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The effects of *Staphylococcus aureus* leukotoxins on the host: cell lysis and beyond

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Abstract

The success of *Staphylococcus aureus* as a leading cause of deadly hospital-acquired and community-acquired infections is attributed to its high-level resistance to most antibiotics, and the multitude of virulence factors it elaborates. Most clinical isolates produce up to four bi-component pore-forming toxins capable of lysing cells of the immune system. Subtle differences in activity and target range of each leukotoxin suggest that these toxins are not redundant, but instead may have specialized functions in attacking and/or evading host defenses. In turn, the host has developed countermeasures recognizing sublytic levels of leukotoxins as signals to activate protective immune defenses. The opposing cytotoxic and immune-activating effects of leukotoxins on host cells make for a complex dynamic between *S. aureus* and the host.

Introduction

Staphylococcus aureus is a highly successful bacterial pathogen that causes significant hospital-acquired infections [1]. Its success is attributed in part to its rapid acquisition of resistance to most available antimicrobial therapies. Even more alarming, it has recently begun causing deadly infections outside of healthcare settings, in relatively healthy and young individuals [2,3]. Strains isolated from both community-acquired and hospital-acquired infections are predominantly methicillin-resistant (MRSA), making successful treatment highly challenging [2]. In addition to its resistance to almost every optimally effective antibiotic, the tenacity of *S. aureus* as a pathogen can be attributed to its arsenal of virulence factors designed to evade or attack host defenses at every level [4,5]. A subset of its virulence factors includes a collection of pore-forming toxins capable of targeted killing of select host cells by creating channels in the plasma membrane. The resulting osmotic dysregulation eventually leads to cell lysis. The pore-forming leukotoxins produced and secreted into the extracellular milieu by *S. aureus* are unique in that they consist of two different protein components that assemble together to form β -barrel pores [6*,7**]. *S. aureus* strains associated with human infections produce four leukotoxins with considerable sequence similarity: the Pantone-Valentine Leukocidin (PVL), gamma (γ)-hemolysin (HlgACB), Leukotoxin ED (LukED), and Leukotoxin AB/GH (LukAB/GH). This review outlines the activities of each leukotoxin, as well as highlights the differences between them (Table 1 and Figure 1).

Leukotoxin pore formation

The two protein subunits of each leukotoxin are classified as ‘S’ and ‘F’. This nomenclature corresponds to the initial purification of PVL where the first eluted subunit was termed fast (F), and the subunit that eluted subsequently was termed slow (S) [8]. The mechanism by

which these toxins bind and/or interact with the target host cells has not been completely elucidated. Initial studies with PVL gave rise to a model whereby LukS-PV serves as the homing component that binds specifically to a host cell receptor, after which LukF-PV is recruited by LukS-PV already docked to its receptor [9]. Conversely, work carried out with γ -hemolysin, indicated that HlgB (the 'F' component of the toxin) binds to its target cell first, followed by the binding of HlgA (the 'S' component) [10]. More recent work suggests the possibility of distinct cellular receptors for both LukS-PV and LukF-PV [11*].

Once localized on the cell surface, the subunits assemble into an octameric prepore with four of each alternating F and S components [6*,7**,12]. Pore formation culminates when the stem domains of the assembled subunits unfold and insert into the cell membrane to form β -barrel pores (Figure 2, steps in pore formation outlined in gray).

Target cells of *S. aureus* leukotoxins

As their names would suggest, these leukotoxins can lyse cells of the leukocytic lineage; however, subtle differences in the susceptibilities of various host cells may explain why *S. aureus* produces multiple highly related molecules that seemingly perform the same function. For instance, all four leukotoxins have been demonstrated to kill human neutrophils [13–15,16**,17**,18], but only γ -hemolysin, and to a lesser extent, LukED, exhibit lytic activity against red blood cells [10,13,18]. Further details of cellular targets of each leukotoxin will be expanded on in the following section.

Sensitivity to leukotoxins also differs between species. For example, human neutrophils are highly susceptible to PVL and LukAB/GH, while neutrophils from mice are largely resistant to lysis by both toxins [16**,19*]. Susceptibility of cells from any one species also varies depending on the toxin, as in the case of monkey neutrophils, which are highly resistant to killing by PVL [16**] but susceptible to LukAB/GH [19*]. In addition, LukED appears to be the only leukotoxin that can kill mouse neutrophils efficiently [20**].

Leukotoxin involvement in *S. aureus* pathogenesis

γ -Hemolysin (HlgAB/HlgCB)

Two protein subunit combinations comprise γ -hemolysin, both sharing the same F subunit (HlgB), but differing in S subunit composition (HlgA or HlgC) [21]. Given that γ -hemolysin is the only leukotoxin within this family that can lyse red blood cells with high efficiency, it was not surprising to find that the *hlg* genes are highly upregulated in human blood. In turn, γ -hemolysin promotes the survival of *S. aureus* in blood [22]. Consistent with these *ex vivo* findings, γ -hemolysin conferred an advantage to *S. aureus* in mouse models of bloodstream infections, including bacteremia and septic arthritis [22,23]. The presence of *hlg* genes in virtually all *S. aureus* strains (99%), not only in isolates from bloodborne infections [24], would suggest that γ -hemolysin could also be an important factor in multiple modes of infection.

