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HOST RESPONSE AND INFLAMMATION

# Cessation from Smoking Improves Innate Host Defense and Clearance of Experimentally Inoculated Nasal *Staphylococcus aureus*

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**ABSTRACT** *Staphylococcus aureus* nasal carriage is transient in most humans and usually benign, but dissemination of *S. aureus* to extranasal sites causes the majority of clinical infections, and *S. aureus* is a major cause of serious infections in the United States. A better understanding of innate nasal decolonization mechanisms is urgently needed, as are relevant models for studying *S. aureus* clearance. Here, we screened a population of healthy smokers for nasal *S. aureus* carriage and compared the participants' abilities to clear experimentally applied nasal *S. aureus* before and after completion of a smoking cessation program. We determined that cigarette smoking increases the mean nasal *S. aureus* load ($2.6 \times 10^4$ CFU/swab) compared to the load observed in healthy nonsmokers ($1.7 \times 10^3$ CFU/swab) and might increase the rate of *S. aureus* nasal carriage in otherwise-healthy adults: 22 of 99 smokers carried *S. aureus* at the screening visit, while only 4 of 30 nonsmokers screened positive during the same time period. Only 6 of 19 experimental inoculation studies in active smokers resulted in *S. aureus* clearance within the month of follow-up, while in the cessation group, 6 of 9 subjects cleared nasal *S. aureus* and carriage duration averaged 21 $\pm$ 4 days. Smoking cessation associated with enhanced expression of *S. aureus*-associated interleukin-1$\beta$ (IL-1$\beta$) and granulocyte colony-stimulating factor (G-CSF) in nasal fluids. Participants who failed to clear *S. aureus* exhibited a higher nasal *S. aureus* load and elevated nasal interleukin-1 receptor antagonist (IL-1RA) expression at the preexperiment study visits. We conclude that smokers exhibit higher *S. aureus* loads than nonsmokers and that innate immune pathways, including G-CSF expression and signaling through the IL-1 axis, are important mediators of nasal *S. aureus* clearance.

**KEYWORDS** *Staphylococcus aureus*, experimental colonization, host defense, humans, nasal carriage, smoking cessation

*Staphylococcus aureus* colonizes healthy humans and various mammals, including cows, pigs, and house pets. Treatment of *S. aureus* infections and the rise in antibiotic-resistant infections inflict a tremendous burden on health care and agriculture. *S. aureus* colonizes most often the nose and pharynx of humans, with the nasal vestibule considered the reservoir (1–3). The majority of nasal *S. aureus* strains classify as methicillin-susceptible *S. aureus* (MSSA), although methicillin-resistant *S. aureus* (MRSA) is hosted and disseminated by healthy and hospitalized *S. aureus* carriers. The overall carriage rate in humans ranges from 20 to 50%, with higher rates reported for children, industrial hog workers, immunocompromised individuals, and patients presenting with clinical extranasal *S. aureus* infections (4, 5). Considering that most invasive

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*S. aureus* isolates are genetically indistinct from corresponding nasal isolates (6, 7), nasal *S. aureus* carriage represents a major risk factor for *S. aureus* disease, postoperative complications, and mortality.

The determinants of nasal *S. aureus* carriage include bacterial, environmental, and host immune factors. *S. aureus* adhesion to the nasal mucosa is governed by wall teichoic acid, clumping factor B, and numerous additional microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (8–11). The ability of *S. aureus* to respond to nutrient limitation also appears to be critical to nasal colonization, although no differences were found between nutrient levels in nasal secretions from carriers and from noncarriers (12). Perhaps most critical to the onset of nasal carriage is the immune status of the host. Atopic dermatitis, HIV, diabetes, and dialysis patients have a higher incidence of *S. aureus* carriage, as do newborns and nondiabetics with a body mass index (BMI) of >30 (13–15). The nasal fluids of human *S. aureus* carriers have less intrinsic anti-*S. aureus* activity than do nasal fluids collected from noncarriers (16, 17). Healthy volunteers who cleared experimentally inoculated nasal *S. aureus* produced a rapid response marked by elevated inflammatory factors in nasal fluid collected 2 days postinoculation, whereas subjects who failed to clear *S. aureus* presented a delayed and dysregulated response (18). Deciphering the roles of specific human nasal host defense factors regulating the onset of nasal *S. aureus* carriage will be pivotal for designing better decolonization strategies.

Despite a decline in recent years, an estimated 16.7% of men and 13.6% of women in the United States (approximately 36.5 million adults) currently smoke cigarettes (19), and smoking-related diseases continue as a leading cause of hospitalizations and death. Whether or not smoking influences nasal carriage of *S. aureus* remains an important question. Some studies associated smoking and secondhand smoke with increased *S. aureus* carriage rates (20–23), and others declared no association (24, 25), while the Tromsø (Norway) Staph and Skin Study 2007-08 indicated that smoking might be protective (26). Notably, *S. aureus* carriage triples the risk for postoperative skin and soft tissue infections (27), and smoking associates with higher infection rates (28). In the current study, we determined the nasal *S. aureus* carriage rate in healthy smokers who attend the University of Central Florida (UCF). We tested the ability of carriers to clear their experimentally inoculated strain both while smoking and after cessation, and we identified nasal host defense factors associated with nasal *S. aureus*. We demonstrate that healthy smokers exhibit a higher nasal *S. aureus* load than nonsmokers and are less likely to clear experimentally inoculated *S. aureus* than nonsmokers and those who successfully quit smoking.

