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REVIEW

More than a Pore: Nonlytic Antimicrobial Functions of Complement and Bacterial Strategies for Evasion

Elisabet Bjanes,[a] Victor Nizet[a,b]

[a]Division of Host-Microbe Systems and Therapeutics, Department of Pediatrics, UC San Diego, La Jolla, California, USA
[b]Skaggs School of Pharmacy and Pharmaceutical Sciences, UC San Diego, La Jolla, California, USA

SUMMARY The complement system is an evolutionarily ancient defense mechanism against foreign substances. Consisting of three proteolytic activation pathways, complement converges on a common effector cascade terminating in the formation of a lytic pore on the target surface. The classical and lectin pathways are initiated by pattern recognition molecules binding to specific ligands, while the alternative pathway is constitutively active at low levels in circulation. Complement-mediated killing is essential for defense against many Gram-negative bacterial pathogens, and genetic deficiencies in complement can render individuals highly susceptible to infection, for example, invasive meningococcal disease. In contrast, Gram-positive bacteria are inherently resistant to the direct bactericidal activity of complement due to their thick layer of cell wall peptidoglycan. However, complement also serves diverse roles in immune defense against all bacteria by flagging them for opsonization and killing by professional phagocytes, synergizing with neutrophils, modulating inflammatory responses, regulating T cell development, and cross talk with coagulation cascades. In this review, we discuss newly appreciated roles for complement beyond direct membrane lysis, incorporate nonlytic roles of complement into immunological paradigms of host-pathogen interactions, and identify bacterial strategies for complement evasion.

KEYWORDS autophagy, bacterial pathogenesis, complement, innate immunity, macrophages, neutrophils, opsonization, phagocytosis, virulence factors

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Address correspondence to Elisabet Bjanes, ebjanes@health.ucsd.edu, or Victor Nizet, vnizet@health.ucsd.edu.

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INTRODUCTION

Complement is an essential weapon in the immune system arsenal against microbial pathogens. The complement cascade consists of three initiation pathways that converge on a common terminal pathway resulting in formation of a lytic pore on the target surface. More than 50 proteins are involved, many of which have multiple names; here, we will use standardized nomenclature (1). The classical pathway (CP) is activated in response to antibody recognition of bacterial surfaces and microbial factors, the lectin pathway (LP) is induced by detection of carbohydrate epitopes characteristic of bacteria, and the alternative pathway (AP) is constitutively active at low levels in circulation, where it surveils for the presence of foreign particles (2). Each pathway induces activation of C3 convertases, enzymatic complexes that cleave and deposit C3 activation fragments on target surfaces and further amplify complement activity by promoting C5 convertase formation (3). C5 convertases direct assembly of asymmetric, flexible pores called membrane attack complexes (MACs) in the target membrane, resulting in bacterial lysis (4). Complement activation is highly regulated, ensuring proper lysis of foreign material while minimizing damage to host cells. However, microbial pathogens have evolved evasion strategies that target complement components and/or coopt its regulatory proteins for their own designs (5). Although the thick peptidoglycan layer of Gram-positive bacteria provides protection from MAC lytic activity, these organisms are susceptible to nonlytic complement functions and have developed evasion mechanisms accordingly.

Beyond lytic activities and clearance of microbial infections, complement has pleiotropic roles that bridge the gap between innate and adaptive immunity during homeostasis and development. Components of the complement system are essential mediators during oocyte fertilization (6), embryonic tissue organization (7), regeneration (8), and metabolism (9). Since the role of complement in fundamental cellular processes has been extensively covered (10), we describe here recent discoveries in lytic and nonlytic complement functions in the context of bacterial infections.

In addition to activating the CP, complement and antibody deposition on bacterial surfaces facilitates phagocytosis by professional phagocytes, including dendritic cells, macrophages, and neutrophils (11, 12). Complement cleavage products act as anaphylatoxins and chemotaxins that recruit immune cells and modulate inflammatory responses (13). Complement synergizes with neutrophils to potentiate their killing activity and induce a proinflammatory feedback loop (14). A recently discovered intracellular niche for complement proteins implicates complement in cellular metabolism, immune homeostasis, and intracellular host-pathogen interactions (15–17), while coagulation pathways are intertwined with complement cascades to promote antibacterial activity and hemostasis (18, 19). The importance of complement in B cell receptor (BCR) activation has long been appreciated, but recent studies have revealed that complement is an essential regulator of adaptive immune cell survival and development during health and infection (20–22) and suggest that future nontraditional clinical therapies might better leverage the antibacterial activity of complement. In sum, our understanding of innate and adaptive host defense has been heightened by incorporating complement into current immunological paradigms. In this review, we will discuss lytic and nonlytic roles of complement in the context of antimicrobial immunity and detail bacterial evasion mechanisms with a particular emphasis on clinically relevant Gram-positive pathogens.

BY THE BOOK: CANONICAL COMPLEMENT CASCADES

Off to the Races—Pathway Activation

The CP is induced by activation of the C1 complex that consists of the hexameric recognition component C1q and two serine proteases, C1r and C1s, in a 1:2:2 molecular ratio. C1 complex is activated by C1q binding to a variety of surfaces, including but not limited to the Fc regions of IgG and IgM antibodies, C reactive protein, microbial components such as lipopolysaccharide (LPS), and self-molecules including DNA and

phosphatidylserine exposed on the surfaces of dying cells (2). High-resolution crystallography studies revealed that C1q binds IgG hexamers through noncovalent Fc interactions (23). C1q undergoes a calcium-dependent conformational change following ligand binding, autoactivating C1r, which subsequently activates C1s (24, 25). However, recent structural studies utilizing small-angle X-ray scattering and single-particle electron microscopy suggest that C1r cleavage results from C1r within a neighboring C1 complex and that C1s activation may also require intracomplex cleavage (26). C1s cleaves C4 into C4a and C4b, liberating a reactive thioester on C4b that covalently binds the target surface. C4b associates with C2, which is cleaved by C1s, forming the C3 convertase: C4bC2b (27, 28). This convertase cleaves nearby C3 molecules to C3a and C3b, exposing a reactive thioester molecule that deposits C3b on the pathogen surface (29) (Fig. 1).

The LP is activated in an analogous fashion to the CP by higher-order oligomers of mannose-binding lectin (MBL), ficolins, and collectins that detect carbohydrate epitopes abundant on foreign surfaces, including mannose, fucose, and *N*-acetylglucosamine (2) (Fig. 1). Unlike the C1 complex that associates with $C1r_2C1s_2$ tetramers, LP recognition components predominantly associate as circulating dimers with either mannan-binding lectin serine protease 1 or 2 (MASP-1 or -2) (30). Separate complexes must bind target surfaces in close proximity to allow for cross-activation of MASP-2 by adjacent MASP-1 molecules (31). MASP-1 cleaves C2, while MASP-2 cleaves both C2 and C4, inducing formation of the C3 convertase, C4bC2b (32, 33) (Fig. 1).

The AP is constitutively active in a process called "tickover" that allows it to maintain constant circulating surveillance (34). Spontaneous hydrolysis of the C3 thioester bond to the fluid phase form $C3(H_2O)$ occurs at steady state (34, 35) (Fig. 1). $C3(H_2O)$ binds factor B (FB), which is subsequently cleaved into Ba and Bb by the constitutively active serine protease factor D (FD), generating the fluid phase C3 convertase: $C3(H_2O)$ Bb (36). Intriguingly, the mannan-binding lectin serine protease MASP-3 was recently identified as the sole activator of pro-factor D in human blood under physiological conditions, linking the initiation phases of the AP and LP (37). $C3(H_2O)$Bb cleaves C3 to C3b, which can covalently bind nearby surfaces or remain in the fluid phase (38). C3b binds FB which is cleaved by FD, generating C3bBb (39). This short-lived convertase is stabilized by binding properdin to generate the amplification C3 convertase, C3bBbP (40). The role of properdin in C3 convertase stabilization is undisputed; however, controversy exists whether properdin also acts as a bona fide recognition molecule capable of AP initiation (41–43) (Fig. 1). Reported differences can potentially be attributed to ready polymerization by purified properdin (44).

More and More—Amplification

Once assembled, the C3 convertases of the CP, LP, and AP rapidly amplify the cascade by cleaving and depositing C3 activation fragments on the surrounding surface (Fig. 1). Each subsequent C3b molecule can interact with factors B and D to produce additional AP C3 convertases. In this manner, the AP can be activated downstream of both CP and LP initiation, amplifying the signal cascade. Once C3 convertases bound to a surface reaches sufficient density, additional C3b molecules bind C3 convertases to form C5 convertases: C4bC2bC3b and C3bBbC3b (3, 45).

End of the Line—Termination

The C5 convertases cleave C5 into C5a and C5b, releasing C5a as a potent anaphylatoxin and chemoattractant (Fig. 1). C5b undergoes a transient conformational change that is stabilized by the binding of C6 (46, 47). Association of C7 with C5b6 provides the complex with lipophilic properties that enable it to bind lipid head groups (47). Recent cryo-electron microscopy studies reveal that C5b-7 binding primes the membrane for subsequent pore formation by physically lowering the energy required for changing the bilayer curvature or the bending modulus (4). C5b-7 recruits C8 which inserts the complex into the lipid bilayer, causing the membrane to stiffen (4, 48). C8 recruits and polymerizes 18 C9 monomers in a split-washer configuration, resulting in the formation of an asymmetric, flexible, lytic pore known as the membrane attack

