IMMUNE RESPONSE TO BIOFILMS

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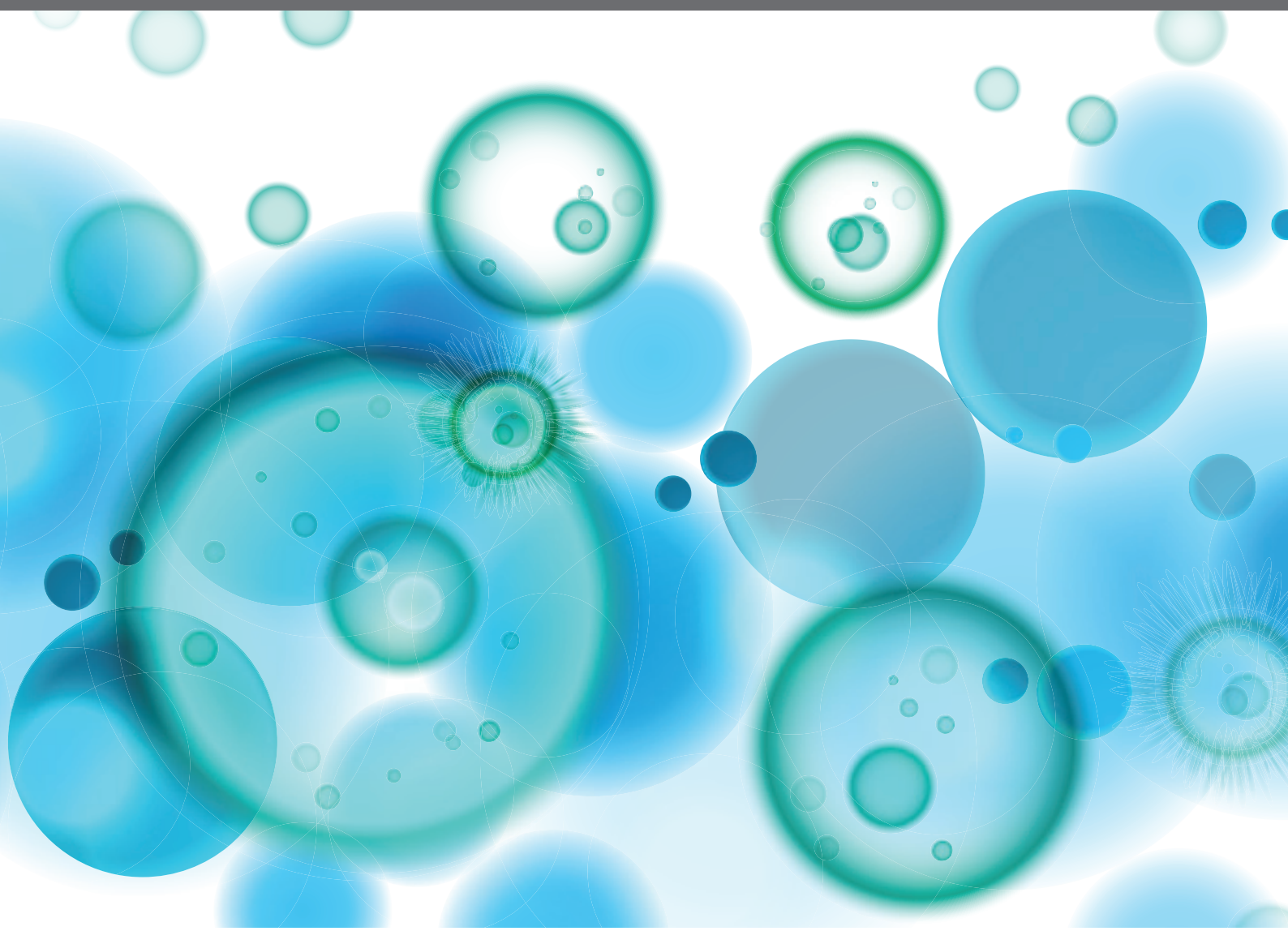

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IMMUNE RESPONSE TO BIOFILMS

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EDITORIAL
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Editorial: Immune Response to Biofilms

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Keywords: biofilms, biofilm-associated infections, innate immunity, adaptive immunity, immune response

Editorial on the Research Topic

Immune Response to Biofilms

Biofilms are one of the most widely distributed and successful form of microbial life and are associated to a significant amount of human infections (1). They typically contain aggregates of microorganisms adhering to a substrate and embedded in a self-produced matrix of extracellular polymeric substances. Importantly, biofilm-associated microorganisms exhibit an altered phenotype with respect to growth rate and gene transcription that provide them with unique characteristics as compared to their planktonic counterparts (2). These include the ability to resist antimicrobial treatments and host immune responses rendering biofilm-associated infections one of the major threats of the modern medicine. Despite the recognized clinical importance of biofilms, the vast majority of studies of the immune response against pathogens focuses on microorganisms in the planktonic state whereas the immune response against infectious biofilms has been far less investigated. There is evidence that the host immune response is only partially beneficial in clearing biofilm-associated infections if not even harmful by accelerating collateral tissue damage, as is seen in *Pseudomonas aeruginosa* biofilm-associated lung infections in Cystic Fibrosis (CF) patients (3). Therefore, it is critical to understand the complex interactions that establish between biofilms and the immune system as this may help in identifying new targets and strategies of immune intervention against biofilm-associated infections. We hope that this Research Topic may contribute to this purpose by collecting a number of papers (9 articles from 60 authors), exploring different aspects of the immune response to microbial biofilms.

The study of the immune response to biofilms is highly dependent on the development of appropriate *in vitro* and *in vivo* experimental models allowing to realistically figuring biofilm-immune cell interactions. In this respect, the Research Topic includes at least three articles. In the first one, Kaya et al., established an *in vitro* host cell-biofilm interaction model suitable to investigate the human peripheral blood mononuclear cell response (PBMC) to *P. aeruginosa* biofilms. Interestingly, the results obtained demonstrated that not only *P. aeruginosa* biofilms induced marked activation and response of PBMC, but also that PBMC or their supernatants caused a significant increase in biofilm-associated *P. aeruginosa*, suggesting a reciprocal complex interaction between host blood cells and the bacterium. A novel murine model of *Staphylococcus aureus* implant-associated infection was developed by Gries et al. Of note, the authors applied for the first time time-lapse intravital multiphoton microscopy to simultaneously visualize in real-time *S. aureus* biofilm formation and immune cell activity. Using this innovative approach, they demonstrated that *S. aureus* biofilms impede neutrophil chemotaxis, redirecting their migration patterns to prevent biofilm invasion. Finally, a RNA sequencing-based approach was used by Heravi et al., to depict the whole transcriptomic profile in diabetic foot infection (DFI) tissues, contributing to clarify the role of the host inflammatory status in the progression of DFIs.

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One of the most studied biofilm-forming bacteria, often taken as model organism in biofilm studies, is *P. aeruginosa*, a key pathogen in CF lung infections and chronic wounds (4). Moser et al. greatly contributed to this Research Topic with an updated and exhaustive review focused on the immune responses to *P. aeruginosa* biofilm infections. The mechanisms involved in the activation of the immune responses, the effector functions elicited by biofilms and their role in tissue damage, as well as the mechanisms by which the biofilms evade immune responses, and potential treatment strategies are discussed in detail in the review.

A number of articles of the Research Topic addresses the inhibitory and/or dysregulating effects of microbial biofilms on the innate immune responses. For instance, in their article, Kernien et al. report that neutrophils collected from patients with invasive candidiasis as well as from healthy donors fail to release neutrophil extracellular traps (NETs) in response to *Candida albicans* biofilms. As NETs exert antifungal activity (5), inhibition of NETs release by *C. albicans* biofilms may very well represent a mechanisms of immune evasion likely contributing to the resilient nature of *Candida* biofilm infections of medical devices (6). The existing knowledge on the role of biofilm-innate immune interactions in driving immune dysregulation and persistent inflammation in chronic wounds was summarized in the comprehensive review of Versey et al. that also illustrates novel treatments currently under development to target these interactions. The article by Miller et al. points out the possible role played by biofilm-products in the genesis or exacerbation of a number of inflammatory human disorders. In particular, the article focuses on amyloid curli, secreted by Gram-negative enteric bacteria, that makes up as much as 85% of the extracellular matrix of enteric biofilms. A thorough discussion on how amyloid-containing biofilms may act as triggers of inflammation and self-assembly of pathological human amyloids, contributing to the pathogenesis of gastrointestinal, autoimmune, and neurodegenerative diseases is provided.

Other two interesting, but relatively poorly investigated aspects of the immune response to biofilms are dealt in the articles of De Morais et al. and Trikha et al., respectively. In the first one, differences in the immune responses elicited by *S. aureus* biofilms in the central nervous system (craniotomy-associated infections) as compared to biofilm infections in the periphery are highlighted, emphasizing the critical role of niche-specific factors in driving *S. aureus* biofilm-leukocyte crosstalk. In the second paper, Trikha et al. demonstrated that angiotensin-converting enzyme inhibitors (ACEi), often utilized for treating hypertension, increases *S. aureus* burden and impairs immune responses in a preclinical model of implant-associated infections, raising the intriguing issue that commonly used drugs may negatively impact the immune response to microbial biofilms.

Overall, we believe that the articles collected in this Research Topic represent a step forward for a better understanding of the host immune response to microbial biofilms and hope that they may stimulate further studies in this interesting research field. Such studies could pave the way for the development of new preventive and/or immune-therapeutic approaches able to dampen the harmful activities of the immune system, meanwhile activating the branches of the immune system that can eradicate biofilm-infections without causing detrimental collateral damage.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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In vitro Interaction of *Pseudomonas aeruginosa* Biofilms With Human Peripheral Blood Mononuclear Cells

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The human immune cell response against bacterial biofilms is a crucial, but still poorly investigated area of research. Herein, we aim to establish an *in vitro* host cell-biofilm interaction model suitable to investigate the peripheral blood mononuclear cell (PBMC) response to *Pseudomonas aeruginosa* biofilms. *P. aeruginosa* biofilms were obtained by incubating bacteria in complete RPMI 1640 medium with 10% human plasma for 24 h. PBMC obtained from healthy donors were added to preformed *P. aeruginosa* biofilms. Following a further 24 h incubation, we assessed (i) PBMC viability and activation; (ii) cytokine profiles in the supernatants; and (iii) CFU counts of biofilm forming bacteria. Cell-death was <10% upon 24 h incubation of PBMC with *P. aeruginosa* biofilms. PBMC incubated for 24 h with preformed *P. aeruginosa* biofilms were significantly more activated compared to PBMC incubated alone. Interestingly, a marked activation of $CD56^{+}CD3^{-}$ natural killer (NK) cells was observed that reached 60% of NK cells as an average of different donors. In the culture supernatants of PBMC co-cultured with *P. aeruginosa* biofilms, not only pro-inflammatory (IL-1β, IFN-γ, IL-6, and TNF-α) but also anti-inflammatory (IL-10) cytokines were significantly increased as compared to PBMC incubated alone. Furthermore, incubation of biofilms with PBMC, caused a statistically significant increase in the CFU number of *P. aeruginosa*, as compared to biofilms incubated without PBMC. In order to assess whether PBMC products could stimulate the growth of *P. aeruginosa* biofilms, we incubated preformed *P. aeruginosa* biofilms with or without supernatants obtained from the co-cultures of PBMC with biofilms. In the presence of the supernatants, the CFU count of biofilm-derived *P. aeruginosa*, was two to seven times higher than those of biofilms incubated without supernatants ($P < 0.01$). Overall, the results obtained shed light on the reciprocal interaction between human PBMC and *P. aeruginosa* biofilms. *P. aeruginosa* biofilms induced PBMC activation and cytokine secretion but, in turn, the presence of PBMC and/or PBMC-derived components enhanced the number of *P. aeruginosa* biofilm associated bacteria. This may indicate a successful bacterial defensive/persistence strategy against immune response.

Keywords: peripheral blood mononuclear cells, cytokines, biofilm, *Pseudomonas aeruginosa*, human immune response to biofilm, natural killer cells

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INTRODUCTION

Pseudomonas aeruginosa is an environmental Gram-negative opportunistic pathogen involved in a large spectrum of infections, especially in immunocompromised and hospitalized patients (Driscoll et al., 2007). Infections caused by *P. aeruginosa* include acute and chronic respiratory infections, hospital-acquired urinary tract infections, chronic infections of wounds, otitis, endocarditis, osteomyelitis, corneal infections, and systemic infections (Driscoll et al., 2007). *P. aeruginosa* plays a particularly critical role in cystic fibrosis (CF) patients, largely contributing to the rapid decline in pulmonary function and representing an important cause of morbidity and mortality (Malhotra et al., 2019). The bacterium is also frequently involved in infections associated to the use of intravascular catheters (Raad, 1998), urinary catheters (Vipin et al., 2019), prosthetic implants (Martinez-Pastor et al., 2009) and other medical devices that represent essential tools of the modern medicine (Tolker-Nielsen, 2014). The pathogenesis of many *P. aeruginosa* infections depends on a striking ability of the bacterium to form biofilms, complex bacterial communities adhering on a substrate, such as mucosal surfaces or invasive medical devices (Tolker-Nielsen, 2014). In biofilms, bacterial cells are typically embedded within a self-produced extracellular polymeric substance (EPS), primarily composed of polysaccharides, proteins, and extracellular DNA (eDNA) (Flemming and Wingender, 2010). EPS plays a crucial role in maintaining the biofilm architecture ensuring a highly hydrated microenvironment and favoring the interactions among bacterial cells. The biofilm mode of growth provides the bacteria with enormous advantages to establish an infection as renders them extremely recalcitrant to both antimicrobial treatment and immune responses (Batoni et al., 2016). In fact, the EPS acts like a barrier that hampers the diffusion of antibiotics as well as host immune cells. In this regard, it has been reported that antibodies or phagocytic cells at most enter the water channels intercalating the micro-colonies that constitute a mature biofilm (Costerton et al., 2003), but hardly penetrate into the deep layers of the micro-communities, especially when biofilms are grown under static conditions (Leid et al., 2002).

Despite the recognized clinical importance of biofilms, the human immune response against infectious biofilms is a research area that necessitates to be thoroughly investigated as the majority of immune research investigations have focused on bacteria in the planktonic state (Moser et al., 2017). A deep understanding of the complex interactions that establish between biofilm bacteria and the immune system may help in identifying new targets and strategies of immune intervention against biofilm-associated infections. In addition, quantitative measurements of the host responses to biofilms may serve as diagnostic tools or possible biomarkers for tracing the course of an infection (Moser et al., 2017; Campoccia et al., 2019).

The host response against *P. aeruginosa* biofilms is particularly complex and it is believed to involve the integrated activity of an array of cell types of both the innate and adaptive immune systems (Maurice et al., 2018). The dynamic interplay between *P. aeruginosa* and the host immune system is one of the major determinants of bacterium's pathogenicity and may shape the phenotype of chronic lung infections, which range from acute exacerbations to sub-clinical slowly progressing conditions characterized by the adaptation of the bacterium to the host and attenuated immune responses (Faure et al., 2018).

The *in vitro* human immune response to *P. aeruginosa* biofilms is a research area still poorly investigated. The cytotoxic effect exerted by the bacterium on a variety of host cell types is one of the main hurdles that hampers the study of the host immune response *in vitro* (Bishop et al., 1987; El-Housseiny et al., 2013). In addition, most of the *in vitro* studies conducted so far have investigated neutrophil or monocyte response to *P. aeruginosa* biofilms (Jesaitis et al., 2003; Walker et al., 2005; Ciornei et al., 2010), while little is known on the human peripheral blood mononuclear cells (PBMC) and biofilm interaction. In the present study, we sought to establish an *in vitro* model of *P. aeruginosa* biofilms and human PBMC co-culture suitable to assess immune responses (e.g., expression of activation markers, cytokine production) of different PBMC subsets. By finely tuning the experimental parameters, such as PBMC and bacteria numbers, incubation times, type of medium used for biofilm formation, we could obtain mature biofilms of *P. aeruginosa* in eukaryotic cell-compatible medium RPMI 1640 and maintain the *P. aeruginosa*-biofilm:PBMC co-culture for at least 24 h, with minimal cell death. Interestingly, the results obtained disclosed a reciprocal interaction between human PBMC and *P. aeruginosa* biofilms. *P. aeruginosa* biofilms induced PBMC activation and cytokine secretion but, in turn, PBMC and/or PBMC components enhanced the number of *P. aeruginosa* biofilm associated bacteria after 24 h of co-culture. These results suggest a successful bacterial defensive/persistence strategy in response to host immune response.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The reference strain *P. aeruginosa* ATCC 27853 was used in the present study. In addition, the reference strain PAO1 (ATCC 15692) and two *P. aeruginosa* clinical strains isolated from abdominal fluid (PA-AF) and CF lung (PA-CF) at the Microbiology Unit of the University Hospital of Pisa, Italy were employed in some experiments. Stock cultures were prepared by growing bacterial strains in Tryptone Soy Broth (TSB) (Oxoid, Basingstoke, UK) at 37°C until mid-logarithmic phase. Bacterial suspensions were then aliquoted and stored at −80°C in the same medium until use.

Biofilm Formation

An aliquot of frozen *P. aeruginosa* culture was thawed, diluted 100 times in TSB, and incubated at 37°C overnight (18 h) with shaking (600 rpm). A volume of 500 μl from the stationary phase culture was centrifuged at 4,000 × g for 5 min. at room temperature (RT) and the pellet was re-suspended in the same volume of complete medium consisting of RPMI 1640 (Euroclone S.p.A, Pero, Milan) supplemented with 10% heat-inactivated pooled human plasma and 2 mM L-glutamine (Euroclone). Preliminary experiments were performed

to establish the optimal bacterial number ensuring biofilm formation in such medium. Addition of a 100 μl bacterial suspension containing ∼1 × 10^6 colony forming unit (CFU) per well to 96-well flat bottom plates (Euroclone) was found to be optimal for the experimental purposes. To this aim, initial bacteria suspension were diluted in complete RPMI medium to reach such bacterial density. To assess the exact number of initial bacteria used in each experiment, an aliquot was plated on Tryptone Soya Agar (TSA; Oxoid, Basingstoke, UK) plates for CFU count. The 96-well plates were incubated at 37°C for 24 h without shaking to allow biofilm formation. Following the incubation, biofilms were gently washed three times with phosphate buffered saline (PBS) to remove non-adherent cells and incubated for further 24 h in the absence or in the presence of PBMC (see below) in complete RPMI in humidified air containing 5% CO_2. Following 24 and 48 h of incubation, biofilm-associated bacterial cells were mechanically detached from the bottom of the wells by scratching 60 s with a pipette tip, subjected to vigorous vortexing, serially diluted and plated on TSA (Oxoid). After an incubation of 24 h at 37°C the number of biofilm-associated bacteria (CFU/ml) was evaluated by CFU counting.

Analysis of *P. aeruginosa* Biofilms Grown in Complete RPMI 1640 by Confocal Laser Scanning Microscopy (CLSM)

P. aeruginosa biofilms grown in complete RPMI were analyzed by CLSM as previously described (Maisetta et al., 2016; Di Luca et al., 2018). Briefly, biofilms were formed for 24 and 48 h in the same conditions as explained above (see "Biofilm formation") but using 8 well ibiTreat polymer coverslips (ibidi GmbH, Gräfelfing, Germany). Following incubation, biofilms were gently washed with sterile MilliQ water (Millipore), and stained with green fluorescent Syto9 (live bacteria) and red fluorescent propidium iodide (dead bacteria) (Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit, Thermo Fisher Scientific). Stained samples were observed under TCS SP5 II (Leica Microsystems Srl, Buccinasco, Milan) confocal microscope (interfaced with a 488 nm Argon laser), using a 60 × 1.25 NA water immersion objective. For each sample, the entire well surface and depth were scanned. 10 μm Axial stacks in the Z plane, with a slice thickness of 1 μm, were taken through representative areas of biofilm.

PBMC:Biofilm Co-culture

Peripheral blood (buffy coat) from healthy subjects was obtained from the donors attending the Transfusion center of Pisa University Hospital or from healthy volunteers. An informed consent was obtained from each donor. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the local Ethical Committee (Protocol 34743, 28/06/2018). PBMC were isolated by standard gradient protocol as described previously (Esin et al., 1996). In brief, blood was diluted with PBS (1:1 ratio) containing 10% sodium citrate (v/v), layered on a density gradient (Lymphoprep, Cedarlane, Canada), and centrifuged at 160 × g for 20 min at RT. Following centrifugation, supernatants were removed, without disturbing the mononuclear layer at the interface, to eliminate platelets. After a further centrifugation at 800 × g for 20 min, PBMC were collected from the interface. After three washes with RPMI, PBMC were resuspended in complete RPMI (RPMI 1640 added with 10% heat inactivated autologous plasma and 2 mM L-glutamine). Preformed biofilms (24 h-old) of *P. aeruginosa* were gently washed three times with PBS to remove non-adherent bacteria and 200 μl of PBMC suspension (2 × 10^6 cells/ml) was added to *P. aeruginosa* biofilms (i.e., 4 × 10^5 PBMC/well). PBMC:biofilm co-cultures were incubated at 37°C in 5% CO_2 for 24 h. PBMC alone were used as a negative control, while PBMC stimulated with 5 μg/ml phytohemagglutinin (PHA, Sigma-Aldrich, St Louis, MO) were used as a positive control of cell reactivity. Wells containing biofilms without PBMC were also established for bacterial count. Following incubation, PBMC suspensions, collected from six wells of each experimental condition were pooled. A 50 μl aliquot was taken from each sample for cell viability assessment prior to centrifugation at 500 × g for 5 min. Supernatants were collected from each condition, sterile filtered (0.22 μm), aliquoted, and stored at −80°C for further assays (i.e., cytokine determination and biofilm stimulation). The PBMC pellet was resuspended in PBS and cell surface staining was performed.

Evaluation of Cell Viability by Trypan Blue Dye Exclusion Test

Viability of PBMC was assessed after 24 h co-culture with *P. aeruginosa* biofilms by the trypan blue dye exclusion test. To this aim, PBMC incubated alone, co-cultured with preformed biofilms, or stimulated with PHA were diluted 5 fold with 0.4% trypan blue, added into a Burker counting chamber (Sigma-Aldrich), and observed under 400× magnification with light microscope (Olympus CH20BIMF200, Olympus optical Ltd, Japan). Alive (clear) and dead (blue) cell numbers were assessed independently by two operators and the counts obtained from six fields were averaged.

Immunofluorescence Staining for Cell Surface Markers

PBMC incubated with and without biofilm or stimulated with PHA were subjected to surface staining for activation and cell surface markers. Two- or three-color immunofluorescence staining was performed as previously described (Esin et al., 2013). Briefly, the cells were washed with PBS by centrifugation at 500 × g for 5 min at 4°C and incubated with saturating amounts of monoclonal antibodies (MAbs) directed against cell surface or activation markers for 30 min at 4°C.

The following MAbs were used for the staining: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 or anti HLA-DR; PE-conjugated anti-CD14, anti-CD19, or anti-CD69 (Miltenyi Biotec, Bergisch Gladbach, Germany); rhodamine-PE-cyanin 5.1(PC5)-conjugated anti-CD56 MAb (Beckman Coulter srl, Milan, Italy). Isotype matched mouse immunoglobulin G (IgG) Mabs (Miltenyi Biotec) were used as negative controls.

Following staining and a wash with PBS, the cells were fixed with 1% paraformaldehyde in PBS (Sigma-Aldrich) for 24 h at 4°C. After a wash with PBS, at least 50000 events were acquired ungated in a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). BD Accuri C6 software (BD Biosciences) was used for computer-assisted analyses. For the analyses, cells of interest were selected by a widely set gate on a two-parameter plot of side scatter (SSC) vs. forward-angle scatter (FSC) excluding debris and events not of interest; among these cells the percentage and/or mean fluorescence intensity (MFI) of the surface markers were calculated according to following panel combinations: negative control-FITC/-PE/-PC5; CD3-FITC/CD69-PE/CD56-PC5; HLA-DR-FITC/CD19-PE.

Determination of Cytokines in Culture Supernatants

The levels of a panel of cytokines (IL-1β, IL-6, IL-10, IL-4, IL-8, IFN-γ, TNF-α) present in the co-culture supernatants were determinated by a flow cytometer based multibead capture assay (LEGENDplex^{TM}Multi-Analyte Flow Assay Kit, BioLegend Inc., San Diego, CA, USA) according to manufacturer's instructions. Sensitivities of the assay were as follows: IL-1β, 0.65 ± 0.47 pg/ml; IL-4, 0.97 ± 0.83 pg/ml; IL-6, 0.97 ± 1.46 pg/ml; IL-8, 1.90 ± 0.65 pg/ml; IL-10, 0.77 ± 1.18 pg/ml; IFN-γ 0.76 ± 0.53, pg/ml; TNF-α, 0.88 ± 0.27 pg/ml. Samples were acquired in a BD Accuri C6 flow cytometer (BD Biosciences), analyzed with LegendPlex v8.0 Software (BioLegend Inc.), and referred to a standard curve. Results were expressed as pg/ml or ng/ml depending on the cytokine.

Incubation of *P. aeruginosa* Biofilms With Supernatants From PBMC-Biofilm Co-culture

Preformed 24 h-old *P. aeruginosa* ATCC 27853 biofilms were washed three times with PBS. Supernatants, obtained from wells of PBMC co-cultured with *P. aeruginosa* biofilms (see "PBMC:biofilm co-culture" above), were diluted 1:1 with fresh complete RPMI and added to the biofilms. Following a 24 h incubation at 37°C, the number of biofilm-associated cells was evaluated by CFU count, as described before. In some experiments, the effect of PBMC-biofilm supernatants derived from two different donors were assessed against 24 h-old biofilms of *P. aeruginosa* PAO1 strain and two *P. aeruginosa* clinical isolates (PA-AF and PA-CF).

Statistical Analysis

The statistical significance of the data was determined by Student's *t*-test for paired samples or by non-parametric Wilcoxon signed-rank test. For multiple comparisons ANOVA for matched samples followed by Tukey-Kramer multiple comparisons test were used. A *P*-value of < 0.05 was considered significant.

RESULTS

Formation of *P. aeruginosa* Biofilms in Complete RPMI 1640 Medium at 24 and 48 h

The biofilm forming ability of *P. aeruginosa* ATCC 27853 in an eukaryotic cell-compatible medium was assessed by incubating stationary phase *P. aeruginosa* in complete RPMI 1640 medium. Following 24 and 48 h of incubation at 37°C biofilm, viability and structure were evaluated by CLSM analysis. As shown in **Figure 1**, at both 24 and 48 h, typical micro-colonies attached on the slide were observed. Moreover, Syto9/PI staining demonstrated that most of the bacterial cells within the biofilm structure were alive, confirming the ability of *P. aeruginosa* to successfully form biofilm in the adopted conditions.

Evaluation of PBMC Viability Upon Incubation With Preformed *P. aeruginosa* Biofilms

The viability of PBMC upon incubation with preformed *P. aeruginosa* biofilms in complete RPMI medium was assessed by trypan blue dye exclusion test. Preliminary experiments were performed to establish an incubation time long enough to measure immune functions of PBMC with minimal cell death (data not shown). As depicted in **Figure 2**, PBMC incubated for 24 h with *P. aeruginosa* biofilms were 90 ± 3% alive and this was comparable to cells stimulated with a mitogen (PHA, 87 ± 9%). The viability of PBMC incubated alone was 95 ± 4%, indicating that the conditions adopted for *in vitro* PBMC:biofilm co-cultures allowed to keep the human cells alive at acceptable levels for 24 h.

Activation of PBMC Subsets Upon 24 h Co-culture With *P. aeruginosa* Biofilms

After gradient separation and before adding on biofilms, the PBMC typically consisted of 72.8 ± 2.9% $CD3^+$ T cells, 8.5 ± 0.8% $CD3^-CD56^+$ NK cells, 9.7 ± 4.4% $CD14^+$ monocytes and 9.0 ± 2.0% $CD19^+$ B cells. To evaluate whether PBMC:biofilm co-culture induced activation of PBMC, cells were harvested at 24 h and stained with MAbs directed against cell surface subset markers ($CD3^+$, T cells; $CD56^+CD3^-$, NK cells; $CD19^+$, B lymphocytes; $CD14^+$, monocytes) and the early activation marker CD69 (for T and NK cells) or for HLA-DR (for B lymphocytes).

As an average of seven different donors, T and NK cells co-cultured for 24 h with *P. aeruginosa* mature biofilms expressed the early activation marker CD69 at statistically higher levels as compared to PBMC incubated alone ($CD69^+$ $CD3^+$ T cells, 11.5 ± 1.6% vs. 1.6 ± 0.3% $P < 0.001$, Student's t-test for paired samples; $CD69^+$ $CD3^-$ $CD56^+$ NK cells, 6.4 ± 2.3% vs. 0.6 ± 0.2% $P < 0.05$, respectively; see **Figure 3** for data from a representative donor). As professional antigen presenting cells, B lymphocytes constitutively express HLA-DR marker whose intensity increases upon antigen-driven activation (Liu et al., 2017). Although the percent of B lymphocytes incubated with

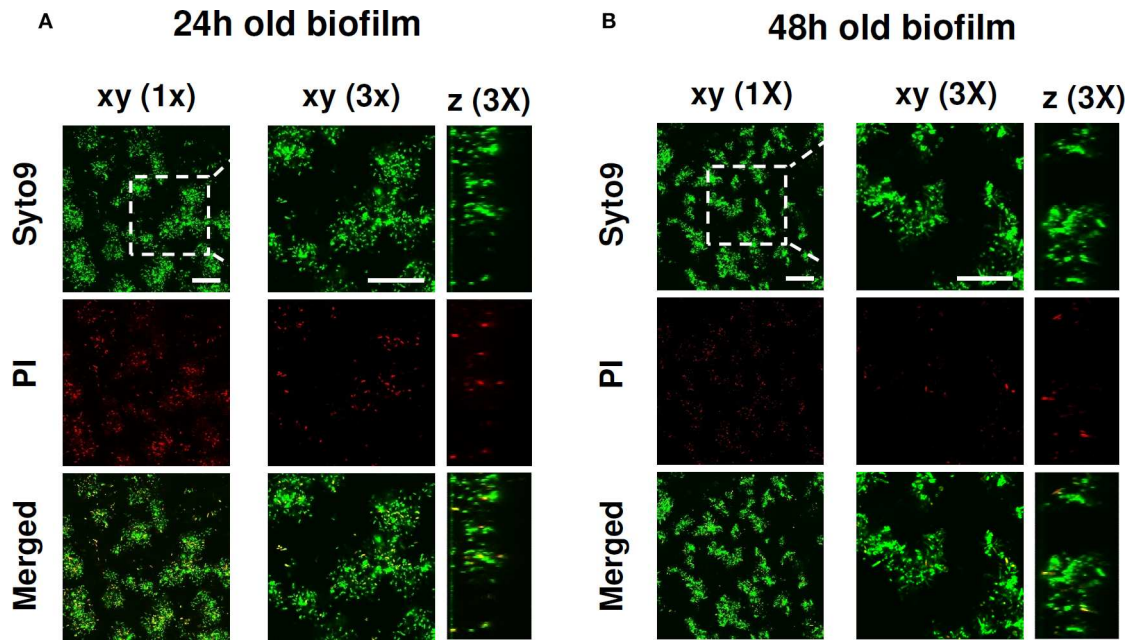

FIGURE 1 | CLSM images of *P. aeruginosa* ATCC 27853 biofilm formed in RPMI medium. The viability of *P. aeruginosa* and the biofilm structure were evaluated upon **(A)** 24 and **(B)** 48 h of culture in complete RPMI 1640 at 37°C. After a wash to eliminate planktonic bacteria, *P. aeruginosa* biofilms were stained with green fluorescent labeled Syto 9 (488/500–540 nm) for alive bacteria and with red fluorescent propidium iodide (PI, 488/600–650 nm) for dead bacteria. Figure shows results from a representative experiment of the two performed. Dashed square indicates the 3x zoomed area. Scale bar = 25 μm.

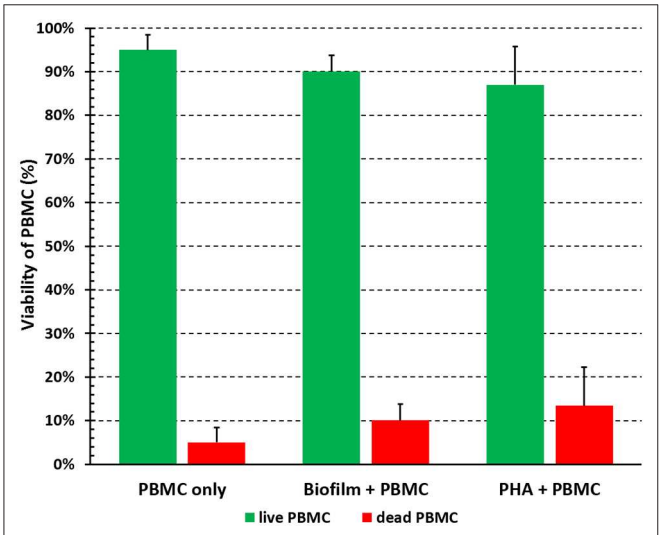

FIGURE 2 | Viability of PBMC incubated with preformed *P. aeruginosa* biofilms for 24 h**.** PBMC were collected after 24 h of incubation in the absence (PBMC only) and in the presence (Biofilm + PBMC) of preformed biofilms, or stimulation with a mitogen (PHA + PBMC) in complete RPMI, and percentage of live and dead cells were assessed by trypan blue exclusion test. Mean values ± SEM from six independent experiments are shown.

P. aeruginosa biofilms was significantly higher than that of PBMC incubated alone, no significant increase in the HLA-DR mean fluorescence intensity was observed on $CD19^+$ cells upon co-culture with *P. aeruginosa* biofilms or incubation with PHA (**Supplementary Figure 1**).

Next, we analyzed the percentage of activated cells within each cell subset (**Figure 4**). Interestingly, upon 24 h of co-culture with preformed *P. aeruginosa* biofilms, 17 ± 3% of all T cells were activated whereas the percentage reached the value of 60 ± 8% in the case of $CD56^+CD3^-$ NK cells ($P < 0.05$). In some donors, as much as 85.7% of $CD56^+CD3^-$ NK cells were activated (**Figure 3I**). Therefore, the NK cell subset represented the most activated cell subset upon stimulation with *P. aeruginosa* biofilms. Only 3 ± 1% of T cells and 9 ± 4% of NK cells, were activated when the PBMC were incubated alone (**Figure 4**). As expected, the stimulation of PBMC with PHA resulted in an increase of activated cells that were mainly expressing the T cell marker $CD3^+$ (**Figures 3M**, **4**). In this case, 29 ± 8% of T cells were activated, while the mean percentage of the NK cells was 17 ± 7% (**Figure 4**).

In all experimental conditions, $CD14^+$ cells among harvested PBMC were <0.5%, suggesting that monocytes adhered or remained entrapped within the biofilm during the incubation or were lysed in the attempt to ingest biofilm cells.

Cytokine Secretion Profile of PBMC Co-cultured With *P. aeruginosa* Biofilms

In order to establish whether cell activation resulted in functional activity of PBMC co-cultured with *P. aeruginosa* biofilms, we evaluated the cytokine profile (IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ) in the co-culture supernatants and compared it to that of PBMC incubated alone or stimulated with PHA.

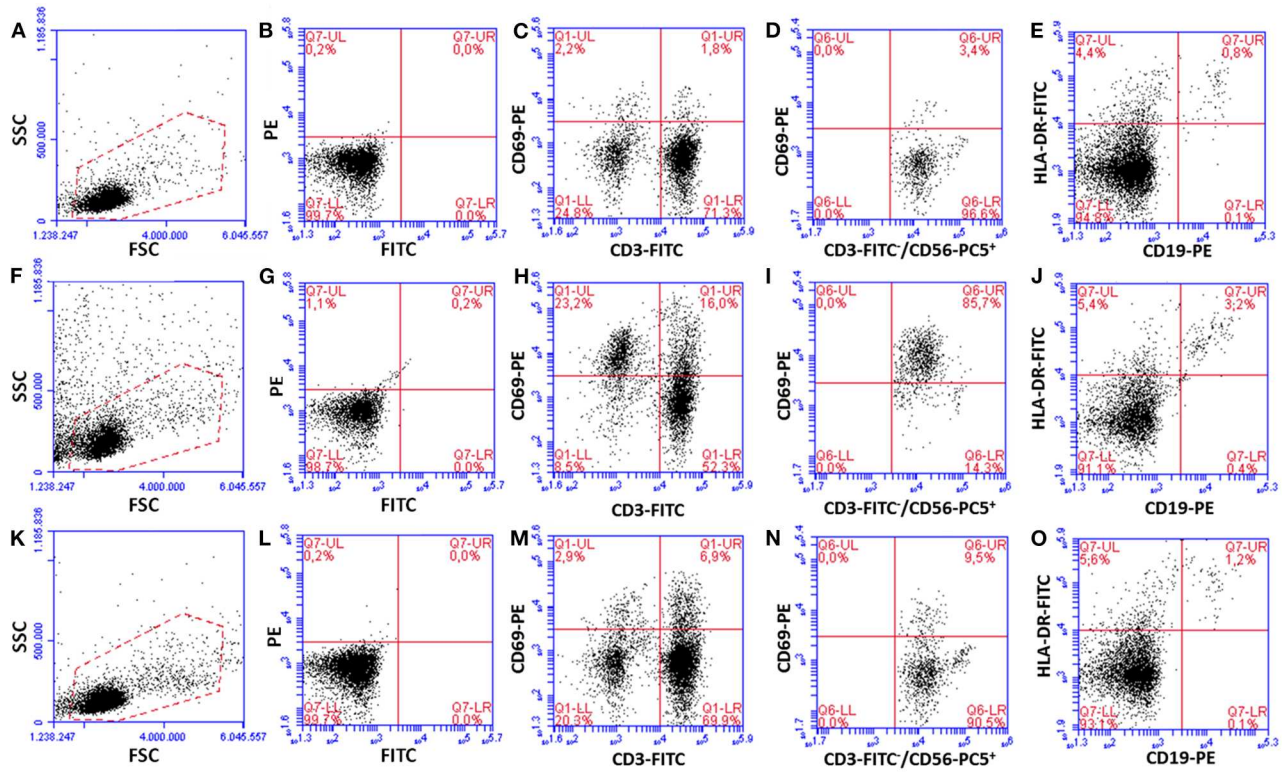

FIGURE 3 | Activation of PBMC subsets upon 24 h of incubation with preformed *P. aeruginosa* biofilms. Dot plots from a representative experiment are shown. PBMC incubated alone **(A–E)**, co-cultured for 24 h with preformed *P. aeruginosa* biofilms **(F–J)**, and incubated with a mitogen [PHA, **(K–O)**] were harvested and surface stained with MAbs against cell subset markers ($CD3^+$ T cells, $CD56^+CD3^-$ NK cells, $CD19^+$ B cells), activation marker CD69 (for T and NK cells), and HLA-DR. **(A,F,K)** A gate (red dotted lines) which includes resting and activated live PBMC was set for the analyses. **(B,G,L)** Isotype matched mouse IgGs, used as negative controls (isotype control for PC5 is not shown). **(C,H,M)** T cells, **(D,I,N)** NK cells (CD69 staining on $CD56^+CD3^-$ cells gated from CD3 vs. CD56 plots are shown), **(E,J,O)** B cells.

Among the pro-inflammatory cytokines tested, IL-1β, IFN-γ, IL-6, and TNF-α were significantly increased in the supernatants of PBMC co-cultured 24 h with *P. aeruginosa* biofilms in comparison to PBMC incubated in the absence of bacteria, whereas there were no significant differences in the IL-8 levels (**Figure 5**). Interestingly, in addition to pro-inflammatory cytokines, anti-inflammatory cytokine IL-10 was also produced at relatively high levels by PBMC co-cultured in the presence of *P. aeruginosa* biofilms as compared to PBMC incubated alone. The amount of IL-4 secreted into culture supernatants was very low overall and without any significant differences. As expected, PHA stimulated the secretion of most of the tested cytokines from PBMC (**Figure 5**).

Effect of PBMC: Biofilm Co-culture on the CFU Number of Biofilm-Associated *P. aeruginosa*

In order to investigate whether PBMC could affect the number of biofilm-associated *P. aeruginosa* upon 24 h of co-culture, preformed *P. aeruginosa* biofilms (24 h old) were incubated in the presence or absence of PBMC from different donors for further 24 h. After gently washing biofilm 3 times with warm PBS to remove planktonic bacteria and PBMC without disrupting the biofilm, biofilm-associated bacteria were harvested by mechanically disrupting the biofilm and plated on TSA. Interestingly, as shown in **Figure 6**, a statistically higher CFU number was obtained from biofilm incubated in presence of PBMC from different donors as compared to the corresponding biofilms cultured without PBMC ($P < 0.01$), indicating that co-cultivation of *P. aeruginosa* biofilms with PBMC enhances the number of biofilm-associated bacteria.

Effect of Supernatants From PBMC:Biofilm Co-culture on *P. aeruginosa* Biofilms

To test the hypothesis that soluble factors produced during PBMC:biofilm co-culture could stimulate the growth of *P. aeruginosa* biofilms, we collected culture supernatants upon 24 h co-culture of PBMC with preformed *P. aeruginosa* biofilms. The supernatans, diluted 1:1 with fresh complete RPMI, were added to 24 h-old biofilms of *P. aeruginosa*. The number of biofilm-associated bacteria was evaluated after further 24 h incubation and compared with that of biofilms incubated without supernatants. As shown in **Figure 7A**, the CFU count of biofilm-associated *P. aeruginosa*, incubated in the presence of the

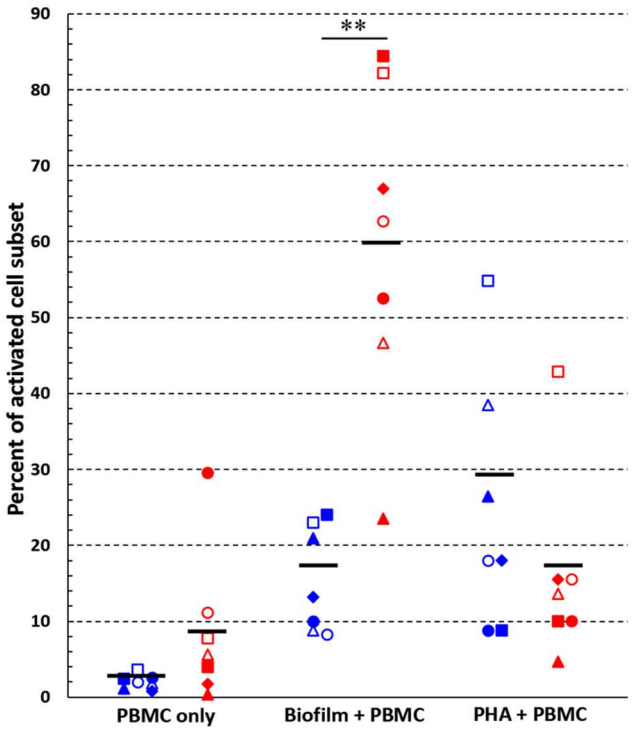

FIGURE 4 | Activated cells among T and NK cell subsets. Percentages of activated ($CD69^+$) cells within T cell- (blue symbols) and NK cell (red symbols) -subsets, respectively, were calculated following 24 h incubation of PBMC alone, with preformed *P. aeruginosa* biofilms, or with a mitogen (PHA). $^{**}P < 0.01$, Student's *t*-test for paired samples, $n = 7$. Black lines indicate mean values. Each symbol indicates a different donor/experiment.

supernatants, resulted two to seven times higher than that of biofilms incubated alone ($P < 0.01$).

In some experiments, we incubated 24 h old *P. aeruginosa* biofilms in the presence of supernatants obtained from PBMC stimulated with PHA, PBMC incubated alone, or from 24 h old *P. aeruginosa* biofilms incubated for an additional 24 h in the absence of PBMC. In all such experiments, no enhancement in the CFU of biofilm associated bacteria was observed (**Supplementary Figure 2**).

Next, we investigated whether the observed effect on *P. aeruginosa* ATCC 27853 biofilm growth was a strain specific phenomenon, or could also be true for other *P. aeruginosa* strains. To this aim, two different supernatants previously demonstrated to have high biofilm-enhancing ability toward the *P. aeruginosa* ATCC 27853 strain were selected (Sup 1 and Sup 2, **Figure 7B**). The two supernatants were added to 24 h old *P. aeruginosa* biofilms of PAO1 strain and two clinical isolates (PA-AF and PA-CF) and incubated for further 24 h. Although with some differences, both supernatants significantly enhanced biofilm growth of all the three strains tested as compared to the corresponding biofilms incubated in the absence of the supernatants (**Figure 7B**).

DISCUSSION

P. aeruginosa is a ubiquitous environmental bacterium that can be isolated from different habitats, including water, soil, and plants. The bacterium is also an important opportunistic human pathogen causing serious infections in immunocompromised or hospitalized patients. The large majority of infections caused by *P. aeruginosa* rely on the ability of the bacterium to form biofilm. The host response, both the innate and the acquired arms, may play a relevant role in the course of biofilm infections (Moser et al., 2017). Although a detailed knowledge of the host immune response to biofilms is still lacking, it appears that the host is mostly unable to eliminate the pathogen in the form of a biofilm. In such form, the pathogen rather persists establishing a chronic infection where the immune response, instead of being protective, may even accelerate collateral tissue damage (Moser et al., 2017). Chronic *P. aeruginosa* infections may persist for months to decades, as in the case of pulmonary infection in individuals with CF, during which a complex and dynamic interplay between the pathogen and the host establishes.

In the present study, in order to get further insights on the intricate relationship between *P. aeruginosa* and host immune cells, we have established an *in vitro* co-culture model of *P. aeruginosa* biofilms and PBMC from healthy blood donors. *P. aeruginosa* biofilms have been previously obtained *in vitro* using different types of bacteriological or synthetic media supplemented with various nutrients or growth factors (Haney et al., 2018; Wijesinghe et al., 2019). It is generally agreed that the nutrient contents of the culture medium may greatly impact the biofilm growth and development of different microbial species (Stepanović et al., 2007; Seneviratne et al., 2013; Weerasekera et al., 2016). In order to create an environment simulating the *in vivo* condition and suitable to assess immune cell responses, herein we obtained mature *P. aeruginosa* biofilms in RPMI 1640 supplemented with human plasma, a medium that is normally used for the growth of eukaryotic cells, but not for bacteria. Such medium resembles the composition of body fluids of the human host, as it contains high concentrations of amino acids, such as L-glutamine, L-arginine, L-asparagine, as well as vitamins and inorganic salts (Kucharíková et al., 2011). Confocal microscopy analyses, performed following 24 and 48 h of incubation of stationary phase *P. aeruginosa* in such medium, clearly demonstrated the presence of adherent bacterial cells organized to form characteristic microcolonies separated by empty spaces, resembling the water channels that are characteristic of *P. aeruginosa* biofilm structure (Rasamiravaka et al., 2015). Vital staining showed that most of bacterial cells were alive, confirming the ability of the RPMI 1640 medium to sustain the development of *P. aeruginosa* biofilms. A co-culture time of 24 h of PBMC with preformed *P. aeruginosa* biofilms was sufficient to induce cell activation with limited cell death. As an average of different donors approximately 12% of the PBMC, harvested following *P. aeruginosa* biofilm co-incubation, were activated T lymphocytes ($CD3^+CD69^+$ cells). Such an activation might result from a non-specific direct stimulation or from cytokines released by monocytes, but it might also be compatible with a secondary response to *P. aeruginosa* antigens,

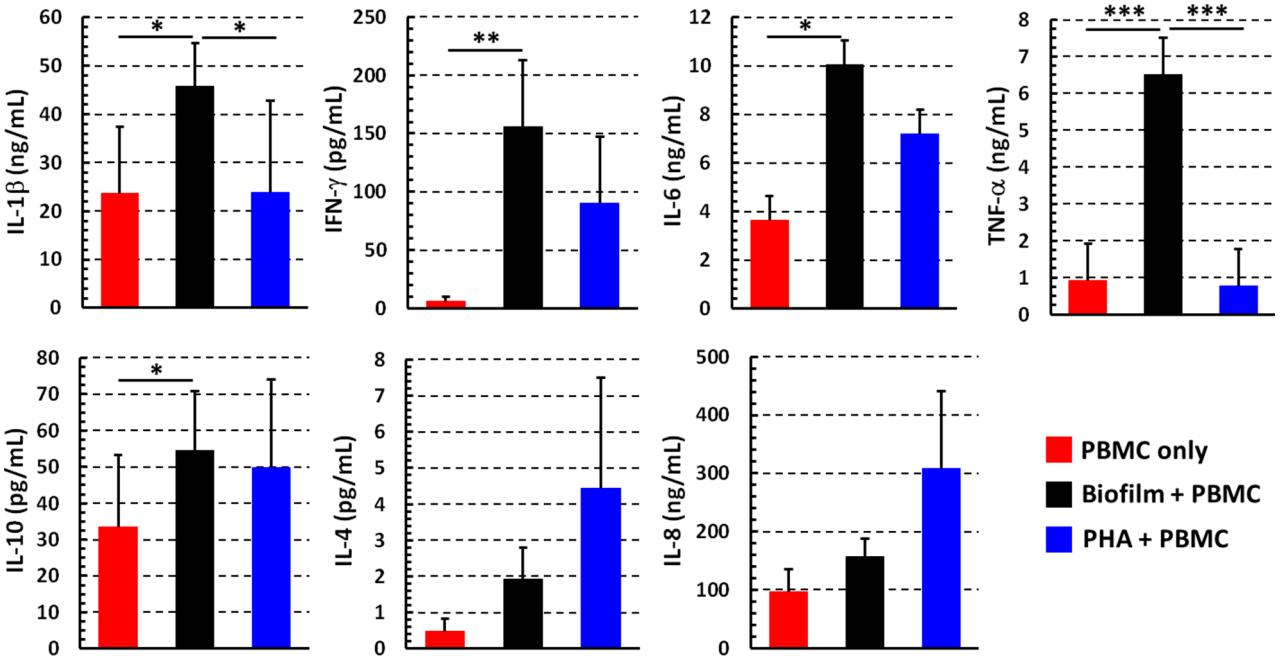

FIGURE 5 | Cytokine profiles of PBMC stimulated with *P. aeruginosa* biofilm**.** The supernatants were collected after 24 h from wells in which PBMC incubated alone (red bars), with preformed *P. aeruginosa* biofilm (black bars), or stimulated with a mitogen (PHA, blue bars) in complete RPMI. Cytokine amount was evaluated by flow cytometer based multibead capture assay. Mean values ± SEM from 10 independent experiments are shown $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA for matched samples and Tukey-Kramer multiple comparisons test.

as the blood donors might have been sensitized by environmental exposure to *P. aeruginosa*, a bacterium widely distributed in the environment. Still little is known about the existence of biofilm-specific antigens and whether they are "seen" by host immune cells. Obvious candidates are components of the EPS, a highly hydrated mixture of extracellular DNA (bacteria- and host-derived), proteins, polysaccharides, and lipids whose effects on host cells appear to be of great complexity ranging from immunogenic to anti-immunogenic according to the microbial species or strains considered (Watters et al., 2016). Only few studies have investigated the T cell response to bacterial biofilms and most of them have been focused on *Staphylococcus* biofilms with some conflicting results (Prabhakara et al., 2011; Hanke et al., 2013). Early work carried out in different mouse strains intratracheally infected with *P. aeruginosa*, has demonstrated that the Th1-reacting C3H/HeN mice show a better disease outcome compared to the Th2-reacting BALB/c mice, suggesting that a Th1 response might be beneficial in chronic *P. aeruginosa* pulmonary infection (Moser et al., 1999). It is possible that different T cell subsets (Th1, Th2, Th17, Treg) take part to the response and that, similarly to other chronic infections, a balance between antigen-specific pro-inflammatory (Th1/Th17) and anti-inflammatory (Th2, Treg) T cells is required for ensuring a protective effect and a limited tissue damage (Ehlers, 1999).

A very interesting result that emerged from our study is the striking activation of $CD56^{+}CD3^{-}$ cells (NK cells) following 24 h PBMC exposure to *P. aeruginosa* biofilm. As an average, 60% of NK cells expressed the early activation marker CD69 upon stimulation with *P. aeruginosa* biofilms and the percentage reached very high levels in some donors (up to 86%). NK cells are traditionally considered as "nonspecific" or "innate" effector cells, meaning that they do not recognize bacteria in an antigen-specific manner and do not generate memory responses. Such view has been challenged over the last years when it became clear that NK cells might be endowed with those properties that for a long time have been ascribed only to cells of specific immunity (e.g., immunological memory) (Narni-Mancinelli et al., 2011; Sun et al., 2011), placing such cells at the interface between innate and adaptive immunity. The main route to the activation and functional activity of NK cells is via soluble factors (e.g., IL-12; IL-1β; TNF-α) released by accessory cells (monocytes; macrophages; dendritic cells) or T cells (e.g., IL-2). Nevertheless, we and others have provided strong evidence that NK cells express functional "pathogen recognition receptors" (PRRs) that can directly interact with "pathogen associated molecular patterns" (PAMPs), resulting in cell activation and functional activity (Chalifour et al., 2004; Esin et al., 2008, 2013). Thus, NK cell activation observed in the present study might be due to soluble factors present in the supernatants of PBMC:biofilm co-cultures, as well as to a direct recognition of yet unidentified biofilm-associated PAMPs by such cells. Putative NK cell receptors involved in the direct recognition of microbial ligands are numerous and include several members of the TLR family (e.g., TLR2, TLR4, TLR5, TLR9), intracellular receptors such as NOD2, or members of the natural cytotoxicity receptor (NCR) family (Esin and

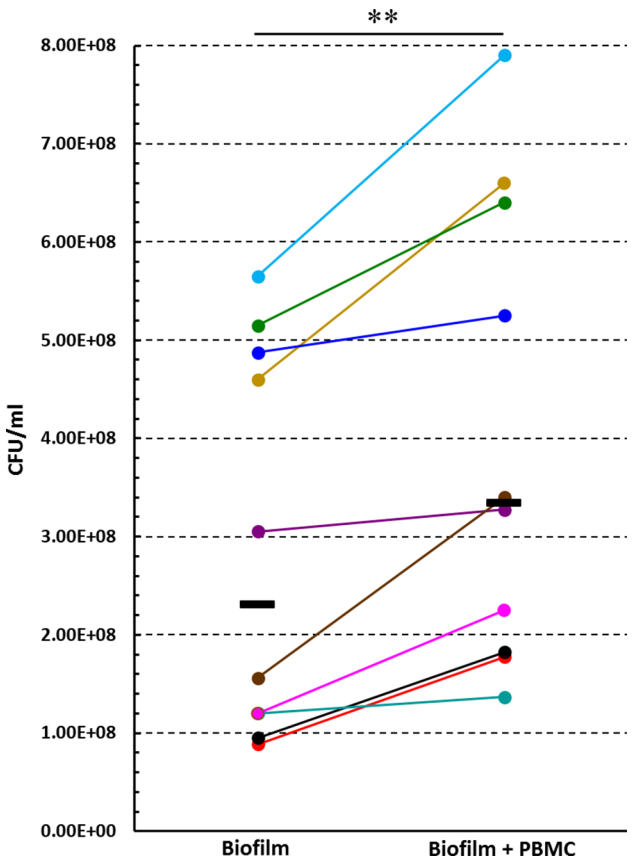

FIGURE 6 | The effect of PBMC:*P. aeruginosa* biofilm co-culture on the number of *P. aeruginosa* biofilm associated bacteria. Preformed (24 h old) *P. aeruginosa* biofilms were cultured in the presence or absence of PBMC obtained from different donors for further 24 h, and the numbers (CFU) of biofilm associated bacteria were assessed. Each donor/experiment is represented by a different color. Black lines represent median values.$^{**}P < 0.01$, Wilcoxon signed-rank test, $n = 10$.

Batoni, 2015). In this regard, we have previously reported that, unlike a number of Gram-negative bacteria, a soluble form of the NCR family member NKp44 is able to bind intact *P. aeruginosa* cells suggesting the existence of putative ligands for this receptor on the bacterium surface (Esin et al., 2008). Interestingly, it has been demonstrated that a fraction of human NK cells express basal levels of TLR9 and respond to bacterial DNA containing a high frequency of unmethylated CpG motifs (Ashkar and Rosenthal, 2002; Roda et al., 2005). Since bacterial DNA is an essential component of the EPS of *P. aeruginosa* biofilms, it might be possible that such component may represent, among others, a biofilm-specific ligand able to activate immune NK cell response (Watters et al., 2016). Another receptor expressed on NK cells (and on other immune cells) thought to be involved in host defense against *P. aeruginosa* infection is NKG2D (Wesselkamper et al., 2008). NKG2D ligands are markedly induced by *P. aeruginosa* on pulmonary epithelial cells and the receptor seems critical for mouse protection following respiratory infection with the bacterium. Importantly, host-cell expression of NKG2D ligands was demonstrated to increase cytokine production by NK cells in response to a bacterial TLR-ligand such as LPS, suggesting a possible synergistic effect between different NK cell receptors in the response of NK cells to *P. aeruginosa* (Wesselkamper et al., 2008).

