Panton-Valentine Leukocidin Facilitates the Escape of *Staphylococcus aureus* From Human Keratinocyte Endosomes and Induces Apoptosis

Chia-Yu Chi,1,2,3 Chia-Chun Lin,2 I-Chuang Liao,4 Yi-Chuan Yao,2 Fan-Ching Shen,2 Ching-Chuan Liu,3 and Chiou-Feng Lin1

1Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, 2National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, and 3Department of Pediatrics and 4Department of Pathology, National Cheng Kung University Hospital, Tainan, Taiwan

Skin and soft-tissue infections (SSTIs) caused by community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) have emerged as major health problems throughout the world. Most SSTI CA-MRSA strains produce Panton-Valentine leukocidin (PVL), but its contribution to CA-MRSA pathogenesis is poorly defined. Here, we used an endemic PVL-positive SSTI-causing CA-MRSA strain from Taiwan, together with an isogenic PVL-knockout mutant (Δpvl) and complemented PVL-positive derivative, to evaluate the role of PVL in the pathogenesis of CA-MRSA in the RHEK-1 human keratinocyte cell line and a rabbit skin infection model. We found that both PVL-positive CA-MRSA and isogenic Δpvl strains attached and were engulfed into endosomes of RHEK-1 cells within 1 hour following infection. However, by 2 hours after infection PVL-positive CA-MRSA more effectively disrupted endosomes, escaped into the cytoplasm, and replicated intracellularly. By 6 hours after infection, the PVL-positive strain caused significantly more caspase-dependent keratinocyte apoptosis than the isogenic Δpvl mutant. In the rabbit infection model, 1 week following infection the wild-type strain produced significantly more widespread lesions and cell apoptosis than the isogenic Δpvl mutant. These findings indicate that PVL is an important virulence factor that enables CA-MRSA to produce necrotizing skin infections by allowing the bacteria to escape from endosomes, replicate intracellularly, and induce apoptosis.

**Keywords.** endosome; keratinocyte; Panton-Valentine leukocidin; *Staphylococcus aureus*.

*Staphylococcus aureus* is an important human pathogen that causes a variety of infections, ranging from localized skin and soft-tissue infections (SSTIs) to severe necrotizing fasciitis and life-threatening disseminated infections. The ability of *S. aureus* to evoke these diverse clinical manifestations is attributed to its production of numerous exotoxins. One of these, Panton-Valentine leukocidin (PVL), is a 2-component (LukS-PV and LukF-PV) pore-forming toxin that targets phagocytic leukocytes [1, 2]. The emergence of community-associated methicillin-resistant *S. aureus* (CA-MRSA) has sparked interest in the potential importance of PVL in SSTIs [3]. Despite considerable progress in recent years in our understanding of the role of PVL in the pathogenesis of CA-MRSA, the mechanisms by which it produces skin damage are not well understood.

There is strong epidemiological evidence that PVL-producing strains are closely linked with necrotizing SSTIs [4–7]. However, there are conflicting reports of the role of PVL in animal models of SSTI, sepsis, and pneumonia [8–11]. The differences among the various studies may be related to the host animal species, microbial strain, target cell and organ, route of administration, and other methodological variations. The experimental host that most closely reflects disease in humans is uncertain. For example, rabbit neutrophils are more sensitive to the cytolytic activity of PVL toxin than those from mice and other species [1]. Intradermal injection of purified PVL in rabbits has been...
shown to provoke skin erythema followed by dose-dependent necrosis [5]. In one study, PVL-producing strains of *S. aureus* were shown to more effectively produce early skin lesions in rabbits than isogenic PVL knockout mutants [12]. In another study, the authors found a lack of uniformity in the ability of PVL-producing USA300 or ST8 strains and PVL-negative USA500 to produce skin lesions in rabbits [13]. It is possible that these findings were confounded by the genetic diversity of the staphylococcal strains.

