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Staphylococcus aureus Panton-Valentine leukocidin (PVL) alters human neutrophil viability and function

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STAPHYLOCOCCUS AUREUS PANTON-VALENTINE LEUKOCIDIN (PVL) ALTERS HUMAN NEUTROPHIL VIABILITY AND FUNCTION

By

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Community-associated methicillin-resistant *S. aureus* (CA-MRSA) infections are the leading cause of community bacterial infections in the United States. Genes encoding Panton-Valentine leukocidin (PVL) are present in most *Staphylococcus aureus* strains that cause CA-MRSA infections. Despite an association of PVL with the most abundant CA-MRSA strains, its role in pathogenesis—if any—remains unknown. PVL is a pore-forming toxin with specificity for myeloid cells, including polymorphonuclear leukocytes (PMNs or neutrophils). It is widely assumed that PVL-mediated pore formation in the plasma membrane of PMNs leads ultimately to cytolysis. However, this hypothesis has not been tested and previous studies indicate that levels of PVL *in vivo* are likely insufficient to cause host cell lysis. On the other hand, sublytic concentrations of PVL prime neutrophils for enhanced production of reactive oxygen species (ROS), and cause secretion of proinflammatory molecules and granule exocytosis. The molecular basis of PVL-mediated priming is unknown. To better understand the role played by PVL during *S. aureus* infection, we used human PMNs to investigate the correlation between PVL-mediated pore formation and cytolysis, and the molecular basis of PVL-mediated priming. Using *S. aureus* culture conditions that favor high production of PVL, there was on average more neutrophil plasma membrane permeability and cell lysis caused by supernatants derived from wild-type (PVL-positive) CA-MRSA strains compared to those from isogenic *lukS/F-PV* negative (PVL-negative) strains. Unexpectedly, there was not always a correlation between pore formation and cell lysis caused by *S. aureus* culture supernatants, and these findings were confirmed using purified PVL. Consistent with published studies, sublytic concentrations of PVL primed neutrophils for enhanced release of superoxide, caused upregulation of CD11b, and accelerated apoptosis. Microarray analysis revealed changes in the PMN transcriptome following exposure to PVL, including upregulation of molecules involved in the proinflammatory response. Consistent with microarray data, multiple proinflammatory molecules were released from neutrophils after stimulation with PVL. We propose that the primary function of PVL is distinct from leukocyte lysis—namely, that it elicits a proinflammatory response that can enhance the host innate immune response to *S. aureus* infection.

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CHAPTER ONE

INTRODUCTION

1.1 Methicillin-resistant *Staphylococcus aureus*

1.1.1 *Staphylococcus aureus* (*S. aureus*) and the emergence of methicillin-resistance

S. aureus is a Gram-positive bacterium that has a long history of causing a wide range of infections in the human host. The pathogen is among the leading causes of mortality by single infectious agents in the U.S. (1,2). *S. aureus* is also notorious for its ability to acquire resistance to antibiotics. For example, penicillin was introduced to treat human infections in the early 1940s and penicillin-resistant strains (PRSA) emerged soon thereafter (by 1942) (3). The molecule responsible for penicillin-resistance was identified as penicillinase (beta-lactamase), a plasmid-encoded enzyme that binds and hydrolyzes the beta-lactam ring of penicillin, rendering the antibiotic ineffective for treatment of infection (4). Strains expressing penicillinase were frequently isolated from patients without prior penicillin treatment, indicating in-hospital transmission of resistant strains (5). As such, the prevalence of penicillin-resistance in *Staphylococcus* increased dramatically within nosocomial settings (6). A penicillin-resistant *Staphylococcus aureus* strain known as phage-type 80/81 became prominent in Australia in hospitals and the community in the 1950s and 1960s (7). Phage-type 80/81 strains ultimately became epidemic worldwide (8,9).

By the mid-1950s, phage-type 80 strains were the cause of 42 percent of all outbreaks (9).

Methicillin was introduced in 1959 to treat *S. aureus* infections, largely because of the increased incidence of PRSA (10). Emergence of methicillin-resistant *S. aureus* (MRSA) occurred rapidly and initial reports of methicillin-resistant strains were published in 1961; however, outbreaks remained sporadic until the late 1960s. Industrialized countries reported increasing numbers of nosocomial MRSA infections in the later part of the decade (11-14) and there were warnings that MRSA would become problematic for hospitals worldwide (15-22). In 1975, 2.4 percent of *S. aureus* strains isolated from US hospitals were methicillin-resistant. By comparison, 29 percent of *S. aureus* isolates in 1991 were MRSA (23).

Methicillin-resistance is conferred by the *mecA* gene, which is encoded on a mobile genetic element known as the *Staphylococcal* chromosome cassette *mec* (SCC*mec*) (24). The *mecA* gene encodes a low-affinity penicillin-binding protein, PBP2a, that enables bacterial cell wall synthesis to continue in the presence of beta-lactam antibiotics (25). Currently, eight different SCC*mec* types have been identified (I-VIII). SCC*mec* types I-III, and VIII encode for resistance to multiple antibiotics, including methicillin-resistance (*mecA*), while types IV-VII encode methicillin-resistance only (26).

In contrast to the PRSA pandemic, MRSA outbreaks remained largely within the hospital setting for 30 years and MRSA is now endemic in hospitals throughout the developed world (27-29). Immune compromised patients or

individuals with a predisposing risk factor, such as surgery, are particularly susceptible to infection. In addition, transmission and spread of MRSA is promoted in the healthcare setting by hospital staff colonized with MRSA (29,30). Nasal carriage of MRSA (endogenous reservoirs) was reported as a risk factor for bacteremia and infection of surgical sites (31-34). MRSA infections in which disease onset originated from a healthcare institution is known as hospital-associated MRSA (HA-MRSA). The use of antibiotics in healthcare settings has

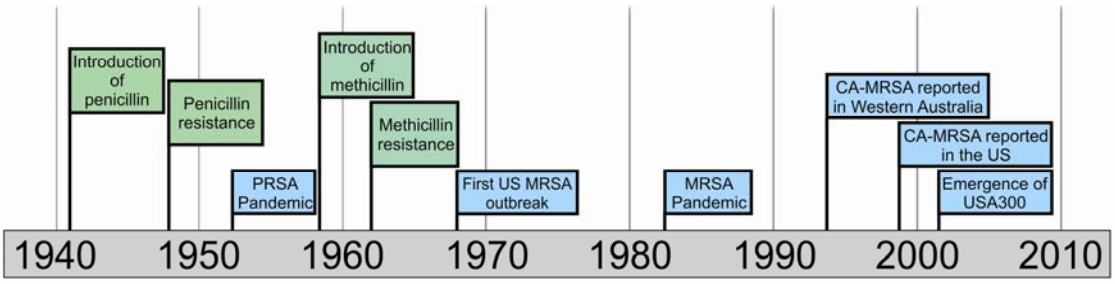

Figure 1. **Timeline depicting the emergence of penicillin and methicillin-resistant *S. aureus* strains from 1940 to 2010.** Green boxes represent introduction of an antibiotic and emergence of antibiotic-resistant strains. Blue boxes represent dissemination of resistant strains.

selected for HA-MRSA strains that are resistant to several antimicrobial agents. As such, HA-MRSA strains typically harbor the larger SCC*mec* types that encode resistance to multiple antibiotics (26). The rapid emergence of strains that are resistant to multiple antibiotics underscores the need to develop new therapeutics for treatment of MRSA infections (11,30,35) (Fig. 1).

1.1.2 Community-associated methicillin-resistant *S. aureus* (CA-MRSA)

The epidemiology of MRSA infections changed in the early 1990s. Although MRSA continued to be a prominent cause of hospital-associated infections, there were reports of MRSA outbreaks involving individuals outside of the healthcare setting. In 1993, Udo et al. reported CA-MRSA infections in individuals from the remote Kimberley region of Western Australia (Fig. 1) (36). The Centers for Disease Control and Prevention (CDC) and Herold et al. subsequently reported severe infections in pediatric patients in the Midwestern U.S. and these individuals also had no prior hospital exposure (37,38). These articles were among the first to report bona fide CA-MRSA infections. The prevalence of pediatric cases treated for MRSA contracted in communities rose from 10/100,000 admissions in 1988-1990 to 259/100,000 admissions in 1993 to 1995 (37).

CA-MRSA infections became widespread after 2003 (39) and ultimately became epidemic in the U.S. (1,29). For example, MRSA was the causative agent of 59 percent of all US community-associated bacterial infections in 2004 (29). These community-associated MRSA (CA-MRSA) infections often occurred in healthy, young populations such as athletic teams, preschool children, and military personnel (2,40,41). Skin and soft tissue infections compromise about 90 percent of all CA-MRSA infections (41). Severe CA-MRSA disease, such as osteomyelitis, sepsis, or necrotizing pneumonia, is infrequent but these syndromes can cause death (38,42,43-45). The ability of CA-MRSA strains to cause infection in otherwise healthy individuals suggests that these strains have increased virulence compared to strains that typically cause hospital infections.

CA-MRSA outbreaks typically occur in close-quarter environments. Direct contact between hosts is considered to be the primary mode of transmission (46). Contaminated fomites, such as towels and whirlpools, shared by multiple individuals may also contribute to spread of CA-MRSA (42,47). The CDC has developed the “Five C’s” or a list of five factors that are associated with risk of infection: 1) crowded environments; 2) frequent skin-to-skin contact with an infected or colonized person; 3) compromised skin (skin injuries); 4) contaminated items or surfaces; 5) lack of cleanliness or personal hygiene (http://www.cdc.gov/niosh/topics/mrsa/). In addition, the CDC provides specific guidelines to prevent further spread of CA-MRSA infections as follows: 1) keep the infected area covered with a clean, dry bandage; 2) wash hands with soap and water or use an alcohol-based hand gel, particularly after touching the infected area 3) maintain good personal hygiene 4) avoid sharing items that were exposed to the affected area with other individuals; 5) consistently launder clothing that has come in contact with the infected area; 6) limit activities involving skin-to-skin contact if infected area cannot be covered with a clean, dry bandage; 7) clean shared surfaces or equipment with detergent/disinfectant that specifies *S. aureus* on the product label (http://www.cdc.gov/ncidod/dhqp/pdf/ar/CAMRSA _ExpMtgStrategies.pdf). Application of the topical antibiotic mupirocin was recommended by Raz et al. to control recurrent CA-MRSA outbreaks (48). Notwithstanding, a study completed within the military population suggested such treatment does not decrease CA-MRSA colonization rates (49).

One method to categorize or type strains is based on pulsed-field gel electrophoresis (PFGE). PFGE banding patterns of *S. aureus* strains with the same or similar patterns belong to the same PFGE type (11,45). Comparison of PFGE banding patterns from different isolates provides information about the relatedness of the isolates and is appropriate for understanding the molecular epidemiology of outbreaks. Isolates from community-associated infections are genetically distinct from those that cause hospital-associated infections, suggesting there has been *de novo* emergence of the most prominent CA-MRSA strains rather than emergence from existing prominent HA-MRSA strains (50). The predominant HA-MRSA strains in the U.S. are PFGE types USA100, USA200, USA500, USA600, and USA800 (52). To date, the CA-MRSA epidemic in the U.S. consists of two predominant PFGE types, USA400 and USA300 (29,30,38,40-42). In addition to PFGE, multi-locus sequence typing (MLST or ST) can be used to determine the molecular epidemiology of MRSA infections. The ST of each strain is determined by single nucleotide polymorphisms (SNPs) present in seven housekeeping genes. Strains that have identical sequences at all seven loci are assigned to the same ST (11,51).

USA400 outbreaks occurred in the late 1990s; however, the occurrence of USA400 infections has diminished in the US and USA300 has become the main cause of CA-MRSA infections (Fig. 1) (29,52). The molecular basis for the success of USA400 and USA300 as human pathogens remains unclear. Whole genome sequencing and analysis of MW2, the prototype USA400 strain (53), and FPR3757, a USA300 strain representative of the epidemic USA300 clone (54),

revealed novel genetic elements that could enhance dissemination and/or pathogenicity. One of the notable findings was the presence of a prophage (phiSA2mw in MW2 and phiSA2usa in FPR3757) containing genes encoding Panton-Valentine leukocidin (PVL). The PVL genes (*lukS-PV* and *lukF-PV*) are present in 2-5 percent of *S. aureus* isolates worldwide (2,55,56). Despite the overall low percentage of *S. aureus* strains that harbor genes encoding PVL, the most prominent causes of CA-MRSA infections in the U.S. and Canada are PVL-positive strains. PVL is an exotoxin that forms pores in the plasma membrane of neutrophils, monocytes, and macrophages, host cells critical for host defense against *S. aureus*. Therefore, the emergence and success of CA-MRSA as a human pathogen was initially attributed to the presence of PVL in these strains (56-59).

1.1.3 *S. aureus* evasion of human innate immunity

In general, the ability of bacteria to cause disease is linked to evasion of the innate immune response. Polymorphonuclear leukocytes (PMNs or neutrophils) are the most abundant cells of the innate immune system and are essential for host defense against invading microbes (60). The importance of these leukocytes in the control and clearance of *S. aureus* infections is exemplified by patients with primary neutrophil defects. Deficiencies in phagocytic function, especially in neutrophils, result in recurrent and possibly fatal bacterial infections in these individuals (61). *S. aureus* is one of the leading causes of infection in patients with primary neutrophil defects.

Release of cytokines and chemokines from the tissue or cells surrounding the site of infection elicits an inflammatory response that includes rapid recruitment of PMNs *in vivo*. Molecules secreted by bacteria or components of the bacterial cell wall may also induce neutrophil chemotaxis and recruitment (62,63). Exposure of neutrophils to cytokines, chemokines, or bacterial components such as lipopolysacharride (LPS) *in vitro* primes these phagocytes for enhanced production of reactive oxygen species (ROS) and granule exocytosis following a secondary stimulus (Fig. 2) (64). N-formyl-methionyl-

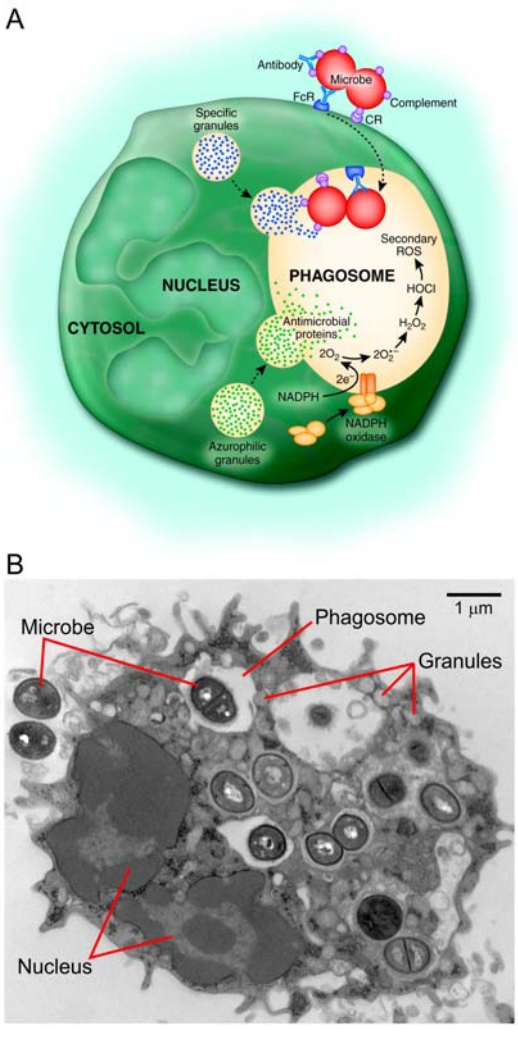

Figure 2. Neutrophil degranulation and release of ROS after phagocytosis. A) Phagocytosis is facilitated by host serum proteins (antibodies and/or complement) that bind to the microbial surface. Following phagocytosis, granules fuse with the phagosomal membrane and release antimicrobial proteins into the phagosome. Superoxide (O_2^-) is also released into the phagosome following phagocytosis of microbes. B) A transmission electron micrograph of a human neutrophil that has phagocytosed *S. aureus* (microbe). Reprinted with permission from Wiley Interdiscip. Rev. Syst. Biol. Med. (64).

leucyl-phenylalanine (fMLP), a representative of bacterial N-formyl peptides, is often utilized as the secondary stimulus in *in vitro* assays. PMN priming also upregulates expression of surface receptors such as CR3 (CD11b/CD18), molecules involved in cell adhesion, phagocytosis, and activation (65-68).

At the site of infection, PMNs rapidly engulf bacteria (a process known as phagocytosis), which are then sequestered within forming phagosomes (Fig. 2). The process of phagocytosis is facilitated by host serum proteins (such as antibody and complement) that opsonize microorganisms. Cytoplasmic granules fuse with the phagosomal membrane and antimicrobial proteins such as alpha-defensins are enriched in the phagosome. Concurrently, phagocytosis activates a membrane bound NADPH oxidase to produce superoxide, which is then converted to numerous secondary ROS within the phagosome (69-71). These secondarily-derived ROS include hypochlorous acid, a primary constituent of household bleach (60,70) Exposure of the pathogen to such harsh, intracellular conditions enables PMNs to kill the ingested microbes while preventing non-specific damage to host tissues.

Neutrophils are widely known to be associated with inflammatory diseases such as rheumatoid arthritis, and can cause significant destruction of host tissues should they undergo necrosis (72,73). Consequently, removal of effete neutrophils from sites of infection is crucial to resolution of inflammation. Phagocytosis ultimately accelerates apoptosis to promote resolution of the inflammatory response and return the immune system to quiescence (Fig. 3).

