Vaccination with Detoxified Leukocidin AB Reduces Bacterial Load in a Staphylococcus aureus Minipig Deep Surgical Wound Infection Model

Fernandez, J1*, Sanders, H2*, Henn, J1, Wilson, JM3, Malone, D1, Buoninfante, A2, Willms, M1, Chan, R4, DuMont, AL4, McLahan, C3, Grubb, K3, Romanello, A3, van den Dobbelsteen, G2, Torres, VJ4 and Poolman, JT2

1Bacterial Vaccines, Spring House, PA, USA
2Janssen Vaccines & Prevention B.V., Leiden, The Netherlands
3In Vivo Sciences, Spring House, PA, USA
4Department of Microbiology, New York University Grossman School of Medicine, New York, NY, USA

*Authors contributed equally to this manuscript

Summary

We developed a robust and translational minipig deep surgical wound infection model to better predict the efficacy of Staphylococcus aureus vaccines in human clinical trials. Leucocidin AB was shown to have potential as a vaccine antigen against S. aureus
Previous presentation of data

The results presented in this manuscript have not been presented previously.

Corresponding author

Jan T Poolman, PhD

Head Bacterial Vaccine Discovery and Early Development,

Janssen Vaccines & Prevention B.V.

Zernikedreef 9, 2333 CK, Leiden, The Netherlands.

T: +31 715197626, Cell +31 631917392, Email: JPoolman@its.jnj.com
ABSTRACT

Vaccines against *Staphylococcus aureus* have eluded researchers for over three decades while the burden of staphylococcal diseases has increased. Early vaccine attempts mainly used rodents to characterize preclinical efficacy, and all subsequently failed in human clinical efficacy trials. More recently, the leukocidin LukAB has gained interest as a vaccine antigen. We developed a minipig deep surgical wound infection model offering three independent efficacy readouts: bacterial load at the superficial and at the deep-seated surgical site, and dissemination of bacteria. Due to similarities with humans, minipigs are an attractive option to study novel vaccine candidates. With this model, we characterized the efficacy of a LukAB toxoid as vaccine candidate. Compared to control animals, a 3-log reduction of bacteria at the deep-seated surgical site was observed in LukAB-treated minipigs and dissemination of bacteria was dramatically reduced. Therefore, LukAB toxoids may be a useful addition to *S. aureus* vaccines and warrant further study.

**Keywords:** Leukotoxin AB, minipig, surgical wound infection, *Staphylococcus aureus*, vaccine
INTRODUCTION

Staphylococcus aureus (SA) is a leading cause of hospital-acquired infections, such as surgical site infections, pneumonia and sepsis, and is a common cause of community-acquired skin and soft tissue infections (SSTI) and bloodstream infections [1, 2]. There is a substantial unmet medical need for an effective vaccine to prevent serious SA infections, many of which are now multi-drug resistant [2]. Although this has been a goal for decades, to date, no vaccine has shown efficacy in humans [3]. Single component (Merck’s V710 vaccine targeting iron-regulated surface determinant B), bivalent (Nabi Biopharmaceuticals StaphVAX vaccine containing conjugated capsular polysaccharides (CP) 5 and 8 and four-component (Pfizer’s CP5 and CP8 conjugates, clumping factor A, and manganese transport protein C [4]) vaccines all failed to demonstrate clinical efficacy [5]. Published theories on why these vaccines were not effective include their focus on generating opsonic antibodies and their reliance on preclinical mouse models of infection [5]. Staphylococcal manipulation of host immune responses is essential for pathogenesis and such bacterial immune evasion factors must be neutralized before opsonophagocytosis can occur [6]. There is also increasing evidence that protection against Staphylococcal disease requires a balance of both cellular and humoral immunity against appropriate targets [5]. Mice are a poor model with which to study this balance, as laboratory mice often have low levels of exposure to SA [7] and, consequently, a very different level of immunological priming compared to humans. Furthermore, SA causes disease in multiple organ systems, and differences in virulence factors among strains may influence the clinical manifestations of the disease [8]. Often, to predict vaccine efficacy, multiple infection models in mice, rats, and/or rabbits are used to mimic different clinical manifestations. The SA strains used in these models are generally either laboratory- or mouse-adapted, and the model requires a high inoculum for a robust infection, resulting in models that do not resemble the disease progression in humans [9].

Dependence on rodent models particularly hinders development of vaccines to virulence factors of SA that are species-specific, such as the bi-component pore-forming leukotoxins, many of which are being investigated as promising vaccine candidates [3, 10-12]. Leukocidin AB (LukAB) has been shown to be the primary toxin responsible for primary human PMNs cell death during tissue culture
infection [13], and impairs function of and kills antigen presenting cells [14], thus potentially reducing the host defense and immunological memory needed to combat current and subsequent infections. Despite potent activity of LukAB towards primary human phagocytes, the higher affinity of the toxin to the human CD11b receptor compared to the murine receptor [13, 15] renders commonly used mouse models incompatible for studying LukAB.

