Staphylococcus aureus and innate immunity

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Abstract

Sepsis is an old and lethal disease caused by immune dysfunction in response to infection. The innate immune system is the first responder to infection and the first to dysfunction. It comprises both humoral and cellular components such as the complement, coagulation and fibrinolytic cascade systems as well as the polymorphonuclear neutrophils and monocytes. These usually recognize and respond to pathogens in an orderly fashion, leading to resolution of infection and restored homeostasis. However, in sepsis the response is first exaggerated and later reduced or even non-existent. As of today, patients suffering from sepsis only receive antibiotic and supportive treatment.

The Gram-positive bacterium *Staphylococcus aureus* is a frequent cause of infection and sepsis. It is well known for its wide antibiotic resistance and ability to survive within humans. Several membrane-bound and secreted proteins promote staphylococcal infection by inactivating complement, surviving within phagocytes and exploiting coagulation to disseminate with the host. However, an overview of the mechanisms and virulence factors involved is currently not found.

This review therefore covers documented interactions between *S. aureus* and the complement system, the coagulation and fibrinolytic systems as well as neutrophils and monocytes in infection and sepsis following a brief introduction to each topic. The aim is to give both the reader and the writer an overview of the current knowledge and ongoing research.

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Sepsis - an introduction

Sepsis is as fascinating as it is old. Its Greek translation reads the "decomposition of animal, or vegetable or organic matter in the presence of bacteria", described by Homer over 2700 years ago as a derivative of "I rot" (1). Today, by definition, sepsis designates a collection of clinical findings on the basis of confirmed infectious origin; a temperature above 38 or below 36 degrees Celsius, a heart rate greater than 90 beats per minute, a respiratory rate greater than 20 breaths per minute (or an arterial carbon dioxide concentration, $PaCO_2$, less than 4.3 kPa), and a white blood cell count either greater than 12×10^9 or less than 4×10^9 cells per mL (2). These are the criteria of the systemic inflammatory response syndrome (SIRS), arising in both sterile and infectious inflammation, but sepsis is only true for the latter, when two or more of the SIRS criteria are met as well.

Importantly, the consensus conference where these criteria were defined marked a turning point in our understanding of sepsis - it is no longer caused by the infectious agent, but rather by the immune system's exaggerated response to it. Since then, this response has been studied thoroughly but is still difficult to fully comprehend. The late Roger C. Bone summarized the septic pathogenesis as "a cascade that is initiated by a focus of infection or injury and ends with severe endothelial damage, profound hemodynamic derangements and, often, death" (3). Indeed, if left unchallenged, sepsis progresses to severe sepsis with organ dysfunction, hypoperfusion and/or hypotension, and ultimately, it progresses to septic shock - a state of refractory hypotension demanding aggressive supportive therapy - multiple organ failure and death (2). Over the last years however, the idea of a singularly exaggerated immune response has been questioned. Partly, because the promising results of immunomodulatory therapy in allegedly flawed animal models failed to apply in the human setting (4, 5), and partly because the pathogenesis of sepsis and septic shock turned out to be both pro- and anti-inflammatory (6). Nonetheless, the hypothesis remains. The infective agent merely topples the first domino. This process is also extensively reviewed elsewhere (7, 8)

The regulated inflammation of infection is primarily a physiological and protective process meant to restore homeostasis (9). It basically revolves around the three R's - *r*ecognition, *r*esponse and *r*esolution: The immune system, recognizes the infective

agent. It then initiates a proportional, well-orchestrated response aimed at destroying the agent with minimal collateral tissue damage. Upon clearance of both the infective agent and the activated immune components homeostasis is restored. In sepsis, however, the response is disproportional.

At first, bacterial endotoxin or lipopolysaccharide (LPS) of the Gram-negative cell wall was the main suspect. LPS was therefore used in several animal models. Circulating and membrane-bound receptors on the cells of the immune system, notably polymorphonuclear neutrophils (PMNs), recognize LPS and transcript and release cytokines, the "hormones" of inflammation (7, 10). The use of LPS alone, however, proved too simplistic. First because bacteria stripped of LPS could initiate similar effects, albeit at higher concentrations (11). Second and more important, because at least half of all cases of sepsis are caused by Gram-positive bacteria (12) lacking LPS altogether. Even so, the immune system does initiate a harmful response to infection in sepsis. It starts with innate immune recognition of the infective agent's pathogen associated molecular patterns (PAMPs). Also the damage associated molecular patterns (DAMPs) exposed by the infective agent's virulence factors. e.g. exposed intracellular proteins in response to pore-forming toxins – could be recognized. Most commonly, the infection originates from either pneumonia or an intra-abdominal or urinary tract infection (13). Together with leukocyte activation, the activation of the innate immune system includes activation of the plasma proteolytic cascade systems: The complement system, the coagulation system, the fibrinolytic system and the kallikrein-kinin or contact system (14-18).

The innate immune system - as any biological system maintaining homeostasis - consists of afferent or sensing components that register disturbances (here pathogens). The effectors or efferent components mount a response aimed at resolving these disturbances (19). The afferent and efferent components consist of both soluble and cellular components. This division is a more common albeit simplified way of portraying the immune system (20). The soluble components include the plasma cascade systems as well as the naturally occurring antibodies and the pentraxins. The cellular components are activated through their pattern recognition receptors (PRRs) in contact with conserved structures of pathogen or damaged self (Fig. 1)

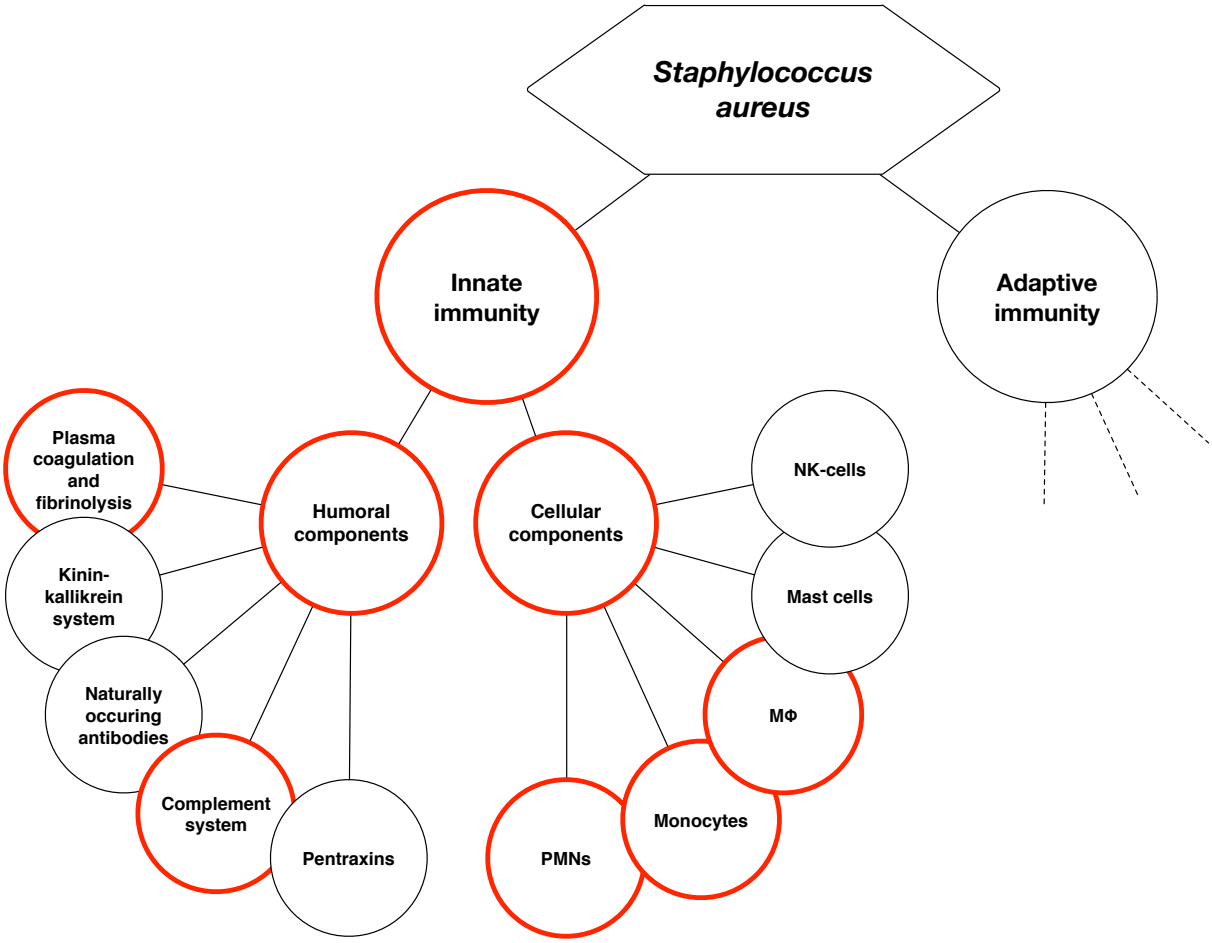

Figure 1: Overview of innate immune components involved in *S. aureus* infection. Topics covered in this review are circled in red.

The aim of this review is therefore to explore the current literature on how some of these key components function and dysfunction in response to the prime Gram-positive pathogen, *Staphylococcus aureus* - the most frequently isolated Gram-positive bacterium in human sepsis and an expert in immune evasion and exploitation. Whereas the introductory paragraphs cites existing solid reviews, the passages concerning staphylococcal interaction with the innate immune system comprises all relevant papers found using the medical subject headings (MeSH). In brief, "Staphylococcus aureus" was combined with the different subjects shown in figure 1. The results were then sorted into different categories that later made up the subheadings to the chapters of this Review. The articles were collected from August 2013 through March 2014, the first draft was written in April 2014 and final revisions were made in May 2014. In addition to professor and supervisor Tom Eirik Mollnes, professor Erik Waage Nielsen also contributed to the final draft.

