Novel Synthetic (Poly)Glycerolphosphate-Based Antistaphylococcal Conjugate Vaccine

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Staphylococcal infections are a major source of global morbidity and mortality. Currently there exists no antistaphylococcal vaccine in clinical use. Previous animal studies suggested a possible role for purified lipoteichoic acid as a vaccine target for eliciting protective IgG to several Gram-positive pathogens. Since the highly conserved (poly)glycerolphosphate backbone of lipoteichoic acid is a major antigenic target of the humoral immune system during staphylococcal infections, we developed a synthetic method for producing glycerol phosphoramidites to create a covalent 10-mer of (poly)glycerolphosphate for potential use in a conjugate vaccine. We initially demonstrated that intact *Staphylococcus aureus* elicits murine CD4⁺ T cell-dependent (poly)glycerolphosphate-specific IgM and IgG responses *in vivo*. Naive mice immunized with a covalent conjugate of (poly)glycerolphosphate and tetanus toxoid in alum plus CpG-oligodeoxynucleotides produced high secondary titers of serum (poly)glycerolphosphate-specific IgG. Sera from immunized mice enhanced opsonophagocytic killing of live *Staphylococcus aureus* *in vitro*. Mice actively immunized with the (poly)glycerolphosphate conjugate vaccine showed rapid clearance of staphylococcal bacteremia *in vivo* relative to mice similarly immunized with an irrelevant conjugate vaccine. In contrast to purified, natural lipoteichoic acid, the (poly)glycerolphosphate conjugate vaccine itself exhibited no detectable inflammatory activity. These data suggest that a synthetic (poly)glycerolphosphate-based conjugate vaccine will contribute to active protection against extracellular Gram-positive pathogens expressing this highly conserved backbone structure in their membrane-associated lipoteichoic acid.

Staphylococcus aureus is the most common cause of surgical site infections in community hospitals in the United States (1). Community-acquired, endemic, and epidemic *S. aureus* infections, which most often manifest as skin infections, are also major clinical problems (2). *S. aureus* is a common cause of osteomyelitis in children and adults (3) and is the most common pathogen isolated from children with pneumonia associated with empyema (4). In the United States, *S. aureus* is a leading cause of infective endocarditis, especially in patients with diabetes, on hemodialysis, or with other chronic illnesses (5). *Staphylococcus epidermidis* is also a major cause of clinically significant infections, largely due to its ability to grow on virtually all biomaterials composing indwelling medical devices (6, 7). Once established, these infections tend to be unresponsive to antimicrobials, largely due to production of a thick biofilm, and often necessitate the removal of the infected device (8). In this regard, *S. epidermidis* is the most common cause of nosocomial bacteremia (9).

Lipoteichoic acid (LTA) is an obligatory component of the membrane of Gram-positive bacteria, including staphylococci (10, 11), and it is capable of eliciting specific antibodies (Ab) (10, 12–14). In this regard, immunization of mice with purified native LTA in adjuvant elicited an anti-LTA antibody response that inhibited adherence of *Streptococcus pyogenes* to pharyngeal epithelial cells (15). LTA structures differ among bacteria but typically contain a core chain of (poly)glycerolphosphate (pgp) or (poly)ribitolphosphate (prp) with a glycolipid tail (16). pgp is a major immunodeterminant of LTA-specific antibody (10). Anti-pgp antibody is generally present in low titers in sera from noninfected humans, and antibody titers often increase during staphylococcal infections (17). A recent study demonstrated that polyclonal rab-

bit anti-LTA antibodies with specificity for synthetic pgp mediate opsonophagocytic killing (OPK) of *S. epidermidis* and *S. aureus* *in vitro* and upon passive transfer reduced mortality in a murine *S. aureus* peritonitis model (18). Major bacterial pathogens expressing pgp-containing LTA include *S. aureus*, *S. epidermidis*, group A *Streptococcus*, and *Enterococcus* (19). Unlike preparations of LTA which activate the innate immune system, pgp itself is noninflammatory (20). Although LTA has been reported to be a Toll-like receptor 2 (TLR2) ligand (21, 22), more recent work suggests that this TLR2 activity might represent contaminating lipoproteins/lipopeptides (20).

