

Immunopathogenesis of *Staphylococcus aureus* pulmonary infection

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Abstract *Staphylococcus aureus* is a common human pathogen highly evolved as both a component of the commensal flora and as a major cause of invasive infection. Severe respiratory infection due to staphylococci has been increasing due to the prevalence of more virulent USA300 CA-MRSA strains in the general population. The ability of *S. aureus* to adapt to the milieu of the respiratory tract has facilitated its emergence as a respiratory pathogen. Its metabolic versatility, the ability to scavenge iron, coordinate gene expression, and the horizontal acquisition of useful genetic elements have all contributed to its success as a component of the respiratory flora, in hospitalized patients, as a complication of influenza and in normal hosts. The expression of surface adhesins facilitates its persistence in the airways. In addition, the highly sophisticated interactions of the multiple *S. aureus* virulence factors, particularly the α -hemolysin and protein A, with diverse immune effectors in the lung such as ADAM10, TNFR1, EGFR, immunoglobulin, and complement all contribute to the pathogenesis of staphylococcal pneumonia.

Keywords Airway · Lung · Epithelial · *Staphylococcus aureus* · Virulence · Signaling

Introduction

Staphylococcus aureus is a ubiquitous pathogen associated with a wide range of infections affecting the respiratory

tract that range from asymptomatic colonization to fulminant necrotizing pneumonia. Despite extensive epidemiological studies that include whole-genome sequencing, it remains unclear exactly which staphylococcal properties are required for invasive infection versus status as a part of the commensal flora. While in the past the analyses of *S. aureus* virulence mechanisms have focused almost entirely upon bacterial factors, it has become increasingly apparent that the nature of the host immune response that is evoked by these organisms is a significant determinant in the ultimate outcome of the infection. This has become especially apparent in the ongoing epidemic of MRSA infection in the USA due to the USA300 clone of *S. aureus* that has been associated with over 18,000 deaths in 2005 [1]. These strains elicit an intense pro-inflammatory response often associated with high morbidity and mortality [2, 3].

The success of *S. aureus* in general as a respiratory pathogen, and the USA300 strains specifically, may be attributed to several factors: substantial metabolic capabilities; genetic flexibility, both the ability to acquire and to mutate specific genetic elements, and the unique ability to exploit the immune responses that are evoked. In this review, we will examine a few of the important metabolic capabilities of these organisms that enable them to flourish in the airways focusing specifically on the genes involved in iron acquisition, which is a critical factor for a human pathogen. We will review the data supporting the contribution of USA300-associated virulence factors in pathogenesis and discuss the relevance of murine models in understanding the pathogenesis of staphylococcal pneumonia in humans. As the interactions of staphylococci and the immune system are likely to be the key factors in the pathogenesis of pneumonia, the many *S. aureus* virulence factors that interact directly with immune components (IgG,

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complement, T cell signaling) or activate specific immune cascades (type I IFN signaling, inflammasome activation) will be reviewed in some detail.

Epidemiology of staphylococcal pneumonia

Historically, staphylococcal pneumonia was an especially virulent infection of young infants and the elderly, particularly following influenza infection [4, 5]. A major cause of concern has been the emergence of methicillin-resistant strains of *S. aureus* (MRSA). Resistance to methicillin is encoded by the *mec* element [6]. MRSA has been typically associated with hospital-acquired strains that infect individuals with preexisting conditions. However, the early part of the twenty-first century saw the emergence of community-acquired strains of MRSA (CA-MRSA) that infect otherwise healthy individuals. The majority of cases of MRSA are caused by a single clonal group USA300. This was initially reported in athletes with close body contact to one another or other close-quartered situations such as in correctional facilities [7–11]. Pneumonias caused by USA300 have been steadily increasing over the past 10 years [12–14]. The typical pathology associated with fatal MRSA pneumonia consists of loss of alveolar architecture, hemorrhage, and consolidation of the lung parenchyma [16, 17] (Fig. 1). With the dissemination of the USA300 clones across the USA in the last decade [1, 6], MRSA pneumonia has become much more widespread [12, 13, 15], responsible for a significant number (13%) of the over 18,500 deaths attributed to MRSA in 2005 [1]. Not only did this group include the expected immunocompromised patients, but a large number were also previously healthy individuals in close contact with infected individuals [7–11].

Animal models of pneumonia have shown that USA300 *S. aureus* is more virulent than other strains of MRSA [3, 18, 19]. There is substantial debate as to which virulence factors are responsible for the increased lethality, and it is

likely that the success of the pathogen is due to the co-expression of several virulence determinants. At the genomic level, USA300 contain mobile elements with unique coding sequences, such as the arginine catabolic mobile element that appears to have been transferred from *Staphylococcus epidermidis*. This may confer a growth advantage in systemic infection [20–22] and effect the expression of other virulence factors [23]. A typical feature of MRSA strains acquired in the community is a large (20%) portion of the genome that contains unique genomic elements from horizontal transfer. These genomic elements include pathogenicity islands and exotoxins that cause host damage as well as counteract the immune response [24]. USA300 strains express the toxin Pantone–Valentine leukocidin (PVL) which, along with the increased expression of core genome virulence factors such as α -hemolysin and phenol-soluble modulins [19], appears to contribute to overall virulence. Perhaps the single most important virulence factor is the α -hemolysin which contributes significantly to the pathogenesis of pneumonia in murine models of infection through mechanisms detailed below [25–27]. However, it is important to note that the susceptibility of mice to staphylococcal infection is significantly less than that of humans due to the inability of *S. aureus* to release iron from murine hemoglobin [28]. Thus, while the data generated in mouse models have been very important, particularly in understanding the genetics of the host response to infection, the data that delineate the importance of specific virulence factors in the pathogenesis of pneumonia are subject to considerable debate [29].