## RESULTS

**Elevated nasal *S. aureus* load in healthy smokers compared to nonsmokers.** From January 2014 to November 2015, we screened 30 healthy nonsmokers and 99 healthy smokers for nasal *S. aureus*. The smoker cohort was young (mean, 28 years; median, 25 years; range, 18 to 57 years), 46% female, and racially diverse (75% white, 13% Hispanic, 7% Asian, 5% black). The average smoking history was 8.9 ± 6.0 years (range, 2.5 to 24 years), and most subjects reported smoking 2 to 5 cigarettes per day. Twenty-two of 99 smokers (22.2%) were *S. aureus* positive in one or both nostrils at the screening appointment, while 4 of 30 nonsmokers (13%) exhibited nasal *S. aureus* positivity at their initial visit. For the smoker group, 18 *S. aureus* carriers consented and enrolled in further study visits to test *S. aureus* clearance rates during active smoking versus after cessation. The 30 nonsmokers submitted nasal swabs every 4 to 7 days for several months (mean, 44 swab visits) to monitor natural *S. aureus* carriage. This group was similarly matched to the smoker group demographically: mean age, 25 years; median, 22 years; range, 19 to 43 years; 43% female; 60% white, 7% Hispanic, 30% Asian, 3% black. Sixteen nonsmokers were nasal *S. aureus* negative at every swab visit, while 14 were nasally colonized with *S. aureus* at least once during their respective evaluation periods (participant information and carriage statistics are shown in Table 1). We compared the nasal *S. aureus* loads from smokers' screening visits (average of 2

**TABLE 1** Nonsmoker participant demographics and nasal *S. aureus* carriage history

| Nonsmoker ID ($n$ = 14) | Gender[a] | Age (yr)[b] | Nasal *S. aureus* carriage (no. of *S. aureus*-positive visits/total no. of visits) | Duration of monitoring (wk) |
|---|---|---|---|---|
| D501 | M | 31 | 1/44 | 42 |
| D20 | M | 43 | 68/71 | 77 |
| D547 | F | 40 | 105/127 | 87 |
| D832 | F | 20 | 63/63 | 58 |
| D833 | F | 29 | 1/66 | 71 |
| D834 | F | 21 | 50/71 | 63 |
| D836 | F | 19 | 9/59 | 64 |
| D837 | M | 22 | 55/55 | 57 |
| D840 | M | 21 | 36/63 | 70 |
| D845 | F | 23 | 87/95 | 67 |
| D848 | M | 19 | 19/68 | 61 |
| D852 | M | 21 | 11/36 | 37 |
| D853 | M | 24 | 1/16 | 23 |
| D855 | M | 21 | 29/29 | 24 |

[a]M, male ($n$ = 8 [57%]); F, female ($n$ = 6 [43%]).
[b]Mean age, 25.3 years; range, 19 to 43 years.

nostril CFU counts for 16 of 18 participants with countable agar plates) with the 14 healthy nonsmokers' average *S. aureus* loads from *S. aureus*-positive visits (Fig. 1A). In nonsmokers, the nasal *S. aureus* load averaged 1,698 CFU/swab and was similar for men and women (Fig. 1A). Smokers' mean *S. aureus* load (25,704 CFU/swab) was significantly greater than that of nonsmokers, independent of gender (Fig. 1A). To determine whether seasonal variation might contribute to this difference, we also plotted the 16 *S. aureus* loads recorded for smokers and the average nasal *S. aureus*

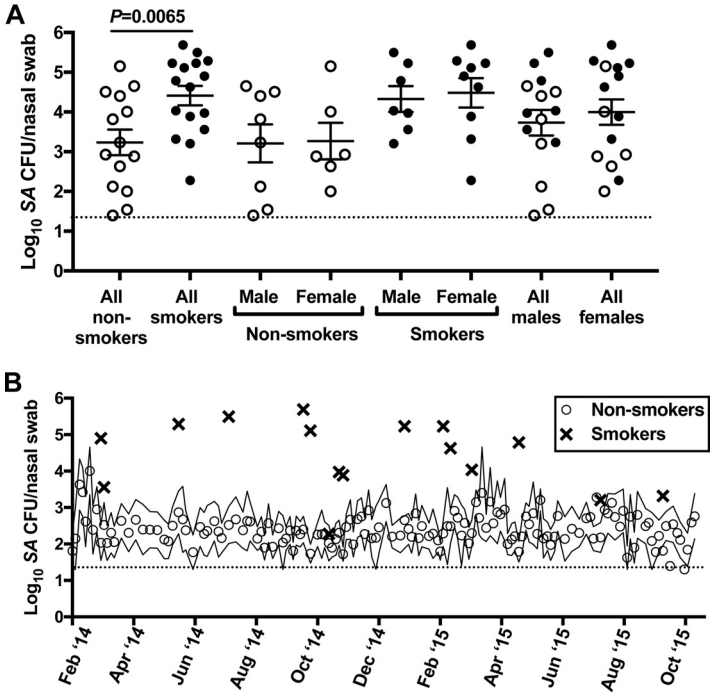


**FIG 1** Cigarette smoking associates with elevated natural nasal *S. aureus* load. (A) Nasal *S. aureus* load (average *S. aureus* CFU/swab for two nostrils) at the screening visit of healthy smokers (solid black circles) versus the average nasal *S. aureus* load per *S. aureus*-positive visit for nonsmokers (open circles) who were monitored longitudinally during the same time period. Error bars represent the means and standard errors of the means (SEM). (B) Seasonal nasal *S. aureus* load for smoker screening visits (bold X's) versus the average nasal *S. aureus* load for 14 nonsmoker carriers (open circles). Solid lines enveloping the open circles indicate the SEM for nonsmoker average nasal *S. aureus* load. The dotted horizontal line in each panel indicates the *S. aureus* detection limit for nasal swab samples (20 CFU/swab).

