**Staphylococcus aureus** Panton-Valentine Leukocidin Contributes to Inflammation and Muscle Tissue Injury

Ching Wen Tseng1,2, Pierre Kyme1,2, Jennifer Low1,2, Miguel A. Rocha3,4, Randa Alsabeh5, Loren G. Miller6, Michael Otto7, Moshe Arditi1,2, Binh An Diep8, Victor Nizet9, Terence M. Doherty1,2, David O. Beenhouwer3,4, George Y. Liu1,2*

1 Division of Pediatric Infectious Diseases and the Immunobiology Research Institute, Cedars-Sinai Medical Center, Los Angeles, California, United States of America, 2 Department of Pediatrics, David Geffen School of Medicine, University of California, Los Angeles, California, United States of America, 3 Division of Infectious Diseases, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California, United States of America, 4 Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, California, United States of America, 5 Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, California, United States of America, 6 Division of Infectious Diseases, Harbor-University of California Los Angeles, Torrance, California, United States of America, 7 Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, Bethesda, Maryland, United States of America, 8 Division of Infectious Diseases, Department of Medicine, University of California, San Francisco, California, United States of America, 9 Department of Pediatrics and Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California, La Jolla, California, United States of America

**Abstract**

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) threatens public health worldwide, and epidemiologic data suggest that the Panton-Valentine Leukocidin (PVL) expressed by most CA-MRSA strains could contribute to severe human infections, particularly in young and immunocompetent hosts. PVL is proposed to induce cytolysis or apoptosis of phagocytes. However, recent comparisons of isogenic CA-MRSA strains with or without PVL have revealed no differences in human PMN cytolytic activity. Furthermore, many of the mouse studies performed to date have failed to demonstrate a virulence role for PVL, thereby provoking the question: does PVL have a mechanistic role in human infection? In this report, we evaluated the contribution of PVL to severe skin and soft tissue infection. We generated PVL mutants in CA-MRSA strains isolated from patients with necrotizing fasciitis and used these tools to evaluate the pathogenic role of PVL in vivo. In a model of necrotizing soft tissue infection, we found PVL caused significant damage of muscle but not the skin. Muscle injury was linked to induction of pro-inflammatory chemokines KC, MIP-2, and RANTES, and recruitment of neutrophils. Tissue damage was most prominent in young mice and in those strains of mice that more effectively cleared *S. aureus*, and was not significant in older mice and mouse strains that had a more limited immune response to the pathogen. PVL-mediated injury could be blocked by pretreatment with anti-PVL antibodies. Our data provide new insights into CA-MRSA pathogenesis, epidemiology and therapeutics. PVL could contribute to the increased incidence of myositis in CA-MRSA infection, and the toxin could mediate tissue injury by mechanisms other than direct killing of phagocytes.

**Citation:** Tseng CW, Kyme P, Low J, Rocha MA, Alsabeh R, et al. (2009) Staphylococcus aureus Panton-Valentine Leukocidin Contributes to Inflammation and Muscle Tissue Injury. PLoS ONE 4(7): e6387. doi:10.1371/journal.pone.0006387

**Editor:** Stefan Bereswill, Charité-Universitätsmedizin Berlin, Germany

**Received** June 19, 2009; **Accepted** June 26, 2009; **Published** July 27, 2009

**Copyright:** © 2009 Tseng et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by a Burroughs-Wellcome Career Award and by National Institutes of Health grant AI074832 to G. Y. Liu. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: george.liu@cshs.org

**Introduction**

Although previously confined to hospitals and nursing homes, methicillin-resistant *Staphylococcus aureus* (MRSA) has encroached upon immunocompetent populations and poses a growing threat to public health worldwide [1–4]. The Panton-Valentine leukocidin (PVL) is a two-component (LukS-PV and LukF-PV) pore-forming toxin secreted by most CA-MRSA strains with demonstrated *in vitro* activity against human leukocytes in its purified form [5]. The toxin is linked in multiple clinico-epidemiological studies to unusually severe disease pathology [6–9], especially in young, previously healthy hosts [8,9], an association that has earned PVL the unproven and controversial reputation of being the major virulence determinant of severe CA-MRSA infections such as necrotizing pneumonia, myositis and necrotizing fasciitis. The virulence of PVL has been formally studied in the laboratory using isogenic *S. aureus* strains (with or without PVL) in murine models of skin infection and necrotizing pneumonia [10–15]. However, the published results from multiple groups have been strongly conflicting. In one notable study, introduction of the *lukSF-PV* genes into a PVL− *S. aureus* laboratory strain significantly enhanced pathogenic potential in a mouse pneumonia model [12]. By contrast, deletion of *lukSF-PV* from the genome of two CA-MRSA strains, MW2 (USA400) and LAC (USA300) had no impact on virulence of the strains in murine models of skin, lung, and bloodstream infection in several published studies [10–13,15]. These subsequent investigations dampened enthusiasm for PVL as a major virulence determinant of CA-MRSA infections.

One caveat regarding analysis of PVL in small animal models is a demonstrable species specificity of toxin susceptibility; for example, human cells have been reported to be 10-fold more susceptible to PVL-mediated lysis than mouse cells *in vitro* [34]. Two groups have...
reported that administration of microgram amounts of purified PVL toxins under the skin of rabbits or into the lung of mice produced significant pathology and inflammation [12,16]. The findings suggest that PVL expression by live CA-MRSA strains during mouse or rabbit infection could be below a critical threshold for tissue injury, thus accounting for the lack of a consistent PVL-related pathology. To probe this possibility, we investigated in a mouse skin and soft tissue infection model whether administration of a higher inoculum of CA-MRSA would uncover a PVL virulence phenotype.