Outbreaks of SSTIs caused by PVL-positive CA-MRSA are often associated with a single clone [14, 15], but there have been nonclonal relationships as well [16]. CA-MRSA are genetically diverse. In some regions, such as Taiwan, the most abundant CA-MRSA strains are quite distinct from those found in other parts of the world. The predominant SSTI-associated CA-MRSA clone in Taiwan is PVL-positive ST59. The PVL gene sequence of CA-MRSA ST59 contains a unique nucleotide substitution [17]. Similar strains have been isolated in the United States, Japan, and Australia recently [18].

The most appropriate target cell in which to study the pathogenesis of SSTIs caused by PVL-positive CA-MRSA is also unclear. Most in vitro studies of PVL have used human neutrophils or alveolar epithelial cells [19–21]. Leukocytes may not provide the most meaningful information since they are recruited to the site of infection after the initial stage of microbial penetration into the skin. Since PVL is strongly associated with SSTIs rather than systemic infections, attention was focused on cutaneous cells in this study. Keratinocytes were selected because they are the principal epidermal cells and have been shown to play an important role in innate and specific immune defense against superficial microbial infections [22, 23].

To determine the potential role of keratinocytes in host defense against PVL-positive CA-MRSA, an in vitro model of CA-MRSA infection was established in human keratinocytes and compared with an in vivo rabbit SSTIs model, using a well-defined representative clinical isolate of PVL-positive CA-MRSA ST59 along with an isogenic Δpvl strain and complemented PVL-positive derivative.

**METHODS**

**Bacterial Strains and Growth Conditions**

*Staphylococcus aureus* isolates were collected from patients with SSTIs at National Cheng Kung University Hospital (Tainan, Taiwan). Community-associated pathogenic strains were selected on the basis of clinical risk factors and manifestations; pulsortype and genotype, including multilocus sequence type (ST); staphylococcal cassette chromosome type (SCC) *mec* type; and the presence or absence of the *pvl* gene. *S. aureus* stains USA300 ST8 and RN4220 were kindly provided by T. L. Yang Lauderdale and L. J. Teng, respectively. Bacteria were grown in trypsin soy broth (Becton Dickinson). Overnight cultures were diluted 1:100 and incubated at 37°C with shaking (250 rpm). *S. aureus* was harvested at the mid-exponential phase of growth (OD600 0.8), washed in Dulbecco’s phosphate-buffered saline (DPBS; Invitrogen), and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) buffered with DPBS. Heat-inactivated *S. aureus* was obtained by incubating bacteria for 20 minutes at 60°C and plating on blood agar to verify the loss of viability.

**Construction of Isogenic lukF/S-PV Deletion Mutant and Complemented Strains**

Standard molecular biological methods, including the isolation of plasmid DNA and transformation into *Escherichia coli*, were performed as described previously [24]. Staphylococcal plasmid DNA was isolated using a HiYield Plasmid Mini Kit (RBC) in accordance with the manufacturer’s instruction, except that bacteria were incubated in PD1 buffer (RBC) that contained 25 µg/mL lysostaphin for 15 minutes at 37°C. To delete *lukF/S-PV* in *S. aureus* parental strains, polymerase chain reaction (PCR)–amplified regions flanking the *lukF/S-PV* locus were cloned into plasmid pESPES, a shuttle vector generated from pE194 and pSP722 (Promega). DNA fragments upstream (PCR fragment 1; 819 bp) and downstream (PCR fragment 2; 1919 bp) of *lukF/S-PV* and a gentamicin resistance gene, *aacA-aphD*, from the Tn4001 transposon (PCR fragment 3; 1644 bp) were amplified with Phusion Flash PCR-Master Mix (Finnzymes) in accordance with the manufacturer’s instructions. PCR-amplified regions flanking the *lukF/S-PV* loci and gentamicin resistance cassette were cloned into plasmid pESP3 and verified by DNA sequencing (Applied Biosystems). The resulting plasmid, pESP3*ΔlukF/S-PV* (Supplementary Figure 1A), was transformed sequentially into *E. coli, S. aureus*

