Panton–Valentine Leukocidin Enhances the Severity of Community-Associated Methicillin-Resistant Staphylococcus aureus Rabbit Osteomyelitis

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Abstract

**Background:** Extensive spread of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) in the United States, and the concomitant increase in severe invasive staphylococcal infections, including osteomyelitis, in healthy children, has led to renewed interest in Panton-Valentine leukocidin (PVL). However, the pathogenetic role of PVL in staphylococcal infections remains controversial, possibly because it depends on the site of infection.

**Methodology/Principal Findings:** We compared the course of experimental rabbit osteomyelitis due to the PVL-positive CA-MRSA strain USA 300 (LAC) and its PVL-negative isogenic derivative (LACΔpvl), using a low and a high inoculum (8 × 10³ and 4 × 10⁶ CFU). With the low inoculum, bone infection was less frequent on day 7 (D7) and day 28 (D28) with LACΔpvl than with LAC (respectively 12/19 and 18/19 animals, p = 0.042). With the high inoculum of both strains, all the animals were infected on D7 and the infection persisted on D28 in almost every case. However, tibial bacterial counts and the serum CRP concentration fell significantly between D7 and D28 with LACΔpvl but not with LAC. Respectively 67% and 60% of LAC-infected rabbits had bone deformation and muscle/joint involvement on D7, compared to 0% and 7% of LACΔpvl-infected rabbits (p = 0.001 and p = 0.005 respectively). Between D0 and D28, the anti-PVL antibody titer increased significantly only with the high inoculum of LAC.

**Conclusions/Significance:** PVL appears to play a role in the persistence and rapid local extension of rabbit osteomyelitis, in keeping with the greater severity of human bone infections due to PVL-positive *S. aureus*. The possible therapeutic implications of these findings are discussed.

Introduction

Panton-Valentine leukocidin (PVL) is a hetero-oligomeric pore-forming toxin composed of two components (LukS-PV and LukF-PV) and produced by *Staphylococcus aureus*. Its prevalence in clinical *S. aureus* isolates used to be low but is currently increasing due to the worldwide spread of PVL-producing community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains [1]. Regardless of methicillin resistance, PVL is particularly prevalent in staphylococcal strains that cause deep skin and soft-tissue infections, severe necrotizing pneumonia and severe bone and joint infections, all of which mainly affect children and young adults [2–5].

Extensive spread of CA-MRSA in the United States, mainly due to the exceptionally infectious strain USA300 [6], and the concomitant increase in severe invasive staphylococcal infections, including osteomyelitis, in healthy children [7–9], has renewed interest in the pathogenetic role of PVL. Studies using various experimental models [10–13] have given conflicting results, however, raising the possibility that the role of PVL might depend on the site of infection, as well as the experimental model [14].

Osteomyelitis has long been recognized as a major clinical syndrome of invasive *S. aureus* disease [15], accounting for 7% of staphylococcal infections among children hospitalized in the United States [9]. A role of PVL in bone and joint infections was initially suspected by Panton and Valentine [16] and has recently been the focus of several studies, mostly in the pediatric setting. In a retrospective study, Martinez-Aguilar et al. [17] noted that musculoskeletal infection due to PVL-positive community-acquired (CA) MRSA seemed to be associated with more fever, longer hospitalization, and more local complications. In a
prospective study comparing pediatric cases of osteomyelitis caused by PVL-positive *S. aureus* and PVL-negative *S. aureus*, Bocchini et al. found that patients with PVL-positive strains had significantly stronger inflammatory responses, more local complications such as myositis and pyomyositis, and more frequently positive blood culture [10]. Independently, Dohin et al compared pediatric cases of bone and joint infection caused by PVL-positive and PVL-negative *S. aureus* and confirmed that PVL-positive cases tended to be more severe and to require longer treatment; in addition, local complications were more frequent and often necessitated repeated surgical drainage [5].

