Clearance of *Staphylococcus aureus* Nasal Carriage Is T Cell Dependent and Mediated through Interleukin-17A Expression and Neutrophil Influx

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The anterior nares of humans are the major reservoir for *Staphylococcus aureus* colonization. Approximately 20% of the healthy human population is persistently and 80% is intermittently colonized with *S. aureus* in the nasal cavity. Previous studies have shown a strong causal connection between *S. aureus* nasal carriage and increased risk of nosocomial infection, as well as increased carriage due to immune dysfunction. However, the immune responses that permit persistence or mediate clearance of *S. aureus* on the nasal mucosa are fundamentally undefined. In this study, we developed a carriage model in C57BL/6J mice and showed that clearance begins 14 days postinoculation. In contrast, SCID mice that have a deficient adaptive immune response are unable to eliminate *S. aureus* even after 28 days postinoculation. Furthermore, decolonization was found to be T cell mediated but B cell independent by evaluating carriage clearance in T-cell receptor β/δ (TCR- β/δ) knockout (KO) and IgH- μ KO mice, respectively. Upregulation of the cytokines interleukin 1 β (IL-1 β), KC (also termed CXC ligand 1 [CXCL1]), and IL-17A occurred following inoculation with intranasal *S. aureus*. IL-17A production was crucial for clearance, since IL-17A-deficient mice were unable to effectively eliminate *S. aureus* carriage. Subsequently, cell differential counts were evaluated from nasal lavage fluid obtained from wild-type and IL-17A-deficient colonized mice. These counts displayed IL-17A-dependent neutrophil migration. Antibody-mediated depletion of neutrophils in colonized mice caused reduced clearance compared to that in isotype-treated controls. Our data suggest that the Th17-associated immune response is required for nasal decolonization. This response is T cell dependent and mediated via IL-17A production and neutrophil influx. Th17-associated immune responses may be targeted for strategies to mitigate distal infections originating from persistent *S. aureus* carriage in humans.

Staphylococcus aureus has reemerged as an important pathogen due to its antibiotic resistance and the increased use of indwelling medical devices. Although *S. aureus* can be transiently found in various locales on the human body, the major ecological niche is the anterior nares. In the human population, 20% are persistently colonized and the remaining 80% are intermittently colonized (1). *S. aureus* is a normal commensal organism of the human nostrils; however, carriage provides a staging ground for *S. aureus* to disseminate to other areas of the body where, once the physical barrier of the skin or epithelial surface is breached, distal infection can occur. These infections result in more than 60,000 deaths per year in the United States alone (2).

S. aureus is one of the most common etiologic agents of nosocomial infections, and it is recognized as the second-leading cause of infections in intensive care units (3). Risk of nosocomial infection increases 3-fold in nasally colonized patients, and for those that succumb to infection, the *S. aureus* genotype cultured from the nose correlates over 80% of the time with the strain at the infection site (4). Therefore, the particular *S. aureus* strain responsible for infection most often originates from the nares.

Differences in colonization status are due to a multitude of factors. There is evidence that colonization is strain dependent, as persistence can be caused by a single *S. aureus* strain (5), compared to presence of genetically unrelated strains over time in intermittently colonized patients (6). Carriage may also be dependent upon the presence of certain commensal bacteria in the nares. A subset of *Staphylococcus epidermidis* organisms was demonstrated to inhibit *S. aureus* growth and nasal colonization through pro-

duction of Esp, a serine protease (7). In addition, patients colonized with Esp-secreting *S. epidermidis* correlated with absence of *S. aureus* in the nasal cavity (7). One study observed that single nucleotide polymorphisms (SNPs) in C-reactive protein and interleukin 4 (IL-4) genes correlated with *S. aureus* nasal colonization (8), whereas another found a correlation with SNPs in the glucocorticoid receptor gene (9).