Macrophages recognize and remove the effete neutrophils through a process called efferocytosis (52,74). Most invading microorganisms are eliminated by the

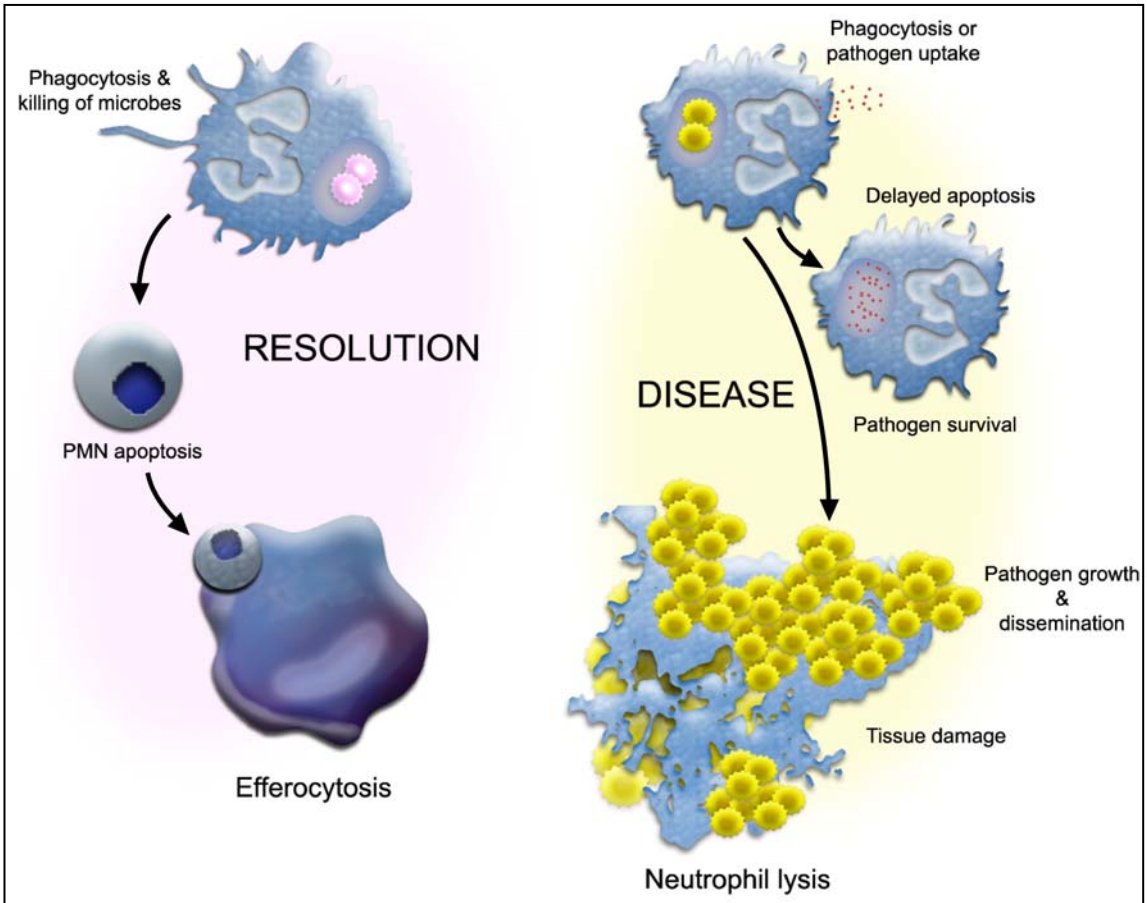

Figure 3. Two possible outcomes of the interaction of PMNs with bacteria. On one hand, bacteria (pink spheres) are ingested, activate PMNs and are killed, and this process ultimately triggers PMN apoptosis and the removal of these cells by macrophages (Efferocytosis). This phenomenon leads to the resolution of the inflammatory response (RESOLUTION). Alternatively, bacterial pathogens (yellow spheres or red particles) are ingested and either delay normal PMN apoptosis and turnover or cause PMN lysis. Typically, these processes facilitate survival of bacteria and thereby promote disease (DISEASE). Reprinted with permission from J. Mol. Med. (52).

oxygen-independent and oxygen-dependent neutrophil killing mechanisms described above. Nonetheless, several bacterial pathogens, including *S. aureus*, are known to evade neutrophil-mediated killing. Pathogens alter neutrophil function or survival to evade the innate immune system and thereby promote disease. As depicted in Fig. 3, some ingested *S. aureus* survive within the neutrophil phagosome and eventually cause destruction of the leukocyte (75-77). Notably, CA-MRSA strains have enhanced capacity to cause destruction of neutrophils compared to representative HA-MRSA strains (78). These findings provide support to the idea that the most prominent CA-MRSA strains are highly virulent as a result of enhanced ability to escape the antimicrobial activity of neutrophils following phagocytosis.

S. aureus produces many molecules that potentially contribute to evasion of the innate immune system, including pore-forming toxins that permeabilize leukocyte plasma membranes (55,79-81). PVL is one such pore-forming toxin and is present in USA300, USA400, and other *S. aureus* strains that are a prominent cause of CA-MRSA infections (52,82). For this reason, significant effort has been made by the infectious disease community to elucidate the role (if any) PVL has in CA-MRSA pathogenesis (52,82).

1.2 Panton-Valentine leukocidin

1.2.1 Panton-Valentine leukocidin (PVL), a bicomponent pore-forming toxin

The observation that PVL is associated with the major CA-MRSA lineages suggests that the toxin may enhance strain virulence or promote enhanced transmission (30,39, 83,84). Characterization of the cytolytic activity of PVL began in 1932 (as described by Panton and Valentine) (85), and thus, PVL was present in *S. aureus* long before the emergence of CA-MRSA. Studies published in 2002 indicate PVL is associated with fatal *S. aureus* necrotizing pneumonia, usually with antecedent influenza (83). These findings, coupled with the coincident emergence of CA-MRSA worldwide, renewed interest in PVL as a major *S. aureus* virulence factor. More recently, studies have confirmed an association of community-associated *S. aureus* pneumonia (MRSA and methicillin-susceptible *S. aureus* or MSSA) with antecedent influenza or an influenza-like illness (86-88). The molecular basis for severe *S. aureus* pneumonia following influenza remains unknown.

PVL consists of two subunits, LukS-PV and LukF-PV, both of which are secreted as water soluble monomers. The crystal structure of LukF-PV (89) and LukS-PV (90) has been solved and each subunit has three regions, the beta-sandwich, the rim, and the folded stem. The LukS-PV subunit binds to the surface of rabbit and human neutrophils, monocytes, and macrophages (56-59). Following LukS-PV binding, a hypothetical pre-pore octomer is formed on the PMN plasma membrane with both subunits present in a 1:1 ratio (Fig. 4). At this point, the rim regions of LukF-PV and LukS-PV interact with the neutrophil plasma membrane. Large variations in conformation between the two subunits are present in the rim (90). Presumably, functional differences between the two

PVL subunits are determined by variances in the rim domain. Complete pore-formation is accomplished by extension of the stem region of both subunits through the plasma membrane, forming a beta-barrel (Fig. 4) (89-93).

Genes encoding PVL, *lukS-PV* and *lukF-PV*, are acquired by horizontal gene transfer via bacteriophage such as phiSA2usa and the subunits are co-transcribed as an operon (94,95). A recent study reported the entire nucleotide sequence of *lukS/F-PV* prophages from 114 clinical MRSA isolates and identified two insertion sites in the *S. aureus* genome (Ins1 and Ins2) (96). The insertion sites are singularly present and either Ins1 or Ins2 occur in specific MRSA lineages. The PVL nucleotide sequence is highly conserved and one genetic polymorphism in *lukS-PV* results in a nonsynonymous amino acid change in LukS-PV. Variants containing an

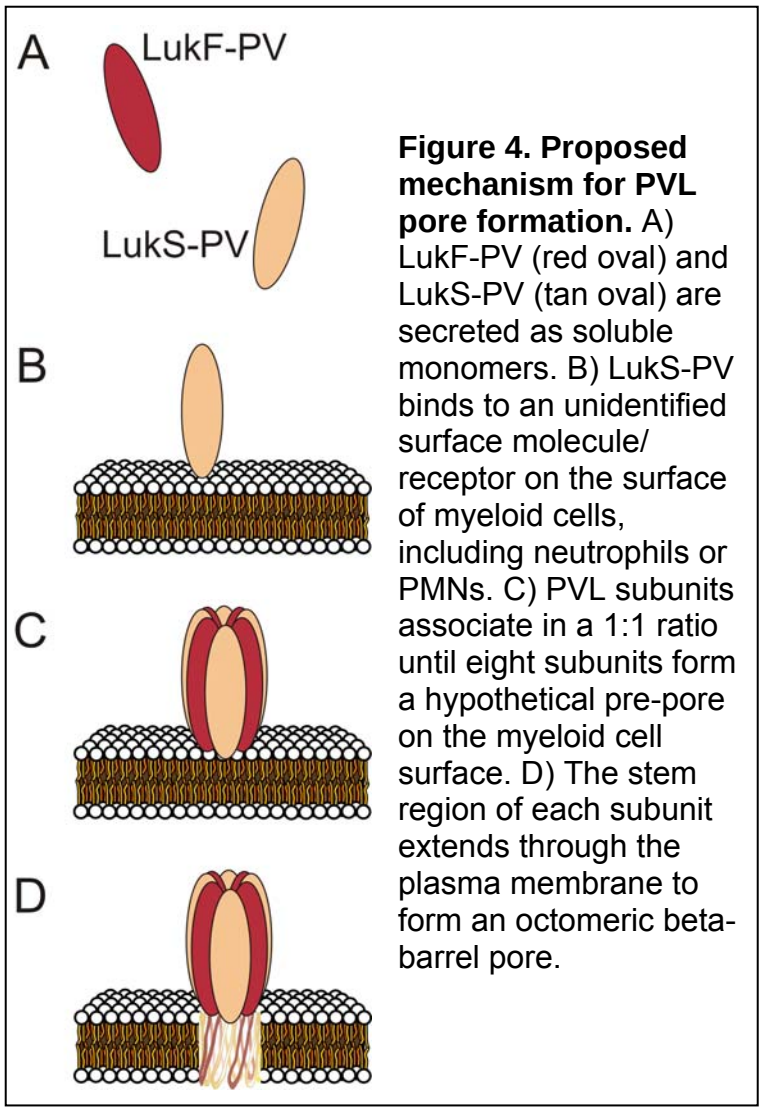

Figure 4. Proposed mechanism for PVL pore formation. A) LukF-PV (red oval) and LukS-PV (tan oval) are secreted as soluble monomers. B) LukS-PV binds to an unidentified surface molecule/ receptor on the surface of myeloid cells, including neutrophils or PMNs. C) PVL subunits associate in a 1:1 ratio until eight subunits form a hypothetical pre-pore on the myeloid cell surface. D) The stem region of each subunit extends through the plasma membrane to form an octomeric beta-barrel pore.

arginine (R variant) or a histidine (H variant) are segregated into specific geographic regions. USA300 expresses the R variant, and therefore this variant is prominent in the US (97). Studies comparing the interaction of PMNs with R and H variants of PVL determined that this amino acid substitution has no impact on PVL activities that are described in greater detail below (98,99).

1.2.2 Panton-Valentine Leukocidin (PVL) and human neutrophil interaction

The potential for PVL to alter neutrophil function or cause cytolysis is of particular interest because PMNs represent the first line of host defense against *S. aureus* infection (60,61,81). Lytic activity of PVL is concentration-dependent (58,100-102) and a concentration threshold of PVL must be reached for lysis of PMNs to occur. Levels of the toxin below this concentration threshold, estimated at 0.2-6 nM, are sublytic (100). Neutrophils exposed to sublytic levels of purified PVL exhibit numerous cellular responses that require the presence of both PVL subunits. A Ca^{2+} influx that requires extracellular calcium precedes the formation of pores in the plasma membrane (58,59). These two events, Ca^{2+} influx and pore formation, are both mediated by PVL; however, they are induced by distinct cellular mechanisms (103). PMNs exposed to sublytic levels of PVL in the presence of Ca^{2+} release several proinflammatory molecules, such as interleukin-8 (IL-8) and leukotreine B_4 (62,104-106). Transcription of IL-8 is upregulated following incubation of PMNs with sublytic concentrations of PVL (105). These findings support the notion that the presence of Ca^{2+} is essential for PVL-mediated release of proinflammatory molecules from human neutrophils. Further,

Ca^{2+} entry into the host cell requires exposure of neutrophils to both PVL subunits (90,92). PVL also induces granule exocytosis, thereby releasing myeloperoxidase, lysozyme, and beta-glucuronidase into the extracellular space (106,107). Although granule exocytosis is consistent with PMN priming, PVL alone causes limited production of ROS by human neutrophils (106,108). However, neutrophils incubated with PVL and then activated with fMLP released 3-4 times more ROS compared to neutrophils exposed to PVL alone (108). This observation indicates that sublytic levels of PVL prime neutrophils for enhanced activation by a secondary stimulus.

Downstream signal transduction pathways induced or inhibited by the interaction of neutrophils with PVL remain largely uncharacterized, although a G-protein coupled receptor has been previously implicated in the process (109). The finding that PVL oligomers associate with neutrophil lipid rafts coupled with the observation that inhibition of protein tyrosine kinases decreases PVL-mediated IL-8 release, supports the notion that PVL triggers neutrophil signal transduction (105,110).

1.3 Contribution to the field

Although the *lukS/F-PV* operon is present in many strains that cause CA-MRSA infections including the epidemic USA300 clone (39,84), the finding is not universal. In addition, multiple studies indicate that the presence of PVL is not a primary determinant of the clinical outcome of *S. aureus* infections (111-114).

Bae et al. actually found that patients with PVL-positive infections were more likely cured after treatment compared to those with PVL-negative infections (113). These findings highlight the need to re-evaluate the role played by PVL during *S. aureus* infection.

PVL-positive strains express differential amounts of PVL during growth *in vitro* or *in vivo* (115-118). Human abscesses caused by PVL-positive strains were analyzed for presence of PVL and expression of 0.27-2 μg/ml of PVL was detected in 15 samples (117). A subsequent study screened 72 methicillin-sensitive and methicillin-resistant clinical specimens (*lukS/F-PV*-positive) for PVL expression (118). Eighty-six percent of the *lukS/F-PV*-positive isolates (62/72 samples) secreted 0-3 μg/ml of PVL during infection. Thus, it is unlikely that a concentration of PVL necessary for cytolysis is attained *in vivo* during infection, except perhaps at the focus of infection.

The overarching objective of my dissertation research is to better understand the role played by PVL during the interaction of CA-MRSA with the host. One hypothesis is that PVL causes cytolysis of PMNs, thereby enhancing *S. aureus* virulence. As a step toward testing this hypothesis, we evaluated human neutrophil pore formation and cytolysis using USA300 and USA400 culture supernatants from multiple *S. aureus* growth conditions *in vitro* or purified PVL, and determined whether there was a direct correlation between pore formation and subsequent cytolysis (Chapter 2).

The level of PVL produced *in vivo* may not be sufficient to cause lysis of PMNs. However, it is possible that sublytic levels of PVL alter the host response

to infection. For example, animal infection studies with wild-type (WT) and isogenic *lukS/F-PV* deletion strains suggest that PVL increases the innate immune response, and thereby augments clearance of infection. We hypothesize that PVL primes PMNs for enhanced function, thus altering the outcome of CA-MRSA infection. To test this hypothesis, we investigated the ability of sublytic concentrations of PVL to prime human neutrophils for enhanced function (Chapter 3). In addition, we assessed global changes in PMN gene expression and identified proinflammatory molecules released from neutrophils following exposure to a sublytic concentration of PVL (1 nM). These results, in combination with previous studies, suggest PVL functions primarily as an immune modulatory agent rather than a cytolytic toxin.

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CHAPTER TWO

RELATIVE CONTRIBUTION OF PANTON-VALENTINE LEUKOCIDIN TO PMN PLASMA MEMBRANE PERMEABILITY AND LYSIS CAUSED BY USA300 AND USA400 CULTURE SUPERNATANTS

2.1 Abstract

Panton-Valentine leukocidin (PVL) is a cytolytic toxin associated with severe community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections. However, the relative contribution of PVL to host cell lysis during CA-MRSA infection remains unknown. Here we investigated the relative contribution of PVL to human polymorphonuclear leukocyte (PMN) plasma membrane permeability and lysis in vitro by using culture supernatants from wild-type and isogenic *lukS/F-PV*-negative (Δ*pvl*) USA300 and USA400 strains. Using *S. aureus* culture conditions that favor selective high production of PVL (CCY media), there was on average more PMN plasma membrane permeability and cell lysis caused by supernatants derived from wild-type strains compared with those from Δ*pvl* strains. Unexpectedly, plasma membrane permeability did not necessarily correlate with ultimate cell lysis. Moreover, the level of pore formation caused by culture supernatants varied dramatically (e.g., range was 0.32–99.09% for wild-type USA300 supernatants at 30 min) and was not attributable to differences in PMN susceptibility to PVL among human blood donors. We conclude that PMN pore formation assays utilizing *S. aureus* culture supernatants have limited ability to estimate the relative contribution of PVL to pathogenesis (or cytolysis *in vitro* or *in vivo*), especially when assayed using culture media that promotes selective high production of PVL.

2.2 Introduction

Staphylococcus aureus is a significant cause of human infections worldwide. The organism also acquires antibiotic resistance readily and methicillin resistant *S. aureus* (MRSA) are endemic in healthcare settings in many countries (1). Prior to the early 1990s, MRSA infections were almost exclusively associated with healthcare settings and disease occurred in individuals with known risk factors for infection. Although healthcare-associated MRSA (HA-MRSA) remain a major problem, MRSA are a leading cause of community-associated bacterial infections in some industrialized countries, such the United States and Canada (2). These so-called community-associated MRSA (CA-MRSA) infections occur in seemingly healthy individuals with no predisposing risk factors for infection, suggesting that they have enhanced virulence by comparison. Experimental data with animal infection models using CA-MRSA strains provides strong support to this notion (3,4). The molecular basis for the enhanced virulence phenotype of CA-MRSA strains, especially USA300 and USA400, which predominate in North America, is incompletely defined.

A methicillin-resistance element known as staphylococcal cassette chromosome (SCC) *mec* type VI (SCC*mec*IV) and genes encoding Panton-Valentine leukocidin (PVL) (*lukS-PV* and *lukF-PV*) are elements common among many CA-MRSA strains worldwide (2). PVL is a cytolytic toxin composed of LukF-PV and LukS-PV subunits that assemble into an octameric pore on the surface of myeloid cells, including polymorphonuclear neutrophils (PMNs). Although sublytic concentrations of PVL cause PMN apoptosis (5), sufficient pore

formation causes a change in the cellular levels of normally impermeable solutes, such as K^+, which can lead to osmotic cell lysis (necrosis) (6,7). The leukocidin is linked by epidemiology to specific types of severe skin infection and severe necrotizing pneumonia (8,9). Inasmuch as PMNs are the primary cellular defense against *S. aureus* infections, molecules such as PVL that have potential to eliminate neutrophils and/or alter neutrophil function might therefore contribute to pathogenesis. USA300 and USA400 strains contain genes encoding multiple pore-forming toxins with high homology or identity to *lukS-PV* and *lukF-PV*. The relative contribution of these molecules to CA-MRSA virulence has not been determined, although this matter has been the subject of intense research over the past several years (10-15). In addition, high expression of PVL in vitro appears optimal only during growth in specific *S. aureus* culture media, which may limit the utility of such assays in predicting activity in vivo.

As a step toward understanding the relative contribution of PVL to lysis of PMNs caused by USA300 and USA400 strains, we evaluated human PMN plasma membrane permeability and lysis using culture supernatants from multiple *S. aureus* growth conditions in vitro.

2.3 Materials and Methods

2.3.1 Bacterial strains and culture.

USA300 (LAC and SF8300) and USA400 (MW2) wild-type and isogenic *lukS-PV* and *lukF-PV* deletion strains (LAC∆*pvl*, SF8300∆*pvl*, and MW2∆*pvl*) were described previously (11,12). Bacteria from frozen, low passage stocks

were cultured overnight in trypticase soy broth (TSB, Difco, Detroit Michigan), CCY medium (3% wt/vol yeast extract, 2% Bacto-Casamino acids, 2.3% sodium pyruvate, 0.63% Na_2HPO_4, and 0.041% KH_2PO_4, pH 6.7), or 100% pooled human serum. Overnight cultures were either used to generate supernatants directly or diluted 1:200 into fresh culture media and incubated for 8 h (to early stationary phase of growth, OD_{600} = 0.75) with shaking at 225 rpm at 37°C. Bacteria were removed from the culture media by centrifugation (2061 g for 10 min at 4°C). Culture supernatants were sterilized by filtration and stored in aliquots at -80°C for future use.

