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Murine Immune Response to a Chronic *Staphylococcus aureus* Biofilm Infection▽†

Ranjani Prabhakara,[1,2] Janette M. Harro,[1] Jeff G. Leid,[3] Megan Harris,[1,2] and Mark E. Shirtliff[1,4]*

Department of Microbial Pathogenesis, Dental School, University of Maryland, Baltimore, Maryland[1]; Graduate Program in Life Sciences, Microbiology and Immunology Program, School of Medicine, University of Maryland, Baltimore, Maryland[2]; Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona[3]; and Department of Microbiology and Immunology, School of Medicine, University of Maryland, Baltimore, Maryland[4]

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Staphylococcus aureus **has reemerged as an important human pathogen in recent decades. Although many infections caused by this microbial species persist through a biofilm mode of growth, little is known about how the host's adaptive immune system responds to these biofilm infections. In this study, *S. aureus* cells adhered to pins in culture and were subsequently inserted into the tibiae of C57BL/6 mice, with an infecting dose of 2×10^5 CFU. This model was utilized to determine local cytokine levels, antibody (Ab) function, and T cell populations at multiple time points throughout infection. Like human hosts, *S. aureus* implant infection was chronic and remained localized in 100% of C57BL/6 mice at a consistent level of approximately 10^7 CFU/gram bone tissue after day 7. This infection persisted locally for >49 days and was recalcitrant to clearance by the host immune response and antimicrobial therapy. Local inflammatory cytokines of the Th1 (interleukin-2 [IL-2], IL-12 p70, tumor necrosis factor alpha [TNF-α], and IL-1β) and Th17 (IL-6 and IL-17) responses were upregulated throughout the infection, except IL-12 p70, which dwindled late in the infection. In addition, Th1 Ab subtypes against a biofilm antigen (SA0486) were upregulated early in the infection, while Th2 Abs and anti-inflammatory regulatory T cells (Tregs) were not upregulated until later. These results indicate that early Th1 and Th17 inflammatory responses and downregulated Th2 and Treg responses occur during the development of a chronic biofilm implant infection. This unrestrained inflammatory response may cause tissue damage, thereby enabling *S. aureus* to attach and thrive in a biofilm mode of growth.**

One of the most common and costly problems for the U.S. health care system is nosocomial infections (22), with *Staphylococcus aureus* being the second leading cause of such infections (5). Methicillin-resistant *S. aureus* (MRSA) is responsible for 40 to 60% of all nosocomially acquired *S. aureus* infections, and these resistant strains are now considered to be endemic in the hospital setting (29). Community-associated *S. aureus* strains may also acquire methicillin resistance (community-associated MRSA [CA-MRSA]), and the modern emergence of such strains is of great concern (18, 23, 47).

Recent studies indicate that *S. aureus* is also the major mediator of prosthetic implant infection (1, 38). The increasing involvement of *S. aureus* in foreign body-related infections, the rapid development of resistance to multiple antibiotics by these organisms, and the propensity of these infections to change from an acute infection to one that is persistent, chronic, and recurrent have led to this organism once again receiving significant attention.

Treatment of prosthetic implant infections is a complicated process, and a number of staphylococcal defense mechanisms may be responsible for this difficulty, as well as the capacity of *S. aureus* to evade clearance by the host immune response. One of the most important mechanisms utilized by *S. aureus* to thwart the host immune response and develop into a persistent infection is the formation of a highly developed biofilm. A biofilm is defined as a microbe-derived community in which bacterial cells are attached to a hydrated surface and embedded in a polysaccharide matrix (13). Bacteria in a biofilm exhibit an altered phenotype in their growth, gene expression, and protein production (15), and prosthetic medical devices are often a site of chronic infection because they present a suitable substrate for bacterial adherence, colonization, and biofilm formation. Biofilm formation by *S. aureus* during prosthetic implant infection makes eradication of this bacteria extremely difficult, due in part to the dramatically increased resistance of bacteria in a biofilm to host defenses (16) and to antibiotics (35, 36) compared to that of their planktonic counterparts.

S. aureus elicits a strong inflammatory response, resulting in the migration of large numbers of neutrophils and macrophages to the site of infection. A majority of *S. aureus* strains have been shown to elicit the production of interleukin-1β (IL-1β), IL-6, and IL-12 p70 in monocytes *in vitro*, and this may result in biasing the immune response toward a Th1-type response *in vivo* (33). Staphylococcal enterotoxin B (SEB) has been shown to induce *in vitro* expression of IL-2 and gamma interferon (IFN-γ) with rapid and intermediate kinetics, respectively, but slow expression of IL-10, a Th2 cytokine (2). The superantigen, staphylococcal enterotoxin A (SEA), also elicits a strong Th1 response *in vitro*, with concomitant production of tumor necrosis factor alpha (TNF-α) and MIP-1α (14). Another staphylococcal toxin, alpha-toxin, has been

* Corresponding author. Mailing address: Department of Microbial Pathogenesis, Dental School, University of Maryland—Baltimore, 650 W. Baltimore Street, 8th floor south, Baltimore, MD 21201. Phone: (410) 706-4720. Fax: (410) 706-0865. E-mail: mshirtliff@umaryland.edu.

