The Bicomponent Pore-Forming Leucocidins of *Staphylococcus aureus*

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SUMMARY

The ability to produce water-soluble proteins with the capacity to oligomerize and form pores within cellular lipid bilayers is a trait conserved among nearly all forms of life, including humans, single-celled eukaryotes, and numerous bacterial species. In bacteria, some of the most notable pore-forming molecules are protein toxins that interact with mammalian cell membranes to promote lysis, deliver effectors, and modulate cellular homeostasis. Of the bacterial species capable of producing pore-forming toxic molecules, the Gram-positive pathogen *Staphylococcus aureus* is one of the most notorious. *S. aureus* can produce seven different pore-forming protein toxins, all of which are believed to play a unique role in promoting the ability of the organism to cause disease in humans and other mammals. The most diverse of these pore-forming toxins, in terms of both functional activity and global representation within *S. aureus* clinical isolates, are the bicomponent leucocidins. From the first description of their activity on host immune cells over 100 years ago to the detailed investigations of their biochemical function today, the leucocidins remain at the forefront of *S. aureus* pathogenesis research initiatives. Study of their mode of action is of immediate interest in the realm of therapeutic agent design as well as for studies of bacterial pathogenesis. This review provides an updated perspective on our understanding of the *S. aureus* leucocidins and their function, specificity, and potential as therapeutic targets.

INTRODUCTION

Staphylococcus aureus is a major bacterial pathogen that causes a significant disease burden in both hospital and community settings (1, 2). The organism can colonize or infect nearly all host tissues, from the skin and nares to bone, joints, muscle, heart, and lungs (1–3). Invasive infections that disseminate via the bloodstream can lead to devastating clinical consequences if treatment is not rapidly initiated. The ultimate success of *S. aureus* in multiple
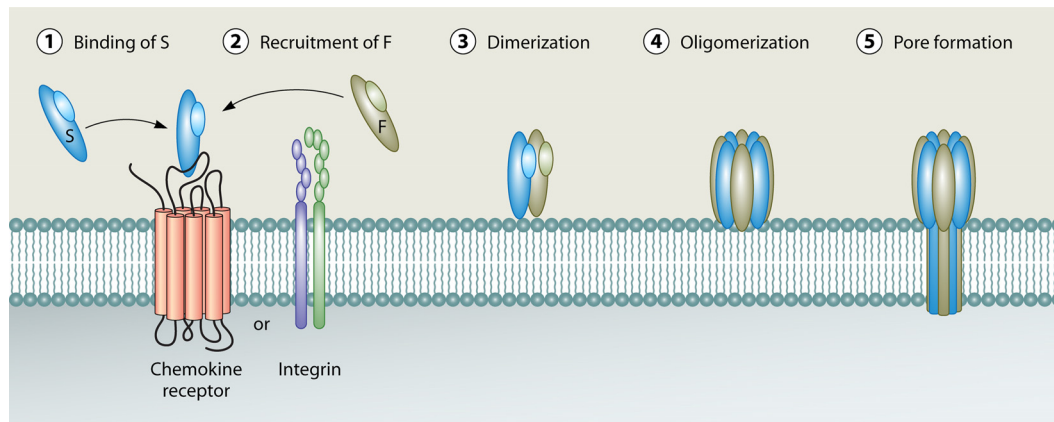
FIG 1 Current model of leucocidin pore formation. Leucocidin pore formation is believed to occur in a stepwise fashion that begins with toxin recognition of cellular receptors on the surface of target host cells. On most host cells, the "S" subunit recognizes a proteinaceous receptor (either a chemokine receptor [LukED and PVL] or an integrin [LukAB/HG]) to facilitate high-affinity binding to the cell surface (1). The S subunit then recognizes and recruits the "F" subunit (2), leading to dimerization on the host cell surface (3). Dimerization is followed by oligomer formation (4). Toxin oligomers assemble into an octameric prepore structure containing alternating S and F subunits. Following oligomerization, a major structural change occurs in the stem domains of the S and F subunits, leading to membrane insertion and the formation of a β-barrel pore that spans the host cell lipid bilayer (5).

disparate host environments and its high incidence among both hospitalized and otherwise healthy individuals make this organism a major concern for public health.

Currently, the clinical standard of care for individuals with invasive *S. aureus* infections includes aggressive administration of antibiotics (4). However, the recent increase in the incidence of multidrug-resistant isolates such as hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) and community-acquired MRSA (CA-MRSA) and the increased dominance of highly virulent clonal lineages that can cause aggressive disease have diminished the success of such therapeutic strategies (5–10). A number of comprehensive reviews addressing the increased incidence of these infectious lineages have already been reported (5, 6, 11–13). It is clear that *S. aureus* exhibits tremendous adaptability when confronted with aversive stimuli (i.e., antibiotics) and harsh environmental conditions (host tissues that deprive the organism of essential nutrients), allowing it to execute a highly pathogenic life-style. Noteworthy examples of its adaptability are the organism's rapid exchange and/or acquisition of DNA and the mutability of its genome, both of which facilitate unrelenting resistance to antibiotics and promote novel and/or enhanced virulence traits (1, 11, 14–18). Such genetic plasticity has left us with limited options for combating the diverse and sometimes deadly conditions associated with *S. aureus* infection.

A major pathogenic attribute of *S. aureus* that facilitates its survival during infection is the ability to secrete a diverse repertoire of immune system evasion factors. Included among these factors are a number of potent cytotoxins (hemolysins, cytolytic peptides, and leucocidins), immunomodulatory proteins (superantigens, superantigen-like proteins, and complement-inhibitory proteins), proteases, and factors that prevent immune cell recognition and killing (protein A, capsule, and catalase, among others) (19–27). Each of these molecules subverts the host immune system in different ways, leaving the organism largely resistant to both innate and adaptive immune defenses. A number of recent reviews and primary research articles serve to highlight the unique mechanisms by which these factors promote immune evasion (11, 19, 25, 28–30). Not only do they work in concert to

effectively inhibit clearance of *S. aureus* by the host immune surveillance, they also prevent the development of immunological memory against this pathogen (31–34). This combined attack on both innate and adaptive immune defenses is believed to be a major reason why current therapeutic and vaccine strategies, which rely on robust innate and adaptive pathogen recognition, have failed (35–38). Clearly, there are major limitations of our current efforts to combat a pathogen such as *S. aureus*, which has adapted to avoid nearly all immune recognition strategies. As a result, recent initiatives are now under way to better define mechanisms by which *S. aureus* subverts the host immune system within a therapeutic framework (39). The underlying themes of these studies are to use our knowledge of *S. aureus* immune evasion tactics to bolster the host immune response, promote natural clearance of bacteria, and enhance the development of immunologic memory against this pathogen.

Of interest in the development of therapeutic modalities aimed at promoting the natural clearance of *S. aureus* by the host immune system are the bicomponent pore-forming leucocidins (19–21). These toxins consist of two separate water-soluble monomeric subunits that target and kill immune cells by binding to host leukocyte membranes and forming β-barrel pores that span the phospholipid bilayer (Fig. 1). All strains of *S. aureus* are capable of producing at least three (HlgAB, HlgCB, and LukAB/HG) of the six known leucocidins, while most highly virulent clinical strains currently infecting humans produce five (HlgAB, HlgCB, LukAB/HG, Panton-Valentine leucocidin [PVL], and LukED). One additional toxin, LukMF′, is present in *S. aureus* lineages that are isolated from ruminants and other mammals but not humans. It is worth noting that other classes of proteinaceous molecules, including the phenol-soluble modulins (PSMs), a family of small amphipathic α-helical peptides with broad-range lytic activities, can also lyse host immune cells, leading to their classification as "leucocidins." Included within the PSM family are the α- and β-type PSMs as well as the historically characterized delta-toxin, a member of the α-type PSMs. These leukocytolytic molecules are distinct from the classically defined bicomponent β-barrel pore-forming leucocidins discussed in this review (for detailed infor-
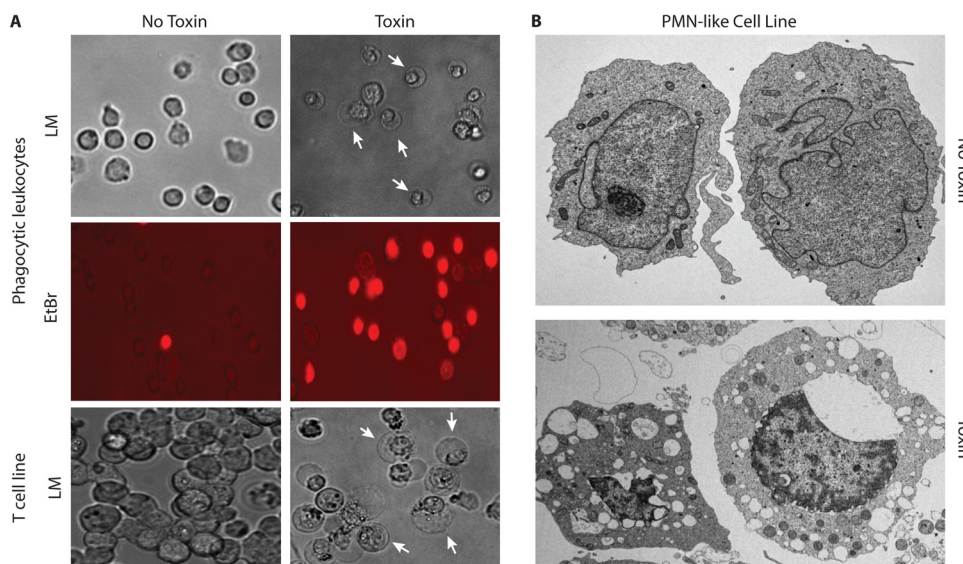
FIG 2 Morphological changes associated with leucocidin-mediated killing of immune cells. (A) Light and fluorescence microscopy images of murine phagocytic leukocytes (macrophages and neutrophils) and light microscopy (LM) images of the human T cell line HUT-R5 (cell line overexpressing CCR5) after exposure to a 90% lethal dose of LukED (5 μg/ml). Characteristic membrane halos and expansion of cellular nuclei are seen, along with increased permeability to ethidium bromide (EtBr) (red), an indicator of pore formation and membrane damage. Arrows point to characteristic cellular morphology changes upon leucocidin intoxication. (B) Electron microscopy images of the human PMN-like cell line PMN-HL60 after exposure to *S. aureus* supernatant containing a 100% lethal dose of LukAB/HG (>2.5 μg/ml). All intoxications and microscopic image acquisition were conducted as previously described (47, 97, 227).

mation on PSMs and their roles in *S. aureus* immune evasion, see references 29, 40, and 41). Reemerging interest in the bicomponent leucocidins has occurred due to their potential as targets in therapeutic design, which could ultimately promote natural clearance of *S. aureus* infection and reduce reliance on antibiotic usage in already resistant strains (20). In recent years, our understanding of the bicomponent leucocidins has changed dramatically. This review will serve to consolidate our current understanding of bicomponent leucocidin biology and provide an outlook for future studies and therapeutic development.

A BRIEF HISTORICAL PERSPECTIVE: IDENTIFICATION AND EARLY CHARACTERIZATION OF THE LEUCOCIDINS

Late 19th Century to Mid-20th Century: Discovery of *S. aureus* Leukolytic Activity and Identification of Panton-Valentine Leucocidin

The study of *S. aureus* leucocidins has a history that spans more than 110 years (42). In 1894, Van de Velde published the first studies to demonstrate the leukocidal activity of *S. aureus* (then called *Staphylococcus pyogenes*) on primary rabbit leukocytes (42, 43). This leukotoxic substance was given the name "leucocidin." At that time, the composition of leucocidin was unknown. Furthermore, it was unclear whether leucocidin was a novel secreted substance or whether the activity was a secondary function of the already described hemolysin (later known as alpha-hemolysin). Additionally, it is not absolutely certain whether Van de Velde's description of leukocidal activity was actually caused by the *S. aureus* leucocidins or whether the observed effects were induced by other toxic molecules present within the complex milieu of bacterial culture supernatant. Observations made in later years, describing a leukocidal activity similar to what was originally described by Van de Velde, support the notion that the "leucocidin" was indeed that of an *S. aureus* bicomponent leucocidin(s) (44–46). His early obser-

vations closely resembled what was later described by Gladstone and van Heyningen and are identical to what we see today upon the intoxication of leukocytes with any of the six known bicomponent leucocidins (Fig. 2) (44, 47). It is therefore likely that the leucocidin described by Van de Velde was that of either one or multiple bicomponent leucocidins. The following year, Denys and Van de Velde also demonstrated that rabbits are able to generate neutralizing antibody against the leucocidin (43). This study was the first to demonstrate the immunogenicity of leucocidin and the utility of neutralizing antibodies in protecting immune cells from the leukocidal activity of *S. aureus* (43).

In the early 1900s, Neisser and Wechsberg followed up on the work of Van de Velde by performing studies to determine whether the leukocidal and hemolytic activities of *S. aureus* were caused by independent toxic substances (48). Through adsorption of hemolysin with red blood cells (RBCs) or leucocidin with leukocytes, those authors determined that each substance could be selectively removed from culture supernatants without influencing the activity of the other (48). Similarly, Julianelle performed studies aimed at correlating leukocidal activity with hemolytic activity within a series of strains and found that in a number of instances, the activities of hemolysin and leucocidin did not coincide with one another (46). As the 20th century progressed, however, more detailed investigations, including those by Weld and Gunther, were unable to conclusively validate whether the *S. aureus* hemolysin (now referred to as alpha-hemolysin) and leucocidin exert functions independently of one another (49, 50). Many researchers were unable to reproduce the adsorption studies of Neisser and Wechsberg, and the conjecture that alpha-hemolysin and leucocidin were independent substances remained dubious at best (48, 50, 51). Much of the reason for the difficulty in deciphering functional differences between alpha-hemolysin and leucocidin would not be appreciated until much later, when the regulatory com-

plexities and breadth of strains expressing more than one leucocidin would be fully evaluated.

In 1932, the conclusion that leucocidin and alpha-hemolysin were unique substances was finally validated by the work of Panton and Valentine. Through detailed phenotypic studies of a number of *S. aureus* strains, some of which had potent hemolytic activity but no leukocidal activity and *vice versa*, it was conclusively established that the hemolytic and leukocidal activities of *S. aureus* were caused by two unique substances (45, 52). Later work validated these landmark studies and prompted a renaming of leucocidin to Panton-Valentine leucocidin or PV leucocidin (PVL), thereby distinguishing it from the suspect Neisser-Wechsberg (NW) leucocidin (which was later found to actually be alpha-hemolysin) (44, 49, 53). Thus, Van de Velde's original definition of leucocidin as a unique entity, insofar as it resembles Panton-Valentine leucocidin, was largely correct. Again, it should be noted that these studies were conducted before it was realized that *S. aureus* strains often encode more than one leucocidin. It is therefore difficult to ascertain whether the leukocidal activity was strictly caused by PVL or a combination of leucocidins present within culture filtrates. We presume that it was likely the latter circumstance for most studies using *S. aureus* culture supernatants, prior to the purification of PVL by Woodin in the 1960s (54, 55). Thus, "PVL" remained the primary toxin of study for much of the mid-20th century (1960s to 1970s). As such, it was the first leucocidin to be purified and served to demonstrate a number of the hallmark features of these toxins (54–71). Of his numerous contributions to the leucocidin field, Woodin provided the first evidence that active leucocidins consist of two separate protein components, designated "S," or slow (LukS-PV), and "F," or fast (LukF-PV), based on their chromatography elution profiles (Table 1). The bicomponent leucocidins are cytotoxic only when both S and F subunits are combined (54, 55).

Mid- to Late 20th Century: Identification of Gamma-Hemolysin

During the same time when PVL was initially being characterized, Smith and Price identified what they described as an alternate hemolysin with distinct biochemical characteristics. This novel hemolysin was derived from an *S. aureus* strain which did not produce appreciable amounts of alpha-hemolysin (72). As such, it was given the name gamma-hemolysin and would later be identified as one of two bicomponent leucocidins with the ability to lyse both leukocytes as well as red blood cells (the other leucocidin being LukED). After its initial identification in 1938, further studies of gamma-hemolysin were hampered due to the difficulty in separating its activity from those of other hemolysins in *S. aureus* (by this time, at least three unique hemolysins had been described, alpha-, beta-, and delta-hemolysins) (73–76). It was not until the 1960s and 1970s that purified preparations of gamma-hemolysin were isolated and its lytic activity was demonstrated on red blood cells of diverse species (76–81). Like PVL, gamma-hemolysin was purified as two separate protein subunits that were active only when combined (77, 78, 82, 83). Fackrell and Wiseman determined that, in addition to red blood cells, gamma-hemolysin exhibited lytic activity on human leukocytes and lymphoblasts, and Szmigielski et al. further measured lytic activity on rabbit polymorphonuclear leukocytes (81, 84). Altogether, these studies served to classify gamma-hemolysin as both a bicomponent leucocidin and a hemolysin (Table 1). This dual lytic activity on leu-

kocytes and red blood cells may explain why early work to determine whether or not the hemolytic and leukocidal activities of *S. aureus* were unique was such a challenging endeavor. It was not until the late 1980s that the gamma-hemolysin genetic locus was cloned and found to actually consist of three genes that comprise two separate toxins, HlgAB and HlgCB. HlgA and HlgC are S components, while HlgB is a common F component (54, 85–90). Given its early identification, gamma-hemolysin, like PVL, has been studied extensively at the biochemical and biophysical levels. Details of the genetic architecture of gamma-hemolysin, its location within the *S. aureus* genome, and its biological activity are discussed below.