Once activated, NK cells express a variety of effector functions including cytotoxicity, production of high amounts of immunoregulatory cytokines (e.g., IFN-γ; TNF-α), production of antibacterial mediators (e.g., NO, α-defensins, granulysin), regulation of other cell type-functions and, in certain circumstances, direct bactericidal activity (Esin and Batoni, 2015) disclosing putative roles of such cells in immunity against biofilms. Of note, consistently with the large proportion of NK cell activation observed in the present study, high amount of IFN-γ were found in the supernatant of PBMC:biofilm co-culture. Interestingly, IFN-γ production by human NK cells stimulated with *P. aeruginosa* has been previously reported by Vourc'h and coworkers who demonstrated that such production is enhanced by NK priming with IL-12, but is STAT-4 pathway-independent (Vourc'h et al., 2017). The same group also demonstrated that direct bacteria-to-cell contact is necessary for IFN-γ production and the type 3-secreted exoenzyme T (Exo T) of *P. aeruginosa* is the main trigger of IFN-γ release (Vourc'h et al., 2017). Another study reported that IFN-γ release from PBMC correlates with an improved lung function in CF patients chronically infected by *P. aeruginosa*, suggesting a possible protective role of IFN-γ *in vivo* and a putative beneficial effect of IFN-γ treatment (Moser et al., 2000). To the best of our knowledge, massive activation of NK cells upon stimulation with *P. aeruginosa* biofilms is a finding previously not reported and point out possible roles for these cells in the response to bacterial biofilms.

Analysis of the cytokine pattern in the supernatants of PBMC:biofilm co-cultures also revealed relatively high levels of the anti-inflammatory cytokine IL-10. Such observation is consistent with the view that bacterial biofilms are able to skew the immune response toward an anti-inflammatory phenotype that promote bacterial persistence at late stage of an infection (González et al., 2018; Campoccia et al., 2019).

Interestingly, the present study demonstrated that not only *P. aeruginosa* biofilms induced activation and response of PBMC, but also the presence of PBMC or supernatant derived from PBMC:biofilm co-cultures caused a statistically significant increase of biofilm-associated *P. aeruginosa*. Such enhancement was observed using PBMC from different donors as well as employing different *P. aeruginosa* clinical strains suggesting that the stimulatory capacity of PBMC on *P. aeruginosa* biofilms might have important implications in the pathogenesis of *P. aeruginosa*-associated colonization of medical devices such as intravenous catheters. The increase in the number of biofilm cells in the presence of supernatants from PBMC:biofilm co-cultures suggests that soluble factors, released in the extracellular environment upon interaction of eukaryotic cells with *P. aeruginosa* biofilms, might be responsible for the observed stimulatory effect. Such effect did not seem to be due to bacterial factors (e.g., inducers of quorum sensing), in fact, biofilm supernatants obtained in the absence of PBMC did not exerted the same biofilm-enhancing effect. Neither

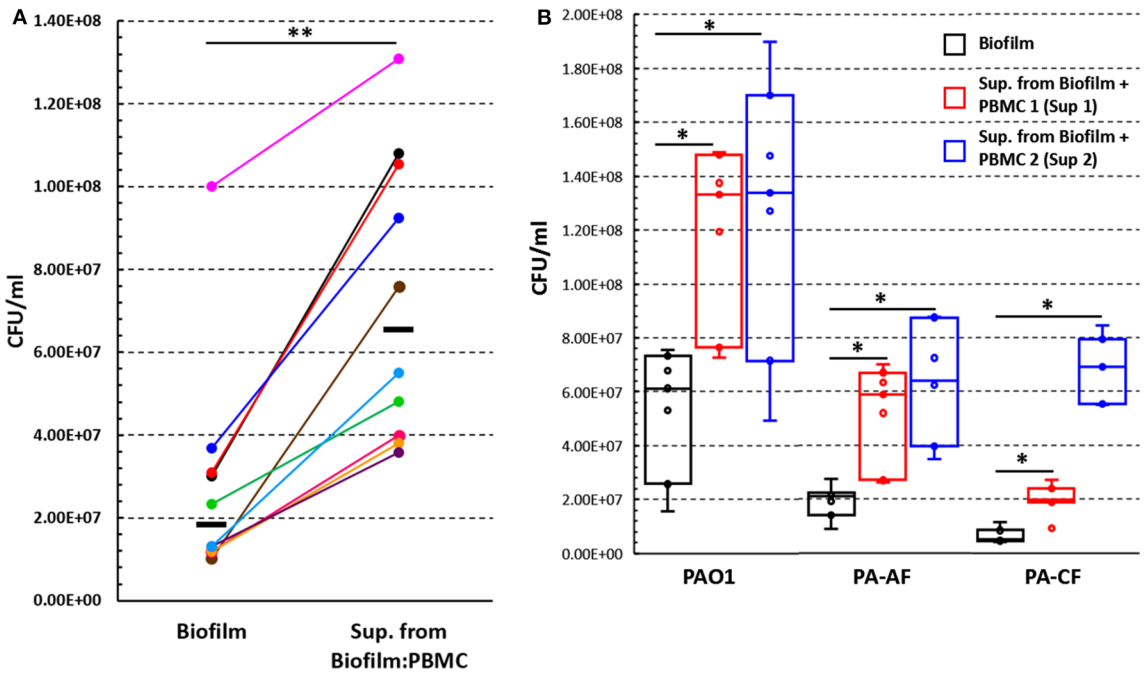

FIGURE 7 | The effect of supernatants, obtained from co-culture of PBMC with 24 h old *P. aeruginosa* biofilms, on *P. aeruginosa* biofilm associated bacteria number. **(A)** Preformed *P. aeruginosa* ATCC 27853 biofilms (24 h old) were incubated for further 24 h in the presence or absence of supernatants obtained from PBMC co-cultured with preformed *P. aeruginosa* biofilm for 24 h ($n = 10$) and the numbers (CFU) of biofilm associated bacteria were assessed. Black lines represent median values. **(B)** Preformed biofilms of clinical *P. aeruginosa* strains (PAO1, PA-AF, and PA-CF) were incubated for an additional 24 h with supernatants obtained from two different donors' PBMC co-cultured with *P. aeruginosa* ATCC 27853 biofilms ($n = 7$); data are presented as the median and interquartile range. $^*P < 0.05$, $^{**}P < 0.01$, Wilcoxon signed-rank test.

supernatants stimulated with the mitogen PHA were able to enhance biofilm growth, suggesting that the stimulatory capacity of the supernatants was biofilm-specific and not due to a non-specific activation of host cells. It has been proposed that *P. aeruginosa* biofilms "sense" the presence of host immune components and actively react to promote its own persistence (Wu et al., 2005; Hänsch, 2012). For instance, Alhede and coworkers demonstrated that *P. aeruginosa* biofilms recognize and respond aggressively to the presence of human polymorphonuclear leukocytes (PMNs) by upregulating the synthesis of rhamnolipids, amphiphilic molecules composed of rhamnose and hydrophobic fatty acid moieties, that represent major virulence factors of the bacterium (Jensen et al., 2007; Alhede et al., 2009). There is also evidence that *P. aeruginosa* increases the formation of biofilm in the presence of PMNs (Walker et al., 2005). Possibly, cytokines or other host factors released in the supernatant upon cell activation/lysis are recognized by bacteria that implement a defensive/persistence strategy against the immune response. This hypothesis is supported by previous findings demonstrating that cytokines such as TNF-α, IL-1 or IL-6 do promote bacterial growth (Porat et al., 1991; Meduri et al., 1999; Kanangat et al., 2001) and that bacteria possess receptor-like molecules for immune mediators (Luo et al., 1993). IL-1β was shown to bind to and induce growth enhancement of *Staphylococcus aureus* biofilms (McLaughlin and Hoogewerf, 2006). Similarly, Mittal and coworkers demonstrated a significant enhancement in growth and virulence factor production when both planktonic and biofilm cells of *P. aeruginosa* were grown in the presence of supernatants containing mouse-peritoneal-macrophage secretory products (MSPs) (e.g., TNF-α, TNF-β, IL-1α, IL-β, GM-CSF, MIP-2, IL-6, IL-12, and IL-18, as well as, reactive nitrogen intermediates) (Mittal et al., 2006). Enhancement of biofilm formation by soluble factors released by adherent PBMC was also described for the fungal opportunistic pathogen *Candida albicans* (Chandra et al., 2007) suggesting that biofilm responsiveness to cytokine production may represent an adaptive response of different types of microbial pathogens to counteract the host attack. Interestingly, Wu and coworkers analyzed the ability of supernatants from antigen-stimulated T cells to induce the expression of the type I *P. aeruginosa* lectin (PA-I or lecA), an adhesin of *P. aeruginosa* taken by the authors as representative quorum sensing-dependent virulence determinant of this organism (Wu et al., 2005). They demonstrated that PA-I expression was increased by supernatants from activated T cell cultures and this effect was essentially ascribable to the presence of IFN-γ in the supernatants. Of note, IFN-γ-mediated increment in both transcription and translation of PA-I started at early stationary phase of growth when bacteria are considered structurally and metabolically assimilable to biofilm cells. Finally, the authors demonstrated that IFN-γ directly binds to an outer membrane protein of *P. aeruginosa* (OprF) resulting in the

activation of quorum sensing, which is known to control the expression of different virulence factors including the ability to form biofilm (Wu et al., 2005). In the present study, when PBMC were co-cultured with *P. aeruginosa* biofilms, high amounts of IFN-γ were found in the culture supernatants, together with elevated proportion of activated NK cells that are known to be producers of IFN-γ. It is tempting to speculate that IFN-γ released by activated NK cells (and/or T cells) upon incubation with *P. aeruginosa* biofilms may represent a double-edged sword that is capable of expressing beneficial effector functions against biofilms, but at the same time potentially able to trigger evasion mechanisms of *P. aeruginosa* through activation of quorum sensing system and enhancement of biofilm formation (Moser et al., 2017).

Overall, the findings from the present study suggest that host PBMC are able to activate and produce cytokines upon interaction with *P. aeruginosa* biofilm. On the other hand, biofilm cells acquire the ability to grow more rapidly when challenged with mediators released from activated immune cells revealing a mechanism that may contribute to the ability of biofilms to resist clearance by host defenses and establish chronic infections. Much research effort is still needed to fully clarify the complex mechanisms and pathways that regulate the mutual interactions between the host immune system and *P. aeruginosa* biofilms. Previously unrecognized elements (e.g., NK cells) may participate to the host immune response to *P. aeruginosa* biofilms, adding complexity to the host:biofilm interaction but also paving the way for the identification of new immunotherapeutic strategies for the control of biofilm infections.

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 ethics committee of Area Vasta Nord-Ovest (CEAVNO). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EK, LG, GM, GB, and SE conceptualized and designed the study. EK, AB, CP, MD, GB, and SE acquired, analyzed, and interpreted the data. EK, GB, and SE drafted the article. EK, LG, AB, GM, CP, MD, GB, and SE critically revised the manuscript and approved it for publishing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00187/full#supplementary-material

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Inhibition of Angiotensin Converting Enzyme Impairs Anti-staphylococcal Immune Function in a Preclinical Model of Implant Infection

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Background: Evidence suggests the renin-angiotensin system (RAS) plays key immunomodulatory roles. In particular, angiotensin-converting enzyme (ACE) has been shown to play a role in antimicrobial host defense. ACE inhibitors (ACEi) and angiotensin receptor blockers (ARB) are some of the most commonly prescribed medications, especially in patients undergoing invasive surgery. Thus, the current study assessed the immunomodulatory effect of RAS-modulation in a preclinical model of implant infection.

Methods: *In vitro* antimicrobial effects of ACEi and ARBs were first assessed. C57BL/6J mice subsequently received either an ACEi (lisinopril; 16 mg/kg/day), an ARB (losartan; 30 mg/kg/day), or no treatment. Conditioned mice blood was then utilized to quantify respiratory burst function as well as *Staphylococcus aureus* Xen36 burden *ex vivo* in each treatment group. *S. aureus* infectious burden for each treatment group was then assessed *in vivo* using a validated mouse model of implant infection. Real-time quantitation of infectious burden via bioluminescent imaging over the course of 28 days post-procedure was assessed. Host response via monocyte and neutrophil infiltration within paraspinal and spleen tissue was quantified by immunohistochemistry for F4/80 and myeloperoxidase, respectively.

Results: Blood from mice treated with an ACEi demonstrated a decreased ability to eradicate bacteria when mixed with Xen36 as significantly higher levels of colony forming units (CFU) and biofilm formation was appreciated *ex vivo* ($p < 0.05$). Mice treated with an ACEi showed a higher infection burden *in vivo* at all times ($p < 0.05$) and significantly higher CFUs of bacteria on both implant and paraspinal tissue at the time of sacrifice ($p < 0.05$ for each comparison). There was also significantly decreased infiltration and respiratory burst function of immune effector cells in the ACEi group ($p < 0.05$).

Conclusion: ACEi, but not ARB, treatment resulted in increased *S. aureus* burden and impaired immune response in a preclinical model of implant infection. These results

suggest that perioperative ACEi use may represent a previously unappreciated risk factor for surgical site infection. Given the relative interchangeability of ACEi and ARB from a cardiovascular standpoint, this risk factor may be modifiable.

Keywords: implant, infection, angiotensin-converting enzyme inhibitor, angiotensin II receptor blocker, bioluminescence

INTRODUCTION

Implant-associated surgical site infections (SSI) represent significant morbidity and mortality for the patient as well as massive economic strain to the current healthcare system (1–7). Despite increasing efforts to prevent SSI through perioperative antibiotic management and the optimization of aseptic surgical technique, infection rates still range from 1.2% in primary joint replacements to 8.6% in ventral hernia mesh repair to as high as 12.9% in ventriculoperitoneal shunts (8–16). Although infection rates and treatment approaches vary by implant type, the overwhelming majority of patients who develop SSI ultimately require surgical implant removal, as bacteria form protective glycocalyx layers on avascular surfaces knows as biofilm (8, 17, 18). In high risk surgeries such as cardiac device implantation and spinal instrumentation, this can lead to catastrophic outcomes such as cardiovascular compromise, spinal column collapse, or death (19, 20). Even in hip and knee replacement surgery, an implant infection carries a worse 5-year mortality rate than breast cancer, renal cell cancer, or HIV/AIDS (21–25). Given the absence of effective treatment for implant infections, prevention is thus paramount. To that end, the identification and optimization of safe and short-term host-targetable risk factors represent crucial, innovative opportunities to prevent SSI.

Angiotensin-converting enzyme inhibitors (ACEi), which block the conversion of angiotensin I to angiotensin II, and angiotensin II receptor blockers (ARB) are two of the most commonly used drugs for the treatment of hypertension (26–29). In 2017, 73 million Americans were prescribed at least one cardiovascular medication, of which, 28 million Americans were prescribed an ACEi and another 15 million were prescribed an ARB (30). Furthermore, according to the CDC, an estimated 11.4% of Americans between 40 and 59 years old, and 21.3% from the age of 60–79 have taken an ACEi in the last 30 days (31). The prevalence of these medications is perhaps even greater in the surgical population. In one multi-institutional study performed across 12 surgery centers, 4,802 out of 14,687 (32.7%) patients who underwent inpatient non-cardiac surgery were taking an ACEi or ARB perioperatively (32).

While the role of the renin-angiotensin system for blood pressure regulation is well-known, emerging evidence suggests this system also has an immunologic function. Of the components involved in this system, ACE appears to have a particularly important role in antimicrobial host defense. Multiple human and animal studies have demonstrated that ACE overexpression increases immune cell response and facilitates host defense against bacterial infections (26, 33–46). In one murine study, selectively reducing ACE expression in neutrophils led to a 6-fold reduction in the clearance of a subcutaneous infection with methicillin-resistant *Staphylococcus aureus* (MRSA) (26). The purported mechanisms underlying any possible immunosuppressive effect of ACE inhibition include dysregulation of TNF-α, IL-6 and/or TGF-β response (47, 48), IL-12 suppression (49), decreased neutrophil superoxide production (26), dysfunctional macrophage activity (34, 35, 40), impaired chemotactic function (34), and decreased pro-inflammatory cytokine production (39, 40). Such mechanisms may impact innate and/or adaptive roles of antimicrobial host defense.

It is crucial to ensure immunocompetency at the time of surgery as the immunoprofile of patients prior to implantation are inextricably linked with the development of a SSI (50–52). It is undoubtedly true that certain patients will not be able to achieve lifelong immunocompetency, however the optimization of the immune system at the time of surgery remains vitally important to minimize SSI, as the majority of implant associated infections occur at the time of surgery (51, 52). Although the immunological impact of ACEi has been studied, to some extent, *in vitro* and in short-term models *in vivo* (33–35, 38, 40–42, 46), there is a lack of longitudinal *in vivo* data to quantify this effect or explore its potential mechanistic basis, and no study to our knowledge has investigated this phenomenon in a surgical model. Thus, the purpose of this study was to assess whether perioperative ACEi treatment impacts the host immune response and determine whether any purported impact would be sufficient to affect infection rates and severity in a well-validated *in vivo* mouse model of implant infection (53–56). This study also aimed to assess whether a reasonable alternative drug with a similar cardiovascular profile could avoid such host immunomodulation, thus optimizing host immunity to minimize perioperative infectious risk.

MATERIALS AND METHODS

Ethics Statement

All animal studies were performed in accordance with protocols reviewed and approved by the Chancellor's Animal Research Committee (ARC) at University of California, Los Angeles (ARC #2012-104-21J). These practices are adherent to National Institute of Health and Public Health Service policies.

Selection and Preparation of Bioluminescent Xen36 *Staphylococcus aureus*

Staphylococcus aureus strain Xen36 (PerkinElmer, Waltham, MA), a bioluminescent strain derived from ATCC-29525 (Wright), was used as the study organism. This strain

expresses a genomically integrated luxABCDE operon (53, 57, 58). Consequently, Xen36 generates a bioluminescent blue-green signal with a maximal emission wavelength of 490 nm from viable, metabolically active organisms. Previous studies demonstrated this strain to be ideal for research targeting the longitudinal monitoring of *S. aureus* infections due to its strength and consistency of signal (57–59).

Bacterial inocula were prepared following previously published protocols (53–57). In brief, Xen36 was isolated on kanamycin to select for purity and affirm possession of the kanamycin-resistance marker integral to the lux operon. The authenticated Xen36 strain was then quadrant-streaked onto tryptic soy agar (TSA; Beckton-Dickinson) and incubated for 24 h at 37°C. Single colonies were then isolated and cultured in tryptic soy broth (TSB) for 16 h at 37°C in a shaking incubator (196 rpm) (MaxQ 4,450, Thermo). A subsequent 2 h subculture of a 1:50 dilution of this culture was used to obtain a mid-logarithmic phase bacteria. Lastly, after centrifugation, cells were pelleted, resuspended, and washed in PBS. Bacterial inocula were quantitated and standardized by spectrophotometry (OD, 600 nm; BioMate 3; ThermoFisher Scientific). A schematic overview of our *ex vivo* and *in vivo* experiments is provided (**Figure 1**).

In vitro Determination of Direct Staphylococcal Growth Effect of ACEi or ARB

In order to confirm that ACEi or ARB do not have any direct antimicrobial effects against *S. aureus*, a Kirby Bauer diffusion susceptibility test was performed (60). Briefly, 20 μL of ACEi, ARB, vancomycin, each at a concentration of 0.5 mg/mL, or normal saline was aliquoted onto separate 6 mm filter paper disks and left to dry for 30 min. One disk from each group was then placed into a separate quadrant of a TSA plate that had been flooded and spread with 200 μL of 1×10^6 *S. aureus* Xen36. This was performed for four total plates. This procedure was replicated on four additional plates using a concentration of 1 mg/mL of study therapeutics. Plates were left to incubate at 37°C for 16 h and zone of inhibition(s) were then analyzed.

Mice and Medication Administration

Eight to twelve-week-old, 20–25 g C57BL/6 wildtype mice (Jackson Laboratories, Bar Harbor, ME) were housed (four mice per standard cage) and stored with a 12 h light and dark cycle with food and water ad libitum. Veterinary staff assessed all mice on a daily basis to ensure well-being throughout the entirety of the experiment.

Mice were randomized to receive treatment with either: an ACEi (lisinopril; 16 mg/kg/day PO; LKT Laboratories, St. Paul, MN) (ACEi group), an ARB (losartan; 30 mg/kg/day PO, LKT Laboratories, St. Paul, MN) (ARB group), or no treatment (control group) with dosing as per prior independent protocols that demonstrated a percent reduction in blood pressure akin to that of humans (40, 61). Medications were suspended in 250 mL containers of drinking water. Ten milliliters of sucralose was added to the drinking water of all mice and intake was recorded daily to ensure each mouse drank 3–5 mL/day. For all *ex vivo* experiments, mice received medication treatment for 4 weeks prior to cardiac puncture and sacrifice. For *in vivo*

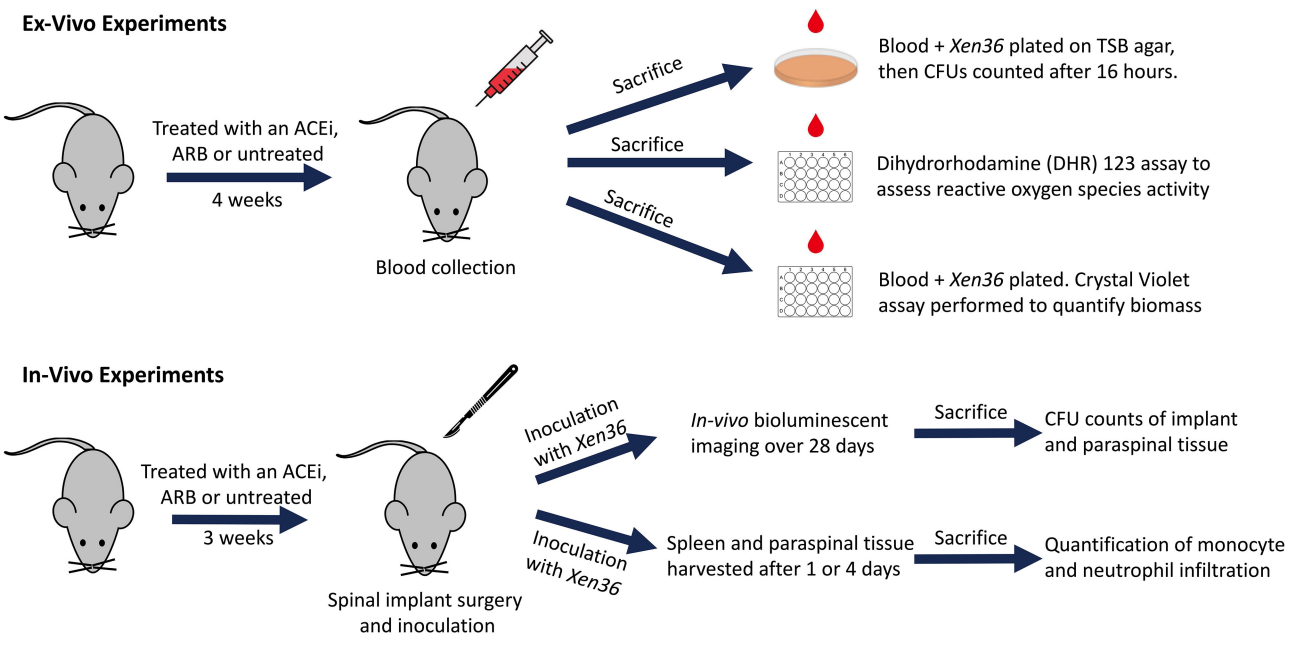

FIGURE 1 | Schematic diagram of *ex vivo* and *in vivo* experiments.

experiments, treatment began three weeks preoperatively and continued postoperatively for 4 weeks until sacrifice. These time points were selected based on previous studies demonstrating sufficiently altered immune profiles of mice blood after 7–10 days (26, 35).

Ex vivo Quantification of Respiratory Burst

Following 4 weeks of medication treatment, blood was collected from six mice in each group via cardiac puncture under 2% isoflurane inhalation anesthesia, followed by immediate euthanasia. Ethylenediaminetetraacetic acid (EDTA) was added to blood samples in a 1:10 ratio to prevent coagulation. One-hundred microliters were added from each mouse to each well within a 96-well flat bottom plate (Corning Costar, Corning, New York). Reactive oxygen species (ROS) activity of whole blood were assessed using a dihydrorhodamine (DHR) 123 assay. Briefly, 10 μL of DHR 123 Assay Reagent followed by 25 μL of Phorbol myristate acetate followed by 2 mL of Red Blood Cell Lysis Buffer was added to each plate. Mean fluorescent intensity was read with an excitation filter of 485 nm and an emission filter of 520 nm using a fluorescent plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany).

Ex vivo CFU Quantification of *S. aureus* Mixed With Whole Blood

Six mice in each group underwent whole blood collection as above. Ten microliters of blood from each mouse were then gently mixed with 10 μL of 1×10^3 *S. aureus* Xen36 and incubated at 37°C for 1 h. After 1 h, the entire 20 μL of solution was quantitatively cultured on TSA and incubated at 37°C for 16 h. Resulting CFUs were then counted for a minimum of $n = 6$ replicates in each group.

Ex vivo Quantification of the Biofilm Biomass

Five mice in each group underwent whole blood collection as above. One-hundred microliters of whole blood from each mouse were mixed with 100 μL of 1×10^7 *S. aureus* Xen36 CFU/mL for a final inoculum of 10^6 CFU in 200 μL. This solution was added to each well within a 96-well-flat bottom plate. Six additional control wells containing 200 μL of saline were also included for standardization. After 24 h of incubation at 37°C, each well was washed with PBS three times to remove residual blood cells and non-adherent bacteria. A well-validated crystal violet assay (Abcam, Cambridge, United Kingdom) (62–64) was performed to quantify the biomass of the residual biofilm formation by OD at 595 nm.

In vivo and Longitudinal Monitoring of Bacterial Burden and Implant and Paraspinal Tissue CFU Quantification

Twenty-six total mice were randomized into the following groups: 2 in the sterile control group, 8 in the infected control group, 8 in the ACEi group and 8 in the ARB group. Mouse spinal implant surgery and inoculation with 1×10^2 *S. aureus* Xen36 was performed as described in prior protocols (53–56). Briefly, a midline dorsal incision was made and dissection was performed through the fascia and muscle directed laterally along the L4 spinous process. The L4 process was manually reamed with a 25-gauge needle. An "L-shaped" surgical grade 0.1 mm diameter titanium implant (Custom Wire Technologies, Port Washington, WI) was then press-fit into the L4 process. The long arm of the implant measured 6.5 mm in length and the short arm measured 3.5 mm in length (**Figure 2**). The IVIS Lumina X5 (PerkinElmer, Waltham, MA) was used to capture bioluminescent images representative of *S. aureus* Xen36 burden

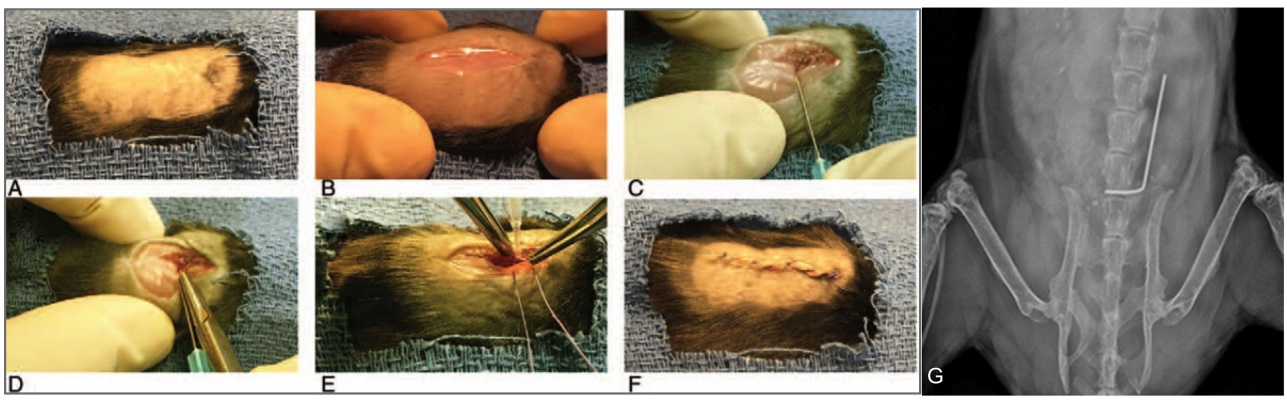

FIGURE 2 | Mouse spinal implant surgery procedures. **(A)** Mice were prepped with six alternating scrubs of povidone-iodine and alcohol and subsequently draped in a sterile fashion. **(B)** A 2 cm midline dorsal incision was made. Dissection was carried through the fascia and muscle and directed laterally along the L4 spinous process. **(C)** The L4 spinous process was reamed with a 25-gauge needle. **(D)** The short arm of the implant was press-fit into the spinous process and the long arm was laid longitudinally parallel along the spine directed cranially. **(E)** The wound was then prepared for closure using polyglycolic acid 5-0 sutures. Prior to these sutures being tied, 1×10^2 CFUs of Xen36 in a volume of 2 μL was inoculated directly onto the long arm of the implant. **(F)** Deep sutures were then tied and a running 5-0 vicryl suture was used to close the skin. **(G)** Proper placement of the implant was confirmed with high resolution X-rays on post-operative day 0 using the IVIS Lumina X5 (PerkinElmer, Waltham, MA).

on postoperative day (POD) 0, 1, 3, 5, 7, 10, 14, 18, 21, 25, and 28. Bioluminescent quantification of bacterial burden was confirmed directly by CFU quantification of the implant and surrounding tissue. On POD 28, each mouse was sacrificed and CFU of bacteria adherent to the implant as well as in the paraspinal tissue quantified. To do so, the implant was sonicated in 500 μL 0.3% Tween-80 (ThermoFisher Scientific) in TSB and the paraspinal tissue was homogenized (Pro200H Series homogenizer; Pro Scientific). Samples from implants and paraspinal tissue were then plated onto a TSA plate and incubated overnight. Resulting CFUs per plate were counted and total CFUs harvested from the paraspinal tissue and implant were expressed as CFUs/mL.

Histologic Analysis and Quantification of Monocyte and Neutrophil Infiltration

An additional 12 mice were randomized into the following groups: four in the infected control group, four in the ARB group and four in the ACEi group. Mice underwent spinal implant surgery and infection as described above. Two mice in each group were sacrificed on POD1 and two mice in each group were sacrificed at POD4. At the time of sacrifice, paraspinal and splenic tissue samples were harvested and stained with hematoxylin and eosin (H&E), F4/80 antibody (representing monocyte infiltration), and myeloperoxidase (MPO) (representing neutrophil infiltration). Histologic images were de-identified and qualitatively reviewed by a board-certified pathologist to assess for F4/80 and MPO signals. Brightfield slides were digitized on a ScanScope AT (Leica Biosystems, Inc., Vista, CA) and morphometric analysis performed with *Definiens* Tissue Studio (Definiens Inc., Parsippany, NJ) to quantify monocyte and neutrophil counts. Briefly, a stain specific algorithm was created using the pre-defined cellular detection module and classification tool, through which positive and negative stained cells within a tissue core were identified. The data were exported to Excel for further statistical analysis.

Statistical Analysis

Probability (p) values were calculated using a Student's t-test (one or two-tailed where indicated), while data analysis among three or more groups were compared using a one-way ANOVA. Longitudinal bioluminescent data were analyzed using a linear mixed effects regression model. Data were expressed as mean ± standard error of the mean (SEM). Stata-14 software (Statacorp, College Station, TX) was used for all statistical analyses and statistical significance was set at $p < 0.05$.

RESULTS

In vitro Determination of Direct Staphylococcal Growth Effect of ACEi or ARB

No zones of inhibition were appreciated in any plates for the normal saline, ACEi or ARB disks at any concentrations. A zone of inhibition of 18.5 ± 0.3 mm was measured around disks with 0.5 mg/mL of vancomycin. A zone of inhibition of 20.5 ± 0.3 mm was measured around disks with 1.0 mg/mL of vancomycin (**Figures 3A,B**).

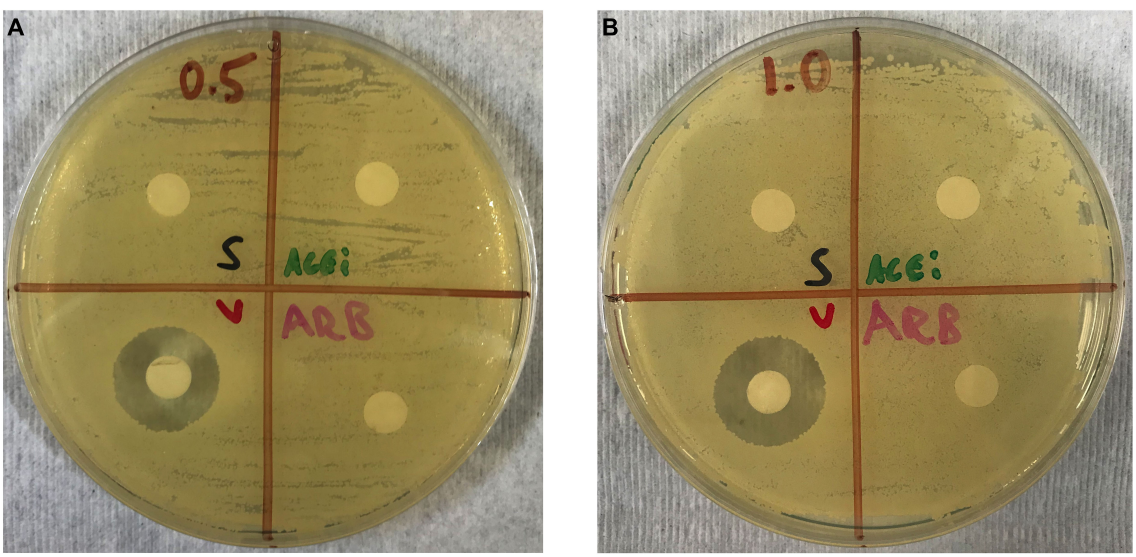

FIGURE 3 | Kirby–Bauer disk diffusion susceptibility test showing no direct effect of ACEi or ARB on *S. aureus* growth when study therapeutics are dosed at 0.5 mg/mL **(A)** and 1.0 mg/mL **(B)**. Thus, any purported effect on bacterial burden in ACEi and ARB-treated mice were not due to any anti-staphylococcal properties of the therapeutics themselves.

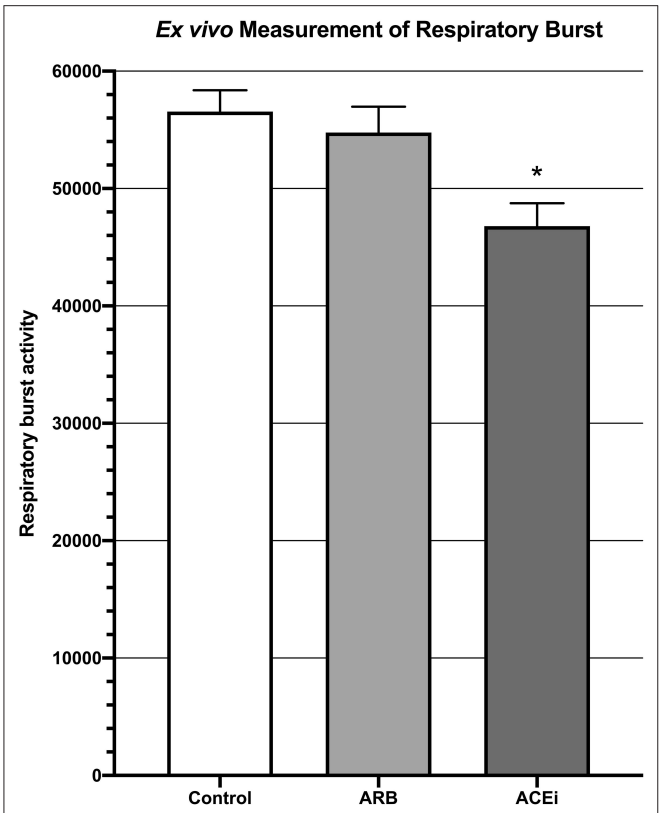

FIGURE 4 | Measurement of respiratory burst *ex vivo* showing significantly decreased respiratory burst in the blood of mice treated with an ACEi as compared to those treated with an ARB and the untreated control group. *$p < 0.05$.

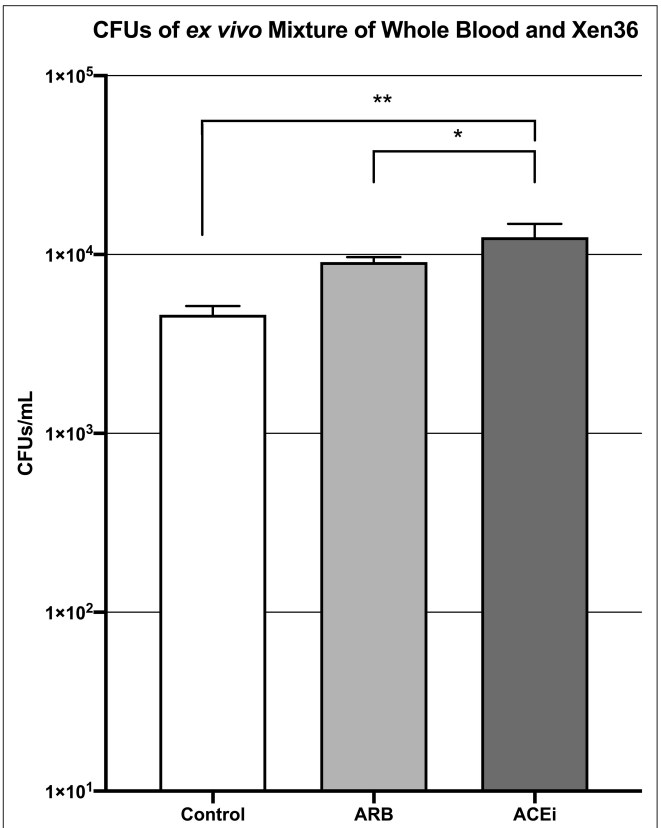

FIGURE 5 | CFU counts demonstrating significantly increased *S. aureus* burden *ex vivo* in the blood of mice treated with an ACEi as compared to those treated with an ARB and the untreated control group. * denotes $p < 0.05$, ** denotes $p < 0.01$.

Ex vivo Quantification of Respiratory Burst, CFU, and Biofilm Biomass

Respiratory burst, represented by mean fluorescent intensity from a DHR 123 assay, was significantly higher in the blood of mice from the ARB group ($5.5 \times 10^4 \pm 2.2 \times 10^3$) and the infected control group ($5.7 \times 10^4 \pm 1.8 \times 10^3$) when compared to the ACEi group ($4.7 \times 10^4 \pm 1.9 \times 10^3$, $p < 0.05$; **Figure 4**).

The mean CFU/mL of *S. aureus* Xen36 in whole blood was significantly higher for the ACEi group than the ARB group ($1.3 \times 10^4 \pm 9.5 \times 10^2$ vs. $9.1 \times 10^3 \pm 2.5 \times 10^2$, $p < 0.05$), which was significantly higher than the infected control group ($4.6 \times 10^3 \pm 2.2 \times 10^2$, $p < 0.05$; **Figure 5**).

Biofilm biomass, represented by absorbance units, was significantly higher in the ACEi group (1.9 ± 0.3) as compared to both the ARB (1.3 ± 0.3) and control (1.1 ± 0.1) groups ($p < 0.05$; **Figure 6**).

In vivo and Longitudinal Monitoring of Bacterial Burden and Implant and Paraspinal CFU Quantification

Bioluminescent signal was higher in the ACEi group than both the ARB and infected control groups at all time points. This difference reached significance at all time points other than POD 0 and 3 ($p < 0.05$). The only significant difference between the ARB and infected control group occurred at POD 14, when the ARB group was significantly higher than the infected control (**Figures 7A,B**).

Viable *S. aureus* CFUs were identified in 0 of 2 (0%) implants from the sterile control group, 2 of 8 (25%) implants from the infected control group, 2 of 8 (25%) implants from the ARB group, and 3 of 8 (37.5%) implants from the ACEi group. The mean CFU/mL cultured from the harvested implant in the ACEi group ($1.1 \times 10^4 \pm 8.5 \times 10^3$) was significantly higher than either the ARB ($9.8 \times 10^2 \pm 7.0 \times 10^2$) or infected control groups ($3.3 \times 10^2 \pm 2.9 \times 10^2$, $p < 0.05$; **Figure 8A**).

The mean CFU/mL cultured from the excised paraspinal tissue in the ACEi group ($1.6 \times 10^6 \pm 5.1 \times 10^5$) was significantly higher than the ARB ($6.1 \times 10^5 \pm 4.2 \times 10^5$) and infected control groups ($6.4 \times 10^5 \pm 3.7 \times 10^5$, $p < 0.05$; **Figure 8B**).

Histologic Analysis and Quantification of Monocyte and Neutrophil Infiltration

Following a review by a board-certified pathologist, there was no qualitative difference in monocyte or neutrophil infiltration to the spleen at POD1 or POD4 between the ACEi, ARB, or control treatment groups based on MPO and F4/80 stains.

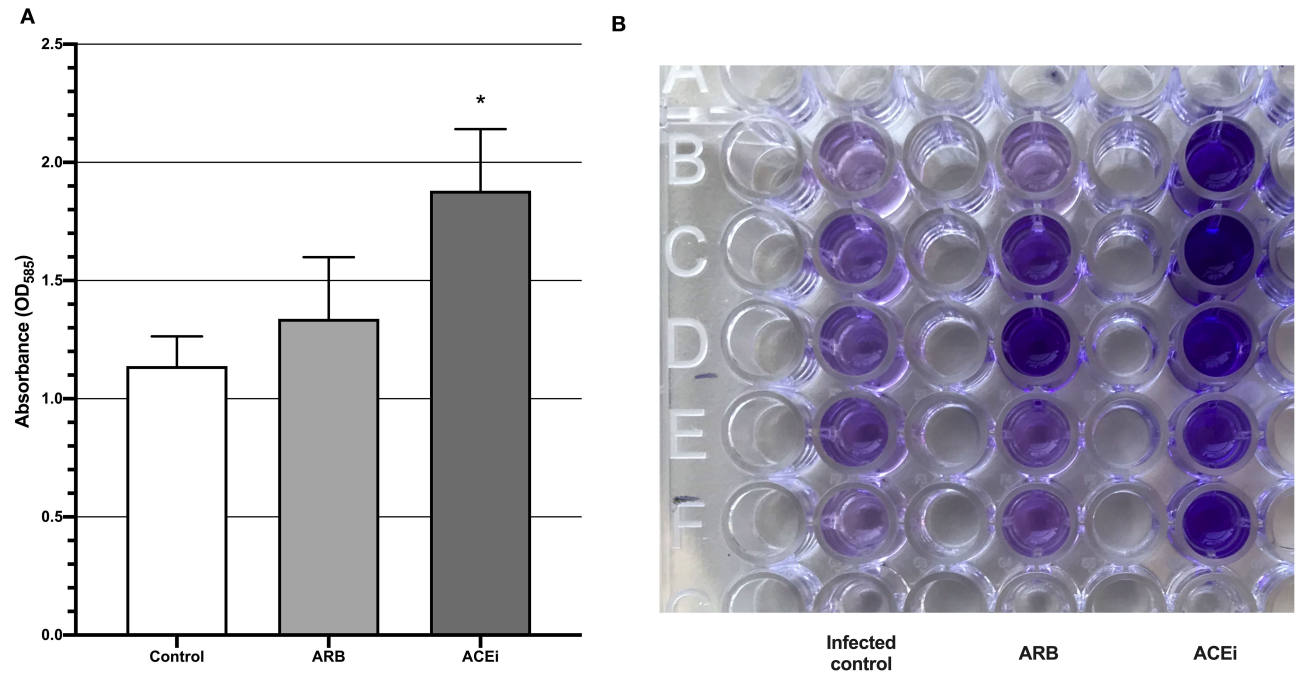

FIGURE 6 | Quantification of biomass of residual biofilm demonstrating a significantly increased *S. aureus* burden *ex vivo* in the blood of mice treated with an ACEi as compared to those treated with an ARB and the untreated group **(A)**. $^*p < 0.05$. Ninety-six well-plate after 24 h of incubation with whole blood and Xen36 for 24 h and stained with crystal violet **(B)**.

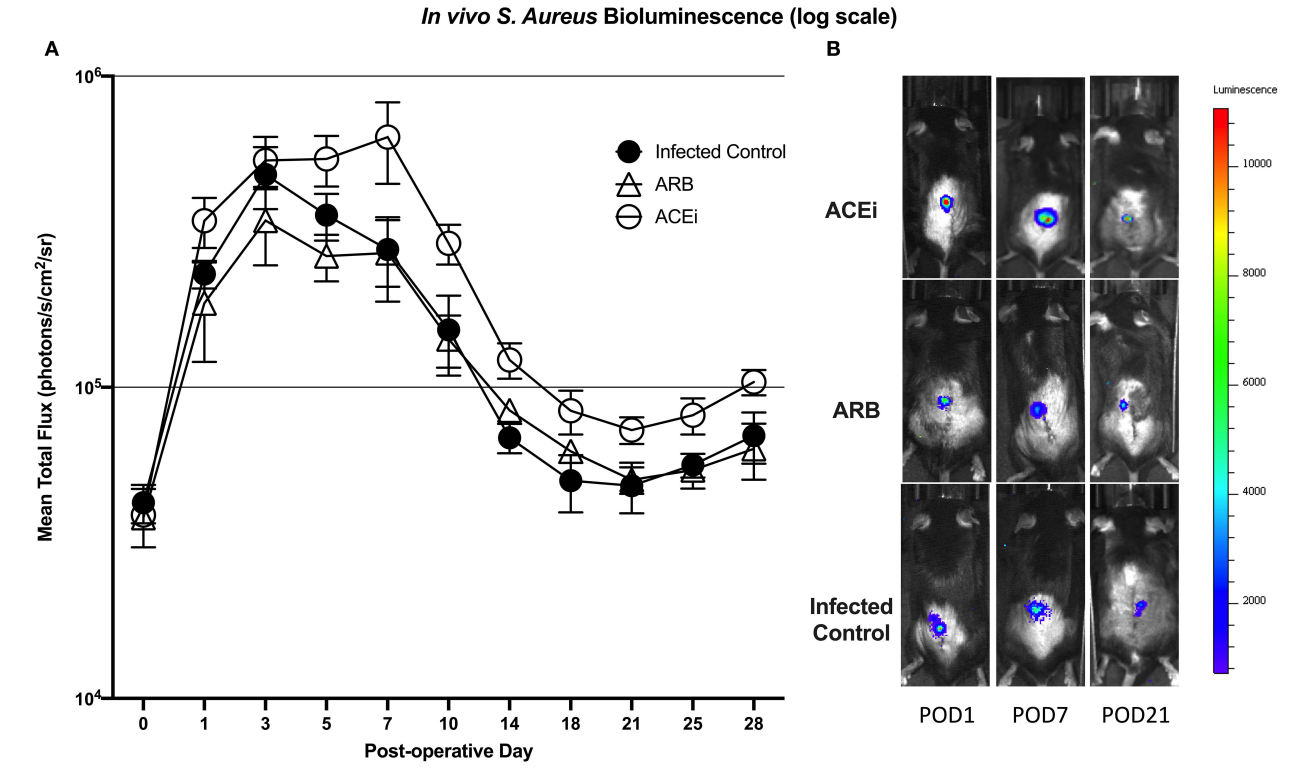

FIGURE 7 | *S. aureus* burden *in vivo* was higher at all time points in mice treated with an ACEi as compared to those treated with an ARB and the untreated group. Statistical significance ($p < 0.05$) was reached at all time points other than POD 0 and 3 **(A)**. Representative Xen36 *S. aureus* bioluminescent images at three selected postoperative time points overlaid on top of grayscale images of mice **(B)**.

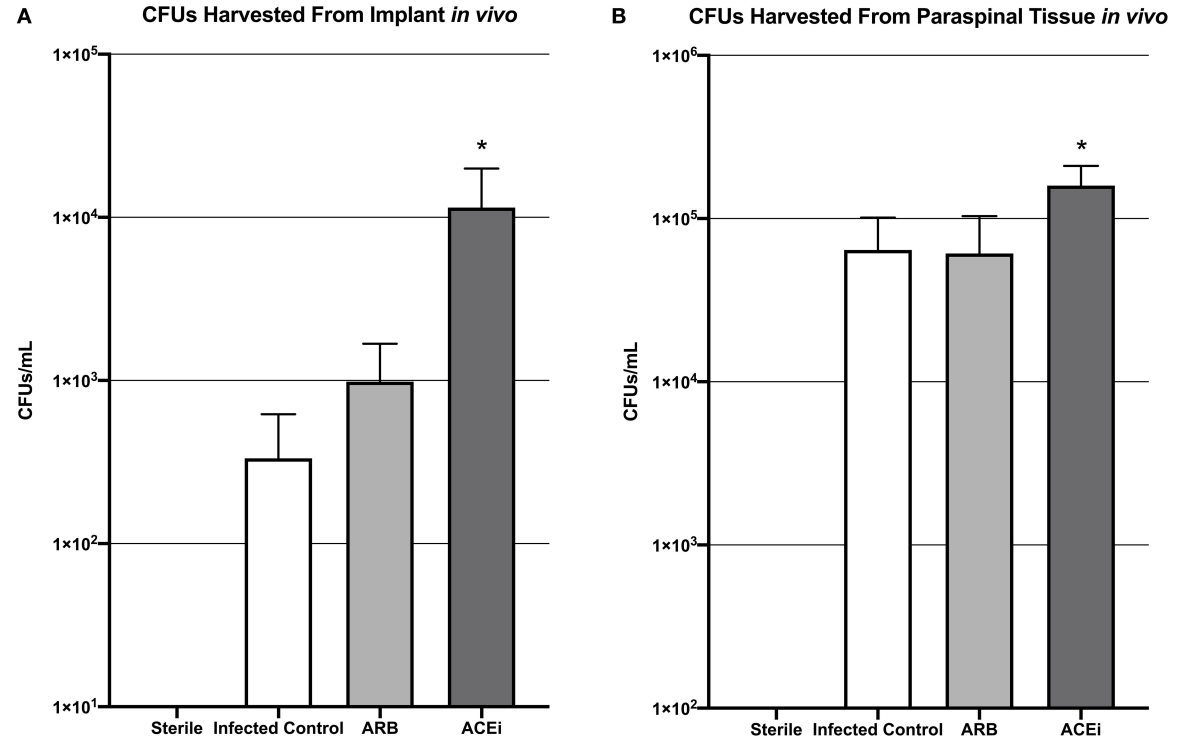

FIGURE 8 | Bacteria harvested from both the implant **(A)** and the paraspinal soft tissue **(B)** demonstrate a significantly increased *S. aureus* burden in mice treated with an ACEi than those with an ARB and untreated mice. *$p < 0.05$.

However, there was a qualitative difference in monocyte and neutrophil infiltration to the paraspinal tissue between the groups at POD 4 (**Figures 9A,B**, **10A,B**). The number of nuclei stained at POD 4 per tissue area sum in samples stained with F4/80 was significantly lower in the ACEi group ($1.9 \times 10^{-4} \pm 5.6 \times 10^{-5}$) compared with both the ARB group ($6.0 \times 10^{-4} \pm 2.7 \times 10^{-4}$) and the infected control group ($5.4 \times 10^{-4} \pm 3.9 \times 10^{-4}$; $p < 0.05$; **Figure 9C**). The number of cells stained at POD 4 per tissue area sum in samples stained with MPO was significantly lower in the ACEi group vs. the ARB group ($1.9 \times 10^{-4} \pm 4.2 \times 10^{-5}$ vs. $3.8 \times 10^{-4} \pm 1.1 \times 10^{-4}$, $p < 0.05$; **Figure 10C**).

DISCUSSION

Implant-associated SSI is a catastrophic complication. Moreover, staphylococcal species represent roughly two thirds of implant-associated SSI and often compound the issue due to multi-drug resistant phenotypes and a high propensity to form biofilms (65–67). Regardless of implant type, conservative, non-invasive treatment measures frequently fail to eradicate an infection and high-risk surgical intervention is often required (17, 19, 20). Thus, there is a significant and unmet need to identify modifiable risk factors that may optimize host immune protection against such infections. ACEi and ARB are amongst the most widely prescribed medications, particularly in the aging surgical population (31, 68). Given the prevalence of both ACEi and ARB, the potential impact of these therapies could be enormous if they modify immune response or efficacy in ways that subvert host defense.

The current findings first showed that neither ACEi nor ARB had any direct anti-staphylococcal activity *in vitro*. The rationale behind this *in vitro* experiment was to show that any purported impact of ACEi or ARB impact on bacterial burden *ex vivo* or *in vivo* would not have been due to any anti-staphylococcal effects of the actual therapeutics themselves. This study also shows that blood from mice treated with ACEi demonstrated significantly decreased respiratory burst capacity as well as a significantly decreased ability to suppress *S. aureus* infection *ex vivo* as compared to ARB-treated or control mice blood. Mice treated with an ACEi also had higher *S. aureus* burden *in vivo* as measured by bioluminescent signal throughout the entirety of the experiment compared to ARB-treated or infected control groups.

Congruent with these findings, CFU burden measured on implants as well as paraspinal tissue in the ACEi group was significantly higher than either the ARB or infected control groups. Furthermore, viable *S. aureus* CFUs were identified in 37.5% of the implants in the ACEi group as compared to 25% in both the ARB and infected control group. Paralleling these microbiologic findings, mice treated with ACEi had significantly decreased monocyte and neutrophil infiltration to the paraspinal tissue on POD 4 compared with infected controls or ARB-treated

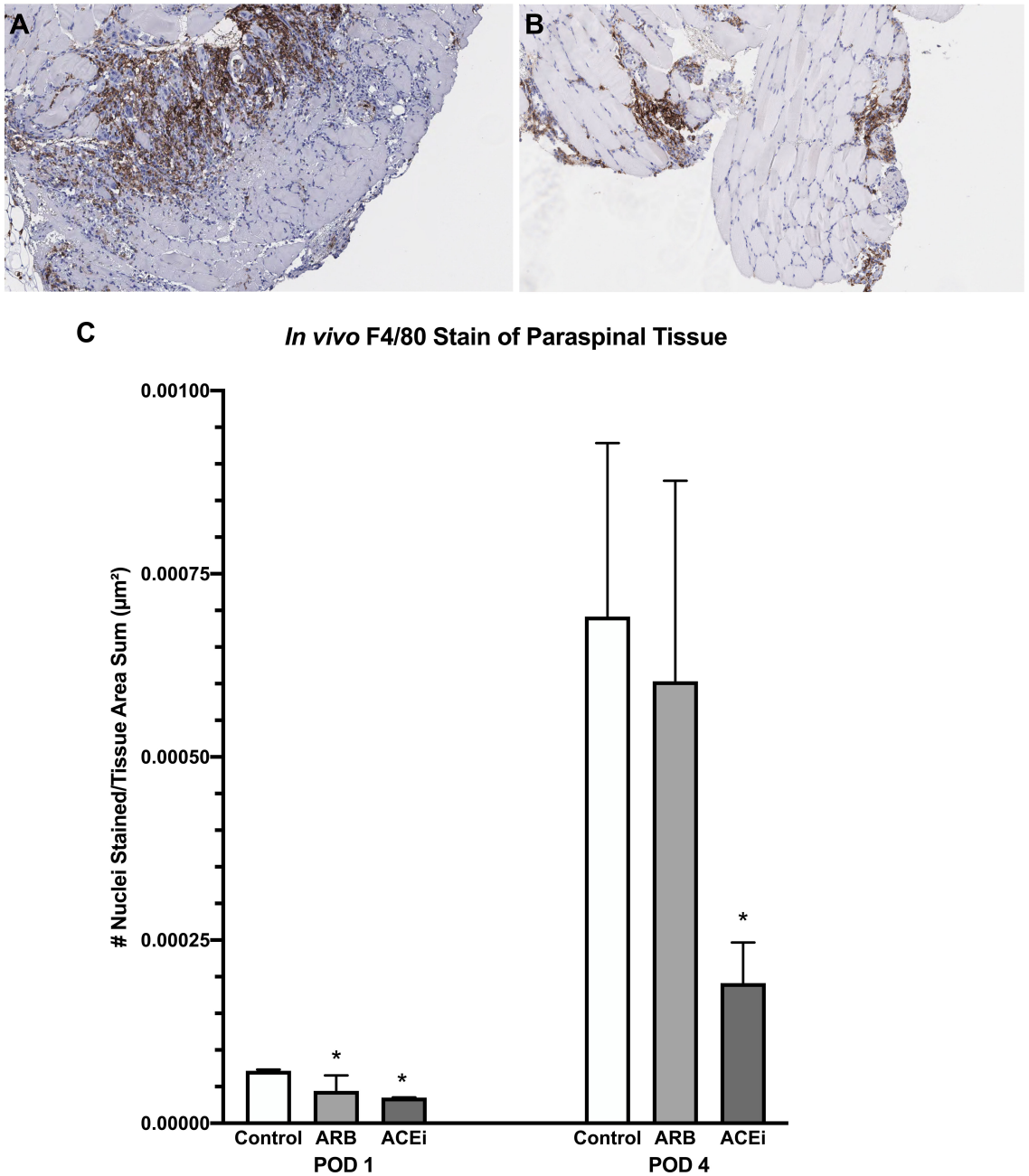

FIGURE 9 | F4/80 stain representing monocyte infiltration to the paraspinal tissue in mice treated with an ARB **(A)** and an ACEi **(B)**. **(C)** Mice treated with both an ARB and ACEi had significantly lower levels of monocyte infiltration than the control group at POD 1. Mice treated with an ACEi had significantly lower monocyte infiltration than mice treated with an ARB and the control group at POD 4 **(C)**. *$p < 0.05$.

mice. These data suggest that monocyte and neutrophil infiltration and/or functional ROS generation might be impaired by ACEi-related mechanism(s) and may be responsible for the increased susceptibility to *S. aureus*. Taken together, these findings suggest that perioperative ACEi treatment may represent a previously unappreciated risk factor to be considered prior to high-risk surgery such as implant instrumentation.