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shown to increase IFN-γ production in $CD4^+$ T cells *in vitro* and to increase binding of DNA to T-bet, a transcription factor involved in commitment to a Th1 response (7). Protein A is also a potent inducer of Th1 cytokines such as IFN-γ, TNF-α, and IL-1 in mice receiving intraperitoneal (i.p.) injections of protein A (42).

While the studies described above hint that a Th1-biased adaptive immune response could result from *S. aureus* infection, relatively little is known about Th2, Th17, and regulatory T cell (Treg) responses in an *in vivo* model of *S. aureus* biofilm infection. Also, it is unknown how the host immune system responds to *S. aureus* as it progresses from an acute to a chronic infection that resists clearance by the host immune system. Determination of the phenotypic characteristics and activation states of infiltrating immune cells, IgG subisotypes produced, and cytokines elicited during acute and chronic *S. aureus* infection may provide insight into the mechanisms of immune evasion used by *S. aureus* to establish a chronic biofilm infection. This knowledge will also further our understanding of why the host does not mount an effective immune response and is ineffective in the clearance of this pathogen.

An augmented Th2 response was previously shown to be effective at preventing a biofilm infection in the early phase of formation (34, 41, 44). However, this antibody (Ab)-mediated response may be downregulated both by the early host cytokine response to *S. aureus* infection and by the *S. aureus* superantigens, capsule, and other toxins, but this has not been previously studied *in vivo*. Although a recent mouse model of prosthetic implant infection was developed (28), the resulting infection did not exhibit all of the hallmarks of a true biofilm infection, as the host and antibiotic therapy were able to clear the infection. Therefore, we developed a mouse model of biofilm infection that is recalcitrant to the host immune response and antimicrobial agent clearance.

In order to characterize the host cellular, Ab, and cytokine responses to *Staphylococcus aureus* biofilm-mediated implant infections, we adapted a mouse model of *S. aureus* implant infection using a biofilm-forming strain of MRSA isolated from an infected prosthetic implant. Biofilms were formed on stainless steel pins and then implanted into C57BL/6 mice (12). After implantation with pins with adherent *S. aureus* cells, viable bacteria could be cultured from the infected pin at 49 days postinfection, even in the presence of vancomycin. In addition, imaging studies demonstrated a well-developed biofilm on the infected pins, thus indicating the development of a chronic biofilm-mediated implant infection. In C57BL/6 mice, implantation of *S. aureus*-coated pins led to the activation of a CD4 response and the early production of IgG2b (the dominant Th1-associated IgG subtype) against the biofilm-upregulated antigen SA0486 (6). In addition, Th1 and Th17 cytokines were present at the implant site, and Tregs were suppressed early in the infection. These studies suggest that staphylococcal infection resulted in the skewing of the host immune response toward proinflammatory Th1 and Th17 responses, which fail to clear the infection.

S. aureus biofilm infections present a very serious and costly problem. Furthering our knowledge of how the host immune system fails to clear this pathogen will help the scientific community to find better control and therapeutic strategies.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 mice (6 to 8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained under microisolator conditions in the animal facility at the University of Maryland School of Medicine (Baltimore, MD), in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Bacterial strain and preparation of implants. The strain of *S. aureus* used in these experiments, MRSA-M2 (M2), is a clinical isolate obtained from an osteomyelitis patient undergoing treatment at the University of Texas Medical Branch (Galveston, TX) and has been used in previous biofilm molecular analyses and animal infection models (6, 26, 32, 39). The well-characterized *S. aureus* strain UAMS-1 was also used in infection studies and antibody isotype studies (see below) in order to ensure that the results were not limited to the M2 strain (4, 10, 11, 17, 37, 43). Autoclaved 0.25-mm insect pins (Fine Science Tools, Foster City, CA) were incubated for 2 h in 10 ml of an overnight culture of *S. aureus* that was diluted 1:100 in sterile Trypticase soy broth.

Cloning, expression, and purification of proteins. Candidate antigens selected by Brady et al. (6) were amplified using the following primers: 5′-ACTCTAGG TCTCACTCCAAAGAAGATTCAAAAGAAGAACAAAT-3′ and 5′-ATGGT AGGTCTCATATCAGCTATCTTCATCAGACGGCCCA-3′. The PCR products were cloned into pASK-IBA14, transformed into *Escherichia coli* TOP10, and sequenced. The clones were then expressed using anhydrotetracycline induction. SA0486 was purified via Strep-Tactin Superflow columns (IBA, Göttingen, Germany). Purity was confirmed by resolving each protein on 15% SDS-PAGE, and quantities were determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Surgical implantation of pins. Four to eight mice per experimental group received tibial implants. Mice were anesthetized via i.p. injection of 100 mg ketamine/kg of body weight (Ketaset; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and 10 mg xylazine/kg (Rugby Laboratories, Inc., Rockville Center, NY). The left leg of each mouse was cleansed with povidone iodine and rinsed with 70% ethanol before surgical implantation of an *S. aureus*-coated or uninfected control pin, according to the methods previously described by Li et al. (28). For antimicrobial efficacy experiments, mice were treated via subcutaneous (s.c.) injection of 50 mg of vancomycin/kg twice daily for 10 days beginning on day 14 postimplantation, which is approximately 10-fold higher than that used in previous studies (9, 24, 45). All other mice did not undergo any additional treatments after surgery until sacrifice. All animal experiments were performed in accordance to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland School of Medicine (Baltimore, MD). Nonimplanted 0.5-mm sections of pins incubated with *S. aureus* were homogenized and cultured to determine the infecting dose upon pin implantation. It was determined that approximately 2×10^5 CFU/pin section (standard deviation [SD] = 5×10^4) was delivered to the tibia for infection.

