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RESEARCH ARTICLE
Host-Microbe Biology

Protection against *Staphylococcus aureus* Colonization and Infection by B- and T-Cell-Mediated Mechanisms

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ABSTRACT *Staphylococcus aureus* is a major cause of morbidity and mortality worldwide. *S. aureus* colonizes 20 to 80% of humans at any one time and causes a variety of illnesses. Strains that are resistant to common antibiotics further complicate management. *S. aureus* vaccine development has been unsuccessful so far, largely due to the incomplete understanding of the mechanisms of protection against this pathogen. Here, we studied the role of different aspects of adaptive immunity induced by an *S. aureus* vaccine in protection against *S. aureus* bacteremia, dermonecrosis, skin abscess, and gastrointestinal (GI) colonization. We show that, depending on the challenge model, the contributions of vaccine-induced *S. aureus*-specific antibody and Th1 and Th17 responses to protection are different: antibodies play a major role in reducing mortality during *S. aureus* bacteremia, whereas Th1 or Th17 responses are essential for prevention of *S. aureus* skin abscesses and the clearance of bacteria from the GI tract. Both antibody- and T-cell-mediated mechanisms contribute to prevention of *S. aureus* dermonecrosis. Engagement of all three immune pathways results in the most robust protection under each pathological condition. Therefore, our results suggest that eliciting multipronged humoral and cellular responses to *S. aureus* antigens may be critical to achieve effective and comprehensive immune defense against this pathogen.

IMPORTANCE *S. aureus* is a leading cause of healthcare- and community-associated bacterial infections. *S. aureus* causes various illnesses, including bacteremia, meningitis, endocarditis, pneumonia, osteomyelitis, sepsis, and skin and soft tissue infections. *S. aureus* colonizes between 20 and 80% of humans; carriers are at increased risk for infection and transmission to others. The spread of multidrug-resistant strains limits antibiotic treatment options. Vaccine development against *S. aureus* has been unsuccessful to date, likely due to an inadequate understanding about the mechanisms of immune defense against this pathogen. The significance of our work is in illustrating the necessity of generating multipronged B-cell, Th1-, and Th17-mediated responses to *S. aureus* antigens in conferring enhanced and broad protection against *S. aureus* invasive infection, skin and soft tissue infection, and mucosal colonization. Our work thus, provides important insights for future vaccine development against this pathogen.

KEYWORDS B-cell responses, *Staphylococcus aureus*, T-cell immunity, adaptive immunity, vaccines

Staphylococcus aureus is a leading cause of community- and healthcare-associated bacterial infections and postsurgical wound infections (1–4). Skin and soft tissue infections (SSTIs) are a common type of community-acquired *S. aureus* infection, which can be recurrent in many individuals (5, 6). *S. aureus* also causes severe invasive disease, such as bacteremia, meningitis, endocarditis, osteomyelitis, pneumonia, sepsis, and

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toxic shock syndrome (4, 7). *S. aureus* bacteremia is associated with high mortality (20 to 40% in adults) despite appropriate antibiotic treatment (8). *S. aureus* colonizes about 20 to 80% of the human population at any given time, providing a reservoir for subsequent infection and transmission (9–12). The rapid increase of *S. aureus* strains that are resistant to multiple antibiotics, such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-intermediate and -resistant strains (VISA and VRSA, respectively), in both community- and hospital-acquired infections (13–15), has complicated the management of these infections.

The development of *S. aureus* vaccines has been challenging. For diseases caused by many bacterial pathogens, such as *Streptococcus pneumoniae*, *Haemophilus influenzae* type b, and *Neisseria meningitidis*, antibodies to polysaccharide (PS) or protein antigens, generated by either natural exposure or immunization, are highly protective (16, 17). A similar approach has been attempted for *S. aureus* vaccine development but yielded disappointing results so far. While multiple candidates targeting various *S. aureus* PSs and/or proteins have shown promise in preclinical studies, no antibody-based *S. aureus* vaccine (via either passive or active immunization) has succeeded in clinical trials (18–23). This failure has then led to further deliberation about the immunological requirements for effective *S. aureus* defense. Indeed, despite the suggestion that individuals with high-titer preexisting anti-*S. aureus* antibody may have better prognosis during *S. aureus* bacteremia and sepsis (24–26) (while *S. aureus*-specific T-cell immunity in those individuals and its contribution to protection were not examined in the same studies), no direct correlation has ever been established between the level of anti-*S. aureus* antibody and the prevention of *S. aureus* infection or colonization (18, 27–29), suggesting that whatever protective role antibodies may play is insufficient to effectively prevent *S. aureus* pathogenesis. Furthermore, a growing body of literature now implicates the importance of cellular immunity in innate and possibly acquired *S. aureus* resistance. Indeed, compared to the general population, HIV-infected individuals have significantly higher rates of *S. aureus* SSTI, bacteremia, endocarditis, and colonization (30–33). A recent study suggests that the decreased *S. aureus*-specific Th1 immunity may be part of the reason for the increased incidence of MRSA SSTI in HIV patients (34). Another classic immunodeficiency associated with frequently recurrent *S. aureus* skin and lung infection is Job's syndrome (i.e., hyper-immunoglobulin E syndrome), which features in defective interleukin-17 (IL-17) production (and thus, Th17 immunity) due to mutations in the *stat3* gene (35, 36). In addition to these observations in humans, studies in mice have also pointed to the importance of innate and memory T cells in resistance to *S. aureus*. Lin et al. showed that deficiency in gamma interferon (IFN-γ) production enhanced mouse susceptibility to *S. aureus* bloodstream infection (37). Brown and coworkers reported that adoptive transfer of *S. aureus*-specific, memory Th1 cells protected naive mice against *S. aureus* peritoneal infection (38). A study in severe combined immunodeficiency (SCID) mice showed that, following immunization with the *S. aureus* antigen IsdB, Th17 cells were critical for protection against lethal *S. aureus* sepsis challenge (39). Furthermore, previous exposure to *S. aureus* protects mice against recurrent dermonecrosis in an antibody- and Th17-dependent fashion (40). The production of IL-17 and IL-22 by innate immune cells was also found to be critically important in the control of *S. aureus* nasal carriage (41–43). These observations therefore suggest that host defense against *S. aureus* may require the involvement of several immune factors rather than humoral responses alone.

