IMMUNE EVASION BY STAPHYLOCOCCI

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Abstract | *Staphylococcus aureus* can cause superficial skin infections and, occasionally, deep-seated infections that entail spread through the blood stream. The organism expresses several factors that compromise the effectiveness of neutrophils and macrophages, the first line of defence against infection. *S. aureus* secretes proteins that inhibit complement activation and neutrophil chemotaxis or that lyse neutrophils, neutralizes antimicrobial defensin peptides, and its cell surface is modified to reduce their effectiveness. The organism can survive in phagosomes, express polysaccharides and proteins that inhibit opsonization by antibody and complement, and its cell wall is resistant to lysozyme. Furthermore, *S. aureus* expresses several types of superantigen that corrupt the normal humoral immune response, resulting in anergy and immunosuppression. In contrast, *Staphylococcus epidermidis* must rely primarily on cell-surface polymers and the ability to form a biolfilm to survive in the host.

Staphylococcus aureus permanently colonizes the moist squamous epithelium of the anterior nares of 20% of the population, and is transiently associated with another 60%[1]. Occasionally, the organism can cause superficial skin infections such as abscesses and impetigo, or serious invasive infections such as septic arthritis, osteomyelitis and endocarditis[2]. Colonization is a known risk factor for invasive disease both in the hospital and the community[3,4]. Hospital patients who have been catherized or who have undergone surgery are at increased risk of infection. Treatment of infections with antibiotics has become increasingly difficult owing to the widespread occurrence of strains that are resistant to multiple antibiotics, known as meticillin (formerly methicillin)-resistant *Staphylococcus aureus* (MRSA). Furthermore, the isolation of MRSA strains that have also become resistant to vancomycin[5,6], the last drug to which the organism had been uniformly sensitive, raises the spectre of a return to the pre-antibiotic era.

The primary defence against *S. aureus* infection is the innate immunity provided by neutrophils. It is now apparent that *S. aureus* has the ability to thwart the neutrophil in various ways. In addition, the organism secretes immunomodulatory proteins that compromise both induced humoral and cell-mediated immunity. This might explain why many individuals can suffer repeated infections. Antibody levels are often too low to be protective, and the host is unable to respond to re-infection with a robust secondary response owing to depletion of T and B cells.

Virulence factors of *S. aureus*

S. aureus expresses a wide array of secreted and cell-surface-associated virulence factors, including surface proteins that promote adhesion to damaged tissue and to the surface of host cells[7], that bind proteins in blood to help evade immune responses, and that promote iron uptake[8]. Most strains express a polysaccharide capsule[9]. The organism can secrete an array of extracellular enzymes such as proteases, a hyaluronidase, a lipase and a nuclease that facilitate tissue destruction and spreading, membrane-damaging toxins that cause cytolytic effects on host cells and tissue damage, and superantigens that contribute to the symptoms of septic shock[10,11].

One major class of surface-located proteins comprises those that are covalently anchored to cell-wall peptidoglycan by sortase, a membrane-associated enzyme that

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recognizes and cleaves the C-terminal LPXTG motif in the sorting signal[7,12]. Other wall- and surface-associated proteins are loosely bound through ionic interactions.

Host defences against infection

When the outer physical barriers of the body, comprising skin and mucous surfaces, have been breached by *S. aureus*, the organism is confronted by the host's immune system, comprising both innate and acquired responses. *S. aureus* infection of the skin stimulates a strong inflammatory response, involving the migration of neutrophils and macrophages to the site of infection. These cells will attempt to engulf and dispose of the invading organisms with the help of available antibodies that are present in the host's serum, and complement. This is where the first important internal confrontation between *S. aureus* and the host occurs.

Complement is a family of proteins and proteolytic fragments derived from them that have many roles in innate and acquired immunity, including direct killing of foreign cells and regulation of other effectors of the immune response[13]. With bacteria such as *S. aureus*, the role of complement is to recruit effector molecules that label cells and target them for destruction by immune effector cells such as neutrophils. This process of complement fixation occurs by three pathways (FIG. 1). The alternative and lectin pathways are components of innate immunity, whereas the classical pathway requires specific interaction with antibody that has bound to antigens on target cells. One of the main purposes of complement fixation is opsonization — to promote phagocytosis by neutrophils and macrophages. Initially, the phagocytes are attracted to the site of infection by chemoattractant molecules