2.3.2 Purification of PVL subunits from USA300 culture medium.

LukS-PV and LukF-PV subunits were purified from culture supernatants of USA300 strain LAC containing deletion of *hlgA*, *hlgB*, and *hlgC* (LACΔ*hlgABC*) as described previously (16,17), but with a few modifications. Briefly, LACΔ*hlgABC* was cultured to early stationary phase of growth in CCY medium and cultures were centrifuged to remove bacteria. Following sterile filtration, supernatant proteins were precipitated with ammonium sulfate (80% saturation) at 4°C for 16 h. Precipitates were centrifuged at 15000 g for 20 min at 4°C and resuspended in Buffer 1 (30 mM sodium phosphate buffer, pH 6.5). Proteins were dialyzed against Buffer 1 for 5 h, subjected to ion-exchange chromatography using a HiPrep 16/10 CM FF sepharose column (GE Healthcare Life Sciences, Piscataway, New Jersey), and eluted with a linear gradient of 0 to 0.5 M NaCl in Buffer 1. Fractions containing LukS-PV were subjected to a

second round of ion-exchange chromatography using a Mono S 5/50 GL column (GE Healthcare Life Sciences) and LukS-PV was eluted with a linear gradient of 0 to 0.25 M NaCl in Buffer 1. Ammonium sulfate was added to LukF-PV and LukS-PV fractions to 1.5 M and these samples were subjected to hydrophobic interaction chromatography using a HiTrap Butyl HP column (GE Healthcare Life Sciences). PVL subunits were eluted with a linear gradient of 1.5 to 0 M ammonium sulfate and aliquots of each subunit were stored at -80°C in 0.2 M NaCl-Buffer 1. Identity and purity of LukS-PV and LukF-PV were evaluated initially by SDS-PAGE and immunoblot analysis, and then by liquid chromatography tandem mass spectrometry (LC-MS/MS) at the NIAID Mass Spectrometry Unit, Bethesda, Maryland.

2.3.3 Human PMN assays.

PMNs were isolated from venous whole blood of healthy individuals using a published method (18) in accordance with a protocol approved by the NIAID Institutional Review Board for Human Subjects. Each human subject included in the study gave informed consent. Lysis of PMNs was assessed by the release of lactate dehydrogenase (LDH) using a Cytotoxicity Detection Kit (Roche Applied Sciences, Pleasanton, California) as described previously (3,12). Culture supernatants were thawed on ice and diluted in RPMI 1640 medium (Invitrogen) buffered with 10 mmol/l HEPES (RPMI/H, pH 7.2). PMNs (1×10^6) in 100 µl RPMI/H were combined with 100 µl of diluted supernatants in 96-well round-bottom plates. Cells were incubated for the indicated times (3-18 h) at 37°C with

5% CO_2. At designated time points, plates were centrifuged at 587 g for 7 min at 4°C. Aliquots (100 µl) from each well were transferred to a 96-well flat-bottom plate and percent LDH release was determined according to the manufacturer's instructions.

PMN plasma membrane permeability (formation of plasma membrane pores) was measured by ethidium bromide (EtBr) uptake as described essentially by Gauduchon et al. (19). Culture supernatants were diluted in RPMI/H as described above and mixed 1:1 with human PMNs (1×10^6) and 4 µmol of EtBr as described (19). At designated time points, PMNs were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, California).

In some experiments, PMNs were incubated with 1 nM purified PVL (LukS-PV + LukF-PV) or individual PVL subunits and EtBr and LDH release were determined as described above.

2.3.4 SDS-PAGE and LukS/F-PV Western Blots.

Proteins present in CCY and TSB culture supernatants were resolved by 12.5% SDS-PAGE and transferred to nitrocellulose membranes using an iBlot Dry Blotting System (Invitrogen, Carlsbad, California). Nitrocellulose membranes were blocked in Tris-buffered saline containing 10% goat serum and 1% Tween 20 overnight. Immunoblots were rotated for 1 h at ambient temperature in diluted blocking buffer containing affinity-purified rabbit IgG specific for a peptide region of LukF-PV or LukS-PV (GenScript USA Inc., Piscataway, New Jersey) or rabbit IgG specific for Hla (Sigma-Aldrich, St. Louis, Missouri). Membranes were

washed three times for 10 min each in wash buffer (250 mM NaCl, 10 mM HEPES, 0.2% Tween-20, pH 7.4) and then incubated with peroxidase-conjugated anti-rabbit donkey IgG secondary antibody for 1 h. Membranes were washed twice in wash buffer and once in Tris-buffered saline and LukF-PV and LukS-PV were visualized using enhanced chemiluminescence (SuperSignal West Pico, Fisher Scientific, Pittsburg, Pennsylvania). LukS-PV was quantified by densitometry with a standard curve of purified LukS-PV using Quantity One Software (Bio-Rad Laboratories, Hercules, California). There was 0.8 μg/ml, 3.8 μg/ml, and 3.6 μg/ml LukS-PV produced by MW2, LAC, and SF8300 in TSB at early stationary phase of growth, and 0.9 μg/ml, 2.0 μg/ml, and 1.2 μg/ml for these strains after overnight growth in the same media. By comparison, there was 30.6 μg/ml, 100.4 μg/ml, and 101.4 μg/ml LukS-PV produced by MW2, LAC, and SF8300 in CCY at early stationary phase of growth, and 31.8 μg/ml, and 32.8 μg/ml for LAC and SF8300 after overnight growth in CCY. PVL subunits were frequently not detectable in MW2 culture supernatants after overnight growth in CCY.

2.3.5 *S. aureus* genomic DNA extraction.

USA300 strain LAC was cultured in TSB to early stationary phase of growth and then bacteria were pelleted by centrifugation as described above. Bacteria were resuspended in 450 μl of P1 buffer (Plasmid Prep Kit, Qiagen, Inc., Valencia, California), to which 50 μl of 1 mg/ml lysostaphin was added. Samples were incubated for 3 h at 37°C to complete lysis. After lysis of bacteria, DNA

was isolated using the DNeasy Tissue Kit (Qiagen) and as recommended by the manufacturer.

2.3.6 RNA extraction and TaqMan Real-Time RT-PCR analysis.

S. aureus strains were cultured as indicated and lysed using FastPrep (FP 120, MP Biomedicals, Solon, Ohio). RNA isolation was completed using the RNeasy Mini Prep Kit (Qiagen) as described previously (20). Each strain and growth condition was assayed in triplicate by TaqMan real-time RT-PCR analysis using an ABI 7500 thermocycler (Applied Biosystems Inc., Foster city, California). Change in expression of target genes was determined by comparison to known quantities of *S. aureus* genomic DNA and relative expression of the housekeeping gene *gyrB*. The primer-probe sequences are as follows: *gyrB* forward primer 5'-CAAATGATCACAGCATTTGGTACAC-3', *gyrB* probe 5'-AATC GGTGGCGACTTTGATCTAGCGAAAG-3', *gyrB* reverse primer 5'-CGGCATCAG TCATAATGACGAT-3', *LukF-PV* forward primer 5'-TTGCTTTTGCTATCCAATAC AGTTG-3', *LukF-PV* probe 5'-TGCAGCTCAACATATCACACCTGTAAGT-3', *LukF-PV* reverse primer 5'-TCGGAATCTGATGTTGCAGTTG-3', *LukS-PV* forward primer 5'-AATAACGTATGGCAGAAATATGGATGT- 3', *LukS-PV* probe 5'-ACTCATGCTACTAGAAGAACAACACACTATGG-3', *LukS-PV* reverse primer 5'-CAAATGC GTTGTGTATTCTAGATCCT-3'.

2.3.7 Statistical analyses.

Data were evaluated using a paired Student's t-test (GraphPad Prism 5, GraphPad Software, Inc., San Diego, California). PMN lysis data in Fig. 7 from wild-type CCY culture supernatants were also compared using a one-way analysis of variance (ANOVA) and Tukey's posttest.

2.4 Results

2.4.1 Membrane pore formation caused by *S. aureus* culture supernatants is highly variable.

To estimate the relative contribution of PVL to formation of membrane pores in leukocytes, we evaluated the ability of culture supernatants from USA300 and USA400 wild-type and Δ*pvl* strains to promote uptake of ethidium bromide (EtBr) by human PMNs (Fig. 5 and 6). EtBr uptake has been used widely to estimate PVL-mediated membrane pore formation with human neutrophils, as the diameter of the pores (2–2.4 nm or 20-24 angstroms) allow free diffusion of EtBr (0.8 nm or 8 angstroms) into cells (21-26). We first tested pore-forming capacity of CCY culture supernatants, since *S. aureus* can produce up to 100 milligrams of PVL per liter of CCY media (16,27,28). There was time-dependent formation of PMN membrane pores with all growth conditions and supernatant concentrations (dilutions) tested (Fig. 5 and 6). On average, there was significantly more uptake of EtBr by PMNs exposed to wild-type culture supernatants compared with those from Δ*pvl* strains (Fig. 5 and 6). However, in many of the individual assays, especially those in which PMNs were exposed to culture supernatants for 30 min, the level of pore formation was comparable

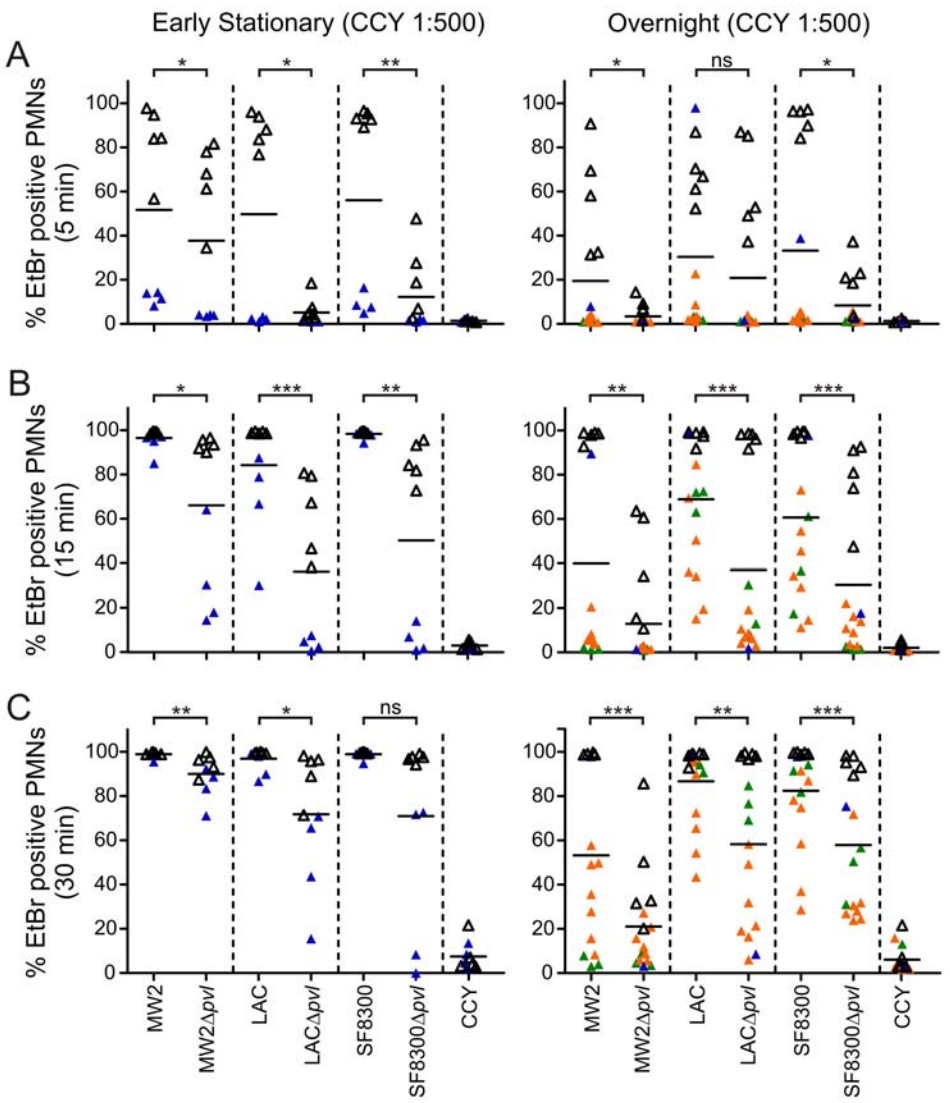

Figure 5. Permeability of PMNs (plasma membrane pore formation) exposed to USA300 and USA400 CCY culture supernatants. PMNs (1×10^6) were incubated with a 1:500 dilution of CCY media alone (CCY) or CCY culture supernatants obtained from growth of *S. aureus* strains as indicated. Each symbol indicates a separate experiment and/or human PMN donor. Different batches of CCY media are indicated by symbol color or fill. Black bars indicate the mean percent ethidium bromide (EtBr) uptake by PMNs at each time point, A) 5 min. B) 15 min. C) 30 min. *: $p \leq 0.05$ vs. wild-type (WT; i.e., MW2, LAC, or SF8300); **: $p \leq 0.01$ vs. WT; ***: $p \leq 0.001$ vs. WT.

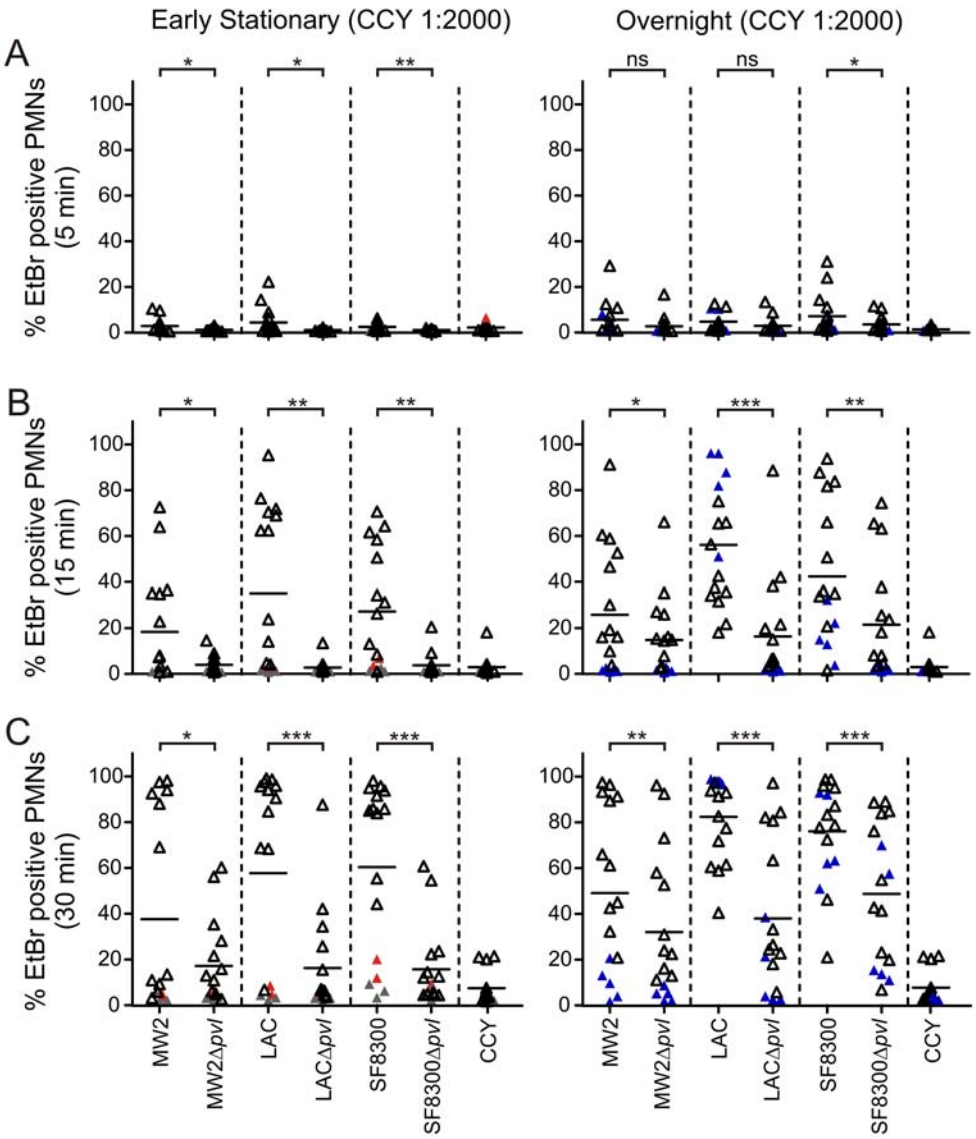

Figure 6. Permeability of PMNs exposed to USA300 and USA400 CCY culture supernatants. PMNs (1×10^6) were incubated with a 1:2000 dilution of CCY media alone (CCY) or CCY culture supernatants obtained from growth of *S. aureus* strains as indicated. Each symbol indicates a separate experiment and/or human PMN donor. Different batches of CCY media are indicated by symbol color or fill. Black bars indicate the mean percent ethidium bromide (EtBr) uptake by PMNs at each time point, A) 5 min. B) 15 min. C) 30 min. *: $p \leq 0.05$ vs. wild-type (WT; i.e., MW2, LAC, or SF8300); **: $p \leq 0.01$ vs. WT; ***: $p \leq 0.001$ vs. WT.

between wild-type and Δ*pvl* strains (Fig. 5B and C, and Fig. 6C). We found that culture supernatants from bacteria grown in trypticase soy broth (TSB), a standard culture medium for *S. aureus*, had little or no PMN pore-forming capacity at the highest concentration used for CCY (1:500 dilution) (data not shown). Membrane pore formation was highly varied using CCY culture supernatants (e.g., the range of pore formation was 0.6–99.9% for the MW2 wild-type strain at 30 min using a 1:500 dilution) (Fig. 5C). Although there was variation in PMN pore formation among individuals using the same batch of culture supernatant, some of the observed variation overall was due to differences among separate batches CCY media (Fig. 5 and 6, symbol colors and fills).