The increased infectious burden that developed with ACEi treatment is consistent with emerging literature that the renin-angiotensin system, and in particular ACE-1, may play an important role in innate pathogen defense (34). Khan et al. (26) recently found that selective neutrophil underexpression of ACE-1 markedly increased the susceptibility of mice to cutaneous methicillin resistant *S. aureus* (MRSA) infection, whereas

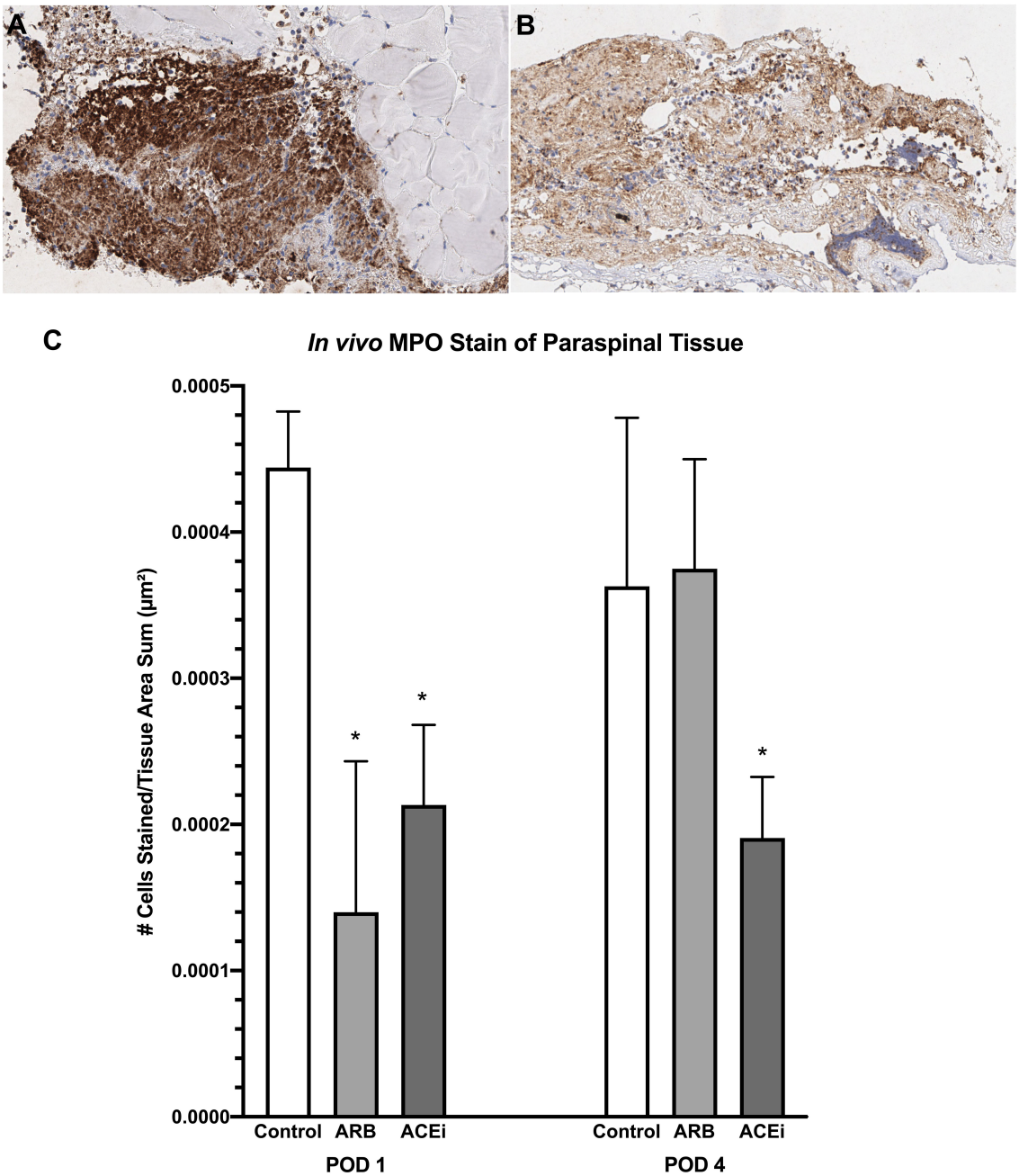

FIGURE 10 | Myeloperoxidase stain representing neutrophil infiltration to the paraspinal tissue in mice treated with an ARB **(A)** and an ACEi **(B)**. **(C)** Mice treated with both an ARB and ACEi had significantly lower levels of neutrophil infiltration than the control group at POD 1. Mice treated with an ACEi had significantly lower neutrophil infiltration than mice treated with an ARB and the control group at POD 4 **(C)**. $^{*}p < 0.05$.

neutrophil ACE-1 overexpression reduced susceptibility. Similar results have been reported with selective ACE-1 expression modulation in macrophages when challenged with both MRSA and *Listeria monocytogenes* (40). In both of these studies, the effect of ACE-1 to enhance pathogen clearance appeared angiotensin type-1 receptor independent, consistent with the majority of the results of the present study.

However, other studies implicate the angiotensin type-1 receptor in diverse leukocyte functions including neutrophil chemotaxis (69) as well as natural killer cell proliferation and chemotaxis (70). Activated neutrophils are also a source of angiotensin-II generation, which are produced both ACE-dependently and independently (71). In our study, ARB-treated mice treated with ACEi showed significantly higher *ex vivo*

CFU counts and significantly lower neutrophil and monocyte infiltration on POD 1 as compared to the infected control group. Therefore, while ARBs may influence pathogen defense-related pathways, these effects appear far less pronounced than ACE1 inhibition. Whether this distinction has clinical relevance warrants future investigation.

There are limitations to this study. It is important to consider the clinical, translational limitations of this implant infection model as it is a simplification of the complex steps involved in human spinal implant surgery. Limitations to this model include being unilateral, involving only the posterior elements of the spine, and use of a single stainless-steel metal implant (53). Furthermore, only *S. aureus* Xen36 was used in this study. Although this has been shown to be a well-validated, representative strain from a clinical isolate (57–59) the authors cannot extrapolate the findings reported to different staphylococcal strains or other microbial organisms. Given that this model allows for a safe, feasible, well-powered, and reproducible way to longitudinally quantify infection *in vivo*, these advantages are widely viewed to outweigh the accepted limitations. Another limitation to this study is the documented differences between murine and human physiology (38, 72, 73). Although doses of study therapeutics have been well-established and verified (40, 61), dose equivalent adjustments to humans as well as murine-specific pharmacological properties of these therapeutics are further limitations. However, mice treated with ACEi have been shown to respond similarly to humans in that they develop hypotension, increased levels of angiotensin I and decreased levels of ACE expression in myeloid cells (34, 38). Lastly, although this study showed that neither ACEi nor ARB exerted any direct antimicrobial effects on the growth potential of *S. aureus in vitro*, the potential direct effects of these study therapeutics on *S. aureus* metabolism, gene expression and/or virulence *in vivo* could also contribute to differences in outcomes observed.

The current findings provide *ex vivo* and *in vivo* evidence that perioperative ACEi treatment as compared to ARB treatment increases *S. aureus* burden in a manner that corresponds to a reduction in immune effector responses in a longitudinal murine implant infection model. These results in conjunction with the overall body of literature on ACEi immunomodulation suggest that perioperative ACEi treatment could pose additional infectious risks to patients. It is, however, important to consider the balance between any purported immunomodulatory effects of ACEi and its protective cardiovascular effects. ACE inhibition has been shown to improve arterial compliance (74, 75) and, by inhibiting angiotensin II formation, decrease left ventricular hypertrophy, generalized coagulability and possibly systemic sympathetic activity in diabetic and hypertensive patients (76–78). Therefore, the discontinuation of ACEi perioperatively is not without cardiologic risk. In patients lacking specific indications for particular antihypertensives, ACEi and ARB are often both considered first line therapy (79). Fortunately, ARB have been shown to exert protective cardiovascular effects to a similar, and perhaps greater, extent than ACEi (76, 78, 80, 81). Thus, the cardiovascular sequelae of switching a patient from an ACEi to an ARB perioperatively may not be substantially different. Moreover, unlike well-established modifiable host risk factors such as obesity and diabetes, switching a patient from ACEi to ARB treatment may be relatively easy, safe, and inexpensive. To this end, it may be possible that certain patients undergoing elective surgery could safely be switched from an ACEi to an ARB during the perioperative period to minimize any purported infectious risk associated with the immunomodulatory effects of ACEi treatment.

Preoperative host optimization is a key component to mitigating the risk of SSI and its devastating sequelae. The results of this study add to the growing body of literature suggesting that ACEi treatment may represent an under-appreciated, modifiable infectious risk factor. Future clinical studies investigating the relation between SSI and choice of antihypertensives are warranted to help develop guidelines regarding the perioperative use of ACEi.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by ARC (Animal Research Committee) at the University of California, Los Angeles (UCLA).

AUTHOR CONTRIBUTIONS

RT, DG, BK, ZM, TS, NC, MY, and NB contributed to conception and design of the study. All experiments were performed by RT, DG, BK, ZM, TS, TO, AC, CM, DL, NC, AS, and NB. Mouse surgical procedures were performed by RT with assistance from DG, BK, ZM, TS, TO, AC, NC, and NB. NC performed the statistical analysis. RT, DG, TS, NC, and DL completed all reference formatting. Figure generation was done by RT, DG, BK, ZM, CM, and NB. RT, DG, BK, ZM, TS, NC, TO, AC, CM, DL, AS, MY, and NB all assisted in writing the first and all subsequent drafts of the manuscript. All authors read and approved the submitted manuscript.

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Intravital Multiphoton Examination of Implant-Associated *Staphylococcus aureus* Biofilm Infection

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Bacterial infections associated with implanted medical devices represents a healthcare crisis due to their persistence, antibiotic tolerance, and immune avoidance. Indwelling devices are rapidly coated with host plasma and extracellular matrix proteins which can then be exploited by bacterial pathogens for adherence and subsequent biofilm development. Our understanding of the host-pathogen interface that determines the fate of biofilm-mediated infections is limited to the experimental models employed by laboratories studying these organisms. Current *in vivo* models of biofilm-mediated infection, while certainly useful, are typically limited to end-point analyses of bacterial burden enumeration, immune cell profiling, and cytokine/chemokine analysis. Thus, with these models, the complex, real-time assessment of biofilm development and innate immune cell activity remains imperceptible. Here, we describe a novel murine biofilm infection model employing time-lapse intravital multiphoton microscopy which permits concurrent and real-time visualization of *Staphylococcus aureus* biofilm formation and immune cell activity. Using cell tracking, we found that *S. aureus* biofilms impede neutrophil chemotaxis, redirecting their migration patterns to prevent biofilm invasion. This approach is the first to directly examine device-associated biofilm development and host-pathogen interactions and will serve to both further our understanding of infection development and help reveal the effects of future antibiofilm treatment strategies.

Keywords: multiphoton microscopy, medical device infection, *Staphylococcus aureus*, biofilm, innate immunity

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INTRODUCTION

Bacterial biofilm infections remain a significant healthcare problem worldwide. Infection risk is substantially increased in the presence of an implanted medical device, such as an orthopedic prosthesis, electronic cardiac device, artificial heart valve, or indwelling catheter (Costerton et al., 2005; Tande and Patel, 2014; Arciola et al., 2018). Over 25% of nosocomial infections are associated with an implanted medical device (Magill et al., 2014), and the incidence of infected hip and knee arthroplasty rates are expected to rise (Kurtz et al., 2007; Tande and Patel, 2014). The current standard-of-care involves surgical debridement and, if necessary, a two-step removal of the infected hardware including placement of a temporary spacer, 4–8 weeks of parenteral antimicrobial therapy, followed by insertion of a new device. This long and debilitating process is associated with significant patient morbidity and financial burden, often exceeding $90,000 per infection (Kurtz et al., 2012). In addition, prior device-associated infection increases patient risk for

infection relapse. Thus, an urgent need exists for novel approaches to prevent device-associated infection and/or facilitate biofilm eradication.

The leading causes of bacterial device-associated infection are *Staphylococcus* sp, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* (Arciola et al., 2018). These pathogens vary widely in their biochemistry, antimicrobial sensitivities, and virulence mechanisms employed to cause disease. Moreover, device-associated infections often pose difficulties in diagnosis using classic culture techniques (Fernandez-Sampedro et al., 2017), however, new molecular methodologies have shown promise in permitting accurate pathogen identification (Costerton et al., 2011). While staphylococci are the leading cause of prosthetic joint infections (Arciola et al., 2018), the causative species of other implant-associated infections largely depend on the implant site and type, patient co-morbidities, geographical location, and time since surgery (Aggarwal et al., 2014).

Medical implants are rapidly coated with host extracellular matrix molecules that help modulate a foreign body reaction, but also represent a surface target for bacterial attachment. As opposed to the planktonic lifestyle, biofilms are adherent communities of bacteria, often encased within a self-produced matrix of DNA, proteins, and/or polysaccharides. The biofilm mode of growth is regulated by a complex network of genetic factors responding to various environmental cues, including available metabolites, host molecules, and quorum sensing (Stewart and Franklin, 2008; Arciola et al., 2012). Moreover, biofilms are notoriously recalcitrant to antibiotic therapy due to metabolic heterogeneity and are well-described for their ability to resist innate immune defenses, including leukocyte invasion and phagocytosis (Thurlow et al., 2011; Mulcahy et al., 2014; Gries and Kielian, 2017). Three-dimensional biofilm structure not only poses a physical barrier to host immune cell infiltration and phagocytosis (Gries et al., 2020), but biofilm products can actively skew immune responses to enable infection persistence (Benoit et al., 2008; Scherr et al., 2014; Gries et al., 2016).

Many *in vitro* and *in vivo* models to have been developed to study biofilm development and host immune responses (Lebeaux et al., 2013). *In vitro* methods often comprise of static or sheer flow models mimicking infections associated with relatively stationary (e.g., internal fixation, peripheral catheters, etc.) or dynamic (e.g., joint arthroplasty, cardiac valve, vascular catheter, etc.) sites, respectively. These models can also include the addition of host factors or immune cells to assess anti-biofilm activity and/or bacterial responses. *In vivo* methods examining biofilm-mediated infections often involve rodent or rabbit models of device-associated infection. These models are largely limited to *ex vivo* analyses; requiring sacrifice of the animal for end-point quantification, including bacteria burdens, tissue histology, flow cytometry, and/or quantifying cytokine/chemokine production. More recently, continuous monitoring of biofilm infection has been demonstrated with bioluminescent bacterial strains and fluorescent reporter animals using whole-animal *in vivo* imaging systems (Thurlow et al., 2011; Wang et al., 2017; Gutierrez Jauregui et al., 2019). These models are advantageous as they do not require sacrifice of the animal to glean useful data, however they are limited by camera sensitivity and associating bioluminescent image data with established bacterial burden standard curves. In addition, these small-animal imaging systems do not permit cellular-level resolution and therefore rely on large number of congregating bioluminescent or fluorescent cells to emit a detectable signal.

To assess the cellular activities and interactions occurring during biofilm infection, several studies have employed confocal or epifluorescent microscopy (Forestier et al., 2017; Abdul Hamid et al., 2020). Unfortunately, these experiments are restricted to a limited depth penetration and single short-wavelength excitatory light that rapidly damages animal tissues. Unlike confocal and epifluorescent microscopy, multiphoton microscopy (MPM) utilizes simultaneous absorption of two or more long-wavelength photons to produce a single, short-wavelength excitatory stimulus. Longer wavelengths of light enable greater tissue penetration without the damaging effects of confocal/epifluorescent light sources (Denk et al., 1990), thereby permitting time-lapse imaging within living tissues. Furthermore, multiple detectors and spectral imaging confer the ability for spatiotemporal multiplexing and second-harmonic generation. MPM technology has rapidly evolved to include high-speed laser scanning and optical sectioning with up to 1 mm depth penetration, allowing 3-D reconstruction of tissue.

While MPM has proven useful in examining bacterial infection in living animals (Hickman et al., 2009; Abtin et al., 2014; Stolp and Melican, 2016), to-date MPM has not been employed to examine bacterial biofilm-mediated infections. Here we report on the use of MPM to simultaneously assess *Staphylococcus aureus* biofilm development and innate immune cell activity. At the time of preparation, this is the first report to examine *S. aureus* biofilm and associated innate immune response using intravital MPM. Furthermore, previous studies have shown a paucity of neutrophil influx at the site of infection (Thurlow et al., 2011; Hanke and Kielian, 2012), despite their enhanced ability to invade *S. aureus* biofilm *in vitro* compared to macrophages (Gunther et al., 2009; Scherr et al., 2013). We hypothesize that *S. aureus* biofilms modulate neutrophil behavior to promote infection persistence. To test this, we utilized cell tracking during time-lapse MPM to monitor neutrophil migration behavior and determined that neutrophils associated with *S. aureus* biofilm infection migrate randomly, indicating that they are redirected to avoid interaction, permitting biofilm persistence.

METHODS

Bacterial Strains and Culture Conditions

The wild-type *S. aureus* strain used in this study was LAC-13C, a USA300 MRSA skin and soft tissue infection isolate cured of plasmid p03 (Fey et al., 2013), widely used in biofilm infection studies with comparable findings to other strains (Vidlak and Kielian, 2016). The plasmid pCM29 was used as a constitutive source of superfolder-GFP (sGFP) expression (Pang et al., 2010). For infection studies, bacteria were prepared by inoculating

freshy isolated colonies into 25 mL Brain-Heart Infusion broth (BHI; Oxoid, UK) containing 10 μg ml^{-21} chloramphenicol and cultured for 16 h at 37°C, 250 RPM. Cells were then washed twice with PBS and diluted to 5×10^5 CFU mL^{-1} prior to infection.

Animals

The wild-type C57Bl/6 and CX3CR1-EGFP knock-in mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The PGRP-S-DsRed mice, where DsRed expression is driven by the PGRP-S promoter (Wang et al., 2011), were bred onto the C57Bl/6 background. All mice were bred in the University of California, Riverside vivarium under specific pathogen-free conditions and were handled in accordance with Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Implant Fabrication

The implant used in this study was shaped from a high-consistency medical-grade translucent silicone elastomer (MED-4780; NuSil, Carpinteria, CA, USA), approved for human implantation for a period of greater than 29 days. A 2 mm thick section of MED-4780 was embedded in optimal cutting temperature compound (OCT; Thermo Fischer Scientific, Waltham, MA, USA) and cut at 250 μm using a cryostat microtome, resulting in a 2×0.25 mm slice. The pieces were further hand-cut to have final dimensions of $3 \times 1.0 \times 0.25$ mm and sterilized by autoclaving.

Ear Pinna Implant Infection Model

Mice were anesthetized using ketamine-xylazine (100 mg/kg-5 mg/kg, IP; MilliporeSigma, St. Louis, MO, USA) and placed on a 36°C heat mat for the duration of the surgery. The left ear was affixed to a petri dish using double-sided adhesive tape and the dorsal side hair was removed using hair removal lotion. The ear was then sanitized with povidone-iodide or 70% ethanol and a small incision was made through the dorsal cutaneous layer with a scalpel. A pocket was then formed by gently separating the dermal layers of the ear pinna using a non-serrated specimen forceps. Next, 10^3 CFUs of *S. aureus* in 2 μL PBS was inoculated directly into the ear pocket. In some cases, the surgery and implant were kept sterile to monitor aseptic conditions. After insertion of the implant, the incision was closed using VetBond tissue adhesive (3M, Saint Paul, MN, USA).

Multiphoton Microscopy (MPM)

To assess both biofilm formation and innate immune cell activity, at least 3 sites directly on and adjacent to the biofilm/implant were imaged in each mouse. Time-lapse Z-stack images were acquired every 1–2 min over a 20–40 min span with 1 μM slices (times varied due to section thickness) using a 40× water immersion lens. MPM was carried out at the UC Riverside Center for Intravital Imaging, equipped with a Nikon A1R Multiphoton Plus (MP+; Nikon, Tokyo, Japan) microscope, including an auto-aligned tunable (700–1,080 nm) infrared laser (Coherent, Santa Clara, CA, USA), resonant and galvano scanners, 3 Gallium-arsenide-phosphide (GaAsP) non-descanned detectors (NDD), and 1 high sensitivity NDD for IR detection. The Nikon A1R MP+ is housed in a procedure room within the specific pathogen-free rodent vivarium and was fitted for BSL-2 usage. Still images and movies were collected, analyzed, and prepared for publication using Nikon NIS-Elements software.

Cell Tracking and Quantification

Neutrophil migration patterns were quantified using Volocity software (Quorum Technologies, Guelph, Ontario, Canada). Automated tracking algorithms to follow cells was confirmed using manual tracking by marking each individual object at each timepoint. The measurements of each neutrophil at each timepoint was taken relative to the centroid of the region of interest and were collectively gathered to form one measurement. Several parameters to define cell behavior were assessed, including velocity, displacement, and meandering index. Velocity represents the average speed of the neutrophil over the track. Displacement represents the average straight line distance from the first timepoint centroid to the last. Meandering index measures deviation from a straight line, with values from 0 to 1.

Post-infection Analyses

After 9 days, animals were sacrificed, and the infected ear was collected for bacterial burden enumeration associated with the implant and surrounding soft tissue. Briefly, the ear was removed, rinsed with 70% ethanol, and the excised implant placed in 100 μL PBS for sonication to dissociate bacteria from the implant surface. The ear was then weighed, cut into smaller pieces, then dissociated in 500 μL of PBS using the blunt end of a 3 mL syringe plunger. Tissue and implant bacterial titers were quantified on TSA and expressed as CFU per mL for implants and CFU per gram for tissue.

Statistical Analyses

Significant differences between experimental groups were determined as described in the respective figure legends. GraphPad Prism 8 (GraphPad, San Diego, CA, USA) was used for all statistical analysis calculations, and a $P < 0.05$ was considered statistically significant. Biofilm images are representative from 8 independent experiments. Neutrophil tracking data are from two independent experiments with statistical analyses performed using an unpaired, two-tailed *t*-test.

RESULTS

The mouse ear pinna is a proven site for assessing immune responses and infection progression (Li et al., 2012; Abtin et al., 2014; Forestier et al., 2017). Thus, we sought to establish an ear pinna interdermal implant model of *S. aureus* biofilm infection. Briefly, for the implant surgery and infection, the left ear of an anesthetized mouse was affixed to a petri dish and the dorsal side hair removed (**Figures 1A,B**). The ear was then sanitized, a small incision made through the outer cutaneous layer, and a small pocket formed by gently separating the dermal layers of the ear pinna (**Figures 1C,D**). Next, 10^3 CFU of *S. aureus* LAC-13C harboring pCM29 was pipetted directly into the interdermal

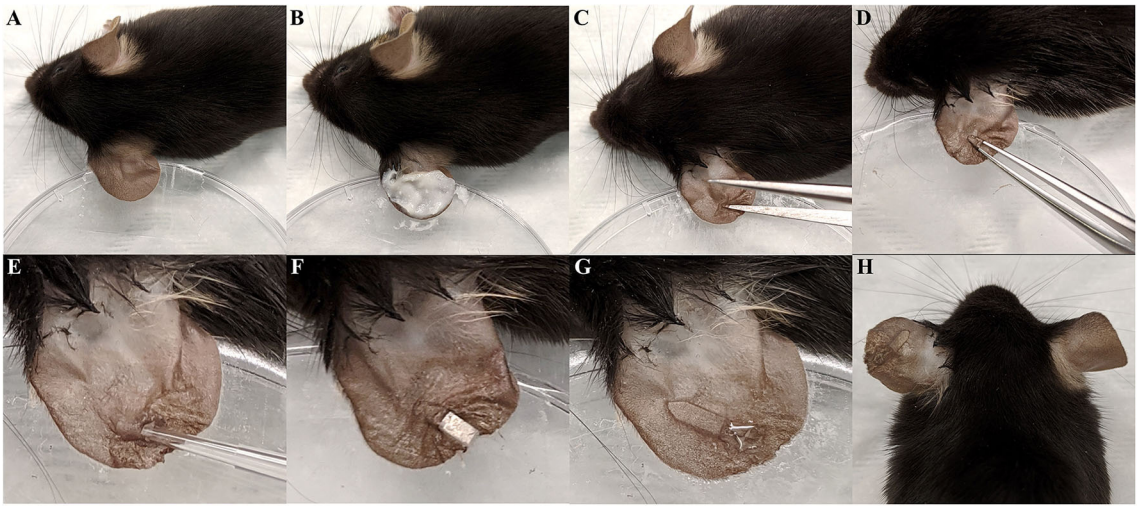

FIGURE 1 | Interdermal implant -associated biofilm infection model surgery. **(A)** The left ear pinna of an anesthetized mouse was affixed to a petri dished using double-sided tape. **(B)** Dorsal side hair was removed from the ear using hair removal cream. **(C,D)** A small incision was made in the dorsal side dermis and widened into a pocket using forceps. **(E)** 10^3 CFU of *S. aureus* in 2 μL PBS was then inoculated into the pinna pocket. **(F)** The 0.25 mm thin slice of silicone elastomer was slid into the pocket. **(G)** The incision was closed with tissue adhesive. **(H)** Final appearance of the ear following surgery and infection.

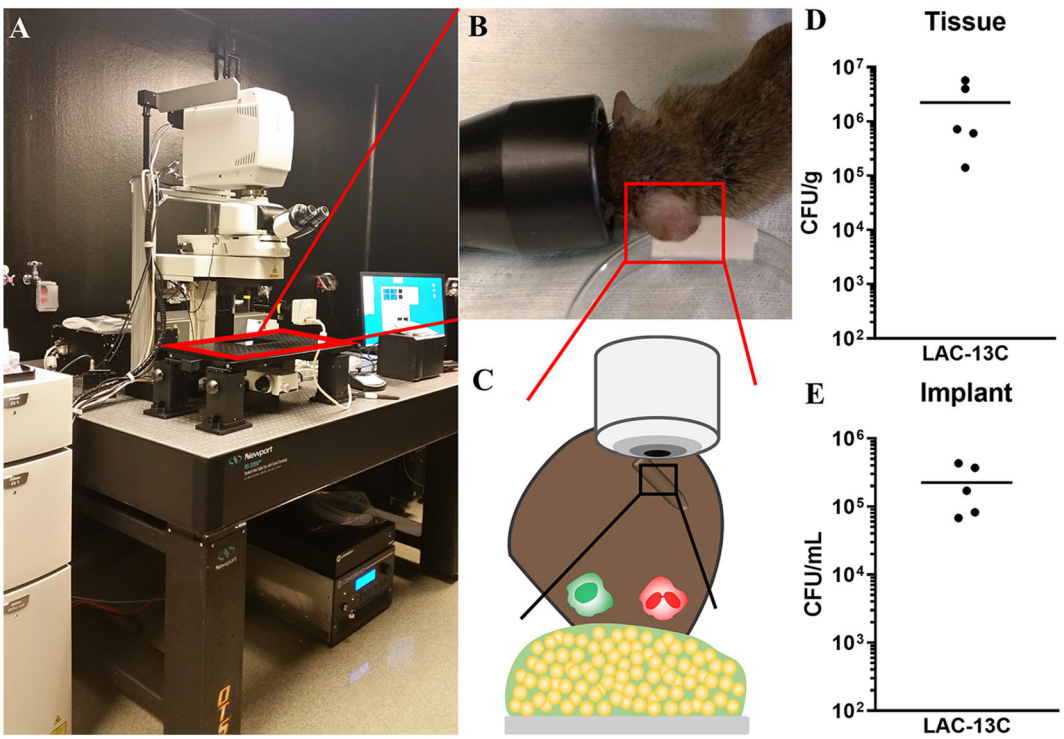

FIGURE 2 | Multiphoton microscopy illustration and post-infection analysis of implant-associated *S. aureus* biofilm infection. **(A–C)** MPM arrangement with a cartoon illustration of biofilm infection visualization. **(D,E)** Day 9 post-infection analysis of bacterial burdens associated with surrounding ear tissue **(D)** and the silicone implant **(E)**. Data are from two independent experiments with the horizontal line representing the mean.

pocket (**Figure 1E**). Finally, a section of translucent medical grade silicone was then inserted into the ear pinna pocket and the incision closed using tissue adhesive (**Figures 1F–H**).

Murine device-associated *S. aureus* biofilm infections typically reach maximum bacterial burdens 3 days post-infection, with *bona fide* biofilms formed by day 7 (Thurlow et al., 2011; Heim

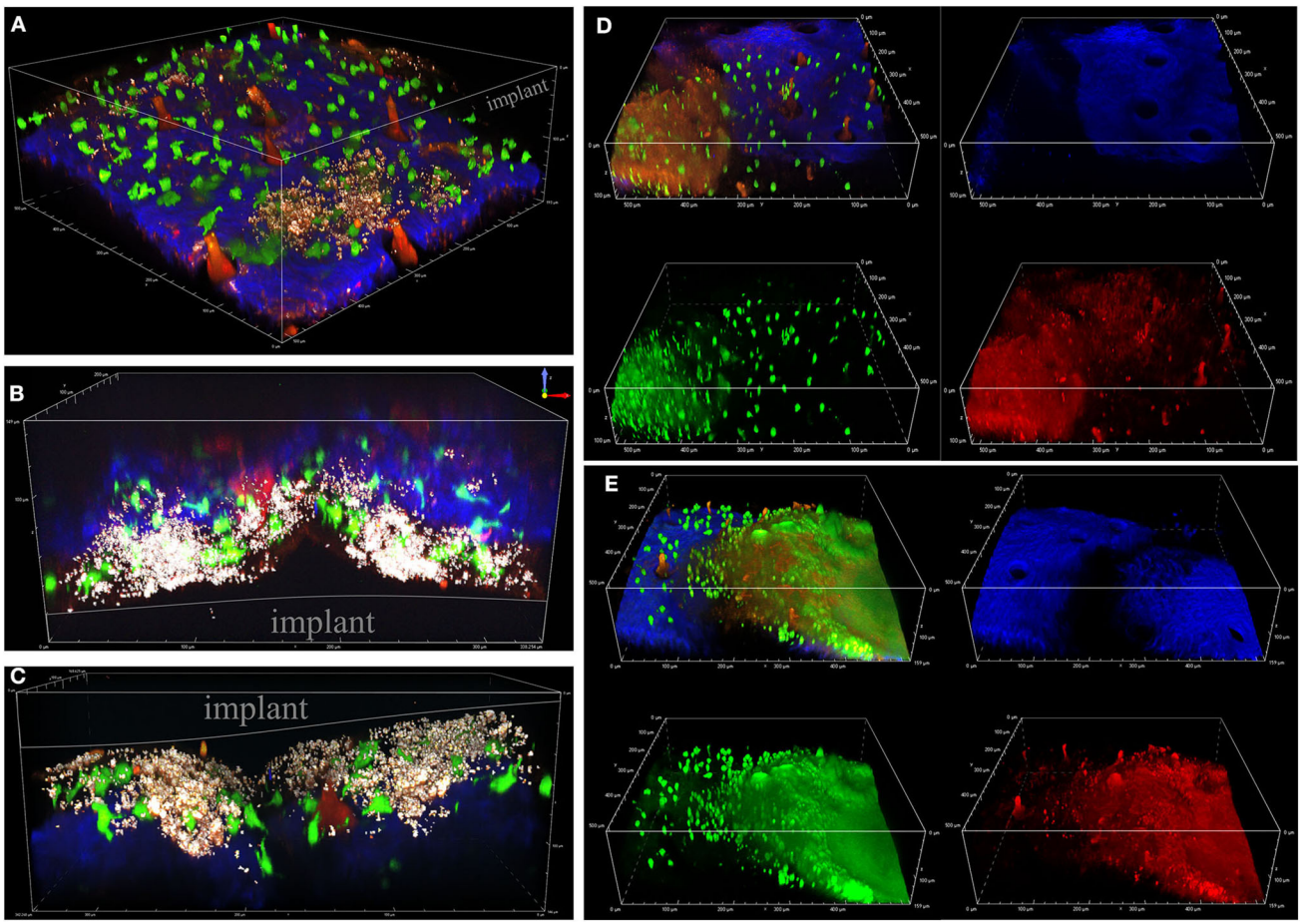

FIGURE 3 | Visualizing *S. aureus* biofilm infection and immune cell interactions. Intravital MPM of a CX3CR1-EGFP reporter mouse infected with *S. aureus*, shown in bright white. EGFP^{+} macrophages/dendritic cells are shown in green, and second-harmonic generated connective tissue (blue) and vasculature (red). Bacterial cells are depicted in red-white as superfolder-GFP expression is detectable in all channels. Representative images depict biofilms at 3 **(A)**, 5 **(B,C)**, 7 **(D)**, and 9 **(E)** days post-infection, taken from 8 independent experiments.

et al., 2015; Yamada et al., 2018). Thus, intravital MPM imaging took place daily starting 2 days and ending 9 days post-infection (**Figures 2A–C**). Importantly, this model and time frame permits the establishment of a robust infection associated with both the implant and surrounding tissue. As shown in **Figures 2D,E**, tissue and implant-associated bacterial burdens after 9 days were comparable to those observed in other *S. aureus* biofilm infection models (Yamada et al., 2018; Gries et al., 2020).

To visualize *S. aureus* biofilm development and associated innate immune activity *in vivo*, we utilized a CX3CR1-EGFP transgenic mouse where EGFP is expressed in monocytes and dendritic cells (Jung et al., 2000). Representative images of *S. aureus* biofilm development over 9 days post-infection are shown in **Figure 3**. *S. aureus* cells are shown in red-white as the bright superfolder GFP emitted fluorescence in both the FITC and TRITC channels. The early stages of infection (days 3 and 5) depict a loose accumulation of individually visible *S. aureus* cells associated with the implant (**Figures 3A–C**). Later stages of infection (days 7 and 9) revealed a larger mass of cells encased in a hazy matrix that largely prevented the visualization of individual bacterial cells (**Figures 3D,E**). While the composition of this extracellular matrix remains to be determined, it is possible that this is the result of mature biofilm matrix development.

To measure neutrophil migration patterns in response to sterile vs. biofilm infected implants, we utilized a PGRP-S-DsRed transgenic reporter mouse. The tracking paths of neutrophils associated with sterile and infected tissues on day 3 post-surgery are show in **Figures 4A,B**. Importantly, neutrophils near but not in direct contact with individual bacterial cells or the biofilm were assessed. Compared to neutrophils associated with a sterile implant, those near the infected implant showed significantly increased cell migration velocity and displacement (**Figures 4C,D**). Interestingly, these patterns were also associated with a significantly increased meandering index, or what appeared to be rapid but aimless neutrophil migration (**Figure 4E**). Together, these data shed

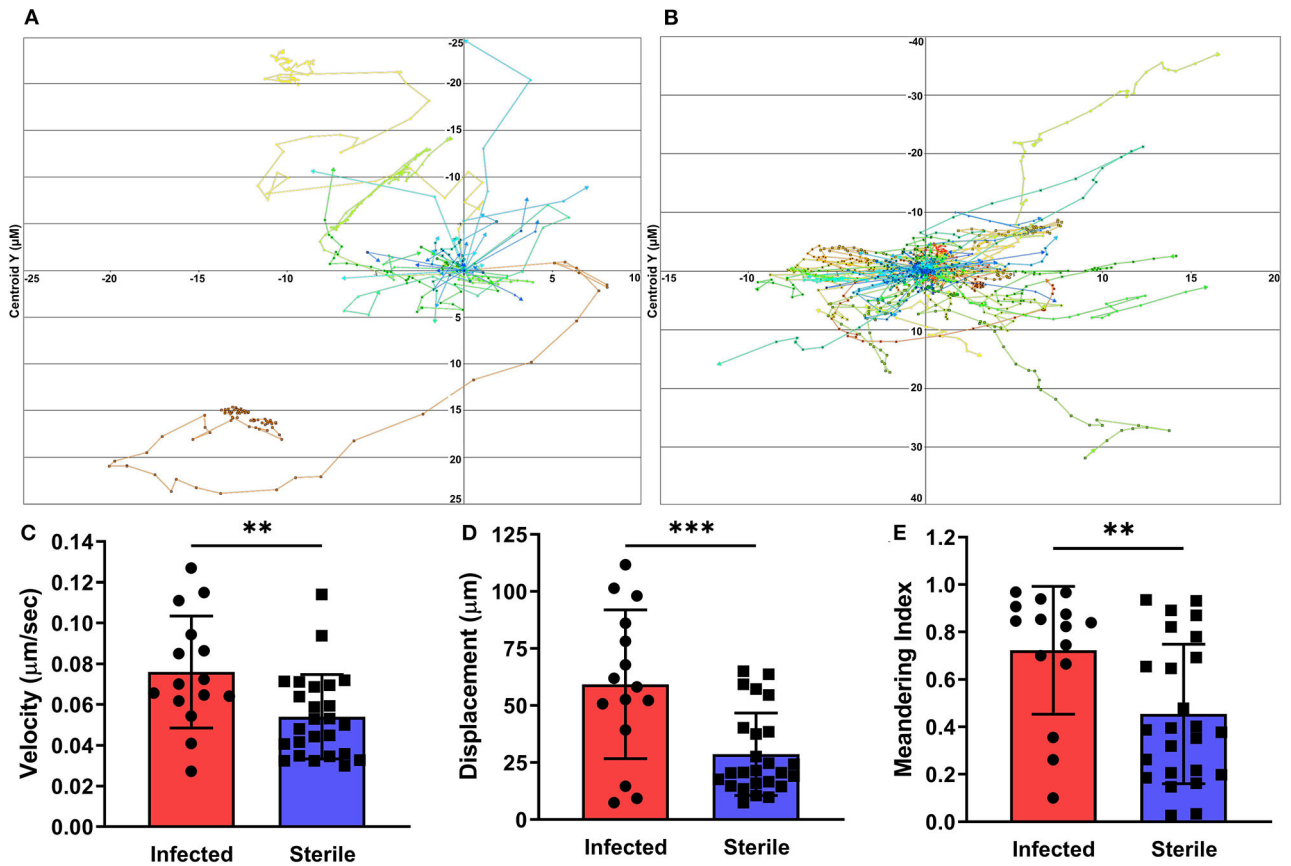

FIGURE 4 | Neutrophil tracking in infected and sterile tissues. Cells immediately adjacent to *S. aureus* infected **(A)** or sterile **(B)** implants were tracked over the given experimental time span with each data point representing a separate time point. Quantification of neutrophil velocity **(C)**, displacement **(D)**, and meandering index **(E)**, from infected and sterile images, normalized to experimental duration and time between data points. Data are from two independent experiments and presented as mean ± standard deviation. Statistical analyses were performed using an unpaired, two-tailed *t*-test; $^{**}P < 0.01$, $^{***}P < 0.001$.

light on the development of *S. aureus* biofilm during device-associated infection and the effects of the biofilm infection on neutrophil activity.

DISCUSSION

Staphylococcus sp. biofilms are the most common cause of medical device-associated infections (Arciola et al., 2018). These infections can arise in otherwise healthy individuals and often lead to chronic, reoccurring infections. Productive innate immune responses are inhibited by *S. aureus* biofilm products, including polarizing infiltrating macrophages and recruitment of myeloid-derived suppressor cells (Scherr et al., 2015; Gries and Kielian, 2017). Moreover, the three-dimensional biofilm architecture itself presents a barrier to immune cell infiltration and phagocytosis *in vitro* (Thurlow et al., 2011; Gries et al., 2020). However, relatively little is known of the host-pathogen interface during *S. aureus* biofilm-mediated infection. In this work we demonstrate the capability of multiphoton microscopy (MPM) to assess bacterial biofilm development and measure associated neutrophil migratory activity. To date, this is the first report using MPM to assess device-associated bacterial biofilm-mediated infection and concomitant innate immune responses.

The mouse ear pinna presents an ideal tissue to examine biofilm development and is a proven site to analyze innate immune activity using MPM (Li et al., 2012). Relative to the mouse body, the ear is sparse in auto-fluorescent hair coverage which can be easily removed prior to surgery or imaging. Furthermore, the implant/infection surgery causes minimal tissue damage and requires few specialized tools. Finally, MPM of the ear pinna is non-invasive and permits extended time-lapse imaging over multiple days using the same animal. In our model, the CX3CR1-EGFP and PGRP-S-DsRed transgenic animals expedite the orientation and focus within the pinna tissue. The *S. aureus* superfolder GFP used in these studies is highly fluorescent and easily detectable in both the FITC and TRITC channels. While this may present some future challenges, it allows easy detection of bacterial cells in the context of second harmonics and mouse fluorescent reporter expression. Notably,

we did not measure any significant loss of the GFP plasmid over the 9-day period (data not shown).

Using MPM, we sought to examine the developmental stages of *S. aureus* biofilm formation *in vivo* and simultaneously examine innate immune cell responses. This is a necessary step as multiple *in vitro* models of biofilm/immune cell co-culture have been employed, but their accuracy to biofilm infection has not been well-established. While many device-associated infections are multispecies, we limited this study to assess *S. aureus* biofilms and their impact on innate immune cell function. We showed that *S. aureus* device-associated infections follow a developmental progression and time frame analogous to that observed *in vitro* using minimal media such as RPMI (Thurlow et al., 2011; Gries et al., 2016). Initially, single *S. aureus* cells are easily observable and closely associated with the implant. By day 5, the number of visible bacterial cells was markedly increased. It remains unclear why maximum bacterial burdens are typically seen by day 3 post-infection, yet a considerable difference exists in the number of bacteria observed between days 3 and 5. A possible explanation is that bacterial viability was substantially decreased between these time points, however further examination of viability would be required to delineate this observation.

Mature *S. aureus* biofilms are characterized as having an extensive extracellular matrix composed of various proteins, sugars, and extracellular DNA (Foulston et al., 2014; Lister and Horswill, 2014). It was therefore not surprising to find that after 7 days, an extensive, hazy covering of the bacterial cells appeared. The matrix would also likely consist of a significant portion of GFP molecules, potentially providing its robust fluorescent appearance. An interesting observation throughout the time course is the overall lack of phagocytosis by monocytes, dendritic cells, and neutrophils. While this is in agreement with *in vitro* findings (Thurlow et al., 2011; Gries et al., 2020), a paucity of phagocytosis was observed beginning on day 3 post-infection, before the robust biofilm structure was formed. Further investigation will be required to assess the role of *S. aureus* biofilm molecules known to affect macrophage and neutrophil phagocytic function (Scherr et al., 2015; Gries et al., 2016; Bhattacharya et al., 2018).

Neutrophils and macrophages are essential cellular components of the innate immune response to bacterial biofilm infection. Their presence also markedly alters *S. aureus* biofilm gene expression *in vitro* (Scherr et al., 2013). Neutrophils are the first line of cellular immune defense against invading bacterial pathogens; their coordinated recruitment and activity are essential to preventing and eliminating infection. Compared to macrophages, neutrophils have shown a much higher propensity for biofilm invasion and phagocytosis *in vitro* (Gunther et al., 2009; Thurlow et al., 2011; Ghimire et al., 2019). We observed significant differences in the cell migration behaviors of neutrophils in response to infected versus sterile implants. Despite neutrophils appearing to respond to biofilm infection by increasing migration velocity and displacement, their movements were less directed toward the implant/infection than neutrophils in sterile tissues. These observation are consistent with a recent report that a delay in neutrophil recruitment to the implant surface may permit *S. aureus* time to grow and form biofilm (Ghimire et al., 2019). We anticipate that neutrophil deviation is a result of *S. aureus* biofilm products interfering with neutrophil cytokine/chemokine signaling and the production of multiple leukocyte inhibitors and toxins (Rooijakkers et al., 2006). The mechanism(s) responsible for neutrophil redirection away from *S. aureus* biofilm are subject of future investigations. Together, these observations further establish the ineffective nature of neutrophil responses to *S. aureus* biofilm-mediated infection.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

CG and DL conceived and designed the experiments. CG, ZR, and JC performed the experiments. CG, ZR, JC, and DL analyzed data. CG wrote the paper. All authors contributed to the article and approved the submitted version.

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Neutrophils From Patients With Invasive Candidiasis Are Inhibited by *Candida albicans* Biofilms

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Invasive candidiasis frequently involves medical device placement. On the surfaces of these devices, *Candida* can form biofilms and proliferate in adherent layers of fungal cells surrounded by a protective extracellular matrix. Due in part to this extracellular matrix, biofilms resist host defenses and antifungal drugs. Previous work (using neutrophils from healthy donors) found that one mechanism employed to resist host defenses involves the inhibition of neutrophil extracellular traps (NET) formation. NETs contain nuclear DNA, as well as antimicrobial proteins that can ensnare pathogens too large or aggregated to be effectively killed by phagocytosis. Given that these neutrophil structures are anticipated to have activity against the large aggregates of *C. albicans* biofilms, understanding the role of this inhibition in patients could provide insight into new treatment strategies. However, prior work has not included patients. Here, we examine NET formation by neutrophils collected from patients with invasive candidiasis. When compared to neutrophils from healthy participants, we show that patient neutrophils exhibit a heightened background level of NET release and respond to a positive stimulus by producing 100% more NETs. However, despite these physiologic differences, patient neutrophil responses to *C. albicans* were similar to healthy neutrophils. For both groups, planktonic cells induce strong NET release and biofilms inhibit NET formation. These results show that a mechanism of immune evasion for fungal biofilms translates to the clinical setting.

Keywords: *Candida*, biofilm, neutrophil, neutrophil extracellular trap, patients, invasive candidiasis, reactive oxygen species

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INTRODUCTION

Candida albicans, a widespread nosocomial fungal pathogen, is an avid biofilm-former and a frequent cause of invasive fungal infection (1). On the surface of medical devices, such as vascular catheters, *Candida* spp. adopt a biofilm lifestyle and grow as adherent communities encased in an extracellular matrix with protective properties (2). *Candida* biofilms infections are notoriously difficult to treat, as they resist high levels of antifungal drugs and withstand host defenses (3–10). Despite advancements in antifungal therapies and diagnostics, the mortality for *Candida*-associated bloodstream infection remains exceedingly high, near 30% (1, 11).

Neutrophils are essential for eradicating many fungal infections, including invasive candidiasis (12–15). Neutropenic patients are at high risk for critical disease, and those remaining neutropenic are more likely to relapse (16). However, in the clinical setting, neutrophils are ineffective at controlling *Candida* device-associated biofilm infections. Eradication of infection most often requires device removal, even for patients with normal numbers of neutrophils (11). Moreover, mortality rates increase for patients when biofilm-infected devices are retained (1, 11).

Ex vivo, *C. albicans* biofilms are approximately 5-fold more resistant to killing by neutrophils when compared to non-biofilm, planktonic cells (7, 8, 17, 18). When no biofilm is present, neutrophils phagocytose the smaller yeast form of *C. albicans* and release neutrophil extracellular traps (NETs) in response to the larger, elongated hyphal form (19, 20). NETs consist of extracellular web-like structures of DNA associated with citrullinated histones and other antimicrobial proteins that contain and kill pathogens. These structures exhibit antifungal activity against both yeast and hyphae (19, 21, 22). NETs are anticipated to be crucial for controlling *Candida* infections because they can target hyphae, which are too large to be phagocytosed (20). Given their role in combatting hyphae and their activity against aggregative pathogens, the formation of NETs would seem to be an ideal response against *Candida* biofilm. However, neutrophils (collected from healthy study participants) fail to release NETs in response to *Candida* biofilms (18, 23, 24). This impaired innate immune response likely contributes to the resilient nature of device-associated *Candida* infections.

While our understanding neutrophil-*Candida* biofilm interactions has advanced significantly using healthy participant neutrophils, studies have not delineated the relevance of these interactions for patients with invasive candidiasis. We considered that infection with *Candida* may influence neutrophil responses. The conventional concept poses that neutrophils are uniformly short-lived, terminally differentiated cells. However, recent investigations reveal phenomena of significant neutrophil plasticity and heterogeneity in a variety of clinical settings (25–27). Here we compare neutrophil responses between healthy participants and those with invasive candidiasis, focusing on NET formation.

METHODS

Organisms and Inoculum

C. albicans (SC5314) was stored in 15% glycerol stock (vol/vol) at -80°C and plated on yeast extract-peptone-dextrose agar (YPD-agar) plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) prior to experiments. Cultures were grown overnight in YPD media (1% yeast extract, 2% peptone, and 2% dextrose) at 200 RPM at 30°C in an orbital shaker. For experiments using biofilms, *C. albicans* was resuspended in RPMI-MOPS at a concentration of 1.5×10^6 cells/ml and added at volumes specified in individual assays, followed by a 24 h incubation at 37°C. For experiments with planktonic cells, 1 ml of an overnight culture was added to 10 ml fresh YPD broth for 2 h at 180 RPM at 30°C. Following the incubation, the planktonic *C. albicans* cells were washed twice with phosphate-buffered saline (-calcium and -magnesium) DPBS (Hyclone Laboratories Inc., Logan, UT) and counted with a hemocytometer. For Sytox Green and reactive oxygen species (ROS) production assays, a burden of planktonic cells (3×10^6 cells/well) equivalent to biofilm burden was established, as described previously (18). For other experiments, planktonic cells were added at concentrations described below.

Study Participants

We obtained blood samples from patients with invasive candidiasis, through protocol 2017-0685 approved by the University of Wisconsin Internal Review Board (IRB). Patients were eligible upon identification of *Candida* growing as a culture from a normally sterile site, in accordance with clinical diagnosis of invasive candidiasis (16). All patients were 18–89 and admitted for hospitalization at the University of Wisconsin Hospital. We collected blood samples following written informed consent. Patient characteristics are presented in **Table 1**. Blood from healthy participants was collected through protocol 2013-1758 approved by the University of Wisconsin IRB. Healthy participants were 18 years of age or older and the group included 9 males and 10 females.

Neutrophil Isolation

We isolated primary human neutrophils from whole blood by negative antibody selection using the MACSxpress Neutrophil Isolation and MACSxpress Erythrocyte Depletion kit (Miltenyi Biotec Inc., Auburn, CA). Experiments with isolated neutrophils were performed in RPMI 1640 media (without phenol red) supplemented with 2% heat-inactivated fetal bovine serum (FBS) and glutamine (0.3 mg/ml). All incubations were at 37°C with 5% CO_2. We collected neutrophils from up to 12 ml of blood, which resulted in 1.0×10^7 to 6.1×10^7 neutrophils per patient.

Sytox Green Assays

For a quantitative measure of NET formation, we used Sytox Green assays, as described previously (18, 23). To measure NET release in the presence of biofilm, we grew *C. albicans* as a biofilm for 24 h in 96-well opaque plates. To measure NET formation in the presence of planktonic cells, we added 3×10^6 planktonic cells/well prior to the addition of neutrophils. For a subset of experiments, phorbol myristate acetate (PMA) was added at a final concentration of 100 nM. Neutrophils were added at a final concentration of 2×10^5 neutrophils/well. After a 4 h incubation, the cell-impermeable free DNA fluorescent dye Sytox Green (Life Technologies, Eugene, OR) was added and read at excitation 500 nm/emission 528 nm, as previously described (18, 28). In experiments with *C. albicans*, fluorescence for *C. albicans* biofilms or planktonic cells alone was analyzed and subtracted as background fluorescence.

TABLE 1 | Characteristics of patients with invasive candidiasis.

Study ID	Age (yr) M/F	Admitting diagnosis	Major comorbidities	Patient location	Antifungal therapy at enrollment	Immune-suppressing therapy prior to enrollment	Central line	*Candida* sp(p). culture source
A	44 M	Catheter-associated bloodstream infection	Renal transplant recipient, short gut syndrome requiring total parenteral nutrition, hypertension	Medical ward	MFG	Tacrolimus Prednisone Mycophenolate mofetil	Yes	*C. glabrata*: Blood Catheter tip
B	61 M	Cholangitis with sepsis	Malignant neoplasm of head and neck, emphysema, recurrent bacteremia, choledocholithiasis	Medical ward	MFG	None	Yes	*C. krusei*: Bile, Biliary drain tip *C. albicans*: Biliary drain tip
C	80 F	Perforated sigmoid colon with abscess formation	End-stage renal disease, chronic pancreatitis, hypertension, left hemicolectomy with colostomy	Surgical intermediate care	MFG	None	No	*C. glabrata*: Abdominal fluid
D	68 M	Cholangitis, empyema	Renal transplant recipient, liver transplant recipient, diabetes mellitus	Surgical ward	FLC	Cyclosporine Mycophenolate sodium	No	*C. albicans*: Bile Pleural fluid
E	55 F	Small bowel obstruction, spinal osteomyelitis	Non-Hodgkin's lymphoma in remission, congestive heart failure, diabetes mellitus	Surgical ward	None	None	Yes	*C. glabrata*: Abdominal fluid
F	51 M	Intra-abdominal abscess	Colon cancer, hydronephrosis, severe thrombocytopenia, diverticulitis with perforation	Surgical ward	FLC	None	Yes	*C. dubliniensis*: Abdominal abscess
G	72 M	Pyelonephritis	Acute kidney injury, hypertension, history of renal cell carcinoma	Surgical ward	FLC	None	No	*C. glabrata*: Renal pelvis Urine
H	67 F	Obstructing pyelonephritis, sepsis, demand myocardial ischemia	Hypertension, hypothyroidism, acute kidney injury, acute pulmonary edema, acute heart failure, rheumatoid arthritis	Medical ward	FLC MFG	Adalimumab	No	*C. albicans*: Blood Urine *C. glabrata*: Blood
I	22 F	Port site infection	Cystic fibrosis, diabetes mellitus, allergic bronchopulmonary aspergillosis	Critical care ward	MFG	None	Yes	*C. dubliniensis:* Port site
J	78 F	Liver abscess	Cholangiocarcinoma, hypertension, dyslipidemia	Medical ward	MFG	None	No	*C. albicans*: Liver abscess

ROS Production Assays

To measure ROS production, we used an oxidative stress assay modified for biofilm (18, 23). Planktonic cells or biofilm were added to 96-well opaque plates, as described above. Neutrophils were stained at room temperature with fluorescent dye CM-H2DCFDA (Life Technologies, Eugene, OR) for 10 min in DPBS prior to addition to a 96-well opaque plate at a final concentration of 2×10^5 neutrophils/well. For some experiments, PMA was added at a final concentration of 100 nM. Fluorescence was recorded prior to incubation at 37°C and at every 30 min for 4 h (excitation, 495 nm; emission, 527 nm). In experiments with *C. albicans*, fluorescence for *C. albicans* biofilms or planktonic cells alone was recorded and subtracted as background fluorescence.

Scanning Electron Microscopy

For electron microscopy experiments, we utilized poly-L-lysine-treated plastic coverslips (13 mm, Thermonax plastic for cell culture). Neutrophil (5×10^5) were added to coverslips containing *C. albicans* biofilm, planktonic cells, or PMA. For biofilm experiments, biofilms were grown on coverslips, as previously described (18, 23). Briefly, 1.5×10^6 cells/ml *C. albicans* were applied to coverslips at 30°C for 30 min. After removing non-adherent cells, fresh RPMI-MOPS was added and biofilms were grown for 24 h at 37°C and then washed with DPBS. For planktonic interactions, planktonic cells were added to coverslips (1.5×10^7 cells/coverslip) for 1 h prior to the addition of neutrophils. For a subset of experiments, PMA was included at a final concentration of 100 nM. Following a 4 h incubation, coverslips were processed for scanning electron microscopy, as described previously (18, 23). Briefly, samples were washed in DPBS and fixed overnight in a 4% formaldehyde and 1% glutaraldehyde solution in DPBS. Following fixation, samples were treated with 1% osmium tetroxide for 1 h, and then washed with DPBS. The samples then underwent ethanol dehydration, which was immediately followed with critical point drying. Samples were then placed on aluminum stubs and sputter-coated with 10 nm platinum. The samples were imaged at 3 kV by a LEO 1530 scanning electron microscope.

Immunofluorescence Microscopy

We utilized immunofluorescence microscopy to visualize the location of neutrophil elastase. Neutrophils were analyzed in 8-well Ibidi devices. Briefly, neutrophils (1×10^5/well) were added to wells containing biofilms, planktonic cells, or PMA (100 nM). To examine neutrophil-biofilm interactions, we seeded 100 µl of *C. albicans* in RPMI-MOPS at 1.5×10^6 cells/ml and incubated for 24 h on a 45° incline to propagate biofilm. After 6 h, 50 µl of

RPMI-MOPS was gently added to prevent biofilm growth at the air interface. Following 24 h of growth, biofilms were removed from incline and gently rinsed with DPBS prior adding neutrophils. For planktonic studies, we added 4×10^5 planktonic *C. albicans* cells per device. Following a 4 h incubation, samples were fixed for 2 h in 1% formaldehyde in DPBS. Samples were then washed with DPBS and incubated with blocking buffer (2% w/v bovine serum albumin (BSA) and 0.02% v/v Tween 20 in DPBS) overnight at 4°C. Samples were exposed to anti-Neutrophil elastase rabbit pAb (MilliporeSigma, Burlington, MA) or anti-Histone 4 (citrulline 3) rabbit pAB (EMD Millipore, Temecula, CA) at a 1:200 dilution in binding buffer (0.1% BSA w/v and 0.005% v/v Tween 20 in DPBS) overnight at 4°C. Samples were washed 3× for 5 min in fresh binding buffer and then incubated with chicken anti-rabbit IgG, DyLight 594 conjugated secondary antibody (ImmunoReagents, Inc., Raleigh, NC) at a 1:500 dilution in binding buffer for 1 h at room temperature and washed 3× for 5 min in binding buffer prior to being imaged in DPBS. Images were obtained on a Nikon eclipse-TI2 inverted microscope equipped with a TI2-S-SS-E motorized stage, ORCA-Flash 4.0LT sCMOS camera, and NIS elements imaging software.

