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Feasibility of using a combination of staphylococcal superantigen-like proteins 3, 7 and 11 in a fusion vaccine for *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is a significant bacterial pathogen in both community and hospital settings, and the escalation of antimicrobial-resistant strains is of immense global concern. Vaccination is an inviting long-term strategy to curb staphylococcal disease, but identification of an effective vaccine has proved to be challenging. Three well-characterized, ubiquitous, secreted immune evasion factors from the staphylococcal superantigen-like (SSL) protein family were selected for the development of a vaccine. Wild-type SSL3, 7 and 11, which inhibit signaling through Toll-like receptor 2, cleavage of complement component 5 and neutrophil function, respectively, were successfully combined into a stable, active fusion protein (PolySSL7311). Vaccination of mice with an attenuated form of the PolySSL7311 protein stimulated significantly elevated specific immunoglobulin G and splenocyte proliferation responses to each component relative to adjuvant-only controls. Vaccination with PolySSL7311, but not a mixture of the individual proteins, led to a $> 10^2$ reduction in *S. aureus* tissue burden compared with controls after peritoneal challenge. Comparable antibody responses were elicited after coadministration of the vaccine in either AddaVax (an analog of MF59) or an Alum-based adjuvant; but only AddaVax conferred a significant reduction in bacterial load, aligning with other studies that suggest both cellular and humoral immune responses are necessary for protective immunity to *S. aureus*. Anti-sera from mice immunized with PolySSL7311, but not individual proteins, partially neutralized the functional activities of SSL7. This study confirms the importance of these SSLs for the survival of *S. aureus in vivo* and suggests that PolySSL7311 is a promising vaccine candidate.

INTRODUCTION

Staphylococcus aureus is an important human bacterial pathogen, responsible for significant morbidity and mortality worldwide.[1] *Staphylococcus aureus* can cause a spectrum of diseases, ranging from local infections such as boils to life-threatening conditions including bacteremia and toxic shock syndrome. There is a heightened risk of contracting bacterial infections such as *S. aureus* in the nosocomial setting,[2] particularly when undergoing surgical procedures or with extended use of indwelling catheters.[3] Resistance to common antibiotics is now widespread in both the community and hospitals,[4] leading to global recognition that development of new treatments for *S. aureus* is a high priority.[5]

Development of an effective vaccine has proved elusive but would be an ideal long-term solution to curtail the significant economic costs, debilitating effects and mortality associated with diseases mediated by *S. aureus*. Both passive and active vaccine candidates have progressed to clinical trials,[6] primarily using increased opsonophagocytic antibody responses as an indicator of

success,[7] but so far have failed to significantly improve patient outcomes. An additional challenge is the lack of reliable immunological correlates of protective immunity to *S. aureus*. Collectively, studies of immune-deficient human patients infer that functional T helper (Th) 17 cells and the production of interleukin-17A (IL-17A) to promote neutrophil chemotaxis and activation play a more important role in containing mucosal infections with *S. aureus* than humoral responses.[7] Similarly, control of a cutaneous infection[8] or stimulation of vaccine-induced protective immunity to a peritoneal challenge with *S. aureus* in mice correlates with the production of IL-17A.[9]

Staphylococcus aureus has a suite of virulence factors capable of subverting many aspects of host immunity,[10] therefore a successful vaccine may need to include multiple antigens—both cell wall associated and secreted—from this versatile pathogen. However, a recent phase II/III trial of the leading multiantigen vaccine candidate SA4-Ag, which was administered without an adjuvant, was terminated because of a lack of efficacy.[11] The value of incorporating an adjuvant has been demonstrated in preclinical studies on 4C-Staph, which targets surface-exposed antigens and several secreted antigens including the alpha-toxin hemolysin (Hla). The choice of adjuvant had a sizeable impact on vaccine efficacy in mouse models, with augmentation of Alum with a Toll-like receptor (TLR) 7 ligand[12] or the use of the squalene-based adjuvant MF59,[13] each shown to confer superior protection from *S. aureus* challenge relative to vaccination in Alum.

Many *S. aureus* vaccine candidates target cell wall components, with the goal of producing opsonic antibodies. However, a strong case has been made for targeting secreted virulence factors, such as superantigens and pore-forming toxins.[14] The presence of these toxins is often linked to more severe forms of staphylococcal disease and preliminary evidence from clinical trials suggests that neutralization of selected toxins with monoclonal antibody treatment may attenuate disease.[14] Conversely, many toxins are not common to all isolates or variably expressed, which limits their utility as vaccine antigens. It may even be necessary to develop several vaccines to target various facets of *S. aureus* pathogenicity, as illustrated in a preliminary study comparing the efficacy of a multicomponent vaccine candidate in murine models of systemic *versus* skin infections.[15]

The staphylococcal superantigen-like (SSL) proteins are a family of 14 secreted immune evasion factors with high structural similarity to superantigens.[16,17] The SSLs are genome-encoded and present in *S. aureus* isolates from both humans and animals,[18,19] suggesting they are fundamental to the survival of *S. aureus*. Collectively they interfere with a broad range of important immune functions including TLR-2 signaling, activation of the complement cascade, binding of immunoglobulin (Ig) G or A as well as hindering neutrophil function and migration.[17] Most studies have focused on the activities of individual recombinant SSLs, but gene expression studies in *S. aureus* have indicated that *ssl* genes are upregulated in response to exposure to human neutrophils.[20] Systemic infection of mice with gene-knockout strains of *S. aureus* lacking the accessory gene regulator (*agr*), which results in over-expression of SSLs, in conjunction with deletion of *ssl1-11*, significantly reduces the *S. aureus* kidney burden, suggesting that in certain contexts the SSLs make a significant contribution to disease.[21] SSLs 3, 7 and 11 are well-characterized family members that each target distinct facets of host immunity. SSL3 is a potent inhibitor of TLR-2 activation in both human and murine cells.[22,23] It blocks lipopeptide binding to TLR-2 and prevents TLR-2 from dimerizing with TLR-1 or TLR-6 to initiate downstream signaling.[24] Systemic infection of mice with an *S. aureus* strain overexpressing SSL3 causes enhanced disease, suggesting that SSL3 contributes to virulence.[25] SSL7 blocks cleavage of complement component 5 (C5), which is required for the production of the potent C5a anaphylatoxin and downstream formation of the membrane attack complex (C5b-9).[26] SSL7 also targets IgA from humans and other species (except murine IgA)[27] to prevent IgA from binding to CD89 (FcαRI).[26] Inhibition of complement C5 cleavage is enhanced in the presence of human IgA,[28] but treatment with SSL7 can still effectively block activation of murine complement C5 to prevent migration of neutrophils in response to *S. aureus*.[29] SSL11 binds to sialic acid–containing glycoproteins including sialyl Lewis x (sLe^x), which enables it to target key receptors expressed on myeloid cells.[30] SSL11 can block the interaction between IgA and CD89, and perhaps more importantly can prevent neutrophil rolling by targeting P-selectin glycoprotein-1[30] as well as locking both neutrophils and monocytes into an adherent phenotype.[31] The glycoprotein profile of murine cells differs relative to human cells,[32] but SSL11 can bind to murine neutrophils (our unpublished data). A combination of crystallography and targeted mutagenesis has been used to identify the amino acids required for the functional activity of SSLs 3, 7 and 11.[24,28,30,33]

To determine the feasibility of using the SSL proteins in a vaccine, we have produced, characterized and tested two fusion proteins comprising wild-type or functionally inactive forms of SSLs 3, 7 and 11. Two different adjuvant formulations, an Alum-based formulation

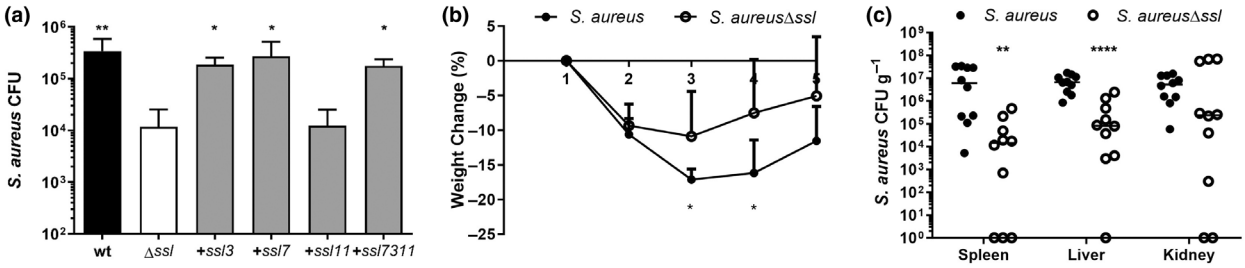

Figure 1. Removal of *ssl* genes from *Staphylococcus aureus* JSNZ results in a reduced ability to respond to immune attack compared with wild-type bacteria. **(a)** Survival of wild-type JSNZ bacteria, the *S. aureus*Δ*ssl* strain and the *S. aureus*Δ*ssl* strain recomplemented with selected *ssl*s in the presence of human whole blood after 20 h; **(b)** attenuated development of disease after intraperitoneal infection of CD1 mice with 10^8 CFU of wild-type or *S. aureus*Δ*ssl* bacteria shown by assessing weight loss relative to baseline over 5 days or **(c)** tissue CFU at day 5. Results in **(a)** are representative of independent experiments with two different blood donors, shown as mean plus standard deviation of replicate samples and statistically significant differences relative to the Δ*ssl* strain determined using a Kruskal–Wallis test with Dunn's multiple comparisons test. The remaining data are combined from two independent experiments, each with $n = 5$ mice. Body weights **(b)** are displayed as mean plus standard deviation and differences over time were compared by running multiple *t*-tests; **(c)** shows individual data points and the median, with significant differences identified by the Mann–Whitney *U*-test (*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$). CFU, colony-forming units.