Staphylococcus aureus

Microbiology

Staphylococcus aureus is a Gram-positive spherical bacterium growing in what resembles golden grape clusters on blood agar, hence the name (21). The bacterium is non-motile, but can grow both aerobically and anaerobically in temperatures from 18 to 40 degrees Celsius. It normally colonizes the anterior nares and perineum of humans (22). It is a feared pathogen because of its increasingly wide antibiotic resistance and coincidental ability to cause in-hospital infections. These range from mere boils to lethal septic shock (23). Structurally, *S. aureus* consists of a thick cellular wall of cross-linked peptidoglycan traversed by lipoteichoic acids (LTAs), covered by a polysaccharide capsule. As in most Gram-positive bacteria, half the cell wall weight consists of peptidoglycan. Eleven different capsular serotypes have been discovered, but serotypes 5 and 8 are the most frequently encountered in human infection (24). The capsule is covered in biofilm, a loose water-soluble slime layer. Both the capsule and slime layer are permeated by a variety of surface proteins collectively termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). These facilitate bacterial adhesion to host tissue (21).

Staphylococcus aureus infections

Staphylococcal infection is a dynamic process. Most of the MSCRAMMs are expressed during the exponential growth phase. During this phase the bacteria aim at colonizing and invading host tissue (23). Once colonization is achieved, the bacterium enters a stationary phase where global regulatory genes switch to increased expression of virulence factors. These facilitate spread within host and immune evasion (25). Staphylococci usually infect the host through small breaches of the body's outermost defense - the skin. They gain access to underlying tissue and the blood stream through wounds, invasive surgery or endovascular catheters. At this point the bacteria are recognized and responded to - confer the three R's in the introduction - entailing a brisk inflammatory response. Toxins also damage and expose endogenous DAMPs which further potentiates this response.

Although beyond the scope of this review, *Staphylococcus aureus* quickly adapts to its surroundings. The different mechanisms of antibiotic resistance obviously provide an

advantage to survival, especially in communities with liberal antibiotic use. For further reading, several reviews on the topic are available (26-28).

Epidemiology

Staphylococcus aureus and especially methicillin-resistant *Staphylococcus aureus* (MRSA) is the leading cause of skin and soft tissue infections in the United States (29). Not surprisingly, *S. aureus* is also a leading cause of invasive infection and sepsis worldwide (30-33). Incidence rates vary, but 30 cases per 100 000 and a 20 per cent mortality seems a fair estimate (34-36). Needless to say, it is a serious and frequently encountered pathogen.

The complement system

Introduction

The complement system is a group of over 30 different circulating and membrane-bound proteins rapidly activated in the vicinity of PAMPs and DAMPs. Next complement also activates the adaptive immune system (37). In the beginning of complement system research, the system was held to be "just an elegant model system" (38). As this research has progressed, evidence suggests it an important system in immune surveillance and therefore homeostasis - It has several other functions beside that of antimicrobial defense (39). There are nearly as many regulators in this system as there are effectors, emphasizing that the system is highly potent and must be restrained.

The complement system has three different pathways of activation - the classical pathway (CP), lectin pathway (LP) and alternative pathway (AP). They all merge at the formation of a C3 convertase (Fig. 2). The classical pathway, also known as the antibody-dependent pathway, is activated by IgM or IgG clusters - hence the name, but also by other pattern recognition molecules such as the pentraxins. After C1q binds, the two pairs of serine proteases C1r and C1s activate each other reciprocally and the pentamer C1 splits. C1s further cleaves C4 into C4a and C4b, the latter opsonizes the cell or bacterium in question. C1s also cleaves C4-bound C2 into C2a and C2b in formation of the classical and lectin pathway C3 convertase, C4b2b.

The LP is similar to the CP ending in formation of the C4b2b. However, the initiating danger signal and recognition molecules differ. Mannose-binding lectin (MBL) and

ficolins recognize mannose sugars on bacteria and IgA. Upon this activation, MBL and ficolins associate with the MBL-associated serine proteases (MASPs), notably MASP-2 which then cleaves C4 and C2 (40).

The alternative pathway represents up to 80-90% of total complement activation (41). The pathway is practically activated immediately as C3b is deposited on bacteria, foreign or apoptotic cells owing to the constant tick-over of C3; A small fraction of C3 is hydrolyzed to $C3_{H2O}$ which then binds factor B (FB). FB is subsequently cleaved by factor D (FD) forming the C3 convertase, $C3_{H2O}Bb$ in plasma. This generates C3b with a thioester moiety that binds amines and carbohydrates on foreign surfaces (39). An initially modest tagging is then greatly amplified on foreign cells and inhibited on cells of self by factor H (FH). Membrane-bound C3b associates with FB which is then cleaved by FD and the convertase further stabilized by properdin (FP), generating the AP C3 convertase, C3bBbP which then activates more C3 for greater opsonization and downstream complement activation.

As soon as AP amplification generates sufficient C3b the C3 convertases also incorporate C3 (C4b2b3b or C3bBb3b) and shift their selectivity towards C5. C5 is cleaved to C5a and b. C5b can then bind C6 through C8 and several molecules of C9. This forms C5b-9 also known as the membrane attack complex (MAC), which is able to punch holes in lipid membranes and lyse microbes. Soluble C5b-9 (sC5b-9) is also formed, and the two forms of C5b-9 are collectively termed the terminal complement complex (TCC).

Complement in sepsis

The smaller fragments of complement activation, particularly C3a and C5a have important functions in infection and inflammation (42). They are known as the anaphylatoxins because of their ability to induce smooth muscle contraction and capillary leakage. C5a is the more potent of the two. Through its two known receptors, C5aR1 and C5aR2, it functions as a powerful chemoattractant, activates phagocytic cells, and induces the release of histamine, granule-based enzymes and oxidants. C5a also activates the coagulation system and impedes vasomotor control (43).

The complement system is dysregulated in sepsis and detectable anaphylatoxins and TCC are proposed markers of complement hyperactivation (14). In this regard, C5a is suggested the primary cause of the complement-mediated effects: On the one hand high

levels of C5a shut down many essential functions of the neutrophil such as chemotaxis and oxidative burst. On the other hand C5a hyperactivates macrophages leading to increased cytokine release (15, 44). C5a also increases cytokine release from endothelial cells alongside increased tissue factor (TF) expression. Lastly, high levels of C5a induce thymocyte apoptosis and may contribute to the immunosuppression observed in late-stage sepsis.

Not surprisingly therefore, complement activation is often presented as a double-edged sword. An exaggerated heave harms the attacker just as much as the foe.

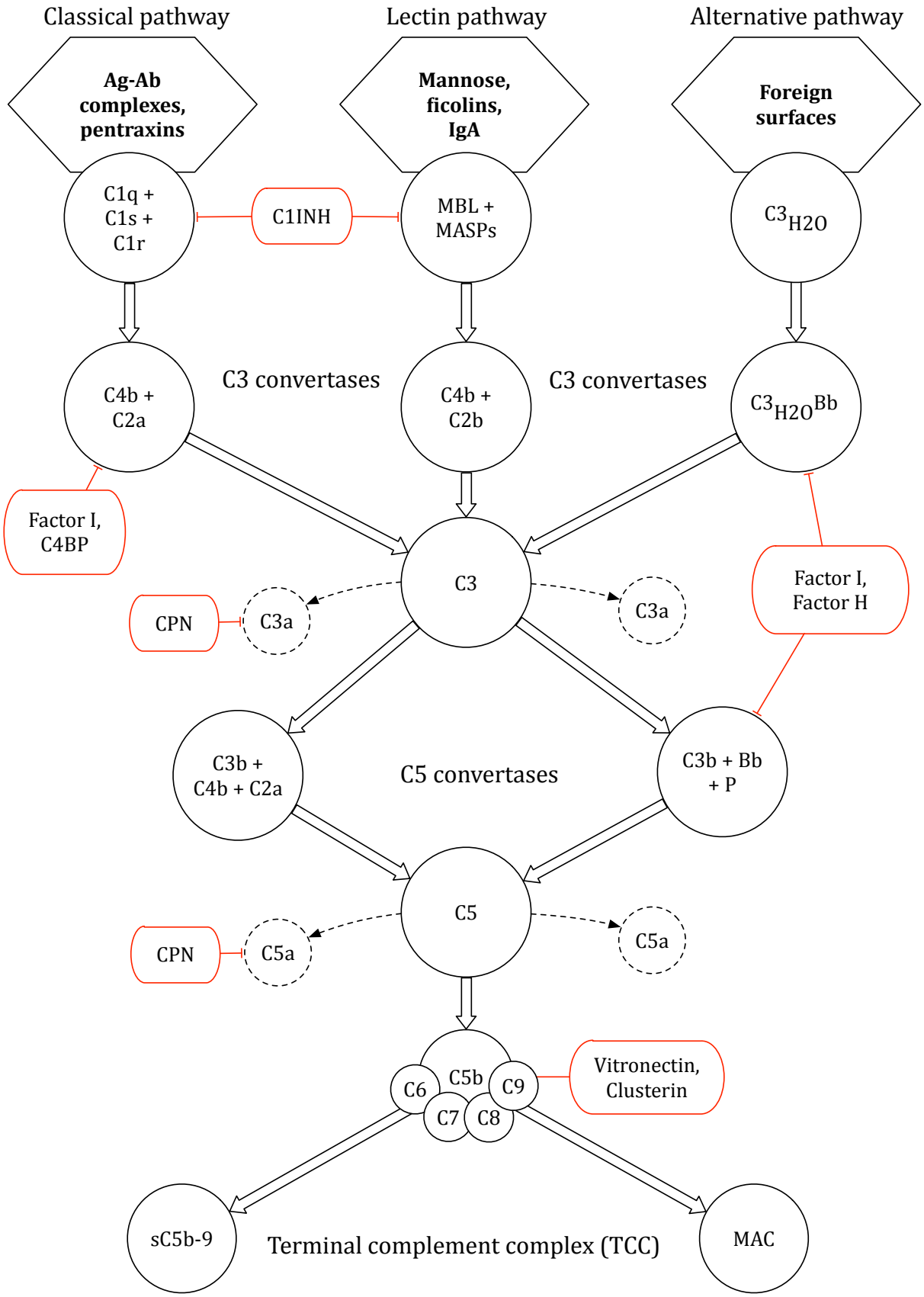

Figure 2: Overview of the complement system including the most common physiological inhibitors in red. CPN = Carboxypetidase N, C4BP = C4-binding protein.

The complement system and *Staphylococcus aureus*

Staphylococcal interaction with the complement system is well studied over the last decades. Obviously, the first studies examined the role of complement in opsonophagocytosis. Later studies are discovering ever-more intricate immune evasive strategies of *S. aureus*.

