ANTIMICROBIAL PEPTIDES AND COMPLEMENT – MAXIMISING THE INFLAMMATORY RESPONSE

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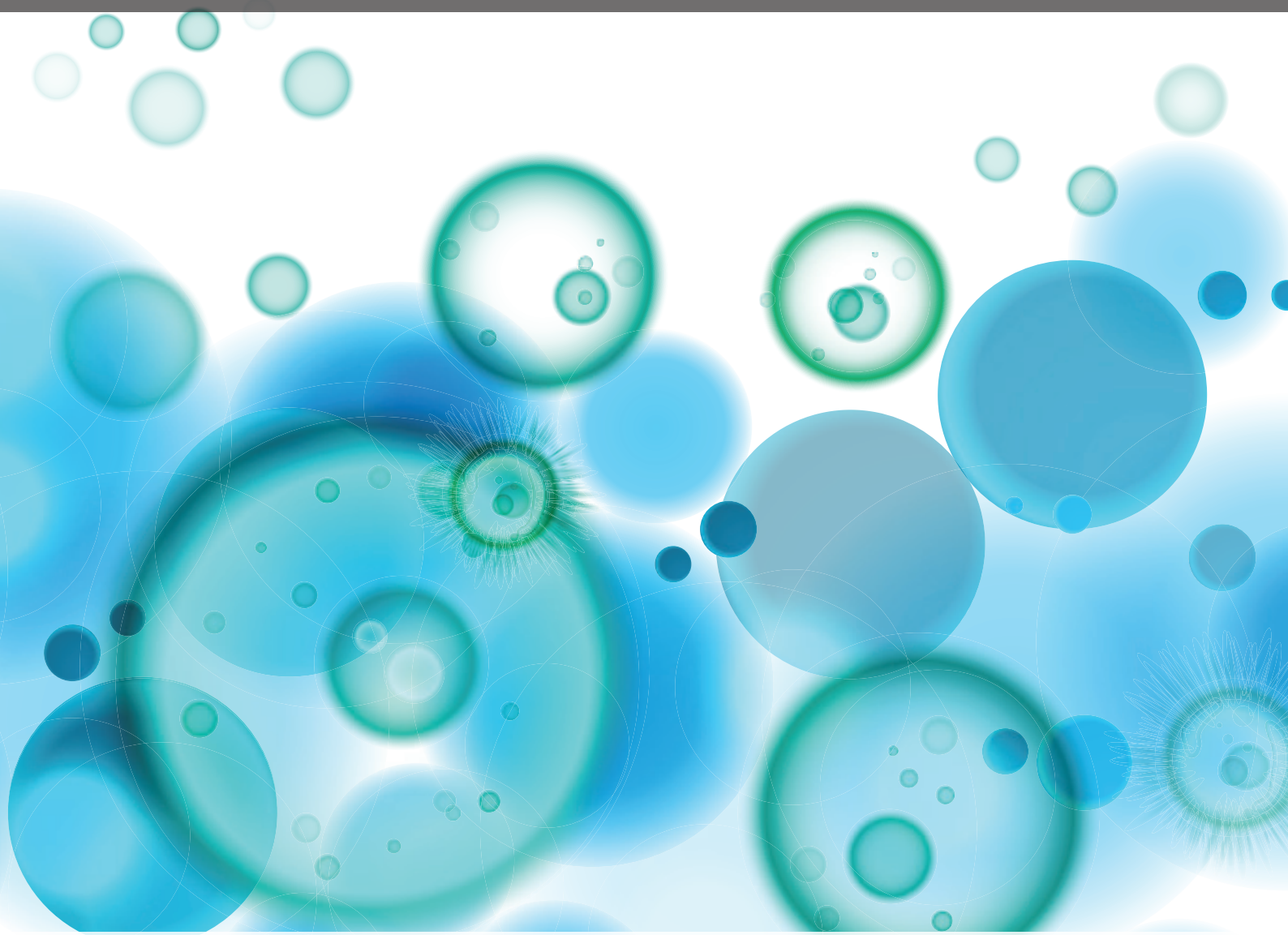

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ANTIMICROBIAL PEPTIDES AND COMPLEMENT – MAXIMISING THE INFLAMMATORY RESPONSE

Topic Editor:
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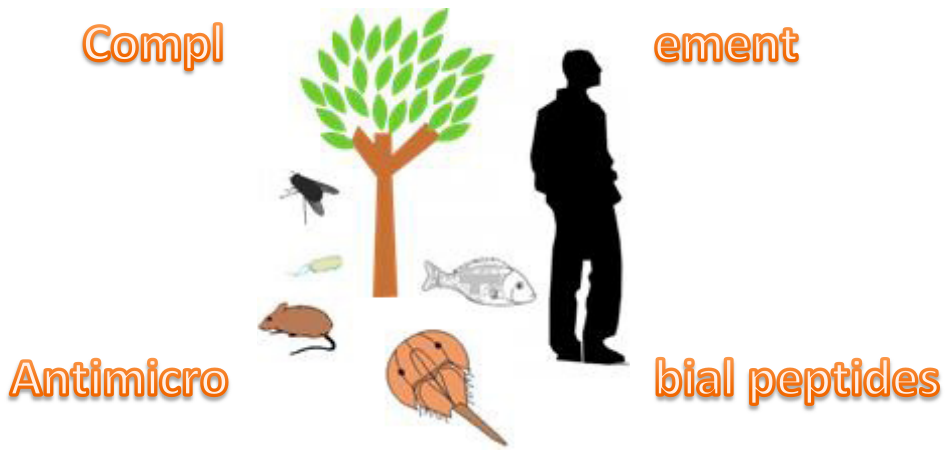

A selection of the diversity of organisms considered within this e-book series is presented. The series taps into biological and medical knowledge to develop an encompassing theme of system activities "Complement / Antimicrobial peptides".

Image by Cordula M. Stover

"Wonder is the seed of knowledge." Francis Bacon, 1605

Antimicrobial peptides and complement are distinct components of the innate immune defence. While antimicrobial peptides, after cleavage of a preproprotein, have the ability to insert directly in non host membranes, complement requires a sequential enzymatic activation in the fluid phase in order to produce a transmembrane membrane attack complex. Its insertion is controlled by membrane bound regulators. Deficiencies are described for both effectors and relate to increased susceptibility of infection. In addition, however, antimicrobial peptides and complement each influence the activity of inflammatory cells as recent data in the respective research areas shows. This series of articles draws together for the entities of antimicrobial

peptides and complement a balance of contributions in the areas of evolution, roles, functions and preclinical applications. By comparing and contrasting antimicrobial peptides and complement, greater cross-disciplinary appreciation will be derived for their individual and overlapping spectra of activity, circumstances of activation and their general ability to more completely inform the inflammatory and cellular response.

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EDITORIAL
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Editorial: Antimicrobial peptides and complement – maximising the inflammatory response

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Keywords: inflammation, complement, antimicrobial peptides, immune response, inflammatory response

Striking commonalities in the roles of complement and antimicrobial peptides have recently been reported; their abilities to apply selection pressures on a bacterial population in the bloodstream (1), to contribute to enhanced phagocytosis of opsonized bacteria (2), and to interactively determine skin microbiome (3). Evolutionary roots for complement proteins and antimicrobial peptides are ancient (4). Predating the avenue of somatic recombination, antimicrobial peptides and complement have further emerged as modulators of cell activities that are part of the adaptive immune response. Therefore, antimicrobial peptides and complement were logical contenders for a focused analysis to distil from a wide complexity a range of overlapping and distinct activities that could serve to maximize local and systemic inflammatory responses.

The task was ambitious. Aiming to draw together experts and junior scientists in two distinct areas of inflammatory responses, an e-book series was produced which in its entirety challenges oligofactorial analyses in health and disease and points to a gain in embracing more fully the interconnection of inflammatory reactions and their components using, as examples, complement and antimicrobial peptides.

Functional analytical approaches may be derived from genomic analyses using cross species comparisons for antimicrobial peptides and is demonstrated in Machado and Ottolini's article (5). Significant copy number variations for defensin genes in and between populations make these exciting modulators of inflammation, mucosal immunity, and infection responses. In the complement system, gene duplication leading to C4A and C4B and functional polymorphisms in the MBL gene (in humans) provide variability in the fluid phase of complement activation.

In two parts, Roumenina's team provide a delicately researched state-of-the-art evaluation of complement activation and its regulation as well as a summary of current understanding of the mutlifaceted roles of complement anaphylatoxins in inflammation (6, 7).

Bevington's group put forward a case in support of further avenues of research to identify pH sensing molecules and understand pH-dependent contact and complement system activation and their interactions (8). The activity of antimicrobial peptides is also influenced by pH conditions (9). It appears therefore that more rigorous measurements of pH in *in vivo* models may help to discern a level of regulation that is currently still underappreciated.

Day and Clark's group remind that sialic acids or glycosaminoglycan structures on the cell surface or in the extracellular compartment provide interfaces, which can determine propagation or inhibition of complement activation and be the basis for tissue-specific susceptibilities to targeting binding of complement proteins (10). Interestingly, in *Drosophila*, engagement of the sulfated polysaccharide chains of heparin sulfate proteoglycans leads to expression of antimicrobial peptides (11).

Stadnyk's group presents a program of work to test hypotheses or inferred models of interaction, which are relevant in understanding pathomechanisms of colitis, but also contribute to our understanding of mucosal tolerance (12). Much has yet to be gained from studying the

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luminal role of antimicrobial peptides vs. the mucosal role of complement to maintain the mucosal barrier (13).

In the periodontal pocket, *Porphyromonas gingivalis*, its reciprocal interaction with complement and antimicrobial peptides during periodontitis is associated with altered local microbiota, bone loss, and evasion to atherosclerotic plaque (14). Chemokines, for which direct antimicrobial activities have been shown, are the focus in Sahingur and Yeudall's treatise on molecular determinants in the development and progression of oral cavity cancers. Produced in response to a polymicrobial insult, locally produced chemokines are relevant to epithelial dysplasia and osteoclast activity and, furthermore, shape the tumor microenvironment (15). Al-Rayahi and Sanyi juxtapose complex activities of antimicrobial peptides and complement components in a wide range of cancers and remind us of early tumoricidal work using bacterial extracts (16).

Rocha-Ferreira and Hristova discuss for the neonatal brain the role of complement and antimicrobial peptides in the dynamics and extent of inflammation and their potential as targetable mediators of hypoxia-induced brain damage (17).

A cautionary tale is told by Schuerholz et al. and Thompson et al., who deal with antimicrobial peptides and complement, respectively, in human sepsis (18, 19). Interactions of host to pathogen are multimodal and immune markers alter over the duration of disease. It seems reasonable to propose that parallel measurement of humorally accessible complement and antimicrobial peptides, players of the innate immunity bridging the adaptive immunity, will yield greater understanding of the dynamic host response during sepsis.

Finally, Zimmer et al. systematically present activity signatures of complement and antimicrobial peptides in homeostasis and disease (20) and point to a need to distinguish other activities, which relate to routine design of recombinant protein expression (21).

In their entirety, the contributions, by providing succinct and critical summaries, primary data and viewpoints, achieve to deepen insight in and understanding of complex matters involving and surrounding antimicrobial peptides and complement. The mind may become more prepared to consider a multipronged approach to health and disease, impacting on both, experimental and therapeutic designs.

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An evolutionary history of defensins: a role for copy number variation in maximizing host innate and adaptive immune responses

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Defensins represent an evolutionary ancient family of antimicrobial peptides that play diverse roles in human health and disease. Defensins are cationic cysteine-containing multifunctional peptides predominantly expressed by epithelial cells or neutrophils. Defensins play a key role in host innate immune responses to infection and, in addition to their classically described role as antimicrobial peptides, have also been implicated in immune modulation, fertility, development, and wound healing. Aberrant expression of defensins is important in a number of inflammatory diseases as well as modulating host immune responses to bacteria, unicellular pathogens, and viruses. In parallel with their role in immunity, in other species, defensins have evolved alternative functions, including the control of coat color in dogs. Defensin genes reside in complex genomic regions that are prone to structural variations and some defensin family members exhibit copy number variation (CNV). Structural variations have mediated, and continue to influence, the diversification and expression of defensin family members. This review highlights the work currently being done to better understand the genomic architecture of the β-defensin locus. It evaluates current evidence linking defensin CNV to autoimmune disease (i.e., Crohn's disease and psoriasis) as well as the contribution CNV has in influencing immune responses to HIV infection.

Keywords: copy number variation, defensins, HIV, psoriasis, Crohn's disease

INTRODUCTION

The defensins represent a class of cationic antimicrobial peptides that play pivotal roles in innate and adaptive immunity as well as roles in non-immunological processes. They constitute an ancient and diverse gene family, present in most multicellular organisms ranging, from plants, fungi, insects, mollusks, and arachnids to mammals, including humans. During their evolutionary history, defensins have become highly diversified and have acquired novel functions in different species. Defensins have evolved to be highly efficient in their antimicrobial responses to a vast array of pathogens.

The term "Defensins" was coined in 1985 after granule rich sediments were purified from human and rabbit neutrophils. This resulted in the characterization of the primary structure of the first six neutrophils defensins (later known as α-defensins) (1–3). These early studies highlighted the structural hallmarks of defensins: that is, despite poor sequence identity across family members, all defensins possesses a highly conserved motif of six cysteine residues that is key to their antimicrobial function. Subsequently, peptides with similar structure were discovered in the early 1990s in bovine (4) and mouse airway first (5) and subsequently in the human intestinal epithelium (6), and became known as β-defensins. The recent ability to interrogate genomic and proteomic data from a diverse array of species allowed the discovery and characterization of further members of the defensin gene family, intensifying interest in unveiling the roles of defensins in physiological and pathological processes.

This review will primarily focus on the role of β-defensins in innate and adaptive immunity. We will highlight the methods currently employed to study the genomic architecture of this multifunctional gene family and how complex genetic variation has an impact on defensin host inflammatory responses.

STRUCTURE OF β-DEFENSINS

The β-defensin family members have poor sequence similarity, suggesting their antimicrobial activity is independent of their primary structure. Nuclear magnetic resonance (NMR) data have been used to evaluate the 3D structure of hBD1, hBD2, and hBD3 (7, 8). These data confirm a high degree of similarity in their tertiary structures, despite their diverged amino acid sequences. The major element of the mature peptides secondary structure is represented by three β-strands arranged in an antiparallel sheet. The strands are held together by the three intramolecular disulfide bonds, formed between the six cysteines. The order of the disulfide bridges can vary, characterizing each family member. The amino-terminal region contains a short α-helical loop (which is absent in α-defensins). α-helical structures are common for protein regions that are incorporated into cell membranes and it has been proposed that this region of the β-defensin protein may anchor to bacteria cell walls (9). This is supported by the presence of two

sites under positive selection located in the N-terminal region that may contribute to β-defensin functional diversity (10).

Defensins do not appear to present a distinct hydrophobic core or a common pattern of charged or hydrophobic residues on the protein surface. This suggests peptide folding is driven and stabilized by disulfide bond formation alone. Moreover, the characteristic β-defensin 3D structure can be preserved and accommodates residues with different properties at most other positions. The first five amino acids of the mature peptide sequence are vital for correct protein folding under oxidative conditions. This favors the formation of the correct disulfide bonded pattern through the creation of a key intermediate (11).

THE EVOLUTION AND DIVERGENT ROLES OF β-DEFENSINS

The evolutionary relationship between vertebrate and non-vertebrate defensins is still unclear; however, phylogeny indicates that a primordial β-defensin is the common ancestor of all vertebrate defensins and this gene family expanded throughout vertebrate evolution (12). This hypothesis is supported by the discovery of β-defensin-like genes in phylogenetically distant vertebrates, including reptiles (13), birds (14), and teleost fishes (15). α-defensins are mammalian specific genes, and in humans α-defensin genes and different β-defensin genes are present on adjacent loci on chromosome 8p22–p23. The organization of this cluster is consistent with a model of multiple rounds of duplication and divergence under positive selection from a common ancestral gene that produced a cluster of diversified paralogous (16, 17). This expansion occurred before the divergence of baboons and humans ~23–63 million years ago (18, 19). The present-day β-defensins probably evolved before mammals diverged from birds generating α-defensins in rodents, lagomorphs, and primates after their divergence from other mammals (20). Recent evidence suggests convergent evolution of β-defensin copy number (CN) in primates, where independent origins have been sponsored by non-allelic homologous recombination between repeat units. For rhesus macaques this resulted in only a 20 kb copy number variation (CNV) region containing the human ortholog of human β-defensin 2 gene. In humans, recent work suggests a repeat unit of 322 kb containing a number of β-defensin genes (21).

Defensin family members possess a plethora of non-immune activities and it is instructive to provide some examples of the diverged nature of defensins function. Some members of the β-defensin family have an important role in mammalian reproduction [reviewed in Ref. (22)]. For example, there are five human defensin genes (*DEFB125–DEFB129*) clustered on chromosome 20, which are highly expressed in the epithelial cell layer of the epididymal duct, which secretes factors responsible for sperm maturation (23). Moreover, human *DEFB118* was shown to be a potent antimicrobial peptide able to bind to sperm, probably providing protection from microorganisms present in the sperm ducts (24). It is noticeable how in long tailed macaque (*Macaca fascicularis*) and in rhesus macaque (*Macaca mulatta*), there is a similar β-defensin, called *DEFB126*, which is the principal protein that coats sperm (25); this coating is lost in the oviduct allowing fertilization to occur. In support of this, the deletion of a cluster of nine beta defensin genes in a mouse model, resulted in male sterility (26). In human studies, a common mutation in *DEFB126* has been shown to impair sperm function and fertility (27).

In a second example, recent studies have suggested that some β-defensin gene products including hBD1 and hBD3, can interact with a family of melanocortin receptors, modulating pigment expression in dogs and possibly in humans (28). Typically, there are two genes that control the switching of pigment types: the melanocortin receptor 1 (*Mc1r*) and *Agouti*, encoding a ligand for the Mc1r, which inhibits Mc1r signaling. Mc1r activation determines production of the dark pigment eumelanin exclusively, whereas Mc1r inhibition causes production of the lighter pigment pheomelanin. In dogs, it was discovered that a mutation in the canine *DEFB103* is responsible for the dominant inheritance of black coat color, which does not signal directly through Mc1r; this insight revealed a previously uncharacterized role of β-defensins in controlling skin pigmentation. Further studies have been conducted on human melanocytes, discovering a novel role of hBD3 as an antagonist of the α-melanocyte-stimulating hormone (α-MSH, a known agonist of Mc1r, which stimulates cAMP signaling to induce eumelanin production). As hBD3 is produced by keratinocytes, it can act as a paracrine factor on melanocytes modulating α-MSH effects on human pigmentation and consequently responses to UV (29). Moreover, it is known that melanocortin receptors are also involved in inflammatory and immune response modulation (30).

EXPRESSION OF β-DEFENSINS

Different β-defensins are present in different epithelial and mucosal tissues and can be constitutively expressed or induced in response to various stimuli (32–52) (Table S1 in Supplementary Material). Their anatomical distribution clearly reflects their ability to neutralize different pathogens and they are more abundant at sites prone to the microbial infections they are specific for. For example, hBD2 is strongly expressed in lung (53); hBD4 is highly expressed in the stomach and testes (54), and hBD3 in the skin and tonsillar tissue (55). hBD1–hBD4 are expressed in the respiratory tract, with constitutive expression of hBD1 (56) and inducible expression of hBD2–hBD4 in response to inflammation or infection (57). In keratinocytes, there is constitutive mRNA expression of hBD1; conversely hBD2 expression is induced by lipopolysaccharides (LPS) or other bacterial epitopes in combination with interleukin-1β, released by resident monocyte-derived cells. hBD3 and hBD4 are inducible by stimulation with tumor necrosis factor (TNF), toll-like receptor ligands, interferon (IFN)-γ, or phorbolmyristate acetates (58). hBD3 is also induced in response to local release of surface-bound epidermal growth factor receptor (EGFR) ligands via activation of metalloproteinases (59, 60).

ANTIMICROBIAL ACTIVITY OF β-DEFENSINS

The most studied function for β-defensins is their direct antimicrobial activity, through permeabilization of the pathogen membrane. Their exact mechanism of action is incompletely understood and two different models have been proposed. The first is a carpet model, where several antimicrobial peptides opsonize the pathogen surface bringing about necrosis, possibly disrupting the electrostatic charge across the membrane (61). The latter is

a pore model, with several peptides oligomerizing and forming pore-like membrane defects that allow efflux of essential ions and nutrients (55).

Defensins *in vitro* are active against gram negative and positive bacteria, unicellular parasites, viruses, and yeast. Cationic peptides including β-defensins are attracted to the overall net negative charge generated by the outer envelope of Gram negative bacteria by phospholipids and phosphate groups on LPS and to the teichoic acid present on the surface of Gram positive bacteria.

β-defensins also possess anti-viral activity, interacting directly with the virus and indirectly with its target cells. Noticeably, in mammals, β-defensins are also produced by the oral mucosa and they are active against HIV-1 virus: in particular, hBD1 is constitutively expressed whereas the presence of a low HIV-1 viral load can stimulate the expression of hBD2 and hBD3 gene products through direct interaction with the virus. More specifically, hBD2 has been shown to down-regulate the HIV transcription of early reverse-transcribed DNA products (62) and hBD2 and hBD3 can mediate CXCR4 down-regulation (but not CCR5) and internalization in immuno-stimulated peripheral blood mononuclear cells (63). This mechanism diminishes the chances of infection (64) and with other salivary gland components, could help to explain the oral mucosal natural resistance to HIV infection. hBD3 also possesses an inhibitory effect on the influenza virus blocking the fusion of the viral membrane with the endosome of the host cell, through cross linking of the viral glycoproteins (65).

Defensins have evolved to maximize their protective role, showing an extraordinary adaptation to different environmental challenges: for instance, plant defensins are particularly active against fungal infections [reviewed in Ref. (66)], slowing down hyphal elongation, and some of them also evolved to gain an α-amylase inhibitory activity that can confer protection against herbivores (67, 68).

IMMUNE MODULATORY ACTIVITY OF β-DEFENSINS

A role for defensins in pro-inflammatory responses and more recently immunosuppression [reviewed in Ref. (69)] has been delineated over the last two decades. An initial important observation was that β-defensins can recruit immature dendritic cells and memory T cells to sites of infection and/or inflammation providing a link between the innate and adaptive arms of the immune system. A mechanism for this was provided by Oppenheim's group where they demonstrated that natural and recombinant hBD2 could chemoattract human immature dendritic cells and memory T cells *in vitro* in a dose-dependent manner. This response was inhibited with the Gαi inhibitor pertussis toxin and suggested the possible involvement of a chemokine receptor(s), which was confirmed using anti-CCR6 blocking antibodies.

T_H17 cells express CCR6 and respond to β-defensins chemoattractant action. Furthermore, T_H17 cytokines (i.e., IL-17 and IL-22) induce expression of defensins from relevant cell types including primary keratinocytes potentially resulting in an amplification of T_H17 responses (70). Increased T_H17 levels have been reported in different autoimmune diseases, such as multiple sclerosis (71), rheumatoid arthritis (72), and psoriasis (73), implicating β-defensin expression in autoimmunity. Given the role of defensins in chemoattracting monocytes and macrophages and the lack of CCR6 on these cell types other receptors were investigated that might mediate this chemoattractant activity. This resulted in the identification of CCR2 as a receptor for hBD2, hBD3, and their mouse orthologs (mBD4 and mBD14) (74).

In addition to signaling through chemokine receptors, defensins have been shown to function through toll-like receptors (75, 76). hBD2 has been shown to be a natural ligand for the toll-like-receptor-4 (TLR-4), present on immature DCs, up-regulating co-stimulatory molecules and leading to DC maturation, and on $CD4^+$ T cells, possibly stimulating their proliferation and survival (77). On bone marrow-derived macrophages pre-treated with a recently identified mBD14 (78), TLR restimulation of these cells resulted in enhanced expression of pro-inflammatory mediators that was Gi protein dependent but independent of CCR2 or CCR6 signaling pathways (79).

β-DEFENSIN COPY NUMBER VARIATION AND DISEASE ASSOCIATION STUDIES

In humans, β-defensins genes are organized into three main clusters at 8p23.1, 20p13, and 20q11.1, with another likely small cluster on chromosome 6p12 (80). At 8p23.1, a number of β-defensins are found on a repeat unit that is typically present at 2–8 copies in the population, with a modal CN of 4. Each chromosome 8 copy can contain 1–8 copies of the repeat unit. The mutation rate at this locus is extremely fast (~0.7% per gamete) (81), indicative of the high level of plasticity in this genomic region. One-copy individuals are extremely rare (82, 83), and suggest that the presence of a null allele might be deleterious and selected against. At the other end of the *DEFB*, CN spectrum lies a proportion of high-copies individuals (9–12 copies) with a cytogenetically visible CN amplification at 8p23.1 that has no phenotypic effect (84). These first experimental observations ignited further interest into the chromosome 8 *DEFB* cluster. Within the repeat unit there is *DEFB4*, *DEFB103*, *DEFB104*, *DEFB105*, *DEFB106*, *DEFB107*, *SPAG11*, and *PRR23D1* (21, 85) (**Figure 1**). The variation in the number of repeat units between individuals in the population and likely sequence variation between copies suggests that CNV of defensins may play a role in modulating defensin expression (86, 87) and function. The consequences of CNV have been explored for a number of years and may include increased gene product, the production of fusion genes, the formation of extra coding domains, or a position effect that alters expression of the gene product (88). This extensive structural genome variation in humans is particularly pertinent to diseases where defensins may be implicated in their pathology. This includes a number of autoimmune and infectious diseases (**Table 1**).

Mapping of the β-defensin CNV region has been challenging but recent data fixes the minimal length of the CNV at 157 kb (103) and a recent study using high density array comparative genomic hybridization combined with paralog ratio test (PRT) assays suggests it may be as large as 322 kb (21). Because of the extensive CNV of defensins, robust methods are required to accurately interrogate CN states in disease cohorts. Various locus specific techniques for CN determination have been utilized including multiplex amplifiable probe hybridization (MAPH) (104), multiple ligation probe amplification (MLPA) (105), and PRT (95). The advantage of such techniques is the ability to obtain data that clusters around integer

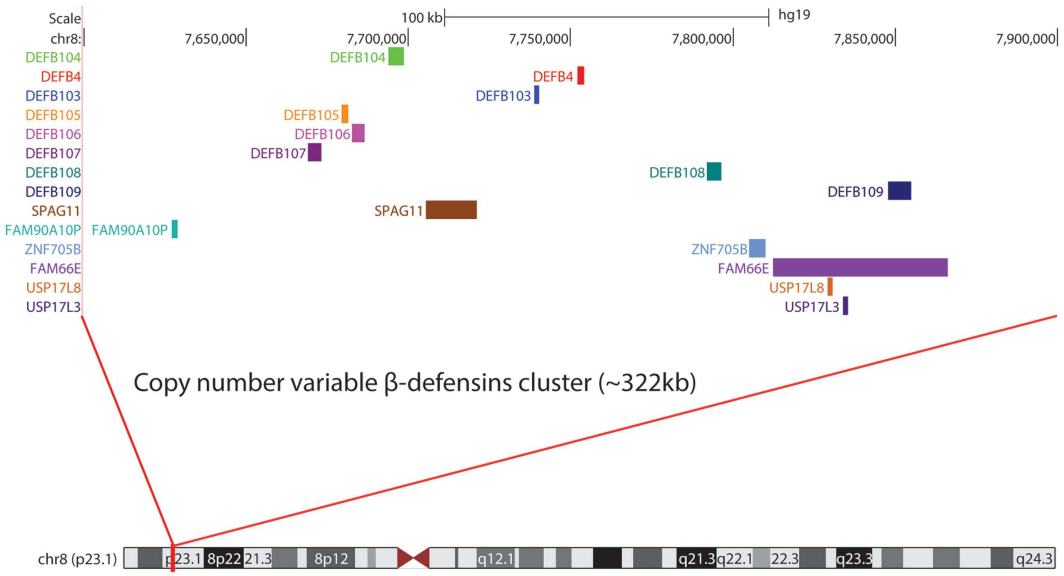

FIGURE 1 | Genome assembly of β-defensin repeat unit at 8p23.1.

CNs providing a high degree of concordance between the methods and confidence in the CN obtained. Association studies investigating some CNVs (i.e., *CCL3L1/CCL4L2* in HIV) have provided conflicting results as the methods used did not generate data that clustered around integer CN values (106, 107). In some cases, initial findings have been replicated in subsequent studies that have utilized more robust methods (108).

In early association studies of multi-allelic CNV and disease, CNV of defensins was implicated in psoriasis. Individuals with more than five β-defensin copies presented a fivefold increased risk of developing psoriasis when compared to two copy individuals. In addition, there was a direct correlation between the number of copies and relative risk (odds ratio of 1.32) (94). This association was replicated (although with reduced odds ratio) in a subsequent study (109). In the case of an autoimmune condition, such as psoriasis, high CN may contribute to the strong induction of hBD2 and hBD3, conferring protection from bacterial infections of the psoriatic lesions (110).

Another disease strongly linked with defensin expression is Crohn's disease (CD) where it has been demonstrated that reduced Paneth cell expression of defensins in the ileum results in ileal CD. Therefore, defensin expression at this site may be important in maintaining the mucosal microbiota. *NOD2* has been strongly implicated in the pathogenesis of CD from GWAS (111) giving a 17.1-fold increased risk for CD in homozygous or compound heterozygous individuals. *NOD2* is a nod like family receptor (NLR) member that controls expression of defensins in CD. Polymorphisms in *NOD2* result in reduced α-defensin expression and exacerbated disease. Polymorphism of the *DEFB1* (non-CNV gene) promoter has been associated with CD (112). So is there a role for CNV in CD? Previous studies indicated that α-defensin CN may be important (113). However, recent work that accurately measured CN using PRTs to determine CN of *DEFA1A3* determined that a SNP (rs4300027) is associated with *DEFA1A3* CN in Europeans (114). This SNP was then used to indirectly interrogate GWAS data and suggested that α-defensins CNV may not be important in CD. A similar outcome was obtained with β-defensin CN whereupon accurate measurement, there was no association with the CD (82) in contrast to previous reports (93, 97). These results, however, do not exclude the role of α and β-defensin expression in the pathogenesis of CD but suggest that the individuals CN state may not be important in this context.

Given the suspected anti-viral role of defensins, it was suggested that defensin CNV may be important in host responses to HIV infection. There are a number of conflicting reports of the association between defensin CN and HIV infection (114–116). A surprising finding from a cohort study that evaluated two sub-Saharan populations with HIV-1 or HIV-1/tuberculosis coinfection was that high CN of β-defensins did not result in the predicted low viral load and did not improve immune reconstitution in patients (98). The converse was found suggesting that the immune modulatory properties of defensins may be subverted during HIV-1 infection. A model suggested to explain this apparently paradoxical result was that high CN may promote increased recruitment of CCR6 expressing cell types that are highly permissive for HIV-1 infection thus amplifying the foci of HIV-1 infection.

CONCLUSION

Defensins play a key role in pathogen host interactions and are at the interface of innate and adaptive immunity. The complex genetic variation that underlies the evolutionary history of defensins and their biology is gradually being elucidated, suggesting defensin CNV is an important contributor to maximizing the host innate and adaptive response. The history of the defensin gene family is particularly paradigmatic given that many CNV loci in

Table 1 | Summary of β-defensin CNV studies.

DEFB cluster CN calls per diploid genome	Sample size	Methods used for CN calling	Association study?	Findings	Reference
2–12	90 Controls 12 Related individuals from 3 families with chr8p23 euchromatic variant (EV)	MAPH SQ-FISH	No	Average CN distribution of 2–7 for controls Average CN distribution of 2–7 for EV carriers	(89)
2–8	27 Unrelated samples	qPCR	No	Concordant CN for *DEFB4* and *DEFB103*	(90)
2–10	355 Patients with cystic fibrosis 167 UK controls	MAPH	Cystic fibrosis	*DEFB* CN is not associated with cystic fibrosis	(91)
2–7 for *DEFB4*	44 Samples	qPCR	No	Discordant CN for *DEFB4*, *DEFB103* and *DEFB104*	(92)
2–10	250 CD patients 252 Controls	Array-CGH qPCR	Crohn's disease	<3 copies associated with CD (OR = 3.06)	(93)
2–12	498 Cases 305 Controls	MAPH PRT	Psoriasis	Higher CN associated with psoriasis RR = 1.69 >6 copies	(94)
2–8	>800 Samples	MAPH/REDVR, MLPA and array-CGH. All validated through PRT	No	PRT is a reliable method for CNV analysis	(95)
2–9	42 Samples	MLPA	No	Strict copy number concordance for all genes in the chr8p23.1 *DEFB* cluster	(96)
1–12	208 Offspring from 26 CEPH families	PRT Microsatellite analysis	No	Fast germline copy number recombination of DEFB cluster (~0.7% per gamete)	(81)
1–12 in CD patients 2–9 in controls	466 CD patients 329 Controls	qPCR	Crohn's disease	>4 copies associated with CD (OR = 1.54)	(97)
1–10	1000 Crohn's disease (CD) patients 500 Controls	PRT on all samples qPCR on 625 samples	Crohn's disease	*DEFB* copy number is not associated with CD (higher accuracy in CN calling and a larger cohort compared with previous studies on CD)	(82)
1–9	1056 Individuals from the HGDP–CEPH panel	PRT	No	Recent selection of high-expressing *DEFB103* gene copy in East Asia	(83)
1–9	1002 Ethiopian and Tanzanian HIV and HIV/TB patients	PRT	HIV viral load in HIV-only and HIV/TB patients	Increased HIV load prior to HAART ($P = 0.005$) and poor immune reconstitution following initiation of HAART ($P = 0.003$)	(98)
2–7	543 SLE patients 112 AASV patients 523 Controls	PRT 515 samples validated with REDVR	Systemic lupus erythematosus ANCA associated small vasculitis (AASV)	Higher CN associated with SLE and AASV (SLE OR = 1.2; AASV OR = 1.5)	(99)
2–8	70 PDAC patients 60 CP patients 392 Controls	MLPA	Pancreatic ductal adenocarcinoma (PDAC) Chronic pancreatitis (CP)	Protective effect of high *DEFB* CN against PDAC (Fisher's exact test $P = 0.027$)	(100)

(Continued)

Table 1 | Continued

DEFB cluster CN calls per diploid genome	Sample size	Methods used for CN calling	Association study?	Findings	Reference
1–9	2343 Samples (689 children and 1149 adults)	PRT	Asthma Chronic obstructive pulmonary disease (COPD)	*DEFB* CN is not associated with lung function in the general population (OR = 0.89)	(101)
2–9	113 Otitis media prone children 259 Controls	PRT	Susceptibility to otitis media	*DEFB* CN associated with nasopharyngeal microbiota composition (with respect to the three predominant pathogens for otitis media: *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae*	(102)

AASV, ANCA associated small vasculitis; array-CGH, array comparative genomic hybridization; CD, Crohn's disease; CEPH, Centre d'Etude du polymorphisme humain DNA panel; COPD, chronic obstructive pulmonary disease. CP, chronic pancreatitis; HAART, highly active anti-retroviral therapy; HGDP, human genome diversity cell line panel; MAPH, multiplex amplifiable probe hybridization; MLPA, multiplex ligation-dependent probe amplification; PDAC, pancreatic ductal adenocarcinoma; PRT, paralog ratio test; REDVR, restriction enzyme digest variant ratio; SLE, systemic lupus erythematosus; SQ-FISH, semi-quantitative fluorescence in situ hybridization; TB, tuberculosis.

the human genome host immunity genes. Further studies should be conducted to better understand the genomic architecture of multi-allelic CNVs. This will aid the development of robust assays that evaluate the overall impact that CNV has on and both physiological and pathological mechanisms of immunity.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2015.00115/abstract

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REVIEW
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Complement system part I – molecular mechanisms of activation and regulation

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Complement is a complex innate immune surveillance system, playing a key role in defense against pathogens and in host homeostasis. The complement system is initiated by conformational changes in recognition molecular complexes upon sensing danger signals. The subsequent cascade of enzymatic reactions is tightly regulated to assure that complement is activated only at specific locations requiring defense against pathogens, thus avoiding host tissue damage. Here, we discuss the recent advances describing the molecular and structural basis of activation and regulation of the complement pathways and their implication on physiology and pathology. This article will review the mechanisms of activation of alternative, classical, and lectin pathways, the formation of C3 and C5 convertases, the action of anaphylatoxins, and the membrane-attack-complex. We will also discuss the importance of structure–function relationships using the example of atypical hemolytic uremic syndrome. Lastly, we will discuss the development and benefits of therapies using complement inhibitors.

Keywords: complement system proteins, complement regulatory proteins, structure–function relationships, anaphylatoxins, membrane-attack-complex, classical complement pathway, alternative complement pathway, endothelial cells

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Introduction

Complement is a central part of the innate immunity that serves as a first line of defense against foreign and altered host cells (1). The complement system is composed of plasma proteins produced mainly by the liver or membrane proteins expressed on cell surface. Complement operates in plasma, in tissues, or within cells (2). Complement proteins collaborate as a cascade to opsonize pathogens and induce a series of inflammatory responses helping immune cells to fight infection and maintain homeostasis. The complement system can be initiated depending on the context by three distinct pathways – classical (CP), lectin (LP), and alternative (AP), each leading to a common terminal pathway. In a healthy individual, the AP is permanently active at low levels to survey for presence of pathogens (**Figure 1A**). Healthy host cells are protected against complement attack and are resistant to persistent low-grade activation. The three pathways are activated on the surface of apoptotic cells, which are constantly generated within the body during normal cellular homeostasis

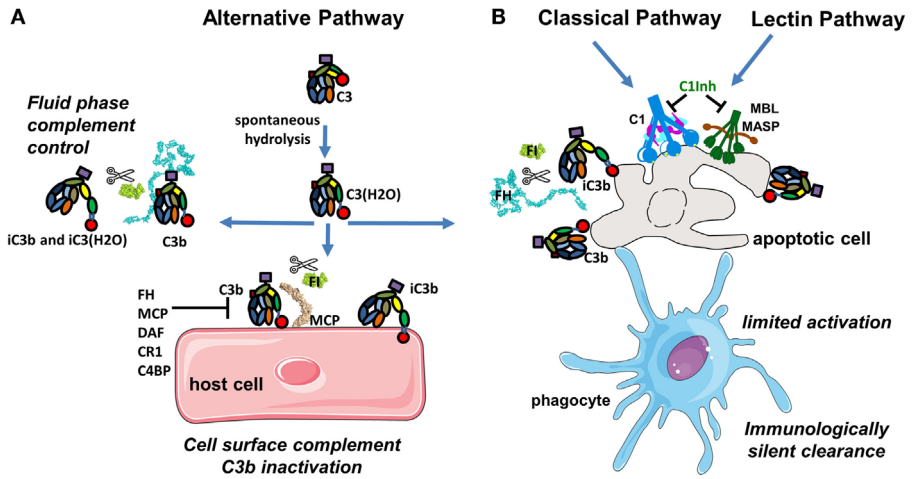

FIGURE 1 | Complement activation in physiological conditions. (A) The alternative pathway is permanently active due to spontaneous transformation of bio-inactive molecule C3 to bioactive C3(H_2O). This allows generation of C3b, which is rapidly inactivated by FH and FI in fluid phase or is covalently bound to the surface and then inactivated on host cells. **(B)** Classical and lectin pathway recognition molecules bind to apoptotic cells and together with C3b from the alternative pathway induce a low level of complement activation. Apoptotic cells are not lysed, but rapidly cleared by phagocytes in an immunologically silent manner. Host cells and plasma contain multiple regulatory proteins to assure that complement activation will be limited in physiological conditions.

(**Figure 1B**). This complement activation is tightly regulated to eliminate dying cells without further activation of other innate or adaptive immune components. Complement is only fully activated in cases of pathogen infection. During an infection, complement leads to inflammation, opsonization, phagocytosis, and destruction of the pathogen and ultimately results in activation of the adaptive immune response (**Figure 2**). Both inefficient and over stimulation of complement can be detrimental for the host and are associated with increased susceptibility to infections or non-infectious diseases, including autoimmunity, chronic inflammation, thrombotic microangiopathy, graft rejection, and cancer.

In this review, we discuss recent advances in the molecular and structural basis of activation and regulation of the complement pathways followed by the discussion of one complement-mediated disease – atypical hemolytic uremic syndrome (aHUS) to illustrate how the knowledge of the structure–function relationships between complement proteins helps to understand aHUS physiopathology and aid in the development of targeted therapy. In the second part of this review, published in the same issue of Frontiers in Immunology, we provide a detailed review of the literature related to the role of the complement system in immunity (3).

Complement Activation During Normal Homeostasis and Pathogen Infection

The central component of the complement system is C3. The activation of each of the three pathways (CP, LP, and AP) results in cleavage of inactive C3 protein into the functional fragments C3a and C3b. C3a is an inflammation mediator and C3b is an opsonin, which can bind covalently and tag any surface in the immediate proximity to the site of its generation.

Complement Tick-Over in the Alternative Pathway

In the plasma, during normal physiological conditions, the dominant active complement pathway is the AP (**Figure 1A**). The AP monitors for pathogen invasion by maintaining a low level of constitutive activation by a process known as tick-over (4). Tick-over is the spontaneous hydrolysis of a labile thioester bond, which converts C3 to a bioactive form C3(H_2O) in the fluid phase (5). The rate of hydrolysis of C3 to C3(H_2O) can be accelerated by interactions between C3 and a number of biological and artificial interfaces, including gas bubbles, biomaterial surfaces, and lipid surfaces and complexes (6). Upon hydrolysis, the thioester domain (TED) of C3 undergoes a dramatic structural change that exposes a binding site for another member of the AP called Factor B (FB). The C3(H_2O)-bound FB is then cleaved by a serine protease (SP) Factor D (FD) allowing formation of a fluid phase C3 convertase complex C3(H_2O)Bb. C3(H_2O)Bb is able to interact and cleave native C3 molecules to C3a and C3b (5, 7–11). During normal physiological conditions, this C3 convertase constantly generates small amounts of C3b, which is able to bind covalently via its TED domain to any adjacent surface containing hydroxyl groups. Nevertheless, not all hydroxyl groups attract equally C3b (12). The -OH in the 6th position appears to be more reactive than the average -OH group in sugars. Therefore, the particular sugars composition of the pathogen surface will determine the efficacy of complement activation. C3b will bind covalently to a surface that is located within about 60 nm from the convertase, due to the fact that the half-life of the thioester in C3b is ≈60 μs with a poor attachment efficiency of 10% (13). On host cells, bound C3b molecules are rapidly inactivated by an army of membrane-expressed or fluid phase-recruited complement regulators (described in detail below). A tick-over

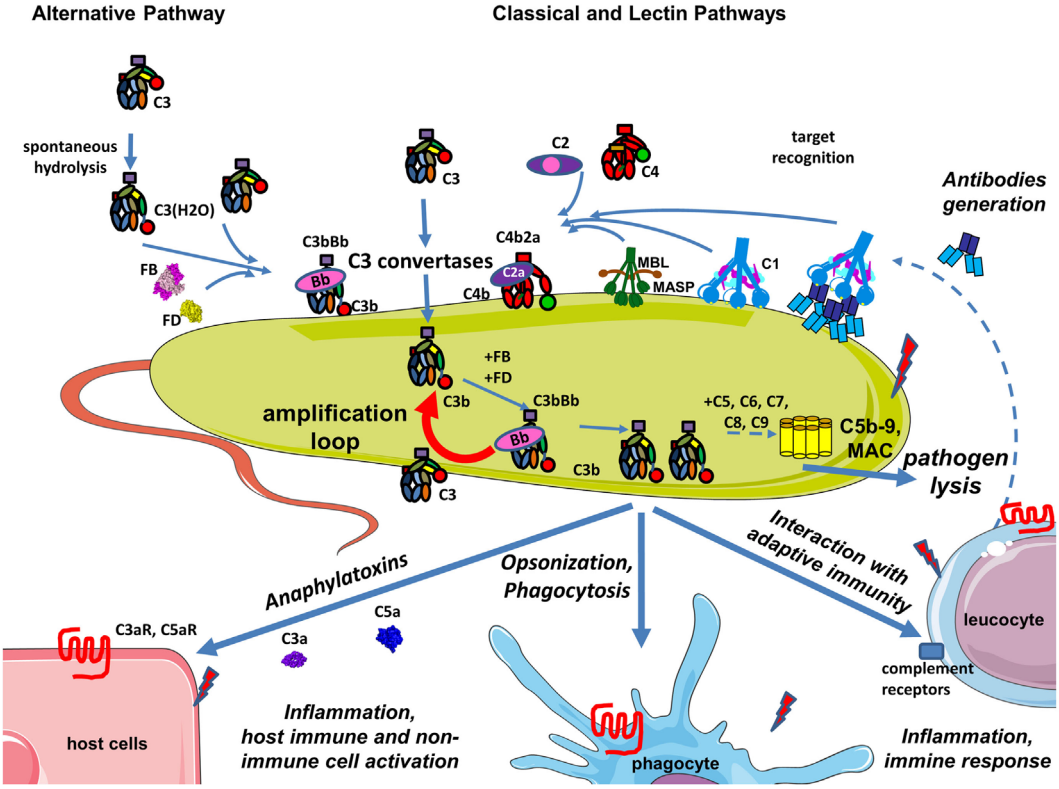

FIGURE 2 | **Complement during infection with a pathogen**. The permanent activity of the alternative pathway allows it to immediately identify pathogens that are not specifically protected against complement. Danger-associated molecular patterns on its surface of pathogens are recognized by C1q, MBL, and ficolins allowing classical and lectin pathway activation, C3 convertase, C4b2a generation, and C3 cleavage. Opsonization due to covalent binding of C3b to the target is accelerated by the amplification loop of the complement pathways. The effector functions resulting from this complement activation are: pathogen lysis by C5b-9 membrane attack complex, opsonization and phagocytosis of the pathogen, activation of host immune and non-immune cells by complement anaphylatoxins, inflammation, stimulation of an adaptive immune response, and antibody generation. Secreted antibodies will bind to the pathogen and create immune complexes that will be recognized by C1q and will activate the classical pathway. Altogether these mechanisms contribute to pathogen elimination.

mechanism for spontaneous activation of the CP has also been suggested, but the molecular interactions of this process are not well understood (14).

Clearance of Apoptotic Cells

Apoptosis, programed cellular death, is a process of normal cellular homeostasis and in healthy individuals everyday billions of cells die by this mechanism. Complement activation occurs on apoptotic cells with low levels of C3b deposition to facilitate their elimination without releasing danger signals, which could lead to further immune responses (15, 16) (**Figure 1B**). This complement activation occurs by membrane alterations and by decreased expression of complement regulators on the membrane of apoptotic compared to resting cells. The silent clearance of the apoptotic cells is assured by the binding of the initiators of the CP (C1q) and LP [Mannose-Binding Lectin (MBL) and ficolins]. These initiator proteins interact with receptors on phagocytic cells (immature dendritic cells or macrophages), which elicit immune tolerance and prevent immune responses toward self-antigens (17–20).

Pathogen Elimination

On pathogens that lack specific regulators of complement, C3b interacts with FB and FD to form a surface-bound C3 convertase as part of the AP, which cleaves C3 into C3a and C3b. Maximum complement activation is achieved during pathogen recognition leading to a pro-inflammatory milieu, contributing to generation of an adaptive immune response and rapid elimination of the pathogen (**Figure 2**). Complement-derived anaphylatoxins have potent inflammatory mechanisms including recruitment of phagocytes to the site of infection and activation of leukocytes, endothelial cells, or platelets. Upon activation, terminal complement components also have direct lytic capacity to kill pathogens.

The CP and LP have a critical role in pathogen recognition and initiation of the complement cascade. However, the AP assures more than 80% of the terminal complement activity during pathogen recognition (21). Additional AP C3 convertases are formed on the C3b molecules generated either by CP activation or the AP C3 convertases. This chain reaction amplifies opsonization of the target and increases generation of anaphylatoxins. This

amplification loop augments the effect of all pathways and is the heart of the complement cascade (22).

Structural Basis of Complement Activation and Regulation

Target Recognition and Initiation of Complement Pathways

The CP and LP have clearly identified recognition molecules, C1q, MBL, and ficolins, which trigger each pathway only when and where it is necessary. The recognition event induces a structural change in the recognition molecule, which in turn induces the activation of enzymes able to cleave the subsequent molecules in the cascade and generate the central enzymatic complexes of complement, CP and AP C3 convertases. The AP lacks a traditional target recognition molecule as an initiator. However, several molecules, such as properdin and P-selectin, can recruit C3(H_2O) and C3b to the cell surface and serve as local initiators of the AP.

Recognition Molecules of the Classical and Lectin Complement Pathways

Complement pathway and LP are triggered after interaction of a pattern-recognition molecule with the target structure. The recognition molecule of the CP, C1q, has an extra-hepatic origin and is produced mainly by immature dendritic cells, monocytes, and macrophages (23). It has a complex, described as a "bouquet of flowers" topology (**Figure 3A**), composed of 18 chains of three types (A, B, and C), forming six globular target recognition domains (gC1q) attached to a collagen-like region (CLR). Each gC1q domain carries a Ca^{2+} ion, which maintains domain stability and regulates the electrostatic field (24). C1q recognizes mostly charged patterns and can bind to more than 100 different target molecules, including IgG and IgM containing immune complexes and surface-bound pentraxins [C-reactive protein (CRP), pentraxin 3 (PTX-3)] (25). Therefore, CP can be activated in either an immune complex-dependent and -independent manner. Mapping of the target molecule-binding sites on gC1q by a site-directed mutagenesis, revealed that charged residues on the apex of the gC1q heterotrimer (with participation of all three chains), as well as, the side of the B-chain are crucial for binding to IgG, IgM, CRP, and PTX-3. These binding sites are not identical, but partially overlapping (24, 26–29). C1q recognizes pathogen-associated molecular patterns including lipopolysaccharide (LPS) (30) and bacterial porins (31). The gC1q domain also recognizes molecules, exposed on the surface of dying cells (32, 33), including phosphatidylserine (34, 35), double stranded DNA (36, 37), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (38), annexins A2 and A5 (39), and calreticulin (35, 40–42). The most well-characterized target recognition molecule of the LP is the MBL, which recognizes carbohydrates (43). MBL has a similar overall structure to C1q, but exists in multiple oligomeric forms (trimers, tetramers, and higher ordered oligomers) (**Figure 3B**). C1q and MBL associate in a Ca-dependent manner with SP complexes, consisting of C1r and C1s for the CP (44, 45) and MBL-associated serine proteases (MASP) for the LP (46, 47). In absence of Ca^{2+} ions (such as in plasma samples collected in EDTA), C1q and MBL cannot interact with $C1r_2C1s_2$ and MASPs, respectively and CP and LP activation is prevented. In the presence of Ca^{2+} ions, after activation, SPs cleave subsequent complement components C4 and C2. The resulting complex C4b2a is the C3 convertase for CP and LP. This C3 convertase has enzymatic activity and is able to cleave the central complement component C3 to bioactive fragments C3a and C3b.

Mechanism of Activation of the Classical Pathway

Recent studies have shed light on the molecular mechanisms of activation of CP and LP. It has long been established that C1q requires one surface-bound IgM or several IgG molecules in close proximity in order to interact with several of its globular domains and to activate complement. However, the molecular mechanisms and the C1-antibody stoichiometry required for optimal activation remain poorly understood (48). IgM is a planar polymeric molecule (pentamer or hexamer), in which C1q-binding sites are hidden. A conformational change occurs upon binding to an antigen (staple conformation), leading to exposure of C1q-binding sites. Contrary to IgM, IgG is a monomer and despite the presence of the C1q-binding sites, only very low affinity binding can be achieved. The epitope distribution of the antigen and the density of the IgG binding determine the level of complement activation, however the molecular mechanisms were unknown until recently. Diebolder and colleagues demonstrated that specific non-covalent interactions between Fc fragments of IgG and formation of ordered antibody hexamers on the antigen surface are needed for efficient C1q-binding (**Figure 3A**) (49). Their proposed model could explain the strong antigen and epitope dependency of complement activation. Efficient C1q-binding could only occur upon formation of a platform of IgG Fc fragments with a steric compatibility for gC1q domains. Clustering of IgG molecules on the antigen surface could be affected by antigen size, density, and fluidity (50, 51) such that smaller antigen-antibody complexes will allow only moderate complement activation. In addition, binding stoichiometry is further complicated by antibodies fluidity on surfaces of regularly spaced epitopes. It has been demonstrated that IgG exhibit "bipedal" stochastic walking forming transient clusters that might serve as docking sites for the C1q-binding and complement activation (52).

Once C1q binds to its target surface, a conformational change is required to transmit the signal from the gC1q domain via the CLR to induce auto-activation of C1r (**Figure 4A**) (53). Molecular modeling, mutagenesis, and disease-associated mutation analysis revealed the structure of the $C1r_2C1s_2$ binding site in the cone of collagenous arms of C1q (44, 54, 55). The $C1r_2C1s_2$ proenzyme tetramer within the C1 complex in a resting state adopts an eight-shaped form (**Figure 4B**). Upon activation, conformational changes in the tetramer allow transition to an S-shaped active form, passing through a transition state. This conformational change allows auto-activation of the C1r SP domain. Subsequently, activated C1r will cleave and activate C1s. The driving force for this auto-activation of C1r is an increase of the angle between the collagen stalks of C1q (48). However, the mechanism for this structural change is poorly understood. Mutagenesis experiments have revealed that residues on the apex and the lateral surface of the B-chain of gC1q are important for IgG, IgM, CRP, or PTX-3

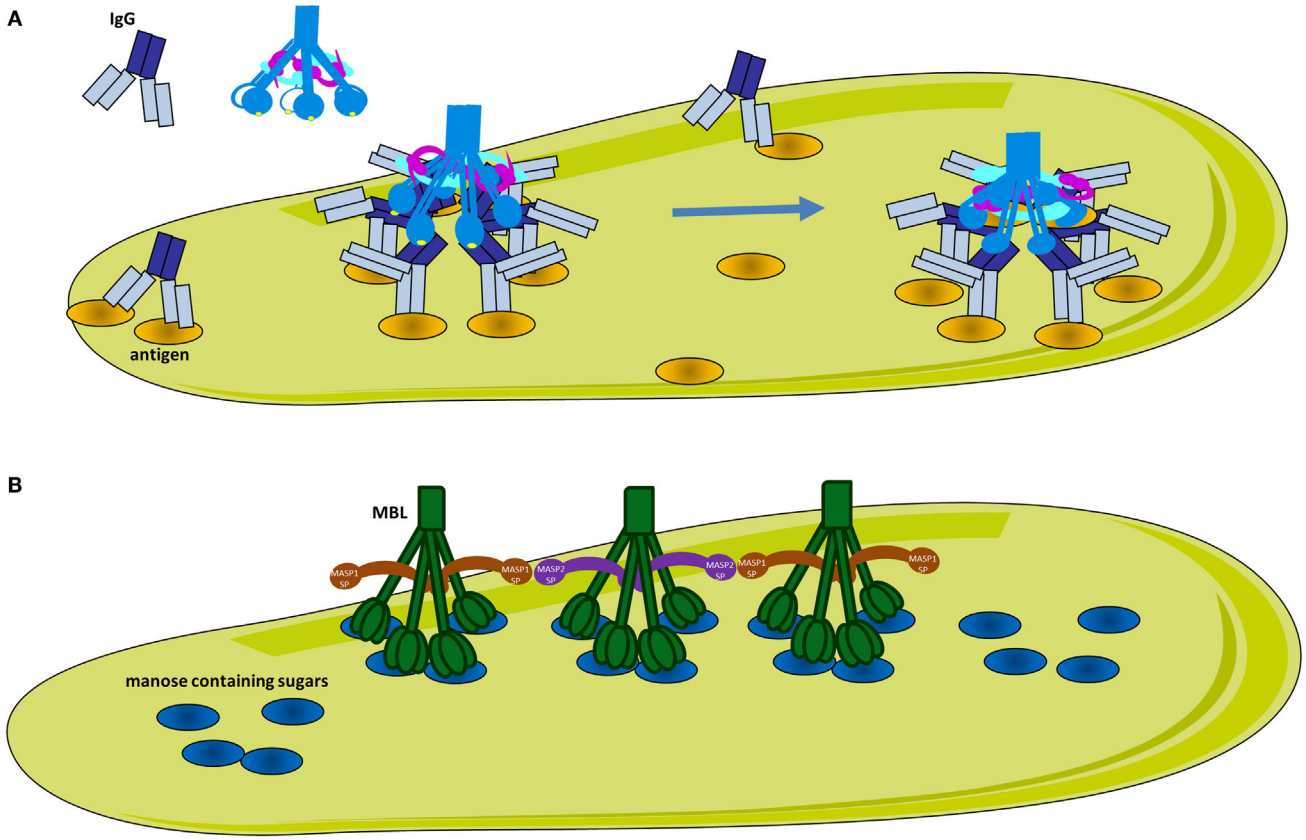

FIGURE 3 | Classical and lectin pathway activation. **(A)** Activation of the classical pathway. Multiple adjacent IgG molecules are needed to bind C1q. IgG interacts with its target antigen forming specific circular structures. A single FAB binds to the antigen, while the other does not. The movement of the Fc domain exposes the C1q-binding sites allowing complementarity with the six globular domains of C1q (gC1q). The number of engaged IgG molecules will determine the compatibility of the immune complex with C1q and hence the strength of classical pathway activation. C1q circulates in plasma-associated with the serine proteases C1r and C1s, forming inactive C1 complex. After binding, the target C1q undergoes a conformational change to increase the angle between its collagenous stalks (CLR). This conformational change activates C1r, which in turn activates C1s. **(B)** Activation of the lectin pathway. MBL recognizes mannose containing sugars on pathogens. MBL circulates associated with serine proteases MASP-1 or MASP-2. Upon target binding, juxtaposition of MASP-2 and MASP-1 containing MBL complexes is required for MASP-1 to activate MASP-2.

interactions (27–29). Taking into account the surface morphology of gC1q and its targets, it is difficult to contemplate how these residues can engage simultaneously in binding. One possibility is that these residues form binding sites, which make contact with the target subsequently and not simultaneously (**Figure 4A**). These data, combined with the importance of the Ca^{2+} ions for the electrostatic field of C1q (24), and the induced conformational change leading to an increased angle between the collagenous stalks by gC1q and target interaction (48, 56–60) has led to the proposal of an electrostatic model for the activation of the C1 complex (**Figure 4A**). This model suggests that the increase in the angle between the collagen stalks occurs because of a rotation of the gC1q domains, driven by electrostatic interactions between gC1q and the target molecule (IgG, IgM, or CRP) (24). Interactions between the negatively charged binding sites on the target may cause a removal of the Ca^{2+} ion from gC1q. Loss of Ca^{2+} changes dramatically the size and orientation of the electric moment vector of gC1q. This electrostatic change can induce the rotation of gC1q allowing it to engage the lateral surface of the B-chain. This rotation may provide the mechanical stress necessary for the transition of the $C1r_2C1s_2$ complex from closed, inactive eight-shaped conformation to an active, S-shaped conformation, allowing C1r auto-stimulation and further C1s activation by C1r. In this active form allows the tetramer to unfold and to extend its C1s ends outside the C1q cone for interaction with C4 and C2. Cleave of C4 and C2 by C1s allows formation of the CP C3 convertase in the immediate proximity to the C1 complex-binding site (44, 45, 53, 61). It is a matter of debate whether the C1s catalytic domain faces the exterior or the interior of the C1q cone. If the recognition and cleavage of C4 occurs inside the cage-like structure of the cone of C1 (62), this may increase the efficacy of the covalent binding of the bioactive cleavage product C4b to the surface. However, it is still unclear what would be the driving force to assure the entrance of both C4 and C2, as well as the substrate molecules of C3, in this confined space. These data imply that one C1 complex will have limited efficacy, generating only one or two C3 convertases as a result of steric hindrance. It is too few compared to the experimental evidence that one activated C1 complex generates about 35 C4 molecules during its lifespan (63).

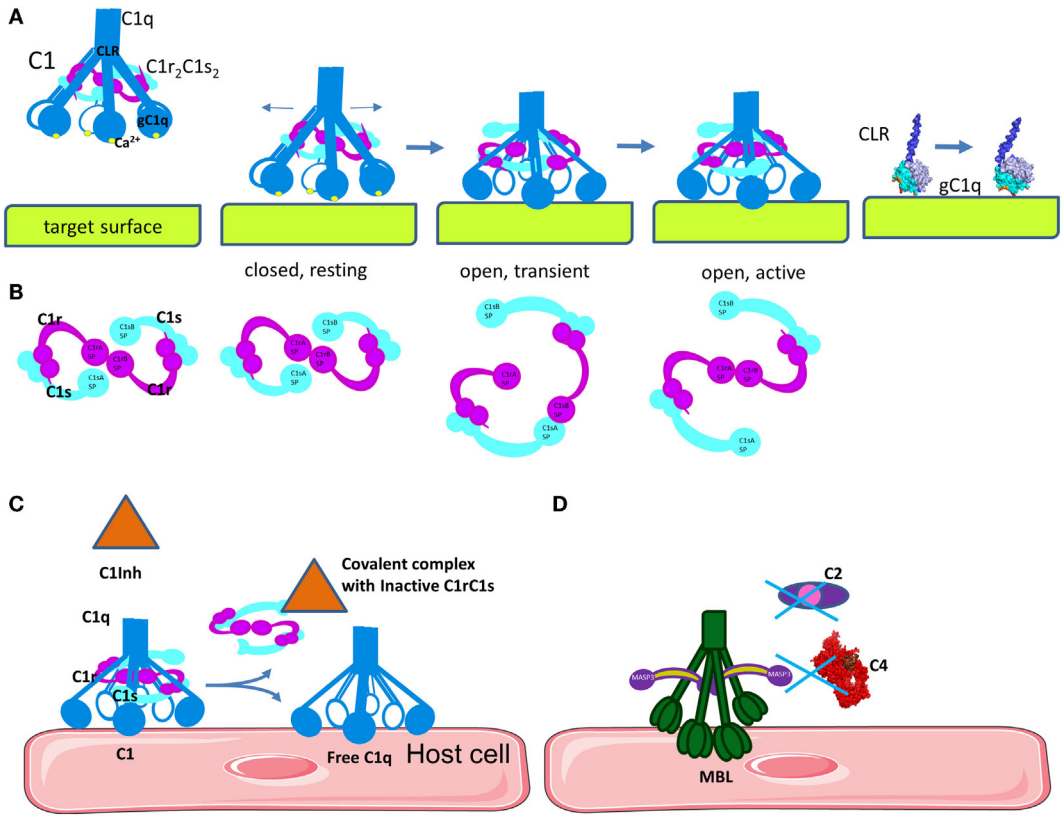

FIGURE 4 | Mechanism of C1 classical pathway activation and regulation. **(A)** Structural changes in C1q are necessary to induce auto-activation of C1r and activation of C1s. Upon binding of the inactive, closed C1 complex, electrostatic interactions with a target surface may alter the electrostatic field of the domain. This will induce a rotation of gC1q, leading to opening of the angle between the CLRs. A part of the binding site on gC1q apex will be lost, but new links will be formed with the side surface of the B-chain. **(B)** Concomitant with the structural changes in C1q, $C1r_2C1s_2$ complex will pass from closed, inactive eight-shaped conformation to an active, S-shaped conformation, allowing C1r auto-activation and further C1s activation by C1r. **(C)** The C1 inhibitor is a serpin that binds covalently to the active site of C1r and C1s, blocking their function. It also dissociates $C1r_2C1s_2$ from C1, releasing free C1q. C1 inhibitor also inhibits the lectin pathway by binding to MASP-1 and MASP-2. **(D)** MBL can bind to MASP-3, MAp44, or MAp19, which cannot cleave C4 and C2.

In order to understand the exact architecture of the C1 complex, further investigation will be required.

Mechanism of Activation of the Lectin Pathway

The pattern-recognition molecules of the LP are MBL, collectins, as well as ficolins H, L, and M (64, 65). The LP pattern-recognition molecules have an N-terminal collagenous region similar to C1q, however their C-terminal domains differ from gC1q. Collectins contain carbohydrate recognition domains, which recognize sugar patterns. MBL, which belongs to the collectin family, recognizes terminal monosaccharide exposing horizontal 3′- and 4′-OH groups (glucose, mannose, and *N*-acetyl-glucosamine) in a Ca-dependent manner. These sugars are rarely present on host proteins and cell surfaces, but frequently expressed on bacteria, viruses, and dying cells. Ficolins are associated with MASPs protein in the circulation and have C-terminal recognition fibrinogen-like domains, which are able to bind acetyl groups, such as *N*-acetyl-glucosamine, on the surface of bacteria. Following binding, MASPs associated with MBL or ficolins are activated and result in the cleavage of C4 and C2 (66, 67). Similar to C1q, stable binding can be achieved only when the ligands are clustered on the surface forming a specific pattern. This complex can simultaneously engage several carbohydrate recognition domains or fibrinogen-like domains for collectins and ficolins respectively.

Despite the similarity between the architecture of the C1 and MBL/MASP complexes, the mechanism of activation of the LP is different than the classical one (64). While in the CP each C1 complex carries both C1r and C1s, the majority (~70%) of the MBL molecules present in plasma are associated with only one homodimer of either MASP-1 or MASP-2 to assure their separation (**Figure 3B**) (68). In physiological conditions, MASP-1 is required for the activation of MASP-2 and both activated proteases can cleave C2 while MASP-2 can also cleave C4. Auto-activation properties confer to MASP-1 a fluctuating state between inactive and active-like conformations, giving it a key role in LP activation (69–74). Auto-activation of MASP-2 provides a residual capacity (~10%) to cleave its natural substrate C4 in zymogen form (75). Since MASP-1 and 2 are associated with different MBL or ficolin molecules, they are required to juxtapose their recognition molecules on ligand surfaces to facilitate activation of different

MASPs (76). Therefore, MASP-1 from one complex will activate MASP-2 from the adjacent complex, allowing C4 cleavage (77). MASP-3 also influences LP activation.

The described mechanisms of activation of the CP and LP illustrate two of the key characteristics of the complement cascade. Complement activation relies on the versatility of the target patterns recognition molecules (C1q, MBL, ficolins) that can discriminate between self and non-self and bind to pathogen- or danger-associated molecules. These molecular patterns are often generated after a specific conformational changes, such as with IgM or particular clustering, such as with IgG, CRP, or pathogen-associated carbohydrates. Complement is driven by these conformational changes that transmit a signal as a result of the recognition event to the subsequent complement components, activating them or modulating their function.

Regulation of the Classical and Lectin Pathways Initiation

Activation of the CP is controlled by a serpin molecule, C1 Inhibitor (C1Inh). C1Inh binds and inactivates C1r and C1s, leading to dissociation of the C1 complex and liberation of free C1q leaving an inactive covalent complexes between C1Inh and C1r or C1s (**Figure 4C**) (78, 79). C1Inh is thought to bind to and stabilize unactivated C1r and C1s in the C1 complex thus retarding their spontaneous activation (80), but this function is poorly studied. C1Inh has additional functions outside complement inactivation, related to kinin pathway (a plasma system related to inflammation, vasodilatation, and pain). Angioedema is disease caused by hereditary or acquired C1Inh deficiency. The edemas are triggered by increased permeability of the blood vessels in response to elevated levels of bradykinin as a result of the C1Inh deficiency. Recombinant and plasma-derived C1Inh are approved therapeutic agents for hereditary angioedema (81).

