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Interleukin-17A (IL-17A) and IL-17F Are Critical for Antimicrobial Peptide Production and Clearance of *Staphylococcus aureus* Nasal Colonization

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Approximately 20% of the population is persistently colonized by *Staphylococcus aureus* in the nares. Th17-like immune responses mediated by the interleukin-17 (IL-17) family of cytokines and neutrophils are becoming recognized as relevant host defense mechanisms for resolution of *S. aureus* mucocutaneous infections. Since antimicrobial peptides are regulated by the IL-17 cytokines, we sought to determine the role of IL-17 cytokines in production of antimicrobial peptides in a murine model of *S. aureus* nasal carriage. We discovered that nasal tissue supernatants have antistaphylococcal activity, and mice deficient in both IL-17A and IL-17F lost the ability to clear *S. aureus* nasal colonization. IL-17A was found to be sufficient for nasal mBD-3 production *ex vivo* and was required for CRAMP, mBD-3, and mBD-14 expression in response to *S. aureus* colonization *in vivo*. These data were confirmed in a clinical study of nasal secretions in which elevated levels of the human forms of these antimicrobial peptides were found in nasal secretions from healthy human subjects when they were colonized with *S. aureus* but not in secretions from noncolonized subjects. Together, these data provide evidence for the importance of IL-17A regulation of antimicrobial peptides and IL-17F in the clearance of *S. aureus* nasal carriage.

It is increasingly recognized that antimicrobial peptides (AMPs) play an important role in the immune system of humans. AMPs are present at all human body sites normally exposed to microbes, such as the skin and mucosa (1). The most prominent mammalian AMPs are the defensins (2). Four human β-defensins (hBD-1 to hBD-4) have been characterized that are produced by mucosa and epithelial cells (3). However, with the exception of hBD-3, their antimicrobial activity is antagonized by increasing concentrations of monovalent or divalent ions found at many body sites (4). In humans, only one cathelicidin, LL-37, is present, characterized by a conserved N-terminal domain that is proteolytically cleaved to generate the mature, active peptide contained within the C terminus (5, 6), and it is mainly produced in leukocytes and epithelial cells (7).

Human cathelicidin and β-defensins have implications for host defense against *Staphylococcus aureus* colonization. LL-37 was determined to be expressed in corneal epithelial cells and to have potent activity against *S. aureus* (8). Expression of hBD-2 mRNA by human keratinocytes was significantly induced by contact with *S. aureus* (9). In conjunction, the activities of hBD-3 and CAP18, the precursor of LL-37, against *S. aureus* were found to be greater than those of hBD-1 and hBD-2 (9). Activity of hBD-2 against *S. aureus* is present but at higher concentrations (10). hBD-3 has a stronger lethal effect on both planktonic and biofilm-grown *S. aureus* than vancomycin and other antibiotics at low concentrations (11, 12). In another study, hBD-3 and hBD-2 were induced in patients with lesional *S. aureus* skin infections but not in healthy patients (13). Additionally, healthy individuals with deficient hBD-3 expression in keratinocytes are more prone to persistent nasal colonization with *S. aureus* (14). The reasons for differences in AMP expression at epithelial surfaces and their relation to nasal colonization remain elusive. Multiple studies have determined that defensin gene polymorphisms, both in sequence and in gene copy numbers, do not seem to be involved in *S. aureus* carriage predisposition (15, 16). In contrast, polymorphisms in the *DEFB1* gene promoter region, a regulator of hBD-1 and hBD-3, were associated with lower hBD-3 expression and persistent *S. aureus* nasal colonization (17).

Cathelicidin and β-defensin antimicrobial peptides are also produced in mouse. Cathelin-related antimicrobial peptide, or CRAMP, is the murine homolog to human LL-37 (18). Analogous to LL-37, neutrophil-derived CRAMP displays effective *S. aureus* killing (19). Mouse β-defensin-3 and -14 correspond to human β-defensin-2 and -3, respectively (20, 21). *S. aureus* cells treated with recombinant mBD-3 showed growth inhibition and morphological and structural changes, including delamination and

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perforation of the peripheral cell walls, porosity, and release of the cytoplasmic contents (22, 23). mBD-14 is expressed in a wide variety of tissues, including spleen, colon, and tissues of the upper and lower respiratory tract (24), is induced in osteoblasts upon stimulation with *S. aureus* supernatants, and exhibits antistaphylococcal activity (25).

Previously, we have shown that clearance of *S. aureus* nasal colonization requires IL-17A-mediated neutrophil influx (26). In addition, we previously found another IL-17 family member, IL-17F, to be upregulated, but its importance during nasal colonization has not been defined. The IL-17 family of cytokines is reported to induce epithelial AMP production in response to *S. aureus* insult (27). Therefore, we hypothesized that IL-17A and IL-17F expression during nasal carriage induces AMPs, and these AMPs promote decolonization. In particular, we were interested in the role of CRAMP, mBD-3, and mBD-14 antimicrobial peptides in *S. aureus* nasal colonization due to their antistaphylococcal activity and inducible tissue expression at epithelial surfaces.

Here, we show that nasal tissue supernatants exhibited antistaphylococcal activity. In addition, IL-17A- and IL-17F-deficient mice are unable to clear nasal colonization and displayed a more severe phenotype than IL-17A-deficient mice alone. IL-17A was found to be required for upregulation of the antimicrobial peptides CRAMP, mBD-3, and mBD-14 during *S. aureus* nasal colonization and to be sufficient for mBD-3 expression in nasal tissue. This observation was confirmed by demonstrating statistically significant upregulation of AMP expression in nasal secretions from healthy subjects colonized with *S. aureus*. This is the first study to provide direct evidence that *S. aureus* induces AMP expression in the nares, suggesting an involvement of antimicrobial peptides in the defense against *S. aureus* nasal colonization.