Late 20th Century to Present: Identification of Leucocidins MF′, ED, and AB/HG

Leucocidin MF′ was first identified in 1995 by Choorit and colleagues (91). The S subunit of the toxin (LukM) was serendipitously isolated during routine purification of the gamma-hemolysin subunits HlgC (LukS) and HlgB (LukF) for functional studies. LukM was initially characterized as an F-type subunit based on functional similarity to HlgB of gamma-hemolysin; however, its high degree of sequence similarity to the gamma-hemolysin S subunits HlgA and HlgC (~70%) and its cross-reactivity with S subunit antibodies of gamma-hemolysin suggested greater similarity to S-type subunits (91). Follow-up studies, which purified and sequenced a novel F component located immediately downstream of the LukM stop codon, served to identify the corresponding F-type subunit of LukMF′, designated LukF′-PV (LukF-PV P83) (92). The activity and limited host range of LukMF′ are discussed below (Table 1).

The identification of an additional leucocidin, LukED, followed in 1998. Gravet and colleagues performed a series of radial gel immunoprecipitation assays to identify molecules in *S. aureus* culture filtrates that were immunologically similar to those of other known leucocidins (93). The use of specified medium conditions led to the increased expression of a novel immunologically reactive molecule while diminishing the expression of other known leucocidins, allowing the purification and subsequent molecular characterization of LukED from *S. aureus* strain Newman (NTCC 8178) (93). Later, Morinaga and colleagues identified what they termed a variant of LukED, LukED$_v$, that was conserved in a remarkable number (>87%) of *S. aureus* strains and was lytic when added to both leukocytes and rabbit red blood cells (Table 1) (94). Ten years after these initial discoveries, it was determined that the original genetic sequence of *lukED* was significantly dissimilar from that of *lukED* found in all other sequenced *S. aureus* isolates, including other sources of strain Newman from which the toxin was first identified. This finding suggests that the originally described *lukED* sequence was likely a sequence anomaly (47). In all other instances, *lukED$_v$* is the dominant sequence type and is remarkably conserved in all sequenced strains (47, 95). Thus, it is suggested for ease of reference that LukED be uniformly used as the primary nomenclature for this toxin here (Table 1).

In 2010, an additional leukocytolytic bicomponent toxin with ~30% amino acid sequence identity to the other known leucocidins was identified (96). Ventura et al. discovered a putative leucocidin during a study that used bacterial cell surface proteomics to identify *S. aureus* proteins that promote the increased virulence of current epidemic strains of CA-MRSA. Some of the most abundant peptides within their cell surface preparations corresponded

TABLE 1 Characteristics of the leukotoxins of *Staphylococcus aureus*

Toxin	Alternate name(s)	Genome location	Toxins with closest similarity (% similarity)	Immune cell type(s) targeted	Strain distribution	Receptor(s) recognized	Role(s) in virulence	Species specificity(ies)	Sublytic activity(ies)	Hemolytic activity
LukSF	PVL, LukSF-PV	ϕSa2	S subunit, HlgC (81.7); F subunit, LukD (82.1)	Monocytes, neutrophils, macrophages	2–3% of all isolates	C5aR and C5L2	Debated; (i) pathogenesis of severe necrotizing pneumonia in rabbits, (ii) conflicting role in skin and soft tissue infection in rabbits and mice, and (iii) may influence the pathogenesis of osteomyelitis	Yes; rabbit and human immune cells only	Yes; (i) neutrophil priming, (ii) induction of apoptosis, and (iii) inflammasome activation	No
LukED	LukED$_v$	Pathogenicity island, vSaβ	S subunit, LukM (77.5); F subunit, LukF' (83.4%)	Monocytes, neutrophils, macrophages, T cells, dendritic cells, NK cells	70% of all isolates	CCR5, CXCR1, CXCR2	(i) Contributes to lethality observed during acute sepsis in mice and (ii) influences bacterial burden in murine renal abscess models	No; toxic to immune cells of rabbits, humans, mice, dogs, and carp	Unclear; (i) potential proinflammatory effects and (ii) lymphocyte proliferation	Yes
HlgAB	For HlgA, Hlg2 and HγII; for HlgB, HlgI, HγI, and LukF; for HlgAB, gamma-hemolysin and gamma-toxin	Core genome	S subunit, HlgC (69.8); F subunit, LukD (75.6)	Monocytes, macrophages, T cells, neutrophils	99% of all isolates	Unknown	(i) Role in pathogenesis of septic arthritis in mice, (ii) influences inflammation and pathogenesis in ocular infection models of rabbits, and (iii) contributes modestly to lethality observed during acute sepsis in mice	Unclear; toxic to immune cells of rabbits, humans, and some cells of murine origin	Not known	Yes
HlgCB	For HlgC, LukS; for HlgB, HlgI, HγI, and LukF; for HlgCB, leucocidin R and leucocidin	Core genome	S subunit, LukS-PV (81.7); F subunit, LukD (75.6)	Monocytes, macrophages, neutrophils	99% of all isolates	Unknown (evidence suggests shared receptors with LukSF-PV)	(i) Role in pathogenesis of septic arthritis in mice, (ii) influences inflammation and pathogenesis in ocular infection models of rabbits, and (iii) contributes modestly to lethality observed during acute sepsis in mice	Unclear; toxic to immune cells of rabbits, humans, and some cells of murine origin	Induction of Ca^{2+} release from neuronal intracellular stores	Yes
LukAB	LukHG	Core genome	S subunit, LukE (36.1); F subunit, HlgB (40.1)	Neutrophils, macrophages, monocytes, dendritic cells	Unknown; likely a significant proportion of isolates	CD11b	(i) Influences bacterial burden in murine renal abscess models and (ii) promotes inflammation in skin of rabbits	Yes; human and rabbit immune cells only	Unclear; (i) potential proinflammatory effects and (ii) NET formation	No
LukMF'	LukS'-PV, LukF'-PV	ϕSa1	S subunit, LukE (77.5); F subunit, LukD (83.4)	Bovine neutrophils and macrophages	Limited to lineages infecting ruminants and other small mammals	Unknown	Debated; believed to be involved in pathogenesis of mastitis	Yes; ruminant immune cells	Not known	No

to a protein with sequence homology to the *S. aureus* leucocidins. They named this novel leucocidin LukHG, where LukH corresponds to the S subunit and LukG corresponds to the F subunit (96). LukHG was found to be both surface associated as well as secreted and contributed directly to the lysis of primary human polymorphonuclear leukocytes (PMNs) *in vitro*, establishing its function as a leucocidin (96). Around the same time, an additional study by DuMont et al. used isogenic leucocidin deletion mutants in *S. aureus* strain Newman as well as proteomic studies of *S. aureus* culture filtrates to identify a novel leucocidin responsible for lytic activity of *S. aureus* culture supernatants derived from bacteria grown in RPMI medium supplemented with Casamino Acids (RPMI-CAS) (97). This toxin, named LukAB (LukA is the S subunit, and LukB is the F subunit) was lytic toward PMNs, macrophages, and dendritic cells; contributed to the cytotoxicity of both methicillin-sensitive *S. aureus* (MSSA) and MRSA strains; protected *S. aureus* from PMN killing; and was required for full virulence in murine models of systemic infection. Sequence comparison of the LukAB toxin to LukHG revealed that both toxins were the same (97). LukAB/HG is the last known bicomponent leucocidin to be identified in *S. aureus*.

Over the past 120 years, our appreciation of the breadth and functionality of the leucocidins produced by *S. aureus* has progressed from that of a single toxic substance, the "leucocidin," to the identification of six unique proteins (LukSF-PV, HlgAB, HlgCB, LukED, LukMF′, and LukAB/HG), all capable of exerting potent lytic activity on a variety of host immune cells (Table 1). The majority of these toxins, except for LukAB/HG, exhibit a remarkable degree of sequence conservation and similar mechanisms of action on host cells (Fig. 1 and 3 and Table 1) (21). However, each toxin exhibits a number of unique characteristics that will likely prove key to enhancing our understanding of leucocidin biological functions on host cells and defining mechanisms of action *in vivo*. Advances in our understanding of both the redundancies and the functional differences among the bicomponent leucocidins, as they relate to toxin lytic activity and cellular signaling, are emphasized in greater detail later in this review.

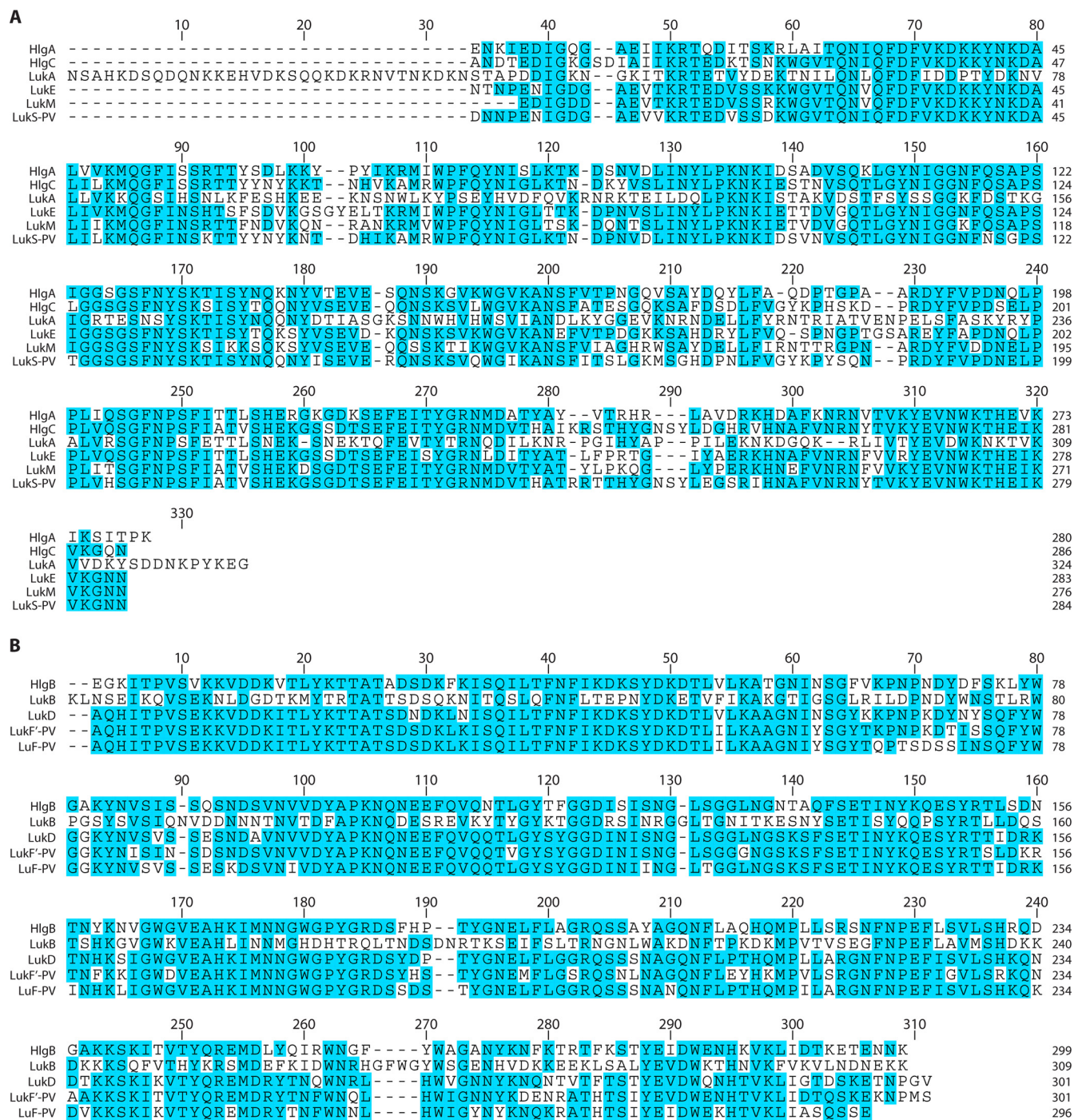## LEUCOCIDIN GENETIC ORGANIZATION AND GENOME DISTRIBUTION

The genetic architecture of the *S. aureus* leucocidins is largely conserved. Typically, S and F subunits of a given toxin pair are cotranscribed from a single promoter. The open reading frame of the S subunit immediately precedes that of the F subunit, with as little as a single nucleotide separating each open reading frame (Fig. 4). The only known exception to this rule is the gamma-hemolysin locus. Gamma-hemolysin comprises a genetic locus consisting of three open reading frames that reside within the core genome of *S. aureus* (85, 89, 98). The *hlgC* and *hlgB* genes consist of a single operon encoding the HlgCB toxin and are thus similar in genetic organization to other leucocidins (Fig. 4) (85). The independently transcribed *hlgA* gene sits upstream of the *hlgCB* operon (Fig. 4). The combination of the gene product of *hlgA* with that of *hlgB*, encoding the F subunit of HlgCB, constitutes the HlgAB toxin (85, 98). Thus, HlgAB and HlgCB share an F subunit. It is worth noting that the gamma-hemolysin subunits are not uniformly named in the literature, which is presumably a consequence of their identification in the pregenomic era but also may have been influenced by the presence of sequence variants isolated from the different *S. aureus* strains used in a variety of studies (90, 99–103). This has led

to considerable confusion when interpreting studies of gamma-hemolysin from the late 1980s through the early 1990s. Table 1 provides a list of alternate names used to describe gamma-hemolysin for reference. In this review, the primary HlgACB nomenclature is adopted for ease of reference. The gamma-hemolysin locus is highly conserved in nearly all *S. aureus* lineages, as the genes are present in 99% of sequenced strains (95, 104). However, there is a degree of genetic diversity within their coding sequences (95). Based on this assessment, it appears that gamma-hemolysin represents a generally conserved set of leucocidins uniformly present in the core genome of *S. aureus*.

Like gamma-hemolysin, LukAB/HG is also core genome encoded. The *lukAB* (*lukHG*) operon sits adjacent to the *hlb* gene (Fig. 4). *hlb* encodes beta-hemolysin, a sphingomyelinase that causes hemolytic activity on blood agar plates in a limited number of *S. aureus* strains (105–107). The gene is inactivated by lysogenic conversion in most *S. aureus* lineages that cause clinical disease (108, 109). As a result, the potential role of beta-hemolysin in pathogenesis is largely unknown. It is typically disrupted by the prophage ϕSa3, which carries genes encoding enterotoxins and the immune evasion molecules staphylococcal complement inhibitor protein (SCIN) and chemotaxis-inhibitory protein (CHIP) (95, 108–110). Importantly, it does not appear that prophage insertion into *hlb* in any way influences the adjacent *lukAB* (*lukHG*) genes. Additional studies are needed to determine whether the presence of *lukAB* (*lukHG*) is uniformly conserved among all *S. aureus* isolates, although the genes are found in all publicly available sequenced genomes, suggesting that it is likely stable. Similarly, additional work is needed to fully evaluate the extent and frequency of *lukAB* (*lukHG*) sequence variants among *S. aureus* lineages. It is well established that a number of genes on the core genome, such as *spa* (encoding protein A), exhibit significant variability within their coding sequences, while others exhibit less frequent variations (*lukED*) (95, 111). Our preliminary assessment of *lukAB* (*lukHG*) suggests that there is genetic diversity within the coding regions of a small subset of isolates, although the extent of this diversity and the functional consequences in terms of cytolytic activity and virulence characteristics remain to be determined (V. J. Torres, unpublished data).

Unlike LukAB/HG, leucocidin ED exhibits little to no sequence diversity among sequenced *S. aureus* strains (95). While its sequence is highly conserved, the presence of the toxin locus is lineage specific. It is completely absent from some *S. aureus* lineages, including clonal complexes (CCs) 22, 30, 42, 45, 75, 398, and 431 (95). Two independent studies predicted the overall frequency of the *lukED* locus to be approximately 70%, with strict lineage dependence (95, 104). Importantly, when present in a given lineage, the gene is fully conserved within all strains of that lineage (95). Higher *lukED* frequencies were predicted in a 2003 study by Morinaga et al. (87%), although this study may not have surveyed as diverse a pool of *S. aureus* lineages, leading to an overestimation of the actual frequency (94, 104). These more recent estimates of *lukED* frequency were derived from sequencing data and are contrary to early studies conducted when LukED was first identified (93). Gravet et al. suggested a *lukED* gene frequency of approximately 30% (93). We suspect that the methods used to identify *lukED* (antibody recognition and DNA hybridization) in this work, coupled with the anomalous sequence of the *lukED* gene, may have led to the underestimated gene frequency (93).

