilute both the peptide and the analog drug in the same concentrations that were present in the combination to compare single compounds vs. combination. Columns 11 and 12 were used as a control, by inoculating column 11 wells with bacteria without any drug and leaving columns 12 only with the same volume of MHB as a media contamination control. Each plate was prepared in triplicates to check for consistency. The bacterial suspension was prepared by growing *S. aureus* SH1000 to mid-exponential phase (2.5 h, with moderate shaking at 37°C) in MHB to an OD_{600} between 0.3 and 0.5. The bacterial suspension was diluted in MHB and $\sim 1 \times 10^6$ bacteria were inoculated in each well. After 24 h of incubation at 37°C in a humid chamber, the plates were visually examined for growth. The Fractional inhibitory concentration (FIC index) for a combination of pexiganan and each antimetabolite drug was calculated as [(MIC of the peptide in combination with a given analog)/(MIC of peptide alone)] + [(MIC of analog in combination with peptide)/(MIC of analog alone)]. The interpretation of the results was as follow: FIC $\leq$ 0.5, synergistic; 0.5 $<$ FIC $\leq$ 1, additive; 1 $<$ FIC $\leq$ 4, indifferent; FIC $>$ 4, antagonistic (36). To ensure that bacteria lost viability while reading MIC values for pexiganan-analog combinations, we used the resazurin colorimetric assay as described previously with minor modifications (37). Resazurin (THK, Germany) was prepared at 0.015 % in distilled water and sterilized by filtration. It was stored at 4°C for a maximum of 1 week after preparation. Resazurin (0.015%) was added to each well (10 μl per well, 1/3 of the original described quantity) and further incubated for 3 h for the observation of color change. Columns with no color change (blue resazurin) were scored as dead culture. In contrast, color change to purple (reduced resazurin) was considered as a sign of viability.

Time-Kill Experiments

Starting from early mid-exponential phase cultures (1×10^7 CFU/ml), bacteria were exposed to growing concentrations of pexiganan ranging from 1 to 8x MIC or pexiganan combined with the nucleoside analogs 6-azauracil, gemcitabine, 5-fluorouracil and 6-thioguanine at their respective 1/2x MIC values. The cultures were incubated with soft shaking at 37°C for 2 h. Samples from each culture (1 ml) were taken at 20-min time-point intervals. The samples were diluted and plated to determine cell viability. The experiments consisted of five independent replicates. Non-treated cells were used as a control.

Statistical Analysis

The effect of treatments on bacterial killing was analyzed using R package nparLD which is designed to perform non-parametric analysis of longitudinal data in factorial experiments modeling the variation over time (38). *P* values of $\leq$0.05, after correction, if needed, were considered statistically significant. All tests were performed with the statistic software R (39).

RESULTS

Changes in Protein Profiles After Pexiganan Treatment

We examined *S. aureus* exposed to pexiganan by studying proteome-wide changes after a 30-min treatment with different pexiganan concentrations (0.125, 0.25, 0.5, and 1x MIC, **Table S1**). Overall, 1160 proteins were identified at a 1% or less false discovery rate (FDR) among which 968 proteins were quantified in at least 3 out of 6 replicates and used for downstream analysis. All identified proteins, their quantification and statistical tests are provided in **Table S2**. A global overview shows a proteome-wide perturbation induced by pexiganan stress for all concentrations compared to control. Many proteins were significantly differentially expressed (**Figure S1**). It is noticeable that as long as the dose increases, the level of expression (fold-change) of both overexpressed and suppressed genes, decreases, making the dot scattering of the volcano plot less disperse (**Figure S1**). This indicates a decrease in the ability of the cell to react with increasing peptide concentration.

Envelope Stress Response to AMPs

Within the upregulated proteome fraction (**Figure 1** and **Figure S1**), a group of proteins related to osmotic stress response shows up. The multi-peptide resistance factor MprF, a protein associated with cationic peptide resistance, which is conserved among many bacterial species (40, 41) is upregulated in all pexiganan doses except in the lowest dose (1/8x MIC). MprF catalyzes the transfer of a lysyl group from L-lysyl-tRNA(Lys) to membrane-bound phosphatidylglycerol producing lysyl-phosphatidylglycerol, a major component of the bacterial membrane with a net positive charge. Hence, a modification of the anionic phosphatidylglycerol with positively charged L-lysine results in the repulsion of the peptides. Changes in the membrane charge is a *per se* resistance mechanism against cationic antimicrobial peptides (42). Thus, MprF increases resistance to moenomycin and vancomycin but also to human defensins (HNP1-3) and contributes to the evasion of oxygen-independent neutrophil killing and other AMPs and antibiotics (43, 44). Another highly expressed protein is CapF, which is involved in the pathway capsule polysaccharide biosynthesis, a mucous layer on the surface of the bacterium that facilitates immune evasion and infection. CapF is an important virulence factor during infections by *S. aureus*. The enzyme CapF is considered a therapeutic candidate to disrupt the capsule polysaccharide biosynthesis (45). Another protein that contributes toward modifying the bacterial envelope and has a significant higher expression is TagG. This protein is part of the wall teichoic acid synthesis during the final steps of the pathway. Wall teichoic acids are important in pathogenesis and play key roles in antimicrobial resistance (41, 46).

Proteases and Chaperones Proteins

The chaperones/proteases ClpL and TreP are among the fifty upregulated genes for the dose corresponding to the MIC (8 μg/ml). ClpL is an ATP-dependent Clp protease. Clp proteases play a central role in stress survival, virulence and antibiotic resistance of *S. aureus* (47). Another protease induced by pexiganan is PepT, also known as Staphopain A. This enzyme is a cysteine protease that plays an important role in the inhibition of host innate immune response. It cleaves host elastins from connective tissues, pulmonary surfactant protein A in the lungs, and the chemokine receptor CXCR2 on leukocytes (48). Proteolytic cleavage of surfactant protein A impairs bacterial phagocytosis by neutrophils while CXCR2 degradation blocks neutrophil activation and chemotaxis (48, 49). Additionally, PepT promotes vascular leakage by activating the plasma kallikerin/kinin system, resulting in patient hypotension (50).

Alteration of Metabolism

For example, NptA, a phosphate transporter, usually induced by phosphate limitation, is highly abundant in post-pexiganan treatment. Inorganic phosphate acquisition via NptA is particularly important for the pathogenesis of *S. aureus*. NptA homologs are widely distributed among bacteria and closely related less pathogenic staphylococcal species do not possess this importer. Another phosphate metabolism-related gene with high expression is SAOUHSC_00480, that codes for a putative nucleoside triphosphate pyrophosphohydrolase (51). Also related to phosphate metabolism, we observed a high level of FruA in different pexiganan concentrations. This protein is a phosphoenolpyruvate-dependent sugar phosphotransferase system (a PTS system) is a major carbohydrate active transport system, which catalyzes the phosphorylation of incoming sugar substrates concomitantly with their translocation across the cell membrane and are potentially important for survival in the respiratory tract of the host (52). GlcB, another PTS system is a phosphoenolpyruvate-dependent sugar phosphotransferase system. This protein catalyzes the phosphorylation of incoming sugar substrates and their translocation across the cell membrane (53). Another two phosphate metabolism related proteins, CarA and CarB, which participate in the L-arginine biosynthesis, were induced. They are involved in the first step of the sub-pathway that synthesizes carbamoyl phosphate from bicarbonate. The elevation of these enzymes could indicate that pexiganan stress may be involved in amino acid depletion.

The gene SAOUHSC_00456 that codes for YabA is significantly increased as well by pexiganan. YabA is involved in the initiation of chromosome replication and is a negative controller of DNA replication initiation in *Bacillus subtillis*. YabA and DnaD inhibit helix assembly of the DNA replication initiation protein DnaA (54). The elevated concentration of YabA could stall the cell division while the bacteria is under severe stress. *S. aureus* upregulates Spermidine/putrescine import ATP-binding protein PotA. This protein is part of the ABC transporter complex PotABCD and responsible for

FIGURE 1 | Heatmap of relative protein expression based on label-free quantification detected by liquid chromatography-mass spectrometry (LC-MS). Only the 50 most significantly up-regulated proteins compared to the control are shown (log2 fold-change). Red rectangle highlights proteins that participate in or depend on nucleotide metabolism. Proteins were extracted after 30 min of pexiganan addition (0.125, 0.25, 0.5 and 1 fractions of the minimal inhibitory concentration). log2 fold-changes are given from highest (green) to lowest (red).

energy coupling to the transport system. Spermidine and putrescine are polyamines whose role in *S. aureus* is not well-defined (55). There are also a set of up-regulated proteins coded by the genes SAOUHSC_01717, SAOUHSC_02581, and SAOUHSC_02581 whose functions remain unknown as described in Uniprot database and showed no homology with any known sequence (51).

One of the hallmarks of our proteomic dataset is that we found a higher level of expression, compared to controls, for proteins related with nucleotide metabolism (**Figure 1**), which is directly connected to the upregulation of phosphate metabolism proteins described above. GmK, for example, is an essential protein for recycling GMP and indirectly, cGMP Guanylate kinase is near four times more abundant than in the control group. GMK is an essential enzyme and a potential antimicrobial drug target owing to its role in supplying DNA and RNA precursors (56). Another nucleobase metabolism-related protein having or exhibiting a higher expression for the 1x MIC treated cells is PyrG. This enzyme catalyzes the ATP-dependent amination of UTP to CTP with either L-glutamine or ammonia as the source of nitrogen. It also regulates intracellular CTP levels through interactions with the four ribonucleotide triphosphates (51).