Progress in the field of Staphylococcal vaccines is likely to stall unless alternative animal challenge/protection models are developed that closely resembles human staphylococcal disease. In this study we describe a SA surgical wound infection model in Göttingen minipigs (Minipig surgical wound infection: MPSWI). Pigs are natural hosts for SA and transmission of disease between pigs and humans has been documented [16-18]. Similarities between the immune systems of humans and pigs include a high percentage of circulating neutrophils, a lack of nitric oxide production following lipopolysaccharide stimulation, similarity of toll-like receptors and dendritic cells, and a comparable response to endotoxin challenge [19]. Pre-existing antibodies to various SA antigens are present in pigs, as seen in humans [20, 21]. The skin of pigs is structurally similar to human skin in terms of thickness, number of hair follicles, pigmentation, and collagen and lipid composition. Pigs have also been used extensively as a research model for wound healing, drug delivery, toxicology, and dermatological conditions [22, 23], and use of the minipig as a higher animal model for toxicology testing has gained regulatory acceptance [23-26]. Additionally, SA has been studied in several pig challenge models [27-30], including a swine model used to evaluate the treatment of SA SSI [31]. To our knowledge, pig models have not been used to date to study deep seated SA infections or to evaluate vaccine efficacy.
METHODS

Animals

Five- to eight-month-old male Göttingen minipigs were group-housed and maintained on a 12-hour light/dark cycle with access to water ad libitum. Blood was collected before prime and boost doses, and 3- and 8-days post-infection.

All animal procedures were approved by the Janssen Spring House Institutional Animal Care and Use Committee and conducted in an AAALAC-accredited facility in accordance with the USDA Animal Welfare Regulations, the Guide for the Care and Use of Laboratory Animals [32, 33], and institutional policies.

Minipig Surgical Wound Model

Fasted minipigs were sedated, intubated, and placed under isoflurane anaesthesia for the duration of the surgery. After sterile preparation of the skin, a skin incision was made to expose the muscle layer on the left thigh and a 5-mm bladeless trocar was advanced to the depth of the femur. 20µl inoculum (approx. 6 log\(_{10}\) colony-forming units [CFU] SA) was injected into the wound via a 6-inch MILA spinal needle through the trocar and the muscle was closed with silk suture and the skin closed with absorbable Vicryl suture (FIGURE 1A).

Eight days later, following euthanasia, the skin was cleaned with 2% chlorohexidine solution and the surgical site skin and full thickness surgical site muscle (2 cm\(^2\)) was removed and cut into three sections - superficial, mid, deep layers, and processed separately. The skin and spleen were aseptically removed and processed separately.
Bacterial Strains and Growth Conditions

Three clinical blood isolates of SA were used to characterize the minipig model: ST398 (OC 26263, MSSA) was used for the LukAB minipig challenge studies (ST398 strains typically colonize pigs but can cause disease in humans [34-36]) ST5 (OC 26245, MRSA) and ST8 strain (OC26260, MRSA) were included because of their high global prevalence among healthcare associated- and community-acquired MRSA infections [37]. Strains were grown in Tryptic Soy Broth (TSB) overnight prior to use.

For opsonophagocytic assays: ST398 challenge strain expressing CP5 and MRSA ST30 clinical strain (BVSA00929) expressing CP8 were used. Strains were grown for 24-hours on Columbia salt agar to induce capsule expression.

Antigen Production and Formulation

Genetically detoxified LukAB (CC8 LukAB sequence where LukA had a deletion at the 10 C-terminal amino acid residues; LukABΔ10C toxoid) and wildtype LukAB CC8 toxin were expressed in SA and subsequently purified as described previously [38].

CP5 and CP8 were purified from SA clinical strains and conjugated to CRM197 through thioether chemistry (CP5/8-CRM).

HlaH35L was produced at GenScript. Briefly, the sequence of SA Newman HlaH35L, cloned behind a N-terminal His-SUMO tag, was expressed in E. coli BL21 by IPTG induction and affinity-purified from the supernatant of the whole cell lysate. After purification, the His-SUMO tag was removed.

Vaccination

Antigens were combined 1:1 (v/v) with AS01B adjuvant 30 minutes prior to vaccination. Minipigs were sedated (Telazol) and bled prior to being immunized (three intramuscular injections separated by three weeks) with CP5/8-CRM, LukABΔ10C toxoid (100 µg) mixed 1:1 with AS01B adjuvant resulting in a ½ human adjuvant dose. CP5/8-CRM was compared to unadjuvanted formulations.
buffer (TBS, pH7.4, 10% glycerol) and LukAB was compared to adjuvanted formulations buffer (separate studies). Surgery and infection occurred three weeks after the last boost.

