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***Staphylococcus aureus* pore-forming toxins: the interface of pathogen and host complexity**

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Abstract

Staphylococcus aureus is a prominent human pathogen capable of infecting a variety of host species and tissue sites. This versatility stems from the pathogen's ability to secrete diverse host-damaging virulence factors. Among these factors, the *S. aureus* pore-forming toxins (PFTs), α -toxin and the bicomponent leukocidins, have garnered much attention for their ability to lyse cells at low concentrations and modulate disease severity. Although many of these toxins were discovered nearly a century ago, their host cell specificity has only been elucidated over the past five to six years, starting with the discovery of the eukaryotic receptor for α -toxin and rapidly followed by identification of the leukocidin receptors. The identification of these receptors has revealed the species- and cell type-specificity of toxin binding, and provided insight into non-lytic effects of PFT intoxication that contribute to disease pathogenesis.

Keywords

Staphylococcus aureus; alpha-toxin; hemolysin; leukocidin; pore-forming toxins; *S. aureus* vaccines and therapeutics

1. Introduction

Pore-forming toxins (PFTs) are a common class of bacterial virulence factors found in many human pathogens including *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Escherichia coli*, and *Staphylococcus aureus* [1]. These toxins belong to a larger family of proteins known as pore-forming proteins (PFPs) which include the mammalian immunity proteins perforin and complement C9 of the Membrane Attack Complex (MAC) [2]. Two groups of PFTs exist, classified on the basis of the structure of their transmembrane

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components: α -PFTs whose pores consist of α -helices, and β -PFTs which form β -barrel pores [3]. Biochemical, structural, and functional studies of these toxins over the past century have contributed to our current understanding of their mechanism of toxicity and role in disease pathogenesis. At the advent of toxin research, PFTs were only recognized for their ability to disrupt eukaryotic membrane barriers to cause host cell lysis. Following the recent identification of multiple toxin receptors, it is now understood that PFTs exert subtle changes in cell activity and host physiology even at sublytic concentrations [3, 4].

The best-studied PFTs to date are arguably those secreted by *S. aureus*, a ubiquitous human pathogen that causes a wide-range of diseases such as pneumonia, sepsis, and skin and soft tissue infections (SSTI) [5, 6]. The success of *S. aureus* can be attributed its broad array of host-targeting virulence factors, most notably its arsenal of seven different β -barrel PFTs: α -hemolysin (α -toxin, Hla) and six bicomponent leukocidins [4, 7]. Deletion mutants lacking one or more of these PFTs demonstrate attenuation in various models of infection and intoxication relative to their isogenic wildtype counterparts [8–15]. However, it is still unclear whether these different PFTs exhibit coordinated activity in the context of human infection to amplify the virulence of the pathogen. The identification of PFT receptors has allowed for unprecedented insight into cell-type specificity of toxin action and a deeper understanding of their role in disease pathogenesis. In this review, we will discuss recent findings with an emphasis on the cognate interactions of the toxin-receptor pairs, and the lytic and non-lytic functions of the PFTs in disease.

2. Overview

2.1 α -toxin

The role of *S. aureus* α -toxin in human and animal disease has been known for nearly a century, incited by an event in 1928 in Bundaberg, Australia where 21 children became seriously ill following inoculation with a toxin-contaminated vaccine, resulting in 12 deaths. The surviving children were found to have abscesses at the site of immunization. Investigation by F. MacFarlane Burnet and others determined that the tragedy was caused by a soluble substance that was hemolytic, dermonecrotic and lethal when injected in rabbits [16], consistent with later studies profiling the toxicity of purified Hla [17]. The cell type specificity of Hla was apparent even in the early years of research, since it selectively lyses rabbit red blood cells (RBCs) [16], in contrast to β -hemolysin which preferentially lyses sheep RBCs [18]. Despite its name, Hla does not lyse human RBCs, but has been demonstrated to lyse human platelets, endothelial cells, epithelial cells, and leukocytes [4].

As a prototype of the small β -barrel PFT, α -toxin is encoded by a single genetic locus, *hla*, and is secreted as a 292 residue, 33.2 kDa water soluble monomer. Upon binding its target cell, Hla oligomerizes to a pre-pore structure, subsequently attacking the cell membrane by extrusion of the β -barrel through the lipid bilayer to form a hydrophilic transmembrane channel (Fig. 1A). Crystallographic studies reveal that Hla assembles into a heptameric pore consisting of three major domains: (1) the extracellular-facing cap domain; (2) the rim domain that interacts with the extracellular leaflet of the host cell membrane; and (3) the stem domain that forms the β -barrel transmembrane channel. The fully assembled toxin is 100Å wide and 100Å in height, with a minimum pore inner diameter of 14Å [19, 20].

The relevance of Hla during *S. aureus* infection is derived from epidemiological data and animal studies. Although most contemporary *S. aureus* strains encode *hla*, there is evidence that increased *hla* expression enhances virulence. For example, the epidemic strain USA300, which outcompeted USA400 during the early 2000s to become the dominant community acquired methicillin resistant *S. aureus* (CA-MRSA) strain in the USA, was found to be significantly more virulent and lethal than USA400 in a rat model of pneumonia, correlating with significant increases in *hla* expression [21–23]. Interestingly, CA-MRSA strains tend to express higher levels of Hla than hospital-acquired (HA) MRSA strains, suggesting that otherwise healthy individuals may require an increased toxin load to become susceptible to *S. aureus* infection [22, 24].

Regulation of Hla expression is primarily governed by the quorum sensing accessory gene regulator (*agr*) system, which is activated in the late-log to stationary phases of growth to induce the transcription and translation of *hla* [25, 26]. Other systems fine-tune the expression of *hla* by modulating the Agr system (eg. Sar family) [27, 28] or in response to environmental conditions (eg. SaeR/S two-component system) [29, 30]. Loss of *hla* expression in some strains stems from mutations in the *agr* locus, associated with less virulent HA-MRSA isolates [22]. Deletion of *agr* or *saeR* resulted in loss of Hla production and reduced lethality in murine models of pneumonia and SSTI [31]. Studies in mice and rabbits using *hla* mutants have been instrumental in demonstrating necessity of the toxin for full virulence. *hla* mutants are significantly attenuated during infection in the bloodstream [32], skin [33–35], mammary gland [36], cornea [37] and pneumonia [38, 39].

2.2 Bicomponent leukocidins

S. aureus leukocidal activity was discovered contemporaneously with its hemolytic activity [40]. However, for many years following this observation, it was unclear if this was a secondary function of α -toxin, or if it was caused by a separate biological product [7]. The independence of the hemolytic and leukocidal features of *S. aureus* was first demonstrated by Julianelle in 1922 [41], then confirmed by Pantón and Valentine in 1932 [42]. In these studies, a careful analysis of multiple *S. aureus* strains revealed that hemolytic activity did not necessitate leukocidal activity [42]. Interestingly, this investigation correlated leukocidal activity with acute pathology in humans, and hemolytic activity with virulence in rabbits [42]. It is notable that the difficulty in assigning the toxic effects of *S. aureus* supernatants to individual toxins is partially attributable to the number of leukocidins that *S. aureus* can secrete, not all of which were identified until the 21st century. The biology of these toxins is further complicated by the fact that different leukocidins can target the same cell types, as well as the same receptors [14, 43–45]. The reader is encouraged to consult other reviews that approach the historical literature with greater detail, such as those by Alonzo and Torres [7].

