HEALTH AND MEDICINE

Globally deimmunized lysostaphin evades human immune surveillance and enables highly efficacious repeat dosing

Hongliang Zhao[1]*, Seth A. Brooks[1]*, Susan Eszterhas[1], Spencer Heim[1], Liang Li[2], Yan Q. Xiong[2], Yongliang Fang[1,3], Jack R. Kirsch[1], Deeptak Verma[4], Chris Bailey-Kellogg[3,4,5], Karl E. Griswold[1,3,5]†

There is a critical need for novel therapies to treat methicillin-resistant *Staphylococcus aureus* (MRSA) and other drug-resistant pathogens, and lysins are among the vanguard of innovative antibiotics under development. Unfortunately, lysins' own microbial origins can elicit detrimental antidrug antibodies (ADAs) that undermine efficacy and threaten patient safety. To create an enhanced anti-MRSA lysin, a novel variant of lysostaphin was engineered by T cell epitope deletion. This "deimmunized" lysostaphin dampened human T cell activation, mitigated ADA responses in human HLA transgenic mice, and enabled safe and efficacious repeated dosing during a 6-week longitudinal infection study. Furthermore, the deimmunized lysostaphin evaded established anti–wild-type immunity, thereby providing significant anti-MRSA protection for animals that were immune experienced to the wild-type enzyme. Last, the enzyme synergized with daptomycin to clear a stringent model of MRSA endocarditis. By mitigating T cell–driven antidrug immunity, deimmunized lysostaphin may enable safe, repeated dosing to treat refractory MRSA infections.

INTRODUCTION

Staphylococcus aureus is one of the most common bacterial pathogens in humans. It causes a wide range of infections, including superficial skin infections, more serious osteoarticular and soft tissue infections, and especially dangerous pneumonia, bacteremia, and infective endocarditis (*1*). In addition, *S. aureus* is notorious for acquiring antibiotic resistance, often within 1 to 2 years of a drug's introduction into the clinic (*2*). Infections by methicillin-resistant *S. aureus* (MRSA), a multidrug-resistant pathogen, have reached epidemic proportions (*3*), and there is an urgent need for new anti-staphylococcal agents with novel cellular targets and mechanisms of action (*4*).

Bacteriolytic enzymes, also known as lysins, are now considered among the top candidates to help address the mounting antibiotic resistance crisis (*5*, *6*). Lysostaphin is a well-known antibacterial enzyme that specifically kills *S. aureus* via hydrolysis of pentaglycine cross-links in its cell wall peptidoglycan (*7*). As a result of its clinically desirable attributes, there has been longstanding interest in lysostaphin as an alternative antibiotic for MRSA infections (*8*). It has undergone numerous human trials as an intranasally administered microbicide to clear endogenous *S. aureus* carriage (*8*), and in one compassionate use case, intravenously administered lysostaphin was used to clear a highly drug-resistant, multifocal MRSA infection (*9*). Despite these successful human trials and a wealth of associated preclinical studies, development and approval of a parenteral lysostaphin therapy has stalled due, in part, to immunogenicity concerns and the observation that lysostaphin treatment elicits antidrug antibodies (ADAs) in human subjects (*8*, *10*).

Classical antidrug immune responses start with uptake and processing of protein therapeutics by antigen-presenting cells. After proteolytic cleavage in lysosomal vesicles, some peptide segments from the protein, termed T cell epitopes, are loaded into major histocompatibility complex [MHC II, or human leukocyte antigen (HLA) in humans] and displayed on the cell surface for subsequent surveillance by T cell receptors (TCRs) on $CD4^+$ helper T cells (*11*). Formation of a ternary MHC II–peptide–TCR complex accompanied by costimulatory signals drives $CD4^+$ T cell activation, maturation of antigen-specific B cells, and ultimately production of high-affinity immunoglobulin G (IgG) antibodies that bind the intact protein therapeutic. Development of ADAs can manifest a range of clinical complications, including altered pharmacokinetics, drug neutralization, immune complex–associated toxicity, and life-threatening hypersensitivity reactions (*12*). Mutagenic deletion of T cell epitopes from protein therapeutics is a well-established strategy for mitigating immunogenicity risks (*13*), although doing so without compromising activity can prove challenging.

To engineer a functionally deimmunized lysostaphin for treatment of genetically diverse patient populations, structure-based molecular design (*14*, *15*) was used to delete putative T cell epitopes restricted by a set of human MHC II supertypes, or alleles, that are broadly representative of global MHC II peptide–binding specificity (*16*). Here, the performance of a deimmunized lysostaphin variant is benchmarked against its progenitor template in a rigorous series of preclinical studies. Standardized in vitro assays and in vivo efficacy studies first assess the parental and deimmunized enzymes' antibacterial potencies. Next, ex vivo cellular immunoassays evaluate activation of human $CD4^+$ T cells upon exposure to the proteins, and the in vivo immunogenicity of each is quantified as ADA titers following immunization of human HLA transgenic mice. The interplay between immunogenicity and efficacy is then tested with a longitudinal bacteremia model in the transgenic mice, where rescue from recurrent, lethal MRSA challenges requires that efficacy is not compromised by antidrug immunity during 6 weeks of repeated drug dosing. Next, the variant's capacity to evade preexisting immunity against the wild-type enzyme is assessed with additional ex vivo cellular immunoassays, and the therapeutic relevance of this property is tested in a recurrent MRSA challenge study using transgenic mice

[1]Thayer School of Engineering, Dartmouth, Hanover, NH 03755, USA. [2]Lundquist Institute at Harbor-UCLA Medical Center, Torrance, CA 90502, USA. [3]Lyticon LLC, Lebanon, NH 03766, USA. [4]Department of Computer Science, Dartmouth, Hanover, NH 03755, USA. [5]Stealth Biologics LLC, Lebanon, NH 03766, USA.
*These authors contributed equally to this work.
†Corresponding author. Email: karl.e.griswold@dartmouth.edu

preimmunized with the progenitor molecule. Last, the translational potential of the deimmunized enzyme is further evaluated using a rabbit model of MRSA infective endocarditis, where synergy with standard-of-care daptomycin chemotherapy is probed in a clinically relevant and refractory infection model. Together, these results suggest that aggressive reengineering of lysostaphin has produced a biotherapeutic candidate that is both highly efficacious and immunologically stealthy in the context of diverse human MHC II haplotypes.

RESULTS

F12 engineering

To create a deimmunized lysostaphin variant for potential clinical application, a structure-based combinatorial library design strategy was implemented in a manner analogous to previous model studies (*15*), but in the present case, a broader set of representative human MHC II alleles was targeted for deimmunization (*16*). Compared to previous model studies, deimmunizing lysostaphin for global patient populations required targeted deletion of a far larger set of putative T cell epitopes spanning many more constituent peptides, rendering maintenance of high-level stability and activity an even greater challenge. To achieve these objectives, a directed evolution approach was used, in which deimmunized library ensembles were designed, constructed, transformed into *Pichia pastoris*, and then screened for anti-MRSA activity using agar plate halo formation assays. The top-performing clone from each round served as a template for the next round of library design and directed evolution, ultimately producing the deimmunized lysostaphin variant "F12." F12 bears 14–amino acid substitutions (fig. S1A), equaling 6% of the native lysostaphin sequence. This high mutation rate was intended to effect global deimmunization, defined here as broad reductions in putative T cell epitope content (fig. S1, B and C) aimed at mitigating immunogenicity for genetically diverse patient populations (fig. S2).

F12 retains potent in vitro and in vivo anti-staphylococcal activity

Although the protein design and deimmunization algorithm explicitly optimized for protein function, loss of lysostaphin fitness was an inherent risk of using such high mutational loads, and this prompted an initial in vitro analysis of F12 stability and activity. Differential scanning fluorimetry (DSF) showed that F12 maintained near–wild-type thermostability (T_m = 55°C versus T_m = 59°C, respectively). Separately, F12's in vitro anti-staphylococcal potency was tested against a panel of drug-resistant *S. aureus* strains, including methicillin-, vancomycin-, linezolid-, and daptomycin-resistant isolates (MRSA, VRSA, LRSA, and DRSA, respectively). F12 exhibited high potency against all study strains, with minimum inhibitory concentrations (MICs) ranging from 40 to 400 ng/ml in standard cation-adjusted Mueller Hinton II Broth synthetic medium (caMHIIB) (table S1). These MIC values were within 5- to 35-fold those of a non-deimmunized lysostaphin counterpart (hereafter "LST") (*17*), and it is noteworthy that F12 exhibited substantially better MIC potency than two clinical-stage anti-MRSA lysins: CF-301 (MIC range, 8000 to 16,000 ng/ml) and SAL200 (MIC range, 800 to 1600 ng/ml) (*18*, *19*).

Having shown potent anti-staphylococcal activity in vitro, we next tested F12's in vivo efficacy following a single bolus administration in a murine model of acute bacteremia. At the highest dose of 500 μg per mouse, both F12 and LST rescued all animals, but at each lower dose F12 achieved significantly better protection (Fig. 1, A and B). Unexpectedly, these in vivo results contrasted with F12's reduced in vitro MIC potency (table S1), and given the fact that mice in the acute infection model were naïve to the enzyme therapies, F12's deimmunized nature could not directly explain its enhanced efficacy. The conflicting in vitro versus in vivo activity trends prompted a new analysis of MICs in serum, a more biologically relevant assay medium. In the serum studies, F12's in vitro potency against MRSA strain MW2 increased more than 10-fold, while that of LST remained unchanged (Fig. 1C). Thus, the deimmunizing mutations in F12 yielded an adventitious increase in the molecule's inherent antibacterial activity in biological environments. While the molecular origins of this effect were not examined here, similar serum effects on in vitro MIC potency have been seen with phage lysin CF-301, and in that case, the origins were traced to the interaction of lysin with serum albumin and serum lysozyme (*20*).