**ELISA and Cytokine Analysis**

To determine total IgG in serum, antigens were coated onto 96-well Maxisorp plates (Nunc) for minimum 1 hour, to maximum overnight, at 2-8°C. Plates were blocked with 2.5-5% (w/v) skimmed milk prior to washing and subsequent addition of serial dilution of serum. Secondary antibody (Rabbit-anti-Pig IgG-HRP [horseradish peroxidase]) was added at 1:40,000 dilution (1:20,000 for LukAB). After further washing, 3,3’5,5’-tetramethylbenzidine was added to detect the HRP. The reaction was stopped with 1M sulfuric acid, and absorbance was read at 450nm.

To measure total IgG against CP5 and CP8, CP5-biotin and CP8-biotin conjugates were used to facilitate plate coating.

Concentrations of 13 cytokines in plasma were determined using the MILLIPLEX MAP Porcine Cytokine/Chemokine Magnetic Bead Panel.

**Opsonophagocytic assay**

HL-60 cells (American Type Culture Collection CCL-240) were differentiated into phagocytes by supplementing culture media with 0.8% Dimethylformamide for 4-5 days. Bacteria were incubated with heat-inactivated sera and IgG/IgM-depleted human sera (PelFreez) as a source of complement, prior to addition of differentiated HL-60 cells (HL-60:bacteria ratio 400:1). After 45 minutes, saponin was added to a final concentration of 1%. CFUs were counted after overnight incubation. The killing titer was calculated as the reciprocal of the highest serum dilution that gave 40% killing compared to a no-serum control.

**Toxin neutralization assay**

Toxin neutralization assays were performed with THP-1 monocytes, using a method adapted from Melhani et al 2015 [39]. THP-1 cells were added to 96-well plates at 1x10^5 cells/well, along with heat-inactivated minipig serum and LukAB CC8 toxin (final concentration 20ng/mL). After 2 hours incubation, lactate dehydrogenase was measured in supernatant using the CytoTox-ONE assay.
Percent cytotoxicity was calculated relative to a cells and toxin-only control. IC50 titers were determined by 4PL curve fitting.

**PMN Isolation and Cytotoxicity assessment**

Human and minipig PMNs were isolated from leukopaks as previously described [40]. PMNs were plated at 200,000 cells per well in 90 µl/well in 96-well plates. CC8 LukAB toxin from SA was diluted to test concentrations ranging from 20-0.015 µg/ml and added at 10 µl/well. After 90 minutes incubation, toxicity was measured by adding CellTiter 96 Aqueous One Solution (10 µl/well), incubating for a further 90 minutes, and reading absorbance at 492nm.

**Statistical Analysis**

Groups were compared using a Tobit regression model to account for possible censoring for the analysis of the CFU response. For the analysis of the IL-6 response, groups were compared using an ANOVA model. The Tobit and ANOVA models both contained treatment group and study number as explanatory factors.

**RESULTS**

**SA clinical isolates produced a robust infection in minipigs**

Bacterial burden at the infection site deep in the thigh was similar at 8 days post-infection (6-7 log_{10} CFU/g tissue) regardless of challenge strain (ST398, ST5, or ST8) (FIGURE 1). At necropsy, there was purulent material found in all three muscle layers and under the skin, and bacteria disseminated from the infection site to the abdominal organs (FIGURE 1).

**Immunization with CP5/8-CRM197 failed to protect minipigs from surgical site infection**

To evaluate the minipig model as a tool to investigate vaccine-mediated protection, minipigs were immunized with a full human dose of CP5/8-CRM197, as previously described [4], in combination with adjuvant, on three occasions prior to challenge with the CP5-expressing ST398 strain. At necropsy, there were no significant differences in bacterial burden at the surgical site or in the
spleen (indication of dissemination) of animals immunized with the CP-conjugate mix vs. animals immunized with saline (FIGURE 2).

All vaccinated animals generated robust IgG titers after vaccination against CP5, CP8 and CRM197 (FIGURE 3), which were not associated with increases in opsonophagocytic assay (OPA) titers in vitro against either the CP5+ ST398 challenge strain, or against a CP8+ strain (FIGURE 3). To confirm that each strain expresses capsule under the growth condition used for the assay, OPA was also performed with monoclonal antibodies against CP5 and CP8 (SUPPLEMENTARY FIGURE S1).

OPA and IgG titers against Staphylococcal Hla in minipig sera were already positive at the beginning of the study and increased over time (FIGURE 3), which is consistent with natural colonization of pigs by SA [41]. IgG against Hla was further measured in serum samples from a larger number of minipigs ranging from 4 months to 18 months of age (SUPPLEMENTARY FIGURE S2). Antibodies against Hla increased with increasing age, indicating that exposure to SA and natural immunity is common in Göttingen minipigs.