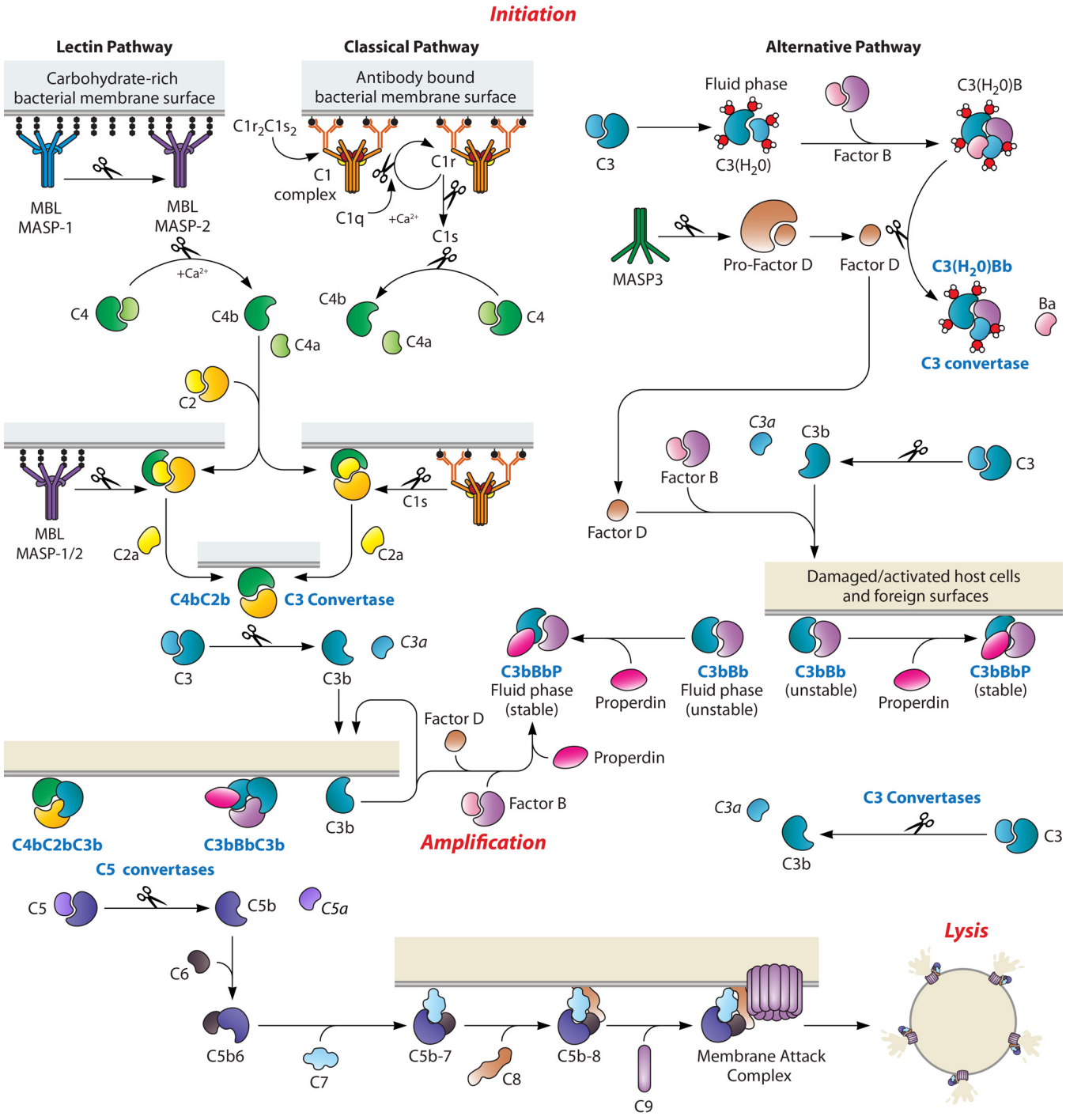

FIG 1 Complement-mediated lysis. The lectin and classical pathways are activated by specific binding of carbohydrate moieties and antibodies to globular mannose binding lectin (MBL)/mannan-binding lectin-associated serine protease-1 and -2 (MASP1/2) and C1 complexes, respectively. These serine proteases cleave C2 and C4 to form the C3 convertase C4bC2b. The alternative pathway is spontaneously activated in circulation and forms the C3 convertase C3(H_2O)B. MASP3 cleaves pro-factor D to its active form factor D, which subsequently generates C3(H_2O)Bb, a C3 convertase. The alternative C3 convertase cleaves subsequent C3 molecules that can interact with factor B to form an unstable fluid or membrane-bound C3bBb. Binding of properdin stabilizes this C3 convertase, C3bBbP. The C3 convertases generate additional C3b, which is deposited on the target surface. Deposited C3b can amplify the alternative pathway or form C5 convertases C4bC2bC3b and C3bBbC3b. The C5 convertases cleave C5 and initiate the enzymatic activation cascade that leads to the formation of the membrane attack complex, a lytic pore, and its insertion into the target surface.

complex (MAC) in the target membrane (49–51) (Fig. 1). Conformational changes during pore formation distort the lipid bilayer structure, ultimately rupturing it (4). While purified terminal complement components efficiently form pores on artificial liposomes and erythrocytes (50), a recent study sought to elucidate the mechanism by

which MAC pores kill bacteria (52). For bactericidal activity, surface-bound C5 convertases must assemble nearby MAC pores capable of perturbing Gram-negative outer and inner membranes. Lacking terminal pathway components, C5b6 rapidly loses its ability to induce pore formation. Use of DNA dyes and bacteria engineered to express periplasmic mCherry and cytosolic GFP revealed that MAC formation resulted in mCherry release and DNA dye nuclear uptake but not GFP release, suggesting a model in which MAC pore formation on the outer membrane induces inner membrane destabilization and eventual rupture through an as-yet-unidentified mechanism (53).

Pump the Brakes—Inhibition

Complement activation is dynamically regulated by numerous inhibitors to prevent inappropriate activation or host cell destruction (Fig. 2). The C1 inhibitor (C1-INH) regulates all three activation pathways. C1-INH irreversibly inactivates C1 and MASP-1/-2 activity, and sequesters C3b to prevent FB binding (54–56). LP activation is also limited by MBL/ficolin-associated protein 1 (MAP-1, Map44), an enzymatically inactive splice variant of MASP-1, which sequesters MBL and ficolins to prevent C4 binding (57). A new regulatory protein was recently identified as Sushi-domain containing protein 4 (SUSD4), which blocks all three pathways of complement activation by preventing C3 convertase formation (58). Membrane-bound form SUSD4a inhibits both the CP and AP, while soluble SUSDb blocks CP and LP activation.

Although the inherent instability and dissolution of convertases within minutes of formation serves as an intrinsic regulatory mechanism, additional forces are required to prevent runaway complement activation. Decay accelerators destabilize C3 and C5 convertases by displacing the enzymatic components of the complex (59). Membrane-bound decay accelerators include C4BP, factor H (FH), FH-like protein-1 (FHL-1), decay accelerating factor (DAF, CD55), and complement receptor-1 (CR1, CD35, C3b/C4b receptor). Importantly, during apoptosis, membrane-bound complement inhibitors are shed from membranes, which allows complement to mark dead and dying cells for phagocytosis and promote tolerogenic immunity (60). Cofactors provide a second level of regulatory activity by enabling the serine protease factor I (FI) to irreversibly proteolytically inactivate surface bound C3b and C4b to split products iC3b/C3dg and iC4b/C4d, respectively. These inactivated fragments are unable to form convertases but function in other biological processes, including phagocytosis. FI cofactors include CR1, C4BP, FH, FHL-1, and membrane cofactor protein (MCP, CD46) (38). FH additionally regulates AP amplification by competing for FB binding with C3b.

Terminal MAC formation is limited by host cell expression of CD59 which binds C8 and C9 and prevents pore formation (61) (Fig. 2). Vitronectin (S-protein), clusterin, and C8 binding protein (C8BP) bind C5b-7, inhibiting assembly of a functional MAC (62, 63). Metabolically active host cells can also shed membrane pores via exocytosis or endocytosis (64).

Proper complement activation is essential for maintaining homeostatic balance between an appropriate response to stressors and development of autoimmunity. Genetic deficiencies of some complement components strongly predispose individuals for development of autoimmune disorders like systemic lupus erythematosus (SLE), while loss of others dramatically increase susceptibility for recurrent bacterial infections (65). Improper activation of complement pathways mediates numerous pathologies including ischemia-reperfusion injury, allograft rejection, autoimmunity, and cancer (66–68). Sepsis, a highly inflammatory multiorgan condition in which the immune system is hyperactivated, is characterized by improper activation of complement pathways and consumption of complement components (69). Complement inhibitors have been considered for sepsis; however, their utility has been hampered by an associated increased risk for bacterial infection (70). Recent clinical and preclinical data have identified several promising candidates that target complement during polymicrobial sepsis, emphasizing the importance of complement regulation during homeostasis, infection, and disease.