Unlike α -toxin, leukocidins are hetero-oligomeric, consisting of two subunits that are encoded within a single open reading frame, with γ -hemolysin AB (HlgAB) as the sole exception [46]. This was demonstrated for the first time by Woodin through the fractionation of the Pantón-Valentine Leukocidin (PVL) using ion-exchange chromatography [47], reflected in the names of the subunits F and S, for fast and slow fractions, respectively. Most

importantly, this study was the first to demonstrate that the two fractions, or subunits, must be combined for maximal cytolytic activity [47]. While reports suggested that the leukocidin oligomer consisted of six or seven subunits [48–50], it is now accepted and confirmed that the assembled leukotoxin is an octamer of four F-subunits and four S-subunits in alternating fashion [51, 52]. Like Hla, the bicomponent leukocidins are generally secreted as water soluble monomers. The S-component confers cell-type specificity by binding to cellular receptors, inducing a conformational change to allow dimerization with F-components (Fig. 1B) [53]. These dimers then oligomerize to form the pre-pore prior to insertion of the β -barrel transmembrane channel [54]. The exception to this is LukAB, which is either secreted as a dimer or dimerizes shortly after secretion, and can also adhere to the bacterial cell wall [55, 56].

2.2.1 Panton-Valentine Leukocidin (LukF-PV and LukS-PV)—The PVL genes (*lukS-PV* and *lukF-PV*) are encoded in several bacteriophages expressing the Sa2 integrase (ex. ϕ PVL, ϕ PVL108, ϕ Sa2mw, ϕ Sa2USA300, ϕ SLT) [57–59]. *S. aureus* strains carrying these phages became prevalent during the CA-MRSA epidemic which began in the early 1990s, and was characterized by the infection of healthy individuals without known predispositions to *S. aureus* disease [60]. Molecular epidemiology studies from this period found that 77–100% of CA-MRSA strains encoded PVL in addition to methicillin resistance genes (*sccmecIV*) [61, 62]. By contrast, *S. aureus* strains found in the nares of healthy patients demonstrated that less than 3% carry the PVL gene [63, 64]. Phylogenetic analysis of the CA-MRSA strains indicated that rather than a single virulent clone, multiple strains containing methicillin resistance gene (*sccmecIV*) and PVL had arisen around the world during the epidemic, corroborating the possibility that the virulence associated with CA-MRSA could be horizontally transferred [60]. Infection with PVL-positive *S. aureus* is associated with SSTIs around the world, and PVL expression has been confirmed in abscesses [65, 66]. While a meta-analysis of epidemiologic data did not find that PVL was associated with pneumonia [65], a clinical study showed pneumonia involving PVL-positive *S. aureus* progressed more rapidly and were more lethal relative to PVL-negative pneumonia [67]. Necropsies revealed that these patients' lungs were ulcerated and hemorrhagic, suggestive of extreme inflammation that the authors attributed to PVL-induced lysis of inflammatory cells, such as macrophages and neutrophils [67, 68].

Despite the epidemiological association, it has been difficult to establish a causal relationship between PVL and disease. Some studies using mouse models show significant reduction of lung and skin pathology by *pvl* strains [69, 70], whereas others report either no change or worsened disease [23, 71–73]. Collectively, these studies are difficult to interpret given that murine PMNs are resistant to PVL-induced toxicity [74]. Recent studies have identified C5aR1 and C5aR2 as the receptor for PVL in humans and rabbits, but murine orthologs fail to bind PVL [74]. It is possible that a yet-unidentified receptor exists in mice, as direct instillation of the purified toxin into murine lungs is highly inflammatory and lethal [69]. Rabbit studies are also inconclusive regarding the role of PVL in exacerbating disease. *pvl* strains caused significantly less damage in models of osteomyelitis, bacteremia, and pneumonia relative to isogenic controls [12, 13, 75, 76], but more severe dermonecrosis in at

least one study [77]. Nevertheless, these studies may still be informative, since PVL-induced necrosis in skin and lung infections mimic pathology seen in human patients [13, 76].

2.2.2 γ -hemolysins (HlgAB and HlgCB)—The γ -hemolysin loci encodes three genes *hlgACB*: two S-subunits HlgA and HlgC, and one F-subunit, HlgB. *hlgCB* forms one transcriptional unit and *hlgA* forms another, each harboring independent promoters [78]. HlgA and HlgB combine to form the classical γ -hemolysin (HlgAB) which was described in 1938 [7], though it was difficult to assign biological or biochemical functions to the toxin until reliable purification methods were developed in the late 1970s [79, 80]. HlgAB is particularly effective at lysing human RBCs [81] and exhibits cytolytic activity towards human and rabbit leukocytes [15, 78]. In contrast, though HlgCB is also referred to as γ -hemolysin, it exhibits limited activity towards RBCs. Instead, HlgCB is primarily leukotoxic; as a result, early studies refer to it simply as ‘Leukocidin’ (LukSF), or as Leukocidin R (LukR) [82, 83].

Little is known about the role of HlgACB in *S. aureus* virulence. 89–100% of strains associated with human or bovine colonization contain the *hlgACB* locus, yet have not been associated with any particular infection type [63, 64, 84]. Studies in mouse models are limited to assessing HlgAB, as HlgCB fails to bind its receptor in mice (see below) [14]. HlgAB has been shown to be required for *S. aureus* survival and proliferation in during bloodstream infection, likely through macrophage evasion and nutrient (Fe^{2+}) release from erythrocytes [14, 15]. This may explain an earlier finding that *hlgACB* mutants caused reduced incidence and severity of septic arthritis following bloodstream infection [85].

2.2.3 LukED—LukED has been a focus of intense investigation owing to its relatively recent discovery. Early studies with recombinant LukED demonstrated its cytolytic activity against rabbit RBC and leukocytes, though it is notable that LukED is less effective than HlgCB on either of these cell types [86]. Subsequent studies showed that LukED was necessary for virulence during systemic infection in mouse models [10, 87].

Epidemiological data shows that *lukED* is widespread, but more prevalent in epidemic strains. A study conducted in Minnesota and Wisconsin found that CA-MRSA strains were likely to contain the *lukED* locus (~99%), whereas MSSA strains collected from either symptomatic patients or asymptomatically colonized human subjects were less likely to contain the locus (44–77%) [63]. These numbers are similar to previous studies that estimated 87–93% carriage rate in Japanese clinically acquired strains (no drug-resistance noted) and 88–99% in CA-MRSA strains acquired around the world [62, 86, 88]. Interestingly, the *lukED* locus is also present in ~90% of *S. aureus* strains collected from cows with subclinical to severe mastitis, supporting a possible role for LukED in virulence or infectivity in different host species [84, 89].

2.2.4 LukAB—LukAB (also known as LukGH) is the most recently discovered member of the bicomponent leukocidin family [9, 56]. It is also the most distantly related member of the bicomponent leukocidin family, with LukA exhibiting only 30% amino acid identity with other S-components and LukB, only 40% identity to other F-components [7, 9]. This toxin appears to be encoded in most sequenced strains of *S. aureus* [7, 90], yet another study noted that three out of ten clinical isolates failed to express LukAB protein [9]. Higher titers

of LukAB neutralizing antibodies were found in convalescent patient sera compared to that of acutely ill patients in the setting of pediatric invasive *S. aureus*, indicating that LukAB is produced during human disease [91].