*LukAB showed comparable cytotoxicity to human and minipig PMNs in vitro*

In contrast to rodent models, LukAB has been shown to bind pig CD11b to a similar level as human CD11b [15]. Here, we evaluated whether LukAB also shows comparable cytotoxicity towards minipig PMNs. When granulocytes isolated from minipig blood were challenged with wildtype LukAB toxin, they showed a similar susceptibility to the toxin as human PMNs (FIGURE 4). By contrast, LukAB exhibits markedly reduced cytotoxicity on rat immune cells (used to estimate mouse) and PMNs isolated from mice and rabbits [42].
LukAB vaccination protected minipigs from surgical site infection

To explore whether the minipig surgical wound infection model could show any protection afforded by vaccination with LukAB, minipigs were vaccinated with three doses of a detoxified LukAB protein lacking the CD11b-targeting domain (LukAB CC8Δ10C, [38]), or an adjuvant control, prior to infection with the ST398 challenge strain. The experiment was performed across two separate studies, each containing 3 LukAB toxoid-vaccinated animals and 3 adjuvant control animals.

Sera from all minipigs contained measurable IgG against the wildtype LukAB CC8 toxin as well as pre-existing antibodies to Hla at Day 0 (FIGURE 5). In the control group, anti-LukAB IgG titers increased between challenge and necropsy, suggesting that, not only is LukAB expressed by the SA strains that naturally colonize minipigs, but also that LukAB is actively expressed during infection in this model.

LukAB vaccination resulted in increased anti-LukAB IgG titers compared to the control group, and these correlated with an increased capacity of the sera to neutralize cytotoxicity of the wildtype toxin against THP-1 cells in vitro (FIGURE 5). This difference was largest at day 63 (day of challenge). By day 71 (necropsy), anti-LukAB IgG and toxin neutralization by sera were similar between the LukAB toxoid-vaccinated and the control animals, due to the boosting effect of LukAB exposure during challenge in the adjuvant group. The same boosting effect during challenge was not seen in the LukAB toxoid-vaccinated group.

At necropsy, animals vaccinated with LukAB toxoid had significantly ($p<0.0001$) lower CFU counts at the surgical site muscle compared to the adjuvant group (FIGURE 6A), with the greatest difference observed in the deepest muscle depth ($p<0.0001$), immediately above the femur (FIGURE 6B), with CFU counts in the superficial muscle (below the skin) being closer to those measured in the adjuvant control group (SUPPLEMENTARY FIGURE S3). Bacterial burden in the skin was similar between the groups; however, protection at the surgical site in the LukAB vaccinated group was clearly visible as a reduction in purulent material throughout the muscle (FIGURE 6C, 7). Furthermore, animals vaccinated with LukAB toxoid had significantly lower CFU counts in the spleen than control animals.
(5/6 at/below the limit of quantification vs 1/6) respectively (FIGURE 6D), indicating that immunization provided protection both at the surgical site, and against dissemination of the bacteria from the surgical site.

As an additional marker of a more systemic response to infection, levels of 13 cytokines were measured in plasma from the minipigs prior to challenge, at days 1, 2, and 3 after challenge, and at necropsy. In control animals, IL-6, a key marker for systemic inflammation during infection, increased sharply within 24 hours after infection (FIGURE 5, other cytokines included in supplementary table S1), before returning to baseline by day 8 post-infection. In general, IL-6 concentrations in plasma of LukAB toxoid-vaccinated animals 24 hours after challenge were lower than those measured in control animals; however, due to low group sizes and high variability among animals this did not achieve significance ($p=0.06$).

This increase in IL-6 was concordant with the rise in body temperature immediately following infection and lasting 48-72h, however there was no difference in body temperature between the LukAB toxoid-immunized animals or the animals treated with adjuvant control.

**DISCUSSION**

To address the need for a pre-clinical model of SA infection that is more representative of the human state than rodents, we have developed a SA deep-seated surgical wound infection model in Göttingen minipigs. Pigs share many characteristics with humans that make them suitable for such a model: for example, similarities in the skin [22], and the immune system [19]. Pigs have been used previously for a variety of SA challenge models, including skin, osteomyelitis, and pyemia [27, 43, 44], demonstrating the versatility of this species, but have not to our knowledge been used as a model for deep-seated surgical wound infection. Pigs are a natural reservoir for SA [45]. Consequently, it is possible to use human clinical isolates in the minipig challenge model. In our model, we studied three human clinical isolates: ST5 (USA100), ST8 (USA300) and ST398. In all cases, we were able to induce a reproducible, high bacterial burden at the deep-seated infection site as
well as at superficial infection sites as two independent measurable endpoints, with low-level infection in other organs.

Göttingen minipigs used in our studies had background antibodies to Staphylococcal Hla, which increased with age, consistent with repeated exposure. This emulates similar changes in circulating anti-Staphylococcal IgG observed in humans [46], but which is absent in mice. This may contribute to the artificially large protective effect of SA vaccines observed in mouse models that has not translated to protection in pre-exposed humans, or indeed in pre-exposed pigs.