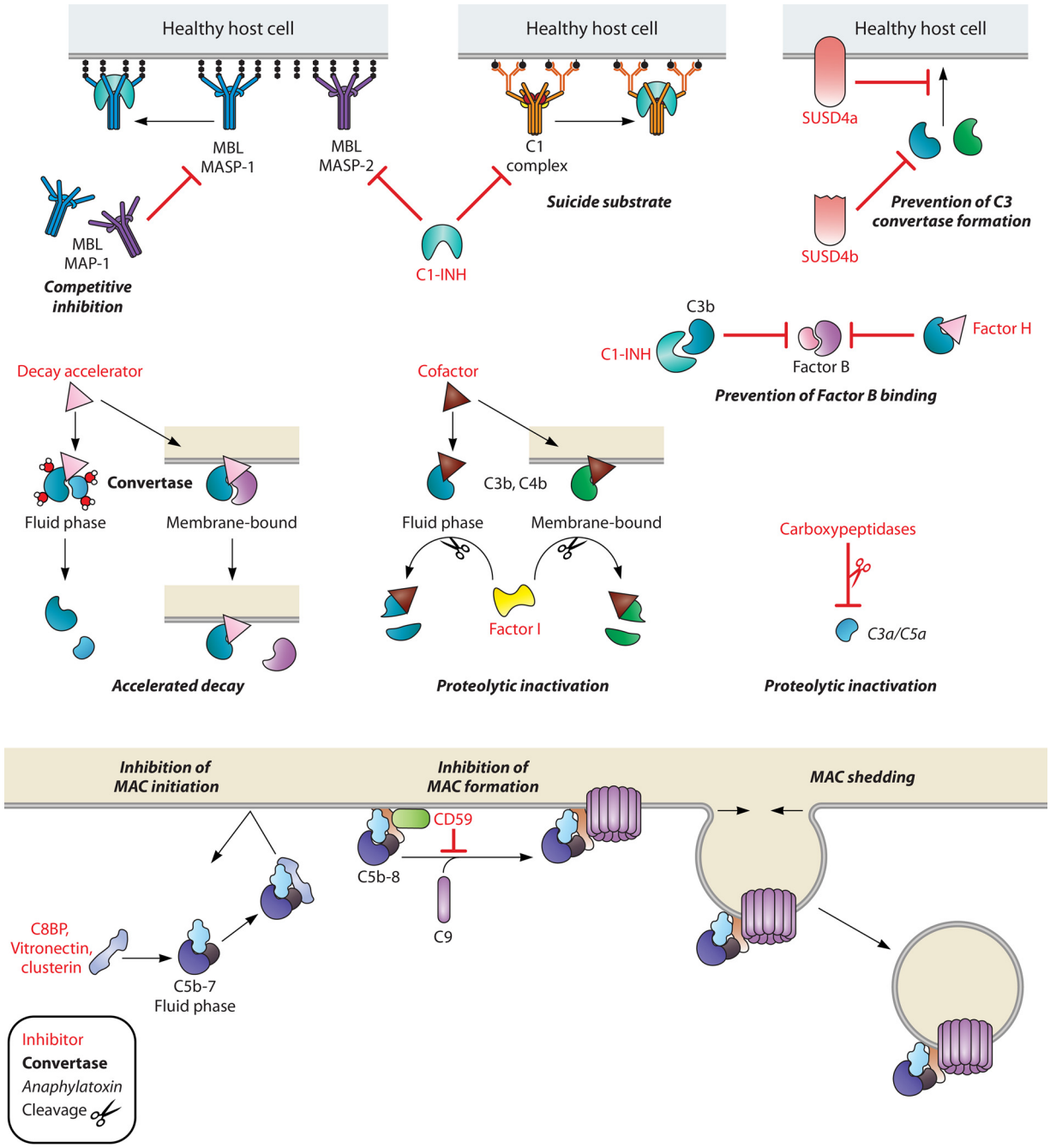

FIG 2 Host complement regulatory activities. Healthy host cells prevent inappropriate complement activation on their surfaces through a variety of mechanisms. MBL/ficolin-associated protein 1 (MAP-1) competes for binding of MBLs and ficolins with MASP-1 and MASP-2. C1-inhibitor (C1-INH) is a suicide substrate for all three complement pathways. Both C1-INH and factor H block factor B binding of C3b. Soluble and membrane forms of Sushi-domain containing protein 4 (SUSD4) prevents C3 convertase formation by preventing C3b and C4b deposition. Membrane-bound and serum circulating decay accelerators dissociate convertases. Cofactors recruit the serine protease factor I which cleaves C3b and C4b to inactive split products. Carboxypeptidases inactivate the anaphylatoxins C3a and C5a. Inhibitory proteins C8 binding protein (C8BP), vitronectin, and clusterin prevent C5b-7 binding to membranes. CD59 blocks membrane attack complex (MAC) formation. Metabolically active cells shed MAC pores by exocytosis and endocytosis.

Surviving MAC Attack—Evasion of Complement Lysis by Gram-Negative Bacteria

Certain clinically relevant Gram-negative bacterial pathogens have evolved strategies to survive complement-mediated lysis, including inhibition or cleavage of complement components, recruitment of inhibitory host proteins to bacterial membranes, steric hindrance by LPS, or membrane modifications (5) (Fig. 3).

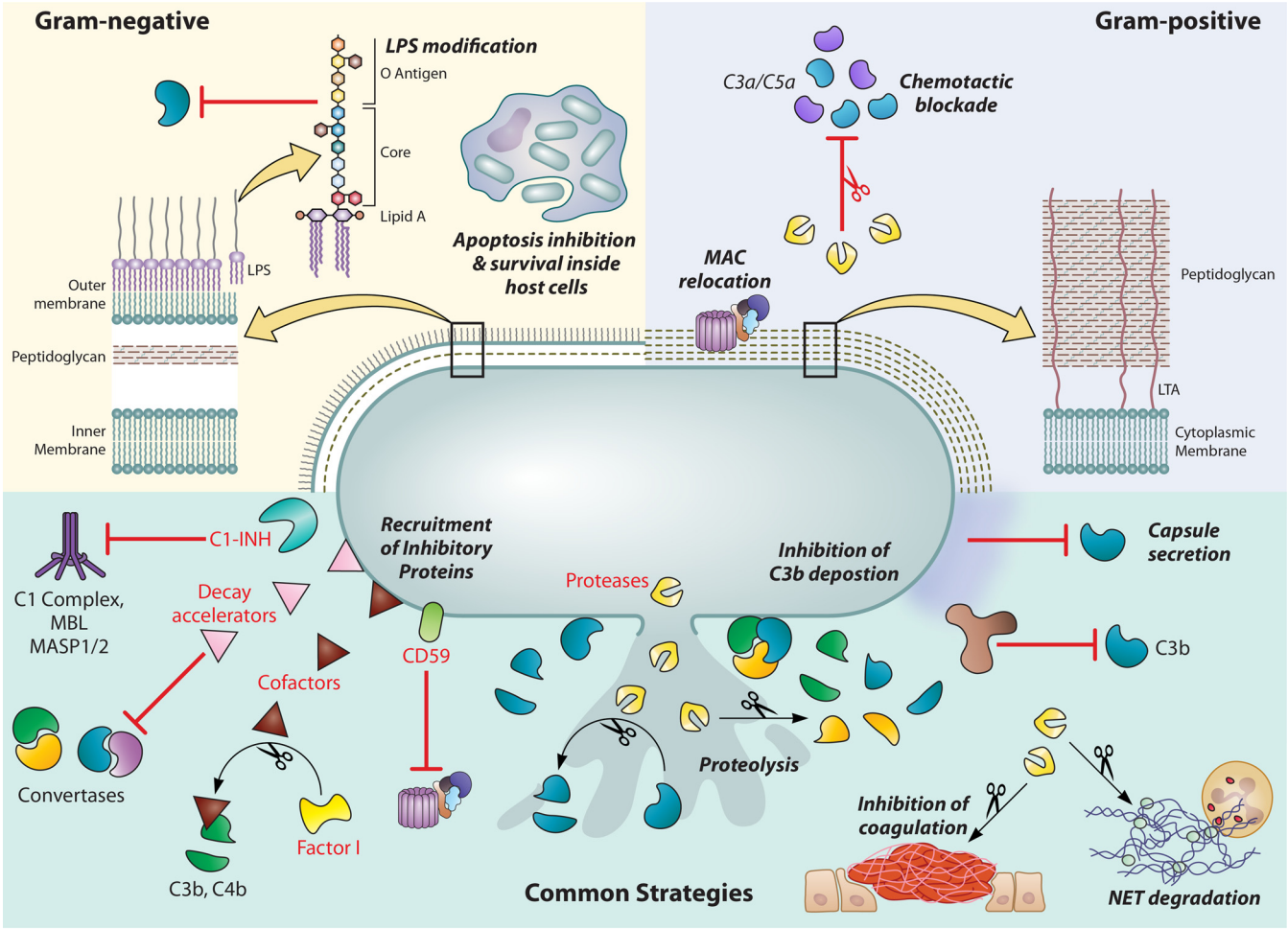

FIG 3 Bacterial complement evasion strategies. Gram-negative membranes consist of a double membrane with a thin layer of peptidoglycan, while Gram-positive membranes have a single membrane and a thick layer of peptidoglycan interspersed with lipoteichoic acid (LTA). Gram-negative bacteria evade complement via lipopolysaccharide (LPS) modifications and by surviving inside host cells to form a replicative niche. Gram-positive bacteria relocate MAC pores to nonlethal sites on their membranes and inhibit neutrophil chemotaxis. Common complement evasion strategies include recruitment of inhibitory proteins to bacterial membranes, proteolysis of complement components, capsule secretion, netosis (NET) degradation, and inhibition of C3b deposition and coagulation. MBL, mannose-binding lectin; MASP-1/2, mannan binding lectin-associated serine protease-1/2; C1-INH, C1-inhibitor.

Proteolytic counterattacks to survive complement include *Escherichia coli* secretion of proteases Pic and EspP, which cleave multiple complement components each (71, 72). Some strains of the opportunistic pathogen *Pseudomonas aeruginosa* produce an alkaline protease that cleaves C2 of the CP and LP to prevent C3b deposition (73), while outer membrane proteases IcsP and PgtE expressed by *Shigella* and *Salmonella*, respectively, cleave and shed C3b deposited on their membranes (74).

Bacteria can coopt host complement inhibitor function by recruiting these factors to their membrane surfaces (Fig. 3). For example, outer membrane protein Ail from *Yersinia pestis*, the causative agent of plague, is necessary and sufficient for serum resistance (75). Ail recruits the cofactor C4BP, resulting in FI-mediated proteolytic inactivation of C4b (76). *Salmonella* Typhimurium outer membrane protein Rck is structurally and functionally homologous to Ail and similarly recruits C4BP (77). Outer membrane proteins OmpA and OmpW from *E. coli* recruit C4BP and FH, respectively, limiting subsequent complement activation (78, 79).

Modification of LPS structures to impair complement recognition is a common resistance mechanism employed by several Gram-negative pathogens (Fig. 3). LPS consists of lipid A fatty acid chains that anchor LPS to bacterial membranes, a core oligosaccharide, and a repetitive O antigen polysaccharide exposed to the environment. "Rough" LPS has a truncated or absent O antigen while "smooth" LPS contains a

complete O chain. Early complement experiments revealed that bacteria with rough LPS were generally much more sensitive to serum killing, implicating LPS as an important bacterial defense against complement (80, 81). *Neisseria meningitidis* utilizes molecular mimicry by modifying the composition of its LPS to contain an epitope identical to carbohydrates found in human glycosphingolipids (82). Similarly, human-like sialic acid modifications to LPS increase complement resistance in *Haemophilus influenzae* by limiting IgM-dependent complement activation (83).

Production of a surface polysaccharide capsule can limit the availability of immunogenic antigens to key serum factors (Fig. 3). The capsules produced by *Francisella tularensis* (84), *Klebsiella pneumoniae* (85), and *E. coli* K1 (86) protect against C3b deposition and complement activation, with the degree of protection dependent on the amount of capsule produced (87).