MATERIALS AND METHODS

Ethics statement. All animals were handled in strict accordance with good animal practice as defined in the federal regulations set forth in the Animal Welfare Act (AWA), the 1996 *Guide for the Care and Use of Laboratory Animals* (28), the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (29), as well as UMB's policies and procedures as set forth in the UMB animal care and use training manual, and all animal work was approved by the UMB Dental Institutional Animal Care and Use Committee (number 12-10-01).

The collection of human nasal secretion samples and *S. aureus* strain SA1108, isolated from the nares of a patient, were approved by the University of Pennsylvania and University of Maryland Baltimore Institutional Review Boards, respectively. Adult subjects provided informed written consent, and no children subjects were used in these studies. In addition, collected human nasal secretion samples and strain SA1108 were deidentified before use. Strain SA1108 was not collected specifically for this study but was received from the Antibodies in *S. aureus* Bacteremia collection at UMB. Only two time points were chosen for collecting patient samples, since patients are often lost to follow-up for multiple sampling due to subject discomfort and the extended time required for the collection of nasal secretions. This two-time-point protocol has been successfully used by Nouwen et al. to effectively determine *S. aureus* colonization rates (30).

Animals. C57BL/6J wild-type (WT) mice were purchased from The Jackson Laboratories. C57BL/6J IL-17A and IL-17A/F knockout (KO) mice were generous gifts from Yoichiro Iwakura (University of Tokyo, Japan). 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.

***S. aureus* strain and nasal colonization model.** *S. aureus* strain SA1108 was isolated from the nares of a 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 (equal to 10^9 CFU/ml). After centrifugation, the pellet was resuspended in sterile phosphate-buffered saline (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 μl (10^8 CFU) was presented intranasally without trauma to the nares.

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-associated lymphoid tissue (NALT) located on the upper palate. The posterior cranium was removed by dissection along the NALT tissue line. The remaining nasal tissue was placed in 1 ml RNAlater or ice-cold sterile RPMI (plus L-glutamine and HEPES) with 10% fetal bovine serum (FBS) and 100 μg/ml streptomycin.

***Ex vivo* stimulation.** Tissue placed in sterile RPMI (plus L-glutamine and HEPES) with 10% FBS and 100 μg/ml streptomycin was incubated in a cell incubator (37°C and 5% CO_2) for 1 h to eliminate endogenous bacteria. Nasal tissue was then washed twice in sterile RPMI (plus L-glutamine and HEPES) with 10% FBS and no streptomycin. After being washed, nasal tissue was placed in 1 ml fresh RPMI (plus L-glutamine and HEPES) with 10% FBS in a 24-well plate. To stimulate tissue, 20 ng/ml of mouse recombinant IL-17A (rIL-17A), rIL-17F, or both was added. Nonstimulated tissue acted as negative controls. At 24 h poststimulation, supernatants were collected and centrifuged at 14,800 rpm and 4°C for 5 min and stored at −20°C until needed. Nasal tissue was washed three times in sterile PBS to remove RPMI medium. Nasal tissue then was placed in RNAlater until needed.

Nasal secretion killing assay. *S. aureus* SA1108 was grown overnight in sterile RPMI (plus L-glutamine and HEPES) with 10% FBS. SA1108 was diluted to an OD of 0.1 (~10^8 CFU/ml) in RPMI. A 100-μl aliquot was added to a clean Eppendorf tube and resuspended in 1 ml RPMI or 1 ml sterile double-distilled water (ddH_2O) (10^7 CFU/ml). Ten microliters of bacterial suspension was added to a 96-well plate. Bacterial suspension was cocultured with either 90 μl nasal secretion or 10 μl nasal secretion and 80 μl sterile ddH_2O. Sterile ddH_2O was utilized to dilute salt concentrations into a range effective for antistaphylococcal activity of antimicrobial peptides. Alternatively, secretions were heat inactivated by autoclaving for 15 min and cooled on ice prior to incubation with SA1108. This provided a final bacterial concentration of 10^6 CFU/ml. RPMI or 1:10 RPMI and RPMI plus streptomycin acted as negative and positive controls, respectively. The bacterial suspension was incubated at 37°C and rotated at 200 rpm for the indicated time. The initial concentration of SA1108 was plated on tryptic soy agar (TSA) to confirm 1×10^6 CFU/ml. After a set amount of time, 10 μl of bacterial suspension was serially diluted in sterile PBS and plated on TSA to measure CFU per milliliter.