Another desirable attribute of antibacterial lysins is their potential to suppress new resistance phenotypes; however, lysostaphin is known to induce resistant *S. aureus* mutants during exposure in synthetic growth medium (>5 log increase in MIC using caMHIIB) (*8*). Given the unexpected increase in F12 potency in serum, it was contemplated that serum-containing medium might also help suppress emergent resistance. To test this hypothesis, MRSA was serially cultured in the presence of subinhibitory nafcillin, F12, or LST for 14 days, and the MICs of cultures against the respective agents were determined after each drug exposure (Fig. 1D). MRSA resistance to nafcillin increased 190-fold after just 5 days of drug exposure, whereas LST and F12 elicited only 50-fold and 3-fold MIC increases, respectively, even after 14 days of selective pressure. The LST and F12 treatments caused a transient loss of bacterial fitness on days 5 (LST and F12) and 7 (F12 only). Specifically, the bacteria failed to produce visible outgrowth in the day 5 and day 7 MIC assays, even in control wells containing no antibiotic. On these days, the no-antibiotic control wells were subcultured despite the lack of visible growth, and this subsequent nonselective subculture recovered viable bacteria, enabling continuation of the experiment. While F12 exhibited significantly better potency than LST on days 3, 6, and 11, this differential was transient and does not necessarily indicate a performance advantage of F12 over LST with respect to resistance induction. In summary, the stringent resistance induction assays performed here suggest that *S. aureus* has limited capacity to acquire F12 resistance in serum, a biologically relevant matrix.

F12 dampens human T cell activation

The strategy for engineering an immunologically stealthy lysostaphin focused on deletion of putative $CD4^+$ T cell epitopes while maintaining native protein fold and function. The success of this specific objective was evaluated by measuring T cell activation in human peripheral blood mononuclear cells (PBMCs). The chosen PBMC assay format included an extended preexpansion period in the presence of protein antigen, allowing amplification of antigen-specific T cells and more robust detection of activated cells compared to standard PBMC assay formats (*21*, *22*). Notably, while standard PBMC assays may consider a stimulation index (SI) of 2 to 3 to be biologically significant, use of the preexpansion methods employed here routinely yields SI of >10 for responding donors, and strong responders may have SI of 100 or more. See Materials and Methods for calculation of SI and a detailed description of responder versus nonresponder criteria.

While donor MHC genotype is a critical determinant of responder versus nonresponder status for a given protein antigen, it bears

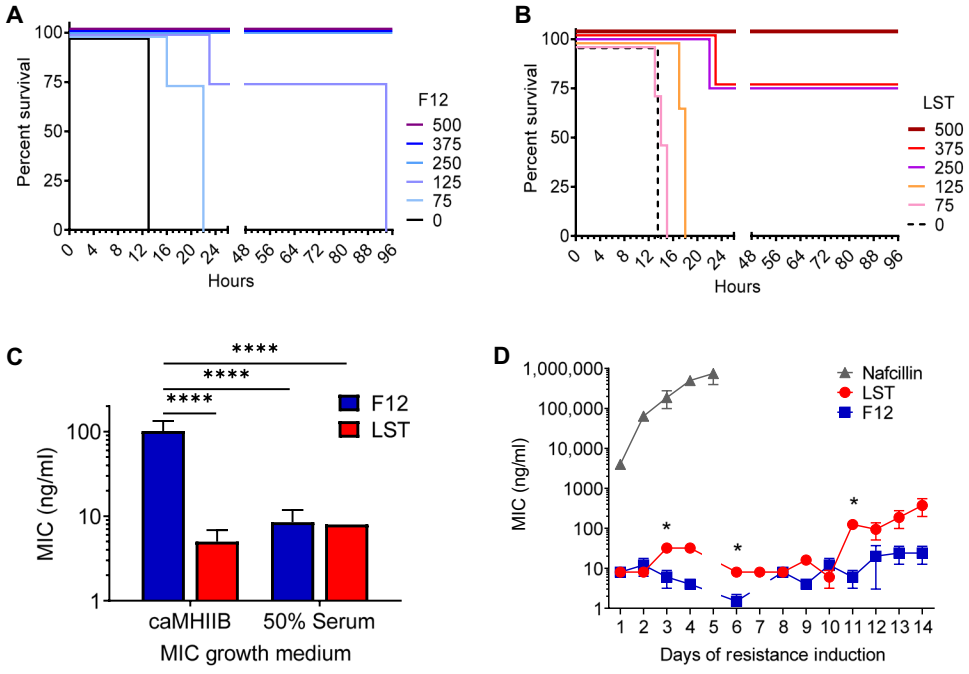

Fig. 1. F12 versus LST efficacy and activity in biological matrices against MRSA MW2. (**A**) F12 dose-response survival curves in murine bacteremia model (dose provided in micrograms per mouse). (**B**) LST dose-response survival curves in murine bacteremia model (dose provided in micrograms per mouse). For matched doses in (A) and (B), F12 was significantly more efficacious at 75 μg, $P = 0.009$; 125 μg, $P = 0.003$; 250 μg, $P = 0.01$; and 375 μg, $P = 0.01$ (Mantel-Cox log-rank test). (**C**) F12 (blue) and LST (red) MIC values in standard caMHIIB medium and in 50% human serum. Mean and SD from three independent experiments, each with two to three technical replicates. For (C), both the enzyme and assay medium resulted in significant differences [two-way analysis of variance (ANOVA)]. F12 in caMHIIB was significantly less potent than all other treatments [$P < 0.0001$, two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli with false discovery rate (Q) = 5%]. (**D**) Serial induction of resistance in 50% serum. Growth wells from MIC assays were serially cultured to induce drug resistance upon repeated and escalating drug exposure. Nafcillin resistance increased 190-fold over 5 days, LST resistance increased 50-fold over 14 days, and F12 resistance increased only 3-fold over 14 days. Missing data at days 5 (LST and F12) and 7 (F12) indicate inability to determine MIC values, as bacteria failed to yield visible outgrowth even in control wells lacking antibacterial agents. Mean and SD of duplicate assays are shown. F12 was significantly more potent than LST on days 3, 6, and 11 [multiple unpaired *t* tests with false discovery rate (Q) = 5%]. *$P < 0.01$; ****$P < 0.0001$.

emphasizing that the central design objective of these studies was mitigating immunogenicity risk for global patient populations; the MHC supertypes targeted during the design process and the MHC genotypes of the tested blood cell donors served as tractable surrogates for genetically diverse MHC types. Therefore, we considered as a first measure of success the relative strengths of responses and overall responder rates for F12 versus LST, irrespective of donor MHC genotype. Among 17 unique donors included in the final analysis, 9 were considered responders to one or both proteins, while 8 failed to show a response to either F12 or LST at any SI cutoff value. The nine responders fell into three categories based on natural breaks in their LST SI values (Fig. 2A). Three strong responders exhibited SI between 40 and 90 for LST, but their corresponding F12 SI values were only 1 to 7, indicating highly effective dampening of the T cell response by F12. Seven moderate responders exhibited SI between 14 and 27 for LST, and all had significantly lower SI for F12 (range, 1 to 10). One weak responder exhibited SI = 3 for LST, with a small 1.7-fold increase for F12.

Responder rates, or the percentage of study donors that are activated by a protein antigen, have been shown to loosely correlate with clinical immunogenicity rates (*23*), and here, LST exhibited 47 to 53% responder rates across a range of SI cutoff values from 2 to 10 (Fig. 2B). In contrast, F12 responder rates were one-third that of LST at SI cutoff values of 2 to 8. At an SI cutoff of >10, F12 had no responders, effecting complete T cell silencing for this small donor panel.

Among nonresponders, donors 1454/1503 and 0101/0407P failed to yield a statistically significant increase in spot-forming cells (SFCs) compared to the dimethyl sulfoxide (DMSO) control ($P = 0.13$ and $P = 0.06$, respectively). These two donors nonetheless exhibited large SI values for LST and muted responses against F12 (Fig. 2C), which was consistent with F12's reduced immunoreactivity among responding donors (Fig. 2A). All other nonresponders failed to meet the criterion of 60 SFCs after background subtraction and, as a result, yielded low SI values for both proteins (Fig. 2C).

One strong responder, two moderate responders, and five of the eight nonresponders encoded MHC II genotypes not targeted by the design process (table S3). Thus, for donor MHC II alleles not explicitly considered during the design, F12 muted the response of three LST-reactive donors and did not elicit neo-responses in donors who failed to recognize LST. These results underscore the utility of deimmunized protein design based on a limited number of MHC II supertypes, and more specifically, the broad dampening of T cell activation among a small but diverse panel of donors represents evidence of successful immunogenicity mitigation with F12.