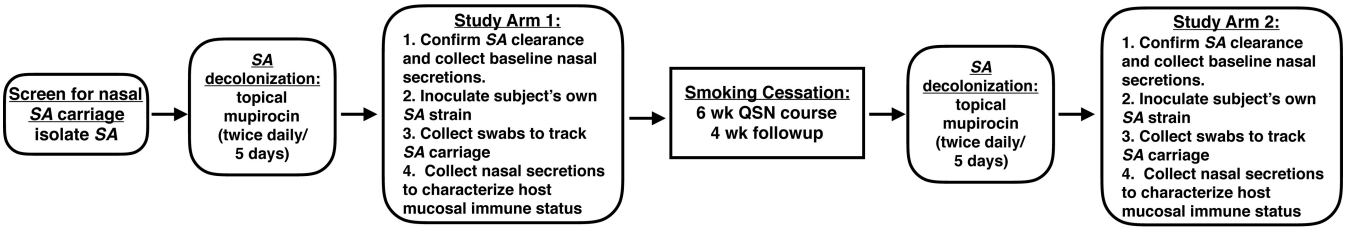


**FIG 2** Experimental design and timeline. Healthy smokers were screened for nasal *S. aureus* (*SA*) carriage, and *S. aureus* isolates were genotyped and prepared as frozen stocks for subsequent inoculations. Participants applied topical nasal mupirocin twice daily for 5 days (days −17 to −13, preinoculation) to clear endogenous nasal *S. aureus*. On day −6 (preinoculation), nasal swabs were collected to confirm *S. aureus* decolonization, and nasal secretions were collected. On days 0 and 1, participants were swabbed for assessment of *S. aureus* carriage status and self-applied $2 \times 10^7$ CFU of their own *S. aureus* strain to each nostril. Nasal swabs were collected every 3 to 4 days postinoculation. Nasal secretions were collected at days 3, 8, 11, 15 or 16, 23 or 24, and 31 or 32 postinoculation. Following the month of follow-up after *S. aureus* inoculation, participants attended a 6-week Quit Smoking Now (QSN) cessation course. Subjects who successfully quit smoking and maintained cessation for an additional 4 weeks repeated the decolonization/recolonization protocol.

loads of 14 nonsmoker carriers over the course of the evaluation period. Figure 1B shows that nonsmokers averaged $10^2$ to $10^4$ *S. aureus* CFU/swab throughout the observation period, with slight peaks noted in February and March of both years. Smokers who screened positive for *S. aureus* exhibited $10^4$ to $10^6$ CFU/swab at visits that spanned both summer and winter seasons. Taken together, these data suggest that smokers exhibit higher nasal *S. aureus* levels than nonsmokers, independent of gender, and even in a young and healthy population and a climate with mild winters.

**Cessation of smoking associates with increased nasal *S. aureus* clearance following experimental inoculation.** To test whether the host's ability to clear nasal *S. aureus* improves upon cessation of smoking, participants underwent an experimental nasal inoculation protocol while actively smoking and then again after having successfully quit cigarettes for at least a month (the study design is depicted in Fig. 2). Participants were cleared of their nasal *S. aureus* using a 5-day/twice-daily treatment with mupirocin ointment. One week after the last application, *S. aureus* clearance was confirmed and nasal fluids were collected for baseline measurements of mucosal immune status. A week later, nasal swabs were again collected in order to confirm the absence of nasal *S. aureus* and the restoration of resident non-*S. aureus* nasal flora to premupirocin levels (18). Each participant then self-applied his or her previously isolated *S. aureus* strain to each nostril (participant and *S. aureus* isolate data are shown in Table 2). Nasal swabs were performed the next day, immediately prior to a repeat inoculation, and swab samples were collected every 3 to 4 days thereafter for a month of follow-up. Nasal fluids were collected at days 3, 8, 11, 15 or 16, 23 or 24, and 31 or 32 postinoculation. We previously reported that in healthy nonsmokers, 10 of 15 studies resulted in the clearance of inoculated *S. aureus* within 1 month (18). For active smokers, only 6 of the 19 studies resulted in clearance of nasal *S. aureus* within the month, and the average ± standard deviation (SD) carriage duration was at minimum 30 ± 2 days, as follow-up did not extend beyond 34 days (Fig. 3A). In the cessation group, 6 of 9 subjects cleared their *S. aureus* and carriage duration averaged 21 ± 4 days (Fig. 3A). To aid with confirmation of active smoking versus cessation, expired-air carbon monoxide (CO) was measured at most of the participants' study visits. A previous study demonstrated that an expired-air CO level of ≤4 ppm indicates abstinence from cigarettes for at least 24 h (29). Nonsmokers' readings on our meter ranged from 2 to 5 ppm (data not shown). For the study participants, expired-air CO was 19.50 ± 5.56 ppm for reported smokers and 4.684 ± 0.44 ppm for reported quitters (Fig. 3B). It should be noted that the active-smoker group contained two subjects with average expired-air CO values that were as low as in the cessation group (Fig. 3B). A pairwise comparison of the 8 participants who completed both studies indicated a trend of shorter *S. aureus* carriage duration for the cessation group ($P = 0.0619$) (Fig. 3C). Interestingly, these 8 participants partitioned into one group that cleared *S. aureus* within 16 days after smoking cessation (Fig. 3C, solid symbols) and another group that could not effectively clear

**TABLE 2** Smoker participant and *S. aureus* isolate details

| Smoker ID ($n$ = 18) | Gender[a] | Age (yr)[b] | MLST/*spa* type[c] | Presence of *mecA*[d] | Study arm participation[e] |
|---|---|---|---|---|---|
| S002 | F | 20 | 59/t216 | − | Smoking |
| S008 | F | 18 | 87/t216 | + | Smoking |
| S012 | M | 22 | 1159/t1685 | − | Smoking |
| S019 | F | 35 | 5/t228 | − | Smoking |
| S036 | M | 24 | 101/t2078 | − | Both |
| S039 | F | 25 | 30/t338 | − | Smoking |
| S042 | F | 18 | 5/t002 | − | Smoking twice (no cessation) |
| S046 | F | 42 | 97/t267 | + | Smoking |
| S051 | M | 38 | 4346/t084 | − | Both |
| S052 | F | 21 | 72/t148 | − | Both |
| S064 | M | 20 | 8/t2866 | − | Both |
| S065 | F | 22 | 8/t2866 | − | Smoking |
| S070 | F | 35 | 59/t1293 | − | Both |
| S072 | M | 28 | 8/t008 | + | Both |
| S076 | M | 27 | 15/t084 | − | Both |
| S080 | M | 26 | 8/t3308 | − | Cessation only |
| S089 | F | 20 | 72/t6759 | − | Both |
| D831 | M | 25 | 22/t852 | + | Smoking twice (no cessation) |

[a]F, female ($n$ = 10 [56%]); M, male ($n$ = 8 [44%]).
[b]Mean age, 25.8 years; range, 18 to 42 years.
[c]MLST, multilocus sequence type; *spa*, staphylococcal protein A gene.
[d]−, absence; +, presence.
[e]There were 2 study arms: active smoking (19 studies) and cessation (9 studies). Subjects participated in one or both arms or as indicated.

