Protective Immunity against Recurrent *Staphylococcus aureus* Skin Infection Requires Antibody and Interleukin-17A

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Although many microbial infections elicit an adaptive immune response that can protect against reinfection, it is generally thought that *Staphylococcus aureus* infections fail to generate protective immunity despite detectable T and B cell responses. No vaccine is yet proven to prevent *S. aureus* infections in humans, and efforts to develop one have been hampered by a lack of animal models in which protective immunity occurs. Our results describe a novel mouse model of protective immunity against recurrent infection, in which *S. aureus* skin and soft tissue infection (SSTI) strongly protected against secondary SSTI in BALB/c mice but much less so in C57BL/6 mice. This protection was dependent on antibody, because adoptive transfer of immune BALB/c serum or purified antibody into either BALB/c or C57BL/6 mice resulted in smaller skin lesions. We also identified an antibody-independent mechanism, because B cell-deficient mice were partially protected against secondary *S. aureus* SSTI and adoptive transfer of T cells from immune BALB/c mice resulted in smaller lesions upon primary infection. Furthermore, neutralization of interleukin-17A (IL-17A) abolished T cell-mediated protection in BALB/c mice, whereas neutralization of gamma interferon (IFN-γ) enhanced protection in C57BL/6 mice. Therefore, protective immunity against recurrent *S. aureus* SSTI was advanced by antibody and the Th17/IL-17A pathway and prevented by the Th1/IFN-γ pathway, suggesting that targeting both cell-mediated and humoral immunity might optimally protect against secondary *S. aureus* SSTI. These findings also highlight the importance of the mouse genetic background in the development of protective immunity against *S. aureus* SSTI.

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have become epidemic in the United States [1]. An increasing percentage of MRSA infections occurs among previously healthy people without identified health care-associated risk factors, so called community-associated MRSA (CA-MRSA) infections [2, 3]. CA-MRSA is now the leading cause of skin and soft tissue infections (SSTIs) in the United States, accounting for millions of patient visits per year [4–6]. These SSTIs are frequently associated with superficial dermonecrosis and abscess formation in subcutaneous tissues.

The CA-MRSA epidemic has provided an impetus to understand the immunopathogenesis of SSTIs in order to support the development of novel strategies to prevent and treat them. Innate immunity is the first line of defense against *S. aureus* SSTIs, including neutrophils, interleukin-1β (IL-1β), and pattern recognition receptors [7]. Recurrent infections, particularly SSTIs, are common, and the role of adaptive immunity against *S. aureus* infections is poorly understood. Furthermore, vaccines against *S. aureus* infection have been unsuccessful; several phase 3 clinical trials have failed despite encouraging preclinical results [8–11]. Interestingly, these vaccines elicited high antibody titers among vaccine recipients, raising the possibility that humoral immunity alone may be insufficient to fully protect against *S. aureus* infections [9, 10].

Evidence supporting a role for cell-mediated immunity in the host defense against *S. aureus* infections is emerging. For example, patients with poorly controlled HIV and low CD4+ T cell counts have high rates of *S. aureus* SSTIs (reviewed in reference [12]). In addition, patients with the hyper-IgE syndrome, in which Th17 function is impaired, are highly susceptible to *S. aureus* skin and lung infections [13], as are mice that are deficient in IL-17 [14, 15]. Therefore, targeting T cell responses against *S. aureus* may also be critical in developing protection against infection.

Investigation of the mechanisms of adaptive immunity against recurrent *S. aureus* infection has been hampered by a lack of an animal model in which "natural" immunity is elicited after primary infection. In this study, we compared the memory response to *S. aureus* SSTI in two genetic backgrounds and found that *S. aureus* SSTI strongly protected against secondary SSTI in BALB/c mice but much less so in C57BL/6 mice. Protection against dermonecrosis was mediated by antibody and IL-17A in BALB/c mice and inhibited by IFN-γ in C57BL/6 mice. Passive transfer of BALB/c immune serum into C57BL/6 mice was sufficient to limit lesion size upon infection, demonstrating a potential prophylactic or therapeutic avenue.

MATERIALS AND METHODS

Mouse model of *S. aureus* SSTI. All animal experiments were approved by and performed in accordance with the regulations of the Institutional Committee on the Care and Use of Animals at the University of Chicago. Our established model of *S. aureus* SSTI has been described [16]. Six-week-old female C57BL/6, BALB/c, T cell receptor (TCR) βδ$^{-/-}$ (B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J), CD4+ T cell-deficient (B6.129S2-*Cd4*^{tm1Mak}/J), and CD8+ T-cell deficient (B6.129S2-*Cd8a*^{tm1Mak}/J) mice

were purchased from Jackson Laboratory. BALB/c and B cell-deficient μMT ($Igh\text{-}J^{tm1Dhu}$) mice were purchased from Taconic.

Bacterial preparation. The USA300 clinical isolate SF8300 (provided by Henry Chambers, University of California, San Francisco [UCSF]) was used for all infections. Its virulence has been described (16). On the day of inoculation, an overnight culture of SF8300 was diluted 1:100 in fresh tryptic soy broth. The cultures were harvested 3 h later (approximate optical density at 600 nm [OD_{600}] of 1.8). The bacterial cells were pelleted by centrifugation, washed, and resuspended in sterile phosphate-buffered saline to achieve a concentration of 1.5×10^7 CFU/50 μl. The inocula were confirmed by plating serial dilutions on tryptic soy agar.

Inoculation and measurement of skin lesions. The mice were sedated, and the flank was shaved and cleaned with ethanol, after which 50 μl of *S. aureus* (or phosphate-buffered saline [PBS control]) was inoculated subcutaneously. Mice were observed to awaken and given access to food and water throughout the experiment. The first inoculation was performed on the right flank, and the second was performed on the left flank. For reinfection experiments, mice were first infected with PBS or *S. aureus*, and all mice were reinfected with *S. aureus* 8 weeks later; therefore, the mice were age matched. Mice were observed and lesions were photographed daily. The raw edge of the lesions was measured using Adobe Photoshop software, and the lesion size was calculated digitally compared with a 100-mm^2 standard. All measurements were performed by an observer blinded to the experimental groups.

Quantification of bacterial burden and local inflammatory response. Mice were sacrificed 3 days after infection, and the skin lesions were removed and homogenized. For bacterial quantification, serial dilutions of the homogenate were plated on mannitol salt agar, and colonies were enumerated 24 h later. The homogenized lesions were centrifuged, and enzyme-linked immunosorbent assay (ELISA) was performed with the supernatants to quantify CXCL-1 (R & D Biosystems), IL-17A (R & D Biosystems), and myeloperoxidase (Hycult Biotechnology). For some mice, skin lesions were removed and fixed in 10% neutral buffered formalin, following which they were paraffin embedded. Sections were stained with hematoxylin and eosin and then were analyzed and photographed using a Zeiss Axioskop microscope (Integrated Microscopy Facility at the University of Chicago).