Unlike gamma-hemolysin and LukAB/HG, the *lukED* genes

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                                                                    10        20        30        40        50        60        70        80
HlgA     ----------------------------------------ENKIEDIGQG--AEIIKRTQDITSKRLAITQNIQFDFVKDKKYNKDA 45
HlgC     ----------------------------------------ANDTEDIGKGSDIAIIKRTEDKTSNKWGVTQNIQFDFVKDKKYNKDA 47
LukA     NSAHKDSQDQNKKEHVDKSQQKDKRNVTNKDKNSTAPDDIGKN--GKITKRTETVYDEKTNILQNLQFDFIDDPTYDKNV 78
LukE     ---------------------------------------NTNPENIGDG---AEVTKRTEDVSSKKWGVTQNVQFDFVKDKKYNKDA 45
LukM     -----------------------------------------EDIGDD--AEVTKRTEDVSSRKWGVTQNVQFDFVKDKKYNKDA 41
LukS-PV  --------------------------------------DNNPENIGDG--AEVVKRTEDVSSDKWGVTQNIQFDFVKDKKYNKDA 45

                 90        100       110       120       130       140       150       160
HlgA     LVVKMQGFISSRTTYSDLKKY--PYIKRMIWPFQYNISLKTK-DSNVDLINYLPKNKIDSADVSQKLGYNIGGNFQSAPS 122
HlgC     LILKMQGFISSRTTYYNYKKT--NHVKAMRWPFQYNIGLKTN-DKYVSLINYLPKNKIESTNVSQTLGYNIGGNFQSAPS 122
LukA     ILVKKQGSIHSNLKFESHKEE--KNSNWLKYPSEYHVDFQVKRNRKTEILDQLPKNKISTAKVDSTFSYSSGGKFDSTKG 156
LukE     LIVKMQGFINSHTSFSDVKGSGYELTKRMIWPFQYNIGLTTK-DPNVSLINYLPKNKIETTDVGQTLGYNIGGNFQSAPS 124
LukM     LIIKMQGFINSTFNDVKQN--RANKRWPFQYNIGLTSK-DQNTSLINYLPKNKIETVDVGQTLGYNIGGKFQSAPS 118
LukS-PV  LILKMQGFINSKTTYYNYKNT--DHIKAMRWPFQYNIGLKTN-DPNVDLINYLPKNKIDSVNVSQTLGYNIGGNFNSGPS 122

                 170       180       190       200       210       220       230       240
HlgA     IGGSGSFNYSKTISYNQKNYVTEVE-SQNSKGVKWGVKANSFVTPNGQVSAYDQYLFA-QDPTGPA--ARDYFVPDNQLP 198
HlgC     LGGSGSFNYSKSISYTQQNYVSEVE-QQNSKSVLWGVKANSFATESGQKSAFDSDLFVGYKPHSKD--PRDYFVPDSELP 201
LukA     IGRTESNSYSKTISYTQNYDTIASGKSNNWHVHWSVIANDLKYGGEVKNRNDELLFYRNTRIATVENPELSFASKYRYP 236
LukE     IGGSGSFNYSKTISYTQKSYVSEVD-KQNSKSVKWGVKANEFVTPDGKKSAHDRYLFVQ-SPNGPTGSAREYFAPDNQLP 202
LukM     IGGSGSFNYSKSIKKSQKSYVSEVE-QQSSKTIKWGVKANSFVIAGHRWSAYDELLFIRNTTRGPN--ARDYFVDDNELP 195
LukS-PV  TGGSGSFNYSKTISYNQQNYISEVE-RQNSKSVQWGIKANSFITSLGKMSGHDPNLFVGYKPYSQN--PRDYFVPDNELP 199

                 250       260       270       280       290       300       310       320
HlgA     PLIQSGFNPSFITTLSHERGKGDKSEFEITYGRNMDATYAY--VTRHR---LAVDRKHDAFKNRNVTVKYEVNWKTHEVK 273
HlgC     PLVQSGFNPSFIATVSHEKGSSDTSEFEITYGRNMDVTHAIKRSTHYGNSYLDGHRVHNAFVNRNYTVKYEVNWKTHEIK 281
LukA     ALVRSGFNPSFETTLSNEK-SNEKTQFEVTYTRNQDILKNR-PGIHYAP--PILEKNKDGQK--RLIVTVEVDWKNKTVK 309
LukE     PLVQSGFNPSFITTLSHEKGSSDTSEFEISYGRNLDITYAT-LFPRTG---IYAERKHNAFVNRNFVVRYEVNWKTHEIK 278
LukM     PLITSGFNPSFIATVSHEKDSGDTSEFEITYGRNMDVTYAT-YLPKQG---LYPERKHNEFVNRNFVVKYEVNWKTHEIK 271
LukS-PV  PLVHSGFNPSFIATVSHEKGSGDTSEFEITYGRNMDVTHATRRTTHYGNSYLEGSRIHNAFVNRNYTVKYEVNWKTHEIK 279

                 330
HlgA     IKSITPK           280
HlgC     VKGQN             286
LukA     VVDKYSDDNKPYKEG   324
LukE     VKGNN             283
LukM     VKGNN             276
LukS-PV  VKGNN             284
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B

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                 10        20        30        40        50        60        70        80
HlgB     --EGKITPVSVKKVDDKVTLYKTTATADSDKFKISQILTFNFIKDKSYDKDTLVLKATGNINSGFVKPNPNDYDFSKLYW 78
LukB     KLNSEIKQVSEKNLDGDTKMYTRTATTSDSDQKNITQSLQFNFLTEPNYDKETVFIKAKGTIGSGLRILDPNDYWNSTLRW 80
LukD     --AQHITPVSEKKVDDKITLYKTTATSDNDKLNISQILTFNFIKDKSYDKDTLVLKAAGNINSGYKKPNPKDYNYSQFYW 78
LukF'-PV --AQHITPVSEKKVDDKITLYKTTATSDSDKLKISQILTFNFIKDKSYDKDTLILKAAGNIYSGYTKPNPKDTISSQFYW 78
LuF-PV   --AQHITPVSEKKVDDKITLYKTTATSDSDKLKISQILTFNFIKDKSYDKDTLILKAAGNIYSGYTQPTSDSSINSQFYW 78

                 90        100       110       120       130       140       150       160
HlgB     GAKYNVSIS-SQSNDSVNVVDYAPKNQNEEFQVQNTLGYTFGGDISISNG-LSGGLNGNTAQFSETINYKQESYRTLSDN 156
LukB     PGSYSVSIQNVDDNNNTNVTDFAPKNQDESREVKYTYGYKTGGDRSINRGGLTGNITKESNYSETISYQQPSYRTLLDQS 160
LukD     GGKYNVSVS-SESNDAVNVVDYAPKNQNEEFQVQQTLGYSYGGDINISNG-LSGGLNGSKSFSETINYKQESYRTTIDRK 156
LukF'-PV GGKYNISIN-SDSNDSVNVVDYAPKNQNEEFQVQQTVGYSYGGDINISNG-LSGGGNGSKSFSETINYKQESYRTSLDKR 156
LuF-PV   GGKYNVSVS-SESKDSVNIVDYAPKNQNEEFQVQQTLGYSYGGDINIING-LTGGLNGSKSFSETINYKQESYRTTIDRK 156

                 170       180       190       200       210       220       230       240
HlgB     TNYKNVGWGVEAHKIMNNGWGPYGRDSFHP--TYGNELFLAGRQSSAYAGQNFLAQHQMPLLSRSNFNPEFLSVLSHRQD 234
LukB     TSHKGVGWKVEAHLINNMGHDHTRQLTNDSDNRTKSEIFSLTRNGNLWAKDNFTPKDKMPVTVSEGFNPEFLAVMSHDKK 240
LukD     TNHKSIGWGVEAHKIMNNGWGPYGRDSYDP--TYGNELFLGGRQSSNAGQNFLPTHQMPLLARGNFNPEFISVLSHKQN 234
LukF'-PV TNFKKIGWDVEAHKIMNNGWGPYGRDSYHS--TYGNEMFLGSRQSNLNAGQNFLEYHKMPVLSRGNFNPEFIGVLSRKQN 234
LuF-PV   INHKLIGWGVEAHKIMNNGWGPYGRDSSDS--TYGNELFLGGRQSSSNANQNFLPTHQMPILARGNFNPEFISVLSHKQK 234

                 250       260       270       280       290       300       310
HlgB     GAKKSKITVTYQREMDLYQIRWNGF----YWAGANYKNFKTRTFKSTYEIDWENHKVKLIDTKETENNK 299
LukB     DKKKSQFVTHYKRSMDEFKIDWNRHGFWGYWSGENHVDKKEEKLSALYEVDWKTHNVKFVKVLNDNEKK 309
LukD     DTKKSKIKVTYQREMDRYTNQWNRL----HWVGNNYYKNQNTVTFTSTYEVDWQNHTVKLIGTDSKETNPGV 301
LukF'-PV AAKKSKITVTYQREMDRYTNFWNQL----HWIGNNYKDENRATHTSIYEVDWENHKVKLIDTQSKEKNPMS 301
LuF-PV   DVKKSKIKVTYQREMDRYTNFWNNL----HWIGYNYKNQKRATHTSIYEIDWEKHTVKLIASQSSE 296
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FIG 3 Amino acid sequence alignment of mature leucocidins without their signal peptide. Amino acid sequence comparisons were generated by ClustalW alignment using Lasergene MegAlign Pro software (DNASTAR). Identical amino acids are shown in blue, and divergent residues are shown in white. (A) Alignment of the leucocidin S subunits HlgA, HlgC, LukA, LukE, LukM, and LukS-PV. Notable distinctions are the unique N- and C-terminal extensions that are present in LukA/H but absent from all other toxins. (B) Alignment of the leucocidin F subunits HlgB, LukB/G, LukD, LukF′-PV, and LukF-PV.

are located outside the *S. aureus* core genome (95, 112, 113). The genes are present in what is known as *S. aureus* pathogenicity island (SaPI) vSaβ (Fig. 4 and Table 1) (112, 114). This pathogenicity island is not a mobile genetic element, and unlike the mobile SaPIs or temperate phages, vSaβ is stable (110, 114–116). However, the gene content of vSaβ is not uniformly conserved (95, 112, 113). It can include a number of genes encoding enterotoxins, LukED, a lantibiotic system, and serine proteases, among other uncharacterized factors (112). The presence or absence of certain genes within any given strain is not universally conserved and is likely a consequence of unique horizontal gene transfer events that occurred within specific *S. aureus* lineages (95, 112, 113). For ex-

S. aureus USA300 FPR3757 Genome
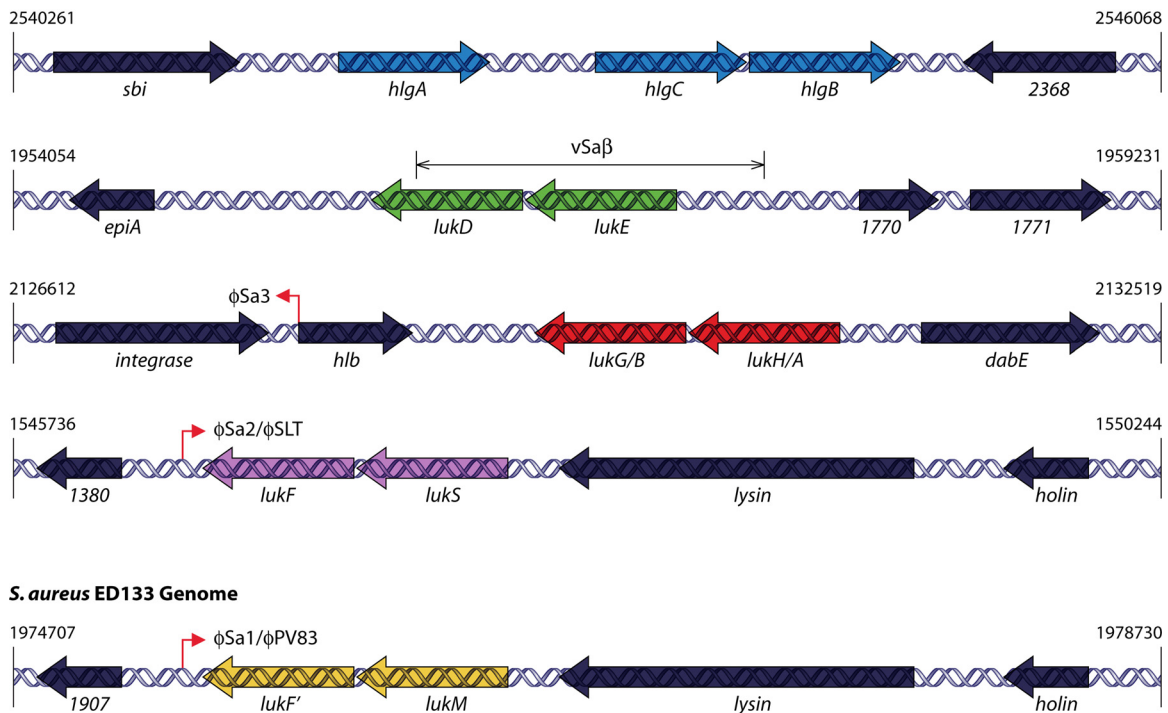
FIG 4 Genome organization of the *S. aureus* leucocidins. Shown is a schematic representation of the *S. aureus* leucocidin genetic loci within the genome of the sequenced USA300 strain FPR3757 (GenBank accession number NC_007793.1) (blue arrows, *hlgACB*; green arrows, *lukED*; red arrows, *lukAB* [*lukHG*]; purple arrows, *lukSF-PV*) or the sequenced genome of bovine isolate ED133 (GenBank accession number NC_017337.1) (yellow arrows, *lukMF'*). Numbers to the right and left indicate the nucleotide base positions of the indicated region designated in the genome repository of the National Center for Biotechnology Information (NCBI). Vertical lines with arrowheads demarcate the location of prophage insertions (φSa3, φSa2/φSLT, and φSa1/φPV83) within the *S. aureus* genome. Flanking genes upstream and downstream of the respective leucocidins are supplied with either their designated nomenclature or their gene number. The *S. aureus* pathogenicity island vSaβ (the site where *lukED* is located) is indicated with branching arrows above the *lukED*-containing region.

ample, a high percentage of strains that lack the *lukED* genes appear to instead contain a locus comprised of enterotoxin-encoding genes (104). Thus, the *lukED* operon is unique from the other leucocidins described thus far in that it exists on a stable yet variable pathogenicity island that was acquired through prior horizontal gene transfer events.

In contrast to the core genome- and vSaβ-encoded leucocidins, Panton-Valentine leucocidin and LukMF' are both located within the genomes of temperate phages (117–119). PVL is located on temperate phage φSa2 (φSLT), while LukMF' is on phage φSa1 (Fig. 4 and Table 1) (110, 120–122). Both PVL and LukMF' are present in an extremely limited range of *S. aureus* lineages (95, 123, 124). The prophage carrying PVL is present in only approximately 2 to 3% of all *S. aureus* isolates (125), although it is found at disproportionately high levels in strains that cause severe necrotizing pneumonia and community-acquired infections (>90%) (126–129). In fact, the predominant strain causing community-acquired disease in the United States, USA300, contains the prophage-carried PVL genes in the majority of isolates (128, 130). Such findings have led to the speculation that acquisition of the φSa2 prophage was at least partly responsible for the enhanced virulence of current epidemic and highly pathogenic strains of *S. aureus* (2, 126, 128, 131). Like LukED, LukSF-PV exhibits limited genetic diversity in its coding sequence among isolates (95, 132). Because of early correlative evidence suggesting a role for PVL in the success of epidemic strains of CA-MRSA, it

has become the predominant leucocidin in clinical and epidemiological studies (126–128, 131, 133). However, it is now becoming less clear whether PVL should even be considered a major factor linked to the enhanced virulence of CA-MRSA (6, 113, 129, 134–137). Compelling evidence for the dominant role of other factors, such as the phenol-soluble modulins (PSMs) and alpha-hemolysin (both of which are highly expressed in CA-MRSA strains), in the progression of skin and soft tissue infection (SSTI) and necrotizing pneumonia highlights the alternate possibility that complex influences from multiple virulence factors have led to the increased incidence of highly virulent CA-MRSA strains (6, 17, 29, 138–141). Nonetheless, the potent activity of PVL on human immune cells together with its epidemiological association with severe disease support the notion that PVL could contribute to pathogenesis in a yet-to-be-identified capacity. Like PVL, the prophage encoding LukMF' is found almost exclusively in strains that are isolated from ruminants, where the toxin is believed to play a significant role in the pathogenesis of mastitis (Table 1) (104, 123, 142–145).