NONLYTIC COMPLEMENT FUNCTIONS AND BACTERIAL EVASION MECHANISMS

Special Considerations in Gram-Positive Bacteria

Gram-positive bacteria have a thick layer of peptidoglycan and a single cell membrane, whereas Gram-negative bacteria have an outer membrane, a periplasmic space containing a thin layer of peptidoglycan, and an inner membrane (Fig. 3). Gram-positive and -negative bacterial outer membranes are studded with lipoteichoic acid (LTA) and LPS, respectively, and induce unique cytokine and chemokine responses (88). Unlike Gram-negative bacteria, Gram-positive bacteria are naturally resistant to direct lytic activity of complement, likely due to the thick outer layer of peptidoglycan. Importantly, this resistance is not due to a lack of binding or recognition by complement. Indeed, numerous Gram-positive bacteria are decorated by C3 activation fragments, including *Streptococcus pyogenes* and *S. pneumoniae*, *Lactococcus lactis*, *Bacillus subtilis*, *Staphylococcus aureus*, and *S. epidermidis* (89). However, MACs are sequestered at specific surface locations on some Gram-positive bacteria instead of randomly dotted across the bacterium, suggesting that complement distribution may impact its bactericidal activity. Recent insight into MAC killing of Gram-negative bacteria reveals that C5 convertases must assemble local MAC pores for bactericidal activity (52). Thus, MAC pores found on Gram-positive bacteria are likely not assembled nor inserted correctly for lytic activity, conceivably due to the thick peptidoglycan layer. Failed lysis notwithstanding, complement serves multiple added functions essential in defense against Gram-positive bacteria, including opsonization for phagocytosis, synergy with molecular effectors of neutrophil-mediated killing (including neutrophil extracellular traps [NETs]), amplifying and modulating broader inflammatory responses, directing T cell development, and coordinating coagulation cascades (Fig. 4). We will discuss the importance of these broader complement roles in host defense and ways in which they are counteracted by clinically important Gram-positive pathogens in detail below.

Preparing the Meal—Complement Aids Opsonization and Phagocytosis

Complement facilitates phagocytosis of foreign material and debris by neutrophils, macrophages, and dendritic cells (Fig. 4). Antibody and complement complexes deposited on bacterial surfaces are recognized by complement receptors (CRs) present on immune and nonimmune cells (90). Complement receptor 1 (CR1, CD35, C3b/C4b receptor) recognizes C3b, C4b, and iC3b. Complement receptor 2 (CR2, CD21) binds C3 split products iC3b, C3d, and C3dg. Complement receptors 3 and 4 are β2 integrin family members that recognize iC3b and consist of a common β-chain and unique α-chain: CR3-CD11b/CD18 and CR4-CD11c/CD18, respectively. Complement receptor of the immunoglobulin superfamily (CRIg, VSIG4) binds C3b, iC3b, and C3c. The expression of CRs varies by immune and nonimmune cell subsets and facilitates homeostatic processes such as metabolism and development in addition to their role in phagocytosis, which we will discuss here (13, 91). Productive phagocytosis yields a membrane-bound phagosome that matures, acidifies, and fuses with endosomes and lysosomes bearing bactericidal cargo that kill and degrade pathogens. This complex process involves

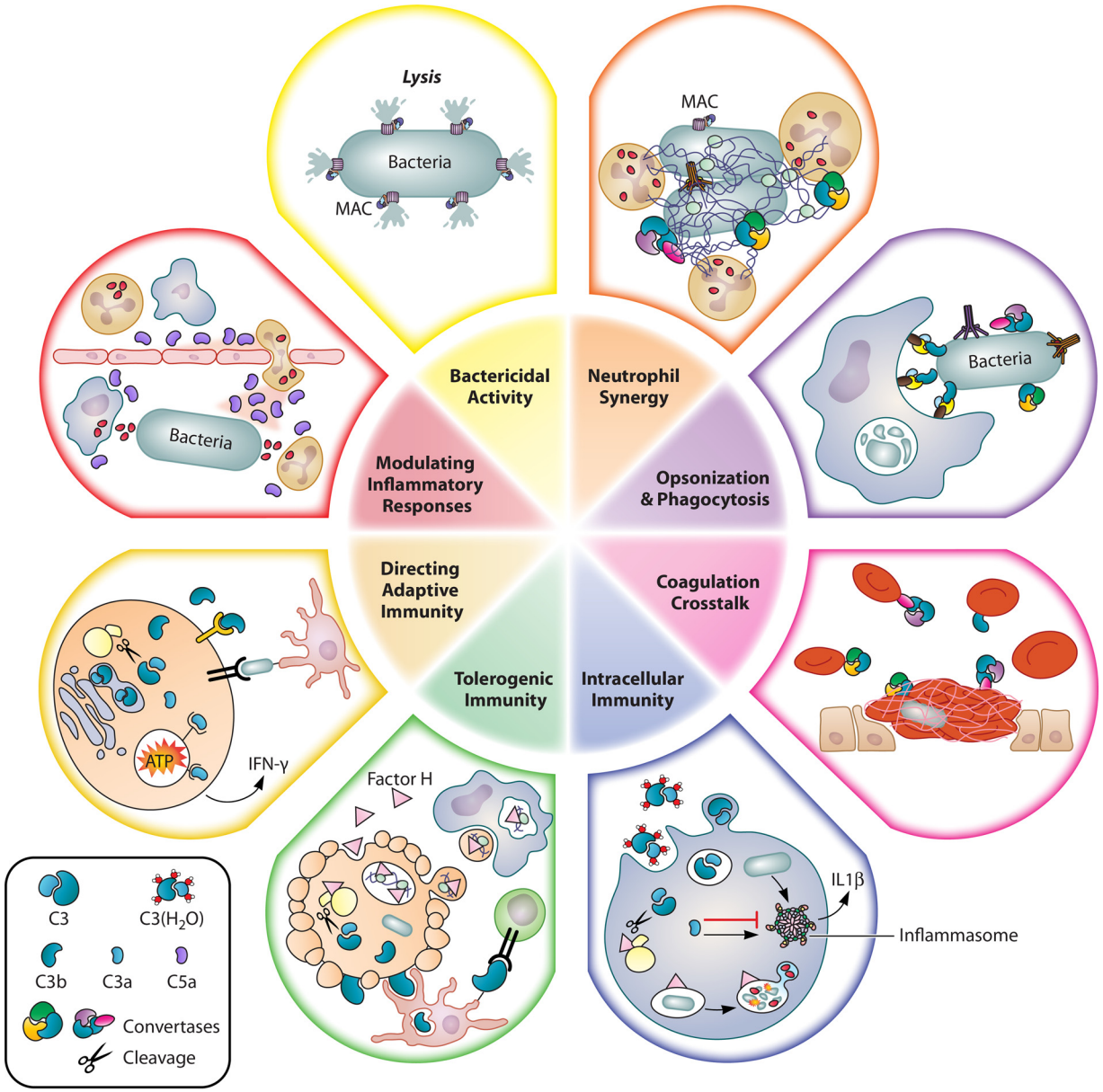

FIG 4 Multiple functions of complement during bacterial infections. Complement is responsible for numerous defense roles during microbial infections including neutrophil synergy, opsonization and phagocytosis, coagulation cross talk, intracellular immunity, tolerogenic immunity, directing adaptive immunity, modulating inflammatory responses, and bactericidal activity.

numerous signaling pathways beyond the scope of this review but is extensively covered by others (12, 92, 93).

Complement-mediated phagocytosis facilitates systemic *in vivo* control of both serum-sensitive Gram-negative bacteria and Gram-positive pathogens resistant to direct killing by MAC attack. CRIg expressed on liver Kupffer cells mediates rapid phagocytosis and clearance of circulating *S. aureus* in mice (94). Intriguingly, CRIg binds directly to lipoteichoic acid (LTA), a major constituent of Gram-positive cell walls, implicating CRIg as a macrophage pattern recognition receptor. Deposition of complement activation products on *Chlamydia trachomatis* infectious elementary bodies facilitates phagocytosis by CR3-expressing human monocytes, resulting in bacterial degradation (11). Thymic stromal lymphopoietin (TSLP), a cytokine known for promotion of allergic responses at barrier sites, was recently shown to enhance neutrophil killing of

methicillin-resistant *S. aureus* (MRSA) and *S. pyogenes in vitro* and in murine skin infections (95). TSLP surprisingly induced increased production of reactive oxygen species (ROS) by neutrophils in an unidentified C5-dependent manner that required phagocytosis. Identification of a previously undescribed connection between TSLP and innate immunity may provide clarity into conflicting evidence on the role of TSLP in murine sepsis models and underscores the complexity and caution required when using anti-TSLP (Tezepulumab) therapeutically. Complement also contributes to bacterial clearance in immune-privileged sites such as the brain. A recent study found *S. aureus* induced microglial production of C3, which mediated maximal IgM opsonization and subsequent phagocytosis by microglia via CR3-IgM interactions (96). During bloodstream infections by *Listeria monocytogenes*, complement balances rapid bacterial clearance by phagocytes and protective immunity. C3 opsonization facilitates bacterial association with platelets that transit to the spleen and are rapidly taken up by CD8α^+ dendritic cells, priming antigen-specific CD8 T cell immunity (97). Thus, complement-mediated phagocytosis can also bridge between innate and adaptive immune systems, simultaneously providing acute clearance and lasting protection.

Many clinically relevant bacterial pathogens have evolved mechanisms to avoid opsonization and phagocytosis, while other well-adapted intracellular microbes have cleverly coopted the opsonin and phagocytic functions of complement to facilitate their own uptake by macrophages and neutrophils whereupon they escape from or remodel the phagosome to a permissive niche (98). Extracellular fibrinogen binding protein (Efb) expressed by 85% of *S. aureus* strains prevents phagocytosis *in vitro* and *in vivo* by disguising the bacteria with a fibrinogen shield that blocks CR1 recognition of opsonized C3b and IgG (99). *S. pyogenes* or group A *Streptococcus* (GAS) prevents phagocytosis by recruiting FI to cleave bacterium-bound C3b (100). The *B. anthracis* poly-γ-D-glutamate capsule blocks C3b deposition and processing to the opsonic split products C3c and C3dg, inhibiting phagocytosis by human macrophages (101). Biofilm formation by *S. pneumoniae* aids evasion of the CP by inhibiting C1q binding and C3b deposition (102). Recruitment of FH to the biofilm further protects *S. pneumoniae* by inhibiting the AP. *Enterococcus faecalis* similarly escapes AP activation through FH recruitment to its surface (103).

In contrast, CR3-mediated uptake of complement opsonized *Mycobacterium tuberculosis* by macrophages is a major mechanism by which the pathogen gains access to its preferred replicative niche (104, 105). Complement opsonization also enables vitronectin-dependent access to nonphagocytic epithelial cells by *Burkholderia pseudomallei*, *B. thailandensis*, and *K. pneumoniae* (106). Uptake of *F. tularensis* by human macrophages is primarily mediated by complement receptors (107), whereupon the pathogen inhibits phagosome maturation, escapes into the cytosol, and replicates intracellularly (108, 109). Thus, efficient phagocytosis must be coupled to equally robust intracellular killing, lest the phagocytic cell become a transport vessel for the pathogen deeper into the body.