Bone cultures. At 4, 7, 14, 21, 28, and 49 days postimplantation, infected and uninfected mice were euthanized, left tibiae were removed, and all soft tissue was dissected from the bone. Using sterile scissors, tibiae were cut into small pieces and placed in 300 μl of 0.85% sterile saline per 100 μg of bone. Bones were homogenized using a Polytron PT 1200 handheld homogenizer (Kinematica, Bohemia, NY), and serial 10-fold dilutions of bone homogenates were plated on sheep's blood agar plates to enumerate the number of viable *S. aureus* cells per g bone. Additionally, 0.5-mm sections of pins representing the lengths inserted into the tibiae of mice were incubated with *S. aureus* as described above and processed for culture in order to determine the infecting dose.

PNA-FISH biofilm detection on explanted pins. Infected and uninfected sterile pins were inserted into the tibia of mice. Pins were carefully removed from the tibiae of infected and uninfected mice to prevent perturbation of biofilm mass at 7 and 21 days postimplantation. Pins were placed in Eppendorf tubes and fixed in 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) before peptide nucleic acid fluorescent *in situ* hybridization (PNA-FISH) with a fluorescein isothiocyanate (FITC)-labeled *S. aureus* probe and a rhodamine-labeled universal eukaryotic cell probe, as per the manufacturer's instructions (AdvanDx, Woburn, MA). Each pin was then examined with a Zeiss LSM 510 confocal scanning laser microscope (Carl Zeiss, Thornwood, NY) for both green and red fluorescence using a FITC/Texas Red dual-band filter and a 63× objective.

Measurement of serum IgG subisotype level. Blood samples obtained from mice that had received tibial implants were collected at 0, 7, 14, 21, and 28 days postimplantation, allowed to clot at room temperature for 20 min, and then centrifuged at $4{,}000 \times g$ for 15 min. Sera were separated from clotted cells and stored at −70°C until ready for use. A high-binding-capacity 96-well enzyme-linked immunosorbent assay (ELISA) plate (Becton Dickinson, Bedford, MA)

was coated with 100 μl of antigen/well (100 ng/well) diluted in PBS and incubated overnight at 4°C. After the wells were washed three times with PBS containing 0.5% Tween 20 (PBST), nonspecific binding activity was blocked by addition of 200 μl of 1% BSA/well and incubation at room temperature for 2 h. After wells were washed three times with PBST, test sera were added at the appropriate dilutions and incubated for 1 h at room temperature. After sample incubation, wells were washed three times with PBST. Rabbit anti-mouse IgG1, IgG2a, and IgG2b secondary Abs (Invitrogen, Carlsbad, CA) were added to the appropriate wells and incubated at room temperature for 1 h. Following five washes with PBST, plate-bound IgG1, IgG2a, and IgG2b were detected with 50 μl of affinity-purified horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA)/well. The colorimetric reaction was developed using OptEIA (BD Biosciences, San Jose, CA) as the substrate (50 μl/well), and color intensity was read at 450 nm. Color intensity was compared to a standard curve for each IgG subtype, and results were expressed as the number of pg/ml.

Measurement of cytokine levels at the implant site. Implanted tibiae and the surrounding soft tissue were harvested from mice at days 7 and 28 postimplantation and stored at −70°C. Samples were homogenized on ice in sterile PBS containing an EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Tissue homogenates were centrifuged for 15 min at 14,000 × *g* at 4°C, and supernatants were analyzed by the Cytokine Core Laboratory at the University of Maryland School of Medicine (Baltimore, MD) using quantitative multiplex sandwich ELISA technology. Cytokines tested included murine IL-2, IL-4, IL-6, IL-10, IL-12 p70, IL-17, and TNF-α.

$CD4^+$ and $CD8^+$ frequency analysis by flow cytometry. Draining lymph node (LN) cells from mice were harvested at days 4, 7, 14, 21, and 28 postimplantation, and single-cell suspensions were prepared. To determine the $CD4^+$ and $CD8^+$ T cell frequency, 1×10^6 LN cells were aliquoted into fluorescence-activated cell sorter (FACS) tubes (Becton Dickinson, Bedford, MA) and surface stained with FITC-labeled anti-mouse CD3, phycoerythrin (PE)-labeled anti-mouse CD8, peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD4, and allophycocyanin (APC)-labeled anti-mouse CD44 MAbs (BD Biosciences, San Diego, CA). Cells were analyzed using either a FACScan or an LSR II flow cytometer (BD Biosciences, San Jose, CA), and results were expressed as the percentages of $CD4^+$, $CD8^+$, and $CD44^+$ cells after gating on the lymphocyte population.