C1q inhibitors released under physiological or pathological conditions such as chondroitin-4 sulfate proteoglycan and the hemolysis derivative heme can bind to C1q and inhibit the CP (55, 82). The mechanism of action of heme, as well as a number of synthetic C1q inhibitors, rely on binding to gC1q and alteration of its electrostatic properties (55, 83). Calreticulin, released during cell death or from parasites can also act as an inhibitor of C1q to prevent CP activation (84, 85).

Inhibition of the LP is influenced by MASP-3, MAp44, and MAp19 proteins, which share high sequence homology with MASP-1 and -2 and have similar binding affinity to MBL and ficolins (70, 86). These proteins may compete with MASP-1 and -2, but are unable to cleave MASP, C2, and C4 preventing further activation of the LP cascade (**Figure 4D**). In addition, C1Inh is able to control LP activation by inhibiting MASP-1 and MASP-2 but not MASP-3 activity (87, 88).

Platforms for Surface Assembly of the Alternative Pathway C3 Convertase

C3b can bind to the cell surface not only via its own thioester bond, but also by interacting with surface molecules that serve as platforms for C3b recruitment (**Figure 5**). Recently, it has been observed that C3b and C3(H_2O) can also bind to the cell surface by these platforms resulting in local activation of the AP. Although activated platelets are the predominant cell type that binds C3(H_2O) (89, 90), the exact molecules it binds are not yet well defined. Nevertheless, several molecules are likely candidates for activated C3 recruitment on different cell types.

Properdin, which stabilizes the alternative C3 convertase (91), is able to bind pathogens, activated or damaged host cells to induce stimulation of the AP (**Figure 5A**) (92, 93). Properdin recognizes heparin and heparan sulfate on tubular cells leading to complement activation (94). Properdin contributes to the AP activation on human neutrophils and has been observed in neutrophil-mediated diseases (95). Degranulation stimuli on neutrophils induce low properdin release and deposition, triggering AP by recruiting C3b and promoting C3 convertase formation. Plasma properdin is also required for an efficient C3b feedback loop and amplified AP activation (95). Generation of C5a also further stimulates properdin release and amplifies complement activation and neutrophil stimulation. Moreover, it has been demonstrated that myeloperoxidase secreted by neutrophils during degranulation binds to properdin and leads to the AP activation (96). Properdin released by activated neutrophils can also bind to activated platelets. In the absence of C3, properdin can also bind directly to platelets after interaction with a strong agonist and serves as a platform for recruitment of C3b or C3(H_2O) and C3 convertase formation (**Figure 5B**) (90). This complement activation may contribute to localization of inflammation at sites of vascular injury and thrombosis.

Another protein, which can recruit C3b to a cell surface is complement Factor H (FH)-related protein 4A (CFHR4A). This protein shares sequence homology with FH and is able to bind C3b via its C-terminal domain. CFHR4A lacks regulatory domains and cannot inactivate C3b. Even more, it has been suggested that CFHR1A can serve as a platform for the alternative C3 convertase formation. Formed convertase on CFHR4A had a higher resistance to FH-mediated decay.

P-selectin (CD62P) recruits leukocytes via binding to P-selectin glycoprotein ligand 1 (PSGL-1) (97) and has been described to bind C3b on the cell surface leading to the activation of AP (**Figure 5C**) (98, 99). Morigi et al. showed the effect of P-selectin as a platform for C3 convertase formation *in vitro* and in a murine model of Shiga toxin (Stx2)/LPS-induced hemolytic uremic syndrome (HUS) (99). P-selectin expression was partially triggered by the anaphylatoxin C3a contributing to a vicious circle of complement activation aggravating microvascular thrombosis HUS pathology (99).

Another activator of C3 convertase, heme, is released from hemoglobin during hemolysis, where it stimulates the AP. Heme induces deposition of C3 activation product in erythrocytes and has been shown to play a role in malaria pathogenesis (100, 101). Heme binds C3 (not C3b), likely near to the TED domain, leading to the generation of C3(H_2O) and homophilic C3 complexes associated with overactive C3/C5 convertases (102). Furthermore, *in vitro* experiments on human EC have shown that heme-induced mobilization of specific EC granules that store von Willebrand Factor and P-selectin called Weibel Palade bodies, is at least in part induced by TLR4 (102, 103). This TLR4 stimulation lead to degranulation of P-selectin accompanied by C3b and C3(H_2O) binding to the cell surface of EC. Heme is a hydrophobic molecule that binds to lipid bilayers and it is hypothesized that cell-bound heme may serve as a platform to recruit C3(H_2O) (102).

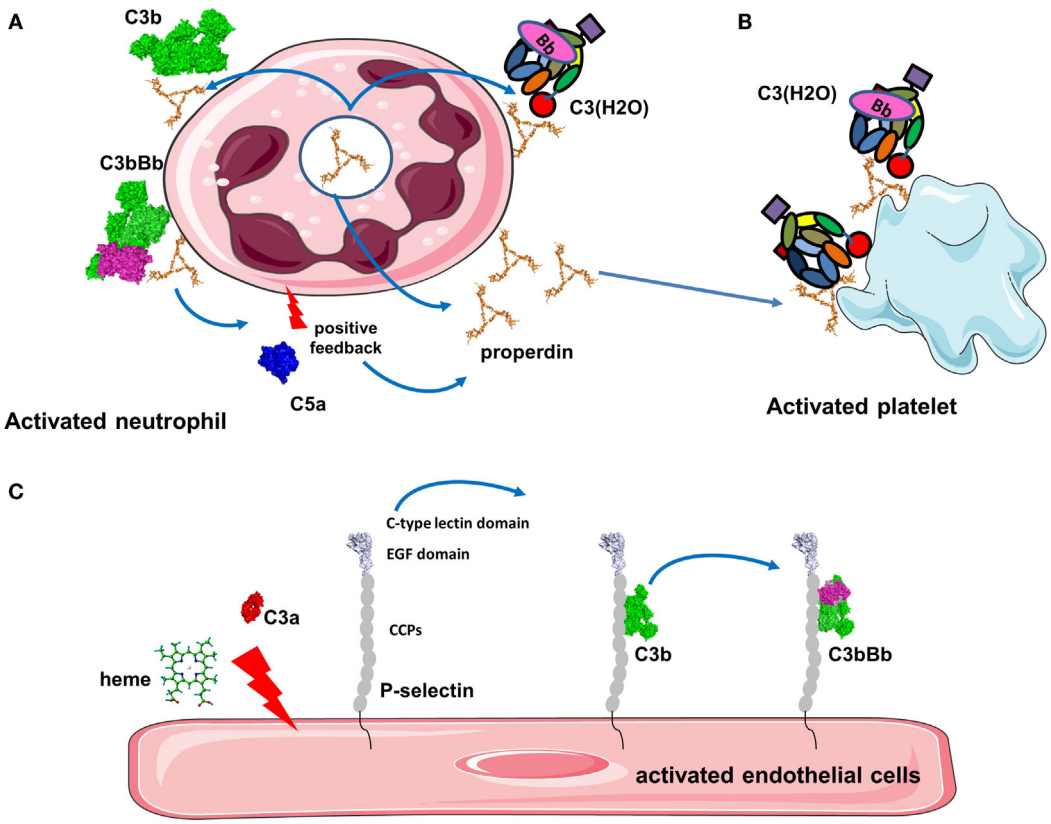

FIGURE 5 | Platforms for complement activation. **(A)** Properdin is released from activated neutrophils and is bound to the cell membrane where it recruits C3b to form the alternative pathway C3 convertases. C5a then activates additionally neutrophils and they secrete more properdin. This installs a vicious cycle of neutrophil and complement activation. **(B)** Properdin released from neutrophils or in the plasma binds to activated platelets promoting C3(H_2O) recruitment and complement activation. **(C)** Stimulation of endothelial cells with C3a, heme, or other agonists induces expression of P-selectin. P-selectin contains CCP domains and binds C3b, promoting formation of C3 convertases that generate more C3a to stimulate cells.

Collectively, these examples lead us to propose a general mechanism for a positive feedback loop implicating protein platforms in tissue damage. An initial trigger will stimulate the cell to either express a platform protein (properdin for neutrophils or P-selectin for EC and platelets) or to bind molecules from the fluid phase (properdin, CFHR4A, or heme in case of hemolysis). The type of the platform will likely depend on the cell type, location of activation, and other yet undiscovered factors. C3(H_2O) will bind to these platforms and will initiate local complement activation and C3b deposition. The amplification loop will generate C3a and C5a, which upon binding to their receptors (described below) will augment cell activation and increase expression of platform proteins stored in intracellular granules or recruited from the plasma. These events will form an intensified circle resulting in local inflammation, thrombosis, and tissue damage.

Structure and Function of the C3 Convertases

Alternative Pathway C3 Convertase

The structure and function of the AP C3 convertase has been dissected during the few last years. Upon cleavage and removal of C3a, C3b undergoes a dramatic structural change (**Figure 6A**) leading to exposure of novel binding sites. This allows recruitment of FB which binds in a Mg^{2+}-dependent manner and yields the pro-convertase C3bB (**Figure 6B**) (104). This interaction occurs via the Von Willebrand Factor A-type (VWF-A) domain and three complement control protein (CCP1-3) domains of FB (104, 105). The catalytic SP domain of FB undergoes large conformational changes oscillating between a closed (loading) and an open (activation) forms (**Figure 6B**) (104–106). In the open (activation) conformation, the scissile bond is exposed and the FD binding site is formed correctly.

Factor D is synthesized in an inactive pro-FD enzyme lacking proteolytic activity (107). It was suggested that this zymogen form can be cleaved by MASP-1/3 into a form with limited activation to support the basal levels found in the AP (108, 109) and becomes fully activated only upon binding to C3bB open complex. The physiological relevance of MASPs-mediated cleavage of pro-FD is still being debated. MASPs cleavage is not the only mechanism for FD activation, since mice deficient in MASP-1/3 have reduced but detectable AP activity (110) and the only patient found to be deficient in MASP-1 and -3 was reported to have a normal AP activity (111). Further studies are needed to elucidate the

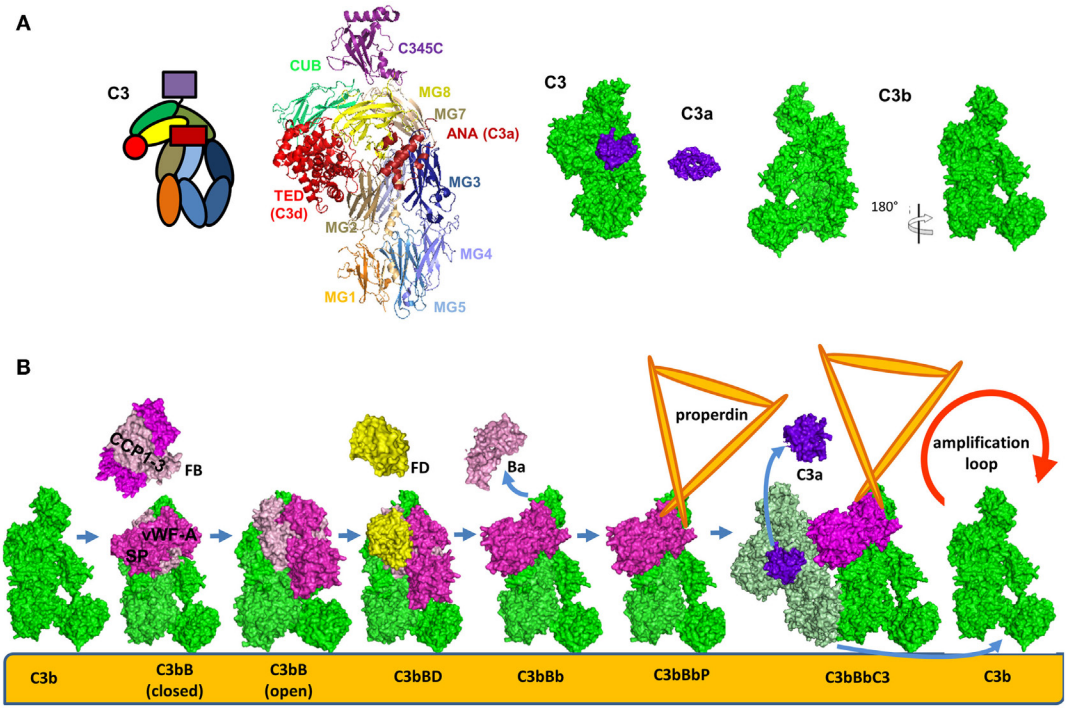

FIGURE 6 | Alternative pathway C3 convertase. **(A)** Structure and domain organization of the central complement component C3 and its cleavage fragments C3b and C3a. C3b is shown in two orientations to illustrate the surfaces containing the ANA domain and the opposite surface, carrying FB and FH binding sites. **(B)** Steps of formation of the alternative pathway C3 convertase. C3b is shown in green, FB in magenta, FD in yellow, C3a is in violet, and the substrate molecule C3 – in light green. For these molecules, the available crystal structures were used for the visualization. The C3bBbC3 complex is visualized based on molecular modeling. Properdin, for which a crystal structure is not available, is depicted in orange.

mechanism of FD activation in mice and men. Insights into this pathway could help lead to the development of MASP-1 inhibitors as a strategy to treat renal diseases associated with uncontrolled AP activation.

Upon activation, FD binds to and cleaves C3b-bound FB releasing the N-terminal fragment Ba (comprising the CCP1-3 and the αL helix). This results in the formation of the C3 convertase of the AP C3bBb (**Figure 6B**) (112). The Bb fragment consists of a VWF-A and a SP domain. The SP domain undergoes a new structural change and is positioned in a conformation, similar to the closed form of the pro-convertase (**Figure 6B**) (113). A substrate molecule of C3 binds then to the alternative C3 convertase and is cleaved generating new C3b and C3a molecules (**Figure 6B**) (113).

Factor B binds in a similar manner to C3(H_2O) to form the alternative C3 convertase [C3(H_2O)Bb] in the fluid phase (114). Even if FB has a higher affinity to C3(H_2O) than to C3b, the convertase activity remains lower than the C3bBb complex, as measured by C3a released. The fluid phase convertase C3(H_2O)Bb also has higher resistance to decay by complement inhibitors (114).

Classical Pathway C3 Convertase

The structure and mode of action of the CP C3 convertase is not well understood. C4 and C2 share high degree of sequence and structure homology to C3 and FB respectively, thus the mechanism of formation of the classical C3 convertase may be similar to the well-studied AP C3 convertase, described above (**Figure 6**). C4 is cleaved by activated C1s or MASP-2 to bioactive fragment C4b and a small fragment C4a (**Figures 7A,B**). C4b contains an internal thioester bond, similar to that in C3b, and forms covalent amide or ester linkages with the antigen–antibody complex or the adjacent surface of a cell. C4b binds C2 in Mg^{2+}-dependent manner. C2 is then cleaved by C1s or MASPs. Since the concentration of C2 is about 20–30-fold lower compared to C4, one active C1 complex can cleave about 35 C4 molecules, while only 4 C2 will be cleaved for the same time (63). The larger fragment C2a remains bound to C4b and forms the CP C3 convertase C4b2a (**Figure 7C**) (115) and the smaller fragment C2b is released in the circulation. Historically, C2b was considered to have kinin-like properties, but recent reports failed to confirm this function (116, 117).

The C3 convertases are an excellent example of a general mechanism that governs different steps of the complement cascade. Each subsequent step can only occur after a conformational change, triggered by the preceding step, thus assuring the temporal and specific control of this powerful destruction cascade (118).

Stabilization of the Alternative Pathway C3 Convertase

The C3bBb is a short-lived complex with a half-life of about 90 s (119) and, therefore a stabilization of this complex is required to assure

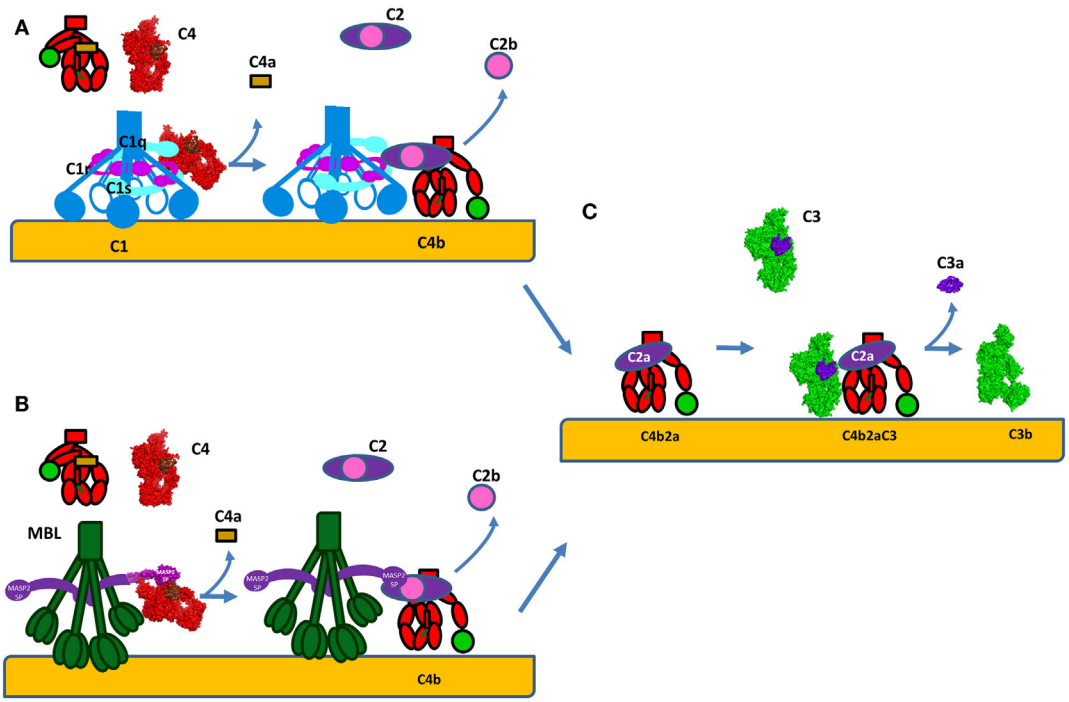

FIGURE 7 | Formation of the C3 convertase by the classical and lectin pathways. **(A)** C1s or **(B)** MASP-2 will cleave C4 into bioactive fragment C4b that bind covalently to the surface of cells and interacts with C2. The small fragment C4a is released. Following, the same enzyme will cleave C2 to generate the classical pathway C3 convertase C4b2a. **(C)** C4b2a will interact with C3 cleaving it and releasing the bioactive fragments C3a and C3b. C3b will bind covalently to the surface and allow formation of alternative pathway C3 convertases C3bBb via the amplification loop. The C3a generated is a pro-inflammatory anaphylatoxin. The C4 molecule is presented in red, with brow colored the ANA domain, which will become C4a after cleavage and in green – the TED domain, which will become C4d after cleavage. The crystal strictures of C4, C4b, and C2a were used for the representation. The CP C3 convertase C4b2a is modeled based on the structure of the AP convertase C3bBb, with which it shares high homology.

efficient host defense (**Figure 6B**) (93). The AP C3 convertase is stabilized 5- to 10-fold by association with properdin (91). Properdin (120) is secreted by monocytes/macrophages and T lymphocytes (121, 122) and is stored in secondary granules of neutrophils (95, 123) and mast cells (124). Properdin is composed of identical rod-like protein subunits (125) that are associated head-to-tail to form cyclic dimers, trimers, and tetramers that resemble rods, triangles, and squares, respectively. The function of properdin is dependent on its level of polymerization, with the tetramer being approximately 10-fold more active than the dimer (126). Purified properdin results in aggregates and has artificially high binding activity (127, 128). Properdin binds C3bBb, as well as, the pro-convertase C3bB and C3b (92). Properdin interacts both with the C345C domain of C3b and the VWF-A domain of Bb, in order to stabilize the convertase (129). Interestingly, electron microscopy studies visualized that properdin binding induces a large displacement of the TED and CUB domain of C3b (129). These structural changes distort the FH binding site (130–132), which may explain the relative resistance of the stabilized C3 convertase to decay by FH.

Recent studies of Hourcade and colleagues demonstrated that properdin is not merely a stabilizer of the C3 convertase, but also a pattern-recognition molecule that binds to microbial surfaces including glycosaminoglycans (GAGs), apoptotic, and necrotic cells providing a platform for C3 convertases assembly (93).

Negative Regulation of the C3 Convertases

The amplification loop is the balance between two competing cycles, acting on the C3b–C3 convertase formation, which enhances both amplification and downregulation via the C3 breakdown cycle. Complement amplification depends on the balance between binding rates of each reaction (22). To regulate activation, several inhibitors of complement pathways, primarily against AP, exist in the fluid phase and on host cells. Complement inhibitors have overlapping functionality. FH works as a soluble inhibitor of the alternative C3 convertase, while membrane cofactor protein (MCP), decay acceleration factor (DAF), and complement receptor 1 (CR1) work as membrane inhibitors. FH is a specific cofactor for C3b and C4 binding protein (C4BP) primarily is a cofactor for C4b, while MCP and CR1 act as cofactors for the inactivation of both C3b and C4b via Factor I (FI).

Inactivation of C3b and C4b by Factor I

Factor I is a SP found in the plasma that cleaves C3b in presence of different cofactor molecules, such as FH, MCP, CR1, or C4BP (**Figure 9A**). The protease activity of FI leads to the generation of degradation product of C3b, iC3b, which is unable to bind FB (133). A zymogen form of FI has not been detected in the

circulation. In fact, FI circulates in a proteolytic form but in inhibited conformation. The activity of the light chain of FI is allosterically inhibited in the circulation by a non-catalytic heavy-chain (134). In presence of its cofactors, the non-catalytic heavy-chain of FI is released and it is able to cleave C3b between Arg1281 and Ser1282 to give the iC3b fragment (134, 135). Molecular modeling suggested that FI binds FH1–4 and C3b simultaneously in a groove between two proteins, which is in agreement with the hypothesis that FI binds CCP1–3 of FH and C345C of C3b (132).

Factor I Cofactors

Membrane cofactor protein, DAF, and CR1 (CD35) serve as cofactors for FI-mediated proteolysis of C4b and C3b. MCP is composed of 4 extracellular CCP domains expressed on all nucleated cells (136) and CR1 contains 30 extracellular CCP domains and is expressed on leukocytes, erythrocytes, and glomerular podocytes. MCP N-glycosylation on CCP2 and CCP4 is essential for MCP inhibitory activity (137). CCP1–4 of MCP binds to C3b and C4b and is structurally similar to the four N-terminal domains of FH (described below), both proteins serve as cofactors for FI for C3b inactivation (**Figure 9A**) (132, 138–140). FH only induces C3b-degradation and is inefficient for C4b. CCP-3 and -4 of MCP are responsible for binding to C3b and C4b, while CCP1–2 only interacts with C4b (**Figure 8A**) (141). The binding site for MCP on C3b is partially overlapping with the site for CCP-3–4 on FH (142).

The first 28 CCP of CR1 are 4 long homologous repeats (LHR) for 7 CCP domains containing the binding sites for C3b and C4b (143, 144). C3b and C4b-binding sites located in CCP8–10 (LHR2) and CCP15–17 (LHR3), respectively, are responsible for FI cofactor activity (143, 145). CCP15–17 has a major role in C3b/C4b inactivation (146). CCP15 carries a positively charged region essential for the C4b-binding and a basic region in the CCP16 that is necessary for C3b-binding. Although the architecture of the CR1/C3b and CR1/C4b is not well defined, it is known that CR1 interacts with the α′NT region of C3b (residues 727–767), which overlaps with the FH CCP1-binding site, but not MCP-binding site (132, 133, 142, 147).

C4b can be inactivated by the action of C4BP, a plasmatic cofactor for FI (**Figure 8B**) (148–151). C4BP has a complex, octopus-like structure comprised of CCP domains containing α- and β-chains (152, 153). The first three CCP domains of each α-chain are involved in cofactor and convertase dissociation functions. A

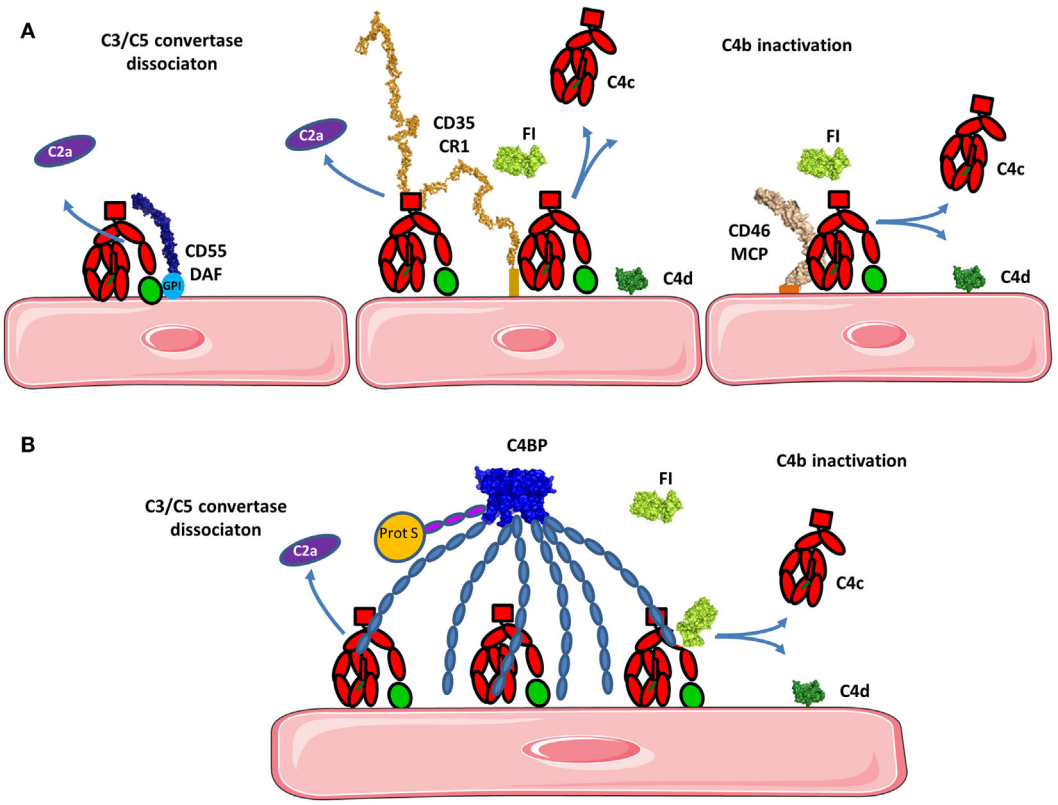

FIGURE 8 | Regulation of the classical and lectin pathways C3 convertase. To avoid overactivation, the CP and LP tightly regulate signaling. **(A)** If a C4b2a C3 convertase is formed, it will be rapidly dissociated by DAF and/or CR1 depending on the cell type. Bound C4b will be inactivated by FI in presence of cofactors such as CR1 and/or MCP. C4d will remain bound to the surface and C4c will be released. **(B)** C4BP can act in fluid phase as well as on the cell surface. C4BP has an octopus structure and interacts with several C4b molecules. It dissociates the C3 convertase and serves as a cofactor for FI in the cleavage of C4b to inactive fragments C4c and C4d. The structures of the complexes of C4b with the regulatory proteins have not been resolved yet, therefore the proteins are depicted in proximity one to another, represented by their known structures, but no complex could be reliably modeled.

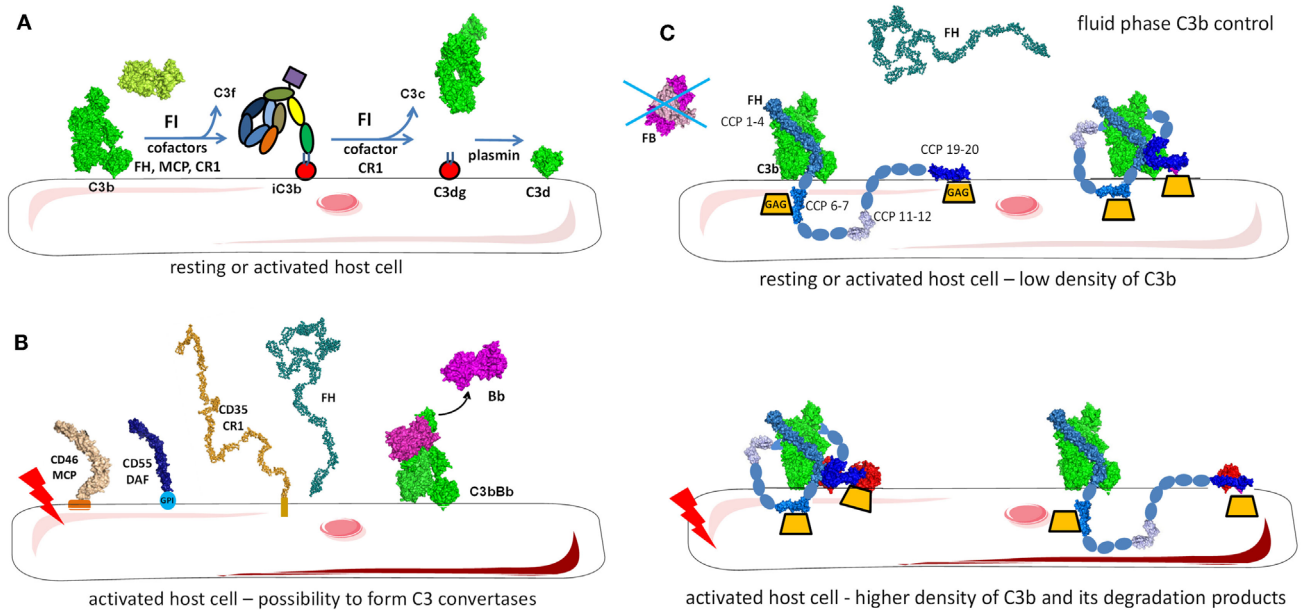

FIGURE 9 | Regulation of the alternative pathway. **(A)** FH as a master regulator of C3b in the fluid phase and on the cell surface. FH binds to C3b in fluid phase preventing novel convertase formation. FH may bind to C3b and GAGs on the cell surface and the architecture of the complex depends on the level of activation of the cell and the density of deposited C3 fragments. Resting cells have only a few C3b molecules that are deposited and FH binds to them with the regulatory domains CCP1-4. CCP7 and CCP20 interact with GAG on the membrane. Alternately, CCP19 may bind to the TED domain of C3b allowing CCP20 to interact with GAGs. If the cell is activated and C3b and C3d (or two C3b molecules) are deposited in close proximity, FH may bind to two of these molecules, allowing GAG binding by CCP20. **(B)** On resting cells, C3b will immediately be inactivated to iC3b by the action of FI and the assistance of cofactors (FH, MCP, CR1). iC3b cannot bind FB and forms C3 convertases. Only the cofactor CR1 allows FI to execute a second cleavage generating C3c (released in the fluid phase) and C3dg, which remains bound to the cell. C3dg is rapidly transformed to C3d by tissue proteases. **(C)** If the host cell is activated, the complement control will not be sufficient to prevent any complement deposition and C3 convertases could be formed. To avoid cell damage, these convertases need to be dissociated. Multiple complement regulators such as DAF, CR1, and FH decay the C3bBb complex formed on host cells. Remaining C3b will be inactivated by FI, using FH, MCP, or CR1.

maximum of four C4b molecules can simultaneously interact with the α-chains of one C4BP molecule (154). The β-chain binds coagulation protein S and contributes to regulation of the coagulation cascade (155).

Complement receptor 1 is a unique cofactor of FI due to its ability to induce further cleavage of iC3b, generating C3c and C3dg degradation fragments (**Figure 9A**) (133), where FH, MCP, and C4BP only induce the cleavage of C3b to iC3b.

Several proteins have been shown to enhance FI-mediated cleavage in the presence of cofactors. Thrombomodulin binds to C3b and FH and negatively regulates complement by accelerating FI-mediated inactivation of C3b in the presence of FH and C4BP (156). Von Willebrand factor also enhances the efficacy of FH as a cofactor for FI (157). In specific *in vitro* buffer conditions, Von Willebrand factor has been suggested to have direct cofactor activity, but the physiological function of this interaction requires further validation (158).

C3 Convertase Dissociation

The AP C3 convertase is dissociated and Bb is released primarily by the action of CCP1–4 in FH (described below). CR1 dissociates the C3 and C5 convertases. CCP1–3 of CR1 carries a binding site for C4b allowing it to induce accelerated decay of the C3 convertase in the CP and AP (**Figures 8A** and **9B**) (143, 159). C4BP prevents the formation of the classical C3 and C5 convertases (148–151).

Decay acceleration factor accelerates decay of the C3 and C5 convertases in the CP and AP (**Figures 8A** and **9B**). DAF has four extracellular CCP domains, a highly glycosylated domain and a GPI anchor (160). DAF inhibits the AP C3 convertase by binding to Bb on the vWA domain with its CCP2 domain (161). CCP2 of DAF has a higher affinity for Bb compared to the intact FB. As a consequence, the active convertase is more sensitive to rapid decay, compared to C3bB (162, 163). DAF also binds to the C3 convertase on C3b via its CCP4 domain (163) and CCP-3 contributes to the accelerated decay function (164).

Factor H – The Master Regulator of the Alternative Pathway

Factor H regulates the AP and the amplification loop of the complement pathways (**Figure 9**). It is a soluble inhibitor of the C3 convertase competing with FB for binding to C3b (**Figure 1A**) (165). It also serves as a cofactor for C3b inactivation by FI (**Figure 9A**) and induces C3bBb complex dissociation (**Figure 9B**) (166, 167). FH is composed of 20 CCP domains arranged as beads on a string (168) (**Figure 9C**). CFHL1 is a shorter protein, containing the seven

N-terminal domains of FH, generated via alternative splicing from the same gene (169) and functions as a fluid phase complement regulator. The fluid phase convertase C3(H_2O)Bb is more resistant than the alternative cell-bound convertase and is less susceptible to regulation by FH (114). FH has two main ligands, C3b and the GAG, found on host cells surface. Recent crystallographic and mutation analysis resulted in precise mapping of the C3b and GAG-binding sites in FH.

Factor H binds to C3b and C3(H_2O), but not to uncleaved C3. The conformational changes in the TED and CUB domains accompanying the transition from C3 to C3b expose the FH-binding sites. FH binds to C3b in at least two regions, at the N-terminus and the C-terminus of the protein. The N-terminal four CCPs contain the complement regulatory activity (132) and the CCP1–4 interacts with the MG ring of C3b. CCP-3 interacts with the CUB domain and CCP4 interacts with the TED domains (132, 142). CCP1–2 competes with FB causing its dissociation from C3b (**Figure 9C**). Both Bb and CCP1–2 are negatively charged leading to electrostatic repulsion. CCP19–20 carry a second binding site for C3b and can also interact with C3d (130, 131, 170, 171).

It has been difficult to unravel questions regarding the stoichiometry and architecture of the C3b–FH complex in the physiological fluid phase and on the surface of cell (**Figure 9C**). Does FH interact in the same way with C3b and C3(H_2O) or with C3b in fluid phase and on cell surface? Could FH bind with its N- or C-terminal domains on the same C3b molecule or can it interact with two adjacent C3b or C3b and C3d molecules or with one C3b molecule via CCP1–4 at the C-terminus and engage in cell membrane binding? If it is assumed that FH CCP1–4 and CCP19–20 bind to the same C3b molecule and if there is a liner arrangement of the C-terminal CCPs, could there be a steric clash between CCP18 and CCP4? The crystal structure of CCP18–20 indicates a bend-back conformation of CCP18, allowing binding on the TED domain of both CCPs 1–4 and CCPs 19–20 (172). Studies of the C3b/C3d binding site on CCP19–20 showed that it may be overlapping, but it is not identical with the GAG-binding site (173–177). The C3b/C3d-binding site is extended toward the CCP19 and the GAG binding is extended toward the apex of the CCP20. It is possible that FH19–20 domains may bind both C3d and GAG at the same time (131, 172, 174, 177). Site-directed mutagenesis of the FH19–20 domains indicates that a ternary complex between C3d/FH19–20/heparin can be formed and is essential for the functional activity of FH (177). The formation of a ternary complex was confirmed by the crystal structure of FH19–20 with C3d and a model sialylated trisaccharide, where a surface area extending from SCR19 to the beginning of CCP20 binds C3d and CCP20 carries a is highly conserved binding site, which may accommodate GAGs and sialic acid containing glycans (174). Structural analysis of the complex of FH19–20 with C3d showed that CCP20 also may interact with C3d suggesting potential competition between C3d and GAG at this site of FH (130). Analysis of published PDB files indicates that this CCP20–C3d interaction is present in the other FH CCP19–20 crystals, but was considered a crystallization artifact. Nevertheless, mutations in CCP20 appeared to affect the interaction with C3b and C3d, suggesting that a C3d–CCP20 interaction is possible. Based on the accumulating structural and functional data, it can be hypothesized that the architecture of the C3b–FH complex is governed by the target surface and the density of the C3b and C3d molecules (**Figure 9C**). On host cells, one isolated C3b molecule will bind CCP1–4, while SCR7 and the C-terminus will interact with the GAG of the cell membrane. Since FH may circulate in plasma in a folded back hairpin conformation (178–181), simultaneous interactions with the N- and C-termini to the same C3b molecule could be possible, while CCP7 and 20 bind to the GAGs of the membrane. Indeed, CCPs 10–13 are involved in the inclination of FH, allowing both CCP1–4 and CCP19–20 binding to C3b (182). Using crystal structures of CCP10–11, CCP11–12, and CCP12–13, the authors also demonstrated that a tilt of 80–100° occurs allowing a hairpin structure formation. The compact architecture of the C3b–FH complex is supported by the existence of a third binding interface involving CCP6–10 in FH and the C3c portion of C3b (171, 183, 184).

Glycosaminoglycans are an important constituent of the cell membrane and play a critical role in complement regulation. In addition to the GAG-binding site in CCP20, FH carries another GAG-binding site located in CCP7 (183, 185–189). These GAG-binding sites in FH allow it to recognize negatively charged heparan sulfate moieties on the membrane and may explain the differences in the affinity of FH binding and a dependence on the expression of GAG and sialic acid on the cell surface (190). The difference in the susceptibility of sheep and rabbit erythrocytes to lysis by human complement, which are at the basis of the classical hemolytic tests for complement activation, is expression level of sialic acid on the surface of these cells. This allows them to bind (sheep) or not to bind (rabbit) to FH (165, 191). Together with the low affinity of properdin for heparin and heparan sulfate (94), GAG expression is a powerful regulator of the complement homeostasis between negative regulation and stimulation of the complement pathways. The two regions anchoring FH to the cell membrane, CCP7 and CCP20, are specialized in binding to unique GAG, expressed in different types of cells, however both are necessary to assure functional activity of FH on the cell surface (189). CCP7 containing construct CCP6–8 binds stronger to heparin than CCP20 containing construct CCP19–20 (192), while CCP6–8 and CCP19–20 do not recognize the same sulfate GAG. GAG and sialic acid are expressed in multiple human organs with different subpopulations and distinct structures that may provide a variation of the binding affinity of complement regulators (193). These differences can potentially explain why polymorphisms or mutations in these regions are associated with complement-mediated diseases.

Mutations or polymorphisms in the GAG-binding sites of FH may create an imbalance in the homeostasis of complement regulation and could explain its association with different diseases. For example, a polymorphism in CCP7 leading to a modification from a tyrosine to histidine at amino acid 402 (Y402H) is the strongest genetic susceptibility factor for age-related macular degeneration (AMD) (194–197). Detailed analysis of this mechanism revealed that CCP7 in FH binds not only to GAG, but also to oxidized lipids, including malondialdehyde (MDA) (198–200). Y402H binds more weakly to MDA and oxidized phospholipids expressed on retinal pigment epithelium compared to the non-mutated protein (198, 200).

Another ligand of CCP7 and CCP19–20 in FH is the CRP, which is secreted by the liver during inflammation acute phase. Binding of FH to CRP can enhance complement inhibition, particularly on apoptotic or damaged cells during inflammatory conditions (201).

The interaction of FH with different cell surfaces is controlled by CFHR proteins. CFHRs belong to the FH family and comprise five different members (CFHR1–5). CFHRs are composed of five to nine CCP domains, present a high sequence homology with FH, and are recently described in detail (202). CFHR1, CFHR2, and CFHR5 share high homology in their two N-terminal domains, allowing them to form homo- and heterodimers (**Figure 10**) (203, 204). CFHR3 and CFHR4 do not form dimers, the C-termini of each share a high sequence homology with FH leading to competition between CFHRs and FH for binding to C3b, C3d, and GAG on the cell surface (203, 205, 206). Therefore, the CFHRs will enhance complement activation, preventing the action of FH.

C5 Convertases and the Terminal Complement Pathway

C3b binds to the C3 convertase to form a new enzymatic complex – C5 convertase which cleaves C5 to bioactive fragments C5a and C5b (**Figure 11**). C5b recruits complement components C6, C7, C8, and C9 which polymerize to form the membrane-attack-complex (MAC) ring (**Figure 12**) (207). The structures of individual components and overall architecture of the C5b-9 complex are starting to be elucidated, while, the structure and the mechanism of action of the CP and AP C5 convertases is not fully understood and require further investigation.

C5 Convertases

The C3 convertases C4b2a and C3bBb are the precursors of the C5 convertases. The C3 convertases are able to bind C5, but with a very low affinity and cleavage rate. These C3 convertases switch their specificity and start to bind and cleave C5 only after binding of an additional C3b molecule in the immediate proximity or most likely over the C3 convertase itself (**Figure 11**) (208). The structure of this trimolecular complex is unknown, but has been suggested that the covalent C3b-binding site on C4b is the residue Ser1217 (p.Ser1236) within the TED domain (209). This residue is not conserved in C3b and the binding site seems to be located outside of the TED domain, in the α43 fragment (residues 1268–1641) (210), suggesting different topology of the two trimolecular complexes. Whether the newly bound C3b molecule interacts with C5 or affects the conformation of the C3 convertase subunits is currently unknown. Laursen and colleagues propose a model by which the MG1, MG4, MG5, and TED domains of C3b will be able to contact the CUB and TED domains of C4b. The CUB, MG2, MG6, and MG3 domains appear to be capable of reaching mainly the rest of C4b, while the MG7, MG8, and C345C domains potentially could be in direct contact with C5 (**Figure 11**) (211).

The dramatic conformational change of C4 upon release of C4a is very similar to C3 (212). TED domain, MG8, and CUB are exposed after C4 cleavage, allowing covalent bond with Gln1013. The crystal structure of C4b confirms the implication of the flexibles residues 1231–1255 in the interaction with the TED domain of C3b (209, 212). This model supports the idea that C3b has no direct interaction with C5 in the classical (and most likely in the alternative) C5 convertase (**Figure 11**). C4b and CVF-C5 structures suggest that C4b-binding area for C5 is within the domains MG4, MG5, and MG7, supporting Laursen hypothesis.

The loss of affinity to C3 and the acquisition of affinity to C5 results in cessation of C3b opsonization and initiation of MAC-induced membrane damage. Upon covalent binding of a C3b molecule to a CP convertase C4b2a, there is a formation of a trimolecular complex with about 1000-fold increased enzymatic activity toward C5 cleavage (213) compared to the bimolecular complex. The CP C3 convertase cleaves on average 1.5 C5 molecules per minute. The gained activity of the trimolecular complex

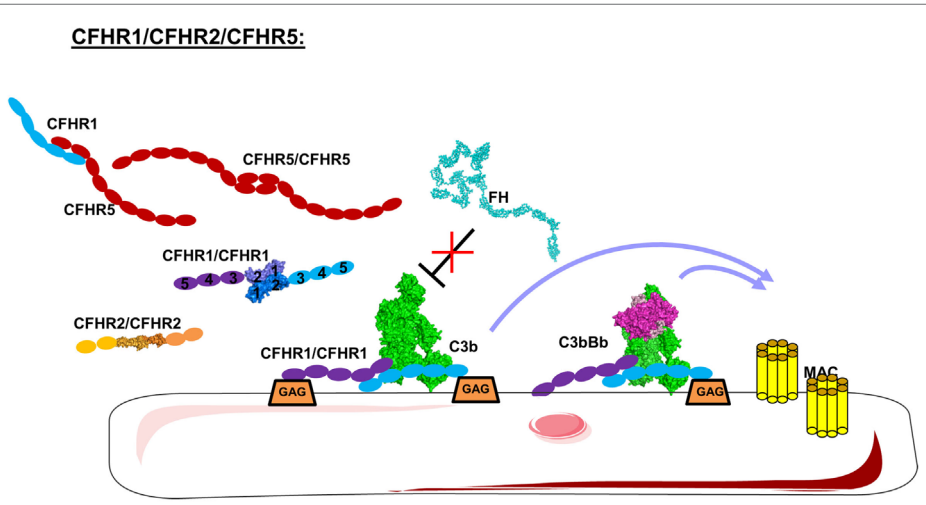

FIGURE 10 | Domain organization and mechanism of CFHRs. CFHR1, 2, and 5 carry dimerization N-terminal domains allowing them to form homo and heterodimers. These CFHRs, particularly CFHR1, are downregulators of FH, competing with FH for C3b and binding on the cell surface. This allow C3 convertases and MAC formation.

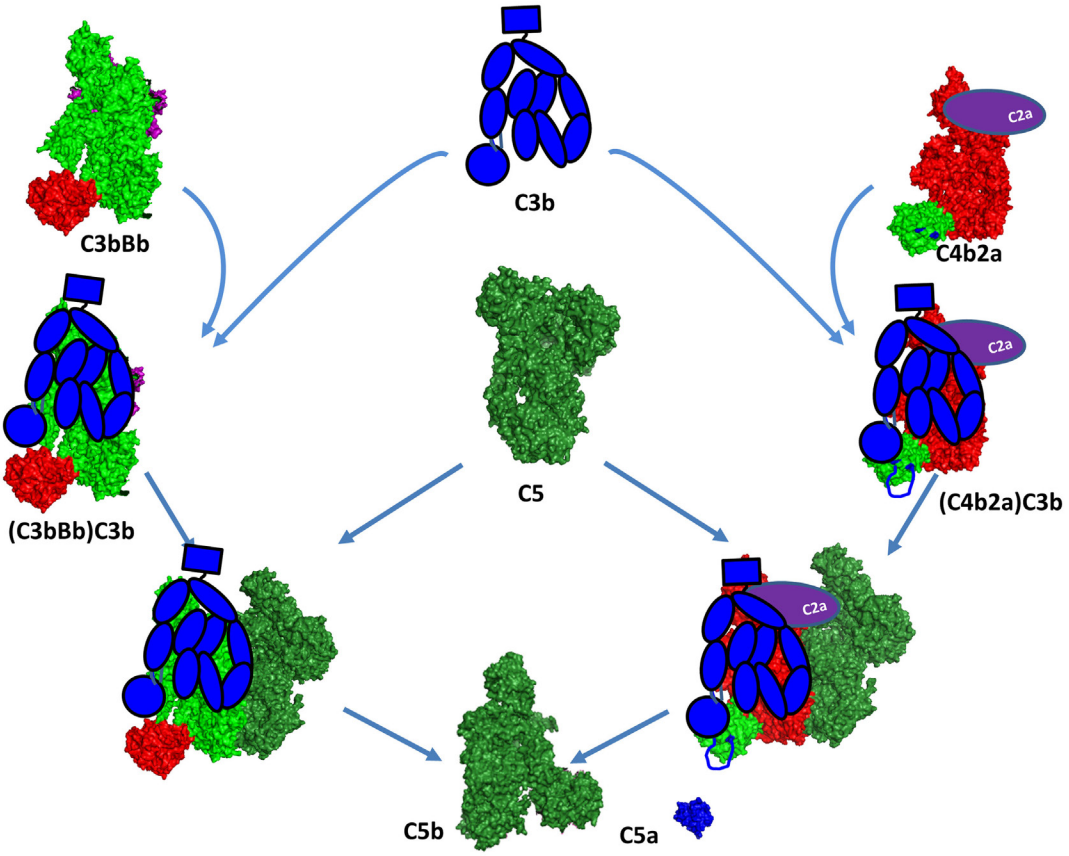

FIGURE 11 | C5 convertase formation. A C5 convertase is generated when a C3b molecule binds covalently in the vicinity or directly to a C4b or C3b, already engaged in a C3 convertase. This new enzyme loses its capacity to cleave C3 and starts to cleave C5. The binding site of the second C3 molecule is unclear, but it has been suggested to bind to the TED domain of C4d and to the CUB domain of C3b. Since the atomic coordinates of the two C5 convertases have not been published yet, this figure represents the current model for their organization. The CP C3 convertase is modeled here on the basis of the structures of C4b, C2a, and the AP C3 convertase C3bBb. The second C3b molecule is depicted in a schematic representation in blue to be distinguished from the C3b molecule interacting with FB to form the C3bBb complex. The Bb and C2a fragments are depicted in magenta and violet, respectively. They are partially visible behind the C3b and C4b molecules.

of the AP C3bBbC3b is six to ninefold weaker compared to the classical, cleaving less than 0.3 C5 molecules per minute (214). To compensate for the weaker efficacy, the AP C5 convertase can be stabilized by properdin (215, 216), which increases its half-life from approximately 3–30 min. This is different than the classical C5 convertase, for which a natural stabilizer has not been described. Together with the fact that the AP amplification loop generates a large number of C3b molecules makes the AP C5 convertase the main source of the terminal pathway complex C5b-9. In 2002, Pangburn and Rawal proposed a ring model for the amplification of the complement activation on the cell surface (213). In this model, any deposited C3b molecule coming from the classical or AP initiation can form an AP C3 convertase that will cleave one or several molecules of C3 (depending on the stabilization by properdin). A ring of new C3 convertases is formed or C3b binds on top of the C3 convertase itself to form a C5 convertase. As the activation spreads, the older enzymes will be inactivated by FH and FI leaving an expanding inner core of inactivated C3b surrounded by an active band of C5 convertases. The outermost band of newly formed C3 convertases is responsible for the growth of the site as it expands across the surface of the target.

Complement-Independent Cleavage of C5 and C3

Accumulating evidence suggests the existence of additional complement activation pathways in the plasma (217) aside from the three established pathways. Thrombin, human coagulation factors IXa, XIa, Xa, and plasmin were all found to effectively cleave C3 and C5 (218). C5a and C5b can be generated from C5 via thrombin, independently of the plasma complement system (217, 219). Thrombin cleaves C5 poorly at R751, which is the site of action of the C5 convertase (220). However, it does cleave C5 at an alternate site, R947, generating intermediates C5T and C5bT. These activation fragments will be generated at sites of activation of the coagulation cascade. Interestingly, C5bT formed a C5bT-9 MAC with significantly more lytic activity than with C5b-9.

Complement-independent cleavage of C3 by plasmin has also been suggested in the literature (221–223). The fragments generated by plasmin-mediated cleavage (C3a-like, C3b-like, iC3b-like,

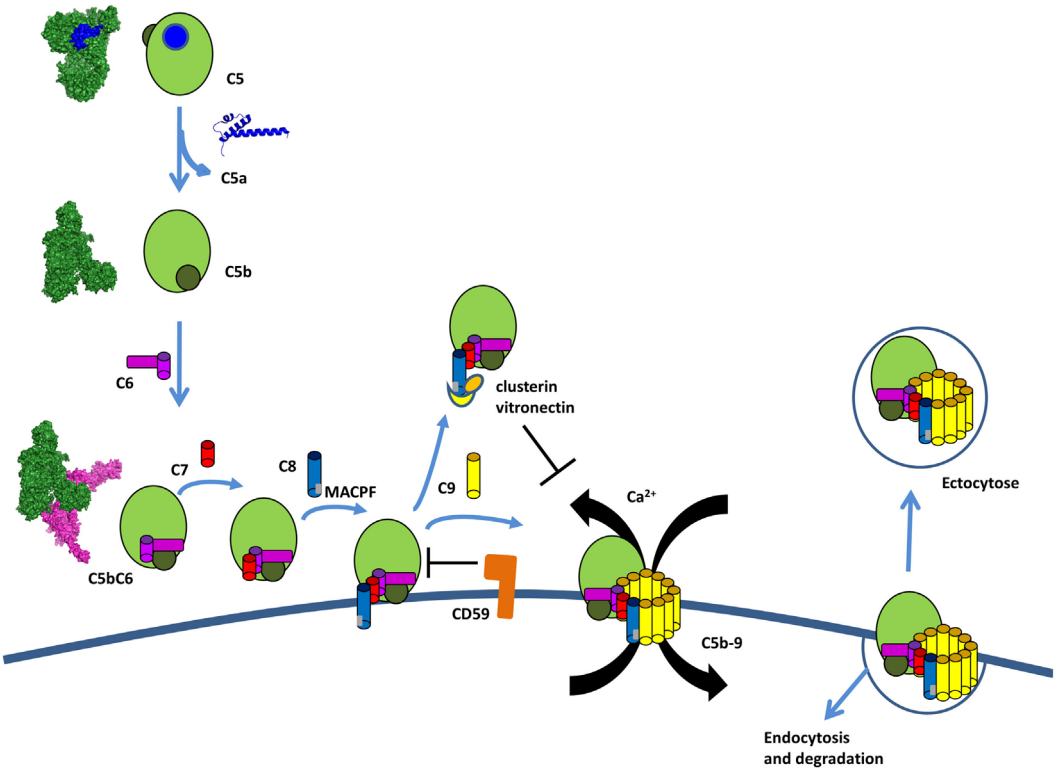

FIGURE 12 | The terminal complement pathway. The C5 convertase cleaves an inert molecule of C5 into a potent anaphylatoxin, C5a, and a bioactive fragment C5b. C5b interacts with C6, C7, C8, and multiple copies of C9 to form the membrane attack complex C5b-9 (MAC). C5b-8 inserts into the membrane and C9 polymerize to form a pore inducing Ca flux and pathogen lysis. Host cells are protected from lysis by expression of CD59, which prevents the insertion and by clusterin and vitronectin, which bind to C8 and prevents insertion in the membrane. If MAC is bound to the membrane, host cells can internalize it or remove it by ectocytose.

C3c-like, and C3dg-like) are similar, but not identical to fragments generated by the complement cascade and are biologically active.

Membrane-Attack-Complex C5b-9 Formation

Activation of the terminal complement pathway results in formation of MAC that form large, 10 nm wide, pores in the target membrane (207). These complexes are formed when C5 is cleaved into C5b by the C5 convertase. Upon cleavage, C5b undergoes a dramatic conformational change, similar to C3b, but with a TED domain ending up only halfway the distance to the MG ring (**Figure 12**) (224). This conformation of C5b interacts with C6, which wraps around the TED domain of C5b. C6 to C9 are homologous proteins that share similar central membrane-insertion domains called MACPF. Binding of C7, C8, and multiple copies of C9 results in MAC formation with C5b–C6–C7–C8β–C8αγ–C9 making an arc with a protrusion at the beginning by C5b. C5b-7 is lipophilic and binds to the cell membrane (225). C8 is the first component to penetrate the lipid bilayer upon interaction with the forming MAC. The structure of the MACPF domain of C8α (226) shows homology to perforin, a pore-forming protein released by cytotoxic T and NK cells, as well as, to bacterial cholesterol-dependent cytolysins. This resemblance suggests a common membrane perforation mechanism for MAC, perforin in the mammalian immune system, and bacterial pore-forming proteins (226). A single MAC contains up to 18 C9 molecules forming a tubular channel. However, only one or two C9 molecules are sufficient to form functional pores (227, 228).

Each functional MAC is sufficient to lyse by colloid osmosis in the membrane of metabolically inert cells, like erythrocytes or liposomes (229). Gram-negative bacteria are also susceptible to complement killing, in particular the meningitis causing *Neisseria* species (230, 231). Individuals deficient in terminal complement components are at increased risk for recurrent meningitis. Gram-positive bacteria have an extremely thick cell wall that MAC cannot penetrate leaving them resistant to complement elimination. Metabolically active nucleated cells are also resistant to lysis by complement (228, 232). In order to induce killing in these cells, multiple MACs must be inserted in the cell membrane together with coordination of calcium flux and not well-understood signal transduction (233). Once MACs have inserted in these cells, calcium flux is induced in the pore from the extracellular space or is released from intracellular stores (234). Subsequently, multiple still not well-known signaling pathways are activated leading to cell proliferation or apoptosis, which is dependent on the targeted cell type and experimental conditions.

Membrane-Attack-Complex Regulation

Membrane-attack-complex formation is tightly regulated to avoid accidental host cell damage and activation (**Figure 12**). C8 was suggested to play a dual role in MAC formation and regulation. In the absence of a cell membrane, the binding of C8 to C5b-7 induces conformation changes that result in a loss of ability to form pores, causing it to act as a MAC inhibitor (235). If soluble dead-end products sC5b-7, sC5b-8, and sC5b-9 are generated in fluid phase and do not bind to a membrane, they are scavenged by clusterin and vitronectin. These two regulators bind below the C5b-9 arc rendering it water soluble and preventing membrane binding (224, 236–238).

The GPI anchored protein, CD59 is expressed on most cell types (239, 240) and blocks membrane perforation by C5b-8 and C5b-9 (241, 242). CD59 does not bind free C8 and C9, but does interact with the MACPF domain of each protein upon conformational changes associated with C5b-9 complex formation (243–245). Furthermore, the lytic terminal complex of complement C5b-9 can be removed within minutes of its deposition on the membrane of target cells either by shedding via membrane vesicles (exocytosis) or by internalization and degradation (246–249).

Complement Anaphylatoxins

The anaphylatoxins, C3a and C5a, are constantly released during complement activation. These small (10–14 kDa) peptides play a critical role in supporting inflammation and activation of cells that express anaphylatoxin receptors (250). To enhance inflammation, anaphylatoxins recruit immune cells to the site of complement activation and induce oxidative bursts on macrophages (251, 252), eosinophils (253), and neutrophils (254, 255). However, some studies challenged the concept for the pro-inflammatory role of C3a. C3a has a more complex function, depending on the context, with a balance between pro- and anti-inflammatory roles. The highlight anti-inflammatory properties of C3a re-evaluate its physiological role during inflammation (256). Furthermore, C3a and C5a induce histamine production by basophils (257, 258) and mast cells (259) to provoke vasodilatation. C5a also recruits T-cells (260) and myeloid-derived suppressor cells (261) that constitutively express C5aR. Although the functional activity of C4a is debated, it has been reported to activate macrophage and monocytes (262, 263). However, a lack of cognate C4a receptor identification and unreproducible data (264) warrant further studies to determine the physiological role of C4a.

Structural data show that both human C3a and C5a adopt an alpha-helical conformation with four- and three-helical bundles, respectively (**Figure 13**). The C5a crystal structure has a core domain constituted as an antiparallel alpha-helical bundle and the C-terminal domain links the core domain by a short loop containing two adjacent arginines in position 62 and 74 (265) that both interact at the same binding site on the receptor. In human plasma, these two fragments are rapidly converted by carboxypeptidase N to C3a desArg and C5a desArg by cleavage at the C-terminal arginine (266, 267). C3a desArg has a very similar structure to C3a (268), but is incapable of binding to C3aR. The alpha1-helix of C5a desArg is detached at the three others alpha-helices (269). Due to this conformational change, human C5a desArg has 90% weaker pro-inflammatory activity compared to C5a (270). In contrast, murine C5a desArg is as potent as the murine C5a upon binding to C5aR on murine cells (271), which could be explained by the lack of major structural changes in the murine C5a desArg, compared to C5a. Both murine proteins form a four-helix bundle, contrary to the human C5a desArg, which adopts a three-helix bundle conformation upon cleavage of the terminal Arg residue. These inter-species differences need to be taken into account during analysis of *in vivo* experiments.

C3aR, C5aR, and C5aL2 belong to the G protein-coupled receptor (GPCR) family that contain seven transmembrane domains that are able to interact with C3a and C5a (**Figure 13**). The C3a-binding site of C3aR is located in the large second extracellular loop that contains a sulfotyrosine 174, which is critical for C3a docking (272). This interaction induces phosphorylation of intracellular pathways including PI3K, Akt, and MAPK leading to chemokine synthesis (273).

There are two sites in C5aR that are essential for C5a binding. The first sight consists of basic residues from human C5a that interact with sequences rich in aspartic residues located on N-terminal extracellular domain of C5aR (274). The second site is in a binding pocket located near the fifth transmembrane domain (275) and interacts with the C-terminal region of human C5a (276). Then, two distinct clusters of hydrophobic residues allow a molecular switch in C5aR leading to G protein activation (277). This mechanism exposes preserved residues clustered in two intracellular and two transmembrane domains that participate to the initial interaction with G proteins (278). The second extracellular loop plays a role of a negative regulator of C5aR activation and may stabilize the inactivated form of the receptor (279). By binding human C5a, C5aR induces downstream effects including activation of PI3K-γ (280, 281), phospholipase Cβ2 (282), phospholipase D (283), and Raf-1/B-Raf-mediated activation of MEK-1 (284).

Human C5a and C5a desArg are also able to bind C5L2 (285). C5L2 is again composed of seven transmembrane domains, however it is not coupled with G protein due to an amino acid alteration at the end of the third transmembrane in the DRY sequence (286). C5a has lower affinity to C5L2 compared to C5a desArg (287) and recently it has been demonstrated that C5L2 is a negative regulator of anaphylatoxin activity (288). It has also been reported that C5L2 and C5aR form a heterodimer (289) and this complex induces internalization of C5aR upon C5a binding (290). This internalization is essential for the induction of the late stage of ERK signaling (291, 292).

Complement Receptors for C3 Activation Fragments

Complement participates actively in the opsonization of pathogens and dying host cells, in addition to the clearance of immune complexes. Recognition molecules in the CP and LP, as well as cleavage fragments of C3, opsonize the target structure and serve as bridging molecules with receptors on the surface of the phagocytes. Depending on the type of the opsonin present (C3b, iC3b, or C3d), the phagocyte will generate a pro-inflammatory response or tolerogenic suppression.

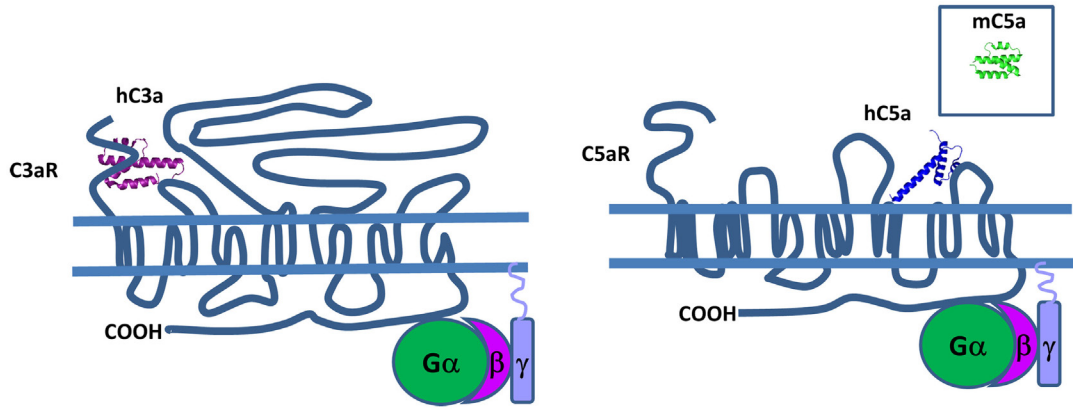

FIGURE 13 | Complement anaphylatoxins. C3a and C5a have a four and three helical bundle topology. Mouse C5a (in the square) is different from its human counterpart, because it has four helical bundle structure. These anaphylatoxins bind to G protein-coupled receptors C3aR and C5aR and stimulate pro-inflammatory signaling pathways.

Pathogens, immune complexes, and cell debris opsonized by C3 cleavage fragments can be recognized by CR (**Figure 14**) with three different structural organizations: containing CCP modules (CR1 and CR2), integrin family members (CR3 and CR4), and the immunoglobulin superfamily member (CRIg) (293). CR1 is expressed on monocytes, macrophages, neutrophils, erythrocytes, and renal podocytes, CR2 is found on B-cells, CR3 and CR4 are expressed by macrophages, monocytes, dendritic cells, neutrophils, and NK cells and CRIg has restricted expression and is found mainly on Kupffer cells in the liver and resident tissue macrophages (294). Interestingly, the expression of CRIg on macrophages in inflamed tissue is lower compared to macrophages outside of an inflammatory area (295).

Complement Receptor 2

Complement receptor2 (CD21) is expressed on B-cells interacting with C3d and iC3b on the surface antigens (**Figure 14**) forming a co-receptor complex with CD19 and CD81 (296). C3d serves as a molecular adjuvant by lowering the threshold for B-cell activation by 1000–10,000-fold (297). The TED domain of C3 has a completely different conformational environment in the native protein as compared to its degradation products C3b, iC3b, C3dg, and C3d. Recent studies showed that the binding site for CR2 in iC3b and C3d lies within the common TED domain (298). The C3d-binding site is located in the two N-terminal CCP domains of CR2 (299). Two different crystal structures had been proposed for the complex CR2:C3d; the first one, described in 2001 by Szakonyi et al. (300), showed that only the CCP2 domain of CR2 interacts with C3d. Contrary to this result, biochemical studies showed that mutations on several basic residues on CCP1 domain affected C3d binding to CR2 (301). In 2010, a second structure was proposed (302) in agreement with the mutagenesis data (301) where both CCP1 and CCP2 are involved in the interaction (**Figure 14**). One possible explanation for the discrepancy between structures could be due to the high concentration of zinc in the crystallization buffer from 2001 leading to a non-physiological complex.

Integrin Family Complement Receptors CR3 and CR4

Integrin family CR3 (also known as CD11b/CD18, $\alpha_M\beta_2$ or Mac-1) and CR4 (also known as CD11c/CD18 or $\alpha_X\beta_2$) are heterodimeric transmembrane complexes, composed of a unique α-chain and a common β-chain. They bind multiple ligands participating in phagocytosis, cell adhesion to the extracellular matrix, leukocyte trafficking, synapse formation, and co-stimulation. Ligand binding and signaling through integrin receptors is governed by a complex cascade of conformational changes, known as inside-out signaling (303). A receptor in its inactive, bend-closed conformation can respond to a cytoplasmic signal, transmitted inside-out through the β-chain, passing to a low affinity binding extended-closed and high affinity binding extended-open conformation. Upon ligand binding, another signal is transmitted outside-in, leading to raid cellular response, including actin remodeling, phagocytosis, degranulation, or slow responses involving protein neosynthesis. CR3 and to lesser extent CR4 are essential for phagocytosis of C3 fragments, opsonized immune complexes, and pathogens (304). CR3 and CR4 differ in their profile of recognized C3 fragments because both receptors bind to iC3b, but CR3 recognizes C3d, while CR4 binds to C3c, suggesting that the two receptors have distinct binding sites on the iC3b molecule (**Figure 14**) (305, 306). The iC3b and C3d binding site of CR3 is located in the VWF-A domain of the α-chain, also called αI domain, and binds in a divalent metal ion-dependent manner (307). This type of domain is present also in FB, which requires divalent ions (Mg^{2+}) in order to interact with C3b. However, the two VWF-A containing molecules do not bind to the same fragments of C3b (113, 305). FB interacts only with C3b, while CR3 binds to iC3b and C3d and the binding sites for these VWF-A domains are distinct (**Figure 14**). VWF-A domain of CR3 binds to the TED domain (C3d part) of iC3b and the CUB and C345C domains of iC3b may also contribute to the interaction with the β-propeller and βI domains of the α-chain of CR3 (305).

