Staphylococcus aureus Corneal Infections: Effect of the Pantan-Valentine Leukocidin (PVL) and Antibody to PVL on Virulence and Pathology

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PURPOSE. Community-associated methicillin-resistant *Staphylococcus aureus* strains expressing Pantan-Valentine leukocidin (PVL) are associated with severe skin and soft tissue infections, necrotizing pneumonia, and eye infections. We determined PVL's toxicity on infected mouse and cultured human corneal epithelial cells and the role of PVL and antibody to PVL in pathogenesis of murine keratitis.

METHODS. Cytotoxicity on corneas and corneal epithelial cells was evaluated by LDH assays. Scratched corneas of female A/J mice were inoculated with approximately 10^7 CFU/eye of either WT *S. aureus*, isogenic Δ PVL, or strains overproducing PVL. Antibodies to PVL or control sera were topically applied to infected corneas 0, 24, and 32 hours postinfection, corneas scored for pathology and tissue levels of *S. aureus* were determined.

RESULTS. PVL expression augmented the cytotoxicity of *S. aureus* on infected mouse corneas and human cultured corneal epithelial cells. Variable effects on leukocyte recruitment, pathogenesis, and immunity were obtained in the in vivo studies. Inactivation of PVL in USA300 strains caused reduced pathology and bacterial counts. Results were variable when comparing WT and Δ PVL USA400 strains, while USA400 strains overproducing PVL caused increased bacterial burdens. Topical treatment with polyclonal antibody to PVL yielded significant reductions in corneal pathology and bacterial CFU in corneas infected with USA300 strains, whereas effects were inconsistent in eyes infected with USA400 strains.

CONCLUSIONS. PVL enhanced the virulence of a subset of MRSA strains in a keratitis model. Coupled with a variable effect of antibody treatment, it appears that PVL plays an inconsistent role in pathogenesis and immunity to *S. aureus* corneal infection.

Keywords: keratitis, MRSA, PVL, antibody

Staphylococcus aureus is among the leading causes of ocular infections, including keratitis, endophthalmitis, and conjunctivitis.¹⁻⁴ The occurrence of *S. aureus* as an eye pathogen is particularly worrisome given the major increase in infections of all types caused by methicillin-resistant *S. aureus* (MRSA), particularly within both the hospital and community settings.⁵⁻⁷ Associated with many of the community-acquired MRSA infections is production of the Pantan-Valentine leukocidin (PVL), whose role in pathogenesis of experimental animal skin and lung infections has led to inconsistent findings and conclusions.⁸⁻¹⁴ PVL-producing MRSA strains have been isolated from community-acquired eye infections, such as the PVL-positive sequence type (ST)772 strain carrying the staphylococcal chromosome mec type V cassette, detected in 22 (67%) of 33 cases from India¹⁵ and an aggressive infection of the eye and orbit reported in individuals in San Francisco, California,¹⁶ infected with the USA300 clone. Some animal studies support the conclusion that PVL production is linked to disease severity in rabbit models of skin¹⁷ and lung infection,¹⁸ as well as in murine models of pneumonia and cutaneous infection principally demonstrated by using USA300 strain LAC and its isogenic PVL-negative strain (LAC Δ PVL).¹¹ In other in vivo experiments using murine models to evaluate the contribution of PVL to virulence, there was either no effect

found,^{9,10,19} or the absence of PVL actually enhanced infection.^{13,14}

PVL has been shown to have two distinct effects on mammalian cells, including pore formation leading to lysis of human, simian, and rabbit phagocytes,^{20,21} and cellular activation of many cell types, including those not susceptible to its lytic activity, such as murine cells.^{22,23} Its cell activation capacity, which causes increases in proinflammatory cytokines and release of antibacterial factors, has been linked to enhanced clearance of *S. aureus* in pulmonary and skin abscess infections.^{13,14} Conversely, inflammatory reactions in the eye result in worsening ocular pathology, a detrimental effect on the host.²⁴⁻²⁷ Thus, the effect of PVL on ocular infections could differ from its effects in other sites of infection.

Neutralizing antibody to PVL is elevated in sera of children with primary or recurrent CA-MRSA-associated skin and soft tissue infection, indicative of a lack of protective efficacy in these human infections.²⁸ Since the effects of PVL on virulence remains controversial and may manifest its properties variably in different tissues or among different PVL-producing *S. aureus* strains, we determined whether deletion of the genes encoding PVL or topical application of antibody to PVL affected bacterial burdens and corneal pathology of *S. aureus* keratitis using

multiple community-acquired (CA)-MRSA strains and a PVL-positive methicillin-sensitive *S. aureus* (MSSA) strain.