Treg frequency analysis by flow cytometry. For Treg analysis, 1×10^6 draining LN cells were aliquoted into FACS tubes (Becton Dickinson, Bedford, MA), and a Treg FACS staining kit (eBioscience, San Diego, CA) was used to determine the frequency of Tregs in draining LN cells. Cells were surface stained with FITC-labeled anti-mouse CD4 and PE-labeled anti-mouse CD25 MAbs and stained intracellularly with PE-Cy5-labeled anti-mouse Foxp3 MAb, in accordance with the manufacturer's protocol. Results were expressed as ratios of the frequency of $Foxp3^+$ $CD25^+$ $CD4^+$ T cells in infected mice to that in uninfected mice. Cells were analyzed using either a FACScan or an LSR II flow cytometer (BD Biosciences, San Jose, CA).

Statistical analysis. Mean and SD values were calculated and analyzed using Student's *t* test, with a *P* value of <0.05 to determine statistical significance. Experiments determining the percentages of mice still infected after vancomycin or PBS treatment were analyzed using Fisher's exact test, with a *P* value of <0.05 to determine statistical significance.

RESULTS

***S. aureus* implant infection results in chronic infection.** Tibiae from mice receiving implants of *S. aureus*-coated and control sterile pins were harvested and processed at days 4, 7, 14, 21, 28, and 49 postimplantation. The numbers of CFU were enumerated from homogenized bone tissue to determine the development of chronic infection and bacterial loads in the tibiae. Results demonstrate that viable *S. aureus* cells were cultured from the infected pin and surrounding bone at all time points tested, as far out as 49 days postinfection (Fig. 1). Bacterial loads initially increased to over 3 logs of the infecting dose to $>10^8$ CFU/tibia but then decreased between 4 and 7 days postinfection. However, at day 7 and beyond, bacterial loads were consistent. Biofilm formation was evident on implanted pins from infected (Fig. 1B) but not uninfected mice (Fig. 1C and D) by confocal scanning laser microscopy. In addition, vancomycin treatment did not clear infection in any of the mice receiving *S. aureus*-coated implants, even though the M2 strain of *S. aureus* is susceptible to vancomycin in planktonic culture. In order to demonstrate that this infection modality was not unique to the M2 clinical strain of *S. aureus*, we also infected mice with UAMS-1, a well-characterized strain derived from osteomyelitis patients (4, 10, 11, 17, 37, 43). This strain was able to produce an implant infection with the similar trend and bacterial concentrations of the chronic infection seen with *S. aureus* strain M2 (see Fig. S1 in the supplemental material).

***S. aureus* implant infection elicits a local CD4 T cell response but not a CD8 T cell response.** Draining LN cells were harvested from mice at 4, 7, 21, and 28 days postimplantation. A single-cell suspension was prepared, and cells were FACS stained to determine $CD4^+$ and $CD8^+$ T cell frequencies and the upregulation of CD44, a classic marker of T cell activation. Results demonstrate that in the draining LN cells of infected mice, there is a significantly higher frequency of $CD4^+$ cells than that in draining LN cells of uninfected mice. This difference was not observed when comparing $CD8^+$ frequencies in draining LN cells of infected mice versus those of uninfected mice (Fig. 2A and B). Further, in infected mice, there is a significant upregulation of CD44 only in the $CD4^+$ population of LN cells. This upregulation of CD44, indicating T cell activation, is not observed in $CD8^+$ LN cells from infected mice (Fig. 2C). In addition, there is no difference in the proportions of $CD44^+$ LN cells observed in either T cell population in uninfected mice (data not shown). At day 7 postinfection, there is a dramatic decrease in both CD4 and CD8 T cells in the draining lymph nodes of infected mice. This is potentially due to activation-induced cell death following polyclonal activation of T cells by *S. aureus* superantigens such as toxic shock syndrome toxin (TSST), which is produced by the MRSA-M2 strain used in this study.

***S. aureus* implant infection elicits differential production of Th1- and Th2-dependent subisotypes against a biofilm-upregulated antigen.** Levels of various IgG Ab subisotypes against the biofilm-upregulated antigen SA0486 were assessed using the commercially available Mouse Typer isotyping kit. SA0486, a staphylococcal lipoprotein of unknown function which is upregulated in *S. aureus* biofilms, was previously determined by our lab to be both immunogenic and expressed by *S. aureus* during the biofilm mode of growth *in vivo* (24). Sera from infected and uninfected mice collected at days 0, 7, 14, 21, and 28 postinfection were tested in this assay. ELISA plates were coated overnight with 0.1 μg of recombinant SA0486/well, and a conventional ELISA was performed to determine the levels of serum IgG1, IgG2a, and IgG2b Abs produced against SA0486. Early during implant infection, at day 7, there were significantly higher levels of IgG2b (a Th1-dependent subisotype) in the sera compared to those of IgG1 (a Th2-dependent subisotype). By day 28, however, there was a major decline in IgG2b levels and a concomitant increase in the levels of IgG1 (Fig. 3). These data indicate that both Th1- and Th2-dependent IgG subisotypes are produced in response to SA0486 by mice receiving infected implants. However, the kinetics of Ab production differ, with the Th1 IgG subisotypes (IgG2b-recognizing microbial polysaccharides) being produced early in the infection and the Th2 IgG subisotype (IgG1-recognized microbial surface proteins) having a delayed pro-