$CD4^+$ T cell depletion. Neutralizing antibody against CD4 (rat IgG2b, clone YTS191) and isotype control rat IgG2b (clone LTF2) were purchased from BioXcell. For *in vivo* $CD4^+$ T cell depletion, mice received antibody (500 μg) via intraperitoneal injection 1 day prior to primary infection with *S. aureus*. An additional dose was given 7 days after infection. To confirm depletion, the mice were sedated and blood was obtained by retroorbital puncture. $CD4^+$ (anti-CD4–fluorescein isothiocyanate [FITC], clone RM4-5; eBioscience) and $CD8^+$ (anti-CD8–peridinin chlorophyll protein [PerCP], clone 53-6.7; BD Biosciences) T cells were quantified using a BD LSR II flow cytometer (BD Biosciences).

Antibody quantification. Enzyme immunoassay/radioimmunoassay (EIA/RIA) 96-well plates (Costar; Corning Inc.) were coated with 5 μg/ml alpha-hemolysin (Hla) (Sigma-Aldrich) or 25 μg/ml iron-regulated surface determinant B (IsdB) (Merck). Mouse serum was prepared from whole blood using serum separator tubes (BD Biosciences). The serum was diluted 1:200 in PBS and added to the antibody-containing wells. Detection was performed using alkaline phosphatase (AP)-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 (1:5,000; AffiniPure, Jackson ImmunoResearch) and AP substrate *p*-nitrophenylphosphate (pNPP) (Sigma-Aldrich), following the manufacturer's recommendations. Absorbance was measured using a GENios spectrophotometer (Tecan).

Serum transfer and antibody purification. Mice were sacrificed 14 days after secondary infection with *S. aureus* (or PBS) by CO_2 inhalation. Blood was obtained by cardiac puncture, and serum was isolated using serum separator tubes (Becton, Dickinson). Antibody was purified from immune BALB/c serum using protein A/G columns (Pierce). In order to fully remove antibody, 3 treatments were performed. Adoptive transfer of

serum or antibody was performed by retroorbital injection (100 μl) on each of the 2 days prior to infection.

T cell transfer. Mice were sacrificed 8 weeks after secondary infection with *S. aureus* (or PBS) by CO_2 inhalation, and the spleens were harvested and placed in sterile medium. T lymphocytes were isolated by negative selection using the Pan T cell isolation Kit II or the $CD8^+$ T cell isolation kit II (Miltenyi Biotec), according to the manufacturer's instructions. One day prior to infection, each recipient mouse received 8×10^6 T cells or PBS in a volume of 200 μl by retroorbital injection.

ELISpot. Enzyme-linked immunosorbent spot (ELISpot) plates (Millipore) were coated with anti-IFN-γ or anti-IL-17 antibody (BD Biosciences) and incubated at 4°C overnight. Wild-type BALB/c or C57BL/6 splenocytes depleted of T cells (rabbit serum complement; Sigma) were used as stimulator cells and incubated with heat-killed *S. aureus* (HTKL-SA) overnight at 37°C with 5% CO_2. The plates were blocked with proliferation medium for 1 h. Responder splenocytes were harvested from BALB/c and C57BL/6 mice and plated at 5×10^5/well with 2.5×10^5 stimulators. The plates were then incubated at 37°C, 5% CO_2, for 24 h. Biotinylated anti-IFN-γ and anti-IL17A detection antibodies (BD Biosciences) and horseradish peroxidase (HRP)-conjugated antibiotin were used as the primary and secondary antibodies, respectively (eBioscience). The plates were washed, and substrate solution was added (BD Biosciences); the reaction was terminated, and the plates were read using an ImmunoSpot series 1 analyzer (Cellular Technology).

Intracellular cytokine staining. HTKL-SA-specific stimulators and responders were prepared as described for the ELISPOT assays and plated in 96-well round-bottom plates with lids at concentrations of 5×10^5 and 1×10^6 per well, respectively, and were incubated at 37°C, 5% CO_2, for 18 h, followed by incubation with brefeldin A for 6 h. Cells were collected from plates and transferred to FACS tubes for antibody viability staining. After viability staining (LIVE/DEAD fixable violet dead cell stain, Invitrogen) and blocking nonspecific binding with anti-FcγR (2.4G2; University of Chicago Immunology Facility Core), the following antibodies were used to stain the cell surface at a concentration of 1 μg antibody per 10^6 cells for flow cytometry analysis: anti-CD90.2–phycoerythrin (PE)–Cy7 (clone 53-2.1; eBioscience), anti-CD4–FITC (clone RM4-5; eBioscience), anti-CD8–PerCP (clone 53-6.7; BD Biosciences), and anti-CD44–allophycocyanin (APC)–Cy7 (clone IM7; BD Biosciences). The cells were washed, and BD fix/perm solution (250 μl per 10^6 cells) was added. The cells were stained with anti-IFN-γ antibody (eBioscience) and anti-IL-17A antibody (eBioscience) (1 μl per 10^6 cells), washed, and analyzed using a BD LSR II flow cytometer (BD Biosciences).

Cytokine neutralization. Neutralizing antibodies against IL-17A (mouse IgG1, clone 17F3) and IFN-γ (rat IgG1, clone XMG1.2), as well as the isotype controls mouse IgG1 (clone MOPC-21) and rat IgG1 (clone HRPN), were purchased from BioXcell. For *in vivo* neutralization, mice received antibody (500 μg) via intraperitoneal injection 1 day prior to infection with *S. aureus*.

Data analysis. Data were compared using Student's *t* test or one-way analysis of variance (ANOVA) with the Tukey posttest, where appropriate. Differences were considered significant when *P* values were <0.05. All data were analyzed using GraphPad Prism software.