In this work, we investigate the respective roles of vaccine-induced *S. aureus*-specific humoral and cellular immunity in acquired protection against *S. aureus* under various pathological conditions, including invasive infection, SSTI (dermonecrosis and abscess), and mucosal colonization in mice. Our results show that depending on the type of *S. aureus* challenge, protection is mediated by different immune pathways, including antibody, Th1 or Th17 response, a combination of the two, or all of the above. Thus, compared to an antibody-based strategy, an approach that elicits all three types of immune responses to *S. aureus* antigens confers more robust and broad protection in mice against both *S. aureus* infection and carriage.

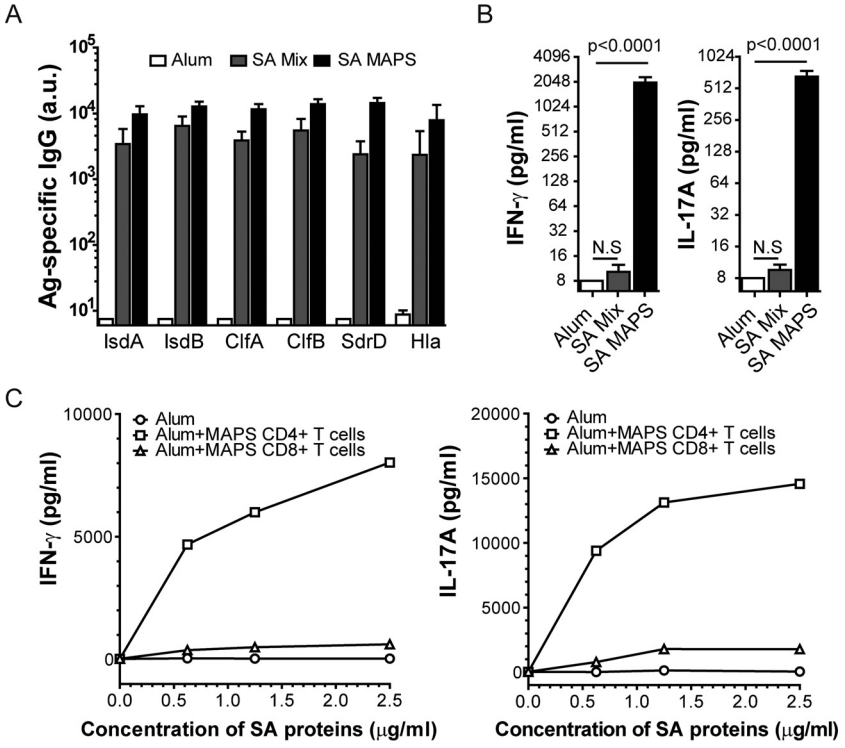

FIG 1 Generation of *S. aureus*-specific immune responses. C57BL/6 mice ($n = 10$ per group) received three subcutaneous immunizations with adjuvant alone (Alum), or Alum-adjuvanted *S. aureus* protein (SA) mix or *S. aureus* MAPS (SA MAPS) vaccine (30 μg of total protein content per dose). (A) Antigen (Ag)-specific IgG antibodies were measured 2 weeks after the third immunization. Antibody titers are expressed in arbitrary units (a.u.) related to a reference serum (as described in Materials and Methods). Bars represent geometric means + 95% confidence interval (CI). (B) IFN-γ and IL-17A production after *ex vivo* stimulation of peripheral blood cells of immunized mice with a mixture of purified *S. aureus* proteins. Bars represent geometric means + 95% CI. Statistical analysis was performed using nonparametric one-way ANOVA (Dunn's multiple comparison test) between indicated groups. N.S, not significant. (C) IFN-γ and IL-17A production after *ex vivo* stimulation of splenocytes isolated from an Alum-immunized mouse with *S. aureus* proteins in the absence or presence of $CD4^+$ or $CD8^+$ T cells purified from the spleen of an *S. aureus* MAPS-immunized mouse.

RESULTS

Generation of *S. aureus*-specific immune responses. To generate different adaptive immune responses to *S. aureus* antigens, we immunized C57BL/6 mice with two antigen formulations. The first formulation consisted of a mixture of six *S. aureus* proteins (referred to as *S. aureus* mix below), including α-hemolysin (Hla) toxoid (see Fig. S1 in the supplemental material), clumping factors A (ClfA) and B (ClfB), serine-aspartate repeat protein D (SdrD), and iron-regulated surface proteins A (IsdA) and B (IsdB) (see Fig. S2 in the supplemental material). When administered with aluminum hydroxide adjuvant (Alum), *S. aureus* mix induces robust antibodies, but no measurable cellular responses to the included antigens (Fig. 1A and B, SA mix). The second formulation consisted of a macromolecular complex (called the multiple antigen presenting system [MAPS] complex) in which the same six *S. aureus* proteins were coupled to a biotinylated polysaccharide scaffold via affinity interaction between rhizavidin (rhavi) and biotin molecules (44) (see Fig. S2 in the supplemental material). As shown previously with other antigens (44), immunization of mice with *S. aureus* MAPS complexes and Alum induced not only a high level of antibodies, but also antigen-specific adaptive cellular responses, as indicated by robust production of IFN-γ and IL-17A upon *ex vivo* stimulation of peripheral blood with the target protein antigens (Fig. 1A and B, SA MAPS). Further analysis indicated that both cytokines are primarily produced by $CD4^+$ T helper cells (Fig. 1C), representing Th1 and Th17 responses.