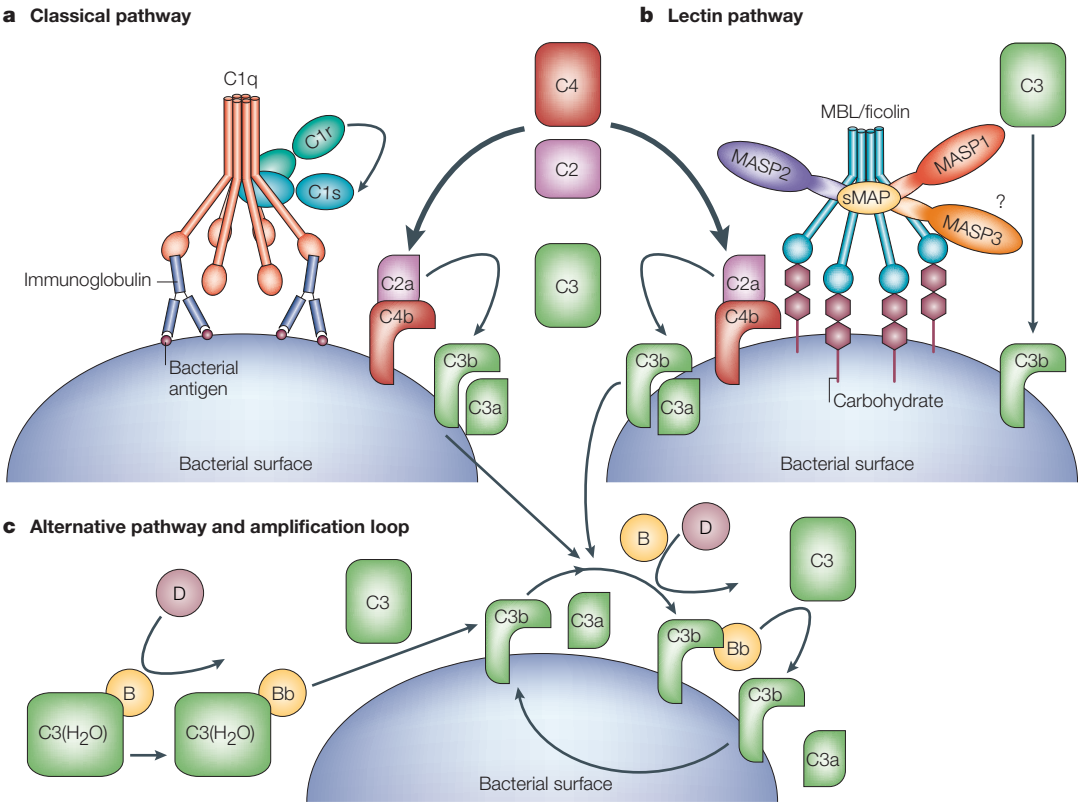

Figure 1 | **Pathways for complement activation. a** | The classical pathway is initiated by the binding of the C1 complex to antibodies that are bound to antigens on the surface of bacteria. The C1 complex consists of C1q and two molecules each of C1r and C1s. The binding of the recognition subcomponent C1q to the Fc portion of immunoglobulins results in autoactivation of the serine protease C1r. C1r then cleaves and activates C1s, which translates the activation of the C1 complex into complement activation through the cleavage of C4 and C2 to form a C4bC2a enzyme complex. C4bC2a acts as a C3 convertase and cleaves C3, which results in products that bind to, and cause the destruction of, invading bacteria. **b** | The lectin pathway is initiated by the binding of either mannose-binding lectin (MBL) or ficolin — associated with MBL-associated serine protease 1 (MASP1), MASP2, MASP3 and small MBL-associated protein (sMAP) — to an array of carbohydrate groups on the surface of a bacterial cell. Similar to C1s, MASP2 is responsible for the activation of C4 and C2, which leads to the generation of the same C3 convertase (C4bC2a). As in the classical pathway, C3 convertase cleaves C3 to C3b and the chemoattractant peptide C3a. The C3b–C2a–C4b complex then cleaves C5 to C5a and the chemoattractant peptide C5b, which stimulates assembly of factors C6, C7, C8 and C9 (not shown). MASP1 is able to cleave C3 directly. **c** | The alternative pathway is initiated by the low-grade activation of C3 by hydrolysed C3 ($C3(H_2O)$) and activated factor B (Bb). The activated C3b binds factor B (B), which is then cleaved into Bb by factor D (D) to form the alternative pathway C3 convertase, C3bBb. Once C3b is attached to the cell surface, the amplification loop consisting of the alternative-pathway components is activated, and the C3-convertase enzymes cleave many molecules of C3 to C3b, which bind covalently around the site of complement activation. Image reproduced with permission from *Nature Reviews Immunology* REF. 133 © (2002) Macmillan Magazines Ltd.

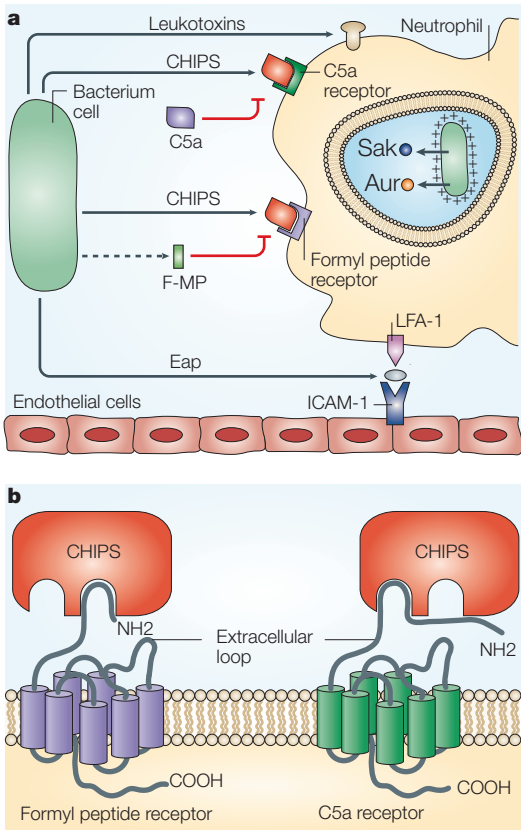

Figure 2 | **Inhibition of the neutrophil response to infection. a** | The chemotaxis inhibitory protein of staphylococci (CHIPS) and the extracellular adherence protein (Eap) interfere with neutrophil chemotaxis and extravasation. Resistance to killing by antimicrobial peptides in the neutrophil phagosome is promoted by D-alanine and L-lysine modifications to cell-wall components (indicated by +), by secretion of staphylokinase (Sak) and aureolysin (Aur), and by the creation of 'spacious' phagosomes in which bacteria can survive. The pore-forming leukotoxins are shown by the mushroom-shaped insertion in the neutrophil membrane. **b** | Model for interactions between CHIPS and the formyl peptide receptor (FPR) and C5a receptor. Two distinct but closely linked binding domains in CHIPS are indicated, one for the extreme N terminus of FPR involving residues F1 and F3, the second for a domain located between residues 10–20 of the C5a receptor. F-MP, *N*-formyl-methionyl peptide; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte-function-associated antigen.

such as small peptide fragments (C3a and C5a) that are released during complement activation, and by formylated peptides released by growing bacteria. The membranes of phagocytic cells have specific receptors for fragments of complement and formylated peptides that enhance the efficiency of phagocytosis. The neutrophils also carry specific receptors that can recognize the Fc region of immunoglobulin G (IgG) and complement proteins bound to the bacterial surface that facilitate efficient uptake and killing.