RT-PCR. For reverse transcription-PCR (RT-PCR), RNA from nasal tissue immersed in RNAlater was purified with an RNeasy minikit (Qiagen). cDNA was produced from 500 ng RNA with a QuantiTect RT kit (Qiagen). PCR was performed in a 25-μl reaction mixture containing 1 μl of 1:10-diluted cDNA, 12.5 μl SYBR green quantitative PCR (qPCR) master mix (Invitrogen), 10.7 μl diethyl pyrocarbonate-treated water, and 0.4 μl of 50 nM forward (F) and reverse (R) primers. To detect specific antimicrobial peptides, primers for mBD-14 (F, 5′-GTATTCCTCATCTTGTTCTTGG-3′; R, 5′-AAGTACAGCACACCGGCCAC-3′), CRAMP (F, 5′-GCCGCTGATTCTTTTGACAT-3′; R, 5′-ATTCTTCTCCCCACCTTTGC-3′), and mBD-3 (F, 5′-CTTTGCATTTCTCCTGGTGC-3′; R, 5′-GCCTCCTTTCCTCAAACAACT-3′) were utilized. RPL-19 served as the ribosomal housekeeping gene (primers: F, 5′-GCATCCTCATGGAGCACAT-3′; R, 5′-CTGGTCAGCCAGGAGCTT-3′). Real-time PCR was performed on an ABI 7500 Fast system (Applied Biosystems, USA) under the following conditions: 95°C for 10 min and then 40 cycles of 95°C for 10 s and 60°C for 30 s. This was followed by the default dissociation cycle for

melting curve analysis. The $\Delta\Delta C_T$ method was utilized by software to calculate the relative expression.

Patient *S. aureus* nasal colonization determination. Nasal swabs collected from patients were placed in 1 ml sterile PBS and vigorously vortexed. Patients were deemed positive for *S. aureus* colonization if they were positive with either a culture- or PCR-based method. To determine *S. aureus* positivity by culture, a 100-μl aliquot was plated on CHROMagar-*S. aureus* for 24 h at 37°C. A sample was considered positive for *S. aureus* if red colonies were present after incubation. To determine *S. aureus* positivity by PCR, DNA from the remaining sample was purified with the QIAamp DNA minikit and lysostaphin (200 μg/ml) by the manufacturer's protocol. PCR was performed with 1 μM primers specific for the *S. aureus* protein A (*spa*) gene (spa-1113f, 5′-TAAAGACGATCCTTCGGTGAGC-3′; spa-1514r, 5′-CAGCAGTAGTGCCGTTTGCTT-3′) (31) under the following conditions: 95°C for 15 min; 30 cycles of 95°C for 30 s, 59°C for 1 min, and 72°C for 1 min; and a final step at 72°C for 10 min (32). PCR products were run on a 1% Tris-acetate-EDTA gel containing 0.5 μg/ml ethidium bromide, and bands were visualized with an Alpha Innotech FluorChem 8900 imager. A sample was considered positive for *S. aureus* if a PCR product was visualized.

ELISAs. This was an institutional review board-approved study prospectively collecting nasal secretions from 60 patients, as described before (33). All patients were greater than 18 years of age and had no active upper respiratory infection. Exclusion criteria included active smoking history, recent antibiotic use (in the last month), a history of chronic rhinosinusitis, or active use of nasal steroid, antihistamine, or saline sprays or saline lavage. Briefly, using a sterile bayonet forceps, a Merocel pope ear wick (catalog number 400141; Medtronic) was placed bilaterally along the nasal cavity between the nasal septum and inferior turbinate under endoscopic guidance. These were left in place for 5 min. They were then removed and spun down at 4°C and >14,000 × *g* for 10 min in Eppendorf tubes to separate nasal secretions from wicks. Nasal secretions were snap-frozen in liquid nitrogen and kept at −80°C until utilized. Secretion samples were normalized for total protein, and enzyme-linked immunosorbent assays (ELISAs) for hBD-2 (catalog number 900-M172; Peprotech), hBD-3 (catalog number 900-M210; Peprotech), and LL-37 (catalog number HK321-01; Hycult Biotech) were utilized to measure protein levels in nasal secretions and done by following the manufacturers' protocols. This procedure was subsequently performed in the same manner 6 weeks from the initial collection date.

Statistical analysis. The data were analyzed by *t* test (one-tailed), Fisher's exact test, or Mann-Whitney test as indicated, utilizing GraphPad Prism version 5.0 (GraphPad Software, Inc.). Results are expressed as means ± standard errors of the means (SEM). A P value of <0.05 was considered significant.

RESULTS

***Ex vivo* supernatants have antistaphylococcal activity that is salt and heat sensitive.** In order to determine the antimicrobial peptide response against *S. aureus* nasal colonization, we developed an *S. aureus* killing assay with supernatants from *ex vivo* nasal tissue. Due to the salt sensitivity of most antimicrobial peptide activity, we included a group of 1:10-diluted supernatants in sterile ddH_2O (34). *Ex vivo* supernatants were able to diminish *S. aureus* over time (Fig. 1A); however, dilution of supernatants 1:10 in sterile ddH_2O resulted in enhanced killing of *S. aureus* (Fig. 1A and B). Additionally, *S. aureus* colonies incubated in diluted supernatants were 1/5 the size of diluted controls (Fig. 1C), and heat inactivation of diluted supernatants partially restored CFU counts to those of diluted controls (Fig. 1D).

IL-17A and IL-17F expression is critical for nasal decolonization. We previously reported that IL-17A-deficient mice have a defect in nasal decolonization and IL-17F is upregulated in response to *S. aureus* nasal carriage (26). Since both IL-17A and IL-17F can induce AMP expression in epithelial cells and IL-17A and IL-17F deficiency can promote spontaneous *S. aureus* abscesses *in vivo* (35), we hypothesized that mice deficient in IL-17A and IL-17F (IL-17A/F KO) would be defective in clearance and have a more severe phenotype than IL-17A-deficient mice alone. In order to determine the combined importance of IL-17A and IL-17F during colonization, WT, IL-17A KO, and IL-17A/F KO mice were inoculated intranasally with *S. aureus*. IL-17A and IL-17A/F KO mice were found to have a significant increase in intranasal *S. aureus* CFU compared to WT mice and an intermediate or complete inability to clear colonization, respectively (Fig. 2A and B). IL-17A/F KO mice have a significantly higher *S. aureus* burden and a trend toward increased colonization rate compared to IL-17A KO mice. In conjunction, the inability to clear colonization in IL-17A/F mice is similar to the phenotype of SCID and β/δ-TCR KO mice based on our previously published data (26).