Statistics

Experiments were performed at least 4 times using neutrophils from different donors on different days. Statistical analyses were performed by ANOVA or Student's t-test using GraphPad Prism8 software. Differences of $p < 0.05$ were considered significant.

RESULTS

Neutrophils From Invasive Candidiasis Patients Exhibit a Heightened Capacity for NET Formation

To delineate neutrophil responses for patients with invasive candidiasis, we collected blood from hospitalized patients and performed ex vivo neutrophil studies (**Table 1**). We also included neutrophils from healthy participants as a comparison, as prior *Candida*-neutrophil work has primarily utilized neutrophils from this population (6–8, 17–20, 23, 29, 30). In the absence of a stimulus, neutrophils from both study groups appeared rounded and NETs were only rarely observed by scanning electron microscopy over the course of 4 h (**Figure 1A**). To quantify NET release, we utilized a Sytox Green assay to measure free DNA (**Figure 1B**). Consistent with the scanning electron microscopy images, unstimulated neutrophils released very little free DNA. Neutrophils from patients with invasive candidiasis trended toward a higher background level of NET release, but this did not reach statistical significance. We next examined neutrophil responses to PMA, a strong inducer of NETs. Strikingly, in response to PMA, neutrophils from patients with invasive candidiasis released nearly 100% more DNA than neutrophils from healthy participants (**Figure 1B**). Scanning electron imaging revealed the formation of NET structures for both groups of neutrophils exposed to PMA, with more frequent NETs in the patient neutrophil group (**Figure 1A**).

During NET formation, neutrophil elastase translocates from the granules to the nucleus, where it cleaves histones and ultimately releases extracellularly with DNA as part of the web-like structures (30, 31). Using immunohistochemistry, we analyzed the location of neutrophil elastase. As described previously for unstimulated neutrophils from healthy people, neutrophil elastase remained intracellular and appeared punctate (**Figure 1C**). This is consistent with prior work that has shown an intracellular, granular localization for neutrophil elastase in resting neutrophils (**Figure 1C**) (30). While unstimulated neutrophils from patients also displayed intracellular neutrophil elastase, in a subset of neutrophils, staining was more diffuse. This suggested that neutrophil elastase may be translocating to the cytosol or nucleus, as has been described for NET release, even in absence of a stimulus. In response to PMA, both neutrophil groups exhibited web-like structures of extracellular neutrophil elastase, consistent with NET formation (**Figure 1C**). The extensive formation of NETs limited a semi-quantitative comparison.

ROS triggers the release of neutrophil elastase into the cytosol (30). Because ROS production is a key step in PMA-induced NET release, we considered the possibility that activation of this pathway might differ between neutrophils from patients and healthy participants (32). We prelabeled neutrophils with an oxidative stress indicator and measured ROS production over the course of 4 h in the presence and absence of PMA (**Figure 1D** and **Supplementary Figure 1A**). In the absence of a stimulus, neutrophils from patients with invasive candidiasis generated more than 3-fold higher ROS levels compared to neutrophils from healthy people. In response to PMA, ROS production mirrored the pattern observed for NET formation, with neutrophils from invasive candidiasis patients generating twice the levels of ROS (**Figures 1B, C**).

Because of the heighten ROS production observed for patient neutrophils, we considered if they may exhibit higher ROS levels at baseline. We found neutrophils from invasive candidiasis patients to generate approximately 2-fold higher levels of ROS compared to healthy participant neutrophils (**Supplementary Figure 1B**). Considering the role of ROS in PMA-induced NET formation, this trait may contribute to the increased NET formation observed for patient neutrophils. To further explore NET formation pathways, we examined histone citrullination. This enzymatic modification of histone arginine residues to citrulline residues mediates chromatin decondensation and NET formation (33). By immunofluorescence, we did not detect histone citrullination in healthy participant neutrophils (**Supplementary Figure 1C**). However, we did observe histone citrullination for a small number of neutrophils from patients with invasive candidiasis, suggesting some cells may have activation of NET formation pathways in the absence of a stimulus. Taken together, the studies reveal that neutrophils from patients with invasive candidiasis display an increased capacity for ROS production and PMA-induced NET formation that differs from neutrophils collected from healthy participants.

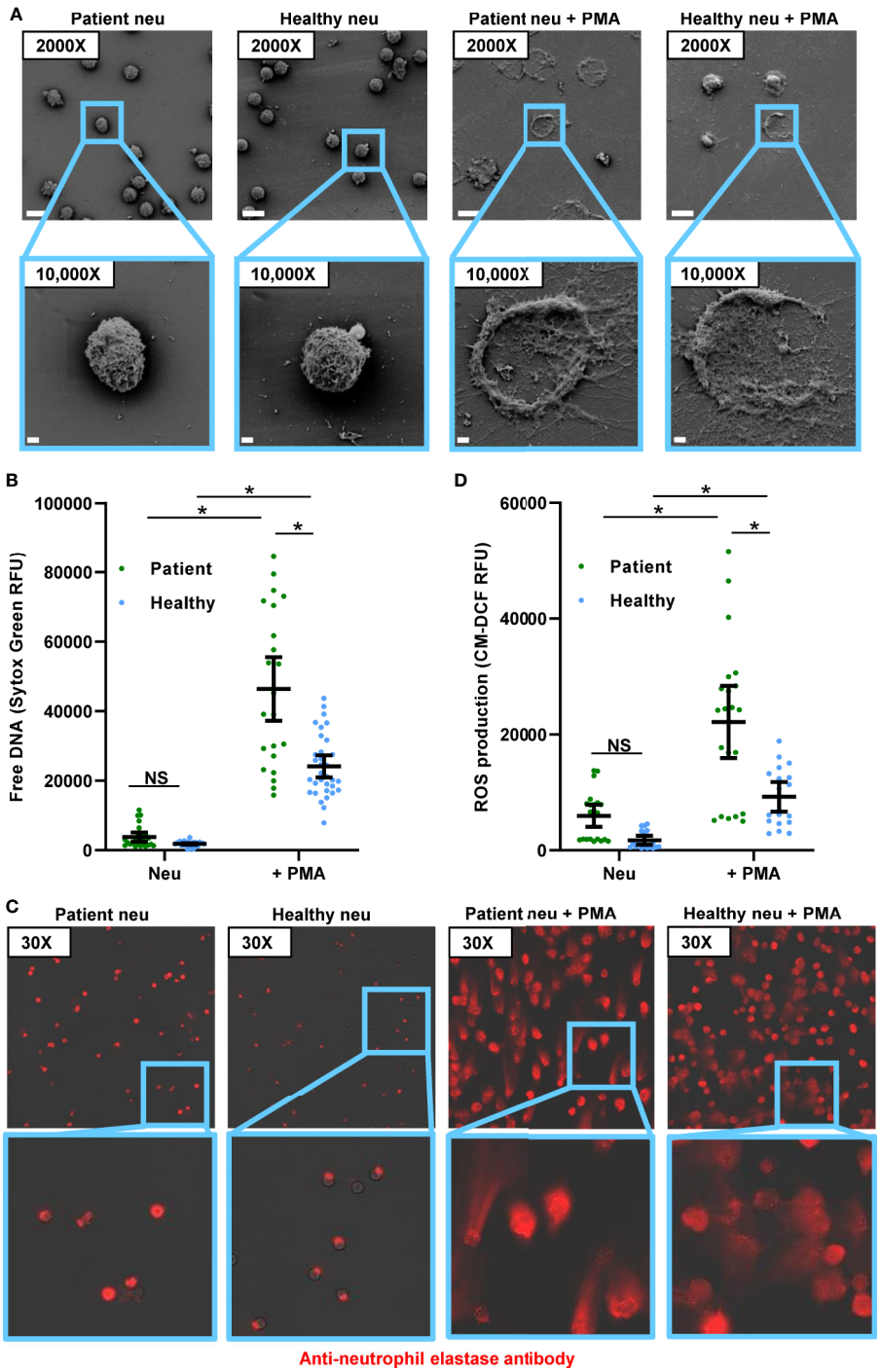

FIGURE 1 | Comparison of PMA-induced NET formation and ROS production by neutrophils from healthy participants and patients with invasive candidiasis. **(A)** Scanning electron microscopy of patient and healthy neutrophils in the absence and presence of the NET stimulus PMA for 4 h. Measurement bars represent 10 μm and 1 μm for images taken at 2000X and 10,000X, respectively. **(B)** Patient and healthy neutrophils were incubated with or without PMA for 4 h and NET release was estimated by free DNA measurement following staining with cell-impermeable Sytox Green. **(C)** Neutrophils were incubated with or without PMA for 4 h, fixed, and stained with anti-neutrophil elastase rabbit primary antibody and chicken anti-rabbit IgG, DyLight 594 conjugated secondary antibody. **(D)** To measure ROS, neutrophils were pre-incubated with free radical sensor CM-H2DCFDA prior to incubation with or without PMA for 4 h, and fluorescence was measured. Statistical significance was analyzed by two-way ANOVA with Sidak's multiple comparisons test, $*p < 0.05$, NS, not significant, mean with 95% confidence interval shown.

Patient and Healthy Participant Neutrophils Respond Similarly to *C. albicans*

We next questioned how neutrophils from invasive candidiasis patients respond to *C. albicans* and if these interactions differ from those that have been observed for healthy people. As previously reported, healthy participant neutrophils form NETs upon encounter with planktonic *C. albicans* (19, 20). Consistent with this, scanning electron microscopy imaging revealed healthy participant neutrophils forming web-like structures coating planktonic cells (**Figure 2A**), and the free DNA of NETs was detected at levels similar to PMA induction (**Figures 2B** and **1B**). Neutrophils from invasive candidiasis patients formed an equivalent amount of NETs upon encounter with planktonic *C. albicans* (**Figures 2A, B**). This was somewhat surprising in light of the heightened capacity of patient neutrophils for PMA-induced NET formation (**Figure 1B**). For both neutrophil groups, immunofluorescence imaging revealed strands of extracellular neutrophil elastase indicating the presence of NETs in response to planktonic *C. albicans* (**Figure 2C**). Upon examination of NET pathways following exposure to planktonic cells, we found that invasive candidiasis patient neutrophils generated 3-fold more ROS (**Figure 2D** and **Supplementary Figure 2A**). The finding that this elevation of ROS did not translate to increased NET formation by patient neutrophils is consistent with the involvement of ROS-independent pathways, as has been described for *C. albicans* (29, 31).

Upon co-culture with *C. albicans* biofilms, both groups of neutrophils failed to form NETs (**Figures 2A, B**). By scanning electron microscopy, neutrophils remained intact without the extrusion of extracellular structures (**Figure 2A**). Although neutrophils from invasive candidiasis patients released slightly more free DNA than neutrophils from healthy people, this was not above the background detected in the neutrophil only control (**Figure 2B**). Neutrophil elastase remained primarily intracellular for both neutrophil groups (**Figure 2C**). The staining appeared slightly more diffuse for the invasive candidiasis patient neutrophils, suggesting early translocation of neutrophil elastase from the granules, similar to the appearance of these neutrophils without a stimulus present (**Figure 1C**). Similarly, biofilms did not induce ROS above the background for either neutrophil control group (**Figure 2D**). Thus, the lack of NET production in response to *Candida* biofilm is a clinically relevant phenotype that translates to patients.

C. albicans Biofilms Inhibit The Activity of Neutrophils From Patients With Invasive Candidiasis

Prior work with healthy participant neutrophils has shown that not only do *C. albicans* biofilms fail to induce NET formation, but also that NET release to other stimulants is suppressed by biofilm (18). Here, we wanted to determine if this phenotype translated to neutrophils from patients with invasive candidiasis. To test this, we measured NET formation by patient neutrophils in the presence of *C. albicans* biofilm and/or a stimulus for NET release (PMA). Consistent with our other experiments with patient neutrophils, PMA induced NET formation and biofilms did not (**Figure 3A**). When both biofilm and PMA were present, neutrophils failed to form NETs, indicating that biofilm suppresses PMA-induced NET formation. Similarly, biofilm formation inhibited PMA-induced ROS production (**Figure 3B** and **Supplementary Figure 2B**). These data support the finding that *C. albicans* biofilms suppress NET formation, even in the presence of a strong stimulant.

DISCUSSION

Invasive candidiasis is a widespread nosocomial infection with high mortality (1, 11). The vast majority of cases involve medical devices that become infected with *Candida* biofilms. While prior work has shown poor neutrophil responses to *Candida* biofilm, the role of these processes for patients with invasive candidiasis had not been elucidated (7, 8, 18, 23). Here, we characterize NET formation for patients with invasive candidiasis. With these patient neutrophils, we observe a heightened capacity for PMA-induced NET formation and activation of NET signalling pathways when compared to healthy participant neutrophils. Despite these different characteristics of patient neutrophils, we found that the neutrophils respond to *C. albicans* in a manner that mirrors healthy participant neutrophils. Both patient and healthy participant neutrophils formed NETs in response planktonic *C. albicans* but not upon encounter with *C. albicans* growing as a biofilm. These findings help explain why *Candida* biofilm infections are difficult to eradicate clinically. The studies also support the use of neutrophils from healthy people to model neutrophil responses during invasive candidiasis.

We were somewhat surprised to find that neutrophils from invasive candidiasis patients exhibited a heightened capacity to form NETs in response to PMA, but not to planktonic *C. albicans*. These differences suggest neutrophils from patients with invasive candidiasis might be primed for certain NET release pathways, but not others, depending on the stimuli (34). For example, PMA-induced NET release is ROS-dependent, whereas planktonic *C. albicans* can induce NETs through ROS-independent pathways (29, 31, 35, 36). We did observe a difference in ROS levels for patients and healthy participants, with patient neutrophils exhibiting elevated baseline ROS levels and higher ROS upon PMA treatment (**Figure 1D** and **Supplementary Figures 1A, B**). The current studies were not designed to determine the cause of the higher ROS production in patients. While this may be mainly due to candidiasis, our population included many patients with polymicrobial intra-abdominal infections (**Table 1**). Therefore, we are not able to exclude a contribution from bacterial infection. Furthermore, the studies did not include patients with serious bacterial infections or inflammatory conditions who did not have candidiasis. It is quite possible that neutrophils from these patient cohorts would also exhibit activation of NET pathways. Bacterial species, including Enterobacteriaceae and *Streptococcus* spp. have been shown to induce NETs through ROS-generating pathways (32, 37, 38). Furthermore, multiple factors may be influencing the neutrophil ROS production in these patients.

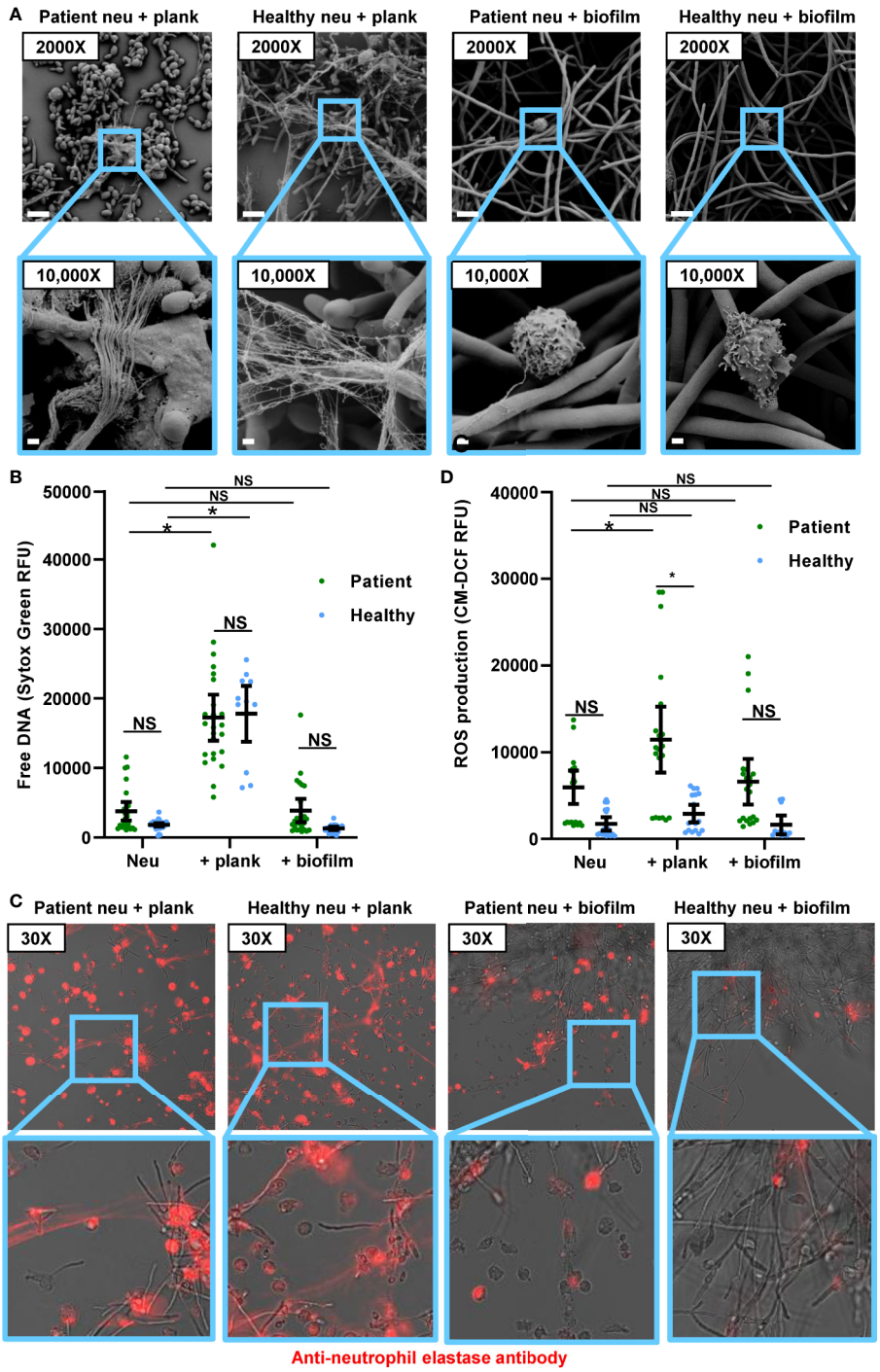

FIGURE 2 | Response to planktonic and biofilm *C. albicans* for neutrophils from healthy participants and patients with invasive candidiasis. **(A)** Scanning electron microscopy of patient and healthy neutrophils co-incubated with planktonic or biofilm *C. albicans* for 4 h. Measurement bars represent 10 μm and 1 μm for images taken at 2,000× and 10,000×, respectively. **(B)** Patient and healthy neutrophils were co-incubated with planktonic or biofilm *C. albicans* for 4 h, and NETs were estimated by measurement of free DNA using Sytox Green. **(C)** Patient and healthy neutrophils were incubated with *C. albicans* planktonic cells or biofilm for 4 h, fixed, and stained with anti-neutrophil elastase rabbit primary antibody and chicken anti-rabbit IgG, DyLight 594 conjugated secondary antibody. Following fixation, neutrophils were stained for neutrophil elastase. **(D)** To measure ROS, neutrophils were pre-incubated with free radical sensor CM-H2DCFDA prior to incubation with planktonic or biofilm *C. albicans* for 4 h, and fluorescence was measured. Statistical significance was analyzed by two-way ANOVA with Sidak's multiple comparisons test, $^*p < 0.05$, NS, not significant, mean with 95% confidence interval shown.

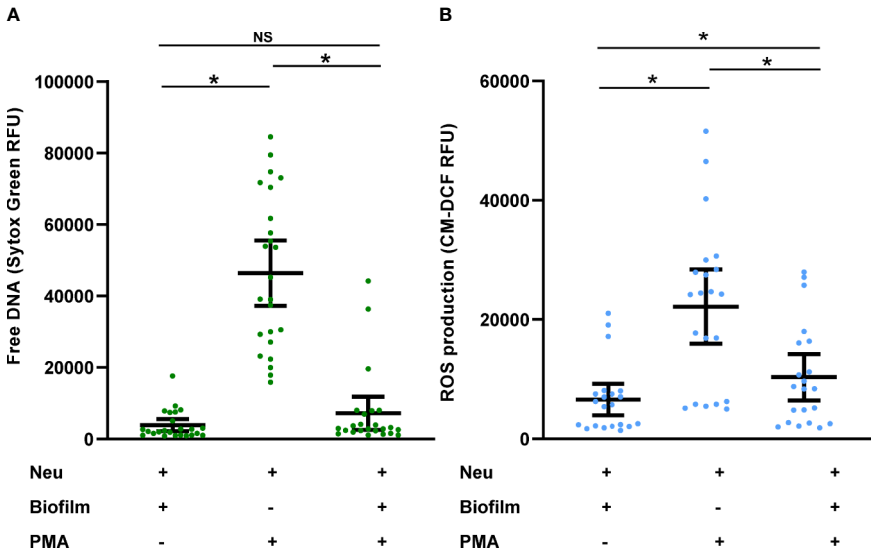

FIGURE 3 | *C. albicans* biofilms inhibit PMA-induced activation of neutrophils collected from patients with invasive candidiasis. **(A)** Patient neutrophils were co-incubated with *C. albicans* biofilm in the absence or presence of PMA for 4 h and NET formation was estimated by measurement of free DNA using Sytox Green. **(B)** To measure ROS, neutrophils were pre-incubated with free radical sensor CM-H2DCFDA prior to incubation with *C. albicans* biofilm in the presence or absence of PMA for 4 h, and fluorescence was measured. Statistical significance was analyzed by one-way ANOVA with Tukey's multiple comparisons test, $^*p < 0.05$, NS, not significant, mean with 95% confidence interval shown.

However, despite the intrinsic heterogeneity of the patient population, the consistency of ROS elevation suggests this phenotype may be common among invasive candidiasis patients.

Prior work has shown that *C. albicans* biofilms inhibit NET release in healthy neutrophils, even in the presence of the potent stimulus (PMA) (18). Here, we show that biofilms also inhibit PMA-induced NET release in the neutrophils of patients with invasive candidiasis. This inhibition is striking in light of their increased baseline activation. Beyond this lack of activity against biofilms, suppression of neutrophils by *C. albicans* biofilm may impair the activation of other cells involved in innate and adaptive immunity (39, 40). Further study is needed to understand how fungal biofilms may influence immune responses to other pathogens in the clinical setting.

The subset of invasive candidiasis patients enrolled in our study are representative of larger cohort studies (1, 11, 41). For example, the study participants carried the diagnosis of immune-compromising diseases, received immunosuppressive drugs, and frequently required central lines (11, 41). However, as expected, we observed differences in co-morbidities, sites of infection, and *Candida* species. Despite the varied patient characteristics, we observed rather uniform neutrophil responses for all studies. This suggests that the findings may be broadly applicable to variety of patients with invasive candidiasis.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Wisconsin Internal Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JK, CJ, and JN planned the study and designed the experiments. CJ, JK, and MB performed the experiments. JK, CJ, JC, and JN analyzed data, performed statistical analyses, and wrote the manuscript. JN supervised the work. All authors offered input to the manuscript and approved the final draft. 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/fimmu.2020.587956/full#supplementary-material

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Evaluation of Host Immune Response in Diabetic Foot Infection Tissues Using an RNA Sequencing-Based Approach

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The normal continuity of skin tissue can be affected by invading pathogens and lead to a series of complicated physiological events. Using an RNA sequencing-based approach, we have captured a metatranscriptomic landscape from diabetic foot infections (DFIs). The hierarchical clustering of the top 2,000 genes showed the expression of four main clusters in DFIs (A, B, C, and D). Clusters A and D were enriched in genes mainly involved in the recruitment of inflammatory cells and immune responses and clusters B and C were enriched in genes related to skin cell development and wound healing processes such as extracellular structure organization and blood vessel development. Differential expression analysis showed more than 500 differentially expressed genes (DEGs) between samples with a low number of virulence factors and samples with a high number of virulence factors. Up-regulated and down-regulated genes were mainly involved in adaptive/native immune responses and transport of mature mRNAs, respectively. Our results demonstrated the importance of inflammatory cytokines of adaptive/native immunity in the progression of DFIs and provided a useful groundwork for capturing gene snapshots in DFIs. In addition, we have provided a general introduction to the challenges and opportunities of RNA sequencing technology in the evaluation of DFIs. Pathways identified in this study such as immune chemokines, Rho GTPases, and corresponding effectors might be important therapeutic targets in the management of DFIs.

Keywords: diabetic foot infections, RNA sequencing, chemokines, cytokines, immune system, wound healing 2

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INTRODUCTION

Diabetic foot infections (DFIs) are the most severe and costly complication of diabetes developing in fifty percent of people with diabetic foot ulcers worldwide (Sadeghpour Heravi et al., 2019) and causing high mortality and disability (International Diabetes Federation, 2019). Sequential physiological events and several molecules and pathways controlled by wound-adjacent cells and affected cells are involved in DFIs development (Bekeschus et al., 2017).

Identification of physiological events and differentially expressed genes across conditions in DFIs have been limited to the hybridization-based microarray methods which can only profile predefined transcripts. Background hybridization noises and low sensitivity are other limitations in microarray studies (Rao et al., 2019).

Recently, RNA sequencing approach has emerged as an alternative technique for transcriptome-based applications beyond the limitations of hybridization-based microarray methods for gene expression profiling (Zhang et al., 2015). The recent advances in RNA sequencing technology not only saves cost and time but also enables transcriptional landscape in clinical samples.

The identification of genes, chemokines, and immune cells involved in DFIs is required for targeting the most relevant pathways (Rees et al., 2015). However, there is no information to provide a complete view of the host gene expression profile in DFIs. In this study, using an RNA sequencing-based method, we broadened our understanding of host inflammatory status that contributes to the development of DFIs.

It should be noted that with the quick growth of RNA sequencing application, considering an appropriate number of replicates is a critical step in experiment design. However, due to unpredicted technical challenges we encountered during conducting this study, the evaluation of DFIs gene expression was limited to a low number of sample size. Although this study laid the groundwork for the evaluation of gene expression analysis in DFIs, further investigation using a larger sample size is needed to verify the application and effectiveness of this method in DFIs management.

To the best of our knowledge, this is the first study to apply an RNA-sequencing analysis to evaluate host gene expression profiles in DFIs.

MATERIALS AND METHODS

Patient Population

In this prospective study, forty-three consecutive patients with DFIs referring to the Liverpool Hospital High-Risk Foot Service were recruited over a 6-month period. Using the International Working Group of the Diabetic Foot (IWGDF), Perfusion, Extent, Depth, Infection, and Sensation (PEDIS) classification system, DFI severity was determined in patients (PEDIS 2: mild infection, PEDIS 3: moderate infection, PEDIS 4: severe infection) (Monteiro-Soares et al., 2020). Recruited patients did not receive any antimicrobial therapy 2 weeks prior to the sample collection.

Sample Collection

Diabetic foot infections were cleaned using sterile 0.9% NaCl and a sharp debridement was collected using a sterile single-use punch from the affected area. Collected tissue samples were preserved immediately in a 2 ml RNA*later* stabilization solution (Thermo Fisher Scientific, Waltham, MA, United States) for 24 h at 4°C and then stored at −80°C until extraction. The overview of the RNA analysis pipeline applied in this study is shown in **Figure 1**.

Sample Preparation, RNA Extraction, and Illumina Sequencing

A detailed description of initial steps regarding sample preparation, RNA extraction, and sequencing process can be found in the previously described study (Heravi et al., 2020b).

Processing of Host Transcripts

Read quality was assessed using Cutadapt v1.18 (Martin, 2011) by trimming adapters and removing flanking "N" bases in the paired-end sequences. The mapping of the trimmed sequencing was carried out with STAR v2.5.2a (Dobin et al., 2013) using the human GRCh37_ICGC_standard_v2 reference. Quality control was performed using RNA-SeQC v1.1.8 (DeLuca et al., 2012). To determine the expected read counts FPKM, RSEM v1.2.30 was used with a forward probability of 0 (Li and Dewey, 2011).

Gene Ontology of Host Transcriptomes

To evaluate the potential biological role of the top 2,000 genes identified in the host, K-means was used to identify clusters of the highly expressed genes using iDEP.90 (9).

Differentially Expressed Genes and Enrichment Analysis

Differentially expression analysis was performed between samples with a low number of virulence factors (control) and samples with a high number of virulence factors (perturbation) using BioJupies (Torre et al., 2018). The signature of gene expression was produced by comparing gene expression levels in the control and perturbation groups using the limma R package and visualized in a volcano plot (Ritchie et al., 2015). Functional analysis of identified genes was carried out in Enrichr (Chen et al., 2013).

RESULTS

Although RNA sequencing analysis is rapidly evolving, selecting the appropriate number of replicates has a significant impact on performance characteristics. However, due to unforeseen challenges including low RIN in extracted RNA samples, we had to select a few samples which met the metatranscriptomics requirements. After the initial assessment of extracted RNA, samples from sixteen patients presented with either a mild (25%), moderate (31.25%), or severe diabetic foot infection (43.75%) with high integrity and quality RNA required for RNA sequencing were selected. All patients suffered from peripheral neuropathy. No patient was present with immunosuppressive disease or cancer at the time of sample collection. Patients data can be found in **Supplementary Table 1**.

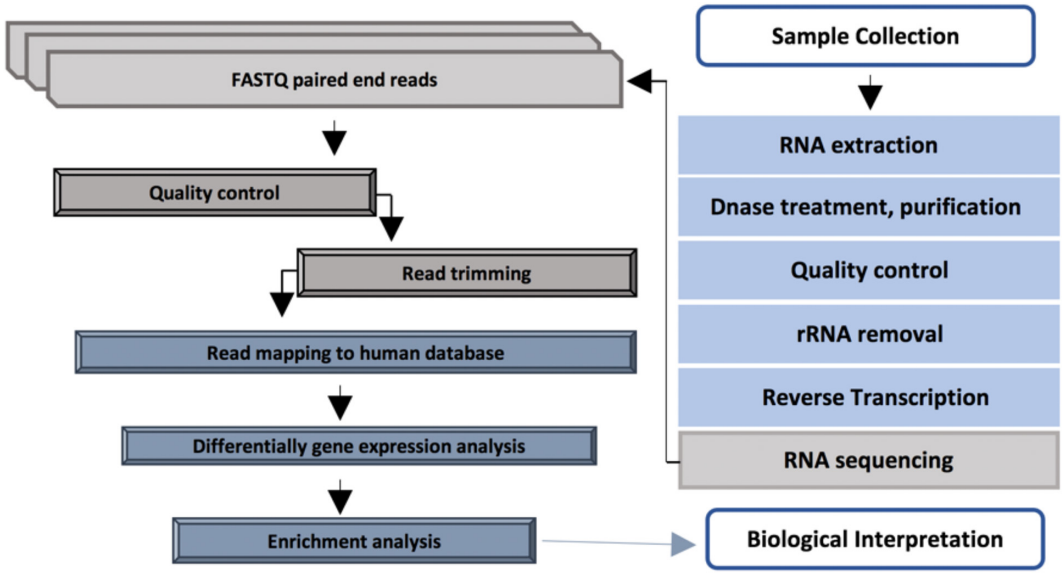

FIGURE 1 | RNA sequencing pipeline applied in this study.

Gene Ontology Analysis of Host Transcriptomes

K-means clustering divided the top 2,000 genes into four main clusters including A, B, C, and D. Samples with mild and moderate infections had a relatively similar pattern compared to samples with a severe infection which may indicate similar expression pattern in the early stages of infection compared to late stages (**Figure 2**). Gene enrichment analysis indicated that cluster C as the biggest cluster (1,363 genes, $p < 8.06E-34$) and cluster B (106 genes, $p < 6.76E-13$) were strongly enriched in genes related to biological molecular mechanisms of skin cells such as extracellular structure organization, cell adhesion, and blood vessel development. Cluster A (284 genes, $p < 6.13E-29$) and cluster D (247 genes, $p < 2.14E-13$) comprised genes involved in immune and defense responses (**Supplementary Table 2**). Cluster D with high expression in mild/moderate samples and lower expression in severe samples showed the probable contribution of molecules and proteins leading to skin cell activities including enzyme production, cell movement, activation of cell receptors, and immune cell chemotaxis/migration. Likewise, Cluster C showed a similar pattern to cluster D regarding the mild/moderate and severe samples. This cluster with the probability of the presence of pathways involved in the development of anatomical structures of blood vessels and skin cells showed higher expression in mild/moderate and lower expression in severe samples. Clusters A and B showed variable patterns across the samples which were enriched with pathways involved in activation of granulocytes/immune cells and muscle cell development, respectively. However, it should be noted that enrichment analysis only reflects the detection of RNA transcripts and does not necessarily imply functional activity and the presence of related proteins.

Differentially Expressed Genes (DEGs) and Enrichment Analysis

Our investigation identified the highest difference and the most interesting findings between samples with a low number of virulence factors (control) and samples with a high number of virulence factors (perturbation) which were defined previously (Heravi et al., 2020b). Differential gene expression analysis between control (DFI121, DFI109, DFI111, and DFI167) and perturbation groups (DFI166, DFI161, and DFI126) showed more than 500 differentially expressed genes (DEGs). Up-regulated and down-regulated genes were mainly involved in innate/adaptive immune systems and transport of mature mRNAs and histone mRNA, respectively ($p < 0.05$) (**Figure 3**).

The top ten enriched terms for up-regulated genes were mainly associated with the innate/adaptive immune systems, expression of Rho family protein, Fc gamma receptors (FCGRs), and re-arrangement of the actin cytoskeleton ($p < 2.26E-09$). Also, differentially down-regulated genes were mainly involved in transport of mature mRNA ($p < 5.12E-04$) (**Figure 4** and **Supplementary Tables 3, 4**).

DISCUSSION

Diabetic foot infections are the most serious complication of diabetes and cause a high rate of morbidity, mortality, and healthcare costs (Heravi et al., 2020a). DFIs are the main cause of lower-extremity amputations and have debilitated many people with diabetes around the world (Vatankhah et al., 2017).

Although different adjuvant treatments have been introduced to manage DFIs, targeting essential molecular pathways involved in the development of DFIs is still a major challenge. By using hybridization methods (microarray), previous studies focused

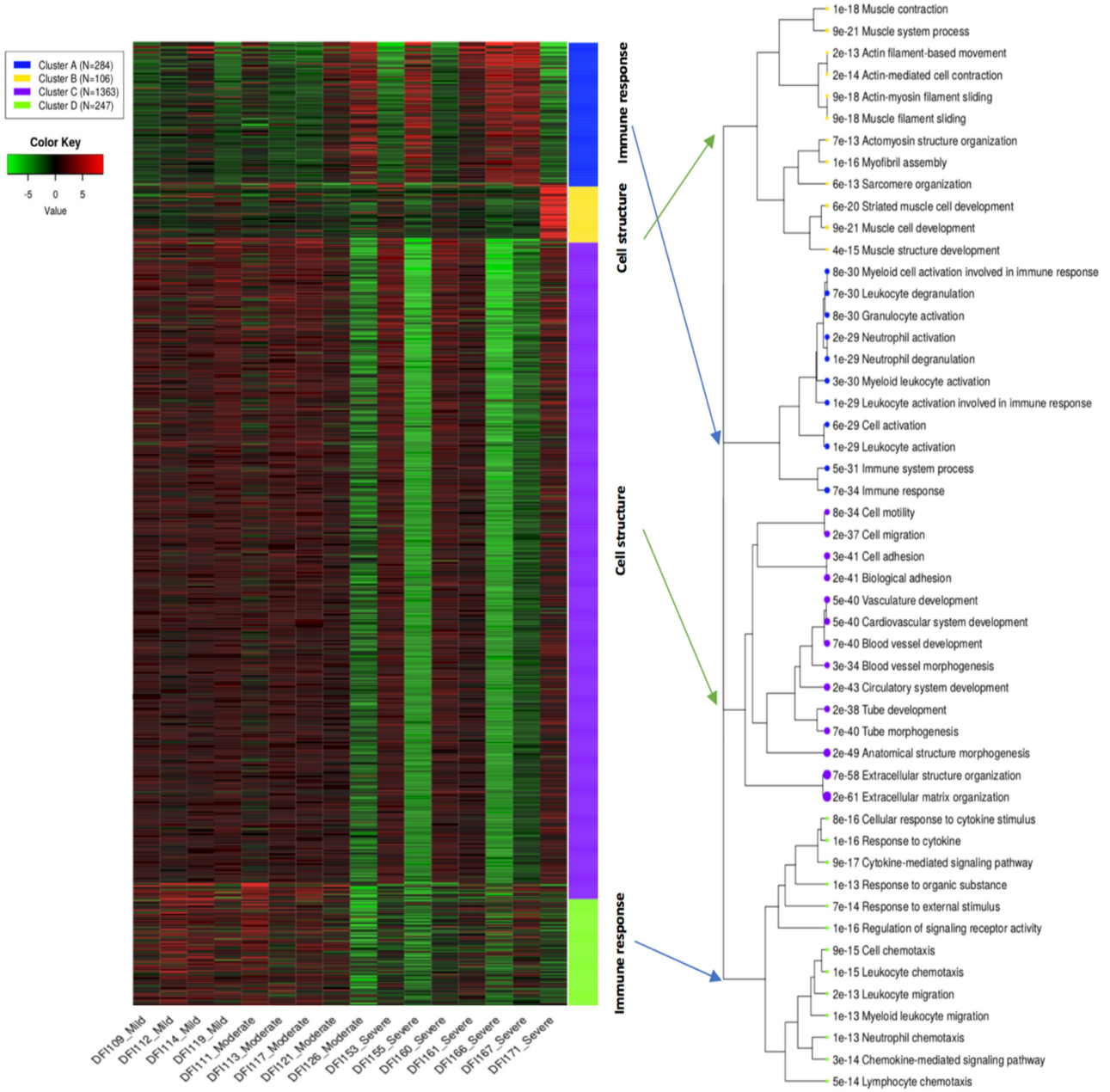

FIGURE 2 | K-means clustering shows gene ontology enrichment analysis of the top 2,000 genes in DFIs. Four main clusters (A, B, C, and D) were associated with innate/adaptive immune responses, and biological functions in skin cells. The hierarchical tree indicates the relationship between gene ontology terms.

on the investigation of a limited number of chemokines and pathways involved in DFIs which may not reflect the complexity of gene expression profile and chemokine systems in DFIs. Also, in hybridization-based methods, prior knowledge of the gene sequence is a prerequisite that may not be possible in large scale studies. Background noises, cross-hybridization, and low sensitivity are other limitations in microarray studies (Nagalakshmi et al., 2008).

RNA sequencing has revolutionized our understanding of gene expression far beyond that of microarray studies. This technique is likely to become the frontline in transcriptome analysis as RNA sequencing pipelines continue to improve and costs continue to decrease. This approach has enabled the possibility of gene expression analysis of all involved genes at once in a complex condition and overcome limitations in hybridization-based methods (Bjornson et al., 2020).

Previous studies showed G proteins and chemotactic cytokines (C, CC, CXC, and CX3C) as major signaling molecules in the initiation and progression of DFIs (Pease and Williams, 2006; Vaseghi et al., 2017). Manipulation of signaling G proteins

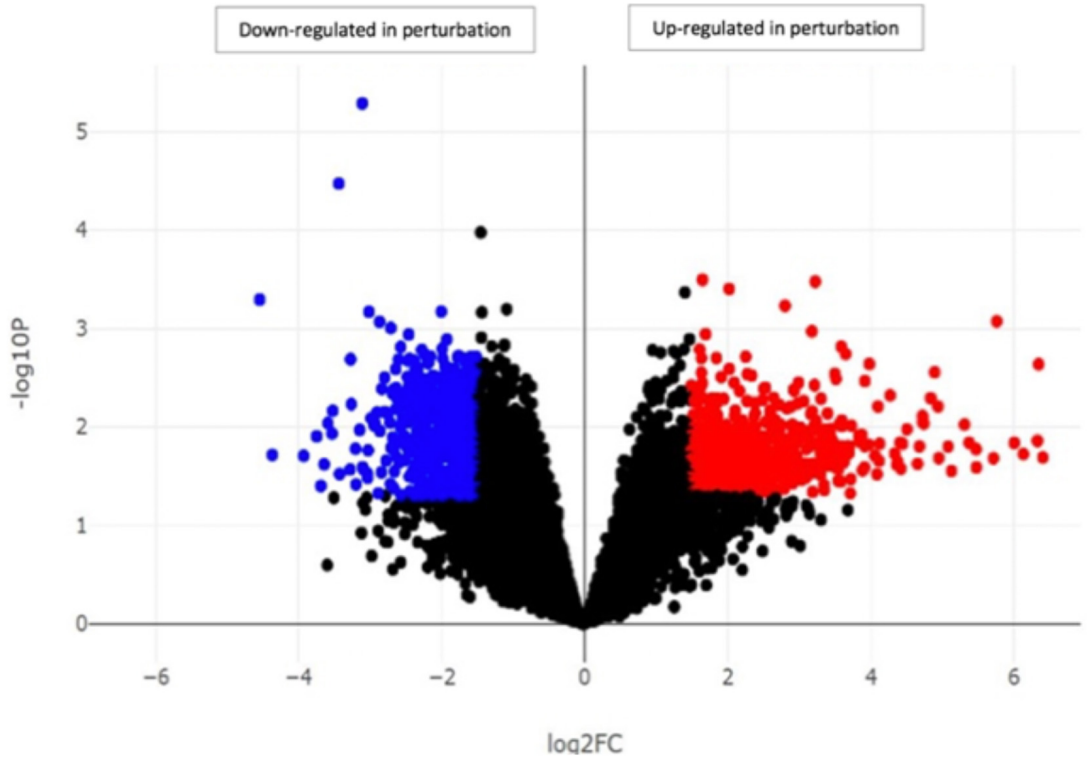

FIGURE 3 | Identification of genes with significantly different expression in DFIs between samples with a high number of virulence factors (perturbation) and samples with a low number of virulence factors (control). Each point in the scatter plot shows a gene. The axes represent the significance vs. fold-change resulting from differential gene expression analysis. All the genes above the line log2FC $x = 2$ and below the line log2FC $x = -2$ were determined as up-regulated and down-regulated genes, respectively.

(Rho GTPases) and chemokine systems has led to many great achievements in the wound healing process (Satish, 2015). Also, recent studies have shown that over-expression of the CXC chemokine family (CXCL12) in wounds of diabetic mice has led to improved healing (Restivo et al., 2010).

However, these studies only focused on the expression of a limited number of genes and chemokines in DFIs, in the present study we took advantage of metatranscriptomic analysis and provided a comprehensive view of gene expressions in DFIs.

We have identified several genes encoding different chemokines and growth factors involved in the progression and wound healing process in DFIs in this study. The identified genes are mainly involved in the recruitment of immune and inflammatory cells and overlapping phases of wound healing (such as hemostasis, inflammation, proliferation, and maturation) and promotion of angiogenesis.

The overall expression profile of the top 2,000 genes in DFIs showed four main clusters (A, B, C, and D). Clusters A and D were enriched in genes involved in the immune system responses such as leukocyte activation, neutrophil degranulation, and chemotaxis/migration of leukocytes. Clusters B and C were enriched in genes related to molecular mechanisms in skin cells (such as the development of muscle cells, extracellular structure organization, and blood vessel development). Cluster D showed a high expression of genes involved in the migration of immune cells in early infections (mild/moderate) while cluster A showed high expression of genes associated with activation and degranulation of leukocyte in severe infections. However, it should be underlined that protein translation and functional activity can not necessarily be suggested by the existence of genes/transcripts.

Cluster D mainly contained genes coding the CXC chemokine family [e.g., CXCL6, CXCL9, and CXCL5 (neutrophil chemotaxis), CXCL3 (monocytes migration), and CXCL10 (macrophages, monocytes, NK cells, T cells, and dendritic cells chemotaxis)]. This cluster had a high expression in mild/moderate samples and lower expression in severe stages which may indicate a slow healing process in severe wounds. Low expression of the CXC chemokine family (CXCL6, CXCL9, CXCL5, CXCL3, and CXCL10) has also been previously associated with the development of non-healing wounds (Satish, 2015). Previous findings have also shown a predominance of CCR5, CCR3, CCR2, and CCR1 in early infections, while expression of CXCR1 and CXCR2 occurred in late infections possibly released by activated monocytes and other inflammatory cells (Baggiolini, 2001; Borish and Steinke, 2003). Clusters B and C with high expression in mild/moderate samples and low expression in severe samples were mainly comprised of genes involved in blood vessel morphogenesis and extracellular matrix organization

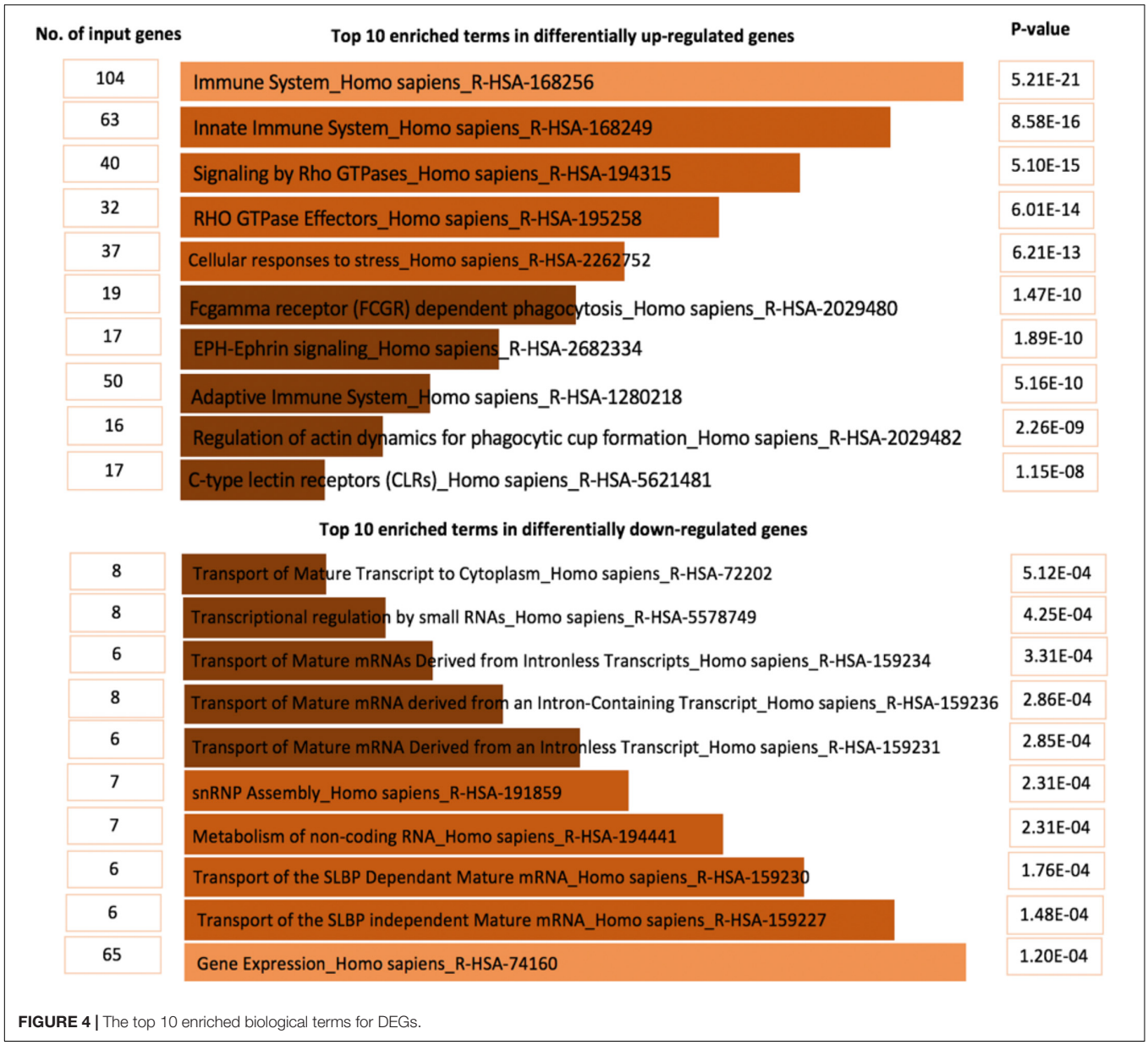

FIGURE 4 | The top 10 enriched biological terms for DEGs.

(such as TIE1, 2, LMOD2, NEB, MYBPC1, ACTN2, and TNNC2). Although the progression of non-healing wounds is multifaceted, there is a strong correlation between the development of non-healing wounds and poor vascular network (Aronow and McClung, 2015). Among circulating signaling molecules and angiogenesis regulating factors, Tie1, 2 belonging to the angiopoietin family have a major role in vessel maturity (Sundberg et al., 2002). Based on previous findings high expression of microvesicular receptors Tie1, 2 have been associated with the normal repair while downregulation of angiogenesis regulating factors was associated with severe ulcers which were in accordance with our findings (Okonkwo and DiPietro, 2017).

Differentially expressed genes analysis between samples with low and high number virulence factors showed that up-regulated genes were mainly involved in innate/adaptive immune systems, while down-regulated genes were mainly associated with the transport of mature mRNAs/histone mRNA. The top 10 up-regulated terms were mainly involved in innate immune systems, Rho GTPases signaling pathway, Fc gamma receptor-dependent phagocytosis and adaptative immune systems (**Figure 4**) which indicated the significant role of inflammation mediated by chemokine and cytokine signaling pathway in DFIs development. High expression of genes involved in signaling G protein pathways, cytoskeletal function, and recruitment of inflammatory chemokines confirmed the necessity of immune cytokines and Rho family GTPases in the development of DFIs and were consistent with previous findings (Abreu-Blanco et al., 2014; Ridley, 2016).

Although RNA sequencing has played a significant role in transcriptome profiling, there are still limitations in this approach that requires to be addressed. When designing an RNA sequencing study, a few considerations should be taken into account. Budget management and the number of sample sizes have always been controversial in the RNA sequencing approach (Geraci et al., 2020). A larger sample size is contributed to increased statistical power and reduced undesired noises. Also, it should be noted that the extraction of high-quality RNA is a challenging process and requires careful precaution. Although after sampling, tissue samples had been immediately preserved in RNAlater solution in this study, after the initial assessment, we were limited to a few RNA samples which met the metatranscriptomics requirements. It is worth pointing out that all the precautions should be applied during the sample collection, storage, and RNA extraction procedure according to the sample type to minimize RNA degradation and improve the outcomes in metatranscriptomic studies.

CONCLUSION

In this study, we evaluated host immune response in DFIs using an RNA sequencing-based approach. Identification of whole transcriptomic profiles using an RNA sequencing-based technology provided a molecular insight of host response in DFIs. Our results demonstrated the importance of inflammatory cytokines in adaptive/native immunity in the progression of DFIs. Pathways identified in this study particularly immune chemokines, Rho GTPases, and corresponding effectors might be important therapeutic targets through modulation of cytoskeletal function and manipulation of cytokines in the improvement of DFIs. However, further investigation in a larger scale is warranted to validate the findings in this study. Moreover, to investigate host-pathogen crosstalk in detail, pre-clinical studies using cell culture and animal models is required.

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 below: https://www.ncbi.nlm.nih.gov/, PRJNA563930.

ETHICS STATEMENT

This study was approved by the South Western Sydney Local Health District Research and Ethics Committee (HREC/14/LPOOL/487, SSA/14/LPOOL/489) and Macquarie University Human Ethics Committee (Reference No. 5201500839). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FH conducted the laboratory experiments and data analysis and drafted the manuscript. MZ and HA performed the data analysis and reviewed the manuscript. KV reviewed the manuscript. HH designed the project, monitored laboratory experiments, and reviewed the manuscript. 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.2021.613697/full#supplementary-material

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REVIEW
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Immune Responses to *Pseudomonas aeruginosa* Biofilm Infections

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Pseudomonas aeruginosa is a key pathogen of chronic infections in the lungs of cystic fibrosis patients and in patients suffering from chronic wounds of diverse etiology. In these infections the bacteria congregate in biofilms and cannot be eradicated by standard antibiotic treatment or host immune responses. The persistent biofilms induce a hyper inflammatory state that results in collateral damage of the adjacent host tissue. The host fails to eradicate the biofilm infection, resulting in hindered remodeling and healing. In the present review we describe our current understanding of innate and adaptive immune responses elicited by *P. aeruginosa* biofilms in cystic fibrosis lung infections and chronic wounds. This includes the mechanisms that are involved in the activation of the immune responses, as well as the effector functions, the antimicrobial components and the associated tissue destruction. The mechanisms by which the biofilms evade immune responses, and potential treatment targets of the immune response are also discussed.

Keywords: biofilm infections, *Pseudomonas aeruginosa*, innate immune response, adaptive immune response, novel treatment possibilities

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INTRODUCTION

Biofilms consist of microbes located in densely packed slow growing microcolonies embedded in a self-produced protective biopolymer matrix. In this life-mode, the microbes attain the highest levels of resistance to our present assortment of antibiotics and the immune system (1, 2). Accordingly, biofilms are a common cause of persistent infections (3), and biofilm-based infections are a major socio-economic burden implicating hospitalization, patient suffering, reduced life quality, increased mortality risk and lost employment. It is estimated that about 60%–70% of hospital acquired infections are caused by microbial biofilms (4). The immune response to biofilms results in collateral damage of adjacent tissues and therefore is an important aspect of biofilm infection pathology (5).

The vast majority of studies of the immune response against bacteria have focused on infections caused by bacteria in the planktonic state. Accordingly, considerably less is known about the immune response to bacteria growing in biofilm-based infections. However, recent *in vivo* and *in vitro* studies have begun to reveal the nature of both the innate and adaptive immune responses to biofilms (5, 6).

Planktonic bacteria are recognized by the innate immune systems pathogen recognition receptors (PRRs) through interaction with pathogen-associated molecular patterns (PAMPs), such as the flagellum and lipopolysaccharide (LPS) recognized *via* Toll-like receptor 5 and 4, respectively (7). Basically, biofilm growing bacteria activate the immune system through the same pathways as planktonic growing bacteria (5, 6). However, when residing in a biofilm the bacteria are embedded in extracellular polymeric substances and the classical PAMPs are less exposed to the immune system. In addition, PAMPs can be down-regulated in biofilm growing bacteria, as has been shown for flagella in *P. aeruginosa* (8, 9). Thus, in the case of biofilm infections the extracellular matrix components of the biofilms play an important role for the immune response (5, 6, 10).

The inflammatory state induced by biofilm unusually involves activation of both the innate and the adaptative immune response due to the chronic nature of biofilm-associated infections. Neither immune response is capable of eradicating biofilm, but they instead lead to extensive secondary damage.

The present review is focused on interactions between *P. aeruginosa* biofilms and the immune system (**Figure 1**). *P. aeruginosa* is involved in several persistent biofilm infections, including cystic fibrosis (CF) lung infections, chronic wound infections, urinary tract infections with or without catheters, and tracheal tube related ventilator-associated pneumonia (11–13). These infections are difficult or impossible to eradicate with antibiotics alone due to the special physiological state of bacteria in biofilms (2). The immune response has detrimental effects, as it causes destruction of the lungs of CF patients and maintains the inflammatory state of chronic wounds (11, 14). Knowledge about the mechanisms involved in activation, regulation, and evasion of the immune responses, as well as the nature of the antimicrobial components produced by the immune cells, and the associated tissue destruction has increased in recent years and will be discussed in the present review. Organ-system specific immune responses can differ substantially due to significant differences in tissue anatomy and physiology and is discussed when appropriate. Measurement of adaptive immune response during chronic persistent infections has proven an important clinical tool and will be described. Even though the role of the adaptive immune response has long been well recognized as being crucial during healing of wounds and in particular in inflammatory skin disease, the study of the role of the adaptive immune response in chronic wounds with *P. aeruginosa* biofilm infection has only just recently taken off (15, 16). Therefore, we have not included a detailed description of *P. aeruginosa* biofilm in chronic wound infections in the section of adaptive immune response. The understanding of all these components of host responses during biofilm infections

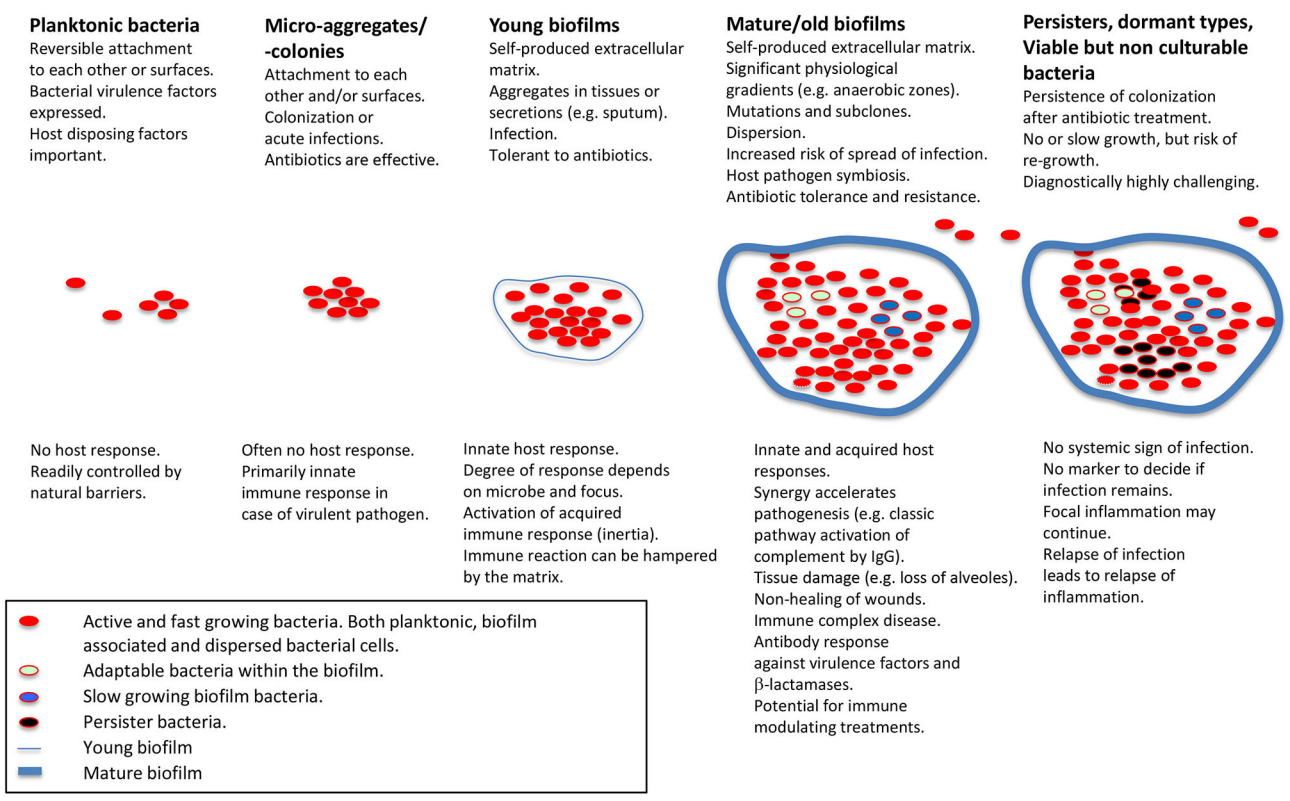

FIGURE 1 | Schematic presentation of biofilm stages and host response. Applies for non-foreign body-related biofilm infections, which is the main focus of the present review. Modified from Moser et al. (5) with permission from John Wiley & Sons, Inc.

may eventually form a basis for development of new and effective treatments against biofilm-based infections.