Several experimental models, using mainly mice but also rabbits, have been developed in recent years to investigate the pathogenic role of PVL in necrotizing pneumonia, skin infections, and sepsis [10–12,19], and also to test a PVL vaccine [19]. However, there have been no experimental studies of PVL in bone and joint infections.

The purpose of this study was to compare the virulence of the PVL-positive strain USA300 and its isogenic *pvl*-negative derivative in a rabbit model of acute osteomyelitis. Rabbits were chosen because their leukocytes are as susceptible as human polymorphonuclear neutrophils to PVL [20]. Strain USA300 was chosen because it is the most prevalent PVL-positive CA-MRSA strain in the United States. Our results clearly showed that PVL plays a role in the aggressive course of experimental CA-MRSA osteomyelitis, a result highly consistent with previous clinical observations.

**Methods**

**Bacterial strains**

We used a clinical *S. aureus* strain belonging to the USA300 lineage, and its isogenic Δ*δS/F-PV* derivative (LAC and LACA Δ*pvl*, respectively), both kindly provided by Frank DeLeo. They are described in detail elsewhere and have been used in murine models of skin infection and pneumonia [11,13,19] and in a rabbit model of bacteremia [12]. PVL production by the LAC strain was checked by using a specific ELISA method [21] in the supernatants of inoculum samples.

**Preparation of bacterial inocula**

The organisms were stored at −80°C until use. Prior to experiments they were cultured in casien hydrolysate and yeast extract medium (CCY) at 37°C for 18 h with shaking. After centrifugation the supernatants were passed through 0.22-μm filters and stored at −20°C until PVL quantification by ELISA. The pellets were washed and resuspended in phosphate-buffered saline solution (PBS) to the desired bacterial density (multiplicity of infection, MOI) immediately before inoculation. The desired MOI was quantified by plating serial dilutions on tryptic soy agar (bioMérieux- France).

**Experimental model**

Norden’s method [22] was used to induce osteomyelitis in female New Zealand white rabbits weighing between 2 and 3 kg. The rabbits were housed in individual cages and received food and water *ad libitum*. The experimental protocol complied with French legislation on animal experimentation and was approved by the Animal Use Committee of Maison Alfort Veterinary School. The animals were anesthetized by intramuscular injection of 25 mg/kg ketamine (Vibrac France) and 25 mg/kg Xylazine Rompun® 2% (Bayer Santé, Division Santé Animal, Puteaux, France). Before *S. aureus* challenge (on day 0), 500 μl of venous blood was drawn and serum was stored at −20°C. An 18-gauge needle was inserted percutaneously through the lateral aspect of the right tibial metaphysis into the medullary cavity. Infection was induced by direct injection of sclerosing agent (0.1 ml of 3% sodium tetradecyl sulphate (Trombopar®), followed by 0.2 ml of inoculum and 0.1 ml of saline. Patch analgesia (Durogesic®) was given for 7 days following surgery.

Animals were assigned to receive a low inoculum (8×10⁵ CFU) or a high inoculum (4×10⁶ CFU) of LAC or LACA Δ*pvl* in order to detect a possible inoculum effect on PVL expression. These inocula were selected on the basis of pilot experiments designed to determine the dose necessary to induce persistent infection with each strain in more than 85% of animals 28 days after inoculation. LAC and LACA Δ*pvl* challenge was always performed simultaneously in order to minimize the influence of experimental conditions. Since a chromosomally-restored derivative of the LACA Δ*pvl* was not available, no complementation group was included in the experiment.

**Macroscopic aspect and bacterial density of bone**

The animals were monitored daily for general and local signs of infection (mobility, aspect of the legs) and were weighed weekly. Moribund animals (immobile, unable to be aroused from a recumbent position, and unable to access food and water) were euthanized by rapid intravenous injection of pentobarbital [23].