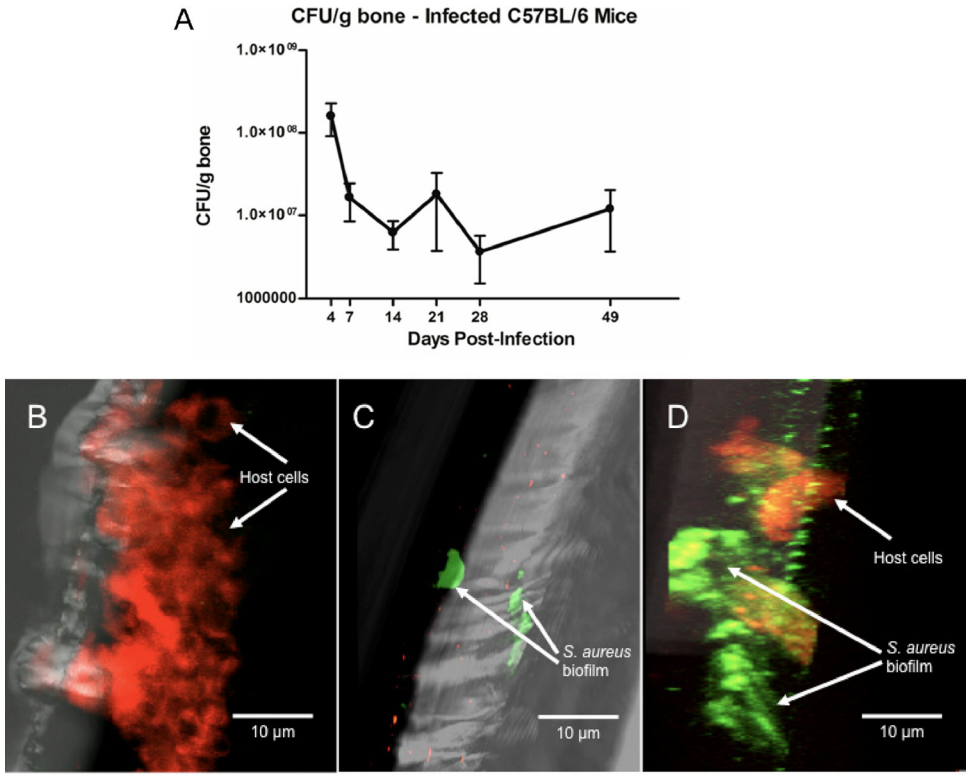

FIG. 1. (A) Development of chronic, biofilm-mediated infection that is recalcitrant to antimicrobial therapy. Number of CFU/g bone over time, indicating the development of a chronic infection. Tibiae from infected and uninfected mice were removed at 4, 7, 14, 21, 28, and 49 days postinfection. No CFU were found in uninfected mice. Serial dilutions of bone homogenates were plated on blood agar plates. The numbers of CFU/g bone were calculated and plotted over time. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ compared to controls by Fisher's exact test. Bars represent SDs. (B to D) Confocal scanning laser microscopic images of uninfected pins removed at 21 days postimplantation (B) and *S. aureus*-infected pins removed at 7 (C) and 21 (D) days postimplantation. Pins were labeled using a FITC-labeled PNA-FISH probe. Biofilm formation is evident on the pin removed from the infected mouse.

duction, coming too late for clearance by the host. We also analyzed the IgG subtypes at multiple time points following infection using a well-characterized strain of *S. aureus*, UAMS-1 (see Fig. S2 in the supplemental material). Infection with the UAMS-1 strain also produces a similar IgG response, in which IgG2A and -B are first activated and it is not until day 21 that the IgG1 subtype begins to increase. While this response to UAMS-1 seems to be somewhat delayed compared to the response to the M2 strain, possibly due to strain-to-strain differences, the trend of an early Th1 Ab response followed by a delayed Th2 response is similar and confirms the response seen in the M2 clinical isolate.

***S. aureus* implant infection elicits Th1 cytokines at 14 days postinfection.** At days 7 and 28 postimplantation, tibiae were removed from infected and uninfected mice. Tibiae were homogenized, and cytokine levels in supernatants were analyzed. Results indicate the significant upregulation of several Th1 cytokines at day 7, including IL-2, IL-12 p70, and TNF-α. There is also significant upregulation of the Th17-associated cytokines IL-6 and IL-17 (Fig. 3). At day 28 postimplantation, the levels of several cytokines drop off, but there is still significantly greater production of IL-2, TNF-α, IL-6, and IL-17 (Fig. 4B).