In contrast to CR3, CR4 binds to the C3c portion of iC3b (**Figure 14**). The architecture of the complex has only been observed at low resolution, by electron microscopy and displays

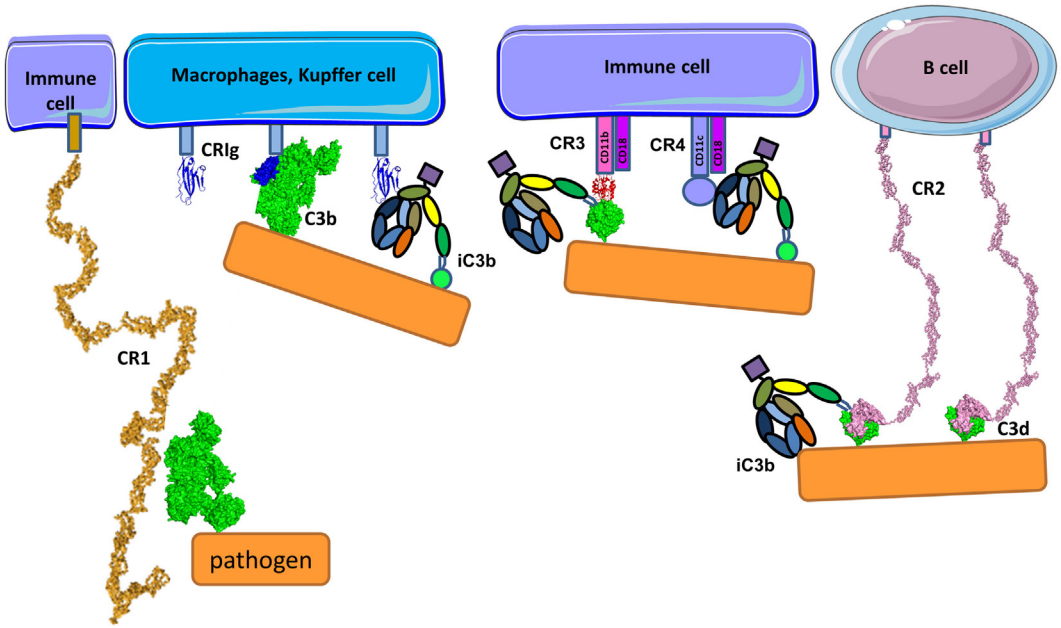

FIGURE 14 | Complement receptors. CR1 is composed of CCP domains and is expressed primarily by immune cells and erythrocytes. Apart from being cofactor of FI, CR1 is also a complement receptor facilitating immune complex clearance and phagocytosis. CR1 interacts with C3b. CRIg has immunoglobulin-like structure in its C3b recognition domain. CRIg binds to C3b and iC3b and is expressed on macrophages and Kupffer cells. Immune cells also express CR3 and CR4 containing integrin domains that bind to iC3b (and C3d for CR3) on different binding sites on iC3b molecule. CRIg, CR3, and CR4 facilitate phagocytosis and modulate the activation state of cells. CR2 is expressed primarily on B-cells and recognizes C3d using the first two CCP domains. It serves as a co-stimulatory molecule for the B-cell receptor upon binding C3d-opsonized pathogen.

a CR4 binding site at the interface between MG3 and MG4 (306). The flexibility of the CUB domain after cleavage by FI allows reorientation of the C3c part of iC3b leading to better exposure of the MG3–MG4 interface (9, 308). Initial studies of the structure and molecular details of the complex conformational changes in CR4 are starting to be elucidated (309) showing that CR4 binds iC3b through the αI domain on the face known to bear the metal ion-dependent adhesion sites similar to CR3 (306).

Immunoglobulin Superfamily Receptor CRIg

Immunoglobulin superfamily receptor CRIg is a CR expressed on macrophages and Kupffer cells in the liver that binds to C3b and iC3b (**Figure 14**) and mediates the phagocytosis of opsonized particles and pathogens (310, 311). CRIg acts as an inhibitor of the AP preventing the entry of the substrate molecule C3 and C5 into the C3 convertase (312). The CRIg-binding site on C3b is located in the MG ring of the β chain, engaging MG3 and MG6 domains. A conformational change in MG3 during the transition of C3 to C3b contributes to the formation of the CRIg-binding site and explains why CRIg binds to C3b and iC3b, but not to intact C3.

Understanding Complement-Related Diseases Using Structure–Function Relationships

The importance of the complement system in physiology is illustrated by the severe and life threatening diseases occurring due to inefficient or excessive complement activity. Abnormal complement activity is associated with many inflammatory, autoimmune, neurodegenerative, and age-related diseases. Here, we will describe the role of complement dysfunction in aHUS.

Atypical Hemolytic Uremic Syndrome

The aHUS is a rare thrombotic microangiopathy that predominates in the kidney. Without appropriate treatment, it leads to end stage renal disease in approximately 60% of patients (313, 314). This thrombotic microangiopathy is different than typical HUS and thrombotic thrombocytopenic purpura because it is not associated with infection by Shiga toxin-producing bacteria or ADAMST13 deficiency, respectively. aHUS occurs at any age and has a poor prognosis (prior to the development of Eculizumab). In contrast, typical HUS is predominantly a pediatric disease and has a favorable renal outcome. aHUS is characterized by a triad of hemolytic anemia due to fragmented erythrocytes, thrombocytopenia, and acute renal failure. Renal failure is a result of platelets rich microthrombi, formed in the small vessels (capillaries and arterioles) of the kidney resulting in a prothrombotic state. The hallmark of the aHUS is the association with alternative complement pathway mutations (**Figure 15A**). Endothelial damage is known to be related to complement dysregulation. Screening for and characterization of mutations in the components of the C3 convertase (C3 and FB), its regulators (FH, FI, MCP, CFHR1, and thrombomodulin), or anti-FH antibodies has become an indispensable part of the diagnostic of the disease (315).

The importance of screening for mutations in complement factors can be observed in the examples of FH and C3. The majority of the mutations in FH found in aHUS patients does not induce protein quantitative deficiency and are located in the C-terminus of the protein (CCP19–20). These mutations affect either the interaction with C3b, GAG, or both ligands leading to impaired cell surface protection against complement attack [(175, 177, 316); summarized in Ref. (191, 315, 317, 318)]. Mapping of these disease-associated mutations on the complex of C3d with FH19–20 (130, 131, 174) revealed that the residues which decreased C3b-binding mapped to the C3b/C3d-binding site of FH in CCP19. The residues that affected both C3b and GAG interaction were located in the FH CCP20 interface (**Figure 15B**). These structural analyses help to explain the mechanism by which genetic abnormalities in FH induce impairment of C3b and/or GAG binding and hence a predisposition to develop aHUS. A similar phenomenon is observed with C3 mutations found in aHUS. These genetic changes are not randomly distributed but cluster alongside FH CCP1–4 and CCP19–20 binding sites (**Figure 15C**) (142, 315, 319, 320). Functional analysis revealed that mutations located in the FH-binding sites resulted in decreased FH binding, thus showing the link with aHUS. Currently, there is a debate as to whether or not in physiological conditions FH CCP20 can interact with an adjacent C3d molecule (130, 131, 174). It is possible the observed interaction between the second molecule C3d and FH CCP20 may be a crystallization artifact and in turn the functional consequences of certain aHUS associated FH and C3 mutations will be more difficult to explain. However, these mutations fall within this suggested C3b/C3d binding site and indeed decrease FH/C3b interaction. This case exemplifies how structural analyses can aid in understanding disease physiopathology and how disease physiopathology improves our understanding of complement.

Disease-associated mutations also has resulted in the mapping of the MCP binding site on C3b. C3 mutations that decreased MCP binding mapped in an area overlapping with the FH CCP-3–4-binding site (142). However, these mutations did not affect CR1 binding suggesting that the CR1 binding site is distinct and that CR1 is not involved in aHUS (142). Experimental and structural analysis revealed that for FH and C3 almost all studied genetic changes mapped to the ligand binding site and had clear functional consequences. aHUS mutations in FB seem to be exception of this rule. Mutations in FB were located in multiple

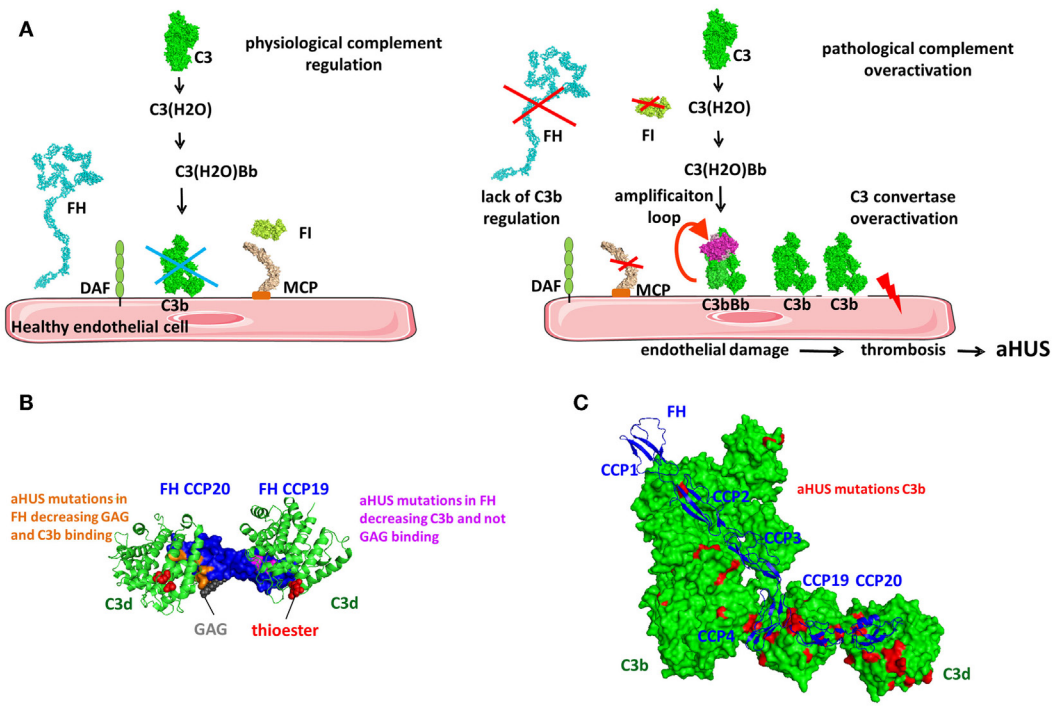

FIGURE 15 | Understanding aHUS using structure–function relationships. **(A)** The role of complement alternative pathway in the physiopathology of aHUS. On healthy endothelial cells, deposited C3b is rapidly inactivated by regulatory molecules including FH, MCP, and FI. For FB binding and C3 convertase formation, FB is dissociated by FH and DAF preventing excessive host tissue damage. Mutations in the complement regulators FH, MCP, and FI can result in inefficient complement regulation. Mutations in the components of the C3 convertase (C3 and FB) induce the formation of overactive C3 convertase or a convertase that is resistant to regulation. In both cases, the complement cascade is activated on glomerular endothelial cell surface leading to endothelial damage, thrombosis, erythrocyte lysis, and aHUS. **(B)** FH mutations in the CCP19–20 region are mapped on the structure of the C3d–C3d–FH19–20 complex. A model GAG bound to FH CCP20 is indicated in gray. FH disease-associated mutations that decrease only C3b-binding are indicated in orange and mutations decreasing both C3b and GAG binding are in magenta. Reduced C3b and/or GAG binding will cause inefficient endothelial cell protection and complement overactivation. **(C)** C3 mutations found in aHUS patients. The majority of the mutations (in red) are not randomly distributed, but mapped to the FH binding sites on C3b. These mutations correlated with decreased FH and MCP binding allowing characterization of the MCP binding site, which overlaps with the FH binding site in CCP-3–4.

domains of the protein and in more than half of the cases they were far from any known binding sites (321). Mutations located within the C3b-binding site did induced formation of an overactive C3 convertase or a convertase resistant to regulation (321–323). The mutations far from this binding interface showed no functional defect as observed by FB functional tests (321). As shown for the majority of complement mutations in aHUS, mapping of disease-associated mutations together with detailed functional analysis should be performed to understand the mechanism of complement dysregulation associated with a disease.

Structural Basis of Therapeutic Complement Intervention

We have described in depth the known molecular mechanisms of the complement system and this unleashes many possibilities for rational design of complement inhibitors for treatment of disease (324, 325). Here, we will give a few examples that illustrate this concept. Blockade of the late effector functions of complement can be obtained if the cleavage of C5 by the two C5 convertases is prevented. The therapeutic monoclonal antibody, Eculizumab, targets human C5 and blocks cleavage by C5 convertases (326). The Eculizumab binding epitope on C5 interacts with the contact interface between C5 and the C5 convertase (211) preventing the entry of C5 into the C5 convertase and blocking further cleavage and generation of the bioactive fragments C5a and C5b-9. Eculizumab blocks the terminal complement pathway but leaves the C3 convertases unaffected. Eculizumab showed significant improvement in clinical outcome and has been accepted for treatment of complement-mediated diseases including paroxysmal nocturnal hemoglobinuria (PNH) (327) and aHUS (328, 329) and clinical trials are ongoing for other diseases.

In order to control complement at an earlier step, inhibitors acting at the level of C3 have also been designed. Compstatin is a 13-residue peptide that is being tested pre-clinically. It binds to C3 and blocks its cleavage (330, 331). Compstatin binds to MG4 and MG5 of C3c and C3b where it undergoes a large conformational change upon interaction (332) causing steric hindrance of the substrate C3 to the convertase complexes and blocking complement activation and amplification. Compstatin has shown efficacy in complement blocking *in vitro* and in animal models including extracorporeal circulation (333), sepsis (334), and PNH (335). Both Compstatin and Eculizumab are species-specific and act only in humans and monkeys, but not in mice or rats, reflecting subtle differences in the structure of human and murine complement components.

Another strategy of rational design of complement inhibitors is to target the regulatory domains of FH or CR1 to the cell surface via potent C3b, iC3b, C3d, or membrane recognition domains, derived from FH or CR2. Mini FH molecules, containing CCP1–4 and CCP19–20 bind C3b and C3d with high affinity and show better efficacy compared to native FH in *in vitro* models of aHUS (336) and PNH (337). A hybrid molecule TT30, containing FH CCP1–5 and CR2 CCP1–4 is designed to accumulate preferentially at sites already under complement-mediated attack (338). TT30 interacts simultaneously with C3b and C3d merging the functionality of fluid phase FH binding to C3b with CR2 interaction to C3d on the surface of host cells (339). TT30 and its murine analog showed significant improvement in models of AMD, ischemia/reperfusion injury, and PNH (340–342).

Conclusion

Currently, we know that complement is not only a simple lytic system, but rather a powerful innate immune surveillance tool, serving as a sentinel against pathogens, modulator of the adaptive immune response, and as a regulator of host homeostasis. This cascade of enzymatic reactions is driven by conformational changes induced after a recognition event assuring that complement will be activated only when and where needed. This special and temporal control of complement activation is guaranteed also by the high specificity and selectivity of the enzymatic reactions, where the involved enzymes cleave only a single substrate and have a single ligand-binding site. In contrast to this high specificity of the propagation of the chain reaction, complement activation relies on target patterns binding by versatile recognition molecules, such as C1q, MBL, ficolins, and properdin. The activation of the three complement pathways leads to the generation of C3b, the Swiss army knife of complement, which interacts with a large variety of ligands and receptors with multiple distinct binding sites. The balance of these interactions determines whether full-blown activation will occur by the amplification loop of the complement pathways, with a generation of one of the most potent inflammatory mediators C5a or the effect will be attenuated by the C3b breakdown cycle. Again, the attenuation relies in large part on the capacity of a versatile recognition molecule FH to discriminate between self and non-self and to stop the amplification loop. C3b and C3(H_2O) may bind to the cell surface via proteins-platforms, like properdin and P-selectin. Expression/binding of these molecules accelerates the complement activation and anaphylatoxin generation. The anaphylatoxins increase the expression/binding of P-selectin and properdin contributing to a vicious circle of complement activation, enhancing local inflammation, thrombosis, and tissue damage.

The knowledge of these molecular mechanisms that has been accumulated during recent years has allowed for better understanding of complement-related diseases. It also opens up the possibility for a rational design of novel molecules with therapeutic potential to control steps in the complement cascade. Clinical application of the anti-C5 blocking antibody Eculizumab has already demonstrated that controlling complement can revolutionize the treatment of patients with overactive complement-mediated diseases.

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Complement system part II: role in immunity

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The complement system has been considered for a long time as a simple lytic cascade, aimed to kill bacteria infecting the host organism. Nowadays, this vision has changed and it is well accepted that complement is a complex innate immune surveillance system, playing a key role in host homeostasis, inflammation, and in the defense against pathogens. This review discusses recent advances in the understanding of the role of complement in physiology and pathology. It starts with a description of complement contribution to the normal physiology (homeostasis) of a healthy organism, including the silent clearance of apoptotic cells and maintenance of cell survival. In pathology, complement can be a friend or a foe. It acts as a friend in the defense against pathogens, by inducing opsonization and a direct killing by C5b–9 membrane attack complex and by triggering inflammatory responses with the anaphylatoxins C3a and C5a. Opsonization plays also a major role in the mounting of an adaptive immune response, involving antigen presenting cells, T-, and B-lymphocytes. Nevertheless, it can be also an enemy, when pathogens hijack complement regulators to protect themselves from the immune system. Inadequate complement activation becomes a disease cause, as in atypical hemolytic uremic syndrome, C3 glomerulopathies, and systemic lupus erythematosus. Age-related macular degeneration and cancer will be described as examples showing that complement contributes to a large variety of conditions, far exceeding the classical examples of diseases associated with complement deficiencies. Finally, we discuss complement as a therapeutic target.

Keywords: complement system, adaptive immunity, crosstalk TLR-complement, pathogen strategies for immune evasion, complement in cancer, complement and innate immunity, complement-related diseases, anaphylatoxins

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The Complement System

Complement system represents a major part of the innate immunity. It is a cascade of soluble proteins and membrane expressed receptors and regulators (**Figure 1**), which operates in plasma, in tissues, on cell surface, and even within the cell. It is composed of more than 40 proteins, the soluble ones being produced mainly by the liver. Complement was discovered at the end of the nineteenth century and described as a "factor" or "principle" capable to induce bacterial lysis.

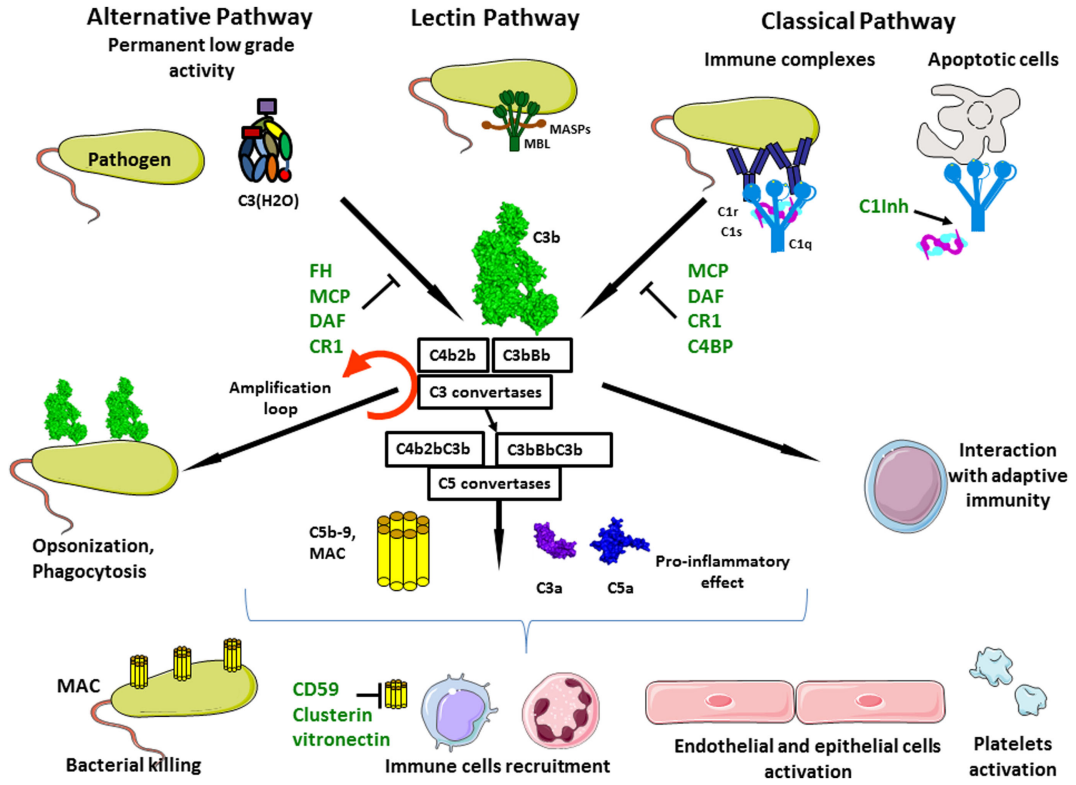

FIGURE 1 | Complement activation. Complement system is composed of three different pathways. CP is activated by immune complex formation on pathogen surface and by calreticulin expressed on apoptotic cells, leading to C1 complex association. LP recognizes mannose-terminating glycan on pathogens leading to MBL MASP complex activation. Both induce formation of the classical C3 convertase C4b2a. AP is permanently activated at low level by spontaneous hydrolysis of C3 into C3(H_2O). Lack of complement inhibitor on pathogens induces alternative C3 convertase activation C3bBb. Complement activation leads to opsonization and phagocytosis by C3b deposition, bacterial lysis by C5b–9 complex formation and inflammation by recruitment of immune cells, endothelial and epithelial cells activation, and platelets activation.

After that, for a long time, complement system has been considered as a supportive part of the innate immunity and received relatively little attention from the immunologists. Over the years, it became clear that complement has versatile functions and that its action extends far beyond the simple bactericidal activity. In a healthy individual, it orchestrates the immunologically silent clearance of host cells after their programed cell death. Complement cascade is activated immediately after encountering the pathogen. Hence, complement participates in pathogen opsonization, tagging it for engulfment by antigen presenting cells (APC); it plays a central role in the inflammatory process and modulates the activity of T- and B-cells. After generation of pathogen-specific antibodies, complement contributes in the clearance of immune complexes and pathogen elimination. Studies over the years demonstrated that complement takes part in nearly every step of the immune reaction and that it deserves a central position in the immunological research. Unfortunately, the lack of coherence in complement proteins nomenclature and the complexity of the enzymatic cascade render complement one of the "most complicated and incomprehensible" parts of immunology and is frequently avoided by students and scientists. With this review, we aim to underline the crucial importance of complement in physiology and pathology.

Complement in Physiology

Sampling for Foreign and Altered Cells

The main complement rule is that everything that is not specifically protected has to be attacked. Host cells carry an armamentarium of "don't attack me" molecules, which are either expressed by the cell or recruited to the cell membrane from the plasma. Therefore, any cell, debris, microorganism, or artificial material lacking these molecules (and carrying $-OH$ or $-NH_2$ chemical groups, which is the case of all biological and most synthetic materials) will represent an "activating surface" and will support complement deposition, i.e., covalent binding of C3b, an activated form of the central complement component C3. This action is provided by the so-called alternative complement pathway, which is permanently active and constantly probes the environment for the presence of activating surfaces (**Figure 2A**). In addition, several pattern recognition molecules recognize material that has to be eliminated, such as apoptotic cells, debris, pathogens, immune complexes, and activate the classical and lectin complement pathways (CP and LP) (**Figure 2B**). All pathways converge at the point of C3 cleavage, which results in generation of bioactive fragments C3a and C3b. C3b binds covalently to any surface and, if it is not specifically protected. The level of C3 deposition is increased by the so-called

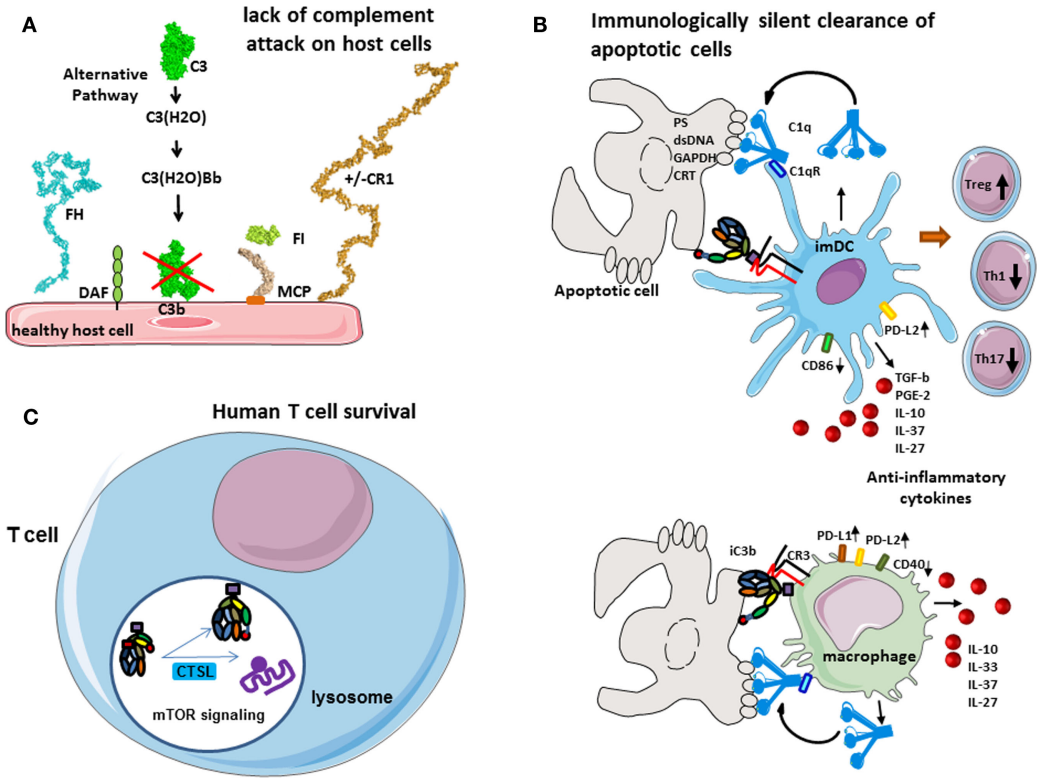

FIGURE 2 | Role of complement in physiology. **(A)** Protection of host cells against complement. Complement AP is permanently activated and deposits C3b molecules to any surface. On host cells, these C3b molecules are rapidly inactivated by different membrane expressed or plasma complement regulators. **(B)** Immunologically silent clearance of apoptotic cells. Limited complement activation occurs on apoptotic cells. C1q recognizes "eat-me" signals on the surface of dying cells. It serves as a bridging molecule, facilitating the phagocytosis by dendritic cells or macrophages. Uptake of C1q-opsinized cargo induces an anti-inflammatory program, increased expression of immunological checkpoint molecules, and prevents up-regulation of maturation markers. iC3b on apoptotic cells interacts with CR3 on phagocytes and induces anti-inflammatory response. **(C)** Complement in human T cell homeostasis. Cathepsin L cleaves C3 intracellularly, generating C3a and C3b. C3a binds on C3aR, expressed in the lysosomes, and stimulates mTOR signaling pathway. This signaling is important for the cell survival in resting state.

"amplification loop" of the alternative pathway (AP). Detailed description of the mechanisms of activation and regulation of the complement system are described in "Complement system part I – molecular mechanisms of activation and regulation" (1), from the same research topic of Frontiers in Immunology.

Briefly, in a healthy organism, the sentinel role is assured by the permanent activity of the AP (1 and 2). A number of complement components are produced in biologically inactive form, which allows them to co-exist in plasma, or to be produced by the same cells, without interacting with one another and inducing unnecessary and undesired complement activation. Complement components are activated in a cascade fashion after a triggering event, each step of the chain reaction, resulting in a conformational change or a cleavage of the downstream component, which becomes activated and gains the capacity to activate the subsequent component in the cascade. Spontaneous hydrolysis, called tick-over, of C3 plays an important role in the immune surveillance and AP activation (2). C3 is present in plasma at high concentrations (~1 mg/ml) and a small portion of it undergoes spontaneous hydrolysis of a particular thioester bond between the side chains of two aminoacids, located in the thioester domain (TED). This hydrolysis induces a dramatic conformational change in C3 and renders it biologically active due to exposure of novel binding sites. In this new form – the C3(H_2O), it recruits two other plasma molecules – factor B (FB) and factor D (FD). FD cleaves C3(H_2O)-bound FB to generate an enzymatic complex C3(H_2O)Bb, called fluid phase C3 convertase. The name convertase indicates that this enzymatic complex can cleave (convert) a native C3 molecule into bioactive fragments C3a (the small fragment) and C3b (the big fragment). C3a is an anaphylatoxin – a pro-inflammatory molecule, which activates the surrounding cells when reaching a threshold concentration. Upon releasing the C3 ANA domain, which becomes the C3a molecule, the remaining part C3b undergoes a dramatic conformational change, similar to that of C3(H_2O). In the newly generated C3b, contrary to C3(H_2O), the thioester bond is not hydrolyzed but becomes transiently exposed, allowing, for a very short time, a covalent reaction with OH^- or $NH2^-$ groups on any molecule or cell in its immediate surroundings. If this bond is not formed, the very short-lived nascent form is hydrolyzed and inactivated in few milliseconds, leaving the inactivated C3b molecule in the fluid phase. When covalently bound to a cell, C3b has different fates depending if it is on a host cell or on a foreign surface.

Protection of the Host Cells Against Complement Attack

The deposition of C3b is highly controlled on host cells. Host cells express complement regulatory molecules on their surface (membrane bound such as membrane cofactor protein, MCP, CD46 or complement receptor 1, CR1, CD35), or recruit plasma regulators, like factor H (FH), which bind to C3b. These proteins serve as cofactors, allowing interaction with a plasma serine protease factor I (FI), which cleaves C3b into iC3b (**Figure 2A**). This results in a conformational change, which suppresses the ability to interact with FB but exposes of novel binding sites in the iC3b molecule, allowing it to interact with other complement molecules. FH also blocks the binding of FB and FD to C3b, thus preventing the formation of C3 convertases. The overall action of all these regulators results in the prevention of complement activation on host cells. In the absence of surface regulators, such as in the case of pathogens or other foreign surface, C3b interacts with FB and FD, forms C3 convertases, which cleave more molecules of C3 to C3a and C3b, thus fueling the amplification loop and allowing full-blown complement activation and, finally, pathogen elimination.

Immunologically Silent Phagocytosis of Apoptotic Cells

Between these two extremes (healthy cell and pathogen) remains the case of stressed and apoptotic host cells. The human body is composed to a myriad of different cells, forming a highly organized system. The proliferation, differentiation, activity, and also the death of cells in this system are tightly controlled. Programed cell death induces major cellular modifications. During a classical cell death, the cell surface undergoes many structural and molecular modification, leading to "eat-me" signals expression. Phagocytes recognize these signals and execute the degradation of apoptotic cell without mounting of an immunologic response. Among these modifications, a major one is the expression of phosphatidylserine (PS) on the external side of the cell membrane, which is normally sequestered in the inner surface of the cell membrane (3). Also, the expression level of some complement regulators (such as MCP) can be reduced. Clearance of apoptotic cells is critical for many physiological processes, including development, tissue remodeling, and maintenance of homeostasis.

The complement system plays a major role in the tolerogenic perception of apoptosis, which is in part mediated by opsonization with C1q and iC3b and subsequent clearance of dying cells (4, 5) (**Figure 2B**). C1q, which is the recognition molecule of the CP, is produced by macrophages and dendritic cells (DCs) and binds to a variety of ligands that can be expressed on the surface of apoptotic cells such as PS, double stranded DNA, GAPDH, or calreticulin (6–9). C1q has complex immune-modulatory effects and a failure of C1q to opsonize apoptotic cells results in defective phagocytosis by monocytes (10) and activation of the DCs (11). Consistently, a quantitative or functional deficiency in C1q may be related to improper apoptotic cell clearance and autoimmunity (12, 13). Similar functions are described for mannose binding lectin (MBL), one of the recognition molecules of the LP (14).

C1q coated apoptotic cells suppress macrophage inflammation through induction of interleukine 10 (IL-10), IL-27, IL-33, and IL-37, inhibit inflammasome activation and increase the expression of negative regulators ASC2 and NLRP12 (15). It has recently been demonstrated that opsonization of apoptotic cells by C1q induced an increase of the expression of PD-L1 and PD-L2 and a diminution of CD40 at the surface of macrophages (16). Similar effects are observed also on dendritic cells. Presentation of self-antigens by DC in the presence of C1q promotes the development of regulatory T cells (Treg) and the production of anti-inflammatory cytokines such as TGF-b, IL-10, PGE2, IL-37, and IL-27 and thus confers tolerance. In addition, opsonization of the apoptotic cells with C1q induced a high expression of PD-L2 and less CD86 on dendritic cells surface after phagocytosis. This "polarization" by C1q-induced decrease of T helper 1 (Th1) and Th17 and proliferation. The non-maturation of the phagocytes, which is showed by the expression of CD40 and CD86 on macrophages and dendritic cells, respectively, and by the secretion of anti-inflammatory cytokines, makes the phagocytosis immunologically silent (16). Therefore, C1q is of critical importance for the silent, non-immunogenic clearance of apoptotic cells (17) (**Figure 2B**).

The inactivated fragment of the central complement component C3–iC3b participates in the clearance of apoptotic cells via interaction with CR3 on monocytes, macrophages, DC, and microglial cells (18–20). iC3b opsonization and CR3-dependent phagocytosis is accompanied by a down-regulation of the pro-inflammatory mediator IL-12 and a lack of oxidative burst in macrophages (21, 22) or by a reduction in the expression of costimulatory molecules and impaired maturation of DC (23, 24). This confers anti-inflammatory properties and supports tolerogenic apoptotic cell clearance.

Cell Homeostasis

In the native state, different cell types secrete complement components and generate C3a and C5a in their microenvironment, an important fact for their viability and function. Liszewski et al. demonstrated that C3 activation can occur continuously within human T cells, mediated by cathepsin L (CTSL) and in a C3 convertase-independent manner (25) (**Figure 2C**). In resting T cells, this C3a binds to intracellular C3aR, expressed in lysosomes, but not on the cell surface. Intracellular C3aR signaling sustains basal mTOR activation (25), required for homeostatic cell survival (26). Of note, C3a generated outside the cell cannot restore the mTOR signaling in cells with inhibited CTSL, suggesting the importance of intracellular generated C3a (25). Upon T cell receptor (TCR) activation, C3aR is expressed on the cell surface and contributes to the mounting of Th1 response in concert with CD46 signaling.

Of note, the phenomenon of intracellular C3 cleavage seems to be species specific, because it does not operate in mice. In mice, resting T cells synthesize complement components constitutively, complement activation occurs in their microenvironment, and the resultant C5a and C3a signaling, through the C5aR and C3aR, participates in cell viability by maintaining the level of phosphorylated AKT (PKB), a T cell activation intermediate that suppresses apoptosis (27). In the absence or after blockade of C5aR and C3aR, the expression of MHC class II and costimulatory-molecules is decreased on dendritic cells (27, 28). The exact contribution to

T cells survival of the intracellular and extracellular C3a generation and C3aR signaling is still not well defined. It is important to note that AKT (PKB) and mTOR belong to the same signaling cascade, which has anti-apoptotic, survival, and proliferation effects (29). The intracellular complement activation is not restricted to T cells (25), and may have an important role in the physiology of other human cell types.

Complement as a First Line of Defense Against Pathogens

Direct Killing

Pathogens are attacked by all complement pathways, but the prevalence of one or another pathway depends on the exact membrane composition. C3b generated by the spontaneous activation of the AP tag all pathogens and, when no regulator is present, the cascade and the deposition of C3b are accelerated (**Figure 3A**). In addition, the pathogen-associated molecular patterns can be recognized by the recognition molecules of the CP and LP C1q and MBL or ficolins. C1q recognizes mostly charged patterns and can bind to more than 100 different target molecules (30), including pathogen-associated molecular patterns such as lipopolysaccharide (LPS) (31) or bacterial porins (32). MBL binds to a wide range of repeating sugar arrays normally presented by many microorganisms, including mannose structures on fungal and micrococcal surfaces, and *N*-acetylglucosamine residues in cell walls of bacteria, in order to initiate neutralization of these organisms (33). C1q may bind also on natural antibodies, which due to their polyreactivity can recognize the pathogens (34). Upon target recognition, C1q undergoes conformational change and activates the two serine proteases C1r and C1s, associated with it in the context of the C1 complex (35, 36). Despite the similarity between C1 and MBL/MASP complex architectures, the mechanism of activation of the CP and LP differ (37). In particular, the serine proteases of the LP, MASP-1, and 2, are associated with different MBL (or ficolin) molecules, thus requiring a juxtaposition to allow MASP-1 from one complex to activate MASP-2 from the adjacent complex (38, 39). However, MASP-2 alone provides about 10% to cleave its natural substrate C4 by auto-activation (40). Activated serine proteases of the CP and LP cleave C4 and C2 to allow formation of the CP C3 convertase C4b2a, which cleaves C3. If this convertase is not regulated, C3 deposition will be accelerated and

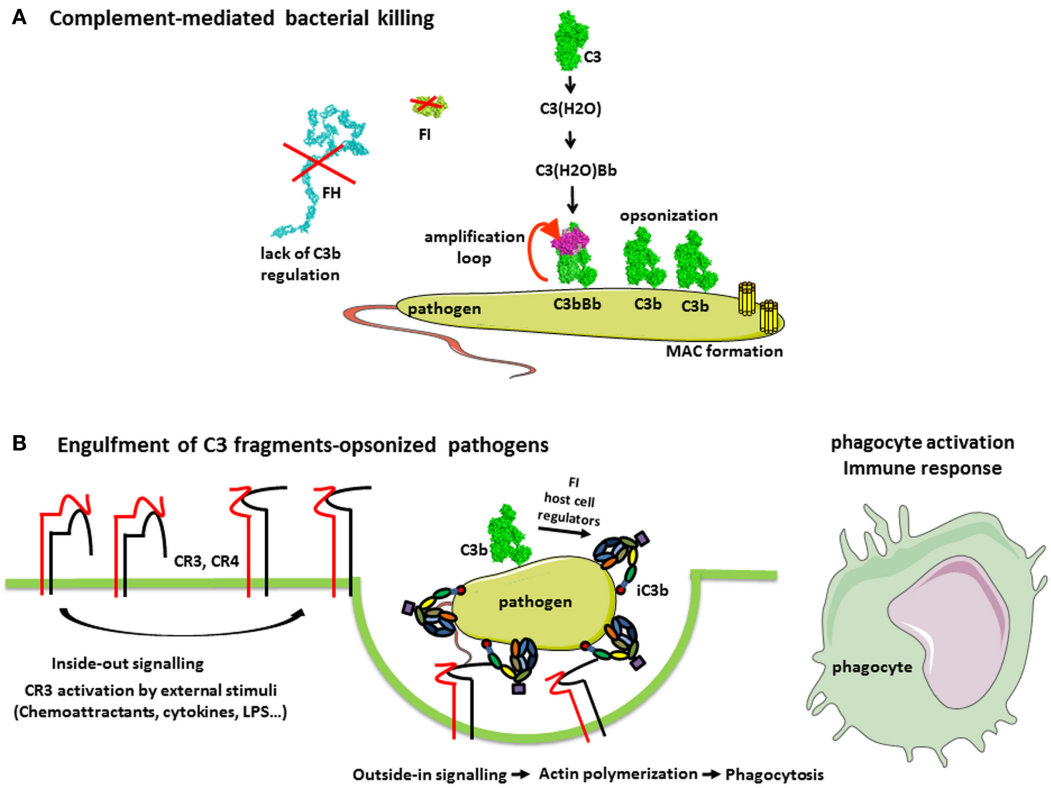

FIGURE 3 | Complement in the defense against pathogens.
(A) Complement-mediated bacterial killing. C3b is deposited on any pathogen surfaces due to the constant activity of the AP. Since most pathogens do not have complement regulatory molecules, C3b is not inactivated and interacts with FB and FD to form a C3 convertase C3bBb. This enzymatic complex cleaves more C3 molecules, resulting in pathogen opsonization with C3b. Further, the cascade proceeds to a C5 convertase and MAC formation, contributing to bacterial killing. **(B)** Complement receptors-mediated phagocytosis of C3b and iC3b-opsonized pathogens. Extracellular stimulatory signals, which are necessary for the CR3-mediated phagocytosis, include chemoattractants (not only chemokines but also bacterial formylpeptides and C5a for neutrophils), cytokines (e.g., TNF-α), and bacterial products (e.g., lipopolysaccharide). External stimuli activate the integrin CR3, (i.e., change to a conformation with high affinity for iC3b) by a Rap-1-mediated signaling. Stabilization of CR3 high-affinity conformation by its engagement with iC3b triggers a RhoA-mediated signaling, which drives the actin polymerization to engulf iC3b-coated target. Complex actin movements are then involved in the intracellular movement of the phagosome during its maturation to the phagolysosome.

the amplification loop will be turned on. Binding of an additional C3b molecule in the immediate proximity, or most probably on the C3 convertase itself, modifies the specificity of the enzyme (41). It then starts to cleave C5, thus turning on a C5 convertase of the classical (C4b2aC3b) or alternative (C3bBbC3b) pathway. Cleavage of C5 gives rise to a powerful anaphylatoxin C5a and to a C5b fragment, which initiates the terminal part of the cascade, common for all activation pathways.

Activation of the terminal complement pathway results in the formation of the membrane-attack complexes (MAC), which forms large, 10 nm wide, pores in the target membrane (42). In fact, most pathogens are able to repair MAC-induced lesions and are resistant to complement lysis by MAC, as Gram-positive bacteria (43). Nevertheless, some Gram-negative bacteria are relatively sensitive to complement killing, particularly the meningitis causing *Neisseria* species (44, 45). This is illustrated by the susceptibility of individuals, deficient in terminal complement components as well as in properdin, to recurrent meningitis. Gram-positive bacteria have a very thick cell wall, which MAC cannot penetrate, therefore being resistant to complement-mediated lysis. Metabolically active nucleated cells are also resistant to lysis by complement (46, 47). However, increase of Ca flux and signal transduction have been described as the result of the insertion of multiple MAC in the membrane, inducing either apoptosis and cell killing or leading to cell proliferation depending on the cell type (48, 49). The molecular mechanisms of complement activation on pathogens are reviewed in detail and illustrated in Merle et al. (1).

Opsonization and Phagocytosis

The main role of complement in pathogen elimination is indirect, namely, the deposition of complement fragments on the surface of pathogen targets, so-called opsonization that allows their recognition, ingestion, and destruction by phagocytic cells, neutrophils, monocytes, and macrophages (**Figure 3B**). Both IgG antibodies and C3 fragments are the classical opsonins. But complement opsonization, resulting from the direct activation of the AP on pathogens surface allows their elimination by phagocytes before the mounting of an adaptive immune response and the appearance of antibodies (**Figure 3**). Phagocytes express specific receptors for C3 fragments, described in "Complement system part I – molecular mechanisms of activation and regulation" (1).

CR1 is a complement component molecule (CCP) domain containing molecule, involved in the control of C3 convertases. It is present on erythrocytes, on phagocytes, and on kidney glomerular podocytes and binds C3b and C4b. CR1 on erythrocytes plays a major role in the clearance of soluble immune complexes, by transporting them to the liver and spleen, where they are cleared by macrophages. The binding of C3b-coated targets to phagocyte CR1 is not sufficient to trigger phagocytosis, but C3b–CR1 interaction enhances the FcγR-mediated phagocytosis of targets bearing both IgG and C3b. Moreover, immune mediators that activate phagocytes, such as fibronectin (50) or LPS (51), induce the phagocytosis of targets opsonized with C3b only. However, this is probably partially mediated by CR1 in that case, since elastase, a major protease released by activated phagocytes, is able both to degrade CR1 and to cleave C3b into iC3b, allowing then iC3b-coated targets to be recognized by the efficient phagocytic receptor CR3 (52).

CR3 and CR4 are specific receptors for iC3b, among C3 fragments, able to induce the phagocytosis of iC3b-coated targets (53, 54). CR3 and CR4 belong to the integrin family, involved in cell adhesion processes, due to their ability to interact, in particular, with intercellular molecule-1 (ICAM-1), present on many cells, including endothelial cells. Integrins are formed from two chains, alpha and beta bearing magnesium ions necessary for their function. CR3 (also called MAC-1, αMβ2 or CD11bCD18) and CR4 (p150,95, αxβ2 or CD11cCD18) form, with LFA-1 and αDβ2, the leukocyte-specific integrin subfamily sharing the β2 chain (CD18). The CR3 also bear a lectin site, different from the iC3b and ICAM-1 binding site, and able to recognize microorganism-derived sugar ligands. If both the lectin and the iC3b-binding sites of CR3 are engaged, the strength of the CR3-mediated phagocytic response is enhanced (55).

CR3 is present on monocytes and its expression is up-regulated upon monocyte to macrophage differentiation. Resting neutrophils express low levels of CR3 on their surface but these levels increase dramatically following cell activation, due to the externalization of large intra-granular pools of receptors. CR4 is expressed poorly on neutrophils and monocytes, but its expression increases upon monocyte differentiation to macrophage or to dendritic cells.

The cellular processes involved in the phagocytosis of IgG- or C3-opsonized targets are different. When phagocytosis is mediated primarily via CR3, the reaction is slow and pathogens gently sink into the phagocyte, by a process involving actin polymerization dependent on the small GTPase Rho (53). By contrast, the Fcγ receptors provide a very vigorous engulfment of IgG-coated pathogens, with membrane extensions driven by small GTPases Rac and Cdc42. However, the CR3-mediated phagocytic response to iC3b-coated targets is boosted by the participation of additional receptors such as toll-like receptors (TLRs) recognizing pathogen patterns.

Indeed, it is worth noting that CR3 and CR4 induce phagocytosis mainly in conjunction with stimuli, such as pro-inflammatory cytokines, which activate phagocytic cells (56). These stimuli induce an inside-out signaling, which switches integrins to an active conformation with a higher affinity for iC3b and ICAM-1 (53). This may represent a control mechanism to avoid unwanted responses to host cells passively coated with a few iC3b molecules resulting from the continuous low-grade activation of the AP.

Apart from CR3 and CR4, some resident macrophages such as liver Kupffer cells express CRIg, a receptor for iC3b belonging to the immunoglobulin family and able to mediate the phagocytosis of iC3b-coated pathogens (57).

The main consequence of phagocytosis is the elimination of pathogens. Internalized microorganisms are killed both by toxic reactive oxygen compounds, generated through a NADPH oxidase complex assembled at the phagosomal membrane, and by microbicidal components, such as lysozyme and proteases, present in phagocyte granules fused with the phagosome to form the phagolysosome. Finally, CR3-mediated phagocytosis results in the apoptosis of phagocytic cells, an important step of the resolution of infection and inflammation (58).

Complement in Inflammation

Anaphylatoxins and Their Receptors

Complement anaphylatoxins C3a and C5a play a critical role in the modulation of immune system activity by complement. C3a and C5a are the small fragments released after cleavage of C3 and C5 by the C3 and C5 convertases of the classical and APs. They contribute to the inflammation and activate immune cells and non-myeloid cells, which express G-protein coupled anaphylatoxin receptors C3aR and C5aR (59, 60) (**Figure 4**). Anaphylatoxins stimulate inflammation by inducing an oxidative burst in macrophages (61), eosinophils (62), and neutrophils (63). Moreover, C3a and C5a induce histamine production by basophils (64) and mast cells (65), resulting in vasodilatation. Even if pro-inflammatory effects of C3a are not in question, studies highlight the anti-inflammatory role of C3a in different contexts (66). Neutrophil migration and degranulation are prevented by the presence of C3a, whether others granulocytes are activated by this anaphylatoxin (63, 67, 68). Thus, this suggests an anti-inflammatory role in acute phase of inflammation, and in cases of ischemia–reperfusion injury and in sepsis mouse models (69, 70).

The activation product of C4, C4a, seems also to have a functional activity on macrophages and monocytes (71, 72). Nevertheless, no C4a receptor has been reported, making it difficult to ascertain the physiological role of C4a (73). More studies are needed to understand whether C4a is an anapylatoxin and what is its mode of action.

In plasma, C3a and C5a are quickly converted by carboxypeptidase N and carboxypeptidase B into C3a-desArg and C5a-desArg, by cleavage of the C-terminal arginine (74–76). Recent study determined a central role of C3a in carboxypeptidase B2 (CBP2) negative mice, which are unable to convert C3a and C5a to C3a-desArg and C5a desArg (77). Using a treatment with blocking antibody against C3aR or C5aR, mice with only a functional C3a/C3aR axis present better survival after sepsis induction, whereas mice with only a functional C5a/C5aR axis present a less survival compare to wild-type mice. These data demonstrate two opposite effects between C3a and C5a, highlighting the complex role of C3a depending on the context. Another particularity of C3a and C5a is that C3a-desArg loses its ability to bind to C3aR and C5a-desArg has 90% weaker pro-inflammatory activity compared to C5a, as shown in human astrocyte model (78). By contrast, murine C5a-desArg is as potent as murine C5a upon binding to C5aR on murine cells (79). These inter-species differences have to be taken into account when *in vivo* experiments are analyzed.

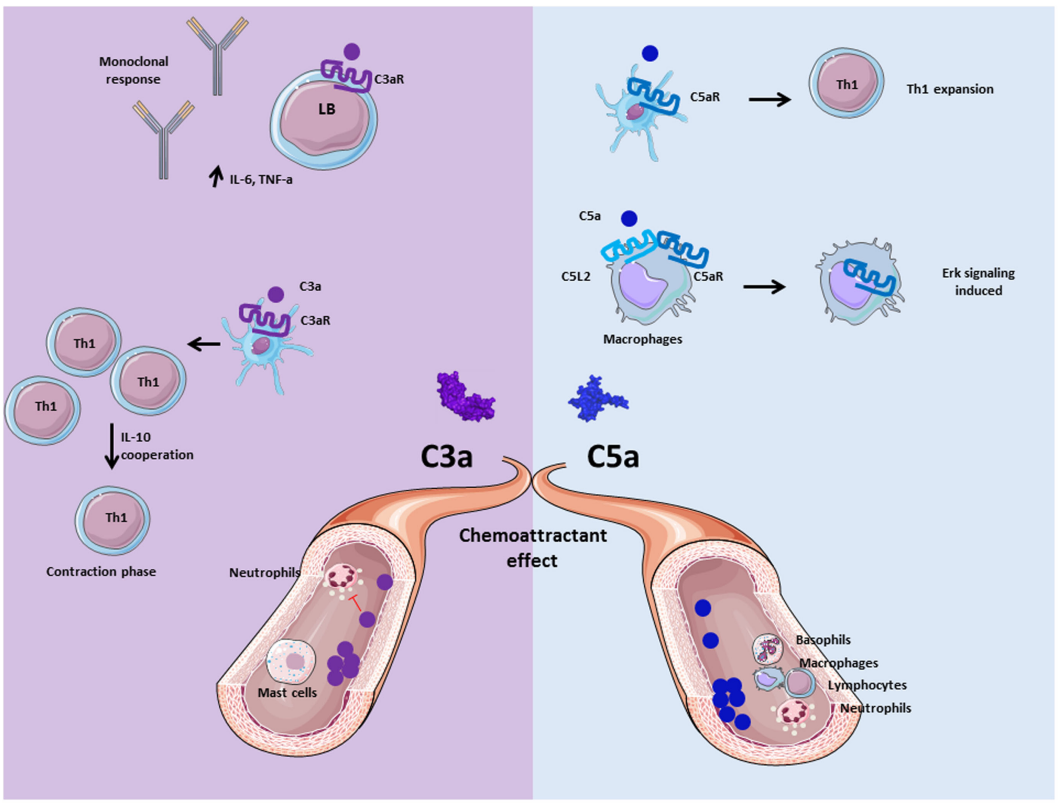

FIGURE 4 | Role of anaphylatoxins C3a and C5a. Anaphylatoxins C3a and C5a participate in inflammation by interacting and activating immune cells via C3aR and C5aR, respectively. C3a is implicated in the adaptive immunity by inducing monoclonal response from B cells and up-regulation of pro-inflammatory cytokines. Moreover, C3a facilitates the contraction phase of T cells by increasing IL-10 synthesis. C5a is implicated in Th1 expansion to improve adaptive immunity response, and allows C5aR internalization in presence of C5L2 to induce ERK signalization and pro-inflammatory effect of macrophages. Both are chemoattractant molecule, and allow mast cells migration for C3a, basophils, macrophages, neutrophils, and lymphocytes recruitment for C5a at the inflammatory site. Nevertheless, C3a has an anti-inflammatory effect on neutrophils by inhibiting their degranulation and recruitment.

Human *C3a* specifically binds its receptor *C3aR* (80), whereas C3a-desArg and C5a cannot bind to this receptor (81). C3aR belongs to the G-protein coupled receptor (GPCR) family with seven transmembrane domains. C3a binding leads to transduction of intracellular signals via heterotrimeric G-proteins and phosphorylation of PI3K, Akt, and mitogen-activated protein kinase (MAPK), leading to chemokine synthesis in human (82). Moreover, in human mast cells, C3a plays a role of chemoattractant molecule and can play an important role in hypersensitivity and inflammatory process (83). In case of chronic inflammation, C3a has pro-inflammatory activity and contributes to disease progression (60). In human monocytes and monocyte-derived macrophages, C3aR and TLR-4 costimulation induce the production of pro-inflammatory mediators, such as IL-1β, tumor necrosis factor alpha (TNF-α), IL-6, and PGE2 (84, 85). Contrary to these pro-inflammatory functions, in acute inflammation conditions C3a prevents mobilization and degranulation of neutrophils (66, 68). The difference in the response of inflamed tissues to C3a, between the acute and chronic phases of inflammation, may well be due to the differing cell types involved (e.g., neutrophils versus monocyte/macrophages) (66).

C3a modulates also the responses of the cells of the adaptive immunity. Human C3a has been described to regulate B cell function by suppressing the polyclonal immune response, IL-6 and TNFα release, in a dose-dependent manner (86). Mice C3aR signaling on DCs is important for the generation of Th1 cells (28, 87). A lack of C3aR activation on DC induces a Th2 polarization and favors the emergence of Treg. C3aR is also expressed on adaptive immune cells, such as T lymphocytes. Indeed, the importance of C3aR and C5aR expression for Th1 induction has been demonstrated in mice (27, 88, 89). Moreover, C3 deficiency induced a decreased expression, on T cells, of the α and β chains of the IL2 receptor. This may explain the aberrant Th1 response observed in patients with C3 deficiency. C3a also participates in the contraction phase of Th1 by regulating IL-10 expression (88).

C3aR is expressed not only by the immune cells but also by endothelial and epithelial cells. Stimulation of C3aR on endothelial cells induces a rapid mobilization of intracellular granules, Weibel–Palade bodies, containing von Willebrand factor and P-selectin. Hence, the cell acquire a pro-inflammatory and prothrombotic phenotype, since P-selectin helps the recruitment of leukocytes via binding to PSGL-1 (90) and von Willebrand factor mediates platelet adhesion (91). Moreover, human and mouse P-selectin binds to C3b on cell surfaces and serves as a platform for the formation of C3 convertases and the activation of the AP (92, 93). C3a activation of endothelial cells thus forms an amplification loop of complement activation, implicated in microvascular thrombosis, including in a mouse model of Shiga-toxin (Stx2)/LPS induced hemolytic uremic syndrome (HUS) (93).

Human and mouse *C5a* bind to *C5aR*, which also belongs also to the GPCR family (94). C5aR stimulation induces downstream effect such as activation of PI3K-γ (95, 96), phospholipase Cβ2 (97), phospholipase D (98), and Raf-1/B-Raf-mediated activation of MEK-1 (99). C5a is known to be a powerful chemoattractant molecule and plays a critical role in the inflammatory response by recruiting immune cells such as macrophages (100), neutrophils (101), basophils (102), and myeloid-derived suppressor cells (MDSCs) (103). Human C5a also recruits adaptive immune cells, such as T cells, which express constitutively C5aR on their surface (104). Human B cells express C5aR but respond to C5a only in case of activation (105). In a $CD55^{-/-}$ mouse model, it has been demonstrated that C5a production by APC is essential for differentiation of T cell in Th1 effector cells (106). Indeed, addition of C5a on $CD80^{-/-}$, $CD86^{-/-}$, and $CD40^{-/-}$ APC restored T cell activation, providing a link between GPCR, costimulation signals via CD28 and T cell survival (27). The axis C5a/C5aR appears to have a crucial role in sepsis, based on the observation that the inhibition of C5a/C5aR interaction decreased the mortality in a rat model (107). Another function of C5a is to induce vascular endothelial growth factor (VEGF) expression and to promote angiogenesis in a human retinal model (108). In a model of enteric infection, C5a is generated thanks to the stabilization of the AP C3 convertase by properdin and leads to IL-6 release from colonic epithelial cells (109). Then, IL-6 regulates inflammation induced by bacteria. In properdin deficient mice, the C5a-dependent IL-6 production is impaired, aggravating the infection. This provides evidences of the implication of C5a in the defense against bacteria-triggered epithelial injury.

C3a, C5a, and C5a desArg are also able to bind *C5L2*. In human, C3a-desArg is able to bind C5L2 and regulates triglyceride synthesis rate (110). C5a has a lower affinity for C5L2, as compared to C5a desArg in human basophil cell lineage (111). As C5a- and C3a-receptors, C5L2 is composed by seven transmembrane domains but it is not coupled with G protein (112). Previous work on human neutrophil determined a role of C5L2 as a negative regulator of anaphylatoxin receptor activity after activation and interaction with β-arrestin, this was confirmed in mouse model (113, 114). However, its role remains unclear. C5L2 has been described as an anti-inflammatory receptor, because $C5L2^{-/-}$ mice have increased production of pro-inflammatory cytokines IL-6 and TNF-α (115). Nevertheless, deficiency of C5L2 on macrophages, neutrophils, and fibroblasts decreased their proinflammatory capacity *in vitro*. C5L2 is also essential for the C5a-mediated cell infiltration in *in vivo*, in a mouse model, suggesting a role of positive modulator of C5a-induced responses (67).

C5L2 expression in human atherosclerosis lesions is correlated with an increase of IL-1β and TNF-α release, supporting the pro-inflammatory effect of C5L2 (116). It has recently been reported that human C5L2 and C5aR are able to form a heterodimer (117). This complex induces internalization of C5aR upon C5a binding and promotes ERK and MEK signaling in a mouse model of acute experimental colitis (118). This internalization of GPCR has already been demonstrated in *in vitro* assays as essential for the induction of the late stage of ERK signaling (119, 120).

Crosstalk Between C3aR and C5aR Signaling Pathways with Toll-Like Receptor Signaling

C3a and C5a are able to induce potent inflammatory pathways via their receptors C3aR and C5aR. The implication of intermediates such as NF-kB, MAPK, and c-Jun N-terminal kinase (JNK) in their transduction pathways suggests a potential crosstalk with other pathways, such as those of TLRs. Indeed, complement is involved in TLR-induced inflammation (121) (**Figure 5**).

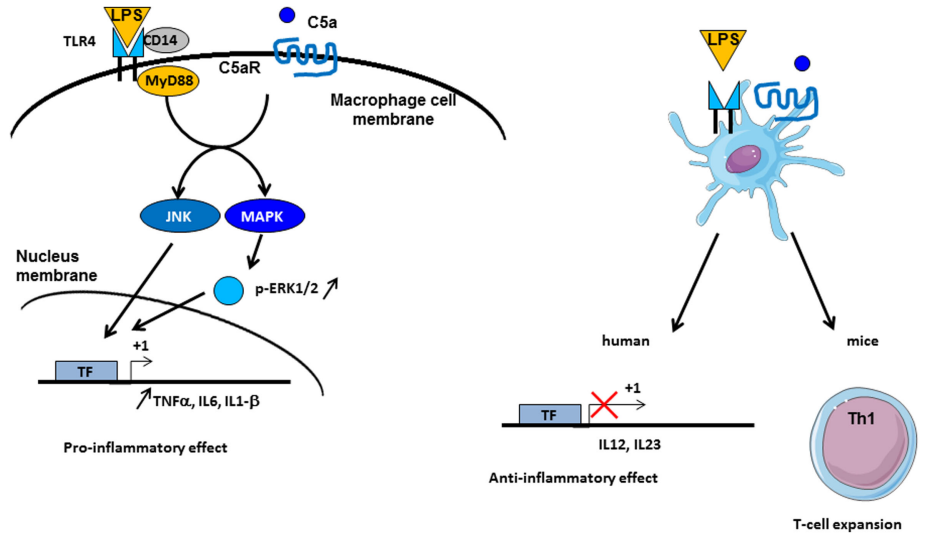

FIGURE 5 | Crosstalk between complement and TLR pathways. C5a/C5aR signaling pathway can cooperate with TLR-4 activation by LPS on macrophages. Intermediate signaling pathways JNK and MAPK are activated and thus lead to pro-inflammatory effect by TNF-α, IL6, and IL1-β synthesis. On dendritic cells (DCs), TLR-4 and C5aR cooperate in different manner between mice and human. *In vivo* experiments have demonstrated an implication in Th1 cells expansion, whereas in human, an anti-inflammatory role of TLR-4/C5aR collaboration has been described by an antagonized effect on IL-12 and IL-23 synthesis by DC.

Co-activation of MyD88-dependent TLRs (TLR-2, TLR-4, and TLR-9) and complement in $CD55^{-/-}$ knockout mice increased plasma inflammatory cytokines such as IL-6, TNF-α, and IL-1beta. Moreover, complement activation by the fluid phase cobra venom factor (CVF) synergizes with LPS to cause a dramatic increase of IL-6 production. These results suggest a strong interaction between TLRs and complement signaling *in vivo* to promote inflammation and modulate adaptive immunity (121). Complement may interact with TLR signaling by C3aR and C5aR, because of the involvement of MAPK, extracellular signal-regulated kinase (ERK1/2), and the JNK, but not p38 MAPK (121). TLR2 also crosstalks with CR3, since TLR2 can transactivate CR3 via PI3K by an inside-out signal (122). In turn, CR3 can regulate TLR2 signalization by recruiting TIRAP and facilitating the recruitment of MyD88 signaling adaptor to initiate TLR signaling (123). Several studies have shown a crosstalk between complement and TLR-4. It is known that TLR-4 activation by LPS can induce C5aR up-regulation on hepatocytes mediated by IL-6 (124). Another, immune-modulatory function of C5a is to reduce the production of IL-12 family cytokines by mouse inflammatory macrophages stimulated by TLR-4 ligands (125). In turn, this resulted in regulation of Th1 cells polarization and a limitation of their expansion. A synergy between C5a and TLR ligands on mouse DC stimulation was found upon stimulation of these cells with a fusion protein composed of C5a and an endogenous ligand for TLR-4 (extra domain A) and an antigen (126). This induced strong antigen-specific T cell responses *in vivo*, without production of immunosuppressive molecules. In humans, the C5a-mediated immature DC stimulation appears more complex. C5a increases the cytokines production in immature DCs, but upon TLR-4 stimulation, C5a inhibits the production of IL-12, IL-23, and TNFα, thus having an anti-inflammatory role (127). These results emphasize that the effects of anaphylatoxins on immune response depend on the crosstalk not only with TLRs but also with other receptors.

Complement and Adaptive Immunity

Discoveries over five decades have shown that the complement system plays an important role not only in the inflammation but also in the adaptive immunity.

The Complement System and Its Interplay with B Cells

The relationship between complement and B cells has already been demonstrated 40 years ago *in vitro* and using *in vivo* models. C3 plays important role in antibody generation by B cells (**Figure 6**). In case of C3 depletion [by a structural analog of C3 from snake venom (CVF), which is capable to bind to FB and to activate complement in fluid phase], the humoral immunity toward certain thymus-dependent antigens and the lymphocyte cooperation was impaired (128, 129). These and other early studies demonstrated that complement binds and localizes foreign antigens within sites where the lymphocytes response takes place. B cells express complement receptor CR2 (CD21), which interacts with C3d and iC3b on the surface of the antigen and forms also a co-receptor complex with CD19 and CD81 (130). Thus, the complex C3d:CR2 induces an increase of B cell receptor (BCR) signaling in the presence of C3d-opsonized antigen on B cell surface (131). *In vivo*, C3 is required for the induction and maintenance of memory cells of the B cell lineage within the microenvironment of germinal centers (GCs), where B cells encounter antigen–antibody–C3 complexes on the surfaces of follicular dendritic cells (FDCs) (132). When C3d-opsonized antigen

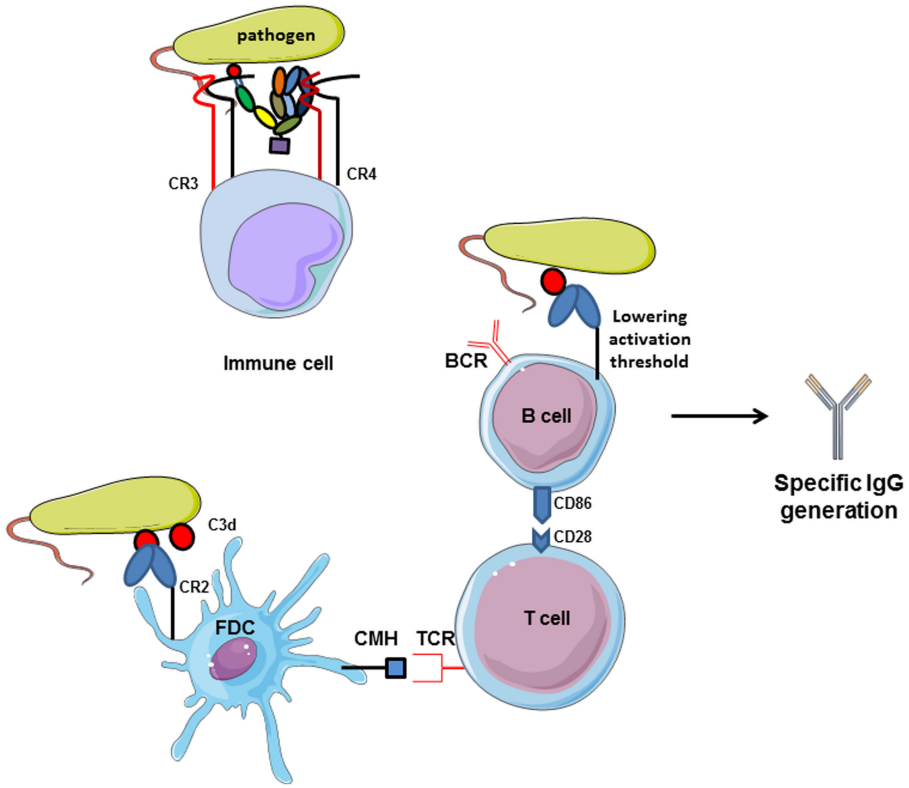

FIGURE 6 | Complement receptors implication in adaptive immunity. CR2 activation by interaction with C3d-opsonized antigen on follicular dendritic cells increases CMH expression and allows antigen presentation to TCR. Then, costimulatory molecules are expressed and T lymphocytes help in memory B cells maturation in germinal centers. Moreover, C3d/CR2 interaction lowers the activation threshold of B cells and increases BCR signaling activity. Cumulated, C3d/CR2 interaction induces specific IgG generation by B cells, and C3d works as a natural adjuvant. CR3 and CR4, expressed on immune cells.

binds to CR2 on FDCs, they can present the antigen in the GC and induce effector and memory B cells (133). This underlines the importance of CR2 expression on FDCs and B cell surface for the generation of antigen-specific IgG. C3d serves as a molecular adjuvant by lowering the threshold for B cell activation by 1000 to 10,000 times (134).