MATERIALS AND METHODS

Bacterial Strains

Strains MW2, NRS 193, and NRS 194 belong to the USA400 lineage, whereas strains LAC and SF8300 belong to the USA300 lineage, and NRS 158 is a PVL-producing MSSA clinical isolate. All of these strains were obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) repository. Isogenic strains lacking the *lukF* and *lukS* genes encoding the two components of PVL (Δ PVL strains) were constructed in strains MW2, NRS 193, and NRS 194 as previously described by deletion of the *lukF* and *lukS* genes.¹³ *S. aureus* strain LAC containing a *Bursa aurealis* marinerlike transposon insert in the genes for the *lukS* component of PVL was kindly provided by Ken Bayles, Omaha, Nebraska (strain NE1848 from The Nebraska Transposon Mutant Library, accession number SAUSA300_1382, Tn inserted into gene 1,547,578, whole genome insertion site). This Tn-insert produced no detectable PVL. Additionally, wild-type (WT) *S. aureus* strains MW2, NRS 193, and NRS 194 harboring either the pOS1 vector or overexpressing PVL from the pOS1-*pvl* plasmid into which we had cloned the *lukS* and *lukF* genes under the control of their own promoter, were used as previously described.¹⁴

Murine Corneal Infection Model

All animal studies were approved by the Harvard Medical Area Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Corneal infection was initiated on scratch-injured eyes of anesthetized mice, as previously described.^{29,30} Bacteria were grown to log phase at 37°C in yeast extract-casamino acid-sodium pyruvate (YCP) broth and the OD_{650nm} adjusted to achieve inocula of approximately 10⁷ colony-forming units (CFUs) per eye. Female A/J mice 6 to 7 weeks old were anesthetized with ketamine and xylazine and then three 1-mm scratches were made on eyes with a 26-gauge needle, which were then inoculated with the bacterial strains in a 5- μ L volume. Following infection, eyes were assigned a pathology score every 24 hours using the following scheme: 0, eye macroscopically identical to the uninfected contralateral control eye; 1, faint opacity partially covering the pupil; 2, dense opacity covering the pupil; 3, dense opacity covering the entire anterior segment; 4, perforation of the cornea, phthisis bulbi (shrinkage of the globe after inflammatory disease), or both. Pathology scores were determined by two independent observers unaware of the experimental conditions. Scoring was congruent more than 95% of the time; in cases where there were discrepancies, the lower score was used. In general, maximal pathology scores were observed 48 to 72 hours after infection, so this time point is shown in all figures depicting corneal pathology scores.

Determination of Viable Bacterial Counts

To determine the infectious level of *S. aureus*, animals were euthanized by carbon dioxide overdose, and the eyes removed, the corneas dissected from the surface then homogenized in tryptic soy broth containing 0.5% Triton X-100. The homogenate was then diluted and plated in the homogenizing medium for bacterial enumeration.

Administration of Antibody to PVL

Rabbit polyclonal antibody to PVL was produced as previously described¹³ and had a neutralizing titer against purified PVL in a polymorphonuclear (PMN)-cytotoxicity assay of approximately 2000. Controls received normal rabbit serum (NRS) with no detectable neutralizing activity. Sera were applied undiluted in a 5- μ L volume topically to the eyes of mice while they were lightly anesthetized by isoflurane inhalation. This was done at time 0 (approximately 5 minutes postinfection), 24, and 32 hours postinfection.

Lactate Dehydrogenase Assay for Corneal Cell Cytotoxicity

Primary cultures of human epithelial corneal cells were established from corneal rims prepared and cultured in flasks as described by Zaidi et al.³¹ Cells were seeded at 2×10^5 cells per well in 24-well plates and after 18 hours exposed to a 1:20 or 1:10 dilution of the cell-free, sterile supernatant from *S. aureus* strains LAC, LAC Δ PVL, MW2, or MW2 Δ PVL grown overnight at 37°C in YCP broth. Aliquots from the wells were collected every 5 minutes and lactate dehydrogenase (LDH) release determined by a commercially available assay (Tox-7 in vitro cytotoxicity assay; Sigma, St. Louis, MO).

The LDH release assay was also used on corneas harvested from infected mice 48 hours postinfection. Female A/J mouse corneas were scratched and infected with 10⁷ CFUs of *S. aureus* strains LAC, LAC Δ PVL, MW2, MW2 Δ PVL, NRS 193, NRS 193 Δ PVL, NRS 194, NRS 194 Δ PVL, SF8300, NRS 158, MW2pOS1, MW2pOS1-PVL, NRS 193pOS1, NRS 193pOS1-PVL, NRS 194pOS1, or NRS 194pOS1-PVL for 48 hours. To recover the extracellular LDH present, mouse corneas were washed with 200 μ L PBS containing protease inhibitors (Complete Mini; Roche Diagnostics, Mannheim, Germany) and this solution used to determine the LDH activity.

Histopathology

Mouse eyes were infected with approximately 5×10^5 *S. aureus* and 48 hours later euthanized, and the eyes enucleated and fixed in 2% formaldehyde. Tissues were embedded in paraffin and anterior sections of the corneal epithelium prepared and stained by hematoxylin and eosin at the Harvard Medical Area rodent pathology core facility. Sections were analyzed by a veterinary pathologist and micrographs prepared.

Statistical Analysis

Differences in CFU levels were compared by unpaired *t*-tests for normally distributed, log-transformed data or with the Mann-Whitney *U* test for ordinal (i.e., corneal pathology scores) and nonparametric data using Graphpad's Prism software (Graphpad Software, Inc., La Jolla, CA).