Animals were killed 7 days (D7) or 28 days (D28) after infection in order to assess the impact of PVL on the time course of osteomyelitis. Before sacrifice, venous blood was drawn for blood culture and serum samples (≥500 μl) were stored at −20°C for later determination of anti-PVL antibody and C-reactive protein titers. The right leg was visually examined, the tibia and femur were dissected out from the surrounding soft tissues, and the knee joint space, soft tissues, compact bone and medulla were grossly inspected. The following variables were evaluated: the presence and location of purulent exudates in the joint space; soft tissue and bone abscesses; the severity of tibial metaphysis deformation; and spread of the infection to the tibial diaphysis. The macroscopic aspect was noted and photographed. Several evaluations were made on photographs, by an investigator who was unaware of the group attribution. The blinded evaluation was reproducible and consistent with autopsy findings.

The upper third of the tibia was frozen in liquid nitrogen, crushed in a pulverizer (Spex 6700; Freezer/Mill Industries Metuchen, NJ), suspended in 10 ml of sterile saline, and quantitatively cultured on tryptic soy agar. Samples of pulverized tibia and subcutaneous abscess fluid were also stored at −20°C for microbiological studies of bone isolates.

**Imaging studies and histopathological examination**

Serial MRI was performed on 6 rabbits infected with the low inoculum (3 with LAC and 3 with LACA Δ*pvl* at 7, 14 and 21 days post-infection. These rabbits were killed 28 days post-infection for macroscopic examination and tibial bacterial counts.

MRI, coupled with plain radiography and histopathological examination of the infected legs, was also performed on two animals injected with Thrombovar® alone, 6 animals challenged with the high inocula and killed 7 days post-infection (3 with LAC and 3 with LACA Δ*pvl*) and 8 animals killed 28 days post-infection (4 with LAC and 4 with LACA Δ*pvl*). In these rabbits, muscle and joint involvement was recorded on the basis of gross signs and histological examination.

MR imaging was performed with a 1.5-Tesla device (Intera; Philips Medical System, Eindhoven 5600 PB, Netherlands) equipped with a surface coil (Sense-Flex-S). The anesthetized animal (Ketamine and Xylazine Rompun® 2%) was placed on its back with the lower limbs extended. The MR imaging protocol included several classical sequences (T1, T2, PD SPIR, STIR, and 3D WatSF), using a 140-mm field of view. The 3D WatSF sequence (TR: 20 ms, TE: 7.5 ms, flip angle of 50°, matrix...
166×512, section thickness 1.5 mm, every 0.8 mm) yielded acceptable reformatted images in the frontal or sagittal plane of the tibia. Moreover, this water-selective sequence was most sensitive for changes due to the infection, the replacement of yellow (lipid) marrow by water representing the earliest abnormality. Images were acquired and examined by a radiologist who was unaware of the group attribution.

For histopathological studies, the right leg, including half the femur, the entire tibia and the foot, was excised. The skin was removed and the leg was fixed for 48 hours in phosphate-buffered 10% formaldehyde. The tibia and femur were then decalcified in 10% nitric acid for 48–72 hours. A perpendicular section was cut through the knee and patella and was used to prepare serial macroscopic slices including the knee, the entire tibia and the distal femur. These slices were dehydrated in alcohol and embedded in paraffin. Histological sections 4 μm thick were then cut and stained with hemalun-eosine-safran (HES). Some samples were also Gram stained. The slides were examined by a pathologist who was unaware of the group attribution.

Serum antibody assay
Antibodies against PVL (before *S. aureus* challenge and at sacrifice) were measured with a specific ELISA method. Specific anti-LukS-PV antibodies were quantified with a protocol adapted from Croze et al [24], with a peroxidase-conjugated swine anti-rabbit polyclonal IgG diluted 1:1000 (DAKO). Serial dilutions of anti-luk-S polyclonal rabbit serum (bioMe´rieux) were used for calibration. The results were expressed in arbitrary units per milliliter (AU/mL), one arbitrary unit corresponding to the amount of anti-LukS-PV antibodies contained in a 1/107 dilution of the polyclonal rabbit reference serum.