At the initial stage of the host immune response to infection, the invading microorganism and its products are taken up by macrophages and other antigen-presenting cells and transported to lymph nodes, where B cells are stimulated to differentiate and secrete antibodies that neutralize toxins and promote more efficient phagocytosis of bacterial cells. It is clear that this system does not work properly in the case of *S. aureus*. Antibodies to *S. aureus* antigens are present in all humans, and there is evidence that titres rise following infection[14–16]. However, these antibodies and immunological memory seem to be insufficient to prevent subsequent infections.

S. aureus has been regarded as a non-invasive pathogen but it is now evident that the bacterium can invade many types of host cells by a mechanism involving the formation of a fibronectin bridge between the bacterial fibronectin-binding proteins and host $\alpha5\beta1$ integrin molecules that triggers internalization[17–19]. The organism can survive in host cells in a semi-dormant form referred to as small colony variants[20]. A cell-mediated immune response is required to dispose of cells bearing intracellular bacteria. However, there is little known about the role of cell-mediated immunity in combating chronic staphylococcal infections.

In this article, I review the recent advances in our understanding of the various mechanisms employed by staphylococci that allow them to avoid innate and acquired immunity. In particular, the immune-evasion strategies of *S. aureus* and the less virulent *Staphylococcus epidermidis* are compared, and reference is also made to streptococci. This analysis supports the notion that *S.aureus* is a well adapted pathogen that has evolved its pathogenic potential as a result of thousands of years of coexistence with man.

Inhibition of neutrophil chemotaxis

Immediately as the bacterium gains a foothold in the host and starts to grow, several chemoattractants are liberated which are specifically recognized by neutrophils at low concentrations, resulting in a strong chemotactic response. The peptide fragments C3a and C5a, released by complement activation, as well as formylated peptides secreted from growing bacterial cells, are recognized at high affinity by specific transmembrane G-protein-coupled receptors on the neutrophil surface[21]. These are stimulated and activate intracellular signalling cascades, resulting in migration of neutrophils from the blood to the site of inflammation.

About 60% of *S. aureus* strains secrete the chemotaxis inhibitory protein of staphylococci CHIPS that can bind avidly to both the formyl peptide receptor (FPR) and the C5a receptor (C5aR) to block the cognate agonist from binding[22] (FIG. 2; TABLE 1). Recent studies identified a FPR-binding domain in the N terminus of CHIPS, and showed that Phe residues at positions 1 and 3 are crucial for activity[23]. Furthermore, the C5aR-binding domain of CHIPS is distinct from the FPR-binding domain (FIG. 2). The C5aR- and FPR-binding activities of CHIPS were separated by specific amino-acid substitutions and the specificity of blocking monoclonal antibodies[24,25]. In the case of C5aR, CHIPS blocks the N-terminal C5a-peptide-binding domain but does not occlude a second activation domain located towards the C terminus.

Table 1 | **Prevalence of factors responsible for immune avoidance by *Staphylococcus aureus***

Factor	Abbreviation	Distribution (% strains tested)	Refs
Protein A	Spa	90/94*	29
Clumping factor A	ClfA	98/100*	29
Capsular polysaccharide serotypes 5 and 8	Cps	Type 5: 16–26‡, Type 8: 55–65‡	124–127
Chemotaxis inhibitory protein	CHIPS	62*§	22
Staphylokinase	Sak	67%‡	128
MHC Class II analogous protein/extracellular adherence protein	Map/Eap	93/96*; 97*	29,129
Extracellular fibrinogen binding protein	Efb	60/68*, 80*	29,130
Aureolysin	Aur	100¶	131
Panton-Valentine leukocidin	PVL	2/4*, 2‡	29
Leukocidin E–D	LukED	33‡	132
γ-haemolysin	Hlg	89/97*	29
Enterotoxin A	Sea	17/32*, 54‡	29,30
Enterotoxin B	Seb	7/9*, 4‡	29,30
Enterotoxin C	Sec	11/10*, 5‡	29,30
Enterotoxin D	Sed	5/5*, 10‡	29,30
Enterotoxin G	Seg	64/55*	29
Enterotoxin H	Seh	10/15*	29
Toxic shock syndrome toxin-1	TSST-1	25/30*, 33‡	29,30

*Detected by PCR. ‡Detected by expression of the protein or polysaccharide. ¶Southern blotting. Failure to detect an amplimer by PCR without confirmation by DNA hybridization might give an underestimate of the presence of a gene. Variation in the incidence of genes from different studies might reflect differences in the sources of the strains tested. A large number of randomly selected nasal-carriage isolates will give an even representation, whereas those selected from nosocomial infections from a single hospital might be biased by the presence of endemic strains (for example, endemic meticillin-resistant *S. aureus* clones). In the study by Peacock *et al.*[29], the first of the two numbers refer to the percentage of strains from carriage isolates that were positive by PCR, whereas the second number is the percentage of invasive disease-causing strains from both community-acquired and nosocomial infections. For Cps5 and Cps8, the two numbers refer to the range determined by several different studies. §The distribution of CHIPS is similar to Sak and Sea because the three factors are encoded by closely linked genes associated with a family of lysogenic bacteriophages.

One of the many ligands recognized by the extracellular adherence protein Eap (otherwise called the major histocompatibility class II analogue protein Map) is intercellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells[26]. Binding of Eap to ICAM-1 blocks binding of the lymphocyte-function-associated antigen LFA-1 on the surface of neutrophils and prevents leucocyte adhesion, diapadesis and extravasation. The Eap protein will likely act in concert with CHIPS to inhibit neutrophil recruitment to the site of infection

Toxins that kill leukocytes

One of the cardinal features of *S. aureus* is the ability to secrete toxins that damage the membranes of host cells. The expression of cytolytic toxins that damage leukotoxins contributes to development of abscesses by the killing of neutrophils that are attempting to engulf and kill bacteria.