RESULTS

Effect of Deleting PVL-Encoding Genes on MRSA Pathogenesis in Murine Corneal Infections

We initially compared the corneal pathology scores and bacterial CFUs recovered from infected corneas using isogenic pairs of four *S. aureus* strains containing intact or deleted genes encoding PVL production. For the USA300 *S. aureus* strain LAC, which had a high level of virulence in the murine keratitis model, we found at both 48 hours (Fig. 1A) and 72 hours (Fig. 1B) after infection that the PVL-producing WT

FIGURE 1. Effect of deletion of *lukF* and *lukS* genes encoding PVL components on pathogenesis of *S. aureus* corneal infection. At both 48 hours postinfection (A) and 72 hours postinfection (B) with USA300 *S. aureus* LAC Δ PVL there were reduced bacterial levels and corneal pathology scores compared with WT strains. (C–E) Outcomes at 48 hours postinfection with *S. aureus* USA400 strains. (C) In strain NRS 193, there were reduced bacterial levels and corneal score at 48 hours postinfection in the Δ PVL strain compared with WT. (D) In strain NRS 194, there was no difference in bacterial

strain was recovered at higher levels and caused more corneal pathology compared to the LAC Δ PVL strain. Using the other *S. aureus* strains, however, most mice had only low levels of infection by 72 hours, hence bacterial burdens and pathology were ascertained at 48 hours postinfection in these studies. For the USA400 strain NRS 193, we found that the Δ PVL mutants yielded significantly lower bacterial levels in the infected corneal tissues and showed reduced corneal pathology scores compared with those of the WT parental strain at 48 hours postinfection (Fig. 1C). With two other USA400 strains, NRS 194 (Fig. 1D) and MW2 (Fig. 1E), there was no significant difference between the WT and Δ PVL mutant in the bacterial levels in the infected corneas and no significant difference in the corneal pathology for strain MW2 but a modest difference in pathology ($P = 0.04$) for strain NRS 194 (Fig. 1D).

Loss of PVL Reduces Cytotoxicity of MRSA on Infected Mouse Corneas and Cultured Human Corneal Epithelial Cells

Next we determined the cytotoxic effect of PVL on infected mouse corneas and cultured human corneal epithelial cells growing in vitro. Corneas removed from mouse eyes 48 hours postinfection that had been infected by WT PVL-producing strains all had a higher level of LDH release compared with the isogenic strain unable to produce PVL, with differences being maximal with USA300 strain LAC and lesser with the three USA400 strains. To ascertain if a similar effect would be seen with human corneal cells, we applied sterile culture supernates from cultures of *S. aureus* strains LAC or MW2 and their respective Δ PVL mutants (final CFU counts for all four strains were within 10% of each other) to the cultured cells (Fig. 2). We found that the WT *S. aureus* LAC and MW2 strains both had a greater cytotoxic effect on human corneal cells in culture compared to isogenic Δ PVL mutants, as shown by LDH release assay. In initial studies, the cytotoxic activity from strain LAC was more quickly manifest on corneal epithelial cells in culture and thus to see an effect over time, the supernatants from strain LAC were diluted 1:20 for analysis of cytotoxic activity, whereas a 1:10 dilution was used for supernates from strain MW2 and its Δ PVL mutant (Fig. 2). By 10 minutes, the plateau of cytotoxic activity was obtained with strain LAC while WT strain MW2, tested at a more concentrated level, took 20 minutes to reach a cytotoxic activity plateau. The Δ PVL strains took 30 to 60 minutes to reach the cytotoxic plateau, indicative of activity from other *S. aureus* factors on corneal cells but nonetheless showing a clear decrease in cytotoxic activity from loss of PVL production.

Effect of Loss of PVL on Leukocyte Recruitment to the Infected Cornea

As elaboration of PVL induces proinflammatory host responses that affect recruitment of leukocytes to infected tissue,^{13,22,32} we examined corneas from mice infected with WT or Δ PVL *S. aureus* by histopathology. A clear difference could be seen in leukocyte levels in corneas from mice infected with USA300 strain LAC, with fewer leukocytes observed in the LAC Δ PVL-infected tissue (Fig. 3). This finding is consistent with the differences in bacterial burdens, corneal pathology, and

levels, but a somewhat reduced level of corneal pathology in the Δ PVL strain compared with WT. (E) With strain MW2 there was no difference between WT and MW2 Δ PVL in bacterial levels or corneal pathology score. Points indicate values for an individual mouse, bars indicate medians, and P values were calculated by the Mann-Whitney U test.

FIGURE 2. Cytotoxic activity in infected mouse eyes and on human corneal epithelial cells of supernates from WT and paired isogenic PVL-deficient (Δ PVL) *S. aureus* strains. (A) Relative cytotoxic effect on mouse corneal epithelium detected by LDH release at 48 hours postinfection. n = four mice per group, bars = means from duplicates of each sample, and error bars indicate the SEM. (B) Cytotoxic activity on cultured human corneal epithelia cells of supernatants obtained from *S. aureus* MW2 (diluted 1:10) and *S. aureus* LAC (diluted 1:20) after growth in YCP medium overnight. Bars indicate means of triplicate samples, and error bars indicate the SEM. * P < 0.05 at indicated time point, t -test.

cytotoxicity measured in the infected mouse eyes (Figs. 1, 2) wherein strain LAC has the most consistent and largest difference between WT and the isogenic Δ PVL strain. Differences among the three USA400 strains were not as pronounced, with the exception being NRS 193 where a slight reduction in leukocyte numbers was seen in corneal tissue infected with its Δ PVL counterpart. Of note, NRS 193 was the only USA400 strain that exhibited a significant difference in CFU and pathology scores between WT and Δ PVL in Fig. 1.