**Results**

**PVL contributes to muscle but not skin injury**

Bacterial strains applied to our infection model included two PVL\(^+\) USA300 strains isolated from wounds of patients with necrotizing fasciitis (CST5 and CST6), a PVL\(^-\) strain (Newman) and PVL\(^+\) or PVL\(^-\) isogenic strains engineered from these bacteria [22] (Table S1). A Western blot confirming PVL expression by PVL\(^+\) strains, but not PVL\(^-\) strains, is shown in Figure 1A.

Previous investigations have demonstrated that a minimum inoculum of 10\(^7\) bacteria is typically required to induce a consistent skin lesion in mice [10], and inocula between 10\(^7\) and 10\(^8\) CFU have been routinely used in S. aureus skin infection studies. To determine whether a higher inoculum would unveil a PVL virulence role, CD1 mice were inoculated subcutaneously on one flank with 10\(^9\) CFU of a PVL\(^+\) strain, and on the opposite flank with an equal inoculum of an isogenic PVL\(^-\) strain. The mice were euthanized on day 3 for analysis of lesions. As shown in Figure 1A and B, mice exhibited no differences in skin lesion size at the site infected with PVL\(^+\) or PVL\(^-\) strains. Unexpectedly however, PVL\(^+\) strains induced larger muscle lesions compared to isogenic PVL\(^-\) strains (Figure 1A and C), and complementation of the mutant with a PVL expression vector restored the ability to cause more severe muscle injury (Figure S2A). The number of bacteria recovered from lesions produced by PVL\(^+\) and PVL\(^-\) bacteria were comparable (Figure 1D and S2B); hence the increased lesion severity associated with PVL\(^+\) strains could not be explained by increased bacterial survival in vivo. CD1 mice, infected with 10\(^7\) or 10\(^8\) PVL\(^+\) or PVL\(^-\) isogenic strains, showed no difference in muscle tissue injury (Figure S1).

**Histology and PVL expression in infected tissue**

To determine whether PVL\(^+\) and PVL\(^-\) strains contribute to differences in tissue histology, H&E stain was performed. Overall, H&E stain showed marked necrosis and neutrophil infiltration within the central focus of infection produced by PVL\(^+\) or PVL\(^-\) strains, but beyond lesion size differences, a clear-cut difference in pathology was not discernable (Figure S3).

To evaluate PVL expression in injured tissues, infected skin and muscle were excised for PVL immunofluorescence and histologic analyses. As evidenced by diffuse staining throughout the tissue

---

**Figure 1. PVL promotes muscle injury.** CD1 mice were injected subcutaneously with 10\(^9\) PVL\(^+\) S. aureus (Newman+PVL vector, CST5, CST6) on one flank, and 10\(^8\) isogenic PVL\(^-\) S. aureus (Newman+empty vector, PVL\(^-\) CST5, PVL\(^-\) CST6) on the opposite flank. Mice were sacrificed 3 days post-infection. For data set B-D, graphs on the left display ratios of lesion sizes or ratios of CFU (PVL\(^+\)/PVL\(^-\)) based on measurements produced in individual mouse; graphs on the right show lesion sizes or CFU grouped according to bacterial strains. (A) Left: Western blot showing expression of LukF-PV, LukS-PV, and \(\alpha\)-toxin by CST5/\(+\)/PVL and CST6/\(+\)/PVL; right: Representative images of skin and muscle lesions. Arrows point to muscle lesion. (B) Skin lesion sizes (C) muscle lesion sizes. (D) Total tissue CFU. In 1C (right panel), muscle lesions harvested from the same mice are joined by a line. * \(p<0.05\), ** \(p<0.01\).

doi:10.1371/journal.pone.0006387.g001
slices, the PVL⁺ necrotizing fasciitis clinical isolate (CST5) and PVL⁺ Newman strain both expressed the toxin in vivo (Figure 2A). PVL staining was particularly prominent around PVL⁺ S. aureus clusters, but was also noted on select muscle bundles, particularly at early time points of infection (Figure 2A and B). Based on measurement of PVL concentration by ELISA (Figure 2C), PVL⁺ Newman secretes lower levels of PVL compared to CST5, even though Newman showed a more prominent PVL effect on muscle injury compared to CST5. The measured toxin level in tissues was in the range of 10–20 ng/ml at day 3, and never significantly exceeded those levels at earlier time points based on a time course experiment performed using PVL⁺ Newman (data not shown). Of note, the ELISA is likely to underestimate the actual PVL concentration in infected tissue since much of the toxin that intercalated into host cell membranes might not have been adequately solubilized to permit measurement in an ELISA.

**PVL-specific antibodies block PVL mediated muscle injury**

To investigate whether blockade of PVL could reduce the extent of muscle injury, mice were injected intraperitoneally with PVL-specific antibodies or DPBS, then challenged the next day with ~10⁹ bacteria. As shown in Figure 3A and B, pretreatment with PVL-specific antibodies significantly reduced the size of muscle lesions induced by PVL⁺ strains, but did not alter lesion sizes caused by PVL⁻ strains. Results of these experiments corroborate the pathogenic role of PVL in severe soft tissue infections in the mouse model, and once again indicate that damage inflicted by PVL predominantly affects deep muscle tissues but not superficial skin layers.

**PVL contributes to inflammation**

It is well established that host inflammation can have devastating consequences during infection [23]. Because PVL cytolysis of PMN is difficult to demonstrate in vitro or in vivo using live bacteria, we next asked whether PVL induces inflammation during infection, by excising lesions from infected CD1 mice and measuring expression of select cytokines and chemokines by ELISA. As shown in Figure 4A–C, PVL-expressing S. aureus Newman induced higher levels of chemokines KC, MIP-2, and RANTES in the injured tissues than its isogenic PVL⁻ mutant; no differences in TNF-α and IL1β induction were observed between the two strains (Figure 4D and E). These results are consistent with report by König and coworkers that sublytic PVL toxin concentration could induce secretion of IL-8 (human equivalent of KC) by PMN [24].