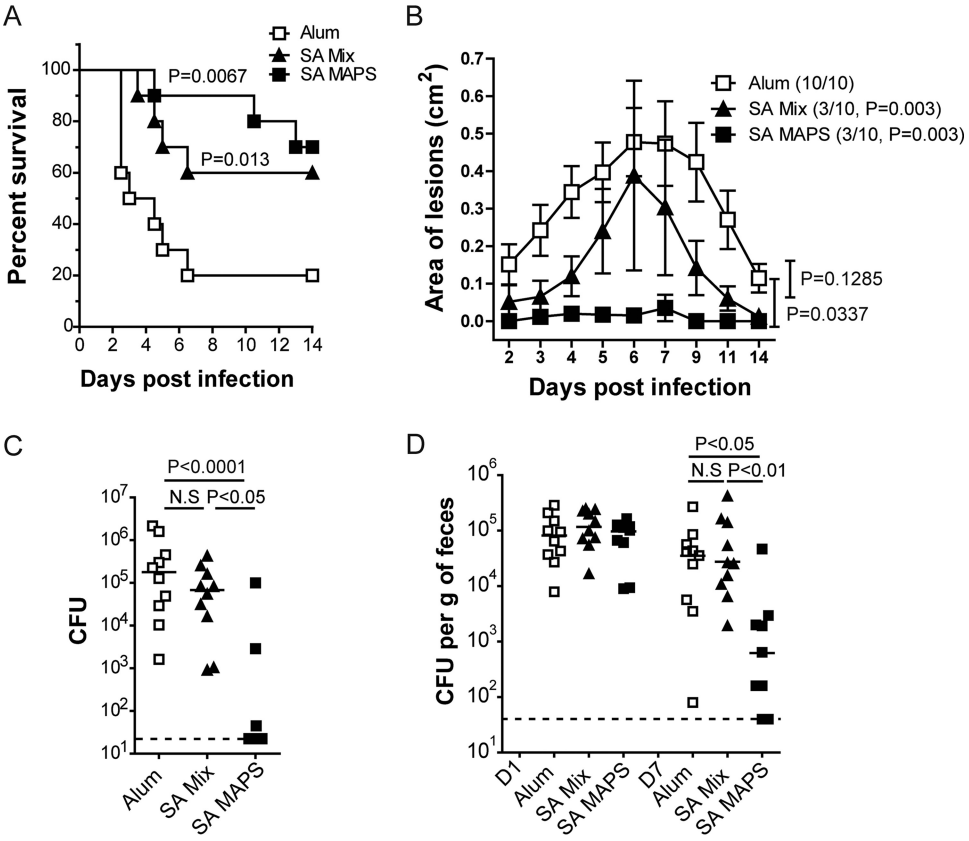

FIG 2 Acquired protection against *S. aureus* invasive disease, SSTI, and GI colonization. Mice ($n = 10$ per group) were immunized as described previously. Three weeks after the last immunization, mice were challenged in models of bacteremia (A), dermonecrosis (B), skin abscess (C), and GI colonization (D). (A) Survival curves after infection. Statistical analysis was performed by the Mantel-Cox test in comparison to the Alum group. (B) The incidence of dermonecrosis in each group is indicated in the symbol key. Individual curves show progression of lesions over time in those animals that developed dermonecrosis postinoculation. Symbols represent means ± standard errors of the means (SEM). Statistical analysis was performed using Fisher's exact test (for incidence) or two-way ANOVA (for lesion size) in comparison to the Alum group. (C) Bacterial CFU recovered from skin abscesses 4 days postinoculation. Each symbol represents one mouse, and lines indicate medians. The dashed line indicates one-half of the lower detection limit (22.5 CFU). Statistical analysis was performed using nonparametric one-way ANOVA (Dunn's multiple comparison test) between indicated groups. (D) Bacterial CFU recovered from feces 1 day (D1) and 7 days (D7) postinoculation. Each symbol represents one mouse, and lines indicate medians. The dashed line indicates one-half of the lower detection limit (40 CFU). Statistical analysis was performed using nonparametric one-way ANOVA (Dunn's multiple comparison test) between indicated groups.

Protection against *S. aureus* is mediated by different adaptive immune responses. Acquired protection mediated by anti-*S. aureus* immune responses was evaluated in four challenge models. For invasive infection, we used a bacteremia model, in which mice were injected intravenously with 2×10^7 CFU of *S. aureus* (ATCC 29213 strain, type 5 capsule-expressing). Protection was evaluated by comparing survival curves over 14 days (Fig. 2A). For SSTI, we used two different models. In the dermonecrosis model, subcutaneous inoculation of mice with 1×10^7 CFU of *S. aureus* (USA300 TCH959 strain) leads to severe skin damage and the formation of necrotic lesions (45). Protection was assessed with respect to the overall incidence of lesions (Fig. 2B, symbol key) and the surface area of the dermonecrotic lesion in those animals that were affected (Fig. 2B, curves). In the skin abscess model, mice were infected with a lower inoculum ($\sim 2 \times 10^5$ to 5×10^5 CFU), which induces enclosed subdermal abscesses with minimal skin breakdown (45). Protection was assessed by comparing densities of *S. aureus* recovered from abscesses dissected 4 days postinfection (Fig. 2C). Finally, *S. aureus* gastrointestinal (GI) colonization was examined following intranasal inoculation of mice with 5×10^7 CFU of the USA300 LAC^{Strep} strain; this results in stable GI colonization in naive mice for >21 days (see Fig. S3 in the supplemental material).

Protection was assessed by comparing bacterial densities in feces at indicated time points postinoculation (Fig. 2D).

We found that mice that developed only anti-*S. aureus* antibodies (i.e., those in the *S. aureus* mix group) were significantly protected in two of the four models, with reduced mortality following intravenous (i.v.) infection (40 versus 80% in the control group [Fig. 2A]) and a decreased incidence of lesions in the dermonecrosis model (30% versus 100% in the control group [Fig. 2B]). However, with respect to skin abscess or GI colonization, they were equally susceptible as the control group (Fig. 2C and D). In contrast, mice that developed both antibody and cellular responses to *S. aureus* antigens (i.e., those in the *S. aureus* MAPS group) demonstrated broad resistance to all four *S. aureus* challenges, including bacteremia (reduced mortality and delayed disease onset) and dermonecrosis (reduced incidence and symptoms of lesions) (Fig. 2A and B), as well as in the abscess and GI colonization models. In the skin abscess model, 7 out of 10 *S. aureus* MAPS-vaccinated mice had no detectable abscess (and no recoverable bacteria) 4 days postinfection, whereas mice in the control group or the *S. aureus* mix group all had skin abscesses and recoverable CFU ranging from 10^3 to 10^6 (Fig. 2C). In the GI colonization model, with an initial inoculation density of 10^5 CFU per g of feces (median, 1 day postchallenge), *S. aureus* MAPS-vaccinated mice were able to rapidly clear bacteria from the GI tract: *S. aureus* could not be detected in 2 out 10 mice 7 days postchallenge, and the group had a median bacterial density of 641 CFU per g of feces, 40- to 50-fold lower than bacterial densities at the same time point in the control group or the *S. aureus* mix group (Fig. 2D).

Differential roles of antigen-specific antibodies and Th1 and Th17 responses in protection against *S. aureus*. The results above suggest that adaptive humoral or cellular responses may contribute differentially to protection against *S. aureus* infections or colonization. Next, we dissected the role of each immune pathway in individual challenge models.