(widely recognized as the gold standard for inducing antibody responses),[34] and an analog of MF59 (which elicits a broader range of humoral and cellular immune responses)[35] were assessed for their capacity to elicit a specific antibody response and reduce the *S. aureus* burden in a model of peritoneal infection.

RESULTS

SSLs are important for bacterial survival

To determine the role of selected SSLs in bacterial survival, all the *ssl* genes were knocked out of the *S. aureus* JSNZ strain to create a Δ*ssl* strain. Survival of the Δ*ssl* strain was significantly reduced when compared with wild-type bacteria ($P < 0.01$; Figure 1a) in a human whole blood killing assay. Recomplemented Δ*ssl* strains confirmed to express SSLs 3, 7 or SSLs 3, 7 and 11 proteins (Supplementary figure 1) had significantly enhanced survival compared with *S. aureus*Δ*ssl* bacteria ($P < 0.05$), whereas restoration of SSL11 did not increase bacterial colony-forming units (CFU) in this assay (Figure 1a). *In vivo* survival of wild-type and *S. aureus*Δ*ssl* strains was compared in three standard mouse models of infection. Mice infected intraperitoneally with *S. aureus*Δ*ssl* developed attenuated disease relative to mice given wild-type bacteria. Mice infected with the *S. aureus*Δ*ssl* strain had significantly reduced weight loss on days 3 and 4 of infection (~5%, $P < 0.05$; Figure 1b), accompanied by a significant reduction in median *S. aureus* CFU in the spleen ($P < 0.01$) and liver ($P < 0.0001$) on day 5 of infection (Figure 1c). By contrast, pilot studies in nasal colonization and subcutaneous abscess models of infection showed no significant differences in either the course of disease or bacterial burden (Supplementary figure 2). These results suggest that the SSLs have an important role in systemic, but not local, *S. aureus* infections.

Design, construction and characterization of an SSL7311 fusion protein

Two 70-kD recombinant fusion proteins containing the wild-type protein SSL7311, or SSL7311M composed of the functional mutants, were made. The residues mutated in SSL7311M have side chains that are surface exposed and interact with ligands; these do not contribute to the structural integrity of the SSL. The SSL7311 proteins had equivalent solubility and stability after purification, were confirmed to be ≥ 95% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the presence of all three SSLs confirmed by Western blot (Supplementary figure 3).

To verify normal protein conformation, the wild-type SSL7311 protein was tested for individual functional activity. The SSL7311M protein was tested in parallel to confirm loss of function. SSL3 inhibits TLR-2 function and human myelomonocytic THP-1 cells were stimulated with the TLR-2 ligand lipoteichoic acid, and the production of tumor necrosis factor (TNF) was measured. This activity was ablated in the presence of SSL3 or SSL7311, but largely unaffected by the presence of a similar concentration of SSL7311M (Figure 2a). SSL7 inhibits end-stage complement. SSL7311 and SSL7 completely inhibited the deposition of C5b-9 onto the surface of *S. aureus*, while SSL7311M showed no inhibition (Figure 2b). Wild-type SSL7 and SSL7311

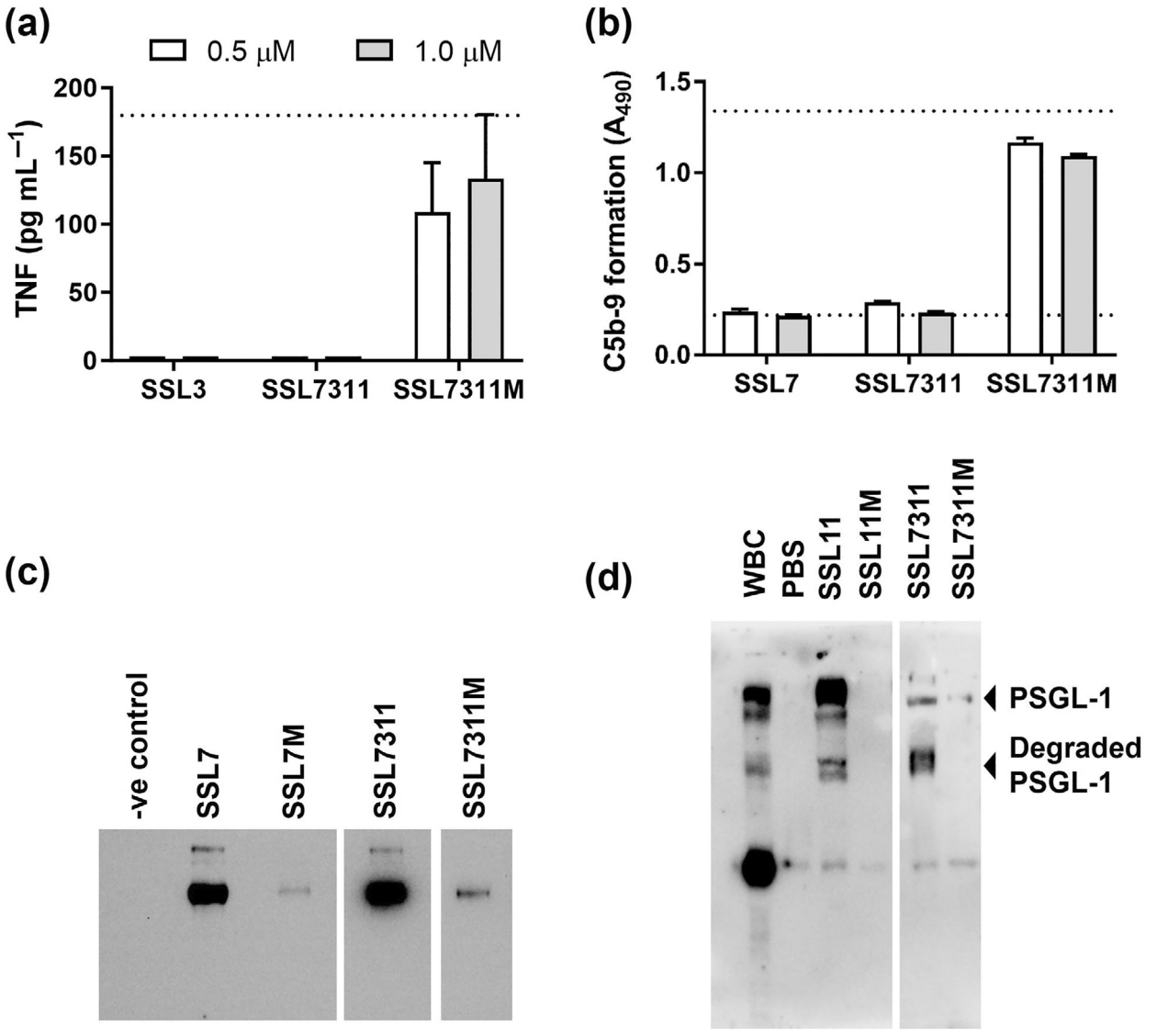

Figure 2. The SSL7311 protein has all the functional activities of the individual components, whereas the SSL7311M protein has attenuated activity. **(a)** THP-1 cells were incubated with the SSL3, SSL3711 or SSL7311M protein and 2 μg mL^{-1} lipoteichoic acid overnight. TNF levels in the supernatants were quantified by ELISA. The dotted line indicates the quantity of TNF made in the presence of LTA alone. **(b)** SSL7, SSL3711 or SSL7311M protein was preincubated with human serum and cleavage of complement C5 was determined by measuring deposition of C5b-9 onto heat-killed *Staphylococcus aureus*. The upper dotted line shows maximum C5b-9 formation in the presence of serum only, and the lower dotted line shows background levels produced in the presence of heat-inactivated serum. **(c)** Serum proteins bound to SSL7, SSL7M, SSL7311 or SSL7311M coupled to sepharose beads, or a negative control of sepharose beads alone, were tested for the presence of human IgA by SDS–PAGE and Western blot. **(d)** WBCs bound to SSL11, SSL11M, SSL7311 or SSL7311M coupled to sepharose beads, or a negative control of sepharose beads alone (PBS), were analyzed for the presence of human PSGL-1 by SDS–PAGE and Western blot. Panels **(a)** and **(b)** show mean plus standard deviation from a minimum of two independent experiments; panels **(c)** and **(d)** are representative of two or more independent experiments. C5, complement component 5; Ig, immunoglobulin; LTA, lipoteichoic acid; PBS, phosphate-buffered saline; PSGL-1, P-selectin glycoprotein-1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SSL, staphylococcal superantigen-like; TNF, tumor necrosis factor; WBC, white blood cell.

