Host innate inflammatory factors and staphylococcal protein A influence the duration of human *Staphylococcus aureus* nasal carriage

Amy L. Cole[1], **Gowrishankar Muthukrishnan**[1,3], **Christine Chong**[1], **Ashley Beavis**[1], **Colleen R. Eade**[1,4], **Matthew P. Wood**[1,5], **Michael G. Deichen**[2], and **Alexander M. Cole**[1,*]

[1]Laboratory of Innate Host Defense, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL, USA

[2]Health Services, University of Central Florida, Orlando, FL, USA

Abstract

Human *Staphylococcus aureus* (*SA)* nasal carriage provides a reservoir for the dissemination of infectious strains; however, factors regulating the establishment and persistence of nasal colonization are mostly unknown. We measured carriage duration and nasal fluid inflammatory markers after nasally inoculating healthy participants with their previously isolated *SA* strains. Ten out of 15 studies resulted in rapid clearance (9±6 days) that corresponded with upregulated chemokines, growth factors, and predominantly Th1-type cytokines, but not IL-17. Nasal *SA* persistence corresponded with elevated baseline levels of MIP-1β, IL-1β, and IL-6, no induction of inflammatory factors post-inoculation, and decreased IL-1RA:IL-1β ratio. *SA*-expressed staphylococcal protein A (SpA) levels correlated positively with carriage duration. Competitive inoculation studies revealed that isogenic SpA knockout (SpA) strains were cleared faster than wild-type only in participants with upregulated inflammatory markers post-inoculation. The remaining participants did not mount an inflammatory response and did not clear either strain.

 SpA strains demonstrated lower growth rates in carrier nasal fluids and lower survival rates when incubated with neutrophils. Collectively, the presented studies identify innate immune effectors that cooperatively modulate nasal carriage duration, and confirm SpA as a bacterial co-determinant of *SA* nasal carriage.

Keywords

*Correspondence: Dr. Alexander M. Cole, 4110 Libra Blvd. Bldg 20, Rm 236, Orlando, FL 32816, (tel): 407-823-3633; acole@ucf.edu.

[3]Present address: David H. Smith Center for Vaccine Biology and Immunology, University of Rochester Medical Center, Rochester, NY, USA

[4]Present address: Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

[5]Present address: Center for Infectious Disease Research, formerly Seattle Biomedical Research Institute, Seattle, WA, USA

Introduction

Complications from *Staphylococcus aureus* (*SA*) skin and soft tissue infections, surgical site infections, and bacteremia, remain an important public health concern as antibiotic resistant strains continue to emerge and healthcare costs continue to rise. Interestingly, *SA* colonization of humans is nearly always non-pathogenic and most people become colonized with *SA* transiently throughout their lives. Approximately 30% of healthy adults carry *SA* asymptomatically at any given moment, regardless of environment, with higher carriage rates and clinical infections in children and those with diabetes, obesity, or certain genetic polymorphisms or drug regimens affecting innate immunity.[1–6] The primary reservoir for *SA* in humans is the nasal vestibule, and it is now realized that clinical and methicillin-resistant *SA* (MRSA) strains are nasally carried by the general public.[7–9] Since nasal carriers of *SA* easily transmit their infectious strains and are themselves at risk for extra-nasal *SA* infections with their nasally carried strain,[10, 11] factors controlling the duration of nasal colonization warrant further investigation.

We have used a combination of *in vivo* and *in vitro* approaches to explore specific determinants of human *SA* nasal carriage. Human autologous nasal inoculation studies revealed that coordinated induction of innate mucosal inflammatory factors associates with nasal *SA* clearance. We also explored the connection between Staphylococcal protein A (SpA) and *SA*'s capacity for survival in the human nasal environment, and determined that levels of expressed SpA protein correlate with longer nasal carriage duration while stains lacking SpA protein exhibited decreased survival upon exposure to nasal fluid and neutrophils.

Results

Distinct *SA* nasal carriage trends among healthy individuals

To investigate early events in nasal *SA* colonization, we designed a human nasal inoculation protocol using a healthy adult cohort that had been monitored for *SA* carriage for 1–3 years by our laboratory.[8] Participants were cleared of nasal *SA* through a twicedaily topical application of the antibiotic mupirocin for 5 days. One week after the last application, clearance of *SA* was confirmed and nasal fluids were collected for baseline (day -7) measurements of nasal mucosal inflammatory factors. One additional week later, participants were inoculated in each nostril with 2×10^7 CFU of donor-matched (autologous) *SA* that had been isolated and genotyped from a prior study visit. Nasal *SA* load was monitored twice weekly for 30–35 days, and nasal secretions were collected at 3–4 day intervals for two weeks, followed by weekly collections for another two weeks.