Downregulation Response to Pexiganan

Pexiganan also negatively impacted proteome-wide gene expression (**Figure S1** and **Table S2**). Among the most affected gene expressions throughout all concentrations are genes such as *dps* (coding for a known iron storage protein), *hld*, *copZ*, *cspC*, *metQ*, *sceD*, *isaA csoB*/*C*, *dltC*, adsA and *sasG*, SAOUHSCA_01134 and SAOUHSCA_02576. The gene *cspB* codes for the downregulated protein CspD, a cold shock protein that accumulates during low temperature or cold shock. This gene is also a component of the stringent response, indicating that it could be a general stress response gene (57). Other genes showing a differentially low level of expression are SAOUHSC_01986, SAOUHSC_01986, SAOUHSC_008020, SAOUHSC_02093, SAOUHSC_02535, and SAOUHSC_01414 which code for uncharacterized proteins (51). SAOUHSC_01030 is a putative glutaredoxin domain-containing protein but it is not characterized either. The gene SAOUHSC_02576 codes for a putative secretory antigen SsaA, identified in *S. epidermidis* but its function is also unknown (51).

In contrast to the upregulation of peptidoglycan synthesis, we observed that putative peptidoglycan hydrolases and probable lytic transglycosylases IsaA and SceD were downregulated. Interestingly, the *isaA sceD* double mutant is attenuated for virulence, while SceD is essential for nasal colonization in cotton rats (58). The gene *moaD* shows also a reduced level of expression and it codes for a molybdopterin converting factor subunit 1. Molybdopterins are a class of cofactors found in most molybdenum-containing and all tungsten-containing enzymes. Molybdopterin pathway reactions consume guanosine triphosphate that is converted into the cyclic phosphate of pyranopterin (59). Another metabolic enzyme, AldA, aldehyde dehydrogenase central carbohydrate metabolism is downregulated in all doses of pexiganan. This is also the case of CopZ, a chaperone that serves for the intracellular sequestration and transport of copper, delivering it to the copper-exporting P-type ATPase A (CopA) (60).

Pexiganan Stress Has a Strong Impact on the Essential Proteome

We visualized the global impact of pexiganan stress (at 1x MIC) on bacterial physiology by using a network analysis based on protein-protein interactions and the function (61) of *S. aureus* essential genes (**Figure S3**). This network analysis provides global view information on protein level alterations and integrates protein-protein interactions, including indirect functional and direct physical associations (61). At this concentration, it is noticeable that the majority of the essential genes are downregulated, and it is possible that this pattern has a strong influence on pexiganan lethality. The majority of upregulated proteins are ribosomal components.

Gene Ontology Analysis Points to an Upregulation of Nucleotide Metabolism

The signature of pexiganan stress on *S. aureus* in the upregulated fraction points to nucleotide metabolism-related genes. GO annotation allows enrichment analysis providing global information based on the gene expression levels by proteomics or transcriptomics or other gene expression datasets (62). We focus this comparative analysis on the protein expression levels of the 100 most upregulated proteins of every pexiganan dosage. We focussed on categorizing by pathways. Some of the upregulated pathways involved genes related to oxidative stress, peptidoglycan synthesis, and N-acetylglucosamine that are expected from cationic antimicrobial peptides since they attack the cell envelopes. In addition, there is a reactivation of the central metabolism by the upregulation of genes from glycolysis, TCA cycle, arginine, and thiamine synthesis. However, the most enriched pathways in the GO analysis for all pexiganan concentrations were related to nucleotide metabolism (**Figure 2**). The nucleotide upregulated pathways include ATP synthesis, Adenine and hypoxanthine salvage pathways, *de novo* synthesis of purines and pyrimidines and S-adenosylmethionine. This result confirms that pexiganan stress induces a scarcity of these metabolites within the cell. Taking into account the previous results, we hypothesized that upregulation of nucleotide-dependent and related genes could create a collateral sensitivity.

Nucleoside Analogs Antimetabolites Act Synergistically With Pexiganan

We designed a simple drug interaction experiment between pexiganan and some nucleoside analogs including the purine and pyrimidine antimetabolites: 6-azauracil, gemcitabine, 5-fluorouracil, and 6-thioguanine (**Figure 3**, **Figure S4** and **Table S4**). This experiment is the classic isobologram, also known as a checkerboard assay (35). All analogs showed a synergistic activity when combined with pexiganan (**Table S4**). The most active ones were 5-fluorouracil and gemcitabine and, while the 6-azauracil and 6-thiogunine showed a milder effect according to their respective fractional inhibitory concentration index (**Table S4**). All the combinations managed to decrease

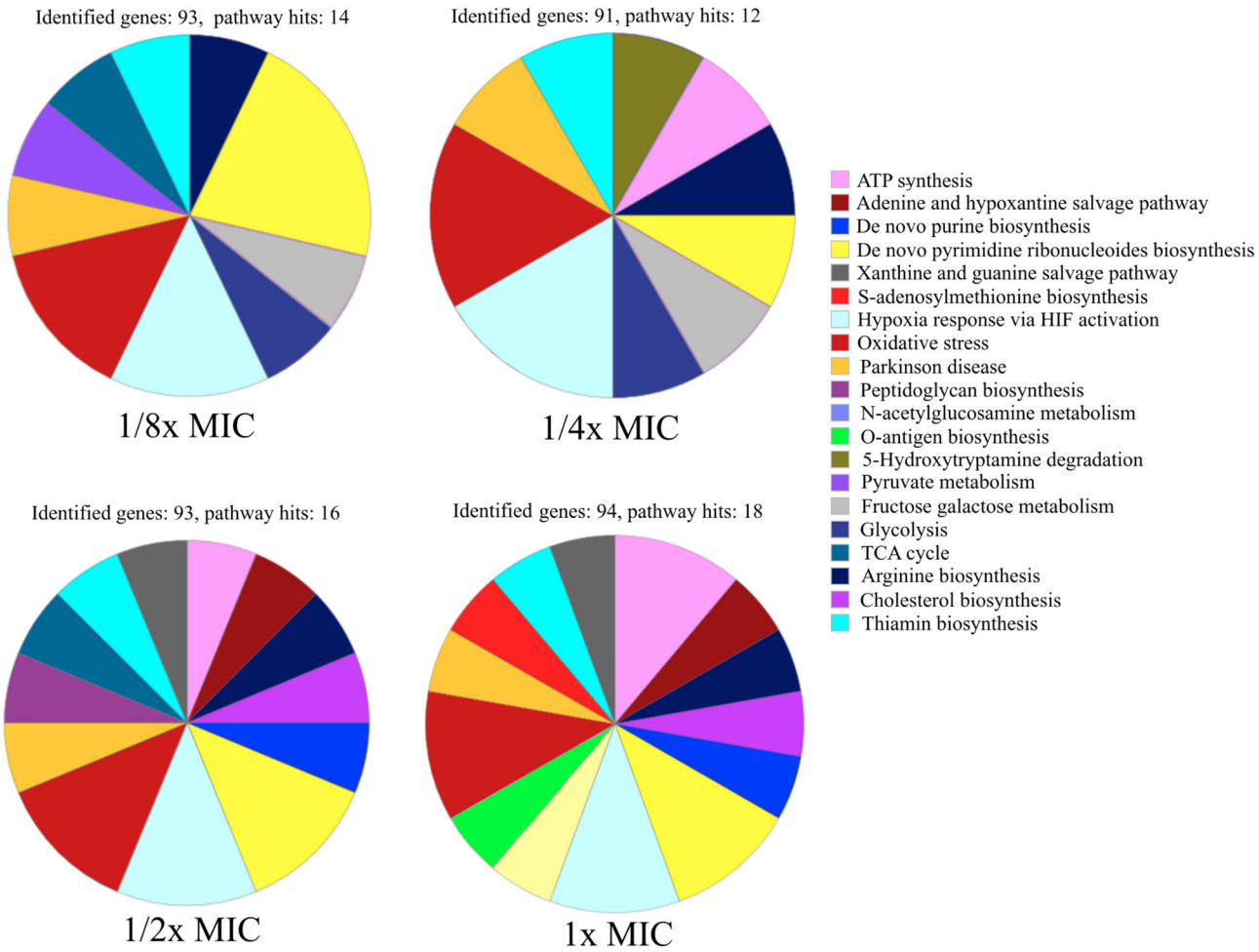

FIGURE 2 | Functional characterization of pathways of up-regulated proteins in *S. aureus* SH1000 at different concentrations of pexiganan (0.125, 0.25, 0.5, and 1 fractions of the minimal inhibitory concentration, MIC). For this analysis, only the 100 most highly differentially expressed proteins for each concentration of pexiganan were used. On top of each chart the number of identified genes and the number of pathway hits (number of genes used for the enrichment analysis) is visible. The analysis was carried out using the online gene ontology analysis software PANTHER (62).

of the minimal inhibitory concentration for each drug when compared to the respective drug alone. These results indicate that pexiganan induces a strong collateral sensitivity to nucleoside analogs.

To study the influence of nucleoside analogs on the killing by pexiganan, we carried out a time-kill experiment combining each of 6-azauracil, gemcitabine, 5-fluorouracil, and 6-thioguanine with pexiganan. We assayed all drugs using half of the minimal inhibitory concentration. We exposed mid-exponential phase *S. aureus* cells to these combinations and sampled the viability of the cultures every 20 min (**Figure 4**). All compounds significantly increased the killing ability of pexiganan, gemcitabine and 5-fluorouracil being the most active drugs. The killing rate was increased by a few orders of magnitude in all combinations. The killing by the combination of gemcitabine or 5-fluorouracil with pexiganan, at their corresponding half MIC values, was more efficient than 8x MIC concentration of pexiganan alone. The viability was assessed not only by the absence of growth but also by the addition of resazurin, a reagent that turns from blue to purple when it is reduced by microbial enzymes that only work within living bacteria (37).