The role of LukAB during infection *in vivo* remains unclear. Studies *in vitro* show that it targets monocytes, dendritic cells and PMNs, and can synergize with PVL when co-expressed [56, 92]. These studies also showed that LukAB plays a role in escaping from phagocytes and neutrophils [93]. To date, few studies have been conducted to assess the role of LukAB in pathogenesis. One study showed that fewer *lukAB* mutants were recovered in a murine kidney abscess model [9]. Another showed that *lukAB pvl* double mutant caused a larger abscess in rabbits and was more lethal during bloodstream infection in mice, though single mutants did not show a phenotype [94]. Unfortunately, like the murine PVL studies, these are difficult to interpret since it was later determined that the LukAB fails to bind its receptor (CD11b) in mice and binds at a low affinity in rabbits [45].

Unlike other leukocidins, which are secreted as monomers and oligomerize following cell-surface binding by the S-component, LukAB must heterodimerize prior to binding its cell-surface receptor. It appears that LukAB dimerization occurs prior to secretion, since its toxic potency is highest when the subunits are co-expressed relative to a mixture of individually purified subunits [55, 95]. The *lukAB* gene is highly polymorphic, with only 82–88% identity between the most divergent strains. In comparison, other leukocidin genes differ by less than 5% between strains, and *lukE* and *lukF-pvl* share 82% identity [95]. Despite this diversity, LukAB pairs still exhibit similar abilities to lyse human PMN [95], though it remains to be seen if the polymorphisms in *lukAB* affect infection in non-human species.

2.2.5 LukMF’—Our discussion of LukMF’ will be limited, as its expression is restricted to *S. aureus* strains circulating in non-human hosts, such as cows, sheep, and goats [96]. This leukocidin is most commonly associated with bovine mastitis, a major cause of milk loss and an economic concern globally [97, 98]. The gene is encoded within the prophage ϕ Sa1, which can be horizontally transmitted across strains, much like PVL [99]. It is notable that LukMF’ is particularly toxic to PMNs of bovine origin relative to other species, indicating a possible host-specific adaptation [96, 100].

3. Cell surface receptors for *S. aureus* PFTs

Receptors for the *S. aureus* PFTs have provided a cohesive explanation of their cellular tropisms, setting the stage for further research into physiologic toxin effects. In this section, we will discuss the PFT receptors identified to date, naming key cell types that are lysed as a result of that interaction (summarized in Table 1 and Fig. 2). It should be noted that future studies will likely identify additional toxin-sensitive cell types on the basis of their receptor expression profile.

3.1 Non-specific Lipid Receptors of α -toxin

Early research showed that α -toxin was capable of binding to and lysing protein-free membranes. This gave rise to the prevailing theory that the α -toxin receptor was a lipid moiety [101]. Evidence suggested that this interaction was specific - for example, pore-

formation on liposomes is dependent on their lipid composition. Liposomes composed of phosphatidylcholine (PC) or a combination of sphingomyelin and cholesterol were susceptible to lysis, whereas liposomes composed of phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), or phosphatidylinositol (PI) were not [102, 103]. Conversely, depletion of sphingomyelin or cholesterol was also able to prevent high affinity binding of Hla on rabbit RBCs and other susceptible cells [103]. Of these, PC emerged as a likely a receptor for Hla, as exogenous PC competes for Hla binding to prevent hemolysis [103]. Moreover, a crystal structure of the Hla oligomer in complex with glycerophosphocholine (GPC) and di-propanoyl phosphatidyl choline (DiC3PC) showed that Hla is able to directly bind lipid headgroups via a crevice between the rim and stem domains [104]. Rim domain residues Y179 and R200 are critical for shaping this binding pocket, and appear to be conserved in bicomponent leukocidins [49, 104].

Several lines of evidence have pointed against the hypothesis that the primary receptor for Hla is a lipid. First, liposomes formed from lipids extracted from human or rabbit RBCs display similar sensitivity to Hla [105]. Second, pronase treatment has been repeatedly shown to reduce rabbit RBC sensitivity to toxin, indicating a proteinaceous receptor [106]. Moreover, Hla surface binding experiments by Cassidy and Harshman estimated ~5000 specific binding sites on rabbit RBCs, far fewer than what might be expected for a lipid moiety [107]. This was later confirmed by Hildebrand and others who estimated 1,200–1,700 binding sites per rabbit RBC at low concentrations of Hla [108]. At high concentrations, both rabbit and human RBCs exhibit non-saturable, non-temperature dependent binding, indicative of the non-specific nature of Hla pore-formation at these concentrations [108].

3.2 Proteinaceous Receptors for α -toxin

3.2.1 Caveolin—Pany and others initially proposed Caveolin-1 (CAV1) as a proteinaceous receptor for Hla, based its ability to bind the rim domain of Hla and co-immunoprecipitate with Hla from lysates [109, 110]. However, other evidence challenged this hypothesis: First, CAV1 is an integral membrane protein expressed on the inner leaflet of the plasma membrane [111]. Second, although CAV1 expression levels correlate to Hla sensitivity, overexpression of recombinant CAV1 doesn't increase sensitivity [112]. Indeed, further investigation found that the rim domain is integral to Hla stabilization on the membrane at the pre-pore stage, rather than to initiate specific receptor binding [110]. These studies and others have shown that Hla and caveolin-1 cluster on lipid rafts, and it has been hypothesized that this facilitates cellular signaling associated with the Hla pore [109, 113].

3.2.2 ADAM10—In 2010, Wilke and Bubeck Wardenburg utilized a biochemical approach to identify the cell surface metalloprotease, A Disintegrin and Metalloprotease 10 (ADAM10), as the specific proteinaceous receptor of Hla [113]. Loss of ADAM10 expression through shRNA knockdown reduced Hla binding and cytotoxicity, and ADAM10 surface expression levels on various cell lines correlated with Hla binding levels [113]. Subsequent studies in the years following the initial discovery have validated this finding. Genome-wide loss-of-function screens in HAP1 haploid or myeloid cells identified ADAM10 as a necessary factor in Hla-mediated cell death, and overexpression of ADAM10

lead to increased Hla binding and toxicity [114–116]. Because ADAM10 is necessary for embryonic development and maintenance of the epithelium, *in vivo* studies are limited to the use of conditional or inducible ADAM10 knockout lines [117, 118]. Nevertheless, these studies have provided valuable insights which are discussed in detail below.

ADAM10 is a cell surface sheddase involved in developmental and homeostatic processes, whose substrates include members of the Notch, ephrin, and cadherin families among others [119–121]. ADAM10 is expressed on cell types already known to be susceptible to Hla intoxication, such as epithelial cells [122], keratinocytes [121], endothelial cells [123], and platelets [124]. Notably, the enzymatic activity of ADAM10 is not necessary for Hla-binding [116]. Maturation and surface expression of ADAM10 is regulated by the members of the TspanC8 family of proteins [125, 126], including Tetraspanin-14 and –33 [114, 115]. Thus, factors that regulate ADAM10 surface expression also regulate the Hla susceptibility [115].

Not only does ADAM10 facilitate pore-formation, but its enzymatic activity is also upregulated by the Hla-pore [8]. Neither binding nor oligomerization of Hla is sufficient for ADAM10 activity, however, blocking the Hla-pore using β methyl-cyclodextrin (M β CD) prevents activation [8, 127, 128]. Specifically, it appears that Ca^{2+} influx through the toxin pore activates ADAM10, a hypothesis that is supported by previous reports that ADAM10 is activated by calcium ionophores such as ionomycin, and requires extracellular Ca^{2+} [129, 130]. In fact, this phenomenon is not specific to Hla- Inoshima and others were able to show that ADAM10 can be activated by pneumolysin (PLY), a cholesterol dependent PFT produced by *Streptococcus pneumoniae* [8, 131].