Opsonization, phagocytosis and intracellular killing are essential steps in the process of eradicating *Staphylococcus aureus*. Initial *in vitro* studies identified complement C2 as an essential factor of this process indicating CP and LP activation (45). Expressed staphylococcal protein A (SpA) was shown to interfere with this activation by binding the Fc-portion of otherwise specific immunoglobulin G (IgG) thereby preventing phagocytosis. However, in absence of IgG, complement activation, opsonization and phagocytosis increased (46). Another unique study in C5 deficient serum found no significant importance of C5 in opsonization and phagocytosis, indicating that other opsonins are just as imporant in response to *S. aureus* (47). Peptidoglycan of the *S. aureus* cell wall, but not teichoic acid, was proven the main activator and target of complement opsonins. However, the opsonins were less efficient at triggering an innate immune response to encapsulated *S. aureus* strains. C3 was found deposited underneath the capsule, hiding both peptidoglycan and C3 from the complement receptors (48-52). The importance of both CP and AP activation in response to Staphylococcal peptidoglycan were then confirmed. The other major cellular components teichoic acid, lipoteichoic acid and protein A only activated complement via the CP (53-54). Complement activation, opsonization and phagocytosis were shown to increase further with the use of some antibiotics. These interfered with staphylococcal protein synthesis at subinhibitory concentrations (here clindamycin and doxycyclin). Antibiotics affecting the cell wall (penicillin, cefotiam, piperacillin and vancomycin) did not help (55). Additional studies showed a rapid degradation of C3b by factor I on *S. aureus* leaving mere 17% in the C3b state to continue AP activation (56).

The importance of complement activation in response to clinically relevant *S. aureus* strains producing the capsule serotypes 5 and 8 was also confirmed in a murine model. Sixty-four percent of the C3 depleted mice succumbed to a challenge of 10^7 CFU *S. aureus*, compared to 8% in the control group. Additionally, when the bacteria were in their mid-

logarithmic phase, they bound 10% as much C3 compared to the stationary growth phase, thus suggesting a direct relationship between capsule production and complement evasion (57). These findings were reproduced by the same research group, identifying C3 and the complement receptor 1 (CR1, CD35), but not C5 as essential in the defense against *S. aureus* (58). However, in a more recent study C5a is proposed to have a protective role. The C5-knockout mouse strain showed significantly reduced survival during staphylococcal bacteremia (59).

Although initially thought redundant in response to *S. aureus*, the LP of complement was later found to increase C4b and iC3b deposition if MBL-deficient sera were reconstituted with purified MBL-MASP (60). Furthermore, ficolin 2 of the LP was shown to recognize lipoteichoic acid of *S. aureus* - among other Gram-positive species - and activate complement (61). However, MBL-deficiency is common, and an interesting bypass of LP activation through specific serum anti-IgG recognizing IgG bound to wall teichoic acid (WTA) was recently described (62). Thus, all complement pathways are involved in the innate immune response towards *S. aureus*.

Staphylococcal components targeting complement

Willem and colleagues discovered several pathogenicity islands located on β-hemolysin-converting bacteriophages that exchange important virulence factors between staphylococci. These included the chemotaxis inhibitory protein of S. aureus (CHIPS), staphylokinase (SAK), staphylococcal enterotoxin A (SEA) as well as the staphylococcal complement inhibitor (SCIN), which all interfere with innate immune functions on different levels (63). Accordingly, they termed the pathogenicity islands innate immune evasion clusters (IECs). From these clusters, expressed SAK recruits and activates plasminogen to plasmin on the bacterial surface where it then degrades human immunoglobulin and C3b (64). SCIN, on the other hand, highly human specific and found in 90% of all *S. aureus* strains, binds, stabilizes, dimerizes and catalytically inactivates the membrane-bound C3 convertases, thus inhibiting the main reactions of complement activation (65, 66). Other relevant virulence factors located on staphylococcal pathogenicity islands include 14 staphylococcal superantigen-like proteins (SSLs). The seventh SSLs bind and inhibit C5 activation (67) - a feat potentiated by also binding IgA (68). The tenth SSLs bind IgG preventing CP activation (69).

Additional immune evasive virulence factors include the C3 binding extracellular fibrinogen-binding protein (Efb) (70), the collagen-binding MSCRAMMs, notably Cna, as well as the SCIN homologues SCIN-B and -C and the Efb homologue, extracellular complement-binding protein (Ecb) (71). Cna and its related molecules are shown to bind C1q, potentially interfering with CP activation (72). Staphylococcal Ecb is shown to bind both C3 and fibrinogen on its C and N termini, respectively, disrupting further phagocytosis by neutrophils Obviously there is a bridge between the complement and coagulation system in innate immune evasion (73).

Clumping factor A (ClfA), also involved with staphylococcal interaction with the coagulation system (covered later), is shown to bind factor I and accelerate factor I-mediated decay of C3b (74, 75). Secreted *Staphylococcus aureus* binder of IgG (Sbi) forms a complex with factor H and C3b particles rendering the factor H moiety intact to dampen complement activation together with factor I (76). Similarly, staphylococcal iron-regulated surface determinant protein (IsdH), expressed in milieus of low iron concentrations, is suggested to reduce phagocytosis by converting C3b to iC3b (77). Also, *S. aureus* is shown to recruit functionally active factor H to its surface to inhibit AP activation and accelerate C3b inactivation (78). *Staphylococcus aureus* surface protein (SdrE) is the proposed binding site for both factor H (79) and C4BP (80) - thereby interfering with all three pathways of complement activation, whereas Ecb has been shown to increase this factor H acquisition to the bacterial surface (81). In this same study factor H and Ecb are shown to mutually increase their C3b binding and ability to inactivate complement activity.

Equally intriguing, secreted staphylococcal proteases are shown to inactivate complement. The metalloprotease aureolysin cleaves C3 into active C3a and C3b but also recruits factors H and I ultimately resulting in quick C3 inactivation (82). Another study confirmed the effect of aureolysin, but also described anti-complement activity of three other staphylococcal proteases: the staphopains A and B and the serine protease V8. They inhibit complement activation in general, and the LP in particular (83). Interestingly, the authors also describe direct C5 cleavage to active C5a by the proteases, especially by aureolysin. However, they also indicate that this C5a is quickly degraded further, minimizing actual complement activation.

Thus, the list of interactions between staphylococci and the complement system seems long and ever changing. Figure 3 provides a brief summary as of today. Perhaps surprisingly, reports on the clinical relevance of the different complement interactions are scarce. Although the murine models of staphylococcal bacteremia suggest complement as an important defense mechanism, these findings will have to be further evaluated in more complex animal models such as porcine models of sepsis. The first observational study of *S. aureus* sepsis including complement analyses detected ambiguous amounts of sC5b-9 in the patient samples, although this potentially should reflect complement activation through all three pathways, no significant relationship with patient outcome was found (84).

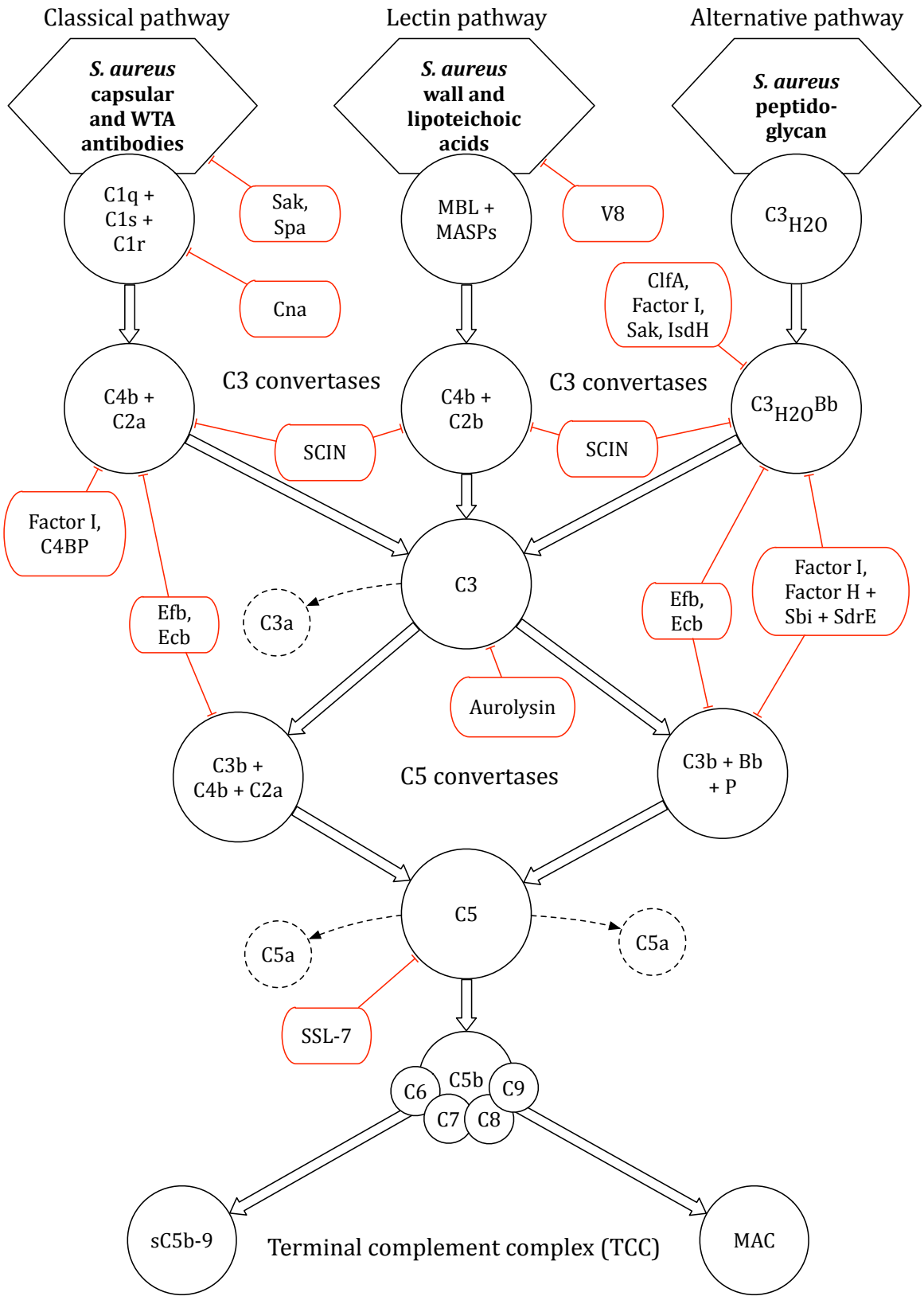

Figure 3: *S. aureus* versus the complement system. Staphylococcal virulence factors are indicated in red whereas the physiological inhibitors are left out of this figure. SpA = Staphylococcal protein A, Sak = Staphylokinase, ClfA = Clumping factor A, IsdH = Iron-regulated surface determinant protein, SCIN = Staphylococcal complement inhibitor, Sbi = Secreted *Staphylococcus aureus* binder of IgG, SdrE = *Staphylococcus aureus* surface protein, Efb = Extracellular fibrinogen-binding protein, Ecb = Extracellular complement-binding protein, SSL-7 = Staphylococcal superantigen-like protein 7.