C-reactive protein (CRP) assay
CRP was assayed in serum by using a specific ELISA method, as recommended by the provider (ALPCO).

Statistical analysis
Percentages (of infected animals, bone marrow involvement, bone deformation, and muscle or joint involvement) were compared with Fisher’s exact test. The non parametric Mann-Whitney U test was used to compare tibial bacterial counts and anti-PVL antibody and CRP titers. P values <0.05 were considered to denote significant differences.

Results
Selection of inoculum sizes
Preliminary studies were performed with three inocula, of $8 \times 10^5$ CFU, $4 \times 10^6$ CFU and $4 \times 10^8$ CFU. Six animals challenged with each inoculum were killed on D7 and D28. The low inoculum of LAC and the high inoculum of LACΔpvl induced persistent infection on D28 in respectively 90% and 86% of animals, and were therefore used in the rest of the study.

Characteristics of osteomyelitis induced by the low inocula
All rabbits challenged with the low inoculum of LAC (n = 19) or LACΔpvl (n = 19), including the animals used in the preliminary inoculum-ranging experiments, were used for macroscopic and microbiological evaluation. One animal each in the LAC and
LACΔpol groups died prematurely, on D4 and D6 respectively. Nine rabbits in each group were sacrificed on D7 and on D28. Mean body weight changed significantly on D7 in the LACΔpol group (+7.5%, \( P = 0.025 \) vs D0) but not in the LAC group (+3.5%, \( P = 0.31 \) vs D0). Significant weight gain was observed in the two groups on D28 (+29% and +34% in the LAC and LACΔpol groups, respectively; \( P<0.001 \) vs D0 in both groups).

Combined analysis of the D7 and D28 results showed that significantly more rabbits challenged with LAC than with LACΔpol had infected bone (18/19 vs 12/19, \( P = 0.042 \)), suggesting that PVL enabled better colonization by and/or survival of the parental strain LAC. However, bacterial counts in crushed bone were similar in the two groups (Figure 1). Bacterial counts tended to be lower on D28 than on D7 (Figure 1).

Macroscopically, the infection mainly involved bone marrow which was yellow/white in all infected animals and necrotic in two animals in each group. Bone deformation was observed in only 4/38 animals overall (3/19 and 1/19 in the LAC and LACΔpol groups, respectively; \( P=0.60 \)) and muscle abscesses were present in only 2/38 animals (2/19 and 0/19 respectively; \( P=0.49 \)).

Serial MRI studies, performed on 3 animals in each group, systematically showed signs of infection on all but the T1 sequences, with a marrow hypersignal initially restricted largely to the metaphysis; it gradually became more intense in 3 rabbits (2 LAC, 1 LACΔpol) (Figure 2A), remained stable in 2 rabbits (1 LAC, 1 LACΔpol), and was already severe on D7 (extending to the diaphysis) in 1 rabbit (1 LACΔpol, Figure 2B). Thus, both the PVL-positive strain and its PVL-negative derivative caused severe bone marrow involvement.

**Characteristics of osteomyelitis induced by the high inoculum**

Respectively 29 and 26 rabbits received the high inocula of LAC and LACΔpol. Six animals (4 LAC and 2 LACΔpol) died or were euthanatized prematurely. Respectively 11 and 12 rabbits infected with LAC and LACΔpol were killed on D7, and 7 and 5 rabbits were killed on day 28 for microbiological evaluation and macroscopic inspection. Macroscopic findings were also recorded in the 14 rabbits (7 in each group) used for imaging and histopathological studies.