In light of the increasing multidrug resistance of staphylococci isolated from human infections (23), there is an urgent need to develop a prophylactic vaccine. A number of antigenic targets are currently being evaluated for active protection against *S. aureus* in clinical trials, but currently there exists no antistaphylococcal vaccine for clinical use (24). LTAs, in contrast to cell wall-associated teichoic acids, are characterized by their relative uniformity (10), a

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FIG 1 (A) (Poly)Glycerolphosphate (pgp) is produced using synthetic glycerol phosphoramidites. (B) A pgp-tetanus toxoid (TT) conjugate is prepared using HyNic/4FB conjugation chemistry.

property that might be advantageous in developing a vaccine that would elicit antibody highly cross-reactive to a number Gram-positive pathogens. However, LTA is a T cell-independent antigen and, as such, exhibits relatively poor immunogenicity (25). In this regard, covalent linkage of T cell-independent polysaccharide (PS) antigens to immunogenic proteins capable of recruiting CD4⁺ T cell help ("conjugate" vaccine) (26) results in the elicitation of high-titer, protective IgG anti-PS responses and the generation of immunological memory, including immunogenicity, in the infant host (27–29). In this report, we describe the development of a synthetic pgp-based conjugate vaccine that is immunogenic, elicits serum antibodies that promote opsonophagocytic killing *in vitro*, and confers protection *in vivo* against staphylococcal bacteremia in a mouse model. In light of a growing consensus that multiple antigenic targets may be required for formulating an effective antistaphylococcal vaccine (24), our data suggest pgp as a potentially promising component.

MATERIALS AND METHODS

Syntheses of 4FB- and biotin-pgp. The synthesis of a 10-mer (poly)glycerolphosphate (pgp) has been described in detail elsewhere (international application no. PCT/US2010/056742; international publication no. WO 2011/060379 [published 19 May 2011; Clifford M. Snapper, Andrew Lees, James J. Mond, David Schwartz, inventors]) (summarized in Fig. 1A). As pgp possesses a phosphate backbone, the design of its synthesis was based on synthesizing a suitably protected chiral glycerol phosphoramidite for its stepwise chain extension to produce a 10-mer pgp polymer using standard solid-phase oligonucleotide synthesis chemistry. The 4-formylbenzamide (4FB) linking group was incorporated on the polymer by addition of the commercially available 4FB phosphoramidite (Solulink Biosciences, San Diego, CA). Biotin was incorporated by the addition of the 5'-biotin phosphoramidite (Glen Research, Sterling, VA) to the terminus of the pgp polymer during its solid-phase synthesis.

Preparation of conjugate vaccines. pgp-tetanus toxoid (pgp-TT) conjugate was prepared using the HyNic/4FB conjugation couple (Fig. 1B) (30, 31). Specifically, TT was modified with amino-reactive S-HyNic to incorporate aromatic hydrazine groups on the side chain amines of the lysine moieties. HyNic-modified TT was conjugated to 4FB-(pgp)₁₀ by

incubation in 100 mM phosphate–150 mM NaCl (pH 6.0) in the presence of 10 mM aniline. The number of pgp polymer units was quantified by measuring the absorbance at 354 nm of the hydrazone bond (molar extinction coefficient = 25,000), and the protein concentration was determined by the bicinchoninic acid (BCA) assay. Covalent conjugates of TT and either meningococcal capsular polysaccharide type C (MCPS-TT) or *S. pneumoniae* capsular polysaccharide type 14 (PPS14-TT) were synthesized as previously described (32).

Mice. BALB/c and C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME), respectively. Female mice between 7 and 10 weeks of age were used. These studies were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, revised 1996, and were approved by the Uniformed Services University of the Health Sciences and University of Maryland School of Medicine institutional animal use and care committee.

Staphylococcus aureus strains. Strain Lowenstein (capsular serotype 5) was purchased from ATCC (catalog no. 49521; Manassas, VA). MRSA-M2 was a methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolate obtained from an osteomyelitis patient undergoing treatment at the University of Texas Medical Branch, Galveston, TX (33). Strains 10833 and MN8M (34) were gifts from Gerald Pier (Harvard Medical School, Boston, MA). MRSA USA300 strain LAC (35) was obtained from Michael Otto (National Institutes of Health, Bethesda, MD). Strains 10833 and MN8M were grown in Todd-Hewitt broth to mid-log phase, heat killed at 60°C for 2 h, and resuspended in phosphate-buffered saline (PBS). Strains Lowenstein and USA300 were grown on Columbia salt agar plates for 24 h, and colonies were resuspended in PBS. MRSA-M2 was grown as described below.