proteins bound to human IgA but SSL7311M displayed minimal IgA binding (Figure 2c). This confirmed that SSL7311M was devoid of both SSL7 functions; similarly, wild-type SSL11 and SSL7311 displayed glycan-mediated binding to P-selectin glycoprotein-1, whereas mutant versions of these proteins lacked this capability

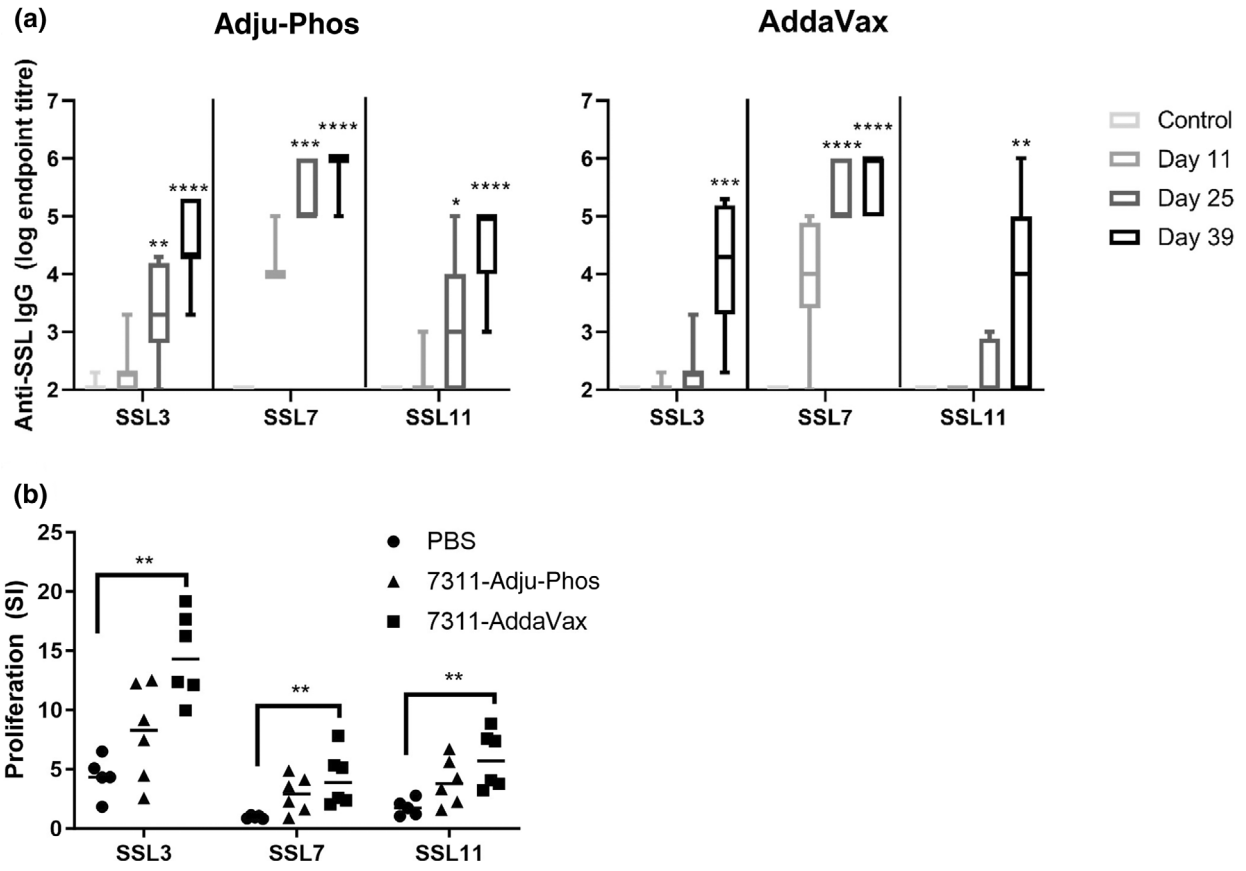

Figure 3. Vaccination with the PolySSL7311 vaccine in adjuvant stimulates humoral and cellular responses to SSLs 3, 7 and 11. Mice were vaccinated subcutaneously on days 0, 14 and 28 with PBS control or 10 μg of PolySSL7311 protein delivered in Adju-Phos or AddaVax and blood was collected on days 11, 25 and 39. **(a)** Serum IgG responses to the individual SSLs were measured by ELISA. **(b)** Splenocytes were stimulated with individual SSL proteins for 72 h, proliferation was quantified by the addition of tritiated thymidine for the final 6 h and results were presented as an SI. Results are from two independent experiments, each containing $n = 6$ mice per treatment group, displayed as box and whisker plots (min–max) **(a)**; or individual data points from two studies, each containing $n = 3$ mice per treatment group, with a horizontal line indicating the median value **(b)**. The development of IgG responses to each SSL over time was compared using a Freidman test with Dunn's multiple comparison test applied. Differences in proliferation responses were assessed using a Kruskal–Wallis test with Dunn's multiple comparisons test applied. Asterisks denote a significant increase in specific IgG or SI relative to adjuvant alone or PBS control groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Ig, immunoglobulin; PBS, phosphate-buffered saline; SI, stimulation index; SSL, staphylococcal superantigen-like.

(Figure 2d). Collectively, these data show that combining the SSLs 3, 7 and 11 into a fusion protein does not inhibit their immune evasion activities, suggesting that this is a suitable format for stimulating anti-SSL immune responses. All further testing used the mutant form of the SSL7311 vaccine, which is referred to as the PolySSL7311 vaccine.

The PolySSL7311 vaccine elicits specific IgG and proliferation responses to all components

The PolySSL7311 vaccine was tested for its ability to stimulate specific humoral and cellular responses to SSLs 3, 7 and 11 in mice after delivery in one of two analogs of adjuvants that are licensed for human use, Adju-Phos, which is an Alum-based formulation, and AddaVax, an oil-in-water–based formulation similar to MF59 (Figure 3a). Robust anti-SSL7 IgG responses were detected on day 25, after two immunizations (mean endpoint titer = 10^5 for Adju-Phos or AddaVax, $P < 0.001$ or $P < 0.0001$ *vs* control). Seroconversion of all mice and production of anti-SSL3 IgG antibodies did not occur until day 39, after three immunizations (mean endpoint titers = ~10^4 for Adju-Phos or AddaVax-treated groups, $P < 0.0001$ or $P < 0.001$ *vs* control). Development of anti-SSL11 IgG responses required three immunizations before significantly increased titers were detectable relative to the control group (mean endpoint

titers = ~10^4 for Adju-Phos or AddaVax-treated groups, $P < 0.0001$ or $P < 0.01$ *vs* control). Therefore, the PolySSL7311 vaccine stimulates antibody responses to all three components when delivered in either adjuvant, with the highest responses to SSL7, then SSL3 and weaker responses to SSL11. Splenocytes were harvested from fully immunized mice on day 39, in conjunction with a phosphate-buffered saline (PBS)-treated control group, and tested for their capacity to proliferate in response to exposure to SSLs 3, 7 and 11 (Figure 3b). Both the PolySSL7311-vaccinated groups had a higher median stimulation index compared with the PBS control group, but only the group vaccinated with AddaVax had a significantly enhanced proliferation response compared with the PBS control group ($P < 0.01$ for SSLs 3, 7 and 11).

The SSL7311 vaccine attenuates *S. aureus* infection

Mice were challenged with *S. aureus via* the intraperitoneal route to determine whether vaccination could reduce signs of disease and the *S. aureus* tissue burden. As delivery in either Adju-Phos or AddaVax was able to elicit specific immunity in vaccinated mice (Figure 3), the utility of each adjuvant was tested in the challenge model. A decline in body weight in response to *S. aureus* challenge is an important objective measure of disease severity. Vaccinated mice lost a comparable amount of body weight compared with the control mice in the first 24 h after the challenge but showed a more rapid recovery at subsequent time points, with significant differences between these groups at day 4 for Adju-Phos ($P < 0.05$) and day 3 for AddaVax ($P < 0.05$; Figure 4). The most significant reduction in *S. aureus* burden was evident in mice vaccinated with the PolySSL7311 vaccine emulsified in AddaVax; the median *S. aureus* CFU was reduced by ~10^2 in vaccinated mice relative to adjuvant-treated controls: liver = 3.5×10^3 *versus* 1.6×10^5 CFU, $P < 0.0001$; kidney = 3.5×10^3 *versus* 1.6×10^5 CFU, $P < 0.05$ (Figure 4). The *S. aureus* burden was reduced to a lesser extent in mice vaccinated with vaccine adsorbed to Adju-Phos; median *S. aureus* CFU was reduced by ~10^1 in vaccinated mice relative to adjuvant-treated controls: liver = 5.2×10^4 *versus* 3.7×10^5 CFU, $P < 0.05$; kidney = 1.9×10^4 *versus* 7.9×10^4 CFU, not significant (Figure 4).