Topical Administration of Antibody to PVL Postinfection Variably Reduces Infection and Corneal Pathology

We next evaluated whether antibody to PVL has therapeutic efficacy on *S. aureus* keratitis. We chose to apply the antibody topically to the cornea, as other results we have published^{13,28} indicate systemic antibody to PVL can enhance MRSA skin infections in mice and high antibody levels to PVL in human sera are associated with recurrent infections.²⁸ Thus, topical antibody might show a local benefit on pathology from PVL-positive MRSA ocular infections without conferring possible negative consequences from systemic administration.

When topically applied onto the eye approximately 5 minutes, 24 hours, and 32 hours postinfection with *S. aureus*,

FIGURE 3. Hematoxylin and eosin stain of corneal sections from *S. aureus*-infected eyes 48 hours postinfection. Sections showing relative accumulation of leukocytes in eyes from mice infected with *S. aureus* USA300 strain LAC and three USA400 strains. Upper panels: $\times 40$ magnification; lower panels: $\times 100$ magnification.

5 μ L of undiluted polyclonal rabbit antibody to PVL yielded a significant reduction in the corneal pathology scores 48 hours after infection for four strains of *S. aureus*: LAC, SF8300, NRS 158, and NRS 194 (Fig. 3). However, topical antibody application reduced bacterial levels in the eye 48 hours after infection for only three of the *S. aureus* strains: LAC, SF8300, and NRS 194, although the reduction for strain NRS 158 did have a borderline P value of 0.066. Antibody to PVL applied to eyes of mice infected with strains NRS 193 or MW2 led to nonsignificant differences in both corneal pathology scores and bacterial levels in the eye (Fig. 4).

Specificity of Antibody Mediated Protection to PVL

We examined the effect of topical therapeutic administration of antibody to PVL on corneal infection due to *S. aureus* LAC and its isogenic Δ PVL mutant to ascertain antigenic specificity of the protection observed. Compared with NRS, antibody to PVL reduced bacterial levels and corneal pathology in mouse corneas infected with WT strain LAC for 48 hours but had no

FIGURE 4. Effect of topical administration of NRS or antibody to PVL on the bacterial burdens and pathology scores of mice infected for 48 hours. *S. aureus* strain indicated on x-axis. There was no effect either on corneal pathology scores or CFU/cornea when NRS 193 or MW2 *S. aureus* were used for eye infection followed by topical administration of antibody to PVL 0, 24, and 32 hours postinfection. Points indicate values for an individual mouse, bars indicate medians, and P values were calculated by the Mann-Whitney U test.

effect on infections caused by the LAC Δ PVL strain (Fig. 5). Notably, comparing the mice treated with NRS and infected with WT *S. aureus* LAC or LAC Δ PVL revealed reduced virulence of the strain unable to produce PVL.

Overexpression of PVL Enhances Bacterial Burdens but Variably Affects Pathology in Infected Mouse Corneas

The above findings showed a variable effect of PVL expression and PVL-specific antibody on corneal pathology and bacterial burdens among the different *S. aureus* strains. However, one might expect strain-specific effects on PVL production, and hence effects on virulence. To see if the USA400 strains, in which there was the least effect on virulence from native levels of PVL, would lead to enhanced bacterial burdens and corneal pathology if PVL expression was increased, we compared WT *S. aureus* strains MW2, NRS 193, and NRS 194 carrying a multicopy cloning plasmid, pOS1, with isogenic strains carrying pOS1 containing cloned *lukS* and *lukF* genes to enhance PVL production. We previously showed pOS1-*pvl* dramatically increased production of PVL compared to the WT strain carrying only the cloning plasmid pOS1 along with single

FIGURE 5. Specificity of antibody mediated protection to PVL. Effect of topical administration of NRS or antibody to PVL on bacterial levels and pathology scores in mouse eyes infected with USA300 *S. aureus* LAC or *S. aureus* LAC Δ PVL for 48 hours. Bacterial levels and pathology scores were significantly reduced for the PVL-producing WT strain following administration of antibody to PVL 0, 24, and 32 hours postinfection, but no effect was obtained in mice infected with the mutant strain unable to produce PVL. Points represent individual mice; bars represent the medians. P values: Mann-Whitney U test. NS, not significant.

chromosomal copies of the *lukS* and *lukF* genes.¹⁴ We have previously noted that there was somewhat reduced PVL expression in the strain carrying the empty vector compared with the WT strain,¹⁴ but this strain was needed to make the proper comparison with the isogenic strain harboring the pOS1-*pvl* plasmid, as they harbor the same base vector, and were cultured under identical conditions.