3.3 Proteinaceous Receptors for the Bicomponent Leukocidins

3.3.1 LukED-CCR5—LukED was the first leukocidin whose receptor was described. Similar to Hla, this was done by identifying cell types with differential susceptibilities to LukED. In this case, the authors treated a panel of human cell lines with purified LukED and found cytotoxicity in T-cell line expressing CCR5, HUT-R5, but not in the Jurkat line with little CCR5 expression. Knockdown and overexpression studies validated CCR5 as the LukED receptor [87]. In agreement with previous studies of bicomponent leukocidin membrane binding, the S-component LukE interacts directly with the receptor, whereas LukD does not. While LukE binding is unable to activate CCR5 signaling, it can act as an inhibitor of CCL5 binding *in vitro* [87].

CCR5 is already a major pharmacological target as an HIV co-receptor [132]. The authors were able to use a variety of antagonists to interfere with LukED toxicity such as monoclonal antibodies and commercial HIV therapeutics, maraviroc and vicriviroc, raising the possibility that existing pharmaceutical compounds may be able to alleviate LukED toxicity in patients [87]. The CCR5 genetic variant that protects humans from HIV infection through the loss of surface expression (CCR5 $\Delta 32$) also prevents LukED toxicity [87].

3.3.2 LukED-CXCR1/2—LukED is capable of killing some monocytes even in the absence of CCR5 [44, 87]. CXCR1 and CXCR2 were identified as two additional LukE receptors that is expressed in PMNs and monocytes [44]. This study identified a key region in LukE (Q182-A196) that is necessary for binding CXCR1/2 but not CCR5, highlighting

the specificity of PFT-receptor interactions [44]. Mutation of this region prevents killing of PMNs, which predominantly express CXCR2, whereas monocytes, which express CCR5 in addition to CXCR1/2, were only partially protected [44]. Furthermore, *S. aureus* strains expressing this mutant LukED were not as virulent as those expressing WT LukED, indicating that LukED interaction with all of its receptors is necessary for full virulence [44].

3.3.3 HlgAB- CXCR-1, -2, -4 and CCR2—HlgAB displays tropism towards human PMNs and macrophages. Human CXCR1, CXCR2, CCR2, and at very high concentrations, CXCR4, were identified as receptors for HlgA [14]. Although it is able to lyse murine macrophages through CCR2, murine neutrophils are not lysed by HlgAB for two reasons: (1) murine CXCR1 but not CXCR2 binds the toxin; (2) mouse neutrophils primarily express CXCR2, unlike human neutrophils which primarily express CXCR1 [14]. Thus, host tropism is predicated not only on structural compatibility, but also on differences in gene expression. As a result, studies using the mouse model are limited to macrophage-toxin interactions. Infection of mice with wildtype bacteria leads to a small but significant reduction in CCR2⁺ monocytes which is not observed during infection with *hlgACB*. This interaction enhances bacterial survival during bloodstream infection, since loss of the toxin (*hlgACB*) or loss of the receptor (CCR2^{-/-} mice) reduces bacterial burden, lending support to the hypothesis that toxins deplete specific immune populations to enhance bacterial survival [14].

3.3.4 HlgAB and LukED-DARC—The Duffy antigen receptor for chemokines (DARC; or Atypical Chemokine Receptor 1, ACKR1) is a receptor present on human RBCs, and used by *Plasmodium vivax* and *Plasmodium knowlesi* for entry. Spaan and others found that it is also the receptor for HlgAB, and to a lesser extent LukED [15]. As with the other leukocidins that target the same receptors, HlgA and LukE have different binding sites on DARC. Notably, both toxins require a sulfated tyrosine residue at position 41, reminiscent of the requirement of sulfated tyrosine moieties for DARC binding by *P. vivax* [133], HIV binding of CCR5/CXCR4 [134], and chemokine binding to their cognate receptors [135, 136].

A number of polymorphisms in the DARC gene exist in the population: a polymorphism in the coding region of the receptor responsible for antigenic differences [137], a promoter polymorphism that eliminates DARC expression specifically in RBCs [138, 139], and a rare missense mutation that reduces overall DARC expression [140]. Spaan and others showed that antigenic differences had no effect on HlgA or LukE binding, but DARC expression levels correlated with hemolysis [15]. The authors suggest that erythrocyte lysis is beneficial to the bacteria via the release of iron. Whether this occurs is less clear *in vivo*, given that both toxins are also associated with immune cell killing and dysfunction [14, 44, 87]. Infection studies in DARC KO mice [141], or an epidemiologic study of *S. aureus* disease and DARC genotypes in humans would further clarify this matter.

3.3.5 LukMF'-bovine CCR1, CCR2, CCR5—Like the other leukocidins, LukMF' is well adapted to its bovine host. LukM is capable of binding bovine CCR1, CCR2, and CCR5, enabling intoxication of bovine macrophages and neutrophils [11]. By contrast, LukM binding is limited to CCR1 and macrophages in humans. Similar to LukED, the tropism of LukM is primarily derived from the DR4 loop of the rim domain to allow it to

bind to bCCR1 and bCCR2, though it does not affect binding of bCCR5. On the host receptor, the 2nd and 3rd extra cellular loops of CCR1 are critical for LukM binding [11].

3.3.6 PVL-C5aR1 and C5aR2—Using a monoclonal antibody panel targeting 56 transmembrane proteins found on PMNs and macrophages, Spaan and others were able to demonstrate that the human C5a receptor 1 (C5aR1) and the related C5aR2 (C5aL2) are both able to bind LukS-PV and facilitate pore-formation [74]. This interaction is inhibited by the C5aR1/2 ligand (C5a) and antagonists of the receptor [74]. It is notable that *S. aureus* produces other toxins that target the complement pathway such as chemotaxis inhibitory protein of *S. aureus* (CHIPS) which targets C5aR1 and staphylococcal complement inhibitor (SCIN) underscoring the importance of inhibiting this pathway for bacterial survival [142, 143].

Neither murine C5aR1 nor C5aR2 bound LukS-PV, confirming older studies that found that murine cells were insensitive to PVL intoxication [74, 144]. This also explains the lack of importance of PVL in murine models of infection [23, 71, 72]. Rabbits remain a viable model for understanding the role of PVL during infection, as rabbit C5aR1 and C5aR2 are only slightly less sensitive to PVL than humans [74]. Recent approaches to addressing PVL pathobiology have attempted to circumvent the lack of PVL interaction with murine receptors by using humanized mice models, in which immune incompetent NSG (non-obese diabetic/severe combined immune deficiency/IL2 γ ^{null} or NOD.Cg-Prkdc^{scid} Il2rg^{IWjl}/SzJ) mice are engrafted with human hematopoietic cells from cord blood (CD34⁺) [145]. These mice are highly susceptible to dermonecrosis and pneumonia caused by PVL-expressing *S. aureus* in a human PMN-dependent manner [146, 147]. These model systems may provide further insights regarding PVL activity involving the immune response.