Protection from the *S. aureus* challenge requires the delivery of SSLs 3, 7 and 11 in the PolySSL7311 vaccine

Fusing all three vaccine-candidate antigens into the PolySSL7311 protein offers benefits in terms of simplifying the vaccine formulation, but it is unclear whether this strategy confers any additional advantages. Therefore, mice were vaccinated with PolySSL7311 (Vaccine) or equivalent quantities of individual SSLs 3, 7 and 11 (SSLs), delivered in Adju-Phos or AddaVax, followed by an intraperitoneal challenge with *S. aureus*. All mice lost ~10% of baseline body weight 24 h after the challenge, but mice given the vaccine in either Adju-Phos or AddaVax recovered more rapidly (Figure 5). Mice vaccinated with PolySSL7311 in AddaVax had a ~10^3 reduction in *S. aureus* in the kidneys (0.9×10^2 *vs* 2.7×10^5 CFU, $P < 0.01$) and a > 10^2 decrease in *S. aureus* in the liver (3.5×10^3 *vs* 2.8×10^6, $P < 0.001$). By contrast, the *S. aureus* burden was comparable in the control and SSLs treatment groups (Figure 5). There was no significant difference in kidney or liver CFU between mice vaccinated with SSL7311, SSLs or controls administered in Adju-Phos (Figure 5).

Immunization with PolySSL7311 elicits enhanced antibody titers to each component

Antibody titers to each of the vaccine components were compared in fully vaccinated control, SSLs or PolySSL7311-treated mice. Similar trends were observed in groups of mice immunized with either adjuvant. Comparable anti-SSL3 IgG responses developed irrespective of whether mice were given SSLs or the SSL7311 vaccine. By contrast, IgG responses to both SSL7 [mean endpoint titer = ~10^6 *vs* ~10^5 for Adju-Phos ($P < 0.01$) and AddaVax ($P < 0.05$)] and SSL11 [mean endpoint titer = ~10^5 *vs* ~10^2 for Adju-Phos ($P < 0.0001$)] and 10^4 *versus* ~10^2 AddaVax ($P < 0.05$) were significantly enhanced in the PolySSL7311 vaccinated mice, when compared with the SSLs group (Figure 6a), suggesting that delivery as a fusion protein enhances their immunogenicity. Additional testing indicated that the antibody response was skewed toward the production of IgG1 irrespective of the SSL, adjuvant or vaccine used, suggestive of a dominant Th2 response (Supplementary figure 4). A preliminary examination of splenocyte responses 4 days after the challenge showed no striking differences in proliferation responses to the SSLs in vaccinated *versus* control animals (Supplementary figure 5). Unstimulated splenocytes primarily produced TNF and IL-6, with lower amounts of Interferon-γ and IL-17A also detected (Supplementary figure 5). Cells from mice given PolySSL7311 in AddaVax produced larger quantities of TNF and IL-6 than animals that received the vaccine in Adju-Phos ($P < 0.05$; Supplementary figure 5). There was no correlation between *S. aureus* kidney CFU and the production of any of these cytokines (data not shown).

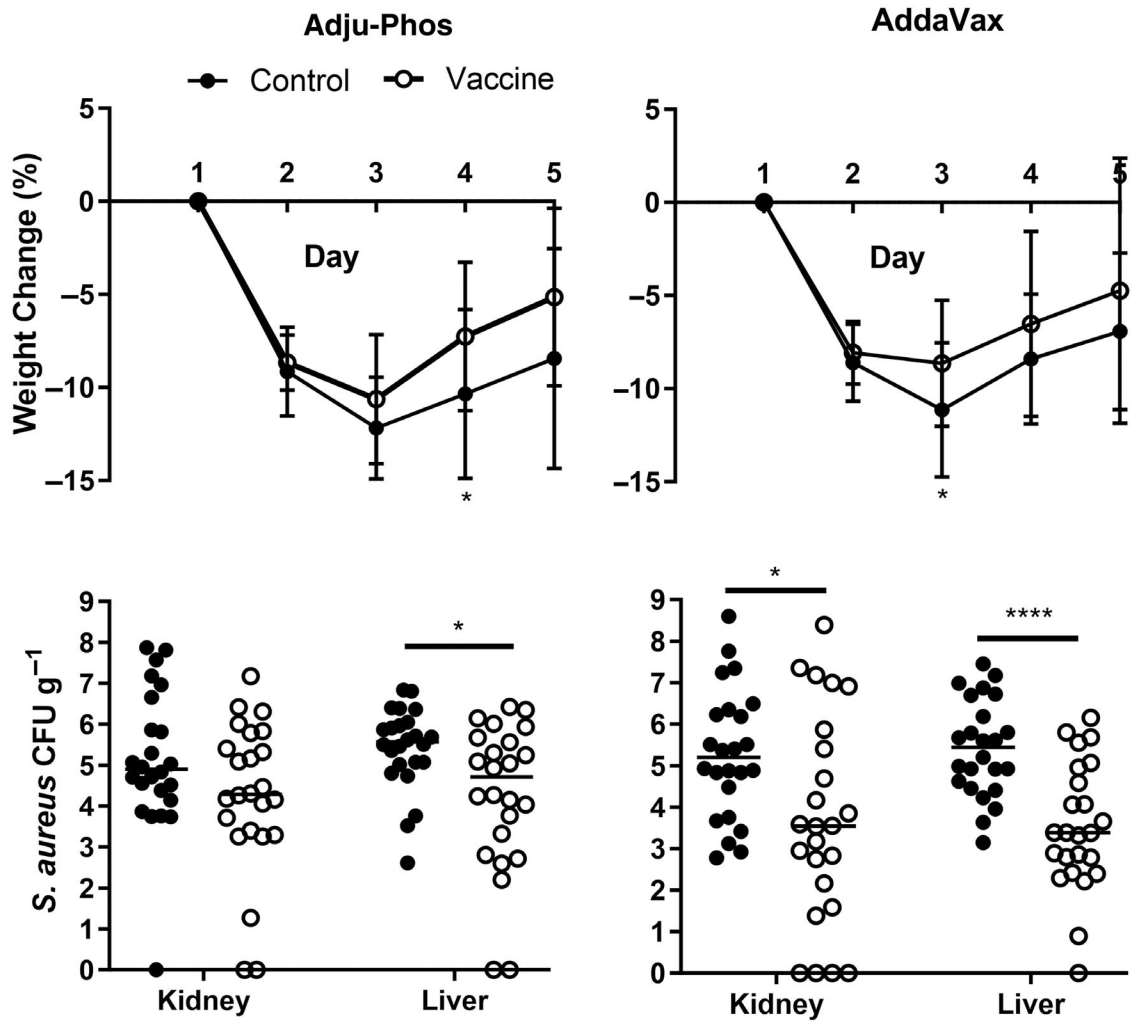

Figure 4. Treatment with the PolySSL7311 vaccine reduces *Staphylococcus aureus* disease. Mice were vaccinated subcutaneously on days 0, 14 and 28 with PBS control or 10 μg of PolySSL7311 vaccine delivered in Adju-Phos or AddaVax, challenged with ~5 × 10^7 CFU *S. aureus* JSNZ on day 42 and tissues harvested on day 46. Data are combined from four independent studies, each containing $n = 6$ mice per treatment group. The horizontal line is a median value. Body weights are shown as mean ± standard deviation. Differences between treatment groups were assessed using the Mann–Whitney *U*-test. Asterisks denote a significant difference between adjuvant-only controls and the PolySSL7311-vaccinated control group for each adjuvant (*$P < 0.05$; ****$P < 0.0001$). CFU, colony-forming units; PBS, phosphate-buffered saline; SSL, staphylococcal superantigen-like.

The PolySSL7311 vaccine elicits antibodies that inhibit SSL7 function

Immune sera were tested for the capacity to inhibit the activity of each component in the SSL7311 vaccine. The quantitative assays used to characterize and confirm the functional integrity of the wild-type version of the SSL7311 vaccine (Figure 2) were modified to incorporate an incubation step with murine sera to determine whether this prevented the interaction between individual SSLs and their respective ligands. Diluted sera from vaccinated or control mice were incubated with SSL3 protein and tested for the capacity to restore TLR-2 signaling by THP-1 cells. Only sera from mice immunized with individual SSL proteins administered in Adju-Phos showed any capacity (median = ~10%, $P < 0.001$) to attenuate the function of SSL3 (Figure 6b). By contrast, incubation of immune serum from PolySSL7311-vaccinated mice was able to partially prevent the binding of SSL7 to human IgA and enabled cleavage of complement C5 in the presence of