Regulation of MRSA virulence factors in pneumonia

An important property of staphylococci is their metabolic flexibility and ability to adapt to environmental pressures. Although typically considered extracellular aerobic pathogens,

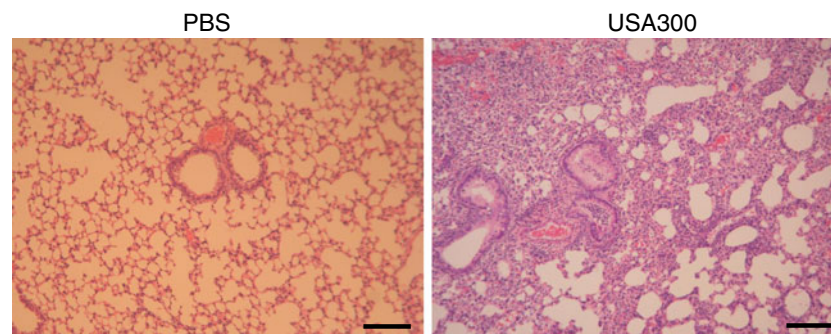


Fig. 1 Pathology of USA300 lung infections. H&E sections of C57Bl6/J mouse lung. Mice were infected with 2×10^7 cfu *S. aureus* USA300 for 24 h. In the uninfected lung, two bronchioles and a blood vessel are visible, along with clear alveolar architecture. The USA300-infected

animal shows loss of alveolar architecture, necrosis, hemorrhage, infiltration of immune cells, and consolidation of the lung parenchyma. Scale bars, 100 μ m

they also grow and disseminate intracellularly [30–32] and tolerate anaerobic conditions [33], all of which are relevant to pulmonary infection.

A number of regulatory systems are present in the genome of *S. aureus* to coordinate virulence factor expression. One of the more important of these is the accessory gene regulator (Agr) system which coordinates the expression of both surface proteins and secreted toxins, divergently (such as the positive regulation of α -hemolysin and the negative regulation of protein A expression) [34]. The regulon encodes a quorum sensing peptide (AgrD) and its export protein (AgrB), a sensor histidine kinase (AgrC), and a response regulator (AgrA) [35]. AgrA autoregulates by binding its own promoter. Regulation by Agr is accomplished by an RNA molecule, RNAIII. The expression of both Agr and RNAIII has been shown to be higher in USA300 strains as well as community- versus hospital-acquired infection, which may provide some rationale for the prevalence and severity of this clonal group [3, 19, 36]. Agr mutations are also found in clinical isolates [37]. Although Agr and its regulon are dispensable for inflammatory signaling in the lung, they are necessary for invasive pulmonary infection [38], and the role of Agr in animal models of pneumonia has been well documented [25, 38, 39].

Intracellular lifestyle of an extracellular organism

One role Agr plays in pathogenesis relates to the intracellular life stage of *S. aureus*. *S. aureus* persists intracellularly within a variety of cells types, including epithelial cells, macrophages, and neutrophils (Figs. 2 and 3) [30–32]. The expression of Agr is required for these intracellular organisms to escape from endosomes. Induction of *agr* expression has been observed just prior to endosome release and no doubt is correlated with the expression of toxins that facilitate

its escape and contribute to virulence in the lung (Fig. 2) [40, 41]. Although epithelial cells are considered non-phagocytic, small numbers of *S. aureus* have been observed in some epithelial cells lines [40, 42–44] and associated with apoptosis (mammary epithelial cells) [32]. In phagocytic cells following uptake, *S. aureus* can persist inside vacuoles for up to 4 days before the escape into the cytoplasm and the induction of cell lysis (Fig. 3) [45]. *S. aureus* can survive for prolonged periods of time in neutrophils and in some cases divide within dying cells [31], an effect which may contribute to a systemic dissemination of the organisms [46]. Staphylococcal isolates from the community (CA-MRSA) display greater propensity to avoid neutrophil killing and also cause more lysis of neutrophils compared with strains from hospital infections [47]. An appreciation for the potential of staphylococci to assume these diverse lifestyles is critical for the development of vaccines as expecting antibodies alone to clear these organisms, which have an intracellular niche, seems unlikely to be successful.

Contribution of specific virulence factors to the pathogenesis of pneumonia

The coordinated expression of many staphylococcal virulence components is required for the establishment of a successful infection. This includes surface proteins (such as the microbial surface components recognizing adhesive matrix molecules, MSCRAMMS) to establish colonization, the expression of iron acquisition systems for proliferation in mammals where iron is tightly sequestered and secreted, and surface components to elude innate and adaptive immunity. There are several toxins whose activity appears to be primarily the lysis of target cells, but increasing data have documented that many staphylococcal virulence factors act by triggering pathological immune response as