IL-17A directly upregulates mBD-3 expression *ex vivo*. Since IL-17A and IL-17F are crucial for *in vivo* clearance of *S. aureus* nasal carriage, we wanted to determine their ability to induce AMP expression in an *ex vivo* nasal tissue model. Nasal tissue from naive mice were harvested and placed in RPMI containing streptomycin to remove endogenous microflora. A concentration of 20 ng/ml of IL-17A, IL-17F, or both IL-17A and IL-17F was added to cultured nasal tissue and then incubated at 37°C and 5% CO_2 for 24 h. Addition of sterile PBS served as a nonstimulated control. Following incubation, RNA was purified from nasal tissue, converted to cDNA, and analyzed by RT-PCR for relative expression of mouse CRAMP, β-defensin-3, and β-defensin-14. Under the conditions tested, mBD-3 was the only AMP upregulated compared to results with the medium control, and it was upregulated by IL-17A alone (Fig. 3B). IL-17F did not promote expression of CRAMP, mBD-3, or mBD-14 (Fig. 3A to C). Stimulation by a combination of IL-17A and IL-17F did not have a synergistic effect but may induce a slight but not statistically significant antagonistic effect on mBD-3 expression over IL-17A treatment alone (Fig. 3B).

Nasal tissue AMP expression is upregulated by *S. aureus* and is IL-17A dependent. We have shown that nasal tissue supernatants have antistaphylococcal properties and can produce AMPs when stimulated with IL-17A *ex vivo*; however, it is unknown if AMPs are induced upon *S. aureus* nasal colonization *in vivo*. In order to determine the antimicrobial peptide response against *S. aureus* nasal colonization, C57BL/6J mice were inoculated intranasally, and nasal tissue was harvested at various time points. AMP expression analysis displayed an upregulation of all antimicrobial peptides tested (Fig. 4A). CRAMP expression was enhanced 7 days postinoculation, whereas mBD-3 and mBD-14 were upregulated 14 days postinoculation.

Since IL-17A was found to induce expression of mBD-3 under *ex vivo* conditions, we decided to test the importance of IL-17A on AMP expression *in vivo*. Therefore, we inoculated IL-17A KO mice with *S. aureus* and harvested nasal tissue for expression analysis as previously described. In contrast to WT C57BL/6J mice, IL-17A KO mice were deficient in upregulation of CRAMP, mBD-3, and mBD-14 at the time points tested (Fig. 4B).

Nasal tissue AMP production correlates with human nasal *S. aureus* carriage state. In order to translate whether AMP production correlates with *S. aureus* colonization in humans, we acquired human nasal secretion samples from healthy volunteers and screened them by culture- and PCR-based methods for the pres-