**Table 1. Frequency of Genes Encoding for Toxins and Virulence Factors of Methicillin-resistant *Staphylococcus aureus* Isolated From Patients With Community-Acquired Skin and Soft-Tissue Infections (SSTIs) and Non-SSTIs**

<table>
<thead>
<tr>
<th>Gene</th>
<th>SSTIs (n = 51)</th>
<th>Non-SSTIs (n = 45)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pvl</em></td>
<td>49 (96)</td>
<td>5 (11)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td><em>tsst-1</em></td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>.47</td>
</tr>
<tr>
<td><em>can</em></td>
<td>1 (2)</td>
<td>6 (13)</td>
<td>.04</td>
</tr>
<tr>
<td>fnb-A</td>
<td>51 (100)</td>
<td>45 (100)</td>
<td>.47</td>
</tr>
<tr>
<td>fnb-B</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>.47</td>
</tr>
<tr>
<td><em>sea</em></td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>1.00</td>
</tr>
<tr>
<td><em>seb</em></td>
<td>46 (90)</td>
<td>36 (80)</td>
<td>.25</td>
</tr>
</tbody>
</table>

*By the Fisher exact test.
strain RN4220, S. aureus 2C-195 (a competent cell belonging to S. aureus clone ST59, kindly provided by F. J. Chen), and the target parental strain of CA-MRSA. The absence of lukF/S-PV genes, transcripts, and protein in the isogenic Δpvl mutant strain was verified using PCR, reverse-transcription PCR (RT-PCR), and Western blot assays, respectively (Supplementary Figure 1B–D). The plasmid pESP-PVL expressing lukF/S-PV was introduced into the Δpvl mutant to complement the deletion of lukF/S-PV. This plasmid was constructed by cloning a 2729-bp DNA fragment including the wild-type lukF/S-PV promoter and coding region into the pESP shuttle vector. After transforming pESP-PVL into the Δpvl mutant strain, the corresponding proteins were verified by Western blotting (Supplementary Figure 1D).

Figure 1. Community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) invades human keratinocytes. A, Confocal fluorescence microscopy images of the Panton-Valentine leukocidin (PVL)–positive CA-MRSA parental strain S2-1696 (top row) and an isogenic Δpvl mutant (bottom row) on monolayers of RHEK-1 cells 1 hour after infection. The bacteria were labeled with an anti-S. aureus fluorescein isothiocyanate (FITC) antibody, and the endosomes were labeled with anti-Rab-5, followed by an Alexa 598–tagged secondary antibody. Both staphylococcal strains were found to colocalize with endosomes. B, Electron micrographs of the same preparation showed that both staphylococcal strains were engulfed by endosomes of RHEK-1 cells. Arrows indicate the endosomal membrane.

Figure 2 continued. RHEK-1 cells 2 hours after infection. The bacteria were labeled with anti-S. aureus fluorescein isothiocyanate (FITC) antibody, and the endosomes were labeled with anti-Rab-5 or anti-EEA-1, followed by an Alexa 598–tagged secondary antibody. Cells infected with the PVL-positive strain developed a more distinct smear-like staining pattern, compared with those infected with the isogenic Δpvl mutant. B, Electron micrographs of the same preparation revealed that PVL-positive bacteria were free in the cytoplasm, whereas Δpvl mutant bacteria remained enveloped by the endosomal membrane. Arrows indicate the parental strain free in the cytoplasm. Hollow arrows show intact endosomal membranes surrounding the mutant bacteria. C, Intracellular PVL-positive CA-MRSA induces endosomal destruction. The fluorescence microscopy images and electron micrographs, described above, were analyzed quantitatively for endosomal destruction. Mean values ± SD were calculated from ≥3 independent experiments by a blinded observer. *P < .05, by the 2-sided paired Student t test. D, PVL protein interacts with the endosome-associated protein Rab-5. RHEK-1 cells were infected with PVL-positive CA-MRSA for 1.5 hours (multiplicity of infection, 100) and incubated for 1.5 hours in the presence of lysostaphin. Crude endosomal membrane extracts were made by passing cells through a needle and performing repeated freeze-thaw cycles. The resulting extracts were immunoprecipitated (IP) with anti-Rab5 antibody or control immunoglobulin (IgG) for 1 hour and incubated with magnetic protein G beads. The beads were then purified and washed on a magnetic column. Specifically bound proteins were eluted in sodium dodecyl sulfate–counting buffer and analyzed by immunoblotting (IB) with an anti-LukS antibody. E, PVL was localized by gold particle staining and transmission electron microscopy (TEM). RHEK-1 cells were infected with wild-type (S2-1696) and isogenic Δpvl mutant bacteria, prepared for TEM, and stained with gold-conjugated anti-LukS-PV antibody.
Figure 2. Panton-Valentine leukocidin (PVL)–positive community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) induces endosomal destruction. A, Confocal fluorescence microscopy images of PVL-positive CA-MRSA parental strain S2-1696 and an isogenic Δpvl mutant on monolayers of keratinocytes.
Eukaryotic Cell Culture
The AD12/SV40 immortalized keratinocyte cell line (RHEK-1) was used for in vitro studies. This cell line has been used in previous studies of keratinocyte proliferation and cytotoxicity [25, 26]. The cell line was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Biological Industries) at 37°C with 5% CO2.