RESULTS

***S. aureus* SSTI strongly protected against secondary dermonecrosis in BALB/c mice but much less so in C57BL/6 mice.** We modified an established model of *S. aureus* SSTI with dermonecrosis (16) to assess the efficacy of a primary *S. aureus* SSTI in protecting against reinfection in two commonly used strains of mice, C57BL/6 and BALB/c. All BALB/c and C57BL/6 mice developed dermonecrotic lesions after primary infection with *S. aureus*, and there were no significant differences in the size of skin lesions observed in the mouse strains (data not shown). In contrast, while some BALB/c mice developed small raised abscesses within 3 to 5 days after the secondary *S. aureus* SSTI (8 weeks after the primary
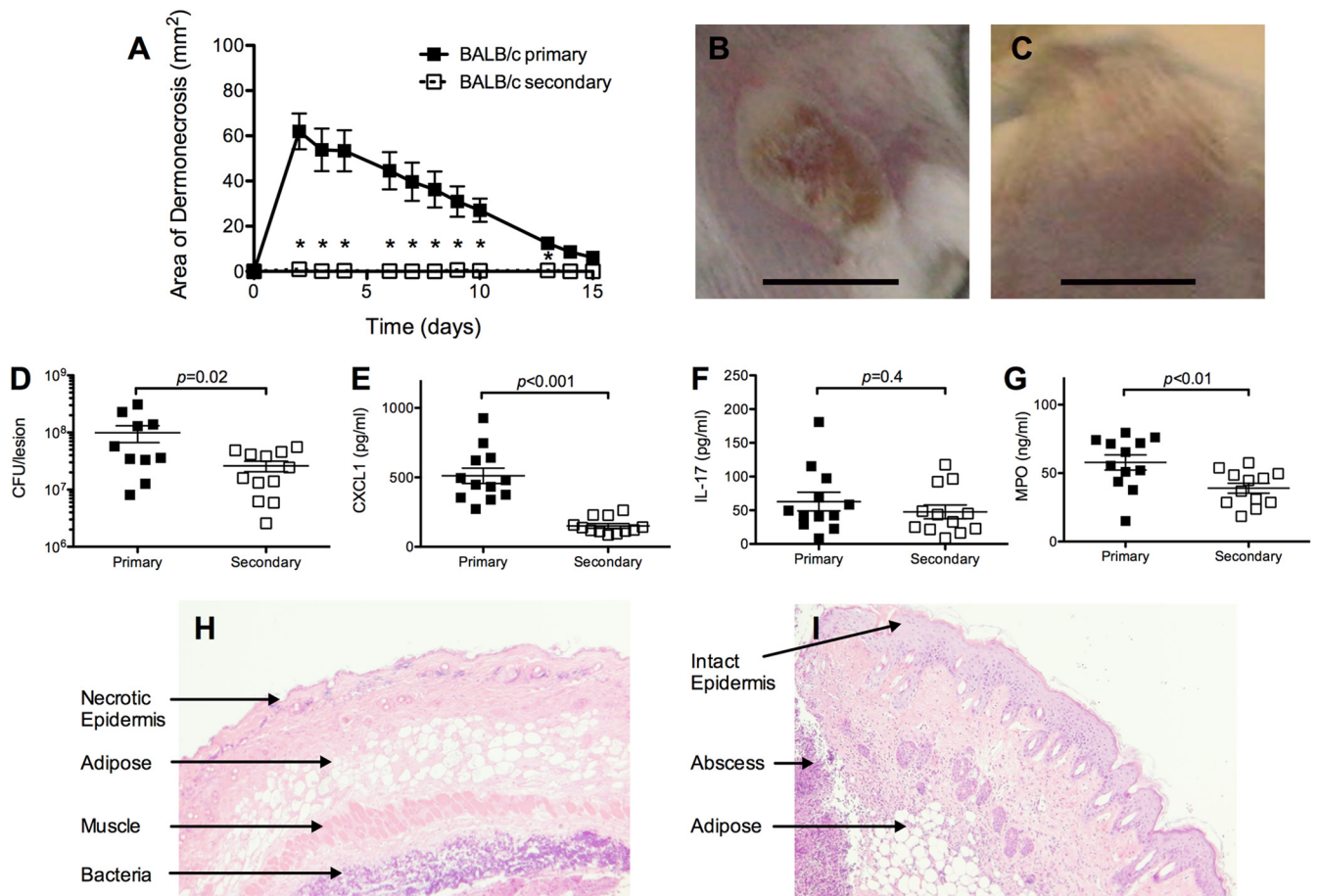
FIG 1 *S. aureus* SSTI strongly protected against recurrent infection in BALB/c mice. (A) BALB/c mice had dermonecrotic lesions after primary *S. aureus* SSTI but not after secondary infection ($n = 8$ to 10 mice/group). (B and C) Photograph of representative lesions after primary (B) or secondary (C) infection. There were fewer bacteria recovered from the lesions (D) and lower levels of CXCL-1 (E) and myeloperoxidase (MPO) (G) but no difference in IL-17A (F) 3 days after secondary infection compared with findings after primary infection. (H and I) Hemotoxylin-and-eosin-stained skin lesions from mice 3 days after primary (H) or secondary (I) SSTI. Data are presented as means ± SEM. ∗, $P < 0.05$ by Student's *t* test. Bars in photographs represent 1 cm. The results of one representative experiment are presented; each was repeated at least twice.

infection), none developed a dermonecrotic lesion, demonstrating that *S. aureus* SSTI induced protective immunity in BALB/c mice (Fig. 1A to C). In support of this, there were fewer bacteria recovered from the lesions of mice 3 days after secondary infection than after primary infection ($P = 0.02$) (Fig. 1D). There were also lower levels of myeloperoxidase (MPO), a marker of neutrophil and macrophage activity ($P < 0.01$) (Fig. 1G) and of the inflammatory chemokine CXCL-1 ($P < 0.001$) (Fig. 1E) but no difference in IL-17A ($P = 0.4$) (Fig. 1F) in the lesions of mice 3 days after secondary infection. The different nature of the skin lesions was evident with histologic analysis; skin lesions of BALB/c mice 3 days after primary infection were characterized by dermonecrosis (Fig. 1H). In contrast, there was evidence of subcutaneous abscess formation with intact epidermis after secondary infection (Fig. 1I). Therefore, the absence of dermonecrosis observed after secondary infection of BALB/c mice was associated with fewer bacteria recovered from the lesions and a less vigorous local inflammatory response.

In contrast, all C57BL/6 mice developed dermonecrotic lesions after secondary infection. Although the skin lesions observed were slightly smaller than those of C57BL/6 mice after primary infec-

tion ($P < 0.05$ on days 3 and 6; $P > 0.1$ thereafter) (Fig. 2A to C), there were no significant differences in numbers of bacterial CFU or levels of CXCL-1, IL-17A, or MPO recovered from the skin lesions of C57BL/6 mice 3 days after primary or secondary infection ($P = 0.2$) (Fig. 2D to G). Collectively, we found that *S. aureus* SSTI induced vastly superior protective immunity against secondary SSTI in BALB/c mice compared with C57BL/6 mice.