LukED

Despite the initial report of this toxin over a decade ago [14,18], research on LukED has remained minimal. As mentioned in the preceding section, in addition to its toxicity toward human neutrophils and rabbit red blood cells, LukED is unique in being highly effective at killing mouse phagocytes [20]. Accordingly, LukED was found to contribute to the lethality observed in a mouse model of *S. aureus* bacteremia. It did so by targeting and killing phagocytes, thereby allowing LukED⁺ *S. aureus* to proliferate. The *lukED* genes have been found in 87% of clinical strains [18], including many MRSA strains responsible for current

pandemics [20], suggesting a potentially significant contribution of this leukotoxin to the pathogenesis of *S. aureus*.

LukAB/GH

LukAB/GH is the most recently identified leukotoxin [17^{**},25^{*}], and is also the most divergent among those produced by *S. aureus*. Sequence identities with other Staphylococcal leukotoxins are below 40% (Figure 1). Despite this low level of sequence identity, LukAB/GH can efficiently kill human neutrophils, monocytes, macrophages, and dendritic cells [17^{**}]. Its toxicity is enhanced further by synergism with PVL [25^{*}]. Interestingly, LukAB/GH is the only leukotoxin reported to associate with the cell surface of *S. aureus*, in addition to being secreted like the other leukotoxins [25^{*}]. The mechanism of LukAB/GH association with the bacterial surface is yet to be investigated. It is also presently unclear if surface-associated LukAB/GH can function as an active toxin. So far LukAB/GH is the only bi-component leukotoxin known to enhance *S. aureus* survival upon phagocytosis by human neutrophils [17^{**}], implying that in addition to 'conventional' cell lysis from outside the neutrophil, this toxin may also facilitate the escape of *S. aureus* from the phagosome. Toxicity of LukAB/GH toward neutrophils is the likely mechanism by which the toxin promotes the survival of *S. aureus* in whole blood, as it has no detectable hemolytic activity [17^{**}]. The contribution of LukAB/GH to virulence was recently demonstrated using a low infectious dose systemic infection model, where presence of the toxin was associated with higher bacterial burden in infected kidneys [17^{**}]. While exhaustive analyses identifying the presence of *lukAB/GH* genes in clinical strains of *S. aureus* are not presently available, all sequenced *S. aureus* strains and all clinical isolates examined in our laboratory are positive for *lukAB/GH*, further suggesting that this toxin is also a highly relevant virulence factor produced by *S. aureus*.

PVL

PVL is present in a small percentage (~5%) of overall *S. aureus* clinical strains, although it is strongly associated with community-acquired MRSA strains (~85%), in particular those causing pneumonia, and skin and soft tissue infections [2,26]. In spite of its apparent linkage to virulent strains capable of causing deadly infections in healthy people, its contribution to virulence has not been conclusively proven. Several multinational studies have concluded that *S. aureus* infection outcomes are not linked to PVL production [27–29], while in some other cases, infections with PVL⁺ strains are even associated with better outcome [30–32]. As such, while PVL is the most studied Staphylococcal leukotoxin, it is also arguably the most contentious.

Because of the resistance of mouse cells to lysis by PVL [16^{**}], murine models of infection examining the contribution of PVL's pore-forming ability yielded inconsistent conclusions by various research groups. Instead, the use of rabbit infection models was considered more appropriate, given that rabbit neutrophils are highly sensitive to PVL [16^{**}]. Nonetheless, data compiled from several rabbit models provided little new insight. One study showed that PVL conferred a modest transient advantage to *S. aureus* in the early stages of bacteremia and in a pneumonia model, but only at very high inocula ($>5 \times 10^9$ CFU) [33,34]. Using rabbit skin infection models, divergent conclusions were reached by two independent groups, despite both using the same MRSA strain. Lipinska *et al.* showed the PVL⁺ strain to cause more significant skin necrosis compared to the isogenic ΔpvI strain [35]. On the contrary, Kobayashi *et al.* reported that larger abscesses were caused by the ΔpvI strain [36]. It should be noted however, that there were subtle variations in experimental design, including the species of rabbit used, slight differences in inocula and site of injection. There was also a study in nonhuman primates (cynomolgus macaques) that compared an isogenic WT and ΔpvI /MRSA strain in causing lower respiratory tract disease [37]. Outcomes of

infection were comparable between WT and Δpv /infected animals, however, the sample sizes were small. Thus, after decades of research by multiple groups, the contribution of PVL to *S. aureus* and MRSA pathogenesis remains inconclusive.