LEUCOCIDIN MECHANISM OF ACTION: PORE FORMATION

Early investigations into the molecular mechanism of action of leucocidins on host cells began with biochemical studies by Woodin in the early 1960s. Although pore formation was not yet known to be the mechanism by which toxin-mediated cell death occurred, Woodin and Wieneke did observe alterations in ion
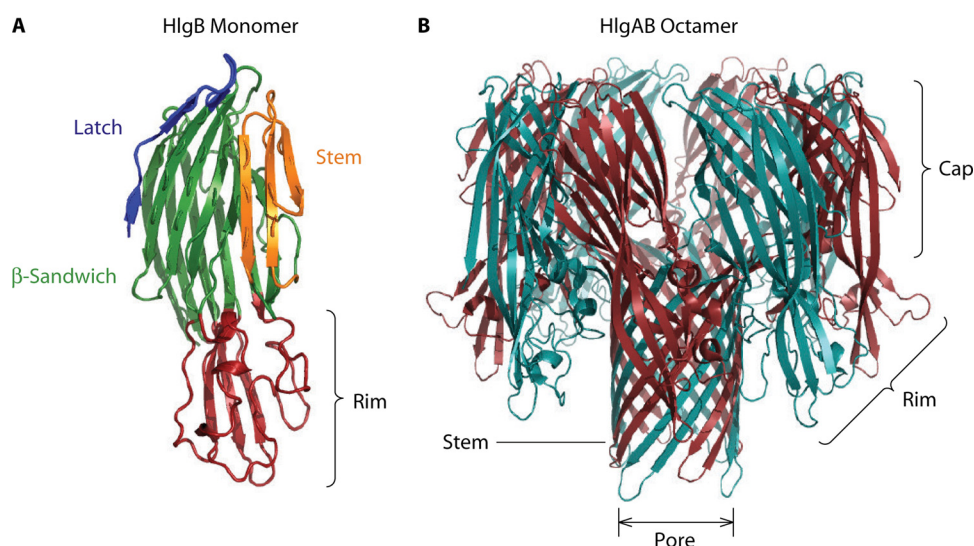
FIG 5 Leucocidin structural features. (A) Crystal structure of the monomeric F subunit of gamma-hemolysin (HlgB) (178). Structural information was acquired from the Protein Data Bank (PDB) (accession number 1LKF), and the major structural domains were colored by using PyMOL software. Blue, amino latch; green, β-sandwich; orange, stem domain; red, rim domain. (B) Crystal structure of the HlgAB octamer (190). The S subunit (HlgA) is in cyan, while the F subunit (HlgB) is in red. The stem, rim, and cap as well as the β-barrel pore are shown. Structural information for the HlgAB octamer was acquired from the PDB (accession number 3B07), and the major structural domains were colored by using PyMOL software.

fluxes, primarily potassium and calcium, and extrusion of cellular contents upon treatment with purified PVL (64, 66, 68). Woodin and Wieneke also observed subunit-dependent binding to cellular membranes and presumed that "polymerization" of the toxin was occurring upon interactions with the cell surface (61, 62). While pore formation was not conclusively demonstrated in any of these studies, a review by Rogolsky in 1979 suggested the possibility that PVL was forming a pore in host cell membranes (146). Follow-up studies by Noda et al. in the 1980s confirmed the increased influx of calcium upon treatment with leucocidin (HlgCB) and binding of the toxin to host cell membranes (147). Additional efforts by both Noda et al. and Morinaga et al. began to assess the ability of HlgCB and PVL to recognize host cell membranes with targeted specificity (148–150). However, none of these studies definitively recognized pore formation as a mechanism of toxin action.

The classification of PVL and subsequent leucocidins as pore-forming toxins was not officially made until 1993, when Finck-Barbancon and colleagues performed a series of kinetic studies to measure calcium and ethidium bromide entry into PMNs (or neutrophils) upon treatment with PVL (151). In this work, it was proposed that ethidium entry into host cells, but not calcium entry, is a definitive marker of pore formation by leucocidins (152, 153). In contrast, calcium entry upon leucocidin treatment is believed to occur prior to pore formation and is mediated by the opening of membrane divalent cation channels (154, 155). In the 10 years following this early assessment of the leucocidin pore, a number of studies using gamma-hemolysin and PVL served to dramatically increase our understanding of the molecular characteristics of pore formation. This work has already been summarized extensively in a series of technical reviews (for details, see references 99–102). Notably, substantial biophysical measures of leucocidin pore formation and structural assembly on host cells were performed (156–159). Key studies included the determination that leucocidins form an octameric pore arranged as alternating S and F subunits (160–163) as well as detailed biochemical

characterizations of residues required for toxin activity, including sites of phosphorylation (164, 165), subunit interactions (166–170), membrane recognition (171–174), and prepore-to-pore transition states (175–177). However, it was the crystallographic determination of toxin subunit structures that was transformative in our understanding of the leucocidins and the mechanism of pore formation on host cells (Fig. 5). In 1999, the F components of both gamma-hemolysin (HlgB) and PVL (LukF-PV) were crystallized, followed by the S component of PVL in 2004 and an engineered dimer of HlgAB in 2008 (178–181). Through these crystallographic studies, the major structural features of the leucocidins were described (Fig. 5). The individual S and F subunits were found to closely resemble the core structure of the previously identified alpha-hemolysin (182). Functional domains include the rim domain, composed of a high degree of aromatic residues that recognize and bind to phospholipids and other molecules; a glycine-rich and hydrophobic stem domain, which undergoes dramatic structural shifts and inserts into the membrane of host cells, forming a presumed β-barrel pore-like structure; the β-sandwich domain, containing key residues for intersubunit interactions; and an amino latch, which is believed to be involved in stem domain positioning during the monomeric-to-oligomeric pore transition for alpha-hemolysin, although the function may not be conserved in the leucocidins (Fig. 5) (169, 183–185).

By as early as 2007, a proposed model of leucocidin function on host cells emerged, which remains largely unchallenged today: (i) monomeric water-soluble toxin molecules are secreted by *S. aureus*; (ii) the S or F components recognize each other as well as proteins and/or lipids on the host surface in a species- and cell type-specific manner; (iii) host cell interactions typically lead to S component recognition and binding to the target cell, followed by F subunit recruitment, although in the case of gamma-hemolysin, the F subunit appears to bind first on red blood cells (186, 187); (iv) the toxin subunits oligomerize into an octameric structure composed of alternating S and F subunits, forming a prepore

(188); and (v) dramatic structural shifts occur, leading to stem domain insertion into the host cell membrane and subsequent pore formation (Fig. 1). Additional studies have substantiated the biochemical characteristics and stoichiometry of the leucocidin pore through the use of photobleaching experiments and fluorescently labeled S or F subunits (189). In either case (LukS labeled or LukF labeled), photobleaching occurred in four intervals, confirming that the pore is comprised of four S subunits and four F subunits (189). Thus far, the majority of the biochemical evidence supports the notion that the leucocidins form octameric pores. Additional structural evidence in support of octameric pores was provided with the description of the crystal structure of a gamma-hemolysin (HlgAB) pore (190). The addition of HlgA and HlgB subunits to 2-methyl-2,4-pentanediol led to spontaneous toxin oligomerization in solution (similar to alpha-hemolysin), allowing crystallographic structural determination (182, 190). The HlgAB pore structure confirmed that the structurally favored orientation of gamma-hemolysin is an octamer and laid to rest a series of prior biochemical inconsistencies that had suggested that the leucocidin pore consisted of mixed hexamers and heptamers (161, 166). It is believed that these hexameric and heptameric structures were actually intermediates captured during the formation of a stable octamer rather than bona fide functional pores (190). Furthermore, atomic force microscopy studies confirmed that octameric pores are the most stable conformation of HlgAB within lipid membranes (191). Based on the sequence similarity of gamma-hemolysin, PVL, LukED, and LukMF′, it stands to reason that the octameric structure described for the HlgAB pore will closely resemble the pore structure of these other leucocidins (Fig. 5). A recent crystallographic assessment of the LukE and LukD monomers further supports this hypothesis, although additional structural studies of the LukED pore itself would provide the most conclusive evidence (192). LukAB/HG, the most divergent of the leucocidins, contains unique features that set it apart from the other leucocidins (Fig. 3), including N- and C-terminal extensions not present in any of the other toxins (97). Interestingly, this unique C-terminal extension was recently shown to contain a single amino acid that is absolutely critical for the recognition of the host cell surface by LukAB/HG and therefore its lytic activity (193). Such findings further support the notion that ascribing functional activities to LukAB/HG from prior research on and/or structures of the other leucocidins will be challenging. As a result, predictions of the structure and biochemical function of LukAB/HG will be reserved until further crystallographic studies are conducted. The divergence of this toxin from others in its class will certainly prove informative in understanding the range of functions associated with these molecules.

ROLES OF LEUCOCIDIN IN PATHOGENESIS

LukSF-PV (PVL)

Since its description in 1932, PVL has long been presumed to contribute to the pathogenic potential of *S. aureus* in humans. Unfortunately, demonstrating this through the use of experimental models has proven exceedingly difficult. Valentine and Butler provided early evidence that rabbits and humans develop antibody titers against leucocidins that are sufficient to block toxin activity *in vitro* (194, 195). Similarly, toxoid preparations of PVL administered to both humans and rabbits demonstrated specific antigenic recognition of PVL by the immune system, suggesting

potential efficacy in promoting the clearance of *S. aureus* infection, although immunization as a preventative strategy was not tested (56, 58, 59, 196, 197). From these studies, it is difficult to ascertain whether PVL is responsible for the significant disease burden caused by *S. aureus* despite the fact that neutralizing antibodies are readily generated and some degree of infection resolution is observed upon the induction of an antibody response. A detailed discussion of the prospects of immunization with leucocidin toxoids as treatment modalities to promote *S. aureus* infection resolution is provided later in this review. Nonetheless, these immunization studies served as an early indicator that the functions of the leucocidins are likely to have relevance to pathogenesis in humans.

Studies of the role of PVL in virulence using animal models and isogenic *pvl* deletion mutants have likewise proven difficult to interpret (6, 19, 198). Murine models, which were originally used in a number of virulence studies, have since been proven unreliable in establishing virulence-associated functions of PVL. This is a direct consequence of the species specificity associated with PVL targeting of host cells (PVL cannot efficiently recognize its cellular receptor, C5aR, on murine cells) (199, 200). Even before the identification of the species-dependent recognition of the PVL cellular receptor, the relative ineffectiveness of the toxin in terms of its lytic activity on murine cells had been noted (201). Such studies presumably should have cautioned against the use of murine models to assess the virulence-associated characteristics of PVL (201). Regardless, murine models were still heavily implemented to evaluate the role of PVL in pathogenesis *in vivo* (139, 202–208). These murine studies are not discussed in great detail, given their exceedingly contradictory results. Instead, readers are encouraged to interpret murine studies of PVL pathogenesis with caution in light of the fact the PVL is unable to exert lytic activity on murine cells, and as such, the biological effects of active toxin are not being considered. Despite its lack of lytic activity on murine cells, there is compelling evidence indicating that PVL is capable of eliciting a proinflammatory response from murine immune cells. Thus, murine studies may be useful toward evaluating the proinflammatory effects of PVL independent of its lytic activity. In fact, the proinflammatory activity of PVL has been linked directly to the pathogenic characteristics of necrotizing pneumonia seen in mouse models (203, 206, 209, 210). Similar effects are seen in mouse models of severe skin and soft tissue infection (SSTI), where muscle tissue injury appears to be a direct consequence of the proinflammatory activity of PVL (205).

In rabbit models of necrotizing pneumonia, where PVL is fully lytic on host immune cells, this toxin dramatically increases proinflammatory responses in the lung (Table 1) (211). Injection of purified recombinant PVL leads to increased immune cell recruitment and increased architectural destruction of the lung due to toxin-mediated recruitment and subsequent lysis of immune cells (211). Compared to wild-type (wt) *S. aureus*, an isogenic deletion mutant of *pvl* exhibits reduced bacterial burden and inflammation in the lungs and leads to an overall increase in rabbit survival (211). Other models of infection using rabbits, such as SSTI, have led to more ambiguous results (Table 1). While some groups have reported a modest but significant role for PVL in the pathogenesis of early-stage SSTIs, others have seen no discernible contribution of PVL to disease (212, 213). In rabbit bacteremia models, PVL plays a negligible role in virulence (214). In contrast, PVL may contribute to *S. aureus* persistence in a model of osteomyelitis

(Table 1) (215). Additional studies in primates were unable to uncover a role for PVL in a pneumonia model (216). The discrepancy in virulence characteristics associated with pneumonia in rabbits and primates likely stems from the fact that primate PMNs are also relatively insensitive to the lytic functions of the toxin (200). In summary, based on a wide variety of animal models, it remains difficult to attribute a specific pathogenic role to PVL. Thus far, it can only be firmly stated that PVL is proinflammatory *in vivo* and directly influences the severity of a limited number of conditions, including necrotizing pneumonia and osteomyelitis in rabbit models. Most other infection models remain largely inconclusive.

From an epidemiological perspective, strains containing the *pvl* genes are associated with more severe invasive disease and poor prognosis and are more likely to be isolated from community rather than hospital settings (2, 126–129, 131, 133). It is important to make the distinction that a higher incidence of PVL-positive (PVL⁺) strains causing community-associated infections and invasive disease does not necessarily imply that PVL alone is the factor responsible for the maintenance of certain infectious isolates within the population or their hypervirulence. In fact, studies now suggest that additional global alterations, including increased expression levels of core genome-encoded factors such as alpha-hemolysin and the phenol-soluble modulins, as well as acquisition of additional mobile genetic elements that confer a metabolic advantage and antibiotic resistance also contribute to the success of current epidemic strains (6, 11, 17, 29, 138–141, 217–219). In line with this supposition, recent studies have identified PVL-negative *S. aureus* strains, including pandemic isolates from South Korea, that are capable of persisting within communities despite their lack of the *pvl*-carrying prophage (113, 198, 218, 220). Thus, in these pandemic strains, other factors are most certainly responsible for the increased virulence and strain maintenance. Clearly, our understanding of PVL as a virulence factor is far from complete. Furthermore, it stands to reason that studies of PVL may directly benefit from investigations of other leucocidins, which have received relatively less attention but whose influence on pathogenesis is more easily evaluated using small-animal models and *in vitro* systems.

Gamma-Hemolysin (HlgAB and HlgCB)

In contrast to PVL, the contribution of gamma-hemolysin to *S. aureus* pathogenesis is less studied (Table 1). In *ex vivo* whole-blood infection models, a gamma-hemolysin mutant (*hlgACB*) is more susceptible to bacterial killing than wt *S. aureus* strains, although this manifests only as modest reductions in mortality rates during systemic infection of mice (221). Additionally, *hlgACB* mutants have been shown to contribute to the pathogenesis of septic arthritis, particularly when alpha-hemolysin (*hla*) is also deleted (222). Relative to wt strains, strains lacking both *hlgACB* and *hla* cause reduced frequency of arthritis and decreased weight loss and elicit lower proinflammatory cytokine (interleukin-6 [IL-6]) levels (222).

The remaining studies evaluating the contribution of gamma-hemolysin to pathogenesis concern ocular models of infection and inflammation (Table 1) (223–226). Intraocular injection of purified gamma-hemolysin into rabbit eyes leads to increased inflammation, hemorrhage, swelling, and retinal necrosis (225). The proinflammatory and pathological characteristics associated with the administration of purified toxin imply a functional role for the toxin in mediating intraocular pathogenesis. Experiments conducted with live bacteria confirm this supposition, as *S. aureus* gamma-hemolysin mutants exhibit reduced pathology and inflammation in a keratitis model of ocular infection as well as reduced eyelid inflammation and CFU recovered from infected vitreous fluid (223, 224, 226). However, *hla* is believed to contribute to an even greater degree to the pathogenesis of ocular infection and causes significant epithelial erosion during corneal infection (224). Together, these studies demonstrate that gamma-hemolysin is responsible for some of the pathological outcomes of ocular infection and associated inflammatory conditions and highlight the major proinflammatory properties of the toxin. Whether the proinflammatory activity of gamma-hemolysin occurs as a result of sublytic cellular engagement and subsequent activation of proinflammatory signaling cascades or is a consequence of overt cellular damage caused by toxin-mediated lysis is not clear from these studies. Additionally, the contributions of gamma-hemolysin to a wide range of infection conditions, including skin and soft tissue infection, osteomyelitis, and pneumonia, have yet to be investigated. More in-depth studies are warranted in this regard.

In addition to defining a role for gamma-hemolysin in the pathogenesis of ocular infection and septic arthritis, these infection models highlight an important point regarding bacterial toxin synergism and redundant functions as they relate to pathogenesis. Both alpha-hemolysin and gamma-hemolysin were shown to influence the pathogenic outcomes associated with ocular infection in similar ways (223, 224). Thus, by virtue of their redundant mechanisms of action *in vivo*, these two unique *S. aureus* cytotoxins have similar influences on overall pathogenic outcomes during infection. Such redundancies have the potential to complicate the assessment of the role of any one virulence factor in a specific disease model and as a result may significantly misrepresent *in vivo* contributions to infection. Later in this review, we describe recent efforts to decipher potential nonredundant functions of leucocidins and evaluate how these unique functional characteristics may drive pathogenic outcomes.

LukED

The implementation of murine infection models has proven particularly useful for studies of LukED function *in vivo*. This is primarily because LukED is one of the only leucocidins to exhibit broad activity on a wide variety of cell types from various species, including mice (HlgCB and HlgAB exhibit specificity for some but not all target murine cells) (Table 1) (47, 94, 227–229). The lytic activity of LukED on murine cells is similar to that on both rabbit and human leukocytes (47, 94). As a result, both murine and rabbit models have been informative in deciphering the mechanism of action of LukED *in vivo*. Purified recombinant LukED elicits inflammation and dermonecrosis upon injection into the skin of rabbits (94). Thus, like PVL and gamma-hemolysin (and possibly all leucocidins), LukED is capable of inducing a proinflammatory response *in vivo*. In murine models of systemic infection, LukED contributes significantly to animal mortality, as mice infected with a *lukED* mutant survive normally lethal acute infection (47, 227, 230). Compared to animals infected systemically with wt *S. aureus*, *lukED* mutant strains exhibit a reduced ability to replicate in target organs (liver and kidneys) and have decreased levels of circulating proinflammatory cytokines in their sera (47, 230). LukED is directly lytic to infiltrating phagocytic leukocytes *in vivo*, as evidenced by decreased cellular viability measured by flow cytom-

etry (47, 227, 230). Much of the ability of LukED to influence pathogenesis *in vivo* is a direct consequence of its targeted killing of specific immune cells, including neutrophils, T cells, macrophages, NK cells, and dendritic cells (Table 1) (47, 227, 230). We discuss mechanisms of leucocidin targeting later in this review.