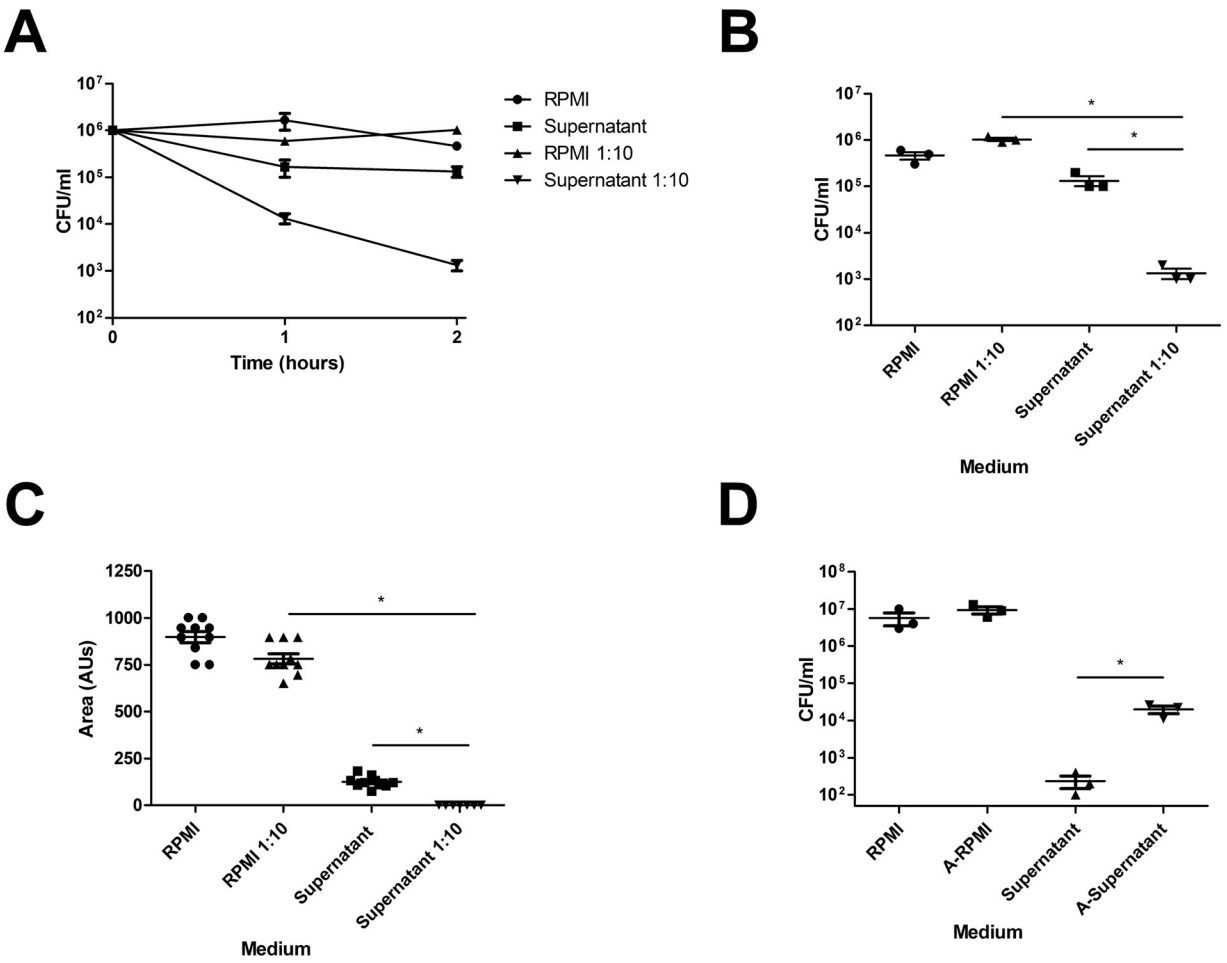

FIG 1 Nasal tissue supernatants have antistaphylococcal activity that is solute and heat sensitive. Naive nasal tissue was harvested from WT C57BL/6J mice. Tissue was incubated in RPMI for 24 h. Tissue supernatants were utilized for *in vitro* killing assays. (A) *S. aureus* (10^6 CFU) was cultured in nasal tissue supernatants or supernatants diluted 1:10 in ddH_2O for various amounts of time. RPMI and RPMI diluted 1:10 in ddH_2O served as controls. (B) CFU counts at 2 h postinduction. (C) *S. aureus* was incubated for 2 h in RPMI or tissue supernatants and plated on TSA. Colony area was assessed by visualization on an Alpha Innotech FluorChem 8900 imager. AUs, arbitrary units. (D) *S. aureus* was incubated for 2 h in diluted RPMI or tissue supernatant or for 2 h in previously autoclaved RPMI (A-RPMI) or supernatant (A-Supernatant). $n = 3$ nasal tissue samples per group. Data are representative of two independent experiments. Statistical analysis was performed with a one-tailed *t* test (*, $P < 0.05$).

ence of *S. aureus*. Two samples were collected over a 6-week period from each subject and grouped by carriage state into noncolonizers (both samples *S. aureus* negative), intermittent colonizers (one sample positive/one sample negative), and long-term colonizers (both samples positive). Since the sampling occurred only over a 6-week period and only two samples were obtained, we could not designate these long-term colonizers as persistently colonized, which are defined as *S. aureus* culture-positive nasal swabs collected on 5 to 10 separate occasions during a 6-month period, and subjects are labeled as persistent carriers if >80% of the cultures are positive (36–38). In our studies with *S. aureus*, we found that 68% of patients were noncolonizers, 17% were intermittent colonizers, and 15% were long-term colonizers (data not shown). Nasal secretion protein levels of human β-defensin-2, β-defensin-3, and LL-37, the human orthologs of mouse β-defensin-3, β-defensin-14, and CRAMP, were measured by ELISAs and grouped by carriage state (Fig. 5A to C). These results showed a significant increase in AMP levels in long-term carriers compared to noncolonizers; however, no statistical difference was found between intermittent colonizers and the other groups. The largest increase in protein expression between noncolonizers and long-term carriers was in LL-37, followed by hBD-2 and hBD-3.

DISCUSSION

There is growing evidence that innate antimicrobial peptides are an important and often required component of the immune system for resolution of infection. Additionally, recent reports support the role of AMPs in host defense against mucocutaneous *S. aureus* infections (39, 40). Here, we show that nasal tissue supernatants have solute and heat-dependent antistaphylococcal activity. Furthermore, we have demonstrated that IL-17F, in conjunction with IL-17A, is required for clearance of *S. aureus* nasal carriage. IL-17A stimulation was sufficient for upregulation of mBD-3 in nasal tissue, and the antimicrobial peptides CRAMP, mBD-3, and mBD-14 were upregulated in an IL-17A-dependent fashion during *S. aureus* nasal carriage. Importantly, human nasal secretions were found to have increased protein levels of hBD-2, hBD-3, and LL-37, the human orthologs of mBD-3, mBD-14, and