Adaptive immunity was necessary for protection against secondary skin infection. To determine if T cell responses contributed to protective immunity against secondary SSTI, BALB/c mice were treated with anti-CD4 neutralizing antibody prior to primary infection, resulting in depletion of CD4$^+$ T cells from the spleen, blood, and draining lymph nodes for up to 8 weeks (see Fig. S1A in the supplemental material). This depletion of CD4$^+$ T cells had no effect on primary SSTI (data not shown) but resulted in abrogation of protection against secondary SSTI; there was no significant difference in the size of skin lesions between CD4-depleted mice after secondary SSTI and control mice after primary infection ($P > 0.2$) (Fig. 3A). Because antibody responses require CD4$^+$ T cells, we quantified antibodies against alpha-hemolysin (Hla) and iron-regulated surface determinant B (IsdB), two anti-
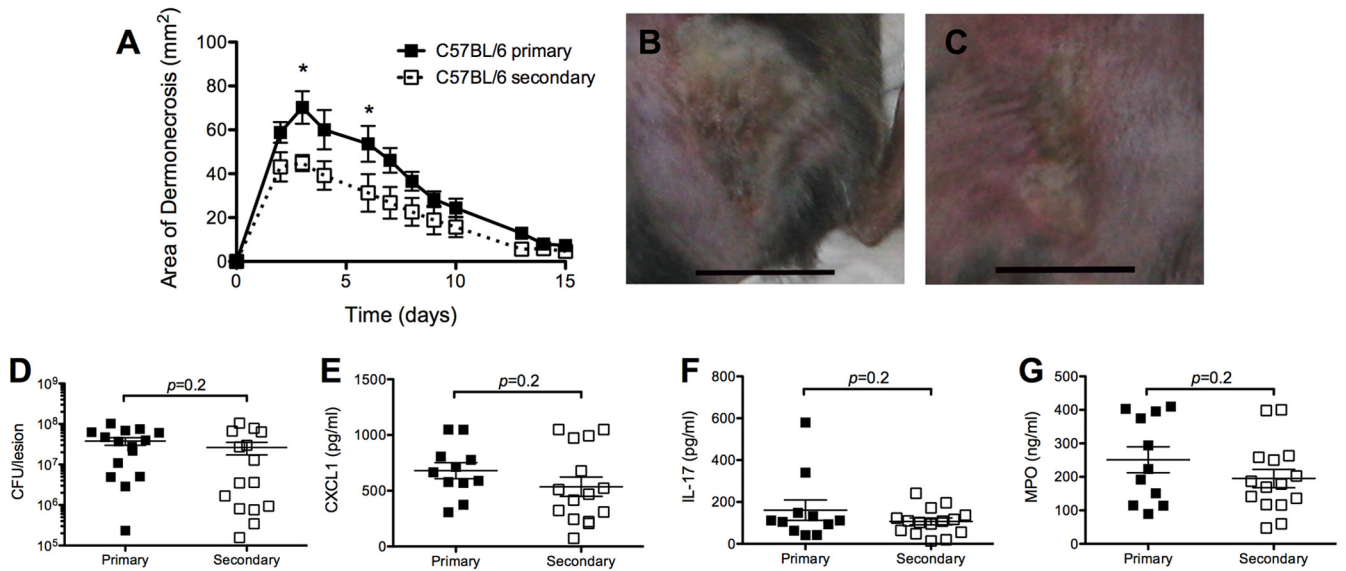
FIG 2 *S. aureus* SSTI protected minimally against recurrent infection in C57BL/6 mice. (A) C57BL/6 mice had smaller lesions 3 and 6 days after secondary SSTI than C57BL/6 mice had after primary infection ($n = 8$ to 10 mice/group). (B and C) Photographs of representative lesions after primary (B) or secondary (C) infection. There were no significant differences in the numbers of bacteria recovered from the lesions of C57BL/6 mice (D) or levels of CXCL-1 (E), IL-17 (F), or myeloperoxidase (MPO) (G) after secondary SSTI, compared with results after primary infection. Data are presented as means ± SEM. *, $P < 0.05$ by Student's *t* test. Bars in photographs represent 1 cm. For each experiment, the results of one representative experiment are presented; each was repeated at least twice.

gens reported to be important in the immune response against *S. aureus* and included in prospective vaccines (10, 17). While anti-Hla and anti-IsdB IgG were present in control mice after primary infection, both were undetectable in CD4-depleted mice (see Fig. S1B). We also found that the modest protection observed in C57BL/6 mice was not present in TCR $\beta\delta^{-/-}$ mice ($P = 0.8$, Fig. 3B). Therefore, protective immunity in both mouse backgrounds required T cells. However, because antibody responses were dependent on CD4$^+$ T cells, the relative contributions of antibody and T cells to protective immunity were examined.

Protective antibody was induced in BALB/c mice but not in C57BL/6 mice. To determine if differences in the antibody response were sufficient to explain the superior protection observed in BALB/c mice, naive and immune BALB/c serum was adoptively transferred to naive BALB/c mice prior to primary infection with *S. aureus*. Mice that received immune serum developed smaller dermonecrotic lesions than those that received PBS or naive se-

rum prior to infection ($P < 0.01$) (Fig. 4A). Adoptive transfer of antibody purified from immune BALB/c serum but not antibody-depleted immune serum conferred protection on naive mice, confirming that antibody mediated the protective effects of immune serum ($P < 0.01$) (Fig. 4B). In contrast to BALB/c mice, adoptive transfer of immune C57BL/6 serum to naive C57BL/6 mice had no effect on lesion size ($P > 0.3$) (Fig. 4C). This observation suggested that protective antibody was not elicited in C57BL/6 mice after *S. aureus* SSTI or that C57BL/6 mice were unable to respond effectively to passively administered antibodies.

To distinguish between these possibilities, we compared antibody responses against IsdB and Hla in BALB/c and C57BL/6 mice 8 weeks after primary and secondary infection. Antibody levels against both antigens increased after the primary infection and increased further after secondary infection in both mouse backgrounds (Fig. 4D and F). There were no significant differences in anti-IsdB total IgG levels between BALB/c and C57BL/6 mice after
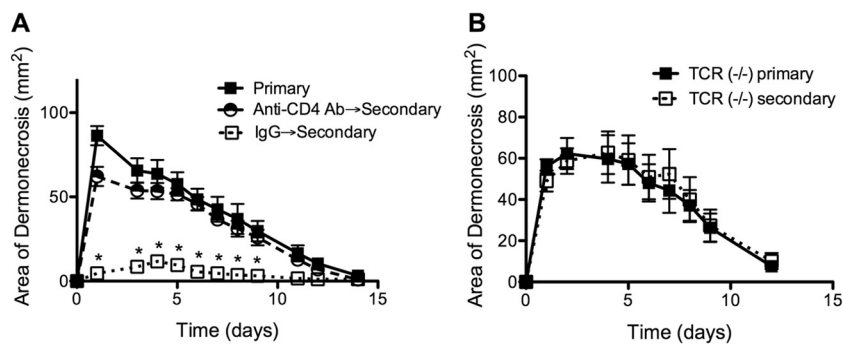