2.4.2 Correlation of membrane pore formation and PMN lysis.

Formation of plasma membrane pores by PVL and other two-component toxins of *S.aureus* is generally considered to result in host cell lysis. To test this notion, we evaluated the ability of culture supernatants from USA300 and USA400 wild-type and Δ*pvl* strains to cause release of lactate dehydrogenase (cell lysis) from human PMNs over time (Fig. 7 and 8). PMN lysis caused by exposure to CCY or TSB culture supernatants from USA300 and USA400 strains was time and concentration dependent (Fig. 7 and 8). In addition, there was significantly more lysis of PMNs exposed to wild-type CCY culture supernatants compared with those from Δ*pvl* strains at the highest concentrations tested (1:250 and 1:500 dilutions) (Fig. 7A and B). In contrast, there was no difference

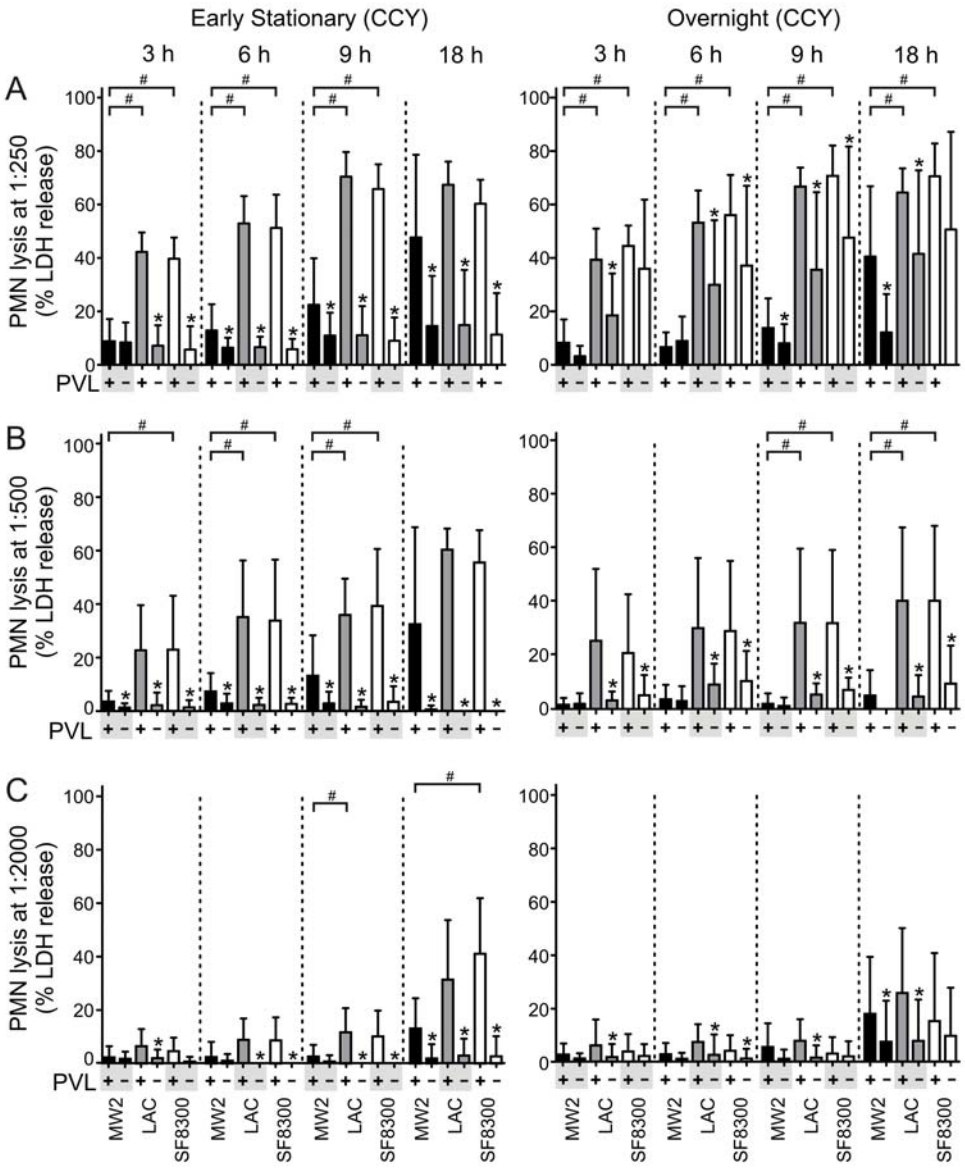

Figure 7. PMN lysis after exposure to USA300 or USA400 CCY culture supernatants. PMNs ($1x10^6$) were incubated for 3, 6, 9, or 18 h with the indicated dilution of culture supernatant. Cell lysis was measured by LDH release as described in Materials and methods. Bars indicate mean ± SD of 8-11 PMN donors; *p ≤ 0.05 vs. WT; #p ≤ 0.05 using a one-way analysis of variance (ANOVA) with a Tukey's posttest. A) 1:250 dilution. B) 1:500 dilution. C) 1:2000 dilution. PVL ''+'' indicates wild-type *lukS/F-PV* positive strain; PVL ''-'' indicates isogenic *lukS/F-PV* negative strain. CCY alone caused no significant lysis of human PMNs over the 18 h culture period (range was 0-1.5% for 3-18 h, n = 4 PMN donors).

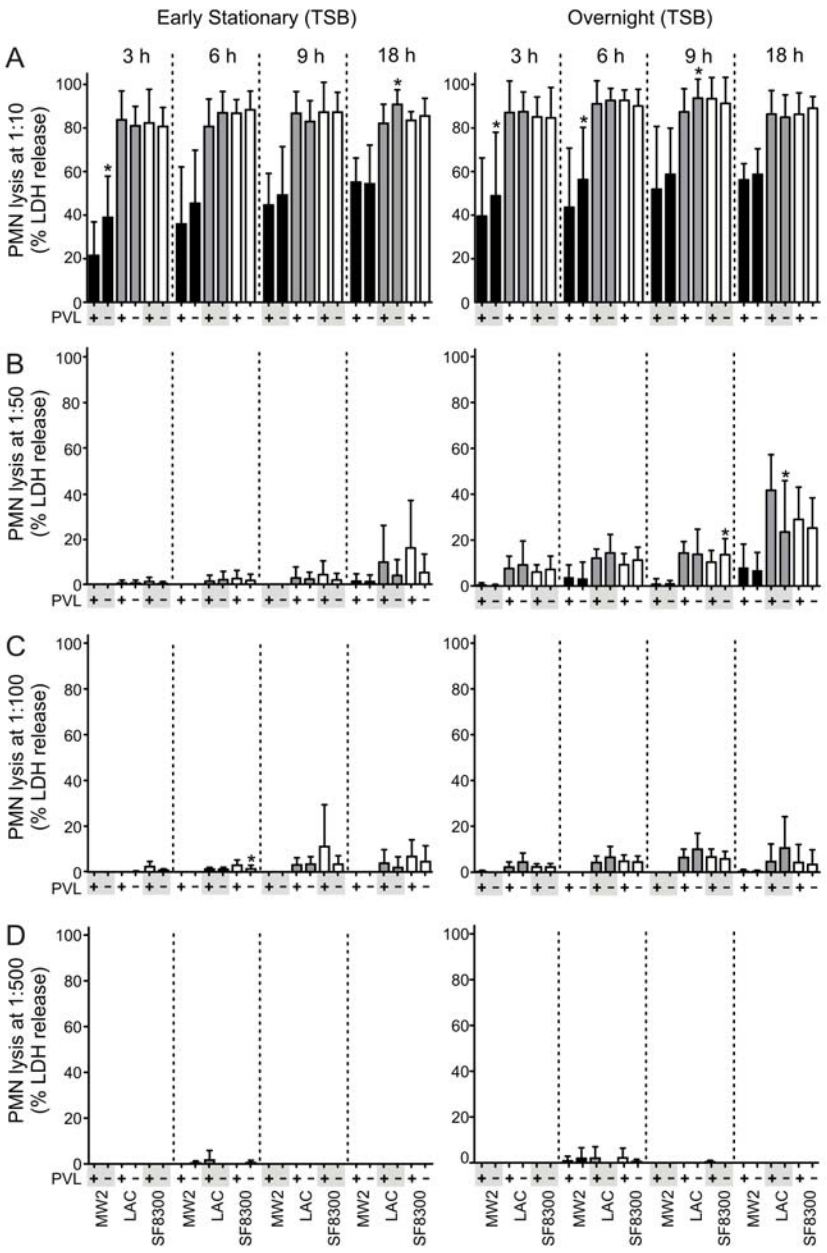

Figure 8. PMN lysis after exposure to USA300 or USA400 TSB culture supernatants. PMNs ($1x10^6$) were incubated for 3, 6, 9, or 18 h with the indicated dilution of culture supernatant. Cell lysis was measured by LDH release as described in Materials and methods. Bars indicate mean ± SD of 6 PMN donors for panels A-C and 4-6 PMN donors for panel D; *p ≤ 0.05 vs. WT. A) 1:10 dilution. B) 1:50 dilution. C) 1:100 dilution. D) 1:500 dilution. PVL ‘‘+’’ indicates wild-type *lukS/F-PV*-positive strain; PVL ‘‘-’’ indicates isogenic *lukS/F-PV* negative strain. TSB alone caused no significant lysis of human PMNs over the 18 h culture period (range was 0-0% for 3-18 h, n = 4 PMN donors).

in PMN lysis between TSB culture supernatants from wild-type and Δ*pvl* strains (e.g., PMN lysis was 87.1 ± 14.5 and 87.5 ± 9.1% after a 3-h exposure to LAC wild-type and Δ*pvl* TSB supernatants from overnight culture (1:10 dilution) (Fig. 8).

Although there was some concordance between pore formation and cell lysis assays, especially with CCY culture supernatants from wild-type strains, there were noted differences. First, at the lowest concentration of CCY culture supernatant used (1:2000 dilution), there was little or no correlation between pore formation and PMN lysis (compare Fig. 6C and 7C). Further, PMN pore formation was at or near 100% after 30 min of exposure to the highest concentration of CCY culture supernatants from Δ*pvl* strains (Fig. 4C), whereas the corresponding cell lysis was < 10% at all time points tested (up to 18 h) (Fig. 7B). We also note that culture supernatants from the MW2 wild-type strain often caused significantly less lysis than those from USA300 wild-type strains (Fig. 7). Collectively, the data indicate that pore formation caused by *S. aureus* culture supernatants does not necessarily correlate with (or result in) cell lysis.

Inasmuch as in vitro culture media (CCY and TSB) are not likely representative of culture conditions during infection in humans, we tested the ability of normal human serum to promote production and activity of *S. aureus* cytolytic toxins. In contrast to CCY and TSB, human serum used as culture media for USA300 and USA400 strains had zero capacity to cause PMN lysis at all concentrations tested (1:1–1:100 dilutions). This finding cannot be explained by the absence of bacterial growth in serum, since *S. aureus* grew reasonably

well in this culture substrate (Fig. 10H). Rather, the lack of PMN cytolysis in serum culture supernatants may be due to the ability of serum lipoproteins and apolipoprotein B to inhibit *agr* signaling (29), a regulator of expression of multiple virulence factors, and thus, synthesis and secretion of PVL.

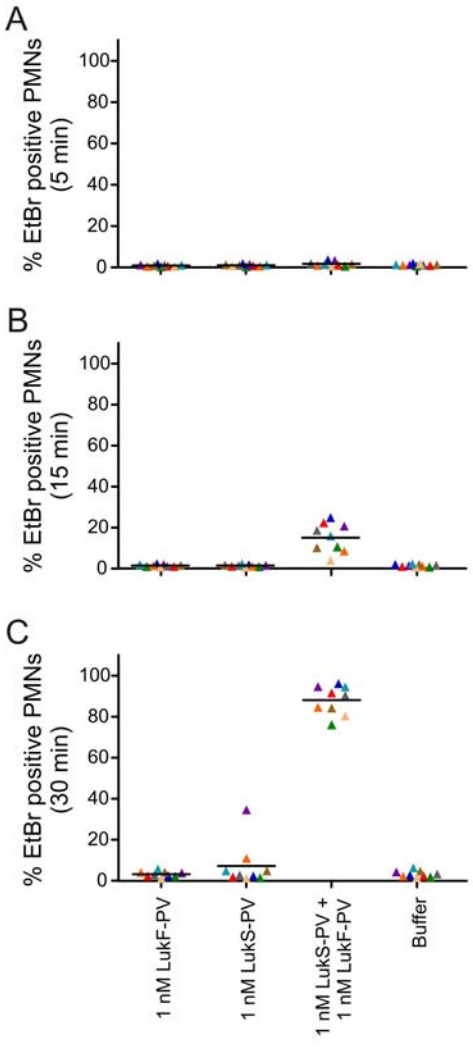

Figure 9. Purified PVL causes relatively consistent levels of plasma membrane pore formation in human PMNs. A-C) PMNs ($1x10^6$) were incubated with 1 nM of purified PVL subunits (LukF-PV or LukS-PV) or the combination of both subunits for the indicated times. Each symbol color indicates a separate human PMN donor (n = 9). Black bars indicate the mean percent ethidium bromide (EtBr) uptake by PMNs at each time point. D) PMNs ($1x10^6$) were incubated with 1 nM of purified PVL subunits (LukF-PV or LukS-PV) and PMN lysis was measured by release of LDH as described in Materials and methods. Red bar indicates mean ± SD of 4 PMN donors.

2.4.3 Cytolytic effects of purified PVL and human blood donor variability.

We next used PVL subunits purified from USA300 to determine whether the variation in PMN pore formation noted in the assays with culture supernatant was due to individual susceptibility to PVL (Fig. 9). Neither subunit alone caused

formation of membrane pores in human PMNs. By comparison, there was time-dependent uptake of EtBr using the combination of 1 nM LukF-PV+LukS-PV (Fig. 9). There was far less variation in the capacity of purified PVL to permeabilize human PMNs compared with culture supernatants (EtBr uptake was 76.1–96.1% at 30 min using purified PVL subunits) (Fig. 9C). Despite the high level of pore formation, there was essentially no corresponding PMN lysis at this time point (LDH release was 0.7 ± 0.7% at 30 min, n = 4 PMN donors) (Fig. 9D). These data indicate that the variation noted in assays with culture supernatants is largely independent of differences in PMN donor susceptibility to PVL.

2.4.4 Levels of *lukS-PV* and *lukF-PV* transcript and corresponding PVL protein subunits are highly varied depending on in vitro growth conditions.

We next compared levels of *lukS-PV* and *lukF-PV* (*lukS/F-PV*) transcripts and corresponding protein subunits following culture of USA300 and USA400 strains in CCY, TSB, or human serum (Fig. 10A–H). Compared with strains cultured in TSB or human serum, there was more *lukS/F-PV* transcript made by LAC and MW2 following culture in CCY (Fig. 10A–C). In accordance with these findings, more LukS-PV and LukF-PV accumulated in CCY culture media compared with that in TSB (Fig. 10D–F). Most notably, there was selective high production of PVL in CCY media, since the level of alpha-hemolysin (Hla, another agr-regulated cytolytic toxin) present in CCY was not increased relative to that in TSB culture supernatants (Fig. 10F). Using a purified PVL standard, we estimate that USA300 strains accumulated ~3–4 μg/ml of LukS-PV in TSB

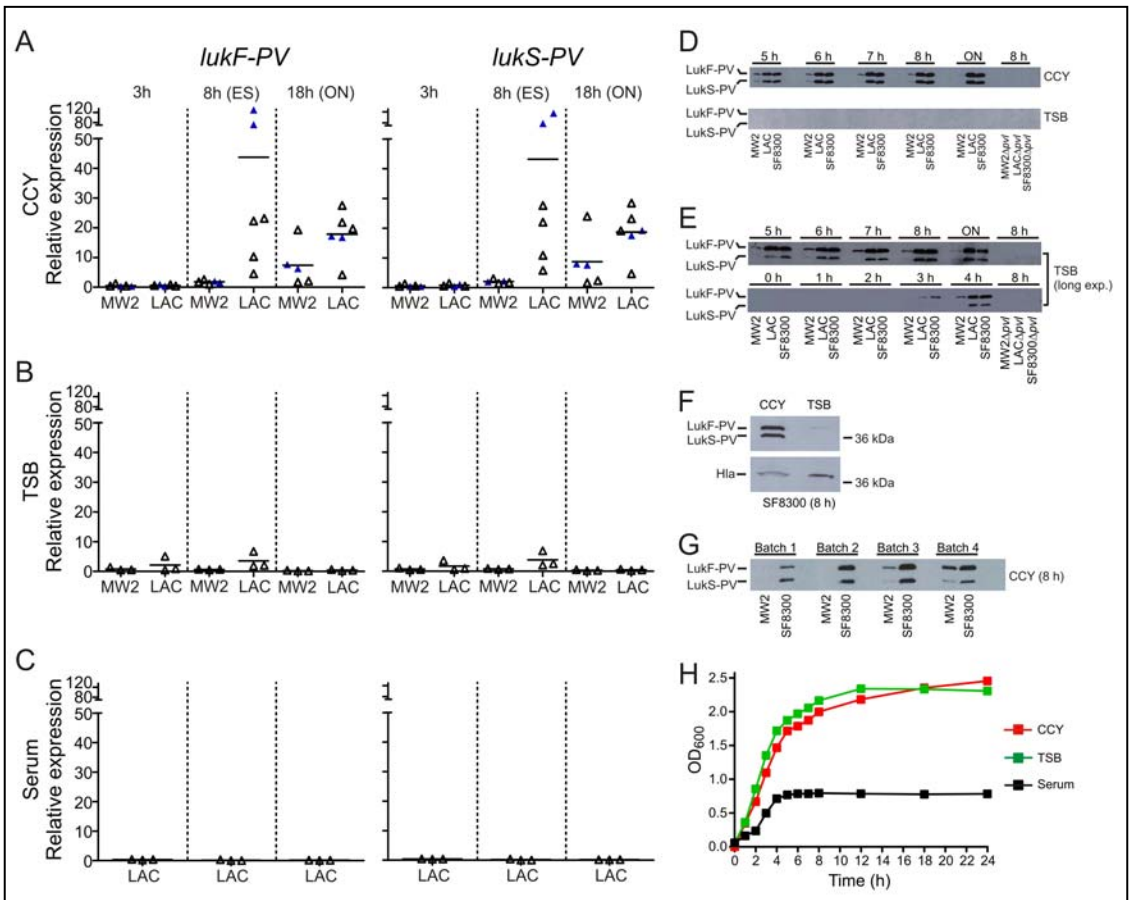

Figure 10. Impact of culture media on *lukS-PV* and *lukF-PV* transcript levels and protein subunit secretion. A-C) Relative expression of *lukS-PV* and *lukF-PV* transcripts during bacterial growth as indicated. Different colors or fill indicate separate batches of CCY. Symbols are the mean of triplicate TaqMan samples. Black bars indicate mean of each strain group. A) Growth in CCY. B) Growth in TSB. C) Growth in normal human serum. D) PVL secretion during growth in CCY (upper panel) or TSB (lower panel). E) Longer exposure of the immunoblot shown in the lower panel of D. Results in panels D and E are representative of 3 experiments. F) Immunoblot of PVL subunits (1.75 µl of CCY or TSB culture supernatants) and Hla (35 µl of CCY or TSB culture supernatants) produced in CCY or TSB culture at early stationary phase of growth. Results are representative of 2 experiments. G) Immunoblot of PVL subunits secreted during MW2 (USA400) and SF8300 (USA300) growth in different batches of CCY. H) Growth of LAC (USA300) in CCY, TSB, and normal human serum.

and ~100 μg/ml in CCY at early stationary phase of growth (see Materials and Methods for details). The finding that there was little or no detectable *lukS/F-PV* transcript made by LAC cultured in human serum (Fig. 10C) is consistent with the observation that supernatants from neither USA300 nor USA400 strains cultured in human serum caused PMN lysis (data not shown) and that serum lipoproteins inhibit *agr* signal transduction (29).

There were also strain-dependent differences in *lukS/F-PV* transcript and PVL protein levels. For example, compared with LAC, there was less *lukS/F-PV* transcript made by MW2 in either CCY or TSB, and there was correspondingly less accumulated PVL protein in MW2 culture supernatants (e.g., there was ~42-fold more *lukF-PV* transcript made by LAC at early stationary phase of growth in CCY compared with MW2) (Fig. 10). These findings are compatible with differences noted between USA300 and USA400 strains in recent studies by Montgomery et al. (30). Although differences in *lukS/F-PV* transcript and PVL protein levels may account in part for the differences in PMN lysis observed between MW2 and USA300 CCY culture supernatants (Fig. 7), there is limited correlation of transcript and protein levels with pore-forming capacity of the strains, which was comparable (Fig. 5 and 6). Taken together, these data provide strong support to the notion that factors present in culture supernatants other than PVL are sufficient to cause formation of membrane pores in human PMNs.