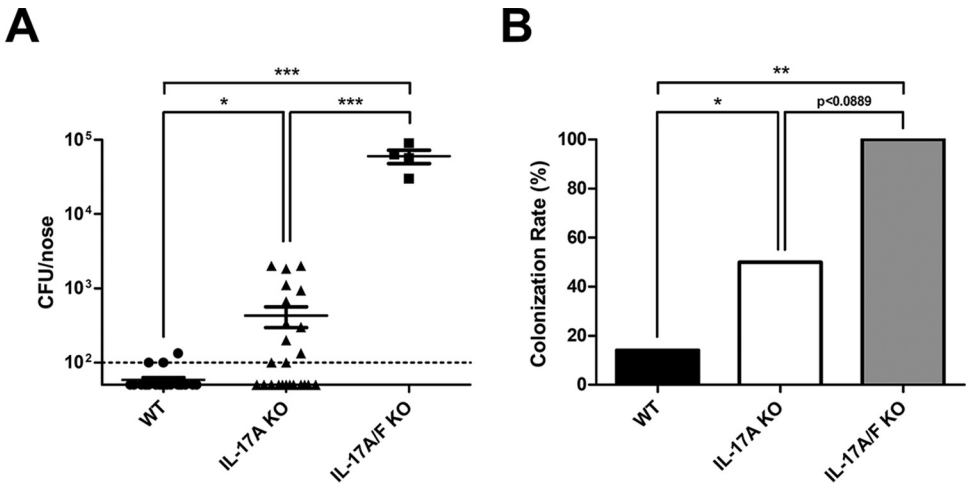

FIG 2 IL-17A and IL-17F are required for clearance of *S. aureus* nasal carriage. C57BL/6J WT, IL-17A KO, and IL-17A/F KO mice were inoculated intranasally with *S. aureus* clinical isolate SA1108. (A) CFU counts/nose 28 days postinoculation (dpi). Nasal tissue was harvested at 28 dpi, homogenized, and plated on CHROMagar-*S. aureus* to determine CFU counts. The detection limit was 100 CFU. (B) Colonization rate 28 days postinoculation. Colonization rate was determined by the number of mice positive for *S. aureus* out of the total number of mice. $n = 4$ to 9 C57BL/6J mice per group. Data are combined from one or two independent experiments. Statistical analyses are in comparison to results for WT mice or between groups, as indicated by lines, and were performed with a one-tailed *t* test or Fisher's exact test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

CRAMP, in patients with long-term *S. aureus* colonization compared to noncolonizers.

Cultured explant nasal tissue supernatant displayed strong antistaphylococcal activity that was solute dependent and heat sensitive (Fig. 1). Our results mimic a study that displayed reduced killing of *S. aureus* in heat-inactivated human nasal secretions (41). As mentioned previously, most antimicrobial peptide activity is sensitive to high salt concentrations. Therefore, the solute-dependent killing effect observed in our *ex vivo* nasal tissue supernatants may be explained by the presence of antimicrobial peptides.

It is important to note that in a previous study, IL-17A-deficient mice had only a partial clearance defect, whereas NOD-SCID mice displayed a complete defect in decolonization (26). We found that depletion of both IL-17A and IL-17F was required to recapitulate the severe clearance defect in NOD-SCID mice, suggesting that IL-17A and IL-17F are critical and nonredundant effector cytokines for elimination of *S. aureus* nasal colonization. Interestingly, a study found that mice deficient in both IL-17A and IL-17F developed spontaneous *S. aureus* mucocutaneous abscesses around the nose and mouth; however, mice depleted of only IL-17A or IL-17F did not produce abscesses (35). Therefore, our data and the literature suggest that both IL-17A and IL-17F are required for host defense and have nonredundant roles against *S. aureus* mucocutaneous infections.

Our data displayed an IL-17A-dependent induction of CRAMP, mBD-3, and mBD-14 upon *S. aureus* carriage. This is not unexpected, as an array of studies currently support the link between Th17/IL-17A and AMP expression (42–44). Interestingly, out of all AMPs and treatments tested, only mBD-3 was upregulated, and it was upregulated only in the presence of IL-17A in our *ex vivo* stimulation experiment. Human airway epithelial cells, upon treatment with IL-17A, had significant upregulation of hBD-2 (mBD-3 equivalent) (45). In addition, stimulation of hu-

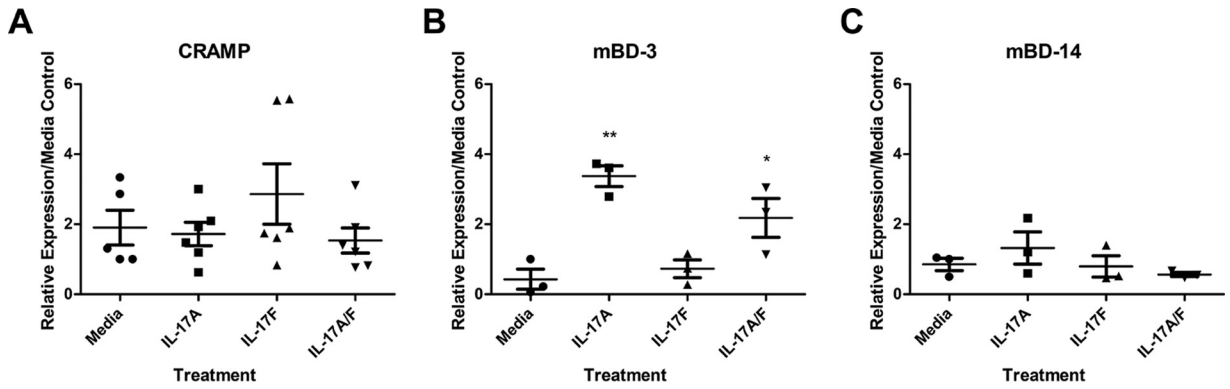

FIG 3 IL-17A is sufficient for expression of mBD-3 in *ex vivo* nasal tissue. Naive nasal tissue was harvested from WT C57BL/6J mice. Tissue was incubated in RPMI for 24 h with 20 ng/ml of mouse rIL-17A, rIL-17F, or both rIL-17A and rIL-17F. Nonsupplemented medium acted as a negative control. Nasal tissue RNA was purified, converted to cDNA, and used for RT-PCR assays. Levels of mouse CRAMP (A), mBD-3 (B), and mBD-14 (C) were measured. Data are presented as RNA expression relative to that of medium (nonsupplemented) controls. $n = 3$ to 6 C57BL/6J mice per time point. Data are representative of two independent experiments. Statistical analyses are in comparison to medium control samples and were performed with a one-tailed *t* test (*, $P < 0.05$; **, $P < 0.01$).