**Effect of host genetic background on PVL virulence**

Next, to facilitate studies of immune response to PVL, we repeated the infection experiments in C57/B6 mice, the back-

---

**Figure 2. PVL expression in infected tissues.** CD1 mice were injected subcutaneously with 10⁹ PVL⁺ S. aureus (Newman+PVL vector, CST5) on one flank, and 10⁹ isogenic PVL⁻ S. aureus (Newman-empty vector, PVL⁻ CST5) on the opposite flank. (A) PVL immunofluorescence staining of uninfected and infection tissues at 72 h post-infection. Left panels: tissues were stained with PVL-FITC; middle panels: DNA was stained with DAPI; right panels: PVL and DAPI stains were merged. E+D:epidermis-dermis layer, SA: S. aureus, and M:muscle. (B) PVL immunofluorescence staining of uninfected and infection tissues. Shown are PVL-FITC, phalloidin conjugated to Texas Red, and DAPI (DNA) stainings of tissues collected at 30 minutes post-infection. D: dermis, and M:muscle. (C) Level of tissue PVL measured by ELISA at 72 h post-infection. ** p<0.01.

doi:10.1371/journal.pone.0006387.g002
Pathogenic effects of PVL in older mice

Previous epidemiologic studies have demonstrated an association between PVL and severe infections among young and immunocompetent individuals [8,9]. To examine whether pathogenic effects of PVL vary with age, 6 month-old mice were injected subcutaneously with isogenic PVL+/− CST5 strains. As shown in Figure 5A, the 6 month old mice had significantly smaller muscle lesions, reduced chemokine secretion, but had a much higher tissue bacterial load compared to 10–12 week old mice. In older mice, PVL had no impact on tissue injury, neutrophil recruitment, or surviving CFU (Figure 6A, B and C), but had a small but significant impact on chemokine secretion (Figure 6D and E). These results are consistent with findings that older individuals have more limited immune response to bacterial challenge [25–28], and have less severe pathology associated with PVL [8,9].

Discussion

Multiple epidemiologic studies have provided compelling evidence linking PVL to pathogenicity of S. aureus infections [6,7,29–31]. Notably, an S. aureus strain responsible for an epidemic in the 1950’s (phage type 81/80) also harbored the LukFS-PV genes; the strain mysteriously disappeared following the introduction of methicillin [32], but PVL resurfaced in the 1990’s in association with severe cases of necrotizing pneumonia and furunculosis in MSSA and CA-MRSA strains [8,33]. However whether PVL itself is a causative factor responsible for increased disease severity has been heatedly debated because of failure by multiple groups to demonstrate that PVL has a virulence role [10–15]. More specifically, in the skin infection model, Voyich et al. subsequently Bubeck Wardenburg et al. infected C57BL/6 and BALB/c mice inoculated with the WT CST5 strains. As shown in Figure 6A, B and C, no PVL phenotype) cleared WT CST5 strains. As shown in Figure 6A, B and C, but had a small but significant impact on chemokine secretion (Figure 6D and E). These results are consistent with findings that older individuals have more limited immune response to bacterial challenge [25–28], and have less severe pathology associated with PVL [8,9].

Discussion

Multiple epidemiologic studies have provided compelling evidence linking PVL to pathogenicity of S. aureus infections [6,7,29–31]. Notably, an S. aureus strain responsible for an epidemic in the 1950’s (phage type 81/80) also harbored the LukFS-PV genes; the strain mysteriously disappeared following the introduction of methicillin [32], but PVL resurfaced in the 1990’s in association with severe cases of necrotizing pneumonia and furunculosis in MSSA and CA-MRSA strains [8,33]. However whether PVL itself is a causative factor responsible for increased disease severity has been heatedly debated because of failure by multiple groups to demonstrate that PVL has a virulence role [10–15]. More specifically, in the skin infection model, Voyich et al. subsequently Bubeck Wardenburg et al. infected C57BL/6 and BALB/c mice with 10^7 WT or PVL KO in the S. aureus LAC USA300 background. In both studies, the authors report either no difference in skin lesion size (in BALB/c or C57BL/6 mice) or a lesser extent) in BALB/c mice at 3 h post-infection, but not in SKH1 and C57BL/6 mice (Figure 5G). No difference was observed between WT S. aureus and the isogenic PVL knockout mutant when MPO was measured at 12 h (Figure S4B). Overall, these results suggest that PVL induction of host immune responses depends on the genetic background of the mouse. We noted interestingly that CD1 and BALB/c mice (PVL phenotype) cleared WT CST5 infection much more effectively compared to SKH1 or C57BL/6 mice (no PVL phenotype), but developed significantly larger lesions compared to SKH1 or C57BL/6 mice (Figure 5A and B). A possible interpretation of these findings could be that CD1 and BALB/c mice detect and respond to PVL with an exaggerated proinflammatory reaction and neutrophil recruitment, which achieves more rapid bacterial clearance, but at a simultaneous cost of more extensive collateral damage to host tissues.