F12 reduces immunogenicity in vivo

The F12 design specifically sought to delete T cell epitopes via mutagenic disruption of MHC II peptide binding, but the desired practical outcome of any such deimmunization effort is a reduction of ADA responses in human patients (*13*). To evaluate whether deletion of

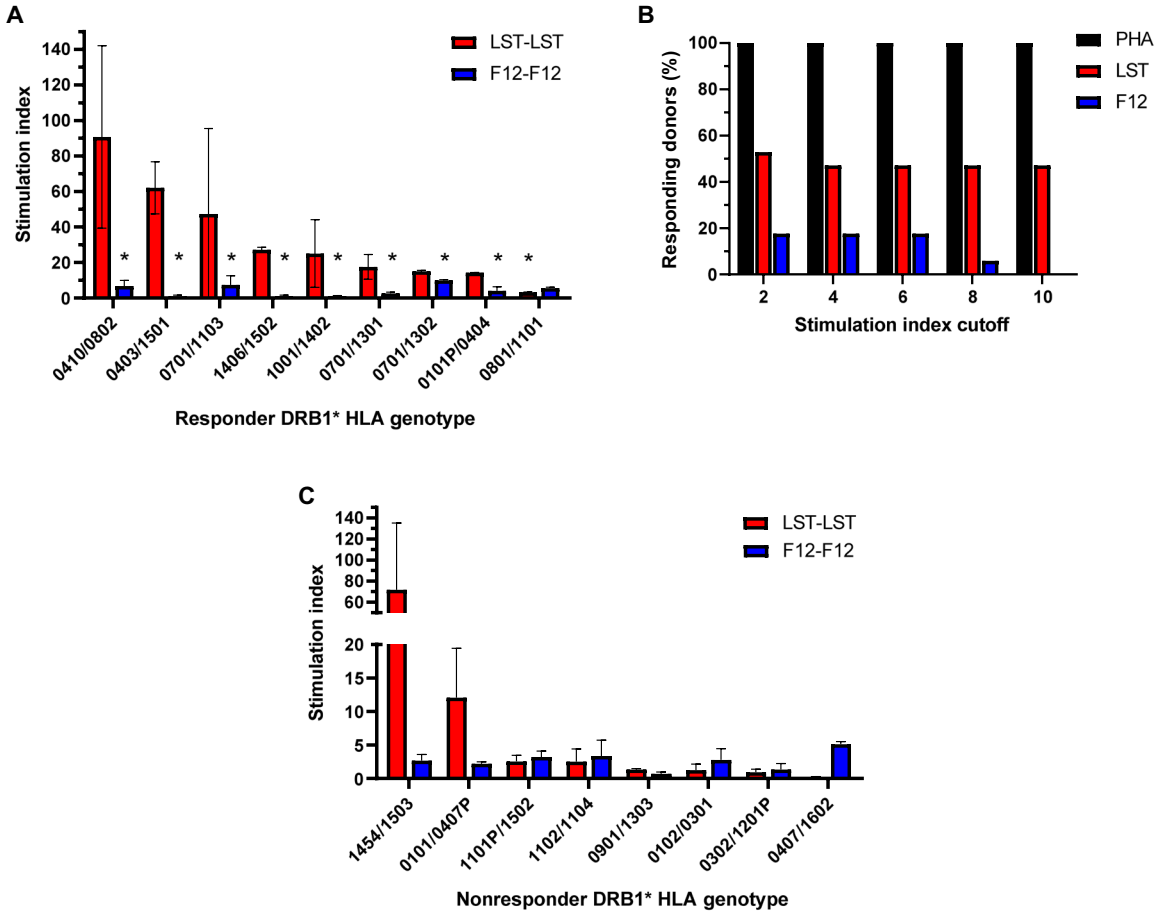

Fig. 2. F12 versus LST activation of human T cells in PBMC preparations. PBMCs were expanded separately in the presence of LST (red) or F12 (blue), restimulated with synthetic peptides matched to the expansion protein, and T cell activation was quantified as SFCs by IL-2 ELISpot. (**A**) SIs for nine donors deemed responders to one or both proteins. Donor DRB1 MHC II genotype is indicated on the *x* axis. Values are average and SD for triplicate ELISpots. F12 is significantly less stimulatory for all responders except 0801/1101 [multiple unpaired *t* tests with false discovery rate (*Q*) = 5%]. (**B**) Responder frequencies as a function of SI cutoff value. Phytohemagglutinin is shown as a positive control (black). F12 had no responders at a cutoff of 10. See Materials and Methods for responder criteria. (**C**) SI for nonresponders. *q value < 0.05.

F12's T cell epitopes mitigated ADA responses in vivo, F12 and LST immunogenicities were tested in human HLA transgenic mice. These transgenic mouse strains are null for endogenous murine MHC II and instead bear a chimeric MHC II derived from human DRB1*0401 (DR4) or DRB1*1501 (DR2) (*24*, *25*). These two alleles are representative of the "DR4" and "main DR" human MHC II supertypes, respectively (*16*). Mice were given four weekly subcutaneous immunizations with either LST or F12 in phosphate-buffered saline (PBS), and serum was collected on study days 14 and 28 for analysis of ADA titers.

Consistent with lysostaphin's known immunogenicity in humans (*8*), LST proved to be strongly immunogenic in both transgenic mouse strains, eliciting high ADA titers in DR4 mice on day 28 [serum dilution 50% effective concentration (EC_{50}) = 1:50 to 1:600; Fig. 3A] and even higher titers in DR2 mice by day 14 (EC_{50} = 1:600 to 1:1800; Fig. 3B). In contrast, anti-F12 ADA titers were 18- and 100-fold lower in DR4 and DR2 mice, respectively. A similar reduction in ADA titers was observed in separate experiments, in which either LST or F12 was administered directly to the airways of DR4 mice once a week for 4 weeks (fig. S3A). This latter route of administration was evaluated given the potential for F12 to be formulated as an inhaled drug for treatment of pneumonia. Overall, these studies demonstrated that the deletion of human MHC II–restricted T cell epitopes in F12 reduced in vivo immunogenicity by one to two orders of magnitude in two different human HLA transgenic mouse strains.

The decreased anti-F12 ADA titers in DR4 and DR2 mice were consistent with MHC II peptide–binding predictions (table S2) as well as reduced T cell activation for human donors encoding DRB1*04 and DRB1*15 alleles (Fig. 2). To more directly probe the relationship between T cell silencing and ADA suppression, additional cellular immunoassays were performed on HLA transgenic murine splenocytes harvested at the conclusion of the immunogenicity studies described immediately above. These antigen-experienced immune cells were restimulated ex vivo with whole LST or F12 proteins, and T cell activation was quantified. Consistent with the observed ADA titers, splenocytes from DR4 mice immunized with LST exhibited significant activation upon LST restimulation, whereas splenocytes from DR4 mice immunized with F12 demonstrated background levels of activation upon F12 restimulation (Fig. 3C). Similarly, in DR2 mice immunized with LST, splenocytes were strongly activated upon LST restimulation, whereas splenocytes from DR2 mice immunized with F12 exhibited significantly lower comparative

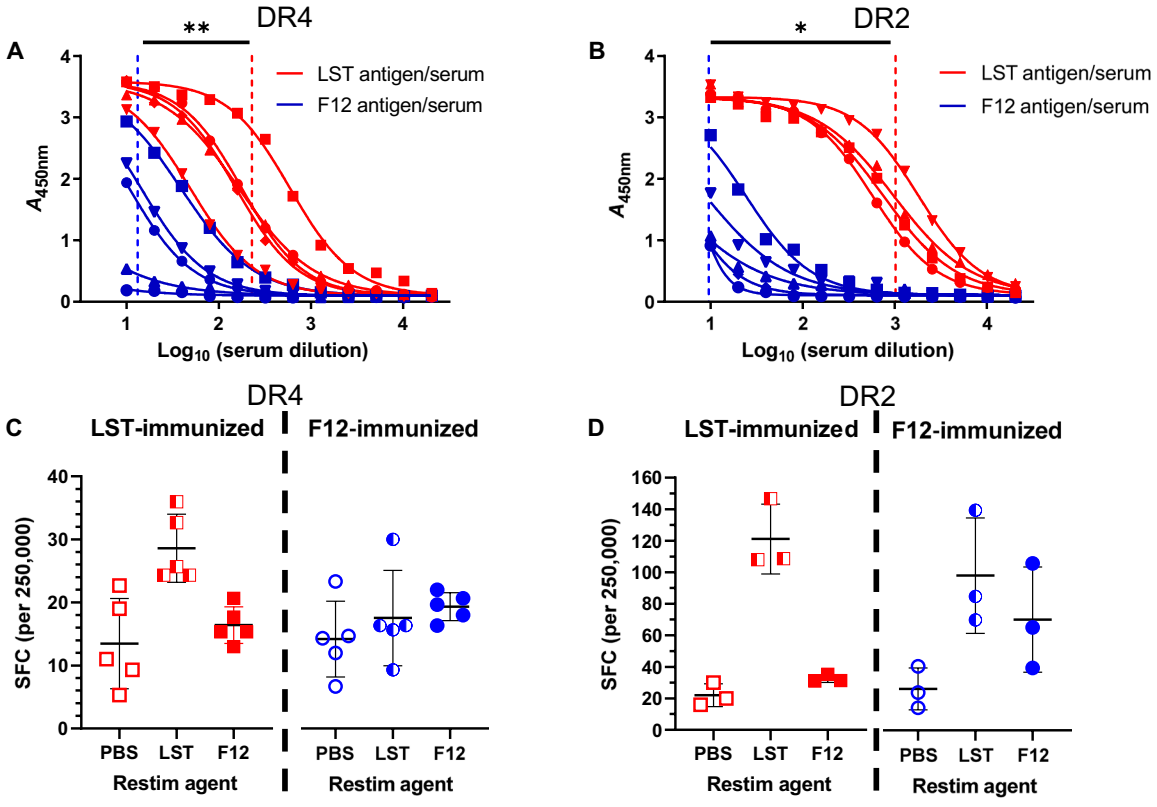

Fig. 3. F12 versus LST immunogenicity in human HLA transgenic mice. (**A**) ELISA of serum dilutions (*x* axis) to quantify ADA titers in DR4 mice following four immunizations with F12 (blue) or LST (red). Mean 50% response titer for each group is indicated with vertical hashed lines. Average titers are significantly different. $P = 0.008$, Mann-Whitney two-tailed test. (**B**) Analogous ADA titers in DR2 mice after two immunizations. Average titers are significantly different. $P = 0.016$, Mann-Whitney two-tailed test. (**C**) Activation of splenocytes harvested from DR4 mice following four immunizations with LST (left, red squares) or F12 (right, blue circles). Splenocytes restimulated ex vivo with buffer control (open symbols), LST (half-open symbols), or F12 (closed symbols). *Y* axis is activated cells based on the ELISpot analysis of 250,000 splenocytes. Mean and SD are indicated for each group. The LST immunized–LST restimulated group is significantly higher than all other groups. See table S4 for two-way ANOVA results. (**D**) Analogous activation of splenocytes harvested from DR2 mice. The LST immunized–LST restimulated group is significantly higher than all other groups except the F12 immunized–LST restimulated group. See table S5 for two-way ANOVA results. A_{450nm}, absorbance at 450 nm. *$P < 0.05$; **$P < 0.01$.

activation upon F12 restimulation, although still above background in the latter instance (Fig. 3D). In total, the immunological analyses with human PBMCs and human HLA transgenic mice provided preclinical evidence that the F12 variant successfully achieved a central design objective: general evasion of human immune surveillance.