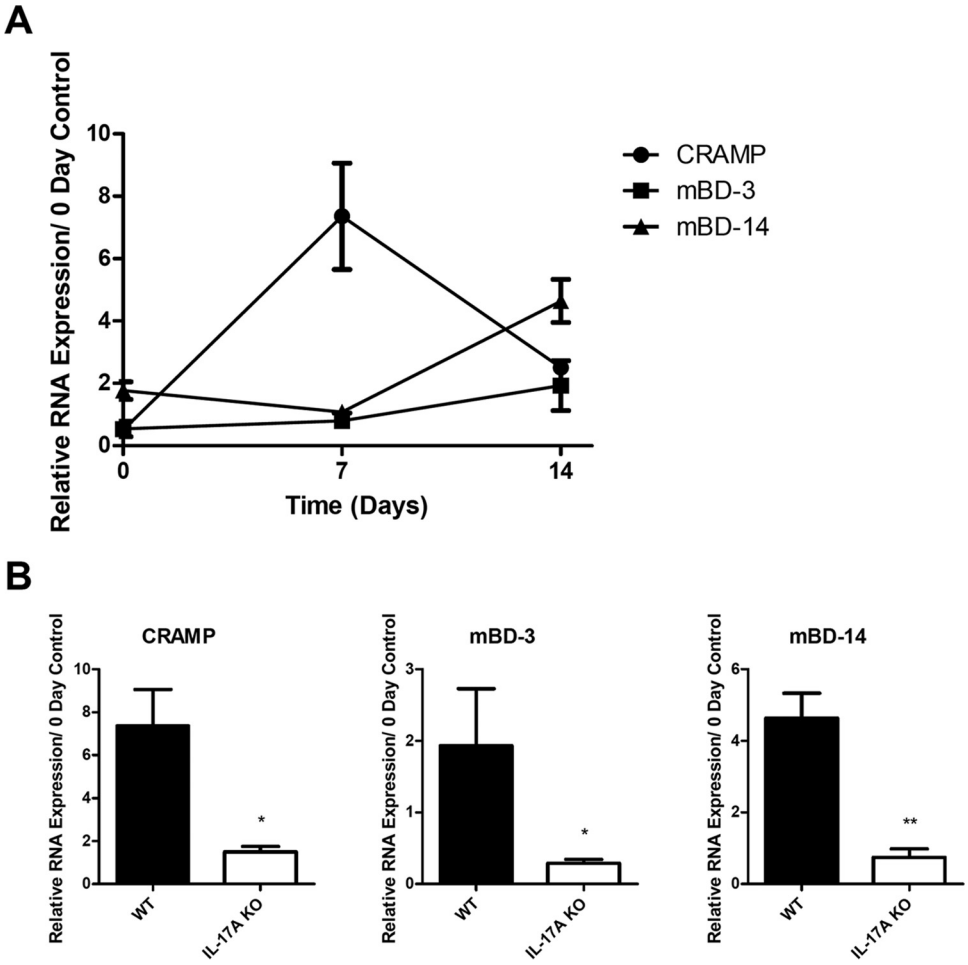

FIG 4 IL-17A is required for AMP expression induced by *S. aureus* nasal colonization. WT C57BL/6J and IL-17A KO mice were inoculated intranasally with *S. aureus* clinical isolate SA1108. Nasal tissue RNA was purified, converted to cDNA, and used for RT-PCR assays. (A) Levels of mouse CRAMP, mBD-3, and mBD-14 were measured in WT mice at various time points. (B) Expression levels of CRAMP, mBD-3, and mBD-14 were compared between WT and IL-17A KO mice at either day 7 or day 14. Data are presented as RNA expression relative to that of day 0 (noninoculated) controls. $n = 2$ to 5 C57BL/6J or IL-17A KO mice per time point. Data are representative of at least two independent experiments. Statistical analysis was performed with a one-tailed *t* test (*, $P < 0.05$; **, $P < 0.01$).

man airway epithelial cells with a myriad of innate cytokines displayed IL-17A as the most potent inducer of hBD-2. This study validates our data showing specific mBD-3 upregulation upon stimulation with IL-17A in murine *ex vivo* nasal tissue.

When given together, IL-17A and IL-17F did not display an additive or synergistic effect on AMP expression. The deficient production of CRAMP and mBD-14 in IL-17A- and IL-17F-stimulated nasal tissue may be due to lack of a costimulatory agent. Peric et al. found that human keratinocytes stimulated with supernatants from T cells isolated from lesional psoriatic skin increased expression of cathelicidin when stimulated in the presence of 1,25-dihydroxyvitamin D_3 ($1,25D_3$; active form of vitamin D) (46). *In vitro*, IL-17A-enhanced cathelicidin mRNA and peptide expression in keratinocytes was dependent on the presence of $1,25D_3$. Another study elucidated that IL-17A and IL-17F, in conjunction with IL-22, synergistically induced the expression of β-defensin-2 and S100A9 and additively enhanced the expression of S100A7 and S100A8 in human keratinocytes (42). Therefore, IL-17A or IL-17F alone may not be sufficient to induce CRAMP and mBD-14 production. The observed upregulation of IL-1β in our previous studies may be a sufficient costimulatory cytokine when combined with IL-17A or IL-17F (26). Liu et al. discovered that IL-1β was required for expression of human β-defensin-4 (47). The inability of IL-17A and IL-17F to induce CRAMP and mBD-14 expression *ex vivo*, while IL-17A is required for their expression *in vivo*, suggests that IL-17A, and possibly IL-17F, are required but not sufficient for AMP expression. Further studies need to be performed in order to determine the cytokine milieu sufficient for CRAMP and mBD-14 production.