The human complement regulator of the CP C4b binding protein (C4BP) has functions extending beyond the dissociation of the classical C3 convertase and serving as cofactor for FI for C4b inactivation. It binds directly to the costimulatory protein CD40 on human B cells at a site that differs from that used by the CD40 ligand. C4BP induces proliferation, the up-regulation of CD54 and CD86 expressions, and IL4-dependent IgE isotype switching in normal B cells. These observations suggest that C4BP is an activating ligand for CD40 and establish another interface between complement and B cell activation (135).

The auto-reactivity of B cell is also tightly regulated by complement. C4 has been demonstrated to be essential to maintain peripheral B cell tolerance (136). Deficiency in C4 promotes the emergence of autoreactive B cells during the maturation in the GCs. These results could be explained by a lack in the clearance of apoptotic cells in GCs that leads to an impairment in host antigen presentation by APC, essential for the education and the peripheral B cell tolerance.

The complement system can contribute to autoimmune diseases by decreasing the threshold for B cell activation. For example, in a collagen-induced arthritis (CIA) mouse model, C3 depletion delays and decreases severity of the disease (137). Complement via C3d plays a key role in B cell function, and C3d antigen can break anergy in autoreactive B cells (138). Complement can modify the antigen-specific B cell response in experimental autoimmune encephalomyelitis (EAE) and possibly in multiple sclerosis (MS). In EAE mouse model, consumption of complement, using CVF, significantly attenuates clinical and histological EAE (139). The authors suggest that complement breaks the anergy of autoreactive B cells, leading to autoantigen-specific IgG production, while the total IgG response remained unaffected.

The Complement System and Its Interplay with T Cells

The importance of complement for the survival of resting T cells has been described above. Upon infection with a pathogen, T cell proliferation and differentiation are controlled by APC and their microenvironment. Complement is able to polarize T cells and participates to the induction and effector phase, as well as to the contraction phase of the T cell response (140–142). In fact, during inflammation, anaphylatoxins C3a and C5a are able to bind their corresponding receptors expressed on the T cells and APC surface, leading to cytokine production by these cells (27)

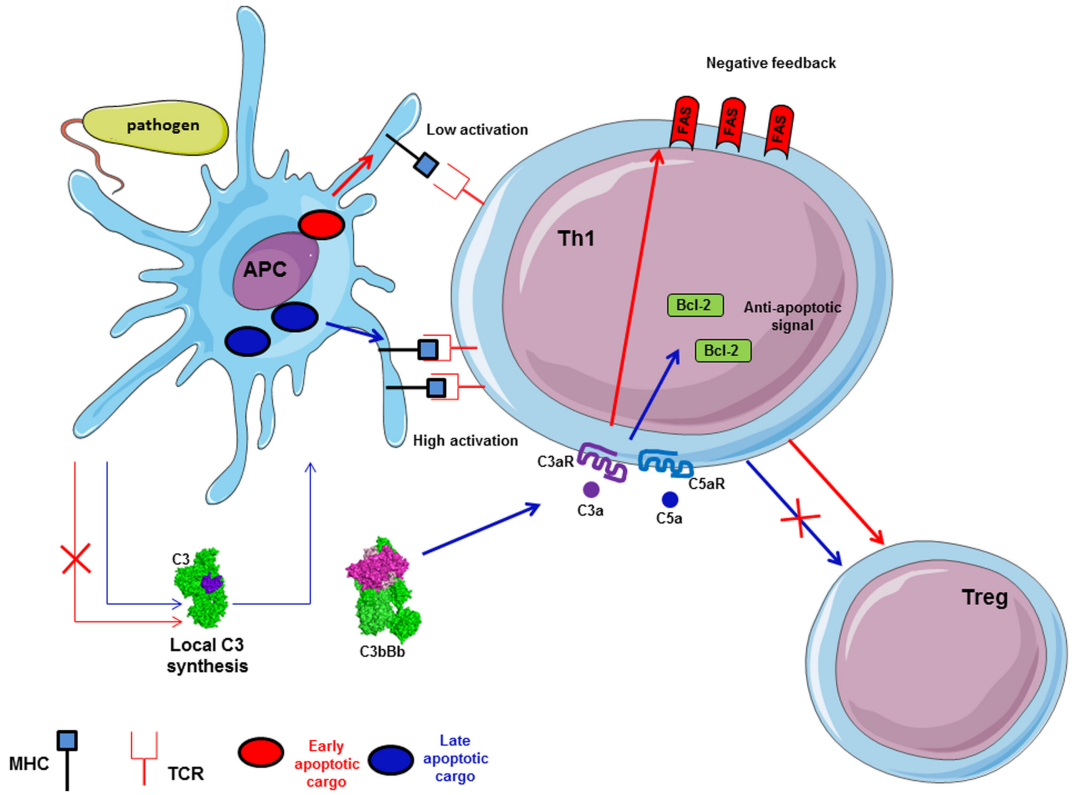

FIGURE 7 | Local synthesis of C3 by antigen presenting cells (APC) is implicated in antigen-specific T cell response. C3 synthesis by APC in case of pathogen internalization induces late apoptotic cargo and allows high expression of MHC on APC surface. Thus, it participates in antigen-specific T cell proliferation and Th1 generation. Local C3 and C5 generate C3a and C5a, which induce up-regulation of Bcl-2 and down-regulation of FAS expression via C3aR and C5aR to facilitate T cell proliferation. Moreover, C3a/C3aR and C5a/C5aR signaling pathways activation promotes Th1 generation and avoid Treg differentiation.

(**Figure 7**). Local C3 synthesis by DC is necessary to induce T cell activation and Th1 response (28). C3 deficiency was shown to accelerate the fusion of the apoptotic cargo with lysosomes and led to impaired antigen-specific T cell proliferation *in vitro* and *in vivo* (143). Moreover, C3a/C3aR is responsible for up-regulation of the anti-apoptotic Bcl2 and down-regulation of the pro-apoptotic molecule FAS during infection on myeloid and lymphoid cells, inducing immune cells survival and proliferation (144). The absence of C3aR and C5aR stimulation during T cell activation induces Treg development (145, 146). Thus, C3-deficient patients, who cannot produce C3a and C3b, present a lack of Th1 response whereas Th2 response remains normal (147, 148).

The complement regulator CD46 plays an important role in the regulation of T cells (149) (**Figure 8**). CD46 has different isoforms affecting its cytoplasmic tail and resulting from alternative splicing. Different isoforms are expressed in different organs. CD46 engagement on $CD4^+$ T cells promotes the effector potential of Th1 cells (150). As IL-2 accumulates, it switches cells toward a regulatory phenotype, attenuating IL-2 production and upregulating IL-10. The interaction of the CD46 cytoplasmic tail with the serine–threonine kinase SPAK plays an important role in this process. The γδ T cells express an alternative CD46 isoform and thus are unable to switch from IL2 to IL10 production. The Treg express different CD46 cytoplasmic tails, as compared to Th1 cells. Therefore, CD46 uses distinct mechanisms to regulate different T cell subsets during an immune response. Recently, the Notch family member Jagged1, which is expressed on T cells, has been identified as a new natural ligand of CD46 (147). Jagged1 binds Notch-1 and this interaction is responsible for cellular activation and proliferation. The *cis* interaction (on the same cell) of CD46 and Jagged1 leads to a competition with Notch, thus controlling the homeostasis of naïve T cells and inhibiting their activation. In the case of TCR stimulation, CD46 is down-regulated, which allows Notch-1 and Jagged1 to bind in *cis* and in *trans* (from two different cells). This induces Th1 proliferation and polarization, leading to IFN-γ and IL2 induction. Interestingly, CD46-deficient patients are unable to produce IFN-γ and have a lack of Th1 response, whereas Th2 response remains normal. Taken together, these results suggest that the expression of CD46 is necessary to induce a Th1 response. Then, CD46 plays a negative feedback when proliferation leads to T cell/T cell contact limiting the expansion of Th1 cells and allows a contraction phase. The binding site for Jagged1 on CD46 has been mapped to the first two CCP domains (147). There is no overlap between this binding site and the C3b binding site, which is located in the CCP3 and 4.

CD55 (decay accelerating factor, DAF) also plays a role in the establishment of the adaptive immune response. In addition to control complement activation, experiments on CD55 deficient

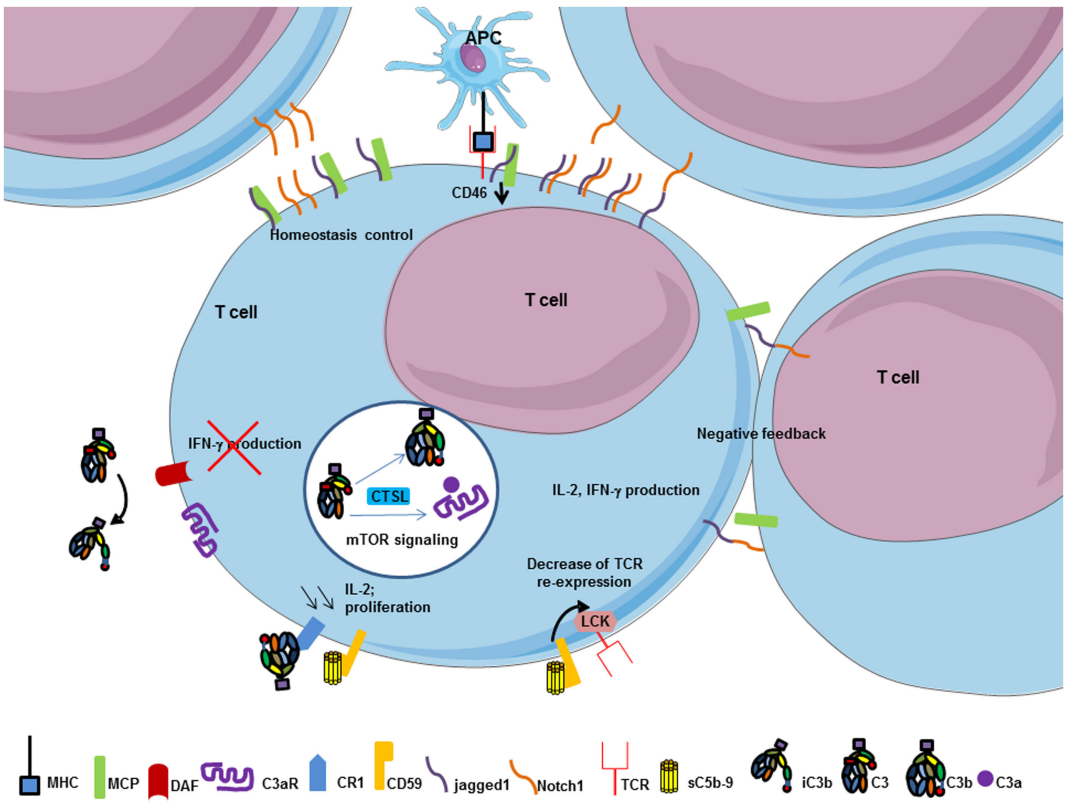

FIGURE 8 | T cell response is modulated by complement components. MHC/TCR interaction between APC and T cell decrease CD46 expression on T cell and allows *cis* interaction between jagged1 and Notch-1 on T cell surface to promote T cell proliferation, IL-2, and IFN-γ production. Thereafter, *trans* interaction between jagged1 and Notch-1, and CD46 work as negative feedback to control T cell homeostasis. Soluble C5b–9 and CR1 regulate T cell activation. Interaction between soluble form of C5b–9 and its specific inhibitor CD59 on T cells decrease TCR re-expression after its internalization to limit T cell activation by transmitting a signal via Lck. CR1 activation by iC3b decreases IL-2 synthesis and proliferation of T cell to promote a negative feedback of T cell activation. CD55 engagement on T cells negatively regulates Th1 induction cells by inhibiting IFN-γ production. Intracellular C3 in T cell is cleaved by CTSL and promotes C3b and C3a intracellular generation. Interaction between C3a and C3aR induces mTOR signaling and survival signal of the immune cell.

mice showed an enhanced Th1 response with a hypersecretion of IFN-γ (151, 152). This may be explained by the overactivation of complement leading to strong local anaphylatoxin production (153).

CD35 and CD59 also participate in T cell regulation. Recent study showed that CD59 is able to modulate T cell activation by transmitting a signal via Lck to TCR/CD3ζ. A knock-down of Lck accelerated the re-expression of CD3 at the cell surface (154). Engagement of CD35 (CR1) on T cells reduces their rate of proliferation and IL-2 secretion (155). Reduction of the expression of CD46 on activated T cells may lead to local complement overactivation, thus generating larger amount of C3b, iC3b, and C5b–9. Therefore, binding of C3b and iC3b to CD35 and C5b–9 to CD59 could contribute to the negative feedback controlling Th1 expansion (149).

In addition, intracellular mechanisms of sensing C3b-opsonized pathogens have recently been described (156). Pathogens that cross the cell membrane, with covalently attached complement C3 on their surface, activate mitochondrial antiviral signaling. This mechanism would represent an autonomous immunity of the cells against non-enveloped viruses and cytosolic bacteria, by inducing signaling pathways, such as NF-kB, IRF3/5/7, and AP-1, leading to pro-inflammatory cytokines production (156).

All these examples illustrate the importance of complement for the mounting of a successful immune response. Therefore, this cascade should not only be considered simply as a humoral factor, mediating innate immunity and inflammation, but also as a potent regulator of cells functions in the adaptive immunity.

Strategies of the Pathogens to Evade the Complement System

Complement system works as a first line of defense and allows in many cases to avoid infections. Nevertheless, the evolution of the pathogens resulted in elaboration of evasion strategies against complement attack. These mechanisms of complement evasion can be classified in different groups (157) (**Figure 9**).

Binding of Host Complement Regulators

Binding of host complement regulators on their membrane allows pathogens to inactivate complement. Bacteria, viruses, fungi, and parasites have been shown to bind high levels of efficient complement regulators such as FH, factor H-like 1 (CFHL-1), and C4

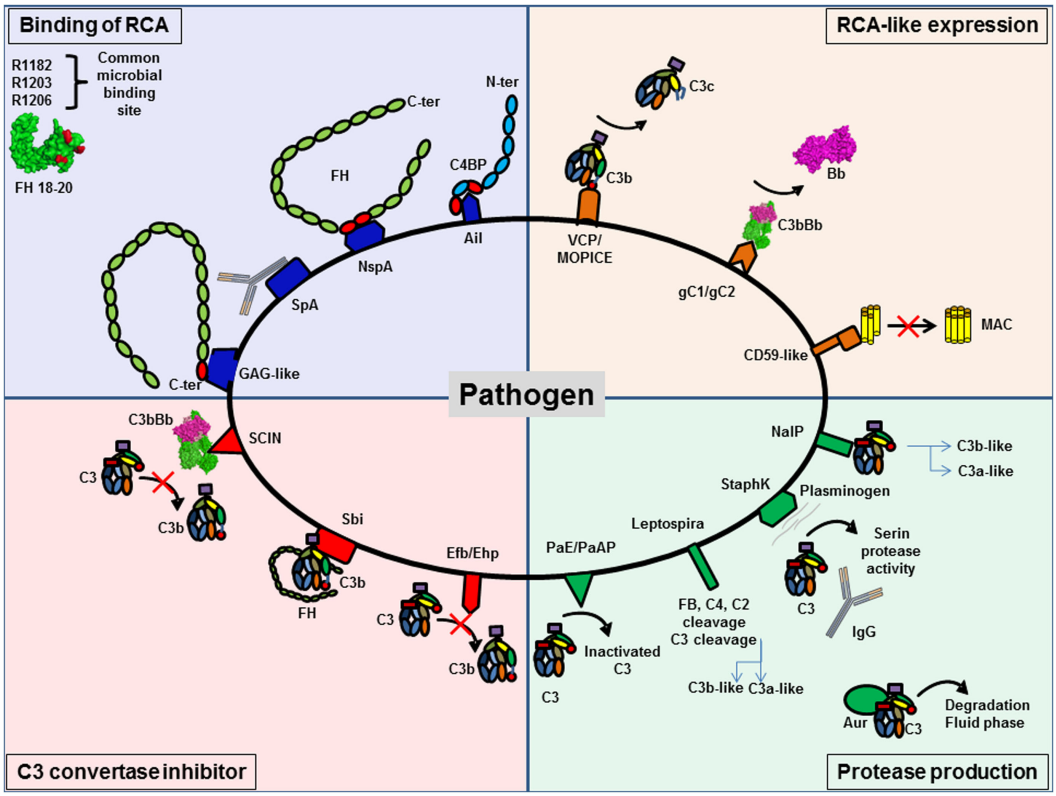

FIGURE 9 | Pathogens are able to protect themselves from complement activation. Pathogens have developed different strategies to inhibit complement activation. They can be classified in four different groups. Several pathogens are able to bind regulators of complement activation (RCA), such as FH and C4BP, to decrease C3 deposition. RCA-like expression allows pathogens to block complement activation without the need to recruit complement regulator. Synthesis of proteases specifically against complement proteins degrades complement components. The last group is pathogens able to express C3 convertase inhibitors.

binding protein (C4BP) (158). Recruitment of these regulators can accelerate the decay of the C3 convertase and provide cofactors for FI, which cleaves C4b and C3b, thus protecting the pathogen against complement attack.

To bind fluid phase complement regulators, pathogens express specific molecular platforms with high affinity, such as PorA in *Neisseria meningitidis* (159), filamentous hemagglutinin in *Bordetella pertussis* (160), Ail in *Yersinia pseudotuberculosis* (161), factor H binding protein (fHbp) and neisserial surface protein A (NspA) in *N. meningitidis* (162), and M protein family in group A streptococci (163).

Factor H binds to pathogens by a "common microbial binding site," located in its C-terminal domain CCP20. This domain binds to heparin and other GAGs on the host cell surface, but the binding sites for GAG and for pathogens surface proteins are not identical (164, 165). Three key amino acids (R1182, R1203, R1206) are critical for this "common microbial binding site" for at least seven Gram-negative and Gram-positive pathogens (165). In addition, the microbial proteins enhanced binding of FH19–20 to C3b, forming a triple complex, and leading to a more efficient complement inactivation on the pathogen surface. This is a unique example of convergent evolution, resulting in enhanced immune evasion of important pathogens via utilization of a "superevasion site" (165).

In addition to CCP 19–20, pathogens trap FH by binding to CCP6–8. *Neisseria* species bind this region of FH. Por1A from *Neisseria gonorrhoeae*, interacts with CCP 6, in addition to CCP 19–20, to escape from complement attack (166). Also, fHbp and NspA from *N. meningitidis* can recruit FH by binding to CCP 6–7 (162, 167). Study of the structure of fHbp by mutagenesis revealed that fHbp binding site for CCP 6–7 overlaps with sucrose octasulfate, a GAG analog. FhbB from *Treponema denticola* binds FH by a similar mechanism. Study of FH/FhbB interaction by site-directed mutagenesis revealed a binding site localized in CCP7 of FH (168).

Staphylococcus aureus has developed a different strategy to prevent complement initiation. Staphylococcal protein A (SpA) recognizes the Fc portion of Igs with high affinity and hide the binding site for C1q, thus blocking the initiation of the CP (169). The outer membrane protein SdrE binds FH and C4BP to *S. aureus* (170, 171). Together, these findings suggest that *S. aureus* is able to inhibit the three complement pathways.

Expression of Complement Regulators-Like Proteins

Expression of complement regulators-like proteins can contribute to pathogens camouflage. Some viruses, such as poxvirus, have found ways to produce soluble proteins that closely mimic the

structure and function of host regulators, such as vaccinia virus, which produce VCP, a protein similar to CD55 and MCP. Thus, these viruses inhibit both CP and AP, dissociating C3 convertases formed on C3b and C4b. Monkeypox virus produces a complement regulator-like protein, MOPICE, which is able to bind human C3b and C4b and serves as cofactor for FI (172). MOPICE is considered as a virulence factor of this virus, since it is expressed in the more virulent strains from Central Africa and absent in the less virulent pathogens from West Africa. Another example of complement regulators-like activity comes from Herpes viruses, which express transmembrane gC1 and gC2 glycoproteins able to bind C3b and to specifically accelerate the decay of the AP C3 convertase (173). *Borrelia burgdorferi* produces a CD59-like protein, which has affinity for C8b and C9 and avoids MAC formation (157).

Production of Proteases that Degrade Complement

Production of proteases that degrade complement proteins is frequently observed in bacteria. Indeed, *Pseudomonas* produce *Pseudomonas* elastase (PaE) and alkaline protease (PaAP) that cleaves immunoglobulins and C1q, thus preventing activation of the CP (174). In addition, these proteases are able to inactivate C3 into a non-functional fragment and inhibit complement activation (174). *Leptospira* strains are able to cleave directly complement proteins and inhibit the three complement pathways. *Leptospira* produced metalloproteases may not only cleave C3 but also FB, C4, and C2 (175). Generated C3 degradation products differ from those obtained by FI, suggesting that they would not result in opsonization and phagocytosis. *S. aureus* is also able to produce distinct proteolytic enzymes against complement proteins. Staphylokinase can form a complex with plasminogen, resulting in a serine protease activity efficient against C3 and IgG. Moreover, plasmin is able to bind the surface of *S. aureus* and inhibits the binding of IgG, C3b, and iC3b and blocks the effect of opsonization (176). This enzyme activity leads to the CP inhibition. *S. aureus* produces four important proteases: cysteine proteases staphopain A and B, serine protease V8, and metalloproteinase Aur. These proteases lead to a drastic decrease in the hemolytic activity of serum and are efficient against the three pathways (177). Aur is responsible for the consumption of C3 in the fluid phase (178). *N. meningitidis* is able to produce a protease NalP, which cleaves C3. This degradation produces two fragments, a shorter C3a-like and a longer C3b-like, degraded by host serum and leading to a decreased C3b deposition on the bacterial surface (179). *N. meningitidis* is able to inhibit the CP by capsular oligosaccharides, which represents a virulence factor of meningococcal infections (180). Capsular oligosaccharides interfere with engagement of C1q by IgG Fc, and lead to decreased C4b deposition and inhibition of CP activation.

Viruses can also produce C3b cleaving enzymes. Nipah virus particles carry a FI-like protease activity able to cleave and inactivate C3b, using FH and CR1, but not CD46, as cofactors (181). These data help to explain how an enveloped virus such as Nipah virus can infect and disseminate through body fluids that are rich in complement.

Production of Inhibitors of C3 Convertase

Production of inhibitors of C3 convertase has been observed in a few cases. *S. aureus* produces extracellular fibrinogen-binding protein (Efb), which inhibits the conversion of C3 to C3b by its affinity for the thioesther domain (TED). It binds to the TED domain on an area, shared by FH and CR2 binding sites (182–185). Efb acts as a C3 convertase inhibitor by blocking C3b formation and inhibits the opsonophagocytosis by granulocytes (186). Ehp (also known as Ecb), a homologous protein of Efb, binds two molecules of C3b and works as an efficient inhibitor of the alternative C3 convertase (187). Thus, it leads to a decrease of the activation of the C5 convertase and of the resulting C5a level. Surface immunoglobulin-binding protein (Sbi) forms a tripartite complex with C3b and functional FH that potentiates inhibition of complement activation (188). Efb and Sbi are able to recruit human plasminogen after binding to C3/C3b. Plasminogen is converted to plasmin by bacterial staphylokinase or by host-specific urokinase-type plasminogen activator and degrades C3 and C3b in the local microenvironment, thus protecting *S. aureus* (184, 189). Of note, the tripartite complex formed by C3, plasminogen, and Efb-C is more efficient than the one with Sbi, probably due to the higher affinity of C3d to Efb than Sbi, (184). The action of these molecules prevents the activity of the C3 and C5, as shown by a decreased C5a generation (190).

Contrary to Efb, staphylococcal complement inhibitor family (SCIN-A, SCIN-B, SCIN-C), can bind C3b in two distinct regions with a primary site at domain MG8 (191–194). By competing with FB and FH, SCIN is able to block the formation of alternative C3 convertase on one side, and to block the generation of iC3b that could induce phagocytosis on the other side. Moreover, SCIN binds and stabilizes the C3 convertase C3bBb, blocks the decay acceleration, and inhibits the cleavage of C3 into C3b. Thus, it avoids opsonization and complement activation in the same time (193).

Targeting binding sites for complement inhibitors on bacterial surfaces and complement-degrading proteases with vaccine-induced antibodies may be used as a strategy for novel vaccines designed to prevent complement escape. On the other hand, some bacterial proteins with anti-complement activity, such as Efb and SCIN, represent potential novel therapeutics, able to control undesired complement activation and tissue injury in multiple non-infectious diseases (195, 196). It is important to consider that by blocking C3 convertase formation, these inhibitors will avoid opsonophagocytosis and, by such, increase the risk of infection in treated patients (197). The increasing knowledge of the complement evasion strategies of pathogens provides novel insights for more efficient vaccine development and for designing novel therapeutic complement inhibitors.

Complement and Non-Infectious Diseases

The importance of the complement system in physiology is illustrated by severe and life threatening diseases, occurring in case of inefficient or exuberant complement activity (**Figure 10**). Abnormal complement activity is associated with a large number of inflammatory, autoimmune, thrombotic, and age-related diseases. The examples of systemic lupus erythematosus (SLE), atypical

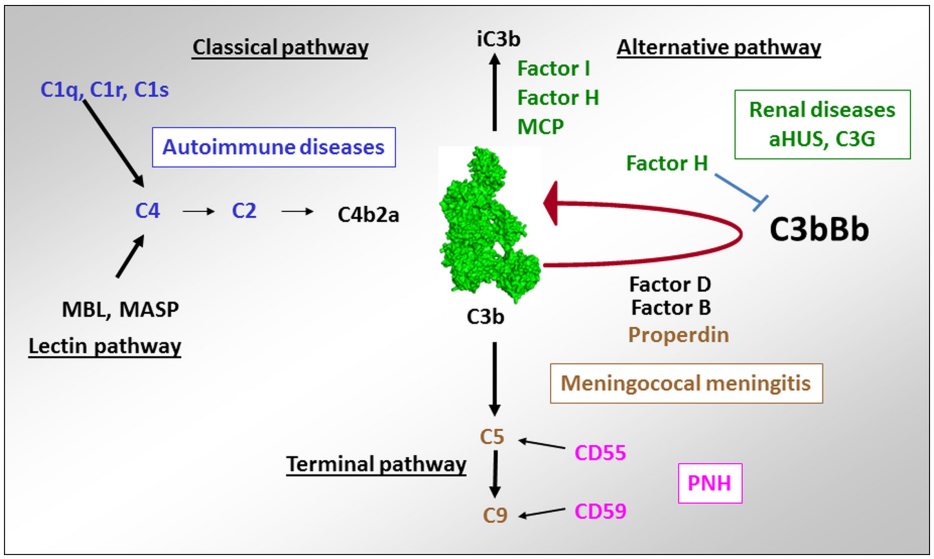

FIGURE 10 | Implication of complement deficiencies in pathologies. Deficiencies of the components of the CP C1q, C1r, C1s, C2, and C4 are associated with autoimmunity. Lack of regulators of the AP FH, CD46, and FI (as well as overactivation of the components of the C3 convertase C3 and FB) is linked to aHUS and C3G. Deficiencies of the terminal complement components C5, C6, C7, C8, and C9 as well as of the only positive regulator of complement – properdin is susceptibility factors to development of meningococcal meningitis. Lack of expression of the regulators of the C3/C5 convertase CD55 and the terminal pathway CD59 on erythrocytes are a cause of red cell lysis in paroxysmal nocturnal hemoglobinuria (PNH).

hemolytic uremic syndrome (aHUS), C3 glomerulopathies (C3G), age-related macular degeneration (AMD), and cancer are treated here in more details, but these are just few among a large list of diseases, including also the paroxysmal nocturnal hemoglobinuria (PNH), graft rejection after transplantation, ischemia/reperfusion injury, Alzheimer and Parkinson diseases, etc.

Diseases Associated with Deficient Complement Activation

Systemic Lupus Erythematosus

Deficiency of components of the classical complement pathway C1q, C1r, C1s, and C4 are very rare but are strongly associated with autoimmune manifestations. The autoimmune disease SLE is characterized by symptoms ranging from skin rashes, chronic fatigue, and arthritis to the more severe glomerulonephritis, serositis, and neurological involvement (198). C1q is the strongest genetic susceptibility factor for SLE, since over 90% of the individuals homozygous for mutations in this protein develop lupus-like symptoms (199). The mutations can cause quantitative deficiency (199) or functional abnormalities (13). A functional abnormality of C1q, due to a mutation in the C1r2C1s2 binding site, resulted in an inability of C1 complex formation and presence of free C1q in the patient serum. The strong association of the mutations of the CP proteins with autoimmunity can be explained by the "waste disposal" hypothesis of Walport (17), suggesting that lack of C1q opsonization and CP activity perturbs the immunologically silent uptake of apoptotic cells and debris, thus resulting in an immune response against self-antigens.

Alteration of the function of C1q can be also induced by presence of autoantibodies against C1q, which are frequently found in SLE, especially in patients with lupus nephritis (200). These antibodies bind to the collagenous tail of C1q (201) or the globular domain (202). These antibodies amplify the effect of C1q-containing immune complexes in the kidneys (203, 204), bind to C1q on early-apoptotic cells, and enhance complement activation (205).

Impaired clearance of apoptotic cells, induced by lack of C1q or inefficient complement activation due to lack of C1r, C1s, C2, and C4 are associated with SLE. At the same time, complement overactivation, due to the presence of anti-C1q antibodies or complement activating immune complexes in the circulation and in kidney glomeruli, is also leading to the same disease, albeit by a different mechanism. Therefore, both lack of activation and a too strong activation of the CP can be linked to autoimmunity.

Diseases Associated with Complement Overactivation

Inherited and acquired quantitative and functional deficiency of the regulators of the alternative complement pathway FH, FI, and CD46 as well as abnormalities inducing overactivation of the AP C3 convertase are associated with rare renal diseases like aHUS (206, 207) and C3G (208, 209).

Atypical Hemolytic Uremic Syndrome

Atypical hemolytic uremic syndrome is a renal thrombotic microangiopathy disease, characterized by glomerular microvascular endothelial cells activation and damage, leading to microthrombi formation and mechanical hemolysis (206). Within the last decade, complement AP dysregulation has emerged as the cause of aHUS (207, 210). In this disease, complement induces glomerular endothelium damage, secondary to unrestricted complement activation with C5b–9 deposits on the endothelial

cells surface. A loss of regulation is a result of mutations in FH, FI, or CD46 genes, found in ~50% of the cases. FH mutations are frequently located in the C-terminal domains and result in reduced capacity of FH to bind C3b and the GAG of the cell membrane (211–213). Mutations in FI impair its catalytic activity (214, 215). Complement overactivation in 10% of the cases is due to mutations in the components of the C3 convertase C3 and FB, forming a more potent convertase and/or a converatase resistant to decay by the regulators (216–221). In addition to mutations in complement genes, autoantibodies against FH, leading to an acquired FH functional deficiency, have been reported in aHUS patients (222–224). The binding epitopes of these autoantibodies are localized to the C-terminal recognition region of FH, which represents a hot spot for aHUS mutations (225).

Altogether complement-associated abnormalities are found in about 60% of the aHUS patients (226, 227). At present, over 200 distinct mutations have been reported in aHUS patients (207). All reported mutations were heterozygous, except in 15 patients (mostly from consanguineous families) with homozygous FH or CD46 deficiency (228). In a particular form of aHUS, mutations were found in DGKE – a gene outside of complement, involved in the protein kinase C signaling pathway (229). Its deficiency induces permanent pro-coagulant phenotype of the endothelial cells (230). aHUS has an incomplete penetrance among the mutation carriers and a triggering event (infection, pregnancy, etc.) and additional genetic predisposing factors (particular at risk haplotypes in FH and CD46) are needed to induce the disease (220, 231, 232). The hemolysis, which accompanies the disease onset, can serve as a secondary hit for the aHUS development, since released heme activates the alternative complement pathway in the fluid phase and on the endothelial cells surface (233, 234). The availability of the first effective anti-complement therapeutic drug, eculizumab, has changed dramatically the outcome of this rare kidney disease.

C3 Glomerulopathies

C3 glomerulopathies are rare chronic kidney diseases, characterized by predominant C3 deposits in glomeruli, in particular, in the mesangium and along the glomerular basement membrane, frequently associated with mesangial cells proliferation. According to the pattern of C3 deposits observed by immunofluorescence and electronic microscopy, two subtypes of C3G are described – dense deposit disease (DDD) and C3 glomerulonephritis (C3GN) (208). The pathogenic process in C3G is presumed to be due to uncontrolled C3 and/or C5 convertases activation, leading to C3 deposits and intra-glomerular inflammation. Autoantibodies targeting the AP C3 convertase, named C3 nephritic factor, are present in more than 50% of C3G patients (235–237). Few genetic abnormalities have been identified. These include mutations of complement genes, coding for C3 (1 case) (238) and the regulator FH (<20 mutations) (237, 239). Mutations affecting *FH* gene result more frequently a protein deficiency in the plasma. More recently, mutations in the *CFHR5* gene were reported, as well as rearrangements and copy number variations in the *CFHR* gene cluster (240–242). A particular form of C3GN is the CFHR5 nephropathy, characterized by a genetic defect in CFHR5, rendering it a strong competitor of FH. It is found in patients from of Cypriot descent (241, 243).

In C3G, genetic abnormalities affect the same genes as in aHUS, with a lesser frequency compared to aHUS, affecting only about 20% of the patients (209, 237). An interesting observation is that the mutations in FH and in FI, which are associated with C3G, are frequently the same, as the ones found in aHUS. They induce either complete or partial FH deficiency or functional defects in the N-terminal part of the protein (237). Mutations in the membrane anchoring C-terminal part of FH are found frequently in aHUS and nearly not in C3G. Therefore, the mutation itself (particularly the deficiencies of FH and FI and the mutations in the N-terminal part of FH) may not be sufficient to determine the type of the renal injury. The triggers of the AP dysregulation remain undetermined.

C3 nephritic factor and FH or FI mutations, inducing an overactivation of the AP are found also in patients with another type of membranoproliferative glomerulonephritis, associated with immunoglobulin deposits in the kidney – MPGN type I (237). Therefore, the role of the AP has to be considered even in diseases, for which CP activation is expected.

Contribution of Complement in Diseases, not Associated with Complement Defects

Age-Related Macular Degeneration

A particular polymorphism of FH (Y402H) is strongly associated with development of AMD, which is the first cause of blindness in the developed countries (244–247). The loss of central vision is associated with loss of photoreceptors and drusen formation in the retina (248). This is due to the constant exposure to light, smoking, the high-metabolic rate in the eye, and its particular sensibility to oxidative stress. Oxidized lipids and malondialdehyde are generated and if not properly handled by FH, they induce complement activation (249, 250) (**Figure 11**). FH binding to oxidized epitopes on altered or dying cells leads to inactivation of C3b to an anti-inflammatory fragment iC3b. Moreover, FH attenuates malondialdehyde-induced IL-8 production by macrophages and retinal pigment epithelial cells and decreases the expression of genes involved in macrophage infiltration, inflammation, and neovascularization in the eye. At risk, FH variant H402 has a weaker capacity to interact with oxidized lipids and malondialdehyde in the drusen, thus allowing constant background complement activation, leading to retinal epithelial cells damage and macrophages activation (249, 250). These results explain the strong genetic association of the H402 with the risk of AMD. Increased risk for AMD is conferred also by polymorphisms in several other genes of the alternative complement pathway, including FI, C3, C2/FB, and C9 (251).

Genetic analyses revealed that the deletion of CFHR1 and CFHR3, which is a polymorphism in the normal population with varying frequency depending on the ethnicity between 2 and 20% (252), is a protective factor against development of AMD (253). CFHR1 and CFHR3 are natural deregulators of FH, competing with it for cell surface binding (241, 254, 255). Therefore, their absence will increase the local binding of FH to oxidized surfaces, leading to a better protection, thus explaining the protective effect of this deletion in AMD.

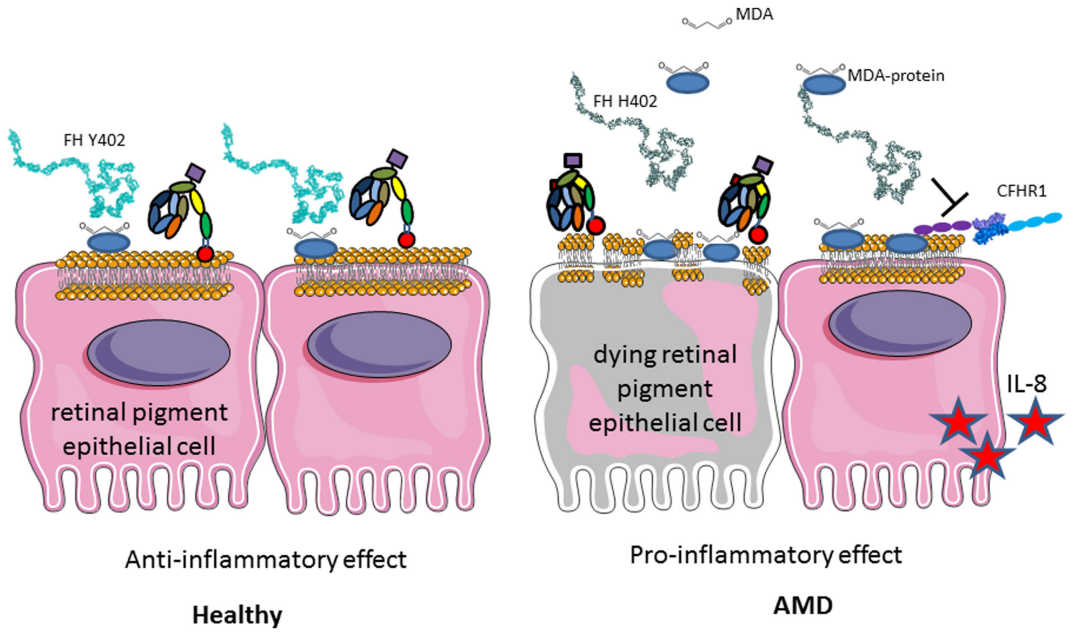

FIGURE 11 | Role of factor H Y402H polymorphism in age-related macular degeneration. FH binds to GAG, oxidized lipids, including malondialdehyde via CCP7 on the membrane of injured retinal epithelial (RPE) cells and protects them from complement attack. H402 variant has a weaker affinity to these products of the oxidative stress and protects less well the cells surface. Deposited C3b is not inactivated to iC3b and promotes complement activation. Less iC3b is generated, which contributes to the silent clearance of the injured cells. RPE cells are activated, secrete pro-inflammatory cytokines, and activate macrophages in their microenvironment. This chronic inflammation predisposes to AMD development with aging.

Cancer

Complement has been considered since a long time as an immune surveillance system against cancer, because complement is activated on the surface of tumor cells. Nevertheless, tumor cells develop inhibitory mechanisms for the terminal steps of the complement cascade, thus preventing complement-mediated cytotoxicity. Surprisingly, recent studies demonstrated that complement activation within the tumor microenvironment can promote tumor growth. Complement activation may support chronic inflammation, promote an immunosuppressive microenvironment, induce angiogenesis, and activate cancer-related signaling pathways. The mechanisms of these phenomena are not fully understood. Prolonged complement activation supports chronic inflammation, promotes an immunosuppressive microenvironment, induces angiogenesis, and activates cancer-related signaling pathways.

Several lines of evidence indicate a role for molecules of the complement system in tumor growth and metastasis, (**Figure 12**). C3, C4, or C5aR deficiencies prevent tumor growth in mice, potentially via inhibition of the CP and the generation of C5a, which has a potent inflammatory potential. In mouse models, the presence of C5a in the tumor microenvironment enhances tumor growth by recruitment of MDSC and increasing T cell-directed suppressive abilities (103, 256, 257). In a breast cancer model, C5aR facilitated metastasis in the lungs through different immune mechanisms in the metastatic niche, including the suppression of effector CD8(+) and CD4(+) T cell responses, the recruitment of immature myeloid cells and the generation of Tregs and a Th2-oriented response (258).

Cancer cells also secrete complement proteins that stimulate tumor growth upon activation via a direct autocrine effect through C3aR and C5aR signaling (256). In patients with ovarian or lung cancer, higher tumoral C3 or C5aR mRNA levels were associated with decreased overall survival. In addition, patients with non-small cell lung cancer have elevated C5a plasma levels (257).

C3a and C5a seem to have opposing effects during tumor development and in case of anti-tumor radiotherapy. While C3a and especially C5a promote tumor growth, radiotherapy-induced tumor cell death and transient local complement activation with production of C3a and C5a (259). The latter appeared crucial to the tumor response to radiotherapy and concomitant stimulation of tumor-specific immunity.

Overexpression of FH has been described in non-small cell lung cancer cell lines and on non-small cell lung cancer biopsies (but not in small cell lung carcinoma and carcinoid cell lines) (260, 261), in bladder tumor cells (262), in cutaneous squamous cell carcinoma (cSCC) and cell lines (263), and in hepatocellular carcinoma tumors (264). Low titer anti-FH antibodies were also found in sera from patients with non-small cell lung cancer (265). Recent studies demonstrated that FH binds to pentraxin 3 (PTX3) in the tumor microenvironment, thus preventing local complement overactivation and generation of pro-tumorigenic C5a (266). Hence, deficiency of PTX3 accelerated tumor development in mouse models of drug-induced cancerogenesis. FI was also suggested to be associated with tumor development of cSCC (267). These results provided evidence for a potential role of FH and FI in the cancer development, but the mechanism of action are still unknown.

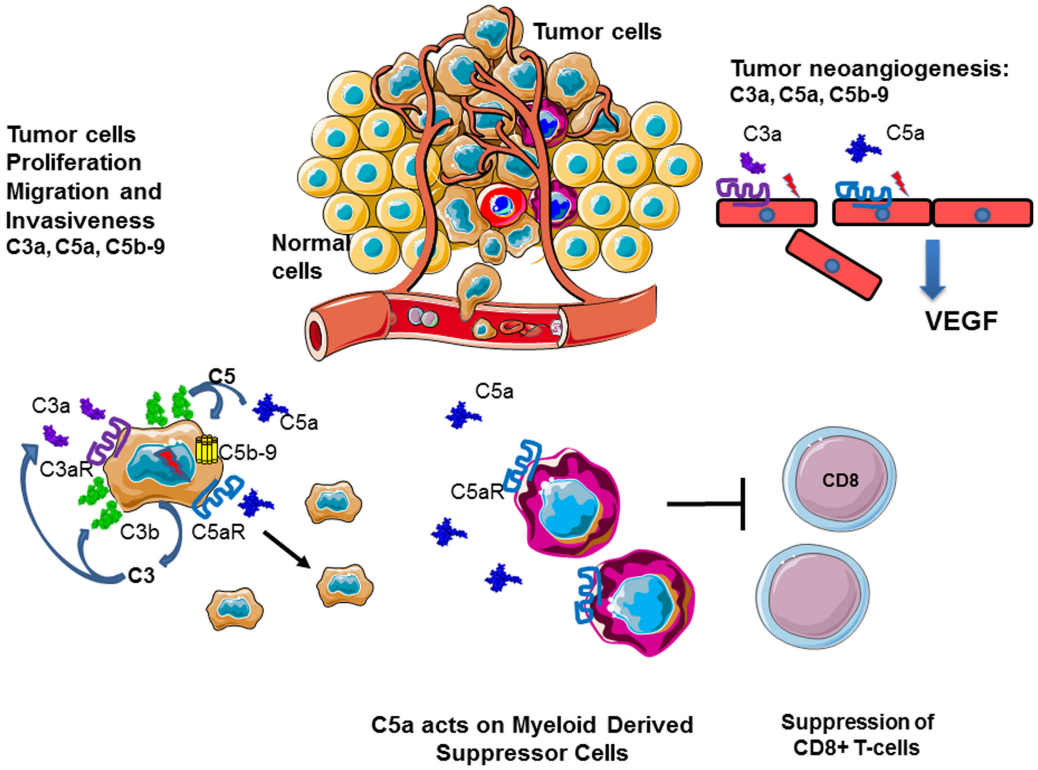

FIGURE 12 | Role of complement in cancer. Complement plays an important role in the chronic inflammation and tumor development. Tumor cells produce complement components and generate C3a, C5a, and C5b–9 in their microenvironment. This result in autocrine tumor cells stimulation, leading to proliferation, migration, and invasiveness. C5a stimulates MDSC, which dampen the immune response, suppressing cytotoxic T cells, and stimulate Treg. C3a, C5a, and C5b–9 promote angiogenesis, helping in tumor nutrient support and dissemination.

These examples clearly indicate that complement is indispensable immunosurveillance system, which needs to function with the right force when and where is needed. Therefore, therapeutic strategies are needed to adjust the level of complement activation in pathological conditions.

Complement as a Therapeutic Target

Since C1q is produced by the myeloid cells, a bone marrow transplantation can overcome this deficiency. Such therapy was efficiently applied to a patient with SLE, resulting in normalization of C1q levels and improvement of the clinical status (268). Deficiency of C1INH in hereditary and autoimmune angioedema (which is not a complement-mediated disease, but induced by excessive production of bradykinin, which is a potent vasodilator) is efficiently treated by plasma derived or recombinant C1INH (269). Alternatively, missing soluble complement components can be introduced to the body by plasma exchange. This was successfully achieved for C1q (270) as well as for FH and FI deficiency in aHUS. Plasma therapy was the first line treatment for aHUS before the development of anti-C5 blocking antibody eculizumab (271, 272). Eculizumab binds to C5 and prevents its entry into the C5 convertase, thus blocking completely the generation of C5a and the formation of MAC (273). This therapeutic is approved for use in PNH and aHUS and gives excellent results (274, 275). Currently, clinical trials are ongoing for many different diseases, where complement is overactivated. Novel inhibitors are under development to block complement at different levels. Particular interest is focused on the blockers of complement at the level of C3 and the C3 convertase. C3b binding peptide compstatin and its derivatives prevent the entry of C3 into the C3 convertase (276, 277). Plasma derived or recombinant FH and recombinant soluble CR1 dissociate the alternative C3 convertase and serve as cofactors for FI (278). Targeted inhibitors like TT30 (FH CCP1–5:CR2) or mini FH (CCP1–4:CCP19–20) bring the regulatory N-terminal domains to the cell membrane, where they are needed to control complement activation (279–282).

Conclusion

Complement plays a central role in the homeostasis and the installation of the adaptive innate immune response. By its supporting role in the clearance of apoptotic and necrotic cells, and its importance in the polarization of T lymphocytes and the humoral response by the cooperation with the B-lymphocytes, complement represents a real keystone of the immune system. This crucial role for the correct functioning of the organism is illustrated by the fact that both complement deficiencies and complement overactivation are associated with severe and life threatening diseases. The improvement of our understanding of the role of complement in health and disease opens up the possibility to use complement modulating drugs in the clinical practice.

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REVIEW
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Complement-coagulation cross-talk: a potential mediator of the physiological activation of complement by low pH

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The complement system is a major constituent of the innate immune system. It not only bridges innate and adaptive arms of the immune system but also links the immune system with the coagulation system. Current understanding of the role of complement has extended far beyond fighting of infections, and now encompasses maintenance of homeostasis, tissue regeneration, and pathophysiology of multiple diseases. It has been known for many years that complement activation is strongly pH sensitive, but only relatively recently has the physiological significance of this been appreciated. Most complement assays are carried out at the physiological pH 7.4. However, pH in some extracellular compartments, for example, renal tubular fluid in parts of the tubule, and extracellular fluid at inflammation loci, is sufficiently acidic to activate complement. The exact molecular mechanism of this activation is still unclear, but possible cross-talk between the contact system (intrinsic pathway) and complement may exist at low pH with subsequent complement activation. The current article reviews the published data on the effect of pH on the contact system and complement activity, the nature of the pH sensor molecules, and the clinical implications of these effects. Of particular interest is chronic kidney disease (CKD) accompanied by metabolic acidosis, in which therapeutic alkalinization of urine has been shown significantly to reduce tubular complement activation products, an effect, which may have important implications for slowing progression of CKD.

Keywords: complement, coagulation, contact system, alternative pathway, lectin pathway, classical pathway, pH

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Introduction – The Physiological and Clinical Importance of pH Effects on Complement

It has been known for many years that complement is strongly activated by low pH, especially when pH falls below about 7.1 (1–7). Owing to the tight regulation of arterial blood pH close to the normal physiological value of 7.4 that occurs even under pathological conditions, complement and plasma proteins in major blood vessels are unlikely to be exposed to such low pH. However, at sites of infection or inflammation, a significant localized fall in pH can occur, reaching pH 6 or even lower (8–10). Furthermore, other fluid compartments in mammals, notably the fluid within the lumen of the renal tubule, routinely maintain pH values below 7.1 (**Table 1**).

TABLE 1 | Summary of intraluminal pH measurements obtained by *in situ* micro-puncture studies in renal tubules of healthy rats.

Reference	Arterial pH	Early PCT	Late PCT	Near the bend of LoH	Early DCT	Late DCT	CD-proximal end	CD-distal rod	Urine
DuBose et al. (11)	7.33 ± 0.02 [18]	6.98 ± 0.03 [26]	6.72 ± 0.02 [47]	N/A	N/A	N/A	N/A	N/A	5.90 ± 0.43 [9]
DuBose et al. (12)	7.34 ± 0.01 [16]	7.06 ± 0.15 [16]	6.70 ± 0.50 [16]	N/A	6.69 ± 0.15 [16]	6.39 ± 0.04 [16]	N/A	N/A	N/A
DuBose et al. (13)	7.38 ± 0.01 [10]	7.06 ± 0.04 [10]	6.80 ± 0.04 [10]	N/A	6.57 ± 0.07 [10]		N/A	N/A	N/A
Karlmark et al. (14)	7.39 ± 0.01 [12]	N/A	6.68 ± 0.02 [10]	N/A	6.51 ± 0.04 [21]		N/A	N/A	5.67 ± 0.03 [12]
Buerkert et al. (15)	7.37 ± 0.01 [25]	N/A	6.92 ± 0.05 [33]	7.34 ± 0.05 [35]	N/A	6.70 ± 0.07 [16]	6.24 ± 0.01 [14]	5.62 ± 0.01 [14]	5.51 ± 0.21 [10]
Buerkert et al. (16)	7.36 ± 0.01 [12]	N/A	6.87 ± 0.08 [12]	7.39 ± 0.06 [30]	N/A	6.67 ± 0.12 [9]	6.51 ± 0.08 [12]	5.98 ± 0.12 [12]	5.60 ± 0.04 [12]
Wnaver et al. (17)	7.34 ± 0.02 [7]	N/A	6.90 ± 0.01 [6]	N/A	N/A	N/A	N/A	N/A	7.84 ± 0.01 [7]

PCT; proximal convoluted tubule, LoH; loop of Henle, DCT; distal convoluted tubule, CD; collecting duct.
Values are mean ± SE; number of observations is shown in parenthesis.

In healthy individuals, the lumen of the renal tubule is not routinely exposed to plasma proteins (including complement proteins). However, in chronic kidney disease (CKD), leakage of such proteins commonly occurs, resulting in proteinuria. There is now abundant evidence (18) that proteinuria is a major factor driving progression of CKD, and that the leakage of plasma proteins into the tubular lumen triggers an array of pathological changes in proximal tubular epithelial cells (PTEC) (18–20), including hyperplasia and epithelial–mesenchymal transition (EMT), which culminate in end-stage tubulointerstitial fibrosis. The complement system is widely recognized as a key mediator of renal injury (21) and there is mounting evidence that activation of plasma complement proteins leaking into the tubular lumen during proteinuria, followed by strong activation of locally synthesized complement (22) leads to progressive tubulointerstitial damage. Significant amounts of complement activation products are excreted in urine of patients with many forms of proteinuric nephropathy (23) and this excretion of activation products is blunted when metabolic acidosis in these patients is treated with sodium bicarbonate ($NaHCO_3$) (23), even though bicarbonate has no long-term effect on proteinuria (23, 24). This implies that, in addition to the well-documented glomerular effects of complement (25), filtered complement, strongly augmented by endogenously expressed tubular complement (22), is activated by the low intratubular pH (**Table 1**). This may explain the important clinical observation (24) that progression of CKD is significantly slowed in response to therapy with oral alkali (sodium bicarbonate), much of which is excreted into the tubular lumen thus raising intraluminal pH.

While renal complement-activation during metabolic acidosis has traditionally been ascribed to covalent activation of complement C3 by ammoniagenesis (26), more recent direct measurements have failed to substantiate this (7), and direct activation of complement by physiological low pH (4–7) is a more likely explanation, possibly through activation of the alternative pathway (AP) (7, 27) and through pH-sensitive cross-talk between the coagulation (contact) and complement systems.

The current article reviews and compares the basic features of the complement and coagulation systems, cross-talk between these two systems, and the mechanisms whereby low pH may activate complement; in particular, the possibility that low pH is sensed initially by the contact system (intrinsic pathway) and that complement is then activated through contact system-complement cross-talk.

The Complement and the Coagulation Systems

The complement and the coagulation systems are two closely linked systems that serve a vital role in maintaining homeostasis. Their activities rely on a delicate balance between activator and inhibitor signals of sequential enzymatic reactions that include activation of zymogens and assembly of new proteolytic complexes. Complement is now thought to be involved in several activities besides its role in fighting infections: these include tissue regeneration (28), clearance of debris (29), and pathophysiology of multiple diseases (30, 31). Likewise, the coagulation system

plays a role in fighting infections (32) and is implicated in pathophysiology of several diseases besides its role in the maintenance of hemostasis. Furthermore, complex cross-talk between complement and the coagulation system has been described that will be addressed in the current review, particularly with regard to mediating the activation of complement by physiologically attainable low pH.

The Complement System

Complement, as an integral part of the innate immune system has a major role in defense against invading pathogens. It achieves this through three main strategies; recruitment of immune cells to sites of infection, labeling of the invading pathogens via opsonization for uptake and destruction by phagocytes, and/or direct lysis of susceptible pathogens. Besides bridging innate and adaptive immunity, complement also bridges the immune and coagulation systems. More than 35 proteins, including circulating zymogens, and an array of fluid phase and membrane-bound regulators and cell-bound receptors, participate in complement activities. Three pathways have been recognized for complement activation; the classical pathway (CP), the AP, and the more recently discovered lectin pathway (LP) (33–35). The CP and LP are analogous, differing only in the initiator molecular complexes and the triggering signals (36). Complement C1q in association with two molecules of each of the serine proteases C1r and C1s makes the initiator complex of the CP (37). The C1qrs complex is activated on binding to antigen–antibody complex; however, it can also be activated in an antigen–antibody independent manner by binding to a number of molecules including C-reactive protein (CRP), lipopolysaccharide (LPS), polyanions, viral proteins, pneumolysin (38), and myelin. The LP-initiating complexes are made up of carbohydrate recognition molecules of either mannose binding lectin (MBL), ficolins or collectin-11 (Cl-11) associated with serine proteases, namely, MASP-1, MASP-2, and MASP-3 (39). LP is initiated upon recognition of certain sugar or acetylated sugar patterns decorating surfaces of invading microbes by the broad-spectrum carbohydrate recognition molecules of the LP. Unlike CP and LP, AP activation does not proceed via specific recognition molecules, but occurs instead through an imbalance between activating and inactivating signals acting on a steady state tick over process (40, 41). This kind of imbalance occurs on susceptible surfaces that lack complement regulators or do not support the binding of such regulators that normally occur on host cells. In addition, AP acts as a loop for amplification of signals from the other two pathways (42, 43).

Binding of recognition complexes from either CP or LP to their target structures leads to conformational changes within the molecules that result in activation of the attached serine proteases; C1r/C1s and MASP-1/MASP-2, respectively. The activated serine proteases C1s and MASP-2 cleave C4 into C4a and C4b. C4a is released into the fluid phase, whereas C4b attaches to the target surface. C2 binds to the attached C4b and is cleaved by C1s or MASP-2 releasing C2b into the fluid phase, whereas C2a remains attached to C4b. The resulting complex C4b2a represents the C3-convertase of the CP/LP that then activates C3. The slow and spontaneous hydrolysis of C3 into C3(H_2O) is considered to provide the flux that maintains the AP activity. The resulting C3(H_2O) is able to bind to factor B (FB), rendering it susceptible to cleavage by factor D (FD). This produces a limited amount of the fluid phase AP C3-convertase [C3(H_2O)Bb] that is able to cleave C3 into C3a and C3b. In absence of surface regulators or surfaces that do not support factor H (FH) binding (the main negative regulator of AP), the C3b formed binds to target surfaces and assembles with FB (in presence of FD) the surface AP C3-convertase (C3bBb) (44) that is stabilized by properdin (45, 46).

All of the three complement activation pathways converge in the proteolytic cleavage of C3, where C3 is cleaved into the anaphylatoxin C3a, and the opsonin C3b that binds covalently to the target surface. Binding of several molecules of the C3b to C3-convertases (either from CP/LP or AP) results in the assembly of C5-convertase that cleaves C5 into the powerful anaphylatoxin C5a and the opsonin C5b. Opsonization of target cells with C5b allows, in certain cases, the assembly of terminal complement components C6, 7, 8, and 9 into the membrane attack complex (MAC) that inserts into target membranes forming channels that disrupt membrane function and lead to lysis of target cells (47, 48).

The Coagulation System

The intrinsic pathway of coagulation was first described by two independent laboratories that proposed the waterfall model (49) and the cascade model (50) of coagulation (**Figure 1**). Following the discovery of tissue factor (TF) (also called factor III or CD142), the waterfall/cascade models were refined to include the extrinsic pathway. However, these models could not fully explain *in vivo* hemostasis or the normal bleeding tendency in patients lacking some of the early components of the intrinsic pathway [the contact system components: FXII, prekallikrein (PK) and high molecular-weight kininogen (HMWK)]. In addition, it was unclear why patients with functional deficiency of the intrinsic pathway FVIII (hemophilia A) or FIX (hemophilia B) have severe bleeding dysfunction despite the presence of the extrinsic pathway activity. In 1990s, the emergence of the cell-based model of coagulation provided plausible answers to these anomalies (51, 52).

The Extrinsic Pathway

Blood clotting through the extrinsic pathway of coagulation depends on the interaction between the circulating active form of FVII (FVIIa) and the membrane-bound TF to form a serine protease complex that activates FX into FXa (see **Figure 1**). FVII is the only coagulation factor found in circulation in active (1%) and inactive forms (53). The mechanism of FVII activation is still unconfirmed; however, autoactivation is suggested to provide the circulating FVIIa (54, 55). Other factors including FXIIa, FXa, FIXa, thrombin, plasmin, and FVII-activating protease also show the ability to activate FVII (56). Under physiological conditions, TF is not accessible to blood components, and only becomes accessible following injury to the endothelial cells lining the blood vessels. TF is expressed in a number of cells, including adventitial cells in the layers surrounding blood vessels. It has been reported that TF is also expressed in a number of activated cells (or cell-derived particles) in the blood, including monocytes, monocyte-derived microparticles, neutrophils, eosinophils, and platelets

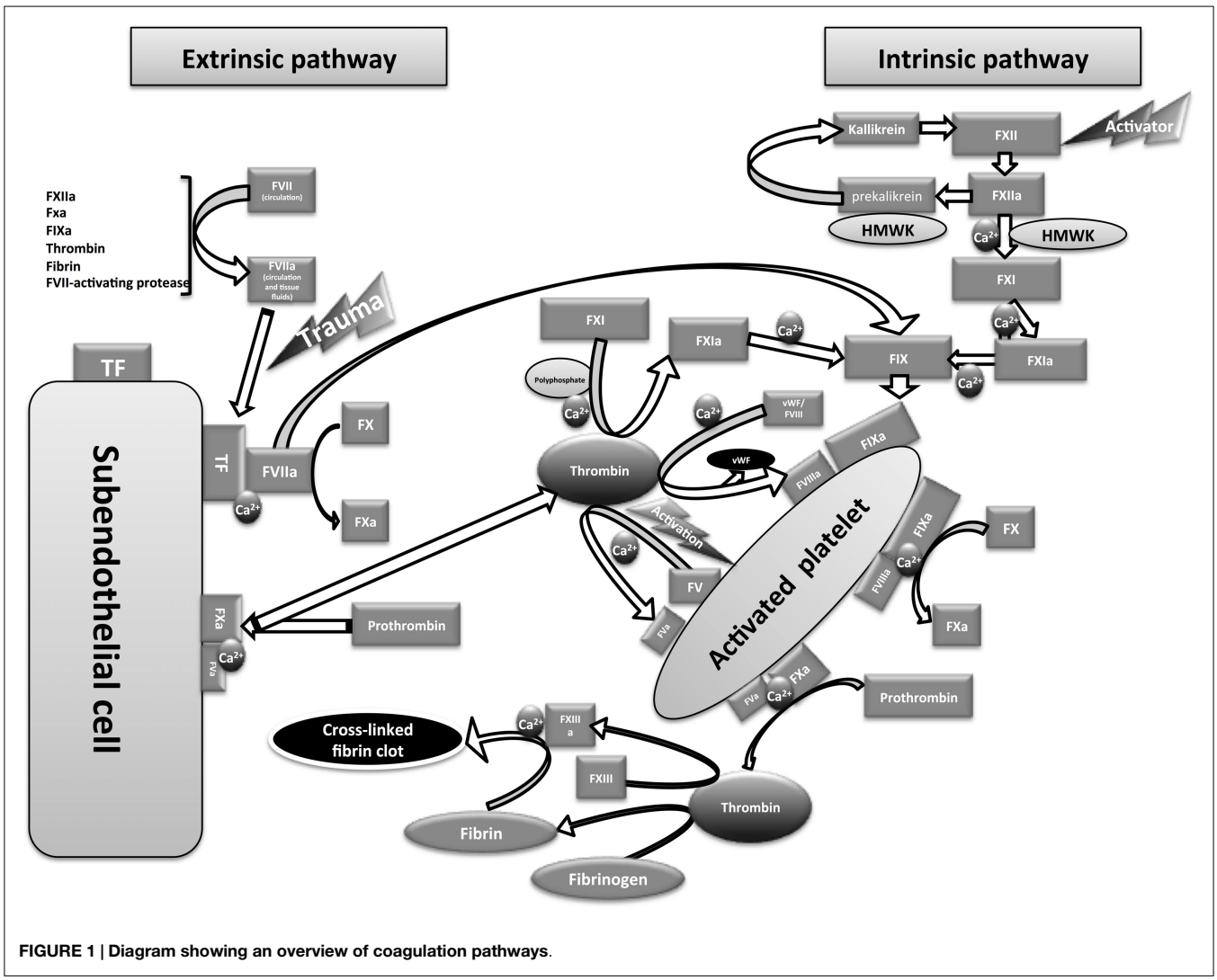

FIGURE 1 | Diagram showing an overview of coagulation pathways.

(57–62). However, intravascular TF exists in an encrypted form that cannot interact with FVIIa unless decrypted, possibly through the enzymatic activity of protein disulfide isomerase (63–65). Intravascular TF is thought to be involved in thrombosis (a pathological form of coagulation) rather than normal hemostasis (57, 59, 66). The extrinsic pathway is believed to be the only physiological trigger of coagulation *in vivo* that is activated immediately upon blood vessel injury (67).

The Intrinsic Pathway and the Contact System

The intrinsic pathway of coagulation is triggered upon the activation of the first component of the contact system "FXII" into FXIIa by an activator surface that is usually a negatively charged surface (68). The *in vivo* physiological activators of FXII are still unclear; however, platelet-derived polyphosphate (54, 69, 70) and mast cell heparin (71) are suggested to be contributory physiological activators of the contact system. Other known activators of FXII include extracellular RNA (72), DNA (73), collagen, Kaolin (74), dextran sulfate (75, 76), oversulfated chondroitin sulfate (77), glass, and plastic. FXIIa activates the second component of the contact system – PK into kallikrein. PK circulates with the cofactor HMWK (the third contact system component). The resulting kallikrein promotes the activation of additional FXII molecules via a positive feedback loop (**Figure 1**). These first two steps in contact system activation do not require the presence of calcium ions. The resulting FXIIa in the presence of calcium ions, phospholipids (phosphatidylserine provided by activated platelet surfaces), and the cofactor HMWK activates FXI into FXIa (78). FXIa then activates FIX into FIXa in the presence of calcium ions and phospholipids. FIXa forms (with the cofactor FVIIIa, in presence of calcium ions and phospholipids) a serine protease "Tenase" that activates FX into FXa. The resulting FXa either from the extrinsic or the intrinsic pathway forms, with the cofactor "FVa," a serine protease called "prothrombinase" that activates prothrombin (FII) into thrombin (the common pathway). The resulting thrombin then acts on fibrinogen, releasing fibrin monomer that is cross-linked in the presence of FXIIIa to form a stable fibrin polymer clot. Besides the procoagulant activity, kallikrein can cleave

HMWK to release the proinflammatory peptide "bradykinin" (kallikrein-kinin pathway) that acts on multiple target cells with the production of inflammatory mediators that include prostacyclin, prostaglandins, leukotrienes, endothelial-derived hyperpolarizing factor, and nitric oxide (79). By generation of bradykinin, the contact system plays an additional role, promoting other immune defense mechanisms during infection [for review, see Ref. (80)].