Cytolytic toxins that form β-barrel pores in the cytoplasmic membranes of target cells cause leakage and, ultimately, lysis. The archetype of this class is the α-toxin, which is secreted as a monomer that associates into a heptamer in the membrane, with β-strands from each monomer assembling into a 14-stranded β-barrel pore[27]. The bicomponent leukotoxins comprise two subunits that are secreted separately and assemble into hexameric or heptameric oligomers with a strong affinity for leukocytes. There are four types of bicomponent leukotoxin, the γ-toxin or γ-haemolysin (Hlg), the Panton–Valentine leukocidin (PVL), leukocidin E/D and leukocidin M/F-PV-like. The γ-toxin lyses both erythrocytes and leukocytes, whereas PVL is toxic only for leukocytes[28]. The *hlg* genes are present in the chromosome of >90% of randomly selected *S. aureus* strains, whereas the *pvl* genes, which are present in a lysogenic bacteriophage, are found in only 1–2%[29,30] (TABLE 1).

There is a strong association between PVL expression and severe skin infections such as recurrent furunculosis[30], indicating that PVL enhances virulence in these infections. Recently, community-acquired MRSA (CA-MRSA) strains have emerged that cause severe necrotizing pneumonia and contagious, severe skin infections in previously healthy individuals[31,32]. These strains are characterized by carriage of the type IV or type V staphylococcal cassette chromosome (SCCmec), encoding resistance to meticillin and other β-lactam antibiotics[33], and often by expression of PVL (encoded by *pvl* genes located in a lysogenic bacteriophage[34]). It is likely that these virulent strains have emerged as the result of horizontal transfer of PVL phages and SCCmecIV and V elements. However, not all CA-MRSA strains express PVL[35].

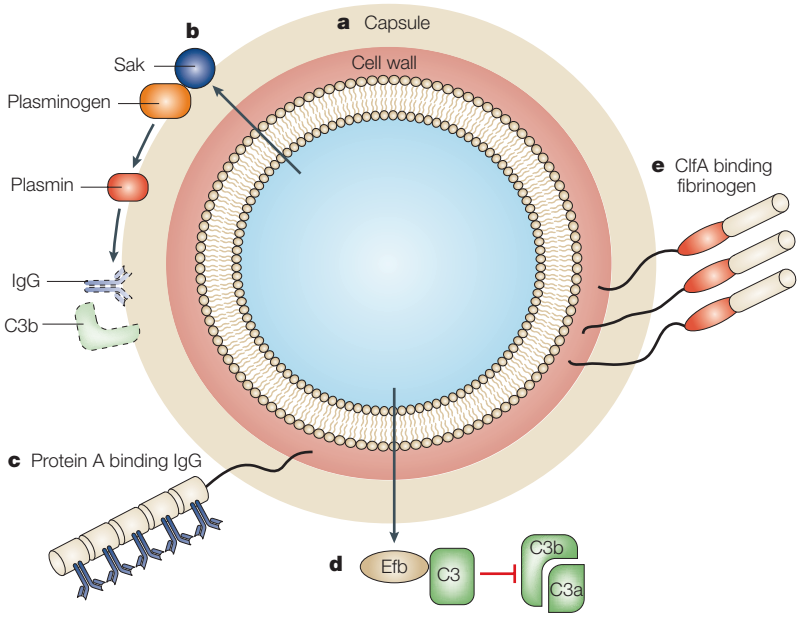

Figure 3 | **Mechanisms by which *Staphylococcus aureus* avoids opsonophagocytosis.** The figure illustrates (**a**) the capsular polysaccharide, which can compromise neutrophil access to bound complement and antibody; (**b**) the extracellular staphylokinase (Sak), which activates cell-bound plasminogen and cleaves IgG and C3b; (**c**) protein A with 5 immunoglobulin G (IgG) Fc-binding domains; (**d**) fibrinogen-binding protein (Efb), which binds complement factor C3 and blocks its deposition on the bacterial cell surface. Complement activation beyond C3b attachment is prevented, thereby inhibiting opsonization. (**e**) Clumping factor A (ClfA), which binds the γ-chain of fibrinogen.

Resistance to phagocytosis

The ability of *S. aureus* to avoid opsonins present in normal serum is an important factor in the success of infection. Antibodies that recognize cell-surface components such as teichoic acid, peptidoglycan and surface-associated proteins are present in sera of most, if not all, individuals. *S. aureus* expresses surface-associated anti-opsonic proteins and a polysaccharide capsule that can both interfere with the deposition of antibodies and complement formation by classical and alternative pathways, or with their access to neutrophil complement receptor and Fc receptor. Therefore, efficient phagocytosis by neutrophils that requires recognition of bound complement and antibody is compromised.

Surface proteins. Protein A is a wall-anchored protein with either four or five domains that each bind to the Fc region of IgG[36]. The X-ray structure of protein A IgG-binding domains in complex with the Fc region of IgG have been solved[37], and residues from helix I that are involved in the interaction have been identified and verified by site-directed mutagenesis[38]. The consequence of the interaction between protein A and IgG is to coat the surface of the cell with IgG molecules that are in the incorrect orientation to be recognized by the neutrophil Fc receptor (FIG. 3). This could explain the antiphagocytic effect of protein A and its role in pathogenesis of *S. aureus* infections. Protein-A-deficient mutants of *S. aureus* are phagocytosed more efficiently by neutrophils *in vitro*[39] and show decreased virulence in several animal infection models[40,41].