The polymorphonuclear leukocytes and macrophages

Together with the complement system, the leukocytes serve as the first line of defense against invasive bacterial disease. The polymorphonuclear neutrophils (PMNs), monocytes and macrophages will be discussed in this review.

Polymorphonuclear neutrophils

Neutrophils are well-reviewed, indispensable effectors of acute inflammation (85-87). They are the most abundant immune cell type, produced and stored in the bone marrow, albeit with a continuous release to the circulation. There they readily await recruitment to areas of inflammation through the fascinating process of tethering, rolling, adhesion, crawling and transmigration (88), also beautifully illustrated in the first figure of ref. 87. This process is enabled by a multitude of chemoattractants, chemokines and homing signals expressed on endothelium in response to infection and inflammation. Histamine, arachidonic-acid metabolites and diverse cytokines induce the expression of selectins (types P and E, in particular) and integrins (such as the intracellular adhesion molecules, ICAMs and the vascular cell adhesion proteins, VCAMs). These bind neutrophils to the lumen surface. Simultaneously, the neutrophils are activated in two-step process by exposure to pro-inflammatory cytokines such as TNF and interleukin (IL-) 1-β and recognition of PAMPs, chemoattractants - particularly IL-8 - or growth factors. Neutrophil activation through G-coupled chemokine receptors induces conformational changes in expressed integrin receptors, including CD11a-CD18 and CD11b-CD18 thereby facilitating neutrophil adhesion to the endothelium. When at the endothelial surface, bound neutrophils crawl and search for the optimal portal of entry to the peripheral tissues. The actual transmigration or *diapedisis* is either paracellular or transcellular, and enabled through interaction with the integrins.

Once the neutrophils have entered the peripheral tissues, they migrate along a chemokine trail to the focus of inflammation. The chemoattractants are either "intermediate" or "end-target" meaning that the intermediate chemokines such as IL-8 and leukotriene B4 (LTB4) have less effect on chemotaxis than the end-stage chemokines, such as C5a and bacteria-derived *N*-fomyl-methionyl-leucil-phenylalanine (fMLP), which are in abundance close to the focus of inflammation.

Activated neutrophils have several potent mechanisms to fight and kill pathogens. Opsonization, as described earlier, enables phagocytosis of cells and organisms with PAMPs or DAMPs through the activation of complement- or immunoglobulin-receptors. The incorporated phagosome is then brutally flooded with either reactive oxygen species (ROS) or antibacterial proteins such as cathepsins, defensins, lactoferrin and lysozyme as the granules containing these effectors fuse with the phagosome (89). The granules containing antibacterial proteins can also be expelled to attack extracellular pathogens. Additionally, neutrophils have recently been shown to degrade their DNA and incorporate it with histones, proteins (lactoferrin and cathepsin to name a few) and enzymes (such as myeloperoxidase (MPO) and elastase) for release to the extracellular milieu as neutrophil extracellular traps (NETs) (90). As the name implies, these engulf and stop pathogen spread, facilitate phagocytosis and possibly kill the pathogen directly with the associated antimicrobial histones and proteases.

Importantly, activated neutrophils have the ability to recruit and activate more neutrophils in a positive-feedback fashion during infection and inflammation (86).

Monocytes and macrophages

Monocytes constitute another group of important innate immune cells. They have both intravascular effector functions of their own (91) and serve as myeloid precursors of the tissue-resident macrophages and dendritic cells, collectively termed MDPs (92). Based on their cell-surface expression of chemokine receptors, CD14 and CD16, monocytes are divided into two main subsets: $CD14^{high}CD16^{-}$ or $CD14^{low}CD16^{+}$, also known as $CD14^{+}$ and $CD16^{+}$ cells, respectively (93). The former represents 80-90% of circulating monocytes and exerts a dominantly anti-inflammatory cytokine profile dominated by IL-10 in response to LPS *in vitro*. The CD16+ cells on the other hand, accounting for about 10% of the circulating monocytes, express TNF and IL-1 in response to LPS and are accordingly termed pro-inflammatory. The number of CD16+ cells increase during infection (94).

Similar to neutrophils, monocytes are recruited by chemokines (95). CC-chemokine ligand 2 (CCL2) is expressed by a variety of cells activated by cytokines, PAMPs or DAMPs. CCL2 binds to the CC-chemokine receptor 2 (CCR2) expressed in high amounts on the $CD14^{+}$ monocytes. Other chemokines also home monocytes to the vessel wall, where they bind and transmigrate by aid of selectins and integrins. This way, depending

on the chemokines, monocytes replenish different tissue-resident macrophages, such as osteoclasts, alveolar macrophages and Kuppfer cells (96)

Circulating monocytes are competent phagocytes. They can destroy pathogens using phagolysosomal enzymes or through the release of reactive nitrogen and oxygen species (93). Additionally, pathogens recognized by monocytes induce cytokine responses to alert and activate other components of the innate immune system. Particularly, monocytes are shown to differentiate into TNF- and inducible nitric oxide syntethase (iNOS) producing (TIP) cells. These cells expel CC-chemokine ligands 2 and 7 (CCL2 and 7) necessary for sufficient monocyte recruitment in response to bacterial infection.

Toll-like receptors

Importantly, both neutrophils and monocytes express Toll-like receptors (TLRs) essential for especially PAMP, but also DAMP recognition (97, 98). These are the best-studied pattern recognition receptors or molecules (PRRs) of the innate immune system (99, 100). PRRs recognize a multitude of structures considered dangerous or foreign to the host. These structures include essential, conserved microbial structures (PAMPs), e.g. the lipid A-portion of LPS in Gram-negative bacteria and peptidoglycan of Gram-positive bacteria (101).

To date, 10 different human TLRs are known. These are transmembrane proteins of either the cell wall or intracellular compartments, characterized by their extracellular leucine-rich repeat (LRR) domains and intracellular Toll/IL-1 receptor (TIR) domains. TLR4 was the first receptor described and recognizes LPS whereas TLR2 is found to recognize a broad range of PAMPs through its association with either TLR1 or TLR6. Notably, several accessory molecules to the TLRs are described, such as CD14, essential to not only proper LPS recognition but cofactor to several other TLRs as well, such as those recognizing peptidoglycan and bacterial DNA (102).

TLR activation results in downstream signaling events culminating in increased expression of cytokines, chemokines, major histocompatibility complexes (MHCs) and co-stimulatory molecules as well as cell-specific activation such as increased ROS production and phagocytic activity in neutrophils. The MyD88-dependent pathway resulting in phosphorylation and subsequent activation of nuclear factor kappa B (NF-

kappa-B) is generally the most known. It is presumably the most important in the cellular innate immune response (103).

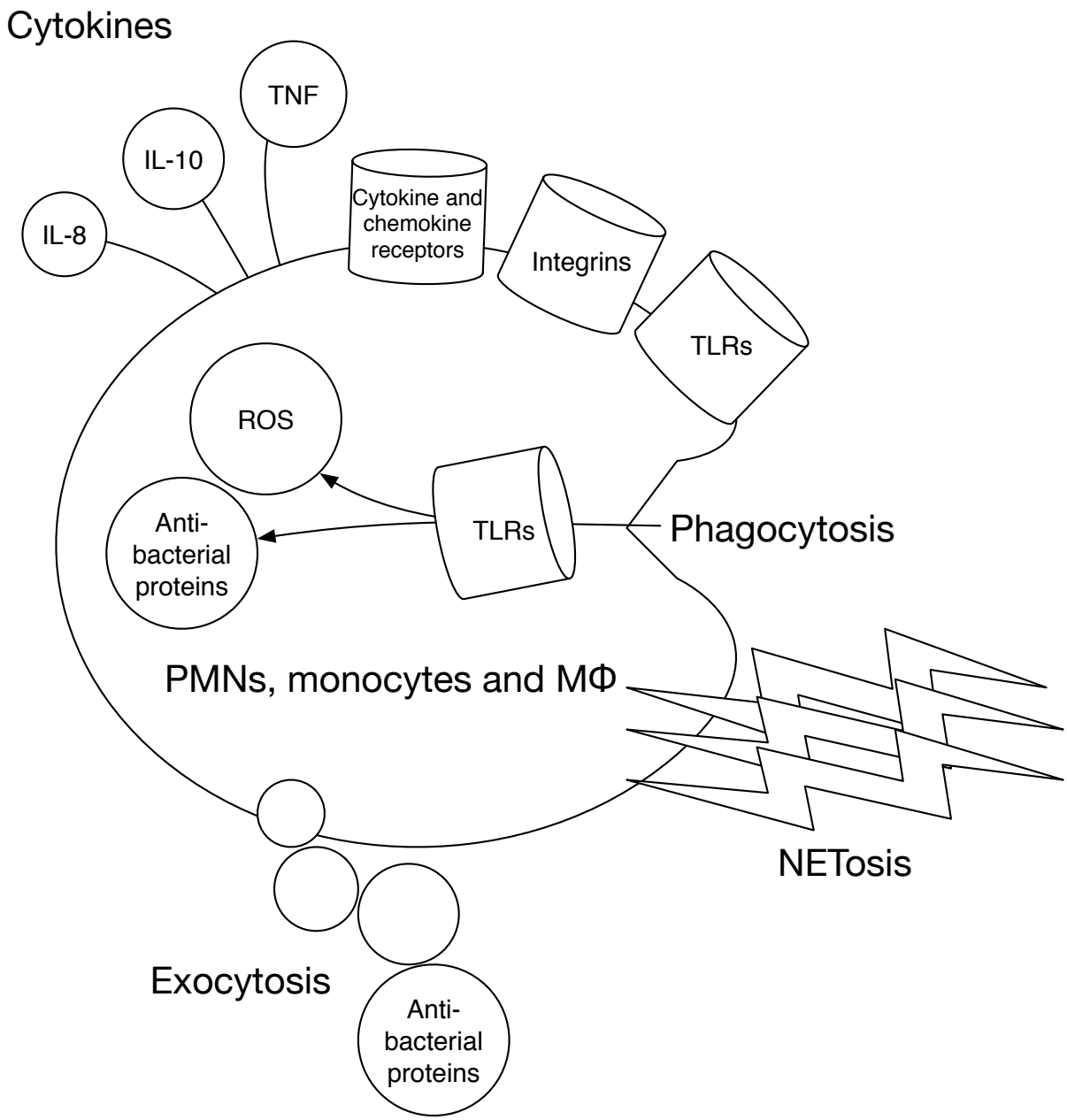

Figure 4: Overview of PMN, monocyte and macrophage effector functions.