There is some correlative evidence to suggest that LukED-producing strains are isolated at a greater frequency from patients with impetigo, antibiotic-associated diarrhea, furuncles, and invasive blood-borne infections (104, 133, 231, 232). However, these studies were conducted when the *lukED* genes were believed to be present in ~30% of all *S. aureus* strains. Since then, additional studies have indicated that the gene itself is lineage specific, but unlike PVL (present in only 2 to 3% of all *S. aureus* strains), it is present in a greater proportion (~70%) of strains (47, 95). Thus, the early clinical associations of *lukED* with impetigo, antibiotic-associated diarrhea, and furuncles are intriguing but should be interpreted with caution, as they were based on a perceived lower global strain distribution of the toxin. It is clear that epidemiological studies of *lukED* (and most other leucocidins other than PVL) are in the early stages, and more detailed assessments of the breadth of strains harboring these genes will certainly provide novel insights into their association, or lack thereof, with various pathological outcomes.

LukAB/HG

Like PVL, the use of murine infection models to evaluate LukAB/HG pathogenesis is complicated by its limited lytic activity on murine immune cells (Table 1) (233). However, in low-dose murine systemic infection models, deletion of LukAB/HG leads to a reduction in bacterial burden (97). Similarly, deletion of *lukAB* (*lukHG*) in a strain of *S. aureus* containing a deletion in the gene encoding the transcription factor repressor of toxins (Rot), which leads to increased toxin production and hypervirulence, modestly reduces the hypervirulence associated with this strain (47). These data suggest that while LukAB/HG exhibits limited lytic activity on murine cells, other yet-to-be-determined functions may still facilitate pathogenic outcomes in murine infection models. In contrast, when using a murine systemic infection survival model, no differences were seen between the wt and a *lukAB* (*lukHG*) mutant (233). Thus, there is conflicting evidence from these models concerning the contribution of *lukAB* (*lukHG*) to pathogenesis using murine infection models. As with PVL, it appears that caution should be taken when interpreting the potential role of LukAB/HG in models where the lytic activity of the toxin is suboptimal. Like the other leucocidins, injection of LukAB/HG into rabbit skin induces inflammation at the site of inoculation albeit to a lesser degree than PVL (233). In contrast, measurements of abscess size and bacterial burden in rabbits infected with wt *S. aureus* or an isogenic *lukAB* (*lukHG*) or *lukAB* (*lukHG*)-*pvl* mutant showed no measureable differences (233). However, it should be noted that LukAB/HG is ~10-fold less potent on rabbit immune cells than on human immune cells; thus, rabbit models may also prove suboptimal for measuring the potential contributions of LukAB/HG to pathogenesis (233).

Ex vivo infection models using primary human cells offer an alternate perspective into the perceived virulence potential of LukAB/HG (97, 234). wt *S. aureus* exhibits greater survival upon infection of PMNs than a *lukAB* (*lukHG*) deletion mutant (97, 234, 235). This is due largely to the greater PMN damage inflicted by LukAB/HG-producing strains (96, 97, 234, 235). One mecha-

nism by which LukAB/HG promotes bacterial survival amid PMNs is by facilitating bacterial escape after phagocytosis. *S. aureus lukAB* (*lukHG*) deletion mutants escape less efficiently from phagocytosed PMNs, leading to reduced bacterial rebound after phagocytosis (234, 235). Similar escape defects are observed with the cytolytic phenol-soluble modulins (236). Such findings again highlight that the redundant effects imparted by cytotoxic molecules produced by *S. aureus* (in this case, PSMs and LukAB) are likely to synergize to promote optimal bacterial escape after PMN phagocytosis. Altogether, *ex vivo* studies demonstrate that LukAB/HG likely has major roles in promoting bacterial survival in the presence of infiltrating PMNs. Unfortunately, the described issues of species specificity make further *in vivo* analyses a challenge. LukAB/HG targets rabbit and mouse immune cells with significantly less potency than human and primate cells; thus, both models appear to grossly underestimate the influences of LukAB/HG on pathogenesis and have thus far led to conflicting conclusions (233). The development of more appropriate animal infection models will help further investigations of LukAB/HG pathogenic functions. Such models are discussed in greater detail later in this review.

LukMF'

LukMF' is not found in any *S. aureus* isolates of human origin and is seen predominantly in strains isolated from cases of bovine mastitis (Table 1) (123, 124, 142). However, whether LukMF' contributes to the pathogenesis of bovine disease is not well appreciated at this time. Purified recombinant LukMF' is toxic to bovine neutrophils and macrophages and is capable of binding to murine neutrophils, macrophages, and T cells (123, 237, 238). It is the most potent leucocidin on cells of bovine origin and can be isolated from the mammary glands of cows with severe mastitis (239). Infected cows can elicit an immunological response to LukMF', as antibody can be isolated from serum during the course of infection (239). Whether these associations indicate that LukMF' plays a significant role in the pathogenesis of mastitis remains to be determined. Unlike the other leucocidins, which elicit strong proinflammatory responses upon administration, injection of LukMF' into the mammary glands of cows does not lead to a pronounced proinflammatory response, as measured by leukocyte infiltration into the toxin-infused mammary glands (240). The administration of sublethal amounts of toxin to bovine mammary macrophages is unable to elicit the production of the proinflammatory cytokine IL-8 or IL-1β, in contrast to what is often seen for other leucocidins (240). Despite its clear association with strains of bovine origin and its absence from human *S. aureus* isolates, it remains difficult to conclude whether LukMF' is a major virulence factor involved in the progression of mastitis or other bovine diseases. In fact, the presence of LukMF' is not exclusively a defining characteristic of bovine isolates, as (i) not all bovine strains contain LukMF' and (ii) ewes, goats, squirrels, and other mammals can be colonized by LukMF'-producing strains (123, 144, 241).

UNDERSTANDING SPECIES AND CELLULAR SPECIFICITY OF THE LEUCOCIDINS

As is clear from the previous section, studies aimed at evaluating the contribution of *S. aureus* leucocidins to pathogenesis have been hampered due to the limited host range associated with many of the toxins. In nearly all instances, this has negatively

influenced the ability to interpret data, establish optimal infection models, and formulate firm conclusions. Additionally, most early studies focused largely on the similarities among the leucocidins, their ability to target PMNs, and the complications of interpreting outcomes caused by functionally redundant molecules. However, it is now becoming apparent that each toxin has evolved to recognize specific cellular subpopulations that may dictate unique pathogenic outcomes (Table 1). A number of recent studies have now more clearly defined the species and cellular specificity of the leucocidins and have had a major impact on our understanding of leucocidin biological functions and their potential diverse roles in infection biology. These studies are summarized below.

Leucocidin Cellular Receptors Dictate Cell and Species Specificity

LukED and CCR5, CXCR1, and CXCR2. As mentioned above, LukED exhibits a broad host range in regard to its tropism for and lytic activity on immune cells. The toxin is capable of killing rabbit, murine, and human leukocytes with similar potency and is also able to target the lymphocytes of canines and fish (Table 1) (47, 94, 227–229). The mechanisms by which LukED, or any other leucocidin, exerts its cellular and/or species specificity have gone largely unrecognized over the past century. Most leucocidins are known to preferentially recognize certain cellular membrane lipids, and this lipid recognition is believed to facilitate the prepore-to-pore transition (61, 99, 178, 180, 242, 243). However, it is difficult to ascertain how membrane lipid recognition could also lead to the precise targeting of well-defined cellular subsets. Thus, it has been proposed that, rather than lipids, the leucocidins recognize distinct proteinaceous receptors on the cell surface to target and kill immune cells. Evidence for leucocidin cellular receptors other than membrane lipids is supported by a number of studies which demonstrate specific and saturable binding of the toxins on target cells (173, 174, 244). In line with this evidence, LukED was recently found to target the chemokine receptor and HIV coreceptor CCR5 to kill inflammatory macrophages, T cells, and dendritic cells (227). Thus, while phospholipid composition may play a role in some aspects of leucocidin cell surface recognition, the demonstration of LukED recognition of CCR5 indicates that the leucocidins are likely to target specific cellular subsets through binding of proteinaceous receptors. In agreement with previous studies that used fluorescently labeled toxins to demonstrate saturable binding kinetics on the surface of host cells, the LukE subunit binds specifically and saturably to CCR5-expressing cells. Binding of the S subunit (LukE) fits all previous models, which had predicted initial binding to the host cell surface via the S subunit of the toxin followed by F subunit recruitment (173, 227). Targeted killing of CCR5$^+$ cells is fully recapitulated using primary human peripheral blood mononuclear cells (PBMCs) as well as upon the isolation of CCR5$^+$ macrophages and T cells from *S. aureus*-infected mice (227). Additional *in vivo* studies indicate that LukED is capable of directly targeting CCR5$^+$ macrophages during acute infection to promote *S. aureus* immune escape and facilitate disease progression (227). Remarkably, clinically approved CCR5 receptor antagonists block the interaction of LukED with its receptor, indicating that toxin-receptor interactions can be directly targeted in order to prevent leukocyte killing (227). The therapeutic potential of blocking of LukED targeting with pharmacological antagonists was not assessed in *in vivo* infection models due to the limited activity of the antagonists on murine receptors. However,

the prospects of leucocidin inhibition using small molecules are promising given the potent inhibitory activity of the antagonists in *in vitro* models (245).

Interestingly, it has been known for some time that LukED targets and kills PMNs, yet these cells do not express CCR5 on their cell surface (47, 93, 94, 230). Murine models of systemic infection suggest that LukED is capable of influencing pathogenesis in a manner that is partially dependent but also independent of CCR5 surface expression (230). Thus, it stands to reason that LukED influences pathogenesis via additional mechanisms that are independent of its activity against CCR5$^+$ cells. It was found that, in addition to CCR5, LukED targets CXCR1/2 on primary human neutrophils, monocytes, NK cells, and a subset of CD8$^+$ T cells (230). Directed targeting of CXCR1 and CXCR2 on host cells is the mechanism by which LukED kills PMNs during systemic infection of mice. Similar to CCR5-dependent targeting of macrophages, CXCR1 and CXCR2 targeting of PMNs leads to more efficient immune escape by the bacterium and enhanced pathogenesis (230). Through the combined targeting of these three cellular receptors, LukED is able to facilitate the disarming of both innate and adaptive arms of the immune system. Interestingly, like gamma-hemolysin, LukED is the only other leucocidin known to lyse red blood cells (Table 1). It remains to be determined whether RBC lysis by LukED is a result of receptor-specific recognition or whether the targeting of RBCs represents a promiscuous nonspecific activity shared by LukED, HlgAB, and HlgCB.

PVL and C5aR and C5L2. PVL and LukED share a high degree of sequence identity (>75%) (Table 1), yet various lines of evidence from early biochemical and receptor-specific studies support the notion that LukED and PVL do not share similar receptors (21). This evidence is as follows: (i) PVL has a more limited host range than LukED (it is not lytic on murine immune cells); (ii) PVL exhibits a more restricted immune cell-targeting profile, as it kills primarily neutrophils, monocytes, and macrophages; (iii) of the leucocidin subunits, only HlgC is capable of competing with fluorescently labeled LukS-PV for binding of the surface of primary leukocytes; and (iv) the most divergent regions of PVL and LukED occur in the rim domain of the protein believed to be most critical for receptor recognition and cellular specificity (Fig. 3 and 5) (153, 174, 200, 230). Interestingly, the ability of HlgC to successfully compete with labeled LukS-PV for cell surface binding suggests that PVL and HlgCB of gamma-hemolysin may target similar receptors on the cell surface, although this has not yet been validated experimentally (174).

A recent study by Spaan et al. used a leukocyte receptor antibody screening strategy to identify cellular factors for which PVL has a high affinity (199). They assessed the ability of LukS-PV to displace fluorescently labeled antibodies against leukocyte receptors present on the surface of host immune cells. These studies led to the identification of the seven-transmembrane G-protein-coupled receptors (GPCRs) C5aR and C5L2 as the receptors required for cellular targeting by PVL. C5aR/C5L2 recognition by PVL is mediated by both the core membrane-spanning portions of the receptors as well as their extracellular N termini (199). The toxin interaction occurs through the S subunit, is highly specific for human C5aR, and is less so for those of other species, including macaque and murine receptors. Thus, C5aR targeting by PVL provides a rationale for the species specificity associated with its lytic activity (199, 200). C5aR also serves as a receptor for the chemotaxis-inhibitory protein of *S. aureus* (CHIPS), which binds to

the receptor and prevents neutrophil activation and recruitment (246–251). Indeed, CHIPS is capable of blocking the PVL interaction with C5aR on host cells (199). In this study, CXCR2 was used as the negative control to which PVL binding of C5aR was compared. In all cases, CXCR2 expression did not promote binding of PVL to the cell surface (199). Such findings are in line with the presumption that LukED (which targets CXCR2) and PVL, despite sharing a remarkable degree of sequence conservation, do not target the same receptors on host cells (199, 230). It is interesting that both LukED and PVL target seven-transmembrane GPCRs. As a result, it was speculated that GPCRs may be a conserved receptor class targeted by these highly similar toxins (199). By virtue of binding C5aR, LukS-PV not only exerts its lytic activity on target host cells but also can facilitate the priming of primary human PMNs for activation by proinflammatory stimuli such as *N*-formyl-Met-Leu-Phe (fMLP). This finding fully supports previous studies that suggested that PVL could promote the proinflammatory responsiveness of PMNs when applied at sublytic concentrations (199, 252, 253).

LukAB/HG and CD11b. LukAB/HG shares only 30 to 40% sequence homology with the other *S. aureus* leucocidins (Fig. 3 and Table 1) (97). Thus, it stands to reason that its marked sequence diversity could result in unique functional characteristics that set LukAB/HG apart from other bicomponent leucocidins. Thus far, early evidence for unique biochemical and/or biological features is limited to three observations. First, unlike the other leucocidins, LukAB/HG appears to associate with the bacterial cell surface in certain bacteriological growth media (tryptic soy broth [TSB] and growth medium containing digested casein or Casamino Acids, yeast extract, and glycerophosphate [CCY medium]) (96, 234). LukAB/HG is the only bicomponent leucocidin known to establish what appears to be a functional reservoir of toxin on the bacterial cell surface. The precise role for surface-associated LukAB/HG is not yet completely defined; therefore, we will reserve speculation on the possible biological significance of active toxin reservoirs in the pathogenesis of *S. aureus* at this time. However, this novel characteristic of LukAB/HG suggests that the toxin is unlikely to behave in the exact manner as the other leucocidins. Second, a critical amino acid required for LukAB/HG receptor recognition was recently identified within a 10-amino-acid C-terminal extension that is not found in any of the other leucocidins. When the glutamic acid at position 323 of LukAB/HG is mutated to an alanine, the toxin is no longer able to recognize its cellular receptor (CD11b) (discussed below), rendering it inactive on host cells (193). Finally, evidence was recently provided to suggest that LukAB/HG exists as a stable heterodimer in solution. The formation of stable heterodimers contrasts with that of the other leucocidins, which are believed to exist primarily as water-soluble monomers in solution. It has been proposed that such heterodimers may enhance the potency of LukAB/HG by eliminating the need for a cell surface dimerization step during pore formation (193). Together, these early findings strongly suggest that there are a number of major structural and functional differences between LukAB/HG and the other bicomponent leucocidins. Additional studies will provide important insights into the ways in which this diversity influences the toxin's activity and role in pathogenesis.

LukED and PVL both recognize GPCRs that are important for immune cell activation and chemotaxis (199, 227, 230). It is thus tempting to speculate that highly similar leucocidins (PVL, LukED, HlgCB, HlgAB, and LukMF′) will also use GPCRs to rec-

ognize and kill host cells. In contrast, recent studies indicate that LukAB/HG does not target a GPCR, in line with the hypothesis that the sequence divergence of this toxin is likely to dictate unique functional attributes. A biochemical approach was recently used to identify the cellular receptor targeted by LukAB to promote its lytic activity on immune cells (235). A pulldown of solubilized membrane proteins incubated with LukAB showed that CD11b, a component of the αM/β2 integrin Mac-1, is required for LukAB targeting of primary human neutrophils (235). The toxin interacts specifically with the I-domain of CD11b, the primary site of endogenous ligand binding. Binding to the I-domain is entirely species dependent, as the affinity of LukAB for the I-domain from murine CD11b is negligible (235). This finding serves to explain why murine leukocytes, many of which express significant levels of CD11b, are largely resistant to even high doses of the toxin (233, 235). Thus, like PVL, the ability of LukAB to recognize its cellular receptor on the host cell membrane dictates cell type and species specificity. The identification of CD11b as the receptor for LukAB has provided mechanistic insight into the toxin's ability to facilitate *S. aureus* intracellular escape upon phagocytosis by PMNs. It was found that after phagocytosis, CD11b is accessible for LukAB-mediated targeting of *S. aureus*-containing phagosomes. This intracellular targeting facilitates rapid bacterial escape mediated by neutrophil lysis (235). The precise biochemical mechanism by which LukAB engages its receptor remains to be determined.