F12 immune evasion enables repeated efficacious dosing

An important practical goal of the F12 engineering effort was enabling safe and efficacious repeat dosing of the antibacterial enzyme, which could be required to treat refractory or recurrent MRSA infections. To assess this outcome, both F12 and LST efficacies were evaluated in a recurrent bacteremia model using transgenic DR4 mice. The recurrent model is analogous to the acute infection model described above, except that rescued mice were given 1 week to recover and then reinfected and retreated in an iterative fashion (Fig. 4A). This experimental scheme enables longitudinal efficacy testing in the context of mounting antidrug immunity.

Three animals in the LST cohort ($N = 10$) succumbed to the initial day 0 infection and one to the second day 7 infection, compared to complete rescue of the F12 cohort ($N = 6$) (Fig. 4B). These early observations, before development of class-switched IgG ADAs, reflected F12's greater inherent efficacy. Subsequently, four of six LST-treated animals succumbed to the third infection on day 14 and both remaining animals to the fourth infection on day 21. These later time points correlate with anti-LST ADA development in DR4 mice (Fig. 3B and fig. S3B). In contrast, the F12-treated group demonstrated 100% survival over the course of seven iterative cycles of infection and treatment. These observations support the conclusion that strong ADA responses undermine the LST parental enzyme's in vivo efficacy, whereas the deimmunized nature of F12 results in no apparent diminution of efficacy over at least seven cycles of serial infection and treatment.

F12 evades established anti-LST immunity

In addition to evading naïve immune responses, another challenging goal for deimmunized biotherapies is efficacious treatment in the presence of preexisting immunity. Such a scenario could occur during switching between alternative drugs or as a result of previous environmental exposure, both of which are of direct clinical relevance. Two separate ex vivo cellular immunoassays were used to assess F12's evasion of established anti-LST immunity. First, ex vivo crossover assays were conducted with F12 protein and splenocytes harvested from DR4 and DR2 HLA transgenic mice that had been immunized with LST. While splenocytes from LST-immunized mice exhibited significant activation when restimulated with LST protein, restimulation of the same splenocytes with F12 resulted in

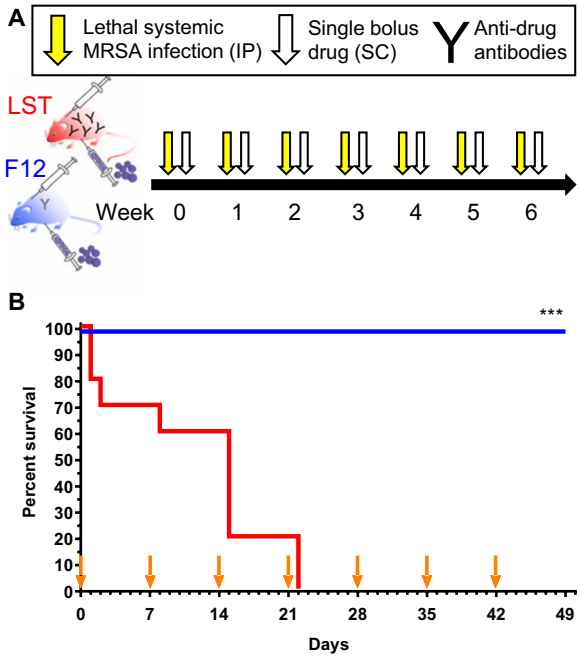

Fig. 4. Recurrent bacteremia model in DR4 human HLA transgenic mice. (**A**) Schematic of recurrent infection model. Mice were given a lethal intraperitoneal (IP) challenge of MRSA clinical isolate USA400 and, 1 hour later, were treated with either 500 μg of LST (red) or F12 (blue) subcutaneously (SC) administered. Surviving mice underwent serial cycles of infection and treatment. (**B**) Kaplan-Meier survival curve for F12 versus LST treatments. Infection dates are indicated with orange arrows. F12 provides significantly better protection than LST. $P = 0.0003$, log-rank test. ***$P < 0.001$.

no detectable signal above background (Fig. 3, C and D). Second, analogous ex vivo crossover assays were conducted with human PBMCs. Specifically, PBMCs expanded in the presence of LST antigen were restimulated separately with LST or F12 peptide pools, and T cell activation was compared. T cell activation following restimulation with F12 peptides was universally lower for all LST-responding donors (Fig. 5A). Thus, F12 efficiently evades surveillance by helper T cells primed to recognize the LST protein.

Next, an in vivo efficacy study was designed to test the extent to which F12 evasion of preexisting anti-LST immunity would result in improved efficacy. DR4 HLA transgenic mice were first preimmunized with the LST protein and then split into two cohorts for testing in the recurrent bacteremia infection model. One cohort was treated with LST ($N = 12$) and one with F12 ($N = 12$). F12 manifested significantly better overall efficacy ($P = 0.0034$, log-rank test), rescuing 33% of animals through four cycles of infection and treatment, compared to no cumulative survival for the LST-treated cohort (Fig. 5B). Notably, compared to infection studies in naïve animals, the reduced efficacy of F12 in this preimmune study may have resulted from cross-reactive antibodies elicited by the LST preimmunizations. In previous immunogenicity studies, 20 to 40% of DR4 mice developed prominent anti-LST ADA after just two subcutaneous immunizations (fig. S3B), and anti-LST antibodies from DR4 mice were further shown to cross-react with F12 (fig. S3C). Nonetheless, the preimmunization study showed that F12 is capable of rescuing mice from a lethal MRSA challenge despite preexisting anti-LST immunity.

F12 clears MRSA infections in a difficult-to-treat endocarditis model

The established clinical development pathway for lysins, and likely their optimal mode of use, is in combination with standard-of-care antibacterial chemotherapies (*26*, *27*). To evaluate F12's potential as a combination therapy, it was tested in a clinically relevant rabbit model of left-sided infective endocarditis (*28*). Transcarotid-transaortic catheters were surgically implanted in rabbits on study day −3, rabbits were given an intravenous challenge with MRSA isolate MW2 on day 0, and drug therapy was provided on days 1 to 4. The efficacy of daptomycin (4 mg/kg, intravenously, once daily × 4 days) and F12 (40 mg/kg, intravenously, single dose on day 1) monotherapies was compared to a combination of the two agents. Mortality rates were 100% for untreated control animals, 14% for daptomycin, 20% for F12, and 0% for the combination therapy. Even more notable, at the doses used here, the combination therapy completely eradicated MRSA from all target tissues, while daptomycin and F12 monotherapies were only marginally effective in reducing bacterial burden in cardiac vegetations, kidneys, and spleen (Fig. 6). These results show that a single bolus of F12 combined with standard-of-care chemotherapy rapidly and completely clears MRSA in a notoriously difficult-to-treat left-sided infective endocarditis model.

DISCUSSION

The U.S. Centers for Disease Control and Prevention recently revealed that previous analysis of the number of MRSA hospitalizations, MRSA-related deaths, and MRSA-attributable health care costs were vastly underestimated (*29*). While the new analysis suggests some progress in combating MRSA infections between 2012 and 2017, MRSA remains the single most deadly drug-resistant bacteria in the United States, and there continues to be an urgent need for innovative therapeutics to combat this and other antibiotic-resistant pathogens (*30*). One such class of antibiotic alternatives is bacteriolytic enzymes, or lysins, and lysostaphin is a prototypical example of these catalytic anti-infectives (*8*).

Lysins manifest numerous powerful and clinically relevant attributes including high potency, rapid onset of action, efficacy against contemporary resistant strains, and the potential to suppress emergent resistance phenotypes (*31*, *32*). However, because of their microbial origins, these nonhuman proteins pose a high risk of immunogenicity in human patients, and putative antidrug immune reactions have the potential to undermine therapeutic efficacy and threaten patient safety (*12*). The two most advanced lysin biotherapeutics have proven to be highly immunogenic in humans even after a single administration. In phase 1 safety trials, SAL200 elicited dose-dependent ADA in 37% of healthy subjects (*33*) and CF-301 in 69% (*34*). Lysostaphin is similarly known to elicit ADA in a wide range of animal models as well as human patients (*8*). Here, an analysis in human HLA transgenic mice showed that lysostaphin elicits high-titer ADA and further that these anti-lysostaphin antibodies completely ablate the enzyme's therapeutic efficacy during longitudinal studies in DR4 mice. This antibody-mediated loss of efficacy combined with immunogenicity-related safety concerns underscore the clinical risks of lysin immunogenicity. Therefore, to realize the full therapeutic potential of these innovative antibiotics, it will be important to mitigate their immunogenicity in humans.