The contribution of antibodies was evaluated by passive immunization. We obtained sera from rabbits pre- or post-*S. aureus* MAPS vaccination (see Fig. S4 in the supplemental material) and passively transferred these to mice before challenge in each model. In the bacteremia model, mice that received postimmune sera had significantly lower mortality at 14 days postinfection compared to the control group (50% versus 90%, $P = 0.0007$) (Fig. 3A). In a separate experiment, we sacrificed the mice 20 h postinfection and measured bacterial burden in their kidneys: as shown in Fig. S5 in the supplemental material, the group that received postimmune sera had significantly lower CFU than the control group, suggesting that antibody-mediated bacterial clearance contributes to protection in this model. Passive immunization also effectively mitigated (but did not fully prevent) *S. aureus* dermonecrosis, resulting in reduced lesion size (Fig. 3B). However, the presence of *S. aureus*-specific antibodies did not provide any protection against either skin abscess or GI colonization (Fig. 3C and D).

The contribution of cellular responses was studied in antibody-deficient ($\mu MT^{-/-}$) mice. Vaccination of $\mu MT^{-/-}$ mice with *S. aureus* MAPS induced Th1 and Th17 responses to *S. aureus* antigens without detectable humoral responses (Fig. 4A) and conferred significant protection in three models: compared to the control group, *S. aureus* MAPS-vaccinated $\mu MT^{-/-}$ mice had smaller lesions during dermonecrosis challenge ($P = 0.03$ [Fig. 4C]), significantly reduced abscess formation ($P = 0.0001$ [Fig. 4D]), and accelerated clearance of *S. aureus* carriage from the GI tracts postcolonization ($P < 0.0001$ [Fig. 4E]). In the case of *S. aureus* bacteremia, the presence of only cellular responses did not provide significant protection (Fig. 4B).

Therefore, we conclude from these experiments that humoral and cellular responses serve as complementary mechanisms in providing protection against different *S. aureus* challenges: antibody-mediated mechanisms confer protection against *S. aureus* bacteremia, but are ineffective in prevention of *S. aureus* skin abscess or colonization, against which antigen-specific cellular responses are both essential and sufficient. Furthermore, humoral and cellular responses, collectively, confer optimal protection against *S. aureus* dermonecrosis.

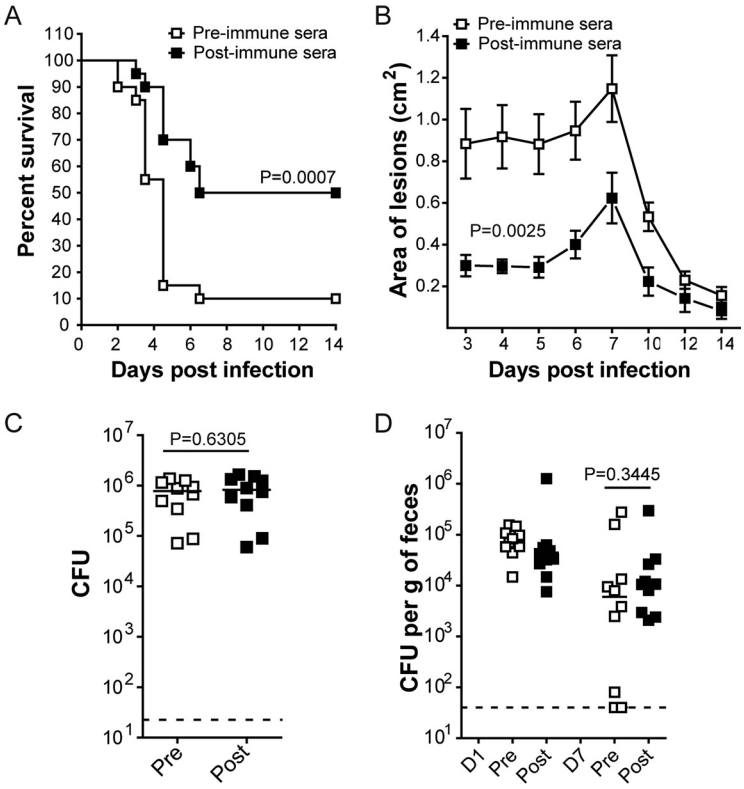

FIG 3 Evaluation of antibody-mediated protection against *S. aureus* in different models. Mice ($n = 10$ per group) received 200 μl of pre- or postimmune rabbit sera 1 day prior to *S. aureus* inoculation. (A) Survival curves during *S. aureus* bacteremia. Differences between groups were analyzed by the Mantel-Cox test. (B) Size of lesions at different time points postinoculation in the dermonecrosis model. Symbols represent mean ± SEM. Differences between the Alum and *S. aureus* protein (SA) mix groups or Alum and *S. aureus* (SA) MAPS groups were compared using two-way ANOVA. (C) Bacterial CFU recovered post-*S. aureus* inoculation in the abscess model. Each symbol represents one animal, and lines indicate medians. A dashed line indicates one-half of the lower detection limit (22.5 CFU). Statistical analysis was performed using the Mann-Whitney *U* test (two tailed). (D) Bacterial CFU recovered from feces 1 day (D1) and 7 days (D7) postinoculation. Each symbol represents one mouse, and lines indicate the median. A dashed line indicates one-half of the lower detection limit (40 CFU). Statistical analysis was performed using the Mann-Whitney *U* test (two tailed).

As immunization with *S. aureus* MAPS construct induces at least two types of cellular responses, Th1 and Th17 responses, we sought to further dissect their respective contributions to protection in the abscess or GI colonization model, using cytokine supplementation or depletion approaches. To evaluate the role of Th1 or Th17 immunity against skin abscesses, we infected naive mice with *S. aureus* inocula that were premixed with recombinant mouse IFN-γ, IL-17A, IL-22, or a combination of different cytokines. The presence of recombinant cytokine(s) did not affect the viability of *S. aureus* in the inoculum (see Fig. S6 in the supplemental material). Mice in the control group were infected with *S. aureus* mixed with buffer vehicle: 9 out of 10 animals (90%) developed skin abscesses with a median bacterial density of $>10^4$ CFU (Fig. 5A, phosphate-buffered saline [PBS]). In contrast, supplementation of the inoculum with either IFN-γ or IL-17A but not IL-22 during infection was able to lower the incidence of abscess to 50% and reduce the median bacterial burden to 55 or 208 CFU, whereas coadministration of both IFN- γ and IL-17A resulted in almost complete protection (9/10 mice were free of abscess 4 days postinfection) (Fig. 5A). This result was further confirmed by cytokine depletion in *S. aureus* MAPS-vaccinated animals during abscess challenge, which showed that *S. aureus* MAPS-induced protection was only slightly impacted by depletion of either IFN-γ or IL-17, but was significantly attenuated when antibodies to both cytokines were administered (Fig. 5B). We noticed that protection was not completely abolished by administration of anti-IFN-γ and anti-IL-17 antibodies.