FIG 3 Adaptive immunity was necessary for protection against secondary *S. aureus* SSTI. (A) CD4$^+$ T cell depletion prior to primary infection of BALB/c mice abrogated protection after secondary SSTI compared with treatment with an isotype control antibody (IgG) ($n = 8$ mice/group). (B) There was no difference in lesion size between TCR $\beta\delta^{-/-}$ mice after primary or secondary infection (C57BL/6 background; $n = 8$ mice/group). Data are presented as means ± SEM. *, $P < 0.05$ by one-way ANOVA with Tukey's posttest. For each experiment, the results of one representative experiment are presented; each was repeated once.
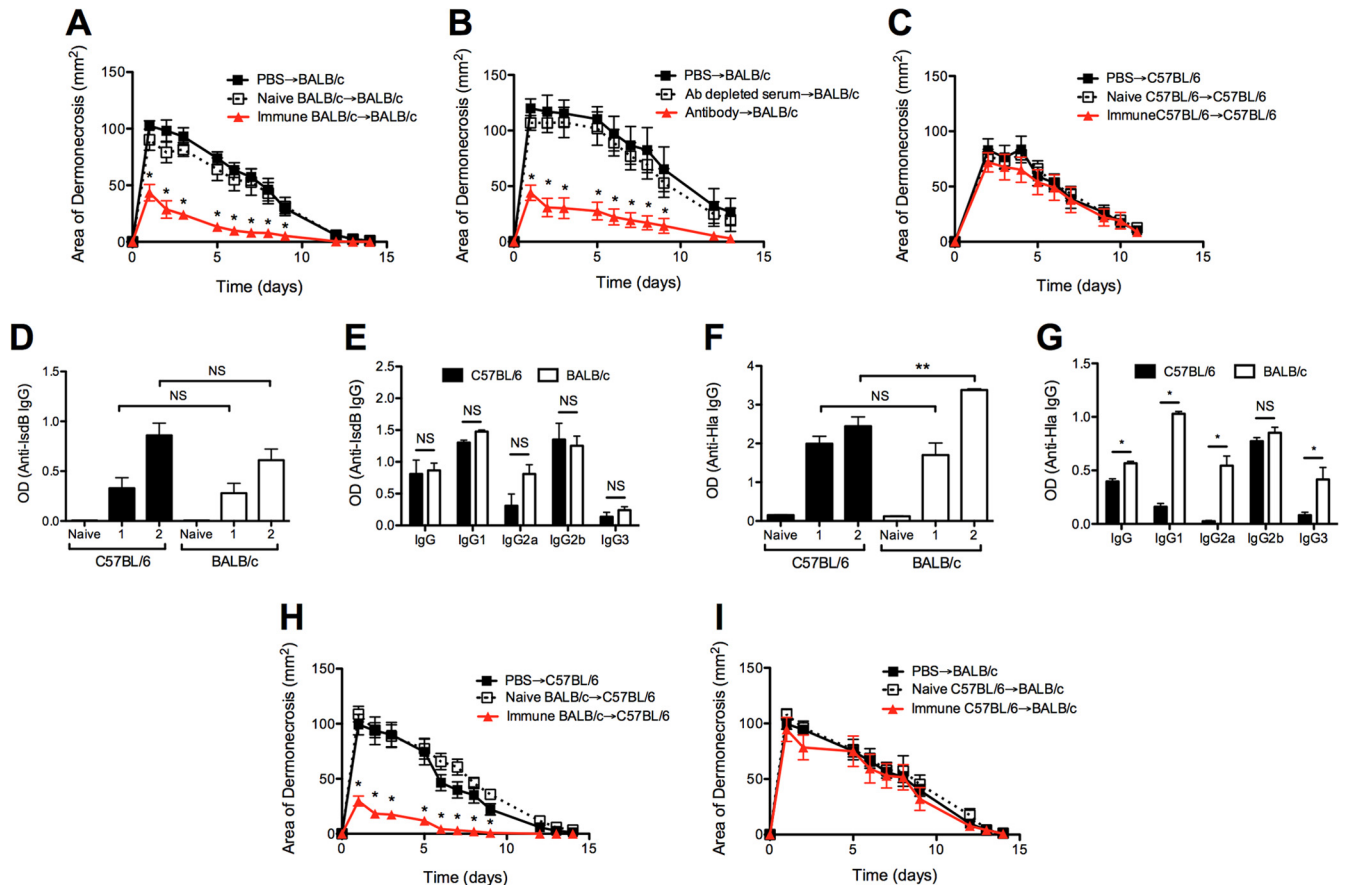
FIG 4 *S. aureus* SSTI elicited protective antibody in BALB/c mice but not in C57BL/6 mice. (A) Adoptive transfer of immune BALB/c serum into naive BALB/c mice prior to primary infection with *S. aureus* resulted in smaller lesions than were seen with naive serum or PBS. (B) Adoptive transfer of antibody purified from immune BALB/c serum but not antibody-depleted serum prior to primary infection resulted in significantly smaller lesions than were seen with BALB/c mice that received PBS. (C) Adoptive transfer of immune C57BL/6 serum into C57BL/6 mice prior to primary infection with *S. aureus* did not result in significantly smaller lesions than were seen with mice that received naive serum or PBS. (D) There were no significant differences in anti-IsdB IgG levels between C57BL/6 and BALB/c mice 8 weeks after primary ("1") or secondary ("2") infection. (E) There were no significant differences in anti-IsdB IgG1, IgG2a, IgG2b, or IgG3 after secondary infection between the groups. (F) Anti-Hla IgG levels were higher for BALB/c mice than for C57BL/6 mice after secondary but not primary infection. (G) There were higher levels of anti-Hla IgG1, IgG2a, and IgG3 but not IgG2b after secondary infection in BALB/c mice. (H) Adoptive transfer of immune BALB/c serum into C57BL/6 mice prior to primary infection resulted in smaller lesions than were seen with mice that received naive BALB/c serum or PBS. (I) In contrast, adoptive transfer of immune C57BL/6 serum into BALB/c mice prior to primary infection did not result in smaller lesions. Each experiment used 5 to 10 mice/group. Data are presented as means ± SEM. ∗, $P < 0.05$; ∗∗, $P < 0.01$ by one-way ANOVA with Tukey's posttest. The results of one representative experiment are presented; each was repeated at least twice.

primary infection or in levels of anti-IsdB total IgG, IgG1, IgG2a, IgG2b, or IgG3 after secondary infection (Fig. 4D and E). Similarly, total anti-Hla IgG levels did not differ between the two backgrounds after primary infection ($P = 0.4$) (Fig. 4F). In contrast, there were higher levels of anti-Hla total IgG, IgG1, IgG2a, and IgG3 ($P < 0.01$) but not IgG2b after secondary infection of BALB/c mice, compared with results for C57BL/6 mice (Fig. 4F and G). These data suggested that a selective difference in the antibody response in BALB/c mice from that in C57BL/6 mice might be the basis for differential antibody-mediated protection. To test this possibility, crossover serum transfer was performed, in which immune BALB/c serum was transferred into C57BL/6 recipients or vice versa. Immune BALB/c sera conferred protection to naive C57BL/6 recipients ($P < 0.01$) (Fig. 4H), while immune C57BL/6 sera did not confer protection to naive BALB/c recipients ($P > 0.3$) (Fig. 4I). Collectively, these results suggested that the superior protection observed in the BALB/c mice was mediated, at

least partly, by the quality of the protective antibody response and not by differences in antibody-mediated effector mechanisms that confer protection.

Protective T cell responses were induced in BALB/c mice but not in C57BL/6 mice. To test whether there also was a role for B cell/antibody-independent protective responses, we infected B cell-deficient (µMT) BALB/c mice. µMT mice had smaller lesions after secondary infection than after primary infection ($P < 0.05$) (Fig. 5A). This confirmed that some protection occurred even in the absence of B cells and protective antibodies, and we hypothesized that this protection was mediated by T cells.