Fifteen experimental inoculations were performed on eight individuals, with five subjects (D528, D547, D720, D830, D831) participating 2–3 times over a one year period (Fig 1). Participant and strain information are shown in Table 1. All but one participant was designated as an intermittent nasal *SA* carrier since repeated samplings demonstrated at least one visit in which *SA* (CFU/swab) was not detected in either nostril. Participant D720 was considered a persistent carrier based on nine out of nine *SA*-positive nasal swab samples

taken during 18 months of monitoring.[8] Non-*SA* nasal bacteria levels decreased expectedly following the topical mupirocin regimen (day -7); however, levels rebounded by inoculation day (day 0), and were steady for the duration of the month-long observation period (Fig 1A). In 10 of the 15 studies, clearance of *SA* from the nares occurred within 9±6 (mean±SD) days, with all participants clearing *SA* by day 20 (Fig 1B). Among the 5 studies in which nasal *SA* was not cleared by the end of the month-long follow-up period (Fig 1C), all exhibited at least a 2-log reduction in *SA* CFUs during the month. Three of the five participants' nasal *SA* decreased below the level of detection at 1–2 visits during days 14–28, although levels rose again by days 31–35 (Fig 1C, participants D547, D720, D831). Participants D547, D720, D830, and D831 all experienced one inoculation study in which nasal *SA* persisted to the end of the follow-up period (Fig 1C), while clearance occurred in replicate studies utilizing the same autologous *SA* isolate (Fig 1B). These different outcomes underscore the complex nature of interactions between *SA* and human nasal mucosa, and suggest that the host response to *SA*, more than *SA* strain genotypic attributes, modulates carriage duration. Furthermore, clearance of nasal *SA* by D720 (D720 inoculation 2 in Fig 1B) indicates that even carriers designated as "persistent" are capable of clearing nasal *SA*.

The combined upregulation of chemokines, growth factors, and inflammatory cytokines associated with nasal *SA* clearance

Previous studies showed that nasal secretions from *SA* carriers support the *in vitro* growth of *SA* more than secretions from healthy non-carriers,[12, 13] suggesting that host factors play a major role in either the onset or duration of colonization, or both. It has also been demonstrated that nasal *SA* carrier fluids have higher levels of antimicrobial peptides HNP-1, HNP-3, and HBD-2 compared to non-carrier fluids,[13, 14] indicating a local innate immune response to colonizing *SA* that might prevent carriage symptoms even without accomplishing complete *SA* clearance. In order to determine additional inflammatory modulators that contribute to human nasal host defense against *SA in vivo*, we performed a 27-plex cytokine/chemokine/growth factor assay on each collected nasal fluid from the 15 autologous inoculation studies, and stratified the data according to whether participants' nasal *SA* was cleared ("clearance" group) or not ("carriage" group) during the subsequent month of follow-up. Figure 2 presents the sum of 16 detectable nasal fluid analytes at day - 7 (pre-inoculation) and day 2 (post-inoculation), for each of the clearance and carriage groups. The total level of expressed host response factors (IL-8, IP-10, MCP-1, Eotaxin, MIP-1β, IFN-γ, IL-6, TNF-α, IL-1β, IL-1RA, G-CSF, VEGF, FGF, PDGF, IL-7, IL-12 p70) was significantly upregulated post-inoculation only during nasal clearance of *SA* (*P*=0.0098, Fig 2). Among the 27 proteins measured in the multiplex assay, the following were either undetectable or expressed at very low concentrations (< 10 picograms/nasal fluid sample) by all participants at all sampling times: IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-15, IL-17, MIP-1α, RANTES and GM-CSF.

Disparate baseline and induced host inflammatory mediator profiles between nasal *SA* clearance and carriage groups

Baseline (day -7) levels of the CC chemokine MIP-1β (CCL4) and the inflammation markers IL-1β and IL-6 were significantly higher in the *SA* carriage group compared to the clearance group (Fig 3). In the clearance group only, levels of IL-6 and IL-8 were elevated at