Figure 3. Anti-PVL antibodies ameliorate severity of muscle injury. CD1 mice were injected intra-peritoneally with PBS or rabbit antisera against LukS-PV and LukF-PV. Twenty four hours post-infection, mice were infected on opposite flanks with either PVL+ or PVL− isogenic S. aureus. Shown are ratios of lesion sizes (PVL+/PVL−) and muscle lesion sizes from individual mice on day 3 post-infection. Bacterial inocula were (A) Newman+/−PVL and (B) CST6+/−PVL. ** p<0.01. doi:10.1371/journal.pone.0006387.g003

ground mouse strain on which most knockouts are maintained. Injection of C57/B6 mice with 10^7 WT CST5 and PVL− CST5 mutant, unexpectedly, elicited muscle lesions of comparable size (Figure 5C), suggesting that the mouse’s genetic background is a further determinant of PVL-induced disease pathology. Previously, Bubeck Wardenburg and colleagues have demonstrated that host background differences could be a determinant of PVL virulence [11,13]. In their study, PVL showed no virulence effect in C57BL/6 mice [11] but paradoxically attenuated pathogenicity of S. aureus in BALB/c AnNHsd mice [13]. To further evaluate whether and how the mouse genetic background and immune system contribute to PVL mediated injury, we examined skin and soft tissue infection in four different mouse strains: BALB/c, C57BL/6, SKH1, and CD1. As shown in Figure 5C, PVL contributed to muscle lesions in CD1 and BALB/c mice, but had no effect on lesion severity in SKH1 and C57BL/6 mice. The presence of PVL related injury in any particular strain of mouse correlated directly to differences in PVL-associated chemokine secretion (Figure 5E and F). Specifically, the CST5 PVL− strain elicited significantly higher levels of MIP-2 and KC in CD1 and BALB/c mice compared with the isogenic PVL+ strain, but this chemokine differential did not occur in SKH1 or C57BL/6 mice. Furthermore, as measured by tissue MPO activity, PVL induced increased neutrophil infiltration in CD1 and (to a lesser extent) in BALB/c mice at 3 h post-infection, but not in SKH1 and C57BL/6 mice (Figure 5G). No difference was observed between WT S. aureus and the isogenic PVL knockout mutant when MPO was measured at 12 h (Figure S4B). Overall, these results suggest that PVL induction of host immune responses depends on the genetic background of the mouse. We noted interestingly that CD1 and BALB/c mice (PVL phenotype) cleared WT CST5 infection much more effectively compared to SKH1 or C57BL/6 mice (no PVL phenotype), but developed significantly larger lesions compared to SKH1 or C57BL/6 mice (Figure 5A and B). A possible interpretation of these findings could be that CD1 and BALB/c mice detect and respond to PVL with an exaggerated proinflammatory reaction and neutrophil recruitment, which achieves more rapid bacterial clearance, but at a simultaneous cost of more extensive collateral damage to host tissues.
coworkers have reported that mouse phagocytes are less susceptible to lysis compared to human phagocytes and show an approximate ten-fold difference in membrane permeability following purified PVL toxin challenge as measured by 51-Cr release assay [34]. Additionally, we have found in our study that the concentration of PVL measured in mouse tissue, following subcutaneous infection with \(10^9\) PVL\(^+\) CA-MRSA, is in the range of 10–20 ng/ml, which even with an underestimate of the PVL concentration in infected tissue for technical reasons (see Results), is still 50–100-fold lower than PVL measured from human abscess samples (median, 1 mg/ml) [35]. The possibility of differential PVL induction in human and mouse tissues in human and mouse response is another potential confounding factor in extrapolating animal data to the human condition. Ultimately, the role of PVL in human diseases will need to be addressed by the use of humanized animals, or possibly by the therapeutic effect of PVL-specific antibodies on human CA-MRSA infections.

Notwithstanding the differences between human and mouse infection, our model has features that mimic the human disease. PVL in the mouse preferentially causes injury of the underlying muscle following subcutaneous CA-MRSA infection, and histologic evaluation showed moderate staining of muscle tissues with anti-PVL antibodies. Consistent with this finding, we have recently submitted a report of a child with myositis caused by a PVL\(^+\) methicillin-sensitive \(S\). \(aureus\) strain, whose muscle tissue stained selectively and strongly with a PVL specific antibody. It is not known how PVL binding to muscle tissues or PVL induced chemokines contribute to muscle injury. However, given the reported association of severe myositis with CA-MRSA and PVL, the parallel findings in human and mouse strongly suggest a causal link between PVL and human myositis [36].

Multiple studies point to a strong association between PVL and severe necrotizing pneumonia and furunculosis in the current CA-MRSA epidemic [8,9]. These infections appear to target young and healthy hosts, who appear to suffer more severe infection compared to older or immunosuppressed individuals. Consistent with these findings, we observed that PVL induced greater inflammation and caused greater pathology in mice without conferring a bacterial survival advantage. Further, a PVL effect on tissue pathology was apparent in those mice with the most effective immune clearance of \(S\). \(aureus\) (10–12 week old CD1 and BALB/c mice), but was insignificant in mice that had a more limited response to the pathogen (C57BL/6, SKH1, and 6 month old CD1 mice). These findings, when taken together with the cytokine results, suggest a parallel between our model and the human infection, and suggest two conclusions: 1) in CA-MRSA, a primary pathogenic effect of PVL is to provoke an overly-robust inflammation, and 2) the severity of PVL-specific pathology may ultimately depend on the capacity of the host immune system for mounting an aggressive neutrophil response to infection. These results are consistent with human epidemiologic reports indicating that younger, immunocompetent individuals infected with MRSA are more susceptible to severe injury [8,9], and suggest that individuals with enhanced immune response to pathogens are at higher risk for more serious pathology.