On D7, body weight loss was significant and similar in the two groups (average −8% with LAC and −10% with LACΔpol; \( P = 0.014 \) and \( P<0.0001 \) vs D0, respectively), reflecting more severe disease than that observed with the low inocula. Body weight returned to baseline by D28, with similar weight recovery in the two groups. All the animals had infected bones on D7, and the infection persisted on D28 in 90% and 86% of animals challenged with LAC and LACΔpol, respectively. As with the low inoculum, mean bacterial counts were lower on D28 than on D7 (Figure 1). However, the decline was significant in rabbits challenged with LACΔpol but not in those challenged with LAC, again suggesting that PVL enhanced bacterial survival.

Osteomyelitis induced by the high inocula was associated with severe local macroscopic changes at autopsy, with bone marrow involvement.
involvement, bone deformation, and extension to the muscle (localized abscesses, myositis, sinus tract) and joint (purulent arthritis) in some rabbits (Figure 3, Table 1). Bone deformation and muscle/joint involvement were seen in respectively 67% and 60% of LAC-infected rabbits on D7, compared to respectively 0% and 7% of LACΔpvl-infected rabbits (P = 0.001 and P = 0.005 respectively) (Figure 3). On D28 the frequency of bone deformation was similar in the two groups (~60%), while muscle/joint involvement was still significantly more frequent with LAC than with LACΔpvl (57% versus 9%, P = 0.033). Thus, PVL+ osteomyelitis was an acute infection with rapid local extension, contrary to PVL− osteomyelitis.

MRI and plain radiography, performed on 7 LAC-infected and 7 LACΔpvl-infected rabbits, showed the same signal modifications as in the rabbits challenged with the low inocula. However, imaging of isolated legs, used for the high-inoculum group, was less informative than imaging of live animals (used for the low-inoculum group), thus explaining why no modifications were seen on Day 7 in the high-inoculum group. Radiographic changes were apparent on D28 in half the rabbits infected with LAC (2/4) and LACΔpvl (2/4), with widening and deformation of the tibial metaphysis and diaphysis, osteolysis, and subperiosteal osteogenesis. Typical radiological and histological aspects in infected rabbits, and their correspondence with macroscopic findings, are shown in Figure 4.

**Anti-PVL antibody titers**
Anti-PVL antibodies were measured in blood drawn before infection (D0) and at the time of sacrifice (D28) (Figure 5). The median anti-PVL titer in rabbits infected with the high inoculum of the LAC strain increased 31-fold on D28 (range 39,190–613,100 AU/mL, median 121,350 AU/mL) versus D0 (range 1,270–4,220 AU/mL, median 3,129 AU/mL). No significant change in the anti-PVL titer was detected on D28 after challenge with LACΔpvl, at either the high inoculum (D0 range 1,490–4,330 AU/mL, median 3,620 AU/mL; D28 range 1,490–6,944 AU/mL, median 3,841 AU/mL) or the low inoculum (D0 range 1,215–3,870 AU/mL, median 2,388 AU/mL; D28 range 1,284–16,290 AU/mL, median 2,905 AU/mL), or with the low inoculum of the LAC parent strain (D0 range 1,233–5,164 AU/mL, median 3,852 AU/mL; D28 range 1,391–18,650 AU/mL, median 3,761 AU/mL).

**PVL production in vitro and in vivo**
PVL production by strains recovered from the inocula and infected bones was measured in vitro and remained constant, ranging from 7.5 to 10 μg/ml LukS-PV. The PVL concentration was also measured in some pus samples. As much as 0.65 μg/ml of

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**Table 1. Macroscopic findings in rabbits challenged with a high inoculum: details of soft tissue and joint changes.**

| Strain     | Days | Purulent arthritis | Abscess | Myositis | Sinus tract |
|------------|------|--------------------|---------|----------|-------------|
| LAC        | D7   | 1/15               | 6/15    | 0/15     | 2/15        |
|            | D28  | 4/14               | 2/14*   | 3**/14   | 2/14        |
| LACΔpvl    | D7   | 0/15               | 1/15    | 0/15     | 0/15        |
|            | D28  | 1/11               | 0/11    | 0/11     | 0/11        |

Results are expressed as number of rabbits with muscle/joint involvement on total number of rabbits challenged with LAC or LACΔpvl.