nasal *S. aureus* in one or both study arms (Fig. 3C, open symbols). Considering our previous findings demonstrating that dysregulation of host defense factors associated with longer carriage duration in individuals who underwent the same experimental inoculation procedure as the one utilized here (18), this partitioning suggests that

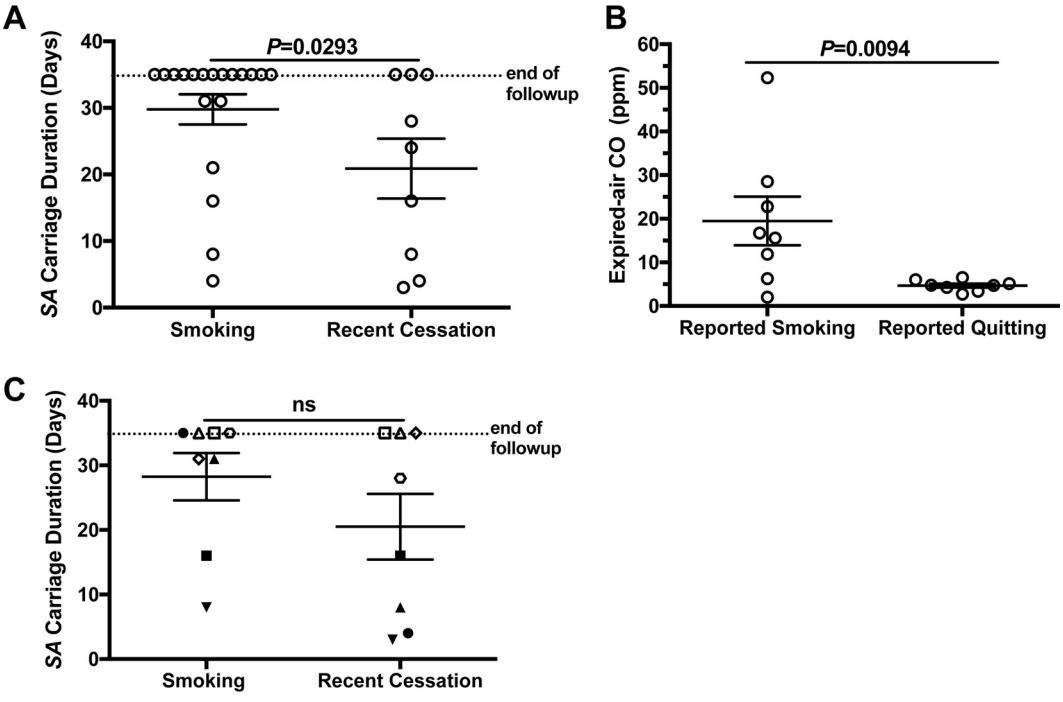


**FIG 3** Cessation from smoking associates with a higher rate of *S. aureus* clearance during experimental inoculation studies. (A) Comparison of experimental *S. aureus* carriage duration between all subjects who were actively smoking and those who recently quit smoking. (B) Average expired-air carbon monoxide (CO) values for participants who reported smoking compared to those who reported cessation of cigarette smoking. (C) Pairwise comparison of experimental nasal *S. aureus* carriage duration for 8 participants (each represented by a unique symbol) who completed both study arms. Open symbols indicate participants who failed to clear *S. aureus* in one or both study arms. Solid symbols indicate participants who cleared experimentally inoculated *S. aureus* in one or both study arms. ns, not significant.

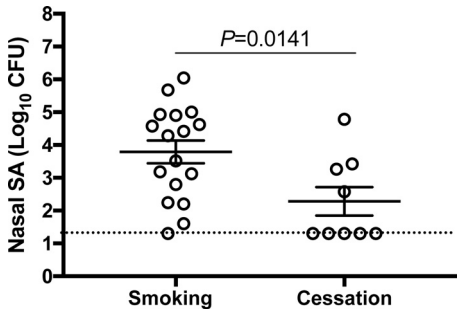


**FIG 4** Decreased natural nasal *S. aureus* load in subjects who successfully quit smoking. Nasal *S. aureus* loads (average *S. aureus* CFU/swab for both nostrils) at day −17 (preexperiment) of the smoking and cessation study arms. Error bars represent the means and SEM. The dotted horizontal line indicates the limit of detection for nasal *S. aureus* (20 CFU/swab).

differences in host mucosal immune effectors might render some individuals susceptible to long-term nasal *S. aureus* carriage regardless of smoking status.

**Decreased natural nasal *S. aureus* load following smoking cessation.** We next compared the natural nasal *S. aureus* loads of participants who completed a smoking cessation program with those of active smokers. Nasal *S. aureus* CFU/swab (average of two nostrils) values recorded at the premupirocin (day −17) study visit demonstrate that smoking cessation resulted in nearly a 100-fold decrease in nasal *S. aureus* load (Fig. 4). Furthermore, all but one of the active smokers' noses maintained *S. aureus* positivity between the screening visit and the premupirocin visit for study arm 1, while only 4 of the 9 noses of cessation subjects swabbed positive at the premupirocin visit for study arm 2 (Fig. 4). This suggests that after smoking cessation, participants were less likely to be naturally recolonized by *S. aureus*.

**Increased expression of *S. aureus*-induced G-CSF and IL-1$\beta$ after smoking cessation.** Innate immunity against colonizing *S. aureus* includes increased neutrophil activity marked by select cytokines and growth factors such as granulocyte colony-stimulating factor (G-CSF), interleukin-1$\beta$ (IL-1$\beta$), and the IL-1 receptor (IL-1R) (18, 30, 31). To assess whether smoking and cessation from smoking associate with different host responses to nasal *S. aureus* inoculation, nasal fluids that were collected before and after mupirocin treatment and every 5 to 7 days following *S. aureus* inoculation were analyzed using a multiplex antibody bead array. We compared nasal fluids collected from *S. aureus*-positive noses with those from *S. aureus*-negative noses and further stratified the data by smoking status. After experimental nasal *S. aureus* inoculation, G-CSF and IL-1$\beta$ were significantly elevated in nasal fluids collected from *S. aureus*-positive noses compared to *S. aureus*-negative noses in the cessation group (Fig. 5A and B). The smoking group, in contrast, exhibited no differences in inflammatory marker levels between nasal fluids collected from *S. aureus*-positive and those from *S. aureus*-negative noses. The IL-1 receptor antagonist (IL-1RA)-to-IL-1$\beta$ ratio was significantly reduced in *S. aureus*-positive versus -negative nasal fluids from cessation subjects, while active smokers exhibited no difference (Fig. 5C). The smoking and cessation groups were comparable in age (means and SD: smoking group, 25.9 $\pm$ 7.4 years; cessation group, 26.5 $\pm$ 6.4 years), and both contained a mix of males and females (smoking, 8 males and 11 females; cessation, 6 males and 3 females). Elevated *S. aureus*-associated G-CSF and IL-1 $\beta$ and a reduced IL-1RA-to-IL-1$\beta$ ratio in nasal fluids from cessation subjects were observed in both male and female subjects (data not shown). Taken together, these data suggest that the increased rate of *S. aureus* clearance demonstrated by the cessation group (Fig. 3) was associated with increased neutrophil activity and IL-1 signaling.