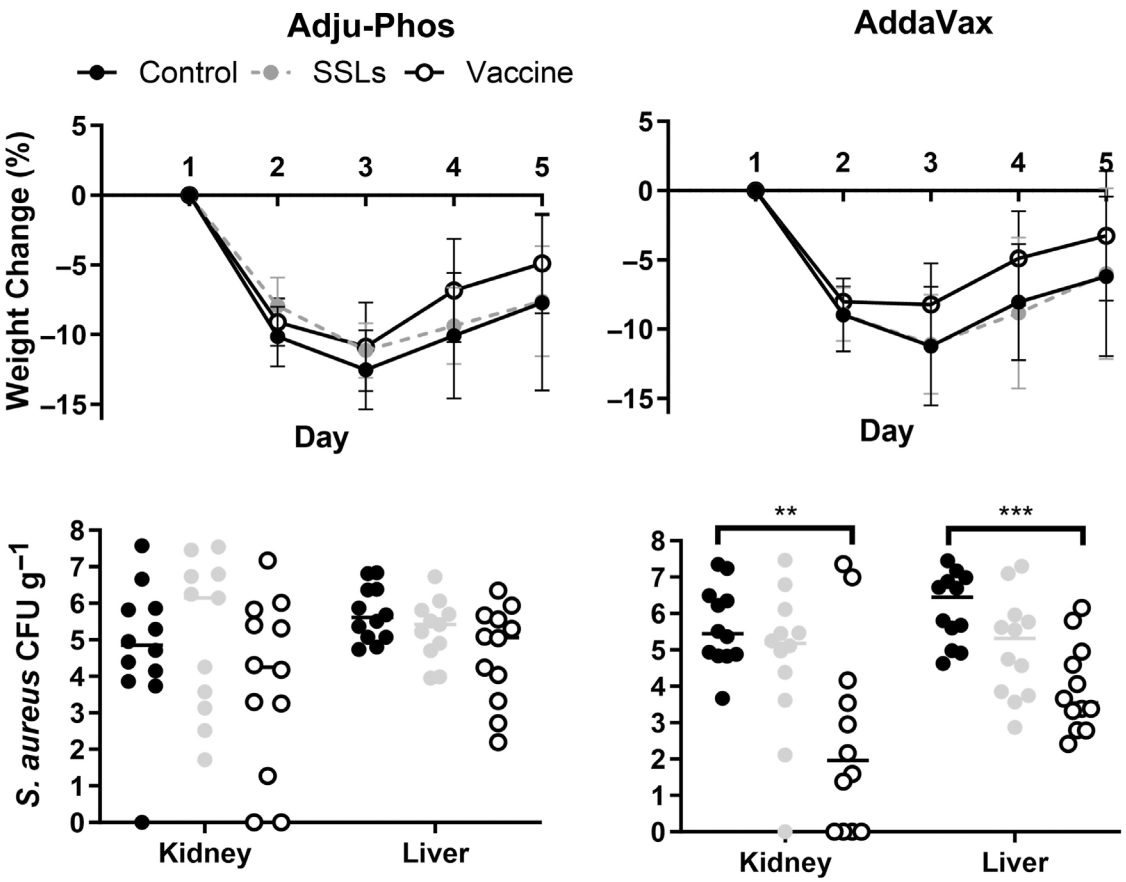

Figure 5. Reduction in *Staphylococcus aureus* disease requires the PolySSL7311 vaccine. Mice were vaccinated subcutaneously on days 0, 14 and 28 with PBS control; 3.3 μg each of SSL7, 3 and 11 (SSLs) or 10 μg of PolySSL7311 protein (vaccine) delivered in Adju-Phos or AddaVax, challenged with ~5 × 10^7 CFU *S. aureus* JSNZ on day 42 and tissues harvested on day 46. Note that data from these two studies are also shown in the plots in Figure 4. Data are combined from two independent studies, each containing $n = 6$ mice per treatment group. Body weights are shown as mean ± standard deviation. The horizontal line is a median value. Differences between treatment groups were assessed using a Kruskal–Wallis test with Dunn's test multiple comparisons test applied (**$P < 0.05$; ***$P < 0.001$). CFU, colony-forming units; PBS, phosphate-buffered saline; SSL, staphylococcal superantigen-like.

SSL7. Sera from mice immunized with either SSLs (median of ~20% in Adju-Phos, $P < 0.05$; ~30% in AddaVax, $P < 0.001$) or PolySSL7311 (median of ~50% in Adju-Phos; ~40% in AddaVax, $P < 0.0001$) partially neutralized binding of SSL7 to IgA relative to controls (~10% IgA binding; Figure 6b). Only incubation of SSL7 with immune sera from PolySSL7311-vaccinated mice was able to restore complement C5 cleavage and deposition of C5b-9 onto the surface of heat-killed *S. aureus* [median of > 90% C5b-9 deposition for mice given the PolySSL7311 vaccine in either adjuvant *vs* negligible detection of C5b-9 in the control group ($P < 0.0001$ for both adjuvants); Figure 6b]. Assays to test for neutralizing antibodies to SSL11 carbohydrate binding were masked by binding of wild-type SSL11 to murine serum glycoproteins at dilutions expected to show an antibody-mediated effect, which prevented quantification of carbohydrate-specific binding by SSL11.

To determine whether an antibody response to these SSLs develops in humans in response to natural exposure to *S. aureus*, sera from 10 healthy human donors were examined for specific IgG responses to SSLs 3, 7 and 11. Healthy individuals have substantial antibody titers to a broad range of *S. aureus* antigens[36] and all donors had median endpoint titers of ≥ 10^4 against each of these SSLs (Supplementary figure 6a). These sera were assessed in the neutralizing assays described earlier. Sera from four of the donors with endpoint titers of 10^5 were able to partially prevent SSL3 from inhibiting signaling through TLR-2 (16–64%). By contrast, none of these donor sera were able to ablate either IgA binding or facilitate

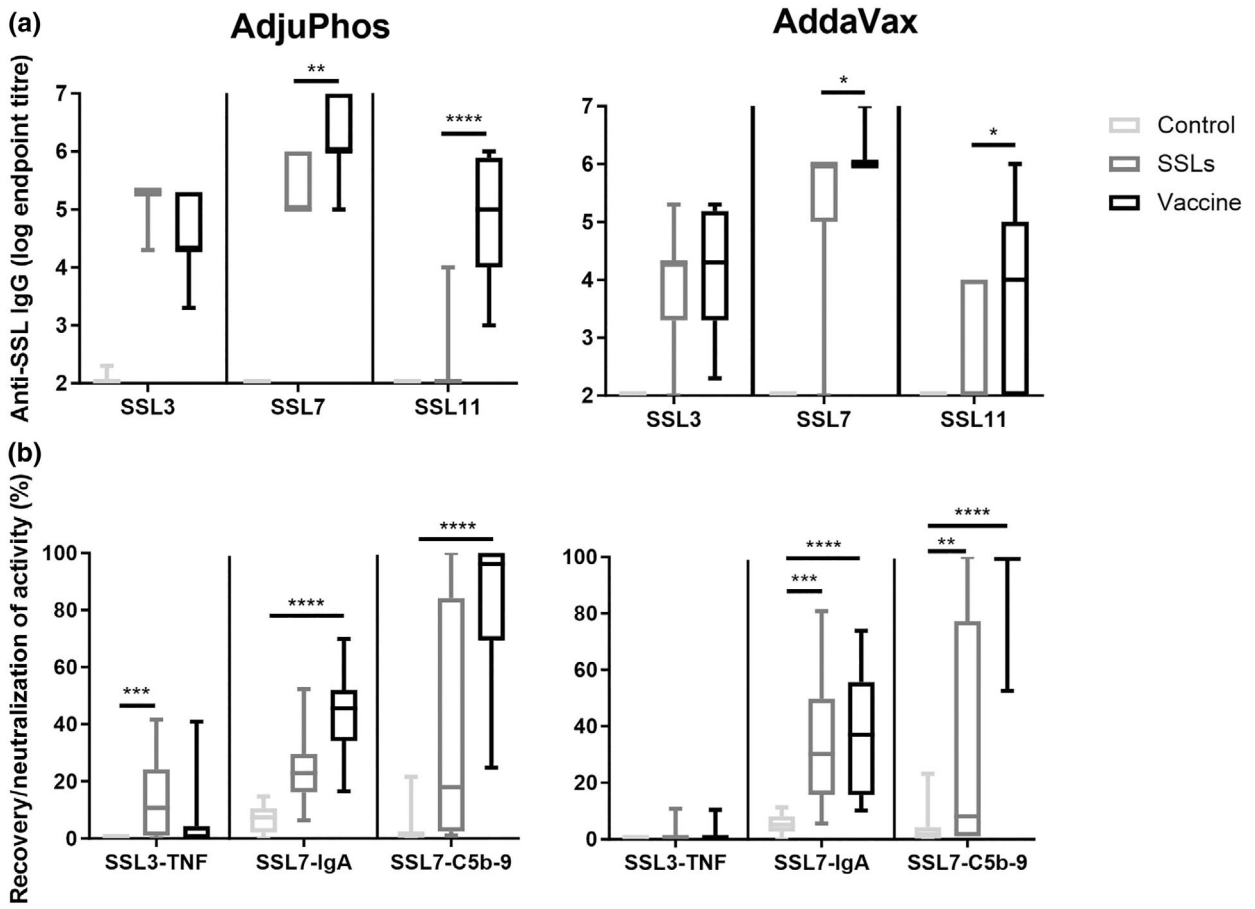

Figure 6. Vaccination with the PolySSL7311 vaccine in adjuvant stimulates increased specific IgG responses and enhanced anti-SSL7 neutralizing antibodies. Mice were vaccinated subcutaneously on days 0, 14 and 28 with PBS control; 3.3 μg each of SSL7, 3 and 11 (SSLs) or 10 μg of PolySSL7311 protein delivered in Adju-Phos or AddaVax (vaccine) and serum antibody responses assessed on day 39. **(a)** IgG endpoint titers to each SSL in the groups vaccinated with SSLs or vaccine, compared using the Mann–Whitney *U*-test. **(b)** Quantification of inhibition of wild-type SSL protein function by incubation with anti-sera from vaccinated or control mice. SSL3-TNF: recovery of TNF production by THP-1 cells incubated with lipoteichoic acid and SSL3; SSL7-IgA: inhibition of SSL7 binding to human IgA; SSL7-C5b-9: prevention of SSL7 from arresting C5b-9 deposition on whole *Staphylococcus aureus*. Results are shown as a percentage of TNF production, IgA binding or C5b9 deposition relative to positive controls. Data are from two independent experiments each containing $n = 6$ mice per treatment group displayed as box and whisker plots (min–max). Statistically significant differences were determined using a Kruskal–Wallis test with Dunn's multiple comparisons test applied. Asterisks denote $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. C5, complement component 5; Ig, immunoglobulin; PBS, phosphate-buffered saline; SSL, staphylococcal superantigen-like; TNF, tumor necrosis factor.

cleavage of complement C5 in the presence of SSL7 (Supplementary figure 6b), suggesting humans infected with *S. aureus* do not develop a neutralizing antibody response to this important virulence factor.