2.5 Discussion

It has long been known that PVL is cytolytic for myeloid cells and therefore, a putative virulence molecule of *S. aureus* (31-34). Finck-Barbançon et al. provided direct evidence that PVL is a pore-forming toxin and reported that membrane pore size is dictated by ionic conditions of the extracellular environment (21). Notably, these studies set a precedent for using an ethidium bromide uptake assay to evaluate membrane permeability following exposure to PVL (21). Plasma membrane pores that allow ethidium bromide uptake by PMNs are in general considered to result ultimately in cell lysis through osmotic imbalance (6,7,21,25). The assay or modifications thereof have since been used to evaluate precise kinetics of pore formation caused by purified or recombinant PVL (19,23), pore-forming capacity of heterologous combinations of LukS and LukF proteins (PVL, gamma-hemolysin, and LukD and LukE subunits) (24), the ability of intravenous immunoglobulin or specific anti-PVL antibody to block pore formation (19), and to estimate the relative contribution of PVL to cytolytic capacity of *S. aureus* culture supernatants (12,19,35).

We reported previously that PMNs exposed to supernatants from Δ*pvl* strains cultured overnight in YCP media, a culture media similar in composition to CCY, promoted uptake of EtBr at levels comparable to the wild-type strains (12). Although these findings were unexpected, especially given the high level of PVL produced in either YCP or CCY media (up to 20% of the protein in culture filtrates (27)), they are perhaps explained by the high variation reported here as intrinsic to the pore formation assay. Some of the variation is due to differential levels of *lukS/F-PV* transcript and PVL protein levels produced in separate batches of the

same media (Fig. 10A and G). Most importantly, there is not a direct correlation between EtBr uptake and LDH release by human PMNs, which is a widely accepted determination of cell lysis.

There is clearly more PMN lysis caused by CCY supernatants from wild-type USA300 strains compared to that from Δ*pvl* mutant strains (not observed with MW2 wild-type and Δ*pvl* strains), but this difference is presumably due to the very high concentration of PVL in CCY culture supernatants. Therefore, diluting CCY culture media to obtain PVL-specific cytolysis is in essence similar to using purified PVL, since such an approach decreases the level of other *S. aureus* molecules that would have otherwise contributed to pore formation and/or lysis. This notion is consistent with the observation that there are limited differences in PMN pore formation between wild-type and Δ*pvl* strains using lower dilutions of CCY culture supernatant (Fig. 5C), and the finding that PVL is selectively overproduced in CCY media relative to Hla (Fig. 10F). Furthermore, the concentration of PVL produced in TSB (3-4 μg/ml), which is more representative of that found in human abscesses (0.3–1.8 μg/ml) (36), is perhaps a better gauge of the relative contribution of PVL to PMN lysis caused by *S. aureus*. In any case, factors present in TSB culture supernatants other than PVL, such as alpha-type phenol-soluble modulins (37) and/or gamma-hemolysin (17), were sufficient to cause lysis of human PMNs (Fig. 8).

We conclude that the PMN pore formation assays described herein, although appropriate to evaluate effects of purified PVL or estimate whether cytolytic

capacity exists in culture supernatant, have limited ability to estimate the relative contribution of PVL to membrane pore formation or PMN lysis *in vivo*.

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CHAPTER THREE

SUBLYTIC CONCENTRATIONS OF *STAPHYLOCOCCUS AUREUS* PANTON-VALENTINE LEUKOCIDIN (PVL) ALTER HUMAN NEUTROPHIL GENE EXPRESSION AND FUNCTION

3.1 Abstract

Community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) infections are often caused by strains encoding Panton-Valentine leukocidin (PVL). PVL causes lysis of neutrophils and other myeloid cells *in vitro*, a function considered widely as the primary means by which PVL might contribute to disease. However, at sublytic concentrations PVL can function as a neutrophil agonist. To better understand this phenomenon, we investigated the ability of PVL at sublytic concentrations (1 nM) to alter human neutrophil function and survival. PVL-primed neutrophils for enhanced release of superoxide following activation by fMLP, caused upregulation of CD11b, and accelerated apoptosis. Priming of neutrophils with other agonists such as IL-8 or GM-CSF altered the ability PVL to cause formation of pores in the plasma membrane. Microarray analysis revealed significant changes in the human PMN transcriptome following exposure to PVL, including upregulation of *STAT3*, *SOCS3*, *CXCL1*, and *CXCL2*. Consistent with microarray data myeloperoxidase, IL-8, IL-6, VEGF, TNFR2, and IL-1RA were released from neutrophils after stimulation with PVL. We conclude that exposure of human PMNs to sublytic concentrations of PVL elicits a proinflammatory response that is regulated in part at the level of gene expression. We propose that PVL-mediated priming of neutrophils enhances the host innate immune response.

3.2 Introduction

Staphylococcus aureus is a Gram-positive bacterium that causes a significant number of infections worldwide (1). Methicillin-resistant *S. aureus* (MRSA) is currently a leading contributor to hospital-acquired (HA-MRSA) infections (2,3). MRSA infections that occur outside of hospital settings, known as community-associated MRSA (CA-MRSA) infections, were reported in the early 1990s and became epidemic in the United States and Canada (4-6). In contrast to HA-MRSA, CA-MRSA causes infections in individuals with no known risk factors for infection. The ability of these strains to cause disease in otherwise healthy individuals suggests that they have enhanced virulence compared to traditional HA-MRSA strains. *In vitro* and *in vivo* work supports this hypothesis (7-9). Although progress has been made, the molecular basis of the enhanced virulence phenotype of CA-MRSA remains incompletely determined.

Genes encoding Panton-Valentine leukocidin (PVL) are present in the genome of many CA-MRSA strains, including the epidemic USA300 strain (10-12). PVL consists of two subunits, LukS-PV and LukF-PV, the genes for which are transcribed as an operon. It is a cytolytic toxin specific for myeloid cells, including polymorphonuclear leukocytes (PMNs or neutrophils). The presence of both subunits is required for formation of pores within the PMN plasma membrane (13,14). Neutrophils are the most prominent cellular component of the innate immune system and thus the primary defense against *S. aureus* infections (15). Therefore, it is possible that PVL contributes to virulence by causing lysis of PMNs and other myeloid cells. However, previous studies have shown that

cytolysis *in vitro* requires a concentration of PVL that may not be achieved *in vivo* (16,17). Sublytic concentrations of PVL elicit numerous cellular responses, including release of myeloperoxidase (MPO) and chemotactic molecules such as interleukin-8 and leukotriene B_4 (18-21). PMNs exposed to PVL undergo granule exocytosis and produce reactive oxygen species (ROS) following stimulation with fMLP (22). These observations suggest that sublytic levels of PVL prime neutrophils for enhanced activation by a secondary stimulus. The molecular basis for PVL-mediated PMN priming remains unknown.

PMNs primed with cytokines or proinflammatory molecules produced by bacteria have prolonged survival compared to unprimed cells (23-25). By comparison, sublytic concentrations of PVL accelerate neutrophil apoptosis (26). Therefore, the observation that PVL primes PMNs for enhanced function and induces rather than delays apoptosis differentiates PVL-mediated priming from that induced by other molecules.

To elucidate the molecular basis of PVL-mediated PMN priming, we measured PMN gene expression following exposure of these cells to sublytic concentrations of PVL. In addition, we identified proinflammatory molecules secreted by PMNs following exposure to the leukotoxin. Our results provide new insight into the role played by PVL during human infection.

3.3 Materials and Methods

3.3.1 Heat-killed USA300 strain LAC.

USA300 strain LAC was cultured overnight in trypticase soy broth (TSB) (Difco, Detroit, MI) from frozen bacterial stocks. Overnight cultures were diluted 1:200 in fresh TSB media and cultured to mid-logarithmic growth phase ($O.D._{600}$ = 0.75) as described (17). Bacteria were centrifuged at 3000 rpm for 10 min, washed once with PBS, and centrifuged again to pellet bacteria. The bacterial pellet was resuspended in RPMI 1640 meida buffered with 10 mM Hepes and boiled at 95 ˚C for 10 min.

3.3.2 Purification of PVL subunits from USA300 culture supernatant.

PVL subunits (LukF-PV and LukS-PV) were purified from culture supernatants of a USA300 *hlgABC* deletion strain (LAC∆*hlgABC*) as described previously (17). Purified LukF-PV and LukS-PV were aliquoted separately and stored at -80˚C in 0.2 M NaCl-Buffer 1 (30 mM sodium phosphate buffer, pH 6.5).

3.3.3 Human PMN isolation.

PMNs were isolated from venous whole blood as previously published (27). Each subject gave informed consent prior to participation in the study. Use of healthy individuals for collection of blood was approved by the Institutional Review Board for Human Subjects, NIAID, NIH.

3.3.4 PMN membrane permeability and lysis assays.

Formation of plasma membrane pores was measured by uptake of ethidium bromide (EtBr) as previously described by Gauduchon et al. (28).

Purified PVL subunits (LukF-PV and LukS-PV) were diluted at the desired concentrations in RPMI/H. PVL-mediated pore formation was evaluated by incubating human PMNs (1×10^6) with 4 µmol of EtBr and 1 nM, 2 nM, or 5nM active PVL for 30 min. LukF-PV and LukS-PV were boiled at 95 ˚C for 10 min to produce heat-inactivated PVL (iPVL). iPVL was used as a negative control. EtBr uptake was analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

Human IL-8 and GM-CSF were purchased from eBiosciences (San Diego, CA). N-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA), and lipopolysaccharide (LPS) were purchased from Sigma Aldrich (St. Louis, MO). IL-8, GM-CFS, fMLP, PMA, and LPS were diluted in RPMI/H. PMN agonists, 20 µM IL-8, 100 ng/ml GM-CFS, 1 µM fMLP, 1 µg/ml PMA, 100 ng/ml LPS, or heat-killed LAC (10^7 cfu), were added to human PMNs (1×10^6) suspended in RPMI/H containing 4 µmol of EtBr. Samples were incubated for 30 min at 37 °C. LPS was sonicated at a frequency of 40 kHz in ice water for 15 min before use (Branson 2200, Danbury, CT). The PMNs were then incubated with 1 nM PVL (LukF-PV and LukS-PV) for the indicated times. EtBr uptake was assessed by flow cytometry (FACSCalibur).

PMN lysis was determined by lactate dehydrogenase release (LDH) using the Cytotoxicity Detection Kit (Roche Applied Sciences, Pleasanton, CA) as previously described (7,29). Human PMNs (100 µl, 1×10^6) were combined with 1 nM, 2 nM, or 5 nM active PVL or iPVL in a 96-well plate (Costar, Corning, NY) and incubated at 37 ˚C for 3 h (total 200 µl/well). Plates were centrifuged at 1600

rpm for 7 min at 4 ˚C and 100 µl aliquots of each well were transferred to a new 96-well plate. Percent LDH release was measured and analyzed as instructed by the manufacturer.

3.3.5 PMN proinflammatory assays.

The release of superoxide (O_2^-) was measured as previously described by DeLeo et al. (30) with modifications. PMNs (1 x 10^7/ml) were incubated at 37 ˚C for 30 min with fMLP, LPS, IL-8, GM-CSF, active PVL (LukF-PV and LukS-PV), heat-inactivated PVL (iPVL), or PVL subunits separately at concentrations described above (priming). Each agonist (1 µM fMLP, 20 µM IL-8, 100 ng/ml GM-CSF, or 1 nM PVL final concentration) was aliquoted into wells of a 96-well microtiter plate before the addition of primed PMNs (1 x 10^6). LPS is a known PMN priming agent (31) and was a positive control for PMN priming. Unprimed PMNs were combined with 1 µg/ml PMA as a positive control for release of O_2^-. All wells contained ferricytochrome c (cyt c, Sigma Aldrich) at a final concentration of 100 µM. All assays were performed in triplicate ± 40 µg/ml superoxide dismutase (SOD, Sigma Aldrich). O_2^- production was determined by measuring the SOD-inhibitable reduction of cyt c at 550 nm for 20 min by using a microplate spectrophotometer (Synergy MX, Bio Tek, Winooski, VT) as described (30). Superoxide production was determined using the molar coefficient for cyt c (32) as described.

Surface expression of CD11b was determined after PMNs (1 x 10^6) were exposed to 1 nM PVL for 0, 15, and 30 min at 37 ˚C. After exposure to PVL, cells

were washed twice with Stain Buffer (BD Biosciences) and incubated on ice for 60 min with a 1:6 dilution of phycoerythrin (PE)-conjugated anti-human CD11b primary antibody or PE-labeled mouse IgG1 isotype control (BD Biosciences). PMNs were washed 3 times with Stain Buffer and analyzed by flow cytometry (FACSCalibur).

Identification of molecules released from human PMNs following incubation with PVL was performed by Rules Based Medicine (RBM, Austin, TX). In brief, human PMNs from 4 different blood donors (1×10^7) were incubated ± 1 nM PVL at 37 ˚C for 4 h. Cell suspensions were centrifuged at 1800 rpm for 10 min at 4 ˚C. Supernatants were analyzed by Rules Based Medicine (HumanMAP v. 1.6) as described (http://www.rulesbasedmedicine.com/productsservices/human-maps.aspx).

3.3.6 PMN apoptosis assays.

PMNs were exposed to 1 nM PVL or individual PVL subunits (LukF-PV or LukS-PV) as described above. Apoptosis was determined using published methods (33,34). PMNs (1×10^4) were analyzed using the Cytospin Octospot system (Thermo Scientific Shandon, Waltham, MA) as described by the manufacturer. PMNs were stained with Wright-Giemsa (Sigma Aldrich) and condensed nuclei were visualized by light microscopy (Zeiss, Axioskop 2 plus, Thornwood, NY) at x100 magnification. A total of 250 cells were scored from 5 fields of view for each sample. Images were acquired with an AxioCam digital camera (Zeiss). Alternatively, PMN apoptosis was assessed using a modified

TUNEL assay (Apo-BRDU Apoptosis Detection Kit, BD Biosciences) as described by Kobayashi et al. (27).

3.3.7 PMN microarray analysis.

PMNs (1 x 10^7) in RPMI/H were cultured with 1 nM PVL or iPVL at 37 ˚C for 30, 60, or 180 min. At each indicated time point, PMNs were lysed with RLT buffer and RNA was purified and used to generate ≥ 12 µg of biotin-labeled cRNA target as previously described (27,34). Samples from 3 different PMN donors were analyzed on HU133+2 GeneChips (Affymetrix, Santa Clara, CA). cRNA labeling, GeneChip hybridization, and scanning were completed according to GeneChip manufacturer protocols (http://media.affymetrix.com/support/downloads/manuals/expression_analysis _technical_manual.pdf). Gene chip hybridization and subsequent scanning was performed by the NIAID-RML Genomics Core Unit at Rocky Mountain Laboratories (Hamilton, MT). Vehicle controls were analyzed to determine levels of background signal for each donor at every time point.

Microarray data was normalized using GeneChip Operating Software (GCOS v1.4). At each time point, PMNs cultured with active PVL were compared directly to those treated with iPVL and a principal component analysis (PCA) was performed using Partek Genomics Suite (Partek, Inc., St. Louis, MO). Genes were defined as differentially expressed if they were significantly different from the iPVL control ($p \leq 0.01$, two-way analysis of variance, ANOVA), changed two-fold in expression, and had signal levels above background. The Venn diagram

was generated using the iVenn program (NIAID Genomics Core Unit, RML, Hamilton, MT). Analysis of signal transduction pathways was performed with Ingenuity Pathway Analysis (IPA, Redwood City, CA).

3.3.8 Statistical analysis.

Rules Based Medicine data were evaluated using a Student's t-test (Microsoft Office Excel 2007, Microsoft Corporation, Redmond, WA). PMN apoptosis data in Fig. 11 were assessed using a Student's t-test (GraphPad Prism 5, GraphPad Software, Inc., San Diego, California). PMN pore formation data in Fig. 12 were compared using a one-way analysis of variance (ANOVA) and Dunnett's posttest to correct for multiple comparisons (GraphPad Prism 5).

3.4 Results

3.4.1 Sublytic concentrations of PVL alter human neutrophil function.

We first determined the concentration at which PVL from USA300 strain LAC culture supernatants causes formation of plasma membrane pores but limited cytolysis (Fig. 11A). By 30 min, greater than 90% of PMNs were EtBr-positive at each of the PVL concentrations tested (pore formation was 93%, 98%, and 99% for 1 nM, 2 nM, and 5 nM PVL, respectively). PMN lysis was measured by release of lactate dehydrogenase using conditions identical to the pore-formation assays, but incubation time was extended to 3 h (Fig. 11C). Despite high levels of pore formation with 1 and 2 nM PVL, subsequent cell lysis was

limited (9 ± 3% and 15 ± 11%, respectively). We note that cell lysis increased significantly between 2 nM and 5 nM (e.g., lysis was 52 ± 6% using 5 nM PVL). Heat-inactivated PVL (iPVL) caused no pore formation and had zero cytolytic capacity (Fig. 11A and C).

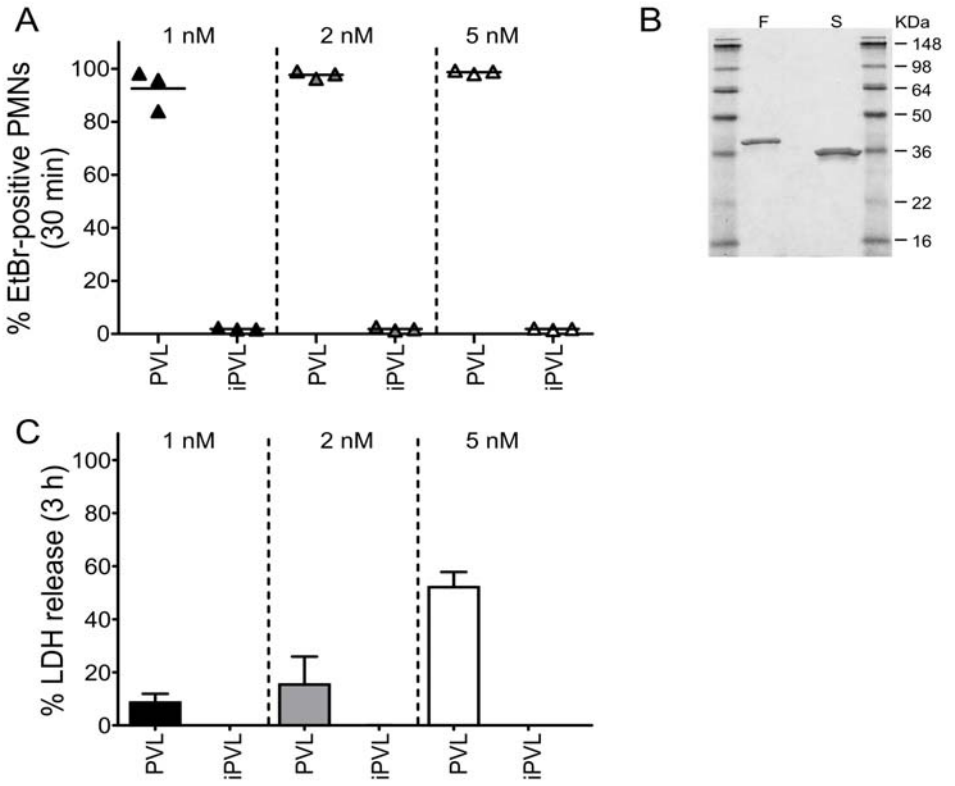

Figure 11. Determining concentrations of purified PVL that remain sublytic. A) Pore formation. PMNs (1 X 10^6) were incubated with 1 nM, 2 nM, or 5 nM native or denatured PVL for 30 min and plasma membrane permeability was evaluated using an ethidium bromide (EtBr) uptake assay. Each symbol represents a separate experiment and black bars indicate the mean. iPVL, heat-inactivated PVL. B) PVL purified from USA300. FPLC-purified PVL subunits (LukF-PV = F and LukS-PV = S) from USA300Δ*hlgABC* culture supernatants were analyzed by SDS-PAGE. The gel was stained with GelCode Blue Stain. C) PMN lysis. PMNs (1 X 10^6) were incubated with 1 nM, 2 nM, or 5 nM native or denatured PVL for 3 h. Lysis was determined by a standard assay that measures release of lactate dehydrogenase (LDH). Bars indicate the mean ± SEM of three donors.