3.3.7 HlgCB-C5aR1 and C5aR2—HlgC competes with LukF-PV for binding on leukocytes [148]. C5aR1/2 were identified as the HlgC receptor shortly after they were determined to be the PVL receptor [14]. Though it has a broader species tropism than PVL, HlgC is still unable to bind murine C5aR1/2, precluding its study in mouse models [14, 53]. Structure-function analyses indicate that LukS-PVL and HlgC require different regions of C5aR1/-2 for binding; thus, C5aR1 antagonists such as Chemotaxis inhibitory protein of *S. aureus* (CHIPS), PMX205, and AS-65122 are effective inhibitors of PVL binding but not HlgCB [53].

3.3.8 LukAB-CD11b—Given the relative dissimilarity of LukAB to the other bicomponent leukocidins, it is not surprising that this leukocidin is the only of the family that does not bind a GPCR. DuMont and colleagues used a pulldown assay with human PMNs to determine that the LukA receptor is CD11b (Integrin α_M), a component of the Complement Receptor 3 (CR3; Mac-1; $\alpha_M\beta_2$ integrin) [45]. Receptor specificity is conferred by the 10 AA C-terminal tail that is unique to LukA. Importantly, LukA does not bind murine CD11b, and its affinity to rabbit CD11b has not yet been tested. Like PVL and HlgCB, LukAB pathobiology cannot be investigated in mouse models, severely limiting the ability to understand its role during infection [55].

4. Cellular Effects of Toxin Action

The identification of the PFT receptors has improved our comprehension of the mechanism of toxin assembly and pore-formation, and simultaneously expanded our understanding of each toxin's host cell tropism. Target cells can now be identified on the basis of receptor expression profiles, allowing for the study of toxin activity on less sensitive cell types and the non-lytic effects of the PFTs.

4.1 Intracellular signaling

A common paradigm for PFT action is disruption of the plasma membrane leading to osmotic imbalance that precedes cell death. Intracellular increase of Ca^{2+} is one of the earliest observations made in PFT intoxicated cells [149]. It is thought that this initiates intracellular signaling associated with intoxication. In the case of Hla, Ca^{2+} entry appears to occur directly through Hla pores formed on the plasma membrane, as this is inhibited by the channel blocking inhibitor, β -methyl cyclodextrin (βMCD), and by depletion of extracellular Ca^{2+} [8]. While this study ruled out a possible role for intracellular stores for Hla-induced Ca^{2+} increase [8], PVL and HlgCB were shown to stimulate Ca^{2+} release from intracellular stores [150]. Notably, both toxins involve Ca^{2+} stores in the endoplasmic reticulum, but only HlgCB acts on acidic stores such as lysosomes [150]. Unexpectedly, this study showed that this differentiation may be determined by the F-subunit, though the exact mechanism remains unclear. This raises the intriguing possibility that the conformational change of the S-subunit induced by F-subunit binding may trigger host receptor signaling, an aspect of toxin activity that will likely differ between each PFT [150]. All in all, these studies highlight the divergence in intracellular signaling events between PFTs.

In addition to initiating Ca^{2+} signaling, Hla pore-formation causes extracellular Na^+ influx into the cell and K^+ efflux [151]. Experiments with HlgCB indicated that ion flux did not occur through toxin pores, but occurred indirectly through Na^+/K^+ ATPases [152]. In Hla treated lung epithelial cells, K^+ efflux is sufficient to induce p38-MAPK signaling [153]. Similarly, K^+ efflux was responsible for initiating inflammasome activation in monocytes following LukAB intoxication [92]. Other signaling pathways that are initiated by Hla-pore formation include the p38, Erk, and c-Fos signaling pathways, which are associated with inflammatory or immune signaling, as well as the initiation of cell survival pathways [153, 154]. In particular, the p38 pathway has been shown to be activated in cells treated with Hla-concentrations that were insufficient to induce detectable levels of cell death, indicating the sensitivity of the pathway [116]. Ion flux associated with pore-formation in general, rather than specific PFTs, may be a sufficient damage or "danger" signal to initiate cellular signaling cascades.

Hla intoxication in adherent cells has been demonstrated to affect cytoskeletal structures. Sub-cytolytic concentrations of Hla resulted in dephosphorylation of FAK, Src, p130Cas and paxilin, decoupling integrin-mediated extracellular signals from intracellular signals that control focal adhesion turnover and cytoskeletal rearrangements [113]. It is unknown if these cytoskeletal alterations are the result of ADAM10 activity on junctional proteins, such as E-cadherin [8], or the result of pore-induced signaling independent of ADAM10. The disruption of adherens junctions by Hla may also initiate signaling cascades that are

detrimental to cell survival. A recent study found that deletion of several components of the adherens junction reduced the susceptibility of cells to Hla [114]. Cells lacking an intracellular component of the junctional complex, the plekstrin-homology domain containing protein 7 (PLEKHA7), were similarly susceptible as WT cells to pore-formation, but recovered hours after the initial intoxication event [114]. Importantly, PLEKHA7 knockout mice were resistant to skin and lung infections. [114]. While the exact mechanism by which PLEKHA7 confers susceptibility is still unknown, there is an intriguing possibility that the adherens junction may control the response to cellular injuries and resultant cell death [114]. All in all, cytoskeletal restructuring and alteration of the adherens junction may contribute to the immune evasion strategy of *S. aureus*, as has been observed in numerous other pathogens such as *L. monocytogenes* [155, 156] and *Y. pestis* [157].

4.2 Inflammatory signaling

Inflammation during *S. aureus* infection is a double-edged sword. On one hand, uncontrolled inflammation is associated with tissue damage and lethality [158, 159]. On the other, failure to receive inflammatory signals, primarily through the IL-1R/MyD88 signaling axis, leads to damaging bacterial overgrowth [160]. PFTs have long been implicated in inflammation during infection. Bhakdi and others were the first to note that Hla treatment caused IL-1 β secretion by human monocytes in culture [161]. It is now confirmed that Hla and all leukocidins are capable of inducing IL-1 β secretion through the induction of K⁺ efflux [14, 92, 162, 163]. In all cases, pore-formation alone is insufficient; macrophages must be primed with a TLR agonist, such as peptidoglycan (PG) or lipotechoic acid (LTA), in order to induce transcription of pro-IL-1 β , in agreement with other studies showing a two-step regulation of IL-1 β secretion [163, 164].

Conditional ADAM10 KO mice have been particularly useful in understanding the role of PFT-induced inflammation on disease [165]. Myeloid-lineage specific ADAM10 KO mice secrete less IL-1 β during lung and skin infection with Hla-competent *S. aureus* [165]. Interestingly, the reduction in IL-1 β secretion has different effects depending on the site of infection. During pneumonia, myeloid-lineage ADAM10 KO mice exhibit increased host survival despite the fact that neither bacterial numbers or lung pathology markedly improved in this condition [165]. This is consistent with previous studies demonstrating that inflammation during invasive disease is host-detrimental [166]. By contrast, during infection of the skin, loss of Hla action on myeloid lineage cells results in increased lesion sizes and increased bacterial growth [165], underscoring the importance of IL-1 β in resolving skin infections [160, 167]. In this context, damage caused by Hla is a crucial danger signal sensed by macrophages to initiate host-protective immune responses. Hla or PVL intoxicated PMNs also secrete leukotriene B₄ (LTB₄), a potent inflammatory mediator, although the exact mechanism by which this occurs is unknown. GTPases may participate in this phenomenon, since inhibition using pertussis toxin or cholera toxin prevents PVL-induced LTB₄ production, whereas overactivation of GTPases using *Clostridium botulinum* exozyme C3 enhances production [168]. It is, however, unclear whether this occurs as a result of intracellular signaling triggered by pore-formation, or as a result of C5aR engagement by the toxin.