S. aureus osteomyelitis model. The model was established based on a previous report (36). Briefly, autoclaved 0.25-mm-diameter insect pins were incubated overnight with *S. aureus* (strain MRSA-M2) under conditions that favor biofilm formation (33, 37). The inoculating dose of *S. aureus* was determined to be 3.0×10^5 CFU/pin (standard deviation [SD] = 5×10^4). C57BL/6 mice ($n = 6$) received tibial implants of infected pins, and sera were collected at various times postinfection.

In vitro induction of IL-6 secretion from peritoneal cells. PBS (10 ml) was injected into the peritoneal cavity, through the fat pad, using a

23-gauge needle. After 3 min, lavage fluid containing the peritoneal cells was withdrawn using an 18-gauge needle. Cells were pelleted by centrifugation at 1,600 rpm for 10 min at room temperature. The cell pellet was suspended in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 50 µg/ml penicillin, and 50 µg/ml streptomycin) and adjusted to 1×10^6 total cells/ml. The cell suspension was added at 0.2 ml per well to a 96-well plate and incubated for 3 h at 37°C in a 5% CO₂-containing incubator. Plates were then washed to remove unbound cells. Fresh medium (0.2 ml) containing various concentrations of PGP-TT (amount reflecting pgp content) or purified, natural LTA (Sigma, St. Louis, MO) was added to the cultures of bound cells and incubated for 48 h at 37°C, followed by plate centrifugation and collection of culture supernatant for determination of interleukin-6 (IL-6) concentrations by enzyme-linked immunosorbent assay (ELISA) (R & D Systems, Minneapolis, MN).

Immunizations and measurement of serum pgp-specific IgM and IgG titers by ELISA. Heat-inactivated *S. aureus* (strains MN8M and 10833) cells were intraperitoneally (i.p.) injected into BALB/c mice at 1×10^9 CFU/mouse in saline solution with boosting on day 21. Selected mice were i.p. injected, 1 day prior to bacterial immunization, with rat IgG anti-mouse CD4 monoclonal antibody (MAb) (clone GK1.5) (0.5 mg/mouse) that was purified from ascites. Polyclonal rat IgG (Sigma) was used as a control for GK1.5. pgp-TT, PPS14-TT, and MCPS-TT conjugates were i.p. injected (5 µg/mouse) in 13 µg of alum plus 25 µg of 30-mer phosphorothioated stimulatory CpG-oligodeoxynucleotide (CpG-ODN) (5' AAA AAA AAA AACGTT AAA AAA AAA AAA 3') (38) with boosting in a similar fashion. Sera were collected from the tail vein. Serum pgp-specific IgM and IgG titers were measured by ELISA, essentially as previously described (39). Specifically, Immulon 4 ELISA plates (Dynex Technologies, Inc., Chantilly, VA) were precoated with avidin (10 µg/ml) followed by biotin-pgp (5 µg/ml). Three-fold dilutions of serum samples starting at 1/50 were then added, followed by alkaline phosphatase-conjugated goat anti-mouse IgM and IgG, and developed using *p*-nitrophenyl phosphate (disodium).

OPK assay. The microtiter-based opsonophagocytic killing (OPK) assay was based on that described by Burton and Nahm (40). The HL60 cells were propagated in a 5% CO₂ atmosphere at 37°C and differentiated to granulocytes by culturing in RPMI 1640 with 10% fetal bovine serum, 1% L-glutamine, and 0.8% *N,N*-dimethylformamide for 5 to 6 days. *S. aureus* strains were cultivated for 24 h on Columbia salt agar plates. The OPK assay was performed in round-bottom polystyrene 96-well plates. Each well (80 µl) contained 4×10^5 HL-60 cells, 1×10^3 CFU *S. aureus*, pooled sera from naive or immunized mice ($n = 7$) or monoclonal antibodies (MAb), and 1% guinea pig serum (Cedarlane) as a complement source. Control samples included *S. aureus* incubated with complement and HL60 cells (no antibody), *S. aureus* incubated with mouse antiserum or IgG and complement (no HL60 cells), and *S. aureus* and HL-60 cells only. The 96-well plates were incubated at 37°C with shaking at 500 rpm. After 2 h, 20 µl 1% Triton X-100 was added to each well to lyse the HL60 cells. After a 3-min incubation at 37°C with shaking, 5-µl aliquots of the final reaction mixtures were plated in duplicate on tryptic soy agar plates. Percent killing was defined as the reduction in CFU/ml after 2 h compared with the CFU/ml at time zero. The serum used as our complement source did not exhibit any functional effects of potentially contaminating anti-teichoic acid antibody. Thus, comparison of guinea pig serum and baby bunny serum or adsorption of guinea pig serum showed no difference in experimental results. In further support of this, our control mixture consisting of HL60 cells, *S. aureus*, and guinea pig serum but no antibody source exhibited no OPK activity.