all post-inoculation timepoints compared to baseline. Similarly, IL-1β was not significantly upregulated post-inoculation in participants who carried *SA* but was elevated at post-inoculation days 2 and 7, and again at week 4 in the clearance group. IL-1β activity is counteracted at mucosal surfaces by high levels of IL-1 receptor antagonist (IL-1RA). Levels of IL-1RA in nasal fluid were similar between the clearance and carriage groups (averaging 100 pg per collected nasal fluid (ng/mL levels) for most participants); however, only the clearance group exhibited induced nasal IL-1RA post-*SA* inoculation (days 7, 10, and 23–30; Fig 3). Nasal fluid G-CSF was elevated post-*SA* in the clearance group and in the majority of participants in the carriage group, supporting the notion that neutrophil recruitment to the nasal mucosa keys the human innate nasal response to *SA* exposure. The remaining detectable nasal fluid analytes are displayed in Supplementary Figure 1. Notably, IL-7 and IL-12 p70 (T cell stimulation/maturation factors), IP-10 (CXC chemokine CXCL10), TNF-α, IFN-γ, FGF, and PDGF were induced at most post-inoculation timepoints in the clearance group but not in individuals experiencing prolonged carriage. Collectively, figures 2 and 3 suggest that a coordinated acute inflammatory response in the nasal mucosa shortens the duration of *SA* carriage.

Low IL-1RA:IL-1β ratio is associated with nasal *SA* persistence

We next calculated the ratio of IL-1RA to IL-1β for each collected nasal fluid, and compared the clearance and carriage groups. Participants who successfully cleared nasal *SA* exhibited a mean IL-1RA:IL-1β ratio   100 at baseline and at day 7 and onward post-*SA* inoculation (Fig 4), with the decreased ratio at day 2 corresponding with the observed induction of IL-1β (Fig 3) immediately post *SA*-inoculation. Participants who failed to clear nasal *SA* presented a mean nasal fluid IL-1RA:IL-1β ratio ranging from 10 to 30 at all days except day 16 (ratio ~80, Fig 4), which correlated with a decrease in mean expression of IL-1β at that timepoint (Fig 3). The nasal fluid IL-1RA:IL-1β ratio may therefore represent a metric for anti-*SA* function in the nasal mucosa, with the observed differences between the clearance and carriage groups indicating that dysregulated IL-1β-mediated signaling, or the introduction of non-*SA* subclinical nasal stimuli capable of augmenting IL-1β levels, may contribute to *SA* persistence.

SA SpA levels correlate with human nasal carriage duration

Previous proteomic analyses of *SA* nasal carriage strains revealed a potential correlation between levels of *SA*-expressed staphylococcal protein A (SpA) and carriage persistence.[15] For each of the strains used in the autologous inoculation experiments presented above, we obtained the *spa* type, *spa* polymorphic X region short sequence repeat (SSR) number, and SpA protein level (Table 1). Mean SpA protein levels correlated positively with nasal carriage duration observed in the 15 nasal inoculation studies (Fig 5A; r=0.45, *P*=0.046), while there was no correlation between strains' *spa* SSR number and carriage duration (Fig 5B; r=0.03, *P*=0.522). We next evaluated whether SpA protein level correlated with induction of inflammatory markers observed two days post-inoculation compared to baseline. Fold induction values calculated for each of the 16 detectable analytes were averaged and plotted against the mean SpA protein value for the corresponding *SA*. There was no correlation between SpA protein level and the average induction of inflammatory markers (Fig 5C; r=0.0371, *P*=0.4478), nor was there a correlation between SpA level and

the fold induction of any individual inflammatory marker (data not shown). Since SpA has been demonstrated by other groups to promote activation of epithelial cells through TNFR1 signaling and NFκB activation,[16–18] we likewise assessed the effect of each *SA* strain on human nasal epithelial cell (NEC) NFκB activity *in vitro*. When NECs were grown at the air liquid interface and topically applied with *SA* at MOI=5, there was indeed a strong correlation between SpA level and phosphorylation of the NFκB subunit p65 (Fig 5D; r=0.84, *P*=0.002). Conversely, the amount of SpA in individual strains did not impact the invasion of NECs *in vitro* (Fig 5E; r=0.29, *P*=0.483). Strains 2098 and 2024 weren't included in the analysis of the *in vivo* inoculation experiments due to missed visits and incomplete carriage data, but were of interest because of their vastly different SpA levels (Table 1). Notably, they also induced NEC NFκB activity proportionally to their SpA levels (27.3±1.6-fold induction of NFκB by 2098 and 8.8±0.5-fold by 2024, data not shown). Taken together, these studies provide human *in vivo* evidence that elevated expression of SpA at the protein level supports *SA* nasal colonization. Since a failed acute inflammatory response to nasal *SA* also correlated with carriage (Figs 2–3), the *in vitro* assays indicating a role for SpA in activating epithelial NFkB activity may not adequately model the asymptomatic (subclinical) *SA*-host interactions that occur in human nasal mucosa.