Covalent protein modification with polyethylene glycol (PEGylation) reduces immunogenicity by physically shielding immunogenic epitopes,

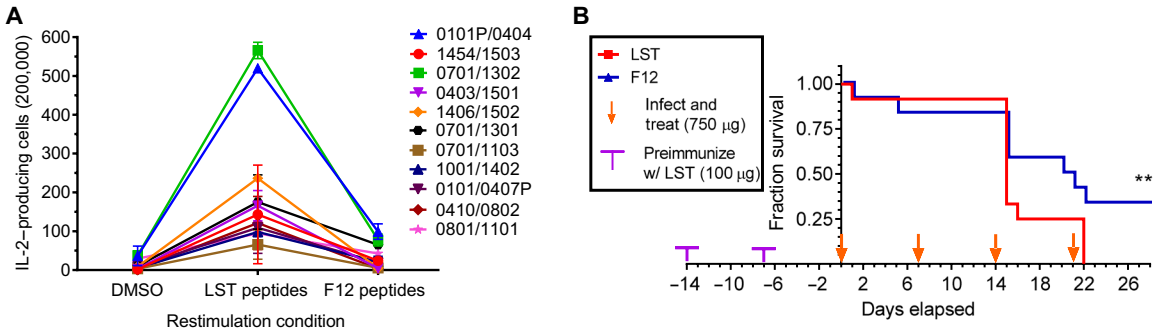

Fig. 5. F12 evades established anti-LST immunity. (**A**) Crossover PBMC assays. Human PBMCs were expanded in the presence of LST protein and, following expansion, were split and restimulated separately with peptide pools corresponding to either LST or F12. The number of SFCs by ELISpot is provided for each responding donor after DMSO control, LST, or F12 peptide restimulation. Donor DRB1 MHC II genotype is indicated in legend. F12 restimulation is significantly less activating than LST restimulation for all donors except DRB1*0801/1101 (two-way ANOVA). See table S6 for summary statistics. (**B**) F12 manifests improved efficacy in DR4 mice preimmunized with LST. DR4 mice were preimmunized twice with 100 μg of LST, split into two groups ($N = 12$ each), and tested in the MRSA recurrent bacteremia model treated with either 750 μg of LST (red) or F12 (blue). Preimmunization is indicated with purple "T," and infections are indicated with orange arrows. F12 provides significantly better protection. $P = 0.0034$, log-rank test. **$P < 0.01$.

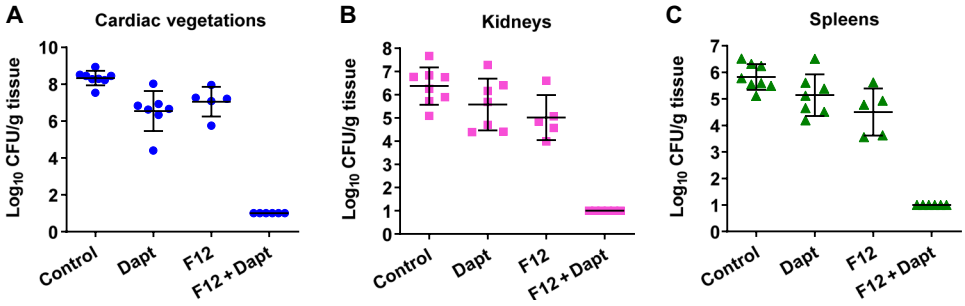

Fig. 6. F12, daptomycin, and combination therapy in a rabbit model of left-sided infective endocarditis. Daptomycin dosed at 4 mg/kg once daily × 4 days, F12 dosed at 40 mg/kg once daily × 1 day, or a combined treatment using the same dosing regimens. Viable bacterial counts recovered from (**A**) cardiac vegetations, (**B**) kidney, and (**C**) spleen at the study conclusion are shown. Lines and bars are mean and SD. The combination therapy eradicated bacteria in all target tissues such that the limit of detection was assigned as an upper bound. The combination therapy is significantly different from all other groups (two-way ANOVA). See table S7 for summary statistics. Mortality was 100% for control animals, 14% for daptomycin, 20% for F12, and 0% for the combination therapy.

and it is a well-recognized strategy for mitigating biotherapeutic immunogenicity (*35*). Lysostaphin itself has been modified by PEG to improve its pharmacokinetics and reduce immunogenicity (*36*), but it was subsequently found that PEGylation abolished the enzyme's bactericidal activity (*8*). More sophisticated site-specific PEGylation of the lysin Cpl-1 likewise undermined antibacterial activity, ultimately leading to the conclusion that lysins are not generally amenable to modification with PEG (*37*).

Here, mutagenic deletion of putative T cell epitopes was used to mitigate lysostaphin's immunogenicity risk while maintaining high enzymatic activity. Analyses with human PBMCs and two different strains of human HLA transgenic mice provided strong preclinical evidence that F12 is evasive of human immune surveillance and further that this reduced immunogenicity translates into improved efficacy. In particular, F12's capacity to rescue DR4 human HLA transgenic mice from seven serial MRSA challenges over a 6-week period is evidence that the globally deimmunized enzyme can be safely and effectively dosed multiple times. This distinguishes F12 from the immunogenic anti-MRSA lysins CF-301 and SAL200, each of which is undergoing clinical development based on a single-dose administration. By dosing only once, the risks associated with adaptive immunity can be circumvented but at the potential cost of suboptimal efficacy against refractive infections such as left-sided endocarditis, osteomyelitis, and pneumonias. A single dose of CF-301 failed to improve outcomes for left-sided endocarditis patients in a phase 2 clinical trial: Only 2 of 11 patients responded to the CF-301 + daptomycin combination therapy versus 2 of 3 patients responding to daptomycin alone (*38*). Notably, left-sided endocarditis patients have been excluded from the CF-301 phase 3 trial (*39*). Other indications that are likely to necessitate repeated long-term dosing include comorbidities that render patients susceptible to recurrent *S. aureus* infections, for example, cystic fibrosis, chronic obstructive pulmonary disorder, and diabetes (*40*–*43*). For these patient groups, a single dose of lysin therapy, even if effective, would provide only a short-term benefit. In contrast, by mitigating adaptive antidrug immunity via evasion of T cell surveillance, F12 has the potential for safe and efficacious repeat dosing to treat both refractory and recurrent *S. aureus* infections.

Beyond refractory and recurrent infections, there exist other scenarios in which adaptive immunity could limit lysins' clinical utility. For example, previous environmental exposure to a wild-type protein may result in patients with established antidrug immunity before receiving a recombinant biotherapy. In the case of lysostaphin, its source organism *Staphylococcus simulans* can colonize human

skin (*44*), and as a result, some patients may have preexisting anti-lysostaphin immunity. This scenario is of direct and immediate clinical relevance. The U.S. Food and Drug Administration placed the CF-301 phase 1 trial on temporary clinical hold due, in part, to concerns over preexisting immunity, which were brought to light by repeat-dose preclinical safety studies (*45*). The phase 1 trial ultimately proceeded after development of assays to prescreen and exclude subjects with preexisting anti–CF-301 antibodies, but these individuals represent another patient subset that will not benefit from the lysin drug. Here, F12 was shown to be highly evasive of human T cells primed to recognize the progenitor LST enzyme, and separately, F12 exhibited significantly enhanced efficacy against recurrent MRSA bacteremia in DR4 mice that were preimmunized with the LST progenitor. These results suggest that, in addition to evading naïve immune responses, F12 has the potential to evade memory immune responses directed against wild-type lysostaphin. If proven true in a clinical setting, this could enable safe and effective treatment of subjects with preexisting anti-lysostaphin immunity due to environmental exposure.

F12's capacity to evade established anti-LST T cell immunity also suggests a previously unexploited benefit of T cell epitope depletion. Some biotherapies have several competing drug products in the market, but these analogs can manifest varying levels of clinical immunogenicity due to differences in production hosts, glycosylation, formulation, route of administration, or other factors. Interferon beta (INFβ) is one example, wherein intramuscular INFβ-1a is significantly less immunogenic than subcutaneous INFβ-1a or INFβ-1b. In a clinical study, multiple sclerosis patients with high-titer ADA against subcutaneous INFβ-1a or INFβ-1b were randomly assigned to continue subcutaneous INFβ therapy or be switched to the low-immunogenicity intramuscular INFβ-1a therapy. A year later, there was no differential change in ADA titers between the two groups, indicating that the low-immunogenicity product is beneficial for drug-naïve patients only (*46*). Here, the combined preimmunization and efficacy study of LST and F12 in human HLA transgenic mice showed a significant benefit to treating with the T cell epitope–depleted variant after exposure to the LST progenitor. This raises the interesting question of whether T cell epitope–depleted biobetters might represent rescue therapies for patients with established memory B and T cells directed against a non-deimmunized progenitor drug. A T cell epitope–depleted variant's evasion of binding by high-titer circulating antibodies would be fortuitous, as T cell and antibody epitopes need not, and often do not, overlap (*13*). However, IgG class ADAs have a typical half-life of just 21 days (*47*), and therefore, evasion of circulating ADAs would be most relevant for patients in whom immunogenic drug exposure was recent or ongoing. In contrast, memory B cells, which do not themselves secrete high-titer antibodies, can circulate for decades, becoming rapidly reactivated via a T cell–dependent process upon reexposure to the protein antigen (*48*). Evasion of this long-term memory ADA response might be feasible via use of a T cell epitope–engineered biobetter, as described here.

It is important to note that the immunological assays used here are preclinical in nature and have limitations with respect to predicting clinical ADA responses. PBMC assays have been shown to correlate with overall clinical immunogenicity for monoclonal antibodies (*23*), and they have separately enabled identification of individual T cell epitopes that drive clinical immunogenicity (*49*, *50*). However, the human *mhcII* gene locus is one of the most polymorphic regions in the human genome, and the small panel of donors used here does not recapitulate the global diversity or prevalence of human MHC II haplotypes. Similarly, the HLA transgenic mice used here each encode only a single human MHC II allele, although it should be noted that the respective DRB1*0401 and DRB1*1501 alleles are MHC supertypes that are broadly representative of peptide-binding preferences among other alleles of those supertypes (*16*). Last, while HLA transgenic mice restrict T cell epitopes using human MHC II binding domains, the remainder of their cellular and molecular immune system is murine, and their predictive power with respect to human studies remains an area of active research. Nonetheless, the work described here provides strong preclinical evidence that design-based approaches to T cell epitope deletion can effectively mitigate biotherapeutic immunogenicity risk. Compared to experimentally driven T cell epitope mapping and deletion strategies, computational design can accelerate development of highly active deimmunized candidates for testing (*13*). In particular, F12 represents a promising lead in the ongoing effort to combat multidrug-resistant *S. aureus*, and F12's deimmunized nature might ultimately enable safe, repeated dosing to treat refractory or recurrent MRSA infections.