In infected mouse eyes the increased expression of PVL enhanced the bacterial burdens achieved compared to the WT/pOS1 strain (Fig. 6A), with the mean magnitude of the effect ranging from a log_{10} difference of 0.38 to 1.9. We saw an effect of overproduction of PVL with strain MW2, as determined by enhanced bacterial burdens (1.9 log_{10}) and increased corneal pathology (Fig. 6B). Measurement of the cytotoxic effect on corneal cells by LDH release showed that overexpression of PVL modestly enhanced cytotoxicity (Fig. 6C). Similarly, antibody to PVL had a variable ability to reduce bacterial levels and corneal pathology associated with the three USA400 PVL overproducing strains (Fig. 7). Even when there was a statistically significant reduction in bacterial levels, the overall reduction was modest, approximately 0.25 log_{10} . Thus, when PVL-positive strains of *S. aureus* are able to produce a level of PVL that enhanced bacterial burdens (Fig. 6), topical, passive antibody therapy still had a variable and modest effect on pathogenesis of keratitis. Considering the high copy number pOS1-*pvl* plasmid led to a more than 45-fold increase in PVL compared with strain MW2/pOS1,¹⁴ the small volume of PVL antisera that could be applied to the cornea may have been insufficient to neutralize the significantly elevated levels of

FIGURE 6. Effect of overproduction of PVL on bacterial burdens, pathology scores, and corneal cytotoxicity in mice infected for 48 hours with three different USA400 strains of *S. aureus*. (A) Bacterial burdens in the eyes were determined 48 hours postinfection for the indicated *S. aureus* strain expressing either WT levels of PVL in the presence of control plasmid pOS1 or increased levels of PVL from plasmid pOS1-pvl. Bar graphs: columns represent means, number of mice indicated for each column, error bars the SEM; *P* values determined by unpaired *t*-tests. (B) Scatter dot plots of corneal pathology scores: points represent individual mice, lines indicate the medians, and *P* values determined by Mann-Whitney *U* test. (C) Cytotoxicity measured by LDH release in mouse corneas; bars represent means of four mice, and error bars indicate the SEM.

PVL. However, PVL-independent effects unique to the USA400 lineage also cannot be ruled out.

DISCUSSION

In the present study we determined the contribution of PVL to bacterial burdens, corneal pathology, and corneal cytotoxicity in experimental *S. aureus* keratitis. We found a variable effect on pathogenesis when testing strains elaborating their natural levels of PVL. The USA300 strain LAC showed reduced pathology and cytotoxicity following deletion of the PVL genes and reduced infection and corneal pathology when antibody to PVL was used to treat the infection. Another USA300 strain, SF8300, also had reduced infection and pathology in the presence of antibody to PVL. For a PVL-positive MSSA strain, a borderline effect of antibody to PVL was

FIGURE 7. Effect of NRS or antibody to PVL on bacterial levels and pathology in the corneal epithelium of mice infected with three USA 400 *S. aureus* strains overexpressing PVL. (A) Compared to NRS, antibody to PVL administered 0, 24, and 32 hours postinfection reduced bacterial burdens in the scratch-injured eye 48 hours postinfection for strains MW2 and NRS194 but not strain NRS193. (B) Antibody to PVL reduced corneal pathology only in eyes of mice infected with strain NRS194. *P* values: unpaired *t*-tests for column graphs (bars represent means, and error bars indicate the SEM) or Mann-Whitney *U* test for scatter graphs (each point is an individual mouse; lines indicate medians).

found, suggesting some potential use of antibody to PVL in treating a subset of infections caused by PVL-producing *S. aureus*. Among USA400 strains, there was a variable effect of deletion of PVL genes on virulence, and antibody to PVL similarly had a variable effect on bacterial levels and corneal pathology following *S. aureus* infection. Increasing PVL production in the USA400 strains modestly enhanced bacterial burdens and corneal cytotoxicity 48 hours postinfection, indicating that under circumstances when elaboration of high levels of PVL can be achieved in these strains, the toxin had measurable deleterious effects. However, enhanced PVL production in USA400 strains only resulted in a more severe corneal pathology using *S. aureus* MW2. For strains NRS 193 and NRS 194, there was a modest increase in bacterial levels and no major change in pathology scores and borderline increases in in situ cytotoxicity. Strains lacking PVL production nonetheless had notable cytotoxic effects in the corneas during infection and on cultured human corneal epithelial cells, highlighting the existence of other bacterial factors capable of damaging host cells, and possibly occluding the effects of PVL production by the WT strains. The difference in in situ and in vitro toxicity of WT and Δ PVL strains is consistent with the conclusion that there are strain-specific differences in virulence independent of PVL.