Clumping factor A (ClfA) is the dominant fibrinogen-binding protein present on the surface of *S. aureus* cells in the stationary phase of growth[42,43]. Unlike most surface proteins, which are expressed predominantly during the exponential phase, the *clfA* gene is switched on in the stationary phase from a σ^B-dependent promoter[43], which ensures that about 10-fold more ClfA is present compared to the exponential phase[42], when the *clfA* gene is expressed at a lower level from a weaker σ^{70}-dependent promoter. The N-terminal A domain of ClfA binds to the γ-chain of fibrinogen molecules[44]. When cells are densely packed together in suspension, the γ-chain C termini, which are located at either end of the bivalent molecule, can bind simultaneously to two ClfA molecules on different cells, which results in cell clumping. However, *in vivo* the density of cells is too low for clumping to occur. Instead, bacterial cells are coated with fibrinogen molecules as shown in FIG. 3.

ClfA is a virulence factor in the murine model for sepsis and arthritis[45]. It was postulated that virulence is enhanced during the bacteraemic phase of the infection as well as during growth in infected joints because bacterial cells became coated with fibrinogen, which in turn inhibited deposition of, or access to, opsonins. This notion is supported by the observation that ClfA protects *S. aureus* from phagocytosis by murine macrophages[46] and by human neutrophils (J. Higgins and T.F., unpublished results) and that protection is at least partly dependent on fibrinogen. It is likely that fibronectin-binding proteins and ClfB, which also bind fibrinogen[47,48], can protect the bacterium in a similar way during the exponential phase of growth, when these proteins are expressed in greater abundance than ClfA.

Capsule. Most *S. aureus* clinical isolates express a thin microcapsular layer that is composed of serotype 5, serotype 8 or serotype 336 capsular polysaccharide[9,49] (TABLE 1). Whether the small proportion of untypable strains express other types of capsule or are non-capsulated is not known. Expression of type 5 and type 8 capsule is associated with increased virulence in animal infection models[50–53]. *In vitro* phagocytosis assays revealed that the presence of the capsule reduced the uptake of cells by neutrophils in the presence of normal serum opsonins, indicating that capsule is anti-opsonic[51,52]. Complement factors can assemble on the cell-wall surface underneath the polysaccharide, but presumably these are inaccessible to complement receptor on the surface of neutrophils. By contrast, high levels of specific anti-capsular polysaccharide antibodies promote opsonophagocytosis and protect against infection[9,54]. Many strains of *S. aureus* carry genes that specify the polysaccharide intercellular adhesin (PIA), an extracellular polysaccharide that is of particular importance for *S. epidermidis* infections. The role of this polymer in biofilm formation and avoidance of phagocytosis is discussed in the section on *S. epidermidis*.

Box 1 | **Antimicrobial peptides**

Peptides with antimicrobial activity are important components of innate immunity. In humans, they are produced in tissues and by cells such as platelets and neutrophils, and are present on mucosal surfaces, in the airways and on skin. Antimicrobial activity is generally due to disruption of the integrity of lipid bilayers, but in some cases more specific inhibitory modes of action might occur. Antimicrobial peptides can be classified as follows[123]:

- **Small anionic peptides, for example, dermicidin and peptides found in airway surfactant fluid.**
- **Small cationic peptides lacking cysteine residues, for example, human LL-37, a cathelicidin found in neutrophils. These peptides are often disordered in aqueous solution but form an α-helix in a hydrophobic environment such as a lipid bilayer.**
- **Cationic peptides that form disulphide bonds, for example, protegrin, α-defensins such as human neutrophil peptides, and human β-defensins.**
- **Anionic and cationic peptide fragments derived from larger proteins, for example, lactoferricin from lactoferrin, cascocidin from casein and antimicrobial domains of haemoglobin, lysozyme and ovalbumin. The role of this class in innate immunity is untested.**

Inactivation of complement. Assembly of C3 convertases on the surface of bacteria is a prerequisite for complement activation. The structurally and functionally related C4bC2a (classical and lectin pathways) and C3bBb (alternative pathway) carry out the essential function of cleaving C3, which results in release of soluble C3a and covalent attachment of C3b to the bacterium. *S. aureus* secretes a 9.8-kDa protein called *Staphylococcus* complement inhibitor (SCIN), which binds to and stabilizes both C4bC2a and C3bBb, resulting in inhibition of further C3b formation[55]. Normally, the C3 convertases are transiently active, with dissociation leaving microorganism-bound C4b and C3b to act as cofactors for further cleavage of C2 and factor B, respectively. Stabilization of the complexes by SCIN blocks this crucial amplification loop and is a potent mechanism for preventing complement activation. SCIN can therefore block phagocytosis and killing of *S. aureus* cells by human neutrophils.

The extracellular fibrinogen-binding protein Efb was recently shown to bind to complement factor C3 and to block C3 deposition on the bacterial cell surface[56–58]. Therefore, complement activation beyond C3b attachment (FIG. 3) was prevented and opsonization was inhibited.

S. aureus also has the ability to inactivate complement factor C3b and IgG molecules that are bound to the surface of opsonized bacterial cells. Host plasminogen molecules attach to the bacterial cell surface, where they are bound in 1:1 stoichiometry by staphylokinase, a plasminogen activator protein that is secreted by *S. aureus*. The potent serine protease of plasmin is activated and cleaves surface-bound C3b and IgG, resulting in reduced phagocytosis by neutrophils[59].

Resistance to killing by antimicrobial peptides

If *S. aureus* is successfully engulfed by a neutrophil, it is well endowed with surface modifications to help it survive in the phagosome. In *in vitro* neutrophil phagocytosis assays, a significant fraction of engulfed bacterial cells survive killing mechanisms[60,61]. This is in part due to natural modifications to wall teichoic acid (WTA), lipoteichoic acid and to a membrane phospholipid. The Dlt proteins result in D-alanine substitutions of ribitol teichoic acid and lipoteichoic acid that partially neutralize the negative charge of the cell surface that attracts cationic molecules[62] (FIG. 2). Similarly, the MprF protein adds an L-lysine residue to phosphatidylglycerol that is exposed on the outer face of the cytoplasmic membrane[63,64]. In both cases, the modifications reduce the affinity of the cationic, antimicrobial defensin peptides (BOX 1) that are secreted into the neutrophil phagosome and repel them from cytoplasmic membrane. In addition, these modifications serve to protect bacteria from positively charged antimicrobial proteins in serum, such as phospholipase A2 and lactoferrin. Mutants defective in Dlt and MprF are more susceptible to killing by cationic antimicrobial proteins and neutrophils *in vitro*, and have markedly reduced virulence in several animal infection models[65,66].