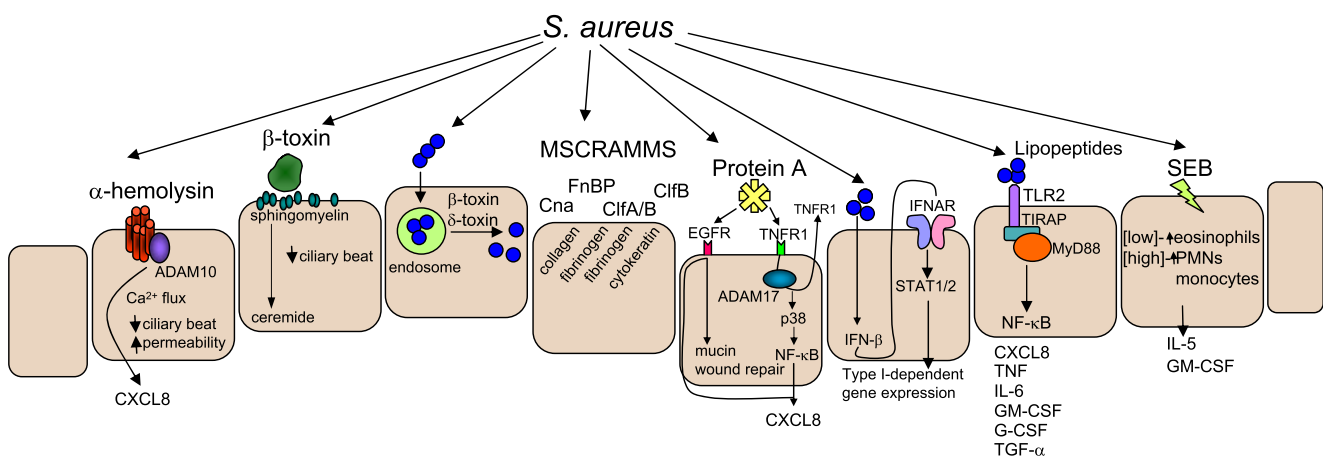


Fig. 2 Virulence factors and host signaling cascades activated by *S. aureus* in the airway epithelium. Depicted are some of the virulence factors mentioned in the text and their effects on the host epithelium. See text for details

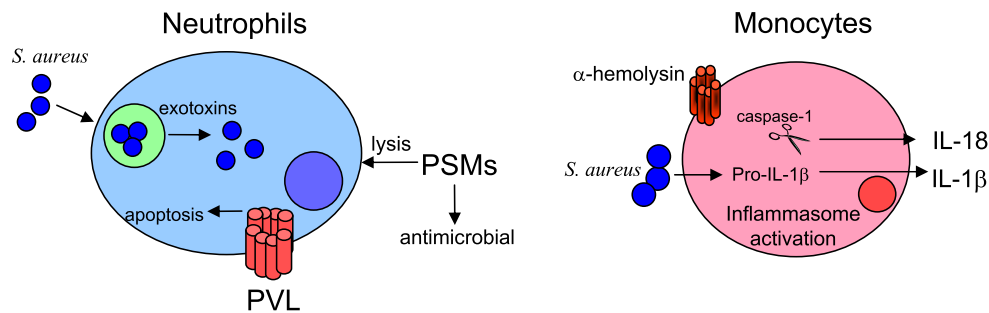


Fig. 3 *S. aureus* interactions with neutrophils and monocytes. *S. aureus* is able to persist intracellularly in neutrophils for days; their escape from vacuole compartments is reliant upon exotoxin production. Both PVL

and PSMs have shown an ability to lyse neutrophils. In monocytes, interaction with *S. aureus* and the action of α -hemolysin lead to the activation of the inflammasome

well as avoid immune clearance by thwarting complement and IgG-mediated phagocytosis.

Iron acquisition

Iron is essential for both prokaryotic and eukaryotic metabolic pathways, and as such, numerous mechanisms have evolved to assure its acquisition from the environment. Mammals normally express a number of high-affinity binding proteins such as transferrin and hemoglobin to sequester iron for storage and prevent access by invading pathogens. *S. aureus* produces siderophores that bind free iron for utilization by the cell [48–50]. *S. aureus* express two heme acquisition systems that are able to utilize hemoglobin as an iron source and a transport heme, heme transport system (Hts) and iron-regulated surface determinant (Isd) groups of proteins. Both of these systems contribute to virulence in mouse models of infection [51, 52]. One disadvantage of working with murine models of staphylococcal infection is that *S. aureus* has greater affinity for human as compared with murine hemoglobin. Mice that express human hemoglobin are more susceptible to systemic *S. aureus* infection [28]. *S. aureus* expresses a homologue of the iron regulatory protein ferric uptake regulator (Fur) that senses iron limitation and regulates a number of virulence factors, including α -hemolysin and PVL [53]. In a murine pneumonia model, Fur was required for full virulence, with *fur* mutants expressing increased levels of exotoxins and reduced immunomodulatory proteins (Fig. 4). These immunomodulatory proteins include factors that decrease the organisms' ability to avoid neutrophil killing as well as evade the complement cascade.

Microbial surface components recognizing adhesive matrix molecules

The pathogenesis of staphylococcal pneumonia is usually initiated by aspirated organisms that first colonize the nasal

cavity. There are substantial epidemiological data confirming the greater likelihood of colonized patients to develop invasive infection than those who are non-colonized, and the “de-colonization” of such patients prior to surgery is common practice and cost-effective [54–56]. Thus, there is significant interest in identifying the critical staphylococcal proteins that mediate nasal colonization. Magnus Hook first coined the term MSCRAMMS to describe the numerous surface proteins that specifically recognize host components, especially those relevant to soft tissue infection, such as collagen, fibrinogen, and fibronectin (Fig. 2) [57]. These have been extensively analyzed in models of murine infection, trying to identify a “critical” factor for staphylococcal adherence. However, the obvious redundancy in the binding capabilities of the staphylococcal surface components suggests that a single entity is unlikely to emerge as “THE” adhesin required for the pathogenesis of either nasal colonization or invasive infection.