When community-acquired MRSA infections first emerged in the 1990s, PVL-producing USA400 clones were predominant, but around the turn of the millennium they were replaced by PVL-producing USA300 strains in the United States, although this replacement did not occur in Europe or other more economically advantaged parts of the world. Differences between USA300 and USA400 strains have been analyzed indicating USA300 strains have specific sets of genes distinct from other *S. aureus* lineages that could facilitate virulence^{19,33} and may have higher levels of production of alpha-toxin, phenol-soluble modulins, and other core-genome-encoded virulence determinants.¹⁹ Schlievert and colleagues³⁴ analyzed virulence factor production among MRSA lineages and found both USA300 and USA400 strains synthesized inflammatory cytolytic factors including alpha-, delta-, and gamma-toxins, but

USA300 isolates produced an enterotoxin Q-like superantigen and a highly pyrogenic variant of toxic shock syndrome toxin 1. In contrast, USA400 strains produced different superantigens, including staphylococcal enterotoxins B and C. The degree to which these differences affect the virulence of various *S. aureus* lineages in ocular infections is not known, but overall indicate that the variability in virulence factor production among isolates of this organism might make it nearly impossible to define a single or limited set of virulence factors that have the most profound impact on pathogenesis in corneal infections. It is conceivable that some factor or factors expressed by USA300, but not USA400, could synergize with PVL to exacerbate ocular infections. Although USA300 clones did not emerge outside of the United States, emerging clonal complexes in Asia and Europe include the ST772 group, which was recently reported to be the cause of a majority of severe eye infections in India. The recent emergence of both USA300 and ST772 clones saw a concomitant increase in severe eye infections worldwide,¹⁵ suggesting that these emerging clones may be better equipped to cause eye infections.

With regard to whether topical application of antibody to PVL might be beneficial in the setting of ocular *S. aureus* infection, one must take into consideration the difficulty of ascertaining in a rapidly progressive human MRSA corneal infection if a patient is infected with a strain wherein PVL production might be contributing significantly to pathology. When it appears to do so, such as with the USA300 strains, topical administration of antibody to PVL could have a salubrious effect in these infectious settings. However, for other infections, including those with strains not making PVL, antibody to this toxin may not prove beneficial. Importantly, the animal studies did indicate antibody to PVL was not enhancing corneal infection, as it does murine skin infections,¹³ raising an interesting situation wherein use of antibody to PVL as therapy for *S. aureus* keratitis would likely be beneficial in treating infections with some strains but have neither a positive or negative effect on other strains.

The controversy over the effect of PVL on pathogenesis of MRSA infections is complicated and confounded by a number of factors. There is an epidemiologic association of the production of PVL with highly virulent CA-MRSA infections,^{35,36} but animal studies have yielded a range of outcomes regarding whether PVL is a factor in *S. aureus* virulence.⁸⁻¹⁴ More recent studies in infected humans generally do not support a role for PVL in enhancing pathogenesis or severity of infection³⁷⁻⁴¹ and a recent systematic review and meta-analysis of studies ascertaining a role for PVL-producing *S. aureus* strains in infection found that outside of some increased morbidity in musculoskeletal infections, infection with PVL-producing strains did not notably affect outcomes.⁴² Additionally, we have reported that children with recurrent PVL-positive MRSA skin and soft tissue infections had the highest levels of cytotoxin-neutralizing antibody among various groups of pediatric patients and uninfected controls,²⁸ indicating, at a minimum, neutralization of PVL is not beneficial in this setting.

PVL also has variable effects on host cells and thus is able to lyse polymorphonuclear neutrophils from rabbits and humans, but not mice.²¹ At low concentrations, PVL activates the inflammatory properties of neutrophils and granulocytes^{22,32,43} and thus increases host resistance to experimental infection.^{13,14} While these disparate results may seem confusing, they are also consistent with the findings reported here that there is a variable effect of PVL production on pathogenesis of corneal infection, with outcomes dependent on strain background.

Nonetheless, in spite of these variable findings in our murine studies, it is still possible that PVL could contribute to pathogenesis of human corneal keratitis given the often unique

response of the eye to microbial infection and virulence factors, and the contribution of PMN-dependent inflammation to worsening corneal pathology.^{24,44-47} This means that a factor that lyses PMNs responding to infection can paradoxically show a beneficial effect by reducing the measurable corneal opacity and pathology, which is an effect we might have missed in mice whose PMN resist lysis by PVL. Interestingly, mouse corneal tissue infected with WT *S. aureus* strains released higher levels of LDH than mice infected with the corresponding Δ PVL counterparts, indicating effects of PVL outside of lysis of PMN. On the other hand, there may be more serious consequences from PVL-mediated lysis of human PMN responding to an *S. aureus* eye infection, since we showed with experimental *Pseudomonas aeruginosa* keratitis there were improved pathologic outcomes from reductions in PMN that were coupled with increased bacterial levels in the eye and brain and spread of systemic infection.⁴⁷ Thus, an overall conclusion from the results of the mouse studies on a role for PVL in *S. aureus* keratitis would indicate a strain-dependent effect on pathology due to a contribution from PVL to the cytotoxic activity on corneal epithelial cells, as well as strain-dependent effects of topical treatment of these infections with antibody to PVL. However, as antibody treatment did not enhance infection even when it had no effect on bacterial levels or corneal pathology, it is possible that antibody to PVL could still make a useful contribution to new treatments for *S. aureus* corneal infections, manifesting a positive outcome in those instances in which it can sufficiently neutralize the toxin with concomitant decreases in infection and pathology.