*including one rabbit with concomitant purulent arthritis.

**Figure 3. Bone and muscle/joint involvement after challenge with a high inoculum of LAC and LACΔpvl.** Percentages of rabbits with bone marrow involvement, bone deformation and muscle/joint involvement 7 (D7) and 28 (D28) days after challenge with a high inoculum of LAC and LACΔpvl. n is the total number of animals. P values for differences between the c and LAC groups were obtained with the non parametric Mann-Whitney U-test.

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LukS-PV was detected in the pus of a knee abscess in a LAC strain-infected animal.

CRP

C-reactive protein levels were measured on D7 and D28 after challenge with the high inocula of LAC and LACΔpvl. CRP levels were high in both groups on D7 (LAC: 100.6–924.0 mg/L, median 287.0 mg/L; LACΔpvl: 91.3–879.9 mg/L, median 306.8 mg/L), and fell on D28, significantly with LACΔpvl (p = 0.002) but non significantly with LAC (p = 0.085) (figure 6).

Discussion

Hematogeneous osteomyelitis has long been recognized as a major clinical syndrome of S. aureus disease in children. Since the antibiotic era, acute osteomyelitis has become a less frequent cause of hospital admission and death [15]. Concomitantly with the recent worrying emergence of CA-MRSA strains, the incidence of S. aureus osteomyelitis, which represents 7% of infections due to staphylococci in hospitalized children in the United States, doubled between 2002 and 2007 [9].

Panton and Valentine themselves suspected a role of PVL in osteomyelitis [25], reporting that the leukocidin was produced in large amounts by staphylococcal strains causing severe infections [16]. Yet no experimental studies of the impact of PVL on the course of osteomyelitis have been conducted to date. Here we provide evidence that PVL plays a role in rabbit CA-MRSA osteomyelitis, i) by enhancing the persistence of the infection, even with a low inoculum, and ii) by facilitating local extension during the early phase of infection after challenge with a high inoculum.

More rabbits had infected bones on D7 and D28 after challenge with a low inoculum of LAC than with a low inoculum of the PVL-negative isogenic derivative, suggesting that PVL enhances bacterial persistence at the site of infection. PVL had a similar but less marked influence after challenge with the high inoculum. Our findings are consistent with the recent report by Diep et al, which suggests that PVL plays a role in the early stages of rabbit CA-MRSA bacteremia by enhancing the survival and spread of the strain USA300 [12].

Figure 4. Typical radiological and histological findings after challenge with a high inoculum of LAC (PVL+). A, B, C: Radiological and histological findings 28 days after challenge with a high inoculum of LAC (PVL+). Note the major deformation and widening of the entire diaphysis, compared to the control animal injected with sclerosing agent alone. Histological studies showed an intramedullary abscess and signs of chronic osteomyelitis. B: Bone abscess: the bone marrow space is filled with altered neutrophilic PMN, accompanying bone destruction and necrosis. C: Sequestrum: area of necrotic bone surrounded by an acute inflammatory exudate (pus). D, E, F: 28 days after challenge with a high inoculum of LAC (PVL+), note the soft tissue abscess surrounded by a fibrous layer on MRI and histological studies. G, H, I: Histological findings in a rabbit (PVL+) that died on D8 after challenged with a high inoculum of LAC. Histological sections (H) show muscle involvement with diffuse necrosis and dystrophic calcification, and (I) a purulent exudate in the joint cavity.

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PVL-positive osteomyelitis induced by the high inoculum caused bone deformation and muscle/joint involvement in more than 60% of rabbits, as early as 7 days post-infection. In contrast, the PVL-negative isogenic derivative failed to cause bone deformation or muscle involvement on D7, while muscle involvement was infrequent and less severe 28 days post-infection than with the parent strain.