The collagen-binding protein of *S. aureus* (encoded by the *cna* gene) binds collagen substrates and tissues. While the involvement of collagen binding in virulence has been shown in models of endocarditis and keratitis [58, 59], with respect to the airway, only an association with PVL-positive isolates that cause increased pulmonary pathology has been observed [60]. Fibronectin-binding proteins are

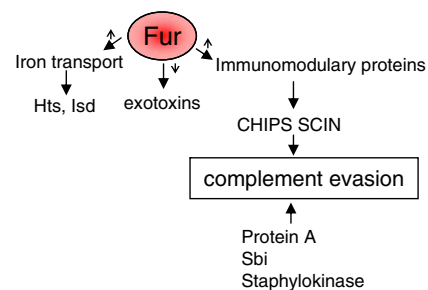


Fig. 4 Iron-dependent regulation of virulence by Fur. In the presence of iron, Fur is able to increase the expression of iron transport systems (Hts, Isd) and the complement evasion proteins CHIPS and SCIN while decreasing exotoxin production. Other proteins known to aid in complement evasion are also shown

involved in the adherence of *S. aureus* to undifferentiated human airway epithelial cells. This does not extend to differentiated cells, where binding is reduced [61]. The ability of *S. aureus* to bind fibronectin does not facilitate nasal colonization, but does mediate internalization into epithelial cells [62]. Lung injury, as well as bacterial load, was increased in a rat model of pneumonia with a fibronectin null strain [63]. Adherence to fibrinogen by *S. aureus* is accomplished by clumping factors A and B. Clumping factor A (ClfA) plays an antiphagocytic role in both neutrophils and macrophages [64, 65]. ClfA is important in animal models of arthritis, sepsis, and endocarditis, but not in models of respiratory tract infection [66–69].

Clumping factor B

Clumping factor B (ClfB) is involved in respiratory infection, mediating the attachment to cytokeratins on nasal epithelial cells. Mutants lacking ClfB reduce the ability of *S. aureus* to adhere [70]. Similarly, a sortase mutant lacking the gene that links surface proteins during cell wall assembly via cleavage of an LPXTG motif [71] was also reduced in nasal colonization. Immunization with ClfB or treatment with monoclonal ClfB antibody both decreased the ability of *S. aureus* to persist in the nares [62]. The role of ClfB in nasal colonization was further confirmed in human subjects as those infected with a *clfB* strain cleared *S. aureus* faster (median clearance, 3 days) than the wild-type organism (7 days) [72]. Following nasal colonization, via ClfB or other staphylococcal ligands, the participation of several conserved gene products, both surface components and secreted toxins, all contribute to invasive infection.

α -Hemolysin

Following aspiration into the lower respiratory tract, the secretion of multiple cytolytic toxins has been thought to contribute to pathology, as reviewed below. It is important to note that many studies have relied upon recombinant or purified toxins delivered at concentrations unlikely to be achieved in vivo. Studies comparing mutant versus wild-type strains seem more likely to reflect the impact of specific virulence factors expressed by *S. aureus*.

The α -toxin or α -hemolysin (encoded by *hla*) of *S. aureus* is a major pore-forming toxin that assembles into heptamers at the cell membrane to create small pores sufficient for the movement of ions [73]. Hla expression is Agr-dependent and increased upon interaction with epithelial cells, infection in vivo, and is higher in USA300 strains

[3, 19, 32, 74]. In airway epithelial cells α -hemolysin has been associated with calcium fluxes, pro-inflammatory signaling [75], and alteration of ciliary beat frequency of cells (Fig. 2) [76]. α -Hemolysin binds to the metalloproteinase ADAM10, which is necessary for the toxin to cause cytotoxicity at low concentrations [77]. The interaction of ADAM10 with α -hemolysin activates a number of intracellular signaling events. The application of α -hemolysin to perfused lungs results in increased vascular leakage and damage, and increased permeability of airway epithelial cells [74, 78–80]. Infection with α -hemolysin null strains is associated with significantly lower mortality compared with wild-type strains in pneumonia models [25–27], associated with reduced pulmonary inflammation, neutrophil influx, and bacterial counts. The mechanism of α -hemolysin toxicity is associated with the activation of pyroptosis, induction of the inflammasome via caspase-1 activation, its targeting pro-IL-1 β , and the generation of IL-1 β and IL-18, highly pro-inflammatory cytokines (Fig. 3). This has been well described in immune cells and is likely to be important in other cells in the infected lung [81–86]. The lack of pathology associated with murine infection with *hla* mutants is consistent with studies demonstrating a correlation between α -hemolysin expression levels and virulence in the lung [87]. The use of α -hemolysin as a vaccine target for pneumonia has shown promise. Immunization of mice with mutant forms of α -hemolysin that cannot form pores protect mice from lethal pulmonary challenge [87]. Mice also show a decreased bacterial burden when immunized with an inactive α -hemolysin variant. Antibodies raised against α -hemolysin can be transferred and subsequently protect naive mice, while the treatment of epithelial cells in vitro with α -hemolysin antibody reduces epithelial injury [87]. Monoclonal antibodies raised against α -hemolysin as well as β -cyclodextrin compounds (that have symmetry to α -hemolysin) are also effective in preventing mortality in mice and in decreasing epithelial cell injury [88, 89].

β -toxin

The contribution of the beta and delta toxins of *S. aureus* to pulmonary infection is less well studied. As both target erythrocytes, they may contribute to the iron-scavenging mechanisms for the organism, but that has not been demonstrated experimentally. The β -toxin of *S. aureus* (expressed in 96% versus 56%) bovine versus human carrier isolates [90] is a sphingomyelinase that targets the membranes of host cells to generate ceramide (Fig. 2) [91]. β -toxin lyses a variety of cells types, including red blood cells, monocytes, lymphocytes, and neutrophils [92–94]. In airway epithelial cells, purified beta toxin has been shown to inhibit ciliary beat frequency [95]. Hayashida et al. [96]

observed a β -toxin-associated pathology of the lung with increased airway permeability as well as a neutrophilic response, which was decreased in infections with β -toxin null strains. Instillation of a recombinant toxin caused comparable loss of alveolar architecture as in *S. aureus*-infected mice. The effect of beta toxin in the lung may not be direct as injury was ameliorated in neutropenic mice, indicating that the immune response played a significant role in injury.