BIOFILM FORMATION OF *P. aeruginosa* DURING CHRONIC INFECTION

Biofilm formation by *P. aeruginosa* occur along with the production of several extracellular matrix components such as type IV pili (17–19), Cup fimbria (20), exopolysaccharides (21–23), CdrA adhesin (24), extracellular DNA (25), LecA/LecB lectins (26, 27) and Fap amyloids (28). The selection during chronic infection of *P. aeruginosa* variants that over-produce some of these biofilm matrix components is strong evidence for the involvement of biofilms in chronic infections (9, 29–32). Moreover, the presence of biofilms in CF lungs and chronic wounds has been demonstrated by microscopy (33, 34). *P. aeruginosa* can synthesize three different exopolysaccharides designated Pel, Psl, and alginate, although some strains only produce a subset of these exopolymers (21–23, 35). Overproduction of alginate enables mucoid *P. aeruginosa* strains to form persistent infections in the lungs of cystic fibrosis (CF) patients (29). Moreover, *P. aeruginosa* rugose small colony variants that overproduce Psl and Pel exopolysaccharide show enhanced persistence in CF lungs (9, 30, 31), and in chronic wounds (32). Evidence has been presented that Psl protects *P. aeruginosa* from host defenses in the initial phase of infection of the CF lung (36). Thus, it is possible that an extracellular biofilm matrix dominated by Psl is important in the initial stage of chronic lung infection before the bacteria mutate to produce a biofilm matrix dominated by alginate.

The host immune response plays an important role in the course of biofilm infections, and substantially affects the environment faced by the bacteria. The initial response to the presence of pathogens is an accumulation of activated neutrophils that may reduce the local O_2 concentration due to O_2 consumption accelerated by the respiratory burst and the production of reactive O_2 species (ROS) and nitric oxide (NO) (37–39). Thus, O_2 consumption by the neutrophils may result in O_2 depletion in infected parts of the body (40). The restricted O_2 availability accelerates stratified growth in *P. aeruginosa* biofilms, resulting in low metabolic activity in the center of biofilm as a consequence of nutrient depletion. However, micro-oxic conditions are sufficient to support growth of *P. aeruginosa* due to a highly flexible respiratory apparatus (41, 42) Moreover, bacteria may obtain energy under the anaerobic conditions prevalent in biofilm infections *via* anaerobic respiration or fermentation (43). Anaerobic respiration can occur by denitrification, where nitrogen oxides are utilized as alternative terminal electron acceptors (44, 45). The source of these N-oxides is suggested to originate from the rapid reaction of NO and O_2 produced by activated neutrophils (44) resulting in the formation of peroxynitrite ($ONOO^-$) (46), which may dismutate to nitrate (NO_3^-) and nitrite (NO_2^-) (47). The concentration of NO_3^- and NO_2^- in CF sputum (43, 48–50) may support *P. aeruginosa* growth at rates similar to those found in CF pulmonary biofilm (45). These findings suggest that the growth rate of *P. aeruginosa* during chronic CF lung infection is determined primarily by the number of surrounding neutrophils (51) which deplete O_2 and produce NO_3^- and NO_2^- which can be used by the bacteria for anaerobic respiration. As biofilm formation, neutrophil accumulation and O_2 depletion are common factors in multiple chronic infections, this interaction between host cells and pathogen is likely to occur also in other infections (44).

INNATE IMMUNE RESPONSES DURING *P. aeruginosa* BIOFILM INFECTIONS

Innate immunity fights infections from the moment of first contact and is composed of germline-encoded, non-clonal cellular and humoral mechanisms. These mechanisms enable nonspecific defense against pathogens without former interactions with infectious microbial invaders (52). The main components of the innate immune response engaged in response to *P. aeruginosa* biofilm include neutrophils, macrophages, dendritic cells, NK cells, and the complement system.

The most solid demonstration of a role of innate immune responses to bacterial biofilm has been obtained by introducing human neutrophils and macrophages to *P. aeruginosa* biofilms devoid of planktonic bacteria (53–56). The observed response comprises neutrophil accumulation, respiratory burst, penetration, phagocytosis, production of cytokines and eradication of the biofilm bacteria. In addition, *P. aeruginosa* cultures with increased bacterial aggregation induced stronger respiratory burst by neutrophils and cytokine release by macrophages (57).

Likewise, early sampling of mouse lungs challenged with *P. aeruginosa* biofilms has shown that the innate immune response involves intense accumulation of activated neutrophils in the airways (54, 56, 58–60). Early accumulation of neutrophils at the site of *P. aeruginosa* biofilm infection is also evident from experimentally infected chronic wounds in mice (14).

INNATE IMMUNE RESPONSE IN CF PATIENTS WITH CHRONIC *P. aeruginosa* LUNG INFECTION

The innate immune response has gained particular attention in patients with CF and chronic *P. aeruginosa* lung infection, due to the association between accumulation of neutrophils in endobronchial secretions and reduced functionality of the lungs (61). The recruited endobronchial neutrophils display inflammatory activity as indicated by continuing respiratory burst (37, 62) and generation of nitric oxide (44). Accordingly, destruction of the lung tissue has been correlated with oxidative and proteolytic lesions of endobronchial neutrophil activity (63, 64). Chronic lung infections in CF patients are associated with defective apical ion transport due to mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (65). Infected CF lungs are dominated by *P. aeruginosa* growing as endobronchial biofilms surrounded by

numerous neutrophils (33) and scarce planktonic bacteria, which are subject to phagocytosis by neutrophils (33, 37). The neutrophil response in infected endobronchial secretions in CF resembles the response in experimental *in vitro* and *in vivo* biofilms, where high numbers of neutrophils accumulate close to the biofilm (33) and depletion of molecular oxygen (O_2) is accelerated (37). This is caused by the reduction of O_2 to superoxide (O_2^-) during the neutrophils' active respiratory burst (66). Thus, the response of neutrophils to biofilms during chronic lung infection in CF may contribute considerably to the O_2-depletion in infected CF lungs (40). Furthermore, as active neutrophils primarily rely on ATP generated by anaerobic glycolysis (67), the high intake of glucose by neutrophils in CF lungs (68) as well as the enhanced level of L-lactate in sputum from CF patients with chronic *P. aeruginosa* lung infection (69), is in agreement with a high activity of neutrophils during biofilm infection in CF lungs. The neutrophil response to planktonic *P. aeruginosa* likewise includes stimulation of the respiratory burst (37), suggesting that neutrophil activation may also include a response to planktonic *P. aeruginosa* in infected CF lungs. Moreover, activation of neutrophils in infected CF airways may be triggered by alginate (70), LPS or immune complexes (71). The intensity of the neutrophil response may be enhanced by priming with LPS (72) and soluble factors of the innate immune response, such as platelet-activating factor, TNF-α, IL-8 and leukotriene B4 (73–77). Additionally, the migration through inflamed tissue may lead to stimulation of neutrophils due to multiple engagements of integrins and inflammatory cytokines (78). The presence of infectious agents is actually not needed to stimulate the respiratory burst, as seen in response to injury of the intestine in mice (79). The apparent lack of significantly disturbed capacity of neutrophils in CF patients (76) suggests that the reaction of neutrophils to *P. aeruginosa* biofilms seen in CF patients may also apply to infectious *P. aeruginosa* biofilms in non-CF patients. Accordingly, biopsies from chronic wounds have revealed biofilm surrounded by high numbers of neutrophils (60, 80–82). Similarly, neutrophils accumulate in high numbers at infectious biofilm in prosthetic knees (83, 84), and the accumulation of neutrophils was intensified and prolonged by *P. aeruginosa* biofilms in experimental chronic wounds and peritoneal infection (14). Thus, the induction of the biofilm life style observed during interaction between *P. aeruginosa* and neutrophils *in vitro* (85–87) may be highly relevant for the formation of biofilm *in vivo*.

The capability of the innate immune system to recognize invading microorganisms is aided by PRRs that recognize and bind to conserved microbial PAMPs leading to stimulation of the host response. Numerous varieties of PRRs, and their matching ligands are known, but PRRs reacting with PAMPs specifically expressed in microbial biofilm have not been described. PRRs may exist as intra- and extra-cellular membrane-bound receptors, cytoplasmic receptors, or soluble receptors. Since their discovery Toll-like receptors (TLRs) have advanced to become a very well-known family of PRRs. One group of TLRs is expressed on the surface of host cells where they mainly recognize microbial membrane components including lipoproteins, proteins and lipids, while other TLRs are intracellular and recognize microbial nucleic acids (88).

In the airways of chronically infected CF patients, TLR5 was the only MyD88-dependent TLR that was increased on neutrophils (89). This increased expression is possibly facilitated by G-CSF, IL-8 and TNF-α, and by the interaction of bacterial lipoprotein with TLR2 and TLR1 (88). TLR5 is a flagellin receptor (90) and its augmented expression on neutrophils in CF lungs is challenging to explain since flagella are lacking in mucoid biofilms *P. aeruginosa* isolated from CF lungs (91). The absence of flagella in nonmucoid biofilms, however, intensifies the bactericidal activity of neutrophils *in vitro* due to release of bactericidal amounts of lactoferrin (92), which may prevent biofilm formation (93, 94). Even though the significance of TLR5 expression for the outcome of biofilm infections is unclear, it may reinforce phagocytosis of planktonic, flagellin-intact *P. aeruginosa* subpopulations in the CF lungs (94). In support of this, neutrophils only ingested planktonic bacteria in infected airways of CF patients (33, 37), and *P. aeruginosa* with dysfunctional flagella survived for longer time during lung infection in mice (95). The capability of planktonic *P. aeruginosa* to provoke a stronger TLR-mediated response than biofilm *P. aeruginosa* has also been observed for the expression of IL-8 by epithelial cell lines (96). Bacterial eDNA, which is a matrix constituent of biofilms (25, 97), may stimulate neutrophils without involving TLR9 resulting in increased IL-8 production and intracellular signaling (98, 99). Alginate is an abundant component of the matrix in biofilm formed by mucoid *P. aeruginosa*, and is regarded as the strongest virulence factor in chronic lung infection in CF patients (100). Alginate may increase the respiratory burst of neutrophils (101), and monocytes may respond to alginate by initiating the production of cytokines (102). The activation of monocytes by alginate generated by *P. aeruginosa* may be mediated by TLR2 and TLR4 (103), while the PRRs involved in the activation of neutrophils remain elusive. The matrix of *P. aeruginosa* biofilms may contain other polysaccharide components, such as Psl and Pel, which may stimulate an innate response to biofilm (104). Recent evidence suggests that the specific exopolysaccharide composition of *P. aeruginosa* biofilms is a determinant of the neutrophil response (10). A biofilm with a matrix composed primarily of Psl and alginate polysaccharides was found to be particularly efficient in activating neutrophils (10). It remains, however, to be determined if the innate response against exopolysaccharide expression in biofilm is distinctly stronger than the innate response against exopolysaccharide expression in planktonic cells. In that case, we suggest considering exopolysaccharide as a subgroup of PAMPs termed "biofilm associated molecular patterns" (BAMPs) (**Figure 2**).

Although the soluble and the membrane-bound receptors of the complement system are among the most studied PRRs, a pivotal role of the complement system for the outcome of biofilm infections remains to be firmly established. Infectious biofilm may establish in spite of complement activation even in patients with intact complement systems. In this respect, *P. aeruginosa* may secrete elastase and alkaline protease that inactivate the

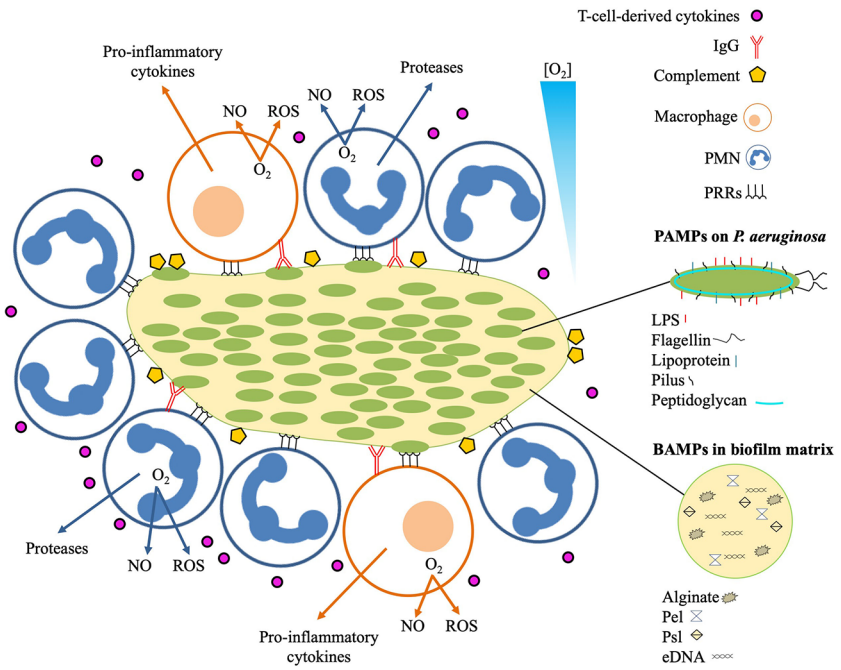

FIGURE 2 | Local immune response to infectious *P. aeruginosa* biofilm. The innate immune response recognizes pathogen associated molecular patterns (PAMPs) expressed on *P. aeruginosa*, and biofilm-associated molecular patterns (BAMPs) present in the biofilm matrix. Detection of BAMPs and PAMPs by PMNs and macrophages is mediated by pattern recognition receptors (PRRs). Binding of BAMPs and PAMPs to PRRs stimulates the PMNs and macrophages resulting in consumption of O_2 for liberation of tissue-toxic reactive oxygen species (ROS) and nitric oxide (NO). Additional responses by the PMNs include secretion of proteases that may cause proteolytic tissue lesions while the macrophage may further enhance the inflammation by emitting pro-inflammatory cytokines such as TNF-α, Il-1, IL-6, IL-8, and IL-12. The effector cells of the adaptive immune response mainly reside distantly such as the T-cells and the B-cells in the secondary lymphoid organs and the plasma cells in the bone marrow. Activated T-cells may release cytokines that further reinforces the inflammation by stimulating the accumulation and activation of PMNs and production of IgG. The contribution of the increased accumulation of activated PMNs to the local inflammation is further accelerated by binding of antigens to IgG, leading to immune complex mediated stimulation of the PMNs and activation of the classical complement pathway.

complement system (105). Further protection may be provided by alginate with O acetylation which prevents complement opsonization of mucoid *P. aeruginosa* biofilms (106). The involvement of the complement system in CF lung infections has been demonstrated by the frequent isolation of activated complement (C3c) in the sputum from chronically infected CF patients (107). Furthermore, the matrix polysaccharide, Psl, protects mucoid bacteria from opsonization and killing by complement components in human serum (108). However, whether complement activation requires biofilm formation is unlikely since planktonic bacteria induce stronger activation of the complement system (109). However, *P. aeruginosa* isolated from CF sputum may escape activated complement system (110).

The intense buildup of neutrophils associated to *P. aeruginosa* biofilm infections in CF, chronic wounds and implanted devices, would be anticipated to eliminate the biofilm. However, specific defects may weaken the immune defense. Thus, as a consequence of the basic defect in CFTR, both neutrophils and macrophages in the CF lungs exhibit blunted phagocytic capacity that could contribute to poor bacterial clearance and altered efferocytosis (111, 112). Moreover, the failing bactericidal activity of the summoned neutrophils may rely on rhamnolipids produced by *P. aeruginosa* (56). Synthesis of rhamnolipid depends on quorum sensing (QS) (60) indicating the ability of *P. aeruginosa* biofilm to contain bacterial densities necessary to achieve the quora required to activate QS-dependent rhamnolipid production (56, 59, 60) in chronic wounds (81) and lungs of infected CF patients (33). Rhamnolipids protects the biofilm against approaching functional neutrophils by inducing cellular necrosis (60). Intriguingly, the molecule OdDHL may attract neutrophils (113) and may thus attract and lure the neutrophils to the site of infection where they are killed by rhamnolipids. The QS-regulated attenuation of the host response may facilitate the initial establishment of biofilm infection (6). However, succeeding lung infection in CF patients involves extensive genetic adaptions with frequent mutations, e.g. in the QS regulator gene *lasR* (114). Dysfunctional QS of the *lasR* mutants may result in defective proteolytic neutralization of chemotactic cytokines allowing the pro-inflammatory cytokines to attract increased numbers of neutrophils to the lungs leading to intensified pulmonary inflammation (115). The size of bacterial aggregates may also contribute to the protection of bacteria against the immune response offered from biofilm formation. In fact, when the size of aggregated *P. aeruginosa* with deficient QS exceeded diameters of 5 μm, phagocytosis by human neutrophils was inhibited (116).

P. aeruginosa in biofilms can produce additional virulence factors, such as pyocyanin, that may cause cellular damage and immune modulations in cystic fibrosis lungs (117). Pyocyanin has been associated to broader functions, such as impairment of ciliary beat frequency and mucin hypersecretion, which in turn create a positive loop for biofilm formation and dysregulated immune responses in the CF lung (118).

It may be expected that the infectious biofilm in CF lungs would succumb due to the potent antibiofilm activity of antimicrobial peptides produced by neutrophils and lung epithelial cells (119). However, the low pH in CF lungs may impair the antimicrobial activity of antimicrobial peptides (120, 121). In addition, the defective distribution of salts in CF lung may have crucial effect on the optimal functionality of some antimicrobial peptides (122). Other environmental conditions in CF lungs may contribute significantly to the reduced activity of antimicrobial peptides. These conditions include proteolytic degradation of antimicrobial peptides by bacterial proteases (123) and by host proteases (124) and inhibition of antimicrobial peptides by binding to complexes of LPS, F-actin, mucins, and host derived DNA (125).

INNATE IMMUNE RESPONSE TO *P. aeruginosa* INFECTION IN CHRONIC WOUNDS

Whereas the majority of our knowledge on immune responses to *P. aeruginosa* biofilms comes from studies of CF lung infections, studies of chronic wound infections has recently shed additional light on the topic. The prevalence of recalcitrant wounds is expanding epidemically alongside with obesity and lifestyle diseases. The host response to bacterial intruders in chronic wounds is hallmarked by a persistent inflammatory phase. This phase comprises continuous oxidative damage, senescence of fibroblasts and skewing of constructive growth factors required for tissue resolution. The pathoetiology also includes low mitogenic-activity, high protease combined with low inhibitor-activity, microbiota changes, the etiology behind the original insult and the specific invading pathogen. Accumulating evidence emphasizes the paramount impact of infectious bacterial biofilm on the host response in the wound and the implication for recovery.

Unfortunately, it is challenging to achieve appropriate numbers of participants for conducting randomized studies on intervention in patients with recalcitrant wounds due to different chronicity definitions and patient heterogeneity. In addition, it is not feasible to extrapolate the results from chronic wounds of one etiology to another since many patients may suffer from several diseases (126).

The impact of infection with *P. aeruginosa* on wound chronicity is well described in clinical settings and experimental models (80, 81, 127). The presence of biofilm is now commonly recognized as a leading cause of chronic infections with persisting pathology despite antibacterial therapy and continuous induction of the host response (128). Certain components of *P. aeruginosa* biofilms, such as rhamnolipids, are likely playing important roles for persistence of infection as it causes cellular necrosis and killing of neutrophils (56, 59, 60). Other studies support the capability of *P. aeruginosa* to attenuate bactericidal components of the host defense (53, 129)

The endogenous antimicrobial peptides (AMPs) are phylogenetically ancient and constitute a crucial part of the skin's innate defense to infection (130). AMPs may be made by keratinocytes and infiltrating granulocytes and macrophages in response to infection, wound healing, trauma, or chronic inflammation. In addition, AMPs possess regenerative properties (131). AMPs are amphipathic molecules (132), which enables interaction with phospholipids of microbial membranes leading to pore formation and bactericidal cell lysis (133). The endogenous antimicrobial host defense protein S100A8/A9 belongs to the alarmin group and displays various activities. S100A8/A9 is expressed in actively healing wounds in human and murines (134, 135), but S100A8/A9 is absent in chronic, colonized venous leg ulcers in humans (136, 137) possibly resulting from the distorted local host response. This is suspected to cause deterioration of wound healing.

Relevant animal models are valuable tools for obtaining knowledge on the interplay between host and pathogen. Accordingly, animal models have enabled detailed descriptions of disposing factors, infectious agents and host response to infection. There are obvious limitations when comparing murine to humane wound healing and regeneration. Mice heal with predominantly contraction in a looser attached skin with higher hair density and thinner dermis versus the humane granulation healing. There are also significant differences in the immune response, with more neutrophils in the humane circulating blood versus a higher number of lymphocytes in mouse blood in addition to substantial differences with regards to the antimicrobial peptides. Despite this, mice represent a generally accepted experimental animal of choice.

To study the interaction between biofilm and the host response, we have established a chronic wound model which enables examinations of *P. aeruginosa* biofilm-infected wound closure in two mouse strains. One strain is relatively resistant to *P. aeruginosa* infection and consists of C3H/HeN mice. The other strain is made up of BALB/c mice which are susceptible to the infection (14, 138). The C3H/HeN mice have Th1-dominated response towards the infectious agents *Leishmania* major and *Candida* species. On the contrary, the response of the BALB/c mice against these agents is Th2-dominated. The direction of the Th response has essential effects on mortality rates and clearance of infection (138). A dichotomized early response in the mouse model of chronic wounds has been indicated by the attenuated local IL-1β inflammatory response to *P. aeruginosa* biofilm during the first 5 days of infection in C3H/HeN mice as compared to the BALB/c mice (14). Furthermore, our group recently demonstrated that *P. aeruginosa* biofilm may decrease the intensity of local neutrophil response in several murine wounds which may compromise the control of infection. The connection between the slow healing and the genotype in BALB/c mice has been confirmed by another group (198), which makes this

strain of mice an excellent choice of animal model for wound healing. In this context, comparing the spontaneous healing of *P. aeruginosa* biofilm infected wounds in C3H/HeN and BALB/c mice with the S100A8/A9 expression, could be highly valuable for further evaluation of the significance of S100A8/A9.

ADAPTIVE IMMUNE RESPONSES DURING *P. aeruginosa* BIOFILM INFECTIONS

The adaptive immune system discriminates the host proteins and other potential antigens from foreign molecules, to ensure that the lymphocytic and humoral antibody mediated effector functions do not result in excessive damage to the infected organism. However, the adaptive immune reaction is extensively superior in the specific response, as compared to the innate responses. Furthermore, recognition of the identical or similar pathogen upon reinfection by the adaptive immune system advances rapid clonal expansion of up to a 1000-fold antigen specific effector and central memory cells at subsequent exposures. The developed memory is the premise for immunity to subsequent infections. Compared to innate responses, which cannot discriminate between primary and secondary responses, the secondary responses of the adaptive immune system is substantially faster, more potent and with enhanced affinity as compared to primary exposure (139, 140). Activation of the adaptive immune system often results in clearance of the infection by planktonic bacteria, due to the combined activity of the innate and adaptive immune systems augmenting both the immune reactions. However, in the case of chronic biofilm infections the pathogens are not eliminated. Instead, the synergy of the innate and adaptive immune mechanisms, the latter with inertia at first encounter, is a central component of biofilm pathogenesis (5, 141–143).

Activation of the adaptive host responses is facilitated through dendritic cells (DC) required for sufficient activation at the first pathogen encounter and macrophages (Mφ) (144). Immature DCs in the peripheral tissue are effective in antigen uptake and are especially abundant at pathogen exposed regions, as the mucosal surfaces and in the secondary lymphoid tissue (145, 146). DCs mature following antigen uptake, and from inflammatory cytokine impact, into mature DCs dedicated in antigen processing and presentation (145, 146). Therefore, the DCs are essential in linking the innate and adaptive immune systems, and have the exclusive capacity to prime naïve T-cells into subsequent Th1, Th2, or Th17 cells and responses (145–147). Due to the limited presence of DCs in tissues, isolation is highly challenging, especially in human studies. Our own studies using a chronic *P. aeruginosa* lung infection model revealed commitment of pulmonary DCs during the infection (148). Pulmonary DCs was demonstrated as early as 2 days of initiation onset (148). Interestingly, an increased number of DCs in the regional lymph node was not detected until day 7 (148). The fraction of activated pulmonary DCs increased during the 10-day observation period, when demonstrated by CD80 and CD86 expression (148). In contrast, the percentage of activated DCs in the lymph node decreased at day 10 (148). The cytokine release of the DCs from the lung and lymph node were in general paralleled. Interestingly however, the initial release of the pro-inflammatory cytokines IL-6 and IL-12 reached a maximum at days 2–3, followed by an increased IL-10 production at day 7 (148). This observation, likely represents an essential controlling role of the DCs in induction of the adaptive immune system effector functions, impacted by the adjacent innate responses (148). This is supported by observations from another study, where *P. aeruginosa* QS signal molecules diminished the murine DC IL-12 production, while the IL-10 release remained. In addition, antigen specific T-cell proliferation was down regulated by QS exposed DCs. These results indicates that DCs are inhibited in T-cell stimulation by the *P. aeruginosa* QS signals, and by this mechanism contribute to the *P. aeruginosa* biofilm pathology (6, 149).

From previous observations of GM-CSF and G-CSF on DCs, we hypothesized that the increased G-CSF would impact the DC response in chronically pulmonary *P. aeruginosa* infected CF patients, besides recruiting PMNs from the bone marrow (150). Indeed, the GM-CSF/G-CSF ratio and the IFN-γ response correlated, and interestingly also correlated to a better lung function. In contrast, IL-3 and IFN-γ responses correlated inversely (150–156). DCs seem to impact host responses in biofilm infections and represent a potential therapeutic target.

As mentioned above, the innate and adaptive immune effector elements function in collaboration. As a consequence of the persistent biofilm infection, the adjacent tissue is impacted by the injurious oxidative radicals and enzymes originating from the inflammatory cells. Besides the pathogen related virulence factors, elastases, proteases, and other exoenzymes resulting from the inflammation expedites degradation of crucial surface molecules of the immune cell, further adding to impaired anti-biofilm mechanisms of the host responses (107, 157–160). The ineffective host response is considered the key basis of the biofilm related pathology, since antibodies against several bacterial virulence factors, such as elastase, lipopolysaccharide, and flagella have been reported, which presumable should improve biofilm outcome (161–163). However, these virulence factors are considered to be involved in pathogenesis, predominantly during the initial phases and to support development from microbial colonization to infection *per se*. Although, the bacterial virulence factors are less involved in the direct chronic biofilm pathology, the antibody mediated precipitation of virulence factors and other microbe antigens results in formation of immune complexes deposited in the tissues. Since, this leads to activation of the complement system and PMN opsonization, tissue damage is the consequence (100).

A special situation of the adaptive immune response and chronic *P. aeruginosa* infection of airways is the induction of a mucosal antibody response represented by specific secretory IgA (sIgA). The IgG responses can be regarded as an element of the systemic immune response, and primarily get access to mucosal surfaces through inflamed epithelium. In contrast, sIgA is the primary antibody of mucosal surfaces, and it is produced in double the amount of IgG, and is secreted to the mucosal surfaces as dimeric sIgA bound to the secretory component (164). At the

surfaces, sIgA functions through immune exclusion by binding to the pathogen and its PAMPs without activation of complement and opsonization. In CF sIgA has been found in sinuses and correlating to chronic sinusitis, whereas IgG dominates in the lower airways, where it correlates to inflammation of the respiratory airways (165). sIgA was also found to correlate to an early detection of *P. aeruginosa* of the lower airways of CF patients (165).

T-CELL RESPONSE AND CLINICAL OUTCOME IN CF PATIENTS WITH CHRONIC *P. aeruginosa* LUNG INFECTION

The biofilm infection and host response interplay has been best characterized for CF patients with pulmonary chronic *P. aeruginosa* biofilm infections (6). Early intensive antibiotic therapy, maintenance antibiotic treatment strategy between exacerbations, and planned elective intravenous antibiotic courses has become standard of care in CF (11). However, the natural course of the pulmonary chronic *P. aeruginosa* infection revealed a dichotomized outcome. A poor outcome, and a pronounced or rapid escalation in antibody response, was reported for most CF patients (166). However, for a small group of CF patients the humoral response was modest and these patients had a beneficial outcome (166). In addition, the intensified antibiotic treatment strategy in CF, resulting in significantly superior outcomes correlates to less pronounced antibody responses in CF (167).

By investigating specific cytokine release from re-stimulated peripheral blood mononuclear cells (PBMCs), and later on cytokine measurements from unspecific stimulated T cells, a Th1/Th2 cytokine dichotomy in chronically infected CF patients was revealed (168, 169). Chronically infected CF patients had a Th2 dominated cytokine response with increased IL-4 (and IL-5, IL-10) production and diminished IFN-γ production. In addition, a similar Th1/Th2 cytokine dichotomy was later demonstrated in bronchoalveolar lavage fluid from subgroups of CF patients (170, 171). Interestingly, IFN-γ release from PBMCs correlated to an improved lung function, suggesting a potential beneficial effect of IFN-γ (168). Inbred mouse strains with chronic *P. aeruginosa* lung infection showed a pronounced pulmonary IFN-γ level in the relatively resistant C3H/HeN mouse (138, 172). Reinfection of the susceptible BALB/c mice resulted in a pulmonary Th1 response similar to the C3H/HeN mice and resembled the course of a primary infection in the C3H/HeN mice (173).

The explanation for the improved outcome of a Th1 dominated response in CF patients with chronic *P. aeruginosa* lung infection is incomplete, especially since the Th1 dominated response would be more appropriate towards intracellular pathogens. However, phagocytosis of apoptotic PMNs by alveolar macrophages before the PMNs progress into necrosis and thereby increase inflammation, is believed to be involved (174). Reduction of IL-8, the most important PMN chemoattractant is another likely mechanism (175, 176). A diminished Th2 response would presumably result in a reduced antibody response, due to reduced B and plasma-cell stimulation, and subsequently decreased immune complex formation and tissue damage.

Additional T cell subsets have been described, including the Th17 subset, characterized by production of IL-17 and sometimes IL-22 (177). Th17 cells are induced by TGF-β (178) and may be of interest in CF, since IL-17 induces the PMN mobiliser G-CSF and chemoattractant IL-8 (179, 180). In this way, Th17 may add to pulmonary pathology of chronic *P. aeruginosa* lung infections (179, 180). In sputum from stable CF patients and in chronically infected CF patients, IL-17 and IL-23, was increased as compared to CF patients without chronic *P. aeruginosa* lung infections (179). Interestingly, such difference was not observed in CF patients infected with *Staphylococcus aureus* (179). A substantially decreased fraction of peripheral Th17 cells in CF patients has been reported, and interpreted as augmented homing of the cells to the lungs, increasing the pulmonary inflammation (181). Determinations of cytokines related to Th subsets were conducted in children with CF, and demonstrated increase of both IL-17A and the Th2 related cytokines IL-5 and IL-13 in children with symptoms (180). In contrast, such relationship was not observed for Th1 related cytokines, indicating a correlation between Th2 and Th17 subsets in CF (180). Such a Th2-Th17 axis could dispose for *P. aeruginosa* lung infections, but this has not been clarified yet (171, 180, 182). Interestingly, T cell suppressive neutrophil myeloid-derived suppressor cells (MDSCs) has recently been reported in CF (183, 184). The presence of neutrophil MDSCs in peripheral blood correlated to improved lung function in CF in contrast to what would be expected (183). Down regulation of the harmful and dominating Th2 and Th17 response axis, could be the mechanism behind this observation.

The role of regulatory T cells (Treg), Th22, and additional T cell subsets has only been sparsely studied in biofilm infections. However, decreased levels and reduced functions of these immune cells in CF patients have been suggested and may result in augmented IL-17 and IL-8 production (182, 185).

NOVEL POTENTIAL TREATMENT OPTIONS TOWARDS *P. aeruginosa* BIOFILM INFECTIONS

The administration of preformed antibodies or immunoglobulins to treat various infectious diseases is known as passive immunization therapy. Passive immunotherapy using avian IgY immunoglobulins (yolk) targeting *P. aeruginosa* represents an alternative to conventional antibiotic therapeutics. IgY is the predominant serum antibody in chickens and is the avian homologue of mammalian IgG (186). It accumulates in the egg yolk from the blood and provides the offspring with humoral immunity. Hyperimmunization of chickens with specific antigens, provides high yields of specific IgY antibodies in the egg yolk (187). *In vitro* studies with IgY targeting *P. aeruginosa* showed

firm binding to flagella and interference with the adhesion of bacteria to epithelial cells (188). Potentially, such effect could prevent bacteria from colonizing the respiratory tract. Additionally, our group has also observed promising effects of anti-*P. aeruginosa* IgY. In *in vitro* studies, respiratory PMN burst and bacterial killing of *P. aeruginosa* were shown to be significantly increased in the presence of anti-*P. aeruginosa* IgY (189). Anti-*P. aeruginosa* IgY seems to affect aggregation of bacteria resulting in immobilization and increased surface hydrophobicity, enhancing non-Fc receptor mediated phagocytosis (190). The observed *in vitro* effects of anti-*P. aeruginosa* IgY, were in accordance with *in vivo* observations in an acute murine pneumonia model, where we demonstrated a 2-log reduction in pulmonary bacteria, which was paralleled by decreased inflammation in the airways of anti-*P. aeruginosa* IgY treated mice as compared to mice receiving non-specific IgY (191).

Potentially, anti-bacterial immunotherapies by means of pathogen specific IgY augments PMN mediated phagocytic effects and reduce the level of airway colonization in CF and may even potentiate the action of anti-pseudomonal antibiotics (192). Moreover, a clinical study examining the effects of oral prophylactic immunotherapy with anti-*P. aeruginosa* IgY in non-chronically infected CF patients has shown promising results (193).

Recombinant S100A8/A9 also show promising therapeutic properties. Our group found that immune modulation of *P. aeruginosa*-biofilm infected wounds on BALB/c mice by 4-days local application of recombinant S100A8/A9, combined with systemically administered ciprofloxacin, significantly reduced the bacterial load of the wounds (194). Since *in vitro* synergistic effect between S100A8/A9 and ciprofloxacin was not observed, the effect is highly dependent on host cells (194). Human studies and animal experiments indicate impairment of the S100A8/A9 response and that the level of S100A8/A9 is inappropriate in non-healing wounds. We are currently investigating this area to improve the understanding of the pathophysiological multifaceted role of S100A8/A9 in biofilm-infected wounds.

In adjunctive therapies of non-healing wounds with an inappropriate anti-biofilm host response, autologous fibrin rich patches containing thrombocytes and leucocytes are a promising treatment strategy (195). A three layered 3C patch, is produced by centrifugation of the patient's whole blood in a specially developed device (195). The 3C patch is subsequently applied to the chronic wound (196). In an open study on chronic wounds of various backgrounds, an accelerated healing with 3C patches was revealed in the majority of the patients (197). The effect is most likely caused by production of healing growth factors and cytokines, e.g. PDGF-bb, from thrombocytes (195). In support of these observations, a substantial PMN activity was observed inside 3C patches in terms of respiratory burst, PMN phagocytosis activity and anti-biofilm action (196).

CONCLUSIONS AND PERSPECTIVES

Knowledge of the immune responses and bacterial defense mechanisms under conditions of biofilm infections is important as it constitutes an important part of the pathology of biofilm infections. As documented in the present review, our knowledge of immune responses to biofilm infections has increased considerably in recent years and is likely to provide important treatment tools against biofilm infections in the future. We may eventually be able to damping harmful immune system activities, or to activate parts of the immune system that can eradicate biofilm infections without causing detrimental collateral damage. In addition, antibiotic augmenting effects of the immune system could be identified. Alternatively, we may be able to manipulate the bacteria and down-regulate or eliminate the components of biofilms that are responsible for the recalcitrance towards immune system activities.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Immunopathogenesis of Craniotomy Infection and Niche-Specific Immune Responses to Biofilm

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Bacterial infections in the central nervous system (CNS) can be life threatening and often impair neurological function. Biofilm infection is a complication following craniotomy, a neurosurgical procedure that involves the removal and replacement of a skull fragment (bone flap) to access the brain for surgical intervention. The incidence of infection following craniotomy ranges from 1% to 3% with approximately half caused by *Staphylococcus aureus* (*S. aureus*). These infections present a significant therapeutic challenge due to the antibiotic tolerance of biofilm and unique immune properties of the CNS. Previous studies have revealed a critical role for innate immune responses during *S. aureus* craniotomy infection. Experiments using knockout mouse models have highlighted the importance of the pattern recognition receptor Toll-like receptor 2 (TLR2) and its adaptor protein MyD88 for preventing *S. aureus* outgrowth during craniotomy biofilm infection. However, neither molecule affected bacterial burden in a mouse model of *S. aureus* brain abscess highlighting the distinctions between immune regulation of biofilm vs. planktonic infection in the CNS. Furthermore, the immune responses elicited during *S. aureus* craniotomy infection are distinct from biofilm infection in the periphery, emphasizing the critical role for niche-specific factors in dictating *S. aureus* biofilm-leukocyte crosstalk. In this review, we discuss the current knowledge concerning innate immunity to *S. aureus* craniotomy biofilm infection, compare this to *S. aureus* biofilm infection in the periphery, and discuss the importance of anatomical location in dictating how biofilm influences inflammatory responses and its impact on bacterial clearance.

Keywords: biofilm, *Staphylococcus aureus*, craniotomy, myeloid-derived suppressor cell, neutrophil, macrophage, microglia

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INTRODUCTION

Craniotomy and decompressive craniectomy are neurosurgical procedures where part of the skull (i.e. bone flap) is removed to access the brain (**Figure 1**). Craniotomy involves the temporary removal of the bone flap for procedures that include tumor resection, localization and resection of epileptogenic foci, and aneurysm clipping, where the bone is replaced intraoperatively (1). Decompressive craniectomy refers to the excision of the bone flap for an extended period following traumatic brain injury, ischemic stroke, or intracranial hemorrhage to treat intracranial

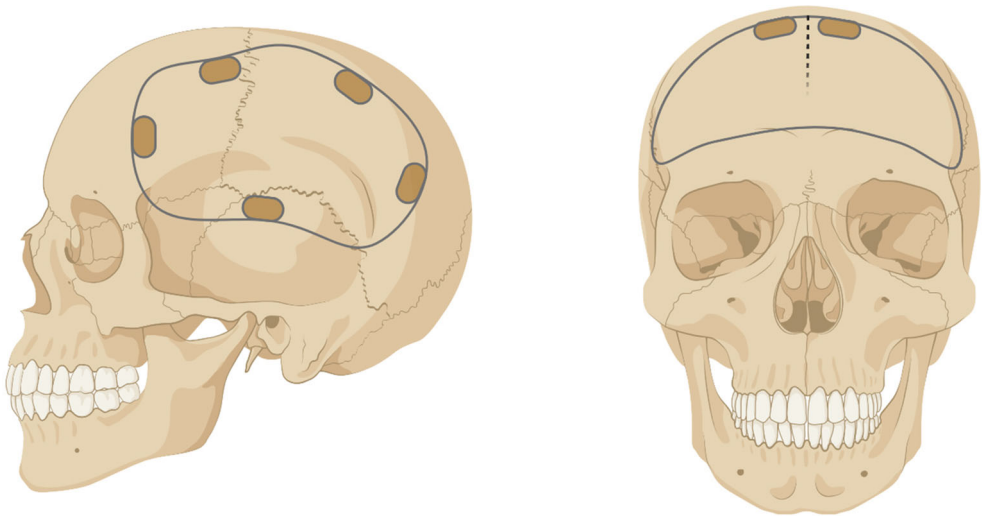

FIGURE 1 | Example of a unilateral (left) or bilateral (right) craniotomy/craniectomy. Figure created with BioRender.

hypertension (2). Upon removal, the bone flap is typically cryopreserved or implanted subcutaneously in the abdomen of the patient to preserve vascularization and replaced after cerebral edema has resolved (3, 4). However, complications can occur with prolonged absence of the bone flap including extracranial herniation, trephine syndrome, hydrocephalus, seizures, and neurological dysfunction (5, 6).

Despite peri- and post-operative prophylaxis, infectious complications occur in approximately 1% to 3% of craniotomy and craniectomy procedures (7, 8). These infections are associated with a high mortality rate and poor prognosis if not treated early (9–11). In terms of therapy, the decision of whether to salvage or discard the bone flap is left to the neurosurgeon and is often dictated by the length of time from the initial surgery to the presentation of clinical signs of infection. The first option is to salvage the infected bone flap with a combination of debridement and long-term antibiotic therapy. Alternatively, the bone flap can be discarded and, following extended antibiotic treatment, a cranioplasty is performed to correct the acquired skull defect with an autologous bone graft or prosthesis (12–15). Prior craniotomy infection increases the risk for re-infection, which may result from the outgrowth of residual bacteria that were not eliminated due to biofilm formation (see below).

Approximately one half of craniotomy/craniectomy infections are attributed to *S. aureus* (7, 16–18), a gram-positive pathogen that forms a biofilm on native bone (19). Infections can be caused by other bacteria and fungi, although these occur at a much lower rate (8, 17). Around 30% of the human population is colonized with *S. aureus*, typically in the nares and skin, and colonized individuals have an increased risk for invasive *S. aureus* infection (20). Although pre-surgical screening for *S. aureus* carrier status is routinely performed to decolonize carriers prior to orthopedic surgery (21), this approach has not been universally adopted in neurosurgery for patients that require a craniotomy/craniectomy.

S. aureus is a versatile pathogen, which is attributable to several features. First, the organism is prone to genetic adaptation, particularly the ability to acquire antibiotic resistance. An example is the *mecA* cassette that affords *S. aureus* resistance to the entire class of methicillin antibiotics (22). Second, *S. aureus* expresses an extensive repertoire of virulence factors that promote its pathogenesis and interfere with host immune recognition and bacterial clearance. These include cell surface attachment factors, capsular polysaccharides, enzymes, pore-forming toxins, superantigens, and numerous immune modulatory molecules (23–28). In addition to encoding a myriad of virulence factors, *S. aureus* can form biofilm that represents another virulence determinant (29). Biofilms are complex microbial communities surrounded by a matrix composed of extracellular DNA (eDNA), protein, and polysaccharide (29). The organization of bacteria within a biofilm creates microdomains with differential access to oxygen and nutrients leading to a sub-population of organisms that are less metabolically active, referred to as persisters (30, 31). Because most antibiotics target bacterial cell wall and protein synthesis, the metabolic dormancy of some biofilm-associated bacteria is responsible for the well-known antibiotic tolerance of biofilm. Compared to planktonic bacteria, the mechanisms responsible for *S. aureus* biofilm to evade immune-mediated clearance are only beginning to be understood. Work from our group and others has shown that *S. aureus* biofilm evades Toll-like receptor (TLR)-mediated recognition (32, 33), inhibits phagocytosis (33–37), and induces the recruitment of granulocytic-myeloid-derived suppressor cells (G-MDSCs) that inhibit monocyte/macrophage proinflammatory activity (38–40). Recent work has demonstrated that *S. aureus* metabolites (D- and L-lactate) play a key role in inducing epigenetic changes

in G-MDSCs and macrophages to promote the production of the anti-inflammatory cytokine IL-10 and biofilm persistence (41).

Novel therapeutic approaches for *S. aureus* infection continued to be explored, since most antibiotics have poor efficacy against biofilm and a fine balance must be achieved with currently available antibiotics to reach an optimal minimum inhibitory concentration (MIC) during chronic administration while minimizing toxicity (31, 42). An effective vaccine against *S. aureus* has remained elusive (43, 44). This is likely explained by the fact that the organism can cause a wide range of infections with distinct attributes, and that it expresses numerous virulence factors that impair host immunity. The latter point has recently been shown to play an important role in a mouse model of *S. aureus* bacteremia where immunization with *S. aureus* toxoids reduced mortality, bacterial burden, and organ dysfunction (45). A better understanding of *S. aureus* colonization dynamics, how the organism interacts with different leukocyte populations, and influences of the local tissue milieu will be necessary to develop improved therapeutics for infections caused by *S. aureus*.

MOUSE *S. AUREUS* CRANIOTOMY MODEL: SIMILARITIES TO HUMAN INFECTION

As mentioned above, *S. aureus* is a major cause of infectious complications following craniotomy (7, 9, 10); therefore, our laboratory developed a mouse model of *S. aureus* craniotomy infection to understand the immune mechanisms responsible for bacterial persistence (19). In the mouse model, a craniotomy is performed and the bone flap is colonized with *S. aureus*, which leads to biofilm formation on the bone and chronic infection in both the brain and subcutaneous galea that cannot be cleared with systemic antibiotics (46). Importantly, the mouse model shares several features with human craniotomy infection. This includes a conserved biofilm structure on the bone flap as revealed by scanning electron microscopy with similarities in extracellular matrix deposition, foci of bacterial aggregates on the bone flap, and complex tower-like structures (19). In addition, magnetic resonance imaging (MRI) revealed galeal inflammation with superficial cortical brain involvement (19), which is also an attribute of human infection and supports the translational relevance of the mouse model.

COMPARTMENTALIZATION OF IMMUNE RESPONSES DURING *S. AUREUS* CRANIOTOMY INFECTION

The CNS was once considered immune privileged based on the restrictive attributes of the blood brain barrier (BBB) (47–49). However, it is now clear that immune responses do occur in the CNS in a wide range of neurodegenerative and infectious diseases, and immune surveillance of the CNS takes place in the absence of pathology (50–52). Over the past decade, our laboratory has characterized the immune responses to *S. aureus* biofilm infection in both the CNS (craniotomy-associated infection) and periphery (prosthetic joint infection (PJI)) (19, 40, 41, 46, 53–56). Comparisons between these models clearly show that the immune responses elicited are distinct, which will be discussed later in this review, reflecting influences of the local tissue milieu. The impact of infection site and how this shapes the subsequent immune response has also been reported by other groups and emphasizes the need to understand niche-specific factors that influence *S. aureus*-immune crosstalk (57–61).

Even within a given infection, compartmentalization of immune responses can be observed. An example is the *S. aureus* craniotomy model where patterns of leukocyte recruitment and inflammatory mediator expression are distinct in the brain vs. subcutaneous galea despite both tissues being exposed to bacteria on the bone flap. For example, monocytes, innate lymphoid cells (NK and γδ T cells), and T cells are preferentially recruited to the brain, whereas G-MDSCs and neutrophils (PMNs) are the main leukocyte infiltrates in the galea (**Figure 2**) (19, 56, 62). The attributes of these cell types and their role during *S. aureus* infection will be described in more detail below. Likewise, the expression of chemokines, such as CCL2 (monocyte chemoattractant protein-1; MCP-1) and CXCL10 (interferon-inducible protein 10 kDa; IP-10) are higher in the brain (19, 56), which coincides with the enhanced recruitment of monocytes and lymphocyte populations. In the galea, chemokines responsible for PMN and G-MDSCs influx (CXCL2; macrophage inflammatory protein-2; MIP-2) are generally enriched (19, 56) in agreement with the preferential recruitment of these populations to this compartment.

Interactions between the immune system and CNS are not only important for controlling infection, but also for maintaining homeostatic functions including neurogenesis, behavior, and neuronal activity (63–66). Therefore, a delicate balance must be achieved to elicit sufficient inflammatory responses to clear infection without becoming overactive, which can lead to collateral tissue damage. Indeed, many bacterial infections in the CNS, including those caused by *S. aureus*, result in areas of tissue necrosis that vary according to infection severity. CNS biofilm infection represents an interesting dilemma since the chronicity of these infections is not characteristic of an overactive immune response, but instead one that is non-productive or anti-inflammatory. In this instance, CNS pathology may be mediated by products released from the biofilm, such as bacterial proteases, nucleases, or *via* the consumption of metabolites that are critical for CNS function (i.e. glucose). In the following section, we present an overview of the different immune populations associated with *S. aureus* craniotomy infection and their functional attributes.

MICROGLIA AND MYELOID CELLS ASSOCIATED WITH *S. AUREUS* CRANIOTOMY INFECTION

Microglia

Microglia comprise approximately 10% of brain parenchymal cells, which undergo slow proliferation throughout the lifespan

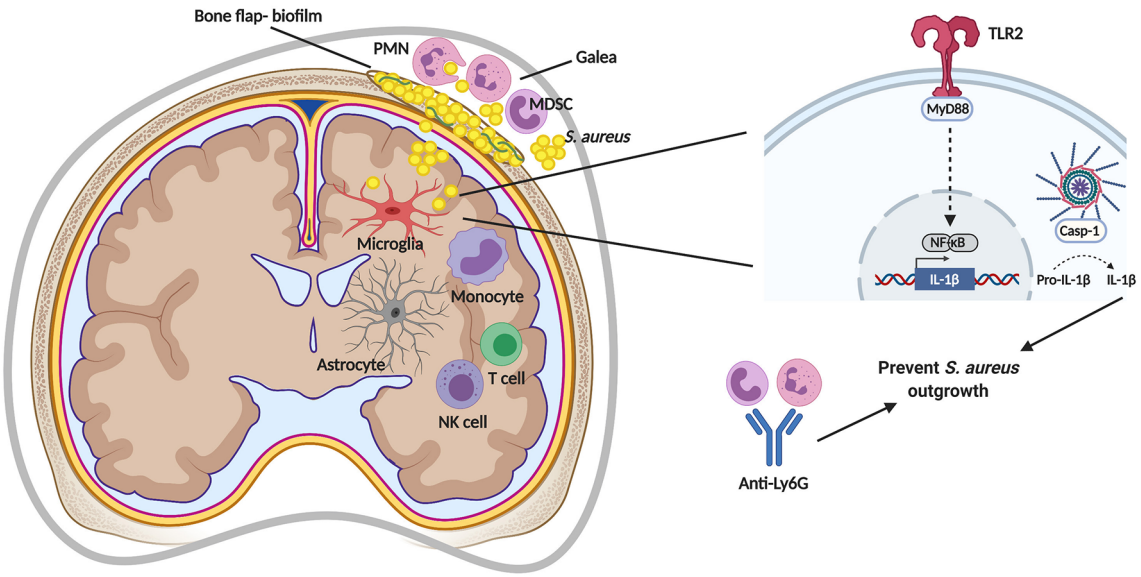

FIGURE 2 | Immune responses during *S. aureus* craniotomy infection. *S. aureus* biofilm formation of the bone flap elicits a unique inflammatory response in the subcutaneous galea and brain. TLR2-mediated signaling *via* MyD88 induces pro-IL-1β production that is cleaved by a caspase-1 (casp-1)-containing inflammasome for secretion and to prevent *S. aureus* outgrowth. *S. aureus* containment is also mediated by neutrophils (PMNs), as shown by depletion using anti-Ly6G. MDSC, myeloid-derived suppressor cell; NF-κB, nuclear factor-kappa B; NK, natural killer. Figure created with BioRender.

of an organism to maintain their numbers (67, 68). Historically, there was much debate about the origin of microglia, where earlier dogma considered microglia to be bone marrow-derived; however, this has now been definitively disproved (69, 70). It is now well established that microglia arise from erythromyeloid precursors in the primitive yolk sac that migrate to the brain where they differentiate into microglia (71, 72). Microglia continuously survey the CNS parenchyma by expanding and retracting their processes to monitor the extracellular milieu, surrounding neurons and other glial cells, as well as to detect invading pathogens and CNS damage (73–75). Microglia play a key role in the phagocytosis of microbes, apoptotic cells, and protein aggregates, and produce a wide array of inflammatory mediators based on their diverse repertoire of pattern-recognition receptor (PRR) expression. Pertinent to *S. aureus*, microglia express 1) TLR2 and TLR9 that recognize bacterial lipoproteins and non-methylated CpG DNA motifs, respectively; 2) nucleotide-binding oligomerization domain-containing protein 2 (NOD2), an intracellular PRR that senses muramyl dipeptide, a component of peptidoglycan that is abundant in the cell wall of *S. aureus* and other gram-positive bacteria; and 3) CD14 (76–79). Following PRR activation, microglia produce a wide array of proinflammatory cytokines and chemokines (i.e. TNF-α, IL-6, IL-1β, IL-12, and CCL2) as well as reactive oxygen and nitrogen species (ROS, RNS). These mediators have pleotropic effects including promoting BBB permeability (TNF-α, IL-6, IL-1β), leukocyte recruitment (CCL2) and activation (TNF-α, IL-6, IL-1β, IL-12) and bactericidal activity (ROS/RNS), but they can also negatively impact neuronal function and survival if not tightly regulated (80). Therefore, the induction of anti-inflammatory mechanisms are critical to resolve inflammation and promote tissue repair, which are largely mediated by cytokines such as IL-10 and transforming growth factor-beta (TGF-β) (81–83). The chronicity of *S. aureus* craniotomy infection suggests a potential imbalance towards an anti-inflammatory state. This is supported by the presence of immune suppressive G-MDSCs as well as PMN and monocyte infiltrates that also possess anti-inflammatory activity as reflected by their ability to inhibit T cell activation (62).

Although as myeloid cells microglia and bone marrow-derived macrophages have distinct origins, they share many attributes including similarities in marker expression, cytokine production, and dependence on macrophage colony-stimulating factor-1 (CSF-1) for survival and proliferation (84–87). During CNS inflammatory conditions, it is not possible to discriminate between microglia and infiltrating macrophages in histological sections since activated microglia transform to an amoeboid morphology that is indistinguishable from macrophages. However, microglia can be discerned from infiltrating monocytes and macrophages by flow cytometry based on CD45 expression (macrophages are $CD45^{high}$ whereas microglia are $CD45^{low/intermediate}$) (88). Furthermore, advances in next generation sequencing (NGS) and scRNA-seq have identified unique transcriptional profiles of resident microglia versus infiltrating macrophages (89–92), which has led to the identification of markers that are either uniquely (Tmem119, P2YR12, Hexb) or more highly expressed (CX3CR1) in microglia compared to macrophages to aid in their discrimination. Emerging studies from our laboratory have established the transcriptional heterogeneity of resident microglia and macrophage infiltrates in the brain during *S. aureus* craniotomy infection with the goal of identifying unique

markers that will enable the purification of distinct microglial/macrophage clusters to understand their functional role and whether this shapes the chronicity of CNS biofilm infection (62).

Monocytes and Macrophages

Monocytes are bone marrow-derived and invade the inflamed CNS in response to injury or infection primarily *via* a CCR2-dependent pathway (93–95). Studies have implicated monocytes in the pathogenesis of several neurological diseases, including experimental autoimmune encephalomyelitis (EAE) an animal model of multiple sclerosis (MS), where preventing monocyte recruitment or monocyte depletion reduced disease severity (96, 97). To date, fewer studies have examined monocyte responses to *S. aureus*. TLR2 has been shown to regulate *S. aureus* intracellular survival in monocytes *via* a type I IFN pathway and induce IL-10 production to limit T cell responses (98, 99). Future studies are needed to assess the role of monocytes during craniotomy infection since they represent the predominant leukocyte infiltrate in the brain following *S. aureus* invasion (56).

Upon migrating into tissues, bone marrow-derived monocytes differentiate into macrophages. Macrophages are professional phagocytes that, along with microglia, play an important role in eliminating debris and apoptotic cells during inflammation in the brain parenchyma, which is critical for maintaining CNS homeostasis (100). There are three resident macrophage populations associated with the CNS, namely perivascular, meningeal, and choroid plexus macrophages. Perivascular and meningeal macrophages are derived from yolk sac progenitors, whereas choroid plexus macrophages originate from both yolk sac progenitors and the bone marrow (71). Each macrophage population possesses unique phenotypes with different capacities for self-renewal (71, 101), which is likely influenced by the local tissue microenvironment.