Growing evidence suggests that the contact system has surprisingly little impact on physiological hemostasis. Deficiency of FXII in humans and other animal species is not associated with deficient hemostasis. Moreover, non-mammalian vertebrates and cetaceans do not have FXII (81). On the other hand, it is strongly suspected that the contact system is associated with thrombosis (82). Deficiency of the contact system is protective against development of thrombosis and stroke (32, 83, 84). It is now believed that the mechanisms of physiological hemostasis are different from those of thrombosis (82) and thrombosis can be triggered via the activation of FXII by platelets and erythrocyte-derived microparticles (85). Current therapeutic strategies in thrombosis focus on targeting the contact system, hence affording protection from thrombosis or embolism without interfering with the hemostatic capacity. In view of the apparent links between the contact system and the activation of complement at low pH (discussed below), the contact system might also be a suitable therapeutic target in blocking the detrimental effects of complement activation under acidic conditions.

The Cell-Based Model of Coagulation

A more complete understanding of *in vivo* coagulation requires the cell-based model of coagulation, in which cell surfaces play a role distinct from providing the phospholipids required for assembly of protease complexes via platelet surfaces. The initiation step is believed to occur continuously in the extravascular tissue fluids and lymph, into which coagulation factors such as FVII, FIX, FX, prothrombin, and other low molecular-weight coagulation factors can diffuse from blood (51). In this step, FVIIa binds to TF on TF-bearing cells to form TF/FVIIa complex that activates FX to FXa. The resulting FXa activates prothrombin to thrombin before being rapidly inactivated by tissue factor pathway inhibitor (TFPI) and antithrombin III (86, 87). At the same time, TF/FVIIa complex activates FIX into IXa (88) that is required in further steps. Thus, this initiation step provides a continuous supply of trace amounts of extravascular thrombin that is required for further steps in the event of blood vessel injury. If such injury occurs, the following amplification and propagation steps will be triggered, involving blood coagulation factors and platelets that cannot normally diffuse from blood vessels into surrounding tissues. This occurs when these come into contact with the thrombin generated from the initiation phase (as described above), and the TF-bearing cells and subendothelial collagen.

Subsequently, platelets will be activated at the injury site by thrombin and collagen (89), releasing FV that becomes activated by thrombin on the platelet surface (see **Figure 1**). At the same time, thrombin will release FVIII from von Willebrand factor (vWF)-FVIII complex and activate it to FVIIIa. During this amplification step, the activated platelets are covered with the cofactors FVIIIa and FVa. The amount of thrombin generated at this stage is not sufficient to drive clot formation; however, it is very important for the amplification of the procoagulant signal.

In the subsequent propagation step, FIXa produced either from TF/FVIIa complex or from FXIa generated via thrombin action on FXI [in the presence of polyphosphate (90)] will bind to FVIIIa on platelets in the presence of calcium ions to form tenase that is 50 times more efficient than TF/FVIIa complex in FX activation (87), thus releasing large amounts of FXa. This FXa assembles with FVa and calcium to form prothrombinase on the activated platelet surface that will generate copious amounts of thrombin, which drive fibrin clot formation. Accordingly, TF/FVIIa appears to be the primary physiological trigger of *in vivo* coagulation (67) and contact system components (FXII, PK, and HMWK) do not seem to have a major role.

Cross-Talk between Complement and Coagulation Systems

The complement and coagulation systems share a number of common features. Activation of both systems leads to conversion of zymogens and assembly of proteolytic complexes that are mostly serine proteases of high-substrate specificity. Interactions between complement and coagulation systems have been described in a number of publications. For example, some complement regulators such as complement C1 inhibitor are involved in regulation of the contact system (91), and serine proteases from either of the two systems may act on substrates from the other system. For this reason, severe trauma and acute blood loss are not only associated with disseminated intravascular coagulopathy (DIC) but also with massive complement activation. This generates the potent anaphylatoxins C3a and C5a, and these may in their turn intensify coagulation (92–94). Activated platelets, which are critical participants in coagulation, can also activate both the CP (95) and the AP (96, 97); however, the physiological impact of this activation is still unknown, although complement activation products are known to activate platelets (97), which may lead to a positive feedback loop.

Thrombin generated from the coagulation system can activate complement C3 and C5 independent of the established complement activation pathways (98). Furthermore, C3 and C5 activation can proceed independent of each other (98). Similarly, Amara et al. (92, 99) reported the cleavage of C3 and C5, with the generation of C3a and C5a, respectively, by the coagulation factors FIXa, FXa, FXIa, and plasmin (100) independent of the known complement activation pathways. Thrombin and plasmin have been suggested to activate complement during liver regeneration in the absence of C4 and AP activity (28). In addition, FXIIa has been shown to activate the CP of complement via activation of C1qrs complex (101). Surprisingly, fluid phase activation of FXII by oversulfated chondroitin sulfate activated not only the contact (Kallikrein-Kinin) system but also C3 and C5 in the presence of EDTA (77). EDTA is a well-known inhibitor of complement via all of the known activation pathways by sequestering the divalent cations (Ca^{2+} and Mg^{2+}) necessary for complement activation. Thus, C3 and C5 activation can proceed

by a mechanism that is independent of the known C3- and C5-convertases. Depletion of FXII from plasma abolished this activation without affecting the normal complement activity; and reconstitution of depleted plasma with purified FXII restored complement activation (77).

However, not all such effects involve FXII: the use of aprotonin – a protease inhibitor of kallikrein and plasmin (but not of FXII) – inhibited C5 activation by oversulfated chondroitin sulfate (77). Furthermore, plasminogen-depleted plasma also failed to induce C5a production by oversulfated chondroitin sulfate (77). Thus, it seems that the contact system activity – not exclusively the individual activities of FXIIa or kallikrein – may drive complement C3 and C5 activation through generation of plasmin that has previously been reported to cleave C3 and C5 (77, 92, 100).

Wiggins et al. (102) showed that purified rabbit kallikrein was able to generate from rabbit C5 an activity that was chemotactic for rabbit neutrophils, suggesting that Kallikrein may cleave C5. Besides that, kallikrein was shown to play a role similar to factor D in cleaving C3bB, generating the AP C3-convertase C3bBb. However, this activity required the presence of divalent cations (103).

Conversely, complement components have been shown to influence coagulation activity in multiple ways. The key enzyme of LP activation (MASP-2) is able to generate thrombin through direct cleavage of prothrombin (104). Likewise, the terminal complement component complex C5b–9 has similar activity toward prothrombin even in absence of FV (105). Moreover, both the sublytic MAC and the cytolytically inactive terminal complement complex exhibit procoagulant activity mediated via the induction of TF expression by endothelial cells (106). The anaphylatoxin C5a promotes procoagulant activity by several actions on cells. C5a induces the upregulation of TF expression by endothelial cells (107) and by neutrophils (108). In addition, C5a induces the switch of mast cell and basophil activities from profibrinolytic to prothrombotic through the upregulation of plasminogen activator inhibitor-1 (PAI-1) (109). Interaction between the two membrane receptors TF and complement C5a receptor (C5a R) further suggests a cross-talk between the two systems (108). Accordingly, there is considerable evidence for two-way communication between complement and coagulation system components, influencing the activities of both systems.

Complement and Contact System Activities at Acidic pH

Several *in vitro* studies have demonstrated potent activation of complement activity, at acidic pH values, some of which are in the physiologically relevant range shown in **Table 1** (1–7). However, care is needed in interpreting these effects for two reasons: first because *in vivo* the low pH may have multiple sites of action (as in the case of renal tubules or inflammatory foci); and second because not all such effects observed *in vitro* may be directly physiologically relevant because they require extremely low pH or non-physiological temperature. For example, Hammer et al. (1) reported that acidification of serum or C5 and C6 to pH 6.4 at 0°C followed by neutralization was associated with complement activation that led to lysis of non-sensitized red blood cells in presence of terminal complement components C7, C8, and C9. They attributed the observed activity to the formation of a complex between C5 and C6 similar in activity to that of C5b6 generated via the AP or the CP. This complex was thought to be formed as a result of C6-mediated cleavage of C5 α-chain aided by low pH, which changed the tertiary structure of either or both of C5 and C6. However, the physiological significance of this complex is uncertain, as it was unstable at physiological temperature (37°C).

Low pH may also exert multiple effects through the AP. AP hemolytic activity on erythrocytes from paroxysmal nocturnal haemglobinuria (PNH) patients (110, 111) and rabbit erythrocytes was enhanced at pH 6.4 compared to pH 7.4 (2), which forms the basis of Ham's test used in diagnosis of PNH (110). They explained the enhancement of activity through the increased formation of the two C3-convertases; C3(H_2O)Bb and C3bBb, in addition to the enhanced binding of FB and C5 to C3b deposited on erythrocytes at pH 6.4. At the same time, the inhibitory effect of CR1 and factor I (FI) was also diminished at this pH. Similarly, Peake et al. (7) suggested that maximal complement deposition on cultured PTEC occurred via the AP at acidic pH.

Complement activation under mildly acidic conditions has also been attributed to human CRP via the CP (3). CRP is known to trigger CP activation upon interaction with phosphocholine-containing or polycationic agents. However, even in absence of these agents, CRP has been reported to activate complement, with optimal activity at pH 6.3. Furthermore, Hammond et al. (112) showed that CRP was able to interact with FH, which may be a way of partially regulating the enhanced complement activity mediated by CRP at low pH. However, it should be noted that this interaction of CRP with FH required more acidic pH (5.2–4.6).

Contact System-Complement Cross-Talk as a Mediator of the Effect of Low pH on Complement

Apart from the evidence cited above, relatively little is known of direct effects of mildly acidic pH on complement proteins or complement regulatory proteins. An alternative explanation of the activation of complement by low pH is that pH is initially sensed by component(s) of the contact system and this then leads indirectly to complement activation through complement-coagulation cross-talk. In the study involving CRP cited above (3), complement activation occurred in glass tubes, but not in polypropylene tubes. Addition of Kaolin to polypropylene tubes restored complement activation, suggesting a requirement for the presence of negatively charged surfaces to support the pH-dependent CRP-mediated complement activation (3). These authors attributed these effects of low pH to the conformational changes that CRP underwent at this pH. However, a possible alternative explanation is that the contact system is involved in this CRP-mediated complement activation at mild acidic pH (3), as activation occurred only in the presence of negatively charged surfaces, conditions that activate the contact system as well.

Further evidence in support of a role for the contact system in mediating the activation of complement by low pH (through complement-coagulation cross-talk) comes from the observation

that low pH results in accumulation of FXIIa as well as complement activation products. Generation of complement activation products C3a and C5a was observed by Emeis et al. (4) upon acidification of blood with either HCl or lactic acid. This complement activation was attributed to the effect of acidic pH itself, not to the lactate anion, as addition of lactate at control (non-acidic) pH had no such effect. Likewise, respiratory acidosis of blood was also associated with increase in C3a and C5a levels. Lactate acidosis could activate not only C3 and C5 but also more distal steps in the complement system, with the formation of the soluble terminal complement complex (sC5b–9) either in adults or neonates (113). In addition, Sonntag et al. (5, 6) showed that acidification of blood and plasma with lactic acid was associated with dose-dependent increase in complement C5a and contact system FXIIa generation, even in the absence of cellular components. Similarly, Renaux et al. (114) and Thomas et al. (115) reported increased contact system activity with increased kallikrein activity and bradykinin formation during hemodialysis upon lowering the pH of diluted plasma from pH 7.4 to 7.1.

In addition to the *in vitro* studies, parallel activation of complement and the contact system has also been observed *in vivo* in conditions associated with tissue acidosis, for example, in cases of myocardial infarction, shock, and perinatal asphyxia, where the anaphylatoxins C3a and C5a, and FXIIa levels were elevated (5, 6, 116, 117).

The molecular basis of this apparent strong relation between complement activation and contact system activation under mildly acidic pH conditions, where the contact system is probably the driving force for the complement activation, is still unclear. The pH sensor molecule(s) involved are unknown. A highly pH-sensitive plasma protein is the histidine proline-rich glycoprotein [HPRG – reviewed in Ref. (118)] in which much of the pH sensitivity is attributable to the "histidine rich" region of the molecule. Even though HPRG is known to associate with complement proteins (118, 119), no direct acid-dependent activation of complement by this molecule has ever been demonstrated. However, an interesting potential link with the contact system arises from the observation that HPRG and HMWK show about 50% sequence identity in their "histidine rich" region (118), indicating a possible direct pH-sensing role by HMWK.

In view of the apparent links between acid-dependent complement activation and the progression of CKD that were reviewed at the start of this article, the molecular basis of this effect of low pH, and the involvement of possible pH sensing in the contact system, merits further investigation; particularly as conventional therapy for correction of low pH by administering oral sodium bicarbonate to CKD patients may carry with it cardiovascular risks associated with sodium loading. Selective inhibition of the contact system (which may be possible without impairing hemostasis) might be a suitable alternative therapeutic target. However, before this possibility is pursued, two important points about the activation of complement at low pH remain to be clarified:

First, if the complement activation ultimately arises from complement-coagulation system cross-talk, with the cleavage of C3 and C5, by FIXa, FXa, FXIa, and plasmin (100), it needs to be confirmed that these proteases are still active at the relevant low pH. If this is important in the renal tubular lumen during proteinuria, it also needs to be shown that the failure of glomerular permselectivity during proteinuria is sufficient to allow the relevant contact system components (and not just complement) to leak into the acidic renal tubular lumen.

Second, it needs to be confirmed that the effect of elevated pH *in vitro*, or the therapeutic effect of alkalinizing the tubular lumen with bicarbonate therapy *in vivo* (24) arises from inhibiting the contact system, rather than through blocking the other proposed effects of low pH on complement, such as acid-induced amplification of spontaneous AP activity or enhanced binding of FB and C5 to C3b (2).

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Complementing the sugar code: role of GAGs and sialic acid in complement regulation

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Sugar molecules play a vital role on both microbial and mammalian cells, where they are involved in cellular communication, govern microbial virulence, and modulate host immunity and inflammatory responses. The complement cascade, as part of a host's innate immune system, is a potent weapon against invading bacteria but has to be tightly regulated to prevent inappropriate attack and damage to host tissues. A number of complement regulators, such as factor H and properdin, interact with sugar molecules, such as glycosaminoglycans (GAGs) and sialic acid, on host and pathogen membranes and direct the appropriate complement response by either promoting the binding of complement activators or inhibitors. The binding of these complement regulators to sugar molecules can vary from location to location, due to their different specificities and because distinct structural and functional subpopulations of sugars are found in different human organs, such as the brain, kidney, and eye. This review will cover recent studies that have provided important new insights into the role of GAGs and sialic acid in complement regulation and how sugar recognition may be compromised in disease.

Keywords: sialic acid, heparan sulfate, glycosaminoglycan, complement factor H, properdin, innate immunity, tissue specificity, complement regulation

INTRODUCTION

The complement system plays a vital role in the protection of a host from invading bacteria and other microorganisms. However, this potent immunological weapon must be tightly regulated, or there is a risk of attack of host tissues leading to damage via an inappropriate inflammatory response (1). Sugar molecules provide a diverse and complex means by which the complement system can not only identify bacteria and other invading pathogens as a threat but also identify host surfaces that require protection (2). With three activating pathways of complement, it is the alternative and lectin pathways that utilize sugar molecules the most (1). The lectin pathway is activated by the recognition of carbohydrate moieties, such as mannose or glucose, on the surface of bacteria, by the mannose-binding lectin or ficolins (3, 4). On the other hand, the alternative pathway of complement is modulated in host tissues by glycans such as sialic acid [the predominant form being *N*-acetylneuraminic acid (Neu5Ac)] or the glycosaminoglycan (GAG) chains of proteoglycans. The presentation of specific sialic acid or GAG structures on the surface of a cell, or within the extracellular matrix, can dictate whether positive or negative regulation of an immune response occurs, including complement activation. This is because the sugar compositions of both GAGs and sialic acid can vary greatly from one organ to another and even between different regions/microenvironments within the same tissue (2, 5, 6).

Glycosaminoglycans and sialic acid play an important role in the recruitment and control of a wide range of innate/cellular immune system regulatory proteins, as well as proteins involved in tissue remodeling following an inflammatory response (7, 8). For example, GAGs are key regulators of pulmonary inflammation during lung infection through their binding of cytokines, chemokines, and growth factors, which leads to leukocyte adhesion and accumulation (9). Interestingly, the protein tumor necrosis factor-stimulated gene-6, which plays a role in protecting tissues from the damaging effects of inflammation, has recently been found to antagonize the interaction of the chemokine CXCL8 with the GAG heparan sulfate (HS) on the surface of endothelial cells and thereby inhibit neutrophil extravasation (10). In this mini-review, we will concentrate on the role of sulfated GAGs (particularly HS) and sialic acid on the recruitment and regulation of components of the complement cascade.

Abbreviations: AMD, age-related macular degeneration; aHUS, atypical hemolytic uremic syndrome; BM, Bruch's membrane; CCP, complement control protein; DS, dermatan sulfate; FH, factor H; FHL-1, factor H-like protein 1; HS, heparan sulfate; GAG, glycosaminoglycan; GlcA, glucuronic acid; GlcN, *N*-glucosamine; IdoA, iduronic acid; Neu5Ac, *N*-acetylneuraminic acid.

MODULATION OF COMPLEMENT BY SULFATED GAGs

There are four different types of sulfated GAGs that are found ubiquitously in human tissues – namely chondroitin sulfate, dermatan sulfate (DS), HS, and keratan sulfate – all of which are attached to proteoglycan core proteins and have considerable diversity in their "sequence" of sugars (11). Of these, HS is the most structurally diverse and plays a vital role in cell differentiation, signaling, and immune homeostasis (12–16). The HS chain comprises repeating disaccharide units of a glucuronic acid (GlcA) or iduronic acid (IdoA) linked to *N*-glucosamine (GlcN) (17, 18). As shown in **Figure 1A**, each disaccharide has four positions that can be variably modified with sulfation (or acetylation in the case of the *N* position of GlcN) and, along with the epimerization of some GlcA sugars to IdoA, this allows for immense structural diversity of HS chains that are typically 50–200 disaccharides in length. This diversity is made more complex by the subdivision of HS chains into *N*-sulfated (NS) regions and *N*-acetylated (NA) regions (of variable length) separated by small "transition" (NS/NA) zones (see **Figure 1B**). Overall, it is this complexity that provides a broad range of structures that can be recognized differentially by proteins, such that the biosynthesis of distinct "sequences" at particular tissue sites can promote/regulate their binding within a particular microenvironment (2, 19). For example, the complement regulatory proteins factor H (FH) and factor H-like protein 1 (FHL-1), a truncated version of FH generated through alternative splicing [that has 7 rather than 20 complement control protein

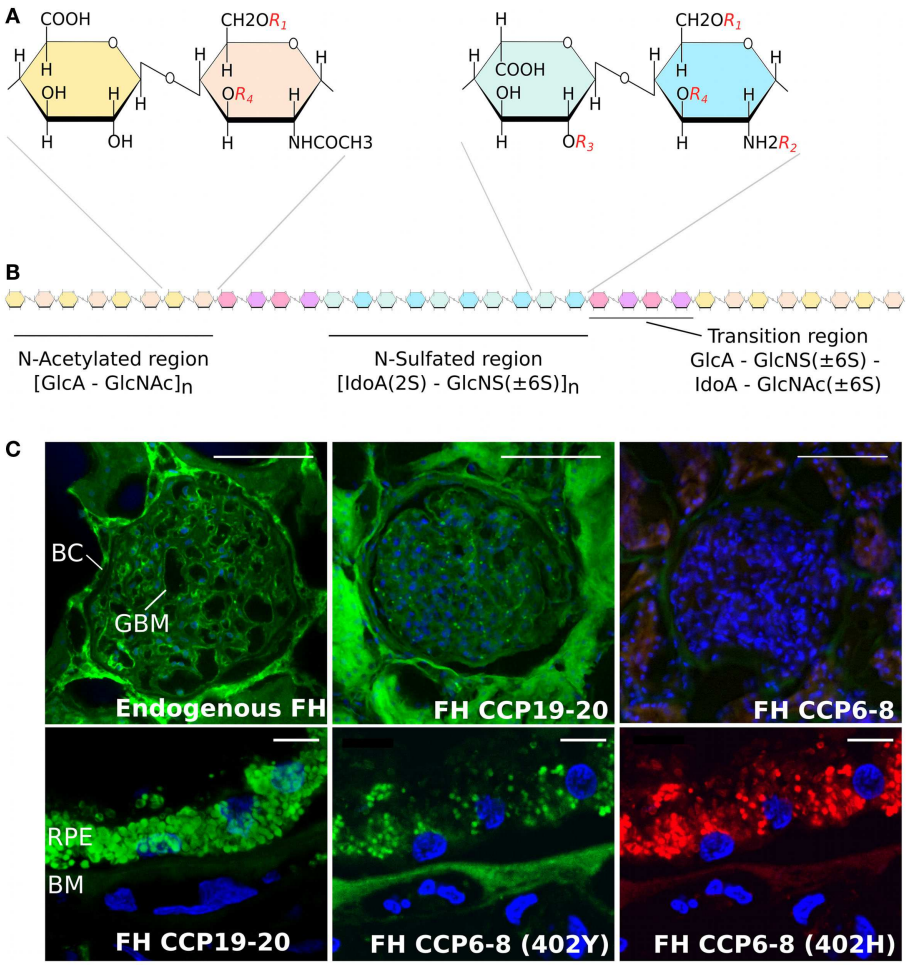

FIGURE 1 | Structure of heparan sulfate and the binding of factor H to human kidney and eye tissue. **(A)** Schematic showing disaccharide structures found in the HS chain. These are comprised of glucuronic acid (GlcA) and *N*-acetylated glucosamine (GlcNAc), found predominately in the *N*-acetylated region, and iduronic acid (IodA) and *N*-sulfated glucosamine (GlcNS) that are found in the *N*-sulfated region. The four possible sulfation positions are listed as: R_1, 6-*O*-sulfation; R_2, *N*-sulfation; R_3, 2-*O*-sulfation; and R_4, 3-*O*-sulfation. **(B)** Diagram demonstrating the distribution of the *N*-acetylated and *N*-sulfated regions of HS and their separation by short transition regions. **(C)** Staining of human kidney glomeruli (top panels) and the macula region of the human eye (lower panels) for endogenous FH and FH CCP6–8 and CCP19–20 binding sites; for full details, see Ref. (21). Endogenous FH (green staining) can be seen in both the Bowman's capsule (BC) and glomeruli basement membrane (GBM) in the human kidney, where this binding is predominately mediated by the CCP19–20 region of the protein. However, the CCP19—20 region of FH binds poorly to the Bruch's membrane (BM) of eye, where the interaction of FH is predominantly mediated by CCP6—8. The Y402H polymorphism, found in CCP7, alters the binding of FH to BM, demonstrated by the lack of red staining in the bottom right hand side panel. Scale bars in the top panels of **(C)** represent 100 μm, and in the lower panels represent 10 μm.

(CCP) repeats], prevent inappropriate alternative pathway activation/amplification in host tissues; in part, this is mediated by their binding to HS (and DS) on cell surfaces and within the surrounding matrix (20–22). One particular variant of FH/FHL-1 (termed 402H; that has a histidine at residue 402 in CCP7) is associated with an increased risk of age-related macular degeneration (AMD), a common cause of blindness in developed nations, and requires a high level of HS sulfation for its binding (23, 24). Because such highly sulfated sequences are rare within the human Bruch's membrane (BM) (an extracellular matrix of the eye), this might be the underlying cause of why complement dysregulation occurs at this site; i.e., due to insufficient FH/FHL-1 binding in 402H individuals (20, 22), leading to local inflammation that drives AMD pathology. FHL-1 has been found to be the major form of FH within BM (22) and unlike FH does not have a second GAG-binding domain (in CCP19–20) to compensate for its impaired tissue recognition; FHL-1 also lacks the sialic acid-binding site in CCP20 (see below). Importantly, the recent finding that the overall amount of HS in BM falls during normal aging (accompanied by a significant reduction in the level of sulfation) might explain the age-related nature of AMD (25); i.e., further impairing binding of the 402H variant of FH/FHL-1. Age-dependent changes in the sulfation patterns of HS have also been reported in tissues such as in the aorta (26) and in outgrowth endothelial cells (27); in the latter, a decrease in the amount of 6-*O*-sulfation with age results in a decrease in the migratory capacity of these cells toward vascular endothelial growth factor and stromal cell-derived factor 1α.

Properdin has an opposing role to FH/FHL-1 in that it is a positive regulator of the complement system (28). Properdin stabilizes the alternative pathway C3 convertase (C3bBb) allowing more conversion of C3 into C3b and thus amplification of complement activation. Because properdin exists as oligomers (dimers, trimmers, and tetramers), which can bind multiple C3b molecules, it can therefore act as a platform for the assembly of additional C3 convertases (29, 30). It has also been demonstrated that properdin can bind to HS and chondroitin sulfate on apoptotic T cells, thereby aiding their clearance by promoting complement-mediated opsonization/phagocytosis (31, 32). Furthermore, it has been shown that properdin and FH bind distinct HS sugars on renal tubular epithelial cells (33, 34) demonstrating the power of GAGs to mediate immune homeostasis on tissues by recruiting both positive and negative regulators of complement through the presentation of different sulfation patterns [reviewed in Ref. (28)].

MODULATION OF COMPLEMENT BY SIALIC ACID

Sialic acid also mediates complement interactions and this family of sugars is typically found at the termini of the *N*- and *O*-linked glycans substituting mammalian cell surface and secreted proteins (35, 36). The basic nine-carbon structure can be modified at the 4, 5, 7, 8, and 9 positions to generate a large amount of structural diversity (see **Figure 2**). It is the C2 carbon that forms the glycosidic bond to the neighboring sugar, i.e., at multiple different positions, allowing for variation in its orientation of presentation (35, 37, 38). Like GAGs, sialic acids can also control the activation of complement through binding FH; e.g., on erythrocytes, conferring protection from the spontaneous tick over of the alternative pathway (39). The binding of FH to sialic acid results in an increased affinity for C3b and thus enhances its cofactor and decay accelerating activities. However, FH binding can be influenced by the type and modifications of sialic acid, e.g., 9-*O*-acetylation of sialic acid reduces the affinity for FH (39, 40). In this regard, the molecular mechanism by which FH can attach to surfaces via sialic acid, while simultaneously binding C3b, has recently been elucidated (41); crystal structure analyses identified the amino acid residues in the CCP20 domain of FH that bind the glycerol side chain (C7–C9) and carboxyl group of *N*-acetylneuraminic acid (Neu5Ac). Furthermore, it was shown that there is a high level of specificity in the interaction of FH with Neu5Ac since this is dependent on the type of glycosidic bond present; i.e., FH binds α2–3, but not α2–6 or α2–8 sialic acid linkages. Mutations in the residues in FH that are responsible for recognizing sialic acid are associated with the rare kidney disease atypical hemolytic uremic syndrome (aHUS) (42). These changes perturb the Neu5Ac binding pocket and reduce the affinity of FH for sialic acid, providing a biochemical explanation for poor complement regulation on the glomerular endothelium in aHUS (41).

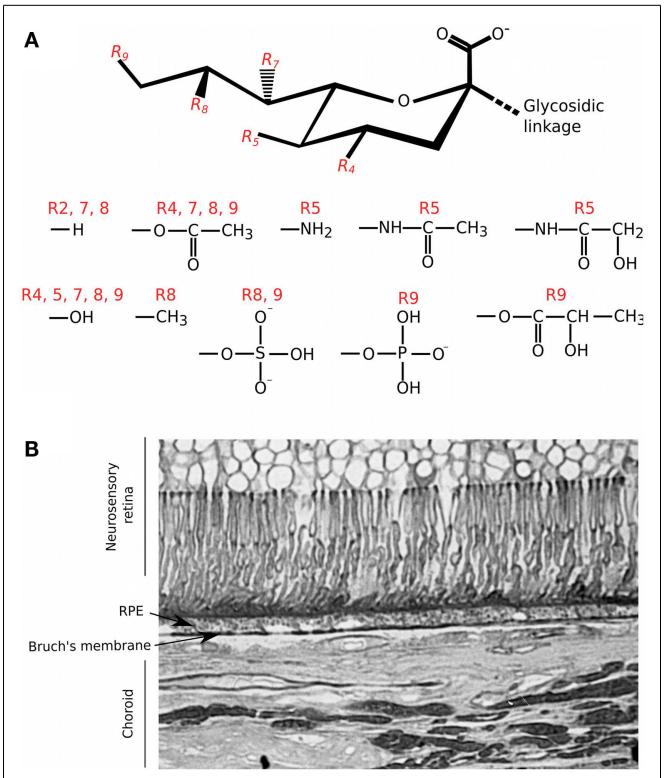

FIGURE 2 | Structural diversity of sialic acids and their distribution in the human eye. **(A)** Schematic of the basic 9 carbon structure of sialic acid and some of the possible substitutions (35); it is the C2 position that forms the glycosidic linkage to other saccharides within *O*- and *N*-linked glycan chains. R4, R5, R7, R8, and R9 groups are variably modified with the chemical groups illustrated. **(B)** Staining of a tissue section of human macula with the *Maackia amurensis* (MAA) lectin was carried out as described in Bishop et al. (5); MAA has high affinity for sialic acid linked α2–3 to galactose (Neu5Acα2–3Gal). Staining with MAA is seen throughout different structures of the eye but with particular intensity on Bruch's membrane.

The CCP19–20 region of FH is also known to bind HS (43), most likely at an interaction surface overlapping that for sialic acid (41); thus there is the possibility that these sugars might compete for binding, e.g., on cell/matrix surfaces where both are present. Although this sialic acid/GAG-binding site in CCP19–20 does not contribute greatly to FH's binding to BM (21), it is known that sialic acid is present throughout the human eye (5); this includes Neu5Acα2–3 (see **Figure 2B**). Therefore, it is possible that sialic acid may contribute to the binding of FH through other, as of yet uncharacterized, sites. Indeed, treatment of eye tissue with HS/DS-degrading enzymes only reduced endogenous FH levels by ~50%, consistent with the possibility that sialic acid could also be mediating binding to structures including BM (20).

TISSUE SPECIFICITY

As described above, both GAGs and sialic acid display considerable molecular diversity. However, importantly, there are differences in the populations of structures/sequences of these sugars found within different tissues. For example, HS is thought to play a regulatory role in many physiological processes (13–15) through the tissue-specific (or least tissue-restricted) biosynthesis of particular sulfation patterns as a form of "zip code" [reviewed in Ref. (2, 19)]. Its variations in sequence pattern can even be seen between different regions of the same tissue, as illustrated by the distinct HS epitopes mapped within the human macula (6), within pancreatic islets (44), and in the human kidney (45).

There is also evidence that functional HS "area codes" are different in the human kidney to those found in the human eye (21); i.e., those that mediate FH binding. Like BM, the glomerular basement membrane is an extracellular matrix that protects itself from complement attack by recruiting FH, in this case, through its CCP19–20 domain binding (at least in part) to HS (see **Figure 1C**). It has been shown previously that, while the CCP19–20 region mediates the binding of FH to glomeruli, it is the CCP6–8 region that is mainly responsible for binding to HS (and DS) in BM (20, 21). This demonstrates a level of specificity in the biosynthesis of functional HS sequences in the different tissues, or alternatively, that the binding specificity of these two regions of FH has become tuned to the different "compositions" of HS found in the two locations. This is also consistent with the observation that mutations in the CCP19–20 region of FH (46, 47), which are mainly associated with aHUS, do not present with an ocular phenotype but frequently effect heparin/HS binding [see Ref. (21, 48–50) for further discussion]. Similarly, the Y402H polymorphism in CCP7 of FH/FHL-1, a major risk factor for AMD, does not predispose individuals to kidney disease.

Transgenic mouse studies demonstrate that knocking out expression of FH causes aHUS (51) as well as some features that resemble AMD (52). Furthermore, by expressing a form of murine FH without CCPs16–20, it was demonstrated that this region of FH is important in the development of aHUS (53); this is consistent with the recent findings that the CCP19–20 region of FH likely plays a critical role in self recognition in kidney glomeruli through its binding of HS and/or sialic acid (21, 41). In fact, it has been proposed that FH is held in an inactive "latent" conformation by intramolecular interactions and upon binding to HS or sialic acid the conformation changes to one that has higher affinity for C3b and increased co-factor activity (54, 55). Therefore, the presence of HS or sialic acid on host cells may regulate not only the localization of FH but also the affinity for C3b.

Infection with enterohemorrhagic *E. coli* can also cause typical (or infection-induced) HUS; the shiga toxin produced by the bacteria can bind directly to the CCP6–8 or CCP18–20 regions of FH and impairs cofactor activity on cell surfaces but not in the fluid phase (56). Thus, it seems likely that surface recognition mediated by these regions of FH is inhibited through their binding to shiga toxin, although this requires further investigation. Similarly, the condition dense deposit disease can be caused by systemic loss of FH, normally due to mutations affecting the protein structure or its secretion. The resulting global dysregulation of complement results initially in progressive nephropathy with dense drusen-like deposits in the glomerular basement membrane, and later with drusen formation in BM of the eye (55, 57). However, the Y402H polymorphism in the HS-binding site of FH is associated with increased risk of dense deposit disease (58), so a role for GAGs (or sialic acid) is not an impossibility, but this coding change does also affect other functional activities of FH [see Ref. (12)].

The exciting work from Blaum and co-workers (41) has demonstrated that the CCP20 region of FH mediates considerable specificity for particular sialic acid structures (i.e., for Neu5Acα2–3), where amino acid residues involved in their recognition are associated with complement dysregulation in the kidney (46, 47). This suggests that there may be parallels with FH's tissue specificity for GAG binding (21). In this regard, we know that distinct sialic acid structures are present in different parts of the eye, including within BM (5) and, therefore, it will be interesting to see whether different regions of FH differentially recognize sialic acids in a tissue-specific manner.

The brain is another organ where interactions of complement with host sugars have been found to contribute to immune homeostasis and become dysregulated in disease; in this context, it is believed that complement proteins, including FH, are synthesized locally within brain tissue (59). For example, FH has been shown to associate with the brain lesions of Alzheimer's disease patients through the binding of HS (60), changes in HS structure are associated with disease progression (61). HS has been shown to bind amyloid-β (62) where this is modulated by the level of HS sulfation (63). In fact, it is believed that neurotoxic amyloid-β competes with neuroprotective fibroblast growth factor 2 for a common HS binding site (63). Furthermore, it has been suggested that the presence of amyloid-β prevents the heparanase-mediated turnover of HS chains (64), which could lead to enhanced binding of FH to HS structures within brain lesions, hindering their clearance by complement. HS has also been shown to regulate the processing of the amyloid precursor protein to amyloid-β by the Alzheimer's beta-secretase, BACE-1 (65). This is mediated via direct binding of HS to this enzyme, where the specificity of the interaction, e.g., with regard to sulfation pattern, has allowed the generation of heparin derivatives and HS oligosaccharides with therapeutic potential for Alzheimer's disease (66, 67).

The presence of sialic acid on neuronal cells can prevent the activation of the classical complement pathway by masking the binding sites for C1q (68). The removal of sialic acid results in

C1q binding, activation of the classical pathway, and opsonization of the neuronal cells with C3b; microglial cells in the brain can then recognize C3b via Complement Receptor 3 (CR3) and activate the phagocytosis of these labeled cells. It has been postulated that the presence of sialic acid on the cell surface acts as a marker of cellular health that may be lost/impaired during inflammation and oxidative stress (69).

MODULATION OF THE COMPLEMENT RESPONSE BY PATHOGENS

As described already, FH has two HS-binding regions and at least one site for interaction with sialic acid and with its flexible, modular, structure FH is capable of interacting with several self-ligands on the host surface simultaneously (70), which is believed to enhance its binding avidity. This allows for the recognition of a diverse range of cell and tissue types as well as making it harder for microorganisms to recruit FH to avoid host defense. However, the interplay between host and pathogen is like a constant weapons race. It is therefore not surprising that pathogens have evolved ways to mimic these self-associated molecular patterns (SAMPs) (71). Many human pathogens, including *Pseudomonas aeruginosa* have in common with human cells the sialic acid, Neu5Ac, on their surface (72), which allows them to recruit FH from the blood and thereby prevent a complement-mediated response (73). *Neisseria gonorrhoeae* also have surface sialic acid and this was shown to bind FH in the CCP16–20 region (74), and in light of recent discoveries, the sialic acid is likely to bind CCP20 (41). Bacteria either synthesize the sialic acid *de novo* or acquire it from their host by secreting a sialidase enzyme that cleaves sialic acid from host cells, which can then be taken up and presented via bacterial transporters (75). Currently, no pathogens have developed the ability to create sulfated GAGs (71, 76). However, bacteria have developed proteins that mimic host carbohydrates such as *Neisseria meningitides*, which produces a FH-binding protein that has been shown to bind to the CCP6–8 region of FH (77).

MODULATION OF THE COMPLEMENT RESPONSE BY CANCER

Like pathogens, cancer cells can also protect themselves from complement-mediated immune activation (78). FH and FHL-1 expression is up-regulated in some cancers (79) and inhibition of their expression reduces the growth rate of the cells *in vivo* (80). Cancer cells also commonly up-regulate sialic acid synthesis (81), possibly by up-regulating sialyltransferases (78), to reach a state that has been coined "super-self" (82). It is thought that increased surface levels of sialic acid confer protection against complement by recruiting FH (83) – removing sialic acid from cancer cells enhances their complement-mediated lysis (84) – and contributes to immune evasion from NK and other immune cells by non-complement-mediated mechanisms (78). Interestingly, many breast cancer cells have an increased amount of HS proteoglycans on their surfaces compared to normal mammary cells (85), and therefore, it is tempting to hypothesize that the up-regulation of this SAMP, like sialic acid, confers increased protection of cancer cells to complement by recruiting FH.

CONCLUSION

The structural diversity of GAGs and sialic acids makes a significant contribution to the regulation of immune homeostasis through the formation of "sugar postcodes" in human tissues. In particular, these sugars represent molecular signals capable of specifically recruiting either complement inhibitors, or activators, to a host surface in a tissue-specific fashion. Recent evidence suggests that changes to the GAG/sialic acid "repertoire" in a particular tissue, whether caused by disease or normal aging, can result in an inappropriate complement response and tissue damage. In some circumstances, it may be possible to correct this dysregulation of the innate immune system; e.g., the use of modified GAGs that interfere with the binding of properdin (but not FH) to HS on renal tubular epithelial cells might be of benefit in proteinuric renal disease (33, 34). As such, drugs aimed at modifying complement–sugar interactions in a tissue-specific manner could represent a viable therapeutic option in a number of disease contexts.

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Do antimicrobial peptides and complement collaborate in the intestinal mucosa?

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It is well understood that multiple antimicrobial peptides (AMPs) are constitutively deployed by the epithelium to bolster the innate defenses along the entire length of the intestines. In addition to this constitutive/homeostatic production, AMPs may be inducible and levels changed during disease. In contrast to this level of knowledge on AMP sources and roles in the intestines, our understanding of the complement cascade in the healthy and diseased intestines is rudimentary. Epithelial cells make many complement proteins and there is compelling evidence that complement becomes activated in the lumen. With the common goal of defending the host against microbes, the opportunities for cross-talk between these two processes is great, both in terms of actions on the target microbes but also on regulating the synthesis and secretion of the alternate family of molecules. This possibility is beginning to become apparent with the finding that colonic epithelial cells possess anaphylatoxin receptors. There still remains much to be learned about the possible points of collaboration between AMPs and complement, for example, whether there is reciprocal control over expression in the intestinal mucosa in homeostasis and restoring the balance following infection and inflammation.

Keywords: antimicrobial peptide, defensin, cathelicidin, Paneth cell, anaphylatoxin, lectin pathway, intestine, colitis

INTESTINAL EPITHELIUM AND INNATE DEFENSE

The cell boundary of the intestinal mucosa, interfacing the environment through the lumen, is comprised of a single-layer columnar epithelium, which in turn is composed of multiple cell types. These cells are undergoing constant renewal from epithelial stem cells in the crypt, with support of other epithelial and stromal cells in the niche. Progeny from the stem cells differentiate into the four specialized epithelial cell lineages; absorptive enterocytes with metabolic/digestive functions, mucus-secreting goblet cells, digestive-hormone secreting enteroendocrine cells, and Paneth cells. Paneth cells differ from other intestinal epithelial cells (IECs) in that they remain at the base of the crypts instead of migrating up the crypt during differentiation. Paneth cells are the major producer of antimicrobial peptides (AMPs) and they live longer than other IECs, surviving at the base of the crypt for approximately 20 days (1). IECs are interconnected through multiple molecular links but paramount among these are tight junctions, which control the permeability of the epithelial monolayer. Finally, scattered within the epithelium is a peculiar population of lymphocytes, the intraepithelial lymphocytes (IEL). IEL are squeezed between the basolateral borders of IECs and the two cell types communicate in maintaining the epithelial barrier (2). Mouse IEL reportedly express AMPs following exposure to bacteria (3). There undoubtedly remains more to be learned about IEL in defining the antimicrobial properties of the epithelium.

In addition to the cellular barrier, the innate defenses in the intestinal tract include highly glycosylated mucins (muc), secreted by goblet cells (4). The epithelium of the small intestine is overlayed with a single unattached mucus layer while two defined layers of mucus protect the colonic epithelium. In the colon, the inner layer is physically attached to the epithelium while the outer layer is unattached. Commensal microorganisms inhabit the outer, lower density mucus layer of the colon. Not surprisingly, degradation of the mucus layers permits contact between the IEC and bacteria. Illustrating the outcome of a comprised mucus layer, mice lacking MUC-2 develop colitis (5). In addition to mucins, goblet cells also produce trefoil factors, in particular trefoil factor 3 (TFF3), which facilitates mucin crosslinking and promotes epithelial repair, as well as resistin-like molecule-B (RELM-B), which stimulates MUC-2 secretion (4). TFF3 also induces a complement regulatory molecule, decay accelerating factor (DAF) on IEC (6). The mucus layer(s) are further impregnated with soluble factors that fortify the defensive capabilities. Secretory IgA, synthesized by B lymphocytes in the lamina propria, is transported into the mucus layer by IEC. Finally, AMPs and complement are found in the lumen, in the mucus.

AMPs OF THE IEC

There are multiple families of AMPs suggesting an evolutionary divergence in the intestinal mucosa, a rich habitat for microbes and a principle route of infection of the host. AMPs are active against a variety of organisms including gram-positive and gram-negative bacteria, parasites, fungi, and enveloped viruses (**Table 1**) (7).

Although there are many AMPs, the majority share a few common structural features including an overall positive charge (due to lysine and arginine residues) and an increased attraction to the hydrophobicity of bacterial membranes, due to an abundance of hydrophobic amino acid residues (7). Mentioned

Table 1 | Properties of AMPs in the intestines.

	Antimicrobial mechanism	Specificity	Murine version
α-defensins			
HD-5	Pore-forming	Gram-negative, gram-positive, viruses, fungi, parasite	Cryptidins
HD-6	Nanonet	Gram-negative, gram-positive, viruses, fungi, protozoa	Cryptidins
β-defensins			
hBD-1,2	Pore-forming	Gram-negative	mBD-1 (hBD-1) mBD-3 (hBD-2)
hBD-3	Pore-forming	Gram-negative, gram-positive	mBD-14
hBD-4	Pore-forming	Gram-negative, gram-positive, fungi	
C-type lectin			
RegIIIα	Unknown	Gram-positive	RegIIIγ
Cathelicidin			
LL-37	Pore-forming	Gram-positive, gram-negative, viruses, fungi, protozoa	CRAMP
Others			
Secretory phospholipase A2	Degradation of membrane phospholipids	Gram-positive	–
Lysozyme	Peptidoglycan hydrolysis	Gram-positive	–

earlier, Paneth cells are the main though not the exclusive source of AMPs. In response to IL-22, Toll-like receptor (TLR) and nucleotide oligomerization domain (NOD-2) signaling Paneth cells secrete lysozyme, secretory phospholipase A2 (sPLA2), α- and β-defensins, the C-type lectin regenerating islet-derived proteins (Reg), angiogenin 4, and cathelicidins in the small intestine, with the α-defensins being the most abundant (1, 4, 8). Paneth cell secretion of AMPs is important in maintaining spatial segregation of the intestinal microbiota from the epithelium (9). RegIIIγ-deficient mice consequently exhibit a defect in this segregation and microbes penetrate the mucus layer making intimate contact with host cells (10). In addition to providing AMPs, Paneth cells also help maintain crypt stem cells through the production of pro-growth factors such as WNT3 and Notch ligands (11).

Enterocytes are widely reported to produce AMPs including β-defensins (hBD-1,2,3,4), RegIIIα, and LL-37/human cationic AMP 18 (4, 12, 13). In fact, in mice temporary enterocyte expression of cathelin-related antimicrobial peptide (CRAMP) is important in allowing neonatal small intestinal colonization prior to the establishment of Paneth cells (14).

DEFENSINS

Defensins, arguably the most studied and well understood family of AMPs, target the surface membrane of microbes and function by forming pores leading to increased permeability of the membrane and the interruption of electrochemical gradients (**Table 1**) (15). The polypeptides are translated as an inactive precursor, which is cleaved to an active form. The primary protein sequence is a 87–94 residue peptide including a hydrophobic leader sequence, a short acidic pro-piece (which neutralizes the peptide), and a highly cationic mature sequence (7). Subtypes of defensins undergo different post-translational processing into an active cationic peptide, for example, in mice enteric α-defensins (cryptidins) are activated by matrix metalloproteinase matrilysin (MMP-7) (16). MMP-7 is a member of the metalloproteinase family of proteolytic enzymes produced by stromal fibroblasts and Paneth cells that degrade the extracellular matrix (17). Contrasting the situation in mouse cells, human Paneth cells only contain the pre-form of HD-5 and MMP-7 is undetectable (1). Thus, while human HD-5 was reported to be susceptible to MMP-7 cleavage, detection of a human homolog of the enzyme in the intestinal mucosa remains to be reported (18). Instead, an isoform of trypsin produced by Paneth cells was found to activate the protein resulting in multiple intermediates with variable levels of bactericidal activity (18, 19). Finally, a shorter amino-terminal extension in the human β-defensins permits bactericidal activity of the pre-forms (19). Otherwise the active defensins are 20–40 amino acids in length with three intramolecular disulfide bonds formed by a six-cysteine consensus sequence. The position of these intramolecular bonds is used to classify the family into α, β, and ζ defensins, with ζ defensins restricted to Rhesus monkeys (1).

α-Defensins

Alpha-defensins are classified based on a conserved pattern of six cysteines, which are linked 1–6, 2–4, and 3–5 (20). There are six subtypes of human α-defensins, four of which are found exclusively in neutrophils (HNP-1,2,3,4) and two of which are found in Paneth cells (HD-5,6), called the "enteric defensins" (1). Enteric defensins are found in rodents but the leukocyte α-defensins are not (1).

HD-5 exhibits direct bactericidal activity through a pore-forming mechanism but in an interesting contrast to the typical permeability-altering property of AMPs, HD-6 exhibits antibacterial activity indirectly (**Table 1**). HD-6 reportedly forms trap-like structures, which do not kill but instead immobilizes bacteria. HD-6 polymerizes to form peptide nanonets, which inhibit microbes from translocating across the intestinal barrier (21). Another difference is that HD-5 exhibits anti-parasite activity while HD-6 does not. Otherwise both human α-defensins share similar molecular structures, both exhibit anti-viral activity and

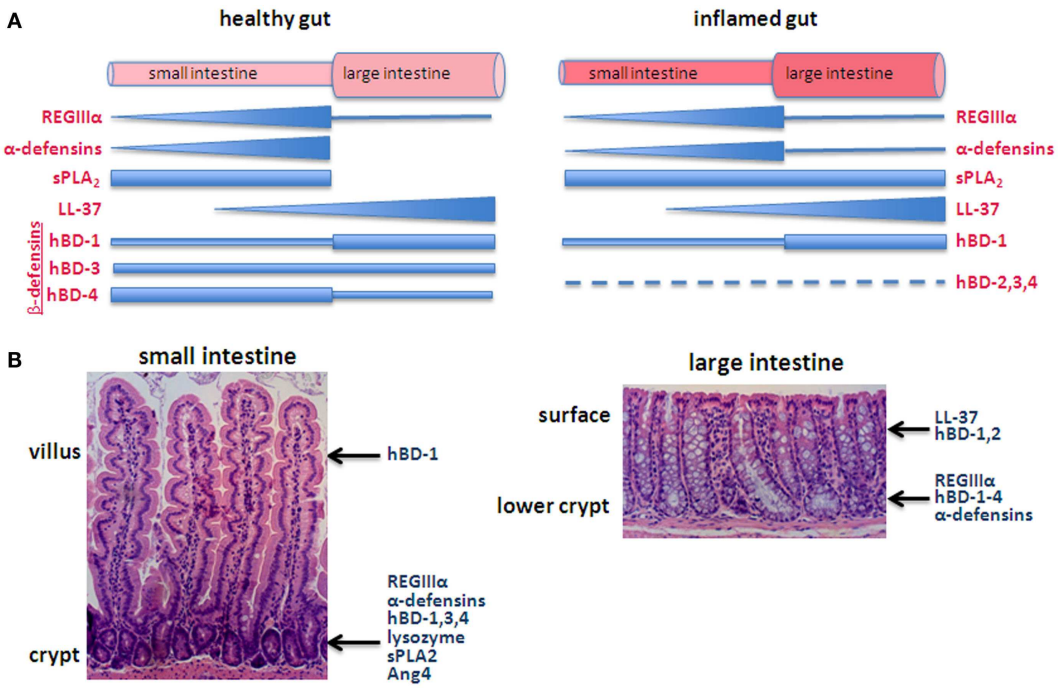

FIGURE 1 | Generalized depiction of AMP expression along the axes of the intestines. **(A)** Pattern of expression in the longitudinal axis, comparing the healthy (left) with inflamed (right) intestines. The thickness of the bar/triangle for each AMP roughly depicts the relative concentration of that particular AMP. For example, RegIIIα is found along the small intestine with highest levels in the ileum and low levels in the large intestine. α-Defensins are also predominantly expressed in the small intestine with levels corresponding with the increasing abundance of Paneth cells from the duodenum to ileum. The longitudinal distribution of complement has not been characterized. **(B)** The epithelium of the small intestine is organized into crypts and villi, or in the case of the large intestine, crypts, and surface epithelium (e.g., lacks villi), which create a vertical axis along which differentiating cells migrate. Some AMPs are secreted from different cell types along this axis. For example, β-defensins are secreted by goblet cells, Paneth cells, and enterocytes and thus are produced in locations along the crypt-villus axis.

both have been reported to be restricted to Paneth cells. HD-5 and HD-6 mRNAs are most highly concentrated in the ileum where Paneth cell abundance is highest (**Figure 1A**) (22). Relatively high levels of HD-5 are also detectable in the jejunum while levels of both HD-5 and 6 are low in the colon (21) (**Figure 1**).

β-*Defensins*

There are four human β-defensins (hBD-1,2,3,4), which are all expressed in keratinocytes and epithelial cells in a variety of human tissues (7, 20). The human β-defensins share a similar molecular structure with conserved cysteine residues linked 1–5, 2–4, 3–6 (20). With the exception of hBD-2, all human β-defensins are constitutively expressed in the small and large intestine (**Figure 1**). hBD-2 is unique in that it is detectable in low amounts in healthy tissues but is inducible with IL-1 (13). The molecules are expressed in Paneth cells and enterocytes and are all active against gram-negative bacteria. hBD-3 and hBD-4 additionally are active against gram-positive bacteria and are chemotactic for monocytes. hBD-1 mRNA is present in IEC at low levels throughout the intestines with highest expression in the colon (22).

RegIIIα

RegIIIα (also known as human hepatocarcinoma-intestine pancreas/pancreatitis-associated protein), is an AMP expressed in the liver, brain, and intestines of humans (23). It is present in the duodenum, jejunum, ileum, and colon, with expression highest in the crypts of the small intestine (**Figure 1**) (24). RegIIIα is a member of a large family of Reg genes but is one of only two RegIII genes found in humans (23). All are members of the C-type lectin family that bind glycan chains of peptidoglycan on the cell wall of gram-positive bacteria (25). The murine C-type lectin, RegIIIγ, is 65% identical to human RegIIIα and exhibits similar peptidoglycan binding (25). Paneth cells and enterocytes but not goblet cells express RegIIIγ (24, 25). Noteworthy, RegIIIγ lacks the complement recruitment domains present in other microbe-binding mammalian C-type lectins [such as mannose-binding lectin (MBL)] suggesting it is limited to direct anti-bactericidal activity (25).

LL-37

LL-37 is an AMP expressed in epithelial cells, keratinocytes, neutrophils, mast cells, monocytes, NK cells, B-cells, and γδ T cells (20). LL-37 belongs to the cathelicidin family of AMPs and functions in a similar way to defensins, by puncturing holes in the surface membrane of microbes (**Table 1**). LL-37 is unique in that in addition to antimicrobial activity, it has other immunological activities, acting through various receptors on cells (26). These activities include chemotaxis, wound healing, angiogenesis,

degranulation of mast cells, and neutralizing lipopolysaccharide (LPS) and lymphotoxin-A (LTA) (26). LL-37 expression can be either constitutive or inducible. In the intestine, LL-37 is produced constitutively by cells above the transit-amplifying zone in colonic crypts, with lower levels detected in the small intestine (**Figure 1B**) (27). Production can be increased when these cells are stimulated with short chain fatty acids (27).

Thus a picture emerges in which the mucus layer resting on the healthy epithelium is rich with AMPs, although the pattern of expression along the intestines implies specializations among the different molecules (**Figure 1**) (11). The idea that AMPs show differential distribution is also evident at another level, roughly along the crypt-to-villus axis (**Figure 1B**). For example, β-defensins are secreted by goblet cells, Paneth cells, and enterocytes and thus are produced along the crypt-villus axis (22) while hBD-3,4 expression is highest in cells of the lower crypt (12). RegIIIα is produced by Paneth cells in the crypts of the small intestine and detected in the crypt and lower villi (24, 28). Similarly, α-defensins are predominantly expressed by Paneth cells in the base of the crypts although HD-5 has been detected in villous epithelial cells of the jejunum and ileum (13, 29).

EPITHELIAL AMP EXPRESSION DURING INFLAMMATION

The cellular composition of the intestinal mucosa changes significantly during inflammation. This is in part due to the large numbers of infiltrating AMP-producing leukocytes as well as differences in the relative abundance of epithelial cell types resulting in altered expression of constitutive AMPs. One reported difference between the healthy and chronically inflamed intestinal epithelium impacting on AMP expression is an increased abundance of metaplastic Paneth cells in the colon (1). Colonic metaplastic Paneth cells produce α-defensins, lysozyme, and $sPLA_2$ (1). $sPLA_2$, in particular, is not detected in the healthy colon but is expressed by metaplastic Paneth cells as well as some colonocytes during inflammation (30). Other AMPs are also increased during inflammation. Murine RegIIIγ expression was increased during bacterial exposure and mucosal inflammation, and human RegIIIα expression was reported increased in patients with IBD (25, 31). Alpha-defensins are also induced in the large intestine during inflammation, associated again with metaplastic Paneth cells (1). Considering the importance of Paneth cells in providing AMPs, mutations in microbe sensing molecules in Paneth cells are thought to directly impact defensin production. However, the specific microbiome has emerged as an important factor in influencing defensin production even in mice with defects in these sensing molecules. This was demonstrated when defensin secretion by Nod2 gene knockout mice reverted to wild-type levels after exposure of the knockout mice to wild-type microbiota in co-housing experiments (32). Mixed findings have been reported for other AMPs and inflammation. hBD-1 levels have been reported to not change between the healthy and inflamed gut but contrarily, have also been shown to decline in ulcerative colitis (21, 33, 34). hBD-2,3, and 4 levels reportedly increase during ulcerative colitis but not Crohn's disease (12). LL-37 expression reportedly does not change during inflammation (27, 35). Thus, the impact of inflammation on AMP expression varies based on the AMP and the specific disease, and the generalizations in **Figure 1A** should not be understood to apply to all diseases.

Studies from gene knockout mice

Much of what we know about the role of AMPs in the intestines is derived from research done using mice. The importance of α-defensins in gut homeostasis was shown by examining the gut microbes of matrilysin deficient ($MAT^{-/-}$) mice, recalling that MMP-7 is required for α-defensin activation in mice (16). The lack of active α-defensins resulted in an impaired ability to control levels of both non-invasive and invasive bacteria in the intestines (16). Additionally, the oral lethal dose of an invasive strain of *S. typhimurium* was 90% less than that of the wild-type mice (16). Similar findings of increased susceptibility to bacteria were observed with mice experiencing graft versus host disease, which includes injury to Paneth cells resulting in reduced α-defensin production. Reduced α-defensin expression in turn was associated with changes in commensal bacteria populations and lower numbers of the major enteric commensals and higher numbers of minor enteric commensals (*E. coli*) led to septicemia in the mice (36).

Alpha-defensin expression in mice has been reported to be Nod2 dependent and consequently the Nod2 gene knockout mouse ($Nod2^{-/-}$) has been a popular model to study (37). $Nod2^{-/-}$ mice reportedly have higher levels of commensal bacteria as well as a reduced ability to prevent pathogenic enteric bacterial colonization (38). $Nod2^{-/-}$ mice infected by *L. monocytogenes* had lower numbers of specific cryptidins in their terminal ileum and were less successful in defending against the pathogen than wild-type mice. The mice also had larger populations of bacteria in their livers and spleens (37). $Nod2^{-/-}$ mice challenged with *Helicobacter hepaticus* suffered from granulomatous inflammation of the ileum but $Nod2^{-/-}$ expressing transgenic HD-5 killed the bacteria (39). However, again, these experiments were conducted without necessarily controlling for the impact of the microbiota.

Transgenic expression of HD-5 was used in other infectious models. Compared to wild-type mice, overexpression of HD-5 protected mice from infection by *S. typhimurium*. Wild-type strain mice died while the transgenic mice experienced less severe disease, less colonization, and recovered (40). These outcomes support the idea that defensins mediate protection beyond the regulation of commensal populations of microorganisms.

The role of cathelicidins has also been explored using mouse models. In addition to the role of CRAMP in colonization of the neonatal gut, CRAMP is also involved in the response to injury. Mouse colons inflamed with DSS showed increased levels of mCRAMP. $mCRAMP^{-/-}$ mice experienced worse colitis, which was reversible using exogenous mCRAMP or mCRAMP-encoding plasmids, confirming that the cathelicidins are protective in the colon (41, 42). In another study, cathelicidin deficient mice ($camp^{-/-}$) displayed a thinner inner colonic mucus layer than wild-type mice and had lesions on the surface epithelium due to a higher incidence of penetration and colonization by *E. coli O157* (43).

Similar to the $camp^{-/-}$ mice, $RegIII\gamma^{-/-}$ mice also presented with changes in mucus distribution and incidence of bacteria

on the mucosa of the ileum (44). A significant reduction in the amount of mucus was detected in RegIIIγ$^{-/-}$ mice due to changes in MUC-2 expression (44). Bacteria were observed in contact with the surface epithelium in the knockout mice; however, it is unclear whether this was due to the absence of the bactericidal activity of RegIIIγ or a consequence of changes in mucus distribution. RegIIIγ$^{-/-}$ mice were also reported to have higher numbers of gram-negative bacteria in their feces (44).

Taken together, the evidence that AMPs are important in the defense of the healthy and inflamed intestines is compelling. Yet it is not entirely clear why there is such a diversity of AMPs, some with varying patterns of expression along the length of the intestines. Additionally, even in the studies using gene knockout mice, it cannot be concluded that the AMPs act alone and directly to affect the phenotypes reported (for example, the role of the microbiome in shaping the AMP response was illustrated in Nod2$^{-/-}$ mice). There remains a high likelihood that AMPs act in concert with other defenses to achieve homeostasis and recover following injury and inflammation. The complement system is now emerging as one such parallel defense mechanism.

COMPLEMENT AND THE INTESTINE

Complement comprises a set of soluble proteins and membrane receptors and regulators that function in a highly coordinated manner to destroy microbes and facilitate removal of apoptotic/necrotic cells. Split complement molecules link the innate and adaptive immune systems, indirectly by acting on antigen presenting cells and directly by acting on leukocytes including lymphocytes. Despite the known crucial involvement of these functions in modulating the local response to microbes, the role of complement in the intestines is not completely understood.

Complement activation primarily occurs through one of the three pathways: the classical pathway (CP), lectin pathway (LP), and/or alternative pathway (AP) – all converging at the C3 convertase step. C3 convertases cleave C3 into C3a and C3b. C3b then associates with the C3 convertase to form a C5 convertase, which cleaves C5 into C5a and C5b. C5b become the nidus for binding C6, C7, C8, and C9 molecules to form the membrane attack complex (MAC), the lytic machinery of complement.

Each route of activation has proximal effectors that double as pattern recognition molecules. C1q, a proximal CP protein, combines with immune complexes forms a multimolecular complex with serine proteases, C1r and C1s. This complex cleaves C4 then associates with the product C4b into a complex, which cleaves C2 to form the CP C3 convertase. The LP is initiated by binding of MBL/ficolins to the mannose residues on microbial surfaces. Bound MBL recruits MBL-associated serine proteases (MASP-1 and MASP-2) that function similar to C1r and C1s by cleaving C4 and C2 to form the classical C3 convertase. The AP is unique as it does not require pattern recognition molecules to become activated. Instead, a "tick over" mechanism involves the spontaneous hydrolysis of C3 into C3(H_2O), which behaves similar to C3b and binds factor B (fB). Through a series of reactions involving factor D (fD) and properdin, the C3bBb complex forms the AP C3 convertase [reviewed in Ref. (45)]. Two additional models of AP activation have been proposed; (1) properdin, acting as a pattern recognition molecule, binds to a surface and provides a platform for C3 convertase assembly and, (2) C3b attached to a surface binds properdin, which in turn promotes AP convertase formation (46–48). In addition to the three principal pathways, evidence has emerged showing that complement may be activated through other mechanisms. For example, MBL can cleave C3 through a C2 by-pass activation mechanism (49, 50). MASPs reportedly cleave C3 to C3b thereby triggering the AP (51). Additionally, MASP-1, without the requirement of MBL, may cleave factor D from the pro- to mature form, again leading to AP activation (52). Finally, some coagulation pathway proteases can directly cleave C5 and C3 (53). Finally, an opinion has emerged that the AP may not be an entirely independent pathway but rather is responsible for complement amplification that was initiated by other pathways (54, 55).

COMPLEMENT IN THE INTESTINAL LUMEN

With regard to bolstering innate defenses in the mucosa, it is important to know whether complement proteins are present in the lumen. This question has not been systematically or comprehensively studied and the current understanding is incomplete. In support of complement in the lumen are published accounts of split complement molecules on the mucosa. In one example, C3b and MAC proteins were detected on the surface epithelium in patients with Crohn's disease and ulcerative colitis (56, 57). In another report that measured complement in lumen samples, higher C3 and C4 levels were found in jejunal secretions from Crohn's disease patients compared to healthy participants (58). Complement proteins were also detected in lumen samples collected from the small intestine of patients experiencing bacterial overgrowth (59). Noteworthy, and at odds with complement in the lumen, these authors failed to find MAC proteins, suggesting the MAC is not present (active) in the lumen. When considering where complement in the lumen may be derived from the pancreatic epithelium has been identified as a source of complement, with exocrine secretions arming at least the duodenum (60). Otherwise multiple reports identify epithelial cells as a source, for example, C4 mRNA was detected in both healthy participants' and Crohn's disease patients' mucosa while C3 expression was limited to crypt cells in inflamed samples (61). A lack of C3 detection in epithelial cells was repeated in another study that did detect C3 in colonic subepithelial myofibroblasts (62). Factor B proved to be among the most highly increased complement molecules in epithelial cells in inflamed mucosa of IBD patients compared to healthy donor mucosa, suggesting that inflammation leads to arming the AP in the intestine (63). On the other hand, MBL has not been detected in mouse or human mucosa suggesting this proximal activator of the LP may not be active (64). Despite the lack of C3 detection using *in situ* techniques, C3 has commonly been found in epithelial cell lines. Bemet-Camard et al. reported that T84, Caco-2, HT-29, and a non-transformed IEC line (INT407) were all positive for C3 and C4 (65). Another study repeated the finding that Caco-2 cells produce C4 and fB but also C3 (66). In addition to constitutive production, stimulation of IEC with cytokines such as TNF, IL-6, and IL-1β increased the production of complement proteins (66). However, consistent with the lack of MAC in the luminal secretions from small intestine, this study failed to detect MAC mRNAs. How is the lack of local MAC

synthesis compatible with the detection of MAC on the mucosa? Bleeding in ulcerated parts of the intestines could result in complement in the lumen. Additionally, infiltrating leukocytes make complement and could act as a source. Neutrophils are the leading source of properdin and by providing properdin following infiltration these cells, neutrophils may mediate AP amplification in the lumen (46, 67). Altogether, these findings suggest that the intestinal epithelium does not synthesize all the complement molecules and this raises questions over what role complement therefore plays in the healthy intestines. During inflammation, the remaining proteins are perhaps provided by infiltrating cells and/or blood resulting in complete pathways, and local activation becomes possible. Finally, the idea that complement activation occurs, including through the MAC, is indirectly supported by epithelial cells possessing CD55 and CD59 on the apical surface. These membrane proteins are negative regulators of the convertases and MAC, respectively (68). As for complement impacting the intestinal epithelium from the lumen, we reported that cell lines apically express the C5aR and respond to C5a with increased CXCL8 mRNA, introducing the possibility that anaphylatoxins provide danger signals to the epithelium (69). In summary, many complement proteins are present in the uninflamed mucosa and complement is activated during inflammatory conditions, opening the possibility that AMPs and complement may collaborate to enhance the innate defenses in health and disease in the intestines.

COMPLEMENT AND AMP CROSS-TALK AT THE INTESTINAL MUCOSA

Secreted into the mucus layer, there is good reason to suppose these two antimicrobial systems interact on microbial targets but also possibly through the reciprocal regulation of expression, whether agonistically and/or antagonistically (**Figure 2**). The fact is that such interactions have not yet been described in the intestines and we can only hypothesize on the manner of interaction. Noteworthy is a recent report from Chehoud et al. who found that antagonizing C5aR resulted in a decline in diversity of the skin microbiota of healthy mice associated with changes in immune effectors including AMPs (70). Such a relationship between split complement effectors, AMPs, and microbes could certainly be active in the intestines.

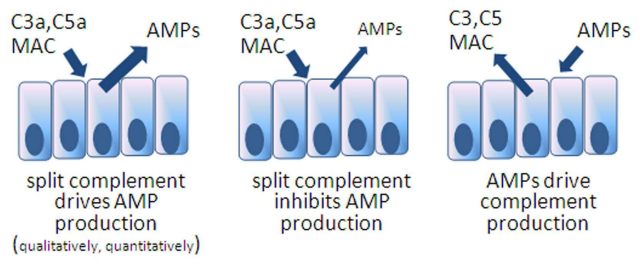

FIGURE 2 | Models speculating on reciprocal interactions between AMPs and complement expression in the intestines. The first model is inferred from the example of C5aR blocked mice showing deficiencies in skin AMPs. The others remain to be tested.

AMP/COMPLEMENT COLLABORATION?