Phenol-soluble modulins

Phenol-soluble modulins (PSMs) are small peptides originally isolated in the phenol-soluble lysate during extraction from *S. epidermidis* [97]. They are referred to as modulins due to their ability to induce cytokine release. The PSMs identified in USA300 MRSA [98] include four short (alpha, ~20 amino acids) and two longer (beta, ~40 amino acids) types that are produced in greater amount than in other *S. aureus* strains [19]. The PSMs are sensed by the formyl peptide 2 receptor and lead to the recruitment, activation, and lysis of neutrophils and play a role in virulence in skin models of infection (Fig. 3) [99, 100]. PSMs also possess antimicrobial activity against other species such as *Streptococcus pyogenes* [101]. This antimicrobial function may be a factor that has influenced the prevalence of USA300 within the community. It has also been proposed that PSMs impact upon biofilm development, as seen in *S. epidermidis*. Due to their surfactant-like properties, they may be involved in biofilm structure as well as detachment and dissemination [102]. Since the initial identification of PSMs in *S. aureus*, another PSM has been identified in the methicillin resistance locus (*mec*). This *mec*-PSM also plays a role in skin models when the expression of the other PSMs is low [100]. The role PSMs play in pneumonia models of infection have yet to be determined, but an increased PSM expression has been detected in epidemic (USA300) isolates. The δ -toxin of *S. aureus* is a 26-amino acid cytolysin encoded by RNAIII [103] and is similar to the α -PSMs. δ -toxin is lytic to a range of cell types and structures such as erythrocytes, bacterial protoplasts, and lysosomes [104]. δ -toxin acts synergistically along with β -toxin to facilitate staphylococcal escape from endosomes in airway epithelial cells (Fig. 2); however, its role in vivo is yet to be determined [42].

Panton–Valentine leukocidin

PVL is a toxin encoded by two co-transcribed genes, *lukF-PV* and *lukS-PV*, carried on a bacteriophage [105]. PVL forms octomeric protein pores at the cell membranes of

neutrophils and macrophages and is specific to humans and rabbits, but not mice or non-human primates [106–112]. PVL causes the apoptosis of neutrophils via caspases 3 and 9 (Fig. 3) [113], and a role for TLR2 in causing inflammation in the lung by PVL has been observed [114]. TLR2 null mice displayed reduced production of IL-1 β , TNF, and KC in the lung after the administration of PVL [114], and the toxin alone has dermonecrotic capabilities [115].

While PVL is epidemiologically linked to invasive pneumonias caused by USA300 strains, there has been significant controversy over its role in pathogenesis [5, 21, 29, 116–118]. It is clearly not required for severe CA-MRSA infections in humans [119]. PVL has shown variable roles of involvement in murine models of pneumonia [26, 120–123]. Since PVL has specificity for neutrophils from humans and rabbits [106–112], rabbit models of USA300 do show a necrotizing pneumonia that suggests the contribution of PVL to pathology [112] with lung necrosis, pulmonary edema, and other outcomes consistent with the necrotizing pneumonia seen in humans. Rabbits infected with *pvl* strains showed decreased mortality, and complementation was able to restore virulence to the *pvl* strain. However, the extremely high inocula used in these studies and the length of infection have led many to remain skeptical of PVL being important in the pathogenesis of acute MRSA pneumonia in humans [29].

Staphylococcal gene products that activate immune signaling

In addition to the immunological effects of the hemolytic toxins, other *S. aureus* proteins activate T and B cells and numerous other immune effectors. These include T cell activation via superantigens (TSST and SEA/SEB) [124–126], complement evasion [127], polyclonal B cell and platelet activation via protein A [128, 129], immunoglobulin sequestration by *S. aureus* IgG-binding protein (Sbi) and protein A [130–132], and activation of the inflammasome by α -hemolysin [83, 84]. The production of the staphylococcal superantigens that are able to activate clones of T cells through V beta recognition alone has been well characterized for decades (Fig. 5). It is unclear whether this is a direct cause of pulmonary pathology, but more likely, the massive immunoactivation caused by *S. aureus* superantigen production is likely to contribute to pulmonary pathology. Superantigens evoke a massive immune response, stimulating T cells by cross-linking T cell receptors with MHC class II molecules on target T cells [124–126], resulting in T cell proliferation, cytokine production, and apoptosis [133]. Macrophages are accessory cells in this process [134], although a direct interaction with superantigens can induce

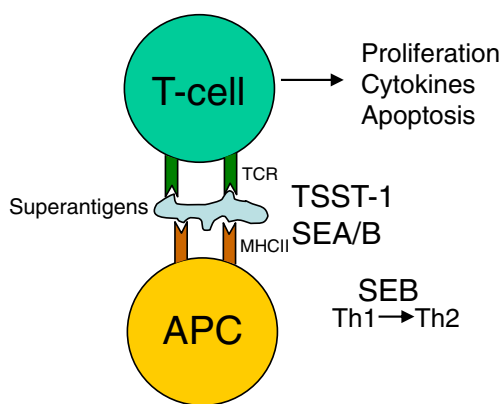


Fig. 5 *S. aureus* superantigens. *S. aureus* superantigens cause T cell proliferation, cytokine production, and apoptosis by simultaneously cross-linking MHCII molecules on antigen presenting cells with T cell receptors

cytokine production [135]. Two groups of superantigens produced by *S. aureus* are the toxic shock syndrome (TSS) toxin (TSST-1) and the enterotoxins.

TSST-1 is the toxin responsible for TSS, a syndrome defined by fever, rash, hypotension, and multisystem involvement, initially associated with the use of highly absorbent tampons during menstruation [136–138] but is now well recognized in many patient populations [139]. Cases that involve the respiratory tract have been reported, including pneumonia, post-influenza infection, pharyngitis, and laryngotracheitis [140–144], although it is unclear what direct role TSST-1 has in pathogenesis. The enterotoxins (SE—A and B) of *S. aureus* are a group of structurally related toxins [145–147] suggested to play a role in the allergic response to infection in model systems [148]. A correlation exists between the levels of IgE antibodies to enterotoxin and asthma [149]. A similar observation is seen between enterotoxin IgE antibodies and higher eosinophil cationic protein, a marker for asthma and rhinitis as well as IgE levels for dust mites [150], suggesting that the presence of enterotoxin can mediate or potentiate the inflammation associated with asthma. Cytokine release by epithelial cells in response to SEA has also been observed [151].