Food for Thought—Complement Traffics to Autophagy

A recently appreciated mechanism by which bacterial uptake is connected to clearance is through complement-mediated autophagy (110). Recent studies show complement opsonized *Listeria* are trafficked to autophagy pathways that restrict bacterial replication, also known as xenophagy (74, 111). Furthermore, mice lacking intestinal expression of ATG7, a key autophagy mediator, exhibited increased *Listeria* replication similar to those seen in C3-deficient mice; chemical induction of autophagy in *C3*$^{-/-}$ mice rescued bacterial burdens. Interestingly, *Shigella* was not impacted by C3-driven autophagy; indeed, both *Salmonella* and *Shigella* expressed proteases capable of cleaving C3 and escaping complement-mediated autophagy (74). Other clinically relevant bacteria are known to be controlled by autophagy pathways. A globally disseminated GAS serotype evades autophagy by degrading ubiquitination adaptor proteins using the serine protease SpeB (112). However, SpeB expression is highly variable among GAS serotypes, and those lacking SpeB expression were successfully targeted by autophagy

machinery in epithelial cells and mouse embryonic fibroblasts. Ubiquitylation is similarly required for xenophagy and control of *M. tuberculosis* in macrophages and murine models of infection (113). However, whether complement contributes to autophagy-dependent control of GAS or *M. tuberculosis* remains to be discovered.

Surprisingly, the link between complement and autophagy extends beyond host-pathogen control. Recent studies reveal intracellular C3 interacts with ATG16L1, a crucial autophagy mediator, in pancreatic β islet cells (114). Furthermore, C3-mediated autophagy was essential for homeostatic islet cell turnover and proved protective during diabetogenesis. CD46 cross-linking in cultured primary human epithelial cells induced protective autophagy and reduced oxidative stress, highlighting a potential therapeutic target for allergic asthma models (115). In contrast, receptors for C3a and C5a inhibited mitochondrial autophagy (mitophagy) in dendritic cells following hematopoietic cell transplantation (116). Receptor inhibition prevented subsequent development of graft-versus-host disease, implicating complement-autophagy interactions as mediator of pathology. Furthermore, sublytic MAC formation on kidney podocytes induced protective autophagy; however, internalization of a functional pore may prevent efficient autophagy and lead to pathology as seen in patients with idiopathic membranous nephropathy (117). Thus, the complement-autophagy connection appears to be an advantageous defense strategy against pathogens; however, the balance between homeostasis and pathology may be a difficult line for these two pathways to walk together.

Fueling the Fire—Complement Modulates Inflammatory Responses

During complement activation C3a and C5a, potent anaphylatoxins, are released by proteolytic cleavage by C3 and C5 convertases, respectively (Fig. 4). These highly inflammatory molecules stimulate trafficking of key immune cells to the site of infection by binding to receptors C3aR, C5aR1 and C5aR2, respectively (118). C4a is structurally similar to C3a and C5a; however, until recently its biological function as a nontraditional protease activated receptor (PAR) 1 and 4 agonist was unknown (119), the implications of which will be discussed below in the section on complement and coagulation interactions. C3a is a chemoattractant for dendritic cells, macrophages and many others, whereas C5a promotes migration of macrophages, neutrophils, and monocytes (13). In addition, C3a and C5a stimulate oxidative burst activity in neutrophils and macrophages and trigger degranulation of mast cells and basophils. C3a and C5a augment the production of multiple inflammatory cytokines by both innate and adaptive immune cell populations (120). Furthermore, C3a and C5a play an important role in remodeling the infection microenvironment by increasing vascular permeability and smooth muscle contraction. To prevent indiscriminate activity by anaphylatoxins, C3a and C5a are rapidly converted to inactive forms C3a-desArg and C5a-desArg, respectively, by circulating carboxypeptidases (118).

Complement components directly influence the inflammatory response to bacterial pathogens *in vivo* (Fig. 4). For example, FH binds an unknown ligand on *M. bovis* during infection and induces upregulation of critical proinflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-6, while downregulating anti-inflammatory cytokines IL-10 and TGF-β (121). Murine survival after infection by the obligate intracellular bacteria *Rickettsia australis* requires C3-mediated proinflammatory signaling, attributed to anaphylatoxins that drive gamma interferon (IFN-γ), *Rickettsia*-specific IgG, and adaptive responses (122). C5aR1 blunts the *in vivo* immune response to *L. monocytogenes* by suppressing type 1 IFN gene expression via the second messenger cyclic di-AMP (123, 124). In contrast, blockade of C5aR1 and C5aR2 of mid- and high-grade sepsis improves patient mortality, suggesting that anaphylatoxins can contribute to lethal levels of acute inflammation (125). Indeed, treatment with a neutralizing C5a peptide protected mice in a pneumococcal pneumonia sepsis model even when administered 24 h postinfection (126). Multiple pharmacological candidates targeting complement-mediated inflammation are currently undergoing preclinical development (127, 128).

The importance of C3a and C5a in microbial immunity is underscored by pathogens with mechanisms to disrupt anaphylatoxin function (Fig. 3). GAS secretes the serine protease ScpA which inhibits anaphylatoxin immunomodulatory functions by enzymatic cleavage of C5a and C3a (129). Chemotaxis inhibitory protein of *S. aureus* (CHIPS), found in more than 50% of clinical isolates, potently inhibits C5a-mediated neutrophil recruitment, and the metalloprotease, aureolysin, prevents release of C5a altogether (130, 131). In addition, a multidrug resistant outbreak strain of *M. tuberculosis* coopts C5aR1 to manipulate monocyte and macrophage cytokine secretion and inhibit expansion of IFN-γ -producing lymphocytes (132). Furthermore, the periodontitis-associated bacterium *Porphyromonas gingivalis* subverts a protective antimicrobial signaling response to remodel the oral microbiome to a dysbiotic state where it can thrive on the uptake of tissue degradation products (133). *P. gingivalis*-expressed gingipains cleave C5, generating C5a and inducing C5aR/Toll-like receptor 2 (TLR2)-dependent signaling that inhibits neutrophil phagocytosis and enhances dysbiotic inflammation in periodontal tissue. Intriguingly, local C3 inhibition in nonhuman primates protects against *P. gingivalis*-induced periodontitis (134). C5a recruitment of neutrophils is particularly important during *Neisseria* infections; eculizumab is a U.S. Food and Drug Administration (FDA)-approved anti-C5 therapy that treats paroxysmal nocturnal hemoglobinuria, a rare blood disease. Despite antibiotic prophylaxis and immunization with meningococcal vaccines, patients receiving eculizumab have significantly increased *Neisseria* infections (135). Thus, complement is an attractive target for pathogens, pathobionts, and novel therapeutics.

Casting a Wider NET—Complement and Neutrophil Synergy

Neutrophil extracellular traps or NETs are increasingly appreciated as a major aspect of innate immune and inflammatory function. During NETosis, neutrophil DNA decondenses and is expelled from the nucleus coated with cytotoxic granules, peroxidases, and proteases (136), with the potential to entrap pathogens and restrict their spread, although the direct killing contribution of NETs is controversial. A recent study clarified the bactericidal activity of NETs, demonstrating that captured bacteria remain largely viable but are subsequently killed by complement (137). Interestingly, NETs were found to shield *S. aureus* and *P. aeruginosa* from complement killing until after NET degradation (138). Human serum contains NET-degrading DNase I, and many bacteria evade NETs via protease secretion. Thus, complement may act as a cleanup system to capture bacteria who escape or destroy NETs.

Furthermore, complement opsonization promotes NET formation, and NETs provide a scaffold for complement activation implying a reciprocal relationship to promote pathogen clearance at the foci of infection. Complement opsonization of *S. aureus* and the oral pathogen *Actinomyces viscosus* significantly enhanced NET release compared to serum-free controls in a manner dependent on CR1 (139). During *P. aeruginosa* infection, neutrophils release properdin, FB, and C3 and undergo NETosis (140). Properdin is subsequently deposited on the forming NETs and entrapped bacteria, inducing terminal complement activation via the AP. *In vivo*, complement and neutrophils synergize to induce pathogen clearance. C3-deficient mice are unable to NETose in response to a *S. aureus* skin infection and suffer severe bacteremia and dissemination compared to control mice, despite normal neutrophil trafficking (141). Transfer of wild-type serum restored NETosis function, indicating that complement opsonization is essential for NET formation and systemic control of bacteremia. C3aR-deficient neutrophils similarly lack NET-forming capacity (142).

Complement-neutrophil synergy extends beyond NET induction. Neutrophil activation of the AP induces a feedback loop that further amplifies proinflammatory neutrophil responses (14). C3 fragments covalently bind the surface of TNF-α-activated neutrophils in an FB-dependent manner and induce CD11b expression and neutrophil oxidative burst. Deposited C3 activation products formed the C5 convertase which generated C5a, hyperactivating neutrophils and amplifying further inflammatory responses (14). Furthermore, addition of purified FD and properdin rescued neutrophil

opsonophagocytosis defects in sera from MBL-deficient individuals, confirming the importance of the AP in augmenting neutrophil activity (143). During some infections by intracellular pathogens, macrophages undergo an inflammatory form of cell death called pyroptosis and trap viable bacteria in their cellular "corpse," termed a pore-induced intracellular trap (144). Neutrophils subsequently efferocytose and kill the released bacteria in a C3-dependent process as evidenced by C3-deficient mice failed to recruit neutrophils to the site of infection and displayed diminished efferocytosis and bacterial clearance.

In contrast, improper amplification of complement-neutrophil pathways has been implicated in poor outcomes and tissue damage during sepsis, in which neutrophils are the dominant responding cell type (145). Unregulated release of C3a and C5a stimulates a two-sided neutrophil response that suppresses neutrophil oxidative burst, chemotaxis, degranulation, and bactericidal activity while simultaneously inducing excessive release of NETs, proteases, and other inflammatory mediators that contribute to tissue damage and amplify complement activation. In the context of autoimmunity, during antineutrophil cytoplasmic antibody associated vasculitis NETs provided a platform for AP activation that mediated endothelial cell damage (146). Hence, the intersection of neutrophil and complement is a double-edged sword that can both mediate immunopathology and facilitate pathogen control.