The host adaptive immune response is another factor with implications for carrier status of the host. HIV-infected patients with a CD4⁺ T-cell count of $\leq 200/\mu\text{l}$ have an increased prevalence of persistent colonization (10). In addition, colonized children have significantly higher levels of IgA and IgG against *S. aureus* proteins than do noncolonized children (11). Despite the evidence provided by these studies, the specific immunological mechanisms for clearance of *S. aureus* nasal carriage are undefined.

In this study, we developed a murine model of nasal colonization in order to elucidate the host immune response required for elimination of *S. aureus* carriage. We evaluated the lymphocytic

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FIG 1 *S. aureus* nasal colonization is intermittent in C57BL/6J mice. C57BL/6J mice were inoculated intranasally with *S. aureus* clinical isolate SA1108. (A) CFU count/nose for the indicated days postinoculation. Nasal tissue was harvested at various days postinoculation, homogenized, and plated on CHROMagar-*S. aureus* to determine CFU counts. Detection limit = 100 CFU. (B) Colonization rate for the indicated days postinoculation. Colonization rate was determined by number of mice positive for *S. aureus*/total number of mice. $n = 5$ or 6 C57BL/6J mice per time point.

response and measured nasal tissue cytokine levels following inoculation via a cytokine multiplex assay. Decolonization was found to be T cell and IL-17A dependent. Additionally, nasal lavages were performed to measure cellular influx into the lumen, and *in vivo* depletion assays were performed to determine the requirement for cellular migration in promoting clearance.

MATERIALS AND METHODS

Animals. C57BL/6J (wild type [WT]), SCID, IgH- μ KO, and T-cell receptor β/δ (TCR- β/δ) knockout (KO) mice were purchased from The Jackson Laboratories. C57BL/6J IL-17A KO and IL-23p19 KO mice were generous gifts from Yoichiro Iwakura (University of Tokyo, Japan) and Nico Ghilardi (Genentech, South San Francisco, CA), respectively. Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore Dental School. Female mice aged 6 to 8 weeks were used for each study.

***Staphylococcus aureus* strain and nasal colonization model.** *S. aureus* strain SA1108 was isolated from the nares of a persistently colonized patient enrolled in an epidemiological carriage study. SA1108 was grown overnight in 15 ml tryptic soy broth and diluted to an optical density (OD) at 600 nm of 0.1 (10^9 CFU/ml). After centrifugation, the pellet was resuspended in sterile PBS to a final density of 10^{10} CFU/ml.

Prior to inoculation, mice were anesthetized with isoflurane followed by intraperitoneal (i.p.) injection with 0.1 ml of a ketamine (20 mg/ml) and xylazine (2 mg/ml) cocktail. An inoculum of $10 \mu\text{l}$ (10^8 CFU) was presented intranasally without trauma to the nares. After a predetermined number of days, mice were sacrificed.

To harvest nasal tissue, mice were decapitated using surgical scissors (Roboz, Gaithersburg, MD). The facial tissue and lower jaw were dissected along with the nasal mucosa-associated lymphoid tissue (NALT) located on the upper palate. The posterior cranium was removed by dissection along the eye line. The remaining nasal tissue fragment was placed in 1 ml ice-cold sterile phosphate-buffered saline (PBS) alone or with protease inhibitor cocktail (Roche, Madison, WI).

Harvested tissue was homogenized for 1 min on ice. *S. aureus* SA1108 organisms were quantified by plating $10\text{-}\mu\text{l}$ serial dilutions of tissue homogenate on *S. aureus*-CHROMagar plates (CHROMagar, Paris, France) and incubated for 24 h at 37°C. Colonization rate was determined by number of mice positive for SA1108 per total mice inoculated.

Cell differential quantification. Following sacrifice, the mouse trachea was exposed and cannulated, followed by a 500- μl lavage of ice-cold sterile PBS injected from the trachea toward the nose. Nasal expunged fluid was collected and immediately placed on ice. Samples were applied to Fisher Scientific Superfrost Plus gold slides using a Thermo Shandon Cytospin 3 at $700 \times g$ for 7 min. Slides were allowed to air dry and then fixed and stained using a Diff-Quik stain kit (Fisher Scientific, Pittsburgh, PA). Cells were quantified by standard morphological criteria at a magni-

fication of $\times 200$. Ten random fields of view were chosen, and the sum value for each cell type was used for data analysis.