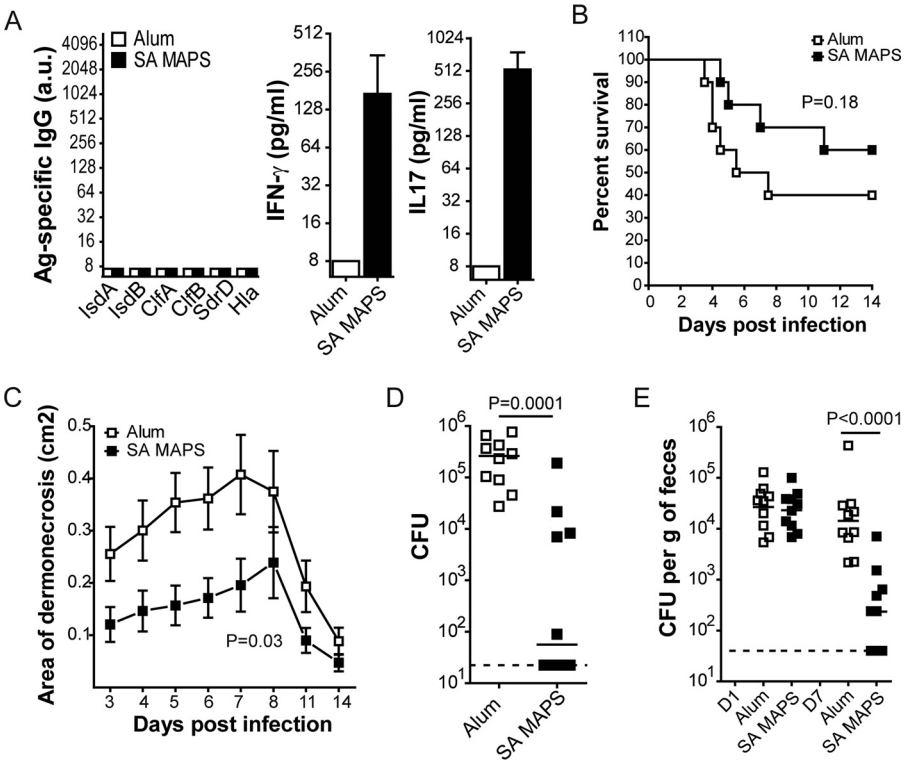

FIG 4 Evaluation of *S. aureus* MAPS-induced protection against *S. aureus* infection and colonization in antibody-deficient mice. $\mu MT^{-/-}$ mice ($n = 10$ per group) were immunized three times with adjuvant alone (Alum) or *S. aureus* (SA) MAPS. (A) Antigen-specific antibody and cellular responses were examined 2 weeks after the last immunization. (B) Survival curves following intravenous infection. Differences between groups were analyzed by the Mantel-Cox test. (C) Sizes of lesions at different time points postinoculation in the dermonecrosis model. Symbols represent the mean ± SEM. Differences between groups were analyzed using two-way ANOVA. (D) Bacterial CFU recovered from skin abscesses. Lines indicate medians. Statistical analysis was performed using the Mann-Whitney *U* test (two tailed). (E) Bacterial CFU in feces 1 day and 7 days postinoculation. Lines indicate medians. Statistical analysis was performed using the Mann-Whitney *U* test (two tailed).

This result may be due to an insufficient concentration of administered antibodies and/or the inability of antibodies to effectively access and neutralize locally produced cytokines at the site of abscess formation. Taken together, these results suggest that either Th1 or Th17 responses to these antigens may be sufficient to prevent *S. aureus* skin abscess.

In contrast to what was observed in the abscess model, when a cytokine depletion was performed in the GI colonization model we found that depletion of either IFN-γ or IL-17 completely abolished *S. aureus* MAPS-induced protection in mice, suggesting that the clearance of *S. aureus* carriage may require both Th1- and Th17-mediated immune responses (Fig. 6).

DISCUSSION

Humoral immunity and cellular immunity are two important arms of host defense against microbial invasions. While the strategy of generating antibodies has been widely used in bacterial vaccinology and highly successful in the prevention of several diseases, there is a growing recognition that adaptive cellular responses, such as Th1 and Th17 responses, may play a critical role in protection against infection and/or colonization by certain extracellular bacterial pathogens (46). Studies in mice showed that Th17 memory responses specific to *S. pneumoniae* could facilitate nasopharyngeal clearance (47, 48) and provide cross-serotype protection against invasive pneumococcal disease (49). Moreover, a recent work using a nonhuman primate model revealed an important role of Th17/Th1 memory generated by the whole-cell pertussis vaccine in