Some bacterial pathogens have evolved strategies to block the activity of complement and neutrophils. For example, *B. pertussis* suppresses NET formation by producing adenylyl cyclase toxin that inhibits ROS production (147). Healthy neutrophils are short-lived and undergo immuno-quiescent apoptosis after a day or two in circulation. Complement proteins FB, C1q, and C3, as well as CR3 and CR4, are required for maximal clearance of apoptotic cells by human macrophages (148). Some pathogens coopt this homeostatic process to either remove neutrophil populations or to delay turnover and provide a replicative niche. Streptolysin O and multiple leukocidins produced by GAS and *S. aureus*, respectively, induce enhanced neutrophil cell death (149, 150). In contrast, the facultative intracellular pathogen *Coxiella burnetii*, which causes Q fever, suppresses neutrophil apoptosis upon infection, allowing continued access to a replicative niche (151).

Inside Scoop—Novel Roles of Intracellular Complement

Although complement is best described as an extracellular proteolytic cascade, recent studies have identified novel intracellular functions for complement (152) (Fig. 4). The liver is the major source of complement proteins; however, monocytes, macrophages, and nonimmune cells, including endothelial and epithelial cells, contribute to complement production (2). A recent study identified a recycling pathway in which spontaneously hydrolyzed C3(H_2O) is "loaded" by primary and cultured human cells, stored in endosomes, and recycled to the extracellular milieu (15). Most of the internalized C3(H_2O) was secreted within 24 h; however, approximately 20% was retained and processed by cointernalized FH, FI, and cathepsin L (CTSL), resulting in *de novo* intracellular C3a. In $CD4^+$ T cells, this process induced IL-6, indicating that intracellular complement recycling pathways may skew immune proinflammatory profiles (15). C3 and C3a have been found within many cell populations, including monocytes, neutrophils, B and T cells, fibroblasts, and endothelial and epithelial cells, suggesting that complement may be a general contributor to cellular homeostasis (16). The role of intracellular complement in metabolism and cellular homeostasis has been largely studied in T cells, to be discussed further below in a section concerning complement and adaptive immunity (22).

Inflammasomes are cytosolic signaling scaffolds that detect intracellular stressors and pathogens, inducing pyroptosis, a highly inflammatory form of cell death, and proinflammatory IL-1 cytokine release (153). Complement plays dual roles in inflammasome regulation that depends on the initiating stimuli and cell type, providing either amplification of inflammatory signals or inhibition of inflammasome activity (154). Analogous to a bacterial pore-forming toxin, sublytic MAC formation on epithelial and

dendritic cells induces calcium dysregulation, potently triggering the NLRP3 inflammasome, which consists of the sensor NLRP3, the adaptor ASC and the cysteine protease caspase-1 (155, 156). In addition, complement-opsonized zymosan phagocytosed by macrophages strongly induces NLRP3-mediated IL-1β release (157). Intriguingly, MAC complexes on eaten particles were transferred to the membranes of the phagocytes, resulting in "bystander" sublytic MAC deposition that induced ionic flux and inflammasome activation and promoted Th17 polarization in T cells. In monocytes, C3a-C3aR interactions potentiated LPS-induced ATP efflux that activated NLRP3, driving IL-1β release capable of enhancing human $CD4^+$ Th17 responses (158).

Intracellular C5 is activated following T cell receptor and costimulation during which C5a interaction with intracellular C5aR induces ROS generation and inflammasome assembly (159). Interestingly, C5a plays opposing roles during inflammasome formation in monocytes and macrophages. C5a-C5aR1 ligation activates NLRP3 inflammasomes and IL-1 secretion in monocytes but inhibits inflammasome assembly in macrophages, suggesting that complement modulates innate immunity as a protective adaptation to limit excessive bystander damage caused by tissue resident macrophages while enhancing the sentinel function of circulating monocytes (160). In addition, C1q bound to apoptotic cells negatively regulates NLRP3 inflammasome formation by macrophages and promotes tolerogenic immunity through inhibition of caspase-1 cleavage and increased expression of inhibitory cytokines (161). Complement-inflammasome interactions underlie an evasion mechanism exploited by *F. tularensis*. CR3 mediates bacterial uptake into human monocytes whereupon C3-opsonized *F. tularensis* inhibits inflammasome priming by recruiting Ras GTPase activating protein (RasGAP) to suppress signaling pathways upstream of inflammasome activation (162).

Intracellular complement signaling contributes to anti-pathogen immunity. A recent paper described NF-κB signaling induced by internalized C3 during infection with a variety of viruses, as well as *S.* Typhimurium. Complement-mediated signaling required an unknown intracellular receptor that promoted proinflammatory cytokine production and pathogen restriction (17). *L. monocytogenes* efficiently escapes phagosomal killing in macrophages by means of the pore forming protein listeriolysin O. However, complement opsonized *L. monocytogenes* that escapes into the cytosol is recognized by CRIg and targeted for autophagy-mediated degradation (163). Mechanistically, binding of CRIg to the chloride intracellular channel 3 (CICL3) on macrophage vacuoles containing *L. monocytogenes* promotes successful phagosome maturation, fusion with lysosomes, and bacterial killing (163). Mice deficient for either CRIg or CICL3 are unable to control *L. monocytogenes* and succumb rapidly to infection, confirming the importance of intracellular complement in mediating pathogen clearance.

Since the discovery of intracellular complement is a relatively recent finding, bacterial evasion strategies that target this newly appreciated role of innate immunity have not yet been reported. Although the biological relevance of cytoplasmic complement is not fully known, the presence of complement in intracellular spaces surely poses a particular threat to obligate intracellular bacteria. Recent studies provide evidence for speculation that microbes may employ a variety of mechanisms to escape intracellular complement. For example, complement-opsonized *L. monocytogenes* is targeted for intracellular autophagy; however; *Shigella* and *Salmonella* both employ proteases that cleave deposited activation products and enable xenophagy evasion (74). Furthermore, cytoplasmic sensing of C3-opsonized viruses and bacteria induced mitochondrial antiviral signaling protein-dependent signaling and antipathogen responses (17). Notably, antiviral induction varied among the tested viruses, suggesting the viruses employed strategies to antagonize intracellular complement-mediated immunity. Therefore, it stands to reason that bacteria might behave similarly. Alternatively, intracellular complement signaling may also provide an attractive target for pathogens to hijack immune responses. Since many intracellular bacteria remodel the environment of their host cell to suit their metabolic and replicative needs, it is plausible that these activities could also subvert the function of intracellular complement. Given the importance of intracellular

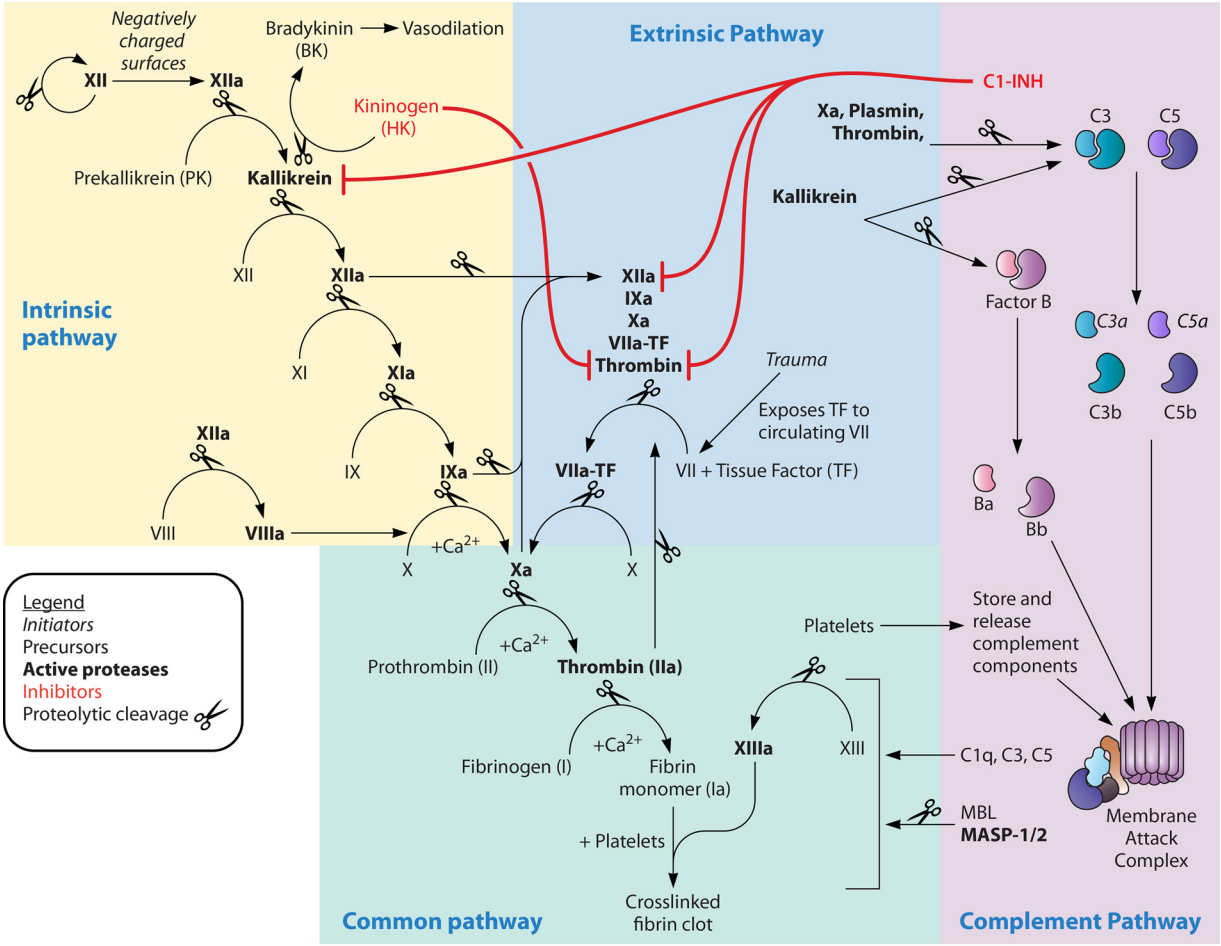

FIG 5 Coagulation and complement cross talk. The intrinsic coagulation pathway is triggered by interaction of negatively charged surfaces with factor XII which initiates the subsequent enzymatic cascade. The extrinsic pathway is initiated when tissue damage exposes tissue factor to circulating factor VII. Both pathways converge on the activation of factor X which induces thrombin and fibrinogen cleavage to form a cross-linked fibrin clot. C1-inhibitor (C1-INH) inhibits kallikrein, factors XIIa, IXa, and thrombin. Factor Xa, plasmin, and thrombin intersect with complement pathways by cleaving C3 and C5. Kallikrein cleaves C3 and factor B. Platelets store complement components in alpha-granules that are released upon activation. C1q, C3, and C5 promote thrombus formation. MBL/MASP-1/2 complexes cleave terminal coagulation components to facilitate clot formation.

and autocrine complement signaling for T cell survival and effector differentiation (16), it would not be surprising if a pathogen coopted the pathway to alter T cell responses to promote a more hospitable niche or dampen the adaptive response. Bacterial evasion mechanisms that target intracellular complement pathways are an exciting topic for future investigations.