One obvious functional convergence between AMPs and complement is the common antimicrobial activity. Complement activation leads to antimicrobial activity with the MAC but surprisingly, C3a also has microbicidal activity. The antimicrobial specificity of C3a and other peptides following cleavage of C3 is broad, including gram-negative and gram-positive bacteria (71) and fungi (72). C3 has a long history of evolution and was present before elements of the MAC evolved. Perhaps primitive organisms depended on C3 split products having direct antimicrobial activities. Perhaps also related to this long evolutionary history is the discovery that cells, including epithelial cells, constitutively make and cleave C3 into C3a as an autocrine survival signal (73). It will be interesting to know whether there is a relationship between C3a antimicrobial potency and organisms which depend more on innate defenses, compared to higher vertebrates with adaptive immune defenses.

In addition to acting independently to repel microbes, these two systems may collaborate by reducing the effective concentration needed for lysis when both are present. Experiments to test this potential have not yet been reported but are certainly achievable. In another manner, HD-6 presents an interesting possibility for collaboration with complement, drawing on an example from neutrophils. Mentioned earlier, HD-6 forms a lattice in the mucus; how closely this lattice resembles neutrophil extracellular traps (NETs) is not clear but NETs do collaborate with complement. NETs are the discharged nuclear and cytoplasm contents of dying neutrophils giving their best final effort to impede microbes. C1q binds to NETs leading to complement activation (74). The HD-6 lattice could function as a foundation for focusing complement activation by complement pattern recognition molecules like properdin. By this mechanism the contribution of HD-6 may be ensuring complement activation occurs, in addition to the physical impedance of microbes.

Contradicting the speculation that the two systems may act in synergy is a report showing that an AMP can interrupt complement activation, at least *in vitro*. Bhat and co-workers found that HBD-2 (but not HBD-1) bound C1q and prevented activation of the classical pathway *in vitro* (75).

RECIPROCAL CONTROL OVER EXPRESSION

Another likely point of interaction between complement and AMPs is the possibility that there is reciprocal control over expression of the two systems (**Figure 2**). An example can be drawn from more primitive organisms, mosquitoes, where C3-like molecules drive AMP expression in order to control viruses (76). In this regard, it would be interesting to know whether mice deficient in specific AMPs, as discussed earlier, respond by increasing local complement concentrations or possibly respond with reduced levels if the AMP directly impact complement synthesis. On the other hand, and considering that some AMPs are constitutively produced and that complement is present but not activated (with perhaps cytoplasmic C3 being the exception), the reciprocal situation may be true: that activated complement products impact AMP expression. Anaphylatoxin receptor gene knockout mice have been applied in various models of colitis with an incomplete understanding of whether other innate defenses have been compromised. A rational line of investigation will be to determine

the impact of anaphylatoxins on AMP expression in IEC, including Paneth cells.

CONCLUSION

Considerable attention and progress has been made in understanding the cell sources and contribution of AMPs to defense of the intestines. Multiple AMPs show regional patterns of expression in both axes of the intestines, longitudinally and within the crypt-to-villus axis. The other antimicrobial system, complement, is present and becomes activated in the mucosa but we are only beginning to explore the impact of this activation, including on AMP activity and expression. We offer some hypotheses for further investigation into what is certain to be a closely coordinated collaboration between the two systems in the defense of the host.

AUTHOR CONTRIBUTIONS

All authors researched the content, composed a section of the review, edited multiple drafts, and gave final approval of the manuscript.

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P. gingivalis in periodontal disease and atherosclerosis – scenes of action for antimicrobial peptides and complement

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According to the NHS, it is estimated that over 50% of the adult population are, to some extent, affected by gum disease and approximately 15% of UK population have been diagnosed with severe periodontitis. Periodontitis, a chronic polymicrobial disease of the gums, causes inflammation in its milder form, whereas in its severe form affects the surrounding tissues and can result in tooth loss. During periodontitis, plaque accumulates and sits between the junctional epithelium and the tooth itself, resulting in inflammation and the formation of a periodontal pocket. An interface is formed directly between the subgingival bacteria and the junctional epithelial cells. Bacterial pathogens commonly associated with periodontal disease are, among others, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, together known as the "red complex." This review will mostly concentrate on the role of *P. gingivalis*, a Gram-negative anaerobic bacterium and one of the major and most studied contributors of this disease. Because periodontal disease is associated with the development of atherosclerosis, it is important to understand the local immune response to *P. gingivalis*. Innate immune players, in particular, complement and antimicrobial peptides and their effects with regard to *P. gingivalis* during periodontitis and in the development of atherosclerosis will be presented.

Keywords: *P. gingivalis*, complement system, antimicrobial peptides, periodontitis, atherosclerosis

MODE OF ACTION OF *P. GINGIVALIS* DURING PERIODONTITIS

Porphyromonas gingivalis is an anaerobic Gram-negative bacterium involved in the onset of inflammation and tissue destruction during periodontal disease. It can be found in small numbers in the oral cavity of healthy individuals (1, 2). Pathology occurs when *P. gingivalis* binds to and accumulates on the tooth surface, leading to the development of a mixed biofilm, the expansion of the bacteria into the gingival sulcus, and the formation of a periodontal pocket (3). Inside this periodontal pocket lies the gingival crevicular fluid, an inflammatory exudate – source of essential nutrients for *P. gingivalis* growth – present in low abundance in healthy individuals, but drastically increased during gum inflammation. In this milieu, *P. gingivalis* invades gingival epithelial cells via the binding of its fimbriae to β1 integrin on the host cell surface followed by a rearrangement of the host actin cytoskeleton (4, 5). It then blocks apoptosis through the PI3K/Akt and JAK/Stat pathways, allowing intracellular bacterial proliferation (6, 7). In addition, it inhibits IL-8 expression by epithelial cells, creating what is known as the "local chemokine paralysis" (8). This mechanism induces a delay in neutrophil recruitment, which allows the proliferation of bacteria in this new niche, leading to an alteration of the subgingival microbiome with respect to its composition and total bacterial count (9, 10). The emergence of this dysbiotic assembly of microorganisms is believed to be partly responsible for the pathology observed. This is supported by findings in a murine model of *P. gingivalis*-induced periodontitis, where *P. gingivalis* was shown to contribute to periodontal bone loss by reshaping the normal commensal microbiota, while it failed to induce bone loss in germ-free animals (11). Its activity as a "keystone pathogen" may well arise directly from its atypical LPS, which does not activate TLR4 – acting either as a weak TLR4 agonist or even a TLR4 antagonist according to the local inflammatory state – and rendering it immunologically silent, potentially facilitating the initiation of the colonization (12).

MANIPULATION OF THE COMPLEMENT SYSTEM BY *P. GINGIVALIS*

Early studies documented the activation and regulation of complement components in the gingival crevicular fluid where complement is believed to be present at 70% of its serum concentration (13, 14). *P. gingivalis* has developed different strategies to evade killing by the complement system. First, its surface anionic polysaccharide confers *P. gingivalis* serum resistance (15). Moreover, two types of cysteine proteases – known as gingipains – are produced by *P. gingivalis*: the lysine specific Kgp and the arginine specific RgpA and RgpB (16). While these proteases take part in the destruction of the extracellular matrix, they are also able to cleave the complement components C1, C3, C4, and C5, as well as to capture C4b-binding protein (17–20). This leads to the inhibition of complement activation, but intermittently also to a local accumulation of the anaphylatoxin C5a, the only bioactive fragment present after the actions of gingipains (20). While the

massive degradation of complement proteins does not directly benefit complement resistant *P. gingivalis*, it could allow the colonization and proliferation of other bacterial strains with a higher sensitivity toward complement killing.

The local gingipain-induced accumulation of C5a at the site of infection then activates C5aR. $C5aR^{-/-}$ mice have been shown to be resistant to age dependent as well as *P. gingivalis*-induced experimental periodontitis (11, 21). Similarly, periodontal inflammation and subsequent bone loss could nearly be abrogated by treating conventional wild-type animals with a C5aR antagonist, underlining the important role played by this anaphylatoxin during periodontitis (22). In neutrophils, *P. gingivalis* has been shown to inhibit bacterial killing in a Mal/PI3K, C5aR-, and TLR2-dependent manner (23). This could explain the increase in anaerobic oral bacteria and the change in microbiota observed after infection with *P. gingivalis* in conventional, but not $C5aR^{-/-}$ mice (11). In macrophages, a C5aR-TLR2 crosstalk has been demonstrated to activate the cAMP-dependent PKA pathway, leading to a reduction of intracellular nitric oxide, which permits intracellular bacterial survival (19). The presence of CXCR4 activation further accentuated this synergism (19, 24). This C5aR-TLR2 crosstalk seems particularly important in understanding how *P. gingivalis* can directly dampen the immune response in an already immunologically tolerant tissue such as the mucosa. In addition, C5aR activation in macrophages inhibits the TLR2-induced IL-12p70 production (21).

The interaction of *P. gingivalis* fimbriae with TLR2 leads to the inside-out activation of the β2 integrin CR3 (CD11b/CD18) via PI3K (25). Direct interaction of *P. gingivalis* fimbriae with the chemokine receptor CXCR4 similarly results in CR3 activation (26). In macrophages, *P. gingivalis* uses this TLR2-activated CR3 as a port of entry as well as to survive intracellularly (25). In fact, inside-out activation of CR3 has been shown to suppress IL-12p70 production in macrophages (21, 25, 26). Also, the pro-inflammatory cytokines IL-1β, IL-6, and TNFα, known to induce bone resorption, are up-regulated in a C5aR/TLR2- and CR3-dependent manner by *P. gingivalis* (21, 23, 27). The resulting inflammatory breakdown products may then further strengthen the dysbiosis as recently suggested by a study underlining the inflammophilic character of the periodontitis-associated microbiota (28). Taken together, these results highlight the role played by the complement system during periodontitis: *P. gingivalis* manipulates the host complement components to escape immune clearance, colonize its new niche, and reshape the local microbiota.

ANTIMICROBIAL PEPTIDES OF THE ORAL CAVITY

The oral cavity is home to various peptides with antimicrobial activity, secreted by epithelial cells, neutrophils, and salivary glands. Their expression often increases during periodontitis [reviewed in Ref. (29)]. One of these molecules, the cathelicidin LL-37, plays a major role in oral health, as illustrated by the severe periodontitis observed in patients suffering from either Kostmann or Papillon–Lefèvre syndromes, two rare conditions characterized by the absence of mature bioactive forms of LL-37 (30, 31). Various studies have nevertheless suggested that cathelicidins only possess a very limited direct microbicidal activity *in vivo* and instead exert a plethora of immunomodulatory effects [reviewed in Ref. (32)]. More recently, LL-37 has been shown to promote phagocytic uptake by macrophages, which could be used at its advantage by *P. gingivalis* (33). Alpha (HNP1-3) and beta (hBD1-3) defensins are another class of antimicrobial peptides present in the gingival crevicular fluid (29). During periodontitis, the expression of cathelicidins, α, and β defensins is increased in the gingival crevicular fluid, most particularly in the presence of *P. gingivalis* (34–36). However, *P. gingivalis* has been shown to be highly resistant to killing by LL-37 *in vitro*. Similar observations were made for defensins, suggesting that the higher antimicrobial activity observed during periodontitis may have very little direct effect on *P. gingivalis*, but most probably has a major impact on other more susceptible bacteria (36–38). This could represent another way by which *P. gingivalis* shapes the local microbiota thereby selecting for periodontopathic strains, non-periodontopathic strains having been shown to be more susceptible to the activity of antimicrobial peptides (36, 38). Importantly, LL-37 can act as a pro-inflammatory trigger during periodontitis. In fact, as well as being a chemoattractant for neutrophils expressing FPRL1 receptor, LL-37 was demonstrated to induce the production of leukotriene B4 (LTB_4), a potent chemotactic agent, in human neutrophils via binding to the cathelicidin receptor FPR2/ALX (39, 40). LTB_4 can then trigger LL-37 release by neutrophils in an autocrine manner, thus creating a pro-inflammatory loop eventually leading to bone tissue destruction. This inflammatory response is eventually dampened by lipoxin A_4 – a ligand for the FPR2/ALX receptor produced during the resolution phase of inflammation (39, 41, 42). Determining copy number variation in antimicrobial peptides and screening for relevant SNP may help to stratify those at risk of developing aggressive periodontitis who would benefit from early periodontal management (43–45).

EVASION FROM THE ORAL CAVITY: LINK TO CARDIOVASCULAR DISEASES

Numerous studies have associated chronic periodontitis with various diseases, such as rheumatoid arthritis, diabetes, and cardiovascular diseases (46–48). Similarly, *P. gingivalis* has been observed at other sites than the oral cavity (49–51). While the exact mechanism used by *P. gingivalis* to reach distant anatomical locations has not yet been defined, *P. gingivalis* has been shown to survive intracellularly in macrophages, epithelial, endothelial, and smooth muscle cells and to be able to spread from one cell to another (4, 19, 25, 52). *P. gingivalis* could therefore potentially use these cells as means of transportation to travel to peripheral tissues.

Atherosclerotic disease has long been viewed as a manifestation within disease complexes such as metabolic syndrome, renal failure, and other chronic inflammatory conditions. The atherosclerotic plaque is a site of inflammation within the arterial intima, where inflammatory cells and lipids accumulate. Viable periodontic pathogens, including *P. gingivalis*, have been found in atherosclerotic plaques in mice and in humans (49–51). Antimicrobial peptides and complement activation products are both constituents of the plaques (53–55). The abilities of *P. gingivalis* to manipulate the complement and the antimicrobial systems in remote location could putatively contribute to the progression of

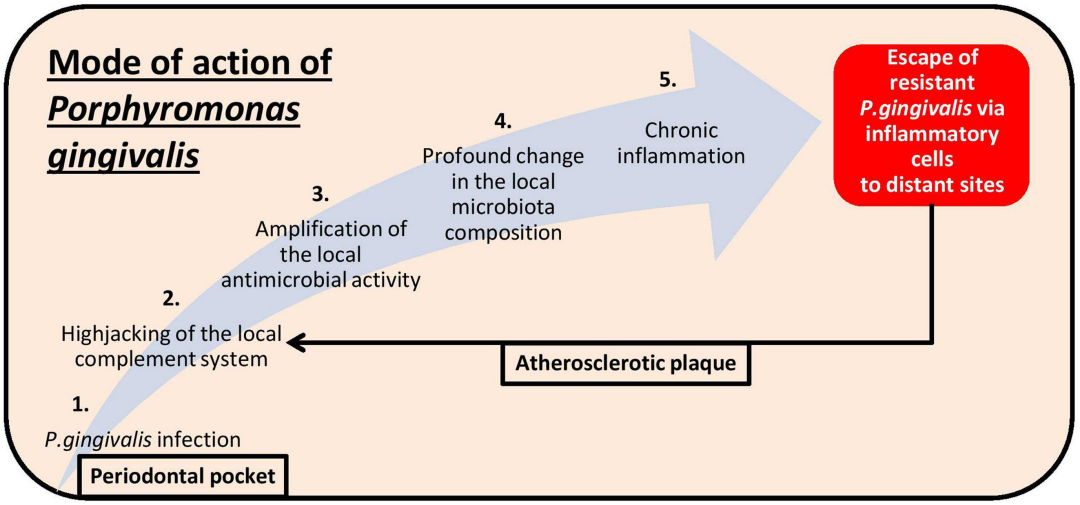

FIGURE 1 | Pathomechanistic sequence of events leading to periodontitis following *Porphyromonas gingivalis* infection (light blue arrow) as well as to the exacerbated pathophysiology observed in atherosclerosis plaques after evasion of the bacteria from the oral cavity (black arrow).

atherosclerosis. In fact, *P. gingivalis* has been shown to accelerate plaque formation in an apolipoprotein $E^{-/-}$ mouse model (56).

C5a is present in atherosclerotic plaques and acts as a proatherogenic molecule (57). While it does not seem to play a role in the initial development of the pathology, C5a has been shown to promote apoptosis in endothelial and smooth muscle cells as well as to induce the expression of the metalloproteases MMP1 and MMP9 in macrophages in atherosclerotic plaques. This leads to the degradation of the extracellular matrix and to the rupture of the plaque (57–59). Similarly, reduced plaque size was observed after treatment of $ApoE^{-/-}$ mice with a C5aR antagonist (60).

Elevated expression level of LL-37 has been reported in atherosclerotic lesions, where it is thought to modulate the local immune response and induce apoptosis in vascular smooth muscle cells (54, 61). The presence of LL-37-resistant *P. gingivalis* in the lipid plaque could lead to an increase of the local concentration of antimicrobial peptides. Together with the gingipain-dependent local accumulation of C5a in the vicinity of *P. gingivalis*, this could be responsible, at least in part, for the exacerbated pathophysiology observed in the mouse model as well as in human disease.

POTENTIAL THERAPEUTICS

The molecular actions involving complement and antimicrobial peptides (and others) in the oral cavity are now well known but the systems are not easily amenable to therapeutic targeting. Treatments against periodontitis consist mainly on reducing the formation of bacterial plaque in the oral cavity using physical and chemical forces. Antibiotics may be given as a short course but they usually only accompany periodontal treatment, as they have difficulties to penetrate periodontal biofilms. Various isolates of oral bacteria such as *Lactobacilli* have been shown to reduce *in vitro* the growth of different periodontopathogens including *P. gingivalis* (62, 63). Clinical trials confirmed the potential of these probiotic agents to be used as a complement to periodontal treatments (63–65). Vitamin D supplementation with its beneficial effect on bone mineralization and its anti-inflammatory potential (inhibition of IL-6, IL-8, and TNFα) may as well be an additional therapy to consider (66, 67). Another option consists on the use of proresolving mediators; in fact, topical applications of the resolvin RvE1 molecule were able to reduce and even to some extent restore periodontitis-associated bone loss in a rabbit model of experimental periodontitis (68, 69). However, the most promising therapy, to date, remains the periodontal vaccines as immunization has been shown to protect against experimental periodontitis in different animal models and could potentially prevent the overt inflammation observed in associated diseases (56, 70).

CONCLUSION

Porphyromonas gingivalis is a good example of a bacterium able to shape the composition of its microbial environment and to subvert the immune system toward chronic inflammation (**Figure 1**). Evidence of periodontopathogens in atherosclerotic plaques implies a direct role – which might have justified the recent broad population health advice of increasing oral hygiene – but the concomitant presence of oral and gut commensals in biopsies of atherosclerotic arteries begs as well the question of how leaky our mucosal tolerance barrier is.

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Chemokine function in periodontal disease and oral cavity cancer

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The chemotactic cytokines, or chemokines, comprise a superfamily of polypeptides with a wide range of activities that include recruitment of immune cells to sites of infection and inflammation, as well as stimulation of cell proliferation. As such, they function as antimicrobial molecules and play a central role in host defenses against pathogen challenge. However, their ability to recruit leukocytes and potentiate or prolong the inflammatory response may have profound implications for the progression of oral diseases such as chronic periodontitis, where tissue destruction may be widespread. Moreover, it is increasingly recognized that chronic inflammation is a key component of tumor progression. Interaction between cancer cells and their microenvironment is mediated in large part by secreted factors such as chemokines, and serves to enhance the malignant phenotype in oral and other cancers. In this article, we will outline the biological and biochemical mechanisms of chemokine action in host–microbiome interactions in periodontal disease and in oral cancer, and how these may overlap and contribute to pathogenesis.

Keywords: chemokine, periodontitis, inflammation, oral cancer, host–pathogen interactions, Toll-like receptor

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Introduction

The human body is constantly under assault from a plethora of environmental factors that includes chemical, physical, and microbiological agents. In turn, cellular mechanisms have developed to combat these noxious stimuli, and cells of the innate immune system are central players. Sophisticated molecular regulatory pathways exist to coordinate the host response to bacterial infection and other microbiological challenges. Innate immunity also plays a fundamental role in the pathogenesis of malignant disease, acting as a surveillance mechanism to prevent tumor establishment. On the other hand, the cellular immune response is now recognized as a key promoter of tumor progression and metastasis through potentiation of chronic inflammation at tumor sites. Today, it is widely accepted that deregulated inflammation within the cellular microenvironment is one of the key elements driving tumorigenesis (1–4). Therefore, how immune and inflammatory processes are regulated and how they may result in different outcomes to the host are intriguing questions that are beginning to be understood in some detail.

The oral cavity is home to a diverse microbial community of more than 700 microbial species including commensal and opportunistic bacteria, viruses and fungi living in a symbiotic relationship with each other and the host immune system (5–7). The host–microbiome interactions at the oral mucosal surface are critical to maintain periodontal tissue homeostasis, and the balance between

microbial cell turnover and host pro-inflammatory and anti-inflammatory responses eventually determines the clinical outcome. Deregulated host immune responses resulting from environmental and systemic exposures (e.g., smoking, obesity, stress, aging, diabetes), host genetic, and epigenetic defects and/or dysbiotic oral microflora subverting the host defense mechanisms lead to chronic periodontitis (8–13). Thus, today it is believed that although microbial insult initiates the periodontal disease, deregulated immune response mechanisms determine the progression of the lesion and the extent of tissue destruction.

Periodontal disease affects 47% of the population (14). It is defined as inflammation of the periodontium involving the supporting tissues of the teeth and it is characterized by loss of epithelial attachment, connective tissue, and alveolar bone. Chronic periodontitis also serves as a constant reservoir of inflammatory mediators and microbial products that can act upon host tissues. Thus, besides its destructive local effects, persistent forms of the disease are also associated with several systemic conditions, including cardiovascular diseases, adverse pregnancy outcomes, rheumatoid arthritis, diabetes, and pulmonary diseases (15–21).

Emerging evidence also suggests a link between periodontitis and oral cancer, the rationale being that chronic inflammation is a major factor in both diseases (22, 23). The ability to re-route immune cells to a site of infection within the body relies in large part on the action of chemotactic cytokines, or chemokines. These are small polypeptides that are secreted into the microenvironment, and which serve to recruit leukocytes and other immunological mediators to their point of action, such as periodontal inflammatory foci. However, as well as these functions, some chemokines and their receptors have been implicated in cancer development and progression by promoting cell proliferation, motility, angiogenesis, and metastatic spread (3).

In this review article, we will outline the pathogenesis of periodontal disease and oral cancer and the plausible biological mechanisms that may link these, focusing on chemokine ligand and receptor function, and how this might promote tumorigenesis through modulation of the microenvironment.

Periodontal Disease Pathogenesis

The development and progression of periodontitis is a complex process initiated by a dysbiotic polymicrobial insult and involves multiple host cells of myeloid and non-myeloid origin including oral keratinocytes, neutrophil polymorphs (PMNs), macrophages, monocytes, dendritic cells, osteoblasts, and osteoclasts. These cells possess cytosolic, membrane-associated, and secreted pattern recognition receptors (PRRs) including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLR), and C-type lectin receptors, which can engage with periodontal microbial associated molecular patterns (MAMPs) [e.g., lipopolysaccharide (LPS), lipoproteins, fimbriae, BspA, nucleic acids] and damage/danger associated molecular patterns (DAMPs) (e.g., nucleic acids, fibrinogen, heat-shock proteins). This interaction releases inflammatory mediators that aid in the development of an efficient innate immune response to eliminate the pathogen and coordinate development of an adaptive immune response. Although activation of the immune system is crucial to combat microbial insult, deregulated, persistent immune responses due to factors that are still not completely understood result in chronic inflammation, eventually leading to periodontal tissue destruction (24).

In its most general classification, periodontal disease can be categorized as either *gingivitis* or *periodontitis*. Gingivitis defines the inflammation of gingival tissues without alveolar bone loss, whereas periodontitis is accompanied by destruction of alveolar bone. Periodontal disease progression follows four histological stages: *initial*, *early*, *established*, and *advanced* (25). These definitions are based on the distinctive histological features of the pathological site with regard to the cells and the extent of tissue destruction involved. However, it is important to note that disease progression is a dynamic and highly interactive process where there is overlap between each stage, with common cells and inflammatory mediators.

The *initial* sequela to the polymicrobial insult is an acute inflammatory response with increased vascular dilation and blood flow. There is activation of complement and kinin systems and arachidonic acid pathways. There is also increased PMN migration toward the lesion due to chemotactic stimuli originating both from microbial cells as well as host-derived inflammatory mediators. These include IL-8 (CXCL8), complement components C5a and C3, and leukotrines. Some collagen loss is also apparent. The character and intensity of the inflammatory response determine whether the *initial* lesion resolves rapidly, with restoration of the tissue to a normal state, or whether it evolves into a chronic inflammatory lesion.

Early lesion is characterized by accentuation of the features of the *initial* lesion with increased vascularization, accumulation of more PMNs and lymphocytes (mainly T-cells), as well as continuous activation of complement and arachidonic acid pathways. Macrophages, plasma cells, and mast cells start to appear at the site of acute inflammation. These cells produce pro-inflammatory mediators including IL-1β, IL-6, IL-8, IL-17, and TNF-α, which may exacerbate the inflammatory response and promote progression to more advanced stages of disease. There is further loss of the collagen fiber network around the inflammatory infiltrate due to activation of the local immune system.

As the disease progresses into the *established* phase, there is a transition from an acute to a chronic state. The manifestations of *early* and *initial* changes still persist, along with the appearance of B-lymphocytes and continuingly increased numbers of PMNs, macrophages, monocytes, and T-cells. There is also the presence of extravascular immunoglobulins in the connective tissue and in the junctional epithelium. In the *established* lesion, which is clinically diagnosed as *chronic gingivitis*, there is substantial loss of gingival extracellular matrix due to increased collagenase activity and activation of the local immune system, but without bone loss.

The *advanced lesion* constitutes the final stage of the transition to periodontitis. At this stage, there is progression of inflammation to involve the alveolar bone. Production of inflammatory mediators such as cytokines, chemokines, arachidonic acid metabolites (prostaglandins), and complement proteins by activated PMNs, macrophages, monocytes, lymphocytes, fibroblasts, and other host cells can cause oxidative damage by promoting the release of tissue-derived enzymes such as matrix metalloproteinases (MMPs). Furthermore, cytokines can act on stromal and

non-stromal cells causing increased expression of receptor activator of nuclear factor kappa-B ligand (RANKL) while decreasing osteoprotegerin (OPG) production. If the inflammation is not resolved, destruction of extracellular matrix and irreversible alveolar bone loss occur.

In summary, a polymicrobial insult initiates the periodontal disease process while the progression and clinical presentation of the disease involve complex interactions between host cells and the oral microbiome, triggering persistent inflammation, which eventually destroys the local tissues and further impacts distant sites due to the continuous access of inflammatory mediators and microbial components into the systemic circulation. The biological and biochemical pathways that may link chronic periodontitis with various systemic diseases are reviewed elsewhere (15–21).

Pathogenesis of Oral Squamous Cell Carcinoma

Squamous cell carcinoma is the most frequently occurring malignancy of the oral cavity and adjacent sites, representing over 90% of all cancers. Worldwide, 200,000 new cases of oral cavity and lip cancer are diagnosed annually, with around 98,000 deaths (http://globocan.iarc.fr/Pages/fact_sheets_population.aspx). The predominant etiological factors for oral cavity cancer are alcohol and tobacco use, with carcinogens impacting on the oral mucosa to create a field that is predisposed to undergo malignant transformation, so-called "field cancerization." Although, with early detection, "cure" rates may be as high as 50%, most lesions are diagnosed at a late stage and have a much poorer prognosis due to locoregional or distant spread, with 5-year survival as low as 16%. Disappointingly, clinical outcomes have not improved significantly in decades, in spite of advances in surgical, chemotherapeutic, and radiotherapeutic management, as well as the advent of targeted therapies.

The molecular pathogenesis of oral cavity cancer is, in many cases, the result of dysregulation of common signaling pathways that actively drive oncogenesis, on a background of tumor suppressor inactivation. The basis for this may be a combination of somatic mutations, as described recently (26), together with epigenetic and transcriptomic alterations. As just one example, hyperactivation of epidermal growth factor receptor (EGFR) signaling through receptor overexpression, or mutation of either the receptor or downstream signaling components, can lead to enhanced cell proliferation and motility, thereby contributing to tumor growth and metastasis. Inactivation of the *P53* and *CDKN2A* tumor suppressors also occurs with high frequency in oral cancer. In addition to molecular dysregulation as a result of chemical carcinogens in tobacco and alcohol, infectious agents play a role in development and progression of oral cancer. Although considerably less frequent than in the oropharynx, human papillomaviral-related carcinogenesis contributes to a small proportion of oral cavity cancers [around 10%: (26)]. What is less clear, however, is a mechanism that might explain the long-standing notion that poor oral hygiene is related to OSCC development. As in other cancers, though, there is no doubt that chronic inflammatory conditions underpin oral carcinogenesis, and perturbations of cytokine- and chemokine-dependent immunoregulatory pathways are evident in oral cancer. Of course, the oral cavity is prone to a number of bacterial infectious diseases, such as periodontitis, and it is possible that oral bacteria may serve to initiate or promote tumor development, analogous to the association of gastric cancer with *Helicobacter pylori* infection. In fact, a number of periodontal bacteria including *Prevotella intermedia*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* have been associated with OSCC (27, 28) The plausible biological mechanisms, including microbial and inflammatory, that may link periodontitis and oral cancer are discussed below.

Chemokines

Members of the chemokine superfamily are secreted polypeptides, which range in molecular mass from around 5 to 20 kDa. Historically, these were named based on their function (e.g., macrophage inflammatory protein: MIP-1α, β), but a generic nomenclature is now in use. This categorizes chemokines into four subfamilies based on the relative position of conserved cysteine residues within the polypeptide. Thus, C, CC, CXC, and CX_3C chemokines are recognized (**Figure 1**).

Chemokine ligands bind to cell surface receptors and the interactions may be unique (single ligand and single receptor) or promiscuous (single ligand/multiple receptors, or multiple ligands/single receptor). For example, IL-8 (CXCL8) binds to both high- and low-affinity receptors (CXCR1 and CXCR2, respectively), whereas CXCL5 only binds to CXCR2. Conversely, the CXCR2 receptor binds multiple CXC-chemokine ligands, whereas CXCR1 binds IL-8 exclusively. Intracellular signaling pathways

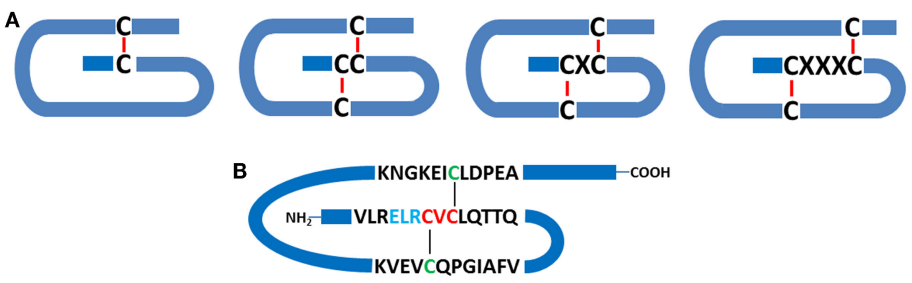

FIGURE 1 | Chemokine structure. **(A)** Schematic indicating the relationships between conserved cysteine residues (C), together with intrachain disulfide bridges. C-, CC-, CXC-, and CX_3C- classes of chemokines are depicted. **(B)** Schematic representation of human CXCL5, a pro-inflammatory and pro-angiogenic ELR^+ chemokine, which contains the ELR motif N-terminal to the CXC consensus sequence.

activated by chemokine ligand-receptor binding include those mediated by extracellular signal regulated kinases (ERKs), phosphoinositide 3-kinase (PI3K), AKT, small GTPases (Rac, Rho, cdc42), and NFκB family members. Thus, there is good potential for molecular crosstalk with growth factor signaling pathways.

In general, the CXC chemokines primarily activate neutrophils, whereas CC chemokines are mainly chemotactic for monocytes/macrophages and lymphocytes, and play a role in the class switching of macrophages from M1 to M2 during the transition from acute to chronic inflammation. CC chemokines are also crucial in the development of adaptive immunity by propagating lymphocyte recruitment and antigen presentation, and they play a critical role in bone metabolism by providing signals for the trafficking of osteoblast and osteoclast precursors.

Several inflammatory mediators including cytokines are abundantly expressed in the course of periodontitis (29). With regards to cancer risk, chemotactic cytokines or chemokines are of particular interest to us as they are involved in almost every stage of periodontal pathogenesis and can also be expressed in healthy sites. In addition to their role in immune cell trafficking to the site of infection, they also regulate angiogenesis, cell proliferation, apoptosis, and tumor cell homing. The next section will give a brief overview of chemokines involved in chronic periodontitis. Later, we will discuss how they may modulate the oral microenvironment to promote cancer progression.

Chemokines in Periodontal Disease

As noted above, the host immune response to infection is largely regulated by cytokine signals that drive initiation and progression of the inflammatory process. Their involvement with periodontal disease and their possible systemic effects have been well-reviewed elsewhere (24, 30). Here, we will focus specifically on chemokines that are expressed in periodontal tissues and their potential modulatory role in the tumor microenvironment.

Chemokine expression can be triggered by MAMPs, DAMPs, inflammatory mediators, host factors, and mechanical stress. In the oral tissues, chemokines can be synthesized by a variety of cell types including fibroblasts, osteoblasts, endothelial, epithelial and mast cells, PMNs, lymphocytes, and monocytes/macrophages. Thus, they play a major role in immune cell trafficking to sites of periodontal infection (29).

IL-8 (CXCL8) is one of the most abundantly expressed chemokines in the oral cavity and can be detected in both healthy and periodontally diseased tissues (31–36). IL-8 is primarily produced by gingival keratinocytes, fibroblasts, endothelial cells, and macrophages in response to periodontal bacteria and bacterial components. It functions to direct cell trafficking, mainly PMNs, to the site of infection. Inflammatory cell recruitment eventually results in more cytokine production, thus contributing directly to progression of the periodontal lesion. Although clinical studies consistently show that increased IL-8 levels are associated with periodontally diseased sites (31–36), *in vitro* studies with individual periodontal pathogens show variations in IL-8 production depending on the bacteria and the cell type being studied. For example, *P. gingivalis*, a key-stone periodontal pathogen, shows a distinct ability to manipulate local immune responses for its survival, either by increasing or suppressing IL-8 production (37–42). These unique features, possessing both pro- and anti-inflammatory properties, give *P. gingivalis* the ability to create a dysbiotic environment and escape immune surveillance. However, the possible biological mechanisms through which this bacterium may promote tumor development have yet to be investigated in depth.

Besides being a major chemoattractant for PMNs, IL-8 can also affect bone metabolism through its direct actions on osteoclast differentiation and activity by signaling through the high-affinity CXCR1 receptor (43). Additionally, the results of a meta-analysis investigating the risk of oral cancer in patients with polymorphisms of the IL-8-251A>T locus revealed that Caucasian populations harboring the AA genotype had a higher risk of developing malignancy (44), whereas a separate meta-analysis also indicated increased risk for individuals with either the AT or AA genotype (45). The functional significance of this finding is yet to be determined. Thus, many periodontal bacteria and bacterial components can trigger IL-8 production in different cells of the periodontal tissues and IL-8 is considered as one of the major chemokines associated with periodontal disease. Therefore, future investigations are warranted to elucidate the biological mechanisms triggered by IL-8 that modulate the oral environment and increase susceptibility for tumor development and/or progression.

Besides IL-8, another CXC-chemokine, CXCL12 is reported to provide trafficking signals for osteoblast and osteoclast precursors, and it also enhances the activity of MMP9 in osteoclasts, promoting bone resorption. CXCL10, which is a chemoattractant for activated Th1 cells, is found in inflamed gingival tissues (29) and is expressed by human gingival fibroblasts (46) in response to interferon-γ, tumor necrosis factor-α, and IL-1β, suggesting this as a mechanism to recruit Th1 cells to inflammatory foci in periodontitis. Notably, these authors also found that the anti-inflammatory cytokine IL-10 represses CXCL10 expression. B-lymphocytes are also plentiful in periodontal lesions, and it is not surprising that CXCL13, which is chemoattractant for B-cells, is highly expressed in diseased tissues, suggesting a role for this chemokine in the local humoral response to periodontal pathogens.

CC-chemokines are also documented to play various roles in the pathogenesis of periodontitis. CCL2 and CCL3 are chemotactic for monocytes and lymphocytes, CCL4 is chemotactic for $CD4^+$ T cells, and CCL5 attracts Th1 cells. All of these ligands have been found at higher levels in chronic periodontitis patients compared to healthy controls, and are associated with disease severity (47–49). Increased expression of the CC-chemokines, CCL2, and CCL20 was also correlated with increased numbers of dendritic cells (50). Macrophages are one of the key cell types found in large numbers in periodontitis lesions, and are involved in phagocytosis of pathogens and production of inflammatory mediators. However, increased accumulation of macrophages and deregulated inflammatory responses can disrupt tissue homeostasis, thereby contributing to periodontal disease. Like IL-8, CCL3 and CCL5 also play roles in bone metabolism and induce migration and activation of osteoclasts. Thus, it is likely that they exacerbate periodontal disease severity.

CCL20 is involved in Th17 cell recruitment and may promote periodontal disease. Hosokawa and coworkers (51) demonstrated that IL-22 increased CCL20 production in human gingival fibroblasts through an NF-κB-dependent mechanism when the cells were pre-challenged with IL-1β. Similarly, IL-6-stimulated periodontal ligament cells showed elevated secretion of CCL20 in a STAT3-dependent manner (52).

CCR4 is a high affinity receptor for both CCL17 (53) and CCL22 (54) and is expressed at higher levels in chronic periodontitis, as well as being associated with higher levels of the anti-inflammatory IL-10 in the periodontium (55). CCL17 and CCL22 are also expressed in diseased periodontium, and it may be possible that expression of these ligands serves to limit periodontal disease severity by attracting Th2 and Treg cells. Stimulation of human gingival fibroblasts with a combination of TNF-α and IL-4/IL-13 was found to increase expression of CCL17. While CCL17 expression was inhibited by *Escherichia coli* LPS and Pam3CSK4, which are activators of TLR4 and TLR2 signaling, respectively, in TNF-α/IL-4 stimulated gingival fibroblasts, CpG DNA (which activates TLR9) enhanced CCL17 production induced by TNF-α/IL-4. Thus, it remains unclear whether CCL17 is likely to reduce or exacerbate inflammation in periodontitis.

Chemokines in Oral Cancer

Although the normal function of the chemokine system is immunomodulation, there is an ever-increasing body of evidence that documents subversion and dysregulation of this intricate network of signaling molecules during the onset and progression of malignant disease. Overall, altered chemokine function in cancer promotes cell survival, enhanced proliferation, neovascularization, motility and metastasis in multiple tumor types, and this is comprehensively reviewed elsewhere. Several studies have implicated a number of chemokines and their receptors in oral squamous cell carcinogenesis [reviewed in Ref. (56)]. Most frequently, this appears to involve chemokines of the CXC subgroup such as CXCL1 (Gro-α), CXCL8 (IL-8), CXCL5, and CXCL12 (SDF-1). IL-8 has long been recognized as an autocrine regulator of OSCC growth (57), and it also contributes to enhanced cell motility (58). Indeed, salivary IL-8 has been proposed to be a discriminative biomarker for oral cancer (59). The closely related CXCL5 protein, normally chemotactic for neutrophils, has also been shown to drive oral cancer cell growth and motility as well as enhance tumor angiogenesis. Moreover, *in vivo* studies demonstrated complete inhibition of the tumorigenic phenotype when CXCL5 expression was suppressed in OSCC cells (60). Studies by Khurram and colleagues (61) reported elevated expression of both CXCR1 and CXCR2 in oral cancer. The former is a high-affinity IL-8 receptor, and the latter is a low-affinity IL-8 receptor that is also able to bind a number of CXC-chemokines including CXCL1, CXCL2, CXCL3, CXCL5, and CXCL6. These authors reported coincidental activation of signaling pathways by IL-8 and CXCL1, suggesting that simultaneous stimulation of cancer cells by multiple chemokines in the tumor microenvironment is likely to occur.

The CXCL12 (SDF-1)/CXCR4 axis also appears to play critical roles in OSCC development and progression. Expression of the CXCR4 receptor is elevated in tongue cancers (62), with metastatic tumor cells expressing higher levels than non-metastatic. This is consistent with the homing model proposed by Muller et al. (63), where tumor cells that overexpress a chemokine receptor migrate preferentially to organs that express the cognate ligand *via* a chemokine gradient. Perhaps of high relevance to this review, bacterial products have been reported to increase the expression of CXCR4 on oral cancer cells (64).

SDF-1/CXCR4-driven invasion of oral cancer is reported to be dependent upon NFkB signaling (65, 66). Further studies have demonstrated release of the CXCR4 ligand, SDF-1, from bone cells adjacent to the tumor. This has been proposed to contribute to bone turnover as a result of CXCR4-mediated upregulation of IL-6, which is then secreted by the tumor cells to stimulate osteoclastogenesis (67), thereby enhancing tumor invasion. Studies by Oue et al. (68) provided further evidence of a role for IL-6 and RANKL in OSCC, mediated at least in part through a CXCL2-dependent mechanism, as the effects were diminished by a CXCL2 neutralizing antibody.

Of course, there is always the potential for repression of malignant properties by chemokines, as some have noted anti-tumor effects. The chemokine CXCL14, also known as BRAK to signify the tissues (*br*east *a*nd *k*idney) from which it was originally isolated (69), was shown to be expressed in normal squamous epithelium but reduced or absent in malignant oral tissues (70), and is also repressed by EGFR signaling (71). Ectopic expression of CXCL14 completely blocked OSCC growth *in vivo* (72), suggesting that loss of expression may contribute significantly to the pathogenesis of oral cancer.

The CC-class of chemokines has also been implicated in oral carcinogenesis. Studies by Ferris and coworkers (73) documented loss of CCR6 and upregulation of CCR7, the receptor for CCL19 and CCL21, on oral cancer cells, and demonstrated that this was related to lymph node metastasis. CCL5–CCR5 signaling is reported to enhance OSCC motility, as well as increasing production of the gelatinase, MMP9 (74). An interesting study from Li and colleagues (75) demonstrated CCL2 production by cancer-associated fibroblasts (CAFs), which stimulated production of reactive oxygen species (ROS) in cocultured oral cancer cells. In turn, ROS promoted further CCL2 production in CAFs, thus generating a mutually beneficial microenvironment in which both tumor cells and fibroblasts could thrive.

Evidence is also present in the literature to support a role for XCR1, a receptor for lymphotactin (XCL1), in epithelial biology. Khurram and colleagues (76) reported expression of this receptor on oral cancer cells as well as on normal keratinocytes and neutrophils. Based on immunohistochemical analysis, low but detectable expression in normal mucosa was primarily restricted to the basal layer. In contrast, XCR1 expression was more marked in oral cancer tissues and could also be found in metastatic deposits. Interestingly, positive staining was observed in lichen planus, a potentially premalignant condition of immunological etiopathogenesis. Stimulation of XCR1 activity led to elevated expression of the gelatinases MMP2 and MMP9, which was more marked in cancer cells compared to normal keratinocytes. Moreover, cancer cells expressed MMP7, which was not observed in normal cells treated with lymphotactin. Potentially, then, XCR1

may mediate matrix remodeling in oral epithelium, and potentiate migration and invasion in malignant disease.

Potential Biological and Biochemical Mechanisms Linking Periodontal Disease and Oral Cancer

It has been estimated that 15–20% of tumors are driven by chronic infection and inflammation, and that individuals who are prone to chronic inflammatory disorders have an increased risk of cancer development (77). Recent advances suggest that the tumor microenvironment plays an important role in the initiation and progression of malignant disease (78). This can be mediated through biological factors related to genetic and epigenetic make-up of the cancer cells or through interaction of tumor cells with the surrounding milieu, which is composed of cells and soluble molecules of both microbial and host cell origin as well as extracellular matrix.

Several studies have reported an association between chronic periodontitis and cancer both in the oral cavity as well as at distant sites (22, 79, 80). Studies that have investigated the association between periodontal disease and oral cavity cancer are summarized in **Table 1**. While plausible biological mechanisms exist that can link the two diseases (**Figures 2** and **3**), the exact etiology is yet to be established. Tezal and coworkers reported that patients with periodontitis had a 5.23-fold increased risk of tongue cancer for each millimeter of alveolar bone loss (81). A further case-control study suggested a similar trend, with a stronger association between periodontitis and oral cavity cancer, compared to oropharyngeal or laryngeal tumors (82), and this was still noticeable in non-smokers. In addition, SCC lesions were more likely to be poorly differentiated in periodontitis patients compared to patients without periodontitis. Furthermore, a study of base-of-tongue cancers indicated that patients with HPV-positive tumors had greater bone loss than those with HPV-negative lesions (83), with the authors concluding that chronic periodontitis may influence HPV infection. Interestingly, a cross-sectional study revealed a relationship between alcohol consumption (a recognized risk factor for oral cavity cancer development) and clinical attachment loss (84). Thus, it may be possible that alcohol promotes oral carcinogenesis through multiple mechanisms, including potentiation of periodontal inflammation. As outlined above, constant polymicrobial insult and production of inflammatory mediators drive the initiation, progression, and persistence of periodontitis lesions. Chronic exposure to microbial and host-derived products can likely modify the oral microenvironment and possibly distant tissues, promoting carcinogenesis or at least increasing susceptibility.

TABLE 1 | Studies investigating the link between oral cancer and periodontal disease.

Author	Study design	Oral health criteria	Study population	Results
Bundgaard et al. (85)	Case-control	Missing teeth	161 patients and 400 controls (Denmark)	Significantly increased risk of developing oral SCCA for patients with fewer than 15 teeth
Rezende et al. (86)	Case-control	CPITN[a] and DMFT[b]	50 patients and 50 controls (Cuba)	76% of subjects in cancer group showed greater than 6 mm pockets compared to 10% of control group
Garrote et al. (87)	Case-control	Missing teeth	200 patients and controls (Cuba)	Significantly increased risk of oral cancer for patients missing 16 or more teeth
Hiraki et al. (88)	Case-control	Missing teeth	429 patients and 10,480 controls (Japan)	Significantly increased risk of head and neck cancer with decreased number remaining teeth
Marshall et al. (89)	Case-control	Missing teeth	290 patients and controls (United States)	Significantly increased risk of oral cancer with loss of 11 or more teeth
Michaud et al. (90)	Cohort	Self-reported history of periodontal disease (confirmed by radiographs and missing teeth)	118 patients (United States)	No significant increase in risk for oropharyngeal cancer with history of periodontal disease or increased number of tooth loss
Rosenquist et al. (91)	Case-control	Missing teeth	132 patients and 320 controls (Sweden)	Significantly increased risk of oral and oropharyngeal cancer for missing over 20 teeth
Tezal et al. (92)	Cohort	Clinical attachment loss (CAL)	131 oral tumors and 323 oral pre-cancerous lesions (United States)	Significantly increased risk of oral tumor and pre-cancerous lesion with >1.5 mm clinical attachment loss
Tezal et al. (81)	Case-control	Alveolar bone loss	51 cases and 54 controls (United States)	Significantly increased risk of tongue cancer with increased alveolar bone loss
Tezal et al. (82)	Case-control	Alveolar bone loss	266 patients and 207 controls (United States)	Significantly increased risk of oral cavity SCC with periodontitis
Tezal et al. (93)	Case-control	Alveolar bone loss	124 head and neck SCC patients (United States)	Periodontitis is associated with tumor HPV status
Wen et al. (94)	Cohort	Medical records from insurance claims	96,375 gingivitis and 51,791 periodontitis cases (Taiwan)	Significantly increased risk of oral cancer with the history of periodontitis
Zheng et al. (95)	Case-control	Missing teeth	404 subjects and controls (United States)	Significantly increased risk of oral cancer with increased missing teeth

[a] *WHO guidelines of community periodontal index of treatment needs (depth of periodontal pockets on scale of 1–4).*
[b] *WHO guidelines of decayed, missing, and filled teeth.*

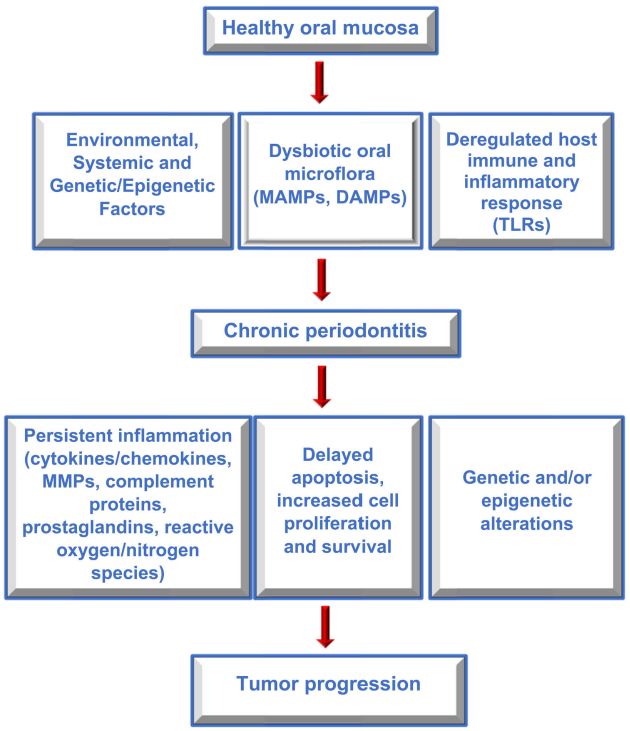

FIGURE 2 | Factors contributing to periodontitis and cancer. Flow diagram outlining the interplay between host and environmental factors that modify the oral microenvironment, contribute to periodontitis and oral cancer, and which may link the two diseases.

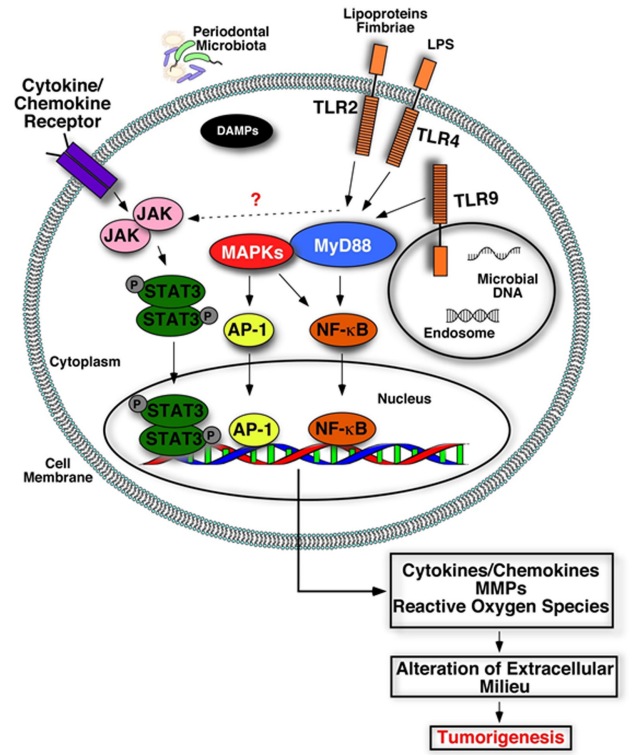

FIGURE 3 | Plausible biological mechanisms that may link deregulated inflammation and cancer. Engagement of microbial components (LPS, lipoproteins, nucleic acids) or damage-associated molecular patterns (DAMPs) with their receptors (TLRs) triggers activation of inflammatory signaling cascades and increases production of inflammatory mediators, tissue destructive enzymes (MMPs), and reactive oxygen/nitrogen species. Accumulation of these host-derived factors within the mucosa due to deregulated inflammation may alter and create a favorable oral microenvironment that promotes tumorigenesis.

Ongoing research efforts focus on the working hypotheses that the chronic periodontitis may be linked with oral cancer risk either through direct toxic effects of the oral microbiome and associated byproducts and/or through the indirect effect of chronic oral inflammation. The next section will give an overview of potential biological mechanisms related to persistent, chronic periodontal disease that may promote cancer development in the oral cavity.

The Contribution of the Oral Microbiome

As mentioned above, a number of factors are considered to play an important role in the genesis of oral cancer (96), including tobacco and alcohol use, dietary factors such as saturated fat and fruit/vegetable intake, and microbiological agents. While tobacco and alcohol are recognized as being primary etiologic factors for oral squamous carcinogenesis, there is a worrying trend in the incidence of oral cancer in patients who have never used these agents (97), raising the possibility that other factors may have a major role in tumor progression in these subjects. Furthermore, many users of tobacco and alcohol do not develop malignant oral disease, suggesting that additional events and/or cofactors are of importance.

A diverse group of microbial species inhabit the oral cavity, including bacteria, viruses, and fungi. For many years, it has been suggested that oncogenic human papillomaviruses may contribute to oral squamous carcinogenesis (98–101), similar to their role in cervical cancer. However, more recent studies have indicated that HPV may be more closely related to particular subsets of disease, specifically oropharyngeal and tonsillar carcinomas (102–105).

In addition to a possible viral etiology of a subset of oral cancers, bacteria have also been implicated in tumor development. For many years, *Treponema pallidum* has been associated with oral squamous carcinogenesis (106, 107). While the incidence of syphilis has declined markedly over the last century, it may still be involved in a small number of cases (108). However, poor oral hygiene has also been documented as a risk factor for development of oral malignancy (85, 89, 91, 109, 110), raising the possibility that some of the many other bacterial species present in the oral cavity may be of importance. For example, in a study of bacterial species associated with oral SCC, Nagy and coworkers reported a significantly higher number of anaerobic periodontal bacteria associated with malignant lesions compared to normal mucosa, including periodontopathogenic *Prevotella* and *Porphyromonas* species (28). Another study investigated the relationship between salivary micro-organisms and oral cancer (27). Interestingly, these authors reported increased levels of bacterial species associated with SCC, including *Prevotella*, *Porphyromonas*, and other species, and demonstrated that the levels of at least three salivary bacterial species were predictive of around 80% of SCCs. Whether

this relationship is merely associative or indicates a role for oral bacteria in neoplastic progression remains to be determined.

The idea of a bacterial etiology for human cancer is by no means limited to the oral cavity. Indeed, it is readily apparent that *H. pylori* infection is causally associated with malignancies of gastric epithelia (111, 112), leading to the categorization of this organism as a WHO class I carcinogen. *H. pylori* causes inflammation of the gastric mucosa, likely by inducing gastric epithelial cells to secrete IL-8, which results in recruitment of inflammatory cells to the site of infection (113). Moreover, IL-8 has the capacity to stimulate proliferation of epithelial cells, at least in part through transactivation of the EGF receptor (114). Further, as mentioned above, IL-8 and other chemokines and their receptors have been implicated in tumor development and metastatic progression in a number of human malignancies (115). This may well be true for oral cavity cancers and that oral bacterial species have the capacity to induce parallel events. In fact, a recent investigation reported that increased IL-8 expression in OSCC significantly correlated with increased serum IL-8 levels and poor clinical outcomes. The study also showed that IL-8 enhanced generation of CD163-positive M2 macrophages, and that CD163 positivity at the tumor invasion front correlated with significantly worse overall survival and disease-free survival in OSCC (116). In further support of the possible contribution of the oral microbiome to tumorigenesis, it was reported that hepatocyte growth factor (HGF) can be induced in fibroblasts by oral bacteria as well as by cytokines (117), while production of CCL20 was upregulated by *Actinobacillus actinomycetemcomitans*, *E. coli* LPS, and TNF-α (118). This provides a link between the oral microflora and factors which are known to influence both the proliferation and migration of epithelial cells. Additionally, studies by Sakamoto and coworkers investigated bacterial colonization of oral tumors and cervical lymph nodes (119, 120). Viable bacteria were recovered from both tumor and lymph nodes, with involved nodes showing higher colonization than uninvolved nodes (120). It was suggested that disruption of the integrity of the epithelial layer by the primary tumor might facilitate bacterial entry and subsequent lymphatic drainage. If, indeed, oral bacteria can colonize and survive in lymph nodes, they might stimulate the production of factors that could aid survival and proliferation of any tumor cells in the immediate environment. Furthermore, the association of *F. nucleatum*, another periodontal bacterial species, with colorectal cancer also supports the notion that oral pathogens can obtain access to distant sites and may participate in tumorigenesis in other organs as well (17, 121). *F. nucleatum* is one of the most frequently identified bacteria both in healthy gingiva as well as periodontitis sites and can also induce production of cytokines and chemokines (121).

Another alternative may be that oral bacteria invade tumor cells at the primary site, influencing their biological behavior. *P. gingivalis* has the ability to invade and propagate intracellularly (122), including in gingival epithelial cells (123). It can also delay apoptosis in gingival epithelial cells through various different mechanisms, such as by activating the JAK1/STAT3/Akt pathway, downregulating caspase-3 and caspase 9 expression, upregulating microRNAs (specifically miR-203), leading to suppression of SOCS3, preventing ATP-dependent apoptosis (124–128). Programed cell death is one mechanism through which cells avoid replicating if DNA damage is excessive and cannot be repaired. Thus, by interfering with this key regulatory process *P. gingivalis* may promote carcinogenesis. Furthermore, *P. gingivalis* also possesses both anti- and pro-inflammatory activities thereby contributing to dysbiotic microflora (129). This feature also gives the bacterium a unique ability to manipulate the immune system for its own survival. *P. gingivalis* can also promote invasion of oral cancer cells by activating the ERK1/2-Ets1, p38/HSP27, and PAR2/NF-κB pathways to induce MMP9 (130). Increased IL-6 and IL-8 production was also reported in HSC-3 and H413 oral cancer cells in response to *P. gingivalis* challenge (131). Another important way in which *P. gingivalis* might contribute to tumor progression is through its ability to suppress angiostatic chemokines. In a key study (132), Jauregui et al. found that this bacterium could suppress the secretion of CXCL9, CXCL10, and CXCL11. Thus, the removal of their regulatory activity could promote neovascularization and enhance tumor growth.

While the bacterial etiology of periodontitis is well established, the contribution of viruses to the pathogenesis of periodontal disease has also been supported by a number of studies (133–137). It is likely that viral/bacterial interactions in the oral cavity can further modulate the oral microenvironment. For example, it has been shown that Herpes simplex virus type 1 (HSV-1), Epstein-Barr virus (EBV), and human cytomegalovirus-infected periodontitis lesions harbor elevated levels of periodontal pathogens. It was also reported that EBV can reside in gingival epithelial cells, which may serve as an oral reservoir for virus infected cells. Importantly, epithelial EBV infection was significantly increased in chronic periodontitis (138). Additionally, both HPV and EBV have been implicated in head and neck carcinogenesis. As mentioned above, a study among patients with base-of-tongue cancer suggested a synergy between periodontitis and HPV status (82), and chronic periodontitis was also associated with HPV positive tumor status in patients with incident primary squamous cell carcinoma of the oral cavity, larynx, and oropharynx (93). The biological and molecular mechanisms defining how bacterial/viral interactions modify the tumor microenvironment and lead to disease initiation or progression are poorly understood, and are an area of active investigation. A plausible possibility is the activation of innate immune sensors such as TLRs (see below). For example, TLR-2, TLR-4, and TLR-9 can be stimulated by oral bacterial species, whereas viruses activate TLR-3, -7, -8, and -9. Thus, there would seem to be scope for synergistic activation of multiple pathways in response to different components of the oral microbiome, resulting in gene activation and production of secreted mediators into the extracellular millieu. However, this is likely to be a complicated scenario as some studies have reported that activation of specific TLRs (for example, TLR-3 and -7) may trigger apoptosis rather than pro-survival and pro-oncogenic mechanisms (139–141).

The Contribution of Chronic Inflammation

Disease progression in periodontitis is a complex process that involves the interaction of multiple components of the host immune response and the oral microbiome, as outlined above.

The engagement of MAMPs and DAMPs with their cognate receptors releases inflammatory mediators that aid in the development of an efficient innate immune response to eliminate the pathogen and coordinate development of an adaptive immune response. However, the impaired tissue homeostasis and deregulated inflammation in periodontitis exposes the local and systemic tissues to noxious metabolic products including living and necrotic cells, cytokines, chemokines, prostaglandins, MMPs, reactive oxygen and nitrogen radicals. Accumulation of these host-derived factors may likely modulate the tumor microenvironment by causing DNA damage, promoting epigenetic and genetic alterations, increasing angiogenesis, cell survival, proliferation, migration, and inhibiting apoptosis (**Figures 2** and **3**). Altered cells may eventually promote production of more inflammatory mediators, further enhancing inflammation and contributing to cancer pathogenesis.

One mechanism through which host cells detect microbial challenge is through engagement of microbial components with innate receptors, specifically TLRs. These receptors can interact with components of bacteria, viruses, and fungi (142). Several cell types found in periodontal tissues express TLRs, and each receptor is involved in the sensing of distinct microbial products. TLRs are type I trans-membrane proteins composed of an extracellular leucine-rich repeat (LRR) domain involved in ligand recognition, a trans-membrane domain, and a toll-interleukin 1 receptor (TIR) domain involved in signaling. The signaling pathways activated by TLRs engage adaptor molecules that are recruited by TIR/TIR domain interactions. These include the myeloid differentiation primary response gene (MyD88), the TIR domain-containing adaptor protein (TIRAP, also known as MAL), TIR domain-containing adaptor inducing interferon-β (TRIF), and the TRIF-related adaptor molecule (TRAM). MyD88 is essential for signaling through all TLRs, except TLR3, and is involved in early activation of NF-κB and MAPKs, leading to pro-inflammatory gene expression. As well as immune cells and oral keratinocytes, oral cancer cells also express TLRs (143–146). Thus, tumor cells or their premalignant counterparts may respond to direct stimulation by oral microbes or their byproducts, activating pro-inflammatory, pro-proliferative, and pro-migratory signaling pathways.

Of high relevance to carcinogenesis, an interesting study by Lappin and colleagues (147) determined plasma concentrations of CXCL5 and IL-6 in systemically healthy subjects with or without periodontitis, and who either did or did not smoke. They found significantly higher levels of circulating CXCL5 in smokers with periodontitis, which correlated with probing depth, attachment loss, and tobacco consumption. Given the potential function of CXCL5 as a pro-angiogenic factor (148), together with its role to promote tumor cell growth and motility (60), high circulating levels of CXCL5 could potentially act to promote tumorigenic progression in concert with local factors.

The loss of periodontal tissue integrity due to chronic inflammation aids viral and bacterial survival and persistence, as well as enhancing the inflammatory response. It is therefore plausible to hypothesize that prolonged inflammation in periodontal tissues due to activation of innate sensors by periodontal microbial products or DAMPs may promote a tumor-favorable environment by exposing the tissues to multiple cytokines/chemokines. In fact, emerging evidence also supports this concept (149). Increased expression of host innate receptors has been reported in periodontitis lesions compared to healthy sites (150–153). Among these, TLR9 has been implicated in oral squamous cell carcinogenesis (149). TLR9 is an intracellular sensor that can activate a range of cell types such as macrophages, dendritic cells, PMNs, B cells, and epithelial cells through recognition of bacterial and viral DNA sequences that are released following phagocytosis. TLR9 activation can trigger production of multiple inflammatory mediators in response to periodontal pathogens, including IL-8 production from gingival epithelial cells and macrophages (154–157). TLR9 expression was also reported to be significantly elevated in the tissues of oral squamous cell carcinoma as well as periodontitis, and increased receptor expression was correlated with increased tumor size and clinical stage (153, 158, 159). *In vitro* studies revealed that activation of TLR9 can mediate oral cancer cell migration by up-regulating MMP2, and tumor cell proliferation by up-regulating cyclin D1 expression, both in an AP-1-dependent manner (159, 160). Increased IL-1α and IL-6 production in OSCC cells treated with a TLR9 agonist was also reported (160). It is therefore possible that TLR9 activation can contribute to tumorigenesis by enhancing inflammation and through modulating cell cycle progression. Another clinical investigation reported a strong correlation between TLR2, -4, and -9 expression and increased tumor invasion in oral tongue squamous cell carcinoma (161). This study also revealed that increased TLR9 expression correlated with advanced tumor size, while high TLR5 expression was associated with a lower tumor grade. It is important to emphasize that all of these receptors are also activated by periodontal bacteria in the course of periodontal disease and trigger production of cytokines and chemokines (162) through NF-κB, AP-1, and MAPKs. Among these molecules, NF-κB expression and activity is often elevated in oral cancers, with protein levels gradually increasing as the lesion progresses from premalignant to invasive form (163–165). Further, aberrant function of NF-κB has also been reported to stimulate STAT3 activation by an autocrine/paracrine mechanism in SCC, suggesting crosstalk between these signaling pathways (166). STAT3 is a critical mediator of the pro-angiogenic and immunosuppressive activities of myeloid cells in the tumor microenvironment, and it can lead to enhanced cell cycle progression and neovascularization, thus promoting tumor growth (167, 168). Collectively, these findings support the hypothesis that aberrant activity of multiple innate receptors and inter-related downstream signaling pathways contribute to oral tumorigenesis. Intriguingly, STAT3 and NF-κB lie downstream of TLR-mediated inflammatory cascades including those activated by TLR-2, -4, and -9 (169). It remains to be determined whether STAT3 signaling is triggered as a result of direct stimulation of these receptors or as a result of the production of cytokines, such as IL-6, and development of a positive feedback loop.

In summary, it is likely that deregulated receptor activity within the oral cavity due to chronic periodontitis may be associated with tumor progression, possibly as a result of exposure of the mucosa to cytokines, chemokines, MMPs, and reactive oxygen (or nitrogen) radicals. Accumulation of these host-derived factors are

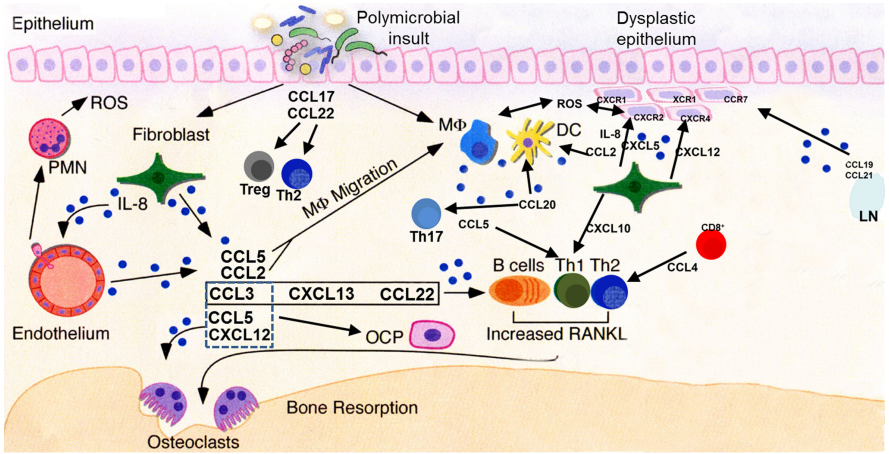

FIGURE 4 | Key chemokine functionalities in periodontitis and oral carcinogenesis. A dysbiotic oral microflora triggers inflammatory processes in the oral epithelium. Release of chemokines, among other molecules, results in progression (or suppression, in some cases) of the inflammatory process and stimulation of both innate and adaptive immune responses through recruitment of cellular mediators. Persistent inflammation extends deeper into the tissues, subsequently leading to osteoclast activation and subsequent destruction of alveolar bone. Multiple chemokines involved in the periodontal inflammatory process may stimulate their cognate receptors present on normal, dysplastic, or malignant epithelial cells, deregulating cellular growth, and promoting the motile phenotype. Pro-angiogenic chemokines, such as IL-8 and CXCL5, act upon endothelial cells to promote neovascularization of developing tumors. LN, lymph node; ROS, reactive oxygen species; PMN, neutrophil polymorph; OCP, osteoclast precursor; DC, dendritic cell; MΦ, macrophage.

likely to modulate the tumor microenvironment by causing DNA damage, promoting epigenetic and genetic alterations, increasing angiogenesis, cell survival, proliferation, migration, and inhibiting apoptosis. Altered cells may eventually promote production of more inflammatory mediators, further enhancing inflammation thereby contributing to cancer pathogenesis (**Figure 3**). Still, the role of TLRs in cancer is ambiguous, as they can either mediate signaling leading to inhibition of apoptosis and altered cell proliferation, or trigger immune responses to cancer. While the observations outlined above provide some evidence for plausible mechanisms linking cancer and periodontal inflammation, future studies are warranted to delineate the specific cellular and molecular pathways that may precipitate tumorigenesis in the oral cavity.