The importance of T cells in protection was tested by the adoptive transfer of T cells from naive or immune BALB/c mice into naive BALB/c mice prior to primary infection. Indeed, BALB/c mice that received immune BALB/c T cells had smaller lesions than mice that received T cells from naive mice ($P < 0.01$) (Fig. 5B). In contrast, adoptive transfer of immune C57BL/6 T cells into
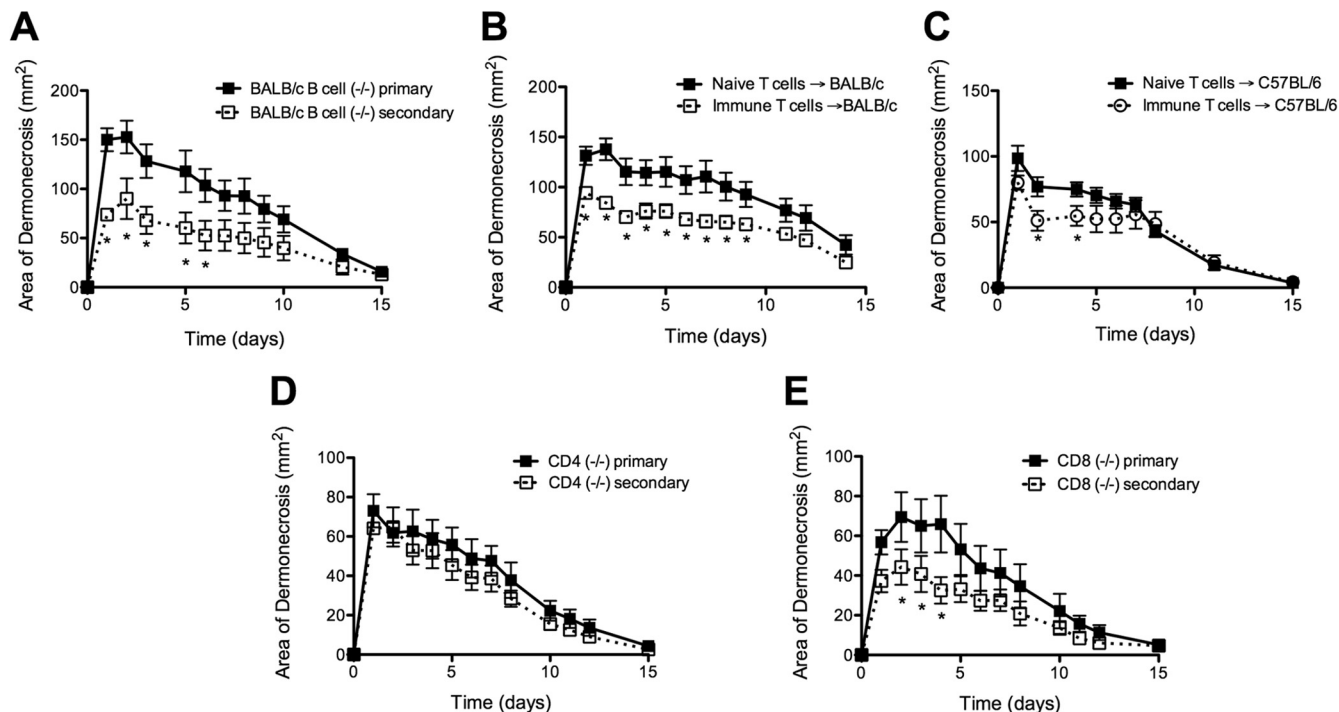
FIG 5 T lymphocytes mediated B cell/antibody-independent protective immunity in BALB/c mice but not in C57BL/6 mice. (A) Secondary infection of B cell-deficient BALB/c mice resulted in smaller skin lesions than those seen after primary SSTI ($n = 8$ mice/group). (B) Adoptive transfer of immune BALB/c T cells into BALB/c mice prior to primary infection resulted in smaller lesions than were seen with mice that received naive T cells ($n = 8$ mice/group). (C) In contrast, adoptive transfer of immune C57BL/6 T cells into C57BL/6 mice prior to primary SSTI resulted in minimal protection; lesions were significantly smaller 2 and 4 days after infection but not thereafter ($n = 8$ mice/group). (D) There were no differences in the size of skin lesions of CD4-deficient mice after primary or secondary SSTI ($n = 8$ mice/group). (E) There was a trend toward smaller lesions in CD8-deficient mice after secondary SSTI than after primary SSTI. Data are presented as means ± SEM. ∗, $P < 0.05$ by Student's t test. The results of one representative experiment are presented; each was repeated at least once.

naive C57BL/6 mice was minimally protective; skin lesions were smaller early after infection ($P < 0.05$ at 2 and 4 days), but these differences did not persist ($P > 0.1$) (Fig. 5C). There were higher numbers of IFN-γ and IL-17A staining cells among the transferred immune BALB/c T cells than among nonprotective naive T cells, but there were no differences in the numbers of CD8$^+$ T cells, suggesting that CD4$^+$ T cells mediated protective immunity (see Fig. S2A in the supplemental material). In further support of a role for CD4$^+$ T cells, adoptive transfer of immune CD8$^+$ T cells had no significant effects on lesion size ($P > 0.1$) (see Fig. S2B and C). Also, CD4$^{-/-}$ mice were not protected against secondary SSTI, while there was modest protection observed in CD8$^{-/-}$ mice (Fig. 5D and E). Thus, CD4$^+$ T cells contributed to the protective responses observed in BALB/c mice, and the protection conferred by immune T cells from BALB/c mice was greater than that of immune T cells from C57BL/6 mice.

To define differences in T cell responses in the two mouse backgrounds, IFN-γ and IL-17A ELISpot was performed with splenocytes isolated from C57BL/6 and BALB/c mice 8 weeks after primary and secondary SSTI. *S. aureus*-specific IFN-γ and IL-17A responses for both mouse backgrounds increased after primary and secondary infection compared with those for naive mice ($P < 0.01$) (Fig. 6A and B). The IFN-γ response was higher in C57BL/6 mice after primary infection than in BALB/c mice ($P < 0.01$) (Fig. 6A), whereas the IL-17A response was higher in BALB/c mice ($P < 0.001$) (Fig. 6B). The IFN-γ/IL-17A ratio was therefore higher in C57BL/6 mice than in BALB/c mice ($P < 0.05$) (Fig. 6C). Similar

responses were observed after secondary infection, with a stronger IFN-γ response in C57BL/6 mice ($P < 0.001$) and a higher IFN-γ/IL-17A ratio in C57BL/6 mice ($P < 0.001$) (see Fig. S3A to C in the supplemental material).

To confirm that IFN-γ and IL-17A were produced by CD4$^+$ T cells, intracellular cytokine staining was performed after secondary infection. Consistent with the ELISpot assay, we observed a higher percentage of CD4$^+$ CD44$^+$ IFN-γ$^+$ cells in C57BL/6 mice ($P < 0.001$) (see Fig. S3D in the supplemental material), a trend toward a higher percentage of CD4$^+$ CD44$^+$ IL-17A+ cells in BALB/c mice ($P = 0.08$) (see Fig. S3E), and a higher IFN-γ/IL-17A ratio in C57BL/6 mice ($P < 0.05$) (see Fig. S3F). Therefore, the superior protection in BALB/c mice, compared with that in C57BL/6 mice, was associated with a higher ratio of IL-17A-/IFN-γ-producing CD4$^+$ T cells after infection with *S. aureus*. Based on these observations, we hypothesized that IL-17A production by T cells was protective, whereas IFN-γ production was not.