Figure 4. Tissue chemokines and cytokines after infection with \(S\). \(aureus\) Newman (+/− PVL). CD1 mice were infected on opposite flanks with either Newman-empty vector or Newman+PVL expression vector. Infected tissues were harvested at the indicated time post-infection and tissue chemokines and cytokines were measured by ELISA. (A) Tissue MIP-2, (B) RANTES, (C) KC, (E) TNF-α, and (D) IL-1β. Controls consisted of DPBS injected mice. * \(p<0.05\).

doi:10.1371/journal.pone.0006387.g004
Prior efforts to link PVL to human pathology have focused primarily on the cytolytic properties of PVL. Lysis of human phagocytes could be readily demonstrated using purified PVL [34], but a study conducted using live WT and PVL knockout S. aureus showed no lytic effect of the toxin on human neutrophils across a range of bacterial concentrations [10]. Our study suggests
that a function of PVL more readily achievable at physiologic
doses is its ability to provoke inflammation and recruit immune
effector cells such as neutrophils through activation of inflamma-
tory molecules (e.g., RANTES, KC, and MIP-2). In vitro, PVL
induction of IL8 could be demonstrated using human neutrophils
at a concentration lower than that required to induce cell lysis
[37]. These findings suggest that PVL induced PMN cytolysis as
the primary explanation of PVL injury may need to be
reevaluated. Under our model, enhanced inflammation, particu-
larly recruitment of phagocytes, could explain PVL-associated
“spider bite” lesions and abscesses (representing phagocyte
accumulation), which have become the most common presenta-
tion of CA-MRSA skin and soft tissue infections [38]. Though our
study highlighted differences in chemokine induction by PVL+ and
PVL− strains, PVL likely induces additional pro-inflammatory
factors during infection. In tissue culture, König and coworkers
have shown that PVL triggers secretion of leukotriene B4 and
oxygen metabolites when incubated in the presence of human
neutrophils [37]. Hence, inflammation initiated by multiple pro-
inflammatory mediators is likely an important contributor to PVL-
mediated pathology in our model.

Finally, our data suggest that application of antibodies against
PVL could limit or even abrogate PVL-mediated injury. However,
anti-PVL antibodies may not be effective for all CA-MRSA
infections. In preliminary experiments, we have tested few
additional CA-MRSA WT and PVL KO strains (LAC and
MW2), but found that under our experimental conditions, PVL
expressed by LAC and MW2 was associated with increased
bacterial survival but similar level of muscle tissue injury on day 3
(unpublished data). We are performing more exhaustive studies in
mice to identify conditions under which PVL expressed in other S.
aureus backgrounds would induce muscle injury.

In summary, we have developed a model of severe necrotizing
soft tissue infection in which PVL shows significant contribution to
muscle injury. Though the model does not by itself resolve the
debate on the relative importance of PVL in the MRSA epidemic, it
unveils surprising parallels between the mouse and human disease,
and provides novel insights towards PVL-related immunopathology;
hence the model could prove to be valuable for further investigation
of PVL functions.

Materials and Methods

Bacterial strains and growth conditions

Two clinical CA-MRSA isolates (CST5 and CST6), their isogenic
PVL knockout strains, one laboratory strain (Newman) transformed
with either an empty vector or a PVL expression vector were used for
this study (Table S1). The PVL knockout of CST5 and CST6 isolates
were constructed by site-directed mutagenesis using primer pairs
GAAAGGAAATGATTTTTAGGTC, GACCTAAAAAT-
CATTTCCTTC, AATATTCTATTGGAAAGGCCACC and
CTCAATATTGTTATCAGCTTTAG to introduce two stop
codons in the lukS-PV open reading frame (ORF). The DNA
fragments containing the two stop codons were cloned into pMAD
[17], and then electroporated into RN4220. The mutant allele was
transduced into CST5 and CST6 using phage λ218 using a previously
established protocol [18]. For complementation and overexpression
studies, the PVL locus was amplified by PCR using primer pair CTTGACCACCGGTATCGAGCCGCATATTGCATCAATTCAC

Figure 6. Effect of innate immunity and mouse age on PVL virulence function. Ten to twelve week old CD1 mice were infected on opposite
flanks with either PVL+ CST5 or isogenic PVL− CST5. (A) Muscle lesions, (B) total tissue CFU 3 days post-infection, (C) tissue MPO activity at 3 h post-
infecition, and (D) and (E) KC and MIP-2 levels at 3 h post-infection. * p<0.05, ** p<0.01, *** p<0.005.
doi:10.1371/journal.pone.0006387.g006

PLoS ONE | www.plosone.org 7 July 2009 | Volume 4 | Issue 7 | e6387
GAGCTGGTTACC and cloned into shuttle vectors (Table S1). S. aureus strains were routinely cultured on sheep blood agar plates and colonies with comparable hemolysis phenotypes were selected for each experiment. Bacteria were also cultured in Todd-Hewitt broth or L-broth at 37°C with shaking at 250 rpm.

Cloning and expression of rLukF-PV and rLukS-PV

lukS-PV and lukF-PV were amplified by PCR using flanking primer sequences (lukS-PV: GACCCGAACTAAGGCTGATACAAT and TCAATTATGCTTTTACATTTTG; lukF-PV: CCCGCTCAACATATCACCTGT and TTAGCTCAATTAGTATTTTTCCTTAG) and cloned into pET151/TOPO-D (Invitrogen). Recombinant PVL proteins were expressed in E. coli at 30°C in the presence of 1 mM IPTG. His6-tagged proteins were purified over nickel/cadmium columns, and quantitated by BCA (Pierce). The His6-tag was removed by Ac/TEV protease per manufacturer’s protocol (Invitrogen), with cleavage confirmed by SDS-PAGE.