In addition to modification of negatively charged surface molecules, *S. aureus* also secretes proteins that neutralize cationic peptides. Staphylokinase, a prothrombin activator that stimulates dissolution of fibrin clots and degradation of IgG and C3, also has potent defensin-peptide-binding activity. It binds defensins with a stoichiometry of approximately 1:6 and contributes to protection of bacteria *in vivo*[67,68]. Also, the extracellular metalloprotease aureolysin cleaves and inactivates the human defensin peptide cathelicidin LL-37 and contributes significantly to resistance to the peptide *in vitro*[69].

Resistance to lysozyme

Lysozyme is a bactericidal protein that is an important part of the innate defences against bacterial infection. It is a muramidase that cleaves the glycosidic linkage between *N*-acetylglucosamine and *N*-acetyl muramic acid of cell-wall peptidoglycan, causing cell lysis. The enzyme is present in many body fluids and is expressed at enhanced levels by phagocytic cells that have been stimulated by proinflammatory signals during infection[70]. The biochemical basis of the complete resistance of *S. aureus* to lysozyme was recently attributed to a membrane-bound *O*-acetyltransferase that modified the C6 hydroxyl group of muramic acid[71]. A mutant in the *O*-acetyltransferase became sensitive to lysozyme, whereas complementation with the wild-type gene restored acetylation and lysozyme resistance.

S. aureus can survive in neutrophil phagosomes

The importance of neutrophils in the defence against staphylococcal infection is reflected by recurrent infections suffered by individuals with chronic granulomatous disease (CGD), a disease caused by defects in genes encoding the subunits of NADPH phagocyte oxidase, which normally generates superoxide radicals during the respiratory burst[72,73], and is supported by studies with neutrophil-depleted mice[74,75]. *S. aureus* has several mechanisms that

contribute to its innate resistance to phagocytic killing, including interference with endosome fusion and release of antimicrobial substances[76] by factors that are dependent on the global regulator SarA. In addition, the bacterium avoids the lethal effects of oxygen free radicals that are formed during the respiratory burst by several mechanisms.

Intriguingly, the yellow carotenoid pigment of *S. aureus* contributes by scavenging oxygen free radicals[77]. A mutant defective in synthesis of pigment was more susceptible to killing by neutrophils *in vitro* and was less virulent in a mouse cutaneous-abscess infection model. Furthermore, neutrophils from a human CGD patient had a similar aberrant effect on the mutant and wild-type bacteria.

S. aureus expresses two superoxide dismutase enzymes that remove O_2^- (REF. 78). Mutants defective in these enzymes have reduced virulence in a murine abscess model, indicating a role for combating oxidative stress *in vivo*. Manganese homeostasis is also an important innate defence mechanism against oxidative stress because of the divalent cation's ability to act as a non-enzymatic superoxide dismutase[79]. Mutants defective in Mn^{2+} uptake also lacked virulence in a murine abscess model. Reactive oxygen compounds can damage proteins by oxidizing the sulphur atom of methionine (to form methionine sulphoxide). *S. aureus* expresses three methionine sulphoxide reductases[80], one of which has been shown to be important for virulence in a mouse bacteraemia infection model[81], indicating that it contributes important functions for survival *in vivo*.

Transcriptional microarray analysis of mRNA from five strains *of S. aureus* following ingestion by neutrophils indicated a large number of differentially regulated genes[82]. The number of genes affected depended on the strain, with CA-MRSA strains involving a greater number than nosocomial MRSA strains. The former were significantly more virulent in a mouse infection model and were more resistant to killing by neutrophils, indicating that their enhanced survival involves a greater number of factors and is more complex that the latter. Many known or suspected stress-response genes were upregulated immediately after ingestion, including superoxide-dismutases, catalase and carotenoid-pigment-biosynthesis genes. The leukotoxin Hlg was strongly upregulated in all strains, indicating an important role in destroying neutrophils. Unfortunately, this study did not address the role of the PVL, about which there are contradictory reports of its association with virulent CA-MRSA[35].

A similar array study has been carried out with *Streptococcus pyogenes*[83]. A smaller number of genes were differentially regulated when compared to MRSA strains, which could indicate that for *S. pyogenes* there is more emphasis on inhibition of phagocytosis, whereas *S. aureus* is more readily ingested[83]. However, there are likely to be common mechanisms for survival involving resistance to oxidants and other intracellular stresses.

S. aureus was previously regarded as an exclusively extracellular pathogen, but it is now evident that it has the ability to survive in neutrophils and macrophages, as well being able to infect non-professional phagocytes such as endothelial and epithelial cells, promoted by the fibronectin-binding proteins forming a fibronectin bridge to the α5β1 integrin on the host-cell surface[17,19].

Avoidance of phagocytosis by *S. epidermidis*

During the past twenty years, *S. epidermidis* has emerged as a leading cause of nosocomial infections[84]. It is normally a harmless commensal of the human skin, lacking the multiple virulence factors that allow *S. aureus* to invade the host and thwart the immune system[85]. It has come to prominence as a pathogen owing to its ability to colonize implanted medical devices and form biofilms[84]. The multilayered, high-density structured biofilm protects bacteria from antibiotics and the human immune system[86]. Formation of a biofilm is initiated when bacteria adhere to a biomaterial surface, a process that is mediated by surface-associated proteins such as the major autolysin AtlE[87] and the fibrinogen-binding protein Fbe[88,89]. Most cells in the biofilm are not in contact with the surface but are held together by PIA, a charged homopolymer comprising β-1,6-linked *N*-acetylglucosamine. As well as providing the glue that holds the multilayered cell complex together, PIA has recently been shown to contribute directly to avoidance of innate immunity by promoting resistance to antimicrobial defensin peptides. A PIA-defective mutant was more susceptible to killing by peptides LL-37, β-defensin 3 and dermicidin[90] and had increased susceptibility to neutrophil uptake and killing.