Concluding Remarks

Emerging evidence argues that mutual interactions between host cells and the oral microbiome (bacteria and/or viruses) in the course of chronic periodontal inflammation likely creates a tumor-favorable microenvironment that may promote cancer development and progression (**Figure 4**). However, studies of the biological and biochemical mechanisms to explain the observed associations between oral cancer and periodontitis are at an early stage, and future investigations are warranted to determine the etiology and to delineate the molecular pathways involved. While the studies are underway exploring the possible cause–effect relationship between periodontal inflammation and oral cancer, it is imperative for health care professionals to make their patients aware of the current evidence that there might be a link between periodontal disease and cancer development. Thus, maintaining good oral health should be considered as part of a healthy lifestyle, not only to prevent tooth loss but also for overall systemic health. Individuals who exhibit periodontal disease and who also have other risk factors related to their lifestyle or family history may benefit from more frequent periodontal maintenance visits to help maintain the infection and inflammation at a minimal level. In general, patients should be encouraged to adopt positive lifestyle habits such as regular physical and dental visits, meticulous oral hygiene, cessation of smoking, healthy eating habits, regular exercise, and elimination of other risks that may predispose to malignant transformation. It is also imperative for dental and medical professionals to communicate with each other and work as a team to manage their patients and reduce or eliminate possible risks, resulting in better overall oral and systemic health.

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The overlapping roles of antimicrobial peptides and complement in recruitment and activation of tumor-associated inflammatory cells

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Antimicrobial peptides (AMPs) represent a group of small (6–100 amino acids), biologically active molecules, which are produced by plants, mammals, and microorganisms (1). An important element of the innate immune response, AMP, possesses potent antibiotic, antifungal, and antiviral activities. Furthermore, AMP may be involved in a number of other processes such as angiogenesis and modulation of the immune response such as stimulation of chemokines and chemotaxis of leukocytes. AMPs have been proposed as alternative therapies for infectious diseases. AMP may also exert cytotoxic activity against tumor cells. Further understanding of the biological function of these peptides during tumor development and progression may aid in the development of novel anti-tumor therapies with refined application of innate molecules. AMP and complement have distinct roles to play in shaping the microenvironment (**Table 1**). Components of the complement system are integral contributors in responding to infection and sterile inflammation. Moreover, complement plays a role in the trafficking of cells in the tumor microenvironment, and thereby possibly in the immune response to cancer. This article will try to outline characteristics of AMP and complement in mobilization and recruitment of cells in tumor microenvironment.

Keywords: defensins, cathelicidins, anti-tumor activity, c5a, cell recruitment and activation

DEFENSINS, AN EXAMPLE OF ANTIMICROBIAL PEPTIDES WITH A CONTROVERSIAL ROLE IN CANCER

The defensin family includes three subfamilies (α, β, and θ defensins). Defensins can be stored in cytoplasmic granules of neutrophils and in macrophages or can be released into the extracellular environment from epithelial cells and mesothelial cells. In humans, there are 6 α defensins and 11 β defensins, exhibiting a wide range of antimicrobial, but also additional biological activities: β defensins possess a proinflammatory effect via binding a variety of receptors. For example, human β defensin 2 (hBD-2) and, to a lesser extent, human β defensin 1 (hBD1) bind to CCR6 resulting in increased chemoattraction of both $CD4^+$ memory T cells and immature dendritic cells (2). β defensins can also play a role in tumorigenesis. Altered expression of β defensins was seen in epithelial cell-derived cancers such as prostatic cancer, basal cell carcinoma, oral squamous cell carcinoma (OSCC) and renal cell carcinoma. Expression of hBD1 was found to be diminished or suppressed in malignant prostate tissue compared to high or moderate expression of hBD1 in surrounding benign tissue (3). This altered expression of β defensins led researchers to investigate anti-tumor activity of β defensins. Results showed that induction of hBD1 expression in prostatic cancer cell line lead to increased cell death inferring that hBD1 is anti-tumorigenic and that loss or decrease in expression may lead to tumor progression. In this sense, it was found that diminished expression of hBD1 was found associated with worse grading of prostate cancer (4).

However, the role of defensins in tumor development and tumor progression has been controversial with some groups reporting overexpression of some β defensins in some types of cancer. High levels of hBD1 and hBD-2 were found in sera of patients with lung cancer. Another example is the overexpression of hBD1 in renal cell carcinoma and hBD3 in OSCC (5, 6).

Further investigation into the role of hBD3 in mediating the tumor-related inflammatory process in oral cancer revealed that overexpression of hBD3 was associated with recruitment and accumulation of tumor-associated macrophages (TAM). These cells represent a significant part of infiltrating immune cells in the tumor microenvironment and are thought to play a role in the development and progression of tumors, but are probably a heterogeneous population (7). Moreover, hBD3 overexpression stimulated the expression of interleukin-1α (IL-1α), IL-6, IL-8, CCL18, and tumor necrosis factor-α (TNF-α). HBD3 chemoattract monocytes to the tumor microenvironment via chemokine receptor 2 (CCR2), further supporting the role of human β defensins in establishing the tumor microenvironment, which leads to tumor progression (8). By increasing vascularization, HBD increase tumor angiogenesis in SCC microenvironment (9). Conejo-Garcia and coworkers described a mechanism in which β defensins support tumor vasculogenesis through recruitment of dendritic cell precursors. Tumor vascularization and growth were enhanced in the presence of increased Vegf-A expression (10).

Table 1 | Tumors develop a complex network of cellular interactions involving both proinflammatory and suppressive cells.

Immunosuppressive	Proinflammatory
C5a/C5aR → MDSC recruitment	C5a attract $CD8^+$ T-cells
AMP involved in angiogenesis	AMP exert cytotoxic activity
↓ Expression of human β defensin 1 → tumor progression (prostate cancer)	↑ hBD3 associate with recruitment TAM (lung cancer, renal cell carcinoma, and OSCC)
HPN1 inhibition Classical and Lectin pathway	β defensins bind to a variety of receptors (CCR2)
Express α defensins (HPN1-3) in endothelial → angiogenesis	hBD1 bind to CCR6 resulting in ↑ $CD4^+$
Express α defensins (HPN1-3) in endothelial → angiogenesis	Blockage of C5aR ↓ decrease tumor growth
Deficiency of LL37aid in inflammation and tumor progression	Overexpression hBD3 → chemoattract monocytes tumor microenvironment
	↑ ↑↑ HPN1-3 → cytotoxic effect on cancer cell line

It was also found that HBD3 has the ability to inhibit the migration of colon cancer cells in a dose-dependent manner, which means that HBD3 could be a potential new drug for treatment of colon cancer (11).

Other reported activities of defensins include activation of the classical pathway of complement. The interaction with the complement system occurs via both C1q-dependent and C1q-independent mechanisms (12, 13). In contrast, other studies have reported that HBD-2, which has a structural homology with a C1q inhibitory molecule, can significantly inhibit the classical pathway of complement (14). Some defensins may inhibit both the classical and lectin pathways of complement. Human neutrophilic peptide 1 (HPN1) was found to bind to C1q and MBL leading to inhibition of these two complement activation pathways (15).

Human β defensins have not been the only group of defensins associated with tumor tissue. In the last few years, α defensins were detected in malignant tissue and fluids (serum and plasma) from patients with cancer. Human neutrophilic peptides 1, 2, and 3 (HNP 1–3), members of α defensins antimicrobial peptides (AMP), are expressed in endothelial cells in several tumor types suggesting that angiogenesis could be effected by α-defensins (16). The role of α defensins in cancer has also been controversial with some *in vitro* studies suggesting that supranormal (>25 μg/ml) levels of HNP1–3 have a cytotoxic effect on cancer cell lines and physiologic levels (6–25 μg/ml) of HNP 1–3 increase the proliferation and invasiveness of cancer cell lines (17), while others show that α defensin expression associates with greater invasiveness *in vivo* and *in vitro* (18).

COMPLEMENT AND CANCER

The complement system is an important element of the innate immunity with a well-established role in acute inflammation and continuous activation in chronic inflammation. In addition, components of the complement system mediate cellular regeneration and growth (bone and cartilage development, neurogenesis, bone marrow engraftment and regeneration of the liver) (19, 20). The complement activated system play a dual role in the tumor microenvironment. Traditionally, it was assumed that components of the complement system play a role in anti-tumor immune response either through complement mediated cytotoxicity or via antibody-dependent cell-mediated cytotoxicity. However, work has suggested that components of the complement system may also cause immune suppression and enhance tumor growth which means that the role of complement components in tumor growth should be revisited. Complement anaphylatoxins C3a and C5a play a complex role in tumor growth by inhibit antigen-specific CD8 + T cell-mediated anti-tumor immune responses. In their study of a murine TC-1 syngeneic model of cervical cancer, blockage of C5aR resulted in decreased tumor growth. Coinciding with an increase in infiltrating $CD8^+$ cytotoxic T cells and inhibition of MDSC recruitment (21), the effect of C5aR blockade was similar to that seen after treatment with a well-known anti-tumor chemotherapeutic agent. Moreover, a similar degree of inhibition in tumor growth was also noted in C5aR-deficient mice. MDSCs isolated from C5aR-deficient tumor bearing mice lacked the ability to inhibit $CD3^+$ T cells proliferation seen in C5aR wild-type mice (21). Overactivation of the infiltrating cells due to high concentration of C5a may result in suppression of anti-tumor T cells, thus leading to progression of tumors. On the other hand, low concentration of C5a can lead to a powerful anti-tumor immune response (22).

These findings present complement inhibition as an option for developing novel anti-cancer therapies, which lacks the side effects of the conventional anti-cancer therapies. The concomitant inhibition of egress of cells from the bone marrow by interfering with C5a/C5aR interactions, however, carries a possible risk of compromised inflammatory response to infection.

C3 deficiency leads to altered immune response in experimental ovarian cancer causing a decrease in tumor development and progression (23).

CATHELICIDINS, A GROUP OF ANTIMICROBIAL PEPTIDES WITH MISCELLANEOUS BIOLOGICAL FUNCTIONS

Cathelicidins constitute a mammalian antimicrobial peptide family. Cathelicidins are characterized by a variable antimicrobial peptide on the C-terminus and a moderately conserved N-terminal cathelin domain. The cationic property of cathelicidin enables it to electrostatically react with the anionic membrane of microbes and some tumor cells resulting in disruption of cell membranes, which eventually leads to cell death. This process results in destruction of tumor cells (and microbes), while normal cells are left intact. In humans, the only known cathelicidin is the hCAP-18/LL37. After stimulation, hCAP-18 is cleaved producing a peptide containing 37 amino acids starting with two leucines termed LL37. Expression of the hCAP-18 gene was found in squamous epithelium of intestine, mouth, cervix, tongue, esophagus, and the airways. In addition,

LL37 is produced by immature neutrophils, natural killer cells, B cells, monocytes, and mast cells. LL37 plays a protective role in preventing bacterial inflammations. This conclusion came from the observation that LL37 is down regulated in patients infected with *Shigella*. A number of studies have pointed out that cathelicidin could play an important role in preventing bacteria-related inflammation and perhaps also carcinogenesis. An example of this is infection of the gut with *Helicobacter pylori*, where LL37 is over-expressed in the early stages of infection providing a protective role. However, the expression of LL37 is reduced as the infection progresses and results in disturbances in cell turnover in the gastrointestinal tract contributing to *H. pylori*-associated carcinogenesis in the GI tract. Promotion of carcinogenesis and inflammation is attributed to dysregulation of cathelicidin (mechanism unknown). Collectively, these findings suggest that deficiency in this peptide may aid in inflammation and tumor progression (24). So what is the precise role of these AMP during inflammation and carcinogenesis? In addition to its antimicrobial ability, LL37 seems to have a chemotactic capability facilitating the migration of neutrophils, monocytes, $CD4^+$ cells, and mast cells. LL37 also has the ability to induce degranulation and release of inflammatory mediators from mast cells, which are one of the first immune cells that encounter invading microbes. It has been suggested that LL37 augments the host's immune response via regulation of the expression of particular genes with anti-inflammatory and proinflammatory roles (25). In addition, LL37 enhances the induction phase of adaptive immunity through recruitment of T helper cells and increased HLA-DR expression by human dendritic cells (26). Thus, Cathelicidins seem to have a role in augmenting both innate host defenses and adaptive immunity and, where activity is high, may aid in establishing an anti-tumor response.

Defining the exact role that LL37 plays during carcinogenesis is not straight forward. In some studies, expression of LL37 was found to increase in a number of tumors such as ovarian, lung, and breast cancers. On the other hand, the same peptide was found to possess a suppressive role in other types of cancers such as GI tract cancer. This observation was further supported by the finding that Cathelicidin-deficient mice had increased susceptibility to carcinogen-induced colonic tumors. Further studies have shown that LL37 exerts an apoptogenic function via a caspase-independent manner (27). Furthermore, it was shown that lung tumor growth is promoted by cathelicidin expressed in tumor cells (28).

The role that LL37 plays during carcinogenesis might be related to whether the cancer is the result of persistent inflammation or if the inflammation is a result of cancer.

THE ANTI-TUMORAL ACTIVITY OF SOME AMP AND COMPLEMENT COMPONENTS AGAINST HEMATOLOGICAL TUMORS

Hematological tumors such as leukemias and lymphomas cause approximately 10% of deaths in cancer patients. Thus, there is a demand to develop new anti-tumoral agents preferably from natural and biological sources, which targets tumor cells. In addition to their antimicrobial properties, some AMPs seem to exert a cytotoxic effect against tumor cells. Recent studies have focused on exploring the anti-tumoral activity of some AMPs and presenting them either as alternatives to conventional anti-tumor therapies or to be used in combination with other conventional anti-tumor drugs. It is thought that the anti-tumoral activity of these peptides occurs mainly via apoptosis and necrosis. However, the exact mechanism by which some AMPs exert their anti-tumoral activity is not fully understood yet. There have been many speculations in this regard; one of the suggestions is that cell death of tumor cells is due to a detergent-like effect and cell permeabilization. On the other hand, one group has demonstrated that tumor cell death may be a result of calcium accumulation in the mitochondria. It has also been proposed that the anti-tumoral activity of some AMP is a result of the cationic nature of most of these peptides. It is thought that the selective killing of tumor cells is the end result of an interaction between the cationic peptides and the anionic cell membrane components. Certain AMPs can be used with conventional chemotherapeutic drugs to enhance their cytotoxic activity. An example of such AMPs is Cecropin A (CA), which has a cytotoxic effect on human lymphoblastic leukemia and can be used as a pharmacological tool with some chemotherapeutic drugs such as CA/S–FU combination (29).

In addition to AMPs, complement components also seem to have an anti-tumoral action as it had been well documented that leukemic cells have the ability to activate both alternative and lectin pathways of complement. This activation leads to opsonization of tumor cells and their subsequent uptake by NK cells and leukocytes [reviewed in Ref. (30)].

ANTIMICROBIAL PEPTIDES AS NOVEL ANTI-TUMORAL THERAPIES

Although a number of studies have demonstrated that some AMPs exhibit anti-tumoral activity against different types of cancers such as leukemia, ovarian, and prostate tumors; however, none of these peptides are currently used commercially to target tumor cells. The idea of using bacteria as anti-tumoral agents was first addressed by two German scientists; W. Busch and F. Fehleisen. These two physicians had individually noted that infections with *Streptococcus pyogenes* in some cancer patients resulted in a regression of tumor cells. However, the American physician William Coley was the first to start a well-documented approach to use bacteria in treatment of cancer back in 1890. Coley had managed to develop a vaccine, which combined two killed bacterial species and used it to treat lymphomas, sarcomas, melanomas, and myelomas with a high success rate. In addition, some anaerobic bacteria have been shown to selectively target solid tumors and leading to tumor lysis. However, this approach was abandoned after the introduction of radiation, chemotherapy, and surgery as standard tools for cancer treatment. Side effects associated with these aforementioned therapies or difficult locations of tumors have stressed the need to look for novel therapies for cancer treatment, which would either replace or supplement conventional cancer therapies. Hence, the use of bacteria and AMPs in cancer treatment has been taken up again. It is thought the cationic nature of the AMPs enable them to interact with the various anionic molecules such as sialic acid residues, heparan sulfate, and anionic phosphatidylserine, which are largely present on the surface of cancer cells. This explains why AMPs are more prone to interact with tumor rather than non-tumor cells. A number of *in vitro* and

in vivo studies have given evidence on the anti-tumoral activity that some AMPs possess (31). Melittin is an AMP extracted from the European honey bee *Apis mellifera* induces cell lysis. A conjugate of melittin/avidin engineered to target cancer cells with high matrix metalloproteinase 2 (MMP2) activities lead to a significant decrease in the size of B16 tumor in mice (32). A recent study has proposed using BD2 in immunogene therapy of tumors. This strategy involves recruitment of immature dendritic cells to the tumor microenvironment and subsequently promoting their maturation, which will provoke a local anti-tumor immune response (33).

Antimicrobial peptides are not selective against tumor cells. Thus, further systemic investigations into these AMPs are needed to understand the molecular properties, which make these peptides appropriate for clinical use.

OUTLOOK

In conclusion, a number of studies have addressed the role of AMP during carcinogeneis and cancer progression. But arrived at contradictary conclusions with regard to anti-tumor or tumor promoting activities, some groups have even suggested that certain AMP might provide an unconventional approach for cancer treatment. In addition to complement components, there is good evidence that certain AMP such as defensins and Cathelicidins influence the mobilization from bone marrow and recruitment of cells in the tumor microenvironment (34).

During inflammation, complement components C5a and C3a are generated leading to chemoattraction of immune cells such as T lymphocytes, monocytes, and eosinophils. Subsequently, the interaction of these components with their receptors leads to release of cytokines and reactive oxygen species resulting in tissue damage. Persistent inflammation leads to the establishment of a microenvironment supportive for tumor growth. Tumor-mediated complement activation provides a continuous source of complement activation bioactive products creating an inflammatory environment supporting tumor growth. It was found that activation of C3aR and C5aR lead to an increase in expression of IL-6 mRNA, which is a potent cytokine capable of stimulating angiogenesis and inhibiting apoptosis in tumor cells (35).

Taken together, both complement components and some antimicrobial peptides of the innate immune defense arm have a role in regulating and trafficking of immune cells during tumorigenesis as well as in modulating the adaptive anti-tumor immune response. Their direct role on tumor cells may be more distinct. Therefore, future studies should attempt to study the role of these two effector systems together rather than in isolation to uncover the relative importance of each and possible additive, exploitable, effects during carcinogeneis and cancer progression.

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Antimicrobial peptides and complement in neonatal hypoxia-ischemia induced brain damage

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Hypoxic-ischemic encephalopathy (HIE) is a clinical condition in the neonate, resulting from oxygen deprivation around the time of birth. HIE affects 1–5/1000 live births worldwide and is associated with the development of neurological deficits, including cerebral palsy, epilepsy, and cognitive disabilities. Even though the brain is considered as an immune-privileged site, it has innate and adaptive immune response and can produce complement (C) components and antimicrobial peptides (AMPs). Dysregulation of cerebral expression of AMPs and C can exacerbate or ameliorate the inflammatory response within the brain. Brain ischemia triggers a prolonged inflammatory response affecting the progression of injury and secondary energy failure and involves both innate and adaptive immune systems, including immune-competent and non-competent cells. Following injury to the central nervous system (CNS), including neonatal hypoxia-ischemia (HI), resident microglia, and astroglia are the main cells providing immune defense to the brain in a stimulus-dependent manner. They can express and secrete pro-inflammatory cytokines and therefore trigger prolonged inflammation, resulting in neurodegeneration. Microglial cells express and release a wide range of inflammation-associated molecules including several components of the complement system. Complement activation following neonatal HI injury has been reported to contribute to neurodegeneration. Astrocytes can significantly affect the immune response of the CNS under pathological conditions through production and release of pro-inflammatory cytokines and immunomodulatory AMPs. Astrocytes express β-defensins, which can chemoattract and promote maturation of dendritic cells (DC), and can also limit inflammation by controlling the viability of these same DC. This review will focus on the balance of complement components and AMPs within the CNS following neonatal HI injury and the effect of that balance on the subsequent brain damage.

Keywords: antimicrobial peptides, complement, neonatal hypoxia-ischemia, microglia, astrocyte, hypoxic-ischemic encephalopathy

INTRODUCTION

Neonatal brain injury resulting from oxygen deprivation around the time of birth affects 1–3/1000 live term births in high-income countries with rates 5–10 times higher in low-resource setting. About 40% of the affected infants die in the neonatal period and additional 30% sustain lifelong neurological deficits, including cerebral palsy, epilepsy, and cognitive disabilities (1). Neonatal hypoxia-ischemia induces a robust inflammatory response in the immature brain, which is considered to play an important role in the development of brain damage and subsequent hypoxic-ischemic encephalopathy (HIE). Initial inflammation involves activation and recruitment of various immune cells into the injured brain. The initial pro-inflammatory response is followed by hypoxic-ischemic (HI) secondary energy failure that may last for days, followed by a switch to anti-inflammatory response and resolution. However, the exact mechanisms involved in the immune response following HIE still remain unknown. Several mediators of the inflammatory cascade include components of both innate and adaptive immune systems, such as cytokines, chemokines, adhesion molecules, as well as antimicrobial peptides (AMPs) and complement (C).

NEONATAL HI

Despite the neonatal period only constituting the first 28 days of life, it accounts for 38% of death in children younger than 5 years of age. Direct causes leading to neonatal death include infection (36%), prematurity (28%), and birth asphyxia (23%). The latter two, combined with congenital defects (7%) account for the majority of deaths occurring within the first week of life (2). This morbidity is generally a result of multiple organ dysfunctions (3) or termination of care. Epidemiological studies have shown that asphyxia is not the most common cause for developmental disorders; however, it poses important clinical problems, as infants who survive an asphyxia episode around the time of birth are at high risk of developing lifelong devastating impairments. Neonatal HIE and the ensuing clinical manifestation cause significant global public health burden (4), with infant sufferers at risk of subsequently developing cerebral palsy and/or other neurological dysfunctions such as cognitive impairment, epilepsy, and autism (4–6).

The pathophysiology of brain injury resulting from birth asphyxia includes evidence of fetal stress in the hours leading to birth, associated with depression at birth, need for resuscitation,

Table 1 | Mechanisms of HI injury.

Primary energy failure
Decline in cerebral blood flow, O_2 substrates, and high-energy phosphate compounds
Initiation of neurotoxic cascade
Reduction of membrane homeostasis leading to calcium influx, mitochondrial dysfunction, brain acidosis, apoptosis, and necrosis
Latent phase
Normalization of oxidative metabolism
Secondary energy failure
Continuation of neurotoxic cascade
Inflammatory response
Caspase activation
Decrease in levels of protein synthesis and growth factors
Continuation of apoptosis and necrosis

evidence of metabolic acidosis as well as clinical and imaging signs of neurological anomalies (7). This phase is classified as primary energy failure, where reduction in cerebral blood flow and oxygen substrates leads to depletion in adenosine triphosphate ATP and phosphocreatine production and a switch from aerobic to anaerobic metabolism, causing accumulation of brain lactate and tissue acidosis (**Table 1**). Additionally, excitotoxic and oxidative cascades cause excessive stimulation of neurotransmitter receptors and cell membrane ionic transport failure, resulting in accumulation of intracellular calcium (8, 9), and successive cell swelling, activation of neuronal nitric oxide, and subsequent release of reactive oxygen species leading to mitochondria dysfunction, apoptosis, and programed cell death (10). As soon as the energy supplies are exhausted, cell necrosis occurs (11). Following successful reperfusion and resuscitation, there is a normalization of cellular metabolism and intracellular pH leading to neurotransmitter reuptake (12). However, in cases where the hypoxic-ischemic episode is severe or prolonged, these cascading events lead to a secondary energy metabolism failure in the mitochondria and subsequent persistence of excitotoxicity, oxidative stress, induction of inflammatory response, activation of caspase enzymes, and further apoptotic and necrotic cell death (12, 13).

NEONATAL HI AND INFLAMMATION

For a long period of time, the central nervous system (CNS) has been regarded as an immune-privileged site. The blood–brain barrier (BBB), formed by the endothelial lining of the cerebral capillaries, the arachnoid multi-layered epithelium, and the CSF-secreting choroid plexus epithelium, in conjunction with neighboring cell types such as astrocytes and pericytes, prevents infiltration of circulating immune cells, including B- and T-cells, and diminishes the influx of neurotoxic and neuroexcitatory agents from the blood flow (14). However, the CNS has the capacity to generate innate and adaptive immune response. In the CNS, the immune roles of peripheral neutrophils, dendritic cells (DC), macrophages, and natural killer cells are replaced by microglia, astrocytes, and oligodendrocyte precursors (15).

HI brain injury induces a robust inflammatory response in the immature brain (16). Furthermore, injury to neurons leads to a rapid change in their gene expression with stimulation of astrocytes and microglial activation and aggregation for survival support (17). Neuroglial activation is a graded response accompanied by secretion of pro-inflammatory cytokines, causing increased production of nitric oxide, reactive oxygen species, activation of the vascular endothelium, and recruitment of peripheral immune cells into the injured brain (18).

CNS IMMUNE CELLS

Microglia

Microglia are considered as the resident macrophages of the CNS and account for 10–20% of total glial population. Under normal physiological conditions, microglia are present in a resting state with highly ramified and motile processes. However, in the presence of environmental changes to the brain, microglia become rapidly activated, undergoing morphological changes involving retraction of processes and increase in cell body size. Depending on the extent of damage, microglial cells will further activate, become phagocytic and migrate to the site of injury (19). Microglia play an important role in HIE. Retrospective post-mortem clinical studies have shown substantial microglial activation and infiltration in the hippocampal dentate gyrus of HIE infants, which was not observed in infants who had died from trauma or sepsis (20). Microglial contribution to secondary energy failure is thought to occur via production of pro- and anti-inflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α) (21, 22), as well as expression of toll like receptors (TLRs) and antigen presentation (**Figure 1**). Microglial cells are also able to release matrix metalloproteinases, thus leading to breakdown of the BBB, allowing influx of leukocytes into the no longer immune-privileged CNS, thus exacerbating inflammation and subsequent brain damage (23). However, there are contradicting experimental mouse data on whether inhibition of microglial activation following neonatal HI is beneficial (24, 25). The microglial innate immune response is characterized by classical or M1 activation with subsequent production of associated pro-inflammatory molecules, followed by resolution and a switch to alternative or M2 phenotype leading to anti-inflammatory signaling and clearance of reactive species and wound healing (26).

Astrocytes

Astrocytes are the most abundant cell type in the CNS. They are essential supporters of brain homeostasis and neuronal function and also regulate synaptogenesis (27). However, post-mortem clinical studies have demonstrated a prevalence of astrogliosis of 15–40% within the white matter of HI infants (28). Under HI conditions, pro-inflammatory mediators, cytokines, and reactive species produced by damaged neurons and oligodendrocytes can lead to astrogliosis. Activated astroglia, despite not being considered as a traditional inflammatory cell, secrete inflammatory cytokines such as IL-1, IL-6, interferon-γ, and TNF-α (29). Increased levels of these cytokines exacerbate nitric oxide toxicity, and both apoptosis and necrosis, thus aggravating HI injury (30). Astrocytes can also produce chemokines, which attract migration of immune cells into the CNS (31). Astrocytes have TLRs and respond to TLR ligands. Following brain injury, astrocytes

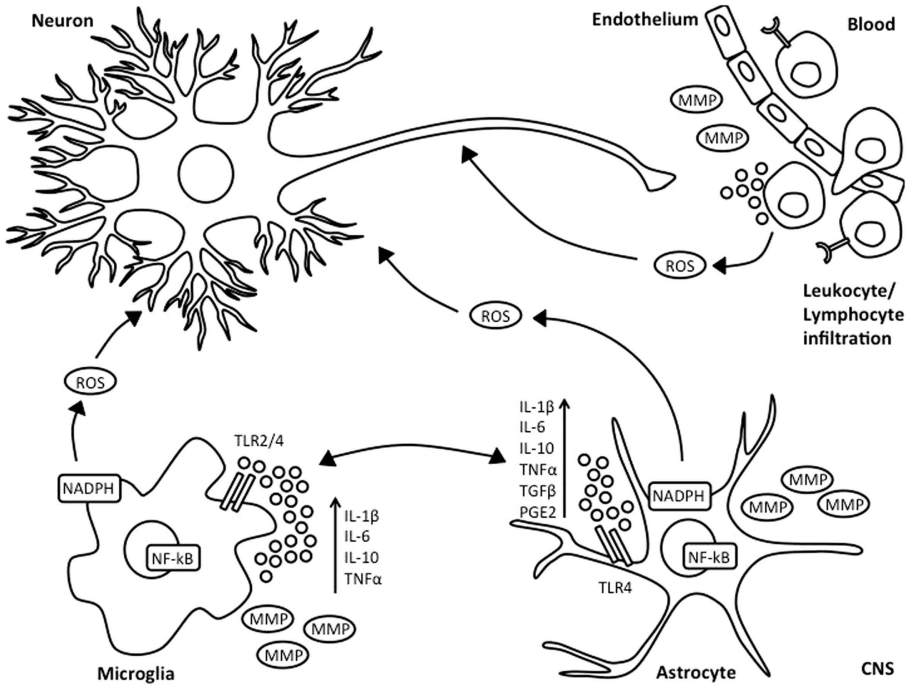

FIGURE 1 | Inflammatory response following HI injury. Neonatal asphyxia leads to activation of microglia and astrocytes, which subsequently results in increased synthesis and secretion of pro- and anti-inflammatory cytokines, reactive oxygen species, and release of matrix metalloproteinases. This is associated with BBB breakdown, as well as influx of leukocyte and lymphocyte immune cells into the injured brain.

also express major histocompatibility complex (MHC) and co-stimulatory molecules, develop Th2 immune response, and inhibit expression of IL-12 (27).

PERIPHERAL IMMUNE CELLS

Neutrophils

It is well established that HI brain injury is associated with infiltration of inflammatory cells into the brain. Neutrophils are the most abundant type of leukocytes and are an integral part of the innate immune system. Although in adult rodent models of ischemic insult, neutrophils are known to accumulate within the brain as early as 4–6 h post injury, and lasting up to 48 h (32, 33), this does not appear to be the case in neonatal HI injury, where infiltration of neutrophils into the injured brain is less marked, with a lesser number present at 42 h post-insult. However, this same study demonstrated that neutropenic P7 rats had 70% reduction in brain swelling at 42 h post-HI when compared to littermate controls (34). Therefore, despite the suggestion that neutrophils do not accumulate in the immature brain following HI, they still play a relevant role in exacerbation of neonatal brain damage.

Lymphocytes

Lymphocytes are granulocyte blood cells crucial in the immune response, and contribute to either adaptive (B- and T-cells) or to innate (NK-cells) immunity. In experimental adult rodent studies, lymphocytes are shown to infiltrate the CNS within a few hours after cerebral ischemia, and to remain within the brain for several days (35, 36). An adult mouse study using RAG1$^{-/-}$ mice deficient in both B- and T-cells has shown a substantial reduction in cerebral infarction in the mutants following cerebral arterial occlusion. Furthermore, the same study demonstrated that mature B-cell negative animals did not show an altered response to ischemic injury (37). Interestingly, in the neonatal mouse model of middle cerebral arterial occlusion (MCAO), T-cell infiltration appears to occur only after 24 h post-insult and persists for up to 96 after injury (38). This could be due to immaturity of lymphoid progenitors at this stage of brain developmental. A clinical study assessing peripheral blood if infants with HIE showed that blood mononuclear cells are still relatively undifferentiated in newborns, with reduced expression of surface markers (39). However, in chronic inflammatory response to HI injury, CD4 lymphocytes are present within the infarcted brain regions 7 days after injury, and persist within the area of damage for a long period of time (40).

Dendritic cells

Dendritic cells are antigen-presenting cells recognized by T-cells and acting as messengers between adaptive and innate immune system. Initial IL-1β and TNF-α response, as well as TLR activation cause translocation of NF-κB inflammation transcription factor into the nucleus of preferentially DC, and also macrophages and endothelial cells, inducing transcription of pentraxin-related protein (PTX3), a soluble pattern recognition receptor from the lectin family (41, 42). Pentraxin not only assists the recognition of microbes and amplification of innate immunity but is also involved in the clearing of self-components and decreased

DC recognition of apoptotic cells (43). A study looking at global pattern of gene expression following neonatal HI has shown activation of PTX3, suggesting a possible role for DC involvement in the subsequent immuno-inflammatory response (16).

INFLAMMATORY MEDIATORS

Cytokines

Both pro- and anti-inflammatory cytokines and their receptors are present in the brain and cerebrospinal fluid, and act as an integral part of the CNS inflammatory response to adverse stimuli (44). In fact, it is widely accepted that cytokines work as a final common pathway to injury from a number of varying insults, including HI. The most widely study cytokines in ischemic models of brain injury are IL-1, IL-6, IL-10, TNF-α, and transforming growth factor-β (TGF-β). From these, IL-1, IL-6, and TNF-α appear to exacerbate brain injury (45), whereas IL-10 and TGF-β may have neuroprotective function following ischemic injury (46). The early response IL-1, IL-6, and TNF-α cytokines are believed to be influential in the progression of injury in the immature brain via stimulation of synthesis of other cytokines and adhesion molecules, and prompting leukocyte infiltration, which in turn will lead to further recruitment of immune cells, as well as induction of neuronal injury mediators such as nitric oxide. This continual and progressive stimulus has influencing modulatory effects on glial gene expression and activation. Depending on the extent of cytokine-mediated cytotoxic inflammatory cellular activation, cell damage and subsequent death occurs (47, 48). Prospective clinical studies have shown an association between high levels of IL-1, IL-6, and TNF-α and infants who are deceased at 1 year of age or diagnosed with cerebral palsy (49). Subsequent clinical studies have also demonstrated a correlation between IL-1 CSF levels and HIE (50). Serum IL-1β, IL-6, IL-8, and TNF-α have demonstrable correlation with the MRS biomarker of anaerobic respiration lactate/choline (51). Additionally, CSF IL-6 levels after neonatal asphyxia are also associated with both early and late neurological outcomes and severity in HIE (52).

Chemokines

Chemokines are chemotactic cytokines thought to act together with different adhesion molecules such as selectins, integrins, and immunoglobulins in order to control immune cell trafficking. These proteins play a detrimental role in various neurodegeneration models, including HI, ischemic stroke, and excitotoxic brain injury (53). A neonatal mouse study of HI injury has demonstrated that mRNA expression of chemokines precedes infiltration of immune cells into the brain, thus proving its relevance in the inflammatory response following insult to the immature brain (31, 40).

Adhesion molecules

Adhesion molecules, including selectins, integrins, and immunoglobulins, play an essential role in leukocyte infiltration to the brain. Initially, adhesion molecules have low affinity binding consisting of rolling of cells, resulting then in high affinity binding and firm adhesion (54). Targeting these molecules in stroke experimental models has demonstrated their importance in brain injury, as inhibition of leukocyte adhesion resulted in improved neurological and histological outcome, whereas over-expression increased tissue infarction (55–57). However, the role of adhesion molecules in HIE still remains largely unknown.

ANTIMICROBIAL PEPTIDES

Antimicrobial peptides are a diverse group of cationic polypeptides containing less than 100 amino acid residues. AMPs were discovered through studies of the insect antimicrobial defense mechanisms and the pathways involved in intracellular phagocytosis of bacteria in different mammalian species (58). For a long time, AMPs were associated with antimicrobial and antifungal activities through opsonization, agglutination, neutralization, or destruction of pathogens (59). Emerging evidence suggests chemotactic and immunomodulatory characteristics of AMPs through chemotaxis, phagocytosis, cytokine production, production of reactive oxygen species, and maturation of DC (59–61). Most AMPs have a positive charge and are divided into several categories based on primary structure and topologies (62), although the most well studied AMPs are cathelicidins and defensins.

Defensins contain six conserved Cys residues forming three disulfide bridges. Depending on the spacing between the Cys residues and the topology of the disulfide bonds defensins are classified in α-, β-, or θ- (61, 62). Defensins are present in many animal species and their expression is associated with cells and tissues involved in host defense against microbial infections (**Table 2**). Depending on the cell type expressing them, defensins act either intracellularly through oxygen-independent destruction of phagocytosed microorganisms or are secreted in the extracellular milieu where they directly attack the microbial membrane. Therefore, defensins are either stored as granules of neutrophils and Paneth cells of the small intestine or secreted by monocytes, macrophages, natural killer cells, keratinocytes, and epithelial cells (61).

α-defensins were first characterized as antimicrobial proteins purified from extracts of cytoplasmic granules of polymorphonuclear leukocytes (PMNs) (63). Human α-defensins are produced by leukocytes, Paneth cells, and epithelial cells of the female urogenital tract. There are six α-defensins, called human neutrophil peptide (HNP) 1–4 and human defensins 5–6 (64). In addition to their antimicrobial activity, some α-defensins (HNP-1) possess also antiviral characteristics. HNP-1 inhibits HIV and influenza virus replication, and inactivates herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and adenovirus (61).

There are four β-defensins known as human beta defensins (HBDs) 1–4 and possessing structural similarity to the α-defensins. HBD-1 was first isolated from human plasma and is constitutively synthesized by epithelial cells of the urinary and respiratory tracts (62), as well as keratinocytes (65). HBD-1 expression can be up-regulated through treatment with lipopolysaccharide (LPS), peptidoglycan, and interferon-γ (62). HBD-2 was first purified from psoriatic scales and its expression overlaps with that of HBD-1, but HBD-2 is also present in skin, pancreas, leukocytes, and bone marrow. HBD-3 was identified simultaneously in psoriatic scales and through bioinformatics, and apart from epithelia is also expressed at lower levels in non-epithelial cells of the heart, liver, fetal thymus, and placenta. HBD-4 was identified by genomics (66) and its expression has been assessed through detection of mRNA and considered to occur primarily in testis and

Table 2 | Cell sources and expression of defensins and cathelicidin.

Name	Defensin type	Cell source	Tissue	Production	Activity
HNP 1–4	α	PMNs	Abundant	Constitutive Inducible	Antimicrobial Antiviral
HBD 5–6	α	Paneth cells Epithelial cells	Abundant	Constitutive	Antimicrobial Chemotactic for PMNs and T-cells
HBD-1	β	Epithelial cells Keratinocytes	Urinary and respiratory tracts	Constitutive and inducible (LPS, peptidoglycan, interferon-γ)	Antimicrobial Chemotactic for monocytes, dendritic cells, and CD4 T-cells
HBD 2–4	β	Epithelial cells Keratinocytes	Psoriatic scales	Inducible (IL-1, TNF-α, LPS)	Antimicrobial
LL-37	Cathelicidin	Epithelial cells, neutrophils, T- and B-lymphocytes, NK-cells, keratinocytes	Thymus, spleen, skin, liver, bone marrow, stomach, intestine and testis	Constitutive and inducible (insulin-like growth factor 1, TNF-α, IL-1 α, IL-6)	Chemotactic for granulocytes and CD4 T-cells

HNP, human neutrophil peptide; HBD, human beta defensin; PMN, polymorphonuclear leukocytes.

epididymis (62). HBD2–4 are inducible and can be up-regulated in response to pro-inflammatory stimuli such as IL-1, TNF-α, and LPS. Multiple defensin genes have been discovered suggesting more HBDs on peptide level (67).

The third family of defensins, the θ-defensins, generate from precursor peptides of α-defensins (68) and have been identified in rhesus macaque monkey leukocytes. The θ-defensins are not expressed in humans due to mutations encoding premature stop codons (69).

Cathelicidins are another major group of structurally and evolutionary distinctive mammalian AMPs constitutively expressed in thymus, spleen, skin, liver, bone marrow, stomach, intestine, and testis, and therefore similar in abundance of expression to the defensins (61, 66, 68). There is only one human cathelicidin gene encoding the amphipathic alpha-helical peptide LL-37 (64). Cathelicidins are constitutively expressed in epithelial cells, neutrophils, T- and B-lymphocytes, NK-cells, and in mouse and human mast cells, and their synthesis can be enhanced by LPS and lipoteichoic acid (66, 70). Cathelicidins have direct antimicrobial effect on Gram+ and Gram− bacteria and their synthesis in keratinocytes can be induced by *Staphylococcus aureus*. Some cytokines (insulin-like growth factor 1, TNF-α, IL-1α, and IL-6) can also induce the synthesis of LL-37 in keratinocytes (61). There are other AMPs, i.e., lysozyme, azurocidin, and bactericidal/permeability-increasing protein, which also possess antimicrobial activities and enhance phagocytosis (64).

Although AMPs are mostly known for their anti-bacterial properties, a great number of them also possess chemotactic features. α-defensins are chemotactic for PMNs and T-cells, HBDs for monocytes, DC, and CD4 T-cells, while LL-37 are chemotactic for granulocytes, as well as CD4 T-cells. All this suggests an essential role of AMPs as a link between innate and adaptive immunity. Generally, AMPs have antimicrobial properties, but are also an essential part of the inflammatory response (71), and different environmental stimuli involving multiple signaling pathways promote their synthesis. Pro-inflammatory molecules (IL-1, TNF-α, IL-6) and bacterial products augment the expression of cathelicidins and defensins through activation of AP-1, JAK2, and STAT3 signaling pathways (61). Altogether AMPs appear to be a crucial component of the antimicrobial host defense, directly inactivating the pathogens and contributing to the immune response associated with the pathogen removal.

COMPLEMENT

The complement system is a crucial component of innate immunity and is responsible for the recognition and elimination of pathogens. Its activation is associated with inflammatory mediation (72, 73) and induction of pro-inflammatory cytokines secretion (72). Activation of the complement system also facilitates clearance of toxic cell debris and apoptotic cells (74–76), as well as immune complexes (72, 76, 77).

The complement system plays an important role in various inflammatory disorders. Its activation can significantly contribute to inflammation-mediated tissue damage following ischemic-reperfusion injury (78), whereas complement deficiencies highly favor the development of autoimmunity (79). The accumulation or unsuccessful removal of cellular debris may contribute to autoimmune disorders like systemic lupus erythematosus (75), as well as various chronic inflammatory diseases like age-related macular degeneration (76), rheumatoid arthritis (80), and asthma (81).

The complement system consists of more than 30 soluble and cell-associated factors and can be activated through three pathways (classical, alternative, and lectin) (**Figure 2**). The components of the complement system are synthesized to a great extent not only by hepatocytes but also by tissue macrophages, blood monocytes, and epithelial cells of the gastrointestinal and genitourinary tracts.

INNATE IMMUNITY OF THE BRAIN

Both AMPs and C components are important factors of the innate immune system. Besides the fact that AMPs and C components are mostly produced in the periphery and that the

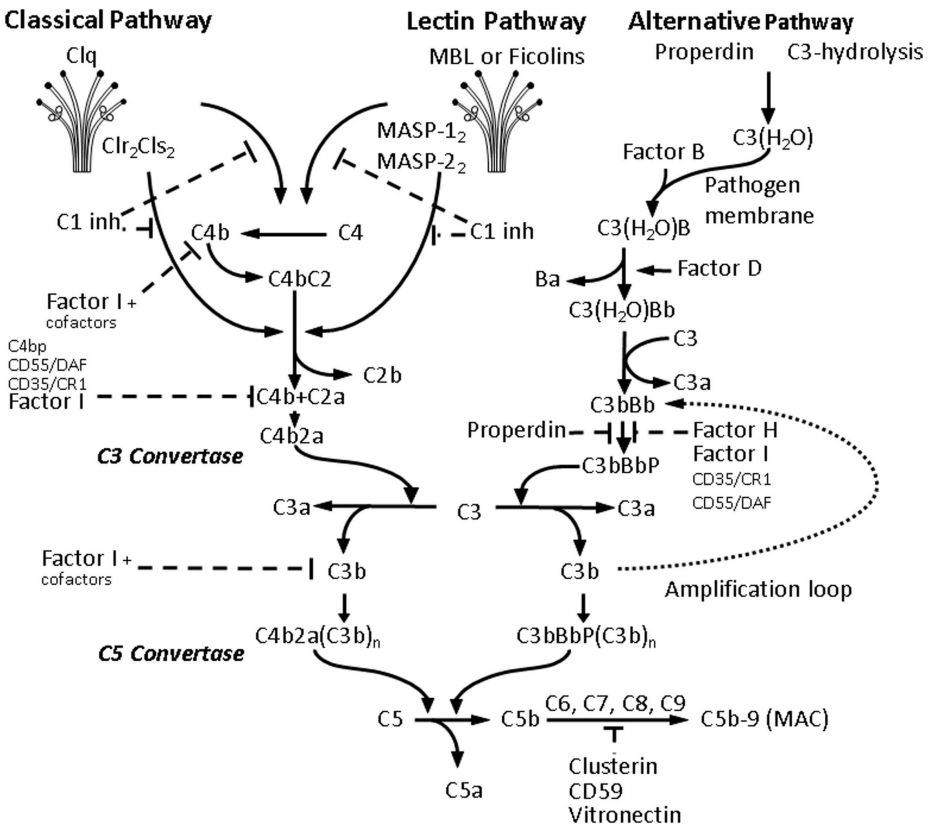

FIGURE 2 | Activation of the complement cascade. Activation of all three C pathways generates homologous variants of C3-convertase cleaving C3 into C3a and C3b, whereas C3a stimulates mast cell degranulation and has chemotactic properties, and C3b acts as an opsonin and binds to the surface of pathogens. Increasing C3b deposition leads to the formation of C5-convertases cleaving C5 into the chemotactic C5a, and the fragment C5b, which together with C6, C7, C8, and the polymeric C9 forms the membrane attack complex (MAC) leading to the formation of transmembrane channel and osmotic lysis of the targeted pathogen. The classical pathway (CP) is initiated by binding of the C1-complex, consisting of a C1q molecule and a tetramer of 2 C1r and 2 C1s molecules, to antigen-bound IgM or IgG. The C1-complex cleaves C2 and C4 into C2a and C2b, and C4a and C4b, respectively. The C2a and C4b fragments form the CP C3-convertase. The lectin pathway activation is due to binding of mannose-binding lectin (MBL) and ficolins (Ficolin-1, -2, and -3) to carbohydrate pattern on microorganisms and dying cells, thus activating the MBL-associated serine proteases MASP-1 and MASP-2, which would in turn cleave C2 and C4. The alternative pathway (AP) is continuously activated through spontaneous C3-hydrolysis, resulting in formation of C3 convertases, which cleave C3 to a C3b-like C3, i.e., $C3(H_2O)$. Complement regulators are typically present on host cells and absent on pathogens, thus allowing $C3(H_2O)$ to bind factor B on the surface of the latter, and form additional C3 convertases after activation by factor D. In the presence of Factor D, $C3(H_2O)B$ is cleaved to Ba and Bb and forms $C3(H_2O)Bb$, which in turn cleaves C3 to C3a and C3b forming C3bBb, which is stabilized by properdin. Properdin bound to microbial surfaces and apoptotic and/or necrotic cells can recruit C3 and also activate the AP (82). The final C3bBbP complex enzymatically cleaves more C3 and amplifies C activation. The C3-convertase of the AP can bind another C3b fragment and the resulting complex $C3bBbP(C3b)_n$ acts as a C5-convertase and triggers the formation of MAC and pathogen elimination.

BBB permeability is not absolute, there is evidence suggesting that both groups of proteins can be also produced in the brain (15, 83, 84).

GLIAL CELLS IN INNATE IMMUNITY OF THE BRAIN

As previously mentioned, both microglia and astroglia are important participants in the innate immune response of the brain and both cell types can produce complement components and AMPs, as well as cytokines (**Table 3**).

NEURONS IN INNATE IMMUNITY OF THE BRAIN

In the brain, immune function and modulatory activity are not features attributed only to immune competent cells, i.e., microglia and astrocytes, but also to non-immune cells. Neurons were originally considered to be just effector cells of C activation and neurodegeneration resulting from glial activation or cytokine influx through the BBB. However, neuronal expression of mRNA for C1q, C2, C3, C4, C5, C6, C7, C8, and C9 has been observed in post-mortem tissue from patients with Alzheimer's disease (AD) (15, 73). Neuronal expression of C1-inhibitor has also been registered in AD cerebral tissue, suggesting expression of C regulator proteins and protection from full C activation associated with membrane attack complex (MAC) formation and cell lysis. Clusterin, C3aR, Factor H, and S protein have also been detected in neurons (73). Therefore, through its capacity of *de novo* synthesis of C components and regulators, the neuronal population appears to be an active player in the innate response of the CNS. So far, there is no data suggesting neuronal AMP production.

Table 3 | Expression of TLRs, complement components, and antimicrobial peptides by microglia and astrocytes.

Cell type	TLRs	C components	C regulators	C receptors	AMPs
Microglia	TLR1, TLR3, TLR5-9 (158) TLR2 (159) TLR4 (160)	C1q, C1r, C1s, C2, C3, C4 (73)	C1-inhibitor (73) CD59, CR1 (15)	C1qR, CR3, C3aR, CR4, C5aR (73)	HBD-1(109) LL-37 (91)
Astrocytes	TLR2 (161, 162) TLR3 (163) TLR4 (162, 164) TLR5 (162, 164) TLR9 (162, 164)	C1q, C1r, C1s, C2, C3, C4, Factor B, Factor D, C5–C9 (73)	C1-inhibitor, Factor H, Factor I, S protein, clusterin (73) CD59, DAF, MCP, CR1 (15)	C1qR, CR2, C3aR, C5aR (73)	HBD-1, HBD-2 (83), LL-37 (91)

OLIGODENDROCYTES IN INNATE IMMUNITY OF THE BRAIN

Oligodendrocytes have also been shown to express C components like C3, as well as C regulator proteins, in particular C1-inhibitor, Factor H, S protein, and clusterin (73).

ENDOTHELIAL CELLS IN INNATE IMMUNITY OF THE BRAIN

Although the data suggesting expression of C components by cerebral epithelium is quite limited and points only toward production of C3 (85) peripheral endothelium has been proven to synthesize C1, Factor B, Factor H, and C5aR. Therefore, there is a possibility that brain epithelium might be also producing these C components. In respect to production of AMPs, synthesis and expression of HBD-2 mRNA and protein have been observed in human brain capillary endothelial cells following exposure to *Chlamydophila pneumoniae* (86). Overall, this data suggest a potential role of brain epithelium in innate immune response and modulation.

COMPLEMENT COMPONENTS AND AMP EXPRESSION IN THE BRAIN UNDER NORMAL CONDITIONS

Some immune proteins such as pro-inflammatory cytokines (TNF-α, IL-6), MHC 1, and MHC receptors, apart from their capacity to trigger and participate in an immune response, also possess non-immune characteristics. Since C components are shown to similarly demonstrate non-immune features, for example, promote proliferation and regeneration in peripheral tissues (76), it is possible that they also execute analogous functions in the CNS. This hypothesis is supported by the observation that C3aR can regulate *in vitro* differentiation and migration of neural progenitor cells (87). In a study looking at the capacity of the classical C pathway to mediate CNS synapse elimination, Stevens and colleagues observed association of C1q and C3 with remodeling of synaptic connections in the visual system of the developing mouse brain (88). Chu and colleagues observed enhanced synaptic connectivity and epilepsy as a result of global deletion of C1q in mice (89). C1q also augments microglial clearance of apoptotic neurons and neuronal blebs and modulates the subsequent inflammatory cytokine production (90).

In the CNS, only HBD-1 is proven to be constitutively expressed. Hao and colleagues detected mRNA for HBD-1 in cultured microglia, astrocytes, and meningeal fibroblasts, but not in neurons (83). Conversely, HBD-2 expression is not constitutive but inducible and can be detected following exposure to LPS and/or pro-inflammatory cytokines (LT-1β, TNF-α) (83). The expression of cathelicidin LL-37 is also inducible and reported in cerebrospinal fluid and serum from patients with bacterial meningitis (91).

COMPLEMENT COMPONENTS AND AMP EXPRESSION IN THE BRAIN UNDER PATHOLOGICAL CONDITIONS

Most of our knowledge of the expression and function of C components and AMPs in the brain is derived from studies of different brain diseases. Both C and AMPs have been registered in bacterial meningitis and cerebral infections, in trauma, stroke, and reperfusion injuries, as well as chronic conditions of brain injury such as AD, multiple sclerosis (MS), Parkinson's, and Huntington's diseases.

COMPLEMENT AND AMPs IN ALZHEIMER'S DISEASE

Cribbs and colleagues observed up-regulation of innate immune system pathways in post-mortem hippocampus from aged and AD patients (92). The C system is associated with the inflammatory response, occurring around the neurofibrillary tangles and amyloid-β (Aβ) plaques in AD. Interestingly, different expression of C components is associated with the different neuropathological progression stages of AD (15). In the early stages of AD, C1q, C4d, and C3d are found, but MAC is absent, while in later stages the levels of C1q, C4d, and C3d are more prominent and MAC is registered in neurofibrillary plaques and neurite tangles (93–98). Yasojima and colleagues observed increased level of C1q in entorhinal cortex, hippocampus, and mid-temporal gyrus, characterized with high density of Aβ-plaques and neurite tangles (99). Additionally, Tooyama and colleagues demonstrated that C1q in the Aβ plaques is endogenously produced in the AD brain (100) suggesting C1q as an important mediator of AD inflammation. Genome wide association studies have allowed considerable progress in understanding AD genetics, identifying loci, including CR1, which are significantly associated with AD susceptibility (101–103).

The Aβ-plaques and the neurofibrillary tangles in AD have been mostly associated with classical C pathway activation, whereas alternative pathway (AP) has been documented only in the Aβ-plaques in human AD patients (104), and in murine AD models (94). The participation of the AP in AD inflammation has been confirmed through AD mouse model using $C1q^{-/-}$ mice, where products resulting from C3 cleavage and properdin were registered in the Aβ-plaques (94, 105). Fonseca and colleagues demonstrated that treatment with PMX205, a C5aR antagonist, significantly

reduces neuropathology in a mouse model of AD (106). However, the data retrieved from mouse models of AD and associated with complement activation should be cautiously considered due to the differences between the mouse models. Fonseca and colleagues observed a much slower progression of the disease in 3xTg mice compared to other transgenic strains and suggested AP activation or a C3-independent cleavage of C5 accounting for the detrimental outcome in these mice (107).

Conversely, the C system might as well play a protective role in AD (93). Osaka and colleagues have demonstrated that C5a may protect against excitotoxicity and activate neuroprotective mitogen activated protein kinase (108).

The data referring to the role of AMPs in AD are relatively limited, although the inflammatory process occurring in AD is associated with increased levels of HBD-1 mRNA in choroid plexus epithelium and HBD-1 protein in hippocampal neurons (109). Therefore, the nature of the AD inflammatory response is complicated and involves both C system and AMPs.

COMPLEMENT AND AMPs IN MULTIPLE SCLEROSIS

C activation in MS is lesion and location dependent. In white matter lesions, C3d and C4d are detected and most likely covalently bound to myelin sheaths, while C3d, C1q, and C5 are associate with disrupted myelin, micro- and astroglia, and vessel walls (15, 110, 111). It is possible that some C factors in the white matter lesions are rapidly turned over as detection of C1q and MAC on myelin sheaths so far has not been successful (15). In gray matter lesions, C activation is very low (110), while in mixed white and gray matter C3d and C4d are detected on the myelin sheaths on the border of the lesions and C3d is only registered in the blood vessels (15). The production of C factors in MS is most likely endogenous, with macrophages considered as a main source of C1q and C3 and astroglia testing positive for C components in all lesions.

Direct link between AMPs and MS has not been yet established. Ultraviolet-B irradiation and vitamin D are important factors explaining the geographic variation and the increased prevalence of MS in areas with lower amount of sunshine (112). Once MS has developed, ultraviolet-B irradiation and vitamin D can reduce the severity of the disease through vitamin D-induced apoptosis of CD4 T lymphocytes (113). Vitamin D enhances innate immunity and the transcription of cathelicidin (114) and different defensins (115). Therefore, the balance of AMPs might be an important factor associated with the control of the inflammatory process in MS.

COMPLEMENT AND AMPs IN STROKE AND REPERFUSION INJURIES

The pathobiology of stroke involves an inflammatory response associated with all stages of the ischemic cascade, starting from the early damaging events triggered by arterial occlusion to the late regenerative processes underlying post-ischemic tissue repair (23). Increased immunoreactivity for C1q, C3c, C4d, and C9, and virtually absent C regulators were registered in ischemic lesions from patients with acute brain ischemia or ischemic stroke, suggesting activation of the classical C pathway (116). Therefore, the combination of increased deposition of C components and decreased expression of C regulators is a possible mechanism of tissue damage during ischemia in human brain. Supporting evidence for this hypothesis is provided by the study of Van Beek and colleagues who characterized the expression of different C components following permanent MCAO in the mouse (117). Their data demonstrate increased levels of C1q and C4 mRNA in ischemic cortex and increased expression of C4 in perifocal neurons suggesting local expression of C components, which (i) may contribute to the inflammatory process and represent a key component in secondary injury and (ii) may result in the formation of MAC and contribute to host cell lysis (117). The role of the AP in ischemic stroke has not yet been fully investigated, although a study by Elvington and colleagues in a murine model of MCAO suggests that the AP propagates cerebral inflammation and injury through amplification of the complement cascade (118).

Conversely, some data suggest protective role of the C system following ischemic injury, proposing that C activation does not appear to be a primary contributor to brain injury in a rabbit model of acute thromboembolic stroke (119) and also that C activation contributes to remodeling during repair in the CNS (73).

There is no data directly connecting AMP expression and stroke, although the inflammatory response resulting from ischemic injury can be associated with AMP production. Williams and colleagues proposed that central to the innate and adaptive immune response, and the prolongation of inflammation within the brain, is a dysregulation of constitutively expressed and inducible AMPs (84). *In vitro* exposure of human primary epithelial cells to high levels of glucose or low insulin results in decreased expression of HBD-2 and HBD-3 (84). Ischemic events in the adult brain are associated with the occurrence of chronic hyperglycemia, which can contribute to glycation of specific amino acid residues on AMPs, resulting in conformational changes and inhibition or prolongation of AMP function (84, 120). Therefore, the balance of AMP expression in the brain might be crucial for the inflammatory processes and subsequent occurrence of brain damage in the CNS.

COMPLEMENT AND NEONATAL HI

Complement is an essential aspect of innate immunity, and plays a role not only in normal brain physiology but also during pathology, including ischemia. Experimental research using rodent models of HI are now starting to clarify its role in hypoxic-ischemic brain injury.

A hallmark of hypoxia-ischemia primary energy failure is acidosis. A study by Sonntag and colleagues looking at umbilical arterial pH 22–28 h after birth has shown that serum C3a and C5a are increased after fetal acidosis (121). Another clinical study has demonstrated that circulating C3 is reduced following neonatal asphyxia (122). Initial experimental rodent studies have shown that C9 administration appeared to be detrimental (123), and that cobra venom factor (CVF) treatment did not affect HI induced brain injury in a study by Lassiter et al. (124). However, this same treatment approach was performed subsequently by Cowell and colleagues, and shown that CVF pretreatment decreases brain infarction following neonatal HI (125). Precise studies using C1q knockout mice were used to investigate the classical C pathway role in neonatal HI. A study by Ten and colleagues revealed that $C1q^{-/-}$ mice had substantial reduction in brain infarction,

as well as neurofunctional impairment when compared to wild type controls. Furthermore, wild type mice demonstrated greater deposits of C1q and C3 deposits within the brain as well as the presence of granulocytes in the area of infarction (126). This study strongly suggests that classical complement activation and subsequent brain deposition of C1q and C3 is not only associated with infiltration of granulocytes but also with HI brain injury. This hypothesis was further strengthened when the same group looked at brain mitochondria, and demonstrated that neurons of $C1q^{-/-}$ mice were resistant to hypoxia-ischemia, with preserved brain mitochondria respiration, and reduced production of reactive oxygen species. Additionally, this study demonstrated that classical complement activation detrimental role in hypoxic-ischemic injury does not involve activation of MAC (127).

C3 is expressed in the brain by both neurons and glial cells (**Table 3**). Its activation and subsequent generation of C3a is known to have pro-inflammatory properties, and its expression appears to be detrimental in several models of CNS injury. However, C3a also has anti-inflammatory properties following LPS administration, by decreasing LPS-mediated cytokine release (128). Additionally, *in vitro* studies have shown its neuroprotective effects by acting on both microglia (129) and astrocytes (130). C3a can bind to its canonical receptor C3aR, and according to some controversial data to the alternative receptor C5L2 (131–133), which is expressed in both neurons and glia cells and has anti-inflammatory properties (134). However, in a study by Järlestedt and colleagues, it was demonstrated that canonical over-expression of C3a in astrocytes resulted in reduction of HI-induction of hippocampal tissue loss, as well as reduced numbers of astrocytes and microglia/macrophages in the ipsilateral striatum, suggesting that C3a protective role following HI is a result of its binding to C3aR (135).

Overall, experimental studies have shown that C1q is highly present in the brain following ischemia (136), and that classical complement pathway activation via C1q generates C3a and C5a pro-inflammatory mediators, which are associated with HI brain injury (137, 138), as well as complement-associated genes (16). Additionally, deletion of C1q not only reduces brain infarction and neurofunctional deficit but also results in protection of mitochondria respiration, indicating a role for classical complement activation and brain oxidative stress (127), and demonstrating a link between innate immunity and oxidative stress. Conversely, C3aR-mediated activation of C3a has also revealed a degree of protection following HI insult.

AMPs AND NEONATAL HI

Currently, there is no direct evidence for the involvement of AMPs in neonatal HI brain damage. In respect of the capability of the CNS to locally produce AMPs and the evidence for their participation in inflammation-associated diseases such as AD and MS, AMP involvement in neonatal HI inflammation and subsequent brain damage is quite possible.

As previously described neonatal HI triggers an inflammatory response including activation of microglia, astroglia, DC, and is associated with release of pro- and anti-inflammatory cytokines, chemokines, and adhesion molecules. According to Bain and colleagues one of the reasons for the occurrence of HI brain damage is the misbalance in the release of pro- (IL-1, IL-6, IL-8, TNF-α) and anti-inflammatory (IL-10) cytokines promoting differentiation of oligodendrocyte precursor cells into astrocytes, but not oligodendroglia thus impairing subsequent myelination (139). In a mouse model of neonatal HI, Shrivastava and colleagues observed up-regulation of pro-inflammatory IL-1β, IL-6, and TNF-α and modulation of anti-inflammatory cytokines IL-1 receptor antagonist, IL-4, IL-13, and IL-10 (140). The up-regulation of IL-1β is to a great extent due to microglial activation in response to the HI injury and subsequently affects astrogliosis. Both IL-1 and IL-6 have been implicated in the induction and modulation of reactive astrogliosis (141). Microglial IL-1β might be directly affecting astroglial activation (142), while the effects of IL-6 on astrogliosis might be either direct or through the JAK2/STAT3 pathway as STAT3 is a critical transcription factor regulating astroglial maturation and GFAP expression (143, 144). Activated astroglia can subsequently produce HBD-2 (83) and alter the innate immune response in the brain following neonatal HI injury.

Neonatal HI might be directly affecting astrocytes promoting IL-1β (142) production, which can amplify astroglial and microglial activation and stimulate both cell types to produce AMPs. Conversely, activated astrocytes can also down regulate microglial activation through production of anti-inflammatory cytokines such as transforming growth factor β and prostaglandin E_2 (145, 146) thus limiting inflammation and subsequent neurodegeneration (142). This can potentially affect microglial production of AMPs.

Post-HI hyperglycemia is harmful for the HIE (147). Although results obtained from adult experiments cannot be directly transferred and used as explanation for neonatal data due to differences in the level of maturation and enzyme development (5, 140), some pathways might be valid in adult as well as neonatal set-up. Therefore, observations of low AMP levels due to hyperglycemia and/or increased insulin resistance associated with many neuropathologies such as AD (84) might be also valid following neonatal HI. Reduction of mRNA for HBD-2 and HBD-3 has been observed after *in vitro* exposure of human epithelial cells to high glucose and/or low insulin (84). Therefore, the detrimental effects of hyperglycemia following neonatal HI might be attributed to alteration of AMP production.

Toll like receptors are important for antigen recognition in the CNS. TLR-regulated responses can control elimination of cell debris and promote repair in the brain (148) and are suggested to play an important part in CNS inflammatory conditions, including ischemia (141, 149). Microglial cells express TLRs and respond to TLR ligands (**Figure 1**) (148). TLR-4 is expressed by astrocytes, endothelial cells, and neurons (150, 151). Hao and colleagues suggested that astrocytic production of HBD-2 depends on cytokine stimulation of TLR, IL-1β, and TNF-α receptors (83). The precise mechanism through which astrocytes produce defensins is unclear, but TLRs induce NF-κB activation in response to cytokines and bacterial toxins, thus stimulating astrocytes to produce AMPs (64) (**Figure 1**). This hypothesis might be also valid in respect to astrocytic AMP production following neonatal HI.

Another mechanism through which AMPs might be affecting the inflammatory response following neonatal HI is through their ability to recruit immature DC to the site of inflammation and

promote DC maturation through TLR-4, thus modulating the adaptive immune response of the brain (152).

All these suggest that misbalance of constitutively expressed (HBD-1) and inducible (HBD-2, HBD-3) AMPs, as well as cathelicidin (LL-37) is likely to occur in cases of inflammation-associated neurodegeneration in the adult brain, as well as following neonatal HI.

So far, there is no evidence suggesting direct damaging effects of AMPs on mammalian cells (83). Supportive evidence for the role of AMPs in the innate immune response following neonatal HI is the use of novel innate defense regulator peptides (IDRs) in animal models of neonatal cerebral inflammation and injury. IDRs are synthetic derivatives of endogenous cationic host defense peptides such as cathelicidin, selectively suppressing inflammation and augmenting protective immunity to pathogens (153–155). Recently, Bolouri and colleagues demonstrated that IDR-1018 suppressed pro-inflammatory gene regulation in a neonatal mouse model of LPS-sensitized HI damage (156). The same group also suggested that post-HI treatment with IDR-1018 reduces LPS-induced HI brain damage. Therefore, IDRs might be promising neuroprotective agents for neonatal HI.