A determining role of PVL in muscle extension has been also observed in a mouse model of skin infection: severe myositis was observed in mice inoculated subcutaneously with LAC but not in those inoculated with LACΔpvl [19]. These experimental findings are consistent with the more severe local disease observed in children and young adults with osteomyelitis due to PVL-producing strains. Bocchini et al [18] have shown that patients infected with PVL⁺ S. aureus isolates are significantly more likely to have concomitant myositis or pyomyositis than are patients with PVL⁻ S. aureus isolates. Dohin et al [5] showed that patients with PVL⁺ infection were more likely to have abnormalities on initial radiographic examination, a finding suggesting a more aggressive course.

PVL targets immune cells such as polymorphonuclear neutrophils (PMNs), monocytes and macrophages. Depending on the concentration, PVL-induced pores cause cytokine release and cell death by apoptosis or necrosis, thus enhancing the inflammatory response and causing extensive tissue necrosis [26]. The leukotoxic activity of PVL could thus explain the differences in soft tissue involvement observed here between the PVL⁺ and PVL⁻ strains. The inflammatory response to S. aureus also seems to be enhanced by PVL in the clinical setting, with a higher erythrocyte sedimentation rate (ESR) and a higher C-reactive protein level [5]. We found no difference in the day-7 CRP level between animals infected with LAC and LACΔpvl. In contrast, the CRP level fell significantly on D28 in animals infected with LACΔpvl but not in those infected with the PVL-positive parent strain LAC. This fall in the CRP level in LACΔpvl-infected rabbits coincided with a decline in bacterial counts in bone. Conversely, the persistently high CRP levels in LAC-infected rabbits were associated with severe extra-osseous involvement and were possibly related to the proinflammatory properties of PVL.

Anti-PVL antibody titers rose markedly in rabbits challenged with the high inoculum of the LAC strain. This fall in the CRP level in LACΔpvl-infected rabbits coincided with a decline in bacterial counts in bone. Conversely, the persistently high CRP levels in LAC-infected rabbits were associated with severe extra-osseous involvement and were possibly related to the proinflammatory properties of PVL.

Anti-PVL antibody titers rose markedly in rabbits challenged with the high inoculum of the LAC strain. This fall in the CRP level in LACΔpvl-infected rabbits coincided with a decline in bacterial counts in bone. Conversely, the persistently high CRP levels in LAC-infected rabbits were associated with severe extra-osseous involvement and were possibly related to the proinflammatory properties of PVL.
Bocchini et al [18] (presence of myositis and pyomyositis) and by Dolhin et al [5] (early cortical bone deformation).

In addition, the choice of adequate experimental readouts is critical. In our study, bacterial counts in bone - the classical parameter used in therapeutic studies but one that is never determined in human infections - were similar with the two strains. In contrast, macroscopic findings, which are also used to evaluate the severity of human osteomyelitis, were strain-dependent and correlated closely with radiological and histopathological findings.

In conclusion, our findings, obtained in a rabbit model of osteomyelitis due to a PVL-expressing strain of *S. aureus* and its isogenic PVL-negative derivative, supports the role of PVL as a virulence factor that enhances bacterial persistence and local spread. This is consistent with clinical reports of severe osteomyelitis due to PVL-producing *S. aureus* strains in children. In addition to clarifying the pathophysiologic role of PVL in osteomyelitis, our experimental model should be suitable for assessing new treatments. The erosion of the antibiotic armamentarium has prompted a search for other ways to prevent and treat serious staphylococcal infections. Our results call for the evaluation of new therapies to combat PVL such as anti-PVL intravenous immunoglobulin.

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**Author Contributions**

Conceived and designed the experiments: ACC GL CV JE FV ASM. Performed the experiments: OD CV JFC MMJ JE FV. Contributed reagents/materials/analysis tools: OD. Wrote the paper: ACC MMJ FV. Revised the article critically for important intellectual content: GL.

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