Tying the Clot—Complement and Coagulation Cross Talk

Coagulation is the process by which a platelet-fibrin-rich clot forms in response to damaged tissue. Analogous to complement pathways, coagulation induces a cascade of proteolytic enzyme activation culminating in clot formation (138, 164) (Fig. 5). Briefly, in the extrinsic pathway, tissue factor (TF) expressed on vascular endothelium normally concealed from circulation is exposed to blood and initiates coagulation by binding the serine protease factor VII. Factor VII is cleaved to factor VIIa by thrombin, factors Xa, IXa, and XIIa, and active TF-factor VIIa complexes. TF-VIIa converts factors IX and X into active proteases IXa and Xa. Xa cleaves prothrombin to thrombin, cleaving soluble fibrinogen molecules to insoluble fibrin strands and activating factor XIII, which cross-links fibrin to form the hemostatic plug in concert with recruited platelets. The intrinsic pathway is initiated when factor XII binds negatively charged surfaces and

cleaves the enzyme kallikrein. This induces the release of bradykinin which catalyzes factor XII conversion to XIIa, initiating the sequential cleavage of factors XI, IX, and X (Fig. 5).

Complement and coagulation cascades intersect at several places. Thrombin was identified as a C5 convertase when C3-deficient mice were surprisingly found to have wild-type levels of C5a (18, 165). Indeed, thrombin, plasmin, and factors Xa and XIa can produce C3a and C5a, enhancing neutrophil recruitment (18), indicating that complement and coagulation pathways cross-activate and compensate for loss of individual components. Furthermore, thrombin was found to additionally cleave C5 at an alternative site and generate a MAC complex more potent than the canonical C5b-9 against erythrocytes (166). Whether it is similarly lytic against bacteria was not tested. A recent study identified the serine protease kallikrein as an enzymatic converter of C3 and FB that yields functional cleavage products, inducing AP activation (167). Platelet α-granules store hundreds of proteins including bactericidal thrombocidins, as well as C3 and other complement components; platelet activation induces degranulation that can activate complement through the release of stored components (168).

C1q has been reported to have multiple conflicting roles in hemostasis; however, a recent study clarified the *in vivo* contribution of C1q to coagulation (169). C1q-deficient mice lost twice as much blood as wild-type controls, a phenotype that could be rescued by reconstitution with exogenous C1q. C1-INH efficiently inhibits multiple components of the coagulation pathways, including factors XIa and XIIa, as well as kallikrein and thrombin (170). Furthermore, C1-INH inhibits homeostatic and *E. coli*-enhanced coagulation in human blood in a dose-dependent manner (171). MASP-1 and -2 of the LP cleave terminal coagulation components to form cross-linked clots (19, 172). Although MASP-2 catalytic activity is weaker than that of factor Xa, activation occurs at physiological concentrations whereby MBL-MASP-2 complexes associated with *S. aureus*, indicating *bona fide* cross talk between complement and coagulation pathways during infection. C4a was recently identified as a nontraditional agonist for protease-activated receptor 1 (PAR1) and PAR4, inducing calcium mobilization, endothelial permeability, and stress fiber formation (119). Since PAR receptors regulate platelet physiology, activation, and angiogenesis, C4a may function in platelet homeostasis, although this remains to be demonstrated. *In vivo*, $C3^{-/-}$ mice displayed increased bleeding times. In contrast, C5-deficient mice had normal bleeding times but higher rates of rebleeding than wild-type mice, which is indicative of impaired thrombus stability (173).

Coagulation-complement intersections mediate pathogen immunity, as well as hemostasis. Platelets aggregate around C3-opsonized *L. monocytogenes* and facilitate their uptake by splenic $CD8^+$ dendritic cells, which present antigen to cytotoxic $CD8^+$ T cells responsible for pathogen clearance and protective immunity (97, 174). Platelet depletion or C3 deficiency impairs antigen-specific T cell expansion. In addition, C3-mediated platelet aggregation occurs during murine bloodstream infections with *S. aureus*, *E. faecalis*, *B. subtilis*, and *S. pneumoniae*, suggesting complement and coagulation promote a common mechanism for directing intracellular adaptive immune responses during bacterial infections (97, 174). Platelet contact with *E. coli* induced degranulation; released C3 fragments bound to both platelet and bacterial surfaces (175).

In contrast, the mutual amplification of complement and coagulation cascades is pathological during sepsis, which is characterized by complement consumption and platelet depletion (69). Unsurprisingly, complement inhibition is of therapeutic interest during sepsis. C5aR1 blockade enhanced murine survival in a meningococcal model of sepsis (176). In addition, C5 inhibition in a baboon model of *E. coli* bacterial sepsis inhibited C3b and MAC deposition and proinflammatory cytokine production, decreased coagulation consumption, and protected against sepsis-induced multiple organ damage (177). Furthermore, 80% of treated baboons survived sepsis, while the untreated group experienced 100% mortality within 48 h. Thus, inhibition of C5 may prove a viable

strategy for sepsis therapies (178); however, given that usage of eculizumab, a humanized anti-C5 monoclonal antibody, predisposes individuals to infection by meningococcus, caution must be exercised prior to administration in the context of existing bacterial infections.

Many pathogenic bacteria possess mechanisms to escape the combined forces of complement and coagulation (Fig. 3). For example, *S. aureus* secretes fibrinogen binding proteins which coopt fibrinogen to form a shield around the bacterium, preventing C3b binding and clearance (99). Surface expressed GAS M protein acts in a similar protective fashion (179). In addition, the GAS virulence factor streptococcal inhibitor of complement inhibits coagulation and fibrinolysis, promoting bacterial survival within clots in addition to its role as an inhibitor of the MAC (180). Furthermore, GAS also subverts platelet-complement interactions to induce platelet consumption during sepsis (181). Released M protein induces formation of a complex between complement factors, fibrinogen, and IgG. Subsequent complex interaction with platelets induces aggregation, apoptosis, and clearance by monocytes, resulting in platelet depletion, a hallmark of sepsis. FH binds sialic acid moieties and is an essential protector of complement-induced killing of host cells, particularly platelets; *S. pneumoniae* neuraminidase A cleaves sialic acid, resulting in increased complement-mediated platelet activation, aggregation and pneumolysin-induced hemolysis (182). *B. anthracis* peptidoglycan activates platelets by direct binding to FcγRII and indirectly through MAC formation on platelet surfaces (183). Thus, the intersection of coagulation and complement pathways serves as another evolutionary battlefield between host and pathogen.

Bridging the Gap—Complement Instructs Adaptive Immunity

Complement bridges innate and adaptive immunity by directing essential T cell and B cell physiology during homeostasis and development, which we will briefly describe before focusing on the intersection of complement and adaptive immunity in the context of bacterial infections (13, 22, 184). In resting T cells, intracellular C3 is constitutively turned over by the protease cathepsin L, which provides essential survival signals that drive IFN-γ secretion and T helper 1 (Th1) development (16, 20). Intracellularly derived C5a plays dual roles in IFN-γ production in $CD4^+$ T cells and mediates T cell differentiation, expansion, and survival (159, 185). CD46 functions as a metabolic switch that regulates T cell subset differentiation depending on the cytokine microenvironment (186, 187). Notably, CD46 augmentation of nutrient uptake and fatty acid synthesis enhances cytotoxic $CD8^+$ T cell responses, amplifying cytokine production and killing capacity, while CD46 glycolytic flux in $CD4^+$ T cells is essential for Th1 effector function (188). Individuals lacking complement components exhibit serious defects in T cell development and function (20, 187, 189). For example, C1q regulates mitochondrial metabolism and survival of $CD8^+$ memory precursor effector cells, restricting tissue damage and autoimmunity in murine SLE, which may explain the propensity for individuals lacking C1q to develop lupus (9).

Complement directs T cell activation and expansion during bacterial infections (Fig. 4). $C3^{-/-}$ mice have substantially fewer activated $CD4^+$ and $CD8^+$ splenic T cells independent of dendritic cell maturation and antigen-presenting cells following infection with *L. monocytogenes* (21). Adoptive transfer of purified $C3^{-/-}$ and $C3^{+/+}$ $CD8^+$ T cells into a C3-sufficient recipient resulted in expansion and activation of both sets of donor T cells following subsequent infection with *L. monocytogenes*. Furthermore, C3a and C5a mediate protection against *L. monocytogenes*-induced apoptosis of myeloid and adaptive cell populations required for bacterial clearance (124, 190). A prospective study of abdominal bacterial sepsis patients found a significant relationship between C3 depletion and T cell immunosuppression (191). These authors found more than 80% of patients who succumb to infection had depleted C3 and increased T regulatory cells (T regs), emphasizing the importance of complement in maintaining balance between T cell subsets during acute bacterial infections.

Some bacteria have coopted complement receptors on T cell surfaces to misdirect T cell responses. The M protein from GAS binds CD46 expressed by human $CD4^+$ T

cells and induces their development into IL-10-producing T-regulatory suppressor cells (192). *M. leprae* exploits CD46 and C3 to dampen dendritic cell maturation and induce IL-10-producing T regs (193, 194).

Complement is also essential for humoral immunity. Human CR1 significantly inhibits B cell proliferation and activation; in contrast, CR2 acts as a coreceptor and molecular adjuvant for the B cell receptor (BCR). CR3 and CR4 promote human B cell migration and proliferation, respectively. C3-antigen-antibody complexes on follicular dendritic cells are required for maintenance of memory B cells within germinal centers. Autocrine C3aR1 and C5aR1 signaling is required for *in vivo* IL-6 and IgG production in response to ovalbumin immunization (195). Impaired complement function is associated with a number of B cell-mediated pathologies (196).