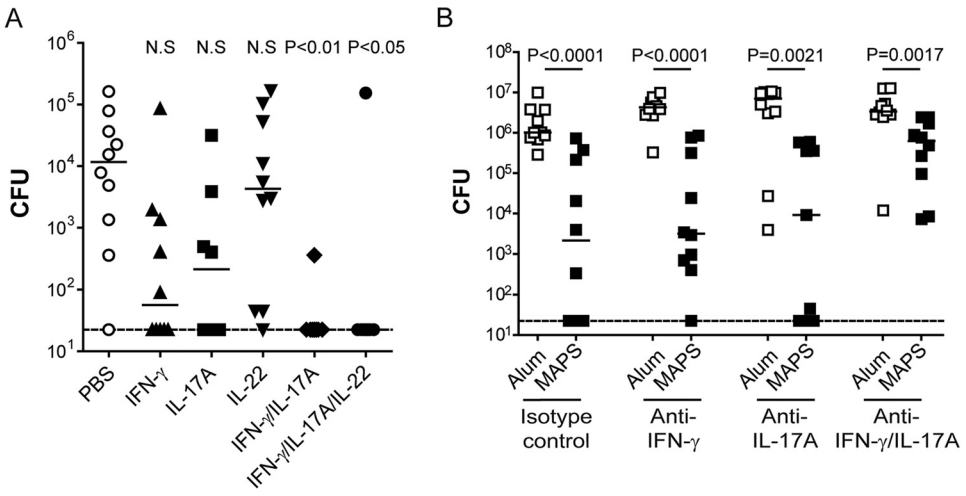

FIG 5 Role of Th1 and Th17 responses in prevention of *S. aureus* skin abscess. (A) Supplementation with recombinant mouse IFN-γ or IL-17A (but not IL-22) mitigates *S. aureus* abscess formation in naive mice. Mice (n = 8 to 10 per group) were injected subcutaneously with 2.5×10^5 CFU of USA300 strain mixed with PBS (vehicle control), rIFN-γ (1 μg per mouse), rIL-17A (0.9 μg per mouse), rIL-22 (1 μg per mouse), a combination of rIFN-γ and IL-17A, or all three cytokines. Skin abscesses were dissected 4 days postinoculation for bacterial CFU quantification. Lines indicate medians. Statistical analysis was performed using nonparametric one-way ANOVA (Dunn's multiple comparison test) in comparison to the PBS group. (B) Depletion of both IFN-γ and IL-17A significantly attenuated *S. aureus* (SA) MAPS-mediated protection against abscess formation. Mice (n = 10) were immunized three times with Alum or *S. aureus* MAPS before skin abscess challenge. Antibodies against IFN-γ and/or IL-17A or the isotype control were administered 1 day prior to inoculation and also on the day of inoculation. Lines indicate medians. Statistical analysis was performed between the indicated groups using the Mann-Whitney *U* test (two tailed).

protection against colonization, transmission, and secondary infection of *Bordetella pertussis* and hypothesized that the absence (or significant reduction) of such cellular responses induced by current acellular pertussis vaccines may be an important reason for their inferior efficacy (50).

The development of *S. aureus* vaccines has been extremely challenging. In addition to the wide variety of diseases for which the organism is responsible, *S. aureus* is a common colonizer of healthy individuals, with a concomitant risk of transmission and skin and soft tissue autoinoculation. It is unclear whether natural immunity to *S. aureus* truly occurs: unlike for other common bacterial pathogens such as pneumococcus, *Haemophilus influenzae* type b, and meningococcus, there is no clear age association with *S. aureus* infections, and recurrence is not uncommon. These findings suggest that the naturally induced immune responses to *S. aureus* infections, antibodies (51–54) and T-cell responses (38, 55), are likely insufficient to protect against this pathogen. Previous preclinical studies of *S. aureus* vaccine candidates had primarily focused on invasive infection models in which antibodies to specific *S. aureus* polysaccharide and/or protein antigens were found to be highly protective. However, the failure of all antibody-based vaccine candidates in clinical trials to date could be interpreted as indicating that immune mechanisms other than antibodies may be required to effectively reduce or prevent *S. aureus* pathogenesis in humans. Another important implication is that preclinical evaluation using different challenge models, including invasive infection, SSTI, and mucosal colonization models, in which protection is mediated via various immune factors (e.g., antibodies and Th1 and/or Th17 responses) (38, 40, 43), may be important to properly assess the potential of vaccine candidates.

In this work, we elucidate the importance of vaccine-induced *S. aureus*-specific antibody and Th1 and Th17 responses in conferring comprehensive protection against *S. aureus* infection and colonization. Our results show that different immune responses mediate protection depending on the site and type of *S. aureus* infection/colonization. For instance, antibodies protect mice against *S. aureus* i.v. infection, and to some extent, dermonecrosis, but are ineffective in the prevention of skin abscess or GI colonization, whereas antigen-specific cellular (Th1 and Th17) responses are critically involved in

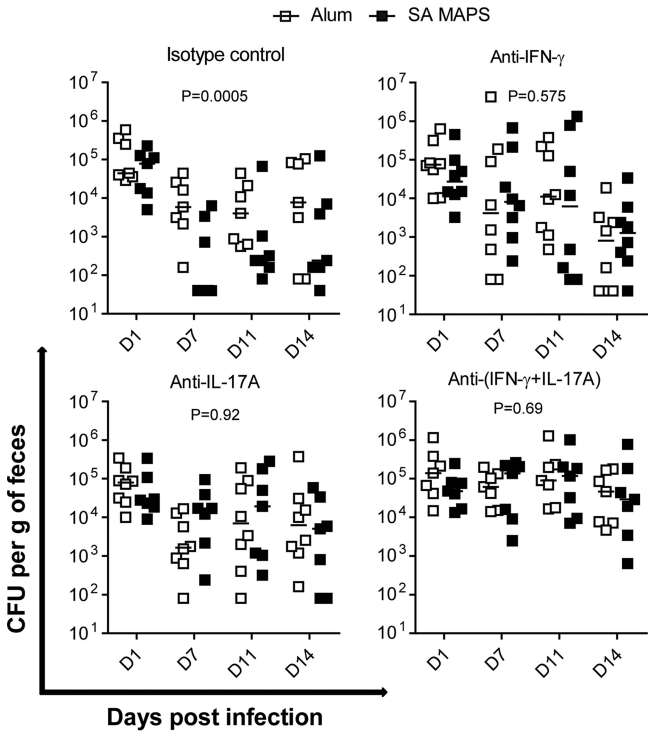

FIG 6 Role of Th1 and Th17 responses in clearance of *S. aureus* GI colonization. Mice ($n = 10$) were immunized three times with Alum or *S. aureus* (SA) MAPS prior to challenge in the GI colonization model. Antibodies against IFN-γ and/or IL-17A or the isotype control were administered 1 day prior to inoculation and also on days 1, 5, and 8 postinoculation. Feces were collected on days 1, 7, 11, and 14 postinoculation for CFU analysis. Lines represent medians. Statistical analysis was performed using two-way ANOVA.

protection against SSTI (dermonecrosis and abscess) and GI carriage but do not provide significant protection following i.v. infection. In this light, it is not surprising that we find optimal protection against *S. aureus* when all three immune pathways are engaged during vaccination. Therefore, our data strongly suggest that vaccine strategies aiming to induce multipronged B- and T-cell responses to *S. aureus* antigens may be critical to prevent different aspects of *S. aureus* pathogenesis and thus provide comprehensive protection against this pathogen.