Analogous to SEA, SEB also induces an inflammatory response in the lungs (Fig. 2). Consistent with SEA and SEB potentiating an allergic response, SEB shifts the T cell response from Th1 to Th2 and suppresses Treg activity to cause a state of persistent inflammation [152]. At low doses, SEB induces cytokine production in the lungs and leads to increases in eosinophil counts, while higher doses lead to increases in neutrophils and monocytes and are capable of producing an interstitial pneumonia [133, 153–156]. SEB also enhances the inflammation caused by allergens [157]. The interaction of human epithelial cells with SEB results in the production of IL-5 and GM-CSF [158].

Evading of complement

The complement system performs a variety of functions to clear invading pathogens. *S. aureus* expresses a number of proteins to circumvent this response [127]. *S. aureus* expresses IgG-binding proteins that are also able to bind complement. Protein A (discussed below) is an IgG-binding protein that can bind to C1q, which is highly expressed on platelets [159]. A second IgG-binding protein, Sbi, binds to the Fc region of IgG and aids in avoiding neutrophil-mediated opsonophagocytosis (Fig. 4) [130, 160]. Sbi is also able to bind complement, interacting with the C3 portion of the cascade [161]. In vivo models have shown that Sbi promotes survival in blood [130].

Two additional complement evasion proteins are staphylococcal complement inhibitor (SCIN) and chemotaxis inhibitory protein of staphylococci (CHIPS; Fig. 4). Both SCIN and CHIPS are present on an immune evasion cluster located on a bacteriophage, which also contains staphylococcal enterotoxin B (described below) and staphylokinase. Staphylokinase activates human plasminogen to plasmin at the bacterial cell surface, creating a surface-bound protease capable of digesting IgG and complement [162]. Staphylokinase also releases α -defensins from neutrophils and interacts with α -defensins directly to inhibit bactericidal activity [163]. This immune evasion cluster is highly prevalent in carrier strains (90%), although it is not essential for nasal colonization [164]. SCIN is produced by the majority of strains. SCIN prevents the formation of the C3 convertases, resulting in a reduced phagocytosis and killing by neutrophils [165]. CHIPS is also capable of complement evasion via binding of the C5a receptors. CHIPS inhibits neutrophil and monocyte chemotaxis by interacting with the formylated peptide receptor [166, 167]. There are a number of other complement evasion proteins in the genome of *S. aureus*, including homologues of SCIN and CHIPS [168]. As with all of the complement evasion proteins, a direct role in pneumonia is yet to be established.

Protein A

Protein A (SpA) is perhaps the most complex of the staphylococcal components in terms of its multiple interactions with host immune signaling. Protein A (SpA) is an abundant surface protein of *S. aureus* expressed by virtually all strains that is shed during growth [169]. SpA contains an N-terminal signal sequence followed by five repeated domains (E, D, A, B, C) that bind IgG [131, 132], a property which is commercially utilized in biochemistry. SpA is anchored to the surface of the bacterial cell through a carboxy terminal LPXTG motif and the action of a sortase enzyme [71, 170]. The carboxy

terminus of SpA contains variable numbers of a 24-bp repeat sequence known as the Xr region. The Xr region of SpA is successfully used in the typing of *S. aureus* isolates and has high concordance with genomic microarray data [171, 172]. This region is associated with a high mutational rate in *S. aureus* isolates from the airways of patients with cystic fibrosis [173]. The Xr region likely participates in immune signaling [174], although the mechanism remains to be defined.

S. aureus also induces the expansion of B cells through the binding of protein A. Protein A interacts with the Fab portion of V(H)3-type B cell receptors, reducing the adaptive immune response [175]. This induces polyclonal nonspecific B cell expansion and is thought to interfere with the use of staphylococcal mutants in vaccine development. Mutant forms of protein A unable to interact with B cells have shown promise as vaccine candidates in murine models of infection [176].

The role of protein A in the virulence of *S. aureus* has been demonstrated in models of sepsis and pneumonia [25, 176–178]. Neonatal mice infected with a *spa* mutant show reduced numbers of bacteria in both the lung and spleen, in addition to a significant reduction in pro-inflammatory signaling [177]. The *spa* strain was also significantly attenuated in its ability to activate signaling (CXCL8) in airway epithelial cells. In an adult model of murine staphylococcal pneumonia, mortality was significantly less with a *spa* mutant compared with the wild-type strain [25]. Several mechanisms have been proposed for the role of protein A in virulence, including its interaction with TNFR1 and epidermal growth factor receptor (EGFR; Fig. 2).

TNFR1 signaling by SpA

The TNF receptor (TNFR1) is expressed on many different cell types and initiates the activation of the pro-inflammatory and anti-apoptotic TNF cascade [179]. In the lung, epithelial TNFR1 is localized on the cell surface before being shed into the extracellular milieu after exposure to *S. aureus* through the action of ADAM17 (which is also activated by protein A) [177]. Protein A directly binds to TNFR1 via its IgG-binding domains, mimicking the TNF–TNFR1 interaction [177, 180]. This TNFR1–protein A interaction induces CXCL8 via TRAF2/p38 mitogen-activated protein kinase (MAPK) and NF- κ B [181].

Protein A–TNFR1 responses play an important role in pathogenesis. *Tnfr1*^{−/−} mice display significantly reduced levels of pneumonia and bacteremia when infected with *S. aureus* compared with wild-type mice. The levels of pneumonia and bacteria observed in TNFR1 null mice with

the wild-type strain are comparable to those observed with the *spa* strain [177]. Wild-type mice infected with the *spa* strain as well as *Tnfr1*^{−/−} mice infected with the wild-type organism both display reduced neutrophil numbers in the lung. TNFR1 signaling in response to *S. aureus* is a primary mechanism for inflammatory signaling since the TLR adaptor molecule MyD88 is dispensable for *S. aureus* pneumonia models in vivo [182].