Bacteremia model. The *S. aureus* bacteremia mouse model has been described previously (41). Live *S. aureus* (strain Lowenstein; serotype 5) was injected i.p. into BALB/c mice ($n = 5$) at 4×10^7 CFU/mouse. Blood was collected from the tail vein 1, 2, and 3 days following injection. Blood (100 µl) was mixed with PBS-heparin for a final dilution of 1:100 and

1:1,000 and distributed evenly on blood agar plates. Bacterial colonies were enumerated after overnight incubation at 37°C.

Statistics. Serum Ig isotype titers or blood bacterial CFU/ml were expressed as geometric means $\pm$ standard errors of the means (SEM) of the results determined with individual mice. Significance was determined by the two-tailed Student *t* test. *P* values of ≤ 0.05 were considered statistically significant. All experiments were performed at least twice with similar results.

RESULTS

pgp was produced using synthetic glycerol phosphoramidites, and a pgp-TT conjugate was prepared using HyNic/4FB conjugation chemistry. Antibody specific for the (poly)glycerolphosphate (pgp) backbone of LTA, expressed by a number of Gram-positive bacteria, including *S. aureus*, has been shown to confer passive host protection in a mouse infection model (18). To explore a potential role for pgp in eliciting active antibody-mediated antistaphylococcal host protection when used as a conjugate vaccine, we first developed a method for efficiently producing large amounts of pure, synthetic pgp. As described in Materials and Methods and summarized in Fig. 1A, a suitably protected chiral glycerol phosphoramidite was synthesized for its stepwise chain extension to produce a 10-mer pgp polymer using standard solid-phase oligonucleotide synthesis chemistry. As illustrated in Fig. 1B, a 10-mer pgp-tetanus toxoid (pgp-TT) conjugate was made using HyNic/4FB conjugation chemistry (30, 31). Specifically, a 4FB linking group was incorporated into pgp by addition of 4FB phosphoramidite, and TT was modified with amino-reactive S-HyNic to incorporate aromatic hydrazine groups on the side chain amines of the lysine moieties.

Intact *S. aureus* induces pgp-specific IgM and IgG responses in vivo. Pgp-specific antibody is generally present in low titers in sera from noninfected humans and increases during staphylococcal infections (17). In this regard, we wished to determine whether we could detect serum pgp-specific antibody following immunization of naive mice with heat-killed *S. aureus* or infection with live *S. aureus* using biotinylated synthetic 10-mer pgp on avidin-coated ELISA plates for detection. BALB/c mice were injected i.p. with 1×10^9 CFU/mouse of heat-killed *S. aureus* (strain MN8M or 10833) in saline solution and boosted on day 21. Antibody titers were measured using a biotinylated synthetic pgp 10-mer on avidin-coated ELISA plates. Both *S. aureus* strains induced a primary pgp-specific IgM response (MN8M, 3.4-fold; 10833, 5.1-fold) that peaked by day 7 (Fig. 2A). Strain MN8M, but not strain 10833, significantly boosted pgp-specific IgM (3.9-fold relative to the primary response) upon secondary immunization on day 21. Although primary induction of pgp-specific IgG was minimal, a significant boost in serum pgp-specific IgG titers was observed following secondary immunization relative to the primary serum titers (MN8M, 15-fold; 10833, 3.6-fold) (Fig. 2A).

Although pgp itself is a T cell-independent antigen, its expression by intact *S. aureus* could confer T cell dependence, as we have previously demonstrated for the IgG responses specific for the capsular polysaccharides expressed by intact *Streptococcus pneumoniae*, *Streptococcus agalactiae*, and *Neisseria meningitidis*, which are dependent on CD4⁺ T cell help (42). To test this hypothesis, we injected naive BALB/c mice i.p. with a rat anti-mouse CD4 MAb (clone GK1.5), known to rapidly deplete CD4⁺ T cells *in vivo*, 1 day prior to immunization with heat-killed intact MN8M. Control mice were injected with rat IgG prior to immunization with killed MN8M. As illustrated in Fig. 2B, consistent with our