Generation of rabbit antisera
Rabbits were hyperimmunized with 100 μg recombinant LukS-PV or LukF-PV protein in emulsion with Freund’s adjuvant to generate specific, high-titer antisera to both LukS-PV and LukF-PV. For each protein, two rabbits were immunized. After 4 immunizations (on days 0, 7, 21, and 35) specific antibody titers increased 500,000-fold for both rabbits immunized with rLukF-PV and ≥2,000,000-fold for one rabbit immunized with rLukS-PV as measured by ELISA in which plates were coated with LukS-PV 10 μg/mL. The sera were tested against alpha-hemolysin or gamma-hemolysin in Western blot and ELISA and did not show cross-reactivity to either.

Murine skin infection model
Ten week old SKH1, CD1, and BALB/c mice and 6 month old CD1 mice were purchased from Charles River Laboratories. C57BL/6 mice were obtained from The Jackson Laboratory. Overnight bacterial culture was diluted 1:1000 in prewarmed media and incubated at 37°C with shaking at 250 rpm until an A600nm~2.5. Bacteria were harvested by centrifugation at 4000 rpm for 10 min and supernatants were stored at −80°C for subsequent analysis by ELISA. For the skin, lesions were defined by darkened areas of necrosis; for muscle, the lesions consisted of raised pale or darkened colored lesions overlying the red colored healthy tissue. An area of hyperemia is often visualized around the muscle lesions. Muscle lesions are further differentiated from occasional fat tissue based on color and consistency.

Our method to measure lesion size has been previously reported [19]. Both skin and muscle lesions were quantitated by multiplying the length and width of the lesion. Irregularly-shaped lesions were broken down into smaller symmetrical pieces, and each piece was measured by the same method. Thickness and weight of muscle lesion proved technically difficult to quantitate and was not measured in this study.

To further evaluate this methodology, we assessed retest reproducibility (i.e., the intra-observer coefficient of variability [CV]), inter-observer variability, and calculated intra-class correlation coefficients (ICCs) [20] in a subset of lesions (n = 20; half were PVL+ and half were PVL−, and lesions spanned a wide range) using two blinded observers. We also performed lesion measurements using an independent measurement method that utilized computer-assisted histomorphometric assessment of lesion area [ImageJ; open-source available from the NIH at http://rsb.info.nih.gov/ij/][21]. Two blinded investigators with experience in mouse anatomy and lesion measurement independently assessed twenty lesions spanning a wide range of lesion sizes using the manual method (i.e., measurement of lesion length and width) and the computer-assisted histomorphometric method (i.e., ImageJ). Each observer then measured each lesion a second time after the lesions were shuffled to control for order effects. Lesion sizes were expressed as both area measurements (in mm²) and as a dimensionless ratio of left (i.e., PVL+ to right (i.e., PVL−). Intra-observer CVs (i.e., retest reproducibility) were between 6% and 11%, inter-observer CVs were 6% to 12%. ICCs were 0.882 for the manual method and 0.960 for the histomorphometric technique. The overall ICC between the two methods was 0.930.

Immunofluorescent assays (IFA) and hematoxylin-eosin (H&E) stain
For IFA, the infected tissue was excised and fixed in 10% formalin (Medical Chemical Corporation) overnight. Paraffin embedding and H&E staining were performed by the Department of Pathology at Cedars-Sinai Medical Center. For IFA, tissue sections were deparaffinized and blocked with 5% goat serum in PBS with 0.05% Tween 20 (ISC Biosciences) (blocking buffer) for 1 hour at 37°C. After incubating samples with rabbit anti-LukS-PV antibody (diluted at 1:200 in blocking buffer) at 37°C for 1 hour, slides were washed 5 min with PBS 3 times and incubated with corresponding FITC-conjugated secondary antibody (Sigma). One unit of Texas Red-phalloidin (Invitrogen) was used for counter staining per slide. After a final wash, tissue sections were mounted with Prolong AntiFade containing DAPI (Invitrogen). A minimum of six mice were used for each condition. Stained slides were examined using Olympus BX51 flouresces microscope.

Enzyme linked immunosorbent assay (ELISA)
Mouse MIP-2, RANTES, TNF-α, IL-1β, and KC (R & D Systems) specific ELISAs were performed according to the manufacturer’s instructions. For PVL ELISA, plates were coated overnight at 4°C with known concentrations of rLukF-PV or test samples. The wells were blocked using 5% skim milk and 0.5% normal goat serum (Sigma) for an hour at 37°C. Rabbit anti-LukF-PV anti-serum (1:20,000 in the blocking buffer) was added to each well. After 1 hour at 37°C, the wells were washed 3 times with PBS plus 0.1% Tween-20 (wash buffer). A goat anti-rabbit IgG conjugated to HRP (Cell Signaling) (1:5,000 in blocking buffer) was next added for an hour at 37°C, and after 3 washes, HRP activity was detected using TMB substrate (Fisher Scientific). Threshold detection level of the assay is 2 ng/mL. PVL ELISA has been validated using supernatant from mouse tissue infected with PVL− S. aureus, spiked with known concentrations of rLuk-PF toxin.
Myeloperoxidase (MPO) Assay

Animals were euthanized at 3 and 12 h post-infection, and the skin and muscle lesions were homogenized in 1 mL of PBS-Trition X-100 (0.5%) with protease inhibitor cocktails (Roche). The homogenized suspension was centrifuged at 15,000 × g for 10 min and supernatants were collected and assayed for MPO activity according to the manufacturer’s instructions using isolated murine polymorphonuclear cells as standard (Invitrogen).