SA mutants lacking SpA were cleared faster than wild-type counterparts during competitive nasal inoculation

We next pursued direct human *in vivo* evidence that SpA is an important bacterial determinant of nasal carriage. In a subset of participant strains in our cohort, representing diverse *spa* types and SpA expression levels, we performed site-directed *spa* gene disruption using the TargeTron gene knockout system.[19] An advantage of TargeTron methodology is the lack of antibiotic-selectable markers, thus eliminating the possibility of introducing antibiotic resistant *SA* strains during nasal inoculation. The absence of SpA protein expression (SpA) in participant strains 547, 830, 2024, and 2098 was confirmed by Western blot (Fig 6A) and commercial ELISA (not shown). Strain fitness was assessed by performing growth assays in nutrient rich tryptic soy broth (Fig 6B), low serum cell culture media containing fewer nutrients (Fig 6C), and on confluent NEC layers grown at the air liquid interface (Fig 6D): each assay demonstrated no growth differences between the wild-type (WT) and SpA strains. We also measured NEC-associated versus extracellular *SA* after 2hr incubation and observed that WT and SpA strains were similarly able to attach/invade nasal epithelium, with 2024 SpA demonstrating more cell association than its counterpart WT strain (Fig 6E).

Next, we performed competitive nasal inoculation studies using equivalent amounts of WT and SpA autologous *SA* in each nostril, monitored nasal *SA* load, and collected nasal fluids for the assessment of host inflammatory responses. Upon analyzing the average fold change in inflammatory factor levels (same 16 analytes listed above in the description of Fig 2) for seven independent inoculation experiments, the experiments were observed to naturally stratify by the demonstrated nasal mucosal host response: four inoculations elicited increased nasal fluid inflammatory factors while three inoculations resulted in decreased levels compared to pre-inoculation (Fig 7A). In the former group, SpA strains were cleared significantly faster than WT (Fig 7B; log rank: X^2=4.051, *P*=0.044). The latter group

resulted in carriage of both WT and ▵SpA strains at similar levels throughout the month-long follow-up (Fig 7C; log rank: X^2=0.004, *P*=0.948).

Nasal *SA* strains lacking SpA exhibited reduced survival in nasal fluid and during neutrophil exposure

We next explored whether ▵SpA strains were less likely to persist in an environment of human nasal fluid. To develop a suitable assay, five natural *SA* strains from our cohort were grown to log phase as in the inoculation studies, then washed and incubated with various nutrient buffers or heat-inactivated nasal fluid (thus no endogenous CFUs) for 24 hr (Fig 8A). With an input of 50,000–100,000 CFU, no live *SA* was recovered when strains were incubated with nasal fluid simulant buffer (NFB) containing electrolytes and supplemented with 6 g/L glucose; while provision of 0.2% serum (a source of iron and hemoglobin known to promote *SA* nasal colonization[20, 21]) permitted a modest glucose dose-dependence, yielding 1.5–3-fold growth with 2–6g/L glucose supplementation (Fig 8A). Incubation of *SA* strains in non-carrier nasal fluid (NCF) and *SA* carrier fluid (CF) promoted 100–300-fold growth, with carrier fluid providing greater growth potential (Fig 8A; *P*=0.013, n=5). *SA* growth in DMEM cell culture media (containing salts, amino acids, vitamins, pyruvate and 4.5g/L glucose) was dependent on serum supplementation: 100-fold growth in 0.2% serum and nearly 1000-fold growth in 2% serum. Next, growth of WT versus ▵SpA strains was tested when suspended in DMEM/2%FBS (Fig 8B) or plain NFB (Fig 8C) then admixed with eight volumes of *SA* carrier nasal fluid. When nutrients were higher, all WT and ▵SpA strains propagated similarly (Fig 8B); however, when incubated in the NFB/nasal fluid blend, the ▵SpA variants of strains 547, 2024, and 2098 exhibited significantly decreased growth compared to their WT counterparts (Fig 8C).

Since nasal *SA* clearance has been demonstrated to be dependent on neutrophil recruitment and phagocytosis,[22] we also tested whether WT and ▵SpA strains differed in their ability to survive neutrophil exposure. Strains were incubated alone or with primary human neutrophils (MOI=1, n=4 neutrophil preparations from separate donors) for 1 or 3 hr. Surviving CFUs were enumerated and percent survival was calculated for each strain. Figure 9 demonstrates a significant trend that ▵SpA strains were more vulnerable to neutrophils compared to WT. Collectively, the results shown in figures 7–9 indicate that mucosal host defense regulation is an important determinant of *SA* nasal carriage duration while SpA levels play a role in co-determining carriage.