Although we demonstrated the induction of CRAMP, mBD-3, and mBD-14 in nasal tissue, we have not determined the source of these peptides. These peptides have broad inducible tissue expression. Human LL-37 (mouse CRAMP) is produced in neutrophil granules, in addition to inflamed skin, lung epithelia, and squamous epithelia of human mouth, tongue, esophagus, cervix, and vagina (48–51). The source of CRAMP expression may originate from nasal lumen-associated neutrophils or nasal epithelial cells that have been activated in response to *S. aureus* insult. Human β-defensin-2 and β-defensin-3 (mBD-3 and mBD-14) are present in oral tissue, gastric mucosa, skin, lung epithelia, infected kidney,

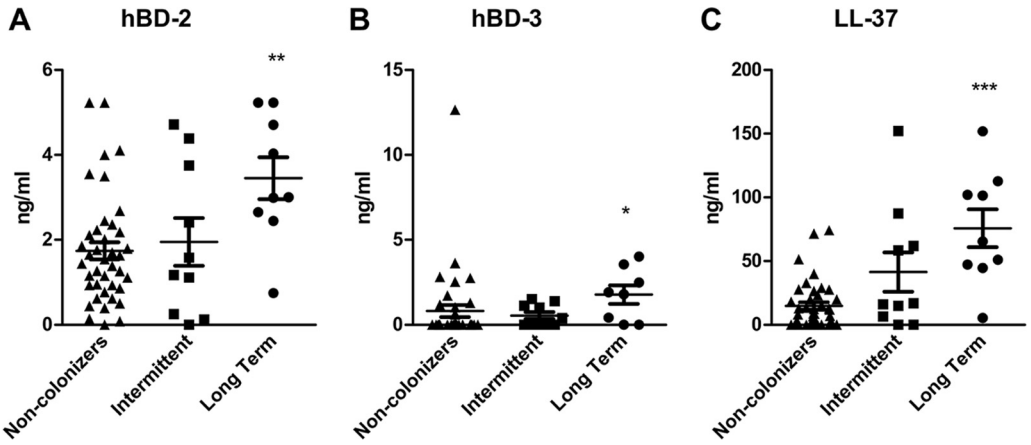

FIG 5 Human *S. aureus* nasal carriage promotes AMP production. Human nasal secretions were determined by culture and PCR to be either from noncolonizers, intermittent colonizers, or long-term *S. aureus* colonizers, and levels of hBD-2 (A), hBD-3 (B), and LL-37 (C) were elucidated by ELISAs. $n = 41$ noncolonizers; $n = 10$ intermittent colonizers; $n = 9$ long-term colonized human samples. Statistical analyses are in comparison to results for the noncolonizer group and were performed with the Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

and trachea (3, 52, 53). The most prominent source of mBD-3 and mBD-14 assembly is likely stimulated nasal mucosa. Further studies are necessary to elucidate the cellular origin of nasal AMP expression in response to *S. aureus* colonization.

These data show that human nasal secretions have increased AMP protein production in response to *S. aureus* carriage and validate our mouse model of AMP expression in response to *S. aureus* nasal colonization. Those with intermittent colonization (culture positive for *S. aureus* at time zero and culture negative 6 weeks later) may have been in the final stages of clearance and had intermediate AMP expression levels. This is in contrast to those patients with long-term *S. aureus* colonization (culture positive for *S. aureus* at time zero and 6 weeks later). However, since human nasal secretion samples were only taken at two time points, the patient *S. aureus* nasal carriage status and AMP secretion levels should be further validated with clinical data involving more frequent sample collection.

These patients had a continuously activated immune response against colonizing *S. aureus* with sustained AMP-upregulated expression. This upregulated AMP expression was shown to be effective at the slow but eventual elimination of *S. aureus* carriage in mice (26). In fact, elevated AMP levels were able to ultimately eliminate *S. aureus* from the nares in greater than 70% of the mice by 28 days after colonization initiation using the controlled environment of the murine model of colonization (26). The sustained colonization seen in humans, even with elevated AMP levels, may be due to a longer time course required for colonization elimination in human subjects. It may also be due to recolonization from the patient's local environment, pets, or human cohabitation. However, since patients were not sampled at later time points, it is unknown if these elevated AMP levels eventually cleared staphylococcal colonization. If these patients were never able to clear *S. aureus* and were persistently colonized, the ineffectiveness of the host may be due to other factors, such as having a suboptimal neutrophil response, a uniquely fit strain of colonizing *S. aureus*, or a permissive nares microbiome. Importantly, we do not have data on AMP levels of noncolonizers given *S. aureus*. It is possible that noncolonizers induce AMPs more robustly than patients colonized long term in response to *S. aureus* carriage or do so in a transient manner.

In conclusion, our data show that IL-17A and IL-17F have nonredundant roles in the clearance of *S. aureus* nasal carriage. Additionally, nasal tissue supernatants display IL-17A-dependent and AMP-mediated antistaphylococcal activity, and patients with long-term nasal colonization have enhanced AMP production. Promotion of antistaphylococcal antimicrobial peptides may be therapeutically beneficial in the elimination of *S. aureus* nasal colonization and thereby prevent distal infections originating from nasal carriage.

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