To illustrate this point, we tested the protective efficacy of a vaccine containing capsular polysaccharides CP5 and CP8, both conjugated to CRM197, as an example of a vaccine that has shown protection in models in rodents but failed to show protection in humans, either alone [47], or in combination with two other antigens [48]. Both the CP5 and CP8 antigens were strongly immunogenic, but in our model, did not show any protection compared to the negative control (as measured by bacterial burden at the surgical site and in the spleen). The ST398 challenge strain used is known to express capsule type CP5 in vitro, so it is expected that capsule is also expressed in vivo.

As antibodies against the capsule are expected to function by promoting opsonophagocytosis, we compared the sera of vaccinated minipigs in an OPA assay against both the CP5-expressing ST398 challenge strain and a CP8-expressing clinical isolate. In both cases, minipig sera showed high levels of background OPA activity prior to immunization, and the presence of anti-Cp5 or anti-Cp8 antibodies in sera post-vaccination did not markedly increase the OPA activity. This lack of increase in OPA over background could explain the lack of protection seen in this model with the CP5/8-CRM197 vaccine.

To further evaluate the model, we assessed a genetically-detoxified LukAB vaccine that cannot be reliably tested in mouse models [13]. SA LukAB-mediated cytotoxicity on minipig granulocytes was similar to that observed with human PMNs, making minipigs a suitable model for evaluation of this toxin. Recently, Karauzum et al described a multicomponent toxoid vaccine containing a LukAB toxoid [12]. Although the authors show efficacy of their vaccine in murine and rabbit models of skin
infection, the contribution of the anti-LukAB response to this effect was not evaluated. Since LukAB does not target murine cells and is only weakly active towards rabbits granulocytes [13, 15, 42], it is unlikely that the reported effect is due to LukAB targeting. Thus, it remains to be determined if targeting LukAB would prevent or ameliorate SA disease in vivo.

While the model described herein enabled us to study LukAB, many Staphylococcal toxins/virulence factors show species specificity [13], and it is likely that there are SA virulence factors that do not ‘work’ comparatively in pigs as in humans. Published examples of these include Staphylokinase [49] and CHIPS (chemotaxis inhibitory protein of SA) [50]. Therefore, although we expect the minipig model to be more suitable for a wider range of SA antigens than mice, there will still be antigens for which it is less effective. Furthermore, some humanized mouse models have been developed for studying particular antigens, such as humanized CD11b mice for studying LukAB [15], which can provide valuable answers to key questions on the antigens for which they were developed.

In contrast to CP5/8-CRM197, LukAB vaccination induced significant protection at the surgical site and in the spleen, and visibly reduced purulent material at the wound. The protective effect was reduced in the skin and superficial muscle, compared to deeper tissues, presumably due to the sutures acting as a nidus for the bacteria and allowing biofilm formation. The reduced bacterial burden in the spleens of the vaccinated group compared to the control group suggests that vaccination was furthermore able to reduce the dissemination of the bacteria from the wound. This reduced dissemination was associated with generally lower circulating levels of the inflammatory cytokine IL-6 in the days following infection.

Serum analysis showed that pre-existing, neutralizing antibodies to LukAB were boosted by vaccination, and by challenge in the control group. Together, these results demonstrate that LukAB is produced by SA during both carriage and infection in pigs, further supporting the use of this model to evaluate LukAB vaccine candidates.
Our study shows that the minipig model warrants further investigation for other disease indications, and evaluation of additional virulence factors as vaccine antigens. This could include comparison of different LukAB toxoid candidates, such as those described by Kailasan et al [10]. However, there are limitations on the number of minipigs that can be used per study depending on the size of the vivarium, and the model might need to be supported by rodent and/or rabbit models to answer specific questions. Additionally, reagent availability for pigs is not as extensive as those for rodents, and the reagents for one breed of pig may not work for others.

In conclusion, Göttingen minipigs are an effective model for SA surgical wound infections and can be used with clinical isolates to evaluate the protective efficacy of SA vaccine candidates. We hope to use this model in future studies to evaluate different vaccine antigen combinations and formulations, as a reliable way to bridge preclinical results in mice to clinical results in humans.
ACKNOWLEDGEMENTS

The authors would like to thank Dr. Rohit Rajoria, Loran Milhaliak, and James Hastings for surgical assistance and clinical care; Timothy van Eijl, Joan van Kregten and Martijn Kremer for performing in vitro assays. We thank Dr. Donna Korvick for giving us the idea of using a trocar to refine the surgical procedure. We acknowledge the work of our biotechnicians for the care and careful observation of the minipigs. We also thank Jan Serroyen for the statistical analysis and Joanne Wolter for her careful review of this manuscript.

Conflict of interest statement

All authors are affiliated with Janssen Research and Development, LLC or New York University.