**The carriage of experimentally inoculated *S. aureus* corresponds with an elevated natural nasal *S. aureus* load and elevated expression of IL-1RA.** While host mucosal immunity is the most likely determinant of nasal *S. aureus* carriage status and duration (1, 18), specific host factors associated with nasal *S. aureus* load or failed *S.*

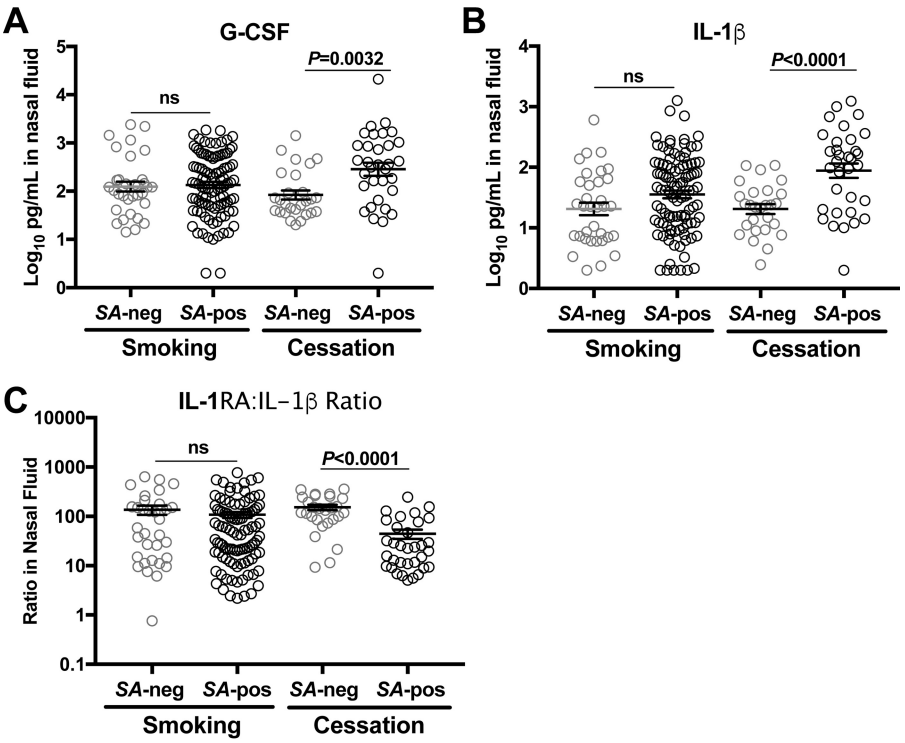


**FIG 5** Smoking cessation associates with augmented innate nasal mucosal defense against *S. aureus*. Participants were cleared of endogenous nasal *S. aureus* and subsequently reinoculated with their own *S. aureus* strain as described for Fig. 2. Nasal fluids were collected from each participant at day −17 (prior to mupirocin application for clearance of endogenous *S. aureus*), day −6 (one week after the last mupirocin application), and at days 3, 8, 11, 15 or 16, 22 or 23, and 31 or 32 postinoculation with autologous *S. aureus*. Fluids were analyzed using Bio-Rad's 27-plex proinflammatory panel, and results are shown for G-CSF (A), IL-1$\beta$ (B), and the Il-1RA-to-Il-1$\beta$ ratio (C), where gray circles represent nasal fluids collected from *S. aureus*-negative noses ($n$ = 34 for smoking, $n$ = 27 for cessation groups) and black circles represent nasal fluids collected from *S. aureus*-positive noses ($n$ = 100 for smoking, $n$ = 33 for cessation groups). Error bars represent the means and SEM.

*aureus* clearance are not known. Using the current cohort of healthy smokers and smokers who recently quit, we next assessed natural nasal *S. aureus* loads and secreted nasal immune factors according to whether the subjects cleared experimentally inoculated *S. aureus* or stayed colonized during the month of follow-up. The "carrier" and "clearer" groups were evenly matched in terms of age and gender (carriers, 24.7 ± 6.1 [mean ± SD] years, 8 males and 9 females; clearers, 27.6 ± 7.4 years, 6 males and 5 females). Consistent with an underlying innate susceptibility for *S. aureus*, carriers presented elevated nasal *S. aureus* levels at both the screening and day −17 (premupirocin) visits (Fig. 6A). Carriers also expressed higher levels of IL-1RA at day −17 than did clearers of *S. aureus* (Fig. 6B), suggesting that carriers' nasal mucosae were naturally "programmed" to antagonize IL-1 signaling. Likewise, nasal *S. aureus* load showed a modest positive correlation with nasal fluid IL-1RA for all subjects who donated both swab and nasal fluid samples on day −17 ($n$ = 22) (Fig. 6C). Collectively, these data suggest that elevated IL-1RA expression supports nasal *S. aureus* survival.

## DISCUSSION

Nasally carried *S. aureus* strains cause the majority of clinical infections, but the events leading to the establishment of human nasal *S. aureus* carriage are not well defined. Thus, methods for studying *S. aureus* interaction with human nasal mucosa are urgently needed in order to identify novel decolonization strategies. In the presented studies, we utilized natural human nasal carriers of *S. aureus* to study early colonization events following participants' self-inoculation with previously isolated autologous isolates.