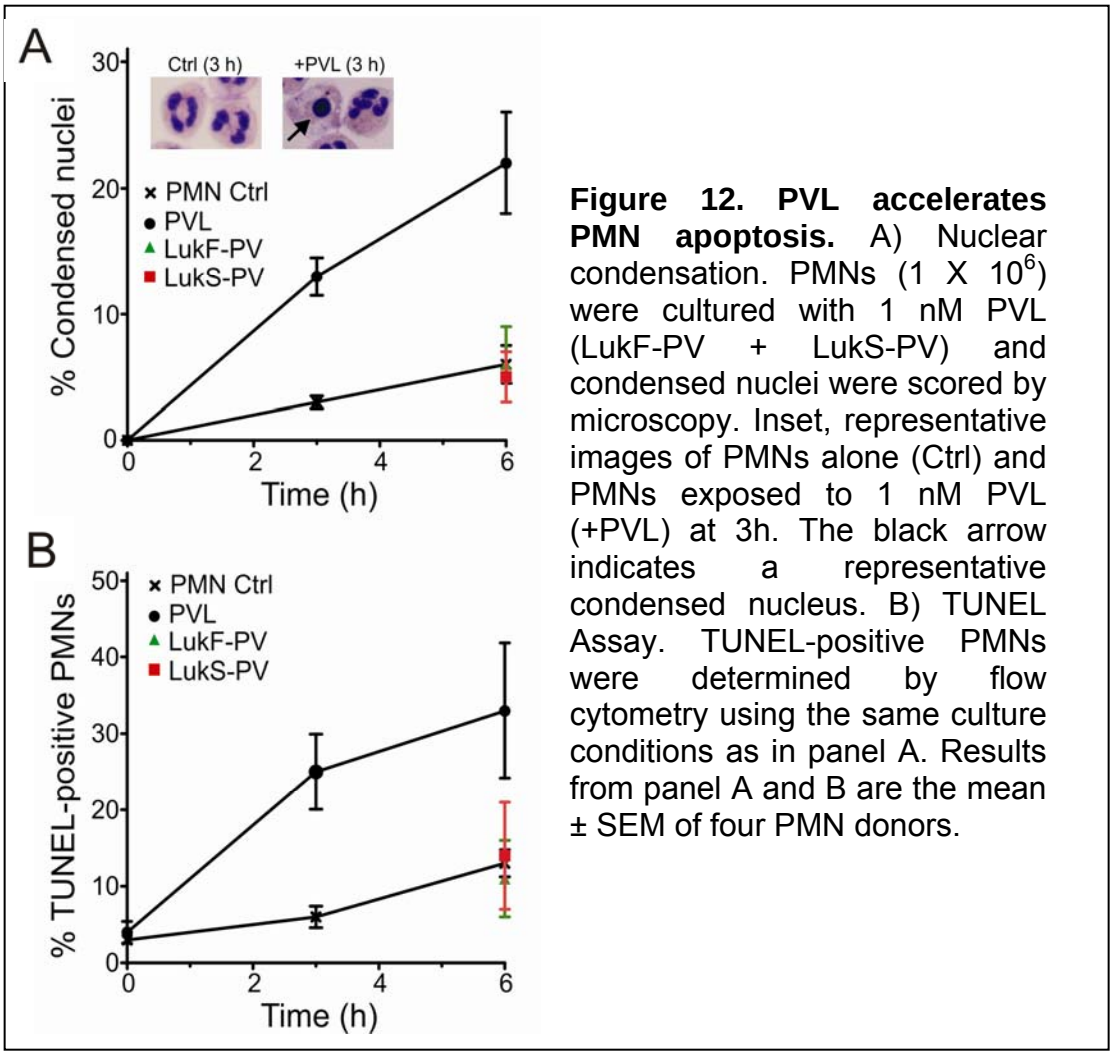

Figure 12. PVL accelerates PMN apoptosis. A) Nuclear condensation. PMNs (1 X 10^6) were cultured with 1 nM PVL (LukF-PV + LukS-PV) and condensed nuclei were scored by microscopy. Inset, representative images of PMNs alone (Ctrl) and PMNs exposed to 1 nM PVL (+PVL) at 3h. The black arrow indicates a representative condensed nucleus. B) TUNEL Assay. TUNEL-positive PMNs were determined by flow cytometry using the same culture conditions as in panel A. Results from panel A and B are the mean ± SEM of four PMN donors.

We next performed a series of experiments to determine whether sublytic concentrations of PVL altered human PMN function as reported previously. Indeed, 1 nM PVL accelerated neutrophil apoptosis (Fig. 12A and B), observations consistent with previous work by Genestier et al. (26). These findings were specific for active PVL, since neither PVL subunit alone (LukF-PV or LukS-PV) altered neutrophil survival.

In accordance with previous studies (22), 1 nM PVL caused enhanced release of superoxide (O_2^-) after stimulation with fMLP (Fig. 13A) and

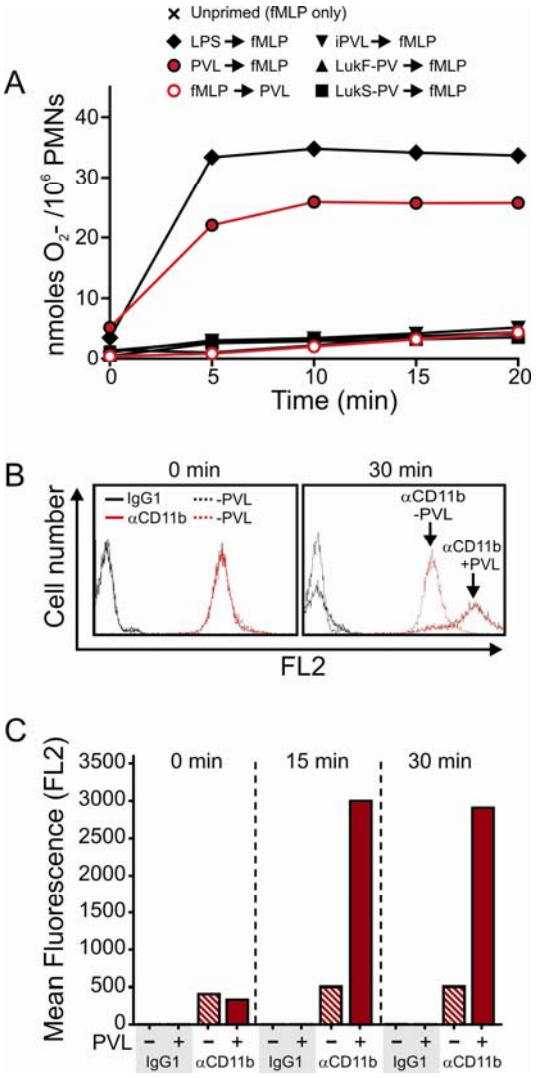

Figure 13. PVL alters human neutrophil function. A) Priming for enhanced release of O_2^-. PMNs (1 X 10^6) were incubated with 1 nM PVL (LukF-PV + LukS-PV), 1 nM heat-denatured PVL (iPVL), or 1 nM of individual PVL subunits (LukF-PV or LukS-PV) for 30 min as indicated. PMNs were incubated for 30 min and then activated with 1 μM fMLP. Superoxide production was measured using standard assay for reduction of ferricytochrome c. Results represent the mean of four separate experiments. B) Surface expression of CD11b. PMNs (1 X 10^6) were incubated with 1 nM PVL (+ PVL) or buffer alone (- PVL) and surface expression of CD11b was measured by flow cytometry. C) Quantification of CD11b surface expression. Bars indicate the mean fluorescence (FL2) of four separate experiments.

upregulation of CD11b at the plasma membrane (Fig. 13B and C). As with other priming agents (31, 35), PVL failed to elicit O_2^- production when used subsequent to fMLP (Fig. 13A). Collectively, these findings are consistent with the ability of PVL to function as a neutrophil priming agent.

3.4.2 Proinflammatory molecules alter neutrophil susceptibility to PVL.

Inasmuch as proinflammatory factors typically prime PMNs for enhanced function, which includes reorganization/redistribution of molecules at the plasma membrane, proinflammatory stimuli could conceivably influence the outcome of the PVL-PMN interaction. To test this hypothesis, we expanded our *in vitro* assays to include stimulation of human PMNs with multiple proinflammatory factors and evaluated PVL-mediated pore formation (Fig. 14A and B). Priming with fMLP or activation with PMA caused a significant decrease in the ability of PVL to form pores in the PMN plasma membrane (e.g., 48 ± 10% of the fMLP primed cells were EtBr-positive following exposure to PVL versus 81 ± 12% EtBr-positive cells following exposure to PVL alone) (Fig. 14A). By comparison, neither LPS nor heat-killed USA300 (HK LAC) altered the ability of PVL to cause formation of membrane pores. On the other hand, priming of PMNs with IL-8 or GM-CSF caused a transient but significant increase in pore formation (e.g., by 15 min 83 ± 5% of the PMNs were EtBr-positive after exposure to IL-8 compared with 65 ± 6% of those incubated with PVL alone, $p \leq 0.01$) (Fig. 14B). These data indicate that remodeling of the neutrophil plasma membrane following exposure to specific proinflammatory agonists alters the ability of PVL to interact with PMNs. One possible explanation for these results is that PVL binds to a PMN receptor whose surface expression changes following exposure to specific agonists.

We next determined if PVL induces O_2^- release from PMNs primed with IL-8 or GM-CSF (Fig. 15). IL-8 and GM-CSF primed neutrophils for enhanced fMLP-mediated O_2^- release (positive control, open circles) as described previously

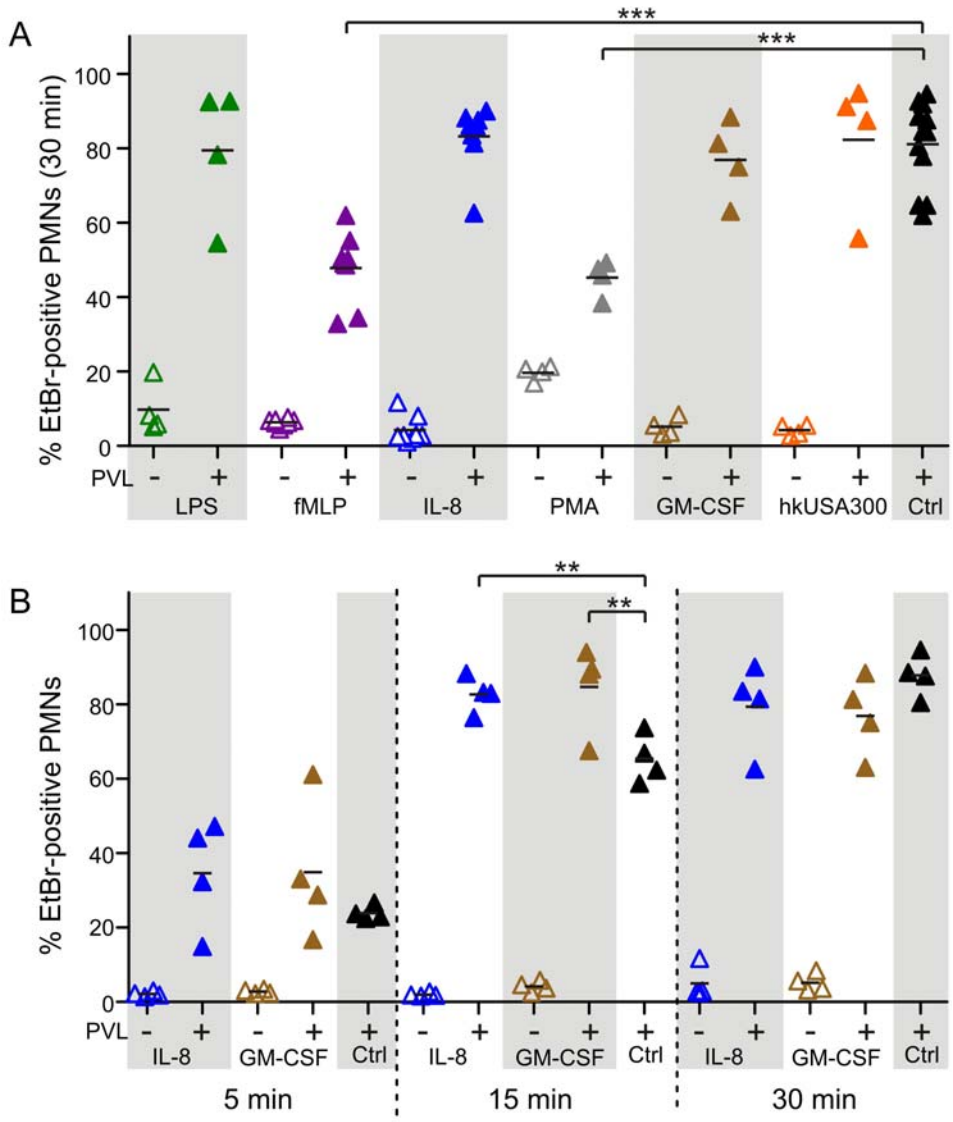

Figure 14. Proinflammatory molecules alter neutrophil susceptibility to PVL. A) Pore formation. PMNs (1 X 10^6) were cultured with 100 ng/mL LPS, 1 µM fMLP, 20 µM interleukin-8 (IL-8), 1 µg/mL phorbol-12-myristate-13-acetate (PMA), 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), or heat-killed USA300 *S. aureus* strain (HK LAC) (LAC, 1:10 bacteria to PMN ratio) for 30 min in Hepes-buffered RPMI 1640. PMNs were then incubated with 1 nM PVL and subsequently assayed for EtBr uptake. Each symbol represents a separate experiment. Colors indicate separate PMN culture conditions as indicated. Red bars indicate mean percent EtBr-positive PMNs. B) Temporal increase in PMN susceptibility to PVL following exposure to IL-8 or GM-CSF. ***, $p \leq 0.001$ vs. ▲ and **, $p \leq 0.01$ vs. ▲ using a one-way analysis of variance (ANOVA) with a Dunnett's posttest. Ctrl, PMNs + 1 nM PVL (positive control).

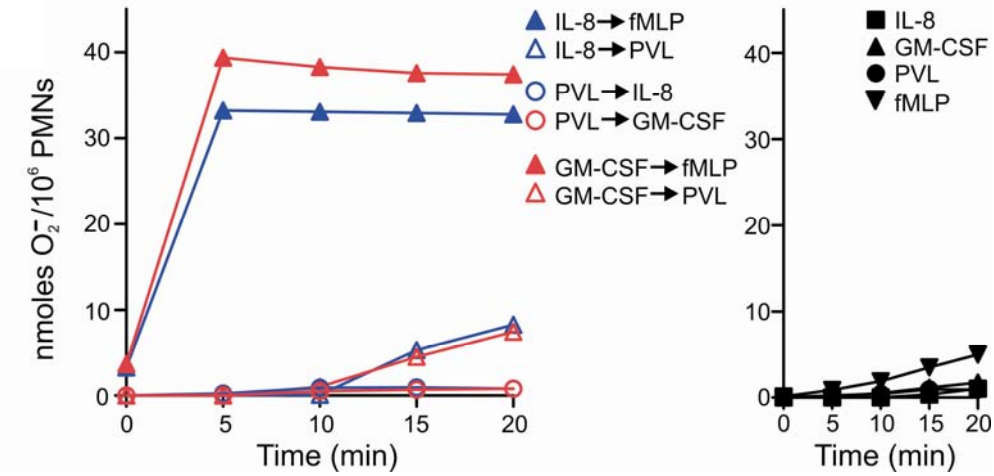

Figure 15. Effects of PVL on PMN superoxide (O_2^-) release. O_2^- production was determined by reduction of ferricytochrome c as described in Materials and Methods. Left panel, PMNs were primed with PVL and then stimulated with IL-8 (blue open circles) or GM-CSF (red open circles). Alternatively, PMNs were primed with IL-8 or GM-CSF and then stimulated with PVL (open triangles) or fMLP (closed triangles). Right panel, assays performed without a secondary stimulus.

(36-38). PVL caused O_2^- release from IL-8 and GM-CSF primed neutrophils, albeit 3-4 times less than that caused by secondary stimulation with fMLP (open triangles). Collectively, these data indicate that the ability of PVL to interact with human neutrophils is dictated by the activation state of the cell, and thus the interaction is specific to some degree.