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References

- Freidlin J, Acharya N, Lietman TM, Cevallos V, Whitcher JP, Margolis TP. Spectrum of eye disease caused by methicillin-resistant *Staphylococcus aureus*. *Am J Ophthalmol*. 2007;144:313-315.
- Asbell PA, Sahm DE, Shaw M, Draghi DC, Brown NP. Increasing prevalence of methicillin resistance in serious ocular infections caused by *Staphylococcus aureus* in the United States: 2000 to 2005. *J Cataract Refract Surg*. 2008;34:814-818.
- Cavuoto K, Zutshi D, Karp CL, Miller D, Feuer W. Update on bacterial conjunctivitis in South Florida. *Ophthalmology*. 2008;115:51-56.
- Chisari G, Accossano S, Reibaldi M. Effect of aminoglycosides (sub-MICs) on adherence of coagulase-negative staphylococci to intraocular lens surface. *J Chemother*. 2002;14:574-578.
- Manzur A, Vidal M, Pujol M, et al. Predictive factors of methicillin resistance among patients with *Staphylococcus aureus* bloodstream infections at hospital admission. *J Hosp Infect*. 2007;66:135-141.
- Klevens RM, Morrison MA, Nadle J, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA*. 2007;298:1763-1771.
- Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*,

- United States, 1999-2005. *Emerg Infect Dis.* 2007;13:1840-1846.
8. Labandeira-Rey M, Couzon F, Boisset S, et al. *Staphylococcus aureus* Pantón-Valentine leukocidin causes necrotizing pneumonia. *Science.* 2007;315:1130-1133.
 9. Voyich JM, Otto M, Mathema B, et al. Is Pantón-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? *J Infect Dis.* 2006;194:1761-1770.
 10. Bubeck Wardenburg J, Palazzolo-Ballance M, Otto M, Schneewind O, DeLeo FR. Pantón-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant *Staphylococcus aureus* disease. *J Infect Dis.* 2008;198:1166-1170.
 11. Brown EL, Bowden MG, Bryson RS, et al. Pediatric antibody response to community-acquired *Staphylococcus aureus* infection is directed to Pantón-Valentine leukocidin. *Clin Vaccine Immunol.* 2009;16:139-141.
 12. Tseng CW, Kyme P, Low J, et al. *Staphylococcus aureus* Pantón-Valentine leukocidin contributes to inflammation and muscle tissue injury. *PLoS ONE.* 2009;4:e6387.
 13. Yoong P, Pier GB. Antibody-mediated enhancement of community-acquired methicillin-resistant *Staphylococcus aureus* infection. *Proc Natl Acad Sci U S A.* 2010;107:2241-2246.
 14. Yoong P, Pier GB. Immune-activating properties of Pantón-Valentine leukocidin improve the outcome in a model of methicillin-resistant *Staphylococcus aureus* pneumonia. *Infect Immun.* 2012;80:2894-2904.
 15. Nadig S, Velusamy N, Lalitha P, Kar S, Sharma S, Arakere G. *Staphylococcus aureus* eye infections in two Indian hospitals: emergence of ST772 as a major clone. *Clin Ophthalmol.* 2012;6:165-173.
 16. Rutar T, Chambers HF, Crawford JB, et al. Ophthalmic manifestations of infections caused by the USA300 clone of community-associated methicillin-resistant *Staphylococcus aureus*. *Ophthalmology.* 2006;113:1455-1462.
 17. Lipinska U, Hermans K, Meulemans L, et al. Pantón-Valentine leukocidin does play a role in the early stage of *Staphylococcus aureus* skin infections: a rabbit model. *PLoS One.* 2011;6:e22864.
 18. Diep BA, Chan L, Tattevin P, et al. Polymorphonuclear leukocytes mediate *Staphylococcus aureus* Pantón-Valentine leukocidin-induced lung inflammation and injury. *Proc Natl Acad Sci U S A.* 2010;107:5587-5592.
 19. Otto M. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annu Rev Microbiol.* 2010;64:143-162.
 20. Li M, Cheung GY, Hu J, et al. Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. *J Infect Dis.* 2010;202:1866-1876.
 21. Kaneko J, Kamio Y. Bacterial two-component and heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. *Biosci Biotechnol Biochem.* 2004;68:981-1003.
 22. König B, Prevost G, Piemont Y, König W. Effects of *Staphylococcus aureus* leukocidins on inflammatory mediator release from human granulocytes. *J Infect Dis.* 1995;171:607-613.
 23. König B, Prevost G, König W. Composition of staphylococcal bi-component toxins determines pathophysiological reactions. *J Med Microbiol.* 1997;46:479-485.
 24. Hazlett LD. Role of innate and adaptive immunity in the pathogenesis of keratitis. *Ocul Immunol Inflamm.* 2005;13:133-138.
 25. Johnson AC, Heinzel FP, Diaconu E, et al. Activation of toll-like receptor (TLR)2, TLR4, and TLR9 in the mammalian cornea induces MyD88-dependent corneal inflammation. *Invest Ophthalmol Vis Sci.* 2005;46:589-595.
 26. Kumar A, Hazlett LD, Yu FS. Flagellin suppresses the inflammatory response and enhances bacterial clearance in a murine model of *Pseudomonas aeruginosa* keratitis. *Infect Immun.* 2008;76:89-96.
 27. Gadjeva M, Nagashima J, Zaidi T, Mitchell RA, Pier GB. Inhibition of macrophage migration inhibitory factor ameliorates ocular *Pseudomonas aeruginosa*-induced keratitis. *PLoS Pathog.* 2010;6:e1000826.
 28. Hermos CR, Yoong P, Pier GB. High levels of antibody to Pantón-Valentine leukocidin are not associated with resistance to *Staphylococcus aureus*-associated skin and soft-tissue infection. *Clin Infect Dis.* 2010;51:1138-1146.
 29. Preston MJ, Fleiszig SMJ, Zaidi TS, et al. Rapid and sensitive method for evaluating *Pseudomonas aeruginosa* virulence factors during corneal infections in mice. *Infect Immun.* 1995;63:3497-3501.
 30. Zaidi TS, Preston MJ, Pier GB. Inhibition of bacterial adherence to host tissue does not markedly affect disease in the murine model of *Pseudomonas aeruginosa* corneal infection. *Infect Immun.* 1997;65:1370-1376.
 31. Zaidi TS, Lyczak J, Preston M, Pier GB. Cystic fibrosis transmembrane conductance regulator-mediated corneal epithelial cell ingestion of *Pseudomonas aeruginosa* is a key component in the pathogenesis of experimental murine keratitis. *Infect Immun.* 1999;67:1481-1492.
 32. König B, Koller M, Prevost G, et al. Activation of human effector cells by different bacterial toxins (leukocidin, alveolysin, and erythrogenic toxin A): generation of interleukin-8. *Infect Immun.* 1994;62:4831-4837.
 33. Tenover FC, McDougal LK, Goering RV, et al. Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. *J Clin Microbiol.* 2006;44:108-118.
 34. Schlievert PM, Strandberg KL, Lin YC, Peterson ML, Leung DY. Secreted virulence factor comparison between methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*, and its relevance to atopic dermatitis. *J Allergy Clin Immunol.* 2010;125:39-49.
 35. Naimi TS, LeDell KH, Como-Sabetti K, et al. Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. *JAMA.* 2003;290:2976-2984.
 36. Lina G, Piemont Y, Godail-Gamot F, et al. Involvement of Pantón-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis.* 1999;29:1128-1132.
 37. Campbell SJ, Deshmukh HS, Nelson CL, et al. Genotypic characteristics of *Staphylococcus aureus* isolates from a multinational trial of complicated skin and skin structure infections. *J Clin Microbiol.* 2008;46:678-684.
 38. Lalani T, Federspiel JJ, Boucher HW, et al. Associations between the genotypes of *Staphylococcus aureus* bloodstream isolates and clinical characteristics and outcomes of bacteremic patients. *J Clin Microbiol.* 2008;46:2890-2896.
 39. Bae I-G, Tonthat GT, Stryjewski ME, et al. Presence of genes encoding the Pantón-Valentine leukocidin exotoxin is not the primary determinant of outcome in patients with complicated skin and skin structure infections due to methicillin-resistant *Staphylococcus aureus*: results of a multinational trial. *J Clin Microbiol.* 2009;47:3952-3957.
 40. Campo M, Hachem R, Jiang Y, et al. Pantón-Valentine leukocidin exotoxin has no effect on the outcome of cancer patients with methicillin-resistant *Staphylococcus aureus* (MRSA) infections. *Medicine (Baltimore).* 2011;90:312-318.
 41. Peyrani P, Allen M, Wiemken TL, et al. Severity of disease and clinical outcomes in patients with hospital-acquired pneumo-