V.J.T. and A.L.D. are inventors on patents and patent applications filed by New York University, which are currently under commercial license to Janssen Biotech Inc. Janssen Biotech Inc. provides research funding and other payments associated with the licensing agreement. All other authors have nothing to disclose relating to this manuscript.

Funding

All costs associated with development of this manuscript were funded by Janssen.
REFERENCES

1. Dayan GH, Mohamed N, Scully IL, et al. Staphylococcus aureus: the current state of disease, pathophysiology and strategies for prevention. Expert Rev Vaccines 2016; 15:1373-92.

2. de M Campos JC, Antunes LC, Ferreira RB. Global priority pathogens: virulence, antimicrobial resistance and prospective treatment options. Future Microbiol 2020.

3. Miller LS, Fowler VG, Shukla SK, Rose WE, Proctor RA. Development of a vaccine against Staphylococcus aureus invasive infections: Evidence based on human immunity, genetics and bacterial evasion mechanisms. FEMS Microbiol Rev 2020; 44:123-53.

4. Begier E, Seiden DJ, Patton M, et al. SA4Ag, a 4-antigen Staphylococcus aureus vaccine, rapidly induces high levels of bacteria-killing antibodies. Vaccine 2017; 35:1132-9.

5. O’Brien EC, McLoughlin RM. Considering the 'Alternatives' for Next-Generation Anti-Staphylococcus aureus Vaccine Development. Trends Mol Med 2019; 25:171-84.

6. Thammavongsa V, Kim HK, Missiakas D, Schneewind O. Staphylococcal manipulation of host immune responses. Nat Rev Microbiol 2015; 13:529-43.

7. Schulz D, Grumann D, Trube P, et al. Laboratory Mice Are Frequently Colonized with Staphylococcus aureus and Mount a Systemic Immune Response-Note of Caution for In vivo Infection Experiments. Front Cell Infect Microbiol 2017; 7:152.

8. Laabei M, Uhlemann AC, Lowy FD, et al. Evolutionary Trade-Offs Underlie the Multi-faceted Virulence of Staphylococcus aureus. PLoS Biol 2015; 13:e1002229.
9. Salgado-Pabon W, Schlievert PM. Models matter: the search for an effective Staphylococcus aureus vaccine. Nat Rev Microbiol 2014; 12:585-91.

10. Kailasan S, Kort T, Mukherjee I, et al. Rational Design of Toxoid Vaccine Candidates for Staphylococcus aureus Leukocidin AB (LukAB). Toxins (Basel) 2019; 11.

11. Tam K, Lacey KA, Devlin JC, et al. Targeting leukocidin-mediated immune evasion protects mice from Staphylococcus aureus bacteremia. J Exp Med 2020; 217.

12. Karauzum H, Venkatasubramaniam A, Adhikari RP, et al. IBT-V02: A Multicomponent Toxoid Vaccine Protects Against Primary and Secondary Skin Infections Caused by Staphylococcus aureus. Front Immunol 2021; 12:624310.

13. DuMont AL, Yoong P, Day CJ, et al. Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. Proc Natl Acad Sci U S A 2013; 110:10794-9.

14. Berends ETM, Zheng X, Zwack EE, et al. Staphylococcus aureus Impairs the Function of and Kills Human Dendritic Cells via the LukAB Toxin. mBio 2019; 10.

15. Boguslawski KM, McKeown AN, Day CJ, et al. Exploiting species specificity to understand the tropism of a human-specific toxin. Sci Adv 2020; 6:eaax7515.

16. Lewis HC, Molbak K, Reese C, et al. Pigs as source of methicillin-resistant Staphylococcus aureus CC398 infections in humans, Denmark. Emerg Infect Dis 2008; 14:1383-9.

17. Bangerter PD, Sidler X, Perrethen V, Overesch G. Longitudinal study on the colonisation and transmission of methicillin-resistant Staphylococcus aureus in pig farms. Vet Microbiol 2016; 183:125-34.
18. Meemken D, Blaha T, Tegeler R, et al. Livestock associated methicillin-resistant Staphylococcus aureus (LaMRSA) isolated from lesions of pigs at necropsy in northwest Germany between 2004 and 2007. Zoonoses Public Health 2010; 57:e143-8.

19. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. Trends Microbiol 2012; 20:50-7.

20. Kolata J, Bode LG, Holtfreter S, et al. Distinctive patterns in the human antibody response to Staphylococcus aureus bacteremia in carriers and non-carriers. Proteomics 2011; 11:3914-27.

21. Verkaik NJ, Lebon A, de Vogel CP, et al. Induction of antibodies by Staphylococcus aureus nasal colonization in young children. Clin Microbiol Infect 2010; 16:1312-7.

22. Summerfield A, Meurens F, Ricklin ME. The immunology of the porcine skin and its value as a model for human skin. Mol Immunol 2015; 66:14-21.

23. Bode G, Clauing P, Gervais F, et al. The utility of the minipig as an animal model in regulatory toxicology. J Pharmacol Toxicol Methods 2010; 62:196-220.