DISCUSSION

We have found that pexiganan, a cationic antimicrobial peptide, can induce a stress response in *S. aureus* that results in a proteome-wide impact. Pexiganan treatment upregulates known virulence factors such as MprF, the capsule synthesis protein CapF, a wall teichoic acid TagG, the proteases ClpL and PepT and other proteins important for the interactions with the hosts. This could lead to a phenotypic cross-tolerance of other immune effectors of hosts and possibly complicate the bacterial infection

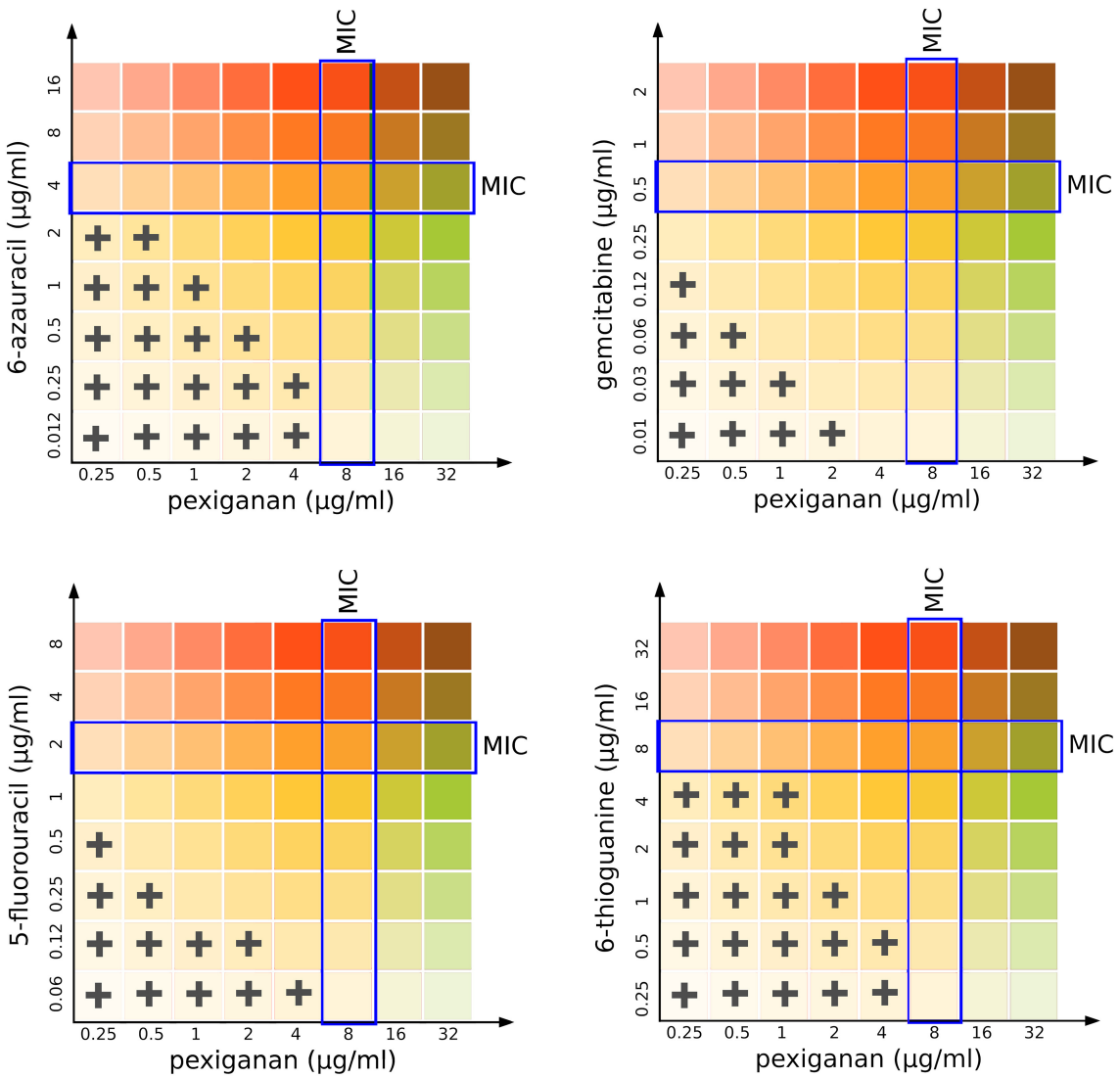

FIGURE 3 | Isobolographical response of pexiganan combination with different antimetabolite nucleosides. The crosses indicate the presence of bacterial growth in the unique concentration combinations of each well. Blue rectangles indicate the MIC value for single drug situations (pexiganan or antimetabolites) and is marked as a reference to visually compare with the actual level of inhibition for each pexiganan-antimetabolite combination.

in case of inefficient treatment where bacteria could be exposed to sub-lethal concentrations. This is a legitimate concern since AMP-resistant variants have been reported to have evolved which have shown some cross-resistance with immune system effectors (63, 64). This risk has been shown for pexiganan as well (12). Our data also provides input about possible induced physiological changes that would help *S. aureus* to adapt to the intra-host environment during its interaction with specific immune system effectors.

It is important to note that, given the coverage of the proteomic data and range of pexiganan doses, we did not find evidence of activation of mutagenic stress pathways or recombination. This indicates that the mode of killing by cationic antimicrobial peptide does not increase genome instability as is typical for classic antibiotics (65). We have previously shown and proposed that antimicrobial peptides, including pexiganan, do not increase the rate of either mutagenesis (66) or recombination (67) in Gram-negative bacteria. Our findings here are consistent with these observations in the Gram-positive model bacterium *S. aureus*.

The elevated level of expression of proteins such as GmK, PyrG, NptA and some amino acids-biosynthesis enzymes such as CarA and CarB that participate in the biosynthesis of L-arginine could be explained by changes in permeability. Amino acids, nucleobases and nucleotides are small molecules that could easily escape from the cellular compartment in case of membrane damage. This is a well-known property of cationic agents, including AMPs (68–70). The fact that only

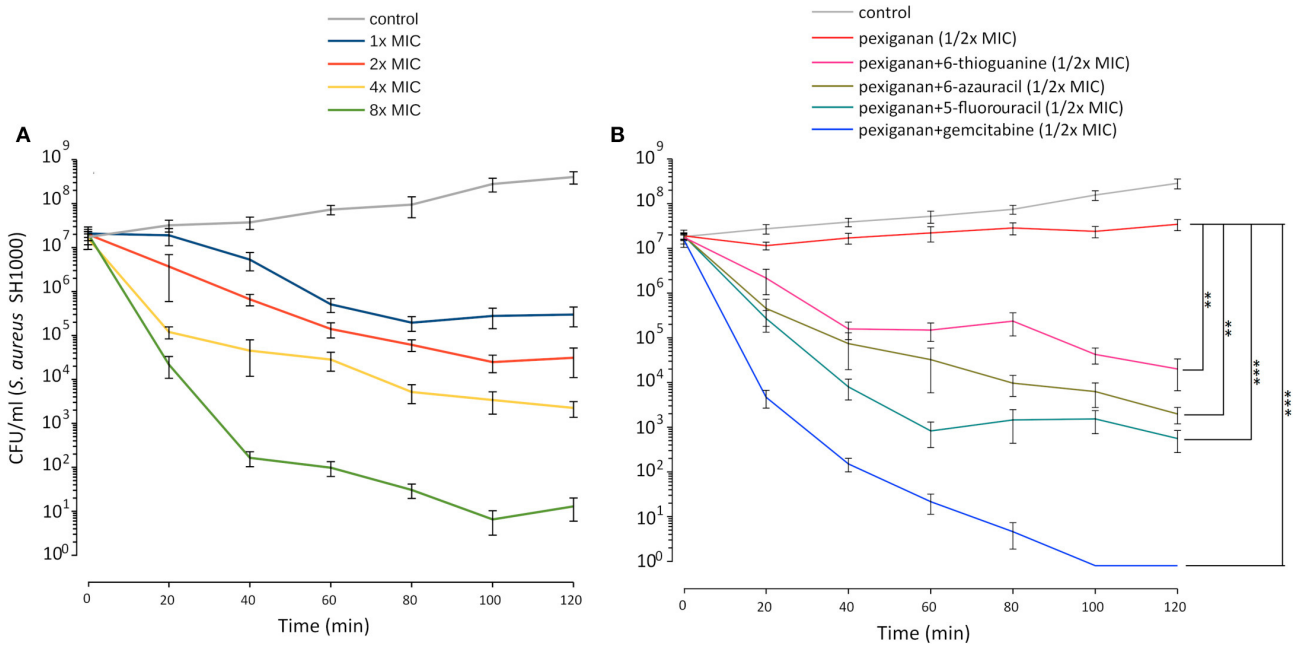

FIGURE 4 | Pexiganan-nucleoside antimetabolite combinations drastically increases the killing capacity of pexiganan. **(A)** Killing dynamic of *S. aureus* SH1000 at different concentrations of pexiganan using the MIC as the starting point. **(B)** Example data of time-kill experiment exposing mid-exponential phase bacterial cultures to pexiganan-nucleoside antimetabolite combinations (both at 1/2x MIC concentrations). The combination has a dramatic effect on the killing ability of pexiganan. Data points were determined by counting colony-forming units (CFU) at different time points. Mean ± SDM, $n = 5$. Asterisks represent significant differences (R package nparLD, one asterisk for $p < 0.05$ and two asterisks for $p < 0.01$ and three asterisks for $p < 0.001$). Only comparisons between pexiganan (1/2x MIC) and pexiganan-analogs combinations are shown.

a few proteins from the amino acids biosynthesis pathways are upregulated could be explained because the experiments were carried out in a complex medium like MHB that contains several amino acids and bacteria would upregulate only necessary pathways. A similar situation might be expected within a host.

The upregulation of the phosphate and nucleotide-related proteins provides a direction to investigate drug susceptibilities created by pexiganan stress. Although the antimetabolites used in this work have good antibacterial activity, if they are used in monotherapy they are also prone to generate resistance (22, 24). Thus, their use in combination could possibly help to prevent resistance (16, 71).