Synergy between complement and humoral immunity mediates antipathogen defenses (Fig. 4). C3b binding to tetanus toxin produced by *Clostridium tetani* protected the antigen from cathepsin D proteolysis and promoted increased B cell presentation that potentiated increased T cell clonal specificity (197). Depletion of CR1 and CR2 in mice inhibited antigen specific IgM and IgG antibody responses to *S.* Typhimurium and sterile antigens (198, 199). Follicular dendritic cells in germinal centers can present C3 opsonized antigens to B cells undergoing affinity maturation, isotype switching and differentiation, although the exact mechanism by which complement promotes these processes remains unclear (200). Depletion of circulating C3 by cobra venom factor injection in mice strongly suppresses B cell antibody responses to multiple antigens, including *S. pneumoniae* pneumococcal capsular polysaccharides (201). Complement opsonization and CR2 are essential mediators of associations of *S. epidermidis*, an opportunistic skin pathogen, with human B cells (202). Interestingly, *S. aureus* blocked complement-induced interactions with B cells via regulation of an unknown virulence factor through the gene regulatory system SaeR/S.

Unsurprisingly, there are examples where bacteria subvert the bridge between complement and humoral immunity. *S. aureus* evades B cell immunity by secreting virulence factors that block interactions between C3 fragments and CR2 (203). A common evasion mechanism utilized by human pathogens is the secretion of proteases active against antibodies (204). For example, several species of *Clostridia* release proteases active against both IgA1 and IgA2 (205). *S. pneumoniae* produces IgA1 proteases in membrane and soluble forms, capable of proteolytically inactivating IgA1 (206). In addition, IdeS, the *S. pneumoniae* virulence factor that cleaves IgG, also cleaves BCRs, inhibiting signaling and memory cell activation (207, 208). *L. monocytogenes* infection induces production of IL-10-producing B cells that inhibit bacterial clearance (209). *F. tularensis* actively induces its own uptake into murine B cells through BCR, CR1, and CR2 cross-linking (210). While connections between complement and adaptive immunity are another mechanism of host protection, they also serve as a target for bacteria.

REFRAMING SCIENTIFIC PARADIGMS AND THERAPEUTIC APPROACHES TO ENCOMPASS COMPLEMENT

Recent discoveries regarding complement activities during homeostasis and pathogen control have provided clear evidence of the many ways in which nonlytic complement is essential. Multiple electron microscopy studies have elucidated the structures of complement complexes and components required for activation. Notably, the mechanism by which MAC pores induce lysis of Gram-negative double membranes was at long last elucidated (4, 52). C5 convertases direct local assembly of MACs on the surface of the outer membrane, resulting in disruption of both lipid bilayers via MAC alteration of membrane bending energetics. These novel findings provoke lingering questions that either (i) MACs on the inner bacterial membranes are smaller or differ in composition to those on the outer membrane, (ii) MAC-mediated disruption of outer membrane bending forces is sufficient to destabilize the inner membrane, or (iii) outer membrane pores allow access of peptidoglycan-degrading enzymes such as lysozyme. The last option was a leading theory by which inner membranes were thought to be

damaged. Heesterbeek et al. demonstrated inner membrane damage occurred independently of lysozyme; however, other enzymes could be involved (52).

Identification of CRIg as a direct receptor for lipoteichoic acid, a major component of Gram-positive bacterial walls, revealed new ways in which complement can act as a pattern recognition receptor to promote phagocytosis and rapid clearance of circulating pathogens by liver Kupffer cells (94). Complement subversion by pathogens is a long-appreciated evasion mechanism, with CD46 even being designated a "pathogen magnet" for its propensity to be coopted by multiple clinically relevant bacteria and viruses (228). While ubiquitously expressed on the surface of nucleated human cells, CD46 is exclusively expressed in murine spermatozoa and ocular tissue, perhaps lost to avoid the fate of its human counterpart (211). New World primates express a modified CD46 to evade viral infection, and it is unclear why human evolution did not follow suit. However, CD46 induction of autophagy machinery in response to measles virus and *S. pyogenes* infection suggests that coupling CD46 to xenophagy may be an alternative strategy to counter pathogen subversion (212).

Recent studies have provided novel appreciation for the role of complement in directing autophagy as a mechanism of bacterial restriction in response to *L. monocytogenes* infection, as well as the ways by which other notable pathogens like *Shigella* and *Salmonella* evade complement-autophagy synergy (74). Interestingly, *Listeria* infection is also controlled by autophagy in the model organism *Drosophila melanogaster* (213). A complement ortholog in *D. melanogaster* was recently described as a regulator of autophagy in neighboring cells (214); thus, complement-mediated autophagy could be a highly conserved defense strategy that mediates antibacterial immunity (215).

The relationships between complement, neutrophils, and coagulation pathways have received significant attention of late (138). Both neutrophils and platelets serve as a source for complement components (140, 175), and complement activation products recruit and activate neutrophils and platelets during hemostasis and infection (14, 174). Notably, C5a was identified as a metabolic master switch for neutrophils by regulating glycolytic flux through pH modifications (216). The cooperative interactions between these pathways is a target for GAS, which cleverly induces a complex made up of antibodies, complement, and fibrinogen, resulting in platelet aggregation and consumption (181).

The paradigm shifting discovery of intracellular stores and *de novo* complement generation provided a significant new avenue of research (16). Intracellular complement, dubbed the "complosome," is tissue and cell type specific with unique functions based on expression (217). Discovery of a C3(H_2O) shuttling cycle revealed novel ways in which complement is stored and processed intracellularly with consequences for cytokine regulation (15). Intracellular complement is now known to be a vital contributor to adaptive immune physiology including development, homeostasis, survival, activation, proliferation, and contraction (22). Given the vital importance of intracellular complement on T cell physiology, the complosome will likely be of similar significance in B cell function. C5a functions as a key rheostat of inflammasome activation in circulating monocytes and tissue resident macrophages, promoting the sentinel capacity of the former while limiting bystander damage of the latter (160). The efficacy of complement in plasma provokes speculation that intracellular complement may be a particular threat to pathogens who rely on survival and replication within host cells (17). Future studies will reveal the specific importance of complement to intracellular antibacterial immunity.

Nonlytic complement functions are the target or basis of several FDA-approved therapies or agents undergoing clinical trials for cancers or autoimmune diseases (128, 218). However, the complexity of pathogen complement evasion mechanisms may have dissuaded exploration of complement-modulating therapeutics for infectious disease indications. Nevertheless, experimental models highlight potential utility of such an approach. C5 inhibition in a nonhuman primate *E. coli*-induced sepsis model dramatically reduced morbidity and mortality relative to untreated animals (177). Indeed,

a recent report described a pediatric patient with sepsis-induced multiorgan failure that was successfully treated with a multipronged therapeutic strategy that included anti-C5 therapy (178). *N. gonorrhoeae*, causative agent of the sexually transmitted infection gonorrhea, evades complement by binding C4BP. Preclinical data revealed that chimeric C4BP-IgM hexamers efficiently outcompetes bacterial C4BP binding and enhances complement-mediated killing *in vitro* and in murine models of vaginal colonization (219). Similar studies have utilized other complement-antibody chimeras, including a FH-IgG chimera that protected against GAS-induced sepsis (220). A nontraditional approach exploited bacteriophage therapy synergy with complement to kill serum-resistant strains of *P. aeruginosa* in a murine model of acute pneumonia (221). Interestingly, some groups have begun to leverage bacterial molecular inhibitors of complement as anti-inflammatory therapeutics (222).

Despite these examples of antibacterial complement-targeted therapeutics, discovery of novel therapies and complement activities is hampered by current testing and scientific paradigms of using conditions in which complement is inactive or excluded. For instance, standard antibiotic susceptibility testing (AST) that determines antibiotic potency (i.e., MIC) is performed exclusively in bacteriological media, which does not mimic *in vivo* physiological conditions in the patient, nor takes into account serum complement (223, 224). This absence facilitates missed therapeutic developments. For example, certain antibiotics deemed ineffective in standard ASTs in fact sensitize multidrug-resistant pathogens to serum or whole blood killing, such as sub-MICs of nafcillin versus MRSA (225). In addition, serum complement assembly of MAC pores was recently recognized to permeabilize the outer membranes of important Gram-negative pathogens, allowing entry and bactericidal inner membrane targeting by vancomycin, a drug thought only to have Gram-positive activity by standard AST (226).

Furthermore, the absence of active serum in fundamental laboratory investigations has missed opportunities to accrue new knowledge on the role of complement in directing immunity and host-pathogen interactions. For example, Schlesinger and others found that C3-opsonized *F. tularensis* hijacks CR3 signaling to limit inflammasome priming and activation (162). A previous study by the authors found that *F. tularensis* induced inflammasome priming under conditions that utilized heat-inactivated bovine serum and overlooked opsonization status (227). Thus, reductionist approaches to basic immunology that omit complement run the risk of excessive simplicity and may fail to identify important physiological processes by both host and pathogen. Classical studies involving circulating immune cells repeated in the presence of intact serum may uncover novel roles for complement in previously established immune pathways.

CONCLUSIONS

Complement is an ancient first line of defense that protects against infection. Canonical activation of three proteolytic cascades converge on the formation of a lytic pore on the offensive surface. While the bactericidal activity of the complement MAC is crucial for host immunity, complement offers multifaceted protection against both Gram-negative and -positive bacteria through its lesser-known functions. Opsonization by complement particles is essential for phagocytosis and destruction of foreign microbes and apoptotic cells. Complement synergizes in a reciprocal manner with neutrophils and coagulation pathways, amplifying clot formation, bacterial killing, and NETosis. Recently discovered intracellular stores of complement play important roles in homeostasis, tolerogenic immunity, and pathogen detection. Tonic complement signaling directs adaptive cell physiology during both health and disease. These nontraditional roles of complement represent opportunities for clinical therapies, including antibiotic agents, that leverage the ability of complement to synergize with the rest of the innate and adaptive immune systems. Future immunological research must include complement in standard assays; drug discovery programs and next-generation AST platforms should be envisioned to consider drug action with active serum present. In

that manner, decades groundbreaking research can be properly leveraged to exploit the full potential of complement to defend the human body.

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