24. Forster R, Bode G, Ellegaard L, van der Laan JW. The RETHINK project on minipigs in the toxicity testing of new medicines and chemicals: conclusions and recommendations. J Pharmacol Toxicol Methods 2010; 62:236-42.

25. Svendsen O. The minipig in toxicology. Exp Toxicol Pathol 2006; 57:335-9.

26. van der Laan JW, Brightwell J, McAnulty P, Ratky J, Stark C, Steering Group of the RP. Regulatory acceptability of the minipig in the development of pharmaceuticals, chemicals and other products. J Pharmacol Toxicol Methods 2010; 62:184-95.
27. Nielsen OL, Iburg T, Aalbaek B, et al. A pig model of acute Staphylococcus aureus induced pyemia. Acta Vet Scand 2009; 51:14.

28. Johansen LK, Frees D, Aalbaek B, et al. A porcine model of acute, haematogenous, localized osteomyelitis due to Staphylococcus aureus: a pathomorphological study. APMIS 2011; 119:111-8.

29. Svedman P, Ljungh A, Rausing A, et al. Staphylococcal wound infection in the pig: Part I. Course. Ann Plast Surg 1989; 23:212-8.

30. Luna CM, Sibila O, Agusti C, Torres A. Animal models of ventilator-associated pneumonia. Eur Respir J 2009; 33:182-8.

31. Mohiti-Asli M, Risselada M, Jacob M, Pourdeyhimi B, Loboa EG. Creation and Evaluation of New Porcine Model for Investigation of Treatments of Surgical Site Infection. Tissue Eng Part C Methods 2017; 23:795-803.

32. Council NR. Guide for the Care and Use of Laboratory Animals: Eighth Edition. Washington, DC: The National Academies Press, 2011.

33. USDA. Animal Welfare Regulations. 2008. 9 CFR § 3.129.

34. Golding GR, Bryden L, Levett PN, et al. Livestock-associated methicillin-resistant Staphylococcus aureus sequence type 398 in humans, Canada. Emerg Infect Dis 2010; 16:587-94.

35. Skallerup P, Espinosa-Gongora C, Jorgensen CB, Guardabassi L, Fredholm M. Genome-wide association study reveals a locus for nasal carriage of Staphylococcus aureus in Danish crossbred pigs. BMC Vet Res 2015; 11:290.
36. Tulinski P, Duim B, Wittink FR, et al. Staphylococcus aureus ST398 gene expression profiling during ex vivo colonization of porcine nasal epithelium. BMC Genomics 2014; 15:915.

37. Monaco M, Pimentel de Araujo F, Cruciani M, Coccia EM, Pantosti A. Worldwide Epidemiology and Antibiotic Resistance of Staphylococcus aureus. Curr Top Microbiol Immunol 2017; 409:21-56.

38. DuMont AL, Yoong P, Liu X, et al. Identification of a crucial residue required for Staphylococcus aureus LukAB cytotoxicity and receptor recognition. Infect Immun 2014; 82:1268-76.

39. Melehani JH, James DB, DuMont AL, Torres VJ, Duncan JA. Staphylococcus aureus Leukocidin A/B (LukAB) Kills Human Monocytes via Host NLRP3 and ASC when Extracellular, but Not Intracellular. PLoS Pathog 2015; 11:e1004970.

40. Reyes-Robles T, Lubkin A, Alonzo F, 3rd, Lacy DB, Torres VJ. Exploiting dominant-negative toxins to combat Staphylococcus aureus pathogenesis. EMBO Rep 2016; 17:780.

41. Smith TC. Livestock-associated Staphylococcus aureus: the United States experience. PLoS Pathog 2015; 11:e1004564.

42. Malachowa N, Kobayashi SD, Braughton KR, et al. Staphylococcus aureus leukotoxin GH promotes inflammation. J Infect Dis 2012; 206:1185-93.

43. Klein P, Sojka M, Kucera J, et al. A porcine model of skin wound infected with a polybacterial biofilm. Biofouling 2018; 34:226-36.
44. Jensen HE, Nielsen OL, Agerholm JS, et al. A non-traumatic Staphylococcus aureus osteomyelitis model in pigs. In Vivo 2010; 24:257-64.

45. Sun J, Yang M, Sreevatsan S, Davies PR. Prevalence and Characterization of Staphylococcus aureus in Growing Pigs in the USA. PLoS One 2015; 10:e0143670.

46. Wu Y, Liu X, Akhgar A, et al. Prevalence of IgG and Neutralizing Antibodies against Staphylococcus aureus Alpha-Toxin in Healthy Human Subjects and Diverse Patient Populations. Infect Immun 2018; 86.

47. Shinefield H, Black S, Fattom A, et al. Use of a Staphylococcus aureus conjugate vaccine in patients receiving hemodialysis. N Engl J Med 2002; 346:491-6.