Discussion

Human nasal carriage of *SA* is established due to a complex interplay between bacterial virulence factors, host surveillance and defense mechanisms, and nutrient availability. Numerous small animal models of *SA* infection have enabled the discovery of bacterial factors influencing nasal colonization events including clumping factor B,[23] wall teichoic acid,[24, 25] and iron-regulated surface proteins IsdA and IsdH.[21] On the host side, younger age, obesity, male gender, tobacco use, vitamin D levels, and certain genetic polymorphisms have been implicated as risk factors for nasal *SA* carriage,[26, 27] although the underlying mechanisms remain elusive. Clinical studies have demonstrated that nasal carriage isolates

match the invasive strain in the majority of cases,[10, 11, 28, 29] and that screening and decolonization procedures associate with decreased extra-nasal infections, mortality, and medical costs.[30, 31] Since the emergence of antibiotic-resistant *SA* is of critical concern worldwide, new protocols for studying early colonization events, immune tolerance, and clearance mechanisms in human nasal *SA* carriage will be essential for development of advanced therapeutics.

We evaluated the role of innate nasal host defense against *SA* by performing autologous nasal inoculations of known *SA* carriers. Participants reported no deleterious effects, and by using this approach we revealed *in vivo* that the nasal carriage state is influenced by the host's early inflammatory response to *SA* colonization. Figures 2–3 indicate that clearance of *SA* from the human nasal mucosa correlates with a coordinated induction of inflammatory mediators IL-1β, IL-6, TNF-α, IFN-γ; chemokines IL-8, MIP-1β and IP-10; and growth factors/T cell maturation factors VEGF, FGF, PDGF, IL-12 p70, and IL-7. In the majority of experiments, hosts mounted this robust defense by day 2, which corresponded with rapid *SA* nasal clearance. *SA* persistence occurred in only 5 of 15 experiments, and corresponded with the host failing to elaborate an induction of these factors following *SA* inoculation. Surprisingly, IL-17, previously shown to mediate *SA* clearance in mice,[22] was not detected in nasal secretions. It is possible that IL-17 could be cell-associated or insoluble in nasal secretions, or that sub-pg/mL levels of IL-17 expression is enough to activate local T-lymphocytes. Nevertheless, the observed Th1-type dominant cytokine response is not surprising on account of its known association with epithelia-derived and neutrophil-mediated immunity. In contrast, the observed 10/15 clearance rate was unexpected, as we initially surmised that upon reintroducing a participant's own *SA* isolate, nasal carriage would be easily restored. Since identical *SA* isolates could persist in one inoculation experiment but not in the next, or vice versa, in the same participant (Fig 1), the collective data suggest that the carriage state is predominantly defined by the host's varied ability to respond to *SA*.

While all participants in the 15 nasal inoculation experiments were healthy and asymptomatic, baseline nasal fluid cytokine and chemokine levels were generally higher for the individuals who failed to clear *SA* than for the clearance group (Figs 2–3), while the IL-1RA:IL-1β ratio was consistently lower in the carriage group (Fig 4). It is not currently understood how or why elevated baseline levels of MIP-1β, IL-1β, and IL-6 wouldn't prime the host for an effective anti-*SA* response. It is possible that a non-*SA* inflammatory stimulator encountered the nasal mucosa during the post-mupirocin phase and contributed to the failed induction of IL-1β and other inflammatory mediators post-*SA* inoculation by shifting the host response toward alternate signaling pathways. Such a scenario, marked by a low IL-1RA:IL-1β ratio, may have provided *SA* with the necessary "window of opportunity" for successful colonization of the nasal epithelial barrier.

We enumerated the non-*SA* nasal microbes but did not identify all of the represented species. Notably, we did check for the presence of Esp-secreting *S. epidermidis (SE)*, reported to inhibit *SA* biofilm formation and nasal colonization,[32] in 8 participants (11 studies, data not shown). 83% of *SE* isolates (103 of 124 colonies) were *esp*-positive, suggesting that this species is common in our cohort of nasal *SA* carriers. Three participants

were co-colonized with *SA* and *esp*-positive *SE* beyond 25 days, while two participants with the fewest number of *esp*-positive *SE* swabs (5 out of 12 and 3 out of 11 respectively) cleared *SA* within one week. Therefore, it cannot be concluded from this subset of individuals that *SA* clearance correlated with Esp-secreting *SE* load. Regardless, the issue of competition between nasal *SA* and endogenous flora will be an important consideration going forward. It was recently observed that increased *SA* clearance occurred after skin-to-skin contact between noncarrier mothers and nasal *SA* positive newborns,[33] and microbiota-induced neutrophil recruitment and T cell maturation correlated with defense against *SA* in mice.[34] Commensal-host cell interaction has additionally been demonstrated to dramatically impact skin immunity.[35] These important studies support the idea that a diverse and dynamic nasal microflora may hamper *SA* colonization, and thus further study of endogenous nasal flora during natural transitioning in and out of *SA* carriage is needed.