PMNs, monocytes, TLRs and sepsis

In sepsis, neutrophil and monocyte function and recruitment is disrupted (104, 105). Higher levels of chemokines in plasma, compared to a single foci of infection, downregulate chemokine receptors. High levels of cytokines from various immune cells, at least in the early stages of sepsis, send ambiguous messages to the immune system.

Altered TLR signaling is an important cause of the altered leukocyte functions seen in sepsis (106). Interestingly, monocytes and neutrophils are shown to respond differently in sepsis according to the disease severity. Cells isolated in the early phases of disease are generally hyperresponsive to PAMPs and thus highly pro-inflammatory whereas cells isolated in the later stages of disease (severe sepsis and septic shock) were hyporesponsive and more anti-inflammatory in nature (107).

Staphylococcal interaction with PMNs and monocytes

There are numerous documented interactions between *Staphylococcus aureus* and the neutrophils, monocytes and their TLRs (as summarized in figure 5 at the end of this section). These include the opsonophagocytosis of S. aureus by leukocytes, the effects of various staphylococcal virulence factors on leukocytes as well as specific staphylococcal TLR interactions. Equally, several recent reports on NET formation in response to *S. aureus* have been published recently.

Opsonophagocytosis

As mentioned, opsonophagocytosis of *S. aureus* is the primary way of eradicating the pathogen. This process is initiated by opsonins such as the complement system, and completed by the phagocytes, neutrophils and monocytes. One early study concluded that neutrophils were better at phagocytosis of *S. aureus* than monocytes, and that neutrophils could ingest roughly 230 bacteria per cell, and kill close to 90% of these within the 20 minute time-frame (108). For monocytes, the corresponding numbers were 50 bacteria and 40%, respectively. The bacteria were, however, pre-opsonized in 5% serum, but a threshold for phagocytosis was documented. Further studies of bacterial uptake kinetics confirmed the threshold using flow cytometry. In general, leukocytes could maximally collect about 80 bacteria, eat 45 and kill 40 (109). The process of bacterial uptake was also confirmed accelerated by opsonins and an endothelial cell surface, in a later study, also showing that opsonization was unrelated to metabolic activity, oxidative burst and degranulation by phagocytes (110, 111). In fact, complement-opsonized *S. aureus* were shown to induce neutrophil aggregation or clumping, i.e. a sign of neutrophil hyperactivation (112). Increased CD66b was later identified as an important mediator of this neutrophil aggregation during sepsis (113).

Concerning phagocyte recruitment, formylated peptides were identified as crucial chemoattractants, but not the only ones as bacteria unable to produce the peptides still recruited neutrophils, albeit at a much lower rate (114). Phenol-soluble modulin (PSM), a staphylococcal exotoxin, was recently identified as a potent stimulator of formyl peptide receptor 2 on neutrophils (115). LTA (116), peptidoglycan (117) and staphylococcal lipase (118) are other proposed chemoattractants, but at least peptidoglycan recruits neutrophil through complement activation. Recently, perivascular macrophages were identified as an important source of neutrophil chemoattractants during staphylococcal skin infection, but that they consequently were a target for *S. aureus* α-toxin, which also lyses the macrophages and reduced neutrophil recruitment (119).

Even so, following ingestion of *S. aureus*, neutrophils release LTB4 (120). Thereby they recruit more neutrophils, and increase their expression of complement receptor 3 (CD11b-CD18) to further increase their capacity to ingest bacteria (121). Neutrophils then use ROS to kill the pathogen. NADPH oxidase and to a lesser extent myeloperoxidase (MPO) generates oxygen radicals, acting in concert for optimal effect (122). This generation of ROS also proved dependent on increased intracellular calcium (123). Lysosomal cathepsin G, a cationic antimicrobial protein possibly binding WTA was identified as a non-oxidative *S. aureus* bactericide, albeit at a limited 7.5 pH interval (124). Equally, phospholipase A2 contributes to staphylococcal degradation in concert with ROS generation (125). Furthermore, neutrophils are now known to secrete calprotectin chelating the micronutrients manganese and zinc to restrict staphylococcal growth within abscesses (126). The two trace elements were later proved essential in staphylococcal superoxide defense by interfering with the superoxide dismutase (127). Likewise, iron-depleted staphylococci were rendered more vulnerable to phagocytosis and killing (128) and microarray-studies of staphylococci suffering from oxidative stress were shown to upregulate genes related to iron and heme uptake, with decreased resistance to oxidation if they were stripped of these genes (129). In return, too much iron was also shown to increase ROS-mediated killing of staphylococci by monocytes (130).

However, killing of *S. aureus* turned out more laborious than with other bacteria, first reflected in increased energy expenditure by neutrophils after ingestion of *S. aureus*

(131). Later *S. aureus* was shown to survive inside neutrophils (132), and exploit them to spread within the host - a notion recently reviewed (133). In fact, *S. aureus* was first shown to promote neutrophil apoptosis (134), later defined as phagocytosis-induced cell death (PCID), a general neutrophil response to several ingested pathogens in the aim of resolution, but *S. aureus* it seems, has found a way to circumvent apoptosis, rather inducing necrosis and lysis of the neutrophil and ensuring its own escape and survival (135). Indeed, a large microarray of staphylococcal genes in contact with neutrophils revealed upregulation of several genes involved in immune evasion, protection from phagocytosis and enhanced virulence (136).

Staphylococcal virulence factors targeting neutrophils and monocytes

Staphylococcal α-toxin, initially identified as a hemolysin, was shown to prime neutrophils at low concentrations (10 hemolytic units), and damage them at higher concentrations (137). Compared to strains lacking toxin production, strains with α-toxin were shown to significantly increase neutrophil count and enhance virulence in a murine model of pneumonia (138). A similar priming effect was observed with TNF, but ingested staphylococci manage to downregulate TNF-receptors on neutrophils thereby reducing their bactericidal capacity (139). Staphylococcal δ-toxin was also shown to prime neutrophils in combination with either TNF or LPS, and induced TNF release and CR3 expression directly (140). Staphylococcal enterotoxins A and B were shown to reduce neutrophil apoptosis and increase phagocyte receptor Fc-gamma expression through T-cell and monocyte activation (141). Enterotoxin A was later identified to protect *S. aureus* from neutrophil killing (142). Equally, a protein in the staphylococcal supernate, later identified as CHIPS (143), managed to downregulate chemokine receptors for C5a and formylated peptides significantly reducing chemotaxis to these two chemokines, but not IL-8 (144). However, high levels of TNF during staphylococcal sepsis were suggested to downregulate IL-8 receptors as well (145). Staphylococcal β-hemolysin, also known as β-toxin, was shown to directly downregulate IL-8 production in endothelial cells (146). In the same regard, staphylococcal extracellular adherence protein (Eap) was shown to restrict neutrophil interaction with endothelium, thereby further limiting recruitment (147). This was also true for the staphylococcal superantigen-like protein 5 (SSL-5) binding P-selectin glycoprotein ligand 1 (PSGL-1). This prevents neutrophil interaction with P-selectin and consequently its rolling along

the endothelium during neutrophil recruitment (148). In fact, SSL-5 was shown to inhibit neutrophil activation by all chemokines and anaphylatoxins and thus a large portion of the chemoattractants. First, SSL-5 binds to the receptors of the various chemokine and anaphylatoxin receptors. Second, SSL-5 scavenges the surrounding milieu for the actual chemokines and anaphylatoxins themselves, and pin them to the neutrophil surface (149). Also, D-alanine modification of staphylococcal LTA and WTA yielded resistance to phospholipase A2-mediated degradation of *S. aureus* (150) and reduced staphylococcal virulence in mice if missing (151).

Yet another staphylococcal exotoxin was proposed as an inductor of neutrophil apoptosis through a p38-mitogen-activated-kinase mechanism (152). Staphylococcal serine protease staphopain B (Sspb) was identified as such a factor. Although apparently unrelated to the p38-pathway, this serine protease is proposed to cleave CD11b on the surface of neutrophils and monocytes inducing a form of apoptosis or necrosis (153). Interestingly, IgG protected the cells from SspB-induced cell death, but staphylococcal protein A (Spa), binding the Fc-fragment of IgG with high affinity, restored the detrimental effect of SspB. Two other important functions of Spa were elucidated. First, Spa was shown to activate the TNF-receptor and increase IL-8 secretion in pulmonary epithelium (154). Second, Spa was shown to exert additive action with Panton Valentine Leukocidin (PVL) in the pathogenesis of lethal necrotizing pneumonia (155). PVL is a pore-forming bi-component cytotoxic factor of neutrophils consisting of components F (lukF-PV) and S (lukS-PV) that insert into the plasma membrane and lyse cells, and especially human neutrophils (156). Spa and PVL were identified as major contributors to the lung injury in the necrotizing pneumonia through recruiting and lysing neutrophils, respectively (157). Additionally, staphopain A (Sspa) was shown to degrade pulmonary surfactant protein A, an alveolar collectin, thus potentially increasing pulmonary virulence further (158).