Immunoblot analysis

Overnight bacterial cultures were standardized by %T_{540} to a concentration of 10^9 CFU/mL. The supernatants were collected by centrifugation and separated using Nu-PAGE system (Invitrogen). Proteins were blotted onto nitrocellulose membranes and probed with specific antibodies against LukS-PV, LukF-PV, and α-toxin (Sigma) and corresponding secondary antibodies conjugated to HRP (Cell Signaling Technology Inc.). Specific proteins were visualized using ECLplus (Amersham Biosciences).

Statistical analysis

Data were analyzed using Prism 4.03 (Graphpad Software, Inc.). The two-tailed Wilcoxon test was used to compare paired samples. Unpaired samples were analyzed using Mann-Whitney test. Unless otherwise indicated, a p value less than 0.05 was considered significant, and noted in the figures. The Kruskal-Wallis test was used when three or more groups of data were compared. In bar graphs, n≥6 and results are presented as mean±SEM.

Supporting Information

Figure S1 Mouse infection using S. aureus inocula of 107 and 108 CFU. CD1 mice were inoculated subcutaneous on one flank with ~107 or 108 PVL+: S. aureus, and on the opposite flank with the same inoculum of isogenic PVL− S. aureus. The injected strains were: CST5 PVL+/−, CST6 PVL+/−, and Newman−empty vector/Newman+PVL expression vector. Mice were sacrificed on day 3 post-infection. (A) and (B) Muscle lesion size and total CFU from mice injected with 107 CST5 PVL+/− or CST6 PVL+/−. (C) and (D) Muscle lesion size and total CFU from mice injected with 108 Newman-empty vector/Newman+PVL expression vector. * p < 0.05, ** p < 0.01. Found at: doi:10.1371/journal.pone.0006387.s001 (0.11 MB TIF)

Figure S3 H&E stain of infected tissues. CD1 mice were infected with either PVL+ or isogenic PVL− S. aureus as previously described. Shown are H&E stainings of uninfected and infected tissues (at day 3 post-infection). E+Depidermidermis-layer: SA. S. aureus, and M:Muscle. Found at: doi:10.1371/journal.pone.0006387.s003 (0.04 MB TIF)

Figure S4 Effect of innate immunity and host background on PVL virulence function. Ten to twelve week old CD1, C57BL/6, BALB/c, and SKH1 mice were infected on opposite flanks with either PVL+ CST5 or isogenic PVL− CST5. (A) Muscle lesion size and CFU on day 3 post-infection. (B) Tissue MPO level at 3 and 12 h after subcutaneous infection of CD1 mice with CST3+/− PVL. Controls consisted of PBS injected mice (negative control) and LPS injected mice (positive control). * p < 0.05. Found at: doi:10.1371/journal.pone.0006387.s004 (0.07 MB TIF)

Table S1 Strains and plasmids used in this study. Found at: doi:10.1371/journal.pone.0006387.s005 (0.06 MB DOC)

Acknowledgments

We thank Dr. Frank DeLeo for helpful discussion of pro-inflammatory properties of PVL, and Catherine Bresee for statistical analyses of lesion measurements.

Author Contributions

Conceived and designed the experiments: CWT PK GL. Performed the experiments: CWT PK JL MAR. Analyzed the data: CWT PK JL MAR RA LM MA BAD VN TMD DOB GL. Contributed reagents/ materials/analysis tools: CWT PK JL MAR RA DOB GL. Wrote the paper: CWT LM MO MA BAD VN TMD DOB GL.

References

1. Chambers HF (2005) Community-associated MRSA-resistance and virulence converge. N Engl J Med 352: 1485–1487.
2. Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Brouil DJ, et al. (2003) Comparison of community- and health care-associated methicillin-resistant Staphylococcus aureus infection. Jama 290: 2976–2984.
3. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, et al. (2003) Community-acquired methicillin-resistant Staphylococcus aureus carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis 9: 978–984.
4. Moran GJ, Krishnadass A, Gorwitz RJ, Fosheim GE, McDougal LK, et al. (2006) Methicillin-resistant S. aureus infections among patients in the emergency department. N Engl J Med 355: 666–674.
5. Kaneko J, Kamio Y (2004) Bacterial two-component and hetero-heptameric protein families: a new connection. FEBS Lett 543: 267–273.
6. Bocchini CE, Hulten KG, Mason EO Jr, Gonzalez BE, Hammerman WA, et al. (2006) Panton-Valentine leukocidin genes are associated with enhanced inflammatory response and local disease in acute hematogenous Staphylococcus aureus osteomyelitis in children. Pediatrics 117: 433–440.
7. Gibb D, Gift Y, Kohler R, Lina G, Vandenesch F, et al. (2007) Pediatric bone and joint infections caused by Panton-Valentine leukocidin-positive Staphylococcus aureus. Pediatr Infect Dis J 26: 1045–1048.
8. Gillet Y, Ioaret B, Vanhems P, Fournet JC, Lina G, et al. (2002) Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet 359: 753–759.
9. Yamasaki O, Kaneko J, Morizane S, Akiyama H, Arata J, et al. (2005) The association between Staphylococcus aureus strains carrying Panton-Valentine leukocidin genes and the development of deep-seated follicular infection. Clin Infect Dis 40: 381–385.
10. Voyich JM, Otto M, Mathema B, Braughton KR, Whitney AR, et al. (2006) Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? J Infect Dis 194: 1761–1770.
11. Bebeck Wardenburg J, Bae T, Otto M, Deleo FR, Schneewind O (2007) Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia. Nat Med 13: 1405–1406.
12. Labandeira-Rey M, Couzon F, Boisset S, Brown EL, Bes M, et al. (2007) Panton-Valentine leukocidin causes necrotizing pneumonia. Science 313: 1130–1133.
13. Bebeck Wardenburg J, Palacios-Ballance AM, Otto M, Schneewind O, DeLeo FR (2006) Panton-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant Staphylococcus aureus disease. J Infect Dis 196: 1166–1170.
14. Brown EL, Dumitrescu O, Thomas D, Badiou C, Koers EM, et al. (2009) The Panton-Valentine leukocidin vaccine protects mice against lung and skin infections caused by Staphylococcus aureus USA300. Clin Microbiol Infect 15: 156–164.