The synergistic combined action of pexiganan with nucleoside antimetabolites could be potentially explained by two underlying mechanisms. First, pexiganan stress forces a response by *S. aureus* that upregulates nucleobase salvage pathways and other nucleotide-dependent metabolic pathways. Second, pexiganan has the potential to change membrane permeability and induce the uptake of such metabolites even at sublethal concentrations possibly leading to much higher intracellular concentrations (**Figure 5**). We have shown previously that cationic antimicrobial peptides can mediate the uptake of small molecules due to changes in permeability at sublethal concentrations (70). The more potent activity of gemcitabine and 5-fluorouracil could be explained because they act on the cell walls as previously reported (24, 72). An additional potential therapeutic advantage of the nucleoside analogs studied here is that all the clinical properties of these drugs are well-known, including toxicological profile, pharmacological activities and metabolizing properties (22, 73). All of them are approved drugs, which should facilitate the introduction of such combinations in clinical practices.

We have shown recently that antimicrobial peptides, including pexiganan, can induce priming in bacteria, an enhanced response to the peptides when bacteria are pre-exposed to low concentrations. We defined the priming response as the ability of bacteria to have better survival to the peptide when it has been exposed to sub-inhibitory concentration in advance. The consequence of priming is not only survival but an increase in tolerance and persistence (74). Tolerance and persistence are a non-genetic path that increase survival to antimicrobials and lead to infection control failure (75). It has been proposed that evolution of tolerance in response to sub-inhibitory concentrations of antibiotics precedes or enhances the emergence of resistance (76). The use of antimetabolites could potentially abolish this property in therapeutic usage.

CONCLUSIONS

The analysis of the pexiganan stress response by *S. aureus* has shown a global response involving several proteins known for their role in the development of resistance against

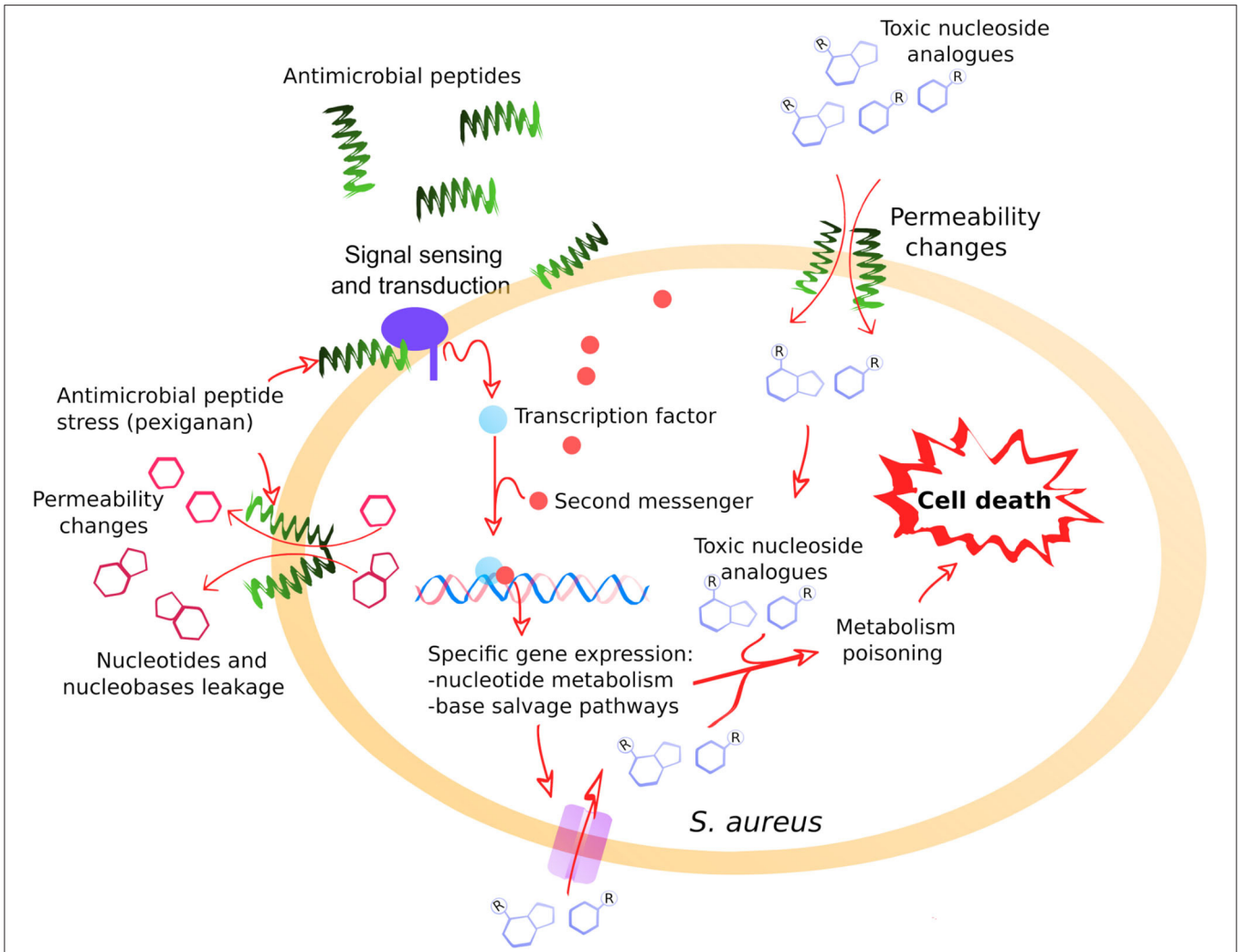

FIGURE 5 | A general model illustrating the positive interaction between pexiganan and nucleoside antimetabolites against *S. aureus*. The interactions of pexiganan with the membrane at sub-inhibitory concentrations lead to transient permeability changes in the envelope that promote leakage of small molecules such as nucleotides, nucleobases or nucleosides. Simultaneously, other small molecules such as toxic nucleoside analogs can increase the diffusion rate toward the intracellular compartment. This stress is sensed by the cell that responds by activating nucleoside metabolism creating an intervention opportunity. In this situation, toxic nucleoside antimetabolites are more efficiently incorporated into RNA, DNA, and other nucleotide depending reactions that may include envelope synthesis, enhancing toxicity and leading to faster cell killing.

antimicrobial peptides and other immune system effectors. Pexiganan has also shown a synergistic increase of antibacterial activity when it is combined with nucleoside antimetabolites. Taken together, our results suggest that pexiganan renders *S. aureus* susceptible to purine and pyrimidine analogs, which are traditionally used for cancer treatment. These antimetabolites can enhance the bactericidal activity of pexiganan against *S. aureus* under the tested conditions. The significant potentiation of the pexiganan bactericidal activity and the decrease of minimal inhibitory concentrations when compared with pexiganan alone could be the basis for new formulations of pexiganan. These results are probably extendable to other antimicrobial peptides and other bacterial pathogens. Thus, the leakage of nucleotides and intermediate small metabolites or cofactors caused by cationic peptides and nucleotide metabolic pathways are common traits of bacteria-peptide interactions as proposed for the symbiont–host interface (77). Our results also show that understanding how antimicrobials operate and how pathogens respond to them is important to guide the design of new effective therapies. Physiological response by bacteria is informative or suggestive about additional drug combinations that can limit the chances of pathogens to evolve resistance while increasing pathogen clearance and decreasing toxicity. This approach should be exploited to rationally design new antimicrobial combinations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AR-R and JR conceived the study. AR-R, AN, BE, GS, and BK performed the experiments and collected the data. AR-R, GS, JK, BK, and CW analyzed the data. AR-R and JR wrote the manuscript and revised the final document. All authors agree to be held accountable for the content therein and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01686/full#supplementary-material

Figure S1 | Volcano plots –log q values vs. log2 fold change of protein intensity measured by LC-MS of pexiganan treated cells with different fractions of the MIC, each compared to an untreated control). Black dots represent not significant expressed proteins while green dots show the upregulated portions and red ones represent the down-regulated fraction.

Figure S2 | Heatmap of relative protein expression based on label-free quantification by liquid chromatography/mass spectrometry (LC-MS). Only the 50 most statistically significant down-regulated proteins are shown, taking as a reference the ones from 1xMIC pexiganan concentration (0.125, 0.25, 0.5, and 1 fractions of the minimal inhibitory concentration). Intensity ranges of the log2 fold-changes are given from highest intensity (green) to lowest (red) sorted by their values for the 1x MIC.

Figure S3 | Network analysis of pexiganan stress (1x MIC) on essential genes interactome of *S. aureus* SH1000. Pale green nodes indicate upregulated proteins while pale red ones represent down-regulated ones. Gray nodes correspond with genes with a high degree of connectivity with this essential network, but they were not labeled. Note the higher proportion of downregulated genes among essential proteome while the majority of unregulated proteins are ribosomal components and thus they aggregate due to physical interaction. The interaction among nodes shows the proteome-wide impact of pexiganan stress at an inhibitory concentration.

Figure S4 | Isobologram showing the synergistic activity of pexiganan and different nucleotide antimetabolite combinations against *S. aureus* SH1000. Columns with no color change (blue resazurin) indicate no viable bacteria while color change to purple (reduced resazurin) was considered as a sign of bacterial growth. Please note that the red rectangle indicates the well of the plates used for the drug combination (8 × 8 wells) while the double arrows indicate the alongside single drug MIC determination. A typical plate is shown from three repetitions. The blue rectangles show the positive (same bacterial inoculum in LB medium) and negative (LB medium alone) controls.