Staphylococcal neurotoxins

Notably, PVL is one of few virulence factors often expressed in community-associated MRSA strains (159). Following its discovery in disease, several other potential leukocyte cytotoxins (leukocidins) were studied. Phenol-soluble modulin alpha 3 (PSM alpha 3) turned out to cause neutrophil lysis *in vitro*, but its true potency was in its synergistic effect in combination with PVL (160). Furthermore, PSMs were shown to activate

neutrophils via the formyl peptide receptor 2 (FPR2) thereby increasing oxidative burst which inactivates PSMs. In this regard PSMs ultimately trigger their own inactivation. However, PSMs also render the neutrophils insensitive to other stimuli and prone to initiate apoptosis through another pathway not yet distinguished (161). The effect of particularly PSM-α peptides on neutrophil lysis following phagocytosis was confirmed in another study, showing that function of the PSM-α-operon alone was sufficient for increased neutrophil lysis and consequent staphylococcal survival (162). Yet another member of the pore-forming leukotoxin family, LukAB, was recently shown to specifically target CD11b of the integrin Mac-1/CR3 (163). As mentioned previously, CR3 is upregulated in response to *S. aureus* and the investigators show that this is necessary for LukAB cytotoxicity, thus it only targets *S. aureus*-activated neutrophils. Likewise, PVL was shown to target the two C5a receptors, not only lysing the cells but also halting C5a-induced immune responses (164). However, in sublytic concentrations, PVL is also shown to prime neutrophils and increase their ability to kill staphylococci (165).

S. aureus is actually documented to release five different bi-component leukotoxins. In addition to PVL and LukAB, LukED, HlgAB and HlgCB have been described (166). LukED concentrations were recently shown to stimulate neutrophils in a dose-dependent manner at nanomolar concentrations *in vitro* (167). Furthermore, both PVL (168) and LukAB (169) are now documented NET inducers. But it seems *S. aureus* has developed evasive mechanisms to NETs through the release of nuclease (170) and adenosine synthase, which degrade NETs. The resulting deoxyadenosine induces caspase-3-mediated macrophage apoptosis (171).

Global regulatory genes

As mentioned, global regulatory genes control the expression of staphylococcal virulence factors. Of these, the agr operon is the most extensively studied, showing that staphylococci in sufficient densities activate a system where they use small peptides to communicate and activate these genes by increasing RNAIII expression. This is known as quorum sensing. One study documented increased activity in the agr quorum-sensing system by measuring the increased amounts of α-toxin and increased neutrophil lysis in response to phagocytosis (172). Another saw reduced release of phenol-soluble modulins and consequently reduced activation of neutrophils by this PAMP (173).

Biofilms

S. aureus also produce a protective biofilm on non-organic surfaces, but phagocytes are shown to activate, penetrate and to ingest bacteria despite of this (174-176). Indeed, staphylococcal biofilms have been considered a more potent virulence factor in *S. epidermidis*, which are also shown to protect themselves form neutrophil recognition and phagocytosis to a greater extent than *S. aureus* (177). Additionally, oxidative burst by phagocytes in response to biofilm was increased through preopsonization with IgG and C3b, but neutrophil adhesion was unaltered either way (178). Nanovesicles produced by neutrophils were shown to inhibit biofilm formation in *S. epidermidis* but not *S. aureus* (179). An analysis of the *S. aureus* transcriptome in response to phagocytes showed a significant downregulation of factors for growth, indicating a form of hibernation inside the biofilms if necessary (180).

Effects of antimicrobials on opsonophagocytosis

The effects of different drugs, chemical compounds and antistaphylococcal agents on leukocyte function in response to *S. aureus* have also been elucidated. The studies on antibiotics, however, may be outdated with the emergence of multi-resistant staphylococci. Although beyond the scope of this review, some immunomodulatory approaches deserves mention. In brief, gamma interferon was shown to increase neutrophil reaction and ROS production in response to staphylococcal formylated peptides *in vitro* (181). In contrast to this, IL-10 decreased neutrophil phagocytosis of serum-opsonized *S. aureus* but left other effector functions unaltered (182). IL-1β was then identified as an important cytokine in the neutrophil recruitment to staphylococcal infection (183). IL-1β also gave abscess formation (184) - a true hallmark of staphylococcal infection. Both studies were conducted in mice.

Staphylococcal TLR interaction

Regarding specific staphylococcal-TLR interaction, there is recent solid documentation that diverse *S. aureus* components are recognized by TLR2 (185, 186). These include the many lipoproteins in particular (187), but LTA (188, 189), peptidoglycan (190) and PVL (191) have also been suggested. However, both peptidoglycan and LTA have been shown to stimulate TLR2 in extremely high concentrations only, or not through TLR2 at all (192, 193). Indeed, Hattar et al. suggested that LTA preparations were contaminated with lipoprotein. Nevertheless, peptidoglycan was proposed to bind intracellular NOD-

receptors and potentiate the effects of other TLR2 agonists such as LTA and LPS through increased expression of CD14, TLR2 and TLR4 (194, 195). Also, whole *S. aureus* has been shown to stimulate dendritic cells via TLR9 (196).

Importantly, much of the staphylococcal sensing through PRRs requires important co-receptors such as CD36 and CD14 (197). For instance, CD14 is now shown to associate with at least TLRs 2, 3, 4, 6, 7 and 9 (102). Yet many aspects of the staphylococcal recognition by PRRs are still unknown.

What we do know is that several of the staphylococcal components activate PRRs to initiate inflammation. This happens through complement activation, as discussed above, and most probably through TLR activation as well. For one thing, TLR2 deficient mice are highly susceptible to staphylococcal infections (198). In this regard, it is interesting that the staphylococcal exotoxin SSL3 blocks TLR2 activation on macrophages and thus prevents downstream cytokine release (199). Peptidoglycan and LTA induce release of TNF, IL-6 and IL-10 in human whole blood (200), IL-1beta (201) and LTB4, IL-8, MCP-1 and G-CSF (116). Furthermore, peptidoglycan and LTA are shown to synergistically promote shock and multiple organ failure in several studies of sepsis, both in rats (202, 203) and pigs (204). Indeed, the involvement of peptidoglycan and LTA in sepsis was recently summarized in an extensive review (205), but an adequate explanation of their role in the septic pathogenesis was not offered.

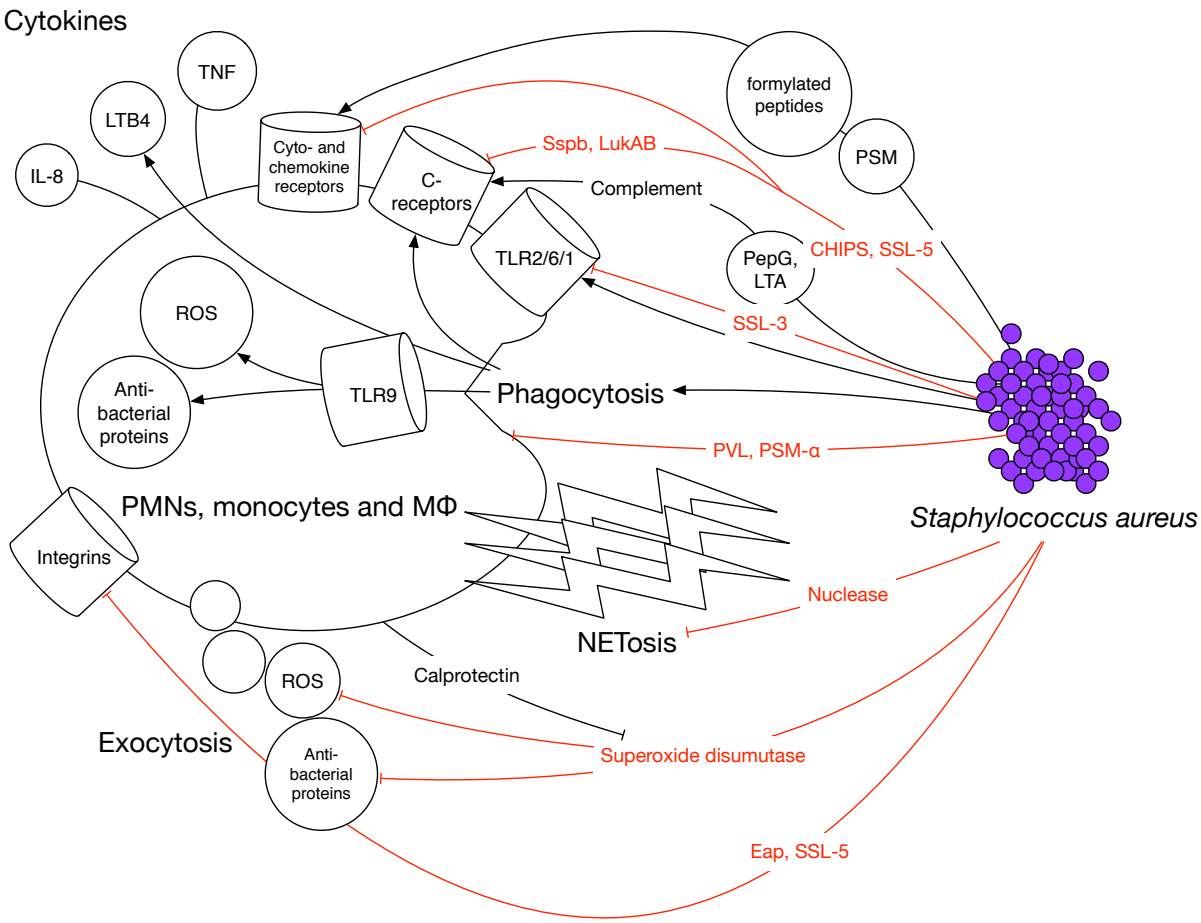

Figure 5: PMNs, monocytes and macrophages versus *S. aureus*. Staphylococcal virulence factors are marked in red. Sspb = Staphopain B, LukAB = Leukotoxin AB, CHIPS = Chemotaxis inhibitory protein of *S. aureus*, SSL-5 = Superantigen-like protein 5, PVL = Panton-Valentin Leukocidin, PSM = Phenol-soluble modulin, Eap = Extracellular adherence protein.

The coagulation and fibrinolytic systems

Coagulation

The last passage of this review concerns the plasma cascade systems of coagulation and fibrinolysis. Closely linked to the other plasma cascades throughout evolution, the process of hemostasis is clearly involved in the resolution of danger, inflammation and homeostasis. Indeed, as with complement activation, the coagulation system balances between hypoactivation and hyperactivation thereby preventing hemorrhage or thrombosis, respectively. However, thrombosis or intravascular clot formation has also recently been proposed involved in physiological inflammation.

The process of coagulation or blood clotting is closely related to the formation of a platelet plug in primary hemostasis. Coagulation may also occur regardless of platelet plug formation (206). Briefly, the formation of a fibrinogen-covered platelet plug is considered the third step in hemostasis following initial vasoconstriction and increased tissue pressure. Blood coagulation or clot formation through strictly controlled proteolysis of plasma coagulation factors is the fourth step.