3.4.3 PVL induces global changes in PMN gene expression.

To gain insight into the molecular basis of PVL-mediated neutrophil priming, we measured global changes in PMN gene expression following exposure to 1 nM PVL (Fig. 16 and 17). The number of differentially expressed genes increased in a time-dependent manner (there were 397 differentially-

expressed genes at 30 min, 1074 differentially-expressed genes at 60 min, and 2850 differentially-expressed genes at 180 min) (Fig. 16). Differentially-

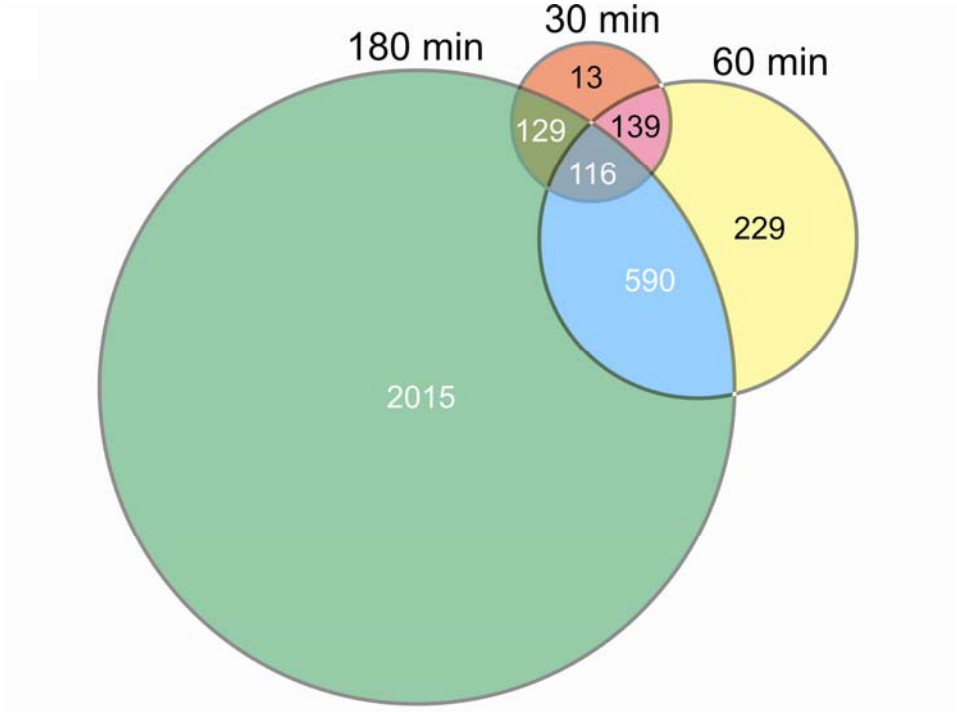

Figure 16. PVL causes global changes in PMN gene expression. PMNs (1 X 10^7) were cultured with 1 nM native or heat-inactivated PVL and changes in transcript levels between the two conditions were measured using Affymetrix HU133+2 GeneChips as described in Materials and Methods. Venn diagram depicting the total number of differentially expressed genes at each time point.

expressed genes were categorized into biological pathways or grouped according to function using IPA software (Fig. 17). Only 9 signal transduction pathways were significantly represented by differentially-expressed genes at 30 min (Fig. 17). However, 30 and 114 signal transduction pathways were significantly represented by differentially-expressed genes at 60 min and 180

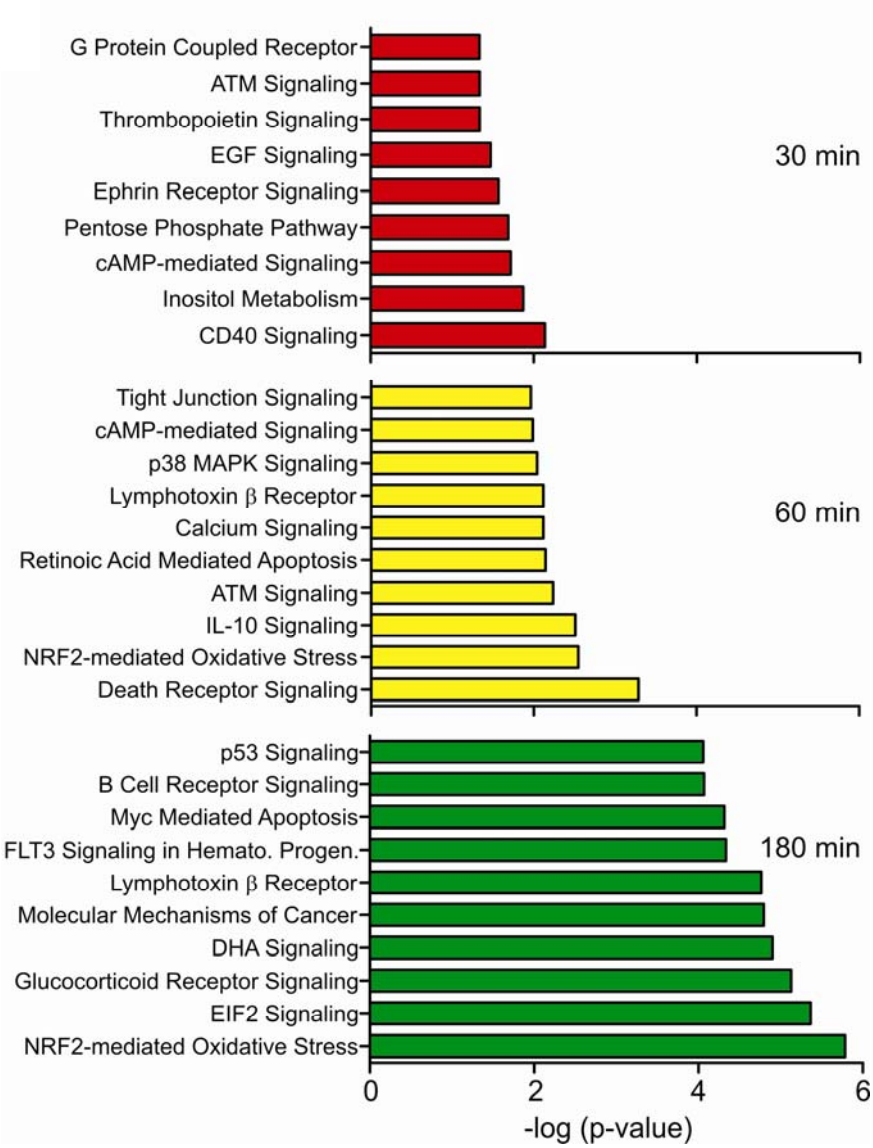

Figure 17. Signal transduction pathways represented by differentially-expressed genes. Pathways or processes were identified using Ingenuity Pathway Analysis as described in Materials and Methods. The p-value is the measure of the likelihood that the association of differentially expressed genes with a given pathway is due to random chance.

min, respectively (not shown). In general, proinflammatory pathways such as CD40 and epidermal growth factor (EGF) signaling are significantly represented early following the PVL-PMN interaction (30 min). By comparison, cell fate

pathways such as death receptor signaling and myc-mediated apoptosis signaling are more highly represented in PMNs by 60 and 180 min after exposure to PVL (Fig. 17).

3.4.4 Expression of PMN genes encoding major transcription regulators is increased following exposure to PVL.

Genes encoding proteins known to mediate the inflammatory response, such as *STAT3*, *SOCS3*, *JUNB*, *FOS*, *JUN*, *FOSB*, *FOSL1*, and *TANK* were upregulated 30 or 60 min after PMN exposure to 1 nM PVL (Fig. 18). Expression of *SOCS3* is regulated by the transcription factor STAT3. Therefore, upregulation of *SOCS3* is consistent with activation of STAT3 (39,40). *JUNB*, *FOS*, *JUN*, *FOSB*, and *FOSL1* encode proteins that dimerize and form the AP-1 transcription factor, which is known to associate with NF-kappaB as a result of signal transduction through proinflammatory pathways (41). *TANK* is a downstream kinase involved in proinflammatory signal pathways that regulate NF-kappaB activation (42,43). Genes involved in NF-kappaB signal transduction were differentially expressed 180 min after exposure of PMNs to PVL. For example, the gene encoding NF-kappaB inhibitor delta (*IkB*δ or *NFKBID*) was upregulated and *MAP3K3* (*MEKK3*) was downregulated, a finding consistent with a decrease in IKKβ activation. Increased expression of *MALT1*, *BCL10*, and *BCL3* by PMNs after exposure to PVL suggests NF-kappaB is activated via nonclassical pathways (42-46).

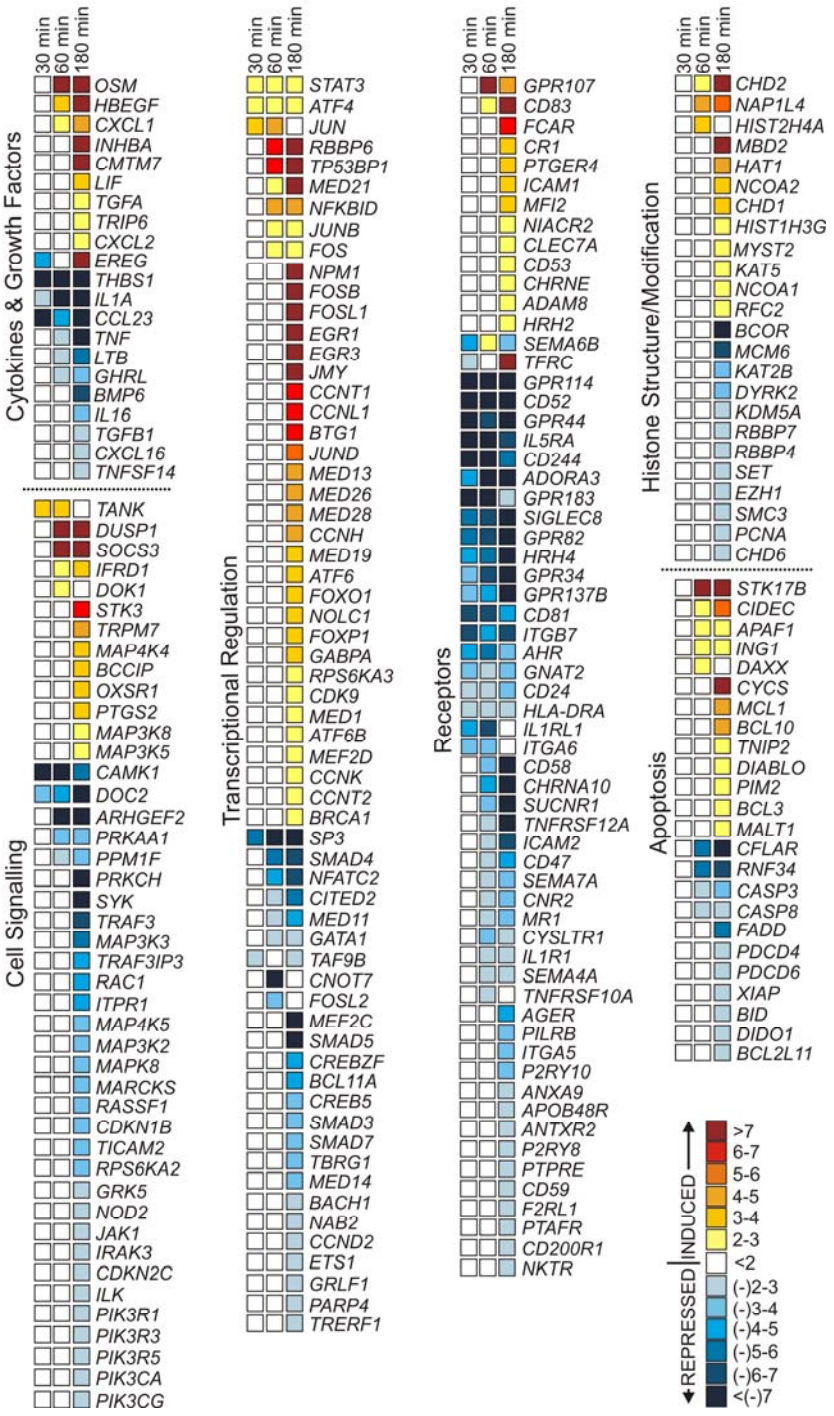

Figure 18. PVL alters expression of genes encoding proteins involved in inflammation and cell fate. Transcript levels were measured as described in Materials and Methods. Colors represent the mean increase or decrease (fold-change) in gene expression in PMNs treated with native PVL relative to those treated with heat-denatured PVL (iPVL). Results are the mean of three separate experiments or donors.

3.4.5 Priming of PMNs with PVL alters expression of transcripts encoding surface receptors and proinflammatory molecules.

Several PMN proinflammatory mediators were upregulated 180 min after exposure to PVL (e.g., *FCAR*, encoding CD89; and *CR1*, which encodes CD35). CD89 is an IgA receptor expressed on phagocytic cells and can promote phagocytosis (47). CD35 binds the serum complement components C3b and C4b at the microbial surface (48,49), thereby facilitating phagocytosis and neutrophil activation through complement receptor activation (50). Genes encoding chemokines GROα (*CXCL1*), GROβ (*CXCL2*), and the cytokine oncostatin M (*OSM*) were upregulated after exposure to PVL. GRO proteins (members of the interleukin-8 superfamily) are expressed and secreted by several cell types including neutrophils (51-54). Oncostatin M, a member of the interleukin-6 family, is stored by mature PMNs as an active protein and released following priming or activation. However, *de novo* synthesis of OSM is known to occur under certain conditions (55) and previous studies have shown transient upregulation of the transcript after phagocytosis (56,57).

Upregulation of PMN proinflammatory surface receptors and molecules after PMN exposure to PVL (described above) is comparable to the increased expression of proinflammatory molecules following priming with LPS and GM-CSF (34,58-63). In contrast to other agonists, exposure of neutrophils to PVL also culminated in relatively early downregulation of genes encoding other surface receptors and cytokines involved in the inflammatory response (Fig. 18). For example, *IL5RA*, *IL1R1*, *IL1A*, *CCL23 (MIP-3)*, *TNFRSF12A*, *TNFRSF10A*,

and TNF (encoding tumor necrosis factor alpha, TNFα) were repressed 60 min after exposure of human neutrophils to PVL. Control of the TNFα-mediated immune response is important for the resolution of inflammation (64-66). Downregulation of transcripts encoding TNF receptors and TNFα at later time points (60 and 180 min) provides support to the idea that there is eventual moderation of the proinflammatory response induced by PVL, a phenomenon consistent with induction of PMN apoptosis (Fig. 12A and B).

Expression of *DIABLO* (*SMAC*), *CYCS*, and *APAF1* was increased after incubation with PVL, findings compatible with activation of caspase 9. This observation is consistent with previous findings reporting activation (proteolytic cleavage) of caspase 9 and caspase 3 following exposure of PMNs to recombinant PVL (26). Furthermore, expression of the anti-apoptotic genes *XIAP* and *CFLAR* (*c-FLIP*) was repressed (67,68). Taken together, these data suggest that exposure of human PMNs to PVL activated proinflammatory signal transduction pathways, an event followed by differential expression of genes involved in cell fate.

3.4.6 Multiple proinflammatory molecules are released from neutrophils following PVL priming.

Inasmuch as PVL primes neutrophils for enhanced function and caused upregulation of transcripts involved in the proinflammatory response, we next measured release of proinflammatory molecules from PMNs following exposure to sublytic concentrations of PVL (Table 1). PVL caused release of multiple

Table 1. PVL-mediated release of proinflammatory molecules. Supernatants were analyzed by RBM using microsphere-based immuno-multiplexing. Asterisks indicate molecules whose concentrations remained below or at the lower limit of detection (LOD) and the LODs were used to calculate a conservative p value.

Protein	Control (ng/mL) (mean ± S.D.) (n = 4)	+1 nM PVL (ng/mL) (mean ± S.D.) (n = 4)	*P* value
Ferritin	0.5200 ± 0.1226	19.50 ± 2.887	0.0009
Vascular endothelial growth factor	0.0198 ± 0.0128	0.5740 ± 0.1740	0.007
Interleukin-1 receptor antagonist	*0.0067 ± 0.0025	0.0408 ± 0.0091	0.009
Tumor necrosis factor receptor 2	0.1860 ± 0.1132	0.4380 ± 0.1282	0.01
Insulin-like growth factor-1	6.630 ± 0.9500	10.40 ± 1.451	0.01
Prostatic acid phosphatase	0.0500 ± 0.0083	0.4200 ± 0.1547	0.02
Myeloperoxidase	342.3 ± 98.10	9003 ± 3642	0.02
Growth hormone	*0.0200 ± 0.000	0.0500 ± 0.0163	0.02
Serum amyloid P component	2.500 ± 2.400	4.000 ± 2.200	0.02
CD40	*0.0044 ± 0.000	0.0120 ± 0.0034	0.03
Plasminogen activator inhibitor 1	0.0500 ± 0.0123	0.0800 ± 0.0149	0.03
EN-RAGE	16.75 ± 10.31	143.8 ± 68.04	0.04
Interleukin-8	0.0061 ± 0.0028	0.0334 ± 0.0166	0.05
Cancer antigen 19-9 (μU/mL)	*320.0 ± 0.000	845.0 ± 349.9	0.06
Tissue inhibitor of metalloproteinases 1	0.2700 ± 0.1857	1.083 ± 0.7054	0.06
Carcinoembryonic antigen	*0.0400 ± 0.0040	0.1700 ± 0.0851	0.06
Factor VII	*0.1100 ± 0.000	0.4000 ± 0.2335	0.09
Adipoliporotein (a)	23.00 ± 8.100	39.00 ± 14.50	0.1
Interleukin-6	*0.0004 ± 0.000	0.0011 ± 0.0007	0.1
Tyroid stimulating hormone (μIU/mL)	*0.0041 ± 0.000	0.0080 ± 0.0041	0.2
Interleukin-1 beta	*0.0002 ± 0.000	0.0048 ± 0.0062	0.2

proinflammatory mediators, including IL-8, IL-6, VEGF, and myeloperoxidase (MPO), from human neutrophils. In addition, we observed significant accumulation of tumor necrosis factor receptor 2 (TNFR2) and IL-1 receptor antagonist (IL-1RA) in RPMI 1640 culture media after incubation of PMNs with PVL. Neutrophils are known to shed TNFR2 following stimulation with fMLP and GM-CSF, thereby decreasing the subsequent PMN response to TNFα (64). PMNs stimulated *in vitro* with LPS or GM-CSF released of IL-1β and IL-1RA; however, concentrations of IL-1RA are significantly higher than those of IL-1β (66). Consistent with these previous studies, 1 nM PVL caused accumulation of 10 times more IL-1RA than IL-1β in PMN culture media (there was 0.0408 ± 0.0091 ng/ml IL-1RA and 0.0048 ± 0.0062 ng/ml IL-1β in culture media after exposure to PVL).

3.5 Discussion

Recent multinational phase III clinical trials indicate that presence of *pvl* genes is not the primary determinant of outcome in patients with MRSA skin and soft tissue infection. In fact, individuals with PVL-positive infections were more likely to be cured (69,70). It is also noteworthy that the concentration of PVL achieved during *S. aureus* infection *in vivo* may be insufficient to cause PMN lysis (71,72), findings that bring into question the role of PVL as a cytolytic toxin. Antibodies against PVL are present in individuals with previous confirmed *S. aureus* infections caused by either PVL-positive or PVL-negative strains (73,74). These observations suggest that some of the antibodies were originally elicited

by two-component toxins other than PVL, but the antibodies cross-react with PVL. Recurrent infections with PVL-positive *S. aureus* strains occur in patients that have anti-PVL antibodies, findings that suggest PVL has little or no role in establishment of infection (75). Furthermore, a previous study demonstrated that administration of anti-PVL antibodies prior to USA300 or USA400 infection (*pvl*-positive) hindered clearance of infection (76). This observation seems at variance with the presumed role of during infection (i.e., cytolytic toxin that enhances virulence), but is consistent with the ability of the molecule to function as a neutrophil priming agent.

The cytolytic properties of PVL are well known from extensive work *in vitro*. However, there is paucity of evidence to indicate that the primary function of PVL *in vivo* is cytolysis of host leukocytes. Furthermore, *in vitro* studies indicate *S. aureus* is rapidly ingested by neutrophils and that these host phagocytes undergo rapid lysis independent of PVL (7,29). Thus, the role played by PVL during infection remains unclear.

Sublytic concentrations of PVL are known to prime neutrophils for enhanced release of O_2^-, and cause release of IL-8, IL-6 and PMN granule contents, such as MPO and lysozyme (18-21). Previous studies demonstrated that granule exocytosis occurs in intact PMNs following incubation with PVL (77). Furthermore, Konig et al. reported that inhibition of protein tyrosine kinases decreases release of IL-8 from PMNs exposed to sublytic levels of PVL (20). Consistent with these previous studies, we found that neutrophils incubated with 1 nM PVL remained intact, as there was little or no release of LDH using these

assay conditions (Fig. 11C). Taken together, these data provide strong support to the idea that PVL-mediated release of neutrophil proinflammatory molecules was caused by activation of signal transduction pathways rather than cytolysis.