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frontiers in Immunology

ORIGINAL RESEARCH
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Expression and Localization of Paneth Cells and Their α-Defensins in the Small Intestine of Adult Mouse

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Paneth cells contribute to intestinal innate immunity by sensing bacteria and secreting α-defensin. In Institute of Cancer Research (ICR) mice, α-defensin termed cryptdin (Crp) in Paneth cells consists of six major isoforms, Crp1 to 6. Despite accumulating evidences that α-defensin functions in controlling the intestinal microbiota, topographical localization of Paneth cells in the small intestine in relation to functions of α-defensin remains to be determined. In this study, we examined the expression level of messenger RNA (mRNA) encoding six Crp-isoforms and Crp immunoreactivities using singly isolated crypts together with bactericidal activities of Paneth cell secretions from isolated crypts of duodenum, jejunum, and ileum. Here we showed that levels of Crp mRNAs in the single crypt ranged from 5 x 10^3 to 1 x 10^6 copies per 5 ng RNA. For each Crp isoform, the expression level in ileum was 4 to 50 times higher than that in duodenum and jejunum. Furthermore, immunohistochemical analysis of isolated crypts revealed that the average number of Paneth cell per crypt in the small intestine increased from proximal to distal, three to seven-fold, respectively. Both Crp1 and 4 expressed greater in ileal Paneth cells than those in duodenum or jejunum. Bactericidal activities in secretions of ileal Paneth cell exposed to bacteria were significantly higher than those of duodenum or jejunum. In germ-free mice, Crp expression in each site of the small intestine was attenuated and bactericidal activities released by ileal Paneth cells were decreased compared to those in conventional mice. Taken together, Paneth cells and their α-defensin in adult mouse appeared to be regulated topographically in innate immunity to control intestinal integrity.

Keywords: Paneth cell, alpha-defensin, cryptdin, Institute of Cancer Research mouse, innate immunity, germ-free mouse

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INTRODUCTION

A monolayer of intestinal epithelial cells is the largest surface exposed to various microbes. Mucosal immunity on the intestinal surface plays a pivotal role in host defense. Paneth cells, one of epithelial cell lineages in the small intestine, reside at the base of crypts of Lieberkühn and have apically oriented secretory granules which contain high levels of antimicrobial peptides, α-defensins (1, 2).

Paneth cells secrete granules containing α-defensins when exposed to bacteria, bacterial antigens, cholinergic stimuli or certain nutrients, and the secreted α-defensins elicit potent microbicidal activities against pathogens (3–5). Paneth cell α-defensins are actively involved in the innate enteric immunity and maintain intestinal homeostasis by controlling the intestinal microbiota to prevent dysbiosis (6–10). In addition, Paneth cells provide survival signals to crypt intestinal stem cells, crypt-base columnar stem cells, and create stem cell niche responsible for regenerating entire lining of small intestinal epithelial cells (11).

α-Defensin in mouse, termed cryptdin (Crp), is a major microbicidal constituent of mouse Paneth cell granules (12, 13). Paneth cells contribute the innate enteric immunity by sensing bacteria and releasing microbicidal activities mostly by activated Crps at effective concentrations (3). Paneth cells contain six Crp isoforms in Institute of Cancer Research (ICR) mouse, and Crp1, 2, 3, and 6 are considered as Crp1-like family with high homogeny of the primary structure (14–17). Among Crps, Crp4 is known to elicit the most potent bactericidal activity *in vitro*, and different functions of six Crp isoforms have been discussed in previous reports. Crp4 is the most bactericidal against *Escherichia coli* as well as *Staphylococcus aureus* (12). In contrast, Crp2 and Crp3 have potent killing activities against *Giardia lamblia* trophozoites, whereas Crp1 and Crp6 have less effect (18). It has been known that Crps show site-specific distribution in the messenger RNA (mRNA) expression in the small intestine. Crp4 mRNA expression is known to be restricted mostly in the ileum (14). A human Paneth cell α-defensin, HD5 is known to have topographic differences in their gene expressions in the small intestinal tissue (19, 20). However, precise special distributions of Paneth cells and their α-defensins in entire mouse small intestine remain to be determined. Furthermore, bactericidal activities released by Paneth cells in different anatomical sites in the small intestine have not been reported and Paneth cell α-defensin expression and function in germ-free mouse remain controversial.

In this study, we analyzed the expression and localization of α-defensins in the adult mouse small intestine by analyzing mRNA expression of six Crp isoforms, Crp immunohistochemistry, and bactericidal activities of Paneth cell secretions using isolated crypts from different anatomical sites of the small intestine. We showed that Paneth cells in the small intestine are specially regulated from duodenum to ileum along with their Crps and revealed that released bactericidal activities by Paneth cells are also regulated in the small intestine consistent with the number of Paneth cells. Furthermore, we revealed that in germ-free mice, bactericidal activities released by ileal Paneth cells are reduced due to decrease of Crp expression. This study reveals anatomical, histological features of mouse Paneth cells and α-defensins, and gives additional insights into the innate enteric immunity.

METHODS

Mice

Cr1j:CD-1 ICR (ICR) adult male conventional and germ-free mice were purchased from Charles River Laboratories Japan, Inc. and propagated at Hokkaido University. All mice were housed under conventional condition maintained under a 12 h light/dark cycle with water and food provided *ad libitum*. All animal experiments in this study were conducted after obtaining approval from the committee on Animal Care and Use at Hokkaido University in accordance with Hokkaido University Regulations of Animal Experimentation.

Preparation of Mouse Isolated Crypts and Paneth Cells

Intact crypts were isolated from mouse small intestine by our previously reported method (3). Small intestine was resected from adult ICR conventional and germ-free mice, and duodenum, jejunum, and ileum were obtained, soaked them with 30 mM EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffer saline (PBS-) with vigorous vibration for every 5 min to separate six fractions. After centrifuge, each fraction was replaced with fresh ice-cold PBS-. Individual crypts were transferred to siliconized microfuge tubes using capillary pipettes. In addition, we collected a crypt-rich pool with more than 80% crypt purity by estimating crypt numbers using hemocytometer. Single crypt from each site of the small intestine was isolated by using glass pipette into the microtube under phase-contrast microscopy (x400) and collected at −80°C (21).

Extraction and Reverse Transcription Reaction of Single-Crypt RNA

Total RNA 100 ng of singly isolated duodenal, jejunal, and ileal crypts were obtained (22). Reverse transcription reaction was conducted on the total RNA for 30 min at 55°C and 5 min 85°C (Transcriptor First Strand cDNA Synthesis Kit, Roche) using anchored-oligo $(dT)_{18}$ primer and transcriptor reverse transcriptase, and synthesized single strand complementary DNA (cDNA).

Real-Time PCR

Using cDNA 5 ng obtained from an isolated crypt from duodenum, jejunum, or ileum as templates, real-time PCR was conducted (LightCycler480 SYBR Green I Master Kit, Roche) using each Crp (Crp1-Crp6)-specific primer and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)-specific primer (**Supplementary Table 1**) and SYBR Green I probe (Roche, LightCycler480) (n = 10). The PCR was performed three steps of 10 s at 95°C denature, 10 s at 63°C annealing, 12 s at 73°C extension for 50 cycles after a 5 min-pre-heat at 95°C. The amplification curve of the PCR product was obtained (23), and confirmed that it was an objective product from the size that compared the marker by the 2% agarose gel electrophoresis including the ethidium bromide. Amplified PCR products were further determined by direct DNA sequencing. Furthermore, we confirmed that there was no non-specific amplification by the fusion curve analysis. Agarose gel (2%) electrophoresis was conducted using the PCR products and GAPDH to obtain standard curve, cut gels of target bands, and purified each cDNA (QIAEXIIGel Extraction Kit) (n = 4 for each) with dilution system of 10^3 to 10^6 copies of the cDNA, and enforced

real-time PCR on similar PCR condition. The copy number of Crp1~Crp6 and GAPDH were calculated. The formula shown below was used to calculate the cDNA amount (primer bp) × 10^{-9}/9.12 μg = 10^5 cDNA copy. A calibration curve was obtained from the average of the obtained CP values, and the copy numbers of Crp1 to Crp6 and GAPDH mRNA were calculated and absolute quantification was performed (n=10 for each). The efficiency showing PCR efficiency was 1.6 or more.

Histological and Immunohistochemical Analysis

For hematoxylin-eosin (HE) staining, the 4% paraformaldehyde-fixed duodenum, jejunum, and ileum from conventional and germ-free mice were embedded in paraffin and cut into 4 μm-thick sections. Then, sections were placed on glass slides and stained by hematoxylin and eosin. In addition, 4% paraformaldehyde-fixed paraffin-embedded sections were cut in serial sections and were immunostained using the following primary antibodies: mouse anti-Crp1 (77-R63) and mouse anti-Crp4 (74-4). The crypt-rich pool and the fraction of intestinal epithelium cells from duodenum, jejunum and ileum from conventional mice were fixed with 2% paraformaldehyde for 40 min at room temperature, and blocked with 0.01% normal horse serum for 30 min. Then, polyclonal anti-Crp1 antibody (rabbit-IgG) which react Crps1, 2, 3, and 6, polyclonal anti-Crp4 antibody (rabbit-IgG) which only react Crp4, and polyclonal anti-lysozyme antibody (rabbit, Dako) were reacted for 60 min. Alexa 488 Fluor (goat anti-rabbit IgG H+L, Invitrogen) as secondary antibody for Crps antibody and rhodamine phalloidin (F-actin, Invitrogen) were reacted for 60 min at 4°C. After nucleus staining by 4',6-diamidino-2-phenylindole (DAPI) for 5 min, adhere to the cover glass which coated Cell-Tak (Corning) for 20 min, and embedded to the slide glass using Fluoromount (Diagnostic BioSystems). Using confocal microscopy (LSM510, Carl Zeiss and A1, Nikon), the samples of duodenal, jejunal, and ileal crypts and isolated Paneth cells were analyzed (n =10 for each).