Gamma-hemolysin. No proteinaceous receptors have been reported for either HlgAB or HlgCB of gamma-hemolysin. Previous studies suggested that HlgCB and PVL likely share a similar receptor based upon the ability of HlgC to compete with LukS-PV for cell surface binding (174). Such direct competition implies that HlgC and LukS-PV share C5aR and/or C5L2 as a cellular receptor, although this has not been experimentally validated. Beyond this assessment, no additional studies have investigated the possibility that gamma-hemolysin targets a proteinaceous receptor to mediate host cell killing. In contrast, a number of studies have suggested that gamma-hemolysin recognizes and binds to the membranes of red blood cells through direct interactions with host lipids and glycolipid derivatives (150, 187, 242, 254). As early as 1980, Noda and colleagues identified the ganglioside GM1 as an efficient inhibitor of HlgCB-dependent (referred to as LukSF or leucocidin here) killing of rabbit leukocytes *in vitro* (150). GM1 is a sialic acid-containing oligosaccharide linked to a ceramide lipid and is found at significant levels on cells within the nervous system but can be found in the membranes of many cell types (255). Preincubation with GM1 for as little as 5 min was sufficient to inhibit toxin activity at a molar ratio of 1:1 (150). This interaction occurred specifically through the S subunit of the toxin, HlgC (150). The isolation of a truncated mutant of HlgC lacking 17 amino acids at its C terminus prevented the recognition of GM1 with the toxin, indicating a potential role for these residues in ganglioside recognition (256). Similar work with HlgAB indicated that GM1 is also capable of inhibiting activity through binding of the S subunit, HlgA, at a similar 1:1 molar ratio (257). The inhibition studies with HlgAB were conducted by using rabbit erythrocytes rather than leukocytes, due to the perceived principal role of HlgAB as a hemolysin at that time. Thus, the influences of GM1 on leukocyte targeting by HlgAB are not known. The fact that GM1 is abundant on the outer leaflet of most cell types further supports the notion that gamma-hemolysin could be using this

ganglioside to associate with cell membranes; however, it does not explain the cell type specificity of the toxin (erythrocytes and immune cells) (255). Based on the recent identification of the proteinaceous receptors for LukED, LukAB/HG, and PVL, it seems unlikely that GM1 is a primary receptor required for gamma-hemolysin targeting. Rather, it may serve as an accessory surface association motif that promotes stabilization of the toxin on the cell surface to facilitate membrane insertion. Further studies of gamma-hemolysin are needed to more clearly incorporate the roles of GM1 and potential proteinaceous receptors into a framework of cellular targeting and pore formation. Regardless, GM1 was shown to display remarkable inhibitory activity against gamma-hemolysin and has proven a useful tool for evaluating regions of the toxin required for cell surface recognition.

Additional studies evaluated the possibility that membrane lipids might serve as major cell surface recognition sites for gamma-hemolysin. Given data that demonstrate saturable and specific binding kinetics on target host cells, it seems unlikely that gamma-hemolysin recognizes lipid motifs exclusively to cause cell lysis. However, enrichment of certain lipids in raft-like microdomains or under artificial conditions may allow for an enhanced pore-forming capacity in a manner that is independent of receptor recognition (191, 258). In fact, synthetic vesicles composed of defined lipids can lead to HlgAB-mediated pore formation in the absence of a protein receptor or GM1 (242). The efficiency of pore formation in this synthetic model is increased in the presence of phosphocholine-containing lipids as well as phosphatidylethanolamine but not sphingomyelin (242). Phosphatidylcholine has been shown to bind specifically to the F component (HlgB) with a molar ratio of 1:1, like that of GM1 with HlgA and HlgC, and the addition of exogenous phosphatidylcholine prevents the lytic activity of HlgCB on rabbit leukocytes (150, 243). The solved crystal structures of both HlgB and LukF-PV further support the notion that lipids are likely recognized by the bicomponent leucocidins, as both structures were cocrystallized in the presence of phosphatidylcholine (178, 179). While the identification of optimal lipid compositions required for pore formation are useful in terms of deducing how lipid chain length and membrane flexibility modulate pore-forming capacity, such investigation bypasses important influences that may occur due to proteinaceous receptor-dependent recognition by gamma-hemolysin on host cells. Based on the evidence provided, it seems likely that a combination of both optimal lipid microenvironments and membrane receptor recognition motifs on host cells dictates the activity of gamma-hemolysin on host cells, although additional studies are needed to determine whether or not this is actually the case.

## INFLUENCES ON CELL SIGNALING AND INFLAMMATION

Inflammation Induced by Lysis

Given that leucocidins exhibit potent lytic activity on host immune cells, it is reasonable to predict that a robust inflammatory response will be induced in response to the cellular damage and release of cytosolic contents associated with cell killing. This toxin-mediated proinflammatory induction of the immune system is believed to be responsible for the pathological features of severe necrotizing pneumonia caused by PVL-producing *S. aureus* (127, 203, 204, 206, 211). Treatment of leukocytes with lytic concentrations of PVL leads to the release of potent proinflammatory mediators such as IL-8, histamine, and leukotrienes (259, 260). IL-8

is a major chemotactic cytokine that influences neutrophil recruitment, and histamine is most commonly associated with proinflammatory allergic reactions and vasodilatation, while leukotrienes, along with prostaglandins (metabolites of arachidonic acid), contribute to acute inflammation (261–263). Beyond proinflammatory mediators, the lytic activity of the leucocidins also leads to the release of major cytoplasmic enzymes that can act locally to cause tissue damage and further elicit proinflammatory mediators (68, 259). Thus, by virtue of their lytic activity on host immune cells, the leucocidins engage in two activities: (i) they prevent host immune cells from phagocytosing and killing *S. aureus*, and (ii) they induce substantial inflammation and cellular damage through the release of proinflammatory mediators and tissue-damaging enzymes, both of which presumably contribute to the severity of disease.

Proinflammatory Receptor Engagement

The lytic capacity of leucocidins is certainly critical to their primary roles in immune cell killing and pathogenesis. However, a substantial body of evidence now suggests that most, if not all, leucocidins have bona fide immune cell-activating properties and/or additional sublytic functions that occur in the absence of cell lysis (Fig. 6) (210, 233, 252, 253, 264–266). Most studies evaluating the proinflammatory signaling properties of the leucocidins stem from work done with PVL and gamma-hemolysin (210, 252, 253, 264–266). To evaluate proinflammatory signaling, the toxins are typically applied at sublytic concentrations or as single subunits so that overt cell lysis does not appreciably obscure other mechanisms by which the proinflammatory response is activated. Noda et al. demonstrated that HlgC of gamma-hemolysin was capable of inducing neutrophil chemotaxis as well as phospholipase A2 activity, which leads to the subsequent release of arachidonic acid and prostaglandins (147). Arachidonic acid is the major metabolite of proinflammatory prostaglandins and leukotrienes; thus, their release by HlgC-treated leukocytes is likely to have significant influences on host inflammation (267, 268). Colin and Monteil first examined the capacity of leucocidins to engage in what was referred to as PMN priming (265). They showed that various combinations of gamma-hemolysin and PVL, when applied at sublytic concentrations (HlgAB, HlgA–LukF-PV, LukSF-PV, and HlgC–LukF-PV), are capable of priming neutrophils for increased production of H_2O_2 upon treatment with fMLP, although at higher concentrations, the leucocidin combinations were perceived to be inhibitory (Table 1) (265). Later studies confirmed that sublytic addition of PVL to primary human PMNs leads to priming for inflammatory reactivity (252). Such enhanced responsiveness includes increased reactive oxygen species production upon the addition of fMLP that is independent of Toll-like receptor 2 (TLR2) and cluster of differentiation 14 (CD14) signaling, increased phagocytosis and killing of *S. aureus*, and increased production of major proinflammatory mediators (Fig. 6) (252). Much of this proinflammatory priming is likely due to engagement of the C5a receptor, the cellular target of LukS-PV (199). Spaan et al. demonstrated that pretreatment with LukS-PV enhances reactive oxygen species production in response to fMLP in a C5aR-dependent manner (199). Thus, toxin-receptor interactions appear to be key to the induction of the nonlytic proinflammatory activities of PVL. In contrast to PVL, LukAB/HG does not appear to induce neutrophil priming, as treatment with the toxin does not lead to an increased production of reactive oxygen
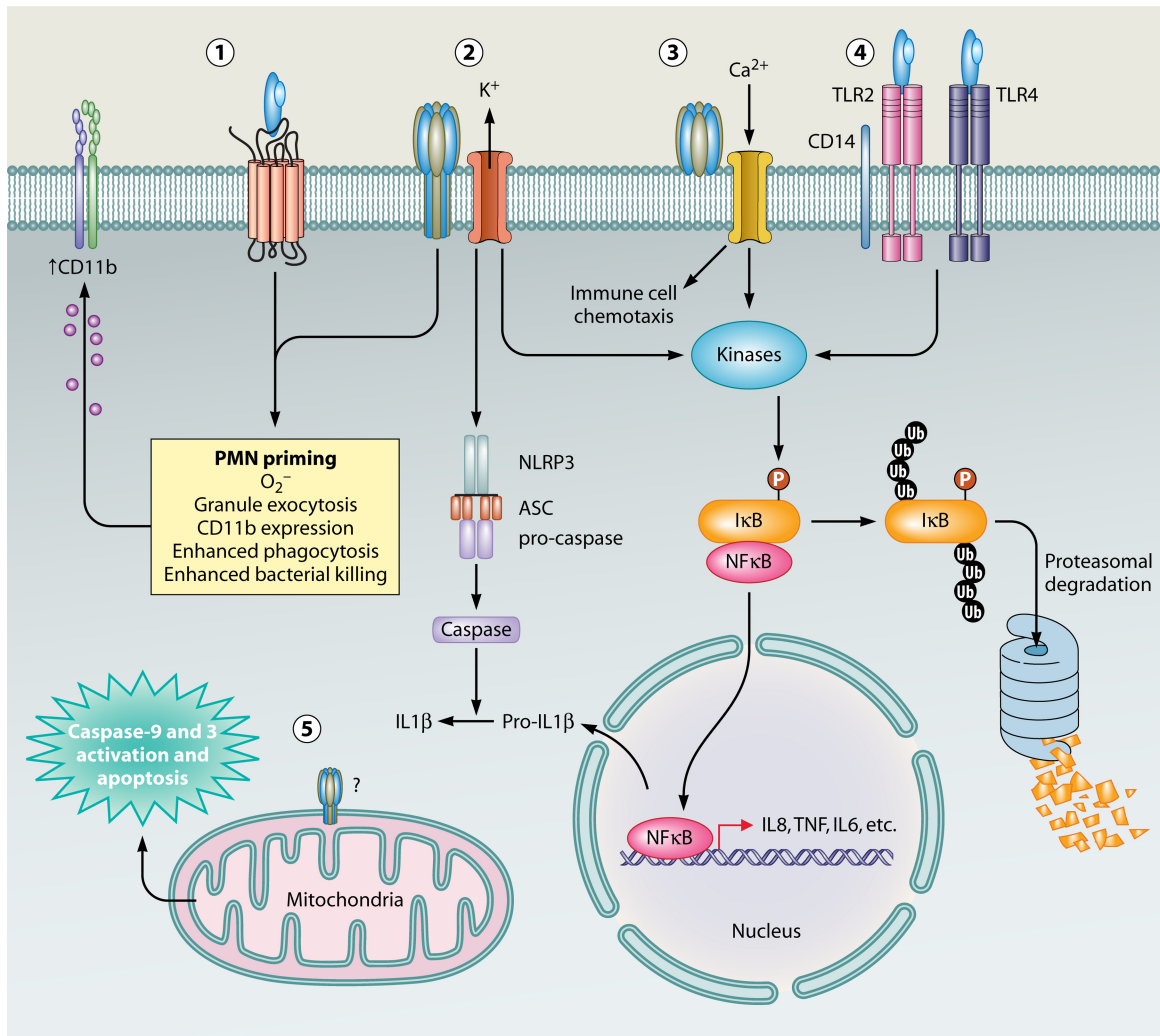
FIG 6 Sublytic effects of *S. aureus* leucocidins. Sublytic activities of leucocidins have been investigated primarily for PVL and gamma-hemolysin. Some sublytic functions are shown. (1) Priming of PMNs through the engagement of cellular receptors and other mechanisms yet to be defined that lead to increased reactive oxygen species formation, enhanced granule exocytosis, robust phagocytosis, and increased bactericidal activity of host neutrophils. (2) Induction of the NLRP3 inflammasome and subsequent IL-1β release mediated by potassium efflux from the cytosol due to pore formation. (3) Stimulation of immune cell chemotaxis and NF-κB activation as a result of calcium influx. The subsequent activation of cellular kinases leads to IκB phosphorylation and targeted degradation, followed by translocation of NF-κB to the nucleus and induction of proinflammatory gene expression. (4) Engagement of Toll-like receptors (TLR2 and TLR4) to stimulate the same canonical NF-κB activation pathway described above (3). (5) Activation of apoptosis via mitochondrial disruption potentially caused by pore formation.

intermediates and does not influence phagocytosis or bactericidal activity, although it may indirectly influence inflammation through the induction of neutrophil extracellular trap (NET) formation and increased CD11b expression (269).

Despite the perceived TLR2/CD14-independent role of PVL in PMN priming, Zivkovic and colleagues reported an *in vivo* immunomodulatory response that appears to rely upon toxin engagement of TLR2, leading to the activation of NF-κB through the canonical pathway (IκB-α phosphorylation that leads to proteasomal degradation and translocation of NF-κB to the nucleus, followed by subsequent NF-κB-dependent gene expression) (Fig. 6) (270). Furthermore, a direct interaction of LukS-PV with TLR2 was demonstrated by enzyme-linked immunosorbent assays (ELISAs), and knockout of both TLR2 and CD14 rendered cells largely unresponsive to the toxin (270, 271). A similar study proposed that HlgCB interacts with Toll-like

receptor 4 (TLR4) (a pattern recognition receptor known for recognizing lipopolysaccharide [LPS] of Gram-negative bacteria to induce inflammation) to induce the production of IL-12-p40 and tumor necrosis factor alpha (TNF-α) from murine bone marrow-derived dendritic cells (Fig. 6) (272, 273). Thus, it appears from these studies that the leucocidins are likely to engage classical pattern recognition receptors, in nonclassical ways, to induce inflammatory responses. More in-depth investigation will serve to validate these findings and determine how such altered proinflammatory signaling through TLR2 and TLR4 impacts global immune responses to *S. aureus*.

Inflammasome Activation

In addition to its described proinflammatory priming of PMNs, PVL is also known to bind to both monocytes and macrophages (likely due to its recognition of C5aR on the cell surface) and elicit

cellular responses (174, 253). When PVL binds to the surface of monocytes and macrophages, significant increases in IL-1β release are observed (Table 1). IL-1β is a major proinflammatory cytokine that is produced during a cellular process known as inflammasome activation (Fig. 6) (253). This cytokine can activate neutrophils and induce the expression of additional proinflammatory cytokines such as TNF and IL-6 (for detailed information on the inflammasome, the production of IL-1β, and its influence on the host response to infection, see references 274–278). One major inflammasome complex, known to respond to pore-forming toxins to induce the release of IL-1β, is the NLRP3 inflammasome (278–280). PVL-dependent induction of IL-1β release from monocytes and macrophages appears to be directly dependent on NLRP3 inflammasome activation, similar to what has been observed for alpha-hemolysin (Fig. 6) (253, 281). In support of this study, others have determined that PVL is the primary leucocidin responsible for the release of IL-1β by primary human macrophages, although it was found that HlgCB also induces IL-1β release albeit to a lesser extent (266). Leucocidin synergism with other toxic molecules produced by *S. aureus* was also found to effectively enhance the release of IL-1β induced by PVL, highlighting the complex nature of the inflammatory response that likely occurs during *S. aureus* infection (266). The increased IL-1β release by macrophages in response to a sublytic administration of PVL was shown to stimulate cocultured cells (in this case, alveolar epithelial cells) to release the proinflammatory cytokines IL-8 and macrophage chemotactic protein 1 (MCP-1) via cytokine-dependent activation of the IL-1 receptor (266). This study is particularly informative, as it directly links the functional consequences of IL-1β release by immune cells in response to PVL to the induction of proinflammatory signaling by epithelial cells, which ultimately leads to increased immune cell recruitment during infection. An important difference between the above-described two studies is their use of moderately lytic concentrations (253) versus sublytic concentrations (266) of PVL to induce IL-1β production. The fact that sublytic toxin concentrations are capable of inducing IL-1β release supports the hypothesis that toxin-mediated signaling events occur through direct cellular recognition strategies and are not simply an effect of overt toxin-induced lysis. Signaling by leucocidins to induce the release of IL-1β by immune cells may also extend into the neuronal compartment, as microglia are known to produce IL-1β in a manner that is partially dependent on the presence of secreted gamma-hemolysin (282). Thus, in addition to the lytic activity of the *S. aureus* leucocidins, the capacity to induce proinflammatory signaling may also have dramatic influences on ultimate infection outcomes.