As with complement activation, blood clotting can be considered a "branching tree" where different pathways of activation converge on a final common pathway. Although this convention is highly simplified by omitting all the cross-talk both between the different pathways and back and forth between "upstream" and "downstream" events, it does offer some perspective.

The so-called intrinsic or contact-dependent pathway is initiated by collagen, a negatively charged surface (such as that of an activated platelet or circulating microparticle) or platelet-bound high-molecular-weight kinogen (HMWK) that mediates the formation of activated factor XII (Hageman factor), i.e. XIIa for short. XIIa then converts prekallikrein to kallikrein, which accelerates the conversion of XII to XIIa in a positive feedback fashion, and also activates factor XI to XIa. Of note, kallikrein also cleaves HMWK to release bradykinin, a potent vasodilator and pro-inflammatory mediator (18). XIa - as Va and IIa - cleaves VII to VIIa. VIIa forms a quaternary complex with XIa, calcium and negatively charged phospholipids on the platelet surface known as the (intrinsic) tenase. The tenase cleaves factor X to Xa.

Similarly, the extrinsic pathway is initiated through tissue factor, TF (prothrombinase or factor III) exposed to the circulation. TF is constitutively expressed in non-vascular cells and is inducible in especially circulating monocytes, neutrophils and microparticles (207), thus exposed following endothelial damage and inflammation. TF mediates VIIa generation and TF, VIIa and calcium form a tertiary (extrinsic) tenase also generating Xa.

The final common pathway is therefore initiated by Xa, which mediates Va formation. Xa, Va and calcium form a prothrombinase complex. As the name implies, prothrombinase generates thrombin from prothrombin. Thrombin, therefore, is at the heart of coagulation. It has three main purposes. First, it converts fibrinogen to fibrin monomers, which spontaneously and immediately polymerize, and then stabilize by XIIIa, also generated by thrombin. Second, thrombin accelerates VIIa and Va generation through positive feedback and third, activates endothelium and platelets (206).

Fibrinolysis and anticoagulation

Reversely, this system is counterbalanced by several paracrine and anticoagulant factors, mainly originating from the endothelium. These include the tissue-factor pathway inhibitor (TFPI), targeting the prothrombinase, the antithrombin III (AT III), targeting thrombin and Xa as well as thrombomodulin binding thrombin to the endothelial surface and subsequently activating protein C, which, by the aid of co-factor protein S, inactivates factors VIIa and Va. Furthermore, in the event of clot formation, it can be degraded through fibrinolysis by plasmin formed from plasminogen by either the endothelial tissue plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). Naturally, these can also be counter-balanced by two other *s*erine *p*rotease *in*hibitors (serpins), namely the plasminogen activator inhibitors 1 and 2 (PAI-I, -II). Of these PAI-I is clearly the most relevant. Figure 6 provides a schematic overview.

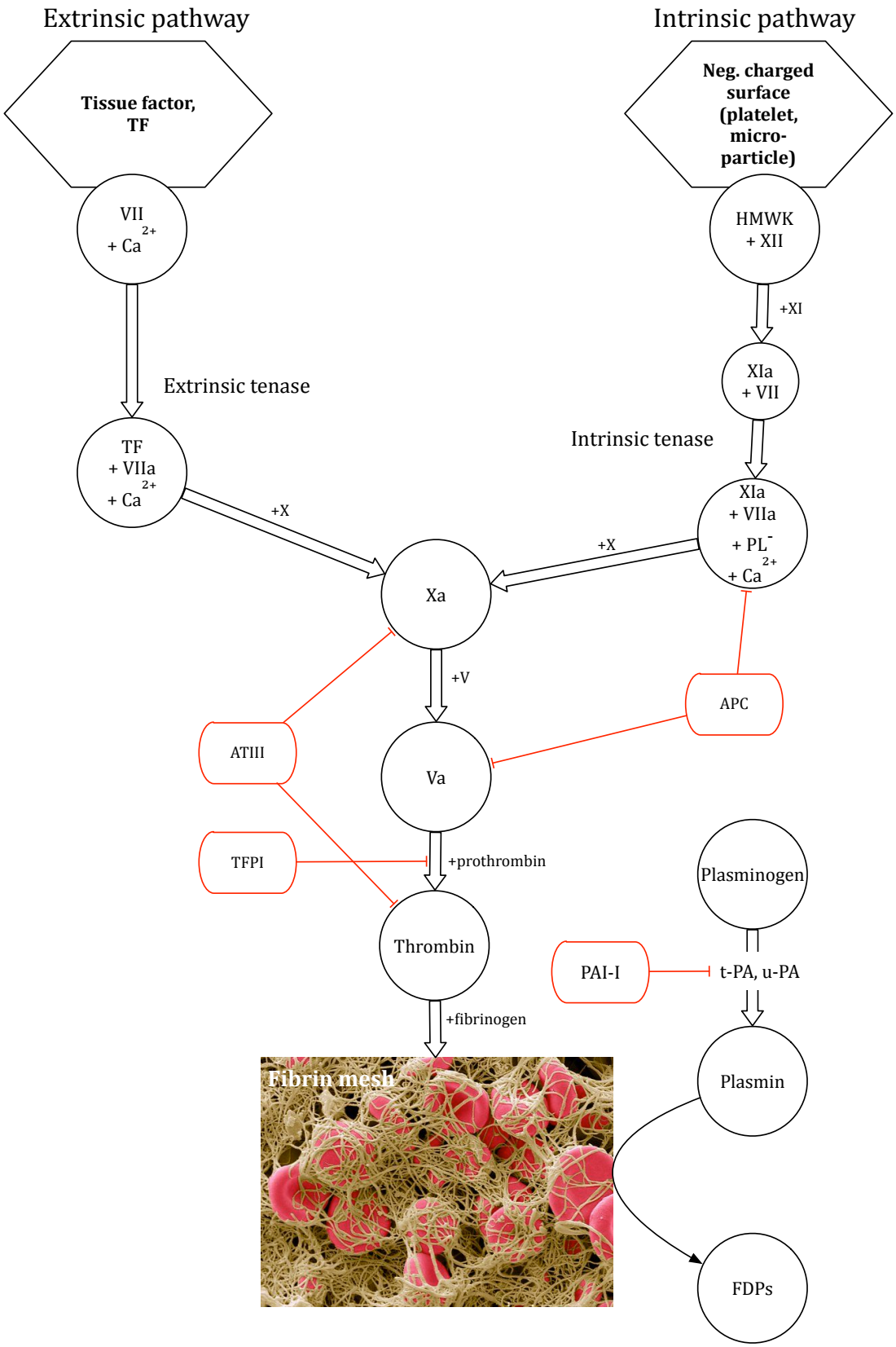

Figure 6: The coagulation system generating fibrin and the fibrinolytic system degrading it to fibrin-degradation products (FDPs). HMWK = High molecular weight-kinogen, APC = Activated protein C, PL- = negatively charged phospholipids, TFPI = Tissue factor pathway inhibitor, ATIII = Antithrombin III, t-PA = Tissue plasminogen activator, u-PA = urokinase-type plasminogen activator, PAI-1 = Plasminogen-activator inhibitor.

Evidently, the clotting cascade and consequent fibrinolysis is potent and must be strictly controlled. However, during inflammation and sepsis this control is lost leading to simultaneous thrombosis and hemorrhage. In severe sepsis, disseminated intravascular coagulation (DIC) is characterized my microthrombi and diffuse fibrin deposition that occlude capillaries and cause multiple-organ failure, but also profuse bleeding in other areas such as the skin. Thus, DIC is strongly associated with higher mortality in sepsis, and its interrelation with inflammation is complex and potentially devastating as the two processes activate and perpetuate each other (208). However, as mentioned, the two may not be different entities. A recent review identifies the intriguing process of immunothrombosis as a novel component of the innate immune system (209). Here, the authors propose that immunothrombosis has several different physiological functions: Pathogens are trapped and unable to disseminate as microvessels are sealed with microthrombi. In doing so, immunothrombosis generates a closed compartment ideal for pathogen recognition and killing. Furthermore, fibrinogen and/or fibrin enhance the immune response through immune cell recruitment whilst NETosis is highly dependent on its procoagulant properties for optimal effect. However, the authors identify DIC as a form of aberrant immunothrombosis. Whereas microthrombi and fibrin deposition allow sufficient organ perfusion and tissue oxygenation under physiological conditions, they may strangulate these same organs in sepsis and DIC, causing multiple organ failure. What favors immunothrombosis to DIC is still unknown, but some clues might be found in the multiple interactions between host coagulation and pathogen - the next topic of this review.

Coagulation, fibrinolysis and *Staphylococcus aureus*

Staphylococcal coagulases

Staphylococci activate the coagulation system through several MSCRAMMs. Notably, the ability of surface-bound staphylocoagulase (Coa) to bind and activate prothrombin, yielding staphylothrombin is widely used to diagnose *S. aureus* infections (210). To date, ten different serotypes of Coa have been described (211). Likewise, the von Willebrand factor-binding protein (vWbp) is identified as a coagulase (212), and both coagulases have to function to resist neutrophils and to form abscesses (213). Indeed, it is proposed that staphylococci secrete Coa and vWbp to generate provisional pseudocapsules on the bacterial surface and avoid recognition by the immune system during their

dissemination (214). It is now shown that the coagulases associate with factor XIII in addition to fibrinogen, von Willebrand factor and prothrombin to enhance fibrin generation (215). Interestingly, most *S. aureus* strains also secrete staphylokinase, a potent activator of the fibrinolytic system (216, 217) and inactivator of neutrophil-derived antimicrobial peptides (218). Equally, secreted staphylococcal aureolysin is proposed to activate the fibrinolytic system at several levels, converting plasminogen to angiostatin and mini-plasminogen, degrading PAI-1 and neutralizing the otherwise inhibitory effects of alpha(2)-antiplasmin (219). It is therefore tempting to speculate that staphylococci first cover themselves in fibrin by way of the coagulases and then undress at suitable locations by way of the staphylokinase, but this is still unclear.