Collection of Paneth Cell Secretion and Bactericidal Assay

Individual isolated crypts from conventional or germ-free mice were incubated in either 30 μl of PBS- or PBS-containing 1,000 bacterial colony-forming unit (CFU)s of *Salmonella typhimurium* per crypt for 30 min at 37°C (n = 3 each). Cellular components were deposited briefly by centrifugation, and supernatants were transferred to sterile microfuge tubes and stored at –20°C as control supernatants and secretions with bacterial exposure. Then, 5 μl of the collected samples were incubated with 1 x 10^3 CFU of *S. typhimurium* (3, 24) for 1 h at 37°C. Surviving bacteria were determined by plating on nutrient agar and counting colony numbers after growth for overnight at 37°C. Bacterial cell killing as the percentage relative to bacteria incubated PBS- alone were determined.

Statistical Analysis

Data were shown in mean ± standard deviation (SD). One-way ANOVA and Tukey *post-hoc* tests were used for statistical analyses and considered $p < 0.05$ as statistically significant.

RESULTS

Quantification of Cryptdin Gene Expression in the Isolated Crypt From Duodenum, Jejunum, and Ileum of Adult Conventional Mice

First, we measured Crp isoform mRNA expression at single-crypt level by using single-crypt derived total RNA of conventional mice. The mRNA expressions of each Crp isoform in the individual single-crypt RNA from duodenum, jejunum, and ileum were Crp1; 8 x 10^4, 5 x 10^4, and 2 x 10^5, Crp2; 3 x 10^3, 5 x 10^3, and 3 x 10^4, Crp3; 5 x 10^5, 3 x 10^5, and 1 x 10^6, Crp4; 1 x 10^4, 2 x 10^4, and 2 x 10^5, Crp5; 6 x 10^4, 3 x 10^4, and 2 x 10^5, and Crp6; 1 x 10^5, 2 x 10^5, and 8 x 10^5, respectively. There was significantly higher gene expression for each Crp isoform in the ileum compared to that in the jejunum (**Figure 1**). In addition, there were significantly higher gene expression for Crp2, Crp4, Crp5, and Crp6 in the ileum compared to those in the duodenum. In contrast, no significant difference was shown on each Crp isoform gene expression between duodenum and jejunum. The gene expression of Crp3 was highest and Crp2 was lowest among Crp isoforms from duodenum to ileum. GAPDH gene expressions in duodenum, jejunum, and ileum were 4 x 10^4, 3 x10^4, and 3 x 10^4, respectively, and no significant differences were observed.

The mRNA expression of each Crp isoform in single-crypt obtained from duodenum, jejunum, and ileum was calculated as the ratio *versus* gene expressions of GAPDH in single-crypt from each corresponding site. The Crp/GAPDH ratios for duodenum, jejunum, and ileum were Crp1; 3.4, 2.8, and 14.2, Crp2; 0.1, 0.2, and 2.3, Crp3; 24.4, and 13.9, 111.6, Crp4; 0.5, 1.5, and 23.7, Crp5; 2.7, 1.3, and 18.6, and Crp6; 14.2, 11.5, and 74.1, respectively. All Crp isoform mRNA expression ratios were remarkably higher in ileum than duodenum and jejunum (**Supplementary Figure 1A**).

The gene expression of Crp1-6 in the single-crypt of the ileum was greater 4.2, 19.7, 4.6, 48.3, 6.9, and 5.2 times compared to those in the duodenum, respectively. The gene expression of Crps in the ileum was increased four times of Crp-1 to 48 times of Crp-4 than those in the duodenum. The mRNA expressions of Crp1 and Crp4 were 5 and 16 times higher in the ileum than those in the jejunum, respectively. The difference of each Crp isoform gene expression was the smallest in Crp1 and the greatest in Crp4 (**Supplementary Figure 1B**).

Immunolocalization of Cryptdin and Number of Paneth Cells in the Isolated Crypts of Adult Conventional Mice

Paneth cells could be recognized with HE staining of the small intestine of conventional mice, showing eosin-positive granules in the cytoplasm (**Figures 2A–I**). Crypts of duodenum, jejunum, and ileum were identified by the microscope with Nomarski method (DIC image) showing Paneth cells which reside at the crypt base with dense intracellular granules. The size of the crypt was almost same among duodenum, jejunum, and ileum. The Paneth cell granules were spheres of the 0.5 ~2 μm, and the

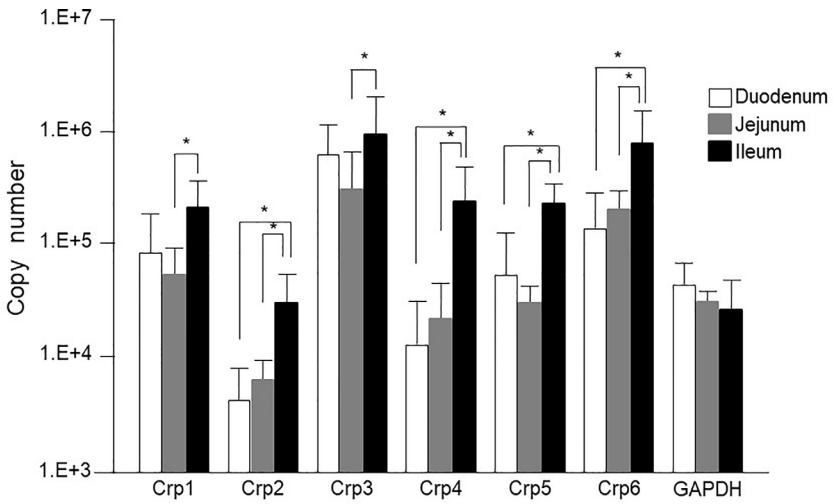

FIGURE 1 | Cryptdin gene expression levels in the isolated single crypt of duodenum, jejunum, and ileum. Crp isoform messenger RNA (mRNA) copy numbers in duodenum, jejunum, and ileum at single-crypt level (n = 10 for each, mean ± SD, *p < 0.05).

number of granules in one Paneth cell was from 3 to 30. Crp1 and lysozyme were immunostained with granules of the Paneth cells (**Figures 2J–L**). Three-dimensional structure of Paneth cells in the isolated single crypts by the image of phalloidin indicating cytoskeleton and DAPI indicating nucleus were shown in **Figures 2M–O**. The number of Paneth cells in a single crypt from duodenum, jejunum, and ileum was 6.6 ± 1.3, 7.1 ± 1.4, and 17.7 ± 2.5, respectively. Paneth cells were significantly rich in the ileum than those in duodenum and jejunum (**Figure 3A**). No significant difference in the Paneth cell number was observed between duodenum and jejunum.

Crp1 antibody reaction was restricted to the granule in the Paneth cell. However, some Paneth cells were negative for Crp. The numbers of Paneth cell (Crp-positive Paneth cell) in the duodenum, the jejunum, and the ileum were 2.0 ± 1.2, 3.2 ± 1.3, and 16.2 ± 2.7, respectively per one crypt, indicating that there are significantly abundant Crp-positive Paneth cells in ileum compare to duodenum or jejunum (**Figure 3B**). In contrast, no significant difference was shown in numbers of Paneth cell between duodenum and jejunum. The Crp-positive ratios in Paneth cells in duodenum, jejunum, and ileum were 33.9, 45.5, and 91.5%, respectively, indicating that ileal Paneth cell contains significantly greater Crps than duodenum and jejunum (**Figure 3C**).

Because α-defensin is packed in Paneth cells granules, we further counted Crp-poisitive granule numbers in Paneth cells. Numbers of Crp-positive granules in single crypt in duodenum, jejunum, and ileum were 3.4 ± 3.1, 7.9 ± 6.2, and 85.1 ± 21.5, respectively. There were significantly abundant numbers of Crp-positive granules in ileum compere to duodenum and jejunum (**Figure 3D**). Lysozyme, an antimicrobial protein, was also known to locate only in Paneth cell granules in intestinal epithelial cells. Therefore, we further determined numbers of Paneth cells containing lysozyme-positive granules in duodenum, jejunum, and ileum, and the numbers were 5.6 ± 1.6, 5.6 ± 1.6, and 15.6 ± 3.0, respectively, indicating significantly abundant lysozyme-positive Paneth cells in ileum. The lysozyme-positive ratios of Paneth cells were similar in entire small intestine; 86.6% in duodenum, 79.8% in jejunum, and 88.1% in ileum (**Figure 3C**). We further showed that the number of lysozyme-positive granules in Paneth cells in single-crypt from duodenum, jejunum, and ileum were 21.0 ± 9.2, 24.0 ± 11.9, and 82.0 ± 26.7, respectively. The numbers of granules which showed Crp1/lysozyme-double positive in duodenum, jejunum, and ileum were 2.1 ± 3.0, 5.6 ± 7.4, and 68.5 ± 21.9, respectively. Taken together, Paneth cell numbers in the small intestinal crypts of conventional mice are greatly increased from proximal toward distal small intestine.

Immunolocalization of Cryptdin and Number of Paneth Cells in Adult Germ-Free Mice and Bactericidal Activities of Paneth Cell Secretions

We further conducted immunohistochemistry of Crp1 and Crp4 in the small intestine of germ-free mice to test whether the intestinal microbiota affect cryptdin expression. We compared cryptdin isoform expression between conventional and germ-free mice, and both Crp1 and Crp4 expressions in each site of the small intestine in germ-free mice were decreased compared to those of conventional mice (**Figures 4A–C**). The number of Crp-positive cells, i.e., Paneth cells in germ-free mice was significantly decreased in ileum compared to that in conventional mice (**Figure 4D**). In contrast, the number of Crp-positive cells in both duodenum and jejunum unchanged in germ-free mice and conventional mice.