Other Accessory Toxin Effects

Thus far, PVL and gamma-hemolysin have been most intensely studied in terms of their nonlytic effects on host cells. However, LukED has been demonstrated to inhibit lymphocyte proliferation at high concentrations but to stimulate lymphocyte proliferation at low concentrations (229). The mechanism by which this occurs is unknown, as this study was conducted on carp lymphocytes, which, to our knowledge, have not been tested for susceptibility to LukED or for receptor recognition (229). Given the known receptor-dependent targeting of lymphocytes of both murine and human origins, it is possible that LukED may also target carp lymphocytes in a receptor-dependent manner to elicit a lymphoproliferative response at sublytic concentrations. Similar

studies conducted on canine lymphocytes demonstrate that high concentrations of LukED limit lymphocyte proliferation, although this is likely due to the lytic capacity of LukED on these cells (228). With the recent identification of the receptors required for LukED immune cell targeting, more detailed studies of the potential influence on cell signaling can be conducted. Thus far, there is no indication that the LukE subunit alone can elicit signaling events through either CCR5 or CXCR1/2 insofar as toxin treatment is unable to elicit calcium signaling through receptor recognition (227, 230). However, recent proteomic studies indicate that the addition of lytic concentrations of LukED to PMNs induces the production of major proinflammatory proteins and support the notion that most, if not all, leucocidins are capable of inducing inflammation to some degree (Table 1) (283).

A unique activity of PVL is its ability to induce apoptosis at sublytic concentrations (Fig. 6) (284). The administration of PVL at low doses leads to characteristic morphological changes associated with apoptosis, including chromatin condensation and cell rounding (284). Intoxicated cells stain positive for annexin V but are not permeable to propidium iodide, a phenotypic hallmark of apoptotic cells. These apoptotic characteristics are linked to mitochondrial disruption and activation of the proapoptotic caspases caspase-3 and caspase-9 (284). Localization of recombinant PVL to the mitochondria after subcellular fractionation suggests that the toxin may exert deleterious effects on the mitochondrial membrane, leading to the induction of apoptosis. While the implications of PVL-dependent initiation of apoptosis are intriguing, it is important to note that studies describing the toxin's proapoptotic effects were limited to the use of recombinant PVL and bacterial culture supernatants. Additional work is needed to evaluate whether PVL-dependent apoptosis is a biologically relevant sublytic function and whether mitochondrial membrane disruption is a direct consequence of pore formation at the level of the mitochondrion or a downstream consequence of pore formation at the cellular membrane.

A novel sublytic activity of the gamma-hemolysin pair HlgCB is its ability to induce the release of calcium from internal stores within neuronal cells (285). Sublytic concentrations of HlgCB, and, to a lesser extent, HlgAB and PVL, are capable of stimulating glutamate release from cerebellar granular neurons. The release of glutamate was subsequently determined to be a direct consequence of alterations in intracellular calcium release that were induced by leucocidin cellular engagement (285). As a result of this study, it has been suggested that the leucocidins could foreseeably play a role in neuronal tissue damage, thereby influencing the perception of pain during invasive infection with *S. aureus*. A recent study suggested that *S. aureus* is indeed able to directly induce sensations of pain through the action of a pore-forming toxin (in this case, alpha-hemolysin) on nociceptive neurons; however, the bicomponent leucocidins were not implicated (286). Thus, the biological functions of glutamate release caused by HlgCB on granular neurons remain to be determined.

MIXED PORES AND TOXIN SYNERGISM

The genes encoding the S and F subunits of a given leucocidin (LukED, LukAB, LukSF-PV, LukMF′, and HlgCB) sit directly adjacent to one another in the *S. aureus* chromosome and are cotranscribed (Fig. 4). The pairing of the S subunit with its genetically linked F subunit always results in the formation of an active toxin on target host immune cells. As such, native pairings of leucocid-

ins are assumed to be the most common biologically active toxins seen *in vivo*. However, the high degree of sequence similarity among the leucocidins and the unconventional pairing of HlgA with HlgB, despite being translated from independent transcripts, imply that other mixed pairings of leucocidins may be able to form biologically active toxins capable of causing lytic activity or influencing the responses of host cells. Indeed, Prevost et al. performed studies with mixed-subunit pairings of gamma-hemolysin and PVL and found that while hemolytic activity was restricted to pairings of gamma-hemolysin subunits only, PMN lytic activity was not (98). Combinations of LukF-PV and either HlgA or HlgC and combinations of HlgB and LukS-PV caused lysis of primary PMNs (98). In addition, Colin and Monteil provided compelling evidence that mixed pairings of gamma-hemolysin and PVL subunits can induce immune cell activation by priming neutrophils with similar capacities (265). Upon the identification of LukED, additional combinations of leucocidin subunits were found to have lytic activity on both RBCs and PMNs. Hemolytic activity was seen for a number of nonconventional subunit combinations, including LukE plus HlgB, HlgA plus LukD, and HlgA plus LukF-PV (93). An even greater repertoire of toxin combinations exhibited activity on PMNs, as determined by induction of calcium mobilization. However, measurement of ethidium bromide uptake as a readout for pore formation demonstrated that mixed subunits of gamma-hemolysin and PVL were the only toxin combinations capable of inducing robust cell lysis, consistent with what had previously been described (93). In contrast, Morinaga et al. demonstrated that their identified "variant" of LukED (later determined to be highly conserved in nearly all sequenced strains of *S. aureus*) could form active toxins through nonconventional subunit pairings of both PVL and gamma-hemolysin subunits, indicating that the overall diversity of active toxins can become quite large (94). It was further demonstrated by Meyer et al. that S subunits efficiently recruit nonconventional F subunits to the surface of host cells (244). For example, HlgB and LukD can be recruited to the surface of cells that have been pretreated with LukS-PV, while LukS-PV or HlgC can recruit LukF-PV to the cell surface (244). This study did not measure whether these mixed pairings form functional toxins; however, it provides further evidence that within mixed populations, leucocidin subunits are capable of assembling the constituents of active toxins albeit comprised of nonconventional subunit pairs. Upon the identification of LukAB/HG, it was also found that the pairing of either the S or F subunit with the respective F or S subunit of PVL leads to the formation of an active toxin on the human macrophage-like cell line THP1 (266). Still, there are major outstanding questions concerning whether nonconventional leucocidin pairings occur *in vivo* and the exact functional consequences of these pairings as they relate to leucocidin synergism and expanding the diversity of immune cell targets.

LEUCOCIDIN REGULATION

Influences of Medium Composition and Growth Conditions

Environmental factors/conditions play a significant role in dictating differential leucocidin expression profiles and can have major influences on the ultimate cytotoxic activity of *S. aureus* (Fig. 7). Early investigations into the complex environmental stimuli that influence leucocidin expression were provided by Panton and Valentine, Woodin, and Gladstone and coworkers, who demon-

strated that specific growth conditions and medium compositions can promote abundant secretion of PVL by *S. aureus* (44, 45, 64, 287). These studies defined optimal conditions that lead to an increased abundance of PVL in culture supernatants for purification purposes. The use of CCY medium strongly induced the production of PVL, in particular by *S. aureus* strain V8 (44, 55, 59). This medium continues to be used by a number of research groups to purify the toxin from culture filtrates (98, 288, 289). Brain heart infusion (BHI) medium also leads to increased expression levels of PVL in broth culture, with comparatively lower expression levels of the other leucocidins, although gamma-hemolysin gene expression and toxin activity can also be measured in this medium (221). In medium containing yeast extract, Casamino Acids, and sodium pyruvate (YCP medium), the expression level of LukED is increased (93). Growth of strain Newman in YCP medium sufficiently increased the production of LukED over that of gamma-hemolysin to allow its initial purification and identification from *S. aureus* culture supernatants (93). In contrast, LukED is expressed at low levels in most other media, including BHI medium, RPMI medium supplemented with Casamino Acids (RPMI-CAS), tryptic soy broth (TSB), as well as lysogeny broth (LB) (47, 97, 221). The level of LukAB/HG expression is highest during growth in RPMI-CAS, whereas other leucocidins are expressed at comparatively low levels in this medium (97, 234). In LB medium, PVL is more abundantly expressed than LukAB/HG, while in TSB, both PVL and LukAB/HG are produced in relatively similar abundances compared to other leucocidins, which are minimally expressed (234). LukAB/HG is also produced at high levels in CCY medium, as evidenced by its contribution, along with PVL, to the cytolytic activity of CCY culture filtrates *in vitro* (96). Additionally, early efforts to purify PVL demonstrated a dependence on CO_2 for optimal leucocidin expression (290). A CO_2 percentage as high as 40% was used for the generation of preparative cultures of *S. aureus* for PVL purification (290). While CO_2 may enhance the production of toxins, it does not appear to be required for abundant secretion of PVL, as most other studies cultured *S. aureus* under normal atmospheric conditions. Interestingly, DuMont et al. used luciferase reporter strains of *S. aureus* to demonstrate that the *lukAB* (*lukHG*) promoter is significantly upregulated over other leucocidins when cultured in the presence of primary human neutrophils. Such findings indicate that additional host-derived signals may directly influence leucocidin gene expression (234). In all cases, the media and growth conditions described above are relatively complex; thus, it is unknown what specific factors may be promoting the production of one leucocidin over another. These studies do highlight a number of critical considerations: (i) *in vitro* growth conditions must be carefully considered when evaluating *in vitro* leucocidin activity, (ii) the perceived dominance of any leucocidin under one growth condition is not necessarily the same under another, (iii) extrapolation of the relevance of one leucocidin to pathogenesis based solely on *in vitro* studies should be made with caution, and (iv) it is likely that complex environmental cues within an infected host may have effects similar to those seen in broth culture experiments, as the diversity of leucocidins in one infectious site may vary considerably from that in another. The described variations in toxin gene expression in complex media are likely a direct consequence of the diverse regulatory inputs known to exist in *S. aureus*, as many of these systems are believed to be highly responsive to environmental
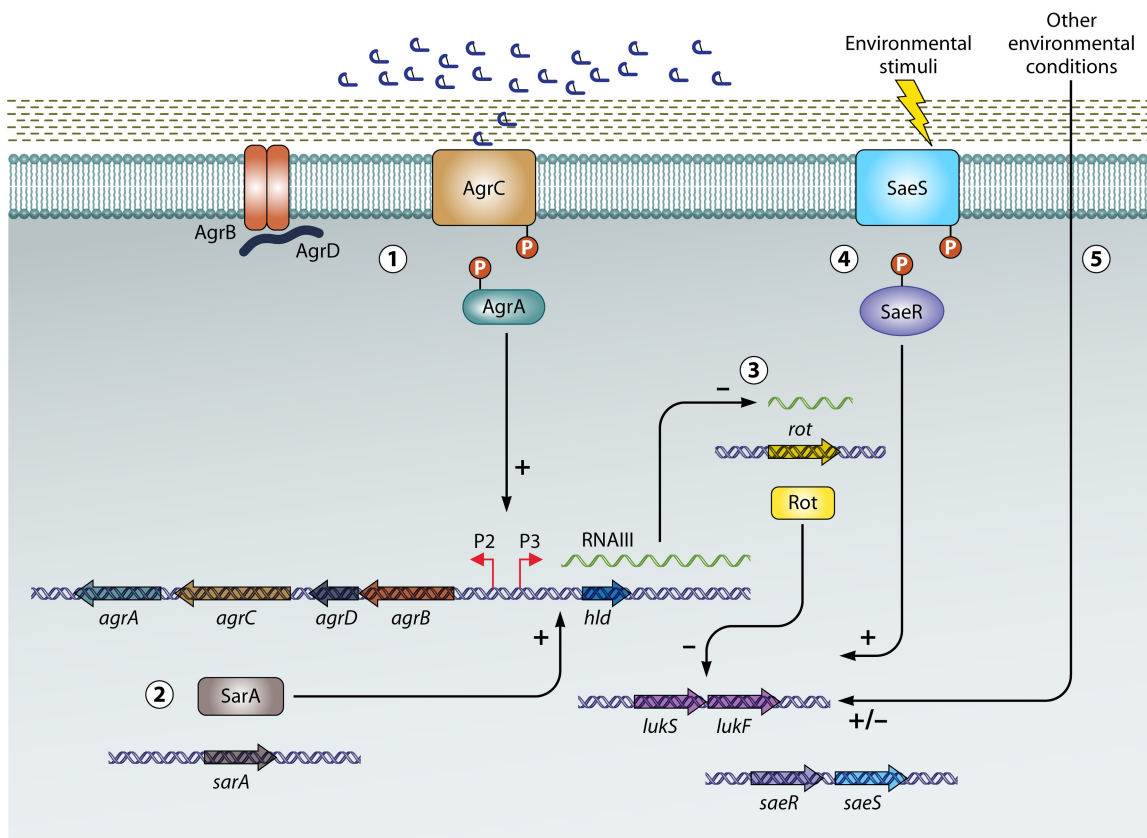
FIG 7 Leucocidin gene regulation. The molecular details of leucocidin gene regulation have not been extensively defined, but a number of master regulators and external signals provide major inputs into their altered gene expression in various environments. Some major regulatory inputs are shown. (1) The Agr quorum-sensing system is activated upon reaching a high bacterial density, leading to AgrA-dependent activation of the P3 promoter, which encodes the regulatory RNA, RNAIII. RNAIII negatively regulates the translation of the leucocidin repressor Rot, leading to increased leucocidin production. (2) The global regulator SarA indirectly facilitates leucocidin expression by positively regulating the expression of the P3 promoter, leading to a similar repression of Rot translation and increased leucocidin production. (3) Rot is believed to bind directly to leucocidin promoters to inhibit toxin gene expression. (4) The SaeRS two-component system recognizes external stimuli from the environment, leading to direct binding of SaeR to leucocidin promoters and subsequent enhancement of gene expression. (5) Other environmental stimuli positively and negatively regulate leucocidin gene expression, although the precise stimuli and their mechanism(s) of activation/repression are yet to be defined.

stimuli. Some major regulatory systems known to influence leucocidin gene expression are discussed below.

Regulation at the Transcriptional Level

Bacterial gene regulation of the bicomponent leucocidins is complex (Fig. 7). Multiple inputs from a variety of global regulatory systems feed into one another to exert precise control over toxin gene expression. It is becoming clear that while there are dominant inputs, the leucocidins display differential regulatory patterns that still require substantial investigation. Semiquantitative reverse transcriptase PCR (semi-qRT-PCR) studies confirmed previous studies indicating that YCP medium increases leucocidin expression compared to that in BHI medium (291). These transcript studies also demonstrated growth phase-dependent expression of the leucocidins, with greater transcript abundances of *hlgA*, *hlgCB*, *lukED*, and *lukSF-PV* as the bacteria entered late exponential and early stationary phases (291). Such growth phase-dependent gene expression is suggestive of regulatory input imparted by the accessory gene regulatory (Agr) system in *S. aureus* (Fig. 7) (292). The Agr system (composed of 4 genes, *agrBCDA*) is a quorum-sensing system that becomes activated upon reaching a

high bacterial density (292). *agrC* and *agrA* encode a histidine kinase and a response regulator, respectively, like other canonical two-component regulatory systems. AgrC is activated upon reaching a high bacterial density due to the increased effective concentration of a small peptide ligand processed from AgrD by AgrB (293–295). This small peptide is known as an autoinducing peptide (AIP) that interacts with AgrC to activate the AgrCA two-component system (294, 296). Agr activation leads to the increased transcription of two RNAs, RNAII (encoding AgrBCDA) and RNAIII, a regulatory RNA that inhibits and/or promotes the translation of multiple gene products, including a transcription factor known as repressor of toxins (Rot) (292, 297–300). Rot is so named due to its ability to act as a repressor at *S. aureus* toxin promoters; however, it is known to also activate promoters of other virulence genes (Fig. 7) (301–304). Rot is negatively regulated at the translational level by RNAIII, such that when the Agr system is activated, RNAIII inhibits Rot translation due to antisense binding to the *rot* transcript, thereby increasing the expression levels of toxins (305–307). Thus, on a global level, the activation of the Agr system leads to increased expression levels of major

S. aureus virulence factors, including proteases, cytotoxins, and cytolytic peptides. Indeed, it was found that when *agr* is deleted, *S. aureus* expresses lower overall levels of leucocidin transcripts (291, 300). This equates to largely reduced leucocidin abundance at the protein level, although some toxins are more dramatically influenced than others. For example, in strain Newman, levels of LukED and Hla are dramatically reduced in an *agr* mutant background, while the abundances of LukAB/HG and HlgCB are reduced but to a lesser degree (47). As described above, the primary input responsible for the reduced production of toxins in an *agr* mutant is believed to be increased repression of gene expression imparted by Rot, which is produced in a greater abundance in an *agr* mutant (in an *agr* mutant, RNAIII levels are low, and thus, Rot translation is derepressed). Indeed, the generation of an *agr rot* double mutant restores most leucocidins to levels similar to or greater than those of wild-type *S. aureus* (47). This suggests that the Agr-Rot axis is critical for optimal leucocidin regulation, although it is not the only regulatory input dictating leucocidin gene expression.

In addition to Agr and Rot, the staphylococcal accessory regulatory protein (SarA) and the *S. aureus* exoprotein (Sae) two-component system has also been shown to positively regulate the expression of a number of *S. aureus* cytotoxins at the transcriptional level (Fig. 7) (300, 308–310). SarA has been shown to positively regulate the gamma-hemolysin genes *hlgCB* and the gene encoding alpha-hemolysin, *hla*, as determined by microarray analysis (300). Like Agr, SarA is a global regulator with influences on the expression of many *S. aureus* virulence genes, including the Agr system itself (311–314). Thus, SarA may impose significant indirect influences on leucocidin gene expression via its positive modulation of the Agr-Rot axis.