15. Montgomery CP, Daum RS (2009) Transcription of inflammatory genes in the lung after infection with community-associated methicillin-resistant Staphylococcus aureus: a role for panton-valentine leukocidin? Infect Immun 77: 2159–2167.

16. Cribier B, Prevost G, Couppie P, Finck-Barbancon V, Grosshans E, et al. (1992) Staphylococcus aureus leukocidin: a new virulence factor in cutaneous infections? An epidemiological and experimental study. Dermatology 185: 175–180.

17. Arnaud M, Chastanet A, Debarbouille M (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl Environ Microbiol 70: 6887–6891.

18. Tseng CW, Zhang S, Stewart GC (2004) Accessory gene regulator control of staphylococcal enterotoxin d gene expression. J Bacteriol 186: 1793–1801.

19. Bunce C, Wheeler L, Reed G, Musser J, Bag N (1992) Marine model of cutaneous infection with gram-positive cocci. Infect Immun 60: 2636–2640.

20. Shrout PE, Fleiss JL (1979) Intraclass correlations: uses in assessing rater reliability. Psychol Bull 86: 420–426.

21. Girish V, Vijayalakshmi A (2004) Affordable image analysis using NIH Image/. ImageJ. Indian J Cancer 41: 47.

22. Miller LG, Perdreau-Remington F, Rieg G, Mehdri S, Perloff J, et al. (2005) Necrotizing fasciitis caused by community-associated methicillin-resistant Staphylococcus aureus in Los Angeles. N Engl J Med 352: 1455–1457.

23. Kash JC, Tumpey TM, Proll SC, Carter V, Perwitasari O, et al. (1996) Activation of human effector cells by different bacterial toxins (leukocidin, alveolysin, and erythrogenic toxin A): generation of interleukin-8. Infect Immun 62: 4831–4837.

24. Konig B, Koller M, Prevost G, Piemont Y, Alouf JE, et al. (1994) Activation of human effector cells by different bacterial toxins (leukocidin, alveolysin, and erythrogenic toxin A): generation of interleukin-8. Infect Immun 62: 4831–4837.

25. Fu YK, Arkins S, Li YM, Dantzer R, Kelley KW (1994) Reduction in superoxide anion secretion and bactericidal activity of neutrophils from aged rats: reversal by the combination of gamma interferon and growth hormone. Infect Immun 62: 1–8.

26. Renshaw M, Rockwell J, Englerman C, Gewirtz A, Katz J, et al. (2002) Cutting edge: impaired toll-like receptor expression and function in aging. J Immunol 169: 4697–4701.

27. Ginaldi L, De Medici A, D’Ostilio A, Marini L, Loreto MF, et al. (1999) The immune system in the elderly. III. Innate immunity. Immunol Res 20: 117–126.

28. Davila DR, Edwards CK 3rd, Arkins S, Simon J, Kelley KW (1990) Interferon-gamma-induced priming for secretion of superoxide anion and tumor necrosis factor-alpha declines in macrophages from aged rats. Faseb J 4: 2906–2911.

29. Boyle Vaara S, Daum RS (2007) Community-acquired methicillin-resistant Staphylococcus aureus: the role of Pantone-Valentine leukocidin. Lab Invest 87: 3–9.

30. Gordon RJ, Losey FD (2008) Pathogenesis of methicillin-resistant Staphylococcus aureus infection. Clin Infect Dis 46 Suppl 5: S350–S359.

31. Dufour P, Gillet Y, Bes M, Lina G, Vandenesch F, et al. (2002) Community-acquired methicillin-resistant Staphylococcus aureus infections in France: emergence of a single clone that produces Pantone-Valentine leukocidin. Clin Infect Dis 35: 819–824.

32. Robinson DA, Kearns AM, Holnes A, Morrison D, Grundmann H, et al. (2005) Re-emergence of early pandemic Staphylococcus aureus as a community-acquired meticillin-resistant clone. Lancet 363: 1256–1258.

33. Lina G, Piemont Y, Godall-Gamot F, Bes M, Peter MO, et al. (1999) Involvement of Pantone-Valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia. Clin Infect Dis 29: 1128–1132.

34. Szumlinski S, Prevost G, Monsteil H, Colin DA, Jejjaseree J (1999) Leukocidal toxins of staphylococci. Zentralbl Bakteriol 289: 185–201.

35. Badiou C, Dumitrescu O, Croze M, Gillet Y, Dobin B, et al. (2008) Panton-Valentine leukocidin is expressed at toxic levels in human skin abscesses. Clin Microbiol Infect 14: 1100–1103.

36. Pannaraj PS, Hulten KG, Gonzalez BE, Mason EO Jr, Kaplan SL (2006) Infective pyomyositis and myositis in children in the era of community-acquired, methicillin-resistant Staphylococcus aureus infection. Clin Infect Dis 43: 953–960.

37. Konig B, Prevost G, Piemont Y, Konig W (1995) Effects of Staphylococcus aureus leukocidin on inflammatory mediator release from human granulocytes. J Infect Dis 171: 607–613.

38. Styrkisson ME, Chambers HF (2000) Skin and soft-tissue infections caused by community-acquired methicillin-resistant Staphylococcus aureus. Clin Infect Dis 46 Suppl 5: S368–S377.