***S. aureus* implant infection decreases the frequency of local Tregs.** To evaluate the levels of Tregs in the development of chronic implant infection, draining LN cells from infected and uninfected mice were harvested at 4, 7, 14, 21, and 28 days postinfection, and single-cell suspensions of LN cells were prepared. LN cells were FACS stained for CD4 and CD25 surface markers and for intracellular FoxP3. Results indicate that the percentage of $CD4^+$ T cells expressing Foxp3 was significantly lower at day 7 postimplantation in mice receiving *S. aureus*-coated implants rather than sterile implants (Fig. 5).

DISCUSSION

It is well established that growth in the biofilm state protects *S. aureus* from clearance by the host immune system. However, cellular and humoral responses to whole-cell *S. aureus* biofilm-mediated implant infections have not been characterized *in vivo*. Determination of the cell types, phenotypic characteristics, and activation states of infiltrating immune cells, as well as the cytokines and Ab subisotypes elicited during acute and chronic *S. aureus* infection, may provide insight into how *S. aureus* evades the host immune response in order to establish a chronic biofilm infection. This present study sought to adapt a mouse model of *S. aureus* biofilm-mediated prosthetic implant infection to accurately mimic chronic infection in humans and to characterize the cell-mediated immune response against this type of infection. We have demonstrated that our model

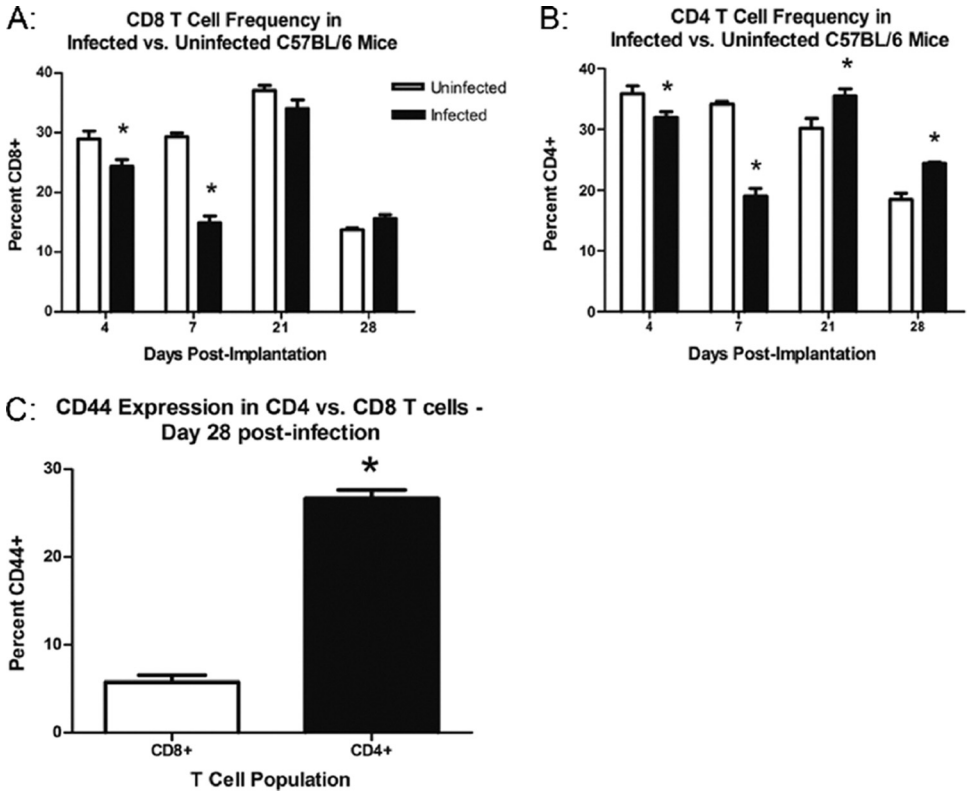

FIG. 2. T cell response to chronic implant infection. Draining lymph nodes were removed from infected and uninfected mice at 4, 7, 21, and 28 days postimplantation. Single-cell suspensions were stained as described in Materials and Methods. (A, B) CD8 (A) and CD4 (B) frequencies were determined by FACS analysis. Both populations are significantly decreased at days 4 and 7, likely due to activation-induced cell death following superantigen activation. At later time points, only the frequency of CD4 T cells is significantly increased in infected versus uninfected mice. (C) At day 28 postimplantation, there is increased expression of the activation marker CD44 only on CD4 T cells from infected mice. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ compared to controls by Student's t test or Fisher's exact test. Bars represent SDs.

results in a chronic, localized infection in C57BL/6 mice that is recalcitrant to treatment with antibiotics or clearance by the host, similar to *S. aureus* prosthetic implant infections in patients. This infection was shown to elicit mainly Th1 and Th17 responses, while Treg responses were suppressed in infected C57BL/6 mice early during infection.