Staphylococci and infective endocarditis

A particular presentation of invasive *S. aureus* infection is the ability to damage and adhere to previously undamaged heart valves causing infective endocarditis. Although the precise mechanisms behind this remains unknown, endothelial cell and platelet activation are key events. Platelet activation and aggregation is promoted by staphylococcal surface components such as the clumping factors A and B (Clf A and B) (220), which bind fibrinogen, and protein A (221), but also through increased monocyte TF expression (222) as well as in a fibrinogen-independent, complement dependent fashion (223). Equally, endothelial cells treated with fibronectin-binding protein A, but not B and not clumping factor A caused a markedly procoagulant response in the vasculature. Later, soluble fibrin was identified as a main bridge between staphylococci and platelets (224). Thus, coagulation activation is an important virulence factor in infective endocarditis.

S. aureus, coagulation and sepsis

Not surprisingly, therefore, coagulation is also activated in staphylococcal sepsis. In experimental whole-blood models, staphylococcal α-toxin is shown to not only lyse platelets but also and more importantly, to promote assembly of the prothrombinase through platelet activation (225). Equally, the staphylococcal superantigens enterotoxins A and B, as well as the toxic shock syndrome toxin 1 (TSST-1) induced an IL-1β-dependent, TF-mediated procoagulant response in whole blood (226). Furthermore, a subsequent study identified peptidoglycan but not LTA as an important procoagulant in human whole blood (227). This was later also shown in human

umbilical vein endothelial cells (HUVECs). Peptidoglycan, LTA and TSST-1 all induced increased TF mRNA transcription and TF expression, but only peptidoglycan increased ICAM and VCAM on the HUVECs as well (228). Furthermore, heat-inactivated *S. aureus* induced an imbalanced increase in PAI-1 expression in peritoneal mesothelial cells in one study (229), whereas decreased fibrinolytic activity in peritonitis was attributed to *S. aureus*-induced mestothelial cell death in another (230). In contrast, the staphopains are shown to increase clotting time through fibrinogen degradation (231). Also, MBL deficiency has been shown to promote DIC in mice (232). Even though MBL and its MASPs are identified as activators of coagulation in the same study, the literature presented in this review suggests that *S. aureus* generally activates coagulation rather than prevents it. Indeed, murine *S. aureus* sepsis induced a hypercoaguable state with increased TF activity (233), whereas in a porcine model of intravenous *S. aureus* sepsis the researchers found pulmonary petecchiae and several thrombotic lesions in association with staphylococcal abscesses as well as increased coagulability over time, measured by thromboelastography (234). The same group later found that the septic pigs in the model developed DIC in accordance with human criteria (235).

In line with these findings, measures have been taken to reduce the hypercoagulability in sepsis. Although recombinant human activated protein C was withdrawn as a licensed drug in non-specific sepsis, a novel study of murine staphylococcal sepsis gave promising results when blocking Coa, vWBp and ClfA (236) - All 20 mice receiving the treatment survived, none in the untreated group. Interestingly, the procoagulant effects of staphylococci may even prevail for a long time following resolution of the infection. A recent large study revealed increased patient risk of a venous thromboembolic episode (VTE) within the first year following *S. aureus* bacteremia (237). Although the exact pathogenesis of VTEs is currently unclear, the many interactions between *S. aureus* and the coagulation system quite probably contribute to the hemostatic dysfunction.

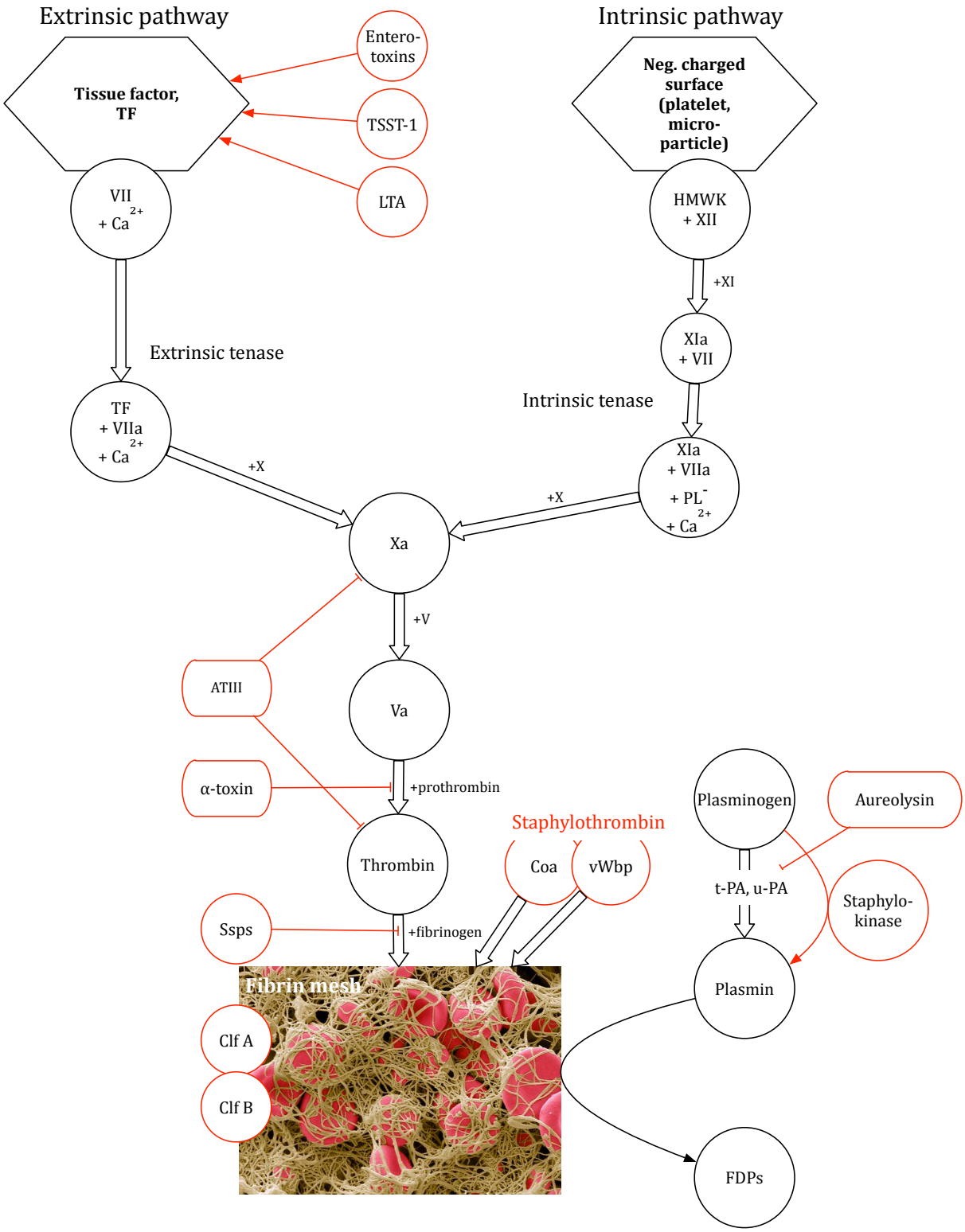

Figure 7: *S. aureus*, coagulation and fibrinolysis. Different points of staphylococcal interaction are marked in red whereas the physiological inhibitors are left out of this figure. TSST-1 = Toxic shock syndrome toxin 1, LTA = Lipoteichoic acid, TF = Tissue factor. vWbp = von Willebrand binding protein, Coa = Coagulase, Ssps = Staphpopains, Clf = Clumping factor.

Discussion and concluding remarks

Evidently, *S. aureus* infection strongly intertwines with the innate immune system, illustrated by the numerous interactions with the complement system, the coagulation system and the leukocytes documented in this review. The question remains, however, if the many immune evasive strategies - inactivating complement, killing neutrophils and activating coagulation is really what makes the staphylococcal infections threatening to the host? Or rather, if the bacteria simply activate these systems and the true damage actually is caused by the body itself? Indeed, the many sophisticated virulence factors of *S. aureus* promote its infection, dissemination and survival during orderly, localized infection. For instance, a recent study demonstrated significant association between increased activity in genes coding for capsular polysaccharides 5 and 8, adhesins, PVL, serine proteases (V8) and SCIN and invasive disease (238). However, this may not be the case in sepsis.

Nonetheless, infection and immune response act in synergy. In line with findings for non-specific sepsis, both delayed antibiotic treatment and septic shock are clearly negative predictive factors in staphylococcal sepsis (239). Equally, in-hospital patients suffering from *S. aureus* sepsis have an excess one-year mortality of 20 per cent compared to non-septic patients, as well as increased morbidity and cost of stays, underlining the difficulties of this particular pathogen (240). However, few studies document the patterns of sepsis caused by different microbes. In general, Gram-positive strains are shown to induce a more delayed release of cytokines compared to Gram-negative strains, as Gram-positive bacteria often demand a more complex immune response, and Gram-negative strains cause a more profound and immediate hemodynamic dysfunction, at least in animal models (241). Furthermore, therapies targeting individual cytokines such as TNF have been more promising in Gram-negative animal models of sepsis compared to Gram-positive models. Yet the hope of finding the "magic bullet" or "one size fits all"-therapy in sepsis is now small. For instance, multiplex analysis of 17 different cytokines over seven days in 30 septic patients did not find a unique mediator related to patient outcome. Although high levels of monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1beta and IL-8 were associated with higher mortality the first three days, all cytokines were generally elevated among non-survivors (242). Therefore, interesting concepts have emerged,

such as the idea of an early and broad attenuation of the immune response through blocking early sensing of pathogen by complement and leukocytes rather than trying to take out single mediators further down the line (243). This concept can be seen as preventing a flood downstream by building a dam upstream or disarming a bomb before it blows. The only trouble is getting to the bomb soon enough - most patients probably enter the clinic long after detonation, at a point where "downstream" intervention is the only possibility. Even so, it is important to identify high-risk patients before they develop sepsis and preventive strategies are currently the best solution to sepsis. Similar strategies are under investigation such as preventing staphylococcal nasal colonization. Equally, sepsis is a very comprehensive disease entity where patients present very differently. It also strikes at the very extremes of age and it is worth considering whether old patients, in particular, are over-exposed to infection and sepsis because of too much invasive intervention. Nonetheless, sepsis causes a huge disease burden through its morbidity, costs and difficulty to manage. More knowledge on the complex interactions between the different pathogens and the immune system in both health and disease is needed. We could then unveil and test suitable targets of intervention to ultimately improve the outcome in sepsis. For the case of *Staphylococcus aureus* we now know a lot about virulence factors but less about how to combat them.

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