DISCUSSION

There is an urgent need to develop alternative strategies to treat or prevent infection with *S. aureus*. We have demonstrated for the first time that a vaccine combining three functionally inactive secreted *S. aureus* immune evasion factors attenuates the development of *S. aureus* peritonitis. These results confirm the important contribution of these SSLs to *S. aureus* survival *in vivo*.

Only delivery of these antigens as a fusion protein significantly reduced bacterial load, suggesting that this format improves the presentation of these antigens to the immune system, potentially through induction of neutralizing antibodies. Vaccination with PolySSL7311 enhanced antibody titers to SSLs 7 and 11 and induced antibodies that neutralized the activities of SSL7. Assessment of antisera from a small set of healthy human

adults detected a robust IgG response but no capacity to inhibit IgA binding or impact on the cleavage of complement C5 by SSL7, suggesting that functional antibodies are not typically produced after exposure to *S. aureus*. SSL7 is a potent inhibitor of neutrophil responses to whole *S. aureus*,[29] dampens immune complex–mediated peritonitis[37] and shows potential for inhibiting complement-mediated disease[38] in murine models. Generation of functional antibodies against SSL7 through the delivery of PolySSL7311 in AddaVax leads to attenuation of bacterial burden, suggesting that SSL7 contributes significantly to *S. aureus* virulence. The impact of SSL7 on infection is likely to be underestimated in the murine model, as the interaction between complement C5 and SSL7 is enhanced in the presence of human IgA.[28,29] By contrast, the location of SSL3 within the SSL7311 protein elicited a strong cellular proliferation response and a robust IgG response, but an impaired capacity to elicit a neutralizing antibody response, potentially a result of masking of key epitopes. The role of SSL3 in conferring protection is unclear, but there is strong evidence that TLR-mediated immune responses, particularly signaling through MyD88, play an important role in controlling *S. aureus* infection in both mice and humans.[25,39,40] We were unable to devise a specific assay to test for inhibitory antibodies against wild-type SSL11 because of its ability to bind to multiple glycoproteins in mouse antisera.

The best adjuvant for a human *S. aureus* vaccine is currently unknown. A standardized comparison of murine responses to vaccines for a variety of pathogens, delivered in different adjuvant formulations, emphasizes that the choice of adjuvant can alter both the immune profile and the level of protection from infection.[41] As there are no robust correlates of protection for *S. aureus*, a comparison of the efficacy of different adjuvant formulations may yield information on potential mechanisms of protection. Vaccination with Adju-Phos or AddaVax produced comparable antibody responses, whereas significant attenuation of the bacterial burden required AddaVax. Similarly, preclinical mouse studies with the 4C-Staph vaccine indicate a superior and enduring protective immune response with MF59 when compared with Alum, linked with the development of specific $CD4^+$ effector T cells, particularly Th1/Th17 cells and a robust antigen-specific antibody response.[13] The efficacy of the 4C-Staph vaccine can be significantly improved by the addition of a TLR-7 ligand to Alum, resulting in enhanced antibody responses, skewing toward a Th1/Th17 profile and induction of consistently high levels of protection after the challenge with a range of clinical *S. aureus* isolates.[12] The importance of selecting the right adjuvant is also illustrated in a recent study with a combination vaccine for *Streptococcus pyogenes*—Alum stimulated a robust specific antibody response (including an opsonic response) but failed to protect mice from systemic infection; the use of alternative adjuvants capable of stimulating a broader immune response, including a Th1 response, significantly attenuated development of disease.[42] A preliminary analysis of the impact of SSL7311 vaccination on cellular responses showed a significant increase in splenocyte proliferation to each vaccine component in mice vaccinated with PolySSL7113 in AddaVax, but no detectable induction of Th1 or Th17 populations, although this latter analysis may have been confounded by the use of splenocytes from *S. aureus*–challenged mice.

Several *S. aureus* vaccine candidates have failed after making substantial progress through clinical trials, which has led to widespread reflection on the process of selecting *S. aureus* vaccine candidates.[43–45] Key issues for validating *S. aureus* vaccine candidates have been identified, including the use of appropriate challenge model(s), the suitability of murine models, determining a meaningful threshold for what constitutes protection from the challenge and confirming cross-strain protection.[44] Ultimately, it may also be necessary to combine multiple vaccine antigens, particularly secreted antigens such as the SSLs or toxins, to effectively target different facets of staphylococcal virulence. The PolySSL7311 vaccine was tested in a systemic (peritoneal) model of infection because the survival of an *S. aureus*Δ*ssl* strain was significantly reduced in this model; however, the potential for this vaccine to confer protection in other settings needs to be addressed. A recent report described a multicomponent vaccine comprising several conserved staphylococcal transporter proteins and α-hemolysin, which had a significant impact on dermonecrosis in a skin infection model, but only conferred partial protection against bacteremia,[15] highlighting the risk of overdependence on a single model. The definition of what constitutes a significant reduction in *S. aureus* burden is also subject to debate. The PolySSL7311 vaccine significantly reduced *S. aureus* tissue burden ($> 10^2$) and attenuated weight loss when administered in AddaVax, suggesting containment of infection, but with room for further enhancement through refinement of delivery systems, adjuvants or by combination with additional vaccine-candidate antigens. Finally, our vaccine candidate was tested using a mouse-adapted *S. aureus* strain (JSNZ) that is both transmissible and virulent in mice.[18] Although the *ssl*s are retained in both veterinary and murine strains,[18,19] suggesting they provide a significant survival advantage across a broad range of species, testing the protective efficacy of the SSL7311 vaccine in clinically relevant strains is an

important step toward demonstrating the broader applicability of this approach.

There are compelling reasons to continue to identify potential vaccine-candidate antigens because of the range of diseases caused by *S. aureus* and its diversity of virulence factors. We have completed initial feasibility tests with a vaccine candidate consisting of a fusion protein designed to target SSLs 3, 7 and 11, which are well-characterized key *S. aureus* immune evasion factors. This strategy has shown initial promise in a mouse model of peritoneal inflammation but requires further investigation and optimization to determine its broader applicability in tackling staphylococcal disease.

METHODS

Ethics statement

Written informed consent was obtained from all donors and blood samples were handled as per the Declaration of Helsinki and University of Auckland Human Participants Ethics Committee guidelines. Animal experiments were approved by the University of Auckland Animal Ethics Committee and were conducted in accordance with the University of Auckland's Code of Ethical Conduct and the Animal Welfare Act 1999.

Bacteria

Staphylococcus aureus was cultured at 37°C in either tryptic soy broth (DIFCO, Sparks, MD, USA) with vigorous shaking or plated onto tryptic soy agar for enumeration of bacteria. Mice were inoculated with *S. aureus* grown to mid-log phase and administered in PBS. The challenge dose was confirmed by serial 10-fold dilution of the inoculum, plating of triplicate spots and enumeration of CFU after overnight incubation at 37°C. Heat-killed *S. aureus* JSNZΔ*spa*[46] was prepared by incubation of an overnight culture of bacteria at 65°C for 1 h, washed in PBS and pelleted bacteria stored at −20°C. *Escherichia coli* was grown at 37°C unless specified otherwise in Luria–Bertani broth [1% w/v Bacto-tryptone (Oxoid, Basingstoke, UK), 0.5% w/v yeast extract (Oxoid), 1% NaCl] with vigorous shaking, or on Luria–Bertani agar (Luria–Bertani supplemented with 15 g/L agar). Where antibiotic selection was required, *S. aureus* was cultured with 0.5 g mL^{-1} streptomycin or *E. coli* with 0.5 mg mL^{-1} ampicillin, 0.03 mg mL^{-1} chloramphenicol, 0.015 mg mL^{-1} kanamycin or 0.0125 mg mL^{-1} tetracycline (Sigma-Aldrich, Sydney, Australia).