Invasion Assay
Invasion of cultured cells was assayed by a modification of the method of Isberg and Falkow [27]. Briefly, RHEK-1 cells (2 × 10⁵) were seeded in 24-well tissue culture plates (Corning) containing RPMI 1640 medium. The bacterial cultures were washed 3 times with PBS and resuspended in the original volume. A multiplicity of infection of 50 S. aureus were added per well. After 1 hour of incubation at 37°C in 5% CO2, cells were detached from the culture plate and washed with growth medium. Extracellular bacteria were killed by adding 10 µg/mL lysostaphin (Sigma) and incubating cells for intervals as indicated.

Statistical Analysis
Data and images were statistically evaluated using the 2-tailed Student t test calculated with GraphPad Prism 4.0 Software.

RESULTS
Clinical Isolates
To define the molecular and clinical characteristics of PVL-positive CA-MRSA, 96 clinical isolates were obtained from patients with SSTIs (furuncles, carbuncles, cellulitis, and necrotizing fasciitis) and non-SSTI CA-MRSA systemic infections (bacteremia and endocarditis). The distribution of 7 staphylococcal toxin genes in each group is shown in Table 1. Almost all CA-MRSA strains (96%) isolated from patients with SSTIs possessed the pvl gene, compared with 11% from patients with non-SSTIs (P < .001; Table 1). All PVL-positive CA-MRSA isolates from patients with SSTIs belonged to ST59 and carried either SCCmec V (88%) or SCCmec IV (12%).

Morphological Characteristics of Human Necrotizing Fasciitis
To define the histological characteristics of severe skin infections caused by PVL-positive CA-MRSA, we examined tissue specimens from a patient with necrotizing fasciitis caused by PVL-positive CA-MRSA ST59 who received appropriate antimicrobial therapy. Marked coagulation, hemorrhagic necrosis, and inflammatory cell infiltrates were noted in hematoxylin-eosin preparations of specimens from the epidermis, dermis, and subcutaneous layers (Supplementary Figure 2). No bacteria were seen on Gram stain in specimens from the sites of necrosis and severe inflammation. We hypothesized that PVL released from invading bacteria, rather than increased bacterial proliferation per se, might have been responsible for the severe inflammatory response, tissue damage, and cell death. The following experiments were designed to explore this observation.

PVL Is a Virulence Determinant in CA-MRSA–Infected Human Keratinocytes

Generation of CA-MRSA PVL-Positive and PVL-Negative Isogenic Strains
To define the role of PVL in causing inflammation and tissue damage, we selected a PVL-positive CA-MRSA strain (S2-1696) that was representative of clinical isolates from patients with SSTIs. An isogenic lukF/S-PV knockout mutant (Δpvl) of S2-1696 was generated by allelic exchange (Supplementary Figure 1A), and lukF/S-PV were introduced on a plasmid to create a complemented derivative. The morphologic appearance, pigment characteristics, and growth rates of the strains in broth medium were identical. The presence or absence of the pvl locus and PVL expression were confirmed by RT-PCR and Western blot (Supplementary Figure 1B–D).