In accordance with previous work, PVL had proinflammatory effects on human neutrophils, including priming for enhanced release of O_2^-, upregulation of CD11b, and secretion of TNFR2, IL-1RA, and IL-1β. The proinflammatory response elicited by PVL is similar to that caused by inflammatory cytokines or bacterial components such as GM-CSF and LPS (34,62,78). One major difference is that exposure of PMNs to PVL caused release of MPO, a protein sequestered in azurophilic granules (15) (Table 1). Exocytosis of azurophilic granules is not typical of priming with GM-CSF (79), IL-8 (80), or LPS (81). Rather, this phenomenon typically indicates full activation of neutrophils. PVL also accelerated PMN apoptosis at a concentration that elicits a proinflammatory response (Fig. 12A and B), which is at variance with the ability of other priming agents to extend PMN survival *in vitro*. Our finding that genes encoding apoptosome proteins, DIABLO (SMAC), CYCS, and APAF1, were upregulated after exposure to PVL suggests that the caspase 9/3 pathway participates in PVL-mediated apoptosis (Fig. 18). Previous studies have linked induction of PMN apoptosis to inactivation of the NF-kappaB pathway (82,83). Differential expression of PMN genes directly transcribed by NF-kappaB subunits (*ICAM1*, *TNF*) and those encoding proteins that in regulate NF-kappaB activity (BCL3, BCL10, and MALT1) suggest this pathway is activated following exposure to PVL.

PMNs are exposed to a multitude of proinflammatory molecules during infection *in vivo* and different combinations of stimuli are known to elicit different PMN responses (84). The application of this idea to PVL-positive *S. aureus* infections is perhaps reflected by our finding that fMLP, GM-CSF, and IL-8 alter the ability of PVL to interact with PMNs (Fig. 14A and B). Data in this study suggest that sublytic concentrations of PVL prime PMNs for enhanced microbicidal capacity. This notion is supported by Yoong et al. (76), in which anti-PVL rabbit sera inhibited PMN killing of several PVL-positive *S. aureus* strains *in vitro.* By comparison, killing of isogenic *pvl* mutants by PMNs was similar in the presence or absence of anti-PVL antibody (76). Collectively, these observations suggest that PVL can enhance rather than hinder the host innate immune response to *S. aureus* infection. Nonetheless, the ability of PVL to alter PMN bactericidal activity *in vivo* merits further investigation. Elucidation of activated secondary messenger proteins following such stimulation with PVL may provide new insight into the ability of specific agonists to elicit differential responses from human neutrophils.

3.6 Acknowledgements

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CHAPTER FOUR

SUMMARY

4.1 Significance of the work presented in this dissertation

4.1.1 *S. aureus* virulence and cytolytic toxins.

The success of *S. aureus* as a human and animal pathogen is largely associated with its ability to produce a multitude of toxins. For example, *S. aureus* produces several cytolytic molecules with well-characterized *in vitro* activity that contribute to virulence. The host range is varied among *S. aureus* toxins and some, such as the alpha-type phenol soluble modulins (PSMs), have the ability to target a broad range of host cells. PSMs are a group of relatively short amphipathic peptides that are encoded in the *S. aureus* core genome (1). Importantly, PSMs are one of the few molecules known to contribute to the enhanced virulence phenotype of CA-MRSA strains such as USA300 (1). Alpha-hemolysin (α-hemolysin, Hla) is an exotoxin that is encoded in the core genome of virtually all *S. aureus* strains. Hla is secreted as a soluble monomer, forms a heptameric pore in the plasma membrane of target cells (2), and has cytolytic activity toward epithelial cells, erythrocytes, monocytes, lymphocytes, fibroblasts, and platelets (3,4). In addition, *S. aureus* produces several bicomponent pore-forming toxins (PFTs) that are tropic for leukocytes such as macrophages, monocytes, and neutrophils. The pore-forming activity of this group of related leukotoxins requires two components, a LukS subunit and a LukF subunit.

Alternating LukF and LukS components form a pore in the plasma membrane of leukocytes. PFTs that are expressed by *S. aureus* include gamma-hemolysin (γ-hemolysin, Hlg), LukG-LukH (LukGH), LukD-LukE (LukDE), and PVL (comprised of LukS-PV and LukF-PV) (4-6). Similar to the *hla* operon, genes that encode Hlg (*hlgABC*) and LukGH (*lukG/H*) are essentially ubiquitous in clinical isolates of *S. aureus* (6,7). Two combinations of Hlg subunits, HlgA-HlgB or HlgA-HlgC, aggregate on the surface of erythrocytes and leukocytes and cause lysis of these cells (8-10). LukGH is freely secreted and bound to the surface of *S. aureus*. This toxin was recently shown to cause lysis of neutrophils (6). LukDE is encoded in the genome of ~30% of clinical isolates (11); however, the *lukDE* operon is harbored by many prominent HA-MRSA and CA-MRSA strains (11-13). This bicomponent PFT is weakly leukotoxic (11). PVL lyses neutrophils and other myeloid cells *in vitro* and many CA-MRSA strains harbor genes that encode PVL (*lukS/F-PV*) (12,14,15). However, only 2-5% of all clinical *S. aureus* isolates contain *lukS/F-PV* (7,16,17).

There is striking redundancy in cytolytic activity among the *S. aureus* leukotoxins. Presumably, destruction of neutrophils and other leukocytes confers an increased ability of *S. aureus* to evade the host innate immune response. It remains a mystery why *S. aureus* in general produce so many molecules that potentially have a similar role in virulence. *S. aureus* causes a wide range of infections with varied degrees of severity (16,18-20). Perhaps such redundancy in toxin function is advantageous for the pathogen, as it might enable *S. aureus* to colonize and/or cause infection in several niches in the human host. For

example, it is known that expression of PVL and Hlg by *S. aureus* is highly influenced by *in vitro* culture conditions (21,22). These findings underscore the notion that specific conditions *in vivo* likely impact expression of cytolytic toxins and could thereby influence the outcome of infection. In addition, the combination of PVL and LukGH causes lysis of human neutrophils that is greater than that caused by either toxin alone, suggesting that specific leukotoxin combinations are additive or synergistic (6). The concerted action of both toxins may further enhance the success of *S. aureus* as a human pathogen.

PVL-positive strains compromise a small fraction of all *S. aureus* clinical isolates, and correspondingly, there is an association of these strains with a relatively small subset of skin infections (e.g. furuncles and carbuncles) and necrotizing pneumonia with antecedent influenza (14,19,23-25). It is therefore possible that PVL confers some advantage to *S. aureus* under specific conditions or niches *in vivo*. The acquisition of *lukS/F-PV* requires horizontal transfer of a prophage (26,27). Therefore, acquisition of *lukS/F-PV* is either uniquely not important (with the possible exception of particular *S. aureus* infections) and/or the genetic background of specific *S. aureus* lineages may be less compatible with prophage insertion into the genome (restriction system), thus limiting the number of PVL-positive strains. Inasmuch as the vast majority of *S. aureus* clinical isolates encode Hla, PSMs, and other molecules that contribute to infection, PVL is clearly not required to cause disease. Additionally, epidemic PVL-positive CA-MRSA lineages remain fairly specific in geographic distribution (28), which suggests that factors other than PVL determine global segregation of

pathogenic strains. *S. aureus*, including PVL-positive strains, presumably have had the opportunity to disseminate to any global region, given the high levels of international travel. The limited prominence of PVL-positive clinical isolates overall supports the notion that PVL may have a role in specific types of infection or function only in the presence of particular host factors.

4.1.2 Does PVL contribute to the lysis of human neutrophils?

Historic studies clearly demonstrated that purified native PVL is cytolytic toward rabbit and human neutrophils (29-31). In addition, PVL-mediated cytolysis is concentration-dependent and concentrations of PVL less than 2 nM cause formation of membrane pores but little or no cytolysis (21,30,31). Genestier et al. (32) demonstrated that cytolysis of human neutrophils occurs following incubaton with 200 nM of recombinant PVL (rPVL). In contrast, exposure of PMNs to a sublytic level of rPVL (5 nM) accelerates neutrophil apoptosis rather than causing necrosis (lysis). Similarly, a study by Loffler et al. (17) confirmed the finding that a low concentration of rPVL (80 ng/ml or ~1.1 nM) accelerates neutrophil apoptosis. However, these authors also reported that 80 ng/ml causes a 90% decrease in intact cells (reported as % of intact cells) at 20 min, a finding consistent with a high degree of PMN lysis under those assay conditions. The apparent discrepancy in these reports is likely explained by differences in methodology used to determine cell lysis. For example, Genestier et al. (32) measured cell lysis by microscopy, whereas Loffler et al. (17) used a less direct method (uptake of propidium iodide) that determines pore formation and not necessarily cell lysis. There was a similar discrepancy between Loffler et al. (17)

and Voyich et al. (33). These studies examined the contribution of PVL to PMN lysis *in vitro* by use of *S. aureus* culture supernatants. Voyich et al. (33) found that culture supernatants derived from USA300 and USA400 wild type (WT) or isogenic *lukS/F-PV* deletion (Δ*pvl*) strains caused comparable levels of PMN lysis (33). By contrast, Loffler et al. (17) reported that culture supernatants from USA300Δ*pvl* caused decreased neutrophil lysis compared to those from the corresponding WT strain. By inspection, the disparities between these data appear to be the result of two factors: 1) utilization of different methods to assess PMN lysis, and 2) use of culture supernatants from different *in vitro* growth media. Loffler et al. (17) determined levels of cytolysis by measuring uptake of propidium iodide (PI) while Voyich et al. (33) measured extracellular release of lactate dehydrogenase (LDH). Although uptake of PI was used as an indicator of neutrophil lysis, such an approach operates under the assumption that PVL-mediated pore formation correlates directly with cytolysis. However, results presented in Chapter 2 of this dissertation demonstrate that there is not a direct correlation between EtBr uptake (pore formation) and LDH release by human PMNs. Therefore, Loffler et al. (17) measured cell membrane permeability but not necessarily necrosis.

Some of the incongruity in these studies *in vitro* is also likely attributed to the use of supernatants from different culture media. This idea is supported by results presented in Chapter 2 indicating that production of PVL is highly influenced by different culture conditions *in vitro*. Growth of PVL-positive strains in CCY, particularly USA300, is optimal for high production of PVL, whereas

growth in TSB or 100% human serum is not. Accordingly, the variability in quantities of PVL present in culture supernatants from different media *in vitro* partly explains why there are disparities in the literature as exemplified by Voyich et al. (33) and Loffler et al. (17). Taken together, these data indicate that differential expression of PVL *in vitro* alters the contribution of PVL to pore formation and cytolytic activity of culture supernatants (Chapter 2). PVL-positive culture supernatants from *S. aureus* growth media that more closely represent *in vivo* growth conditions (e.g., human serum) support little or no accumulation of PVL. Furthermore, culture supernatants that have limited PVL-mediated cytolytic activity (Chapter 2, Fig. 8 TSB) contain quantities of PVL comparable to that in material recovered from human *S. aureus* infections (34,35). These findings indicate that *in vitro* assays with culture supernatants are of limited utility for estimating the contribution of PVL to *S. aureus* virulence. Consistent with this idea, there is no correlation between levels of PVL produced *in vitro* by clinical isolates and disease severity (36). The observation that concentrations of PVL *in vivo* (34,35) are largely limited to those that cause pore formation but limited lysis *in vitro* (Chapter 2) challenges the widely accepted notion that the primary role of PVL during infection is to cause cytolysis of leukocytes.

4.1.3 PVL as an immune modulatory agent.

Given that *in vivo* expression of PVL likely does not achieve a concentration necessary to cause significant neutrophil lysis, we examined the ability of sublytic concentrations of PVL to alter PMN function (Chapter 3).

Sublytic concentrations of PVL prime neutrophils for enhanced release of O_2^-, and induce extracellular release of IL-8, IL-6 and PMN granule contents (37-40). Previous studies indicate that there is optimal release of O_2^- from PVL-primed neutrophils following stimulation with fMLP (41,42, and confirmed in Chapter 3). However, the molecular basis for PVL-mediated priming of neutrophils is unknown.

Our results indicate that PVL elicits a proinflammatory response from human PMNs. One of the major goals of this dissertation (Chapter 3) was to characterize the molecular mechanism by which PVL primes neutrophils for enhanced response to a secondary agonist. Enhanced release of ROS from PMNs primed with PVL requires a specific order of events as indicated by O_2^- release assays (Fig. 13A and 15). Neutrophils primed with IL-8 or GM-CSF have increased susceptibility to PVL-mediated pore formation, whereas cells primed with fMLP or activated with PMA are resistant to pore formation (Fig. 14). These data suggest that the activation state of the human neutrophil determines the extent to which PVL is capable of causing pore formation and O_2^- release. Inasmuch as neutrophils interact with proinflammatory molecules such as IL-8 during *S. aureus* infection, alterations in microbicidal activity under these conditions merits investigation. To further examine the molecular basis of PVL-mediated priming, PMNs were exposed to PVL and the culture medium was analyzed for accumulation of molecules known to be involved in the proinflammatory response. We discovered that several notable proinflammatory agents were released by PMNs following exposure to PVL (Table 1), including

molecules previously reported to be released by PMNs under these conditions (37-40). Collectively, these data provide new insight into how PVL *in vivo* might alter the outcome of infection.

Microarray experiments with PVL-treated PMNs were performed to gain a broad understanding of the molecular mechanism of PVL-mediated neutrophil priming. The microarray data suggest that PMN exposure to PVL triggers activation of STAT3 and NF-kB signal transduction pathways. Both of these pathways are known to mediate the proinflammatory response in multiple cell types, including neutrophils. These data provide a basis for future studies to explore how PVL, as an immune modulatory agent, alters the host-*S.aureus* interaction.

Although our work reveals the potential of PVL as an immune-modulatory agent, such a function remains to be determined *in vivo*. Nonetheless, recent studies have shown that Hla, another *S. aureus* cytolytic toxin, has an immune modulatory function *in vivo*. Hla is essential for mortality in mouse models of pneumonia and the level of Hla expression correlates with severity of infection (43). Production of Hla during experimental *S. aureus* pneumonia in mice increases quantities of CXCL1, CXCL2, and TNFα present in lung tissue, which in turn recruits neutrophils to the lungs (44). This enhanced neutrophil recruitment ultimately results in severe lung tissue destruction and death. It is well accepted that neutrophils can cause significant host tissue damage under conditions in which the inflammatory response is dysregulated and/or there is not timely removal of effete cells (45,46). Hla *in vitro* elicits release of

proinflammatory molecules such as IL-8, IL-1β and TNFα from monocytes and endothelial cells (47-49). These findings indicate that the role of *S. aureus* exotoxins in pathogenesis is not be solely based on cytolytic activity. Rather, the host inflammatory response elicited by secreted exotoxins is likely a major factor in determining the outcome of *S. aureus* infection. This notion is supported further by Bubeck Wardenburg et al. (50) who reported that protection against CA-MRSA pneumonia is associated with different cytokine profiles compared to unvaccinated controls.

In contrast to Hla, there is little *in vivo* evidence to suggest that PVL increases the severity of experimental CA-MRSA pneumonia (43,51), with the exception of a single study (52). Diep et al. (52) reported a moderate increase in mortality and lung inflammation following infection with a high bacterial inoculum. *S. aureus* necrotizing pneumonia is typically associated with *S. aureus* strains that contain genes encoding for PVL (14,23,25), and Diep et al. (52) demonstrated that purified PVL causes lung inflammation. A caveat of these findings is that a large quantity of PVL (12 μg) is needed to cause accumulation of IL-8 and MCP-1 in rabbit lungs (52). Diep et al. (52) proposed that increased severity of *S. aureus* infection is attributed in part to degranulation of PMNs following exposure to PVL *in vivo*, which in turn causes damage to the lungs and prolongs the proinflammatory response. It is notable that Diep et al. (52) utilized a very large inoculum of PVL-positive *S. aureus* in the pneumonia model and a high concentration of purified PVL to elicit a proinflammatory response in the rabbit lung.

Hla clearly contributes to the severity of staphylococcal disease in animal models, while the contribution of PVL is limited or undetectable. Perhaps this difference can be partially explained by the ability of Hla to target a much wider range of cell types as compared to PVL. As demonstrated by Suttorp et al. (53), exposure of pulmonary endothelial monolayers to Hla increases permeability of the monolayer by stimulating cell retraction, i.e. formation of paracellular gaps. These results suggest that Hla disrupts the air-blood barrier in pulmonary tissue not by cytolysis but by altering cell morphology and/or function. Subsequent studies by McElroy et al. (54) and Bubeck Wardenburg et al. (50) demonstrated that expression of Hla in an animal pneumonia model caused disruption of the air-blood barrier via destruction of lung tissue.

In comparison to Hla, PVL may have a more subtle role in the progression of staphylococcal pneumonia and/or may contribute in rare syndromes (i.e., necrotizing pneumonia). It is also possible that particular host factors increase leukocyte susceptibility to PVL or cause an aberrant proinflammatory response following exposure to PVL. Further studies will be necessary to test these hypotheses.

There is redundancy in the cytolytic and proinflammatory function of *S. aureus* cytolytic toxins; however, studies *in vivo* demonstrate that not all are created equal. Hla is a key virulence factor in staphylococcal pneumonia and SSTIs as exemplified by the ability of anti-Hla antibodies to protect *S. aureus*-infected mice from severe or lethal disease (50,55). By comparison, anti-PVL antibodies confer no protection against *S. aureus* infection in animal pneumonia

models (50). Antibodies that cross-react with PVL are present in the general human population, with or without a medical history of *S. aureus* infection (56-59). Notably, anti-PVL antibodies fail to protect individuals from recurrent infections caused by PVL-positive CA-MRSA strains USA300 and USA400 (60,61).

4.1.4 Concluding thoughts.

The ability of sublytic concentrations of PVL to augment the proinflammatory response (Chapter 3 and 37-40) could be detrimental to surrounding host tissues. Increased release of proinflammatory agents, such as IL-8 and IL-1β, from leukocytes would lead to increased inflammation and the potential for unintended host tissue damage. A countering argument to this notion is that the PVL-mediated release of inflammatory molecules enhances the innate immune response, and therefore, expression of PVL *in vivo* could be advantageous to the host for clearance of infection. This idea is further supported by Yoong et al., who demonstrated reduced clearance of USA300 and USA400 infection after administration of anti-PVL antibodies (62). In light of these and other previous findings that indicate anti-PVL antibodies are not protective against subsequent *S. aureus* infection (60,61), it is particularly concerning that a monovalent or bivalent recombinant LukS-PV toxoid vaccine human trial is underway (http://www.clinicaltrials.gov/ct2/show/NCT01011335?term=panton valentine+leukocidin&rank=2). Host production of anti-PVL antibodies after

vaccination with this LukS-PV toxoid may actually worsen the outcome of *S. aureus* infection rather than providing some benefit to the host.

Based on previous studies and work presented in this dissertation, we propose that PVL functions primarily as an immune modulatory agent rather than a cytolytic toxin. How would a PVL-enhanced proinflammatory response be advantageous to *S. aureus* as a commensal organism or pathogen? It is possible that such a proinflammatory response limits dissemination and disease severity, thereby promoting a long-term commensal interaction with the human host. PVL-mediated alterations in the proinflammatory response are perhaps advantageous for *S. aureus*, particularly in certain host niches or time points during infection.

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