HEALTH AND MEDICINE

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

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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...
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 ($T_m = 55^\circ$C versus $T_m = 59^\circ$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 non-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...
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). Seven moderate 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 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 (FDR) = 0.05%).

**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
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 (EC50) = 1:50 to 1:600; Fig. 3A] and even higher titers in DR2 mice by day 14 (EC50 = 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
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 mice that had been immunized with LST. While splenocytes from LST-immunized mice exhibited significant activation when restimulated with LST, restimulation of the same splenocytes with F12 resulted in no apparent diminution of efficacy over at least seven cycles of serial infection and treatment.
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 h 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. ***\( P < 0.001 \).

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). Transectarotic-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.
immunity can be circumvented but at the potential cost of suboptimal 
tration. By dosing only once, the risks associated with adaptive 
immunogenic anti-MRSA lysins CF-301 and SAL200, each of which 
effectively dosed multiple times. This distinguishes F12 from the 
evidence that the globally deimmunized enzyme can be safely and 
mice from seven serial MRSA challenges over a 6-week period is 
that this reduced immunogenicity translates into improved efficacy. 
strains of human HLA transgenic 
mitigate lysostaphin’s immunogenicity risk while maintaining high 
activity. Analyses with human PBMCs and two different 
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 admin-
istration. 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 indi-
cations that are likely to necessitate repeated long-term dosing in-
clude 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 antibiotic in-
immunity via evasion of T cell surveillance, F12 has the potential for 
safe and efficacious repeat dosing to treat both refractory and recur-
rent S. aureus infections.

Beyond refractory and recurrent infections, there exist other sce-
narios in which adaptive immunity could limit lysins’ clinical utility. 
For example, previous environmental exposure to a wild-type pro-
tein may result in patients with established antidrug immunity be-
fore 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
corcerns over preexisting immunity, which were brought to light
by repeat-dose preclinical safety studies (45). The phase 1 trial ulti-
mately 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 naive 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 de-
pletion. 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, glycosyla-
tion, formulation, route of administration, or other factors. Inter-
feron 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 progen-
tor. 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 bind-
ing 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 rele-
vant 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 pre-
dicting clinical ADA responses. PBMC assays have been shown to
 correlate with overall clinical immunogenicity for monoclonal anti-
 bodies (23), and they have separately enabled identification of indi-
 vidual 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 responses 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 experi-
mentally driven T cell epitope mapping and deletion strategies,
computational design can accelerate development of highly active
deimmunized candidates for testing (13). In particular, F12 rep-
resents 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 pur-
chased 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 pur-
chased from Cellular Technology Limited and were chosen to repre-
 sent 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 sam-
ple 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 ob-
tained from the American Type Culture Collection (ATCC). Strains
AIS 080003, AIS 1000505, SÅLinR #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 can-
didate, 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 Biotechnologie) 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 (NH₄)₂SO₄ (pH 7.0)], loaded onto a Phenyl High Performance column (20 ml; GE Healthcare), and eluted with a 1- to 0 M (NH₄)₂SO₄ gradient. Purified wild type or F12 was dialed 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 density of 10⁶ colony-forming units (CFU)/ml of *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 10⁶ 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 10⁶ 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 l per well. Microplates were covered and incubated without incubation without generation in TSB medium at 37°C.

The MICs of LST and F12 were determined by the microbroth dilution assays. 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 10⁶ 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 10⁶ 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 10⁶ 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% CO₂, 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 subtraction (22); 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 × 10⁷ 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 × 10⁶ CFU for weeks 2, 3, and 4 and 8 × 10⁸ 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 pretreated 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 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 × 10⁸ CFU, an inoculum of the MW2 strain that induces infective endocarditis in >95% of catheterized animals (ID₉₅). 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 sterilily removed and quantitatively cultured. Data for each organ for the different treatment groups were calculated as mean log₁₀ 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

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

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