IL-17A-polarized responses contributed to protection in BALB/c mice, whereas IFN-γ-polarized T cell responses inhibited protection in C57BL/6 mice. We tested whether IL-17A was necessary for protection against secondary SSTI in BALB/c mice by treatment with neutralizing antibody against IL-17A prior to reinfection, with the expectation that this would abrogate protection. However, neutralization of IL-17A had no effect on protection in BALB/c mice (data not shown), which could be explained by the contribution of antibody-mediated protective immunity. Therefore, we performed the same experiment with BALB/c μMT
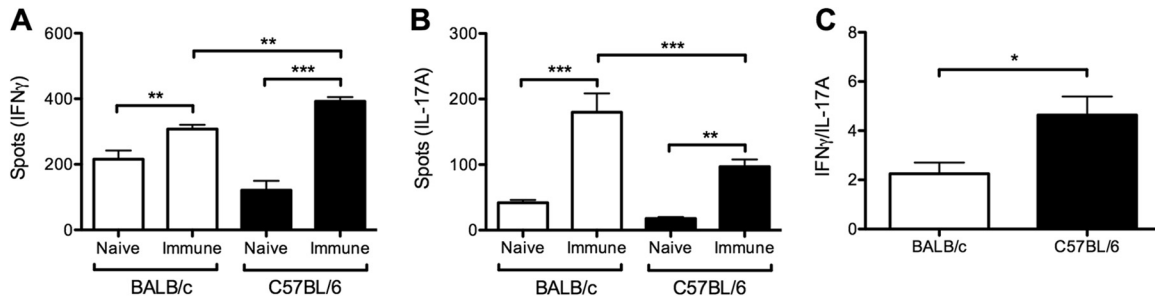
FIG 6 Protective immunity in BALB/c mice was associated with a Th17/IL-17A-polarized response, compared with a Th1/IFN-γ-polarized response in C57BL/6 mice. *S. aureus*-specific T cell responses were assessed 8 weeks after primary infection with *S. aureus* by ELISpot (*n* = 3 to 5 mice/group). IFN-γ (A) or IL-17A (B) produced by *S. aureus*-stimulated splenocytes was increased in C57BL/6 and BALB/c mice after primary infection compared with results for naive controls. (A) IFN-γ responses were higher for C57BL/6 mice than for BALB/c mice. (B) In contrast, IL-17A responses were higher for BALB/c mice than for C57BL/6 mice. (C) IFN-γ/IL-17A ratios were higher for C57BL/6 mice than for BALB/c mice. Data are presented as means ± SEM. ∗, *P* < 0.05; ∗∗, *P* < 0.01; ∗∗∗, *P* < 0.001; by one-way ANOVA with Tukey's posttest. The results of one representative experiment are presented; each was repeated at least once.

mice. Consistent with a role for IL-17A in protection, neutralization of IL-17A prior to secondary SSTI in μMT mice abrogated protection (*P* < 0.01) (Fig. 7A). Furthermore, the protection mediated by adoptive transfer of immune BALB/c T cells into naive BALB/c mice was abolished by the administration of anti-IL-17A antibody at the time of T cell transfer (*P* < 0.05) (Fig. 7B). The importance of IL-17A was limited to adaptive immune responses, because treatment with anti-IL-17A antibody had no effect on lesion size in primary infection (*P* > 0.4) (see Fig. S4A in the supplemental material). These data collectively demonstrate that IL-17A mediated the protection conferred by transfer of immune T cells.

Because the poorer protection in C57BL/6 mice was associated with an IFN-γ-polarized (versus IL-17A) response, we tested whether neutralization of IFN-γ prior to secondary SSTI in C57BL/6 mice would enhance protection and reduce lesion severity. Consistent with this hypothesis, treatment of C57BL/6 mice with neutralizing anti-IFN-γ antibody prior to secondary infection resulted in smaller lesions than those in control mice (*P* < 0.01) (Fig. 7C). As we observed with IL-17A, the effects of IFN-γ neutralization were specific for memory responses, because treatment with anti-IFN-γ antibody prior to primary SSTI had no

effect on lesion size (*P* > 0.4) (see Fig. S4B in the supplemental material). Taken together, these findings demonstrate that the IFN-γ-skewed responses in C57BL/6 mice, in combination with a poor antibody response, resulted in poor protection against secondary SSTI.

DISCUSSION

We have described herein a novel mouse model in which *S. aureus* SSTI successfully elicited protective immunity against dermonecrosis during secondary *S. aureus* SSTI. Furthermore, this protective immunity was mediated by antibody and IL-17A and prevented by IFN-γ. These observations demonstrate that, contrary to popular belief, *S. aureus* SSTIs are in fact capable of eliciting memory responses that protect against subsequent infection, but the development of protective immunity was critically dependent on the genetic background of the host. Although several previous studies have suggested that *S. aureus* infections elicit protective immunity, there are important differences between those and our findings. For example, Agarwal described the protective effects of *S. aureus* SSTI against recurrent infection in Wright-Fleming Institute mice, but the model required the coadministerion of cotton dust to enhance lesion formation (18). Others have found that
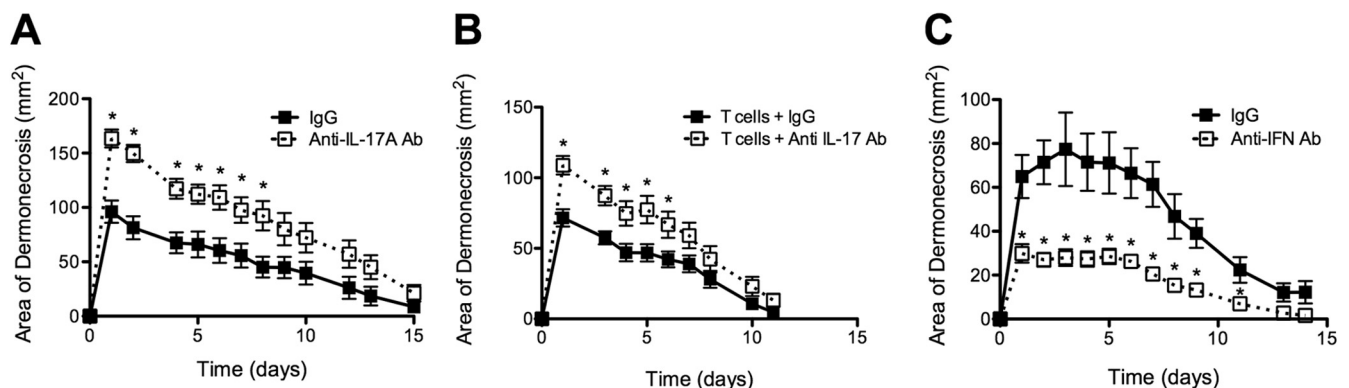

FIG 7 Th17/IL-17A mediated B cell/antibody-independent protection in BALB/c mice, whereas Th1/IFN-γ inhibited protection in C57BL/6 mice. (A) BALB/c B cell-deficient mice treated with IL-17A neutralizing antibody prior to secondary SSTI had larger lesions than mice treated with an isotype control antibody (IgG) (*n* = 9 to 12 mice/group). (B) Treatment with IL-17A neutralizing antibody after immune T cell transfer resulted in larger lesions than were seen with mice that received an isotype control antibody (*n* = 8 mice/group). (C) C57BL/6 mice treated with IFN-γ neutralizing antibody prior to secondary SSTI had smaller lesions than mice treated with an isotype control antibody (IgG) (*n* = 6 to 8 mice/group). Data are presented as means ± SEM. ∗, *P* < 0.05 by Student's *t* test. The results of one representative experiment are presented; each was repeated at least once.

mutant *S. aureus* strains but not wild-type isolates elicit adaptive immunity against *S. aureus* sepsis in single strains of mice (19–22). Therefore, our model, in which a natural SSTI with a wild-type *S. aureus* clinical isolate elicited protective immunity in BALB/c but not C57BL/6 mice, afforded us a unique opportunity to dissect the determinants of protection against recurrent infection.