MATERIALS AND METHODS

Tryptic soy broth (TSB) was purchased from Research Products International. BBL Mueller Hinton II Broth Cation-Adjusted (caMHIIB) was purchased from Becton, Dickinson and Company (BD). Bovine serum albumin (BSA) and human serum (from human male AB plasma, U.S. origin, sterile-filtered) were purchased from Sigma-Aldrich. Ninety-six–well plates with lids, sterile, Greiner Bio-One, were purchased from VWR International. Daptomycin was purchased from Selleck Chemicals. Goat anti-mouse horseradish peroxidase–conjugated IgG antibody was from Thermo Fisher Scientific. Interleukin-2 (IL-2) ELISpot (enzyme-linked immunospot) kits were from Mabtech, and IL-2 was from PeproTech. Human donor PBMCs were purchased from Cellular Technology Limited and were chosen to represent MHC II genotypes that both (i) included MHC II alleles for which the designs were explicitly optimized and (ii) excluded MHC II alleles for which the designs were explicitly optimized. The sample proportion and U.S. population proportion [taken from (*51*)] of the selected donors' DRB1 MHC II alleles are shown in fig. S2.

Bacterial strains

Unless otherwise stated, all bacterial strains were stored in glycerol stocks at −80°C. *S. aureus* strains USA400 and USA300 were obtained from the American Type Culture Collection (ATCC). Strains AIS 080003, AIS 1000505, SALinR #12, and H2138 were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources). Strain ALC6334 was a gift of A. Cheung at the Dartmouth Geisel School of Medicine.

Protein design

Deimmunized lysostaphin variants, including the F12 lead candidate, were designed using structure-based algorithms that predict putative T cell epitopes as well as epitope-deleting mutations that are biased toward maintenance of native protein structure and function. The current design efforts were independent from, but conceptually similar to, previous structure-based deimmunization studies (*14*, *15*). Putative T cell epitopes were predicted for a small

set of human MHC II supertypes or alleles that are broadly representative of global MHC II peptide–binding specificity (*16*). The use of MHC II supertypes facilitates biotherapeutic deimmunization for genetically diverse patient populations without the need to explicitly consider all human MHC alleles.

Protein production and purification

Both LST and F12 were produced from *P. pastoris* that were cultured in a 3-liter Applikon Bioreactor (Applikon Biotechnology) as described previously (*17*). After cultivation, the supernatants were collected by centrifugation and diluted 10-fold with ion exchange chromatography binding buffer [20 mM phosphate buffer (pH 7.5)]. The diluted supernatants were loaded on an SP Sepharose Fast Flow column (100 ml; GE Healthcare) and eluted with a 0- to 250-mM NaCl gradient. Fractions containing LST or F12 were dialyzed into hydrophobic interaction chromatography binding buffer [20 mM phosphate buffer, 1 M $(NH_4)_2SO_4$ (pH 7.0)], loaded onto a Phenyl High Performance column (20 ml; GE Healthcare), and eluted with a 1-to-0 M $(NH_4)_2SO_4$ gradient. Purified wild type or F12 was dialyzed into PBS. The final preparations were approximately 95% pure based on an image analysis of SDS–polyacrylamide gel electrophoresis gels. Before use in cell culture or administration to animals, endotoxin was removed from the protein preparations by Triton X-114 extraction (*52*), and endotoxin levels were verified to be less than 0.1 endotoxin units per milligram of protein.

Melting temperature

The melting temperatures of LST and F12 were measured using DSF as previously described (*53*). Briefly, LST or F12 was prepared with SYPRO Orange (Invitrogen, USA) in 20 μl of PBS to reach a final concentration of 5 μM LST or F12 and 5X SYPRO Orange. Triplicate samples of each protein were run on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), and the results were analyzed by the Bio-Rad CFX Manager 3.0 software.

MIC assays

The MICs of LST and F12 were determined by the microbroth dilution method (*54*). *S. aureus* cultures were grown overnight to saturation in TSB medium at 37°C. Saturated cultures were subcultured 1:100 in fresh TSB, grown to mid-log phase at 37°C, and then serially diluted to a density of 10^6 colony-forming units (CFU)/ml of *S. aureus* in caMHIIB supplemented with 0.1% BSA. Using 96-well polystyrene plates, purified enzymes were serially diluted 1:2 in 50-μl volumes of caMHIIB supplemented with 0.1% BSA. Each well was then inoculated with 50 μl of 10^6 CFU/ml of *S. aureus*, yielding a total volume of 100 μl per well. Microplates were covered and incubated without shaking at 37°C for 20 hours, and MIC was determined as the enzyme concentration yielding no visible outgrowth. MIC in serum medium followed the above method, except that the 10^6 CFU/ml *S. aureus* stock was prepared in human serum, yielding a 1:1 mixture of caMHIIB and human serum in the final MIC assay. Assays were done with one to three technical replicates in two to six independent experiments, and MIC values were averaged.

Resistance induction

Serial induction of resistance was performed following a set of sequential MIC assays as previously described (*54*). Briefly, the MRSA strain MW2 was cultured in 50% human serum with caMHIIB at 37°C with the presence of antibiotic (LST, F12, or nafcillin) following a standard MIC assay methodology. Subsequently, the wells with the highest concentration of antibiotic and visible outgrowth were subcultured into fresh TSB medium and grown overnight to saturation at 37°C. The MIC assay in 50% human serum with caMHIIB was then repeated using the overnight TSB culture as a source for the bacterial stock suspension. Antibiotic concentrations in subsequent MIC assays were increased as required to achieve growth inhibition in the top dilutions. This procedure was repeated for the duration of the experiment (14 days of drug exposure in human serum) or until the MIC of bacteria exceeded 1 mg/ml.

Human PBMCs

Donor HLA genotypes are summarized in table S3. In vitro expansion of LST-specific T cells was carried out as previously described (*22*). Briefly, 2 million live PBMCs were cultured in 1 ml of RPMI 1640 supplemented with 5% human AB serum in 24-well plates and stimulated with either LST (5 μg/ml) or F12 (5 μg/ml). Cells were incubated at 37°C, 5% CO_2, and half the volume of medium was replaced every 3 days with RPMI 1640 supplemented with 5% human AB serum and IL-2 (10 U/ml). On day 14, cells were harvested, counted, washed, and screened for reactivity against LST or F12 peptide pools using IL-2 ELISpot assays (200,000 cells per well) performed according to the manufacturer's protocol. Each donor was expanded separately in the presence of LST or F12, and the expanded cell pool was tested in triplicate with separate pools of LST or F12 peptides (10 peptides per pool at a final test concentration of 1 μM final for each peptide). A negative control containing an equivalent volume of DMSO and a positive control with phytohemagglutinin (PHA) (2 μg/ml) were also tested for each donor expansion condition. After both LST and F12 expansions, donor LP155 (DRB1*0402/DRB1*1301P) yielded poor T cell activation with the PHA control (<200 SFCs) and was excluded from analysis. Donor LP53 (DRB1*0405/DRB1*1202) was excluded from the analysis, because $CD3/CD4^+$ cells could not be quantified due to poor expansion with both LST and F12. SI values for each donor/protein combination were calculated as the ratio of SFCs upon peptide restimulation to SFCs upon DMSO vehicle restimulation. Responders were defined as donor/protein combinations that met all three of the following criteria: (i) a minimum of 60 SFCs per well following DMSO vehicle subtraction (*22*); (ii) statistically significant increase in SFC count relative to DMSO vehicle, determined by two-tailed *t* test with $P \leq 0.05$; and (iii) SI greater than a cutoff threshold, where the cutoff was varied from 2 to 10.

In vivo model studies

All animal protocols were approved by the Institutional Animal Care and Use Committee of Dartmouth College (Hanover, NH) or the Institutional Animal Care and Use Committee of the Lundquist Institute at Harbor-UCLA Medical Center, as appropriate, in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care Guidelines.

Human HLA transgenic mouse immunogenicity

A 100-μl volume of 100 μg of purified LST or F12 in sterile PBS was subcutaneously injected in DR4 or DR2 mice ($N = 4$ to 5 per group) on study days 0, 7, 14, and 21. Serum was collected on days 14 and 28, and antidrug IgG antibody titers (specific to LST or F12 protein) were measured by direct enzyme-linked immunosorbent assay (ELISA) using a standard protocol. Splenocytes were harvested on

day 28, rechallenged ex vivo with 5 μg/ml of LST or F12 protein, and analyzed by IL-2 ELISpot assays (250,000 cells per well) according to the manufacturer's protocol.

Human HLA transgenic mouse in vivo efficacy

All efficacy studies were performed with DR4 mice. For the first cycle of infection and treatment, mice were challenged with an intraperitoneal administration of 2×10^8 CFU of *S. aureus* strain USA400 in a 3% suspension of porcine mucin and, 1 hour later, were treated by subcutaneous administration of 75, 125, 250, 375, 500, or 750 μg of LST or F12 in sterile PBS. For mice in the acute infection dose-response study ($N = 4$ per group, except LST 125 with $N = 3$), surviving animals were euthanized at day 7. For mice in the standard recurrent infection study, infection and treatment cycles occurred at weekly intervals, where follow-up bacterial challenges contained 6×10^8 CFU for weeks 2, 3, and 4 and 8×10^8 CFU for the remaining infections. Animals in the standard recurrent infection study were treated with 500 μg of either LST ($N = 10$) or F12 ($N = 6$). For the recurrent infection study following LST preimmunization, mice were subcutaneously preimmunized with 100 μg of LST on study days −14 and − 7, mice were split into two study arms, and the recurrent infection model was initiated on day 0, using the methods described above. Animals were treated weekly with 750 μg of either LST (matched preimmune treatment, $N = 12$) or F12 (crossover preimmune treatment, $N = 12$). In all efficacy studies, control animals treated with PBS were used to confirm the lethal bacterial challenge for each infection cycle.