Macrophages are critical effector cells during infection, with planktonic *S. aureus* inducing robust proinflammatory cytokine and ROS/RNS production and bactericidal activity (102, 103). However, *S. aureus* expresses a number of virulence determinants to counteract macrophage effector mechanisms. This includes the production of molecules that interfere with TLR2-dependent recognition (104), such as lipase (Geh) (105), staphylococcal superantigen-like protein 3 (SSL3) (106), and molecular mimicry *via* blocking the Toll-interacting receptor (Tir) domain of TLR2 (107, 108). In addition, the paired-immunoglobulin-like receptor (PIR)-B contains an inhibitory immunoreceptor tyrosine-based inhibition motif (ITIM) that, upon binding *S. aureus* lipoteichoic acid, dampens proinflammatory cytokine production (109, 110). Biofilm formation by *S. aureus* also represents another virulence determinant to escape macrophage effector functions. *S. aureus* biofilm evades TLR2-mediated recognition, and macrophage invasion into biofilm is limited *in vitro* and *in vivo*. This biases cells towards an anti-inflammatory profile that prevents bacterial clearance (33). Macrophages are not capable of phagocytosing *S. aureus* biofilm (33), which leads to frustrated phagocytosis and cell death that is mediated, in part, through the action of toxins (α-toxin and leukocidin AB) (37). The adoptive transfer of proinflammatory macrophages in a mouse model of *S. aureus* catheter-associated infection was shown to transform the biofilm milieu into a proinflammatory state concomitant with reduced arginase-1 (Arg-1) expression, which decreased biofilm burden in a MyD88-dependent manner (34). Metabolic reprogramming of monocytes/macrophages to promote their proinflammatory activity was also capable of reducing biofilm burden in a mouse model of PJI (55) highlighting the importance of augmenting macrophage proinflammatory activity as a novel approach to target chronic biofilm infection. The impact of monocytes and macrophages in the pathogenesis of *S. aureus* craniotomy infection and how their metabolic status influences their inflammatory properties remains to be determined and represents an area of investigation in our laboratory.

Neutrophils

PMNs are bone marrow-derived and are released into the circulation at a rate of 10^9/kg body weight per day (111). PMNs are the first leukocytes recruited to sites of bacterial infection by several chemoattractants including IL-8 (functional mouse homologs are CXCL1 and CXCL2), complement split products (C3a and C5a), and formylated peptides released from bacteria (f-Met-Leu-Phe). Upon extravasation, PMNs exert potent bactericidal activity through the action of antimicrobial peptides and granule enzymes, ROS production, neutrophil extracellular traps (NETs), and phagocytic activity (112, 113).

S. aureus encodes an extensive repertoire of virulence factors to escape PMN killing. Molecules such as chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS), formyl peptide receptor-like 1 (FPRL1), staphopain A, and staphylococcal superantigen-like proteins (SSLs) disrupt various aspects of PMN priming, activation, chemotaxis, and adhesion (23, 114, 115). Moreover, *S. aureus* secretes proteins that target complement and opsonophagocytosis (protein A), antioxidants that neutralize ROS (catalase, superoxide dismutase), and numerous toxins with pore-forming properties (phenol soluble modulins, leukocidins, α-toxin) all of which function to diminish PMN antibacterial activity (116–123). Individuals with mutations in NADPH oxidase (chronic granulomatous disease; CGD) are highly susceptible to severe and life-threatening *S. aureus* infections highlighting the critical role of PMNs in bacterial containment (124). Although PMNs are recognized for their beneficial roles during injury or infection (125), dysregulated activity has been implicated in tissue pathology originating from bystander damage *via* products released from activated PMNs (126–128).

The majority of PMNs during *S. aureus* craniotomy infection localize to the galea and bone flap, whereas PMN infiltrates are minimal in the brain. These patterns are similar to the profiles of G-MDSCs recruitment (56). Although earlier work in the *S. aureus* craniotomy infection model suggested an important role for PMNs in preventing bacterial outgrowth, this was with an anti-Gr-1 depletion strategy (19). This approach also targets $Ly6C^+$ monocytes, since the Gr-1 antibody recognizes both Ly6G and Ly6C (129). The functional importance of PMNs during *S. aureus* craniotomy infection was recently demonstrated by our laboratory using a more selective targeting approach (i.e. Ly6G depletion). PMNs were critical for bacterial containment,

although the chronicity of craniotomy infection indicates that PMNs are not capable of eliminating biofilm in the wild type setting (62).

Myeloid-Derived Suppressor Cells

Under physiological conditions, immature myeloid cells undergo maturation in the bone marrow, whereupon they are released and migrate to tissues to become effector macrophages, dendritic cells, or PMNs. During pathologic conditions such as cancer, infection, or chronic inflammation, proinflammatory mediators and/or endoplasmic reticulum (ER) stress drive immature myeloid cell expansion and their conversion into MDSCs (130–132). The growth factors G-CSF and GM-CSF are important for stimulating MDSC expansion with proinflammatory cytokines (IL-1β, TNF-α, IL-6) playing a key role in their activation (133–135). MDSCs exert potent immune-regulatory activity through several mechanisms, including suppressing macrophage and dendritic cell proinflammatory activity, promoting regulatory T cell (Treg) activation, and inhibiting $CD4^+$ and $CD8^+$ T cells. These effects are mediated by the action of several molecules including Arg-1, nitric oxide (NO), TGF-β, IL-10, cyclooxygenase-2 (COX-2), and ROS (133, 134, 136, 137). Through these mechanisms, MDSCs limit inflammation to perpetuate chronic infection by suppressing immune effectors that are important for disease resolution (138).

MDSCs consist of two groups referred to as granulocytic (G-MDSCs or PMN-MDSCs) and monocytic (M-MDSCs) that share phenotypic characteristics with PMNs and monocytes, respectively. Each MDSC subset utilizes distinct mechanisms to attenuate immune responses, where generally M-MDSCs suppress using NO (134, 139, 140), whereas G-MDSCs utilize ROS (141, 142). MDSCs have been best characterized in cancer; however, reports describing their importance during infection and chronic inflammation have emerged in recent years (143–146). Our group has been investigating MDSC-*S. aureus* biofilm crosstalk since 2014, and the role of MDSCs during *S. aureus* infection has been confirmed by other groups (38–41, 147–150). In response to peripheral *S. aureus* biofilm (i.e. PJI), G-MDSCs are critical for inhibiting monocyte/macrophage proinflammatory activity primarily through IL-10 production (38–41). IL-10 is induced by lactate released from *S. aureus* biofilm, which inhibits HDAC11 to induce epigenetic changes at the *Il-10* promoter as well as other genes (41). G-MDSCs are also enriched in humans during PJI, and are expanded in the blood following orthopedic infection (151, 152). This suggests that they may play an important role in dictating infection persistence and/or susceptibility, respectively. MDSCs are the major leukocyte infiltrate in the galea and bone flap during *S. aureus* craniotomy infection, but are rare in the brain parenchyma (56). The transcriptional profiles of MDSCs during craniotomy infection identified them as G-MDSCs, which were shown to inhibit PMN *S. aureus* bactericidal activity (62). The effector molecules that are critical for G-MDSC suppressive activity in the context of *S. aureus* craniotomy infection remain to be identified.

OTHER CELL TYPES IN THE BRAIN DURING *S. AUREUS* CRANIOTOMY INFECTION

Astrocytes

Astrocytes are the most abundant cell type in the CNS parenchyma. They play a key role in maintaining neuronal homeostasis, BBB integrity, and can contribute to immune responses by the production of a wide array of chemokines that promote leukocyte recruitment to the CNS (153–155). *S. aureus* triggers TLR2 signaling in astrocytes and the secretion of NO, IL-1β, and TNF-α *via* NF-kB- and MAPK-dependent pathways (156). Other studies have shown that TLR activation induces astrocyte chemokine production (CCL2, CCL3, CCL5) and augments adhesion molecule expression (157, 158). In astrocytes, the intracellular pattern recognition receptor NOD2 was shown to activate NF-kB leading to IL-6, TNF-α, and co-stimulatory molecule expression, which amplified the anti-bacterial immune response (159). Based on their ability to influence immune responses *via* robust chemokine production, it is possible that astrocytes play an important role in leukocyte recruitment to the brain during *S. aureus* craniotomy infection. Of particular interest would be the production of monocyte, NK cell, and γδ T cell chemokines, since these cell types represent the most abundant leukocyte infiltrates in the brain (62). Studying this will require the use of transgenic mouse models where candidate chemokines are selectively depleted in astrocytes (i.e. Aldh1l1-Cre) (160), since it is not feasible to eliminate astrocytes due to their essential role in brain physiology. However, assigning a biological role to only one chemokine in the context of craniotomy infection might prove difficult based on the known redundancy in chemokine actions (161). An alternative approach would be to identify the chemokine receptors that are required for monocyte, NK cell, and γδ T cell recruitment into the brain and leverage this information to identify the responsible chemokines.

T Cells

T cells participate in CNS immune surveillance and are important for normal learning and memory, behavior, and neurogenesis through IL-4 and IFN-γ production (162–164). It is important to note that these effects occur in the absence of CNS pathology when T cell numbers are low, since it is well recognized that increased T cell recruitment to the brain during diseases such as MS or normal aging is associated with adverse outcomes (164–166).

Interestingly, there are conflicting reports on the role of T cells during *S. aureus* biofilm infection in the periphery. In a model of tibial infection where titanium implants were pre-coated with *S. aureus* (high infectious inoculum), a beneficial role for Th2 and Treg cells in promoting biofilm clearance has been reported (167). In contrast, in a mouse model of *S. aureus* PJI with a low infectious inoculum, few T cells were observed, and tissues from PJI patients have fewer T cell infiltrates compared to individuals with aseptic trauma (39, 151). The reasons for this discrepancy are unclear, but they are likely influenced by

differences in the infectious dose, background strain of mice, or site of implant infection. T cells are observed in the brain during *S. aureus* craniotomy infection, but are largely absent from the galea and bone flap (62). This pattern of recruitment suggests that T cells may play an important role in regulating the host response to craniotomy infection in the brain, but it remains to be determined whether this contributes to infection chronicity, or if T cells are a bystander population and do not significantly influence *S. aureus* biofilm persistence.

IMMUNE RESPONSES DURING *S. AUREUS* CRANIOTOMY INFECTION

As discussed earlier, an intriguing aspect of *S. aureus* craniotomy infection is the generation of distinct immune responses within the CNS (brain) versus peripheral compartments (galea and bone flap; **Figure 2**). Although it might be expected that immune responses would differ in the brain compared to the periphery, prior studies in a mouse model of *S. aureus* brain abscess revealed that inflammatory changes in the brain were similar in nature to peripheral abscesses as described below. Therefore, the *S. aureus* craniotomy model can be leveraged to elucidate signals that orchestrate unique inflammatory events in the brain vs. periphery, which may lead to tailored therapies for each compartment. This would be particularly useful given the fact that CNS neurons cannot regenerate and, as such, eliciting efficient pathogen neutralization without excessive bystander damage that can accompany inflammation is paramount. During *S. aureus* craniotomy infection, PMN influx is significantly higher in the galea compared to the brain despite the presence of CXCL1 and CXCL2 in both compartments (19). This might be explained by the higher bacterial burden in the galea compared to the brain (typically 1-log), although both surfaces of the bone flap are colonized with *S. aureus* (19). Furthermore, the meninges that cover the surface of the brain are patrolled by resident meningeal macrophages that likely serve to limit *S. aureus* invasion into the brain. Interestingly, meningitis is not observed at the histological level in the *S. aureus* craniotomy infection model (19, 56), suggesting that any bacteria that detach from the ventral aspect of the bone flap are prevented from significant expansion in the subarachnoid space. The predominance of PMNs in the galea suggests that they are important for containing infection. This was supported by the finding that mice treated with a Gr-1 antibody became more moribund with increased bacterial burden (19). However, the Gr-1 antibody targets both $Ly6G^+$ and $Ly6C^+$ cells, meaning that not only were $Ly6G^+$ PMNs (and G-MDSCs) depleted, but also $Ly6C^+$ monocytes (168). A subsequent study from our group utilizing selective depletion of $Ly6G^+$ cells has revealed that PMNs are critical for preventing *S. aureus* outgrowth during craniotomy infection, yet mice were not moribund unlike that observed following Gr-1 antibody treatment (62). By extension, this suggests that monocytes/macrophages also play a protective role during craniotomy infection, since they were not depleted with anti-Ly6G. An interesting observation is that although G-MDSCs are also targeted by anti-Ly6G, the removal of this suppressive population did not improve biofilm clearance. Instead, the opposite was observed, suggesting that PMNs are the main driver of biofilm containment in the craniotomy infection model (62).

Based on the chronicity of *S. aureus* craniotomy infection in the mouse model (at least 9 months, the latest time point examined to date), it might be assumed that there is minimal involvement of proinflammatory mechanisms (46). However, as alluded to above, there is some degree of proinflammatory tone during craniotomy biofilm infection because PMN/monocyte depletion with anti-Gr-1 results in rapid *S. aureus* outgrowth in the brain, galea, and on the bone flap (19). Another indication that proinflammatory cytokines are critical for bacterial containment has been through the examination of TLR signaling pathways. Our initial study examined MyD88, the adaptor molecule that facilitates signaling through all TLRs (except TLR3), IL-1R, and IL-18R, and is a critical factor in innate immune defense (169). MyD88 KO mice were extremely susceptible to *S. aureus* craniotomy infection with a significant reduction in PMN infiltrates and proinflammatory mediator production (CXCL1 and IL-1β) that resulted in increased bacterial burden in the brain, galea, and bone flap (19). These phenotypes combined with the enhanced morbidity of MyD88 KO mice were akin to the effects seen during anti-Gr-1 treatment where essentially all innate immune effectors were depleted (PMNs and monocytes) (19). The importance of TLRs and downstream effector mechanisms was further demonstrated by our recent work that revealed a crucial role for TLR2 and caspase-1 during *S. aureus* craniotomy infection, primarily *via* IL-1β action (56). Interleukin-1β is produced in an inactive pro-form that requires proteolytic cleavage by the inflammasome whose active moiety is caspase-1 (170). Inflammasome activation involves two signals; the first being delivered by a PRR, such as TLR2, which leads to the production of inflammasome subunits and pro-IL-1β. The second signal can be delivered by any number of stimuli depending on the type of NLR sensor (i.e. NLRP3, NLRC1, etc.) that results in inflammasome assembly and caspase-1 activation (170). Mice lacking either functional TLR2 or caspase-1 displayed increased bacterial burden in the brain, galea, and bone flap, which coincided with significant decreases in the production of several proinflammatory mediators including IL-1β (56). A critical role for IL-1β in bacterial containment was established by the fact that treatment of caspase-1 KO mice with IL-1β-containing microparticles returned the exaggerated bacterial burden in these animals to levels observed in WT mice (56). These findings revealed the essential role of the TLR2/caspase-1/IL-1β axis in bacterial containment during *S. aureus* craniotomy infection (**Figure 2**). The importance of TLR2 in preventing *S. aureus* outgrowth is intriguing given the number of *S. aureus* virulence factors that target this signaling pathway (28) as described earlier. One explanation is that these TLR2 evasion molecules have been described during planktonic growth, and it is unknown whether they are expressed during biofilm formation. In addition, it is clear that although TLR2-

dependent pathways are capable of limiting *S. aureus* biofilm outgrowth, they are not sufficient to clear infection, since biofilm persists in the wild type setting. This was further demonstrated by the finding that exogenous IL-1β treatment was not able to reduce *S. aureus* burden in WT mice, revealing the recalcitrance of biofilm to proinflammatory signals (56). Interestingly, although TLR9 is an important sensor for staphylococcal DNA that is a major component of the *S. aureus* biofilm matrix (29), TLR9 had minimal impact on the course of craniotomy infection (56). This may result from the fact that TLR9 is an intracellular PRR that requires phagocytic uptake of bacteria or eDNA, and prior studies have demonstrated that *S. aureus* biofilm interferes with macrophage phagocytosis (33, 37). Collectively, these findings highlight the fact that anti-bacterial pathways are operative during craniotomy infection; however, this is difficult to appreciate in a wild type setting based on the chronicity of infection.

There are many unknowns regarding the pathogenesis of *S. aureus* craniotomy infection. One critical point relates to identifying the mechanisms responsible for biofilm persistence despite antibiotic treatment. Second, we know little about the contributions of brain-resident cells, such as microglia and astrocytes, which are capable of influencing immune responses. In addition, we have recently identified a prominent influx of NK and γδ T cells in the brain during *S. aureus* craniotomy infection (62) and it will be interesting to examine the functional significance of each population in future studies. Finally, it will be critical to identify *S. aureus* virulence determinants that are important for promoting biofilm persistence, and to evaluate whether unique *S. aureus* transcriptional signatures are observed in bacteria recovered from the brain, galea, or the bone flap where the physical biofilm resides. These are all topics for future investigation.

S. AUREUS CRANIOTOMY INFECTION AND BRAIN ABSCESS: SIMILARITIES AND DISTINCTIONS

Brain abscesses pose a challenging clinical problem, which can be associated with high mortality rates due to brain compression and neuronal death from associated edema (171). Pyogenic staphylococci and streptococci are among the most prevalent bacterial species associated with brain abscesses emanating from hematogenous spread (172). Abscess formation may also arise as a complication of neurosurgery or head trauma, and is commonly associated with *S. aureus* (8, 173). Anatomically, a brain abscess possesses a well-formed necrotic center containing bacteria and PMNs and is surrounded by a dense capsule composed of macrophages and myofibroblasts (174). Activated microglia and astrocytes are observed in the brain parenchyma surrounding the abscess margins along with extensive edema. Astrocytes have been shown to play an important role in regulating brain abscess pathology, since mice deficient for the astrocytic intermediate filament glial fibrillary acidic protein (GFAP) displayed increased bacterial burden, large lesion size, and diffuse leukocyte infiltration (175). These effects likely resulted from the inability to wall off the abscess, since GFAP is a cytoplasmic protein and its loss does not eliminate astrocytes in the brain parenchyma.

S. aureus craniotomy and brain abscess fundamentally differ based on their chronicity and degree of inflammation. In mouse models, *S. aureus* brain abscesses are shorter in duration typically resolving within 14–21 days (174); whereas during *S. aureus* craniotomy infection, bacteria are detectable on the infected bone flap, galea, and the brain for as long as 9 months (46) despite both models utilizing a similar infectious dose (i.e. 10^3 cfu vs. 10^4 cfu for craniotomy and brain abscess, respectively). Second, there are distinctions at the histological level. Craniotomy-associated infection is not typified by abscess formation in the brain parenchyma of WT animals (19, 56), which is obviously distinct from brain abscess where a solitary lesion is elicited (176, 177). Furthermore, brain abscesses are associated with significant edema (177, 178), whereas edema is not a prominent feature of craniotomy infection in the brain, although a purulent exudate forms in the galea (19, 56). Third, there are distinctions in the patterns of leukocyte recruitment. Craniotomy infections are typified by a more complex immune response owing to differential leukocyte recruitment across the brain, galea, and bone flap. The infected bone flap and galea are dominated by granulocytic infiltrates (i.e. PMNs and G-MDSCs) in regions that coincide with the highest bacterial burden (56). In contrast, the brain is typified by a monocytic infiltrate and an approximate 1-log reduction in bacteria compared to the bone flap and galea. Although brain abscesses are typically a solitary lesion, there is still some degree of specificity in leukocyte homing to particular niches. PMNs migrate primarily to the necrotic core, whereas macrophages are detected along the fibrotic abscess capsule (179).

When comparing the functional roles of TLR2 and MyD88 in both models, some similarities and distinctions are noted (**Figure 3**). First, differences between *S. aureus* craniotomy infection and brain abscesses can be seen at the level of TLR2 and MyD88 involvement in bacterial containment. For example, bacterial burden was similar in brain abscesses of MyD88 KO and WT mice (178); whereas, MyD88 was critical for preventing *S. aureus* outgrowth during craniotomy-associated infection (19). A similar finding was observed with respect to TLR2 where bacterial burden was exaggerated in TLR2 KO animals during craniotomy-associated infection (56), but was comparable in brain abscesses of WT and TLR2 KO mice (180). This is particularly interesting since proinflammatory mediator production was reduced in TLR2 KO animals in both models, revealing the involvement of TLR2-independent pathways in controlling bacterial burden during brain abscess formation.

In terms of similarities between *S. aureus* craniotomy infection and brain abscess three examples are evident. First, neither model is dependent on TLR9, which could explained by the fact that TLR9 is an endosomal receptor that requires phagocytosis for pathogen-derived DNA to engage the receptor (181). *S. aureus* is known to inhibit opsonophagocytosis *via* protein A (SpA) production, and *S. aureus* biofilm attenuates

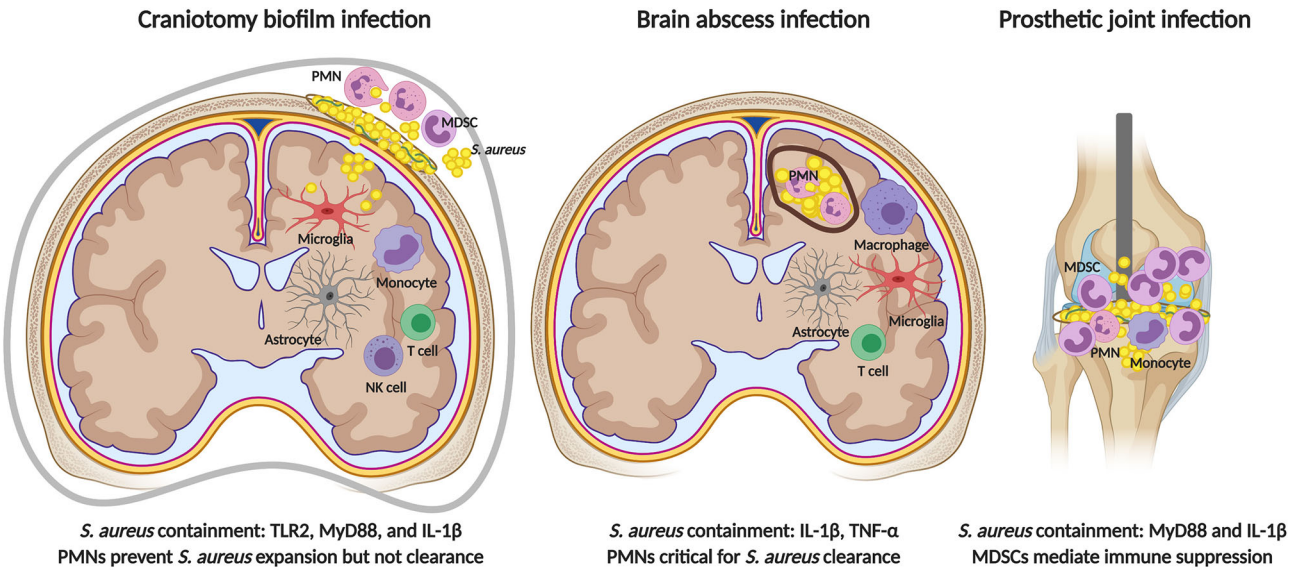

FIGURE 3 | Comparisons in immune responses elicited by *S. aureus* in the CNS versus periphery. TLR2 signaling is critical for preventing bacterial outgrowth during *S. aureus* craniotomy infection (left), but is dispensable during brain abscess (center) and peripheral biofilm infection (right). MyD88-dependent signals are critical for restricting *S. aureus* growth during craniotomy and peripheral biofilm infection, and IL-1β production is key in all three models. PMNs are essential for brain abscess resolution and bacterial containment during craniotomy infection, but do not influence peripheral biofilm infection where infection chronicity is mediated by the inhibitory action of MDSCs on monocyte pro-inflammatory activity. Figure created with BioRender.

macrophage phagocytic activity (33, 37, 182). Second, IL-1β plays an important role in *S. aureus* containment in both craniotomy infection and brain abscess although the pathways leading to IL-1β production differ (**Figure 3**) (56, 183, 184). During *S. aureus* craniotomy infection, caspase-1 was required for maximal IL-1β production; however, there was no role for the well-characterized inflammasome protein NLRP3 or its adaptor molecule ASC (56). Therefore, the NLR sensor that is required for inflammasome assembly and caspase-1 activation during craniotomy infection remains unknown. In contrast, IL-1β release during *S. aureus* brain abscess required the AIM2 inflammasome and ASC but, similar to craniotomy infection, was NLRP3-independent (184). These findings demonstrate the involvement of distinct inflammasome platforms for triggering IL-1β release during *S. aureus* biofilm-associated craniotomy infection versus brain abscess.

Finally, *S. aureus* craniotomy and brain abscess infection share a critical role for PMNs in bacterial containment (**Figure 3**). Mice lacking CXCR2, the receptor for the PMN chemokines CXCL1 and CXCL2, showed minimal PMN recruitment into the infected brain parenchyma and higher bacterial burden in the brain abscess model (185). Likewise, anti-Gr-1 treatment mimicked these findings with exaggerated bacterial burden and a failure to limit the extent of tissue damage during brain abscess development, although monocytes were also targeted with this antibody (185). Similar phenotypes were observed during *S. aureus* craniotomy infection, where anti-Gr-1 administration led to a significant outgrowth of bacteria in the brain, galea, and bone flap within 48 h post-infection concomitant with increased morbidity (19). Subsequent studies to refine cell depletion to only the PMN/G-MDSC populations with anti-Ly6G produced similar findings with exaggerated bacterial burden (62). However, unlike anti-Gr-1 depletion where effects were observed within 48 h the anti-Ly6G phenotype was delayed in comparison, becoming significant at day 7 post-infection, and no morbidity was observed (62). Collectively, these findings reveal the essential role of PMNs in *S. aureus* brain abscess resolution and although PMNs are important for preventing bacterial outgrowth during *S. aureus* craniotomy infection, the fact that animals tolerate PMN depletion suggests the involvement of other immune populations, the identity of which remains to be determined. Furthermore, it is important to emphasize that *S. aureus* craniotomy infection persists even when PMNs are present establishing their ineffectiveness at biofilm clearance *in vivo*. This may result, in part, by the ability of G-MDSCs to inhibit PMN killing of *S. aureus* (62).

S. AUREUS CRANIOTOMY VERSUS PERIPHERAL BIOFILM INFECTION: IMPORTANCE OF TISSUE NICHE

S. aureus is a common etiologic agent of infections associated with prosthetic joints and other indwelling medical devices (186, 187). Over the years, our laboratory and others have identified numerous mechanisms used by *S. aureus* biofilm to evade host immune responses (186, 188, 189). One hallmark of *S. aureus*

biofilm infection outside the CNS is the prevalence of leukocyte infiltrates that display anti-inflammatory properties. One example is MDSCs, immature myeloid cells that have the ability to suppress T cell activation and monocyte/macrophage proinflammatory activity (38). G-MDSCs are the major leukocyte infiltrate in a mouse model of *S. aureus* PJI in addition to patients with PJI (38, 40, 41, 151). Depletion of G-MDSCs and PMNs in the mouse *S. aureus* PJI model using anti-Ly6G transformed infiltrating monocytes to a proinflammatory state that led to a significant reduction in biofilm burden (38). In contrast, although G-MDSCs and PMNs are most abundant in the galea and bone flap during *S. aureus* craniotomy infection, Ly6G depletion of these cells resulted in bacterial outgrowth (62). Therefore, although both models are associated with G-MDSC infiltrates, these results suggest their differential involvement in dictating infection outcome (**Figure 3**). An alternative explanation could be differences in the abundance of PMNs in both infection models. PMN infiltrates are minimal in *S. aureus* PJI (which instead is dominated by G-MDSCs), whereas PMNs are more abundant in the galea and bone flap during *S. aureus* craniotomy infection. Therefore, the inability to contain *S. aureus* following Ly6G depletion in the craniotomy model may result from the loss of the larger PMN population that is a critical bactericidal effector.

Another distinction between PJI and craniotomy infection is the presence of innate and adaptive lymphoid populations in the latter. Both human PJI and the mouse model have few T cell infiltrates (39, 151) in agreement with the ability of the dominant G-MDSC population to inhibit T cell activation/proliferation (190). In contrast, *S. aureus* craniotomy infection is associated with significant NK and γδ T cell recruitment in the brain, with fewer T and B cells (62). These populations are largely absent from the galea and bone flap revealing a unique microenvironment in the brain that is responsible for the recruitment of these lymphoid populations.

A final difference between *S. aureus* biofilm infection in the CNS vs. periphery is demonstrated by the role of TLR2 in disease. As mentioned earlier, TLR2 is critical for bacterial containment during *S. aureus* craniotomy infection (56), whereas the receptor is dispensable during peripheral biofilm infection (**Figure 3**) (33). Despite the differential involvement of TLR2, the TLR/IL-1R adaptor MyD88 is plays an equally important role in preventing *S. aureus* outgrowth during both CNS and peripheral biofilm infection (19, 34, 191). This phenotype is likely driven by IL-1β given that the loss of IL-1β production or signaling results in increased bacterial outgrowth in both CNS and peripheral models of *S. aureus* biofilm infection (32, 56, 192).

THERAPEUTIC STRATEGIES FOR CRANIOTOMY-ASSOCIATED INFECTION

Despite extensive precautionary measures, post-operative complications following craniotomy continue to occur with *S. aureus* responsible for approximately one-half of these infections (18). Multiple surgeries, prolonged hospital stays, and significant mortality confound the complications arising from craniotomy infections (193). Current treatment strategies include the *ex vivo* submergence of the infected bone flap in an antiseptic solution and aggressive debridement prior to re-insertion; however, these have not yet been adopted as standard-of-care practices (194, 195). Surgical drainage in combination with a prolonged antibiotic regimen can often effectively control infection (196), which is largely dictated by the interval between surgery and presentation of clinical signs of infection. In some instances, the bone flap cannot be salvaged and a cranioplasty is performed using a bone graft or alloplastic prosthesis. In either case, patients are subjected to an extended antibiotic regimen lasting for months. Because a second surgery is often required for treatment, and the potential for infection recurrence, devising novel therapeutic approaches may significantly improve the outcome of craniotomy infection without the need for more radical interventions.

Previously, our laboratory demonstrated the efficacy of administering proinflammatory macrophages to promote biofilm clearance *in vivo* (34). We leveraged this observation to evaluate the efficacy of a 3D bioprinted bone scaffold that incorporated viable macrophages and an antibiotic cocktail as a localized delivery system for the treatment of *S. aureus* craniotomy infection (46). The rationale for including viable macrophages was that they might facilitate biofilm dispersal making bacteria more susceptible to antibiotic action. The 3D bioprinted scaffold was capable of reducing established biofilm infection, since scaffold implantation at day 7 post-infection led to a significant reduction in bacterial burden and reduced BBB damage that is associated with chronic *S. aureus* infection (46). Interestingly, although macrophage incorporation into 3D scaffolds was effective at diminishing an established biofilm, this was not the case in a prophylactic paradigm; therefore, subsequent studies focused on increasing the antibiotic dose in the scaffold. This approach mitigated bacterial burden to below the limit of detection for 2 weeks; however, *S. aureus* outgrowth was observed after this period due to the loss of antibiotic from the scaffold. Therefore, second generation 3D scaffolds are currently being developed by our group that incorporate additional bioactive moieties designed to negate the outgrowth of residual bacteria after the antibiotic has exited the scaffold. Other strategies that could be leveraged to enhance therapeutic efficacy include the use of tagged nanoparticles to target a specific immune population to enhance its microbicidal activity. Furthermore, the use of systemic antibiotics once the primary biofilm burden has been reduced by 3D bioprinted scaffolds will be critical to clear residual bacteria, which was supported by our recent study (46).

Other therapeutic approaches have utilized nanoparticle-based delivery systems to augment immune cell function (197, 198). We recently employed a similar strategy to deliver IL-1β-containing microparticles to attenuate bacterial burden in caspase-1 KO mice during *S. aureus* craniotomy infection (56). It will be interesting to see how these rapidly evolving

therapeutics can modulate infection in mouse biofilm models for potential translation to the clinic.

CONCLUSIONS AND PERSPECTIVES

Several studies have highlighted niche-specific differences in the composition of leukocyte infiltrates and their ensuing inflammatory responses during infection (199, 200). One example is a mouse model of visceral leishmaniasis, where parasites are cleared within 2 months following intravenous injection in the liver but are present in the spleen and bone marrow throughout the life of the animal (201). This is thought to result from alterations in adaptive immunity and macrophage function in each of these locations. Tissue-specific cues are exemplified when comparing the immune responses elicited by *S. aureus* biofilm in the periphery that, in general, are characterized by an anti-inflammatory phenotype whereas more proinflammatory responses are elicited during CNS biofilm infection. Deciphering the signals emanating from different tissues will be crucial for understanding the pathogenesis of biofilm infection and for developing selective treatment strategies to avoid adverse side effects. The use of conditional KO mice will be an extremely important tool to understand the role of immune mediators in leukocyte populations enriched in a given tissue niche. This is particularly relevant in the case of *S. aureus* craniotomy-associated infection that is more complex in terms of distinct leukocyte subsets across various tissue domains.

It is intriguing how *S. aureus* can elicit markedly different immune responses depending upon the site of infection. As discussed, *S. aureus* craniotomy infection displays a compartmentalized immune response within affected CNS regions, and recent RNA-seq studies have begun to decipher the pathways that program a given immune population in its unique niche (62). Imaging modalities such as intravital microscopy would provide an unprecedented window into *S. aureus*-leukocyte interactions and migratory patterns associated with CNS resident vs. invading immune cells in real time. Identifying the factors that modulate changes in transcriptional networks, the nature of host-pathogen interactions, and patterns of leukocyte migration would provide a better understanding of *S. aureus*-leukocyte crosstalk and ultimately pave the way for developing tailored therapeutic strategies to mitigate *S. aureus* biofilm infections within the CNS or periphery.

AUTHOR CONTRIBUTIONS

SD and GK contributed equally to writing the manuscript draft with clinical information provided by JM. The initial editing was performed by TK and all authors contributed to subsequent editing. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: A patent has been filed with the US Patent and Trademark Office covering the application of 3D bioprinted scaffolds for the treatment of craniotomy-associated infections that is discussed in this review (PCT/US2020/021440; TK).

The 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.

GLOSSARY

3D	three-dimensional
Arg-1	arginase-1
AIM2	absent in melanoma 2
ASC	apoptosis-associated speck-like protein containing a carboxy-terminal CARD
BBB	blood-brain barrier
CCL2	monocyte chemoattractant protein-1
CNS	central nervous system
CSF-1	macrophage colony stimulating factor-1
CXCL1	keratinocyte chemoattractant
CXCL2	macrophage inflammatory protein-2
CXCL10	interferon-inducible protein 10 kDa
CXCR2	C-X-C receptor 2
EAE	experimental autoimmune encephalomyelitis
eDNA	extracellular DNA
ER	endoplasmic reticulum
G-CSF	granulocyte colony-stimulating factor
GFAP	glial fibrillary acidic protein
G-MDSC	granulocyte myeloid-derived suppressor cell
γδ T cell	gamma-delta
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN	interferon
IL	interleukin
KO	knockout
MAPK	mitogen-activated protein kinase
MDSC	myeloid-derived suppressor cell
M-MDSC	monocyte myeloid-derived suppressor cell
MyD88	myeloid differentiation factor 88
NET	neutrophil extracellular trap
NGS	next generation sequencing
NF-κB	nuclear factor-kappa B
NK cell	natural killer cell
NLR	NOD-like receptor
NO	nitric oxide
NOD2	nucleotide-binding oligomerization domain-containing protein 2
PJI	prosthetic joint infection
PMN	neutrophil
PRR	pattern recognition receptor
RNI	reactive nitrogen intermediate
ROS	reactive oxygen species
S. aureus	Staphylococcus aureus
scRNA-seq	single cell RNA-sequencing
Spa	protein A
TGF-β	transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
WT	wild type

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Microbiome or Infections: Amyloid-Containing Biofilms as a Trigger for Complex Human Diseases

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The human microbiota is the community of microorganisms that live upon or within their human host. The microbiota consists of various microorganisms including bacteria, fungi, viruses, and archaea; the gut microbiota is comprised mostly of bacteria. Many bacterial species within the gut microbiome grow as biofilms, which are multicellular communities embedded in an extracellular matrix. Studies have shown that the relative abundances of bacterial species, and therefore biofilms and bacterial byproducts, change during progression of a variety of human diseases including gastrointestinal, autoimmune, neurodegenerative, and cancer. Studies have shown the location and proximity of the biofilms within the gastrointestinal tract might impact disease outcome. Gram-negative enteric bacteria secrete the amyloid curli, which makes up as much as 85% of the extracellular matrix of enteric biofilms. Curli mediates cell-cell attachment and attachment to various surfaces including extracellular matrix components such as fibronectin and laminin. Structurally, curli is strikingly similar to pathological and immunomodulatory human amyloids such as amyloid-β, which has been implicated in Alzheimer's disease, α-synuclein, which is involved in Parkinson's disease, and serum amyloid A, which is secreted during the acute phase of inflammation. The immune system recognizes both bacterial amyloid curli and human amyloids utilizing the same receptors, so curli also induces inflammation. Moreover, recent work indicates that curli can participate in the self-assembly process of pathological human amyloids. Curli is found within biofilms of commensal enteric bacteria as well as invasive pathogens; therefore, evidence suggests that curli contributes to complex human diseases. In this review, we summarize the recent findings on how bacterial biofilms containing curli participate in the pathological and immunological processes in gastrointestinal diseases, systemic autoimmune diseases, and neurodegenerative diseases.

Keywords: curli, biofilm, microbiome, systemic lupus erythematosus, reactive arthritis, Parkinson's disease, Alzheimer's disease, colorectal cancer

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INTRODUCTION

The community of microorganisms that live upon or within a host are referred to as the microbiota. The human microbiota includes bacteria, fungi, viruses, and archaea that colonize the surface or deep layers of the skin (skin microbiota), the mouth (oral microbiota), the vagina (vaginal microbiota), and the digestive tract (gut microbiota) (1). The human microbiota has received

increasing attention in numerous research fields over the last 15 years. The gut microbiota is of interest as numerous studies have reported that there are changes in the gut microbiota during obesity, diabetes, liver diseases, cancer, and neurodegenerative diseases (2–7).

Studies of the diversity of the human microbiota started as early as the mid-seventeenth century with Antonie van Leeuwenhoek who compared the oral and the fecal microbiota. He observed differences between the microbes in these two locations and also between samples from healthy vs. diseased individuals (8). In 2001, Joshua Lederberg coined the term microbiome to refer to the "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (9). Many fundamental questions concerning the human microbiota have been difficult or impossible to address until recently. In 2017, ∼4,000 publications focusing on the study of the gut microbiota were published, accounting for 80% of the publications on the subject since 1977 (2). The advancement of laboratory techniques and "omics" technologies have allowed researchers to characterize the composition of the microbiota and its functions in human health and disease (10).

The coevolution of humans and their microbial symbionts have selected for a specialized community of microorganisms that thrive in the gut (11). Bacteria comprise the bulk of the gut microbiota with archaea, eukaryotes, and viruses present in much smaller numbers (10). In a healthy human adult, the gut microbiota is dominated by two phyla: Firmicutes and Bacteroidetes (12). Other phyla, including Actinobacteria, Proteobacteria, Verrucomicrobia, and Euryarchaeota, are found in lower abundance (12). The gut microbiota is unlike any free-living microbial communities found in the environment due to the unique environment of the digestive tract (13). The microbiota plays a crucial role in maintaining immune and metabolic homeostasis and protecting the host against pathogens through microbial crosstalk with the mucosal immune system through integrated signaling pathways and gene regulatory networks (13–15). The interactions between the host immune system and the colonizing gut microbiota initiate at birth and are important for host immune system development and homeostasis (16–18). A variety of genetic and environmental factors influence the composition and the function of the gut microbiota including host diet, genetics, age, location, and medication use, especially antibiotics (19). When this homeostatic relationship is disrupted, it can lead to dysbiosis or "an imbalance in the composition and metabolic capacity of our microbiota" (20). Growing evidence indicates that dysbiosis shifts the microbiota in ways that increase inflammation and accelerate the onset or contribute to the pathogenesis of chronic diseases (20) such as cardiovascular disease, obesity, diabetes, cancer, asthma, and inflammatory bowel disease (20–26).

Many species of bacteria that colonize the gut live in biofilms. A bacterial biofilm is a group of bacteria that are encapsulated in a three-dimensional, self-produced extracellular matrix that is adhered to a biotic or abiotic surface (27, 28). The biofilm provides a layer of protection to microorganisms that grow in stressful environments where nutrients are scarce and during changes in temperature, osmolarity, and oxygen availability (29–31). Furthermore, the biofilm blocks access by toxic agents such as antibiotics and the host's immune system (32).

Biofilms can be composed of a single species of bacteria or a consortium of multiple species of bacteria. Biofilms can be formed by a variety of bacterial species including Gram-positive (e.g., *Bacillus* spp, *Listeria monocytogenes*, *Staphylococcus* spp, and lactic acid bacteria including *Lactobacillus plantarum* and *Lactococcus lactis*) and Gram-negative species (e.g., *Escherichia coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa*) (33). The Proteobacteria phylum, which makes up ∼0.1% of the gut microbiota in a healthy individual, expands during inflammation. The Gammaproteobacteria class includes several medically and scientifically important families including *Enterobacteriaceae*, Vibrionaceae, and Pseudomonadaceae. *Enterobacteriaceae* is a large family of Gram-negative bacteria that includes many harmless enteric commensals as well as pathogens such as *Salmonella*, *E. coli*, *Yersinia pestis*, *Klebsiella*, and *Shigella*. All these bacteria have the ability to make biofilms (30, 34–37).

Bacterial biofilms form throughout the human oro-gastrointestinal tract and mixed species biofilms have been observed in dental and gastric infections as well as in intestinal diseases, chronic gut wounds, and colon cancer (38). Biofilms are also formed by species necessary for a healthy gut mucosa, and these biofilms may benefit the host by fortifying host defenses, enhancing the exchange of nutrients between the microbiota and the host, and interfering with colonization by pathogenic bacteria (38). Therefore, biofilms within the human gut can be both beneficial and detrimental to the host depending on whether they are produced by the commensal microbiota or enteric pathogens (38).

Only 10% of the biomass of the biofilm are actual bacterial cells (29, 39). The composition and the structure of the biofilm is dependent upon the bacteria within it and the environment in which it is formed (27, 28). The bacterial cells within a biofilm are physiologically distinct from their planktonic counterparts (40). In a biofilm, cells are embedded within an extracellular matrix (ECM) composed of extracellular polymeric substances such as lipids, polysaccharides, proteins, and DNA (41–43). The ECM accounts for 90% of the total biomass of the biofilm. In *E. coli* biofilms, the major proteinaceous component, which comprises 85% of the ECM, is the amyloid curli. Curli encapsulates individual bacterial cells and forms an interwoven mesh that supports the ECM (44–46). Curli expression is triggered when enteric bacteria are grown under stressful environmental conditions that favor biofilm formation over planktonic cell growth. Curli is responsible for the overall development of the biofilm architecture (47–50) as curli-deficient bacteria do not form mature three-dimensional biofilms and only grow in a single cell layer.

Curli proteins form thin amyloid fibers on the surface of enteric bacterial cells (51–53). These fibers range from 4 to 10 nm in width and have a β-sheet-rich structure in which the β-sheet strands are orientated perpendicular to the axis of the fiber (54). Human amyloids also have a cross-beta structure and share a strikingly similar quaternary structure with bacterial amyloids, including the pathological and immunomodulatory

TABLE 1 | Human diseases exaggerated by bacterial biofilms or biofilm by-products.

Biofilm related condition/disease	Bacteria	Biofilm component/byproduct	Proposed mechanism
Systemic lupus erythematosus (SLE)	*S.* Typhimurium, *E. coli*	Amyloid curli, other bacterial amyloids	Formation of complexes between curli and DNA increases type I interferons and autoantibody production leading to disease flares
Reactive Arthritis (ReA)	*S.* Typhimurium,	Amyloid curli	Increases proinflammatory cytokines and autoimmune response leading to joint inflammation
Parkinson's disease and Alzheimer's disease	*E. coli, Pseudomonas*	Amyloid curli, FapC	Neuroinflammation, increasing fibrillation and deposition of α-synuclein or amyloid-β in the brain
	Commensal Gram-negative bacteria	Endotoxin	During aging, increases blood-brain-barrier permeability, co-localizes with Aβ plaques in the brain, implicated in aggregation of α-synuclein
	Porphyromonas gingivalis	Gingipains	Found within the brains of AD patients, associated with neurotoxicity and neuroinflammation
Colorectal cancer	Fusobacteria, *Alistipes,* Porphyromonadaceae, Coriobacteridae, *Staphylococcaceae*, *Akkermansia*, Methanobacteriale	Unknown	Significant outgrowth of these bacteria in intestinal microbiota of CRC patients
	Bacteroides fragilis, E. coli	*B. fragilis* toxin, colibactin and biofilms	Carcinogenic toxins and biofilms propagate the formation of tumors
	Fusobacterium nucleatum	Adhesin molecule FadA	Induces oncogenic and inflammatory responses in the gut
	Campylobacter jejuni	Cytolethal descending toxin	Induces changes in microbial composition and toxigenic processes

human amyloids such as amyloid-β (Aβ), which is involved in Alzheimer's disease (AD), α-synuclein (αSyn), which is implicated in Parkinson's disease (PD), and serum amyloid A (55–59).

Like other amyloids, curli is a conserved molecular pattern that causes the activation of toll-like receptors (TLR) 1 and 2 as well as intracellular NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome (60–62). Studies have shown that the immune system recognizes both bacterial amyloid curli and human amyloids through the same receptors that facilitate the inflammatory processes (61). Recent studies have demonstrated that curli and curli-associated biofilms in the gut participate in the pathogenesis of human diseases, including colorectal cancer, systemic lupus erythematosus (SLE), and PD (**Table 1**). In this review, we summarize the recent findings that suggest how bacterial biofilms containing curli participate in pathological and immunological processes of these diseases by direct interactions such as cross-seeding of human amyloids and by indirect interactions that trigger inflammation.

CURLI CONTAINING BACTERIA IN THE GASTROINTESTINAL TRACT AND THE URINARY TRACT

Biofilms can occur throughout the entire length of the gastrointestinal tract (38) and can be disease-linked or important for health. Two driver species of pathogenic biofilm formation in the gut are *Salmonella* and *E. coli*. These enteric bacteria thrive in a pro-inflammatory environment, conditions in which they outcompete the commensal microbiota. An outgrowth of enteric bacteria is common during inflammatory states that are associated with many gut disorders (63). It was highly debated whether or not enteric bacteria expressed curli and grow as biofilms in the gut. However, there was indirect evidence in support of this: for example, patients recovering from *E. coli*-induced sepsis harbor antibodies against curli (64). Similarly, antibodies against curli are detected after infection of mice with *Salmonella enterica* serovar Typhimurium (65). A recent study showed direct evidence for curli synthesis in the intestinal tract by *S.* Typhimurium during infection (66). Curli is recognized as a PAMP by the mucosal immune system *via* TLR2/TLR1 heterocomplex (60, 62) leading to the activation of *NF-κB*, eliciting the production of proinflammatory chemokines and cytokines including, IL-6, IL-8, and IL-17A (61, 67). The detection of curli by a healthy gut mucosa leads to reinforcement of the gut barrier preventing the leakage of bacteria and possibly the pathological bacterial amyloids (**Figure 1**) (68, 69).

The urinary tract microbiome is just beginning to be characterized. Uropathogenic *E. coli* (UPEC), the most common cause of urinary tract infections (UTIs) (70, 71), is a member of the family *Enterobacteriaceae* and has the ability to form a curli-containing biofilm (72). UPEC is a frequent colonizer of medical devices and the primary cause of recurrent urogenital infections (73). UPEC forms curli-containing biofilms that are difficult to treat and eradicate with antibiotics leading to multidrug resistance (73). UTI infections can become persistent and result in bacteriuria which can lead to sepsis (71). Expression of *csg* gene cluster, genes that encode curli, is upregulated in UPEC isolated from urine from patients suffering from UTIs, whereas no *csg* expression is detected in urine from healthy controls (74, 75). It was reported that curli promotes colonization and immune

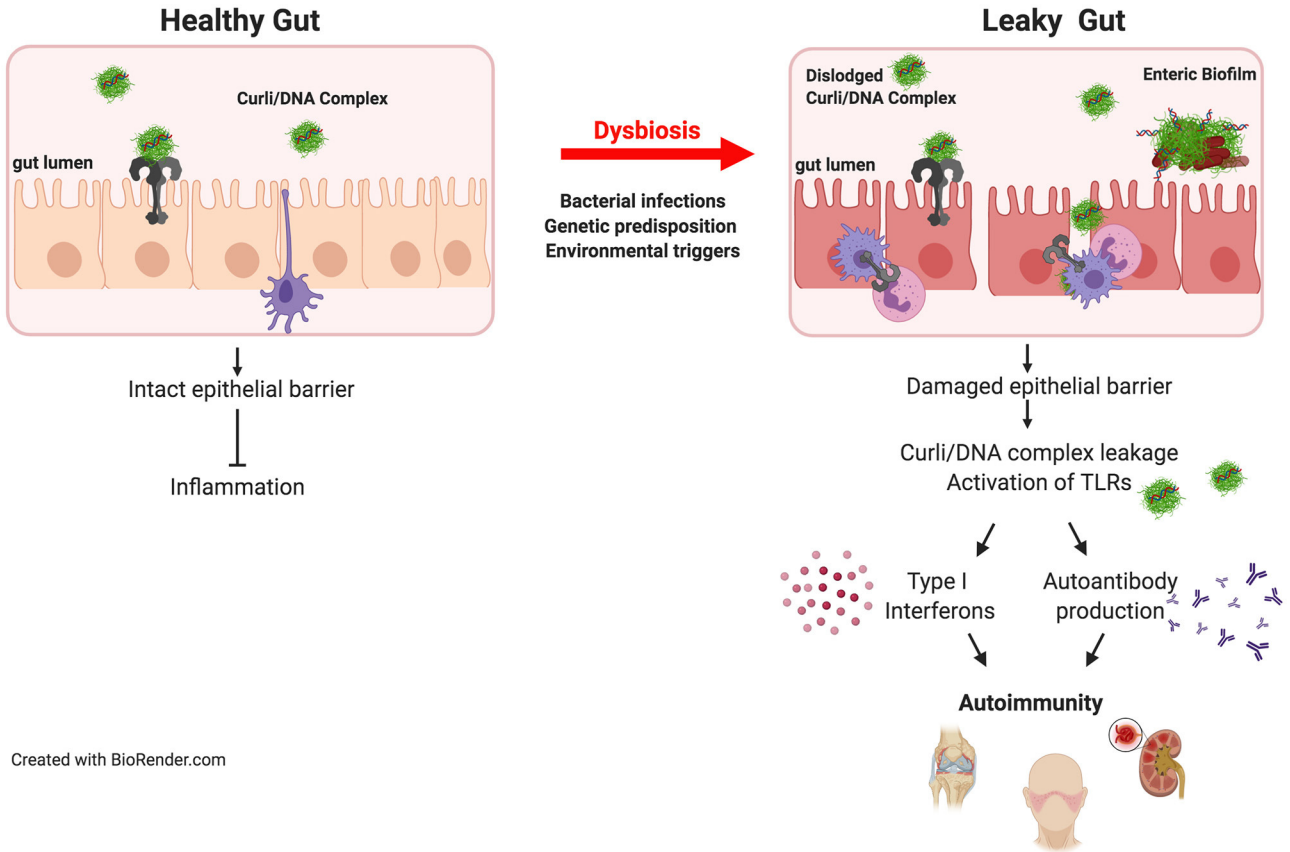

FIGURE 1 | Bacterial amyloids and autoimmunity. Amyloids are produced by the members of gut microbiome. Curli/DNA complexes produced by commensal bacteria are recognized by TLR2/TLR1 heterocomplex which dampens inflammation in healthy intestinal tract. However, when the epithelial barrier is damaged during invasive infections or by other environmental factors or diseases, dislodged curli/DNA complexes from the biofilm activates TLR2/TLR1 heterocomplex and TLR9 leading to the generation of type I interferons and autoantibodies resulting in initiation and exacerbation of autoimmunity. In genetically predisposed individuals, the autoimmune effects of the curli/DNA complexes will likely to be exacerbated.

induction by enhancing bacterial adherence and invasion into the uroepithelium during early stages of UTI (74).

THE INVOLVEMENT OF BACTERIAL AMYLOIDS IN AUTOIMMUNE DISEASES

The primary function of the immune system is to protect the host from possibly harmful substances and pathogens. The first line of defense against non-self-pathogens is the innate immune response. It immediately prevents the spread of foreign pathogens throughout the body. The second line of defense is the adaptive immune response, which is specific to the pathogen presented. This response is long lasting and highly specific. The exact etiologies for autoimmune disorders such as rheumatoid arthritis, SLE, and inflammatory bowel disease remain unknown, but various genetic and environmental factors contribute to their development (76). Furthermore, in those individuals who are predisposed, self-tolerance, the ability of the immune system recognizing self-produced antigens as non-threatening, becomes disrupted. The immune system begins to recognize self-antigens as foreign, leading to the bodies inability to "tolerate" self tissues as it attacks itself causing systemic and organ-specific damage (76–78). Numerous microorganisms use molecular mimicry or mimotopes to avoid detection by the immune system, which in turn could amplify the autoimmune response.

Infections and exposure to pathogens or opportunistic organisms may initiate or exacerbate autoimmune disorders. In addition to true autoimmune diseases mentioned above, a small group of patients experience autoimmune symptoms months and sometimes years after an infection is cleared. These autoimmune sequelae are observed following infections with human pathogens such as *E. coli*, *Borrelia burgdorferi*, *S.* Typhimurium, *Mycobacterium tuberculosis, P. aeruginosa*, Group A streptococci, and *Staphylococcus aureus* (76, 79–87). Joints are affected in many cases and post-infectious arthritis is observed. Most interestingly, all these bacteria express curli-like amyloids and form biofilms during infections. One of best understood examples of post-infectious arthritis is reactive arthritis (ReA). ReA is an inflammatory arthritis that develops in 5–10% of the patients following gastrointestinal infections with *Salmonella, Shigella, Yersinia,* or *Campylobacter* or following

genital infections with *Chlamydia trachomatis* (79). Symptoms usually start 1–4 weeks post-infection and can last more than 5 years (79). Histocompatibility leukocyte antigen (HLA) B27 allele is a risk factor for ReA. About 90% of individuals who develop ReA following *Salmonella* infection carry the HLA-B27 genotype (88). ReA patients are not responsive to antibiotic treatment and cultures of joint fluids yield no bacterial growth. However, one study that employed immunohistochemical staining and mass spectrometry reported the presence of bacterial byproducts in synovial fluid (89–91). A recent study showed that in a mouse model of *Salmonella* infection curli is synthesized in the gastrointestinal tract and leads to increased anti-double-stranded DNA autoantibodies and to synoviocyte proliferation coupled with bone resorption in the knee joints (66). Infection with a curli mutant or a non-invasive strain did not cause such responses suggesting that the presence of curli during invasive infection with *S.* Typhimurium causes ReA (66).

Several mechanisms have been suggested to underlie the development of ReA. T cell-mediated immune responses clearly play a large role in autoimmune diseases. In rheumatoid arthritis, another autoimmune disease that affects joints, the functions of certain subsets of $CD4^+$ T cells with regulatory capacity such as $CD25^+$ regulatory T cells and Th2 cells are severely impaired. As ReA is a rare condition the role of these cells have not been elucidated, but it was proposed that $CD4^+$ T cells that produce IL-17 and generate a type 17-mediated inflammatory response contribute to joint damage (92). Curli binds to and activates TLR2, leading to the production of pro-inflammatory cytokines and chemokines including IL-6, IL-8, TNFα, and IL-17 (60, 61, 67, 93). Therefore, TLR2-mediated IL-17 production is a plausible mechanism for the curli-driven development of ReA. However, recent studies have also shown that curli binds to DNA in the ECM forming highly immunogenic curli/DNA complexes. Curli amyloid acts as a carrier to bring DNA into endosomes where the DNA is recognized by TLR9 and activates type I interferons (94). Curli/DNA complexes also trigger the generation of anti-dsDNA and anti-chromatin autoantibodies following translocation into systemic sites from the gut (**Figure 1**) (41, 66, 94). In autoimmune diseases where anti-dsDNA autoantibodies are observed, DNA seems to be the key component. Nevertheless, it is not known whether the autoantibodies generated during *S.* Typhimurium infection that recognize curli alone or curli/DNA complexes facilitate joint damage directly. Additional studies are needed to assess the role of anti-dsDNA autoantibodies and to determine whether curli without DNA can elicit joint inflammation and damage.

As curli is also be produced commensal strains from the *Enterobacteriaceae* family (69), dissecting the mechanisms by which curli-producing bacteria trigger arthritogenic processes in autoimmune diseases is critical. Studies have shown that bacteria from the normal gut microbiota can also be arthritogenic and cause experimental arthritis in animals (95). Antibiotic treatment can prevent and suppress arthritis in murine models prone to arthritis. Additionally, germ-free animals do not develop arthritis (95). Overall, these studies suggest that amyloids from the gut microbiota contribute to the autoimmune processes. Phylogenetic analysis suggests that the curli assembly machinery is widespread, as homologs of the *csg* genes, which encode curli and are responsible for its biosynthesis and secretion, are found within four phylum, Bacteroidetes, Proteobacteria, Firmicutes, and Thermodesulfobacteria (30, 96, 97). As these members of these bacterial phyla are found in the gut microbiota and biofilms are observed in the intestinal tract, it is likely that the gut microbiota harbors amyloids.

SLE is a classical autoimmune disease in which the immune system causes widespread inflammation and tissue damage in joints, skin, brain, lungs, kidneys, and blood vessels. Bacterial infections are a major cause of morbidity and mortality in patients leading to and exacerbating SLE flares. Epidemiological studies suggest that bacterial infections promote SLE disease in predisposed individuals, but the underlying mechanisms remain unknown. SLE patients are more susceptible to infections, particularly bacterial infections, involving the upper respiratory tract, skin, and urinary tract than subjects without SLE (98–101). SLE patients produce autoantibodies against a wide variety of cellular antigens including double-stranded DNA and nuclear proteins (41). Curli/DNA complexes are powerful immune stimulators (41). When given systemically, curli/DNA complexes trigger immune activation and production of type I interferons as well as autoantibodies in SLE-prone and wild-type mice (41). It was also found that infection of SLE-prone mice with curli-producing bacteria triggers higher autoantibody titers than do curli-deficient bacteria (41). Furthermore, clinical studies of SLE patients with persistent bacteriuria and *E. coli* within their urine were positive for anti-curli/DNA antibodies (102). The levels of anti-curli/DNA IgG correlated with both bacteriuria and flares in the SLE cohort, further suggesting a link between curli/DNA complexes and increased disease severity in SLE (102). These data suggest that enteric bacteria production of curli potentiates disease pathogenesis in individuals predisposed to autoimmune disorders.