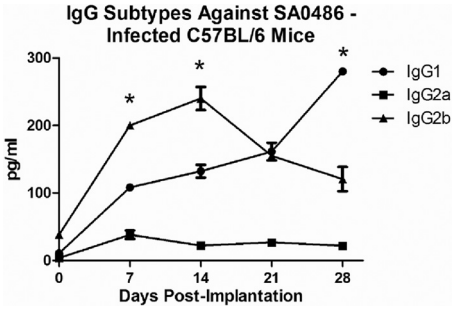

FIG. 3. IgG subtypes against the biofilm-upregulated antigen SA0486. Infected mice were bled at 0, 7, 14, 21, and 28 days postinfection. Sera were collected, and pooled serum samples were analyzed for levels of the Th2 antibody IgG1 and the Th1 antibodies IgG2a and IgG2b against SA0486 over time. IgG2b peaks early during infection on days 7 and 14, whereas IgG1 peaks much later on day 28, by which time mature biofilm formation and chronic infection have developed. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ for IgG1 compared to IgG2b by Student's t test. Bars represent SDs.

In the present study, the implantation of a pin coated with *S. aureus* grown under biofilm-forming conditions results in an infection that is recalcitrant to clearance by both the host immune response and vancomycin treatment. Following implant infection, the acute infection transitioned to a stable chronic infection by day 14 postinfection (Fig. 1). The decrease in CFU counts seen after the early acute expansion of *S. aureus* numbers was likely due to the native immune response killing planktonic bacteria in and around the implant site, since this was before an adaptive immune response was possible. However, during the chronic infection stage, *S. aureus* developed into a mature biofilm and CFU levels plateaued because the immune system is no longer able to clear these biofilm-bound bacteria. In addition, decreased clearance with the antimicrobial therapy of vancomycin confirms that the infection model is clinically relevant, since this is a hallmark of biofilm-mediated prosthetic implant infections in animal models of infection and human patients. Confocal scanning laser microscopy images of infected pins removed from tibiae at 7 and 21 days postimplantation provide further evidence of the presence of chronic infection and biofilm formation, as indicated by the presence of fluorescent green-labeled cocci and biofilm masses on infected (Fig. 1C and D) versus uninfected (Fig. 1B) pins.

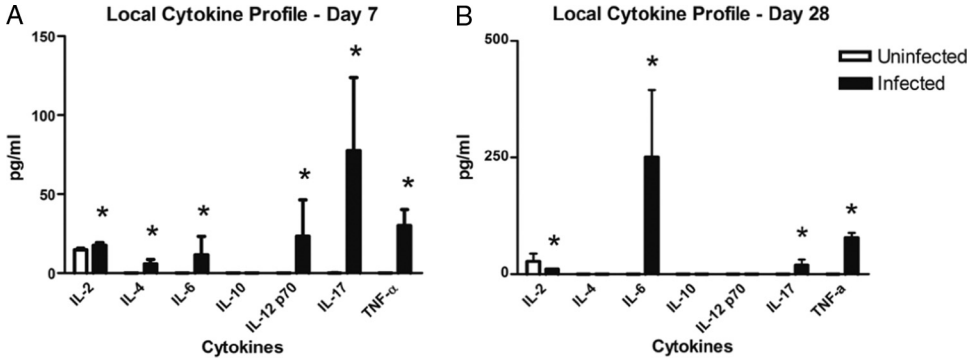

FIG. 4. Local cytokine profile at implant site. Tibiae were removed from mice receiving *S. aureus*-coated or sterile pins. Supernatants from bone homogenates were analyzed for cytokines at day 7 (A) and day 28 (B) postimplantation, as described in Materials and Methods. Significant upregulation of IL-2, IL-6, IL-12, IL-17, and TNF-α indicate a predominantly Th1- and Th17-type response. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ compared to controls by Student's t test. Bars represents SDs.

Other data collected during this study, including T cell subtypes, support the hypothesis that the host responds with a CD4 T cell-mediated response (Fig. 2A and B). Additional support is provided by data derived from the Ab subtype experiments. During the early stages of prosthetic implant infection, the CD4 T cell response is mainly of a Th1 type, as indicated by the IgG subtypes produced against the *S. aureus* biofilm-upregulated antigen SA0486. The host humoral immune response to SA0486 results in IgG2b Abs (Fig. 3), the dominant IgG subtype associated with a Th1 response. These IgG2b Abs may not be able to effectively clear bacteria that are beginning to develop into a biofilm infection, allowing them to form a mature and chronic biofilm. This may be due in part to the poorly opsonizing characteristics of IgG2b Abs (27). Although the immune response does eventually mount a Th2-type response, indicated by the late production of IgG1, this switch does not occur until 28 days postinfection. By this point, IgG1 Abs, which may have successfully cleared an early biofilm, are incapable of clearing the mature biofilm that has formed on the implant and surrounding dead bone.

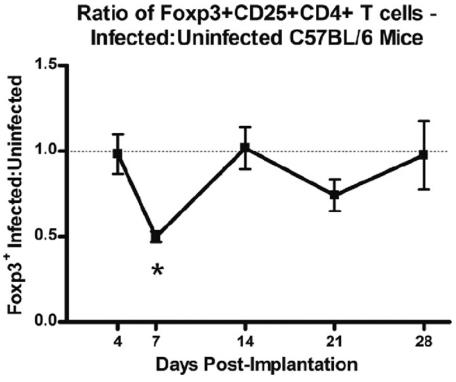

FIG. 5. Treg responses to *S. aureus* implant infection. Draining lymph nodes were removed from infected and uninfected mice at 4, 7, 14, 21, and 28 days postimplantation. Single-cell suspensions were intracellularly stained for Foxp3, as described in Materials and Methods. The Treg frequency, expressed as the ratio of Foxp3 expression in $CD4^+$ lymphocytes of infected mice to that of uninfected mice, was significantly reduced at day 7 postimplantation in mice receiving *S. aureus*-coated implants. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ compared to controls by Student's t test. Bars represent standard errors of the means.