Numerous studies using mouse models or *in vitro* cell culture assays have demonstrated the importance of SpA as a *SA* virulence factor mediating TNFR1-induced inflammatory cascades, opsonophagocytosis, and B cell apoptosis.[16–18, 36] We and others previously showed a relationship between SpA expression levels[15, 37] but not *spa* polymorphisms[38] and human nasal carriage persistence. In the present *in vivo* studies there was a correlation between SpA protein level, but not SpA SSR#, and nasal carriage duration (Fig 5). During competitive inoculation studies using equivalent amounts of WT and SpA autologous *SA*, SpA variants were eliminated from the nose earlier than WT only when the host mounted a coordinated inflammatory defense (Fig 7). Taken together, these findings suggest that the predominant nasal mucosal response to asymptomatic *SA* involves innate immune effectors culminating in neutrophil-mediated clearance, rather than antibody-mediated defenses that play a larger role during clinical infection. We have not characterized the anti-*SA* antibody profiles of carriers in our cohort, and thus cannot speculate about the role of seropositivity in the clearance of WT and SpA strains or the preferential clearance of SpA. However, essentially all people, regardless of carriage status, make immunoglobulins against *SA* antigens, and experimental nasal inoculation did not alter anti-*SA* antibody profiles or humoral responses in healthy participants in a previous report.[39–41] Persistent nasal carriers have a greater risk of clinical *SA* infection with their endogenous strain, but make more *SA*-neutralizing antibodies than non-carriers and have a lower risk of death from *SA* bacteremia.[10, 40] Together, these studies imply that anti-*SA* antibody status is the result of extranasal *SA* exposure, and not a modulator of nasal carriage status or duration. Similarly, the enhanced susceptibility of SpA strains to nasal fluid and neutrophils (Figs 8–9), and the known role of SpA in epithelial TNFR1 shedding[17], suggest that SpA's role in modulating nasal carriage duration is linked to influences on innate rather than adaptive host defenses.

In summary, our findings demonstrate the extent to which innate immune effectors of the nasal mucosa influence the duration of asymptomatic human *SA* carriage. In light of recent evidence suggesting that non-carriers are actually very rare, and that most of the population is colonized transiently,[4, 42] the investigation of clearance mechanisms in *SA* nasal carriers is urgently needed. Current vaccine design efforts show promise for protecting the host from *SA* deep tissue infections and bacteremia[43, 44], however it is likely that development of improved nasal decolonization strategies will involve a distinct set of molecular targets.

Materials and Methods

Participants, informed consent, and ethics

Nine healthy *SA* nasal carriers (5 males, 4 females, range 22–47 years old) were selected from a cohort of 109 healthy individuals whose carriage status was monitored longitudinally for up to three years. Studies were performed with approval of the University of Central Florida's Institutional Review Board, which is fully accredited by the Association for the Accreditation of Human Research Protection Programs. Participants provided consent at each study visit, and there were no adverse effects of nasal *SA* reported during the study.

Isolation, storage, and use of nasal Staphylococcus aureus isolates

To isolate nasal *SA*, the anterior region of each nostril was swabbed with sterile polyestertipped swabs, and each swab was swirled in 2mL of TSB (Bacto tryptic soy broth, Becton Dickinson (BD), Franklin Lakes, NJ) to extract microbes and prepare an initial glycerol stock (overnight incubation at 37°C/250rpm, then stored at –80°C supplemented with 15% glycerol). 0.1mL was plated on tryptic soy agar/5% sheep's blood (TSA II from BD, hereafter termed "blood agar") and incubated ~18hr at 37°C; then colonies were identified as *SA* using the Staphyloslide Latex Test reagent (BD). *SA* colonies were propagated in TSB and then 1) genotyped by MLST and *spa* typing using primers and PCR conditions described previously,[8, 45, 46] 2) stored as colony glycerol stocks at –80°C, 3) prepared as ready-to-use glycerol-free stocks by dispensing overnight liquid culture into microtubes and rapidly freezing the aliquots in liquid nitrogen prior to storage at –80°C. For experiments, stocks were thawed and 1% (v/v) inocula were grown in TSB at 37°C/250rpm for 2.5 hr to achieve log phase growth. *SA* was collected by centrifugation, washed with Hank's buffered salt solution (HBSS, Corning/Cellgro), and then resuspended in HBSS and used for nasal inoculation or treating nasal epithelial cells. At each use, a portion of the *SA* preparation was plated on blood agar to confirm the inoculum. For the assessment of natural SpA levels of each nasal strain, *SA* from 3 representative frozen stocks was handled in the same way as for nasal inoculations, except that HBSS-washed bacteria were further processed for SpA ELISA (details below). Proteins were extracted by adding 10 volumes of 10% acetic acid, then vortexed for 30 min. Soluble extracts were clarified by centrifugation, vacuum-dried (SPD1010 SpeedVac, ThermoFisher), neutralized to pH 7, and added to the ELISA workflow. For Western blot assays (described below), overnight cultures of WT and ΔSpA *SA* were fractionated as described[47, 48] for assessing cell wall-associated and secreted levels of SpA.