In contrast to the presumed indirect influences of SarA on leucocidin gene expression, the *S. aureus* exoprotein regulatory system (SaePQRS) is known to play a direct role in the regulation of a number of *S. aureus* leucocidin genes (Fig. 7) (309, 315). SaeRS is a two-component system that responds to host environmental cues, including PMNs, to upregulate the expression of a diverse repertoire of *S. aureus* secreted proteins, many of which are major virulence factors (304, 309, 310, 316–321). SaeR binds to a consensus sequence within virulence factor promoters to induce gene expression (309, 321). SaeR binding sites have been identified within the promoter regions of nearly all leucocidin genes, including *lukSF*, *hlgA*, *hlgCB*, *lukED*, and *lukAB* (*lukHG*) (309, 321). Experimentally, an *sae* deletion mutant of *S. aureus* has significantly reduced transcript levels of all known leucocidins and is dramatically attenuated in murine infection models (308–310, 315).

In addition to the Agr, SarA, and Sae regulatory inputs, the master regulator of iron acquisition, Fur, provides additional regulatory control over leucocidin gene expression (322). *S. aureus fur* mutants significantly upregulate the production of LukED and HlgCB in broth culture (322). Whether the regulation of leucocidins by Fur occurs via direct or indirect mechanisms is yet to be determined. Nevertheless, the induction of LukED and HlgCB in a *fur* mutant links leucocidin production with nutrient acquisition and suggests that leucocidins may be responsive to other forms of metabolic and nutritional signals. In summary, while we have gained a better appreciation for the fundamental regulatory inputs that influence leucocidin gene expression, the diverse signals and mechanistic details underlying the optimal activity of these complex regulatory pathways remain to be uncovered.

Regulation at the Posttranslational Level

While understanding leucocidin gene regulation at the transcriptional level has been a primary research focus, a small number of studies have assessed the possibility that posttranslational modifications may serve to modulate leucocidin activity. Two studies by Kamio and colleagues suggest that HlgC is phosphorylated in a protein kinase A-dependent manner and that this phosphorylation event is required for the activity of HlgCB on host cells (164, 165). There is a predicted consensus phosphorylation sequence (KRST) within the C terminus of HlgC that is believed to be recognized by protein kinase A (164). In initial studies, it was determined that the threonine at position 246 could be phosphorylated in the presence of protein kinase A but only when HlgC was first denatured (164). When this threonine at position 246 was mutated to an alanine, phosphorylation of denatured HlgC did not occur, and the toxin had no cytolytic activity. However, in this initial study, native HlgC was unable to be phosphorylated in a manner similar to that of the denatured protein, which largely calls into question whether phosphorylation of HlgC at T246 is a biologically relevant phenomenon. However, it is clear from these studies that regardless of the phosphorylation status, T246 is a critical residue for HlgCB function, as mutation to alanine eliminates toxin activity. Follow-up studies using radiolabeled phosphate in the presence of human PMNs demonstrated that HlgC is indeed phosphorylated in the presence of PMNs and that this phosphorylation occurs at T246 (165). Inhibition of phosphorylation by using protein kinase A inhibitors revealed that HlgCB lytic activity is reduced and that this reduction in activity correlates with reductions in levels of phosphorylation. Thus, it appears that phosphorylation at T246 is a relevant posttranslational modification that confers optimal activity to HlgCB in the presence of target cells. Interestingly, LukS-PV contains the same consensus phosphorylation sequence, while LukE and HlgA do not (102). Thus, phosphorylation is not a conserved mechanism by which leucocidin lytic activity is modulated. It is also worth noting that no additional studies have followed up on phosphorylation as a requirement for the activity of HlgCB or any other leucocidin, so readers are cautioned that the relevance of such activity would seemingly benefit from more rigorous investigation.

Recently, studies aimed at identifying the role of *S. aureus* proteases in secreted protein functions demonstrated that leucocidin abundance is likely to be directly influenced by the proteolytic activity of certain *S. aureus* strains. Upon the deletion of all secreted protease-encoding genes, Kolar et al. found that the abundances of LukAB/HG, LukSF-PV, LukED (LukE), and gamma-hemolysin (HlgA) were increased in bacterial supernatants compared to wild-type *S. aureus* (323). Thus, it is clear that proteases are likely to influence leucocidin stability in some capacity. As these studies were conducted with a protease-null mutant of *S. aureus*, it is not possible to attribute leucocidin degradation to the activity of one specific protease. Further investigation is needed to provide additional mechanistic detail and support the biological significance of these authors' findings as they relate to leucocidin activity (323).

TARGETING OF LEUCOCIDINS AS A THERAPEUTIC MODALITY

The leucocidins potently target both innate and adaptive immune cells to promote immune evasion by *S. aureus*. Thus, it stands to reason that blocking of leukocytolytic activity may prove beneficial in promoting natural clearance of *S. aureus* by host immune cells that would have normally been disabled or killed by the toxin. This perspective is not necessarily novel, as in the late 1800s and early 1900s, the potential of Panton-Valentine leucocidin as a therapeutic target was already being realized. Van de Velde et al., Valentine et al., and Gladstone et al. all devised methods by which they demonstrated the immunogenicity of "leucocidin" and the neutralization of leukocytolytic activity by specific antibodies (42, 43, 45, 52, 57, 194, 197, 324). Gladstone and colleagues even conducted a series of studies on both rabbits and humans by using a PVL toxoid (56–59). While there appeared to be some efficacy, as measured by a reduced time to infection resolution, the PVL toxoid left much to be desired in terms of its use as a promising therapeutic for *S. aureus* (56, 58). Insomuch as these PVL immunization studies by Gladstone et al. did not culminate in the development of an effective anti-*S. aureus* therapeutic agent, they did provide suitable evidence that an immune response can be mounted against PVL in humans. Interestingly, much of the immunization efforts by Gladstone and colleagues were conducted on patients with already established skin and soft tissue infections (SSTIs) (56). Thus, immunization during such active infections may have had limited value. Likewise, it is difficult to ascertain whether infection resolution was due to the administration of the toxoid or simply to natural clearance over the course of infection. It was also unknown whether or not the subjects of that study were infected with strains of *S. aureus* that contained the *pvl* genes. More recent research that calls into question the role of PVL in the pathogenesis of SSTIs further limits the interpretation of toxoid efficacy in the studies by Gladstone et al., as the majority of these patients had SSTIs. Regardless, the work of Gladstone and colleagues was at least suggestive of the potential therapeutic value associated with leucocidin immunization and supported further investigation. Importantly, these studies were conducted at a time when there was little appreciation for the diversity of leucocidins present within a given strain of *S. aureus* (19–21). Given the redundant and nonredundant activities of individual leucocidins, it is perhaps not surprising that the administration of a toxoid composed strictly of PVL displayed limited efficacy (19–21). Our current appreciation for the redundancies in cytotoxic molecules expressed by *S. aureus* makes it clear that an effective strategy to promote enhanced resolution of infection by the immune system is going to require the targeting of more than one toxic molecule (19). In particular, the diverse repertoire of immune evasion molecules produced by *S. aureus* will certainly necessitate the consideration of multicomponent therapeutics and vaccines (22, 23). In the past 10 years, our increased knowledge of leucocidin diversity, directed targeting of host immune cells, and their unique cellular specificities has left researchers better poised to test whether the development of novel therapeutic agents targeting multiple *S. aureus* leucocidins will prove to be an efficacious treatment strategy.

In the last 50 years, few studies have sought to directly evaluate the therapeutic potential of antileucocidin-based treatment modalities. One study, which immunized rabbits with PVL in order to prevent the pathological outcomes of mastitis, showed no effi-

cacy (325). In contrast, another study suggested that the administration of intravenous immunoglobulin (IVIG) might have potential therapeutic value, as it is likely to contain a number of antitoxin antibodies. Gauduchon et al. demonstrated that IVIG is capable of neutralizing the toxic activity of PVL *in vitro* and suggested that its use in conjunction with antibiotics may improve outcomes in patients with invasive *S. aureus* infection (326). However, IVIG is currently not recommended as a routine course of therapy, even for invasive *S. aureus* infection, despite its ability to neutralize PVL quite efficiently (4). This is due in part to conflicting evidence surrounding whether or not inhibition of PVL by IVIG is beneficial during infection. In 2010, Yoong and Pier showed that the administration of antisera, isolated from rabbits immunized with PVL, to *S. aureus*-infected mice led to worse disease outcomes (209). This study suggested that the proinflammatory activity of PVL on host immune cells, rather than being detrimental, as described for rabbit pneumonia models, is actually beneficial and assists in establishing more productive immune responses and better infection resolution. When PVL is neutralized (or deleted from the *S. aureus* genome), the overall virulence of *S. aureus* was actually enhanced rather than diminished (208–210, 233). These studies are in direct opposition to work that was reported the year prior indicating that immunization of mice with PVL led to reduced pathological outcomes in models of necrotizing pneumonia and skin and soft tissue infection (204). Similarly, the administration of anti-PVL antibodies to the eyes of *S. aureus*-infected mice using a keratitis model demonstrated increased efficacy for the resolution of CA-MRSA (327). An additional study showed that immunization of mice with rationally designed mutants of PVL leads to significantly improved outcomes in a lethal systemic infection model, although the protection observed in this model is likely due to the broadly neutralizing capacity of the sera (lytic factors other than PVL were blocked by sera obtained from PVL-immunized animals) (328). Currently, it is difficult to reconcile the conflicting evidence both in favor of and against leucocidin immunization strategies as viable therapeutic options. As noted above, the majority of PVL-based virulence studies were conducted by using suboptimal murine infection models. PVL is not lytic toward murine leukocytes, although it is capable of eliciting immune-activating properties on murine cells (200, 210). Thus, a major biological function of the toxin is not considered when using murine models to assess virulence features attributed to PVL and the efficacy of antibody-mediated neutralization of the toxin.

Studies with human subjects have demonstrated that children can produce remarkably high levels of anti-PVL antibodies at a very young age, yet they still remain susceptible to SSTI caused by *S. aureus* (329). Such findings suggest that neutralizing antibody alone does not necessarily protect against *S. aureus* infection. However, it is possible that the presence of PVL antibodies may still have the ability to reduce adverse infection outcomes without necessarily preventing disease. A recent study by Rasigade et al. found that the time to death of patients with necrotizing pneumonia who had previously had PVL$^+$ infections was significantly longer than that of patients who had not previously had a PVL$^+$ *S. aureus* infection (131). However, while the acute onset and pathological outcomes associated with disease were far more rapid in patients who had not experienced PVL$^+$ *S. aureus* infections in the past, both patient groups still showed the same number of deaths overall (131). Thus, PVL antibodies may have slowed the progres-

sion of disease but did not prevent the mortality associated with severe *S. aureus* necrotizing pneumonia. Importantly, both murine and human studies exclusively assessed the therapeutic efficacy of neutralizing the activity of only one leucocidin, PVL. Given the likelihood that any given clinically relevant *S. aureus* strain will produce at least three bicomponent leucocidins, which possess both overlapping and distinct immune evasion functions, it is perhaps not surprising that such low efficacy was witnessed. In an additional study of children with *S. aureus* infection, it was found that those with invasive disease generated a high-titer antibody response to LukAB/HG. The antibodies generated have significant neutralizing capabilities *in vitro* (330). However, like PVL, whether this antibody response to LukAB/HG alone is capable of conferring protection against infection with *S. aureus* remains to be determined. In this study, the titers of LukAB/HG antibody were higher than those of any other leucocidin tested, implying that it may be a dominant antigen seen during infection (330).

When injected into the vitreous of the eyes of rabbits, PVL and gamma-hemolysin are both capable of inducing endophthalmitis (225, 226, 331, 332). Recently, Laventie et al. demonstrated that the administration of LukS-PV and LukF-PV monovalent and divalent heavy-chain-only diabodies are capable of reducing the inflammatory outcomes associated with PVL administration to the rabbit eye (332). Additionally, they demonstrated that one of these neutralizing diabodies, which was originally designed to target only PVL, could also bind to and neutralize HlgCB of gamma-hemolysin (332). Thus, not only are anti-PVL antibodies capable of reducing PVL-induced inflammation in *in vivo* rabbit models, it is also possible to generate antibody molecules that neutralize more than one leucocidin pair. Work by Karauzum and colleagues also demonstrated that the generation of broadly neutralizing antibodies after immunization with PVL can have dramatic effects on pathogenic outcomes using a lethal murine systemic infection model (328). It is likely that antibodies with cross-neutralizing capabilities such as these will prove far more efficacious, highlighting promise toward the development of antitoxin molecules that may be able to target multiple toxins at the same time. By using this same ocular intoxication model, a series of small molecules with broad therapeutic applications known as calixarenes, or SCns (*p*-sulfonato-calix[*n*]arenes), were also tested for their ability to neutralize the activities of both PVL and HlgAB (331, 333). In the presence of the small molecules, the inflammatory pathology associated with toxin administration to rabbit eyes was significantly reduced (331). It has been proposed that this neutralizing capacity of the calixarenes in rabbit endophthalmitis models stems from the ability of the inhibitors to bind LukS subunits with high affinity, thereby preventing cell surface recognition and toxin-mediated killing. The implications of leucocidin-specific calixarenes for use in the treatment of other *S. aureus* infectious conditions have yet to be examined.

The identification of the cellular receptors required for cell surface recognition by LukAB/HG, PVL, and LukED has the potential to further the development of high-affinity leucocidin inhibitors. There is evidence for likely success in this endeavor, in that clinically approved CCR5 receptor antagonists, such as the HIV drug maraviroc, block the cytolytic activity of LukED on CCR5-expressing cells (227, 245). Additionally, the use of antibodies and/or natural ligands as competitors for toxin binding for each of the identified toxin receptors, including CCR5 (LukE), CXCR1/CXCR2 (LukE), C5aR/C5L2 (LukS-PV), and CD11b

(LukAB/HG), indicates that blocking of the initial interaction with the cell membrane is a specific and potent means of inhibiting leucocidin activity (199, 227, 230, 235). Further studies will certainly benefit from a more refined biochemical definition of toxin-receptor interactions. This includes more in-depth investigations into structural features of each toxin that dictate receptor specificity. Importantly, we suggest that receptor recognition motifs within individual toxins are likely to be better therapeutic targets than the receptors themselves. This is due to the fact that normal signaling through the cellular receptors of the leucocidins is, in most cases, critical for normal immune cell function, including phenomena such as chemotaxis to infected tissue and the induction of optimal inflammatory responses (334). Thus, directed targeting of the leucocidins rather than their receptors is likely to prevent negative outcomes associated with diminishing optimal immune responses that could be brought upon by receptor inhibition. Unfortunately, a major complication in the evaluation of the potential efficacy of any leucocidin-based inhibitor *in vivo* continues to be the lack of an appropriate animal model. However, the identification of leucocidin receptors suggests considerable potential toward the development of more appropriate small-animal models to mitigate the complications of species specificity and facilitate therapeutic testing *in vivo*.

CONCLUDING REMARKS

Our understanding of leucocidin function has progressed from the identification of a single toxic substance, the "leucocidin," to the identification of six unique toxic molecules whose biological functions are only now being fully appreciated. It is clear that the study of the leucocidins did not follow a simple path. An initial lack of appreciation for the diversity of leukocidal molecules present within *S. aureus* confounded many early studies, complicated nomenclature, and often led to phenotypic discrepancies among research groups. Similarly, species specificity associated with cellular targeting significantly slowed the pace of novel discovery as it relates to pathogenesis and infection outcomes. Such complications, along with complex epidemiological associations, have left many puzzling over the true roles of the leucocidins in human disease. In contrast, biochemical and biophysical studies have been met with greater success. Over the course of the past 20 years, a comprehensive model of leucocidin pore formation has been developed, which remains unchallenged today. Although PVL is often considered a mainstay in leucocidin research, it is now becoming clear that other leucocidins are equally capable of exerting potent lytic activity *in vitro* and *in vivo* and are certainly deserving of our future research efforts.

In the past 5 years, the leucocidins have received a considerable resurgence in attention. Studies have (i) identified and characterized a novel leucocidin (LukAB/HG), (ii) determined that the leucocidins dictate cellular specificity through the recognition of proteinaceous receptors, (iii) applied murine models to investigate leucocidin lytic activity *in vivo*, (iv) uncovered previously unappreciated proinflammatory functions that occur irrespective of cell lysis, and (v) proposed a number of potential therapeutic methodologies for targeted inhibition of toxin activity. These recent discoveries have opened considerable avenues for future investigation. Some areas of immediate interest include the development of small-animal models that are sensitive to the lytic function of all *S. aureus* leucocidins, investigation into the precise mode of action of all leucocidins in diverse infection settings, fur-

ther determination of sublytic and accessory leucocidin functions that are influenced by receptor-dependent and -independent targeting, and investigation into the therapeutic potential of leucocidin inhibition toward promoting natural clearance of *S. aureus* infection.

Thus, despite having been identified over 120 years ago, current studies of the bicomponent leucocidins continue to provide the *S. aureus* research community with novel insights into the complex underpinnings of toxin-based immune evasion. We are now better poised than ever to develop novel strategies to explore their mode of action *in vivo*, provide a more concrete picture of their contribution to pathogenesis, and determine the therapeutic efficacy of antileucocidin-based treatment strategies.

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