MATERIALS AND METHODS

Mouse and bacterial strains. Wild type and μMT$^{-/-}$ C57BL/6 mice were purchased from Jackson Laboratories. *S. aureus* strains USA300 (TCH959) and ATCC 29213 were purchased from ATCC. The USA300 LAC (JE2) strain was kindly provided by BEI (56). The streptomycin-resistant USA300 LAC (JE2) strain (USA300 LAC^{strep}) was obtained by spontaneous mutation after culturing the parent strain on a blood agar plate containing 0.5 g/liter streptomycin.

Ethics statement. All procedures involving mice were approved by the Boston Children's Hospital Animal Care and Use Committee (IACUC protocol no. 16033133), following the National Institutes of Health guidelines for animal housing and care.

Cytokines and antibodies. Recombinant mouse IFN-γ, IL-17A, and IL-22 were purchased from R&D systems. Cytokines were reconstituted at 100 mg/ml in PBS and then diluted with PBS to the appropriate concentration during infection. Anti-mouse IFN-γ (clone R4-6A2), anti-mouse IL-17A (clone 17F3), and the corresponding isotype control antibodies were purchased from Bioxcell. For cytokine depletion in the skin abscess model, mice received 300 μg of anticytokine antibodies or isotype control via intraperitoneal injection 1 day prior to infection and another 300 μg via subcutaneous injection the same day of infection but at a distinct location from the infection site. For cytokine depletion in the GI colonization model, mice received 300 μg of the indicated antibodies via intraperitoneal injection 1 day prior to inoculation and on days 1, 5, and 8 postinoculation.

Cloning and purification of *S. aureus* antigens. DNA sequences encoding ClfA (positions 221 to 559), ClfB (203 to 542), SdrD (246 to 682), IsdA (47 to 324), IsdB (48 to 477), or Hla (27 to 319) were amplified from *S. aureus* genomic DNA (USA300 TCH959 strain) via PCR and then cloned into a pET-21b vector. A nonhemolytic toxoid of Hla was generated by substitution of residues Asp-Arg-Asp (209 to 211) to Ala-Ala-Ala using PCR. For rhizavidin fusion proteins, DNA sequences encoding the above *S. aureus*

antigens were inserted at the 3′ end of the gene encoding the rhizavidin moiety in a pET-21b vector. All constructs were transformed into the *E. coli* BL21(DE3) strain for expression under isopropyl-β-D-thiogalactopyranoside (IPTG) induction. His-tagged recombinant proteins were purified using nitrilotriacetic acid (NTA) affinity chromatography (Qiagen) followed by size exclusion chromatography using a Superdex 200 column (GE Healthcare Life Sciences). Purified proteins were stored at −80°C until use.

Preparation of *S. aureus* MAPS complexes. Type-1 pneumococcal capsular polysaccharide was purchased from ATCC and used as the scaffold for *S. aureus* MAPS constructs. The polysaccharide was biotinylated using CDAP (1-cyano-4-dimethylaminopyridinium tetrafluoroborate) as the activation reagent as described previously (44). *S. aureus* MAPS complex was assembled by incubation of biotinylated polysaccharide with a mixture of rhizavidin fusions of *S. aureus* antigens (at equal molarity) at room temperature overnight. The input ratio of total proteins to polysaccharide was 3:1 (wt/wt). The assembled complex was isolated by size exclusion chromatography and concentrated by ultrafiltration. The protein concentration of *S. aureus* MAPS complexes was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). The incorporation of *S. aureus* antigens was examined on a reduced SDS-PAGE gel.

Immunization and infection. All vaccines were formulated the day prior to immunization. The antigens were diluted to the appropriate concentration in saline and then mixed with aluminum hydroxide (1.25-mg/ml final concentration [Brenntag]) in 5-ml Eppendorf tubes and incubated at 4°C overnight with rotation (24 rpm) on a Mini LabRoller (Labnet International, Inc.). Four- to 6-week-old female mice received three subcutaneous immunizations on the upper back (30 μg of total protein per immunization per mouse in a 200-μl volume) 2 weeks apart. Animals were bled under isoflurane anesthesia 2 weeks after the last immunization for measurement of antibody and analysis of T-cell responses. The endotoxin concentration in the *S. aureus* mix and MAPS vaccine were 23 and 11 EU per dose, respectively, as measured using the Pierce Chromogenic Endotoxin Quant kit (Thermo Scientific).

For passive immunization experiments, we used rabbit antisera, based on the established finding that rabbit IgG is compatible with mouse Fcγ receptors (57) and to obtain adequate volumes of sera. Rabbit anti-*S. aureus* MAPS sera were generated at Cocalico Biologicals (Reamstown, PA). New Zealand White rabbits were given three intramuscular immunizations, 2 weeks apart, with *S. aureus* MAPS vaccine (300 μg of total protein per immunization per rabbit in 500-μl volume). Sera were collected before the first immunization (preimmune sera) and 2 weeks after the last immunization (antisera). Antigen-specific IgG antibody was detected by enzyme-linked immunosorbent assay (ELISA), and the rabbit serum with highest antibody titer was used for passive immunization in mice. For passive immunization, 8-week-old female mice received 200 μl of heat-inactivated pre- or postimmune sera 1 day prior to infection via intraperitoneal injection.

For preparation of inocula, *S. aureus* strains were streaked onto blood agar plates and grown at 37°C. Colonies were picked and inoculated into tryptic soy broth (TSB [Sigma]) for an overnight culture. In the following morning, bacteria were reinoculated into fresh TSB medium at a 1:100 dilution and incubated at 37°C with shaking. Bacteria were collected 3 h later by centrifugation, washed twice with saline, and adjusted to the appropriate concentration in saline before infection.