Autologous nasal SA inoculation and specimen collection

Since most participants were nasal carriers who usually tested positive for *SA*, all participants underwent the same decolonization protocol prior to initiating the *SA* inoculation studies. Participants self-administered mupirocin nasal ointment (Bactroban, GlaxoSmithLine, Philadelphia, PA) to each nostril, twice daily for 5 days. One week after the last mupirocin application, nasal swabs were collected to confirm that endogenous *SA* had been cleared, and to enumerate non-*SA* CFUs. Nasal fluid was collected by placing a flexible #8 French catheter into the nasal vestibule under low vacuum.[13] Individuals typically collected 0.1–1mL nasal fluid in this manner. Fluids were transferred to

microcentrifuge tubes and stored at −80°C until use. These collections were considered to take place on "day -7." One additional week later, participants' previously isolated strains were prepared as described above and each nostril was inoculated with 2×10^7 CFUs of *SA* on two consecutive days (days 0 and 1, respectively). On day 2, and every 2–4 days thereafter for approximately 35 days, nasal swabs were performed on each nostril. Nasal fluid was collected at days 2, 7, and 10, then every 4–7 days thereafter. All *SA* strains were confirmed for mupirocin sensitivity prior to experimental inoculation; and a subset of enumerated colonies collected during the studies were confirmed by MLST and *spa* typing[8, 45, 46] to match the inoculated strain.

Nasal fluid processing and immunodetection assays

Nasal fluids were thawed on wet ice and vortexed, then pulse-sonicated 30×1sec on wet ice at power level 2 (Fisher Scientific Sonic Dismembrator model 100), and clarified by centrifugation (13000rpm/5min/4°C). Bio-Rad Pro Human Cytokine Assay (Bio-Rad Laboratories, Inc., Hercules, CA) was performed for detection of IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IFN-γ, IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, Eotaxin, FGF basic, G-CSF, GM-CSF, and VEGF. Standard curves were generated by diluting company-provided recombinant standards in each of three buffer preparations to determine whether the total protein content of standard diluent altered assay sensitivity: DMEM/10%FBS to represent a standard cell culture supernatant, keratinocyte serum-free base medium (KSFM, Life Technologies, Grand Island, NY) to represent a low protein buffer, and nasal fluid simulant buffer[12] ("NFB": 85mM Na, 97mM Cl, 20mM K, 1mM Ca, 10mM P, 0.5mM Mg, 0.5mM S) containing 6mg/mL BSA since we determined nasal fluids to have an average of 6mg/mL protein. All standard diluents allowed the same level of sensitivity for each analyte. We set a cut-off of 10 pg/mL as the detection limit since each standard curve demonstrated good fit down to 7–10 pg/mL. Company instructions were followed for incubation times, instrument settings (Bio-Plex 200), and BioPlex Pro II wash station settings. Data (pg/mL) were normalized to the volume of nasal fluid collected and presented as pg per nasal fluid (pg/NF sample), since fluid collections >1mL demonstrated a lower concentration of total protein than typical fluid collections of 0.1–0.5mL. Normalization of the pg/mL data to total protein could not be performed for all of the collected nasal fluids because of volume limitations for certain individuals or collection times.

Human nasal epithelial cell culture, invasion assays, and NFκB assays

Human NEC (nasal epithelial cells "RPMI 2650" from American Type Culture Collection (ATCC), Manassas, VA) were maintained on 100 mm tissue culture dishes, submerged in 10mL/dish DMEM-high glucose supplemented with 10% FBS, 1X penicillin-streptomycin solution, and incubated at 37°C/5% CO_2. For experiments, cells were transferred to the apical compartment of collagen-coated Transwell inserts (0.4 μm pores, Corning Inc., Corning, NY) such that the cells from each confluent 100 mm dish were seeded on twelve 12mm or six 24mm Transwell inserts. After two days, basal medium was changed and apical medium was replaced. On day 3, the apical medium was removed and basal medium was changed daily thereafter. On day 6, antibiotics were removed from the medium. Cells were treated with *SA* on day 9 or 10, when all Transwell cultures demonstrated the ability to seal