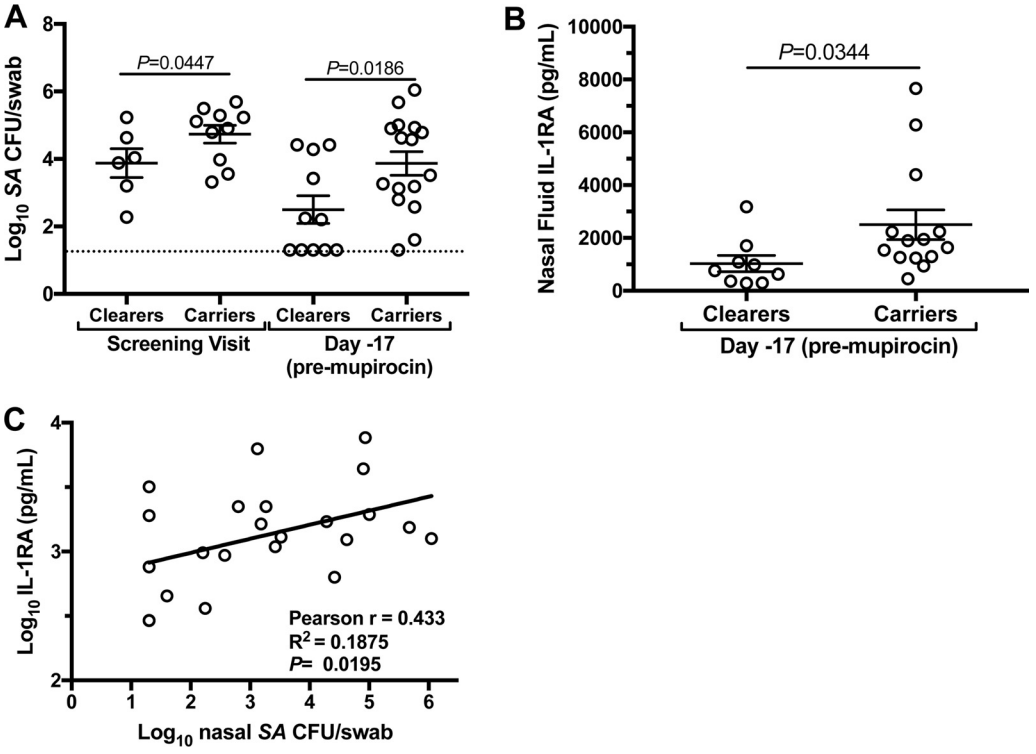


**FIG 6** Clearance of experimentally inoculated nasal *S. aureus* correlated with nasal *S. aureus* load at screening and elevated IL-1RA in nasal secretions. (A) Nasal *S. aureus* levels at the screening and day −17 (premupirocin) visits of participants who eventually cleared ("Clearers") or carried ("Carriers") experimentally inoculated *S. aureus*. The dotted horizontal line indicates the limit of detection. (B) Participants who carried experimentally inoculated *S. aureus* ("Carriers") exhibited elevated natural (day −17/premupirocin) expression of IL-1RA compared to participants who cleared ("Clearers"). In panels A and B, error bars represent the means and SEM. (C) Positive correlation between natural nasal *S. aureus* load (*x* axis) and IL-1RA expression in nasal fluid (*y* axis) collected from subjects at day −17.

We postulated that nasal inflammatory dysregulation might render cigarette smokers more susceptible to *S. aureus* nasal carriage than nonsmokers based on the paradigm that smoking causes both inflammatory and immunosuppressive effects and smokers are at increased risk for infections (32, 33). Several reports have described the effect of cigarette smoke extract (CSE) on innate immunity: CSE-exposed neutrophils exhibit spontaneous superoxide production but attenuated bacterially induced superoxide production and reduced phagocytic function (34, 35). Smokers' macrophages express lower cell surface levels of Toll-like receptor 2 (TLR2) and scavenger receptor, which might be important for recognizing and responding to *S. aureus* and resolving acute inflammation (34, 36). Smoke-exposed *S. aureus* strain USA300 showed reduced susceptibility to killing by macrophages and the antimicrobial peptide LL-37 (37). We observed a 22% nasal *S. aureus* carriage rate for 99 smokers who attended a single screening visit, while only 4 of 30 (13%) healthy nonsmokers were nasal *S. aureus* positive at their first swab appointment during the same period. Therefore, age- and climate-matched healthy smokers might have been more susceptible to nasal *S. aureus* carriage than their nonsmoker counterparts. A limitation of our study design was that while consenting smokers were enrolled in the decolonization/inoculation protocol following *S. aureus* positivity at screening, those who screened negative were not reswabbed at later dates. As *S. aureus* nasal carriage is often a transient state, especially in otherwise-healthy adults (38, 39), a thorough assessment of nasal *S. aureus* carriage rate in smokers, or any population, would require repeated measurements. Notably, we determined that the smoker-carriers' nasal *S. aureus* load was greater than the nonsmokers' load independent of age, gender, or climate (Fig. 1). This might be more meaningful than carriage rate comparisons when considering how smoking and nasal *S. aureus* carriage impact health care, especially in light of the known link between

persistent *S. aureus* carriers (who often exhibit nasal *S. aureus* loads of $10^6$ CFU/swab, like the ones that we observed in smokers) and nosocomial *S. aureus* infections (40).

During experimental nasal *S. aureus* inoculation, active smokers were at a disadvantage compared to individuals who recently quit smoking and healthy nonsmokers with regard to successful *S. aureus* clearance during the month of follow-up (Fig. 3). Smokers also had an elevated premupirocin nasal *S. aureus* load compared to the cessation group (Fig. 4). These findings correspond with smokers' enhanced susceptibility to clinical *S. aureus* infections and postoperative *S. aureus*-mediated complications (28, 33). Combined with the observation that month-long carriers of experimentally inoculated *S. aureus* also presented higher natural nasal *S. aureus* loads (Fig. 6A), this study suggests that the employed autologous inoculation model is physiologically relevant and effective for measuring human mucosal immune activity against colonizing *S. aureus* strains.