FIG 2 Intact *S. aureus* induces pgp-specific IgM and IgG responses *in vivo*. (A) Heat-inactivated *S. aureus* (strain MN8M or 10833) cells were injected intraperitoneally (i.p.) into BALB/c mice ($n = 7$) at 1×10^9 CFU/mouse in saline solution with similar boosting on day 21. *, statistical significance ($P \leq 0.05$) of the results of comparisons between primary and secondary titers. (B) BALB/c mice ($n = 5$) were injected i.p. with 0.5 mg anti-CD4 MAb or control antibody 1 day prior to immunization i.p. with heat-inactivated *S. aureus* (strain MN8M; 1×10^9 CFU/mouse). Mice were similarly boosted on day 21 in the absence of anti-CD4 or control antibody. *, statistical significance ($P \leq 0.05$) of the results of comparisons between the anti-CD4 and control antibody groups. (C) Pins were incubated with *S. aureus* (strain MRSA-M2) under conditions that favor biofilm formation. The inoculating dose of *S. aureus* was 3.0×10^5 CFU/pin. C57BL/6 ($n = 6$) mice received tibial implants of infected pins, and sera were collected at various times postinfection. *, statistical significance ($P \leq 0.05$) of the results of comparisons between infected and noninfected mice. Serum titers (geometric means $\pm$ SEM) of pgp-specific IgM and IgG were measured by ELISA.

earlier studies (42), the secondary, boosted pgp-specific IgM and IgG responses to MN8M were entirely dependent on the presence of endogenous CD4⁺ T cells.

To determine whether pgp-specific antibody was induced during infection with live *S. aureus*, we utilized an osteomyelitis model (36) in which *S. aureus*-infected pins are inserted into the tibias of naive C57BL/6 mice. Sera from infected and noninfected mice were collected on days 7, 14, and 21 postinoculation. As illustrated in Fig. 2C, a transient, significant increase in pgp-spe-

FIG 3 pgp-TT conjugate, in contrast to purified, natural LTA, exhibits no detectable inflammatory activity. Unelicited, adherent peritoneal cells were cultured for 48 h in the presence of various concentrations of LTA or pgp-TT, and IL-6 concentrations in culture supernatants were measured by ELISA. Data are expressed as arithmetic means $\pm$ standard deviations (SD) of the results determined for duplicate wells.

cific IgM was observed on day 14 in infected mice, returning to noninfected levels by day 21. A significant induction of pgp-specific IgG was also observed in infected mice by day 14, with maintenance of similar serum titers on day 21. Collectively, these data confirm observations in humans (17) that humoral immune responses specific for the pgp backbone of LTA occur upon host encounter with *S. aureus*.

pgp-TT conjugate is noninflammatory and elicits a highly boosted secondary pgp-specific IgG response in the presence of adjuvant. Immunization of naive BALB/c mice with unconjugated pgp failed, as expected, to elicit a detectable pgp-specific IgG response (data not shown), consistent with its properties as a T cell-independent antigen. Covalent linkage of T cell-independent antigens to an immunogenic protein carrier to create a conjugate vaccine is a well-established method for converting such antigens into a T cell-dependent form. Conjugate vaccines are capable of eliciting high-titer and boosted IgG responses even in infants, who are largely defective at eliciting T cell-independent humoral immunity. We thus conjugated pgp to tetanus toxoid (TT) (Fig. 1B), a highly immunogenic carrier protein used in several commercial conjugate vaccines. In contrast to preparations of LTA, the (poly)glycerolphosphate backbone is considered noninflammatory (20). To confirm this for the pgp-TT conjugate, we stimulated unelicited peritoneal cells *in vitro* for 48 h with various concentrations of natural purified LTA and equal amounts of pgp that was conjugated to TT. As illustrated in Fig. 3, LTA induced significant IL-6 secretion in a dose-dependent manner, whereas no detectable IL-6 was observed in response to pgp-TT.

Naive BALB/c mice were immunized i.p. with 5 μ g of pgp-TT adsorbed onto alum plus CpG-ODN as an adjuvant and boosted in a similar fashion on day 14. As illustrated in Fig. 4, pgp-TT elicited a primary pgp-specific IgG response that peaked by day 7 with maximal serum titers $\sim$ 5-fold over that observed in naive mice. Of note, secondary immunization resulted in an $\sim$ 20-fold boost in serum titers of pgp-specific IgG relative to primary titers by day 28. In contrast, pgp-TT elicited only a modest primary pgp-specific IgM response with a further modest, though significant, boost upon secondary immunization (Fig. 4). Control mice injected i.p. with 5 μ g/mouse of MCPS-TT in alum plus CpG-ODN showed no induction of serum titers of pgp-specific IgM or IgG (data not shown). Thus, pgp-TT exhibits immunologic characteristics similar to those observed for other conjugate vaccines (43).