Some clinical isolates from device-associated infections form biofilm *in vitro* but do not express PIA[91]. Biofilm formation requires expression of the surface-located accumulation-associated protein Aap. The intact full-length protein must be cleaved by a protease to remove the N-terminal A domain, exposing the more distal repeated region, which then promotes cell–cell interactions. Interestingly, *S. epidermidis* elastase not only activates Aap, but also the neutrophil proteases cathepsin and neutrophil elastase. Therefore, components of host defence cells might actually contribute to biofilm formation and pathogenesis of some *S. epidermidis* strains.

The ability to form biofilm is also likely to be important in the pathogenesis of certain *S. aureus* infections. *S. aureus* encodes genes that are similar to the *ica* genes of *S. epidermidis*, and some strains have been shown to express PIA, particularly when growing under anaerobic conditions[92,93] and *in vivo*[94]. However, the role of PIA in avoidance of innate immunity by *S. aureus* has not been investigated, although the importance of PIA in *S. epidermidis* infection indicates that this is likely.

S. epidermidis also expresses a poly-γ-DL-glutamic acid (PGA) in the form of a surface-located macromolecule[95]. A PGA capsule is required for virulence of *Bacillus anthracis*[96]. A PGA-defective mutant grew

poorly in NaCl compared to the parental strain, indicating that *S. epidermidis* expresses PGA to aid survival in the high-osmolarity environment of the surface of skin. The expression of PGA did not affect the ability of *S. epidermidis* to form biofilm *in vitro*, but did contribute significantly to resistance to killing by antimicrobial peptides and resistance to opsonophagocytosis by human neutrophils.

Immunomodulatory molecules

Modulins. Despite being less virulent than *S. aureus*, *S. epidermidis* can causes abscesses and sepsis. This microorganism expresses a family of small amphipathic peptides with proinflammatory properties called modulins[97]. They are strong chemoattractants for human neutrophils and stimulate their activation[98]. They are more potent than the classic pathogen-associated pattern molecules such as lipoteichoic acid. The modulins of *S. epidermidis* are only expressed when the cell density is high, under the control of the accessory gene regulator (Agr) quorum-sensing system[99]. Expression of modulins could be to the organism's advantage when the cell density is high and the bacteria are resistant to phagocytosis in a biofilm by stimulating local cell damage to provide nutrients.

Protein A. As well as being anti-opsonic, protein A is also a potent immunomodulatory molecule because of its ability to bind to the V_H3 region that is adjacent to the antigen-binding domain of IgM molecules exposed on the surface of B lymphocytes (FIG. 4). Those cells bearing V_H3 IgM are stimulated to proliferate and undergo apoptosis, leading to depletion of a significant proportion of the repertoire of potential antibody-secreting B cells in the spleen and bone marrow[100]. The structural basis of the interaction is known, with this remarkable molecule binding to the V_H3 region through helices II and III, in contrast to the helix-I-binding domain for the Fc region of IgG[101–103].

Enterotoxins and TSST-1. *S. aureus* secretes toxins that act both as superantigens when expressed systemically and cause an emetic response when ingested[104]. The superantigen activity is specified by a distinct domain of the protein from that which determines the emetic response, although the mechanism of the latter is obscure. Some strains also express the superantigenic toxic shock syndrome toxin-1 (TSST-1), which came to prominence through its association with cases of superabsorbent tampon-associated toxic shock syndrome (TSS) in the early 1980s[105]. Clinical isolates of *S. aureus* often express several superantigens. Superantigens have the ability to bind the exterior surface of the MHC class II protein on the surface of antigen-presenting cells and link it to T-cell receptors on the surface of a T helper cell[10,106,107] (FIG. 4). Binding occurs without the requirement for the MHC class II molecule to present an antigenic peptide to a suitable T-cell receptor. Each type of enterotoxin recognizes a specific subset of variable Vβ chains of T-cell receptors and therefore has characteristic Vβ signatures[108]. Also, there are diverse binding sites

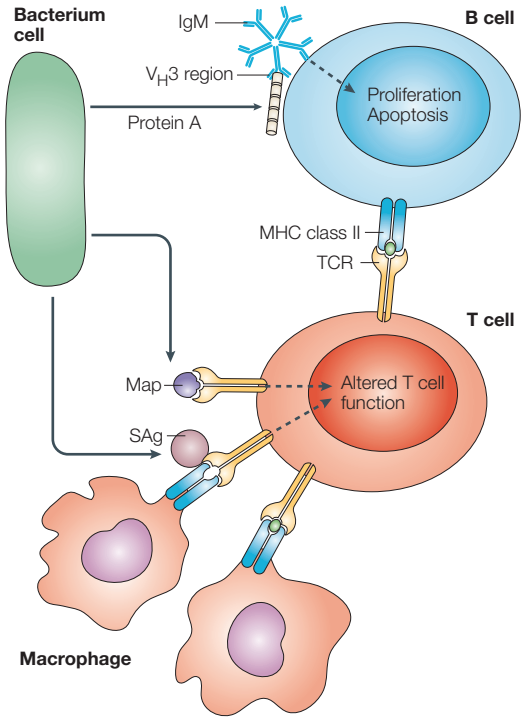

Figure 4 | **Mechanisms of immunosuppression mediated by *Staphylococcus aureus*.** This figure illustrates examples of immunomodulatory molecules used by *S. aureus* to alter the host immune response, including the superantigens (sAgs) enterotoxins and toxic shock syndrome toxin-1 that bind the MHC class II receptor to T-cell receptors; protein A, which binds immunoglobulin M (IgM) V_H3 on B cells; and the MHC class II analogue protein Map, which binds the T-cell receptor (TCR).

on the MHC class II protein[109]. Up to 30% of T cells can become activated, leading to proliferation, with the high levels of cytokines expressed causing TSS[105,106].