Paradoxically, PFTs can enhance bacterial killing by priming PMNs for an oxidative burst upon fMLP treatment. Sublytic concentrations of PVL, HlgAB, or HlgCB is sufficient to increase the magnitude of the oxidative burst, associated with reduced intracellular bacterial survival *in vitro* [14, 73, 74, 169]. This priming effect is non-specific, however, since pores formed by electroporation has also been shown to prime PMNs [169, 170]. Whether this plays a significant role in *S. aureus* disease pathogenesis is a matter of debate. A number of studies in mice and rabbits have shown that infection with *pvl* mutants or administration of PVL neutralizing antibodies resulted in reduced host survival during pneumonia, enhanced skin dermonecrosis, and increased bacterial growth [71–73, 77, 171]. Dermonecrosis is worsened in the absence of LukGH in addition to PVL, suggesting that combinations of PFTs may synergize this effect [94]. Others have found reduced virulence of *pvl* mutants [69, 76, 145, 146]. Regardless, macrophage priming through cell membrane damage may be another mechanism by which hosts sense pathogenic bacteria and eliminate them.

4.3 Activation of programmed cell death pathways

Recent studies indicate PFTs induce necroptosis, a programmed cell death pathway that also causes inflammation. Necroptosis is similar to pyroptosis in that both induce inflammation, but use different molecular pathways. Whereas pyroptosis is downstream of Casp-1 activation, necroptosis is downstream of RIPK1/-3 signaling; these are further discussed in a number of excellent reviews [172–174]. Macrophages treated with necroptosis-specific inhibitors show reduced Hla or LukAB dependent cell death and IL-1 β secretion [175]. During *S. aureus* pneumonia, necroptosis inhibitors and RIPK3 knockout mice are both protected from lung injury and show reduced bacterial burdens [175]. Studies using supernatants from an array of PFT producing bacteria indicate that necroptosis is dependent on an oxidative burst caused by ion efflux, though this has yet to be specifically confirmed for the *S. aureus* PFTs independently [176].

4.4 Receptor inhibition

Whereas Hla acts as an agonist of ADAM10 activity, leukocidins appear to have an inhibitory effect on their respective receptors by blocking native ligand binding. For example, the LukE-CCR5 interaction prevents CCL3, -4, and -5 binding, though LukE itself cannot induce CCR5 activation [87]. HlgA reduces the ability of human PMNs to respond to IL-8, the substrate for CXCR1/2, and for macrophages to respond to MCP-1, the substrate for CCR2 [14].

The S-subunits, HlgC and LukS-PVL both prevent C5aR1 signaling by C5a, as measured by GTPase-induced Ca²⁺ mobilization [14, 74]. It is notable that *S. aureus* produces other toxins that target the complement pathway such as chemotaxis inhibitory protein of *S. aureus* (CHIPS) which targets C5aR1 and staphylococcal complement inhibitor (SCIN) underscoring the importance of inhibiting this pathway for bacterial survival [142, 143]. While these interactions have been shown *in vitro*, the relevance of these findings have not been shown during infection *in vivo*. Nevertheless, leukocidins may prevent interaction with immune cells through both cytotoxic/cytolytic effects and modulation of chemokine function.

4.5 Intracellular survival

Although *S. aureus* has traditionally been studied as an exclusively extracellular bacterium, it is also capable of surviving within phagocytes. Hla expression by internalized bacteria, as well as external application of Hla, have been shown to prevent lysosomal acidification [177, 178]. The mechanism for this process has not been elucidated, though one possibility is via Ca^{2+} influx and subsequent signaling that disrupts phagosomal maturation [178]. This not only increases intracellular *S. aureus* survival, but survival of other pathogens in a model of polymicrobial pneumonia [178]. LukAB and PVL allow phagocytosed bacteria to escape from the phagosomes of neutrophils, macrophages and keratinocytes, then back out to the extracellular space through by killing the host cell [45, 93, 179–181]. LukAB is particularly well-positioned to interact with the phagosomal membrane as it is the only leukocidin that is present on the staphylococcal cell wall [45, 56]. This function of the PFTs is reminiscent of the *L. monocytogenes* PFT, listeriolysin O (LLO), which is necessary for bacterial escape into the host cytosol [182].

Paradoxically, phagocytosis can also be induced by *S. aureus* as an immune evasion tactic. A recent study found that *S. aureus* that survive within phagocytes serve as a reservoir of infection during systemic disease, resulting in increased bacterial outgrowth and reduced host survival [183]. Hla-induced changes in intracellular signaling causes increased $\beta 1$ -integrin surface expression and subsequent phagocytosis, allowing bacteria to escape the extracellular-bactericidal functions of mast cells [184, 185]. In addition, the LukAB receptor CD11b is a component of CR3, a receptor that detects C3bi opsonization or pathogen-associated molecular patterns (PAMPs) to induce phagocytosis and signaling [186]. CR3 bound pathogenic fungi and mycobacteria are thought to be phagocytosed into a pathogen permissive niche instead of a restrictive one [186, 187]. Although LukAB-CD11b interaction does not affect absolute frequencies of phagocytosis [93], it would be interesting to see if LukAB is used to further subvert the phagolysosomal machinery to improve survival.

4.6 Barrier disruption

Barrier disruption is a hallmark of *S. aureus* disease. Hla is capable of doing so at least in part by direct damage to epithelial or endothelial cells. Hla treatment of lung epithelial cells, keratinocytes, gut epithelial cells or pulmonary endothelial cells results in loss of cell adhesion and the appearance of large gaps in between cells [8, 32, 35, 188]. The direct toxic effect of Hla on lung epithelia was recently confirmed in a three-dimensional (3D) lung epithelial tissue culture which recapitulates *in vivo* tissue architecture [189]. ADAM10 knockdown through siRNAs or ADAM10 inhibition is sufficient to prevent Hla-induced degradation of the monolayer. Notably, the burden of disease is reduced in ADAM10 knockout mice. Alveolar epithelium-specific ADAM10 KO mice are protected from lung injury during pneumonia and show increased survival [8]. Similarly, keratinocyte-specific ADAM10 KO mice develop smaller abscesses and have no dermonecrosis [35]. Treatment with the ADAM10-specific inhibitor, GI254023X, replicates these ADAM10 knockout phenotypes, and also prevents vascular leakage caused by Hla during bloodstream infections [32].

The loss in monolayer or tissue integrity caused by Hla may be explained by two possible mechanisms: (1) cell death and (2) loss of intercellular adhesion. In all three cell types, studies have shown that Hla-induced upregulation of ADAM10 enzymatic activity results in cleavage of cadherins, an important component of the adherens junctions [8, 32, 35]. Studies *in vivo* support these findings, as lung alveoli during pneumonia and subcutaneous tissue during skin infection both show loss of E-cadherin staining upon *S. aureus* infection, which is rescued in tissue specific ADAM10 knockout mice [8, 35]. Unfortunately, loss of ADAM10 expression or chemical inhibition of the ADAM10-Hla interaction precludes the possibility of discerning the effects of cell death and ADAM10 activity, as ADAM10 is absolutely required for pore-formation. The recent finding that the protease-dead mutant ADAM10_{E384A} can still bind Hla to allow pore-formation [116] will be an invaluable tool to dissect the role of the two mechanisms during *S. aureus* pathogenesis.