48. Scully IL, Timofeyeva Y, Illenberger A, et al. Performance of a Four-Antigen Staphylococcus aureus Vaccine in Preclinical Models of Invasive S. aureus Disease. Microorganisms 2021; 9.

49. Gladysheva IP, Turner RB, Sazonova IY, Liu L, Reed GL. Coevolutionary patterns in plasminogen activation. Proc Natl Acad Sci U S A 2003; 100:9168-72.

50. de Haas CJ, Veldkamp KE, Peschel A, et al. Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent. J Exp Med 2004; 199:687-95.
Figure 1. Bacterial growth of clinically relevant SA strains in the minipig. Minipig deep surgical wound infection model (A). Bacterial burden after 8 days of infection with ST398, ST5, and ST8 strains in the surgical site muscle (B), skin (C), and spleen (D).

Figure 2. Effect of CP5/8 in the muscle, skin, and spleen of the minipig. Bacterial burden after 8 days of infection (ST398) in the surgical site muscle (A), skin (B), and spleen (C).

Figure 3. Humoral immunity generated after immunization with CP5-CRM and CP8-CRM conjugates. Total IgG was measured by ELISA (A) against CP5 and CP8 (in both cases, polysaccharides were conjugated to Biotin to facilitate plate coating), CRM197 and Hla. Opsonophagocytic activity (OPA) of sera was measured using differentiated HL-60 cells and human complement against a CP5-expressing ST398 clinical isolate (B) and a CP8-expressing ST30 clinical isolate (C), with OPA titers reported as the reciprocal of the serum dilution giving 40% killing compared to a no-serum control. In all cases, results as shown as EC50 values (ELISA) or OPA titers per animal per timepoint (N = 4 animals per group), with a line at the geometric mean per group. Open circles and dashed lines indicate animals in the adjuvant-control group, while closed circles and complete lines indicate animals immunized with the CP5/8-CRM197 vaccine. Study days are measured from the day of the first vaccination, i.e., Days 0, 21, 42 = Dose 1, 2, 3 respectively; Day 63 = Challenge, Day 71 = Necropsy.
Figure 4. Toxicity of LukAB towards human and minipig PMNs. Intoxication of freshly isolated human or minipig PMNs and rat blood immune cells with the indicated doses of LukAB for 1.5 hrs. Cell viability was evaluated with the metabolic dye CellTiter and % cell death was determined by normalizing to cells treated with TritonX-100 set at 100% cell death.

Figure 5. Humoral immunity generated after immunization with LukAB toxoid, and systemic response to infection. Total IgG was measured by ELISA against Hla (A) and LukAB CC8 wildtype toxin (B). Ability of sera to neutralize cytotoxicity of LukAB was measured against THP-1 cells (C). Cytotoxicity was measured by lactate dehydrogenase release, and converted to a percentage relative to a toxin-only control. Concentrations of IL-6 were also measured in plasma prior to challenge and at post-challenge timepoints (D). In all cases, results as shown per animal per timepoint, with a line at the geometric mean (Graphs A, B, C) or mean (Graph D) per group. Open circles/triangle and dashed lines indicate animals in the adjuvant-control group, while closed circles/triangles and complete lines indicate animals immunized LukAB toxoid. The experiment was completed over two studies (3 animals per group per study = 6 animals per group total), with data points from each study shown as triangles (Study 1) or circles (Study 2) on the graphs. For graphs (A), (B) and (C) study days are measured from the day of the first vaccination, i.e., Days 0, 21, 42 = Dose 1, 2, 3 respectively; Day 63 = Challenge, Day 71 = Necropsy. Graph (D) shows days after challenge, i.e., 0 = Challenge (Study Day 63), 8 = Necropsy (Study Day 71).
Figure 6. Effect of LukAB toxoid in the muscle, skin, and spleen of the minipig. Bacterial burden after 8 days of infection (ST398) in the combined surgical site muscle (A), deep surgical site muscle (B), skin (C), and spleen (D).

Figure 7. Effect of LukAB toxoid in the muscle of the minipig. Representative photograph of purulent material at the surgical site 8 days after infection.
Figure 1
Log_{10} CFU/g of Spleen

Saline

CP5/8

limit of detection
$\log_{10} \text{CFU/g of Muscle (All Layers)}$

$p < 0.0001^*$

Control

LukAB (100 ug)

Study 1

Study 2

limit of detection
Graph showing the relationship between log_{10} CFU/g Spleen and treatment groups. The y-axis is labeled as log_{10} CFU/g Spleen, with values ranging from 0 to 4.

There are two treatment groups: Control and LukAB (100 ug).

- Control group has a horizontal line representing the mean value of log_{10} CFU/g Spleen, with a range from 2 to 4.
- LukAB (100 ug) group has two study populations: Study 1 (blue triangles) and Study 2 (blue circles).

The p-value is indicated as *p=0.004*.

The limit of detection is also marked on the graph.