Cytokine analysis. Nasal tissue homogenates were placed in PBS with protease inhibitor cocktail (Roche) and were centrifuged at 14,000 rpm and 4°C for 5 min. Supernatants were collected and stored in a -80°C freezer until needed. Samples were sent to the University of Maryland Cytokine Core Laboratory, and gamma interferon (IFN- γ), IL-10, IL-12p70, IL-17A, IL-1 β , IL-4, IL-5, IL-6, and KC (also termed CXCL1) were measured on a Luminex multianalyte system.

Neutrophil depletion. C57BL/6J mice were injected intraperitoneally (i.p.) with either 200 μg rat anti-mouse Ly-6G or rat IgG2a isotype control antibody (Bio-X-Cell, West Lebanon, NH). Injections occurred 1 day prior to inoculation and every 3 days after initial injection until mice were sacrificed 14 days postinoculation. Neutrophil depletion was confirmed with blood collected 14 days postinfection (d.p.i.) by flow cytometry using phycoerythrin (PE)-labeled anti-Ly-6G antibody (BD Pharmingen, San Diego, CA).

Statistical analysis. The data were analyzed by Fisher's exact test (one-tailed) utilizing GraphPad Prism, version 5.0 (GraphPad Software, Inc.). Results are expressed as means $\pm$ standard errors of the means (SEM). A P value of <0.05 was considered significant.

RESULTS

***S. aureus* nasal colonization kinetics.** Few studies have currently determined the kinetics of *S. aureus* nasal carriage in mice in the temporal context of adaptive immune responses. Therefore, we adapted a murine model of nasal inoculation to elucidate the time course of clearance with a strain of *S. aureus*, termed SA1108, isolated from the nares of a persistently colonized patient (12). Mice were inoculated intranasally, and nasal tissue was harvested at various time points up to 28 d.p.i. for enumeration of CFU/nose and clearance rate. As shown in Fig. 1A, nasal CFU have a log-scale reduction occurring between 4 and 14 d.p.i., followed by a half-log reduction until 28 d.p.i. Complete clearance of *S. aureus* in a subset of mice first occurs at 14 d.p.i. (20% clearance rate) and steadily continues to 28 d.p.i. (50% clearance rate) (Fig. 1B).

Clearance is T cell mediated but B cell independent. The colonization kinetics observed suggests a role for adaptive immune responses in clearance (i.e., CFU reductions 4 d.p.i. and clearance 14 d.p.i.). Therefore, the lymphocyte-deficient SCID mouse strain was utilized to determine the importance of adaptive T-cell and B-cell responses for *S. aureus* carriage. Mice were inoculated intranasally, and nasal tissue was harvested at 28 d.p.i. SCID mice displayed significant increases in *S. aureus* colonization compared

FIG 2 Decolonization is a T-cell-mediated, B-cell-independent response. C57BL/6J WT, SCID, IgH- μ KO, and TCR- β/δ KO mice were inoculated intranasally with *S. aureus* clinical isolate SA1108. (A) CFU count/nose for the indicated days postinoculation. Nasal tissue was harvested at 28 d.p.i., homogenized, and plated on CHROMagar-*S. aureus* to determine CFU counts. Detection limit = 100 CFU. (B) Colonization rate 28 days postinoculation. Colonization rate was determined by number of mice positive for *S. aureus*/total number of mice. $n = 7$ to 9 C57BL/6J mice per group. Data were combined from two independent experiments. *, $P < 0.05$.

to WT mice (Fig. 2A). All SCID mice were colonized 28 d.p.i., compared to 14% of WT mice (Fig. 2B).