PVL Does Not Interfere With the Ability of CA-MRSA to Invade Keratinocytes
S. aureus is generally considered to be an extracellular pathogen, but there is both in vitro and in vivo evidence that it can invade host cells [28, 29]. Intracellular S. aureus has been shown to escape the endosome and induce apoptosis in epithelial cells. [30]. To determine whether PVL might affect the ability of CA-MRSA to be taken up and be internalized by human keratinocytes, we compared the ability of isogenic PVL-positive and PVL-negative strains to invade RHEK-1 cells. The morphological features of the infected cells revealed by confocal fluorescence and electron microscopy are shown in Figure 1A.
Figure 4. Panton-Valentine leukocidin (PVL)–positive community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) induces caspase-dependent apoptosis in human keratinocytes. A and B, Phase-contrast views of a monolayer of normal healthy RHEK-1 cells and those infected with S. aureus PVL-positive wild-type strain S2-1696. Cells with blebs are indicated by arrows. C and D, DAPI-stained image of cell monolayers shown in panels A and B, respectively. Aggregation and condensation of DNA were observed in many infected cells. E, Caspase-dependent cell death of infected cells. RHEK-1 cells were pretreated with z-VAD-fmk at 100 µM for 30 minutes and infected (multiplicity of infection [MOI], 100) with wild-type S. aureus (S2-1696), an isogenic Δpvl mutant, or a complemented mutant strain. Host cell viability was determined by trypan blue exclusion 6 hours after infection. Mean values ± SD were calculated from ≥3 independent experiments. F, Viable PVL-positive CA-MRSA are required to kill human keratinocytes. RHEK-1 cells were infected with viable or heat-inactivated wild-type S. aureus at an MOI of 100. Host cell viability was determined by trypan blue exclusion 6 hours after infection. The results are representative of at least 3 separate experiments. Abbreviations: NS, not significant; RPMI, Roswell Park Memorial Institute 1640 medium. *P < .05 and **P < .01, by the 2-sided paired Student t test.
Figure 5. Skin lesions produced in rabbits following intradermal inoculation of Panton-Valentine leukocidin (PVL)–positive and PVL-negative community-associated methicillin-resistant Staphylococcus aureus. A, Gross appearance of the lesions on days 0–7 after infection in rabbits infected with wild-type S. aureus (S2-1696) (a–h), the isogenic Δpvl mutant (i–p), or the complemented strain (q–x). The unit of measurement is 1 cm. B, Histopathologic appearance.
and 1B. Both the PVL-positive wild-type strain and the isogenic Δpvl mutant were taken up and localized within a Rab5-positive endosome 1 hour after infection.

**PVL Enhances Endosomal Destruction and Escape**

A marked difference in the patterns of fluorescent staining of cells infected with PVL-positive *S. aureus* or an isogenic Δpvl mutant was observed at 2 hours after infection (Figure 2A). Cells infected with the PVL-positive parental strain developed a more distinct smear-like staining pattern, compared with those infected with the isogenic Δpvl mutant. By electron microscopy, most PVL-positive bacteria were observed to be free in the cytoplasm, while most isogenic Δpvl mutant bacteria remained enveloped by an endosomal membrane (Figure 2B). A significantly higher number of cells infected with the parental strain contained disrupted endosomes, compared with cells infected with the isogenic Δpvl mutant (*P* < .05; Figure 2C).

Since PVL is a pore-forming toxin, we wondered whether PVL could directly target and destroy endosomes. To explore this possibility, a coimmunoprecipitation assay was performed using anti-Rab5 and anti-PVL antibodies. As shown in Figure 2D, PVL produced by the parental strain was found to be associated with the endosomal membrane. Furthermore, LukS-PV–specific gold-labeled electron micrographs demonstrated PVL on the endosomal membrane 1 hour after infection (Figure 2E).