NEUROLOGICAL DISEASES

Neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson's Disease (PD) are characterized by proteins such as Aβ, hyperphosphorylated tau, and αSyn misfolded into pathological amyloid aggregates in and around neurons; these aggregates are associated with elevated inflammation (103–105). The gut microbiota impacts the nervous system through the gut-brain axis, a bidirectional "highway" for immune, metabolic, endocrine, and neural signals (**Figure 2**) (106, 107). Gut bacteria can synthesize neurotransmitters and bacterial metabolites, like indoles and short-chain fatty acids that can bypass the blood-brain-barrier and impact the brain (108–110). One hypothesis is that these signals and microbial products are transmitted not through the circulatory system, where they would need to navigate the blood-brain-barrier, but *via* the vagus nerve: the longest cranial nerve in the body, connecting the enteric nervous system to the brain stem, and containing both afferent and efferent fibers (111–113). Thus, it is similarly possible that aggregation of human amyloidogenic proteins may be seeded or indirectly induced by bacterial amyloids that originate in the gut.

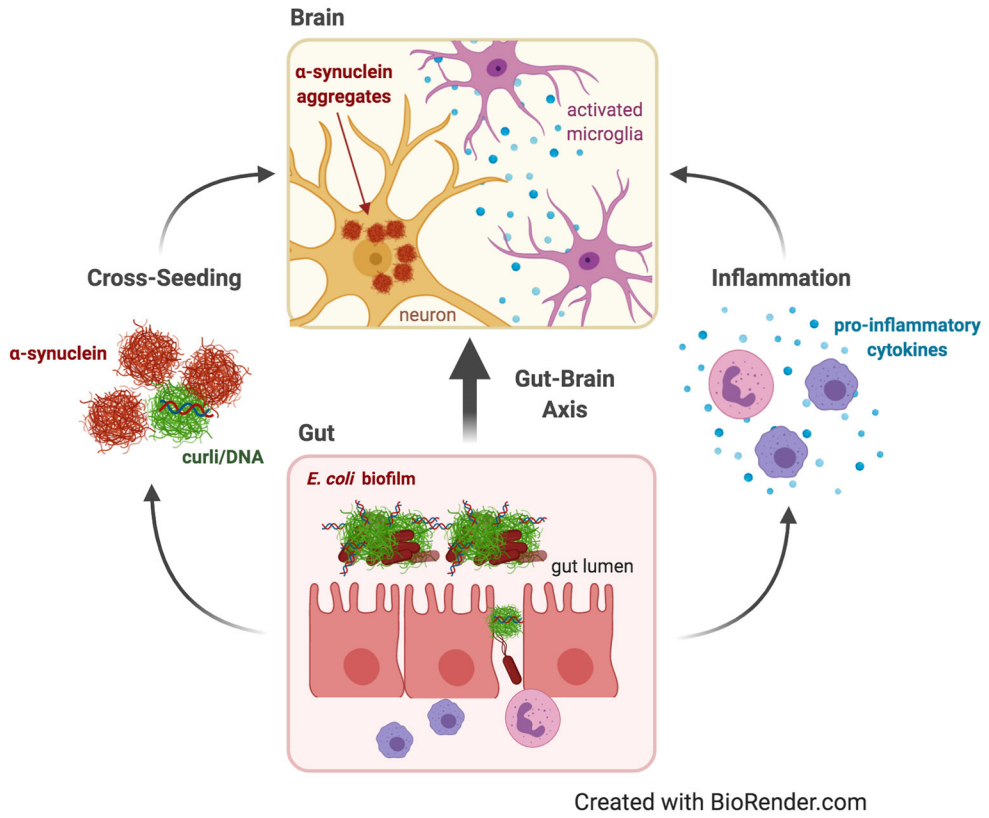

FIGURE 2 | Parkinson's disease. Dysbiosis and leaky gut often occur with age, allowing contents of the gut lumen, such as curli from *E. coli* biofilms, to escape and induce changes in the brain through the gut-brain axis. Curli may contribute to Parkinson's Disease by increasing systemic inflammation and neuroinflammation in the form of activated microglia and astrocytes and elevated pro-inflammatory cytokines. Curli may also cross-seed fibrillation and aggregation of α-synuclein, which is capable of prion-like propagation from the gut to the brain to cause neurodegeneration.

There are two predominant theories about how biofilm-forming gut bacteria might affect neurodegenerative disease: indirectly by provoking neuroinflammation or directly due to cross-seeding of aggregation of human amyloids by bacterial amyloids (**Figure 2**). Neuroinflammation is emerging as a critical component of neurodegenerative diseases. It involves chronic activation of microglia and astrocytes, elevated pro-inflammatory cytokines and chemokines, and accumulation of Aβ and αSyn (104, 105). Dysbiosis of the gut microbiota may cause neuroinflammation by increasing pro-inflammatory cytokines, systemic inflammation, and weakening of the gut barrier by decreasing immune regulatory function (114, 115). Aging is a major risk factor for neurodegenerative diseases, and aging has been shown to change the microbiome composition and increase inflammation (116, 117). Aging also disrupts blood-brain-barrier function, often as a result of dysbiosis, allowing passage into the brain of bacterial cells or inflammatory metabolites that may exacerbate neuroinflammation (118–120).

Bacterial amyloids such as curli share structural and physical properties with human pathogenic amyloids (76, 121). Curli activates the same TLRs that recognize Aβ and αSyn (62, 122, 123). Curli induces elevated pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, which have been shown to impair blood-brain-barrier integrity (64, 124–126). Therefore, it has been proposed that bacterial amyloids cause neuroinflammation and induce protein aggregation, indirectly leading to neurodegeneration (123, 127, 128). In addition, Aβ, tau, and αSyn have antimicrobial properties and so aggregation may be triggered by inflammation and dysbiosis (129–133).

PD is characterized by a loss of dopaminergic neurons in the nigrostriatal pathway. This loss is caused by Lewy bodies and Lewy neurites composed of intraneuronal αSyn aggregates (**Figure 2**) (103). Braak hypothesized that PD begins in the enteric nervous system and spreads up the vagus nerve to the brain (134). The earliest symptoms of PD, which occur up to 20 years before diagnosis in more than 65% of patients, include delayed gastric emptying, gastroparesis, constipation, and other gastrointestinal dysfunctions (135, 136). PD patients were recently demonstrated to have an altered gut microbiome compared to healthy controls (5). Elimination of gut bacteria in transgenic mouse models expressing human αSyn, either by using germ-free mice or treating with antibiotics, resulted in decreased αSyn deposition, neuroinflammation, and motor deficits; an effect that was reversed by recolonizing mice with microbiota from PD patients but not with healthy human microbiota (137). Furthermore, colonization of Fischer 344

rats with *E. coli* led to increased αSyn deposition in gut and brain neurons, increased gliosis, and increased inflammatory cytokines; effects replicated in *C. elegans,* but not observed with *E. coli* incapable of producing curli (**Table 1**) (127). A recent study corroborated these results: colonization of αSyn-overexpressing mice with wild-type *E. coli,* and not with curli-deficient *E. coli*, resulted in elevated αSyn pathology in the gut and brain and exacerbated cognitive, intestinal, and motor deficits and increased neuroinflammation. Interestingly, treatment of these mice with epigallocatechin gallate, an amyloid inhibitor restricted to the gut, prevented the increase in PD pathology and behavioral deficits. The same study confirmed *in vitro* that purified curli accelerated αSyn aggregation (138). Additionally, FapC, a functional amyloid produced by *Pseudomonas* biofilms, contributed to αSyn fibrillation, whereas a mutated FapC inhibited αSyn fibrillation (**Table 1**) (139). Thus, these studies also support the second theory: that bacterial amyloids directly cross-seed the aggregation of human pathological amyloids in the gut, which spread to the brain.

Human amyloids Aβ and αSyn have proven capable of cross-seeding tau (140, 141). Curli homologs from different bacteria strains are also capable of cross-seeding, even between *E. coli* and *Shewanella onedensis* (142). The first paper to propose the idea of cross-seeding by non-mammalian proteins showed that *E. coli* curli accelerated the fibrillation of serum amyloid A in a secondary amyloidosis mouse model (143). Data from *in vitro* studies also suggest that cross-seeding of Aβ aggregation by bacterial amyloids could initiate AD (**Table 1**) (127, 144). Curli is capable of cross-seeding Aβ fibrillation, and treatment of *S.* Typhimurium with D-enantiomeric peptides, known to inhibit Aβ fibrillation, inhibits curli fibrillation and reduces biofilm formation (121). Infection of pulmonary microvascular endothelial cells with clinical *P. aeruginosa,* which produce the bacterial amyloid FapC, induces production of Aβ and tau, capable of prion-like propagation to naïve cells (145). Evidence from animal models will be required to determine whether bacterial amyloids are capable of directly seeding Aβ or tau aggregation in a manner relevant to neurodegeneration.

Numerous recent studies confirmed that αSyn aggregates are capable of propagating from the gut to the brain, causing cognitive and motor deficits. Mice injected with αSyn preformed fibrils into the duodenal or gastric wall developed Lewy body-like αSyn aggregates in the dorsal motor nucleus of the vagus nerve, which spread to the brain and resulted in motor deficits and neurodegeneration (146, 147). The retrograde transport of αSyn pathology to the brain was corroborated in rat models (112, 148). However, studies by one group found that the pathology failed to progress past the dorsal motor nucleus (149, 150). Another group injected baboons with patient-derived Lewy body extracts and found that pathology spread to the central nervous system through circulation rather than *via* the vagus nerve (151). Regardless, there is mounting evidence indicating that bacterial amyloids such as curli, present at high levels in biofilms in the gut, are capable of cross-seeding αSyn aggregation and that αSyn aggregates are capable of spreading from the gut to the brain as a possible initiating event in PD (112, 127, 134, 139, 146–148, 151).

Meanwhile, similar observations are being made in AD, with some important differences from PD. AD is characterized by progressive accumulation of both extracellular Aβ plaques and intraneuronal hyperphosphorylated tau aggregates called neurofibrillary tangles (103). Evidence is inconclusive whether AD pathology begins in the intestines. Some samples from AD patients show increased Aβ deposits in the intestines (152), and several AD mouse models corroborate increased Aβ plaques and hyperphosphorylated tau overexpression in the intestines and enteric nervous system as well as impaired gut motility and function and increased inflammation (152–155). However, one study found no difference in gut motility and absorption between AD and control mice, though they detected intestinal Aβ and tau deposits in AD mice and AD patient samples (156). Another did not find evidence of Aβ in enteric neurons of an AD mouse model (157). Elimination of the gut bacteria by antibiotic treatment of APP/PS1 mice led to reduced Aβ plaque load and associated gliosis, altered cytokine profile, and increased regulatory T cell levels (158, 159). AD patients, and AD mouse models, show altered gut microbiota composition, favoring pro-inflammatory species, compared to healthy patients or wild-type mice (160–162). Microbiome composition affected cognition in APP/PS1 transgenic mice (162). Germ-free APP/PS1 transgenic mice showed reduction in Aβ deposition and pro-inflammatory cytokines in the brain (160). A recent study showed that fecal microbiota transplant from wild-type mice reduced Aβ plaques and tau tangles, gut permeability, systemic and neuroinflammation, and cognitive deficits in an AD mouse model (163). However, there is no *in vivo* evidence directly linking biofilm-forming bacteria and bacterial amyloids in the gut to AD pathology.

Rather than cross-seeding, AD microbiome studies point to a pattern of systemic inflammation and gut leakage that lead to AD pathology and cognitive deficits. Recent studies established that AD neuroinflammation involves NLRP3 inflammasome activation in microglia (164–166). Systemic inflammation induced by endotoxin or by fecal microbiota transplant from AD patients exacerbated microgliosis *via* the NLRP3 inflammasome in a mouse model (167, 168). Interestingly, curli activates the NLRP3 inflammasome in macrophages *in vitro*, suggesting a mechanism of microbiota-induced neuroinflammation (124). The endotoxin hypothesis also posits that microbiota-induced neuroinflammation underlies AD (169). Endotoxin, also known as lipopolysaccharide, produced by Gram-negative bacteria, is elevated in the serum of AD patients and during aging, has been shown to increase blood-brain-barrier permeability, and has even colocalized with Aβ plaques in the brain (**Table 1**) (169, 170). Endotoxin has also been implicated in the aggregation of a strain of αSyn in PD (171).

Another interesting possibility is direct infection of the brain in AD patients. Some have suggested that there is a separate microbiome within the brain, dominated by proteobacteria as well as fungal species (170, 172–174). If true, this would support the antimicrobial hypothesis of Aβ, wherein Aβ is secreted in response to infection as an antimicrobial peptide (129, 130). Still other studies implicate *Porphyromonas gingivalis*, another biofilm-forming bacteria,

which causes chronic periodontitis in the mouth. *P. gingivalis* has been found in the brains of AD patients and is associated with neurotoxicity and neuroinflammation (**Table 1**) (175). Such studies are controversial due to contamination concerns during autopsy (176) and require further investigation. Regardless of how exactly the microbiome affects AD, antibiotics and probiotics have been proposed as treatment options (177–179).

BIOFILMS IN COLORECTAL CANCER AND OTHER DISEASES OF THE GASTROINTESTINAL TRACT

Cancer results from uncontrolled, malignant cell proliferation caused by accumulated genetic and epigenetic mutations. The triggers for these mutations are multifactorial in origin and remain elusive in many cases, but genetics play a critical role. Accumulating evidence also supports the involvement of infectious agents in the development of cancer, especially in those organs that are exposed to microorganisms. Approximately 20% of cancers around the world have been estimated to be caused by microbes (180). For example, human papillomaviruses and the bacterium *Helicobacter* pylori cause cervical and gastric cancers, respectively (181, 182). Studies using *Helicobacter* have demonstrated that the protein encoded by cytotoxin-associated gene (*cagA*) induces DNA damage and that host-derived inflammatory mediators and growth factors are direct risk factors for carcinogenesis (182).

Colorectal cancer (CRC) is the fourth leading cause of cancer-related deaths worldwide (183). About 10% of CRC cases are hereditary, and the rest are sporadic. Risk factors include age, genetics, diet, and environmental factors (184, 185). Unhealthy behaviors such as physical inactivity, smoking, consumption of red and processed meat, and alcohol consumption increase risk of CRC (186–189). Some diseases, including obesity and type II diabetes, are also associated with increased risk of CRC development (190). Chronic inflammation is one of the major risks of CRC. Patients with inflammatory bowel diseases, including ulcerative colitis and Crohn's disease, are at risk for development of colitis-associated CRC (191, 192). The susceptibility of animal models of CRC, such as $APC^{Min/+}$ mice (which carry a germline mutation in *Apc* gene) and azoxymethane-treated mice, is enhanced when dysbiosis is induced by the inflammatory agent dextran sodium sulfate (193, 194).

Further, the intestinal microbiota has emerged as an important factor in CRC initiation and progression. The current view is that CRC initiation is triggered by local mucosal colonization by pathogenic bacteria. Healthy human colon is protected by a mucosal barrier that separates the microbiome from direct contact with the colonic epithelium of the host (195). Reduction of the mucosal barrier increases the contact between the microbiota and colonic epithelial cells and thus constitutes a significant primary step in inciting modifications in the biology of cells and inflammation. Specific changes within the intestinal microbial community are observed in CRC patients, such as increased abundance of Fusobacteria, *Alistipes*, Porphyromonadaceae, Coriobacteridae, Staphylococcaceae, *Akkermansia*, and Methanobacteriales, while representation of *Bifidobacterium, Lactobacillus, Ruminococcus, Faecalibacterium, Roseburia*, and *Treponema* is decreased (196).

Recently, bacterial biofilms were observed in direct contact with CRC tumors upon mucosal degradation. Biofilms exist in the healthy gut mostly within the lumen and away from the epithelium. The fact that the biofilms were in direct contact with the CRC tumors, especially those located in the right colon of humans (determined as proximal host colon to the hepatic flexure) suggests that bacteria or bacterial products are connected to the initiation cell transformation events (197).

Although it was initially thought that biofilms were only on the tumors, patients with biofilm-positive tumors also had biofilms on their tumor-free mucosa. These findings suggest that in patients with biofilm-positive tumors, the luminal environment may provide an ideal landscape for biofilm development. However, the factors that facilitate biofilm development in the colon are difficult to identify as our knowledge of regulatory signals for bacterial biofilm formation is limited. Additionally, presence of bacterial biofilms on tumors is associated with reduced colonic epithelial cell E-cadherin, enhanced epithelial cell IL-6 and Stat3 activation as well as crypt epithelial cell proliferation (197). These observations have generated significant interest over the last few years in how bacterial biofilms facilitate CRC development.

To confirm that the human biofilms are indeed carcinogenic, three murine models of carcinogenesis were evaluated by Tomkovich et al.: germ-free $Apc^{Min\Delta 850/+}$ (129SvEv) mice, (b) germ-free $Apc^{Min\Delta 850/+}$ $Il10^{-/-}$ (129SvEv) mice, and conventional $Apc^{Min\Delta 716/+}$ (C57BL/6) mice. In all three models, inocula prepared from biofilm-covered human mucosa induced colon tumors primarily in the distal colon at 12 weeks after inoculation. In contrast, inocula prepared from biofilm-negative mucosa did not (198). This study suggested that the biofilm itself might contribute to CRC pathology. Facultative anaerobic pathogens and pathobiont strains thrive in an inflammatory environment due to their ability to utilize inflammation-derived molecules such as nitrites and oxides as electron acceptors (199). In addition, microbial metabolism is altered under dysbiotic conditions, conferring new microbial phenotypes such as enhanced cellular adherence and invasion, mucus utilization, and production of metabolites and toxins (200). Microbiome profiling revealed that biofilms of distinct commensal bacteria, including *Bacteriodes fragilis*, *Fusobacterium* spp., and *E. coli*, were enriched in the CRC tumors, and these bacteria were able to promote CRC tumor development in genetically predisposed animals (184, 201–203).

Studies in murine models also showed that enterotoxigenic *B. fragilis* (ETBF) and colibactin-producing *E. coli* (CPEC) secrete carcinogenic toxins that are associated with the propagation of tumors (204–208). Furthermore, CPEC and ETBF were detected in patients with familial adenomatous polyposis, a premalignant disease that can develop into CRC (209).

Fusobacterium nucleatum accelerates tumorigenesis by inducing oncogenic and inflammatory responses in the gut

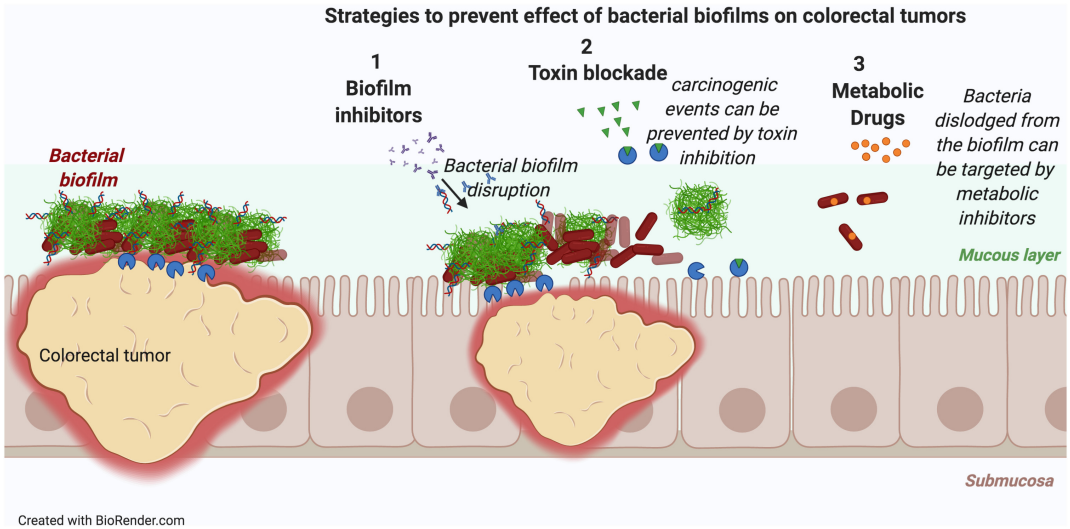

FIGURE 3 | Novel strategies to intercept colorectal cancer. Three novel strategies either alone or in combination could reduce carcinogenesis triggered in biofilm associated colorectal tumors: (i) Inhibition of biofilm formation to dissociate the biofilms from the tumors, (ii) inhibition of toxin production by biofilm forming bacteria, (iii) inhibition of biofilm-associated bacterial growth by metabolic inhibitors.

through the production of the adhesin molecule FadA (210). However, whether FadA has toxigenic properties similar to those of colibactin or *B. fragilis* toxin is currently unknown. Finally, another enteric bacterium, *Campylobacter jejuni*, produces a genotoxin called cytolethal descending toxin (CDT) that induces colorectal cancer and changes in microbial composition and transcriptomic responses. Germ-free $Apc^{Min/+}$ mice colonized with human clinical isolates of *C. jejuni* developed significantly more and larger tumors than the uncolonized mice or mice colonized with the *cdtB* mutant (211). Overall, these studies indicate a role for biofilm and toxin production by certain bacterial species as a driver for CRC pathogenesis.

It is plausible that toxins contribute to the onset of cancer by acting as direct environmental stressors. Toxins may also cause host cell DNA alterations. In germ-free mice that receive fresh feces from CRC patients, colon epithelia is renewed, more precancerous lesions are observed, and there is increased tissue and blood DNA methylation in intestinal tissues (212); this does not occur in mice given feces from healthy controls.

Recent studies showed a striking association between specific host microbes and aberrant DNA methylation in CRC. Addition or removal of acetyl and methyl residues at specific histone regions led to a corresponding gain or loss of DNA methylation at CpG dinucleotides, which led to an altered epigenomic state. CRC tumors can be grouped by CpG island methylator phenotype (CIMP); high CIMP, low CIMP or no CIMP (213). In CRC tumors where *Fusobacterium* species were substantially enriched, the tumors had a unique genetic and epigenetic profiles. The epigenetic changes were associated with high CIMP events and somatic mutations (213–215). However, additional studies are needed to determine whether bacterial toxins or whether certain bacterial species can directly promote oncolytic events by causing changes in DNA methylation. Additionally, dietary and digestive factors that are metabolized by microbiota can cause changes in the metabolic landscape and can alter the immune cell function (216). For instance, some short-chain fatty acids such as butyrate have anti-inflammatory properties and protect the host against colitis by increasing the level of colonic regulatory T cells and change the metabolism of epithelium (217–219). Therefore, dysbiosis of microbiota and localized enrichment of bacterial species may also directly influence the immune metabolism and function acting as a promoter or suppressor of tumor oncogenesis.

These data suggest that restricting the biofilm-mediated colonization by toxin-producing bacteria and reversing the dysbiosis would be a first step to reduce the tumorigenesis. Three strategies are feasible: (i) inhibition of biofilm formation, (ii) inhibition of toxin production or activity, or (iii) targeted inhibition of tumor-associated bacterial growth (**Figure 3**). Consistent with the idea that inhibition of toxin production is a viable strategy, toxin-negative mutants of CPEC and ETBF do not elicit tumor formation in the azoxymethane/dextran sulfate sodium model of CRC (220, 221). Suppression of tumorigenesis has also been achieved by using a small-molecule inhibitor that directly targets the toxin colibactin (222). In support of utility of targeted inhibition of the toxin-producing bacteria, when the growth of CPEC and other *Enterobacteriaceae* are inhibited with the oral administration of sodium tungstate, a metabolic inhibitor that targets molybdoenzymes, development of malignancies was blocked (**Figure 3**) (223).

CONCLUSIONS AND OUTLOOK

In the past decade, it has become increasingly apparent that the gut microbiota and infections with bacterial pathogens profoundly impact complex human diseases. In this review, we highlighted novel findings that indicate that biofilm-forming bacteria that produce the amyloid curli in the gastrointestinal

tract are linked to autoimmune diseases, neurodegenerative diseases, and CRC. In healthy individuals, bacterial biofilms occupy the gut, and the extracellular matrix material educates the immune system to reinforce the epithelial barrier, preventing the leakage of bacterial ligands including pathogenic amyloids. However, the recent studies suggest that biofilms that harbor amyloid proteins like curli can initiate or accelerate pathogenic processes in a number of human diseases. The emerging picture suggests that in individuals that are genetically susceptible to chronic diseases, bacterial amyloids have pathogenic effects. For instance, in individuals who carry genetic risk factors such as HLA-B27, infections with invasive *Salmonella*, a pathogen that expresses curli, triggers autoimmunity and joint inflammation. Furthermore, enteric infections such as those with uropathogenic *Escherichia coli* trigger disease flares in SLE patients. It is important to note that the translocation of curli or curli-expressing bacteria into tissues is critical for the generation of autoimmune responses; this indicates that leakage of other amyloids or amyloid-producing bacteria from the gut or invasive infections may trigger similar responses. Consistent with this idea, infections with curli-producing enteric bacteria and other invasive bacteria that express amyloids, such as *B. burgdorferi*, *S. aureus, Pseudomonas* spp, and *M. tuberculosis*, trigger autoimmune reactions primarily affecting the joints (e.g., arthritis, septic arthritis).

Amyloid proteins are pathogenic in neurodegenerative diseases, and it was anticipated that bacterial amyloids like curli would influence aggregation of human amyloids like Aβ and αSyn. As predicted, recent work has shown that the colonization of the gut microbiota with curli-expressing *E. coli* increases αSyn pathology in mice that are predisposed to develop PD. However, whether curli can directly seed αSyn or whether it indirectly causes neuroinflammation and subsequent neurodegeneration is not known. Given these findings, treatments that reduce the pathological amyloid content in the microbiome or that reverse bacterial amyloid-induced neuroinflammation have potential as treatments for AD and PD.

One of the groundbreaking findings of the past several years was the demonstration that bacterial biofilms are associated with colonic tumors in humans and in animal models of CRC. Carcinogenic toxins produced by bacteria have been identified, but our current understanding of the direct effects of biofilms on the initiation and progression of CRC is limited. Given that we know how toxic amyloid intermediates form during initial stages of biofilm establishment, further work will be required to illuminate how curli or other amyloids contained in biofilms contribute to the onset of CRC by acting as a direct environmental stressor.

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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Biofilm-Innate Immune Interface: Contribution to Chronic Wound Formation

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Delayed wound healing can cause significant issues for immobile and ageing individuals as well as those living with co-morbid conditions such as diabetes, cardiovascular disease, and cancer. These delays increase a patient's risk for infection and, in severe cases, can result in the formation of chronic, non-healing ulcers (e.g., diabetic foot ulcers, surgical site infections, pressure ulcers and venous leg ulcers). Chronic wounds are very difficult and expensive to treat and there is an urgent need to develop more effective therapeutics that restore healing processes. Sustained innate immune activation and inflammation are common features observed across most chronic wound types. However, the factors driving this activation remain incompletely understood. Emerging evidence suggests that the composition and structure of the wound microbiome may play a central role in driving this dysregulated activation but the cellular and molecular mechanisms underlying these processes require further investigation. In this review, we will discuss the current literature on: 1) how bacterial populations and biofilms contribute to chronic wound formation, 2) the role of bacteria and biofilms in driving dysfunctional innate immune responses in chronic wounds, and 3) therapeutics currently available (or underdevelopment) that target bacteria-innate immune interactions to improve healing. We will also discuss potential issues in studying the complexity of immune-biofilm interactions in chronic wounds and explore future areas of investigation for the field.

Keywords: chronic wound, delayed healing, innate immune responses, inflammation, biofilm, host-pathogen interaction, skin microbiome

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INTRODUCTION

Wounds are a broad category of injuries that include everything from minor cuts and scrapes to surgical incisions and serious trauma. In healthy individuals, minor wounds heal quickly without complication. However, larger wounds take more time to heal, increasing risk of infection. Delayed wound healing is a significant issue among ageing individuals, those with immobility and those with chronic diseases such as diabetes, vascular disease, and cancer (1–3). In the most severe cases,

these delays can result in the formation of non-healing or chronic ulcers, which can cause significant pain, prolonged hospitalization, loss of function, and may eventually lead to amputations and/or the development of sepsis (1, 4). Wound care (acute and chronic) is labor-intensive and represents a substantial economic burden on healthcare systems, costing billions of dollars annually in North America (5). As the global population continues to age and experience increasing rates of co-morbid chronic diseases such as diabetes, there is an urgent need to understand the pathophysiology of delayed healing or the formation of non-healing wounds to develop more effective therapies that can repair tissue damage and restore health (3).

There is an increasing interest to understand how dysregulated host-pathogen interactions affect healing processes. For instance, colonization of the wound bed with low levels of bacteria does not affect healing (6). However, local infection with high levels of replicating bacteria plays a significant role in delayed healing and in non-healing ulcer formation (6, 7). Chronic wounds show a considerable diversity in the bacterial species at the site of injury, but it is unclear how these differences contribute to chronicity. Further, these bacteria form robust biofilms, which embed the bacterial cells in a self-produced polymeric matrix, protecting them from host immune responses and antibiotics. This structure provides numerous advantages to the community such as metabolic cooperation, passive resistance, and horizontal gene transfer (8). It has also been shown to impair the tissue repair processes and promote a low-grade inflammatory response (7, 9). In this review we will discuss the current understanding of how interactions between bacterial biofilms and innate immune cells drive damaging inflammatory processes that contribute to delayed healing in chronic wounds. We will also discuss how we can target these interactions to develop novel therapeutics for individuals with difficult to treat chronic wounds.

CONTRIBUTION OF BACTERIAL BIOFILMS TO CHRONIC WOUNDS

Overview of Bacterial Biofilms

Bacteria exist as single, planktonic cells or as multicellular communities and aggregates with or without surface attachment, called biofilms (10–12). Biofilms possess distinct characteristics compared to planktonic cells, including increased antibiotic tolerance, changes in gene expression, and altered host interactions (13). Bacteria within these structures are embedded in a self-produced extracellular polymeric substance (EPS) composed of extracellular DNA, proteins, exopolysaccharides and water. In addition to the microbial components, the EPS can also include host substances such as proteins, DNA, immunoglobulins, and blood components (14, 15).

Biofilms are complex and diverse structures that can be composed of single species or can be polymicrobial (15). They are up to 1,000 times more tolerant to antimicrobial agents and disinfectants than planktonic cells (16). Further, the immune system is often inefficient in combating biofilm-related infections (17, 18). Several factors contribute to the increased robustness of biofilms including low growth rates, high cell density, the presence of persister cells, nutrient and oxygen gradients, horizontal gene transfer, efflux pumps, and high rates of mutation (16, 19). The presence of the EPS matrix is also considered a physical barrier against antimicrobial agents and the host immune responses since it reduces the diffusion of drugs, antibodies and immune cells into the biofilm.

Biofilm-growing bacteria have been shown to colonize medical devices (*e.g.*, contact lenses, cardiovascular valves, implants, ortho-dental prosthetics, urinary and central vascular catheters) and a variety of host tissues, causing many chronic infections, including osteomyelitis, vaginosis, lung infections in cystic fibrosis patients, ventilator-associated pneumonia, device-related infections, and chronic wound infections (15, 20). It is estimated that bacteria in biofilms cause up to 80% of all human infections (21) and are involved in more than 60% of all chronic wound infections (15). These biofilms are composed of bacterial species found in the normal flora of the skin, the gut and oral mucosa as well as in external environments (22).

Human Skin Microbiome

The human body is naturally colonized by thousands of different microbial species that collectively form a complex ecosystem called the human microbiome (23). More than 1,000 different bacterial species can be found on the human body, and it is estimated that there are up to 150 times more microbial genes than human genes within the human body (24, 25). These microorganisms selectively colonize different parts of the body, such as the skin, gastrointestinal tract, conjunctiva, oral cavity, vagina, uterus, and lungs (26). The human microbiome plays essential roles in health, including protection against invading pathogens, metabolism of molecules, nutrient acquisition, control of cellular proliferation and differentiation, and development of the immune system (25, 27, 28). However, the composition and diversity of the microbiome can be altered by several factors (e.g., diet, use of antibiotics, mode of birth, and age) and it can become associated with opportunistic pathogens and specific isolates that cause severe infections (24, 28). For example, certain isolates of *Staphylococcus aureus* can be either commensal, opportunistic or pathogenic.

The highest number of microbes are found in the colon (10^{14} bacteria), followed by the skin (10^{12} bacteria) (26). The skin microbiome is highly adapted to the skin's physiological environment, such as the absence of several nutrients, an acidic pH and temperature. The bacterial microbiota found in each skin region depends on the microbe's ability to thrive in these conditions. For example, bacteria from the *Staphylococci* genus use urea from the sweat as a nitrogen source whereas *Cutibacterium acnes* (formerly *Propionibacterium acnes*) produce fatty acids by metabolizing triglycerides present in sebum (27, 29). The skin is naturally considered a physical barrier against external stressors, and the natural skin microbiota protects the body by competing with pathogenic microbes and impairing their development, catabolizes natural

skin products, such as lipids, and modulates the immune system (29). If the skin is disrupted, or there is an imbalance between pathogenic and the natural microbiota and infections can occur (27, 29).

Overall, most microbes from the skin microbiota belong to four different phyla: Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria (29). While certain species are found in multiple microenvironments (e.g., *C. acnes*, *Staphylococcus epidermidis*, and *Staphylococcus capitis*), the composition of the skin microbiome differs significantly according to the environmental conditions and location. **Figure 1** highlights some of the most common bacterial species found in dry, sebaceous and moist environments. Dry areas represent the most diverse microbial environments. In these regions, streptococcus species are very common but actinobacteria, proteobacteria, firmicutes, and bacteroidetes are also detected including *Corynebacterium tuberculostearicum*, *Staphylococcus* sp., *Veillonella parvula* and *Micrococcus luteus* (27, 30). In moist and sebaceous sites corynebacterium are highly prevalent. However, these microenvironments are also populated with other common species including *Staphylococcus hominis*, *Enhydrobacter aerosaccus*, *Streptococcus mitis* and *Micrococcus lutues* (27, 29, 30).

It is important to acknowledge, that while bacteria are the most prevalent microorganisms found on the skin, fungal species also contribute to the microbiota (27). *Malassezia* is among the most common fungal genera accounting for 53-80% of the fungal population (29). Findley et al. found that *Malassezia* is found on several sites including retroauricular crease, nare, palm, back, and volar forearm. Other fungal species were also detected, with the plantar heel showing the greatest diversity with approximately 80 genera, including *Malassezia, Aspergillus, Cryptococcus, Rhodoturulla, Epicoccum* (31).

From Contamination to Infection

The human skin microbiota can influence the wound healing process and affect the severity of infections. In chronic wounds, microbial contamination and colonization can evolve to an infection, a process that involves different microorganisms (32). Contamination refers to the presence of non-proliferating bacteria originating from the natural microbiota or the environment. All wounds are contaminated by microorganisms; however, if they encounter favorable conditions that support survival, the wounds will become colonized. Colonization is characterized by the presence of multiplying microorganisms on the surface of the wound (33–35). In these initial processes, the host immune

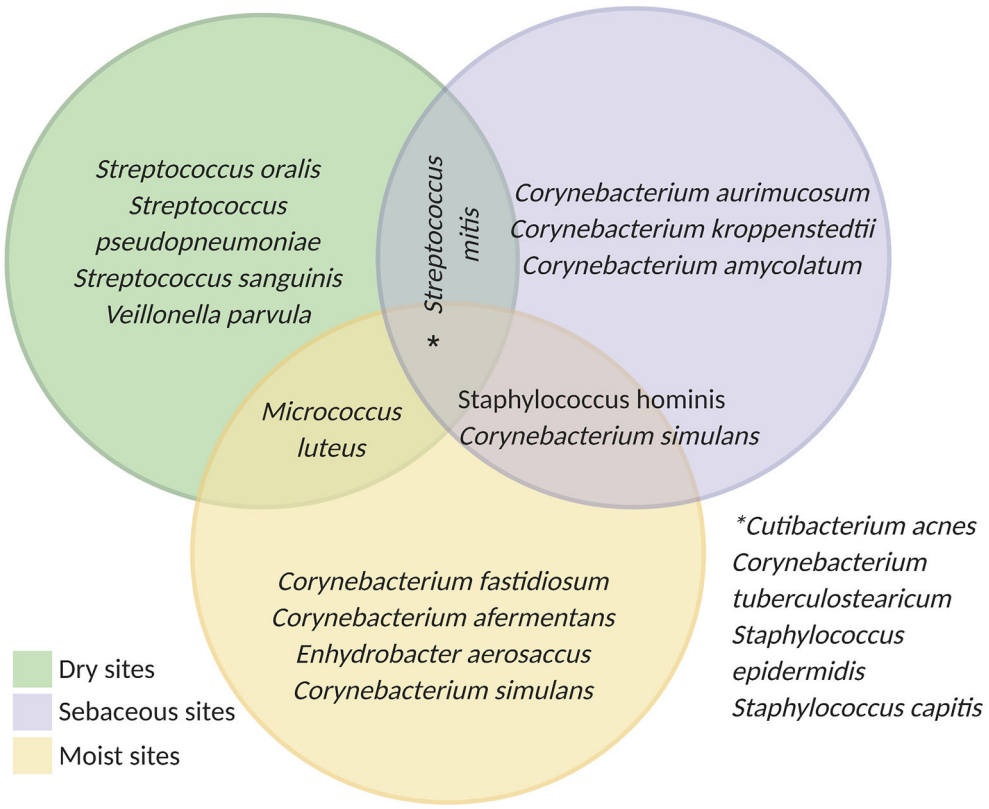

FIGURE 1 | Schematic of skin microbiota according to the physiological sites: dry (green): buttock, volar forearm, hypothenar palm; moist (yellow): plantar heel, popliteal fossa, toe web space, axillary vault, and nare; sebaceous (purple): back, occiput, retroauricular crease, and glabella. Developed using data from (27).

defenses are not triggered, and there are no overt clinical signs of infection. This is likely because the wound is colonized by Gram-positive bacteria, especially those belonging to the *Staphylococci* genus, which do not elicit strong immune responses (32, 36).

In the later stages, Gram-negative bacteria, mainly rod-shaped bacteria such as *Pseudomonas* sp., *Escherichia coli, Klebsiella pneumoniae*, and *Enterobacter* spp. contaminate the wound and become the predominant species in this microenvironment. These species typically originate from the urogenital tract of the patient or from the nearby environment. Colonization of may also be affected by any antibiotic use (local or systemic). This acute colonization is an intermediate stage before deeper tissue infections and only elicits a localized immune reaction (32, 37). In this stage, the bioburden increases, and the presence of these microbes can delay the healing process. Finally, the last step is infection, in which the microorganisms invade the deep tissues, the body cannot control the levels of microbes, and an intense host response is induced (32, 36). This stage is also characterized by the rapid consumption of oxygen by aerobic bacteria, which favors anaerobic microbes (32, 33).

Bioburden

Many aspects of wound bioburden correlate with healing outcomes. These include microbial load, microbial diversity, and presence of pathogenic organisms (38). Microbial load is commonly used to diagnosis a wound infection when clinical manifestations are absent (38). While the skin microbiota contains approximately 1 billion bacteria/cm^2 of tissue (39, 40), a microbial load higher than 10^5 colony-forming units (CFU) per gram of tissue is generally considered the standard reference for diagnosing infection (41). However, this value can vary according to the type of infection and type of wound evaluated. In diabetic foot ulcer (DFU), the microbial load may be higher than in venous leg ulcer (VLU) patients (38, 41).

Microbial Diversity

Microbial composition and diversity are other factors associated with delayed healing. The most common bacteria found in the wound bed are aerobes such as *Staphylococci ssp.*, *Corynebacterium spp*, *Pseudomonas* spp. as well as anaerobes such as *Anaerococcus spp* and *Finegoldia* spp (42–45). However, a higher diversity of microbial species in the wound bed can be associated with impaired healing outcomes (38). In addition to overall diversity, the presence of specific pathogenic organisms or even certain isolates can also contribute to chronic wound formation. Some strains of *S. aureus* and group A streptococci, have been shown to cause severe infections and trigger an intense inflammatory response, affecting the healing process (32). Moreover, high levels of *S. aureus* and *P. aeruginosa* can affect healing outcomes because these organisms are often resistant to different classes of antibiotics, they form biofilms with antiphagocytic activities and they produce several virulence factors, including secretion of toxins and enzymes, which drive further tissue damage (34, 46). Further studies are required to understand how single species vs. poly-microbial biofilms may contribute to these processes.

Methodologies for Characterization

Several factors must be taken into consideration when studying the skin and wound microbiota. Among the most important, are the sample collection technique and the method used to characterize the microbial population (*i.e.*, culture-based or molecular methods) (47). At the level of sample collection, wound swabs and tissue biopsies (or debridement tissue) are commonly used for microbiota analysis. Swabbing the wound surface is often preferred because it is non-invasive, and it can be performed multiple times for longitudinal studies. However, dry swabs generally collect a low biomass and do not capture the diversity of species found in deeper tissues (48). Nakatsuji et al. found the bacterial diversity on the surface was not the same as that in the sub-epidermis, which included high levels of proteobacteria (49). Further, Travis et al. found there was minimal correlation between the number and types of bacteria found in swabs compared to tissue biopsies. They also found tissue biopsies contained an overall greater diversity of bacterial species but that the frequency of potential pathogens was higher in wound swabs (50). While biopsies represent the gold standard to capture the true diversity of bacteria found in skin and wound samples, it is invasive and requires a skilled clinician.

The two techniques most widely used to characterize bacteria in chronic wound infections are: A) culture-based methods, in which the microbial culture is collected from the affected tissue and bacteria are grown on selective or nonselective media; and B) molecular-based methods, in which the bacterial 16S rRNA is sequenced and microbes are identified based on databases (41, 51–53). While the culturing of bacteria is primarily used in the hospital setting, this method is usually limited to growing certain strains of bacteria and does not allow for the detection of fastidious and slow-growing bacteria, viable but nonculturable bacteria (VBNC) or dormant bacteria (12). The use of molecular methods, such as RNA sequencing, has increased in recent years but is still predominantly used for research. In comparison to culturing methods, these approaches allow for the identification of a larger diversity bacterial species in the wound. It can also be used to elucidate microbial activities, behaviors, strategies, and processes during infections (12). However, RNA sequencing is associated with a substantial demand for financial, time and bioinformatic support. Moreover, it cannot distinguish between living, dead or dormant bacteria and might overlook minority species (52). To complement these approaches, microscopy can be used to capture the complexity and the organization of bacterial populations in the wound environment. It can be used to visualize and characterize individual cells within a population/structure (e.g., biofilm) and provide important insights into their interactions and structural organization (48, 54). Regardless of the assay used, several other factors impact the diversity (or our ability of evaluate the diversity) of the wound microbiota including patient demographics, personal hygiene, grade of wound severity, patient's immune status, and ongoing or previous use of antimicrobial therapies (12, 52).

Bacterial Diversity Across Chronic Wound Types

Chronic wounds or non-healing wounds are commonly defined as wounds that have failed to proceed through the normal phases of wound healing in an orderly and timely manner (55). The most common types of these wounds are diabetic foot ulcers, pressure ulcers, venous leg ulcers, abscesses and surgical site infections. While bacteria contribute to the pathophysiology of delayed healing and chronic wound formation, the molecular and cellular mechanisms underlying these processes remain incompletely understood. To date, most studies have been cross-sectional nature (**Table 1**). Few have used longitudinal approaches to evaluate how bacterial communities change in different chronic wounds over the course of infection. Moreover, fewer studies have investigated correlations between the microbiota and specific wound outcomes (53).

Diabetic Foot Ulcers

To date, most studies analyzing microbial communities in chronic wounds have focused on infections from patients with diabetic foot ulcers. These investigations have been recently reviewed in significant detail and are beyond the scope of the current review (52, 56–59). Briefly, the most frequently identified genera in DFU include Staphylococcus, Corynebacterium, Pseudomonas, Streptococcus, Stenotrophomonas, Enterobacter, Escherichia, Enterococcus, Serratia, Acinetobacter, Peptoniphilus, Anaerococcus and Finegoldia (42–45, 50, 60–67). The most common pathogens found were the Gram-positive bacteria *S. aureus*, *S. epidermidis*, *Enterococcus faecalis* and *Streptococcus agalactiae* and the Gram-negative bacteria *P. aeruginosa*, *Stenotrophomonas maltophilia*, *E. coli* and *Acinetobacter baumannii* (43–45, 62).

Other Types of Chronic Wounds

The bacterial communities of other types of wounds, including venous leg ulcers, pressure ulcers, surgical site infections, and abscesses, have not been fully evaluated. Previous studies have shown that like DFU, commonly identified genera include Staphylococcus, Corynebacterium, Streptococcus, Enterococcus, Pseudomonas, Stenotrophomonas, Enterobacteriaceae, Acinetobacter and Finegoldia (**Table 2**, and (43, 60, 78–82). To date, Wolcott et al. have performed one of the largest studies collecting samples from 916 venous leg ulcers, 767 decubitus ulcers, and 370 samples from nonhealing surgical wounds and evaluating bacterial diversity using 16s rDNA sequencing. They found that the most frequent bacterial species found across all wound types were *S. aureus*, *S. epidermidis*, *E. faecalis*, *P. aeruginosa*, *S. maltophilia* and *Finegoldia magna*. They also observed a high prevalence of anaerobic bacteria in wound samples, including *Finegoldia* spp. and *Anaerococcus* spp (present in 24% and 25% of wounds, respectively) and variety of commensal bacteria including Staphylococci and Corynebacteria across wound types (43). Interestingly, unlike DFU, these wounds contained *Cutibacterium acnes* and other Cutibacterium species suggesting an important role for the location of the wounds in spectrum of microbial colonization (43, 83, 84). Further, most of these infections were polymicrobial biofilms consisting of many different species. Only 7% of wound samples were found to be mono-species biofilms with *P. aeruginosa* and *S. epidermidis* being the most common bacteria growing in single-species biofilms in wounds (43). More *in vivo* studies with larger samples sizes are urgently needed to fully understand the importance of microbial diversity, biofilms and the wound microbiome in chronic wounds infections and to elucidate the impact of aerobic, anaerobic, pathogenic and commensal bacteria in driving chronic immune activation and inflammation in situations of delayed wound healing.

CONTRIBUTION OF CHRONIC INNATE IMMUNE ACTIVATION AND INFLAMMATION TO CHRONIC WOUNDS

Overview of Wound Healing

Wound healing has been extensively described elsewhere, and for that reason, we will only briefly summarize the process here (**Figure 2**) (34, 85–92). Normal healing (**Figure 2A**, described as Steps 1-4) is comprised of four coordinated phases: hemostasis, inflammation, proliferation, and tissue remodeling (34). Hemostasis includes platelet aggregation and activation, which initiates the coagulation cascade and the formation of a transient fibrin scaffold (Step 1) (86, 91, 92). During this process, platelet degranulation releases damage-associated molecular patterns (DAMPs), cytokines, chemokines, and growth factors, which accumulate within the scaffold to generate a chemotactic gradient for immune cell infiltration (Step 2) (86, 88, 90). This infiltration is required to clear dead or damaged cells, cellular debris, and any pathogens that colonize the wound bed. It also prepares the wound for the healing phases. The innate immune system plays a critical role in modulating these processes as well as the transition from inflammation to proliferation phases, which includes a transition of inflammatory macrophages (M1) into anti-inflammatory wound healing cells (M2; Step 3) (86, 87). This anti-inflammatory transition activates keratinocytes and fibroblasts in the wound bed where they proliferate and contribute to healing processes (91). Keratinocytes are essential for wound re-epithelialization (86). Fibroblasts deposit collagen to form the extracellular matrix (ECM) or granulation tissue, which replaces the temporary fibrin scaffold (90, 92). During the proliferation stage, angiogenesis also restores tissue vascularity (86). Finally, in the remodeling phase, fibroblasts replace granulation tissue with scar tissue and contraction occurs, resulting in wound closure (Step 4) (34, 86, 88).

Role of the Innate Immune System in the Inflammatory Phase of Healing

Although the inflammatory phase involves a wide range of immune cells, such as mast cells, dermal dendritic cells (Langerhans cells), and T lymphocytes, innate immune cells such as neutrophils and macrophages play a central role in regulating healing process (85, 91–93). For the purposes of this review, we will focus on these two cell types.

TABLE 1 | Summary of most common bacterial species found in chronic wounds (excluding DFU).

Venous Leg Ulcers				
Gram-positive Aerobes	**Gram-negative Aerobes**	**Gram-positive Anaerobes**	**Gram-negative Anaerobes**	
-*Actinotignum schaalii* -*Alcaligenes faecalis* -*Brevibacterium casei* -*Corynebacterium amycolatum* -*C. jeikeium* -*C. simulans* -*C. striatum* -*C. tuberculostearicum* -*Enterococcus faecalis* -*Staphylococcus aureus* -*S. epidermidis* -*S. haemolyticus* -*S. lugdunensis* -*S. pettenkoferi* -*Streptococcus agalactiae*	-*Achromobacter xylosoxidans* -*Acinetobacter baumannii* -*Citrobacter* spp. -*Delftia acidovorans* -*Enterobacter cloacae* -*E. hormaechei* -*Klebsiella oxytoca* -*Proteus* spp. -*Pseudomonas aeruginosa* -*P. fluorescens* -*Serratia nematodiphila* -*Stenotrophomonas maltophilia*	-*Anaerococcus vaginalis* -*Finegoldia magna* -*Peptoniphilus harei* -*Peptostreptococcus assacharolyticus* -*Cutibacterium acnes*	-*Bacteroides tectus* -*Flavobacterium succinicans* -*Fusobacterium gonidiaformans*	
Pressure Ulcers				
Gram-positive aerobes	**Gram-negative aerobes**	**Gram-positive Anaerobes**	**Gram-negative Anaerobes**	
-*C. jeikeium* -*C. striatum* -*C. tuberculostearicum* -*E. faecalis* -*S. aureus* -*S. epidermidis* -*S. haemolyticus* -*S. lugdunensis* -*S. agalactiae* -*S. dysgalactiae*	-*A. baumannii* -*D. acidovorans* -*E. hormaechei* -*Escherichia* spp. -*P. mirabilis* -*P. aeruginosa* -*Serratia* spp. -*S. maltophilia* -unclassified *Enterobacteriaceae* spp.	-*Allobaculum* spp. -*A. vaginalis* -*Eubacterium dolichum* -*F. magna* . -*Peptococcus* spp. -*Peptoniphilus ivorii*	-*B. fragilis* -*Dialister* spp. -*F. nucleatum* -*Prevotella bivia*	
Surgical Site Infections				
Gram-positive aerobes	**Gram-negative aerobes**	**Gram-positive Anaerobes**	**Gram-negative Anaerobes**	**Other**
-*Bacillus* spp. -*E. faecalis* -Coagulase-negative *staphylococci* (CoNS) -*C. striatum* -*C. tuberculostearicum* -*Granulicatella elegans* -methicillin-resistant *S. aureus* (MRSA) -*S. aureus* -*S. epidermidis* -*S. haemolyticus* -*S. lugdunensis* -*S. agalactiae* -*S. mitis* -*S. salivarius*	-*A. baumannii* -*A. lwoffii* -*D. acidovorans* -*Diaphorobacter* spp. -*K aerogenes* -*E. cloacae* -*Enterobacteriaceae* spp. -*E. coli* -*K. oxytoca* -*K. pneumoniae* -*Moraxella* spp. -*Morganella morganii* -*Neorhizobium* spp. -*Novosphingobium spp.* -*Paracoccus* spp. -*P. mirabilis* -*P. aeruginosa* -*Ralstonia pickettii* -*S. nematodiphila* -*Sphingomonas* spp. -*S. maltophilia*	-*A. vaginalis* -*Clostridium* spp. -*F. magna* -*C. acnes*	-*Cloacibacterium* spp. -*F. nucleatum* -*Methylobacterium* spp.	-*Candida albicans*
Abscesses				
Gram-positive aerobes	**Gram-negative aerobes**	**Gram-positive Anaerobes**	**Gram-negative Anaerobes**	
-CoNS -*C.accolens/gurimucosum* -*C. afermentans* -*C. mucifaciens* -*C. tuberculostearicum* -*Enterococcus* spp.	-*Chryseobacterium* spp. -*Haematobacter massiliensis* -*P. aminovorans* -*P. versutus* -*Proteus* spp.	-*F. magna* -*C. acnes*	-*Flavobacterium* spp. -*Porphyromonas* spp. -*Prevetolla* spp.	

(Continued)

TABLE 1 | Continued

Venous Leg Ulcers			
Gram-positive Aerobes	**Gram-negative Aerobes**	**Gram-positive Anaerobes**	**Gram-negative Anaerobes**
-*Micrococcus luteus* -MRSA -Methicillin sensitive *S. aureus* (MSSA) -*S. aureus* -*S. caprae/capitis* -*S. epidermidis* -*S. haemolyticus* -*S. lugdunensis* -*S. petrasii* -*S. agalactiae*	-*Rhodanobacter* spp. -*Sphingomonas* spp.		

TABLE 2 | Summary of studies characterizing host immune responses to bacteria and bacterial biofilms in wound models.

Wound Model	Bacterial Species/ Component	Host Response	Effects on Wound Healing	Reference
Mouse full-thickness excisional wound	*P. aeruginosa* PAO1	Early infection. In skin: ↑ neutrophils, ↓ NK cells, ↓$CD11b^+$ DCs, ↓Gr1-low MoDCs. In spleen: ↓T-cells. In lymph nodes: ↑pDCs. Late infection. In spleen: ↑macrophages, ↓NK cells, ↓IKDCs. In lymph nodes: ↑pre-apoptotic T-cells, ↑pDCs. Planktonic or biofilm infection: ↑TNF, ↑CXCL1, ↑IL-6, ↑IL-1b mRNA expression in skin HK infection: acute ↑neutrophils, ↓$CD103^+$ DCs	↑Bioburden of biofilm-infected wounds compared to planktonic infection	(68)
Kostelec minipig excisional flank wound reaching subcutaneous fat	*S. aureus, E. faecalis, B. subtilis, P. aeruginosa* clinical isolates Preformed biofilm	↑IL-8, ↑CXC-13, ↑arginase-1 ↑oxidative stress response (superoxide dismutase 2, angiopoietin-like 4) ↑MMP-1, ↑MMP-3 ↓collagen-1, ↓laminin-2	↓Granulation tissue formation	(69)
Pathogen-free mouse burn-induced wound	*P. aeruginosa* PAO1 embedded in seaweed alginate to mimic biofilm	↑ IL-1β, ↓AMP S100A8/A9 ↓KC, ↓G-CSF ↓VEGF	↓Wound closure	(70, 71)
Mouse full-thickness excisional wound	Absence of commensal microbiota	↑TNF-α, ↑ IL-10 ↑Alternatively activated macrophage (Dectin-1, Mannose receptor-1, Fizz-1, and Arginase-1) infiltration. ↑Mast cell infiltration ↓Neutrophil infiltration ↑ VEGF, ↑ type III collagen, ↑TGF- β1	↑Wound closure ↑Angiogenesis ↓Scar tissue	(72)
New Zealand white rabbit full-thickness ear wound	*P. aeruginosa* PAO1 *S. aureus* UAMS-1 Polymicrobial biofilm	↑IL-1β, ↑TNF-α mRNA expression compared to single-species biofilm	↓Epithelial and granulation tissue formation	(73)
New Zealand white rabbit full-thickness ear wound	*S. aureus* UAMS-1 Planktonic and biofilm	Low-grade, chronic inflammation (↓IL-1β, ↓TNF-α) mRNA expression compared to planktonic infection	↓Wound healing	(74)
Diabetic mouse full-thickness excisional wound	Wound microbiota	Longitudinal transcriptional shift in wound microbiota correlates with impaired and prolonged host defense response	↓Wound healing	(75)
Mouse full-thickness wound	Bioluminescent *S. aureus* SH1000	↑Systemic and wound infiltrating PMNs	No significant delay in wound healing	(76)
Surgical biopsy of patients with local infection due to a splinter, a bite, an abscess, or thrombophlebitis N=5	*S. aureus* was present in all skin biopsies	↑Granulocytes, ↑T-cells, ↑monocytes/ macrophages in skin ↓IL-8, ↑IL-6 ↑E-selectin, ↑VCAM-1 Keratinocytes: ↑ICAM-1, ↑TNF-α, ↑IL-1α	NA	(77)

NA, not applicable; NK, natural killer cells; DC, dendritic cell; MoDC, monocyte-derived DCs; pDC, plasmacytoid DC; IKDC, interferon killer DCs; HK: heat-killed; PMN, polymorphonuclear leukocytes; MMP, matrix metalloproteinase; AMP, antimicrobial peptide; KC, keratinocyte-derived chemokine; G-CSF, granulocyte-colony stimulating factor; VEGF, vascular epithelial growth factor.