In order to determine the specific lymphocyte response (T cell, B cell, or both) required for decolonization, B-cell-deficient IgH- μ KO and T-cell-deficient TCR- β/δ KO mice were utilized. As described above, mice were inoculated intranasally, and nasal tissue was harvested at 28 d.p.i. IgH- μ KO mice (B-cell deficient) displayed carriage and clearance rates comparable to those of WT mice (Fig. 2). T-cell-deficient TCR- β/δ KO mice, however, displayed significant increases in CFU compared to those in WT mice. Additionally, 14% of WT mice were unable to clear carriage, compared to 87% of TCR- β/δ KO mice.

Clearance is IL-23 independent but relies on IL-17A. To help determine the specific T-cell response required for *S. aureus* decolonization, a cytokine multiplex assay was performed to elucidate upregulated cytokines. Mouse nasal tissue was harvested after various time points postinoculation and homogenized. Tissue homogenate was subjected to multiplex assay testing for levels of various cytokines (IFN- γ , IL-10, IL-12p70, IL-17A, IL-1 β , IL-4, IL-5, IL-6, and KC) (Fig. 3A). IL-17A, KC, and IL-1 β were significantly upregulated following inoculation (Fig. 3B to D). Upregulation of IL-17A and IL-1 β gene expression was confirmed by reverse transcription-PCR (RT-PCR) (data not shown). IL-17A expression peaked at 7 d.p.i. IL-1 β production was significantly increased at 2 d.p.i. and 28 d.p.i. KC was upregulated during all time points tested. IL-6, IL-12p70, and IFN- γ were downregulated postinoculation (Fig. 3A). These results suggested a role for Th17-mediated clearance via the upregulation of IL-17A.

To determine the importance of Th17 responses for decolonization, IL-23p19 KO mice were used. The IL-23p19 subunit, in combination with the IL-12p40 subunit, is required for functional IL-23 cytokine (13). Since IL-23 is important for the maintenance of Th17 cells, the IL-23p19 KO mouse strain has been extensively utilized as a model for Th17-deficient immunity (14–16). Interestingly, the IL-23p19 KO strain displayed a carriage and colonization rate similar to those displayed by WT inoculated mice (Fig. 4). In order to determine the role and importance of the observed IL-17A upregulation in actively decolonizing mice, IL-17A KO mice were inoculated with *S. aureus*. IL-17A KO mice were determined to have a significant

increase in intranasal *S. aureus* CFU compared to that of WT mice (Fig. 4B). In addition, 50% of IL-17A KO mice were colonized, compared to 14% of WT mice.

IL-17A-dependent neutrophil influx enhances clearance. IL-17A expression is important for neutrophil migration and effector function (17). The requirement of IL-17A for decolonization suggests that neutrophil influx into the nasal lumen may occur. To elucidate the cellular components migrating into the lumen of the nares, a nasal lavage was performed. Neutrophils and macrophages/monocytes were identified via cell differential counts from lavage fluid. Neutrophil influx was observed 4 and 7 days postinoculation; however, macrophage/monocyte levels remained constant throughout the time points tested (Fig. 5A).

To determine whether IL-17A expression controls neutrophil migration during colonization, nasal lavages were performed in C57BL/6J WT and IL-17A KO mice. Neutrophil counts were compared between noninoculated controls and mice colonized for 7 days. WT colonized mice displayed significant neutrophil influx compared to noninoculated WT mice. Interestingly, IL-17A KO colonized mice had neutrophil levels comparable to those in noninoculated IL-17A KO and WT controls (Fig. 5B).

A neutrophil depletion assay was performed in order to determine the importance of neutrophil influx and responses during carriage. Mice were given injections of either rat anti-mouse Ly-6G or rat IgG2a isotype control antibody, and nasal tissue was harvested 14 days postinoculation. Neutrophil depletion resulted in increases in *S. aureus* CFU, although not significant (Fig. 5C). Additionally, anti-Ly-6G-treated mice had a significantly greater colonization rate than did isotype controls (Fig. 5D).