Sera from pgp-TT-immunized mice mediate enhanced opsonophagocytic killing of *S. aureus in vitro*. We next wished to

FIG 4 pgp-TT conjugate vaccine elicited a highly boosted secondary pgp-specific IgG response. pgp-TT conjugate was injected i.p. into BALB/c mice (5 μ g/mouse, $n = 5$) in 13 μ g alum plus 25 μ g stimulatory CpG-ODN (30mer) on day 0 and boosted on day 14. Serum pgp-specific IgM and IgG titers (geometric mean + SEM) were measured by ELISA. *, statistical significance ($P \leq 0.05$) of the results of comparisons between primary and secondary titers.

determine whether sera from pgp-TT-immunized mice could mediate opsonophagocytic killing of *S. aureus*. We utilized a micro-titer-based OPK assay in which a human promyelocytic leukemia cell line (HL-60) is cocultured with *S. aureus*, pooled sera (1:8 final dilution) from naive or immunized mice ($n = 7$), and a source of complement for 2 h at 37°C. The percentage of viable *S. aureus* remaining relative to the initial input is determined by lysis of HL-60 cells and enumeration of bacterial colonies on agar. *S. aureus* strain Lowenstein, expressing the type 5 capsular polysaccharide (CP5), and an unencapsulated methicillin-resistant *S. aureus* strain (USA300) were individually tested. As illustrated in Fig. 5, pooled sera from pgp-TT-immunized mice mediated opsonic killing of both the Lowenstein and USA300 strains of *S. aureus* relative to negative-control samples containing preimmune sera or sera from MCPS-TT-immunized mice. Sera from pgp-TT-immunized mice or the BYSX-M110 MABs exhibited a greater degree of killing of the unencapsulated USA300 strain relative to strain Lowenstein, perhaps reflecting some masking of cell wall LTA by *S. aureus* capsular polysaccharide. As expected, sera from CP5-CRM₁₉₇-immunized mice mediated killing of CP5-expressing strain Lowenstein but not the unencapsulated USA300 strain. OPK activity in sera from pgp-TT-immunized mice was demonstrated in 3 independent experiments. These data indicate that the pgp-TT vaccine elicited functional antibodies.

Immunization with pgp-TT protected mice against *S. aureus* bacteremia. To determine whether pgp-TT immunization protected the host against live *S. aureus*, we utilized a model of sublethal *S. aureus* bacteremia in which strain Lowenstein was injected i.p. at a dose of 4×10^7 CFU/mouse. Mice were primed and boosted (day 14 and day 28) i.p. with 5 μ g/mouse of either pgp-TT or a control conjugate of type 14 pneumococcal polysaccharide (PPS14) and TT (PPS14-TT). Both vaccines were mixed with alum plus CpG-ODN as an adjuvant. Mice were challenged 2 weeks following the last immunization. Blood was drawn from the tail vein 1, 2, and 3 days following infection, and bacterial colonies were enumerated on blood agar plates. As illustrated in Fig. 6, by day 1, pgp-TT-immunized mice exhibited >10-fold-lower bacterial numbers in the blood relative to PPS14-TT-immunized mice. On day 2, bacteremia persisted in both groups at levels roughly comparable to that observed on day 1. However, by day 3, bacteremia was essentially cleared in the pgp-TT-immunized group,

FIG 5 Sera from pgp-TT-immunized mice mediate enhanced opsonophagocytic killing of *S. aureus* in vitro. The OPK assay was performed by mixing 4×10^5 HL-60 cells, 1×10^3 CFU *S. aureus* (strain Lowenstein or USA300), 1% guinea pig serum as a complement source, and antibodies in wells of a 96-well plate. Antibody sources included pooled sera (1:8 final dilution) from BALB/c naive (preimmune) mice or mice primed and boosted (day 14 and day 28; sera taken on day 42) with pgp-TT or MCPS-TT (5 μ g/mouse, $n = 7$) in alum plus CpG-ODN. Pooled sera from mice given three doses of CP5-CRM₁₉₇ were diluted 1:40 (final concentration), and purified anti-pgp MAb (BYSX-M110) was used at a 42 μ g/ml final concentration. After 2 h, the HL60 cells were lysed, and the samples were plated in duplicate on tryptic soy agar plates. Percent killing was defined as the reduction in CFU/ml after 2 h compared with that at time zero.