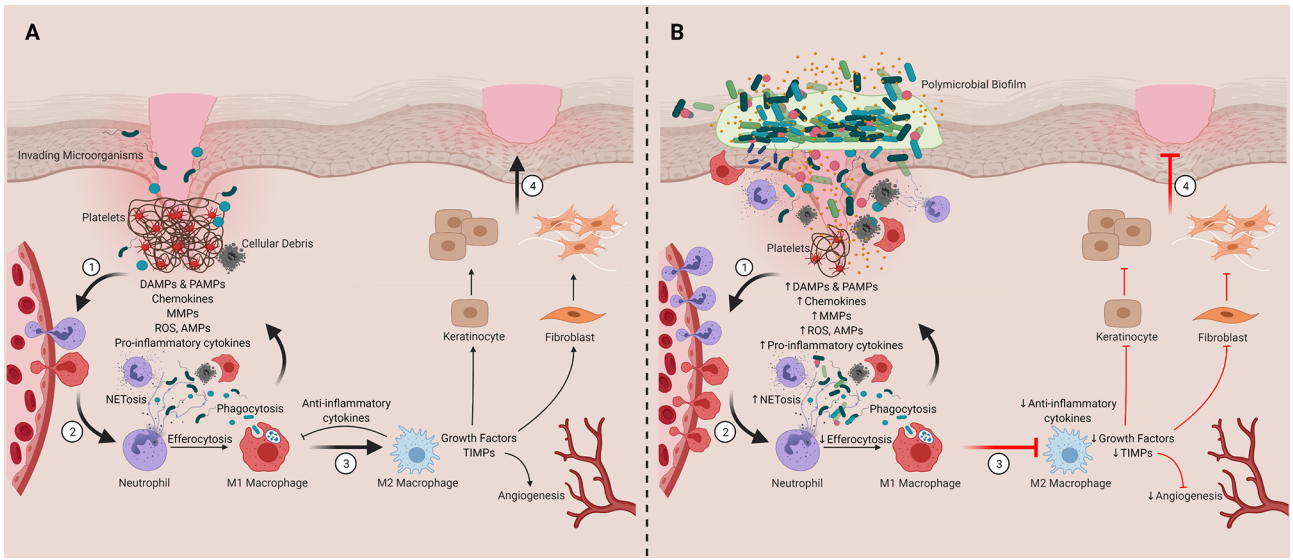

FIGURE 2 | Contribution of innate immune cells and inflammation to timely and delayed wound healing. **(A)** Representation of the four phases of wound healing ([1] Hemostasis, [2] Inflammation, [3] Proliferation and [4] Tissue Remodeling). **(B)** Chronic wounds are stalled in the inflammatory stage. We hypothesize that this inflammation is sustained by chronic activation of the innate immune system, which is driven their interactions and responses to polymicrobial biofilms found in and on the wound bed. DAMPs, damage-associate molecular patterns; PAMPs, pathogen-associated molecular patterns; MMPs, matrix metalloproteinases; ROS, reactive oxygen species; AMPs, antimicrobial peptides; TIMPs, tissue inhibitor of metalloproteinases. Created with BioRender.com.

Neutrophils

Neutrophils play a central role in healing damaged tissues and resolving infections. Early after the initial tissue damage, DAMPs, pathogen-associated molecular patterns (PAMPs), leukotriene B4 (LTB4), and CXCL8 family chemokines induce and augment neutrophil chemotaxis *via* CXCR2 into the wound bed (94–97). As the first cells recruited to the site of injury, neutrophils perform several diverse functions. First, they amplify inflammatory processes by releasing cytokines such as IL-1β, TNF-α, and IL-6 (85, 98) and function to prevent microbial invasion by phagocytosing microorganisms and releasing reactive oxygen species (ROS), proteases, and antimicrobial peptides (99, 100). Further, they undergo NETosis, where they form neutrophil extracellular traps (NETs) made of decondensed chromatin, histones, granular enzymes, antimicrobial peptides and proteases to immobilize and destroy exogenous pathogens (91, 100). They also release serine proteases, such as cathepsin G and elastase, and matrix metalloproteases (MMP), such as MMP2, 8, and 9 that degrade the ECM, facilitating tissue debridement that makes room for additional immune cell influx (92, 99). Neutrophils also contribute to tissue remodeling and repair. Theilgaard-Mönch et al. demonstrated that infiltrating neutrophils activate and induce the transcritption of genes involved in angiogenesis, keratinocyte adhesion, and migration and proliferation of keratinocytes and fibroblasts at the site of injury (98).

Macrophages

After damage has occurred, resident macrophages become activated by DAMPs and PAMPs in the local microenvironment, initiating the inflammatory response required to initiate the healing process (101, 102). Pro-inflammatory chemokines and cytokines such as CCL2, IFN-γ, and TNF-α recruit neutrophils and then circulating monocytes into the wound (102, 103). In mouse models, the expression of chemokine receptors CCR2 and CX3R1 has been shown to be critical for mediating monocyte recruitment to damaged tissues (104, 105). These infiltrating monocytes differentiate into macrophages, and along with resident macrophages, potentiate the inflammatory and healing processes.

Macrophages display incredible phenotypic plasticity, existing on a spectrum of inflammatory and anti-inflammatory activation states (106, 107). During the inflammatory phase of wound healing, macrophages display a proinflammatory or a classically activated M1-like phenotype, producing inflammatory mediators such as TNF-α, IL-6, IL-1β, IL-12, IL-23 to facilitate additional leukocyte recruitment and activation (34, 88, 89, 92). M1 cells also work cooperatively with neutrophils to phagocytose damaged cells and clear the wound of bacteria and tissue debris by producing ROS-containing phagosomes (92). Like neutrophils, they also produce MMPs to degrade the wound extracellular matrix to allow for further immune cell infiltration (92, 102). This digested matrix feeds back as DAMPs to amplify inflammatory signaling (108).

Following the clearance of debris and invading microorganisms, macrophages adopt an anti-inflammatory or an alternatively activated M2-like phenotype to support tissue remodeling and repair (106, 107). In vitro studies have shown that IL-4 and IL-13 drives M2 activation and wound healing functions (107). However, *in vivo*, the mechanisms driving this M1 to M2 phenotypic switch may be more complicated and may

be regulated by IL-10, glucocorticoids, prostaglandins, miRNAs, and adenosine and peroxisome proliferator-activated receptor (PPAR-γ) signaling in the wound microenvironment (86–88, 109). At the end of the inflammatory phase, macrophage phagocytosis of apoptotic neutrophils (i.e. efferocytosis) may also aid in the transition to the repair phases of wound healing (110).

To initiate tissue remodeling and repair, M2 cells produce IL-10, IL-12, and TGF-β to suppress inflammatory effects of TNF-α and IL-1β (90). During the proliferation phase they also release growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor-2 (FGF-2), which promote angiogenesis and keratinocyte and fibroblast proliferation (86, 88, 89, 91, 111). Finally, during the repair and remodeling phase, macrophages stimulate fibroblasts to mature into myofibroblasts that deposit collagen into the wound bed to replace the temporary fibrin scaffold with granulation tissue (86, 92, 112). They also regulate MMPs and tissue inhibitors of metalloproteinases (TIMPs) production to allow for both ECM formation and degradation of excess cellular components to restore homeostasis (92, 113). This M1 to M2 phenotypic switch makes macrophages a central cellular player in regulating the transition from inflammation to proliferation during wound healing (86).

Dysregulated Inflammatory Processes in Chronic Wounds

In chronic wounds, the orderly process of healing is thought to be stalled in the inflammatory phase and is characterized by persistent activation of the innate immune response (**Figure 2B**, described as Steps 1-4) (55, 87). This sustained inflammation drives additional immune cell infiltration and activation (Step 1), which amplifies MMP, collagenase, and elastase activity and suppresses TIMPs (114–119). This imbalance in proteolytic activity leads to excessive degradation of growth factors, anti-inflammatory cytokines, and ECM components, hindering progression through the phases of healing (120–122). This inflammation also drives excessive NETosis and reactive oxygen production (ROS), which contributes to further tissue damage and impaired healing (Step 2) (123–127). Impaired M1-M2 phenotypic switching has also been implicated in chronic wound formation (Step 3). In venous ulcers, iron overload has been linked to sustained proinflammatory M1 macrophage activation (128, 129). Further, studies in diabetic mice have demonstrated that dysfunctional efferocytosis of apoptotic neutrophils by pro-inflammatory macrophages results in apoptotic cell burden that causes sustained inflammation, preventing macrophages from transition into their anti-inflammatory state (130). In further support of these observations, depletion of M2 macrophages in mice with surgical wounds has been shown to increase neutrophil and M1 macrophage infiltration, which prolonged the inflammatory phase and decreased collagen deposition in wounds (131). Collectively these findings suggest sustained innate immune activation plays a central role driving chronic wound formation. Critically, it is unclear what exactly is driving this chronic innate immune activation. Further, it is unclear if the mechanisms driving this innate immune dysfunction including dysregulated M1-M2 switching differs across tissue microenvironments (e.g., wound types), particularly as emerging data suggests differences between antimicrobial and inflammatory responses across wound types (132)

Role of Bacteria in Driving Innate Immune Activation and Inflammation in Chronic Wounds

Emerging evidence suggests the wound microbiome and the formation of bacterial biofilms may contribute to delayed wound healing (45, 133–138). However, the causal relationship between microbiome composition, biofilm formation, dysregulated innate immune activation and persistent inflammation in chronic wounds remains poorly understood (12, 139). It is unclear what comes first – if bacteria and biofilm formation drive innate immune dysfunction, or if innate immune dysfunction makes the wound microenvironment more susceptible to biofilm formation. Understanding the role of bacteria-innate immune interactions in driving persistent inflammation and impaired healing in chronic wounds may offer new opportunities to restore healing processes (140). Previous reviews have explored key findings from *in vitro* studies (141–143). Here, we will focus on *in vivo* evidence from animal models and patient samples. We will summarize the existing knowledge on the role of bacteria-innate immune interactions in driving persistent inflammation in chronic wounds and explore novel treatments currently under development to target these interactions.

General Host Response to Bacteria or Bacterial Components in Wounds

While *in vitro* studies are important for identifying potential cellular and molecular mechanisms underlying innate dysfunction in chronic wounds, these models cannot capture the dynamic and complex nature of the immune response *in vivo*. Animal models and patient samples are better positioned to capture this complexity and can be used to evaluate localized as well as systemic immune responses.

Several studies have shown that wound bacterial infections can alter local and systemic immune responses (**Table 2**). Sweere et al. found that mice with chronic *P. aeruginosa* (PAO1) wound infections showed time-dependent changes in wound, lymph node, and spleen immune cell populations. In this model, early stages were associated with increased skin neutrophil infiltration and a reduction in the number of skin natural killer (NK) cells, CD11b+ dendritic cells (DCs), and Gr1-low monocyte-derived DCs (MoDCs) (68). Similarly, Kim et al. found wound colonization by *S. aureus* increased systemic polymorphonuclear leukocytes (PMN) by twofold and dramatically increased PMN recruitment into the wound bed (76). Systemic immune responses were more pronounced in later stages of wound infection and included increased lymph node plasmacytoid DCs (pDCs), increased splenic macrophages and lymph node pre-apoptotic T-cells and a reduction in NK cells and interferon killer DCs (IKDCs). Based on these findings, authors concluded that

adaptive immune responses might not play a significant role in driving the inflammatory response against *P. aeruginosa* (68). In human skin biopsies, Van der Laan et al. found diverse injuries result in different local inflammatory responses (77). Compared to aseptic conditions, wounds infected with *S. aureus* contained increased granulocytes, T-cells, and monocytes/macrophages in the dermal layer and dermis (77). However, further studies are required to understand changes to systemic responses in humans with chronic wounds.

Animals models have also been used to evaluate differences in immune responses to planktonic bacteria vs. biofilm infections in wounds. Sweere et al. found that *P. aeruginosa* infection was associated with increased TNF, CXCL1, IL6, IL1B mRNA expression in mouse skin but that these responses did not differ between planktonic bacteria vs. biofilms, despite the higher bioburden associated with the biofilm structure (68). However, Trostrup et al. found that *P. aeruginosa* biofilms inhibit wound healing by suppressing VEGF, antimicrobial peptide production (S100A8/A9) and neutrophil effector cytokine production (70, 71). In dermal punch wounds in rabbit ears, Gurjala et al. found that S. aureus biofilms trigger lower levels of inflammation in the wound bed compared to planktonic cells. However, they found that the persistent nature of the immune response to biofilms was shown to impair epithelial migration and granulation over time (74). Interestingly, using a similar rabbit model, Seth et al. found that polymicrobial biofilms containing *P. aeruginosa* (PAO1) and *S. aureus* (UAMS-1) triggered substantially higher inflammatory responses compared to single species structures (73). This elevated inflammatory response impaired wound epithelialization and granulation tissue formation. These authors also found that biofilm-deficient mutant strains of *S. aureus* were associated with reduced cytokine mRNA expression suggesting that the biofilm structure may, at least in part, contribute to the inflammatory response (73). In a porcine model, polymicrobial biofilms containing *S. aureus*, *E. faecalis*, *Bacillus subtilis*, *P. aeruginosa* were shown to prolong inflammation, increase necrosis, delay granulation, and impair the development of the extracellular matrix. Gene expression analysis revealed an upregulation of inflammatory mediators such as IL8, CXCL13, and arginase-1 (ARG1), as well as genes associated with oxidative stress response including superoxide dismutase 2 (SOD2) and angiopoietin-like 4 (ANGPTL4) (69).

Several studies have highlighted that the skin commensal microbiota play important functions in the regulation of wound healing and in the innate immune defense against infection (144–146). In fact, using longitudinal transcriptional profiling, Grice et al. found a shift in the wound microbiota of diabetic mice, and found this shift correlated with impaired healing and a prolonged inflammatory response (75). Alternatively, Canesso et al. showed that in germ-free Swiss mice, the absence of commensal microbiota enhanced TNF-α and IL-10 production, infiltration of alternatively activated macrophages and mast cells, and impeded neutrophil infiltration (72). These effects likely contributed to high levels of VEGF, type III collagen, and TGF-β, thereby accelerating wound closure and angiogenesis, and reducing in scar tissue formation (72). Further investigations are required to understand the intricate balance between the pro-wound healing commensal microbiota and pro-wound impairing pathogenic polymicrobial biofilms, as well as their role in driving a dysregulated inflammatory response in chronic wounds in humans.

Regulators of Immune Responses in Wounds With Bacterial Infections

Several molecules and pathways have been implicated in driving chronic inflammatory responses in non-healing wounds. Most of these have been identified in the context of overt bacterial infections (e.g., abscess) and do not address issues associated with wound chronicity. It is unclear if similar molecules/pathways contribute to delayed healing in wounds with varying levels of bacterial burden (e.g., contamination, colonization, local infection etc.). Despite these limitations, we have summarized these studies below (**Table 3**).

S. aureus is the leading cause of skin and soft tissue infections and is often used in abscess infection models (164, 165). Brandt et al. infected mouse skin with methicillin-resistant *S. aureus* (MRSA; USA300 LAC) and found high levels of LTB4 was produced by macrophages surrounding the abscess (147). This inflammatory lipid mediator is required to create a chemotactic gradient that directs neutrophil infiltration and helps to form an organized abscess architecture. It also aids in bacterial clearance by upregulation of pro-inflammatory cytokines (IFN-γ and IL-12p70) and increased NADPH oxidase activity (147) and has been shown to modulate keratinocyte activity (166). However, in diabetic mice, very high levels of LTB4 were found to be associated with dysregulated cytokine production and excessive neutrophil recruitment (148). This dysregulation was found to correlate with large nonhealing lesion areas and increased bacterial loads (148). Further, inhibition of LTB4 signaling through its receptor (BLT1) was shown to restore a functional inflammatory response, suggesting this molecule may play an important role in derailing the inflammatory milieu (148).

Advanced glycation end products (AGEs) and its receptor (RAGE) have also been implicated in the regulation of skin inflammation and diabetic pathologies (167, 168). Interestingly, Na et al. found that RAGE knockout mice infected with *S. aureus* (SH100) experienced less severe skin lesions and increased abscess formation (149). This milder skin damage was associated with increased neutrophil migration and increased bacterial clearance with reduced inflammation (e.g., monocyte chemoattractant protein-1 (MCP-1), high mobility group box protein 1 (HMGB1), IL-6, and TNF-α) (149). Paradoxically, RAGE-/- were also found to have high baseline levels of inflammation prior to infection (149). However, it was speculated that this priming may be protective and help establish rapid innate immune responses in early infection. Collectively, this data suggests that RAGE may be pathogenic in staphylococcal skin infection, particularly in supporting chronic inflammation.

Accumulating evidence suggests appropriate regulation of neutrophil activation is also critical for effective bacterial killing while limiting inflammation. This process is complex and multifactorial. For example, Cho et al. found that neutrophil

TABLE 3 | Summary of molecules and pathways that regulate the inflammatory responses to bacteria in wounds.

Molecule/ Pathway	Wound Model	Bacterial Species	Host Response	Effects on Wound Healing	Reference
Leukotriene B_4 (LTB_4)/BLT1 activity	Mouse subcutaneous wound	*S. aureus* USA300 LAC	Produced by skin macrophages. ↑Neutrophil chemotaxis (CXCL2, CXCL1, CCL8, CCL4, CCL2, and CXCL1) ↑IFN-γ, ↑IL-12p70 ↑NADPH oxidase bactericidal activity ↓Chronic inflammation (RAGE, TIM, CXCL2, IFN-γ, MMP12, and CCL8)	Organized abscess formation ↑Bacterial clearance	(147)
	Diabetic mouse skin wound (undefined)	*S. aureus* USA300 LAC	↑LTB_4/BLT1 activity ↑Macrophage and neutrophil infiltration ↓Localization to abscess Early infection: ↑ICAM-1, ↑MCP3, ↑IL-33, ↑IL-12p70, ↑IL-1α, ↑RAGE, ↓CXCL1, ↓CXCL2, ↓MIP1β, ↓CCL2, ↓IL-1β, ↓P-selectin Late infection: ↑CXCL1, ↑CCL2, ↑CCL8, ↑MCP3, ↑MIP1β, ↑P-selectin, ↑ICAM-1, ↑IL-1α, ↑IL-33, ↓IL-12p70, ↓RAGE	↑Abscess size with diffuse immune cell organization ↑Bacterial burden	(148)
Receptor for Advanced Glycation End Products (RAGE)	Mouse subcutaneous wound	*S. aureus* SH1000	↓MPO, ↓MCP-1, ↓HMGB1, ↓IL-6, and ↓TNF-α in skin prior to infection ↓Blood neutrophil and peritoneal macrophage infiltration	Severe open skin lesions ↓Abscess formation ↑Bacterial burden	(149)
Myeloid peroxisome proliferation activator receptor γ (PPARγ)	Mouse subcutaneous wound	*S. aureus* SF8300	For inflammation➔ resolution phase Formation of a glucose-depleted, hypoxic fibrotic abscess	↑Bacterial clearance of established infection that failed to clear during the inflammatory phase	(150)
miR-142	Mouse excisional wound	*S. aureus* NBRC 100910	↑*miR-142-3p* and *miR-142-5p* expression by infiltrating neutrophils and macrophages ↑Neutrophil recruitment and timely phagocytosis	Timely resolution of abscess Protection against horizontal transmission of infection	(151)
Myeloid differentiation primary response 88 (MyD88)	Mouse ear pinna intradermal wound	*S.aureus* Newman	Resident dermal macrophages sense *S.aureus via* myD88 For early recruitment and regulation of PMNs	Timely control and clearance of infection	(152)
IL-33	Patients with abscesses due to MRSA. N=3 Mouse intradermal wound	*S. aureus* CMCC(B)26003	↑IL-33 in human skin samples ↑iNOS in murine skin	↓Lesion size ↓Bacterial burden	(153)
Neutrophil-derived IL-1β/IL-1R signaling	Mouse intradermal wound	*S. aureus* SH1000 ALC2906	Induces expression of genes associated with neutrophil chemotaxis IL-1β is produced by neutrophils. TLR2, NOD2, and FPR1 aid in IL-1β production	↑Abscess formation	(154)
Proline-rich kinase (Pyk2)	Mouse skin abscess. Air-filled pouches in the dermis infected with bacteria	*S. aureus* (unknown strain)	↑PMN activation ↑MPO, ↑MMP9	↑Bacterial clearance	(155)
iNOS	Mouse full-thickness skin incisional and excisional wound	HK polymicrobial culture of *S. aureus*, coagulase-negative *Staphylococcus*, *Enterococcal species, P. mirabilis* previously isolated from normal mouse skin flora	↑IFN-γ from lymphocytes ↑iNOS	NA	(156)

NA, not applicable; MRSA, methicillin resistant S. aureus; MPO, myeloperoxidase; MCP-1, monocyte chemoattractant protein 1 (MCP-1); HMGB1, high mobility group box protein 1; FPR, formyl peptide receptor; iNOS, inducible nitric oxide synthase; PMN, polymorphonuclear leukocytes; MMP, matrix metalloproteinase; HK, heat-killed.

recruitment and abscess formation is temporally linked to IL-1β/IL-1R activation, which neutrophils produce as part of an autocrine loop. This loop is driven by α-toxin mediated activation of TLR2, NOD2, FPR1 and the ASC/NLRP3 inflammasome (154). Alternatively, Kamen et al. found proline rich kinase 2 (Pyk2) plays an important role in regulating integrin-mediated degranulation responses (155). Further, a number wound healing-related miRNAs have been identified and recent studies suggest that their dysregulation may contribute to wound pathologies (169). Among these, miRNA-142 has been shown to be an inflammation related miRNA that regulates neutrophil recruitment and S. aureus clearance through the inhibition of small GTPase translation (151). Additional studies are required to better elucidate its role in chronic wounds. Interestingly, resident dermal macrophages have been shown to play a central role in regulating both the timely escalation and eventual termination of neutrophil recruitment. Feuerstein et al. showed that this regulation is dependent on MyD88-dependent sensing of *staphylococci* and the recruitment of Ly6Chigh inflammatory monocytes into the skin (152).

In addition to neutrophils, macrophage responses must be tightly regulated during healing processes. In the inflammatory phase, these cells are proinflammatory (M1) and produce high levels of nitric oxide, reactive oxygen species and other antimicrobial peptides, which can be damaging to the local microenvironment. IL-33 represents a potential target as it plays a central role in activating antibacterial responses by activating the AKT-β-catenin pathway, which induces inducible nitric oxygen synthase (iNOS) and increases NO production (153). Alternatively, Mahoney et al. found lymphocyte derived IFN-γ drives the induction of iNOS in mouse wounds infected with heat-killed polymicrobial culture of *S. aureus*, coagulase-negative *Staphylococcus* and *Enterococcal* species as well as *Proteus mirabilis* (156). Alternatively, Xu et al. have shown that decreased NADPH oxidase activity and ROS production is associated with decreased infiltration of M2 macrophages and delayed wound healing suggesting a dichotomous role for these bioactive molecules (170). PPAR-γ has been shown to facilitate the M1-M2 transition (150). PPAR-γ has also been shown to play a role in MRSA clearance in chronic wounds by forming a glucose-depleted, hypoxic, fibrotic abscess that hinders bacterial growth (150). Finally, Guo et al. have demonstrated that AGEs contribute to excessive macrophage autophagy, which polarizes macrophages towards an M1 phenotype and supports sustained inflammatory processes (171). Moving forward it will be critical to understand how bacterial bioburden and composition contributes to the activation/inactivation of these pathways and to better understand the downstream consequences of dysregulated M1-M2 phenotypic switching in humans.

Other Modifiers of Host Antibacterial Immune Responses in the Wound Microenvironment

Aging is among one of the most significant predisposing factors to delayed healing and chronic wound formation. Older individuals are more commonly affected by vascular disease, venous insufficiency, unrelieved pressure, and post-surgery wound complications (172). Further, various studies have shown that aging affects all stages of the healing process including delayed re-epithelialization, angiogenesis, and collagen deposition (173–175). Changes skin strength may also result in a more pronounced breakdown of skin epithelial barriers, which may increase the bacterial bioburden in the wound microenvironment (175, 176). At the level of the immune system, advanced age is associated with a hyperinflammatory state (177). Innate cells have delayed infiltration, reduced phagocytic capacity, decreased reactive oxygen and nitrogen species production and impaired intracellular killing (178, 179). Interestingly, a recent study observed no age-related changes in TLR2 and FcγRIII expression, phagocytosis, and bactericidal activity in aged mice with cutaneous *S. aureus* infection (157). However, they did find that neutrophils had diminished sensitivity to chemokines (e.g., KC, MIP-2, and MCP-1), which reduced their chemotaxis into the wound bed and delayed healing (157). Additional studies are required to evaluate if similar dysfunction is observed in humans.

Diabetes is also associated with delayed healing and chronic wound formation (**Table 4**). Among the most common manifestations are non-healing foot ulcers (180). In these individuals wound healing is influenced by a predisposition to vascular disease and neuropathy, hypoxia, and hyperglycemia (181). Of particular importance, impaired vascular flow creates a prolonged hypoxic wound microenvironment, which along with hyperglycemia, contributes to oxidative stress (181). Hyperglycemia is also associated with AGE formation, which further delays healing (182). Chronic low-grade inflammatory also defines diabetes pathology and dysregulated healing response in these individuals (183). Emerging evidence suggests altered inflammatory responses to bacteria in the wound microenvironment may contribute to the development of these chronic wounds. In diabetic mice with cutaneous *S. aureus* infection, there is excessive macrophage and neutrophil infiltration into the wound but poor localization to abscess (148). These mice also have altered inflammatory cytokine and chemokine profiles during early and late stages of infection. Despite forming large abscesses, their structure had diffused immune cell organization and higher bacterial burdens (148). In mice infected with *P. aeruginosa*, diabetes was associated with prolonged M1 activation, which impaired healing processes by diminishing re-epithelialization, granulation tissue formation and angiogenesis (158). Alternatively, Nguyen et al. found diabetic mice inoculated with *S. aureus* biofilms had reduced TLR2 and TLR4 mRNA expression and high levels of inflammatory cytokines (IL-1β and TNF-α) (159). They also showed diabetic mice experience poor neutrophil penetration into regions with bacterial aggregates and downregulation of myeloperoxidase activity, a marker of neutrophil oxidative burst (159).

The type of chronic wound and the diversity/number of bacteria found in the wound may also play an important role in determining the magnitude of the inflammatory responses (**Table 4**).

TABLE 4 | Summary of other physiological factors that modify the inflammatory responses to bacteria in wound models.

Physiological State	Wound Model	Bacterial Species	Host Response	Effects on Wound Healing	Reference
Ageing	Mouse with full-thickness excisional wound	*S. aureus* Newman	No age-dependent changes in TLR2 expression, FcγRIII expression, phagocytosis, and bactericidal activity in macrophages and neutrophils ↓Neutrophil sensitivity to chemokines KC, MIP-2, and MCP-1 ↓Neutrophil chemotaxis and infiltration	↑Bacterial colonization, ↓Wound closure	(157)
Diabetes	Mouse full-thickness wound	*P. aeruginosa* ATCC27853	Prolonged M1 activation (TNF-α, IL-1β, IL-6) M2 activation (IL-10, arginase-1, or ym1)	↓Re-epithelialization ↓Granulation tissue formation ↓Angiogenesis ↓Wound closure	(158)
	Mouse full-thickness wound	*S. aureus* UAMS-1 biofilm	↓TLR2, ↓TLR4 mRNA expression ↓TNF-α, ↓IL-1β mRNA expression ↓Neutrophil infiltration in regions containing bacterial aggregates ↓MPO activity	↓Wound closure ↑Bacterial burden	(159)
Chronic venous leg ulcer (CVLU) or diabetic foot ulcer (DFU)	Wound exudate from patients with a CVLU or DFU	CVLU: *Pseudomonas, Staphylococcus, Corynebacterium* spp. DFU: *Corynebacterium, Staphylococcus* spp.	↑Bioburden (≥ 10^7 CFU/ml), CVLU: ↑Angiogenin, ↑ICAM-1, ↑IL-1β, ↑IL-4, ↑IL-6, ↑TNF-α, ↑TNFr2, ↑VEGF, ↑antioxidant capacity DFU: ↓IFN-γ, ↓IL-2, ↓IL-4, ↓IL-5, ↓IL-12p40, ↓IL-12p70, ↓IL-13, ↓TGF-β1 CVLU vs DFU, CVLU: ↑IFN-γ, ↑IL-1β, ↑IL-2, ↑IL-4, ↑IL-13, ↑TNF-α, ↑VEGF, ↑collagenase activity DFU: ↑carbonyl, ↑malondialdehyde, ↑antioxidant capacity	NA	(160)
	CVLU biopsy	*P. aeruginosa* (N=5) and *S. aureus* (N=5) aggregates	↑neutrophil infiltration in *P. aeruginosa* infected wounds compared to *S. aureus* infected wounds	NA	(161)
Recurrent subcutaneous SSSI	Mouse subcutaneous wound	MRSA USA300 LAC	Innate immune memory provides protection against recurrent SSSI: ↑M1 macrophages, ↑LDCs, ↑NK cells, ↑Th17 cells, ↑neutrophil influx to abscess ↑total macrophage population in inguinal lymph nodes. ↑IL-22, ↑IFN-γ, ↑IL-17A, ↑IL-6 ↑MIG, ↑RANTES in the skin and ↑IP-10 in blood AMPs ↑CRAMP, ↑mβD-3	↓Abscesses ↓Bacterial burden	(162, 163)

NA, not applicable; KC, keratinocyte-derived chemokine; Ym1, Chitinase-like 3 protein; MPO, myeloperoxidase; SSSI, skin and soft tissue infection; LDC, Langerhans$^+$ dendritic cell; NK, natural killer; MIG, monokine inducible by IFN-γ; RANTES, regulated upon activation, normal T cell expressed and secreted; IP-10, interferon gamma-induced protein 10; AMP, antimicrobial peptide.

For example, chronic venous leg ulcer (CVLU) exudate commonly contains *Pseudomonas, Staphylococcus, Corynebacterium* spp (160). With increasing levels of bacteria (≥10^7 CFU/ml), angiogenin, ICAM-1, IL-1β, IL-4, IL-6, TNF-α, TNFr2, VEGF, and antioxidant capacity are shown to be elevated (McInnes et al., 2014). Alternatively, diabetic foot ulcers, which lacked *P. aeruginosa*, have diminished IFN-γ, IL-2, IL-4, IL-5, IL-12p40, IL-12p70, IL-13, and TGF-β1 production with increasing bacterial bioburden (160). In comparison of the inflammatory response between CVLUs and DFUs, McInnes et al. determined that CVLUs have higher levels of IFN-γ, IL-1β, IL-2, IL-4, IL-13, TNF-α, VEGF, and increased collagenase activity compared to DFUs. On the other hand, DFUs showed higher levels of carbonyl, malondialdehyde and antioxidant capacity compared to CVLUs (160). Moreover, Fazli et al. found in chronic venous leg ulcer biopsy samples, *P. aeruginosa* aggregates displayed amplified neutrophil infiltration compared to *S. aureus* aggregates in wounds, suggesting that these differences may be due to the intrinsic properties of *P. aeruginosa* to mount a higher inflammatory response (161). Additional studies are required to further explore these associations and differences.

Past exposures to bacterial infections may also alter local immune responses to wound infections (**Table 4**). Upon first exposure to a pathogen, innate immune cells can adapt, such that upon re-exposure, they mount a heightened pathogen-specific inflammatory response to boost host defense and provide long-term protection (184). This phenomenon is known as innate immune memory (184). Emerging evidence suggests innate immune memory may provide protection against recurrent staphylococcal skin infection. In mice primed by prior S. aureus infection, lesion severity was reduced by increased M1 macrophage, Langerhans+ DCs (LDC), NK cells, Th17 cells, and neutrophil influx to the abscess (162, 163). Interestingly, cytokines IL-6, IL-17, IL-22, chemokines MIG and RANTES,

and antimicrobial peptides CRAMP and mβD-3 contributed to the development of innate immune memory in these mice (162, 163). In the context of chronic wounds, innate immune cells are chronically exposed to bacterial biofilms rather than a first exposure followed by recurrent exposure paradigm. Whether persistent activation of innate immune cells constrains development of this protective innate immune memory or not remains to be elucidated.

TARGETING HOST-PATHOGEN INTERACTIONS TO RESTORE HEALING PROCESSES

Managing and treating chronic wounds can be very challenging. It requires a comprehensive wound assessment and the establishment and implementation of a plan of care. These individualized plans aim to optimize the local wound environment and drive healing using four basic strategies: wound cleansing, debridement, moisture control, and bacterial balance (35). Among these, controlling bacterial bioburden is essential for wound healing and can be done by 1) reducing the levels of bacteria found in the wound and/or by 2) optimizing host immune responses to the infection. For the purposes of this review, we will briefly discuss how standard treatments reduce bacterial bioburden and/or restore immune function. Then, we will discuss emerging therapeutics designed to target interactions between bacteria/biofilms and the host immune response to restore healing processes.

Debridement and Negative Pressure Wound Therapy (NPWT)

Both debridement and NPWT have been shown to affect bacterial bioburden and/or inflammation in the wound bed. Many types of debridement technologies exist including biological (maggot/larval therapy), mechanical, hydrosurgical, chemical, autolytic, enzymatic, surgical, and conservative sharp debridement (35). The purpose of these procedures is to remove necrotic or infected tissue to facilitate healing. In addition to removing infected tissue, debridement has been shown to remove and disrupt mature biofilms. Wolcott et al. found serial debridement to continually remove mature biofilms can be used to increase the efficacy of topical antimicrobials on newly forming/immature biofilms, which are more susceptible to treatment (185). NPWT, also known as vacuum assisted closure (VAC) therapy, improves healing by removing excess exudate, maintaining moisture balance, and increasing blood flow into the wound. It has also been shown to control infection and modulate immune responses. In animal models, NPWT has been shown to have anti-biofilm effects (186–188). It has also been shown to modulate growth factor, cytokine expression, and matrix metalloproteinases to support healing (189, 190). This includes decreasing IL-6, iNOS, TNF-α, IL-1β, MMP-1, and MMP-9 and upregulating VEGF, TGF-β1 and TIMP-1 in patients with diabetic foot ulcers (191, 192).

Antiseptics

Irrigation solutions such as sterile normal saline or sterile water are the simplest wound cleansing methods. Antiseptic agents, such as octenidine dihydrochloride (OCT), polyhexamethylene biguanide (PHMB), povidone-iodine, and super oxidized hypochlorous acid (HOCl) and sodium hypochlorite (NaOCl) are widely used in topical wound therapy in solution form or as functionalized dressings due to their high microbicidal and anti-biofilm properties (193–197). OCT and PHMB have surfactant properties to help break apart biofilms and PHMB is particularly useful due to its low toxicity (198). Silver- and copper-impregnated dressing are also widely used in chronic wound management (199, 200). However, a recent scoping review by Rodriguez-Arguello et al. established mixed results in terms of antimicrobial activity and clinical effectiveness of silver agents (201). Moreover, a recent systematic review that evaluated the efficacy of commercially available topical agents containing silver, iodine, PHMB, or hypochlorous acid concluded that a lack of *in vivo* evidence makes it difficult to make recommendations for biofilm-infected wounds (202). Little research is available on the effects of these agents on modulation of the immune/host response. In human ex vivo full-thickness skin injury, OCT has been shown to dampen pro-inflammatory and anti-inflammatory cytokines IL-8, IL-33, and IL-10, but not growth factors VEGF and TGF-β1 (203). In an ex vivo porcine skin model, povidone-iodine, silver lactate, and OCT showed antiprotease activity that was dependent on their wound penetration ability (204). These anti-inflammatory properties need to be further investigated *in vivo*.

Antibiotics

Antibiotics are often also used in the management of chronic wound infections. However, the type of antibiotic prescribed, and the administration route depend on the clinician evaluation and must take into consideration the microbial bioburden, patient clinical condition (e.g., allergies, immunocompetence, comorbidities, and pregnancy), the severity of the infection, and drug toxicity and dosage (35, 205). For instance, contaminated and colonized wounds do not require the use of antibiotics to improve wound outcomes. Alternatively, local infection often involves the use of topical antimicrobials including antibiotics compared to systemic infections that use systemic antibiotics (35, 206).

Topical antibiotics provide a high drug concentration at the infection site and possess low toxicity since the body systemically absorbs a low amount of drug. Moreover, they are easy to apply, and their use can avoid the use of systemic antibiotics. However, topical antibiotics cannot be prescribed to treat deep tissue infections, can affect healing, can cause hypersensitivity, and can select for resistant microorganisms (207, 208). They are often formulated as ointments, gels, creams, and powders, and only a few are available for use (e.g., bacitracin, fusidic acid, gentamicin, mafenide acetate, metronidazole, mupirocin, neomycin, nitrofurazone, polymyxin B, retapamulin, silver sulfadiazine, sulfacetamide Na^+). The antibiotic used also depends on the type of wound. Bacitracin, neomycin sulfate, and polymyxin B are frequently used in combination to treat minor skin injuries. Silver sulfadiazine cream is commonly used as a

topical antibiotic to treat DFUs and pressure ulcers (209, 210). Gentamicin and sulfacetamide are used to treat secondary infections, colistin (polymyxin E) is used for MDR gram-negative infections and metronidazole is commonly used to treat infections caused by anaerobic microbes and to reduce the odor of wounds (207).

Systemic antibiotics are used in patients with more severe infections. However, in these cases, the resistance profile of the pathogen is closely related to the success of the treatment (206). For instance, vancomycin is the first-line treatment to fight MRSA infections, followed by second-line agents, including linezolid, daptomycin, and quinupristin-dalfopristin. Other examples of systemic antibiotics used to treat chronic wounds include macrolides (e.g., erythromycin, azithromycin, and dirithromycin), β-lactams (e.g., cephalosporin, amoxicillin), penicillinase-resistant penicillins (e.g., cloxacillin, oxacillin), trimethoprim-sulfamethoxazole, fluoroquinolone, tigecycline, and clindamycin (35, 206, 211). The type of antibiotic prescribed is also dependent on the type, location and severity of the wound. For mild and moderate DFUs, narrow-spectrum antibiotics are recommended, especially those active against Gram-positive cocci (211). Alternatively, severe DFUs should be initially treated with broad-spectrum antibiotics, such as carbapenem β-lactams or the combination of β-lactam antibiotics and β-lactamase inhibitors (e.g., piperacillin/tazobactam, ampicillin/sulbactam, ticarcillin/clavulanic acid and amoxicillin/clavulanic acid) (210). The use of intravenous antibiotics is recommended to treat pressure ulcers when there is sign of osteomyelitis (209). In these cases, antibiotics that penetrate the bone are required, such as β-lactams (e.g., penicillin and cephalosporin), fluoroquinolones aminoglycosides, and glycopeptides (e.g., vancomycin), linezolid, and rifampin (212).

Several challenges, such as the formation of multi-species biofilms, are implicated in antibiotic treatment success. Wounds are often infected with polymicrobial biofilms formed by several species of resistant bacteria. These biofilms are commonly resistant to topical and systemic antibiotics, which reduces the effectiveness of the antimicrobial treatment. For instance, Shettigar et al. found the authors showed that 60% of the DFU samples investigated were infected with polymicrobial biofilms, in which the isolated *E. faecalis* showed higher resistance to antibiotics than non-biofilm grown cells (213). Furthermore, bacteria within biofilms produce several protective components. Among them, the EPS matrix is an important factor that impairs the penetration of antibiotics into the wound bed. For instance, *P. aeruginosa* EPS contains extracellular DNA and alginate lyase that impairs the diffusion of aminoglycosides (214, 215). Another problem associated with the low permeability of antimicrobial agents through the biofilm structure is the induction of resistance due to the low concentration of antibiotics when they reach the bacterial cells (56).

New Approaches to Treating Chronic Wounds

A variety of new treatments are under development to improve healing and restore tissue homeostasis. Among the most promising are candidates that target or work in conjunction with the innate immune system to improve antibacterial immune responses and/or regulate inflammatory responses. Some of the most promising are highlighted in **Figure 3**. These pathways and molecules represent viable targets because they can be used to modulate both early and late healing processes. There is also a reduced risk of developing drug resistance. Here, we will discuss the potential use of antimicrobial peptides in targeting bacteria levels/biofilm formation and in modulating immune function. We will also describe other strategies under development that seek to develop smarter and controlled innate immune responses by priming the antibacterial immune responses, restoring inflammatory balance, and selectively inducing an M1-M2 transition.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) show tremendous potential for the treatment of severe and chronic infections. These peptides have a broad-spectrum antibacterial activity that usually involves attacking multiple hydrophobic and/or polyanionic targets (216). They have been shown to induce pore formation, disrupt cellular and organelle membrane integrity, inhibit protein and nucleic acid synthesis, block enzymatic activity, inhibit cell wall synthesis, and induce apoptosis through the generation of ROS (217, 218). In addition to their antimicrobial effects, AMPs also modulate immune response to improve bacterial killing by increasing numbers of antigen presenting cells, facilitating the release of NETs, enhancing of phagocytosis, modulating dendritic cell differentiation and T cell activation, suppressing inflammatory signaling and anti-inflammatory cytokines (218). Further, many AMPs have been shown to promote wound healing by modulating of keratinocyte cytokine production and migration, re-epithelialization and angiogenesis (219). In addition, several natural and synthetic AMPs exhibit strong antibiofilm activities, for example by disrupting bacterial communication networks (quorum sensing), inhibiting bacterial cell adhesion or by stimulating biofilm dispersal (220). Here, we discuss just a few examples of these bioactive molecules.

In humans, AMPs are produced by a variety of cell types including skin epithelia cells. In conditions of health, RNase 5, RNase 7, psoriasin and calprotectin are produced and have antimicrobial activity against both Gram-negative and Gram-positive bacteria (221–223). Alternatively, under conditions of inflammation or infection, β-defensins (h-BD) and LL-37 are selectively induced to mount a wide spectrum of antimicrobial activities including antibiofilm activities (219, 224). In addition to their microbicidal activity, hBD has been shown to regulate inflammatory processes by inhibiting TLR signaling pathways and transcriptionally repressing of pro-inflammatory genes expression (225, 226). Further, hBD-3 has also been shown to act as a ligand for the macrophage receptor CCR2, attracting macrophages to sites of epithelial injury (227). LL37 has also been shown to neutralize the activation of macrophages *via* LPS and induce proliferation and migration of endothelial cells (228). It also contributes to multiple phases wound repair including the stimulation endothelia cells and fibroblasts (228, 229) stimulation of keratinocytes (230), neovascularization (228) and angiogenesis (231). A number of approaches are currently under development for administering hBD and LL-37 including poly(vinyl alcohol)/cellulose acetate (PVA/CA) films (232), nanoparticles (233, 234), and nanostructured lipid carriers (235). Further, the efficacy of LL-37

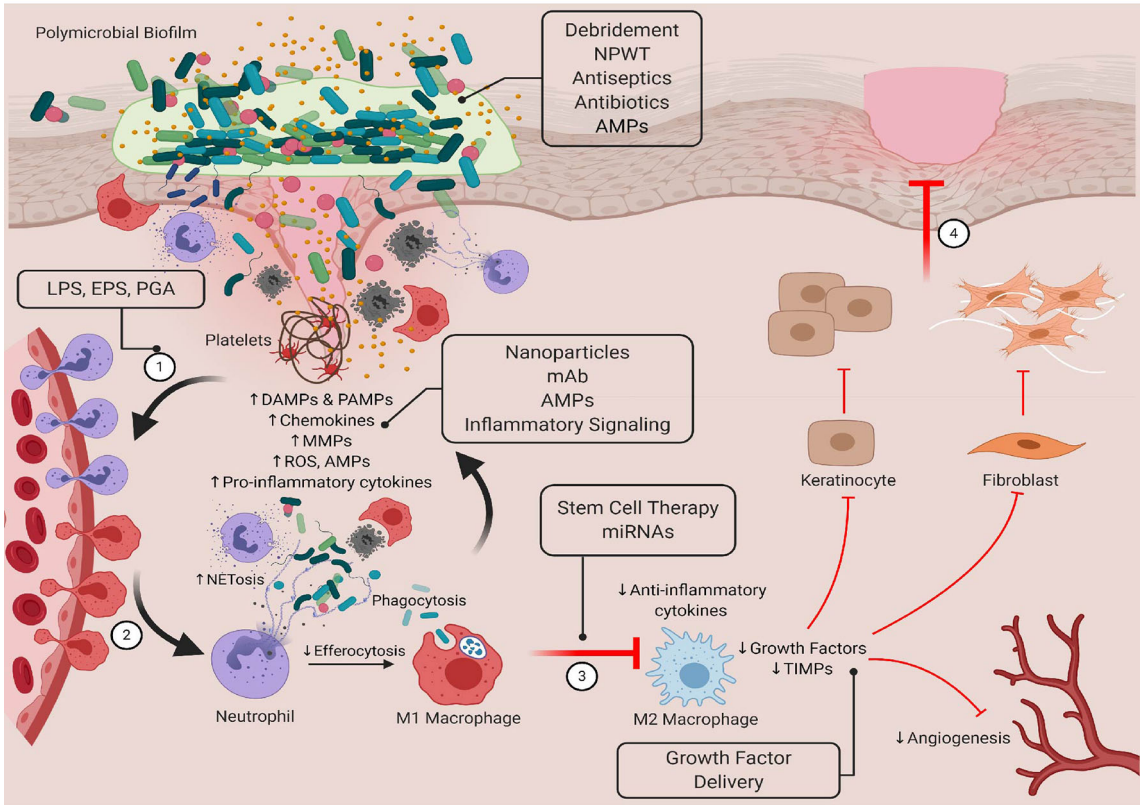

FIGURE 3 | Targeting bacteria-innate immune interactions to restore healing in chronic wounds. Standard therapies such as debridement, NPWT, antiseptics, and antibiotics have been shown to reduce bacterial bioburden in the wound bed, but they do not always restore healing processes. New therapeutics that have both antimicrobial and immunomodulatory properties may be able to overcome the limitations of more traditional treatments. Here, we show novel therapeutics that target these interactions that can be used in early and late stages of healing to restore tissue homeostasis. LPS, lipopolysaccharide; EPS, extracellular polymeric substance; PGA, peptidoglycan; AMP, antimicrobial peptide; mAb, monoclonal antibody; miRNA, microRNA. Created with BioRender.com.

cream in treating DFU is currently in clinical trials (https://clinicaltrials.gov).

Interestingly, wound healing is a relatively conserved evolutionarily process and several species including insects such as *Drosophila*, *Caenorhabditis elegans* and amphibians have been shown produce their own AMPs. For example, Pseudin-2 isolated from the frog *Pseudis paradoxa* has been shown to have a broad-spectrum antimicrobial potency and skin biocompatibility against multidrug-resistant (MDR) *Pseudomonas aeruginosa* (236). It has also been shown to facilitate infected-wound closure by reducing inflammation through suppression of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) (236). Alternatively, the synthetic peptide A3-APO, derived from natural insect products, has shown promise in *in vivo* models. It was shown to efficiently ameliorate resistant aerobic and anaerobic intradermal infections, in part by increased recruitment of epithelial macrophages and their immunomodulatory/anti-inflammatory effects (237). Epinecidin-1 (Epi-1), an AMP derived from grouper *Epinephelus coioides* is also of potential interest. This molecule has been shown to have antibacterial, antifungal, and antiviral activity *in vitro* and *in vivo* (238). In mice with MRSA, Epi-1 has been shown to decrease levels of TNF-a, IL-6, and MCP-1, while also regulating monocyte recruitment during wound healing (239). It also enhances wound closure and angiogenesis (239). These molecules and many others are in early stages of development but represent promising antimicrobial and immunomodulatory therapeutics.

Jump-Starting Innate Immune Responses Using Bacteria/Bacterial Components

Topical application of PAMPs isolated from bacteria has been used to stimulate wound healing by initiating inflammatory processes in early stages of healing. In mice, Kostarnoy et al. have shown that application of lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria and a potent endotoxin, improves healing by accelerating the resolution of inflammation by increasing macrophage infiltration, the expression of proinflammatory cytokines (IL-6, IL-1β, and leukemia inhibitory factor (LIF)), CC-chemokines (CCL2, CCL7, CCL3 and CCL5), growth factors (VEGF, TGF-1β, and FGF-2) in the wound microenvironment and by increasing collagen synthesis in the wound microenvironment (240). Similarly, the exopolysaccharide or extracellular polymeric

substance EPS-S3 derived from the marine bacterium *Pantoea* sp. YU16-S3 has been shown to be a potential biomolecule to promote skin tissue regeneration (241). *In vitro*, EPS-S3 has been shown to increase dermal fibroblasts and keratinocytes, and macrophage activation (241). In vivo, EPS-S3 increases expression of growth factors and adhesion molecules HB-EGF, FGF, E-cadherin suggesting this exopolysaccharide may modulates wound healing through the Wnt/β-catenin pathway (241). Interestingly, other studies have shown that rats subcutaneously implanted with PVA sponges inoculated with non-viable S. aureus or its peptidoglycan have improved healing responses (242, 243). These responses are associated with increased macrophage, neutrophil and fibroblast infiltration, collagen production, and angiogenesis, which contribute to the formation of reparative tissue.

Improving Antimicrobial Responses

Another area of interest is to develop therapeutics that enhance antimicrobial immune responses. Yu et al. have developed iron oxide nanoparticles (IONPs) that are taken up by macrophages to enhance bactericidal activity against intracellular S. aureus. They do this by increasing the expression of pro-inflammatory M1 markers iNOS, IL-1β, and TNF-α and amplifying ROS production (244). They also found these bactericidal effects could be enhanced by coupling IONPs with vitamin C in a Fenton reaction to augment the formation of ROS in the form of hydroxyl radicals (244). Alternatively, Okumura et al. shown that the pharmacological agent AKB-4924 can promote antibacterial immune responses by stabilizing HIF-1α (245). Intracellular HIF-1α accumulation heightens pro-inflammatory responses by increasing expression of LL-37 and IL-8 in human monocytes and enhancing bactericidal activity *in vitro* and *in vivo* (245).

Targeting Inflammatory Balance

As described above, persistent or sustained inflammation is a central drive of dysregulated healing and chronic ulcer formation. Therapeutics that neutralize pro-inflammatory biomolecules such as cytokines, chemokines and bioactive lipids are under investigation to limit tissue damage and restore timely healing processes. Among these, Song et al. have examined the effect of anti-TNF- α monoclonal antibody (MAb) in a primate model of S. aureus-associated skin (246). Systemic administration of anti-TNF-α MAb reduced abscess severity through suppression of circulating proinflammatory IL-8 and IL-12, benefiting host responses to bacterial challenge (246). Alternatively, Brandt et al. have shown that therapeutics that target the LTB4/BLT1 signaling axis can reduce abscess severity and inflammation by limiting neutrophil recruitment, improving chemotaxis, and restoring chemokines and cytokine levels (148). More recently, Vågesjö et al. have developed CXCL12-delivering Lactobacilli to topically administer chemokines with increased bioavailability for wound healing processes (247). In mice models, sustained topical application of CXCL12 increased proliferation of TGF-β expressing macrophages (247) and accelerated wound healing in healthy mice, mice with hyperglycemia, and peripheral ischemia, and in an *in vitro* human skin disk model (247).

Growth factors also play a critical role in modulating inflammation and inducing cell proliferation, angiogenesis, and granulation tissue formation during wound healing. Diminished levels of growth factors, such as VEGF and FGF-2 have been associated with chronic pressure ulcers (248). To restore this imbalance novel therapeutics have been focused on delivering growth factors to the wound bed to promote healing. In a study on diabetic foot ulcer patients receiving intralesional epidermal growth factor (EGF) therapy, García-Ojalvo et al. demonstrated a reduction in systemic proinflammatory biomolecules C-reactive protein (CRP), IL-6, soluble FAS (sFAS), and CCL3, as well as oxidative capacity and nitrosilative (nitrite/nitrate) stress biomarkers (249). Further, intralesional EGF therapy was shown to increase soluble RAGE (sRAGE), which may have protective effects in diabetic patients (249). Another approach to increase growth factors is to provide bone-marrow mesenchymal stem cells (BM-MSCs). Bai et al. developed an injectable hydrogel made from crosslinking N-chitosan and adipic acid dihydrazide with hyaluronic acid-aldehyde to deliver BM-MSCs into the wound bed (250). *In vitro* and *in vivo* investigations in diabetic wound healing showed that the hydrogel was able to stimulate BM-MSC-derived secretion of TGF-β1, VEGF, and FGF-2 as well as inhibiting chronic inflammation through M2 macrophage polarization (250). It also induced granulation tissue formation, collagen deposition, tissue vascularization, and improved wound closure (250). The LeucoPatch uses a similar approach to promote healing. This circular patch is comprised of fibrin, white cells and platelets derived from the patient's own blood, which concentrates cells and growth factors (e.g., PDGF, TGF-β, EGF and VEGF) to support healing. In an observed-masked randomized controlled trial, it was found to improve healing outcomes and shortened time to healing (251).

Promoting Macrophage Polarization Towards an M2 Phenotype

Given that M1/M2 macrophages play a key role in regulating wound healing, reprogramming macrophage polarization towards an M2 tissue repair phenotype represents an attractive target in later stages of the healing process. Stem cell therapy has emerged as a promising treatment for modulating these processes. In a mouse diabetic wound model, treatment with hyaluronic acid spongy hydrogels impregnated with neurogenically conditioned human adipose stem cells (hASCs) increased neoepidermial thickness and accelerated wound closure (252). Moreover, addition of hASCs in comparison to hydrogel treatment alone increased the M2/M1 macrophage ratio, suggesting that hASCs can promote the transition to the repair phase of healing (252). A recent systematic review by Raghuram et al., identified adipose-derived stem cells, bone marrow-derived stem cells, bone marrow-derived mononuclear cells, epidermally derived mesenchymal stem cells, fibroblast stem cells, keratinocyte stem cells, placental mesenchymal stem cells, and umbilical cord mesenchymal stem cells being used *in vitro* and *in vivo* as potential treatments for chronic wounds,

however, clinical effectiveness still requires investigation due to heterogeneity of wound etiology (253).

A multitude of micro-RNAs (miRNAs) have been associated with each phase of wound healing, from pro-inflammatory cytokine signaling to proliferation and remodeling, and offer a potential therapeutic strategy for the treatment of chronic wounds (254). Saleh et al. developed adhesive hydrogels containing miR-223 5p mimic loaded hyaluronic acid-based nanoparticles (255). In vitro, miR-233 5p had the ability to polarize M1 macrophages towards an M2 phenotype, with increased expression of anti-inflammatory gene Arg-1, and suppression of proinflammatory cytokines TNF-α, IL-1β, and IL-6 (255). In vivo experiments on a mouse wound model, miR-233 5p was able to promote tissue vascularization and accelerate wound healing (255).

DISCUSSION

Ageing and immobile individuals as well as those with co-morbid conditions such as diabetes are at high risk for developing non-healing or chronic wounds. These wounds reduce quality of life and increase pain levels, risk for infection and prolong hospital stays. Chronic wounds are also difficult to treat and represent a significant financial burden on all healthcare systems. Globally, as the size of these populations grow, there is an urgent need to understand the pathophysiology of delayed wound healing and to develop effective therapies that repair tissue damage. Critical for the development of these new therapeutics is a comprehensive understanding of the cellular and molecular mechanisms underpinning bacteria-innate immune interactions.

There is still a lot to learn about the microbiological and immunological processes underlying bacteria-innate immune interactions in chronic wounds and their relative contributions to delayed healing. From the microbiological perspective, the use of molecular methods, such as RNA sequencing, has allowed for the identification of a larger diversity bacterial species in the wound. These methodologies have also been used to elucidate microbial activities, behaviors, strategies, and processes during infections (12). However, these approaches are associated with a substantial demand for financial, time and bioinformatic support and cannot be readily transferred into the clinic. Further, they cannot distinguish between living, dead or dormant bacteria and might overlook minority species (52). Moving forward, it will also be critical develop more standardized sampling and analysis to ensure reproducibility across studies (256–258). It is also important to note that, to date, most studies have been performed over an acute timeframe with the longest being over a 28-day period (75). Considering that chronic wounds can take up to 12 months to heal (259), or may not heal at all (55), there is little information about how bacterial populations change over longer time frames. We believe combining single cell analyses (transciptomics, flow, etc.), advanced microscopy and other techniques will provide critical insights into how biofilm structures as well individual cells contribute to chronic wound formation and chronicity across diverse microenvironments and patient populations. Further, we believe more long term longitudinal *in vivo* studies with larger samples sizes and standardized sample collection/analysis are urgently needed to fully understand the importance of microbial diversity, biofilms and the wound microbiome in chronic wounds infections and to elucidate the impact of aerobic, anaerobic, pathogenic and commensal bacteria in inflammation and wound healing across wound types.

To further our immunological understanding, we require clinically relevant model systems that mimic the complex, dynamic interplay between the wound microbiome, innate immune cells, and the various other factors that contribute to dysregulated healing in chronic wounds (260). To date, many studies have investigated interactions in the context of murine *S. aureus* abscess models. While *S. aureus* is a major causative agent of skin and soft-tissue infections, it is a specific type of skin infection. Other studies have evaluated how heat-killed bacteria and planktonic bacteria modulate immune responses but generally only evaluate short term and localized responses. Emerging research has started to evaluate the effects of single-species and polymicrobial biofilms on host immune responses *in vivo*. However, most of these studies have characterized differential responses to *S. aureus* and *P. aeruginosa* planktonic, single-species biofilm, and polymicrobial biofilms (69–71, 73, 74). Given that the wound microbiome is made of a wide diversity of bacterial species in polymicrobial biofilm communities, it is unclear how these findings can be translated into the clinical setting. We also think it is important to note, that much of what we know about the microbial diversity and immune responses in chronic wounds has been derived from models and clinical samples from patients with DFU. In this review, we found a few studies that evaluated interactions in other or non-DFU ulcers such as venous/arterial ulcers and pressure ulcers, but they were somewhat limited in scope (77, 160, 161). Future studies are required to evaluate how host immune responses are modulated by complex polymicrobial biofilms commonly found in wound beds and to better understand if these processes are affected by the wound type/tissue microenvironment.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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