The expression profile of the receptors for the bicomponent leukocidins suggest that they may also target epithelial or endothelial cells to disrupt barrier function. PVL has already been shown to form pores on keratinocytes and mediate cell death, which helps explain its association with necrotizing pneumonia and dermonecrosis in human patients and rabbit models [13, 76, 179]. Intriguingly, ADAM10 may also be involved in leukocidin toxicity, since its enzymatic activity appears to be activated by at least one other PFT, pneumolysin, resulting in E-cadherin cleavage [8, 131]. Thus, ADAM10 may represent a common mechanism by which all PFTs can disrupt barrier function.

5. Toxin Redundancy or Specificity?

The identification of the primary receptors for Hla and the leukocidins have shed light on their cellular targets and informing the molecular mechanisms of cellular action. It is apparent that despite the structural similarity of these toxins, each PFT acts upon a unique set of cells. Here, we offer some perspectives on how PFT activity *in vivo* is highly specific, despite the fact that some cell types, such as PMNs and macrophages, appear to be the target of multiple PFTs.

5.1 Differential regulation

While some toxins can act on the same cell type, differential gene regulation may confer temporal and spatial specificity of toxin action. In general, all *S. aureus* PFTs are upregulated in a growth or density dependent manner, pointing to a partial dependence on the Agr locus [190, 191]. Transcriptional studies using varying growth medium indicate that the PFTs are differentially regulated, reflecting the specific challenges *S. aureus* must overcome for survival in a particular environment. For example, *lukS-PV*, *lukE*, and *hlgB* are upregulated in human cutaneous abscesses, which are replete with leukocytes. Indeed, while these genes are also induced in the presence of PMNs *in vitro*, *hla* is not appreciably induced in either condition [192]. *hlgACB* is highly upregulated during growth in blood, which is not surprising given its hypothesized role in lysing RBCs to aid in *S. aureus* iron-acquisition during infection [15, 193]. Similarly, the promoter for *lukAB* is activated in the presence of PMNs, reflecting its role in escaping from and killing PMNs and macrophages [92, 93]. Although both LukAB and PVL are toxic to PMNs, the relevance of each toxin is

dependent on the growth environment. Whereas PVL is more highly expressed and thus predominates when *S. aureus* is grown in tryptic soy broth (TSB), LukAB predominates when grown in Roswell Park Memorial Institute medium (RPMI 1640) [93]. The transcriptional pathways that regulate these responses, and the response-inducers, are not fully understood. Like other pathogens, nutrient availability is an important environmental cue that regulates virulence genes. One recently identified transcriptional regulator is RpiRc, which down regulates *lukED* and *pvI* loci, probably in response to changes in metabolism [194]. Further delineation of these pathways will be necessary to better understand the physiological relevance of each toxin in the context of the host tissue microenvironment.

5.2 Coordinated toxin action

Although expression profiles of the toxin-receptors suggest that toxins act redundantly, each toxin likely plays non-redundant roles in eliciting inflammation and damage. While individual toxins can be sufficient to enhance bacterial virulence, the combined action of multiple PFTs may be necessary for full virulence. For example, both PVL and LukAB are independently capable of forming pores on PMNs, for example, but their cytotoxic effect is enhanced when combined [56]. Intoxication by one PFT may lead to increased receptor expression for another PFT- for example, PMNs treated with Hla or PVL become activated, resulting in increased CD11b surface expression [169, 195]. In turn, this may increase the susceptibility of PMNs to LukAB, though this has not been empirically tested.

Cooperative effects of the toxins are more apparent in disease models incorporating multiple cell types. A recent study utilizing a three-dimensional lung tissue culture model demonstrated PVL cooperates with Hla to cause lung epithelial damage. Whereas Hla caused direct epithelial damage through cell death and ADAM10 action, PVL could not [196]. However, stimulation of the epithelium with Hla potently induces neutrophil chemotaxis through IL-8 and HMGB1 secretion. Arriving neutrophils, in turn, are activated and lysed by PVL, exacerbating the damage through the secretion of inflammatory or toxic products [189, 196]. PVL is known to cause lung damage through neutrophil activation, and this effect is likely enhanced with Hla [76]. Examining the *S. aureus* PFTs independently from one another obscures the cooperative effects that multiple toxins may have during infection. While it would be ideal to also utilize knockout mice lacking receptors for multiple PFTs, this will be difficult to achieve, given that some PFTs (LukAB, PVL, HlgCB) do not bind murine receptors at all, and others (LukED, HlgAB) bind three or more. Hla has been the sole exception to this, binding only one murine receptor, but not without its own difficulties as ADAM10 is required for development in many tissues [197, 198]. Thus, further research using bacterial mutants lacking multiple PFTs will be the most informative approach to better understand combined effects of PFTs.

Individual *S. aureus* PFTs may represent distinct ‘modules’ of coordinated action that synergize to cause maximal damage. For example, Hla has been shown to be an important virulence factor during *S. aureus* sepsis, since *hla* mutants or ADAM10 inhibitors reduce the severity of infection in mouse and rabbits [32, 199]. Multiple cell types involved in sepsis are susceptible to Hla intoxication, such as the endothelial cells, macrophages, PMNs, platelets, and lymphocytes [200]. Although Hla is able to intoxicate myeloid-lineage and

platelet cells individually, simultaneous intoxication is necessary for the formation of highly inflammatory platelet-neutrophil aggregates (PNAs) which increases lethality from sepsis [201]. By contrast, PVL is unable to induce PNA formation, likely due to relatively low C5aR expression on platelets from healthy humans [195, 202]. Hla-induced cell damage and ADAM10 activation on the pulmonary endothelium contributes to acute lung injury (ALI) during sepsis, however, pathology is only apparent when platelets are also intoxicated, preventing proper adhesion to sites of injury to stop pulmonary vascular leakage [32, 201]. Similarly, during Hla-dependent *S. aureus* pneumonia, intoxication of both the lung epithelium and the myeloid-lineage cells causes lethal immunopathology [8, 165]. This coordination may be even more apparent in PFTs that bind multiple receptors, and therefore may attack a wider range of cells, such as LukED or HlgAB. Combinations of these ‘modules’ of pathology defined by the PFTs may define disease severity and outcomes dependent on the specific constellation of cell types that comprise the infected tissue.

6. Treatment strategies

The *S. aureus* PFTs are prime targets for novel therapeutics, given their multifaceted roles in promoting disease virulence. Therapeutics that directly target the toxin using neutralizing antibodies and vaccines have already been investigated [203–206]. Now that the receptors for each PFT have been identified, it will be possible to develop therapeutics that specifically target the host. Some of these receptors, such as ADAM10 and C5aR1, already have small molecule inhibitors that can be used to prevent toxin binding. The ADAM10 inhibitor, GI254023X, has been used in mouse models to prevent Hla activity during sepsis, SSTI, and pneumonia [8, 32, 35]. In addition to blocking Hla monomers from binding to target cells to initiate pore-formation, GI254023X can also block the catalytic activity of ADAM10 after pores have already been formed [8], raising the possibility that other ADAM10-targeting therapeutics may be efficacious for a long period of time following the initial infection event. Given that ADAM10 may be non-specifically activated by all PFTs, this may be a therapeutic that can be considered for infections by other microbes [8]. Since many of the PFT receptors are also attractive targets for other diseases (ex. HIV for CCR5; auto-immune diseases for C5aR1), existing small molecule inhibitors may effectively block toxin binding, accelerating the drug discovery process.