Rabbit in vivo efficacy

A well-characterized transcarotid artery–to–left ventricle catheter–induced aortic valve endocarditis model caused by MRSA MW2 strain in rabbits (*28*) was used to evaluate the efficacy of F12 alone and in combination with daptomycin. At 48 hours after catheter placement, animals were intravenously challenged with ~2×10^5 CFU, an inoculum of the MW2 strain that induces infective endocarditis in >95% of catheterized animals (ID_{95}). At 24 hours after infection, animals were randomized into one of four treatment groups: (i) control without treatment and sacrificed at 24 hours after infection (the time of therapy initiation, $N = 8$); (ii) F12 at 40 mg/kg, intravenously, once ($N = 5$); (iii) daptomycin at 4 mg/kg, intravenously, once daily, for 4 days ($N = 7$); or (iv) F12 and daptomycin, each dosed as above ($N = 6$). At 24 hours after the last treatment, animals were humanely euthanized, and cardiac vegetations, kidneys, and spleen were sterilely removed and quantitatively cultured. Data for each organ for the different treatment groups were calculated as mean $\log_{10}$ CFU per gram of tissue (±SD).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/36/eabb9011/DC1

REFERENCES AND NOTES

1. S. Y. C. Tong, J. S. Davis, E. Eichenberger, T. L. Holland, V. G. Fowler Jr., Staphylococcus aureus infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* **28**, 603–661 (2015).
2. *Antibiotic Resistance Threats in the United States, 2013* (2013); http://cdc.gov/drugresistance/threat-report-2013/.
3. H. F. Chambers, F. R. Deleo, Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat. Rev. Microbiol.* **7**, 629–641 (2009).
4. M. Z. David, R. S. Daum, Treatment of *Staphylococcus aureus* Infections. *Curr. Top. Microbiol. Immunol.* **409**, 325–383 (2017).
5. V. A. Fischetti, Development of phage lysins as novel therapeutics: A historical perspective. *Viruses* **10**, 310 (2018).
6. D. Gutierrez, L. Fernandez, A. Rodriguez, P. Garcia, Are phage lytic proteins the secret weapon to kill *Staphylococcus aureus*? *mBio* **9**, e01923-17 (2018).
7. H. P. Browder, W. A. Zygmunt, J. R. Young, P. A. Tavormina, Lysostaphin: Enzymatic mode of action. *Biochem. Biophys. Res. Commun.* **19**, 383–389 (1965).
8. J. F. Kokai-Kun, in *Antimicrobial Drug Discovery*, G. Tegos, Ed. (CABI, 2012), chap. 10, pp. 147–165.
9. F. R. Stark, C. Thornsva, E. P. Flannery, M. S. Artenste, Systemic lysostaphin in man--apparent antimicrobial activity in a neutropenic patient. *N. Engl. J. Med.* **291**, 239–241 (1974).
10. K. E. Quickel Jr., R. Selden, J. R. Caldwell, N. F. Nora, W. Schaffne, Efficacy and safety of topical lysostaphin treatment of persistent nasal carriage of *Stahpylococcus aureus*. *Appl. Microbiol.* **22**, 446–450 (1971).
11. E. S. Trombetta, I. Mellman, Cell biology of antigen processing in vitro and in vivo. *Annu. Rev. Immunol.* **23**, 975–1028 (2005).
12. Z. E. Sauna, D. Lagasse, J. Pedras-Vasconcelos, B. Golding, A. S. Rosenberg, Evaluating and mitigating the immunogenicity of therapeutic proteins. *Trends Biotechnol.* **36**, 1068–1084 (2018).
13. K. E. Griswold, C. Bailey-Kellogg, Design and engineering of deimmunized biotherapeutics. *Curr. Opin. Struct. Biol.* **39**, 79–88 (2016).
14. D. Verma, G. Grigoryan, C. Bailey-Kellogg, Structure-based design of combinatorial mutagenesis libraries. *Protein Sci.* **24**, 895–908 (2015).
15. H. Zhao, D. Verma, W. Li, Y. Choi, C. Ndong, S. N. Fiering, C. Bailey-Kellogg, K. E. Griswold, Depletion of T cell epitopes in lysostaphin mitigates anti-drug antibody response and enhances antibacterial efficacy in vivo. *Chem. Biol.* **22**, 629–639 (2015).
16. J. Greenbaum, J. Sidney, J. Chung, C. Brander, B. Peters, A. Sette, Functional classification of class II human leukocyte antigen (HLA) molecules reveals seven different supertypes and a surprising degree of repertoire sharing across supertypes. *Immunogenetics* **63**, 325–335 (2011).
17. H. Zhao, K. Blazanovic, Y. Choi, C. Bailey-Kellogg, K. E. Griswold, Gene and protein sequence optimization for high level production of fully active and aglycosylated lysostaphin in Pichia pastoris. *Appl. Environ. Microbiol.* **80**, 2746–2753 (2014).
18. N. H. Kim, W. B. Park, J. E. Cho, Y. J. Choi, S. J. Choi, S. Y. Jun, C. K. Kang, K.-H. Song, P. G. Choe, J. H. Bang, E. S. Kim, S. W. Park, N.-J. Kim, M.-D. Oh, H. B. Kim, Effects of phage endolysin SAL200 combined with antibiotics on *Staphylococcus aureus* infection. *Antimicrob. Agents Chemother.* **62**, e00731-18 (2018).
19. R. Schuch, H. M. Lee, B. C. Schneider, K. L. Sauve, C. Law, B. K. Khan, J. A. Rotolo, Y. Horiuchi, D. E. Couto, A. Raz, V. A. Fischetti, D. B. Huang, R. C. Nowinski, M. Wittekind, Combination therapy with lysin CF-301 and antibiotic is superior to antibiotic alone for treating methicillin-resistant *Staphylococcus aureus*–induced murine bacteremia. *J. Infect. Dis.* **209**, 1469–1478 (2014).
20. C. Indiani, K. Sauve, A. Raz, W. Abdelhady, Y. Q. Xiong, C. Cassino, A. S. Bayer, R. Schuch, The antistaphylococcal lysin, CF-301, activates key host factors in human blood to potentiate methicillin-resistant *Staphylococcus aureus* bacteriolysis. *Antimicrob. Agents Chemother.* **63**, (2019).
21. R. Mazor, A. N. Vassall, J. A. Eberle, R. Beers, J. E. Weldon, D. J. Venzon, K. Y. Tsang, I. Benhar, I. Pastan, Identification and elimination of an immunodominant T-cell epitope in recombinant immunotoxins based on *Pseudomonas* exotoxin A. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E3597–E3603 (2012).
22. R. S. Salvat, D. Verma, A. S. Parker, J. R. Kirsch, S. A. Brooks, C. Bailey-Kellogg, K. E. Griswold, Computationally optimized deimmunization libraries yield highly mutated enzymes with low immunogenicity and enhanced activity. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E5085–E5093 (2017).
23. M. K. Joubert, M. Deshpande, J. Yang, H. Reynolds, C. Bryson, M. Fogg, M. P. Baker, J. Herskovitz, T. J. Goletz, L. Zhou, M. Moxness, G. C. Flynn, L. O. Narhi, V. Jawa, Use of in vitro assays to assess immunogenicity risk of antibody-based biotherapeutics. *PLOS ONE* **11**, e0159328 (2016).
24. K. Ito, H. J. Bian, M. Molina, J. Han, J. Magram, E. Saar, C. Belunis, D. R. Bolin, R. Arceo, R. Campbell, F. Falcioni, D. Vidović, J. Hammer, Z. A. Nagy, HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. *J. Exp. Med.* **183**, 2635–2644 (1996).
25. N. Kaushansky, D. M. Altmann, C. S. David, H. Lassmann, A. Ben-Nun, DQB1*0602 rather than DRB1*1501 confers susceptibility to multiple sclerosis-like disease induced by proteolipid protein (PLP). *J. Neuroinflammation* **9**, 29 (2012).
26. ContraFect Corporation: Safety, Efficacy and Pharmacokinetics of CF-301 vs. Placebo in Addition to Antibacterial Therapy for Treatment of S. Aureus Bacteremia. In.; 2019.
27. I. B. Inc. (NIH), A Randomized, Double-blind, Placebo-controlled, Multicenter Phase IIa Clinical Study to Evaluate Safety and to Explore Efficacy of N-Rephasin® SAL200, in Patients With Persistent Staphylococcus aureus Bacteremia, vol. 2016.
28. L. Li, A. Cheung, A. S. Bayer, L. Chen, W. Abdelhady, B. N. Kreiswirth, M. R. Yeaman, Y. Q. Xiong, The global regulon *sarA* regulates β-lactam antibiotic resistance