Immune-activating properties of Staphylococcal leukotoxins

In addition to the pore-forming capabilities of these leukotoxins, it has become increasingly apparent that these molecules are also potent activators of innate immunity. Early studies titrating PVL below the threshold of cytotoxicity on its target cells (sublytic concentrations), revealed that PVL activates cells to amplify host immune defenses without causing cell damage. At sublytic concentrations, PVL can cause human neutrophils to secrete proinflammatory mediators (including interleukin (IL)-8 and leukotriene B₄) [38,39] and antimicrobial factors [40,41], as well as enhance phagocytosis and killing of *S. aureus* [42**]. Additionally, PVL can also directly activate cells that are resistant to its lytic activity, including mouse cells and non-immune human cells [40]. Inflammatory cytokines elicited by PVL may in turn activate other cells to secrete additional immune mediators, potentially augmenting inflammation [43**]. As such, host cell activation by PVL has the potential to outweigh its lytic action, possibly explaining why PVL is sometimes associated with better outcomes for the host.

There is ample evidence that other Staphylococcal leukotoxins are also highly effective immune-activators. Like PVL, LukAB/GH can elicit an inflammatory response in rabbits when injected intradermally [19*]. γ -Hemolysin can also synergize with α -hemolysin and β -hemolysin to activate the NLRP3 inflammasome and Caspase-1 [44**]. Interestingly, pore-formation on immune cells can trigger activation of Caspase-1, suggesting that all leukotoxins are capable of doing so [45]. Caspase-1 in turn initiates inflammatory programmed cell death, termed pyroptosis, coupled with the release of large amounts of proinflammatory cytokines IL-1 β , IL-18 and IL-33 [45,46]. Cytokine activation of surrounding immune cells and Caspase-1 triggered pathways involved in membrane biogenesis are also linked to increased cell survival [45] (Figure 2, steps involved in immune activation are outlined with red dashed boxes). Taken together, the contribution of leukotoxins to *S. aureus* infection is likely to be multifactorial, contingent upon toxin concentration.

Because of the potent lytic activity of the Staphylococcal leukotoxins, there is little doubt that they have tremendous potential to cause cellular damage, which in turn will potentiate *S. aureus* pathogenesis. In an attempt to counter this response, the mammalian host recognizes low concentrations of these leukotoxins to amplify immune defenses. There are multiple factors involved in establishing an infection, including bacterial load, bacterial factors elaborated during infection, site of infection, immune status of the host, and antimicrobial therapies, to name a few. Interplay between host defenses and bacterial factors can dynamically tilt the balance between the host response and the pathogenic process, impacting the outcome of infection.

Complexities of multiple leukotoxin production by *S. aureus*

Despite production of numerous related leukotoxins by *S. aureus*, each leukotoxin has a unique repertoire of targets cells, indicating a distinct role for each during the infection process. Additionally, expression and production of each leukotoxin varies independently of each other when *S. aureus* is grown under different conditions [47,48]. This highlights the possibility for differential toxin production at different tissue sites, as the unique environment at each site could alter bacterial growth and toxin production. Adding to the complexity, *S. aureus* clinical isolates also differ in the combination of leukotoxins that they produce [24]. This variability could have major consequences for disease progression when

the potential for synergism between leukotoxins is considered [25^{*}]. The non-lytic, immune-activating effects of these leukotoxins further confound the actual sequence of events during an infection. Further comprehensive study and understanding of the Staphylococcal leukotoxins are needed, as many thought provoking questions remain.

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Figure 1.

Amino acid sequence alignments of *S. aureus* leukotoxin subunits, and percentage identities between them. Panel (a) shows a comparison between 'S' subunits of leukotoxins, while panel (b) shows a comparison between 'F' subunits. Amino acid sequences from the predicted secreted toxin subunits from MRSA USA300 strain FPR3757 were aligned using the Clustal Omega program powered by the European Molecular Biology Laboratory-European Bioinformatics Institute. Red shading signifies fully conserved residues, gray shading indicates conservation between groups of strongly similar properties, and blue letters denote conservation between groups of weakly similar residues. Tables denote the percentage identity between amino acid sequences of the respective leukotoxin subunits.

Figure 2.

Schematic of *S. aureus* leukotoxin pore-formation, and the different cellular pathways it uses to activate host cells. Steps in pore formation are outlined in gray, while steps involved in immune activation are outlined with red dashed boxes and shapes. This model is based predominantly on research and data from PVL [6*,9,11*,38,39,49*]. Activation of the inflammasome and Caspase-1 is based on work studying γ -hemolysin [44**,45]. This figure was adapted from [50].

Table 1

List of *Staphylococcus aureus* bicomponent leukotoxins, and the 'S' and 'F' subunits that encompass each leukotoxin

Leukotoxin	S subunit	F subunit
PVL	LukS-PV	LukF-PV
LukAB/LukGH	LukA/LukH	LukB/LukG
LukED	LukE	LukD
γ -Hemolysin	HlgA, HlgC	HlgB