Treatment strategies must incorporate timing and drug delivery methods that are specific to the site of *S. aureus* injury. The recent identification of the PFT receptors will allow for further studies to dissect the mechanism by which these toxins impart full virulence in their hosts. Investigations using conditional knockout ADAM10 mice have already revealed differences in toxin activity depending on the infection site- specifically, that preventing toxin activity on macrophages in the lung is beneficial to reduce lethal inflammation, whereas toxin activity on macrophages is necessary in the skin to activate anti-microbial inflammation [165]. It may not be appropriate to target toxins with a one-size-fits-all treatment approach that fails to account for local tissue biology.

Targeting toxin-receptor interactions alone may be insufficient during disease. One study utilizing a methyl β -cyclodextrin derivative (IB201) was able to prevent Hla mediated toxicity by functionally blocking the pore [127, 128], thereby preventing cell lysis and

ADAM10 activation [8, 204]. Interestingly, although host survival was enhanced, unlike antibody treated mice, pathology was not improved in IB201 treated mice. It is possible that oligomerization of the toxin without pore activity has adverse signaling effects [204]. Alternatively, IB201 is effective enough to prevent cellular lysis, but is unable to block all pores, leading to a condition phenocopying intoxication with sublytic doses of toxin. Given the plethora of data demonstrating the effects of sublytic doses of PFT, the latter is likely. In addition to toxin-targeted treatment approaches, patients may also require therapeutics that target the molecular pathways that are activated by the toxin, such as anti-inflammatory therapeutics.

In addition to targeting individual toxin-receptor pairs, it will be necessary to also consider treatments that can target multiple toxins simultaneously. This may mean targeting toxin groups that act synergistically, like Hla and PVL, particularly if each toxin has receptor-specific effects. Alternatively, targeting multiple PFTs simultaneously using a mixture of inhibitors/antibodies may be beneficial, as inflammatory signaling induced by these toxins appear to be the result of the pore rather than receptor specific effects. The development of inhibitors that can cross react with multiple PFTs would be ideal to accomplish this goal. Already one inhibitor has been published that binds directly to HlgA, HlgC, and LukS-PV to prevent receptor binding [207]. In addition, a neutralizing antibody that can target Hla in addition to LukED, HlgAB, HlgCB, and PVL has recently been identified. The investigators were unable to use the same antibody to also target LukAB, due to the fact that it exists as dimers in solution rather than monomers [206, 208]. Prophylaxis with this monoclonal antibody was more effective than an antibody targeting only Hla in rabbit and mouse models of pneumonia, reducing the overall bacterial burden [205, 206]. Use of this monoclonal antibody in the clinic as a prophylactic, or incorporation of this information into vaccination strategies will be particularly useful.

7. Concluding Remarks

Despite the discovery of the leukocidins and hemolysins over a century ago, we are only beginning to understand the complexities of toxin activity during disease. Once thought to be generally cytolytic proteins, the recent identification of the primary receptors for all of the *S. aureus* PFTs have highlighted the specificity of each toxin to their target host cells, as well non-lytic effects of the toxins. By either lysing or modifying the function of those cells, *S. aureus* enhances its ability to survive within the host.

An important revelation stemming from receptor identification is the substantive differences in host tropism of each toxin. For some toxins like PVL, HlgCB, and LukAB, structural differences in receptors from other species preclude the interaction. For others such as HlgAB and Hla, differences in receptor expression are responsible for cell-type specific intoxication. Investigators must carefully consider the sensitivity of each model organism to the different PFTs to develop a comprehensive understanding of toxin interactions during *S. aureus* disease. It may be useful to consider the use of humanized mice or human receptor knock-in mice, such as those available for C5aR1 [209].

While many insights on toxin biology have been gained owing to receptor identification, the advantage to *S. aureus* in producing as many as seven different PFTs still remains an enigma. Although the toxins may overlap in their cell-type specificities, their function during disease is not likely to be redundant. Each toxin may drive a specific aspect of *S. aureus* mediated disease, amplified through synergy with the other toxins. The discovery of the PFT host receptors will provide researchers with novel tools to dissect toxin activity *in vivo*, positioning the field to investigate the combined action of multiple virulence factors in distinct host tissue environments.

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A. α -toxin

B. Bicomponent leukocidins

Figure 1. Model of pore-formation mechanism for *S. aureus* PFTs

(A) α -toxin is secreted as a water-soluble monomer (1), which binds to its receptor ADAM10 (2). The toxin then oligomerizes into a heptamer on the plasma membrane to form the pre-pore (3). Caveolin-1 is thought to interact with Hla within the membrane bilayer to stabilize the prepore. The stem domain is then inserted into the membrane form the β -barrel transmembrane channel (4).

(B) The bicomponent leukocidins are also secreted as water-soluble monomers (with the exception of LukAB) (1). The S-component binds the cell surface receptor (2), then recruits the F-component to form heterodimers (3). These dimers oligomerize on the plasma membrane into a hetero-octamer pre-pore (4) prior to transmembrane channel formation (5).

Figure 2. Cellular targets of *S. aureus* PFTs in humans

(A) Receptor targets of Hla and the bicomponent leukocidins. (B) Human cells currently known to be susceptible to *S. aureus* PFTs depicting their expressed receptors. Some cell types, namely monocytes and neutrophils, are susceptible to multiple PFTs, whereas others, such as endothelial and epithelial cells, are currently known to only be sensitive to one toxin, Hla.

Table 1

S. aureus PFT receptors and host cell targets.

Toxin	Other names	Receptor	Human	Rabbit	Mouse	Notes
Hla	α -toxin	ADAM10	Epithelial cells/keratinocytes	Erythrocytes(high)	Epithelial cells/keratinocytes	Monomeric
	α -hemolysin		Endothelial cells Monocytes Neutrophils T-lymphocytes Platelets		Endothelial cells Monocytes Neutrophils Platelets Erythrocytes	
PVL	PVL-LukSF	C5aR1	Monocytes	Monocytes	No (mC5aR1 does not bind)	
HlgAB	γ -hemolysin	C5aR2	Neutrophils	Neutrophils		
		CXCR1/2	Monocytes	Neutrophils	Monocytes	
		CXCR4 (low)	Neutrophils		Neutrophils (medium)	
		CCR2	Erythrocytes		(mCXCR2 does not bind, but mCXCR1 does)	
HlgCB	Leukocidin γ -hemolysin γ -toxin	DARC			Erythrocytes	
		C5aR1	Monocytes	Neutrophils	No (mC5aR1 does not bind)	
		C5aR2	Neutrophils			
		C3R (low)				
LukED		CCR5	T-lymphocytes (memory T-cells)	Neutrophils	T-lymphocytes (memory cells)	
		CXCR1	Monocytes		Monocytes	
		CXCR2	Neutrophils		Neutrophils	
		DARC	Dendritic Cells		Dendritic Cells	
			Erythrocytes		Erythrocytes	
LukAB	LukGH	CD11b	Monocytes	Neutrophils (rCD11b low affinity)	No (mCD11b doesn't bind)	Secreted as heterodimer
Luk MF		CCR1	Neutrophils		T-lymphocytes	Only expressed by ruminant strains of aureus
			Monocytes		Monocytes	Bovine receptors: CCR1, CCR2, CCR5
					Neutrophils	Bovine cellular targets: monocytes, neutrophils