Finally, we tested bactericidal activities from each of 1,000 crypt-derived Paneth cell secretion of conventional and germ-free mice from duodenum, jejunum, and ileum stimulated

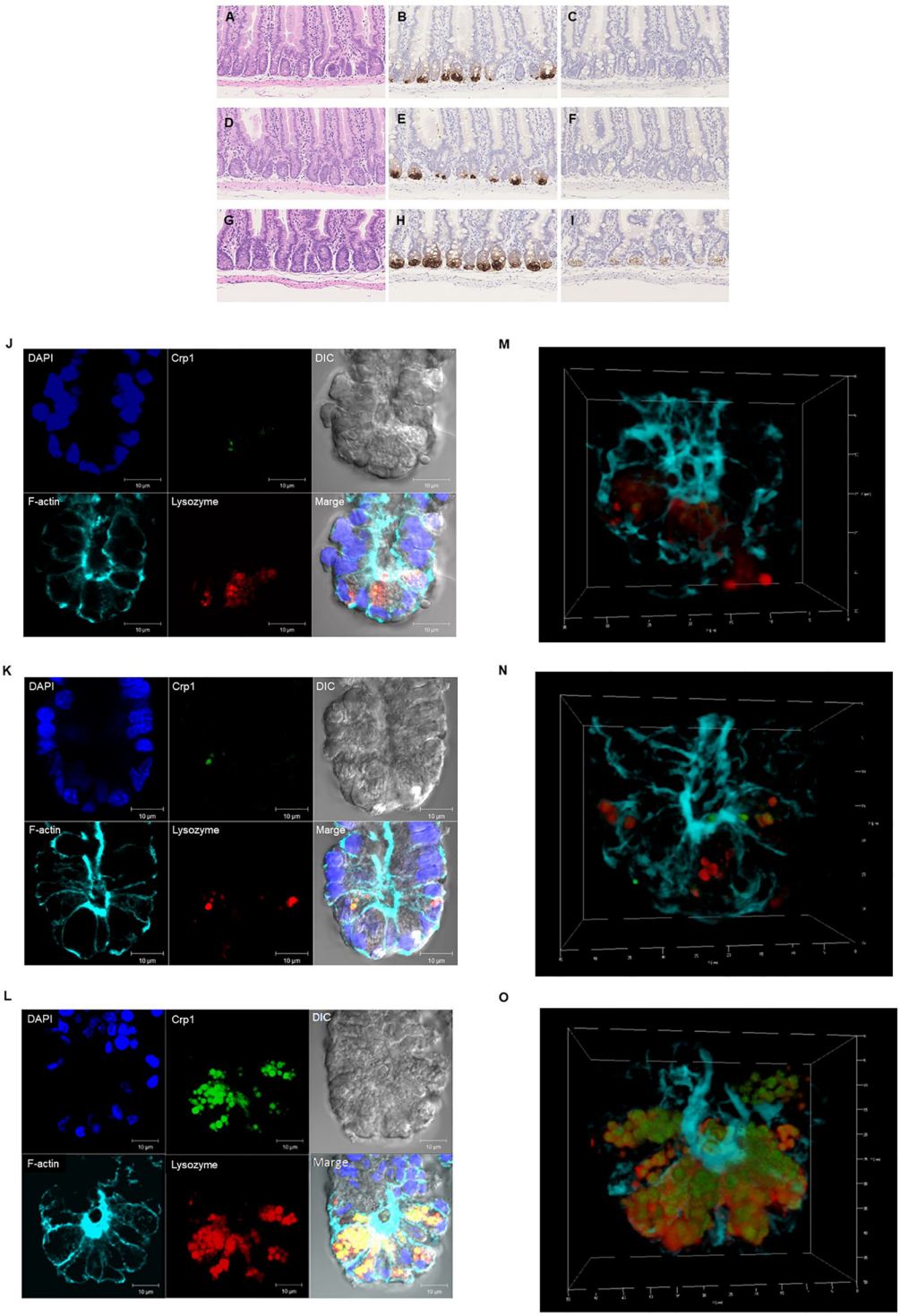

FIGURE 2 | Histological and immunohistochemical analyses of the small intestine. Hematoxylin-eosin staining of the duodenum **(A)**, jejunum **(D)**, and ileum **(G)**. and immunohistochemical analysis of Crp1 in the duodenum **(B)**, the jejunum **(E)**, and the ileum **(H)** and Crp4 in the duodenum **(C)**, the jejunum **(F)**, and the ileum **(I)** of mouse small intestine. Immunohistochemical analyses of isolated single crypt from duodenum **(J)**, jejunum **(K)**, and ileum **(L)** using confocal microscopy. Representative images of each site with Crp1 and lysozyme staining together with 4',6-diamidino-2-phenylindole (DAPI), F-actin, and differential interference contrast (DIC) were shown. The same crypt observed in **(J–L)** with Crp1, lysozyme, and F-actin were shown by 3D images in **(M–O)**, respectively. A representative image of 10 crypts was shown.

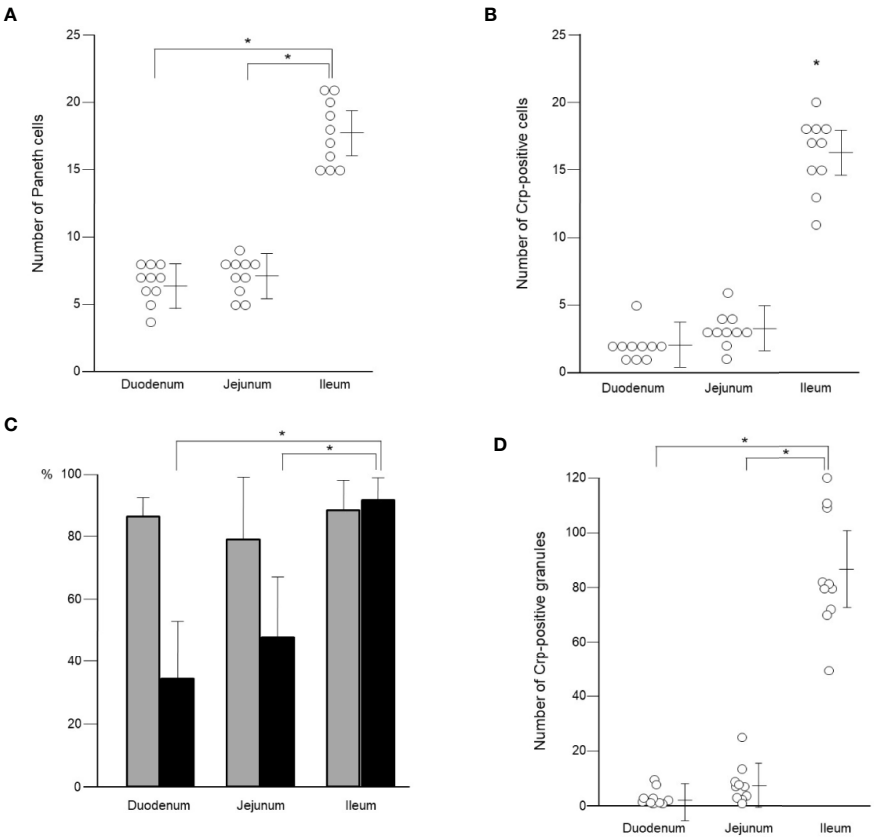

FIGURE 3 | Number of Paneth cells and their granules in single crypt of duodenum, jejunum, and ileum. Number of Paneth cells in single crypt of duodenum, jejunum, and ileum **(A)**. Number of Crp-positive cells in single crypt of duodenum, jejunum, and ileum **(B)**. Percentage of lysozyme-positive (gray column) and Crp-positive (black column) Paneth cells in single crypt of duodenum, jejunum, and ileum **(C)**. Number of Crp-positive granules in single-crypt of duodenum, jejunum, and ileum **(D)**. n = 10 for each, mean ± SD, *p < 0.05.

ex vivo by *S. typhimurium*. Secretions from duodenum, jejunum, and ileum of conventional mice elicit 8.6, 13.6, and 53.7% killing activities against the bacteria, respectively and those of germ-free mice elicit 3.1, 6.9, and 25.2%, respectively (**Figure 4E**). Bactericidal activities of ileal secretions were significantly greater than those of duodenum or jejunum in both mice. In contrast, control supernatants of duodenum, jejunum, and ileum elicit no bactericidal activities (3.1, −0.1, and 1.5%, respectively in conventional mice and 2.8, 1.8, and −0.5% in germ-free mice). Ileal Paneth cells of germ-free mice released significantly lower bactericidal activities compared to those of conventional mice (**Figure 4E**).