The vastly superior protection against dermonecrosis observed in BALB/c mice after secondary SSTI, compared with results for C57BL/6 mice, was characterized by the absence of dermonecrotic lesions, fewer bacteria recovered from the skin lesions, and a less vigorous local inflammatory response. It is noteworthy that despite the absence of dermonecrosis, the decrease in bacterial recovery was less than 1 log, suggesting that limiting local inflammation may also be important in determining the severity of infection. Protection from dermonecrosis was abrogated in BALB/c mice when $CD4^+$ T cells were depleted prior to primary infection and in C57BL/6 TCR $\beta\delta^{-/-}$ mice, demonstrating that a $CD4^+$ T cell-dependent adaptive immune response mediated protection. Humoral immunity, dependent on $CD4^+$ T cells, was critical to the development of protective immunity in BALB/c mice, because adoptive transfer of immune serum into naive mice prior to primary infection resulted in smaller lesions. We confirmed that antibody mediated protective immunity in BALB/c mice by demonstrating that adoptive transfer of antibody purified from immune serum but not antibody-depleted immune serum conferred protection.

Whereas BALB/c serum was protective against dermonecrosis when transferred to either C57BL/6 or BALB/c mice, C57BL/6 serum did not confer protection to either mouse strain. These observations suggest important differences in the serologic response in these two strains of mice but not in their ability to respond to protective antibodies by limiting the severity of *S. aureus* SSTI. In support of this hypothesis, we observed higher levels of anti-Hla total IgG, IgG1, IgG2a, and IgG3 but not IgG2b after secondary infection of BALB/c mice than were seen for C57BL/6 mice. Anti-Hla antibodies have been reported to be protective (17, 23), but whether differences in the anti-Hla IgG response are the basis for the difference in protection or hint at a broader difference in the quality of antibody response between C57BL/6 and BALB/c mice is under investigation.

The findings that *S. aureus* SSTI elicited antibody responses in both C57BL/6 and BALB/c mice, but only BALB/c responses were protective, may have important implications in understanding the mechanisms of protective immunity against *S. aureus*. In particular, the observation that naturally occurring antibodies against many *S. aureus* antigens develop shortly after birth but have not been demonstrated to be protective led to the conclusion that antibodies are not protective (24, 25). Our data suggest an alternative hypothesis that these antibodies may resemble the polyclonal antibody response elicited by *S. aureus* SSTI in C57BL/6 mice, where there is no protection. Our data further suggest that *S. aureus* antigens specifically responsible for protection can be identified by comparing the specificity of the antibody response after *S. aureus* infection in BALB/c and C57BL/6 mice.

Recent findings support a role for specific T cells in defense against *S. aureus* infections, particularly SSTI, primarily by enhancing recruitment of phagocytes. For example, patients with the hyper-IgE syndrome, in whom Th17 pathways are defective, have increased risk of *S. aureus* skin and lung infection (13). These clinical observations are supported by experimental evidence; for example, IL-17-deficient mice develop spontaneous *S. aureus* mucocutaneous infections (15). IL-17 deficiency in mice also specifically inhibited skin and lung defenses against *S. aureus* by limiting the production of antimicrobial peptides and neutrophil-recruiting chemokines in keratinocytes and bronchial epithelial cells (26). Our data also support a role for T cells and IL-17 in protective immunity against recurrent *S. aureus* SSTI. B cell-deficient BALB/c μMT mice were protected against secondary SSTI, and adoptive transfer of immune BALB/c T cells conferred protection to naive mice. As we observed with antibody-mediated protection, *S. aureus* SSTI elicited protective T cell responses in BALB/c mice but not C57BL/6 mice. The *S. aureus*-specific T cell response was strongly skewed to Th1 in C57BL/6 mice, whereas the Th1 and Th17 responses in BALB/c mice were more balanced. Neutralization of IFN-γ improved protection in C57BL/6 mice, whereas neutralization of IL-17A abrogated protection in BALB/c mice. Therefore, our observations are consistent with recent reports that the Th17/IL-17 pathway is protective and that Th1/IFN-γ responses are deleterious (14, 27–31). However, our observations suggest that the ratio of the Th17 responses to Th1 responses rather than the magnitude of the Th17 response *per se* determines protection and that this ratio of T cell responses is determined by the genetic background of the infected host.

Our findings underscore the importance of considering the contribution of the genetic background to protective immunity. Although different mouse strains have been found to have variable resistance against *S. aureus* bacteremia (32, 33), we did not find any difference in lesion size after primary infection between C57BL/6 and BALB/c mice. In contrast, we found that adaptive T cell and antibody responses differed markedly between infected C57BL/6 and BALB/c mice. We speculate that these differences in mice may reflect the potential spectrum of immune responses against *S. aureus* infection in humans. Indeed, the genetic background of the host is increasingly appreciated as being important in defining the immune response. For example, the genomic responses in C57BL/6 mouse models of burn injury, endotoxemia, and trauma poorly reflected those observed in human disease (34), although it is possible that other mouse genetic backgrounds may have responses that are more similar to those of humans. Therefore, it is critical to assess immunity against *S. aureus* infection in multiple mouse backgrounds in order to fully appreciate the translational impact. An alternative approach would be to use outbred mice to more fully define the spectrum of immune responses that are elicited by *S. aureus* SSTI.

The failure of human vaccines against specific *S. aureus* antigens despite high vaccine-specific antibody titers among vaccine recipients has led to a reconsideration of vaccine design (reviewed in reference 8). Our findings support the idea of a multimechanistic (i.e., targeting both humoral and cell-mediated immunity) approach by demonstrating important roles for both antibody and T cells in protecting against secondary SSTI. This notion is further supported by experimental findings that both antibody (17, 19, 23) and T cell/Th17 (35–37) vaccination strategies have shown promise in preclinical studies.

In summary, we observed that primary *S. aureus* SSTI strongly protected against dermonecrosis during secondary SSTI in BALB/c mice, but protection was greatly inferior in C57BL/6 mice. This protection was mediated by antibody and also by B cell/antibody-independent mechanisms dependent on IL-17A and curtailed by IFN-γ. These findings advance our understanding of the

fundamental mechanisms of adaptive immunity against *S. aureus* SSTI, provide insight into why protection is sometimes not observed despite evidence of a strong adaptive immune response, and suggest that vaccine strategies aimed at eliciting the appropriate T cell-mediated and humoral immunity should be prioritized.

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