Expression of superantigens in the infected host also prevents development of a normal immune response[106]. Antigen-specific T cells fail to proliferate in response to antigens that are presented normally by MHC class II due to a phenomenon called anergy[110]. The consequence is immunosuppression due to failure to induce an appropriate antibody response. This also is likely to prevent development of antibodies to superantigen toxins themselves. Lack of antibody to the superantigen is a common characteristic of TSS patients[105].

Interestingly, *S. pyogenes* expresses superantigens that are structurally and functionally related to those expressed by *S. aureus*[106,111]. Indeed, infections by this organism can result in TSS with similar symptoms to the *S. aureus* disease. It is therefore likely that superantigen-producing *S. pyogenes* is also immunosuppressive.

The MHC class II-analogue protein Map (also called Eap, see above) comprises six repeated domains of 110 residues, each containing a 30-residue motif with strong homology to the peptide-binding groove of the MHC class II β-chain[112]. It can bind to the T-cell receptor on T cells, resulting in an alteration

of T-cell function (FIG. 4). Map also causes a reduction in T-cell proliferation, which results in a reduced delayed-type hypersensitivity response[58]. Map causes a shift from a Th1 response to a Th2 response, which affects cell-mediated immunity and might explain the more rapid clearance of a Map-deficient mutant compared to the wild-type strain from internal abscesses in infected mice. Map also shows two distinct effects on peripheral blood mononuclear cells (PBMCs)[113]. At low concentrations, it stimulates proliferation of PBMCs, whereas at high concentrations the protein inhibits the proliferative effect of the superantigen TSST-1 and stimulates apotosis of B and T cells. The receptors recognized by Map in causing these effects are unknown.

Subversion of the humoral immune response

S. aureus cells can bind to platelets and stimulate their rapid activation. Platelet activation and subsequent aggregation is thought to be an important facet of the pathogenesis of endovascular infections, leading to endocarditis[114,115]. The bacteria grow in platelet-fibrin thrombi, where they escape the attention of neutrophils. An infected thrombus on a heart valve is potentially life-threatening, and difficult to treat without surgical intervention. The fibronectin-binding proteins are the dominant surface proteins causing platelet activation for bacterial cells in exponential growth, and ClfA is crucial for cells from the stationary phase of growth[42,116,117]. In both cases, contact with the resting platelet is made through a bridge to the nascent low-affinity GPIIb/IIIa integrin provided by fibronectin or fibrinogen. In addition, for activation to occur, the binding of antibodies specific for the surface protein is required. Bound IgG engages the FcγRIIa receptor on the platelet surface. This causes clustering of receptors and stimulation of intracellular signalling, leading to activation and aggregation. Almost all individuals have low levels of antibodies to surface proteins of *S. aureus*, including ClfA and fibronectin-binding proteins, and these are exploited by the bacterium to promote platelet activation and disease pathogenesis.

Prospects for vaccination

An individual who has suffered from a *S. aureus* infection is usually not protected from a subsequent infection. This is because the host is prevented from mounting a strong antibody response, and immunological memory is compromised by the immunosuppressive activities of superantigens. However, there is mounting evidence that it is possible to generate a robust antibody response to highly purified surface components of *S. aureus* such as capsular polysaccharide[54], the collagen-binding protein Cna and the fibrinogen-binding protein ClfA[45,118]. In each case, active immunization has been shown to protect laboratory animals from infection. Furthermore, immunization of haemodialysis patients with staphVAX, comprising type 5 and type 8 capsular polysaccharide conjugated with *Pseudomonas aeruginosa* exotoxin A toxoid, was successful in reducing the incidence of infection in these vulnerable patients over an 8-month period[119]. A second clinical trial is underway to evaluate the benefit of a booster at 8 months.

Passive immunization of mice with immunoglobulin obtained from human donors with high titres of anti-ClfA antibodies protected against a lethal intravenous dose of the pathogen[45,120]. Immunoglobulin with high titres of antibodies recognizing ClfA of *S. aureus* and SdrG of *S. epidermidis* is currently undergoing a Phase III trial with low-birth-weight neonates, who are particularly susceptible to staphylococcal infection.

Protective immunity in experimental infections was also obtained with a humanized monoclonal antibody directed against the surface protein ClfA[121,122]. The monoclonal antibody also showed therapeutic efficacy in combination with vancomycin when used to treat rabbit endocarditis caused by an MRSA strain. A humanized version is currently undergoing a Phase II trial to treat bacteraemia patients.

Concluding remarks

In this review, I have outlined the multiplicity of mechanisms employed by *S. aureus* to thwart innate and induced immunity. It is particularly instructive to compare *S. aureus* with its close relative *S. epidermidis*, which essentially relies on surface polymers and its ability to form biofilm to survive in the host.

The recently emerged CA-MRSA strains seem to have enhanced virulence compared to nosocomial MRSA strains. This is a major cause for concern because of its propensity to cause rapidly fatal necrotizing pneumonia in healthy individuals. It was originally suggested that carriage of the phage-specifying PVL is a signature of CA-MRSA, with responsibility for enhanced leukotoxicity. However, this cannot always be the case because of the lack of association between possession of *pvl* and leukotoxicity in a diverse collection of CA-MRSA strains[35]. The ubiquitous leukotoxin Hlg is strongly induced in neutrophils and is likely to be important in lysing neutrophils.

Survival in neutrophils is clearly a complex affair, with a large number of genes being differentially regulated. Unravelling the regulatory circuits involved and discovering the mechanisms of enhanced survival in CA-MRSA is a challenge for the future.

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