DISCUSSION

The immune response against *S. aureus* nasal colonization is fundamentally undefined. Therefore, we utilized a murine carriage model to elucidate the immune responses crucial for clearance. We showed that kinetics of clearance followed a time course of adaptive immune responses. Adaptive immunity was found to be necessary for decolonization by inoculation of SCID mice. This was found to be independent of B-cell responses in IgH- μ KO mice; however, clearance was dependent on T-cell lymphocytes

FIG 3 *S. aureus* nasal carriage upregulates proinflammatory and Th17-associated cytokines. C57BL/6J mice were inoculated intranasally with *S. aureus* clinical isolate SA1108. Nasal tissue was harvested at various time points and homogenized. (A) A multiplex enzyme-linked immunosorbent assay was used to determine the cytokine response from noninoculated control and 2-, 7-, and 28-d.p.i. nasal tissue homogenates. (B to D) Levels of IL-1 β , KC, and IL-17A were significantly upregulated during colonization. $n = 3$ to 6 C57BL/6J mice per time point. The detection range of the assay is 3.2 to 10,000 pg/ml. *, $P < 0.05$.

when utilizing TCR- β/δ -deficient mice. Inoculation of mice resulted in upregulation of the innate proinflammatory cytokine IL-1 β , neutrophil recruitment factor KC, and Th17-associated effector cytokine IL-17A. Despite the upregulation of IL-17A, IL-23 (a costimulatory cytokine for Th17 T-cell development) was not necessary for decolonization. However, IL-17A-deficient mice were less able to clear colonization. Additionally, IL-17A-dependent migration of neutrophils into the nasal lumen was observed and was important for decolonization. These data suggest a T-cell-dependent mechanism for *S. aureus* clearance by IL-17A-controlled neutrophil recruitment.

The adaptive immune response was shown to be crucial for decolonization. Previous studies have shown a potential role for antibody-mediated immunity in clearance. IgA and IgG antibodies have been shown to increase in nasally colonized children (11). Additionally, antistaphylococcal antibody levels are higher in persistent carriers than in noncarriers (18). Despite the upregulation of antibody titers in humans, this B-cell-mediated response was not responsible for the clearance observed in our murine model (Fig. 2). Although clearance of *S. aureus* nasal colonization may not be governed by humoral responses, carriage may still be protective for subsequent infections. Holtfreter et al. have shown a

FIG 4 Clearance is IL-23 independent and relies on IL-17A. C57BL/6J WT, IL-23p19 KO, and IL-17A KO mice were inoculated intranasally with *S. aureus* clinical isolate SA1108. (A) CFU count/nose 28 days postinoculation. Nasal tissue was harvested at 28 d.p.i., homogenized, and plated on CHROMagar-*S. aureus* to determine CFU counts. Detection limit = 100 CFU. (B) Colonization rate 28 days postinoculation. Colonization rate was determined by number of mice positive for *S. aureus*/total number of mice. $n = 7$ to 9 C57BL/6J mice per group. Data were combined from two independent experiments. *, $P < 0.05$.

FIG 5 Neutrophil migration into the nasal lumen is IL-17A dependent and crucial for decolonization. C57BL/6J mice were inoculated intranasally with *S. aureus* clinical isolate SA1108. (A) Nasal lavages were performed 0, 4, 7, and 14 days postinoculation. Cell differential counts were done after cytospin of nasal lavage fluid. Cells were counted on 10 random fields of view at a magnification of $\times 200$. Day 0 mice served as noninoculated controls. (B) C57BL/6J WT and IL-17A KO mouse nasal lavages were performed 0 and 7 days postinoculation, and cell differential counts were done to enumerate neutrophil influx. Day 0 mice served as noninoculated controls. (C and D) C57BL/6J mice were injected i.p. with either 200 μ g rat anti-mouse Ly-6G or rat IgG2a isotype control antibody 1 day prior to inoculation and every 3 days after. (C) CFU count/nose 14 days postinoculation. Nasal tissue was harvested at 14 d.p.i., homogenized, and plated on CHROMagar-*S. aureus* to determine CFU counts. Detection limit = 100 CFU. (D) Colonization rate 14 days postinoculation. Colonization rate was determined by number of mice positive for *S. aureus*/total number of mice. $n = 6$ to 8 C57BL/6J mice per group. Data were combined from two independent experiments. *, $P < 0.05$; ns, not significant.

protective effect of *S. aureus* persistent carriage in patients with *S. aureus* sepsis (19). Additionally, persistently colonized individuals had lower mortality rates during subsequent infection than did noncarriers. These data suggest a potential beneficial role for *S. aureus* nasal carriage mediated by higher antibody titers protective against subsequent staphylococcal infections.