Mice were infected 3 weeks after the last immunization. The bacteremia model was performed using the ATCC 29213 strain as described previously (58) with minor modifications. Briefly, mice were anesthetized with isoflurane and injected intravenously with 2×10^7 CFU in 100 μl. Mice were monitored for any sign of illness for 14 days; any ill-appearing animal (presenting with signs of ruffled fur, slow moving, and/or with closed eyes) was immediately and humanely euthanized. In the dermonecrosis model, mice were anesthetized and injected subcutaneously on the shaved lower back with 1×10^7 CFU of the USA300 strain in a 100-μl volume. Mice were monitored for 14 days after infection. Pictures of the infected area were taken at different time points, and the sizes of dermonecrotic lesions were measured using ImageJ software. In the skin abscess model, the backs of mice were shaved, anesthetized, and infected subcutaneously with 2.5×10^5 CFU of the USA300 strain in a 100-μl volume. Mice were then humanely euthanized 4 days after infection. Abscesses were dissected and homogenized in 500 μl of PBS using a bead beater. Serial dilutions of homogenate were plated on mannitol salt plates, and colonies were counted after overnight incubation at 37°C. For animals that were abscess free or for culture-negative samples, the CFU was arbitrarily set as one-half of the lower detection limit (22.5 CFU) to allow for statistical analysis. In the GI colonization model (59), mice were gently restrained and inoculated intranasally with 5×10^7 CFU of the USA300 LAC^{strep} strain in a 10-μl volume. Fecal pellets were collected on days 1 and 7 after inoculation or as indicated. Samples were weighed, resuspended in sterile PBS at 0.1 g/ml, homogenized, and then passed through a CellTrics 30-μm-pore filter. Serial dilutions of the flowthrough samples were plated on mannitol salt plates containing 0.5 mg/ml streptomycin, and colonies were counted after overnight culture at 37°C. For culture-negative samples, CFU was set as one-half of the lower detection limit (40 CFU).

Antibody and T-cell response analysis. Antigen-specific IgG antibody was measured by ELISA using Immulon 2 HB 96-microwell plates (Thermo Scientific) coated with individual recombinant *S. aureus* protein (not containing rhizavidin) (1 μg/ml in PBS, incubated at room temperature overnight). The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. After blocking, serial dilutions of mouse or rabbit sera were added and incubated for 2 h, followed by a 1-h incubation with horseradish peroxidase (HRP)-conjugated secondary antibody against mouse or rabbit IgG. The plates were then washed and developed with SureBlue TMB Microwell peroxidase substrate (KPL). HCl (1 M) was used to terminate the reactions before the A_{450} was analyzed using an ELISA reader. A reference serum was generated by pooling sera from 10 mice that have been immunized three times with *S. aureus* MAPS vaccine. The IgG titer of the reference serum against each target *S. aureus* protein was assigned a value of 12,000 arbitrary units per ml.

Duplicates of 7 serial dilutions of the reference serum were included in each ELISA. The antibody titer of each sample serum was expressed as calculated arbitrary units per ml using standard curves generated by dilutions of the reference serum.

Antigen-specific T memory responses were analyzed by *ex vivo* stimulation of peripheral blood cells or splenocytes with purified *S. aureus* protein antigens (representing a recall response). For whole-blood stimulation, 25 μl of heparinized blood from each mouse was added to 225 μl Dulbecco's modified Eagle's medium (DMEM [BioWhittaker]) containing 10% low-endotoxin defined fetal bovine serum (FBS [HyClone]), 50 μM 2-mercaptoethanol (Sigma), and ciprofloxacin (10 μg/ml [Cellgro]). Cultures were incubated at 37°C for 6 days in the presence of 2.5 μg/ml of the mixture of six *S. aureus* protein antigens (equal weight ratio, non-rhizavidin containing). Supernatants were collected following centrifugation and analyzed by ELISA for IFN-γ and IL-17A concentrations (R&D Systems). Splenocytes were isolated from mice in the Alum- or *S. aureus* MAPS-immunized group. Splenocytes from an *S. aureus* MAPS-vaccinated mouse were then divided into two equal fractions, and from each fraction, $CD4^+$ or $CD8^+$ T cells were purified using a $CD4^+$ or $CD8^+$ selection kit from Miltenyi Biotec. For stimulation, 2.25×10^6 splenocytes of Alum-vaccinated mouse, alone or mixed with 6×10^5 purified $CD4^+$ T cells or 4×10^5 $CD8^+$ T cells that originated from an *S. aureus* MAPS-immunized mouse, were added into each well of a 96-well microplate (250 μl per well) and incubated at 37°C for 3 days in the absence or presence of *S. aureus* proteins at the indicated concentrations. Supernatants were then collected and analyzed for cytokine production.

Hemolysis analysis. The hemolytic activity of wild-type Hla and Hla209 and their respective rhizavidin fusions was measured as follows. Two hundred microliters of heparinized rabbit blood was washed with cold PBS three times. Red blood cells were then resuspended in 10 ml of cold PBS (2% rabbit red blood cells), and 100 μl of a 2-fold serial dilution of Hla samples in PBS with 0.1% BSA, starting from 100 μg/ml, was added to a V-bottom 96-well plate before the addition of 100 μl of red blood cells to each well. PBS containing 0.1% Triton X-100 was used as a positive control (100% hemolysis), and PBS with 0.1% BSA was used as a negative control (0% hemolysis). The plate was incubated at 37°C for 30 min and then subjected to centrifugation at 800 $\times$ *g* for 5 min. The supernatants were transferred into a flat-bottom 96-well plate, and the A_{545} was measured by an ELISA reader. One hemolytic unit (HU) was defined as the activity that causes 50% lysis of 1% rabbit red blood cells after 30 min of incubation at 37°C. The activity of each Hla construct was expressed as the HU of 1 mg/ml of purified protein.

Statistical analysis. All statistical analyses were done using PRISM (version 5.01 for Windows, GraphPad Software, Inc.). Survival curves were analyzed by the Mantel-Cox test. Incidence of dermonecrosis was compared by Fisher's exact test. Development of dermonecrosis postinfection was compared by two-way analysis of variance (ANOVA). Cytokine production and CFU counts in abscesses or in feces were compared using the Mann-Whitney *U* test (two-tailed), nonparametric one-way ANOVA (Dunn's multiple comparison test), or two-way ANOVA, as indicated.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01949-18.

FIG S1, TIF file, 0.7 MB.
FIG S2, TIF file, 2 MB.
FIG S3, TIF file, 0.6 MB.
FIG S4, TIF file, 0.6 MB.
FIG S5, TIF file, 0.6 MB.
FIG S6, TIF file, 0.6 MB.

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F.Z., R.M., and Y.-J.L. designed research, F.Z., O.L., M.J., C. G., and Y.-J. L. conducted experiments and acquired data, F.Z., R.M., and Y.-J.L. analyzed data, F.Z., R.M., and Y.-J.L. provided reagents, and F.Z., R.M., and Y.-J.L. wrote the paper.

F. Zhang, R. Malley, and Y.-J. Lu are named inventors on filed patents related to the MAPS technology and are scientific founders, consultants, and equity owners at Affinivax, a biotechnology company based in Cambridge, MA, that is devoted to the development of vaccines for developing and developed countries, based on the MAPS

technology. Affinivax had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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