Creation of *S. aureus* gene knockout and repair strains

A streptomycin-resistant mouse–adapted strain of *S. aureus* (JSNZ)[18] was used for all gene knockout and *in vivo* studies. *S. aureus* gene knockout strains were produced by allelic exchange with the pIMAY plasmid, and selected genes were reintroduced by complementation.[47,48] Knocking out all 14 *ssl* genes from the genome of strain JSNZ was performed in three steps. First, the flanking sequences of *ssl1* to *ssl10* regions were amplified from genomic DNA using iProof DNA polymerase (Bio-Rad Laboratories, Hercules, CA, USA) and 1–10 upstream forward and reverse primers or 1–10 downstream forward and reverse primers, respectively (Supplementary table 1). These products were mixed and used as templates for PCR using upstream forward and downstream reverse primers. The resulting *ssl1-10* flanking sequence was introduced into pIMAY using *Not*I and *Sac*I. After sequence confirmation, the plasmid isolated from *E. coli* DC10B was electroporated into *S. aureus* JSNZ. Integration of pIMAY into JSNZ and excision of the *ssl1* to *ssl10* regions were performed as described by Monk *et al.*[47] Confirmation of gene deletion in JSNZΔ*1–10* was confirmed by sequencing with the 1–10_out-forward and 1–10_out-reverse primers (Supplementary table 1). The flanking region of *ssl11* was generated by a two-step overlap PCR in the same manner using primers 11 upstream forward and reverse, and 11 downstream forward and reverse. The PCR product was cloned into pIMAY using *Not*I and *Eco*RI and transformed into DC10B. Following sequence confirmation, the plasmid was electroporated into JSNZΔ*1-10*; allelic exchange was performed as previously described by Monk *et al.*[47] Sequence primers 11_out-forward and 11_out-reverse were used to confirm the deletion of *ssl11* in the newly generated strain JSNZ*Δ1-11*. The flanking region of *ssl12* to *ssl14* was generated in two steps in the same manner as above using primers 12–14 upstream forward and reverse, and 12–14 downstream forward and reverse. The PCR product was introduced into pIMAY using *Not*I and *Sal*I and used to transform DC10B. Gene deletion was again achieved by allelic exchange to create the full *ssl1-14* knockout strain JSNZΔ*ssl*. Sequence primers 12–14_out-forward and -reverse were used to confirm the deletion of the *ssl12-14* region.

To generate the SSL7-repaired strain, JSNZΔ*ssl-ssl7Rep*, the *ssl1-10* upstream flanking region was amplified with 1–10_up-forward and -reverse, ssl7 with its promoter region was amplified using *ssl7*-forward and *ssl7*-reverse and the *ssl1-10* downstream flanking region was amplified with 1–10_KIndown-forward and 1–10_down-reverse primers from JSNZ genomic DNA using iProof polymerase. These three products were mixed and used as templates in an overlap PCR using 1–10_up-forward and 1–10_down-reverse. The resulting product was cloned into pIMAY using *Not*I and *Sac*I. This plasmid was electroporated into JSNZΔ*ssl* and integration of *ssl7* back into the genome to create JSNZΔ*ssl-ssl7Rep* was confirmed by sequencing from 1–10_out-forward and -reverse. The SSL3-repaired strain JSNZΔ*ssl-ssl3Rep* was made by overlap PCR using 1–10_up-forward and 1–10_down-reverse with the three products amplified using the primer sets: 1–10_up-forward and -reverse; p*ssl3*-forward and -reverse 1 and 1–10_down-forward and -reverse as a template. The resulting product was inserted into pIMAY and electroporated into JSNZΔ*ssl* for integration of *ssl3* by allelic exchange. The SSL11-repaired strain JSNZΔ*ssl-ssl11Rep* was created by amplification of JSNZ genomic DNA using 11_up-forward and 11_down-reverse. The product was cloned into pIMAY and the plasmid pIMAY:*pssl11* electroporated into JSNZΔ*ssl* for integration of *ssl11*. The SSL3-, SSL7- and SSL11-repaired strain JSNZΔ*ssl-ssl3711Rep* was generated by first introducing

ssl3 into JSNZΔ*ssl-ssl7Rep*. The product of an overlap PCR using primers 1–10_up-forward and 1–10_down-reverse on the three products was amplified using the primer sets 1–10_up-forward and -reverse; p*ssl3*-forward and -reverse 2 and p*ssl7*-forward and -reverse, which was cloned into pIMAY and electroporated into JSNZΔ*ssl-ssl7Rep*. The allelic exchange was performed as described by Monk *et al.*[47] Plasmid pIMAY:*pssl11* was electroporated into the resulting strain JSNZΔ*ssl-ssl37Rep* and allelic exchange was performed to create JSNZΔ*ssl-ssl3711Rep*.

Cloning, expression and purification of recombinant proteins

Escherichia coli strains DH5α, AD494(DE3)pLysS and Rosetta gammi 2(DE3)pLysS were used for cloning and production of recombinant proteins from *S. aureus* JSNZ. Briefly, mutant SSL11 was created *via* the *ssl11*.R179A mutation to attenuate binding to glycoproteins; wild-type SSL7 sequence was mutated in several locations to eliminate interactions with IgA (*ssl7*.L79A.P82A) and C5 (*ssl7*.D115A.H11A.S119A). The N terminus of SSL3 is prone to degradation, therefore a truncated but functional form of wild-type SSL3[24] was amplified from *S. aureus* JSNZ genomic DNA by PCR and subcloned into the expression vector pET32a-3C. Targeted mutations were introduced into SSL3 to prevent interactions with TLR-2 (*ssl3*.F186A.F188A) and attenuate carbohydrate binding (*ssl3*.T327A).

The 3C protease recognition site–containing plasmid, pET32a.3C, was constructed by inserting the *Bgl*II/*Bam*HI-digested sequence (AGATCTGAGGTGCTGTTCCAGGGACCG GGATCC) into the *Bam*HI site of pET32a, followed by the introduction of the *Bam*HI–*Eco*RI region from the pBlueScript MCS between the *Bam*HI and *Eco*RI sites. SSL7311 was generated by amplifying *ssl7jsnz* using iProof DNA polymerase with the primer pair SSL7-combo-forward and SSL7-combo-reverse and cloned into pET32a.3C using *Bam*HI and *Xma*I. Next *ssl11jsnz* was amplified using SSL11-combo-forward and SSL11-combo-reverse and cloned into the *ssl7*-containing pET32a3C vector using *Sac*I and *Xho*I. Lastly, the truncated version of SSL3 starting from residue Q159[24] was amplified from JSNZ genomic DNA using linker-*ssl3*-forward and linker *ssl3*-reverse and cloned into pET32a.3C:*ssl711* using *Xma*I and *Sac*I. To generate SSL7311M, the *ssl7* IgA^{-}/C5^{-} mutant was amplified from *ssl7jsnz* by double overlap PCRs using a combination of SSL7-combo-forward and combo-reverse primers with the mutagenesis primers SSL7-IgA^{-}-forward and -reverse, and SSL7-C5^{-}-forward and -reverse before cloning into pET32a.3C using *Bam*HI and *Xma*I. Next *ssl11* sLacNAc^{-} was generated from *ssl11jsnz* by overlap PCR using SSL11-combo-forward and -combo-reverse, with mutagenesis primers SSL11-sLacNAc^{-}-forward and -reverse and then cloned into the *ssl7* IgA^{-}/C5^{-}-containing pET32a3C vector using *Sac*I and *Xho*I. The *ssl3* TLR2^{-}/sLacNAc^{-} mutant was amplified from *ssl3jsnz* by double overlap PCRs using combinations of linker-ssl3-forward and -reverse with the mutagenesis primers SSL3-TLR2^{-}-forward and -reverse, and SSL3-sLacNAc^{-}-forward and -reverse. The product was cloned into pET32a.3C:*ssl711M* using *Xma*I and *Sac*I. All oligonucleotides were purchased from Sigma-Aldrich Australia and are listed in Supplementary table 1. The genomic sequence of the *S. aureus* JSNZ strain is publicly available (DDBJ/ENA/GenBank accession number QWKQ00000000).

Wild-type and mutant *ssl7*, *ssl3* and *ssl11* genes were cloned into *E. coli* DH5α in tandem. PCR-positive plasmids were sequence confirmed and then transformed into *E. coli* AD494 (DE3)pLysS (Supplementary table 2) to be expressed as N-terminal thioredoxin fusion proteins. Expression of soluble fusion protein required separation of each gene with a glycine linker, use of *E. coli* Rosetta gammi 2(DE3)pLysS and induction of protein production at 28°C. Fusion proteins were purified by Ni_{2+} affinity chromatography followed by cleavage with 3C protease as detailed elsewhere,[30,49] then further purified by ion-exchange chromatography (MonoQ or MonoS; GE Healthcare, Uppsala, Sweden). The polySSL protein was further purified by size exclusion chromatography (Superdex S75; GE Healthcare, Uppsala, Sweden). All recombinant proteins were confirmed to be ≥ 95% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. For functional assays, wild-type SSL3 protein was treated with Endotoxin Removal Beads (Miltenyi Biotech, Bergisch Gladbach, Germany) and confirmed to contain <1 Endotoxin Units/0.01 mg with an Endosafe-PTS reader (Charles River, Wilmington, MA, USA).

Preparation of human whole blood and serum

Peripheral blood from healthy volunteers was used to examine the activity of the *S. aureus*Δ*ssl* strains and purified recombinant proteins. Blood was collected into heparinized tubes and either directly combined with live *S. aureus* or further processed to obtain peripheral blood leukocyte lysates.[26] Human plasma for use in pull-down assays was collected from heparin-treated blood by centrifugation. Blood collected in untreated tubes was left to clot for 30 min at room temperature before centrifugation at 1250 *g* for 20 min at 4°C as a source of normal human serum and stored at −80°C.