The murine model studies described herein suggest a requirement for T-cell responses for the clearance of *S. aureus* colonization. Our data emulate those obtained in experiments involving *Streptococcus pneumoniae* nasal colonization. CD4⁺ T cells were required for immunity against pneumococcal carriage in a murine model, and this occurred in an antibody-independent fashion (20). Another *S. pneumoniae* study discovered the importance of Th17 responses for clearance. CD4⁺ T cells, but not CD8⁺ T cells, were required for reductions in CFU, and depletion of IL-17A resulted in significantly higher nasal carriage of *S. pneumoniae* than in an isotype control, consistent with our IL-17A KO mouse results (21). Additionally, CD4⁺ T-cell and IL-17A-dependent neutrophil influx was observed, and depletion of neutrophils correlated with increased carriage. Similarly, a murine model of *S. aureus* cutaneous infection showed the requirement of IL-17A for neutrophil recruitment and enhanced resolution (22). Our data also suggest Th17-dependent clearance due to IL-17A-mediated neutrophil influx.

It is important to note that in our model, IL-17A-deficient

mice did not ablate immunity against nasal carriage to the degree observed in SCID and β/δ -TCR KO mice (Fig. 2 and 4). This suggests a role for Th17-associated cytokines such as IL-17F in the elimination of nasal colonization. One study found that mice deficient in both IL-17A and IL-17F developed spontaneous *S. aureus* mucocutaneous abscesses around the nose and mouth (23). Further studies will need to be completed in order to elucidate the cytokine milieu essential for carriage eradication.

In our model, we surprisingly observed no effect on clearance in IL-23p19-deficient mice. In concordance with this result, up-regulation of IL-23 gene expression was not observed in colonized WT mice (data not shown). Clearance of *S. aureus* was also independent of IL-17-promoting NKT and γ/δ T cells via use of CD1 and TCR- δ KO mice, respectively (data not shown). Th17-mediated clearance may occur through an IL-23-independent mechanism promoted by observed IL-1 β upregulation. IL-1 β can act directly on naive human CD4⁺ T cells via IL-1 receptors and was shown to be critical for early Th17 differentiation (24, 25). Induction of IL-17A occurred independent of IL-23 in naive murine CD4⁺ T cells stimulated with IL-6 and transforming growth factor β (TGF- β) and was enhanced with addition of IL-1 β (24). These data suggest that Th17 immunity can develop independently of IL-23 in the presence of certain cytokine milieus.

The importance of Th17 responses for epithelial integrity and

protection in humans can be observed in patients with hyper-IgE (Job's) syndrome. Interestingly, Job's syndrome patients are afflicted with recurrent *S. aureus* and *Candida* mucocutaneous infections (26, 27). This disease results from mutations in STAT3 that impede the development of Th17 cells, and consequently, patients have reduced frequencies of IL-17A⁺ CD4⁺ T-cell populations compared to those in healthy individuals (28, 29). Similarly, our data show the importance of Th17-associated responses of IL-17A and neutrophil influx in the clearance of *S. aureus* nasal colonization. Studies involving Job's syndrome patients highlight the importance of Th17 responses in the control of *S. aureus* colonization and infection at epithelial surfaces.

In conclusion, our data show that clearance of *S. aureus* nasal carriage is a B-cell-independent, T-cell-mediated process. The presence of *S. aureus* in the nasal cavity resulted in upregulation of the proinflammatory cytokine IL-1 β , neutrophil recruitment factor KC, and IL-17A. Neutrophil influx governed by IL-17A expression promoted resolution of colonization. Th17-associated immune responses may be targeted for strategies to mitigate distal infections originating from persistent *S. aureus* carriage in humans.

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