**PVL Facilitates *S. aureus* Intracellular Replication**

Since PVL-positive CA-MRSA had a greater effect than an isogenic PVL-negative mutant in disrupting endosomes, we determined whether this was accompanied by a difference in intracellular bacterial replication. The time course of intracellular replication by the 2 strains is shown in Figure 3. Intracellular bacterial concentrations peaked 2 hours after infection and then decreased during the next hour as the cells became disrupted. The number of colony-forming units per cell of PVL-positive wild-type and complemented strains were significantly greater at each time point, compared with findings for the isogenic Δpvl mutant. These findings support the concept that PVL allows bacteria to escape from endosomes and multiply more effectively in the cytoplasm of RHEK-1 cells.

**PVL Triggers Caspase-Dependent Cell Death**

The cytotoxicity of the wild-type, PVL-positive CA-MRSA for RHEK-1 cells was observed by light microscopy. Marked cytotoxicity was produced at 3 hours after infection. Some infected cells were rounded or shrunken, formed blebs, and became detached from the plates. These morphological changes resembled those of apoptosis (Figure 4A and 4B). Infected cell monolayers were stained for DNA with DAPI (4’,6-diamidino-2-phenylindole). The nuclei of infected cells showed DNA fragmentation and chromatin condensation characteristic of apoptosis (Figure 4C and 4D). Host cell viability was determined by trypan blue exclusion 6 hours after infection. Cells infected with the PVL toxin–producing wild-type and complemented strains were significantly less viable, compared with cells infected with the isogenic Δpvl mutant (*P* < .05; Figure 4E). Preincubation of RHEK-1 cells with the pancaspase inhibitor zVAD-fmk was found to prevent cell death, especially in cells infected with the PVL-positive CA-MRSA strain (*P* < .01; Figure 4E). These findings indicate that cytotoxicity produced by the PVL-positive CA-MRSA is associated with caspase activation.

To further elucidate whether viable bacteria are required for CA-MRSA–induced cell death, we cocultured RHEK-1 cells with live or heat-inactivated bacteria and observed cell viability. As shown in Figure 4F, live PVL-positive bacteria had a significantly greater effect on the loss of host cell viability than the isogenic Δpvl mutant. Heat-inactivated bacteria had no effect on viability. These results indicate that live bacteria are required to produce cytotoxicity.

**PVL Is an Important Virulence Determinant in a Rabbit SSTI Model**

**Macroscopic Description of the Development of Lesions**

Female adult New Zealand White rabbits were injected intradermally with the wild-type, isogenic Δpvl mutant and complemented strains at different sites in their flanks. A visible inflammatory reaction was noted in all rabbits and at all sites of injection within the first 24 hours. The lesions enlarged over the next several days to form a central necrotic area surrounded by a region of inflamed skin (Supplementary Figure 3A–C). The PVL-positive wild-type and complemented strains produced broader and deeper lesions than the isogenic Δpvl mutant 7 days after infection (Figure 5A).

**Histological Examination**

Histological findings 7 days after infection are shown in Figure 5B. Rabbits infected with the PVL-positive wild-type

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Figure 5 continued. of skin lesions on day 7 after infection with the wild-type (column 1), isogenic Δpvl mutant (column 2) or complemented mutant (column 3) strains. Sections a, b, and c were stained with hematoxylin-eosin and shown at 20× magnification. Sections d, e, and f were selected from the boxed regions in the upper panels, stained by the Gram method, and examined at 1000× magnification. Section g, h, and i consist of TUNEL assay images photographed with the TissueFAXS system, using a Zeiss microscope. C. The percentage of TUNEL-positive cells was quantified with TissueQuest software (TissueGnostics). Data are mean ± standard error of the mean of 2 rabbits per group, based on pooled triplicate slides for each rabbit. Abbreviation: PBS, phosphate-buffered saline. **P* < .01, by the 2-sided unpaired t-test.
from rabbits infected with the PVL-positive wild-type strain were positively stained by the TUNEL assay, indicative of apoptosis. In contrast, <20% of cells from rabbits infected with the isogenic Δpvl mutant were apoptotic (Figure 5C and Supplementary Figure 3D and 3E).