DISCUSSION

The granules of Paneth cells are rich in α-defensins, and also it has been known to contain other microbicidal or anti-microbial constituents such as lysozyme, secretory phospholipase A_2, and angiogenins (25–27). Recently, there have been emerging evidences that α-defensins secreted by Paneth cells serve vital roles in innate enteric immunity and regulating intestinal microbiota in humans and mice (8–10). Therefore, this study focused on mouse Paneth cell α-defensin, cryptdin, and determined mRNA expression of Crp isoforms, Crp1-6 at single crypt level from duodenum, jejunum, and ileum of conventional mice by conducting quantitative PCR. Previous studies addressing quantity of Crp isoform gene expression in the mouse small intestine showed that Crp1 is most abundant and Crp4 is relatively less in the protein extracted from intestinal tissues of ICR mice (15). It has been also reported that mRNA expressions for Crp1 and Crp4 are 1.2 and 16 times higher, respectively, when compared ileum *versus* duodenum by conducting quantitative RT-PCR of total RNA extracted from small intestinal tissues of FVB mouse (28). Expression levels of Crp mRNA of C57BL/6 mice through an optimized set of primers has been reported that marked differences of the expression are found from the duodenum to the ileum as well developmental stage (29). Another report showed that Crp mRNA expression in the ileal tissue was 1.5 to 20 times higher

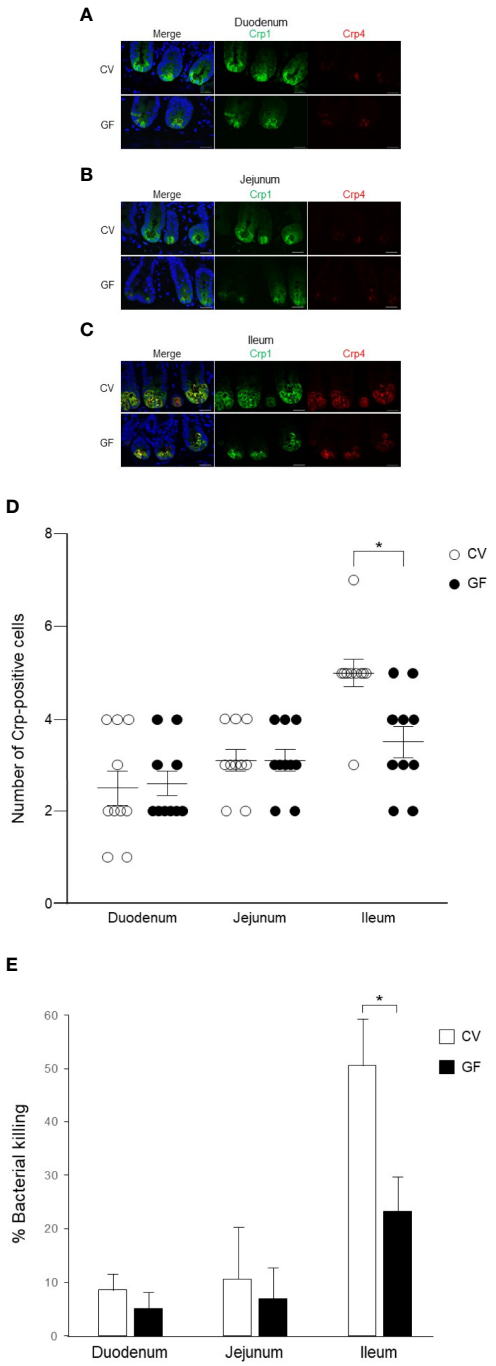

FIGURE 4 | Cryptdin expression and Paneth cell number in the small intestine and bactericidal activities of Paneth cell secretions of conventional and germ-free mice. Representative immunofluorescent staining images for Crp1 (green) and Crp4 (red) in the duodenum **(A)**, the jejunum **(B)**, and the ileum **(C)** of conventional (CV) and germ-free (GF) mice staining together with 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bars indicate 20 μm. Number of Crp-positive Paneth cells in single crypt of duodenum, jejunum, and ileum in CV and GF mice **(D)**. n = 10 for each, mean ± SD, *p < 0.05. Percentage of bacterial killing of Paneth cell secretions in *ex vivo* bacterial infection assay in CV and GF mice. n = 3 each, mean ± SD, *p < 0.05 **(E)**.

than that of jejunum, and the gene expression levels of each isoform are most abundant in Crp1 and fewest in Crp2 in jejunal and ileal tissues of BALB/c mouse (17). Together with previous findings, by the evidence that we showed here using isolated single crypt that each Crp isoform gene expression has no difference between duodenum and jejunum, whereas that is significantly higher in ileum relative to jejunum, suggesting that Crp mRNA expression is controlled topographically in the small intestine. Especially, gene expression levels of Crp4, which is known to elicit most potent microbicidal activities among other Crp isoforms in ileum were 3 and 46 times higher compared to that in jejunum and duodenum, respectively, indicating that Crp4 is the most topographically controlled among Crp isoforms, consistent with previous report (15, 28, 29). Previous study revealed that Crp1 and Crp5 gene expression levels are almost equal in jejunum and ileum (17). We revealed that the gene expressions of each Crp isoform in duodenum, jejunum, and ileum are same in order, as maximum Crp3 > 6 > 1, 4, 5 > minimum Crp2 in single-crypt level. This study further showed that Crp isoform mRNA expression ratios in duodenal, jejunum, and ileum to GAPDH gene expression were from 0.1 for duodenal of Crp2 as minimum to 112 for ileal of Crp3 as maximum.

In this study, all Crp isoform gene expression was significantly elevated in the ileum compare to the jejunum of conventional mice. In addition, as previously reported, although it was faint but Crp4 peptide was present in the jejunum. Crp-1, -2, -3, and -6 are classified as Crp1-like family by sharing highly homologous primary structure. Among these, the homology is the highest in Crp2 and Crp3 (16), which are known to induce Cl- secretion in opening a hole in the eukaryotic cell membrane (30), showing killing activities against *G. lamblia* (18). Crp2 and Crp3 further elicit Cl^- secretion in the intestinal epithelial cells (31). In addition, it has been reported that Crp3 induces inflammatory cytokine secretion (2). In addition, it has been reported that conventional mice showed significantly higher gene expression for all isoforms than germ-free mice, suggesting that the intestinal bacteria affect Crp gene expression (32). We revealed that Crp3 mRNA expression is thirty times higher in the jejunum and the ileum compared to Crp2, suggesting important role in the small intestine of Crp3. It has been reported that Crp4 and 5 have the sterilization activities stronger than Crp1-like-family peptides (12). Importantly, the gene expressions of human Paneth cell α-defensins, HD5 and HD6, in the ileum have been reported to be several times greater compared to those in the jejunum (20).

Intestinal microbiota plays a critical role in maintaining intestinal homeostasis. In this study, we compared Crp1 and Crp4 immunostaining and their function in conventional and germ-free mice. The influence of the intestinal microbiota on Crp expression remains controversial. It has been reported that cryptdin mRNA is equally abundant in germ-free and conventional mice (14), contrary, also reported lower mRNA expression for Crp1 and Crp4 in germ-free mice (28). Our results in peptide level that ileal Paneth cells of germ-free mice express fewer Crps and release decreased bactericidal activities compared

to conventional mice suggest that the intestinal microbiota may be partially required for normal Paneth cell function. Further detailed studies are needed to understand the effects of the microbiota on Paneth cell function. Dysbiosis, a state of disrupted the composition or the amount of microbiota reside in the intestine, gives rise to a variety of diseases such as life-style diseases, neurological disorders, and cancers (33–36). Disruption of α-defensin secretion has been known to cause dysbiosis and result in certain diseases such as obesity, Crohn's disease, and graft-*versus*-host-disease (37–41). In addition, NOD2 mutation leads to a decrease in α-defensin production in Paneth cells in patients with Crohn's disease (42) and amounts of α-defensin peptides decrease in obesity (43). Severe dysbiosis due to lack of Crps in graft-*versus*-host disease model mice reversed by administration of Wnt agonist R-Spondin1 to restore Paneth cells and their Crps, leading to recovery from dysbiosis and resulting in alleviating the disease condition (44).

It has been difficult to observe detail of the intestinal epithelia and hard to count numbers of Paneth cells in the small intestine using the tissue section. Using isolated small intestinal crypts with confocal microscopy, we showed that in the mouse ileum, not only the number of Paneth cells but the proportion of Paneth cells with Crp are significantly higher, and the number of granules with Crp in Paneth cells is also higher. These results suggest that the Paneth cell number and the Crp expression in Paneth cells are spatially regulated in the small intestine. In this study, the ileal Paneth cells which express maximum for both Crp genes in isolated single-crypt level and Crp antibody reactivities in the small intestine elicited most potent bactericidal activities against *Salmonella*, suggesting that gut innate immunity is spatially well-regulated. Obviously, ileum is close to the large intestine, which harbors a huge number of the intestinal microbiota, so that Crp4 having the most potent bactericidal activities may need to be placed predominantly. In Crohn's disease, an intractable inflammatory bowel disease, it is known that lesion formation is predominant in the terminal ileum. The amount and the quality of α-defensins in Crohn's disease have been shedding important insights into the pathogenesis and pathophysiology of the disease (42, 45, 46). Bacterial overgrowth is known to occur not only in the colon but in affected lesions in the terminal ileum in patients with severe ulcerative colitis. It has been reported that α-defensins exert a strong innate immune function in the ileum as well as the large intestine (47–49), suggesting that there may be a relationship between disruption of their functions and pathology of inflammatory bowel disease. Furthermore, not only innate immunity but also symbiosis with the microbiota elicited by Paneth cells in the small intestine have been considered to contribute to maintaining host health and prevent certain diseases (50). In humans, it is known that Paneth cells appear ectopically in response to certain sever chronic inflammation including in gastric mucosa with intestinal metaplasia and colonic mucosa with ulcerative colitis (51). It is also possible that cells adapt to various environmental differences of the lumen of the small intestine, i.e., the intestinal environment. Therefore, it is suggested that spatial control may also be exerted on Paneth cell development and environmental adaptation. Mechanisms controlling the localization of Paneth cell α-defensins in conventional and germ-free mice remain to be determined, and further study is necessary to clarify the underlying mechanisms.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by: The committee on Animal Care and Use at Hokkaido University in accordance with Hokkaido University Regulations of Animal Experimentation.

AUTHOR CONTRIBUTIONS

KN developed the conceptual framework of the study, designed the experiments, conducted experiments, data analysis, interpretation, and wrote and reviewed the paper. YY and RF designed and conducted experiments, data analysis, and interpretation. SO, RS, TN, and MK conducted data analysis and interpretation. TA developed the conceptual framework of the study, data analysis, and interpretation, and reviewed the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.570296/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Ratio of cryptdin mRNA expression levels in the isolated single crypt of duodenum, jejunum, and ileum against GAPDH **(A)**. Ratio of mRNA expression of each Crp isoform in duodenum and jejunum against ileum **(B)**.

SUPPLEMENTARY TABLE 1 | Primer pairs for cryptdin isoforms and GAPDH.

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