whereas it decreased only modestly in mice immunized with PPS14-TT, which continued to exhibit bacterial counts of over 3,000 CFU/ml. Collectively, these data demonstrate that pgp-TT elicits robust, boosted secondary serum titers of pgp-specific IgG that can mediate opsonophagocytic killing of *S. aureus*. The vaccine enhanced *S. aureus* clearance from the blood of infected an-

FIG 6 Mice immunized with pgp-TT were protected against *S. aureus* bacteremia. Live *S. aureus* (strain Lowenstein; serotype 5) cells were injected into BALB/c mice ($n = 5$) i.p. at 4×10^7 CFU/mouse 2 weeks after priming and boosting i.p. (day 14 and 28) with pgp-TT or PPS14-TT (5 μ g/mouse) in alum plus CpG-ODN. Blood was drawn from the tail vein 1, 2, and 3 days following infection. Blood (100 μ l) was mixed with PBS-heparin for final dilutions of 1:100 and 1:1,000 and distributed on blood agar plates for counting of *S. aureus* colonies. For purposes of statistical analysis, colony counts that scored ≤ 100 were assigned a value of 100. Horizontal bars represent geometric means. *, statistical significance ($P \leq 0.05$) of the results of comparisons between pgp-TT- and PPS14-TT-immunized mice.

imals, making it a potential candidate for a prophylactic antistaphylococcal vaccine and worthy of further testing.

DISCUSSION

Lipoteichoic acid expressed by Gram-positive extracellular bacteria (10, 11) is a potential target for antibody-mediated host protection (15, 44). Although LTA has been reported to be a TLR2 ligand and thus to be proinflammatory (21, 22), more recent work suggests that this TLR2 activity might represent contaminating lipoproteins/lipopeptides (20). The backbone of LTA consists of either a highly conserved, noninflammatory (poly)glycerolphosphate (pgp) backbone or a (poly)ribitolphosphate backbone (16, 20). A diverse group of bacterial pathogens of clinical importance, including *S. aureus*, *S. epidermidis*, other coagulase-negative *Staphylococcus* species, group A *Streptococcus* (*S. pyogenes*), and species of *Enterococcus*, among others, express the pgp backbone in their LTA (19). IgG specific for pgp has been shown to confer passive protection against *S. aureus* (18). We thus set out to develop a method to synthesize pgp and to determine its potential value as a conjugate vaccine to mediate antistaphylococcal immunity in the mouse model. The advantages of this approach include the clinical value of efficiently synthesizing large amounts of a chemically well-defined, noninflammatory (21, 45), conserved target antigen and the well-known clinical efficacy of conjugate vaccines for inducing active, antibody-mediated host protection against a number of bacterial pathogens. In particular, a pgp conjugate vaccine has the potential of contributing to host protection against a wide range of Gram-positive extracellular bacteria expressing pgp in their LTA.

We report the synthesis of a suitably protected chiral glycerol phosphoramidite for its stepwise chain extension to produce a 10-mer pgp polymer using standard solid-phase oligonucleotide synthesis chemistry. Subsequent to our initial development of synthetic pgp, several reports were published describing a similar method, although pgp was not evaluated for use as a prophylactic vaccine (18, 46, 47). Utilizing synthetic pgp in an ELISA, we demonstrated that intact heat-killed *S. aureus* elicits a boosted T cell-dependent secondary pgp-specific IgG response *in vivo*. Although isolated pgp has the characteristics of a T cell-independent antigen, a member of a class of antigens that elicit minimal primary IgG responses and no significant IgG memory (25), its expression by intact *S. aureus* converts it into a CD4⁺ T cell-dependent antigen. These data are consistent with our previous studies demonstrating CD4⁺ T cell-dependent polysaccharide-specific IgG responses to intact *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Neisseria meningitidis* (42), and *Acinetobacter baumannii* (unpublished data), although not to their respective isolated polysaccharides. This T cell dependence is likely mediated by coexpressed bacterial proteins that recruit CD4⁺ T cell help for the polysaccharide-specific response and appears to be a general property of polysaccharides expressed by intact bacteria. We further demonstrate that mice with *S. aureus* osteomyelitis respond to the infection with pgp-specific IgG. This indicates that pgp is naturally expressed in a manner that allows immune recognition consistent with that observed in *S. aureus*-infected humans (17).