EGFR signaling by SpA

A slightly different domain of the IgG-binding region of SpA initiates signaling events from EGFR. The apically displayed EGFR mediates a number of signaling events in the airway epithelium and responds to several different ligands.

SpA stimulates EGFR and ERK phosphorylation and stimulates ADAM17 (or TACE-TNF alpha-converting enzyme), the TNFR1 sheddase [180]. ADAM17 activity is also responsible for shedding IL-6R α , *trans*-signaling which cleaves off the cytokine receptors that would perpetuate the pro-inflammatory response. Thus, *S. aureus* activates not only an epithelial pro-inflammatory response via both TNFR1 and EGFR but also ADAM17 that regulates this response.

Additional consequences of EGFR activation include the stimulation of epithelial wound repair and cytoskeletal contraction. Exposure of damaged epithelial cells to heat-killed *S. aureus* results in an increased wound closure and transepithelial resistance. This is in addition to the increased proliferation and survival of uninjured epithelial cells [183]. The initiation of epithelial wound repair is the result of TLR-EGFR signaling. A further consequence of EGFR activation is the induction of mucin production, an important physical component of the innate defenses of the lung [184].

Host responses to *S. aureus* in the respiratory tract

Perhaps the most important factor in the pathogenesis of staphylococcal pneumonia is the intensity of the innate immune response to the aspirated organisms. This is a response to the intact organisms as well as to shed components from the bacteria enmeshed in airway secretions. The initial immune signaling is accomplished by airway epithelial cells that immediately recruit several types of immune cells including dendritic cells (DCs), macrophages, and T cells. The net result of this immune signaling is the recruitment of polymorphonuclear neutrophils (PMNs) which are critical in staphylococcal clearance. It has long been thought that PMNs themselves are the single

most important component in the host response to *S. aureus*. This observation is borne out by the increased susceptibility to staphylococcal infection of humans with deficiencies in neutrophil chemotaxis, oxidative burst, and granule production [185]. However, the cells that actively recruit PMNs and activate them may also be potential targets to modulate the intensity of the inflammatory response to the organisms.

TLR signaling in the airway

The pattern recognition receptors on the surface of exposed airway epithelial cells readily respond to the surface components of *S. aureus*. The airway epithelium expresses the full complement of Toll-like receptors (TLRs). High levels of gene transcription are observed for TLRs 2–6, while the expression for 7/8 is variable, depending upon the cell type [186–189]. Adaptors such as MyD88 and MD2 are also present in addition to RNA receptors like MDA5 and RIG-I [186, 190].

TLR2, which recognizes lipoteichoic acid (LTA) in the staphylococcal cell wall, is important in the response to *S. aureus* airway infection (Fig. 2). The instillation of LTA into human airways results in a significant inflammation and neutrophil recruitment, an observation mimicked in murine models [191, 192]. TLR2 is upregulated in response to *S. aureus* [193]. The virulence factor PVL also directly activates a small group of genes via TLR2 signaling that incorporates the adaptors CD14 and MyD88, leading to NF- κ B signaling [114]. TLR2 is important in some models of systemic *S. aureus* infections [194, 195]. Consistent with this, strains lacking lipoproteins avoid immune recognition and cause increased disease [196]. However, in respiratory infection, MyD88 is dispensable for bacterial clearance, even with a significantly abrogated NF- κ B-dependent cytokine response [182], consistent with the observations that several other pathways (TNF and type I IFN) are also critical for the immune response to *S. aureus*. The airway epithelium responds to this TLR–*S. aureus* interaction with a variety of cytokines, including GM-CSF, G-CSF, CXCL8, and TGF- α , as well as the antimicrobial peptides beta-defensins [180, 193, 197, 198]. The activation of G-CSF and GM-CSF is important in the airway as this promotes neutrophil survival [197]. *S. aureus* is capable of activating CXCL8 production via the MAPK p38 and ERK1/2, which leads to the activation of the NF- κ B transcription factor [199].

Type I interferons

Intracellular receptors, such as those linked to type I interferon (IFN), can also recognize staphylococci in the

airway as bacterial cells often secrete and shed components that may be internalized by airway epithelial cells. Type I IFN signaling has long been known as an important viral defense pathway in the lung [200] resulting in the transcription of hundreds of genes [201]. *S. aureus*, as well as several other extracellular bacterial pathogens, activate this pathway (Fig. 2) [202, 203]. The role type I IFN signaling plays in bacterial infection is variable, depending upon the organisms [204]. Bacteria activate type I IFN signaling through a number of TLR, NOD-like receptors (NLR), and cytosolic receptors that sense products such as cell wall components, DNA, and RNA [205–208]. The activation of type I IFN signaling leads to the production of IFN- β via the phosphorylation of an interferon regulatory factor (IRF), namely, IRF3, IRF5, and IRF7 [209–212]. IFN- β interacts with its receptor, IFNAR (interferon alpha/beta receptor), leading to the phosphorylation of STAT1/2 via the kinases Jak1 and Tyk2 [213]. The activation of type I IFN signaling also activates MAPK [214] and NF- κ B responses [215].

S. aureus activates type I IFN signaling in airway epithelial cells as well as in in vivo models of pneumonia [173]. Incubation of epithelial cells with live organisms activates *Ifnb* as well as a number of downstream genes, including *Lif* and *Mx-1*, as early as 2 h after stimulation via the expected pathways [173]. In vivo, the activation of type I IFN signaling in response to *S. aureus* is detrimental to the host; *Ifnar*^{-/-} mice were significantly less likely to succumb to pulmonary infection than their wild-type counterparts [173]. Consistent with this phenotype being host-derived, *Ifnar*^{-/-} mice had less TNF and IL-6 in their bronchoalveolar lavage (BAL) fluid while having similar bacterial loads when inoculated with non-lethal doses.