DISCUSSION

In humans, SSTIs produced by CA-MRSA strains are strongly associated with PVL [6, 31]. However, the specific contribution of PVL to the pathogenesis of CA-MRSA SSTIs is unclear [11, 32]. In this study, we show that PVL promotes severe SSTIs by exerting toxic effects on host keratinocytes. PVL-positive CA-MRSA or an isogenic Δpvl mutant were equivalently taken up by human keratinocytes and engulfed within endosomes. But thereafter, wild-type PVL-positive CA-MRSA was able to disrupt the endosomal membrane, escape into the cytoplasm, replicate intracellularly, and induce host cell apoptosis. PVL-positive CA-MRSA was also able to produce necrotizing skin infections in a rabbit infection model that were more widespread and associated with more extensive skin cell apoptosis than those produced by an isogenic PVL-negative strain. The specific importance of PVL was confirmed using a complemented PVL-positive strain.

*S. aureus* is generally regarded as an extracellular microorganism, but there is ample evidence that it can be internalized by a variety of host cells [30, 33] in a fibronectin-binding protein (FnBP)–dependent manner [34]. Strain-dependent, FnBP-independent invasion mechanisms have also been reported for primary human keratinocytes [35]. PVL is regulated by the products of the *agr* global transcriptional regulatory locus and expressed late in the growth cycle. In the current study, PVL did not appear to have a role in the initial process of cellular internalization of *S. aureus*.

Destruction of the endosomal compartment has been described for several bacterial species, such as *Rickettsia, Listeria, Mycobacterium*, and *Shigella* organisms. *Listeria* and *Shigella* species produce specific hemolysins (listeriolysin O and IpaB, respectively) that enzymatically degrade the endosomal membrane [36]. ESAT-6, a protein with membrane-lysing properties, is secreted by *Mycobacterium tuberculosis* to produce pores in the phagosomal membrane and facilitate the escape of bacteria to the cytosol [37]. Staphylococcal α-toxin, another pore-forming toxin, has been shown to mediate endosomal escape in phagocytes. [38, 39]. Accordingly, we sought to determine whether PVL, which is a staphylococcal synergohymenotropic exotoxin belonging to the pore-forming toxin family [40], might have similar effects. An additional consideration was that the endosomal compartment provides a restricted microenvironment that could rapidly activate the *agr* system. In this scenario, transcription of the *pvl* locus might occur within this microenvironment. We found that the PVL produced by wild-type CA-

![Figure 6. Model of Panton-Valentine leukocidin (PVL)–positive community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) interaction.](image-url)

**Figure 6.** Model of Panton-Valentine leukocidin (PVL)–positive community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) interaction. Bacteria enter the keratinocyte via the endocytic pathway into a Rab5-decorated endosome. PVL produced by wild-type *S. aureus* interacts with and disrupts the endosomal membrane, facilitating escape of the bacteria into the cytoplasm, where they rapidly replicate and induce caspase-dependent cell death. In contrast, an isogenic Δpvl mutant is trapped and degraded in the endolysosome.

strain exhibited marked acute inflammation in the deep dermis and subcutaneous layer, with well-defined abscess formation and a necrotic center surrounded by a fibrous capsule and granulation tissue. The PVL-positive complemented strain produced extensive epidermal necrosis, marked suppurative inflammation in the deep dermis and subcutaneous tissue, and features of infarction in the deep fascia. Some early granulation tissue was noted surrounding the destructive lesions. In contrast, rabbits infected with the isogenic Δpvl mutant strain exhibited small focal abscesses in the deep dermis and subcutaneous areas, surrounded by a thick fibrous capsule and granulation tissue.

All 3 staphylococcal strains could be visualized in scant amounts by Gram stain at the center of the abscesses, but the most striking finding was the presence of massive numbers of the Δpvl mutant but not the PVL-positive strains at the periphery of the lesions.