Isolated pgp elicited no detectable pgp-specific IgG response *in vivo*. However, active immunization with pgp covalently linked to TT resulted in the induction of a significant primary pgp-specific IgG response and a markedly boosted secondary response consistent with its conversion into a T cell-dependent antigen, as with

other conjugate vaccines (48). Sera from pgp-TT-immunized mice, but not control MCPS-TT-immunized mice, promoted phagocytic killing by a human neutrophil-like leukemia line. This observation is of particular importance given the ability of *S. aureus* to survive for prolonged periods within neutrophils (49). Although OPK mediated by sera from pgp-TT-immunized mice was demonstrated against both encapsulated and unencapsulated *S. aureus* strains, better killing was observed for the latter. This may reflect the previously described ability of the staphylococcal capsular polysaccharide to at least partially mask components of the underlying cell wall, although this phenomenon is highly variable (18, 50–52). Finally, we demonstrated that the ability of pgp-TT to elicit specific IgG capable of opsonic killing is correlated with its ability to promote clearance of live *S. aureus* from the blood following i.p. challenge compared to a control pneumococcal conjugate vaccine. Collectively, these data strongly suggest that pgp-specific IgG elicited *in vivo* in response to immunization with pgp-TT opsonizes *S. aureus* for more efficient uptake and killing by phagocytes with subsequent enhancement in the clearance of bacteremia.

S. aureus, in addition to its role as a commensal in the nose and on the skin, causes a diverse array of infections in humans, including various skin infections, pneumonia, endocarditis, osteomyelitis, arthritis, meningitis, toxic shock syndrome, bacteremia, and sepsis (53). The requirements for inducing host protection in these distinct contexts, including the relative immunocompetence of the host, are likely to differ, and thus a given approach to vaccination may be selective in its clinical efficacy. Thus, the potential value of pgp-TT as a prophylactic antistaphylococcal vaccine will require future testing in other infection models. In this regard, phase III clinical trials involved the use of a conjugate vaccine consisting of *S. aureus* capsular polysaccharides types 5 and 8 (the most common clinical isolates) covalently linked to recombinant *Pseudomonas aeruginosa* exotoxin A (StaphVAX; Nabi Biopharmaceuticals) (54). Although protective in a mouse model, the vaccine failed to confer significant protection when administered to hemodialysis patients. Additional approaches that failed to translate into success in human trials included active immunization with IsdB-derived antigen (55) and passive immunization with human polyclonal antibodies specific for *S. aureus* ClfA (fibrinogen adhesion) and *S. epidermidis* SdrG (fibrin-binding protein) (56, 57). Finally, a recent phase III clinical trial to determine the ability of an anti-LTA MAb (pagibaxumab; Biosynex, Inc.) to confer passive protection against staphylococcal infections in neonates also failed to show efficacy (J. J. Mond, personal communication). The failure of these trials has led to the belief that a multicomponent vaccine may be necessary for significant protection, although vaccine failure due to the immunocompromised nature of the patient populations is also a distinct possibility. In this regard, investigators have identified numerous other virulence factors that may be considered potential vaccine targets (24). Thus, pgp-TT may find its clinical value as part of a multicomponent approach to vaccination.

Several studies have indicated that antibodies against a number of *S. aureus* virulence factors may reduce infection severity but may not prevent disease (58–63). More recent data, while not negating a supporting role for antibody, have nevertheless argued that cell-mediated immunity involving CD4⁺ T cell production of IL-17, a neutrophil recruitment and activation factor, in concert with neutrophils, may be important for antistaphylococcal host

protection (reviewed in reference 55). Thus, patients with Job's syndrome, exhibiting mutations in STAT3 and Th17 development, have an increased incidence in *S. aureus* infections, whereas patients with agammaglobulinemia, who are more susceptible to infections with *Streptococcus pneumoniae* and *Haemophilus influenzae*, do not have increased *S. aureus* infections. Indeed, patients with various defects in cell-mediated immunity generally have a high incidence of infections with *S. aureus*, perhaps reflective in part of diminished Th17 responses (55). Mouse models of *S. aureus* further support these clinical observations. Thus, mice genetically deficient in IL-17A/F (64) have increased susceptibility to *S. aureus* infection, whereas B cell-deficient mice, in contrast to T cell-deficient mice, do not (65). Vaccination of mice with staphylococcal virulence protein IsdB or ClfA was able to protect mice against infection in an IL-17-dependent manner (66, 67). Collectively, these data suggest that a combined multicomponent antibody (including pgp as a target) and cell-mediated approach to vaccination may hold the greatest promise for a prophylactic antistaphylococcal vaccine.

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