Whole blood killing assay

A whole blood killing assay was used to compare the survival of *S. aureus* knockout strains.[48] Briefly, mid-log phase cultures of *S. aureus* were washed, resuspended in Hanks' balanced salt solution (Sigma) and ~ 1×10^4 CFU bacteria were added to 70% v/v whole blood for 20 h at 37°C with gentle shaking. At 0 and 20 h, bacteria were serially diluted in PBS and plated in triplicate onto tryptic soy agar for enumeration of CFU.

Characterization of SSL7311 proteins by pull-down assay

Binding profiles of individual and fusion proteins to target ligands were confirmed using pull-down assays. Recombinant proteins were coupled to cyanogen bromide–activated sepharose (GE Healthcare) and combined with either serum or

peripheral blood leukocyte lysate at a ratio of 1:10 as previously described.[26] Eluted proteins were separated by 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane for detection of bound proteins by Western blotting. Membranes were blocked overnight at 4°C in Tris-buffered saline–0.1% Tween-20 (Sigma) supplemented with 5% w/v skim milk powder. Membranes were probed with primary or secondary antibodies in Tris-buffered saline–0.1% Tween 2.5% w/v skim milk powder and washed three times with Tris-buffered saline–0.1% Tween for 5 min between each antibody incubation. Immobilized protein–antibody complexes were detected with ECL Western Blotting Substrate and visualized with a GelDoc2000 (both from Bio-Rad Laboratories). The identity of bound proteins was confirmed using anti-human antibodies to C5, CD162 or human IgA and bound antibodies were detected with horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit IgG. Further details of all antibodies are shown in Supplementary table 3.

Confirmation of the functional activity of the SSL7311 proteins

Retention or loss of functions associated with SSL7—binding to human IgA and inhibition of complement C5 cleavage—were determined by ELISA.[29] Reagents were added at 50 µL/well to Maxisorb (Nunc, Roskilde, Denmark) plates for all ELISAs unless specified otherwise; samples were assayed in duplicate, and plates were washed in PBS–0.05% v/v Tween-20 three times or more between 1-h room temperature incubation steps. The binding of horseradish peroxidase–conjugated secondary antibodies was detected with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) substrate and the reaction stopped using 10% v/v HCl. Plates were read on an EnSight plate reader (PerkinElmer, Inc., Waltham, MA, USA) and final absorbance was calculated by subtracting the 570 nm from 450 nm values.

Human IgA was purified from pooled healthy donor plasma by SSL7 C5-mutant sepharose affinity chromatography and anti-SSL7 IgG was removed by passing the preparation through a Protein G column (GE Healthcare).[29] Microtiter plates were coated overnight at 4°C with SSL7 protein diluted to 10 µg mL^{-1} in PBS, washed and blocked with 200 µL/well assay buffer PBS–1% bovine serum albumin (Gibco, Grand Island, NY, USA), incubated with purified human IgA at 6.25×10^{-2} mg mL^{-1} and IgA binding detected with anti-IgA:horseradish peroxidase. Inhibition of SSL7-IgA binding was tested by incubation of serum diluted 1:200 from immunized or control (baseline) mice on SSL7-coated microtiter plates for 1 h at room temperature before the addition of IgA. Percent neutralizing activity was calculated as follows: (control – test)/control × 100.

For the detection of membrane attack complex (C5b-9) formation, microtiter plates were coated overnight with ~5×10^6 CFU/well heat-killed *S. aureus*Δ*spa* and then washed and blocked with 200 µL/well 1% w/v human serum albumin (Merck, Auckland, New Zealand) in PBS. Normal human serum (2.5% v/v) and SSL7 were incubated in GHB [150 mM NaCl, 60 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid), 0.1% w/v bovine skin type B gelatin (Sigma Aldrich) pH 7.35 supplemented with 0.06 mM $CaCl_2$ and 0.4 mM $MgCl_2$] for 1 h at 37°C. Deposition of C5b-9 onto the *S. aureus* was detected with mouse anti-human C5b-9 and goat anti-mouse IgG:horseradish peroxidase. Prevention of SSL7-C5 binding was tested by incubating serum (diluted 1:200) from immunized or control mice with 0.5 µM SSL7 for 1 h at room temperature before the addition of 2.5% normal human serum. Percent neutralizing activity was calculated as follows: (test – control serum)/1% inhibition [normal human serum alone (maximum readout) – control serum]/100.

SSL3-mediated inhibition of signaling through TLR-2 was determined using a cytokine stimulation assay. THP-1 myelomonocytic cells (ATCC TIB-202; 10^5 cells/well) were combined with SSL3 and 2 µg mL^{-1} lipoteichoic acid from *S. aureus* (lipoteichoic acid; InvivoGen, San Diego, CA, USA) in a round-bottomed microtiter plate for ~20 h. Supernatants were harvested and tumor necrosis factor (TNF) production was quantified by ELISA (OptEIA Human TNF ELISA Set, BD Biosciences, Franklin Lakes, NJ, USA). Sera from immunized or control mice were incubated with 0.48 nM SSL3 for 1 h at a dilution of 1:25 before the addition of cells and lipoteichoic acid, to assess its capacity to interfere with the interaction between SSL3 and TLR-2. Percent neutralizing activity was calculated as described for the membrane attack complex assay.

Infection and vaccination of mice

Female Crl:CD1(ICR)-Elite certified as *S. aureus*–free specific pathogen–free mice were supplied by the Vernon Jansen Unit (University of Auckland, Auckland, New Zealand), and housed in individually ventilated cages with water and chow given *ad libitum*. Animals were monitored daily by qualified staff.

Mice aged 7–8 weeks were weighed before infection by intraperitoneal injection with ~10^8 CFU *S. aureus* and monitored twice daily. Systemically infected mice were monitored twice daily for clinical signs of infection such as ruffled fur, hunching and reduced activity. Mice that met one or more of the predetermined endpoints loss of ≥ 15% of baseline body weight in 24 h, ≥ 20% weight loss over the course of the study, more than two clinical signs of infection or were found moribund, were killed immediately by CO_2 inhalation. Selected tissues were excised, homogenized and *S. aureus* was enumerated as described elsewhere.[18]

Mice aged 5–6 weeks were briefly anesthetized with isoflurane and vaccinated subcutaneously into the intrascapular region three times, two weeks apart, with 0.1 mL^{-1} material containing 10 µg of 7311 M protein emulsified 1:1 in AddaVax or adsorbed to Adju-Phos (InvivoGen) and then challenged by an intraperitoneal injection with ~5×10^7 CFU *S. aureus* JSNZ 2 weeks after the final vaccination. Blood samples were collected from the tail vein at ~2-week intervals for analysis of antibody responses. Mice were monitored, weighed, killed and tissue processed as described above.

Cages containing 5 or 6 mice were assigned to each treatment, investigators were not blinded to treatment allocations and potential confounders were not controlled for. A negative control group (PBS combined with adjuvant) was included in every immunization study and compared with one or two other treatment groups.

Quantification of anti-SSL3, 7 and 11 IgG

Endpoint titers to SSLs 3, 7 and 11 were measured by ELISA, using the conditions described earlier for SSL7-IgA binding. Sera were serial 10-fold diluted in assay buffer starting at 1:100 for SSL7- or SSL11-, or 1:200 for SSL3-coated wells. Baseline serum samples were included on every assay plate and endpoint titers were calculated as the reciprocal of those values that were $\geq 2 \times$ average baseline values $+2 \times$ standard deviation.

Detection of cellular and cytokine responses

Spleens were collected from individual mice and processed to obtain splenocyte suspensions for the quantification of cell proliferation. Briefly, 2×10^6 splenocytes/well were transferred to round-bottomed microtiter plates and stimulated with 50 μg mL^{-1} of purified recombinant SSL3, 7 or 11 mutant proteins or medium alone for 72 h. Cells were pulsed with 0.25 μCi/well tritiated thymidine (PerkinElmer) for the final 6 h of the culture period before quantification of thymidine uptake. Results are expressed as a stimulation index: stimulated cells/medium alone control from triplicate wells per condition.

Statistics

Data were collated and analyzed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). Data are presented either as means with standard deviation, median values with individual data points displayed or box and whisker plots incorporating group means plus minimum and maximum values. Plots depict data from at least two independent experiments. Statistically significant differences between two treatment groups were determined by the unpaired Student's *t*-test; or three or more by one-way ANOVA with the appropriate test for multiple comparisons applied. Specifics of the statistical analyses performed are supplied in the figure captions.

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AUTHOR CONTRIBUTIONS

Janlin Ying Hui Chan: Data curation; formal analysis; investigation; methodology; writing – review and editing. **Fiona Clow:** Data curation; formal analysis; methodology; project administration; writing – review and editing. **Victoria Pearson:** Data curation; formal analysis. **Ries J Langley:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; supervision; writing – review and editing. **John D Fraser:** Conceptualization; funding acquisition; resources; supervision; writing – review and editing. **Fiona J Radcliff:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; supervision; validation; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.