The amplification of potentially damaging inflammation that is initiated through type I IFN signaling is mediated by the CXCR3 chemokines. The CXCR3 chemokines—CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC)—perform a variety of functions including the recruitment of Th1 and Th17 cells [216–218], increasing PMN accumulation and inflammation [216, 219]. Depletion of macrophages had a detrimental effect on the outcome of MRSA pneumonia in mice, which in part was due to the increased expression of the CXCR3 ligands [219]. Blocking CXCR3 resulted in reduced CD4⁺ cells in the BAL and reduced pulmonary pathology [219]. Given the multiple pro-inflammatory pathogen-associated molecular patterns (PAMPs) associated with virulent staphylococci, strategies to modulate the host responses to these effectors may be useful to control pulmonary damage. While it is not fully understood how *S. aureus* causes lung damage, certainly a portion of the pathology is due to the host itself, as activated by these organisms. Moreover, from the murine models, it seems

that PMNs, despite their damaging release of reactive oxygen intermediates and elastase, are not the sole cause of pulmonary destruction due to staphylococcal pneumonia.

The inflammasome

Much of the pathology caused by infection with *S. aureus* is a result of the intense immune response. A known cause of pulmonary pathology, the cytokine IL-1 β , is produced in response to TLR2 recognition of staphylococcal lipoproteins and membrane perturbation by α -hemolysin as part of pyroptosis, a highly inflammatory form of cell death (Fig. 3) [84, 220]. Pyroptosis involves the activation of the inflammasome, characterized by the activation of caspase-1 and the production of IL-1 β and IL-18 [221, 222]. Activation of the inflammasome is a two-step process that typically involves the activation of a TLR (such as via microbial PAMPs) to induce the production of pro-IL-1 β then membrane perturbation (e.g., via a toxin to generate K⁺ efflux and the proteins NLRP3/NALP3/cryopyrin and ASC) to produce active caspase-1 that cleaves pro-IL-1 β to enable the secretion of mature IL-1 β [223]. This is accomplished in immune cells by *S. aureus* expressing α -hemolysin and TLR2-MyD88 signaling from lipoproteins [83, 84, 224, 225]. An additional *S. aureus* component that activates the production of IL-1 β is peptidoglycan and cell wall degradation products [226]. Inflammasome activation is known to occur in monocytic cells; the IL-1 β generated from this process is likely to contribute to pulmonary pathology. The role epithelial cells play in this process is an area of active investigation.

Staphylococcal superinfection following influenza and the Th17 response

The importance of host immune signaling in the successful clearance of *S. aureus* from the respiratory tract is well illustrated in the common clinical presentation of bacterial superinfection following influenza. The high mortality associated with staphylococcal pneumonia following influenza was first documented during the 1918 influenza pandemic in which many young, previously healthy individuals died of staphylococcal pneumonia, having previously recovered from influenza [227, 228]. Infecting mice with *S. aureus* after influenza exposure significantly increases mortality and lung pathology [18, 229–231]. Several components of immune signaling may contribute to this excess mortality. Influenza-infected mice were observed to have impaired NK cell responses upon *S. aureus* infection [230], which were essential in producing TNF to mediate bacterial clearance.

A major component of the immune response to influenza is the activation of type I IFN signaling, which increases susceptibility to *S. aureus* pneumonia [173]. It seems likely that excessive IFN- β signaling contributes to this pathology [229]. Kudva et al. [231] reinforced the negative impact of type I IFN signaling in staphylococcal pneumonia, linking the significantly higher levels of type I IFNs that resulted in a reduced Th17 response, contributing to a reduced bacterial clearance. When the co-infection was repeated in *Ifnar*^{-/-} mice, an improved clearance of *S. aureus* was observed.

Airway epithelial cells are highly dependent on typical pro-inflammatory as well as Th17 cytokines to produce anti-staphylococcal factors [232]. It has been observed that *S. aureus* can activate IL-17 in DCs and blood monocytes [233, 234]. Deficiency in the Th17 pathway increases susceptibility to cutaneous staphylococcal infections [235] and is also the mechanism attributed to the frequent staphylococcal infection in patients with hyper-IgE syndrome [236]. Hyper-IgE patients possess mutations in the STAT3 transcription factor, resulting in a failure of CD4 T cells to differentiate into Th17 cells. Influenza-infected mice have reduced Th17 cytokines. A decrease in Th17 cytokines (IL-17, 22, and 23) is observed in influenza-infected mice [231] in part due to increased type I interferons. Consistent with this observation, Th17 knockout mice exhibited impaired clearance to *S. aureus* [231]. Transgenic mice overexpressing IL-23 and co-infected with influenza and *S. aureus* had improved outcomes. These studies provide examples of how viral infections (namely influenza) and their profound immunological consequences can predispose individuals to potentially lethal secondary pneumonia.

Conclusion

S. aureus has evolved into the consummate respiratory pathogen able to not only persist in the respiratory tract but also thrive as an invasive pulmonary pathogen. Through the horizontal acquisition of genetic material and positive selective pressure, successful clones of staphylococci, as typified by the USA300 strains, express virulence factors that enable the organisms to successfully exploit the immune response that is evoked in the lung. Strategies to prevent invasive infection will not only need to target the virulence determinants that mediate colonization, iron acquisition, biofilm production, and immune evasion but will also need to be effective against organisms that can disseminate intracellularly. Approaches to the management of staphylococcal pneumonia, perhaps by modulating the host immune response, could be considered as an adjunct to the conventional antimicrobial therapy.

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