- nia due to methicillin-resistant *Staphylococcus aureus* strains not influenced by the presence of the Panton-Valentine leukocidin gene. *Clin Infect Dis*. 2011;53:766–771.
42. Shallcross LJ, Fragaszy E, Johnson AM, Hayward AC. The role of the Panton-Valentine leucocidin toxin in staphylococcal disease: a systematic review and meta-analysis. *Lancet Infect Dis*. 2013;13:43–54.
 43. Hensler T, König B, Prévost G, Piémont Y, Köller M, König W. Leukotriene B₄ generation and DNA fragmentation induced by leukocidin from *Staphylococcus aureus*: protective role of granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF for human neutrophils. *Infect Immun*. 1994;62:2529–2535.
 44. Thakur A, Xue M, Stapleton F, Lloyd AR, Wakefield D, Willcox MD. Balance of pro- and anti-inflammatory cytokines correlates with outcome of acute experimental *Pseudomonas aeruginosa* keratitis. *Infect Immun*. 2002;70:2187–2197.
 45. Chusid MJ, Nelson DB, Meyer LA. The role of the polymorphonuclear leukocyte in the induction of corneal edema. *Invest Ophthalmol Vis Sci*. 1986;27:1466–1469.
 46. Willcox MD. *Pseudomonas aeruginosa* infection and inflammation during contact lens wear: a review. *Optom Vis Sci*. 2007;84:273–278.
 47. Zaidi TS, Zaidi T, Pier GB. Role of neutrophils, MyD88-mediated neutrophil recruitment and complement in antibody-mediated defense against *Pseudomonas aeruginosa* keratitis. *Invest Ophthalmol Vis Sci*. 2009;51:2085–2093.