The Th1 response early after implant infection that is associated with the development of a mature biofilm formation and damage to the host through the action of proinflammatory cytokines is further demonstrated by the cytokine response profile. At day 7 postimplantation, there was significant production of cytokines of the Th1 response (Fig. 4A) at the site of infection. While TNF-α levels continued to be elevated, the Th1 cytokine IL-12 p70 became undetectable (Fig. 4B). The later absence of IL-12 is likely due to the fact that this cytokine is usually expressed early during a Th1 response.

In these cytokine profile studies, there was also significant upregulation of the Th17-associated cytokines IL-6 and IL-17 at both early and late time points postimplantation, indicating that these mice are also mounting a robust Th17 response. Although Th17 cells play an integral role in clearing extracellular bacteria, several studies suggest that the Th17 response and resulting neutrophil activation are detrimental to the host during a biofilm-mediated infection (3, 25, 46). Biofilm-embedded bacteria are largely protected from neutrophil killing, and the concomitant release of inflammatory cytokines from these cells leads to the damage of host tissues and further devitalized surface biofilm formation. In addition, IL-6 has been implicated in promoting Treg insufficiency induced by staphylococcal enterotoxin B *in vitro* (48), thereby allowing the inflammatory response to go on unchecked, as discussed below. There was also a small upregulation of IL-4 early in the infection at day 7. Although IL-4 is a Th2 cytokine, this small and transient increase may not be physiologically relevant.

The last subset of the CD4 T cell-mediated response, Tregs, exhibits anti-inflammatory effects through the suppression of proinflammatory $CD4^+$ T effector cells. Therefore, we hypothesized that *S. aureus* may also target this lymphocyte population during the development of chronic implant infection. In the case of infection with *S. aureus*, Tregs are capable of modulating inflammation induced by staphylococcal enterotoxins, such as SEB (21), but their activity is also actively suppressed by this toxin during infection *in vitro* (8, 21, 48). Our data indicate that *S. aureus* implant infection leads to a significant decrease in the frequency of $Foxp3^+$ $CD4^+$ Tregs in the drain-

ing LN cells of infected versus uninfected mice at day 7 postimplantation (Fig. 5). This early downregulation of Tregs by *S. aureus* can further enhance the ability of *S. aureus* to produce proinflammatory Th1 and Th17 immune responses. Taken together, the activation of the Th1 and Th17 adaptive immune responses and the inhibition of the Th2 and Treg subpopulations seem to provide an ineffective defense against the development of a chronic *S. aureus* biofilm infection. Although somewhat limited, studies of CD4 T cell-mediated responses to *S. aureus* infections in humans have mirrored the studies presented herein. A recent study of patients with chronic rhinosinusitis, a biofilm infection of the sinus mucosa that is often due to *S. aureus*, demonstrated that there is a robust local Th1 response at the site of infection in these patients, as indicated by elevated levels of IFN-γ and MIP-1β (20). In addition, it has also been demonstrated that 34% of patients with atopic dermatitis have skin lesions containing alpha-toxin-producing strains of *S. aureus* and that sublytic levels of *S. aureus* alpha-toxin are capable of activating T cells and increasing IFN-γ production, leading to chronic disease (7). The protective role of the Treg lineage during *S. aureus* infection has also not been well elucidated, although there is evidence that children with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, caused by mutations in the *FOXP3* gene and resulting in a lack of functional Tregs, are susceptible to *S. aureus* sepsis, due mainly to catheter-related infections (19). One reason may be that the tissue damage caused by proinflammatory cytokines may augment the ability of *S. aureus* to form biofilms on areas of devitalized tissue and vascular insufficiency. While this may point to a potential mechanism by which *S. aureus* successfully eludes clearance by the host immune system when progressing from an acute to chronic biofilm infection, more research is warranted. Lastly, although the results described above using the Th1-biased C57BL/6 mice show a strong correlation to chronic disease in patients, these data may not be replicated in other mouse strains such as Th2-biased BALB/c mice. Comparative studies using these two disparate mouse strains are presently ongoing in our laboratory.

Once an implant has become colonized with *S. aureus* and chronic infection develops, the only effective and curative treatment option available is removal of the infected implant (30, 31, 40). This procedure is both costly and traumatic to the patient. Better understanding of the host-adaptive immune response to *S. aureus* biofilm-mediated implant infection in the studies herein may lead to the development of more effective therapeutics and prophylaxes for these types of infections. In addition, these findings may lead to immune adjuvant therapy that will enable the manipulation of the host immune system, either alone or in combination with antimicrobial therapy, to promote the effective clearance of *S. aureus*.

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