CONCLUSION

Many neurodegenerative disorders have similar pathogenic mechanisms and data obtained from one disease may prove valid for another. There is a considerable amount of information in respect to the role of the C system, but there is no direct evidence for the involvement of AMPs in neonatal HI brain injury. Neonatal HI is associated with a robust inflammatory response, involving rapid change in neuronal gene expression associated with stimulation and aggregation of astrocytes and microglia for survival support (17). Activated microglia and astrocytes produce immunomodulatory proteins such as C components (73) and AMPs. We propose that misbalance of those proteins affects the equilibrium between pro- and anti-inflammatory cytokines in the CNS, resulting in prolonged inflammatory response and subsequent brain injury following neonatal HI. The regulation of the innate immune response simultaneously with cellular repair in the CNS is very complex, but the capability of both microglial and astroglial cells to produce C components and AMPs under cytokine stimulation suggests a role for both types of proteins in the brain. Whether this role is associated with the initiation or prolongation of the inflammatory response and subsequent damage following neonatal HI is unclear and needs further investigation. Deletion of some complement components such as C1q, as well as over-expression of others (C3a) has proven protective in neonatal HI brain damage. Application of CVF, C1-inhibitor, C3-inhibition through soluble CR1 or C3-deletion, as well as immunoglobulin treatment, have all shown protective effects in adult stroke animal models (157), suggesting that complement-targeted therapy could prove effective in neonatal HI and needs further investigation. The protective effects of IDRs in neonatal mouse models of HI suggest a potential key role for AMPs in the inflammatory response following neonatal HI brain injury. In conclusion, both C and AMPs appear to be key modulators of the innate and to some extent adaptive immune response following neonatal HI, which makes them potential candidates for neuroprotective strategy.

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Antimicrobial peptides in human sepsis

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Nearly 100 years ago, antimicrobial peptides (AMPs) were identified as an important part of innate immunity. They exist in species from bacteria to mammals and can be isolated in body fluids and on surfaces constitutively or induced by inflammation. Defensins have anti-bacterial effects against Gram-positive and Gram-negative bacteria as well as anti-viral and anti-yeast effects. Human neutrophil peptides (HNP) 1–3 and human beta-defensins (HBDs) 1–3 are some of the most important defensins in humans. Recent studies have demonstrated higher levels of HNP 1–3 and HBD-2 in sepsis. The bactericidal/permeability-increasing protein (BPI) attenuates local inflammatory response and decreases systemic toxicity of endotoxins. Moreover, BPI might reflect the severity of organ dysfunction in sepsis. Elevated plasma lactoferrin is detected in patients with organ failure. HNP 1–3, lactoferrin, BPI, and heparin-binding protein are increased in sepsis. Human lactoferrin peptide 1–11 (hLF 1–11) possesses antimicrobial activity and modulates inflammation. The recombinant form of lactoferrin [talactoferrin alpha (TLF)] has been shown to decrease mortality in critically ill patients. A phase II/III study with TLF in sepsis did not confirm this result. The growing number of multiresistant bacteria is an ongoing problem in sepsis therapy. Furthermore, antibiotics are known to promote the liberation of pro-inflammatory cell components and thus augment the severity of sepsis. Compared to antibiotics, AMPs kill bacteria but also neutralize pathogenic factors such as lipopolysaccharide. The obstacle to applying naturally occurring AMPs is their high nephro- and neurotoxicity. Therefore, the challenge is to develop peptides to treat septic patients effectively without causing harm. This overview focuses on natural and synthetic AMPs in human and experimental sepsis and their potential to provide significant improvements in the treatment of critically ill with severe infections.

Keywords: sepsis, antimicrobial peptides, synthetic, naturally occurring peptides, therapy

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Natural Occurrence in Humans – Change of AMPs in Inflammatory Disease

Antimicrobial peptides (AMPs) are an important component of multicellular organisms' innate immune systems, targeting invading pathogens, including bacteria, viruses, fungi, and parasites (1). The growing relevance of AMPs in recent years is owed to their capability to overcome increasing antibiotic resistance due to their unique combination of anti-inflammatory, antimicrobial, and immunostimulatory qualities (2–4). Generally, AMPs differ greatly in sequence and structure. These peptides are predominantly short (10–50 amino acids) amphipathic molecules. Based on amino acid composition and secondary structures, they can be divided into four groups: (i) α-helical peptides,

(ii) β-sheet peptides stabilized by two to four disulfide bonds, (iii) extended structures, and (iv) loop peptides with one disulfide bond (3, 5, 6).

The most extensively investigated peptides of the mammalian gene family are the defensins and cathelicidins. The defensins, consisting of the alpha and beta subgroups, represent more than 5% of the total protein of human neutrophils (7) and are derived from intestinal Paneth cells, neutrophils, macrophages (1), epithelial cells, mucosal epithelial cells, and keratinocytes (8). Through the stimulation of toll-like receptors (TLRs), including TLR-2, TLR-3, and TLR-5, α-defensins [human neutrophil peptides (HNP) 1–4] are released by their producing cells (4, 8–17). Altogether, six human α-defensins from the granules of neutrophils (HNPs1–4) and Paneth cells (human defensins including HD5 and HD6) as well as four human β-defensins derived from epithelial cells were studied in detail. It has been shown that alterations in expression may influence inflammatory disorders, which emphasizes the importance of these peptides in controlling and preventing microbial infections (18, 19). Some AMPs are constitutively expressed, whereas others can be induced [e.g., HNP 1–3, human beta-defensin (HBD)-2] in response to inflammation (8).

The mechanisms in neutrophil trafficking and function in sepsis have been reviewed previously in Ref. (20). A weakened response to chemotaxis and alterations in neutrophils may result after dysregulation of TLR expression. TLR activation itself results in a downstream liberation of AMP as well as cytokine and chemokine release. This, in turn, activates NF-kB and mitogen-activated protein kinase (MAPK) pathways. A constantly activated TLR may lead to a strongly increased expression of cytokines, thus aggravating sepsis in critically ill patients (20–22).

Cathelicidins are produced by proteolysis of the C-terminus of protein precursors. In humans, only one precursor, hCAP18, is produced mainly in leukocytes and epithelial cells and forms the LL-37 peptide, among others. The application of LL-37 in infection therapy has been hampered by its toxicity. The incubation of smooth muscle cells with 20 μM LL-37 resulted in 20-fold higher DNA fragmentation compared to the control (23). Moreover, human serum inhibited the antimicrobial effects. Another problem is that some multiresistant strains (e.g., USA600-MRSA) showed increased resistance against LL-37, which has been suggested to be responsible for higher mortality rates (24). When the *N*-terminal hydrophobic amino acids of LL-37 were removed, a decrease in cytotoxicity was detected. Furthermore, inhibition of the antimicrobial and lipopolysaccharide (LPS)-neutralizing effects of LL-37 by human serum was reduced. Thus, LL-37-derived peptides may provide a benefit when treating sepsis patients (23). Innate immunity, especially in the case of sepsis, may be influenced by vitamin D status. In turn, vitamin D status regulates the LL-37 levels in sepsis (25). The possible underlying mechanism has been described as a TLR activation of macrophages, which results in increased expression of both the receptor and the hydroxylase of vitamin D, thus inducing AMPs (26). Interestingly, a deficiency in vitamin D is a predictor of sepsis in critically ill patients and results in higher mortality in the intensive care unit (ICU) (27).

Bactericidal/permeability-increasing protein (BPI) is an AMP stored in leukocytes that has a high affinity for LPSs of Gram-negative bacteria. The anti-infective properties of BPI include the permeabilization of bacterial membranes, in addition to the neutralization of LPS (28).

Lactoferrin is a glycoprotein located in the majority of exocrine secretions (e.g., milk, tears, nasal secretions) (29) and in neutrophils (30). Its wide antimicrobial spectrum supports the body's immune response to bacterial, viral, and fungal pathogens (31–34). Anti-bacterial peptides, which are part of the polypeptide chain of lactoferrin, are released after proteolysis and may be developed for new agents in antimicrobial therapy (35, 36). Nevertheless, the exact mechanism of the antimicrobial activity of lactoferrin peptides has not been described to date.

Antimicrobial peptides prevent and control microbial infections by both direct antimicrobial killing and innate immune modulation (4, 9, 37, 38). Direct antimicrobial killing is achieved by the disruption of bacterial cell membranes or translocation into bacteria to affect internal targets (3) (**Figure 1**). The cationic amphipathic AMPs bind to the negatively charged phospholipids of the bacterial cell membranes (3). It is assumed that pore formation and non-specific membrane permeabilization lead to a disruption of the membrane (**Figure 1**). Recently, there has been increasing evidence that molecules on the cell surface act as targets for the AMPs and induce direct killing (10, 39). However, the immunostimulatory properties have been more appreciated for their diversity, including cell migration, survival and proliferation, induction of antimicrobial and immune mediators such as cytokines/chemokines, wound healing, and angiogenesis (**Figure 2**) (4, 40). The results show that HBD-2 seems to be chemotactic for cells that express the human chemokine receptor CCR6 (41). CCR6 is preferentially expressed by immature monocytic dendritic cells (DCs) and CD8+ T-cells that have the memory phenotype (42). Additionally, HNP 1–3 are chemotactic for monocytes, immature DCs and CD4+ and CD8+

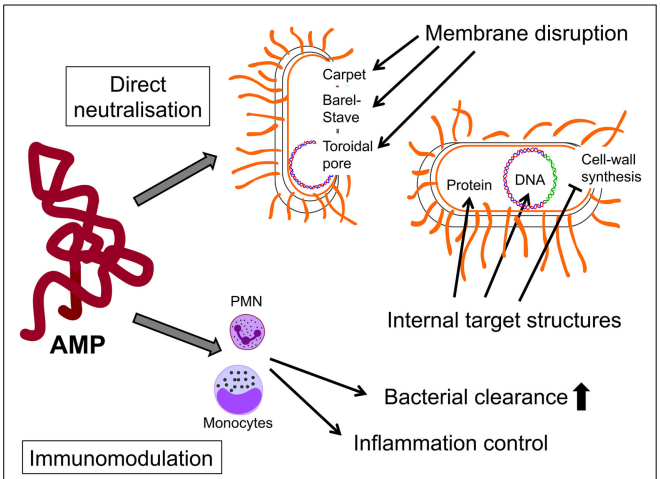

FIGURE 1 | Different modes of action of antimicrobial peptides. AMPs may have direct neutralizing effects on bacteria e.g., by membrane disruption through pore forming or by targeting internal structures of bacteria. In addition to direct effects, AMPs may modulate cells of the adaptive immunity (neutrophils, t-cells, macrophages) to control inflammation and/or to increase bacterial clearance. Modified from Ref. (3). AMPs, antimicrobial peptides.

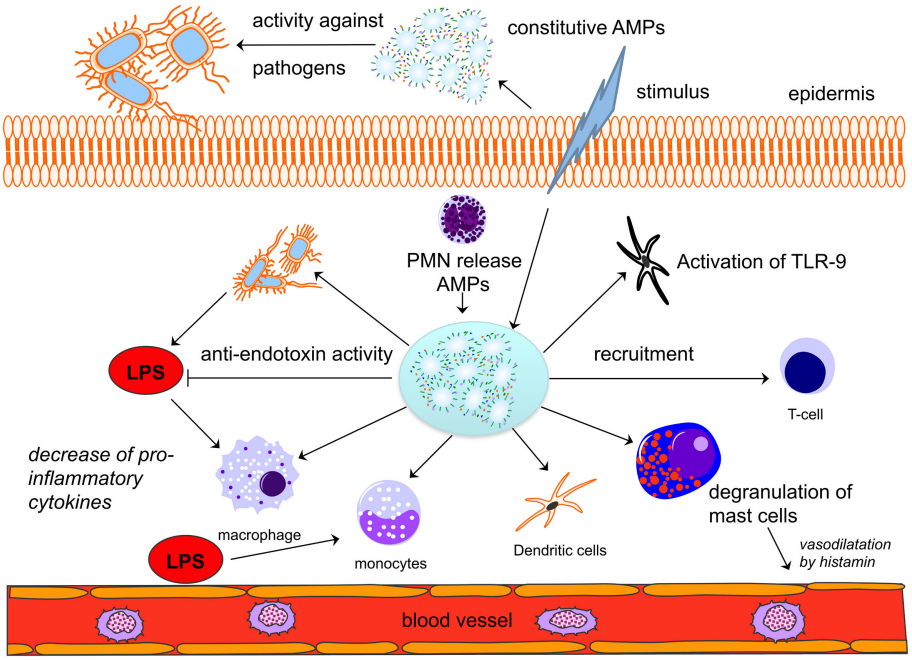

FIGURE 2 | Antimicrobial peptides play a central role in innate and adaptive immunity. A given stimulus by bacteria leads to the release of constitutively expressed AMPs in different cells (here: epidermis). AMPs are released by neutrophils and will activate and recruit macrophages, monocytes, dendritic cells, and T-cells. A direct anti-endotoxin effect of AMPs may decrease the activation of immune cells and thus lead to a decrease in pro-inflammatory cytokine release. Modified from Ref. (26). AMPs, antimicrobial peptides; LPS, lipopolysaccharide; PMNs, neutrophils.

T-cells (43, 44). Furthermore, they induce the release of pro-inflammatory cytokines such as IFN-λ, IL-6, and IL-10 from T-cells as well as TNF-α and IL-1β from monocytes (45, 46). These processes contribute to the maturation of DCs, which link the innate and adaptive immune systems. Stimulation with TNFα contributes to this maturation. DCs activate CD4+ T-cells (and their subsets) and CD8+ T-cells as well as B-cells. In turn, monocytes may be induced by a peptide to differentiate into DCs (47).

Moreover, AMPs protect the organism against harmful pro-inflammatory immune responses, especially against TLR-induced cytokine release. For instance, the above described LL-37 allows free DNA fragments to enter DCs. Consequently, IFN-α is released in reasonable amounts by TLR-9 interaction (**Figure 2**) (48). The free DNA fragments are able to neutralize extracellular LPS and/or stimulate the expression of anti-inflammatory mediators by affecting different signaling pathways associated with, for example, MyD88 and TRIF (49–52).

Role of AMPs in Sepsis

Antimicrobial peptides were identified nearly 100 years ago in body fluids and on body surfaces after inflammatory stimulation (53). However, studies of AMPs in patients with severe sepsis or septic shock are limited. AMPs have been investigated in patients with abscesses, peritonitis, or uninfected body fluid levels of the LPS-binding protein (LBP) and BPI, which prevents endotoxin binding to CD14. The BPI/LBP ratio was significantly elevated in abscesses compared to peritoneal and non-infected fluids. Moreover, the BPI concentration was higher in abscesses with Gram-positive compared to those with Gram-negative organisms. The authors concluded that BPI might attenuate the local inflammatory response and the systemic toxicity of endotoxins released during Gram-negative infections (54). A further study in the same year investigated the levels of polymorphonuclear leukocyte surface BPI, plasma BPI, and plasma LBP in normal human volunteers who were administered *Escherichia coli* LPS and in patients with sepsis and Gram-negative infections. Compared with controls, LPS-challenged volunteers and patients with sepsis both exhibited increased concentrations of polymorphonuclear leukocyte surface BPI and plasma LBP (55). Rintala et al. investigated BPI levels and BPI/neutrophil ratios in 42 healthy controls and 34 patients with severe sepsis. Because of an association between decreased arterial blood pressure and levels of BPI, the authors concluded that BPI might indicate the severity of organ dysfunction in sepsis (56).

As endogenous ligands of TLR-4, HBD 1–3 interact with TLR-4 on immune cells and regulate the expression of inflammatory mediators via the NF-κB pathway (57).

A study determined concentrations of HBD-1, HBD-2, and cathelicidin LL-37/hCAP-18 in tracheal aspirates of mechanically ventilated newborn infants. Concentrations of AMPs correlated with each other and with levels of interleukin-8 and tumor necrosis factor-α in the bronchoalveolar lavage fluid. Pulmonary or systemic infections were associated with significantly increased concentrations of HBD-1, HBD-2, and LL-37 (58). A further study investigated the effect of overexpression of BD-2 on lung injury to evaluate whether the function of BD-2 in the lung could be attributed to both antimicrobial action and modulation of the

immune response. Therefore, recombinant adenoviruses carrying an expression cassette of rat BD-2 or control adenovirus carrying an empty vector were administered intratracheally to Sprague-Dawley rats. After 48 h, acute lung injury was induced by either *Pseudomonas aeruginosa* infection or cecal ligation and puncture (CLP). The amounts of the *P. aeruginosa* in the lung with BD-2 overexpression were significantly lower compared to those of the controls. Furthermore, the overexpression of BD-2 reduced alveolar damage and interstitial edema and also significantly improved the survival rate (59).

A prospective case-control study investigated levels of HBD-2 in 16 patients with severe sepsis. HBD-2 plasma levels in septic patients were significantly higher compared to those in healthy controls and critically ill non-septic patients. Procalcitonin plasma levels and HBD-2 protein plasma levels showed a positive correlation in patients with severe sepsis. Moreover, the study investigated the *ex vivo* inducibility of HBD-2 mRNA in peripheral whole blood cells from patients with severe sepsis compared to non-septic critically ill patients and healthy individuals. Endotoxin-inducible HBD-2 mRNA expression was significantly decreased in patients with severe sepsis compared to healthy controls and non-septic critically ill patients, which may contribute to the complex immunological dysfunction in patients with severe sepsis. The contradiction between the decreased inducibility of HBD-2 in peripheral blood cells of patients with severe sepsis and the elevated levels of HBD-2 in septic plasma may suggest that in addition to peripheral blood cells, circulating endothelial cells or reticuloendothelial cells (e.g., monocytes or macrophages) may serve as a possible source of HBD-2 *in vivo* (60).

A prospective cross-sectional and longitudinal study in a university children's hospital pediatric ICU investigated the systemic release of endogenous HNP 1–3 and lactoferrin in children with severe sepsis. Septic patients showed increased HNP 1–3 and lactoferrin plasma concentrations compared with non-septic critically ill control patients. Furthermore, HNP 1–3 and lactoferrin plasma concentrations correlated with total white blood cell and neutrophil counts. Although increased plasma lactoferrin concentrations were observed with the development of organ failure, there was no association between plasma HNP 1–3 concentration and organ failure or outcome. This observation is weakened by the fact that other mediators such as cytokines and nitrite radicals were not measured. Additionally, lactoferrin concentrations did not differ between non-survivors and survivors and did not correlate with the type of pathogen (61). The enhancement and adherence of neutrophils in damaged tissue may serve as a possible explanation for the correlation between lactoferrin concentration and organ failure. Moreover, high levels of lactoferrin were detected in patients with complement activation (62).

Another observational study determined HBD-2 levels and their impact on sepsis in term and preterm neonates at birth. HBD-2 levels in term neonates were higher compared with preterm infants and correlated with gestational age and birth weight. Of 31 preterm neonates, seven suffered from late-onset sepsis, and this was associated with lower HBD-2 levels (63). Furthermore, it was shown that HNP 1–3, lactoferrin, BPI, and heparin-binding protein (HBP) exerted higher levels in neonates with sepsis (64).

Failed Attempts to Introduce AMP in Sepsis Therapy

Despite their discovery in 1939, AMPs are still rare in daily clinical practice. Currently, there are a significant number of products in development for topical applications of AMPs, such as BL 5010 (BiolineRX) against skin lesions or LTX-109 (Lytix) for the nasal eradication of *Staphylococcus aureus*.

To date, there have been only a few investigations into the therapeutic use of AMPs in sepsis. Promising results of the application of AMPs for meningococcal infection in children have been published (65). Children with suspected meningococcal sepsis were randomly assigned to receive a recombinant 21-kDA modified N-terminal fragment of human BPI (rBPI21) within 8 h of diagnosis (65). The administration of rBPI21 compared to placebo therapy was not superior with respect to mortality. One underlying reason may be the lower-than-expected placebo mortality (10% vs. expected 25%) because most deaths occurred in the interval between identification of patients and rBPI21 administration (65). However, children randomized to rBPI21 treatment showed a trend toward reduced multiple severe amputations and significantly higher physical and neurological abilities according to the pediatric overall performance category (POPC) scale (65).

Another AMP with clinical potential is the human lactoferrin peptide 1–11 (hLF 1–11), a derivative of the human lactoferrin that can be found in neutrophils or in body fluids (66). hLF 1–11 comprises antimicrobial activity and modulation of the inflammatory immune response. In a double-blind and placebo-controlled study to assess the side effects of hLF 1–11, the drug was tested in healthy volunteers and in patient undergoing hematopoietic stem cell transplantation. It showed a favorable side effect profile with only a slight elevation of liver enzymes (66). A planned study for the intravenous application of hLF 1–11 for 10 consecutive days in patients with bacteremia due to *Staphylococcus epidermidis* was withdrawn prior to enrollment for strategic reasons by AM pharma (NCT00509847). According to clinical trials, this decision was based on a strategic company decision. The homepage of AM pharma has no further information about hLF 1–11 or planned trials with the drug, so the future use of hLF 1–11 remains unclear (http://www.am-pharma.com).

A different recombinant form of lactoferrin is talactoferrin alpha (TLF). TLF and lactoferrin possess identical molecular structures, biological activity and in other biochemical properties except for their nature of glycosylation (67). In a phase II study, 194 sepsis patients with at least one organ dysfunction were enrolled and assigned to a TLF or placebo group. Patients under medication with oral TLF showed a lower 28-day mortality rate with a sustained effect on mortality after 6 months. The decrease in mortality was more pronounced in patients with a higher severity of disease as expressed by APACHE-II scores above 25 points (**Figure 3**). Nonetheless, there was no significant difference regarding ICU days or ventilator-free days (67). Due to the promising results, a phase II/III study was initiated (safety and efficacy of TLF in patients with severe sepsis, OASIS; NCT 01273779). Surprisingly, the study was prematurely terminated due to the recommendation of the data safety monitoring board because of a higher 28-day mortality rate in the

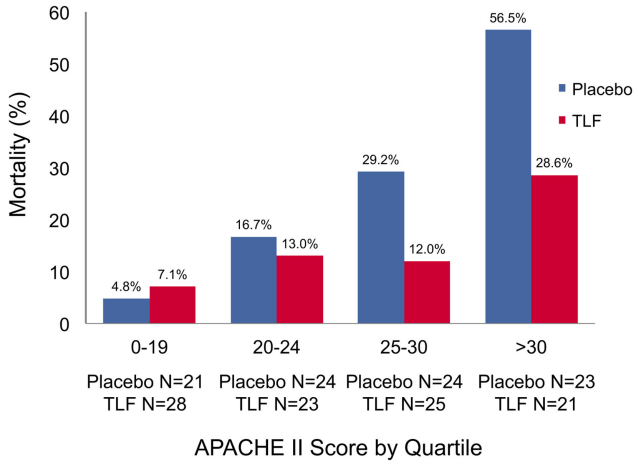

FIGURE 3 | Mortality of patients with severe sepsis and septic shock treated with talactoferrin or placebo. The mortality is reported in relation to disease severity as expressed by the APACHE-II score. A positive effect of oral talactoferrin treatment on mortality in sepsis is detectable in patients with higher severity of disease (APACHE-II > 25). Patients with a lower APACHE-II score benefited less (APACHE-II 20–24) or not at all (APACHE-II < 19). APACHE-II score, Acute Physiology and Chronic Health Evaluation-II score. Reprinted with permission from Guntupalli et al. (67).

talactoferrin group. Here, the reason for failure remains unclear. One could speculate that oral administration is not the ideal route in critically ill patients who often suffer from gastroparesis and disturbed bowel motility.

The last attempt to use AMPs in sepsis was performed by stimulating cathelicidin levels through the administration of calcitriol in 67 patients with severe sepsis or septic shock (46). Though mRNA levels of cathelicidin increased, protein levels were comparable to a placebo group. The authors concluded that the dose and timing of calcitriol treatment might not have been ideally performed (68). Currently, no clinical trial on AMP treatment in sepsis has been initiated (clinicaltrials.gov; accession date February 10th, 2015).

Future Aspects

The growing relevance of AMPs in recent years is because of their capacity to overcome increasing antibiotic resistance, which stems from their ability to decrease pro-inflammation, kill bacteria, and stimulate innate immunity (2–4). Whether one, two, or a combination of all three mechanisms will serve best is still a matter of debate. To date, the development of new drugs predominantly targets only single aspects of the body's response to bacteria rather than bacterial pathogenicity factors (PF).

Synthetic AMPs based on the limulus-anti-LPS-factor (LALF) were designed to bind to the lipid A-moiety of LPS, thus decreasing inflammation and increasing survival in experimental sepsis (69). These synthetic AMPs exerted effectivity against Gram-negative bacteria and additionally against Gram-positive bacteria and mixed infections *in vitro* and in experimental settings *in vivo* (69–71). One further obstacle in the administration of AMPs is the application route. Previous trials used the local or oral route to administer AMPs in sepsis, with encouraging results in early studies. Phase 3 studies could not confirm the first trials (67). The application of designed AMPs is realized via continuous iv infusion and allows decreased inflammation (71), thereby improving the survival rate of septic mice (72). Moreover, virus attachment of enveloped viruses was shown to be decreased by a strong interaction between designed peptides and heparan sulfate (HS) (73). HS is a side chain shed in inflammation from proteoglycans (74, 75). Therefore, HS serves as a danger-associated molecular pattern (DAMP) and triggers a pro-inflammatory cascade in severe sepsis and septic shock (76). Peptide binding to and neutralization of HS may be the underlying mechanism for controlling inflammation (77). This prevents HS from binding to TLR-4, thus inhibiting TLR-4-downstream activation (78).

The combination of different AMPs with anti-inflammatory or bactericidal effects in varying doses may pave the way toward individualized therapy instead of a "one-size-fits-all" antibiotic attempt, which is the standard of care today. Moreover, AMPs offer the unique opportunity to cope with both DAMPs and pathogen-associated molecular patterns (PAMPs) such as LPS or lipopeptides (LPs). Due to this dual mechanism, designed AMPs exhibit their activity in both infectious *and* sterile inflammation (77). The underlying mechanism seems to be a charge-dependent alteration in the secondary structure that attenuates the inflammatory activities of DAMPs and PAMPs (69, 73). This ability distinguishes synthetic AMPs from conventional antibiotics and other attempts in sepsis therapy over the last several decades. Areas other than sepsis are currently the subjects of ongoing clinical studies to control inflammation; for example, talactoferrin, which failed in sepsis therapy (NCT01273779), is now being investigated in cancer studies (NCT00706862).

Thus, newly developed AMPs with decreased toxicity and a broad spectrum efficacy have the potential to improve therapy of bacterial and viral sepsis and to counter the increasing number of bacterial resistances against established antibiotics.

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Properdin levels in human sepsis

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Properdin is a normal serum protein that increases the production of complement activation products by binding C3b integral to convertase complexes and amplifying their activity at the site of activation. Thereby, it not only can aid in the resolution of infection but also contribute to tissue damage. In human sepsis, circulating complement C3 concentrations are decreased, though C3 is described as a positive acute phase reactant. However, properdin levels in human sepsis have not been reported. In this study, serum from 81 critically ill patients (predominately abdominal and respiratory sepsis) were analyzed for properdin levels at defined points of their stay in the intensive care unit (ICU) and compared with 61 age and sex-matched healthy volunteers. Properdin concentrations were significantly decreased in patients with sepsis on admission to ICU, but increased after clinical recovery to exceed levels observed in healthy volunteers. Properdin concentrations at ICU admission were decreased in non-survivors of sepsis compared to survivors, but this did not correlate with APACHE II score. However, pathologically low properdin levels (<7 μg/ml) were related to increased duration of treatment.

Keywords: sepsis, complement, properdin, intensive care, recovery

INTRODUCTION

Sepsis is a life threatening condition in which a systemic inflammatory reaction may progress to shock, hypoperfusion of lungs, kidneys, and intestines and via consumption of coagulation factors to intravascular coagulopathy. Cytokine storm, systemic macrophage activation, complement activation, and activation of the coagulation cascade are factors that drive the organism from a state of overinflammation to immune exhaustion. The complement system is primarily a humoral, hierarchically arranged, system in which activation of recognition molecules is translated to enzymatic activity of protein complexes, which cleave C3 and C5 to generate potent anaphylatoxins, C3a and C5a. The strength of activation also determines cell activities, such as phagocytosis, respiratory burst, and vascular leakage. The contribution of complement activation to tissue damage in septic conditions is thought to be so significant that attempts are made to develop therapeutic blockers in order to rein in its activity (1). However, because complement operates over a wide range of biological functions in health and disease, choosing the window of opportunity in a polymorphic system (2) is likely to be very difficult. In experimental animal models of sepsis, however, treatment with anti-C5a or anti-C5aR antibodies in clear relation to the defined onset of sepsis has proven efficacious (3, 4).

The role of low levels of mannan binding lectin (MBL), one of the lectin pathway recognition molecules, as a single determinant in the outcome from severe shock is not clear; while studies link low MBL levels to an increased risk of developing the systemic inflammatory response syndrome, others assign a beneficial effect to low MBL levels because of the potentially reduced pro-inflammatory reaction (5). A prospective study of septic patients in comparison with healthy controls found depressed levels of complement C3 and elevated complement activation products C3a, Factor Bb and C4d, during the first 2 days of intensive care treatment (6). The observed increase in C5a in septic samples is consistent with previous work analyzed by the Bayesian inference approach (6). However, in sepsis, significant amounts of C5aR are found in serum, most likely shed from neutrophils (7); properdin is produced by neutrophils (8). Properdin is the only amplifier of complement activation but there are no data on properdin levels in human sepsis. A genetic deficiency of properdin predisposes to death from meningococcal septicemia in man (9), and in mice, significant impairment of survival is observed in endotoxin shock (10).

The aim of this study was to investigate properdin concentrations in critically ill patients admitted to the intensive care unit (ICU) with sepsis, using serum samples taken during a recently published observational study (11).

MATERIALS AND METHODS

Patients admitted to ICU at Leicester Royal Infirmary with a diagnosis of sepsis, and healthy volunteers matched for age and sex were recruited, as previously described (11). Permission to measure properdin in these samples was granted by University Hospitals of Leicester NHS Trust (R&D reference: UHL 11348 "Properdin and inflammatory biomarkers in sepsis"). Patients' samples were taken on Days 1 and 2 of ICU admission, and a further sample was obtained after clinical recovery from sepsis. Archived, anonymized serum samples from 81 patients (40 m/41 f; 19-87 years) and of 61 age and sex-matched volunteers (29 m/32 f; 21-85 years) were analyzed. The origin of sepsis was as follows: abdominal $n = 41$, respiratory $n = 25$, neutropenic $n = 5$, urosepsis $n = 2$, and a small group of $n = 8$ made up of line infection ($n = 2$), fasciitis ($n = 4$), postpartum sepsis ($n = 1$), and abscess ($n = 1$).

Properdin was determined from 1/5000 serum sample dilutions using commercial kits (Hycult Biotech, Uden, The Netherlands),

following the manufacturer's protocol. Concentrations from duplicate measurements were calculated from a standard curve set up in duplicate. Those below the lowest dilution of the standard curve (cut off 0.3 ng/ml) were classed as deficient (zero).

STATISTICAL ANALYSIS

Data for properdin concentrations were normally distributed and were analyzed by an unpaired *t*-test between two groups. Duration of ICU or hospital stay was not normally distributed and was compared against properdin concentrations by non-parametric two-tailed Spearman correlation. *P*-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Samples were available for 81, 66, and 45 patients on Days 1, 2 and recovery, respectively. Clinical and other data have been reported previously (11). While consecutive levels were significantly lower at Days 1 and 2 of ICU admission compared to matched volunteers, levels increased significantly at clinical recovery (**Table 1**). Properdin levels did not correlate with survival time in those who succumbed within 1-3 days after admission to ICU. There was no relationship between properdin and total white cell or neutrophil counts. Properdin levels were significantly higher after clinical recovery compared to healthy volunteers. At the timepoint of clinical recovery, however, there was still biochemical evidence of inflammation (CRP and IL-8 concentrations had not completely normalized; increased leukocyte, neutrophil and platelet counts compared to the control group) (11). Properdin levels in the healthy volunteers of European background analyzed as part of this study were higher than those reported in a recent study for healthy South East Asians (12).

The overall 30-day mortality in patients was 21/81 (25.9%). For four patients, in whom there (reproducibly) was no antigenically detectable properdin in their initial serum samples, no subsequent sample existed to exclude a genetic deficiency. But, as properdin is an x-chromosome linked gene and a primary deficiency uncommon, the possibility of a gene defect does not appear likely in the case of the two female patients. When comparing properdin levels determined for samples obtained on day 1 of admission to ICU between survivors and non-survivors, properdin appeared to be lower in non-survivors but this did not reach statistical significance (**Table 1**).

Properdin levels determined on day 1 of ICU admission did not relate to APACHE or SOFA scores (data not shown) or duration of stay in ICU or in hospital, but for the whole group, the ICU stay correlated significantly with hospital stay (80 pairs analyzed, $r = 0.6$, $p < 0.0001$). In other studies, depressed C3 levels found in sepsis were not related to mortality or complications such as pneumonia and hemorrhage (13), but decreased C3 levels were linked to increased hospital stay (14). Therefore, a subgroup analysis was performed for very low properdin levels. These were defined as <7 μg/ml, the lower end of the normal range established in this study. Pathologically low properdin levels on Days 1 and 2 negatively correlated with the duration of intensive care treatment and overall hospitalization, respectively (**Figures 1A,B**).

There are two possible interpretations for these findings: (i) the decline in properdin levels is a descriptor of severity of sepsis due to its ability to bind to LPS of different pathogens (15) or to damaged cells (16) and (ii) properdin is involved in the pathophysiological mechanisms of sepsis.

Recent data indicate that the likely determinants of outcome from sepsis are the cell phenotypes, which emanate from this condition in dependence of the level of complement components.

Table 1 | Properdin levels in sera of critically ill patients with sepsis admitted to ICU and a healthy control group.

Group	Range (μg/ml)	Mean ± SD
Day 1 ($n = 81$)	0–38.8	9.0 ± 7.6
Day 2 ($n = 66$)	0–30.9	8.9 ± 6.9
Clinically recovered ($n = 45$)	2.4–51.2	22.9 ± 11.1[a,b]
Volunteers ($n = 61$)	7.6–34.10	18.4 ± 5.5[c]
Day 1, survivors ($n = 60$)	0–38.8	9.8 ± 8.1
Day 1, non-survivors ($n = 21$)	0–21.2	6.8 ± 5.2

The time point of clinical recovery from sepsis was variable.

[a] *$p < 0.0001$ against Days 1 and 2.*

[b] *$p < 0.0008$ against volunteers.*

[c] *$p < 0.0001$ against Days 1 or 2; Day 1 vs Day 2 n.s.*

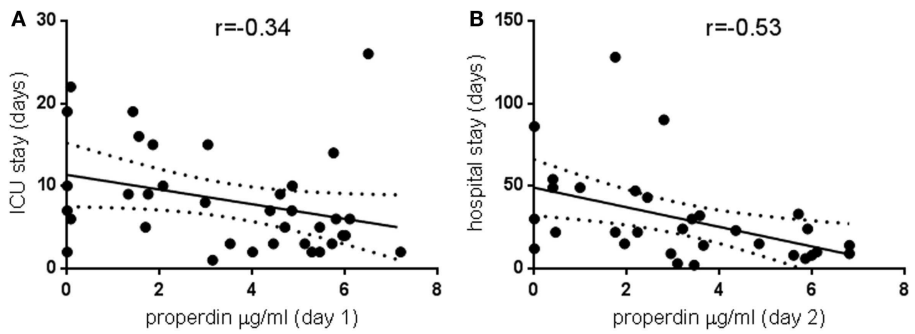

FIGURE 1 | Pathologically low properdin levels in the critically ill and the relationship to treatment duration. Properdin levels lower than 7 μg/ml, the lowest of the normal range established in this study, were plotted against duration of intensive care treatment **(A)** 36 pairs, $p < 0.05$, and total duration of hospitalization **(B)** 31 pairs, $p < 0.005$. Spearman correlation coefficients are indicated; regression lines and 95% confidence bands of the best-fit line are shown.

Depressed serum C3 levels have been linked to the emergence in the critically ill of an immune suppressive T_{reg} population (14). So, altered complement levels may be a mirror of changing cell activities selected during the septic immune response (17). Alternatively, a decline in circulating properdin levels may signify increased complement usage in septic end organs (18) or on inflamed endothelium (19).

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On the functional overlap between complement and anti-microbial peptides

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Intriguingly, activated complement and anti-microbial peptides share certain functionalities; lytic, phagocytic, and chemo-attractant activities and each may, in addition, exert cell instructive roles. Each has been shown to have distinct LPS detoxifying activity and may play a role in the development of endotoxin tolerance. In search of the origin of complement, a functional homolog of complement C3 involved in opsonization has been identified in horseshoe crabs. Horseshoe crabs possess anti-microbial peptides able to bind to acyl chains or phosphate groups/saccharides of endotoxin, LPS. Complement activity as a whole is detectable in marine invertebrates. These are also a source of anti-microbial peptides with potential pharmaceutical applicability. Investigating the locality for the production of complement pathway proteins and their role in modulating cellular immune responses are emerging fields. The significance of local synthesis of complement components is becoming clearer from *in vivo* studies of parenchymatous disease involving specifically generated, complement-deficient mouse lines. Complement C3 is a central component of complement activation. Its provision by cells of the myeloid lineage varies. Their effector functions in turn are increased in the presence of anti-microbial peptides. This may point to a potentiating range of activities, which should serve the maintenance of health but may also cause disease. Because of the therapeutic implications, this review will consider closely studies dealing with complement activation and anti-microbial peptide activity in acute inflammation (e.g., dialysis-related peritonitis, appendicitis, and ischemia).

Keywords: immune cells, innate immunity, histidine tag, deficiencies, acute inflammation

BACKGROUND

The host defense against microorganisms relies on both innate and adaptive elements. Innate immunity is the first line of defense against a microbial pathogen, which exposes a pathogen-associated molecular pattern or more simply a prokaryotic surface membrane, differing from eukaryotic biphospholipid layers in the complete absence of cholesterol. For an efficient and directed response, complement uses both pattern recognition and missing self-recognition strategies [reviewed by Ref. (1)]. Besides, it involves a highly controlled, rapid cascade, and crosstalks with other biological systems, for example, with Toll-like receptors (2). Control of the complement system is maintained by a group of membrane-anchored proteins and soluble, circulating proteins referred to as complement regulatory proteins. Regulatory proteins can act at different points in the complement cascade and help control complement attack and adjust its severity, propagation, and endpoints to the cellular target (3). Cells expose membrane-anchored proteins like membrane cofactor protein (MCP or CD46), decay accelerating factor (DAF or CD55), complement receptor 1 (CR1 or CD35), and CD59 as complement regulatory proteins (4), while properdin and factor H may become membrane associated and then are thought to fine tune locally the extent of complement activation (5).

Defensins are able to kill or eliminate bacteria, fungi, protozoans, and viruses. α- and β-defensins are synthesized as precursors that are proteolytically cleaved into their anti-microbially active forms (6). Human neutrophil peptides (HNP)1 to HNP3, for example, are found in high concentrations in granules of neutrophils (7) and released by degranulation in response to pro-inflammatory or bacterial stimuli (8). Human defensin (HD)5 and HD6 are present in Paneth cells in the crypts of the small intestine (9), whereas β-defensins are induced in epithelial cells by wounding, bacterial products, or pro-inflammatory cytokines (10–13). Based on the chemotactic effect exerted by anti-microbial peptides, much work was spent on identifying a receptor for their actions. It has now emerged that CCR2 and CCR6 are receptors for β-defensins (14), and that the interaction of, e.g., HD6 with glycosaminoglycans may modulate binding of one or the other to CCR2 (15). There are different ways of LL-37 uptake into a cell. The receptors FPRL-1 and P2X7 are important for LL-37 activity and lead to chemoattraction and Il-1β processing, respectively (16, 17). In contrast, cellular uptake of LL-37 into epithelial cells has been shown to be mediated by atypical endocytic processes (18).

COMPLEMENT AND ANTI-MICROBIAL PEPTIDES SHAPE THE LOCAL ENVIRONMENT

Local production of complement components and their role in the inflammatory microenvironment is a currently emerging field. Most of the complement pathway proteins are synthesized in the liver (19); however, extrahepatic biosynthesis additionally occurs in a variety of other tissues and organs (20). Locally produced complement proteins, finely tuned according to the demands of the local environment, may allow differential regulation of inflammation and cellular activation within these tissues. Complement factor H, besides its hepatic expression, is further expressed at low levels in lung, heart, spleen, brain, eye, kidney, pancreas, placenta, as well as neurons and glial cells (21). Production of complement proteins and their regulators directly at sites of inflammation offers an underestimated variety of functions for complement proteins. So far, several cell types have been found to produce complement proteins including macrophages (22), fibroblasts (23), endothelial cells (24), as well as organ specific cells (25–28). Intriguingly, even cells such as peripheral monocytes that were thought to be incapable of synthesizing complement proteins unless activated have recently been shown to produce C1q (29). This suggests a new role for locally synthesized C1q in the immediate local response to pathogen- or danger-associated molecular patterns (PAMPs or DAMPs). Properdin, the positive regulator of the alternative pathway, is produced by a variety of cell types like neutrophils (30), peripheral blood monocytes (31), endothelial cells (32), and T cells (33). Properdin released by phagocytes was shown to bind to apoptotic and necrotic cells (33, 34), contributing to their direct removal or properdin-mediated complement activation. Likewise, local release of properdin may opsonize and kill microorganisms using the same mechanism, if indeed it can operate as a pattern-recognition molecule in its own right (35).

The role of complement in modulating inflammation and maintaining homeostasis is only recently becoming apparent. Local immune responses can be altered by C5a via modulating the local cytokine milieu, especially via the cytokines IL-17 and IL-23. While C5a has been shown to enhance IL-17F, it limits IL-17 and IL-23 production by macrophages or DCs (36). In agreement with these findings, another report determined that IL-17 levels in experimental asthma are reduced by signaling through C5aR (37). So far, little is known about local synthesis and specific function of complement proteins where produced away from the humoral environment that has led to the well-known diagrams of sequential assemblies and enzymatic cleavages. Due to functional studies, there is increasing evidence that locally produced complement proteins are biologically active and have a significant role in local environment. Local synthesis of complement proteins not only contributes to the systemic pool of complement (38) but also influences local tissue injury and provides a link with the antigen-specific immune response (39). The diverse range of extrahepatic sites for synthesis of complement proteins and their regulators suggests the importance and need for local availability of the proteins. It has been suggested recently that plasma-borne complement activation vs. cellular production of complement components sufficient to form convertases may pursue distinct, compartment-selective, biological functions (40). Understanding the relative importance of local and systemic complement production could help to explain the differential involvement of complement in organ-specific pathology.

Locality of production plays an important role not only for complement proteins but also for anti-microbial peptides. Paneth cells in the small intestine have been shown to release granules into the lumen of the crypts thereby contributing to mucosal immunity (41). Those granules contain proteins that are associated with roles in host defense, including lysozyme (42), secretory phospholipase A2 (43), and α-defensins termed cryptidins (44). Anti-microbial peptides secreted by Paneth cells are important for innate immunity as they protect mitotically active crypt cells from colonization by potential pathogens and confer protection from enteric infection (45). Moreover, secretion into the crypt lumen defines the apical environment of neighboring cells (46).

LYTIC ACTIVITIES OF COMPLEMENT AND ANTI-MICROBIAL PEPTIDES

Both complement proteins and anti-microbial peptides share lytic activities. Anti-microbial peptides attack bacteria, fungi, protozoa, and certain viruses by inserting into their membrane manifold causing pore formation and subsequent lysis (47, 48). Due to the cationic character of microbial peptides, electrostatic attraction to the negatively charged phospholipids of microbial membranes occurs resulting in integration into the microbial cell membrane and membrane disruption.

In the absence of regulators, complement proteins contribute to lysis of cells by forming a membrane attack complex (MAC). After cleavage of C5 into C5a and C5b by the highly specific C5 convertase, C5b initiates the terminal complement pathway involving a non-enzymatic assembly of C6, C7, C8, and C9 to form the MAC to cause lysis. Fusion of those proteins brings forth hydrophobic sites that can insert into the membrane to form a transmembrane channel (49). While only one mode of insertion to form a transmembrane channel for the MAC has been described (50), several models exist to explain the insertion of conformationally changed anti-microbial peptides into and across target membranes (51). Pathogens actively interfere with either of these lytic effector processes (52, 53).

Peptides synthesized form the C-terminal portion of complement C3a have inhibitory effect on the growth of *P. aeruginosa, E. coli, B. subtilis*, and *C. albicans*, which does not exceed the activity of equal molar amounts of LL-37 (54). Native human C3a, however, showed inhibitory effect on *C. albicans* growth, which exceeded that of LL-37 at equimolar amounts [50μM; (55)]. While 6 μM C-terminal C3a peptide was needed to observe membrane disruption of *P. aeruginosa* (54), 1 μM native C3a produced leakage of liposomes (55).

PHAGOCYTIC AND CHEMO-ATTRACTANT ACTIVITIES OF COMPLEMENT AND ANTI-MICROBIAL PEPTIDES

The peritoneal cavity is a site in which complement and anti-microbial peptides are key components of the innate immune response and have been investigated with regard to peritoneal dialysis (56, 57). Both mesothelium and leukocytes are the source for this production (58). While the opsonophagocytic activity of complement is well known (via C3b/iC3b), recent findings show that LL-37 can modulate the expression of receptors, which determine

the extent of the phagocytic response of human macrophages *in vitro* (59). Both components of the innate immune response are thereby able to influence the adaptive immune response by altering the phenotype of phagocytic cells to become more mature, i.e., acquire characteristics, which will make them more potent to present antigen in a suitable, germinal center environment. Chemotactic activity of complement *per se* (via generation of C3a, C5a, and engagement with their receptors, C3aR, C5aR, C5L2) has been described (60). In addition, however, bradykinin, which may be released after activation of kininogen by the lectin pathway of complement activation (61), has chemotactic activity (62). Contact and complement system cooperate in a pro-inflammatory way. Interestingly, β-defensins can bind to chemokine receptors, in particular, CCR6 present on dendritic cells and T cells (14) and CCR2 (see above). Complement C3a and CXCL12 cooperate in the chemotaxis of $CD34^+$ progenitor cells in bone marrow, but the receptor has not yet been described, though C3aR has been excluded (60).

CELL INSTRUCTIVE ROLES OF COMPLEMENT AND ANTI-MICROBIAL PEPTIDES

Anti-microbial peptides and complement are constitutively expressed and are upregulated during inflammation. While anti-microbial peptides are commonly known to be synthesized by epithelial cells to partake in the innate host defense (63), the contribution of complement expression in non-lymphoid cells is not well appreciated yet, although the pattern of expression in crypts follows that of anti-microbial peptides (26). Beyond their chemo-attractant ability, complement and anti-microbial peptides may assume immunoadjuvant, i.e., adaptive immunity supportive, properties (63, 64). The type of cellular response is co-determined by the integration of signaling events triggered by mediators. So complement activation products and anti-microbial peptides, which can alter their expression manifold acutely and remain altered chronically, are relevant determinants of this cell activity (65, 66).

Innate lymphoid cells located in the mucosa contribute to the barrier by releasing IL-22, which stimulates the production of anti-microbial peptides (67). IL-22 is also expressed, in the context of TGF-β, by IL-17A and IL-17F expressing $CD4^+$Th17 cells. Synergistically, IL-22 and IL-17A lead to significant induction of mRNA expression for hBD2, S100A7–9 by keratinocytes (68). Because, on its own, IL-17A is a potent stimulator of anti-microbial peptide production (68), those studies reporting a deviation in complement activity, which impact on the Th17 cell population (69, 70), have to be viewed with care. It is likely that a greater component within the immune response is significantly determined by the relative amounts of anti-microbial peptides, which escape attention in the complement field. In this sense, it is a matter of discussion whether the phenotype observed in the properdin-deficient mice when infected with *Listeria monocytogenes* could be significantly influenced by a lack of anti-microbial peptides, which would be due to significantly lower Il-17 levels, which, importantly, do not adequately upregulate during infection (71). C5a and an N-terminal peptide of human lactoferrin with anti-microbial activity, by stimulating macrophages or dendritic cells, respectively, are able to enhance production of Th17 cells (72, 73), which act in a pro-inflammatory, Treg opposing, way.

ROLE OF COMPLEMENT AND ANTI-MICROBIAL PEPTIDES IN ENDOTOXIN CLEARANCE

Intact complement activation in the humoral system (blood) is needed for efficient endotoxin clearance (74), while it exerts at the same time a modulatory effect on cellular, pro-inflammatory activity (75). Anti-microbial peptides may have LPS-neutralizing effect, which is important for the beneficial outcome from sepsis (76). Avoiding exhaustion of these systems would obviate the detrimental development of endotoxin tolerance in sepsis. In severe sepsis, significantly lower levels of plasma C3 have been reported (77) and a failure of PBLs to induce defensins *ex vivo* in response to endotoxins (78). Low Vitamin D3 levels have been linked to mortality in sepsis (79). Interestingly, Vitamin D3 promotes production of LL-37 and β-Defensin (80) as well as C2 and C3 (81, 82) *in vitro*. The complement receptor C5aR is upregulated in lung, liver, kidney, and heart during the early phases of sepsis. Blocking of C5aR has been correlated to improved survival in murine models of sepsis (83).

MONOCYTES AND MACROPHAGES ARE DISTINCT PRODUCERS FOR C3 AND ANTI-MICROBIAL PEPTIDES

Monocytes appear to need LPS stimulation to produce C3 (84), whereas macrophages were shown to produce basal levels of C3 even without stimulation (85, 86). As a recurring point, most of the papers suggest that macrophage differentiation has to have taken place before considerable C3 production occurs (85–92). This observation is also supported by Affymetrix array data (http://www.ncbi.nlm.nih.gov/geoprofiles/60640353), showing more C3 mRNA in macrophages compared to monocytes.

Both monocytes and macrophages are also affected by anti-microbial peptides. The honeybee anti-microbial peptide apidaecin, for example, has been shown to bind both to human macrophages and monocytes (93) without inducing cytotoxic effects. However, apidaecin shows a different subcellular localization in the cytoplasm or in endosomal compartments for macrophages or monocytes, respectively. Besides, the effect upon LPS stimulation differs. Antagonizing LPS-stimulatory effects on both macrophages and monocytes at low concentrations, a high concentration of apidaecin stimulated pro-inflammatory and pro-immune functions of macrophages. Not only for complement production but also for anti-microbial peptides, monocyte to macrophage differentiation plays an important role. The peptide hLF1–11 applied on monocytes during GM-CSF-driven differentiation has been shown to modulate differentiation toward a macrophage subset characterized by both pro- and anti-inflammatory cytokine production and increased responsiveness to microbial structures (94, 95).

Macrophages are considered classically activated (M1) when stimulated by IFNγ or LPS and alternatively activated (M2) when stimulated by IL-4 or IL-13 (96). The arising question is therefore, which subpopulation of macrophages produces C3 predominantly. There were some hints pointing toward M1 macrophages like fact that synthesis of C3 in various organs can be directly upregulated by IFNγ during an inflammatory response (97). In

addition, IFNγ can induce C3 synthesis directly (98) as well as stabilize C3 mRNA (99). Recent studies using guinea pigs deficient for complement C3 showed an impaired antibody response to T-dependent antigens (100), a response dependent on M1 macrophages as well. Those data reveal that C3 production is a highly regulated process and can be modulated by a variety of cytokines, determining whether a macrophage will differentiate into an M1 or M2 macrophage and therefore produce more or less C3, respectively.

Anti-microbial peptides were shown to modulate inflammatory responses as well. LL-37, for example, dramatically reduced levels of pro-inflammatory cytokines such as TNF-α and NO in M1 and M2 bone marrow-derived macrophages, whereas anti-inflammatory functions remained unaltered (101). The same effect could also be observed for human THP-1 cells (102). Another example is the Vitamin D inducible LL-37 anti-microbial peptide, which is expressed mainly by M1 macrophages (103). A recent review sheds light on the feature of monocytes and macrophages to respond differently: they are of heterogeneous origin and do not necessarily follow the differentiation pathway of monocyte–macrophage (104).

DEFICIENCIES OF COMPLEMENT AND ANTI-MICROBIAL PEPTIDES

In humans, genetic deficiencies of the great majority of complement components have been described, giving insights into their functions in both infectious and non-infectious diseases. It is beyond the scope of this article to give a detailed review of genetically determined deficiencies of the complement system. [For a more comprehensive review, see in Ref. (105) or (106).] Deficiencies of most complement components give rise to increased susceptibility to specific pathogens or groups of pathogens. In broad terms, deficiencies of components of the classical pathway (C1q,r,s, C4, and C2) are associated with infections with encapsulated bacteria, such as *S. pneumoniae* and *N. meningitidis*. Deficiencies of lectin pathway components (MBL, MASP-2, and ficolin) have been associated with increased frequencies of (usually less severe) respiratory infections. However, asymptomatic lectin pathway-deficient individuals have also been described. C3-deficient patients suffer from a broader range of pyogenic infections, including more severe respiratory infections and meningitis (e.g., *S. pneumoniae, N. meningitidis, S. pyogenes, H. influenzae, S. aureus)*. Deficiencies of the regulatory proteins properdin and Factor D, as well as of the terminal components of complement activation (C5–C9), are associated with an increase in susceptibility to *Neisserial* infections, reflecting the important role of cytolytic complement activity in the innate immune response against *Neisseriae*. Deficiencies of Factors H and I are associated with increased pyogenic infections (*N. meningitidis, H. influenzae,* and *S. pneumoniae)*. For some complement deficiencies, the lack of complement function in antibacterial immunity may be compensated for by the production of high levels of pathogen-specific IgG antibodies (107). Consequently, the infections may be more prevalent in childhood. Interestingly, deficiencies of some complement components are also associated with non-infectious conditions. For example, deficiencies of C1q,r,s, C4, and C2 are associated with systemic lupus erythematosus (SLE)-like disease, reflecting the important role of the classical complement pathway in clearance of immune complexes from the body. In these complement deficiencies, the autoimmune manifestations may be of greater clinical significance than the increased susceptibility to infections. Similarly, deficiencies of factors H or I most commonly present with atypical hemolytic uremic syndrome. The most obvious example of a non-infectious condition associated with a complement component deficiency is the association between C1 inhibitor deficiency and hereditary angioedema, in which patients suffer from (potentially life threatening) episodic attacks of tissue edema, due to loss of the inhibitory role of C1 inhibitor in cleavage of high molecular weight kininogen to produce bradykinin.

Deficiencies of anti-microbial peptides are less well defined. Anti-microbial peptides play an important role in immune defense in *Drosophila* (108). LL-37-knockout mice have been generated, and are described as having an increased susceptibility to a number of Gram-negative bacterial infections (109–113), suggesting a broad role for anti-microbial peptides in the immune response to infections in mammals. To date, genetic deficiencies of anti-microbial peptides have not been defined in humans. However, reduced expression of anti-microbial peptides in patients has been associated with increased susceptibility to infections of skin and periodontal gingiva (114–116). As we move toward an era in which exome sequencing becomes a feasible approach for defining genetic defects predisposing to immune deficiencies in patients, the significance of deficiencies of anti-microbial peptides in defense against infections may become apparent.

ROLE OF COMPLEMENT AND ANTI-MICROBIAL PEPTIDES IN ACUTE INFLAMMATION

Activation of complement reveals beneficial functions such as pathogen sensing and defense and clearing injured cells on the one hand; however, complement has been shown to play a major role in pathogenesis of various inflammatory processes on the other hand. In response to pathogens or tissue damage, complement is highly capable of inducing all classical signs of inflammation such as redness, pain, hyperthermia, and swelling. Complement products lead to a release of pro-inflammatory mediators, upregulation of adhesion molecules, and increased vascular permeability of endothelial cells (117). Besides the beneficial effect of clearing an infection locally, complement activation may also contribute to a life-threatening systemic inflammatory response (118). Both the classical and alternative complement pathways appear to be activated during sepsis (119) resulting in elevated levels of the complement activation products C3a, C4a, and C5a (120). Among those, C5a appears to be the most harmful molecule (121). Complement activation seems to play a role in acute inflammation in lung and liver, where it has been correlated to acute respiratory distress syndrome and to acute humoral rejection, respectively (122, 123). Part of its detriment complement activation derives from the crosstalk to other activation systems, such as the kininogen pathway and coagulation cascade (124). Besides, systemic complement activation has been confirmed in stroke patients (125). The anaphylatoxins C3a and C5a exert both protective and harmful functions in the central nervous system (126, 127). Direct contact between blood and cerebrospinal fluid in blood–brain barrier dysfunction leads to production of C1q and generation of C3a,

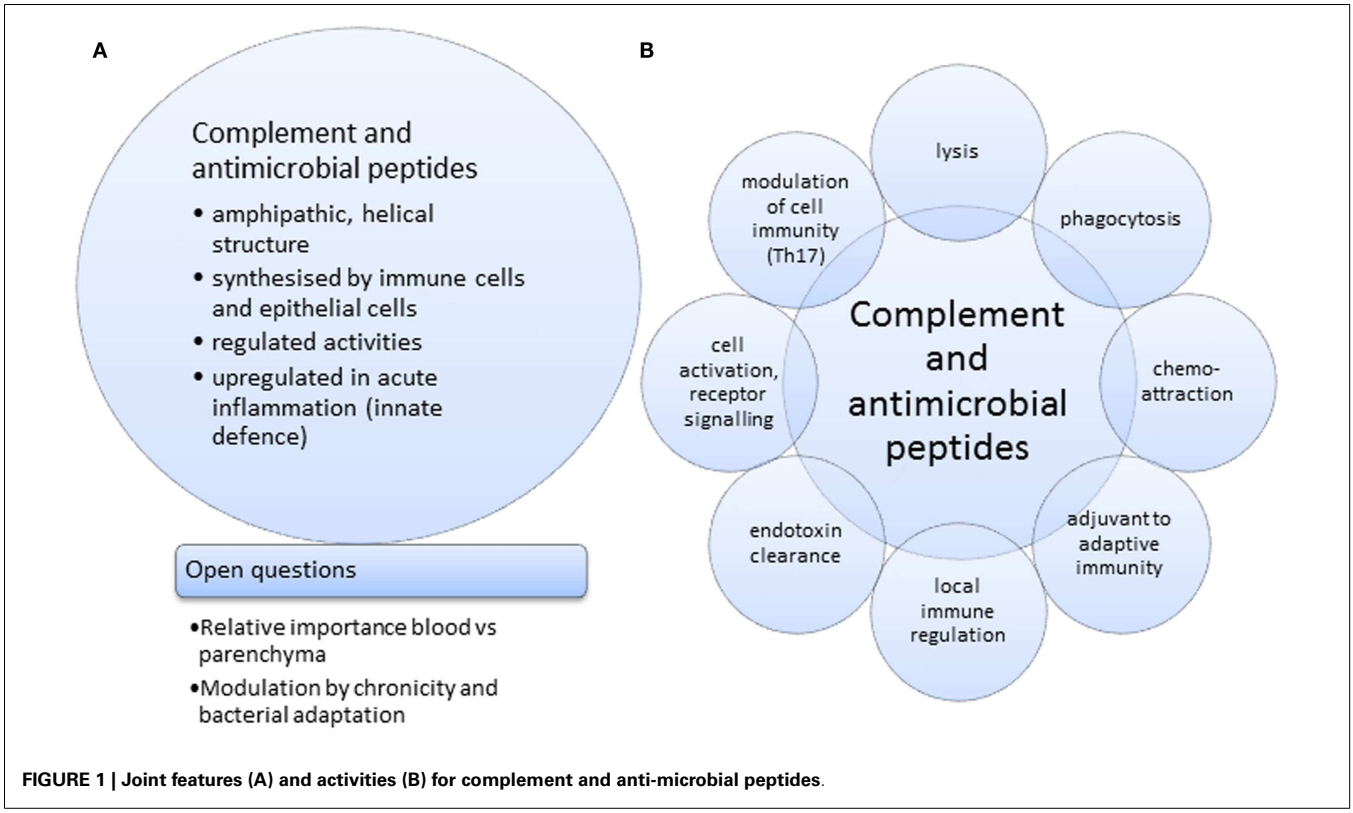

FIGURE 1 | Joint features (A) and activities (B) for complement and anti-microbial peptides.

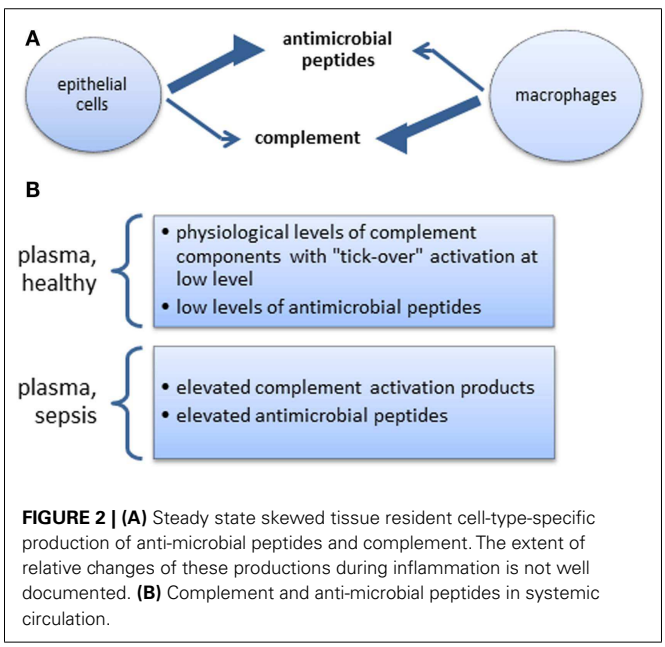

FIGURE 2 | (A) Steady state skewed tissue resident cell-type-specific production of anti-microbial peptides and complement. The extent of relative changes of these productions during inflammation is not well documented. **(B)** Complement and anti-microbial peptides in systemic circulation.

and C5a, which in turn contributes to intracranial inflammation by induction of blood–brain barrier damage and increase in vascular permeability (128, 129). Another example for complement activation is ischemia–reperfusion injury. In ischemia and during reperfusion, complement is activated via the classical, the alternative, and the MBL pathway (130–132). Inhibition of the complement cascade greatly reduced myocardial damage after myocardial infarction (133–135). The role of complement in atherosclerosis remains controversial. Several studies revealed a protective role of complement activation in cardiovascular diseases such as atherosclerosis or vasculitis. The protective effect of complement in the pathogenesis of atherosclerosis has been shown by $C3^{-/-}$ mice exhibiting accelerated development of atherosclerosis (136). We have previously reported on the complexity in design and analysis of complement-targeted mouse models (137). However, a recent population based cohort study showed that unlike C3a, C3, and C5a are not associated with atherosclerosis (138). This suggests that C3a and C3 have distinct roles in pathways leading to cardiovascular diseases. In contrast, a murine study reported that systemic inhibition of complement by Crry–CR2 reduced development of atherosclerosis (139).

Anti-microbial peptides play a modulatory role in acute inflammation via modulation of cytokine production, recruitment of immune cells to the site of injury, and enhancement of phagocytosis (140). Stimulation with IL-4 or IL-13 – classical Th2 response cytokines – leads to rapid Paneth cell degranulation and subsequent release of anti-microbial peptides (141). Anti-microbial peptides play an important role in maintaining the skin barrier and protection against infections. This has been experimentally underlined by mice deficient for LL-37 (142). In addition, LL-37, HBD-2, and 3 are highly expressed in epidermal keratinocytes in response to injury or infections of the skin (143). It has been further shown that LL-37 prevents sepsis by directly dampening pro-inflammatory signaling initiated by LPS (102). Therefore, it may also play a role in dialysis-related peritonitis where endotoxins are

present. Defects in defensin expression have been shown to contribute to a number of mucosal inflammatory diseases, including necrotizing enterocolitis and inflammatory bowel disease (144). Moreover, differentially regulated expression of epithelial-derived anti-microbial peptides has been shown in acute appendicitis. Arlt et al. (145) showed that the anti-microbial peptide HBD-1 is downregulated in patients with acute appendicitis, whereas HNP1–3, HD5 and HD6, and HBD2 and 3 are upregulated, suggesting that differential regulation of the innate immune system is coincident with altered bacterial diversity.

THE CASE OF C3a AND OTHER ANTI-MICROBIAL AGENTS

Structural criteria together with functional *in vitro* data suggest that C3a and C4a, but not C5a (all split products of complement activation), may qualify as anti-microbial peptides *per se* (51). C3a (9 kDa), $C3a_{desarg}$, and synthetic peptides derived from C3a were compared to LL-37 (5 kDa when processed) for their inhibitory effect on *E. coli*, *E. faecalis*, and *P. aeruginosa*, their heparin binding, liposome permeabilization and were found to be strikingly similar (146). Structurally, C3a contains α-helical regions characteristic of anti-microbial peptides, which were found represented in proteolytic fragments generated by the enzymatic activities of cells involved in the acute inflammatory response, such as neutrophils and mast cells (147).

Anti-microbial activity and heparin binding ability are described for histidine-rich peptides (148). Histidine-rich motifs in peptides that relate to anti-microbial activity are conserved (149) and as artificial tags are indeed exploited in subcellular targeting (150). Non-removal of histidine tags after expression of recombinant proteins for the purpose of testing anti-microbial activity bears inherent problems, and findings have to be viewed with utmost caution (151–154). Awareness of this potential pitfall was raised in a very pertinent article in 2013 (155).

By contrast, proteolytic cleavage of high molecular weight kininogen during bacterial infection generates an internal peptide, which has antibacterial activity that compares to LL-37 (156). Similarly, in bovine plasma, activated kallikrein releases from high molecular weight kininogen a histidine-rich fragment (157). Nordahl et al. (152) demonstrated effective antibacterial activity of a histidine-rich peptide generated from high molecular weight kininogen. However, the effect may be potentiated by the presence of the uncleaved histidine tag.

CONCLUSION

In conclusion, much is to be learnt from cross-specialty comparisons.

Apart from refining one's experimental design (cave histidine tags), greater clarity was gained in the use of the term "anti-microbial peptide." Often, an analog (functionally similar gene product), not homolog (shared ancestry) is meant, and sometimes, a recombinantly expressed or proteolytically generated section only of a protein.

While having important functions in maintaining tissue homeostasis, anti-microbial peptides and complement are both involved in shaping the immune response and transcend from the purely innate immunity realm to adjuvant the adaptive immune response.

In many aspects of health and disease, complement and anti-microbial peptides are remarkably similar in function, sharing certain features and broad range of activities (**Figures 1A,B**). They may, however, operate at differing preponderance in separate niches, e.g., blood/tissue, epithelial cells/macrophages (**Figures 2A,B**), supporting the view that two specialist systems are operating in a complementary way. In the context of beneficial activity of immune modulators applied clinically in sepsis, such as Vitamin D (158) and more recently omega-3 fatty acid preparations (159), parallel measurements of, e.g., C3 and LL-37, produced by cells, which express Vitamin D receptor (VDR) and ω-3 fatty acid receptor (GPR120), would provide the type of comparative analyses needed to direct this overlapping field.

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