Production of IL-1$\beta$ and chemokine-mediated neutrophil chemotaxis are demonstrated hallmarks of the acute anti-*S. aureus* response in a mouse cutaneous infection model (30). We and others have shown that IL-1$\beta$ attenuates *S. aureus* adhesion and growth on nasal epithelial cells (41) and induces *S. aureus* killing by neutrophils (42). The present study demonstrates that augmented *S. aureus*-induced IL-1$\beta$ and G-CSF and a decreased IL-1RA-to-IL-1$\beta$ ratio in *S. aureus*-positive noses associate with cessation from smoking (Fig. 5) and improved *S. aureus* clearance (Fig. 3). This corroborates our previous investigation of experimentally inoculated healthy nonsmokers that showed the importance of early IL-1$\beta$ and G-CSF signaling in mediating *S. aureus* clearance (18). This is also the first human study to show that elevated natural nasal IL-1RA levels correspond with elevated nasal *S. aureus* loads (Fig. 6B and C). Levels of nasal IL-1RA ranged from hundreds of picograms per ml to nearly 10 ng/ml over time and between donors, which likely contributed the statistically modest positive correlation (Pearson's $r = 0.433$; $R^2 = 0.18$) (Fig. 6C), but the significant nonzero slope ($P = 0.0195$) (Fig. 6C) and 3-fold-increased nasal IL-1RA in carriers versus clearers of experimentally inoculated *S. aureus* (Fig. 6B) suggest a role for IL-1RA in supporting nasal *S. aureus* survival. Interestingly, elevated IL-1RA levels are associated with obesity and diabetes (43), two conditions that predict higher rates of *S. aureus* carriage, skin infections, and postoperative complications related to *S. aureus* infection. Little is known currently about IL-1R signaling specific to human nasal mucosa, but IL-1R-deficient mice and MyD88-deficient mice had a higher *S. aureus* burden and a decreased neutrophil recruitment in a cutaneous infection model than did TLR-deficient mice or wild-type mice (31). The authors also discovered that IL-1R/MyD88 signaling in resident but not newly recruited bone marrow-derived skin cells promoted neutrophil-mediated host defense against *S. aureus*. Taking these past and present studies in aggregate, it is tempting to speculate that classified "noncarriers" and "intermittent carriers" of nasal *S. aureus* possess innate IL-1$\beta$/IL-1R signaling cascades that mediate the rapid eradication of diverse *S. aureus* strains. Persistent carriers of nasal *S. aureus*, in contrast, elaborate a delayed but sustained cytokine response to *S. aureus* (18) leading to enhanced antimicrobial peptide expression (17, 44), which likely serves to keep nasal carriage asymptomatic. Further studies are needed to test the role of IL-1RA levels across diverse populations of nasal *S. aureus* carriers, especially when taking into account the known role for elevated IL-1RA in predicting the onset of type 2 diabetes (45) and the link between IL-1R polymorphisms and *S. aureus* osteomyelitis (46).

Participant attendance at frequent sampling visits, repeated over weeks or months, is a challenge, particularly when subjects are otherwise healthy and thus must volunteer their time and compliance. A limitation of the presented studies is sample size, begetting the question of whether valid conclusions may be drawn from 19 smoker and 9 cessation studies. The field is in need of an animal model of *S. aureus* nasal carriage that exhibits physiological *S. aureus* load and mucosal host defense, including neutrophil-mediated clearance and antimicrobial peptide production. Until a relevant animal model is validated, it is our view that human studies of *S. aureus* nasal carriage are valuable regardless of sample size. Mice and rats do not naturally harbor nasal *S.*

*aureus*, and rodent noses are different structurally and immunologically from human noses (47, 48). Their continued use might complicate the progression of new drug and vaccine candidates from the laboratory to human clinical trials. For now, the utilized cohort of young healthy adults demonstrated that high nasal *S. aureus* burden associates with cigarette smoking. Participants who successfully quit smoking expressed elevated levels of *S. aureus*-associated nasal IL-1$\beta$ and G-CSF and cleared *S. aureus* faster than active smokers. Participants who did not clear experimental *S. aureus* presented with higher natural nasal *S. aureus* load at screening and preexperiment visits, and their nasal *S. aureus* load was positively correlated with mucosal IL-1RA levels. These findings support the concept that nasal *S. aureus* carriage depends largely on host immune status rather than bacterial genotype. Furthermore, the enhanced nasal *S. aureus* load measured in active smokers might warrant the broadened use of healthy smokers in mucosal host defense and nasal microbiome studies.

## MATERIALS AND METHODS

**Ethics statement.** A protocol for recruitment of healthy participants, collection of nasal swabs and nasal fluid, self-application of topical mupirocin, and self-inoculation of autologous *S. aureus* was approved by the Institutional Review Board of the University of Central Florida. Participants provided consent at each study visit, and no adverse effects were reported.

**Subject screening, sample collection, and inoculation protocol.** In collaboration with UCF Student Health Services, we recruited active smokers who considered themselves healthy, were not taking any medications or oral or topical antibiotics for at least 1 month, and were interested in smoking cessation to be screened for nasal *S. aureus* carriage. Participants sampled each nostril by rotating a sterile polyester-tipped swab around the anterior vestibule 10 times. Swabs were dipped and swirled into 2 ml Bacto tryptic soy broth (TSB; Becton Dickinson [BD], Franklin Lakes, NJ) to extract and culture the microbes. From each 2-ml mixture of microbes and broth, 0.5 ml was added to 0.5 ml TSB–30% glycerol and frozen immediately at −80°C as a noncultured "early" glycerol stock, 0.1-ml and 10-fold serial dilutions were spread onto BD CHROMagar Staph aureus (Fisher Scientific catalog number 14-432-41) or Remel mannitol salt agar (Fisher Scientific catalog number R01580) for identification of *S. aureus*, and 0.1-ml and serial 10-fold dilutions were spread onto TSAII agar containing 5% sheep blood (Fisher Scientific catalog number B21261X) for enumeration of *S. aureus* and non-*S. aureus* CFU. The remaining broth-microbe mixture was placed in culture overnight (total, 4 ml TSB; 250 rpm, 37°C) for preparation of a cultured "late" glycerol stock the next day. Agar plates were incubated 18 h in a 37°C bacterial oven. *S. aureus* colonies on TSAII–5% sheep blood agar were confirmed using the BD Staphyloslide Latex Test kit (Fisher Scientific catalog number B4340953) and subcultured overnight to make *S. aureus* colony glycerol stocks and aliquots of glycerol-free stocks to be used on experiment days. Participants' nasal fluids were collected by suction catheter as described previously (17, 18) and stored at −80°C immediately.