in methicillin-resistant *Staphylococcus aureus* in vitro and in endovascular infections. *J. Infect. Dis.* **214**, 1421–1429 (2016).
29. *Antibiotic Resistance Threats in the United States, 2019* (2019); http://cdc.gov/drugresistance/Biggest-Threats.html.
30. L. Czaplewski, R. Bax, M. Clokie, M. Dawson, H. Fairhead, V. A. Fischetti, S. Foster, B. F. Gilmore, R. E. Hancock, D. Harper, I. R. Henderson, K. Hilpert, B. V. Jones, A. Kadioglu, D. Knowles, S. Olafsdottir, D. Payne, S. Projan, S. Shaunak, J. Silverman, C. M. Thomas, T. J. Trust, P. Warn, J. H. Rex, Alternatives to antibiotics—A pipeline portfolio review. *Lancet Infect. Dis.* **16**, 239–251 (2016).
31. D. Dams, Y. Briers, Enzybiotics: Enzyme-based antibacterials as therapeutics. *Adv. Exp. Med. Biol.* **1148**, 233–253 (2019).
32. M. Schmelcher, D. M. Donovan, M. J. Loessner, Bacteriophage endolysins as novel antimicrobials. *Future Microbiol.* **7**, 1147–1171 (2012).
33. S. Y. Jun, I. J. Jang, S. Yoon, K. Jang, K. S. Yu, J. Y. Cho, M. W. Seong, G. M. Jung, S. J. Yoon, S. H. Kang, Pharmacokinetics and tolerance of the phage endolysin-based candidate drug SAL200 after a single intravenous administration among healthy volunteers. *Antimicrob. Agents Chemother.* **61**, e02629-16 (2017).
34. C. Cassino, M. Murphy, J. Boyle, J. Rotolo, M. Wittekind, paper presented at the 26th European Congress of Clinical Microbiology & Infectious Diseases, Amsterdam, The Netherlands, 9 April 2016.
35. A. Kolate, D. Baradia, S. Patil, I. Vhora, G. Kore, A. Misra, PEG - a versatile conjugating ligand for drugs and drug delivery systems. *J. Control. Release* **192**, 67–81 (2014).
36. S. Walsh, A. Shah, J. Mond, Improved pharmacokinetics and reduced antibody reactivity of lysostaphin conjugated to polyethylene glycol. *Antimicrob. Agents Chemother.* **47**, 554–558 (2003).
37. G. Resch, P. Moreillon, V. Fischetti, PEGylating a bacteriophage endolysin inhibits its bactericidal activity. *AMB Express* **1**, 29 (2011).
38. V. Fowler, paper presented at the European Congress of Clinical Microbiology & Infectious Diseases, Amsterdam, The Netherlands, 16 April 2019.
39. Contrafect Corporation: Direct Lysis of Staph aureus Resistant Pathogen Trial of Exebacase (DISRUPT). In.; 2019.
40. O. Equils, C. da Costa, M. Wible, B. A. Lipsky, The effect of diabetes mellitus on outcomes of patients with nosocomial pneumonia caused by methicillin-resistant *Staphylococcus aureus*: Data from a prospective double-blind clinical trial comparing treatment with linezolid versus vancomycin. *BMC Infect. Dis.* **16**, 476 (2016).
41. S. Esposito, G. Pennoni, V. Mencarini, N. Palladino, L. Peccini, N. Principi, Antimicrobial treatment of *Staphylococcus aureus* in patients with cystic fibrosis. *Front. Pharmacol.* **10**, 849 (2019).
42. E. R. Narewski, V. Kim, N. Marchetti, M. R. Jacobs, G. J. Criner, Is methicillin-resistant *Staphylococcus aureus* colonization associated with worse outcomes in COPD hospitalizations? *Chronic Obstr. Pulm. Dis.* **2**, 252–258 (2015).
43. N. Saltoglu, O. Ergonul, N. Tulek, M. Yemisen, A. Kadanali, G. Karagoz, A. Batirel, O. Ak, C. Sonmezer, H. Eraksoy, A. Cagatay, S. Surme, S. A. Nemli, T. Demirdal, O. Coskun, D. Ozturk, N. Ceran, F. Pehlivanoglu, G. Sengoz, T. Aslan, Y. Akkoyunlu, O. Oncul, H. Ay, L. Mulazimoglu, B. Erturk, F. Yilmaz, G. Yoruk, N. Uzun, F. Simsek, T. Yildirmak, K. K. Yasar, M. Sonmezoglu, Y. Kucukardali, N. Tuna, O. Karabay, N. Ozgunes, F. Sargin; Turkish Society of Clinical Microbiology and Infectious Diseases; Diabetic Foot Infections Study Group, Influence of multidrug resistant organisms on the outcome of diabetic foot infection. *Int. J. Infect. Dis.* **70**, 10–14 (2018).
44. W. E. Kloos, K. H. Schleifer, Isolation and characterization of Staphylococci from Human Skin II. Descriptions of four new species: *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus hominis*, and *Staphylococcus simulans*. *Int. J. Syst. Evol. Microbiol.* **25**, 62–79 (1975).
45. Securities and Exchange Commission, United States Securities and Exchange Commission Form S-1 Registration Statement (S-1 1 d609128ds1.htm FORM S-1, 2014); https://ir.contrafect.com/sec-filings/content/0001193125-14-149004/d609128ds1.htm.
46. C. Gneiss, P.-M. Koudouovoh-Tripp, S. Ropele, T. Gotwald, R. Ehling, A. Lutterotti, F. Aichner, G. Ladurner, C. Eggers, F. Schautzer, B. Kunz, A. Millonig, E. Aspeck, M. Reindl, T. Berger, F. Fazekas, F. Deisenhammer, Influence of interferon-beta therapy switching on neutralizing antibody titres: Results from the Austrian switch study. *Mult. Scler.* **15**, 1481–1488 (2009).
47. F. A. Bonilla, Pharmacokinetics of immunoglobulin administered via intravenous or subcutaneous routes. *Immunol. Allergy Clin. North Am.* **28**, 803–819 (2008).
48. A. E. Hauser, U. E. Höpken, in *Molecular Biology of B Cells (Second Edition)*, F. W. Alt, T. Honjo, A. Radbruch, M. Reth, Eds. (Academic Press, 2015), pp. 187–214.
49. A. Cassotta, V. Mikol, T. Bertrand, S. Pouzieux, J. Le Parc, P. Ferrari, J. Dumas, M. Auer, F. Deisenhammer, M. Gastaldi, D. Franciotta, C. Silacci-Fregni, B. Fernandez Rodriguez, I. Giacchetto-Sasselli, M. Foglierini, D. Jarrossay, R. Geiger, F. Sallusto, A. Lanzavecchia, L. Piccoli, A single T cell epitope drives the neutralizing anti-drug antibody response to natalizumab in multiple sclerosis patients. *Nat. Med.* **25**, 1402–1407 (2019).
50. K. Lamberth, S. L. Reedtz-Runge, J. Simon, K. Klementyeva, G. S. Pandey, S. B. Padkjaer, V. Pascal, I. R. Leon, C. N. Gudme, S. Buus, Z. E. Sauna, Post hoc assessment of the immunogenicity of bioengineered factor VIIa demonstrates the use of preclinical tools. *Sci. Transl. Med.* **9**, eaag1286 (2017).
51. J. R. McGill, O. N. Yogurtcu, D. Verthelyi, H. Yang, Z. E. Sauna, SampPick: Selection of a cohort of subjects matching a population HLA distribution. *Front. Immunol.* **10**, 2894 (2019).
52. S. Liu, R. Tobias, S. McClure, G. Styba, Q. Shi, G. Jackowski, Removal of endotoxin from recombinant protein preparations. *Clin. Biochem.* **30**, 455–463 (1997).
53. Y. Choi, J. M. Furlon, R. B. Amos, K. E. Griswold, C. Bailey-Kellogg, DisruPPI: Structure-based computational redesign algorithm for protein binding disruption. *Bioinformatics* **34**, i245–i253 (2018).
54. C. M. Kusuma, J. F. Kokai-Kun, Comparison of four methods for determining lysostaphin susceptibility of various strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **49**, 3256–3263 (2005).

Acknowledgments: We thank M. Ackerman for helpful comments on the work and S. Fiering and J. Fields for maintenance of mouse colonies with support from 1R01AI123372. **Funding:** This work was supported by NIH grants 1R41AI118133, 2R42AI118133, 5R42AI118133, 5R21AI119741, and 2R01GM098977 and an award from the New Hampshire Innovation Research Center. Protein purification was supported, in part, by NIH grant P20-GM113132.
Author contributions: K.E.G., H.Z., S.A.B., S.E., Y.Q.X., Y.F., S.H., and J.R.K. designed the experiments, and C.B.-K. and D.V. designed the deimmunized enzyme. H.Z., S.A.B., S.E., L.L., Y.F., S.H., and J.R.K. conducted the experiments. K.E.G., H.Z., S.A.B., S.E., Y.Q.X., L.L., Y.F., S.H., and J.R.K. analyzed the data. K.E.G. and H.Z. wrote the manuscript, and all authors provided input and comments for the manuscript. **Competing interests:** K.E.G., C.B.-K., H.Z., and D.V. are co-inventors on multiple patents relating to engineered variants of lysostaphin and therapeutic use thereof, and these patents have been licensed to Lyticon LLC. K.E.G. and C.B.-K. are managers of Lyticon LLC and Stealth Biologics LLC, and Y.F. has an equity interest in these companies. All other authors declare that they have no competing interests. Potential conflicts of interest for K.E.G. and C.B.-K. are under management at Dartmouth. The authors declare that the work presented here is free of any bias. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. Patented and patent-pending materials from this article can be provided by Dartmouth pending scientific review and a completed material transfer agreement. Requests for any materials should be submitted to K.E.G. at karl.e.griswold@dartmouth.edu.

Submitted 25 March 2020
Accepted 21 July 2020
Published 2 September 2020
10.1126/sciadv.abb9011

Citation: H. Zhao, S. A. Brooks, S. Eszterhas, S. Heim, L. Li, Y. Q. Xiong, Y. Fang, J. R. Kirsch, D. Verma, C. Bailey-Kellogg, K. E. Griswold, Globally deimmunized lysostaphin evades human immune surveillance and enables highly efficacious repeat dosing. *Sci. Adv.* **6**, eabb9011 (2020).