**Detection of Apoptosis**

An in situ TUNEL assay was used to determine the presence of apoptotic cells in the skin lesions. We found that >30% of cells were positively stained by the TUNEL assay, indicative of apoptosis. In contrast, <20% of cells from rabbits infected with the isogenic Δpvl mutant were apoptotic (Figure 5C and Supplementary Figure 3D and 3E).
MRSA interacted with and permeabilized the endosomal membrane to release bacteria into the cytosol within 2 hours after infection. Endosomal escape may be mediated by different staphylococcal pore-forming toxins, depending on the genotype of the strain and the susceptibility of the host cells. This study did not examine effects of the α-toxin. However, PVL was found to play a critical role in destroying human keratinocytes. Interventions to inhibit the production of PVL or its ability to disrupt endosomal membranes might be able to ameliorate skin infections caused by PVL-positive CA-MRSA.

We observed that PVL-positive CA-MRSA induces apoptosis in RHEK-1 cells and rabbit skin. These observations are reminiscent of previous studies showing that Shigella flexneri induces apoptosis in macrophages, T cells, and B cells [41]. Induction of apoptosis by S. flexneri ingested in macrophages is mediated by binding of the IpaB invasin to interleukin 1 (IL-1)–converting enzyme caspase-1 [42]. An IpaB-like invasin has not been identified in S. aureus, but recombinant PVL has been shown to target mitochondria and induce Bax-independent apoptosis of human neutrophils [19]. In the present study, the pan-caspase inhibitor zVAD-fmk prevented most of the PVL-induced cell death, suggesting that PVL promotes the induction of apoptosis by a caspase-dependent pathway in human keratinocytes. Some pathways of cell death have been shown to trigger the release of proinflammatory cytokines [43] and initiate an acute inflammatory response. Keratinocytes are known to be major contributors to epidermal cytokine production [44]. In response to external stimuli, including microbial infection, keratinocytes rapidly produce chemokines and cytokines to recruit and activate phagocytes [45]. In a model recently reported by Soong et al, significant activation of caspase-1 and production of IL-1β by keratinocytes was observed in response to the accumulation of intracellular S. aureus [46]. Increases in IL-1β secretion would be expected to increase polymorphonuclear leukocyte recruitment to the skin and enhance proinflammatory cell death [47]. This concept is supported by our histopathological observations that apoptotic cells were associated with infiltrating polymorphonuclear cells in the skin of rabbits infected with the PVL-positive wild-type CA-MRSA and the complemented strain.

The more limited necrosis and inflammation produced by an isogenic Δpvl mutant suggests that additional staphylococcal virulence factors, such as phenol-soluble modulins or α-toxin, might also contribute to the development of the skin lesions. It is possible that these factors act in concert with PVL to enhance keratinocyte cell death and the inflammatory response but may not be critical virulence determinants in the PVL-positive CA-MRSA ST59 strain used in these studies. These observations contrast with findings for the USA300 strain [11, 32]. It is postulated that specific redundant cytotoxins expressed in the USA300 strain are not expressed in the ST59 strain, resulting in the differences in the dependence on PVL for cutaneous pathology. To gain enhanced understanding of genetic characteristics in USA300 and ST59 strains, comparative whole-genome sequence analysis is ongoing. Collectively, our observations support the notion that host cells sense the presence of bacteria in the cytoplasm as a danger signal, activate the apoptosis cascade, and stimulate the innate immune system to control the infection. These inflammatory responses are overwhelming in PVL-positive CA-MRSA infected hosts.

This study has revealed an important role of PVL in the pathogenesis of S. aureus SSTIs, which are strongly associated with PVL in MRSA strains. The following model based our findings is proposed (Figure 6). PVL released from CA-MRSA facilitates the escape of the bacteria from the endosome into the cytoplasm of infected keratinocytes, allowing bacteria to replicate in the cytoplasm. This stimulates induction of the apoptotic cascade, followed by the release of inflammatory cytokines, recruitment of leukocytes, and further cell damage.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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