In the first arm of the study, smokers positive for nasal *S. aureus* at the screening visit were sampled (day −17 nasal swab and nasal fluid collection) as described above and decolonized via self-application of mupirocin nasal ointment (Bactroban; GlaxoSmithKline, Philadelphia, PA) twice daily for 5 days. One week after the last mupirocin application (day −6), participants were sampled to confirm clearance of nasal *S. aureus* and recovery of commensal nasal microflora and to collect "preinoculation" nasal fluid. At day zero, participants inoculated their nasal mucosa with their own *S. aureus* isolate, which had been cultured in TSB for 2.5 h (250 rpm, 37°C), washed three times with sterile Hanks' buffered salt solution (HBSS; Invitrogen/ThermoFisher catalog number 21-023-CV), and resuspended such that each nostril received $2 \times 10^7$ CFU. Nasal swabs were performed the next day, immediately prior to a repeat inoculation. Swab collections occurred every 3 to 4 days thereafter for a month of follow-up. Nasal fluids were collected at days 3, 8, 11, 15 or 16, 23 or 24, and 31 or 32 postinoculation. Expired-air CO measurements, for confirming smoking status, were obtained at most visits using the Smokerlyzer (coVita LLC, Santa Barbara, CA) according to the manufacturer's instructions. In total, 19 experimental inoculation studies were completed with active smokers.

Smokers who successfully completed the UCF Student Health Services' "Quit Smoking Now" (QSN) cessation course and/or remained smoke free for at least 1 month and consented to a second study underwent a repeat mupirocin decolonization protocol followed by inoculation with autologous *S. aureus* and month-long sampling as described above. The QSN program consists of six 1-h weekly sessions that provide participants with the knowledge, techniques, and support necessary to quit using tobacco. There is an option for participants to receive free nicotine replacement therapy (NRT) in the form of patches or lozenge or gum. Expired-air CO was measured at each visit to confirm smoking status. Eight participants completed both study arms, while a ninth subject successfully quit smoking before the screening visit (and thus participated only as a member of the cessation group).

**Bacterial genotyping and assessment of mupirocin sensitivity.** Donor *S. aureus* strains were genotyped by multilocus sequence typing (MLST) and *spa* typing as described previously (49). Isolates were screened for the *mecA* gene by performing PCR as described originally by Tokue et al. (50) using the primers MR1, 5′-AGACGATCCTTCGGTGAGC-3′, and MR2, 5′-GCTTTTGCAATGTCATTTACTG-3′. The PCR conditions were as follows: 2 $\mu$l (~200 ng) bacterial DNA template was added to 400 $\mu$M deoxynucleoside triphosphate (dNTP) mix (Invitrogen catalog number18427088), 200 nM primers, 2 mM $MgSO_4$, 2%

dimethyl sulfoxide (DMSO), 1× buffer, and 1 U Platinum *Taq* (Platinum *Taq* DNA Polymerase High Fidelity kit; Invitrogen catalog number 11304029). Thermocyler (Bio-Rad T100) settings were 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min, and a final extension step of 72°C for 10 min. A 1,339-bp PCR product or no amplicon indicated the presence or absence, respectively, of *mecA*, which encodes the penicillin-binding protein (PBP2A) that confers resistance to β-lactam antibiotics. Donor *S. aureus* strains were also tested for functional mupirocin resistance by performing turbidity (growth) assays (51). *S. aureus* isolates were grown to log phase in TSB, diluted to $10^4$ CFU/90 μl in Mueller-Hinton broth (Sigma-Aldrich, St. Louis, MO) containing 5% sucrose, and loaded to a 96-well culture plate. Ten microliters of 2-fold serial dilutions of either mupirocin (catalog number M-7694; Sigma-Aldrich) or vehicle (volume-matched DMSO) was added to the diluted *S. aureus* culture so that the final concentration of mupirocin ranged from 1 to 16 μg/ml. The culture plate was covered with ThermoSeal A film (catalog number TSA-100; Excel Scientific, Inc.) and placed into a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) programmed for a 16-h kinetic assay at 37°C. Turbidity measurements at optical density at 550 nm ($OD_{550}$) were taken every 5 min following 15 s of agitation. Growth curves ($OD_{550}$ plotted against time) were generated for all wells, and both the input and 16-h incubation cultures were plated on TSAII–5% sheep blood agar for enumeration. All strains grew to $10^7$ to $10^8$ CFU/0.1 ml in antibiotic-free medium. All strains were killed in the presence of ≤1 μg/ml mupirocin.

**Measurement of cytokines, chemokines, and growth factors.** Nasal fluids were thawed, processed, and analyzed for cytokine, chemokine, and growth factor levels as described in reference 18. All nasal fluids were assayed with Bio-Rad's Bio-Plex Pro Human Cytokine group I 27-plex panel (Bio-Rad catalog number M50-0KAF0Y). All analytes were detectable in all or some participants' nasal fluids, except IL-2, IL-4, IL-15, and GM-CSF. The detection limits of the analytes in the utilized kits typically ranged between 1 and 8 pg/ml.

**Statistical analysis.** Data were analyzed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). In most cases, *S. aureus* counts (CFU/nasal swab) were log transformed. Nasal fluid analytes were analyzed and are presented as raw picograms/milliliter or $\log_{10}$ picograms/milliliter. All participants produced similar volumes of fluid (0.4 to 0.8 ml). Groups (smokers versus recent cessation, carriers versus clearers, males versus females) were compared using unpaired *t* tests (two tailed). For cytokine analyses, we performed planned comparisons of *S. aureus*-associated and *S. aureus*-independent G-CSF, IL-1β, and IL-1RA levels based on previous findings (18). Correlations (Pearson's *r*, $R^2$, *P* value) between nasal *S. aureus* counts and cytokine levels were considered significant at *P* levels of <0.05 and if linear regression lines were significant for a nonzero slope.

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