the basal medium away from the apical chamber, creating an air-liquid interface. For *SA* invasion assays, each strain was topically applied at MOI=5, which was confirmed by plating an aliquot on blood agar immediately after application to the cells. After 2 hr, extracellular bacteria were rinsed off the nasal epithelia and plated on blood agar in serial dilutions. Cell-associated bacteria was enumerated by scraping cells off the Transwells, pulse-sonicating on ice (10×1 sec, power level 4) to disrupt the epithelial cells without harming *SA*, and plating on blood agar for CFU enumeration the next day. For NFκB activation assays, treated NEC were rinsed and then lysed and processed for Bio-Plex Pro Cell Signaling assays using Bio-Rad's Pro Cell Signaling Reagent kit. Protein content of each lysate was measured by micro BCA assay (Thermo Scientific Pierce, Pittsburgh, PA), and 20 μg was used for multiplex analysis of phosphorylated-p65 (Ser536) and GAPDH (for normalization of NEC content in each sample).

Targeted disruption of SpA in SA nasal carrier strains

Site-directed disruption of the staphylococcal protein A gene (*spa*) was performed using TargeTron-based insertion of mobile group II introns into *spa* (Sigma-Aldrich, St. Louis, MO), as per the *SA*-specific procedure described by Yao.[19] Because naturally colonizing nasal *SA* strains were refractory to genetic transformations due to Type I and Type IV restriction barriers, we modified the TargeTron methodology. *spa*-pNL9164 intron insertion plasmids were passaged through high-efficiency *E. coli* cloning strain DC10B[49] prior to electroporation into *SA*. Intron insertion was confirmed by PCR and sequence analysis. Loss of SpA protein was confirmed by Western blot of *SA* cell wall and exoproteome fractions (monoclonal antibody #ab49734-200, Abcam, Cambridge, MA); and also by ELISA (protein A EIA kit from Enzo Life Sciences, Plymouth, PA). Four different *spa*disrupted (SpA) *SA* nasal strains were generated. Supplemental Table S1 lists all plasmids and oligonucleotides. Growth assays to confirm that SpA strains were not compromised during sub-cloning and selection were carried out in both liquid culture and on nasal cells as detailed above.

SA CFU microassays with nasal fluids or various nutrient mixtures

SA stocks were thawed and incubated as described above to achieve log phase growth. Bacteria were washed and resuspended in NFB such that $OD_{625} = 0.2$; then diluted 1:100 with either NFB, NFB supplemented with varying amounts of glucose and 0.2% FBS, or DMEM supplemented with 0.2–2% FBS. These diluents or nasal fluids, sonicated as described above and then incubated at 55°C for 20 min to prevent the growth of endogenous bacteria, were then premixed with *SA* in microtubes (12 μL NF or diluent +1.5μL 1:100 *SA*); then 5μL/well was plated in duplicate wells of 72W Terisaki plates (Thermo Scientific Nunc) and overlayed with 3μL/well Chill-out liquid wax (Bio-Rad) to prevent evaporation. One well was immediately plated on blood agar (time 0), and the remaining samples were incubated at 37°C for 24h. Sample wells were collected and rinsed, and serial dilutions were plated on blood agar. CFUs were enumerated, and 24h counts were divided by time 0 counts to obtain fold growth values. Incubations of nasal fluid alone were performed as control to verify that the heating step eliminated the growth of endogenous bacteria. Each *SA* strain was tested against 3–5 individual nasal fluids, or with the different nutrient mixtures in 3–5 independent assays.

Neutrophil assays

Neutrophils were obtained from freshly drawn whole blood from healthy volunteers[50, 51] and incubated overnight at 10^6/mL in RPMI 1640 supplemented with 10% FBS, using 12-well dishes coated with 1 mg/well poly-L-lysine (Sigma #P-9404). The next day, neutrophil media was changed to RPMI/0.5% FBS (R0.5). Log phase *SA* was washed and diluted 100-fold in R0.5, then 0.1mL was added to neutrophils in order to achieve MOI=1 (confirmed by plating on blood agar). Plates were incubated at 37°C/5% CO_2 for 1 or 3 hr, then cell-free *SA* was removed from each well, and cell-bound/phagocytosed *SA* was collected by scraping the cells in a solution of 0.05% Triton X-100 in PBS (applied 2 min before scraping). All collections were immediately mixed and diluted in R0.5 and plated on blood agar for CFU enumeration. Parallel wells containing *SA* but no neutrophils were used to control for *SA* growth, and duplicate wells were used to confirm that Triton treatment didn't impact *SA* CFU counts. Percent survival was calculated for neutrophil-exposed *SA* relative to cell-free *SA* ((SA_{PMN}/SA_{alone})×100) for each strain. Assays were performed using 4 separate neutrophil donations.

Statistical Analysis

Data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). For multiplex cytokine data obtained from nasal secretions, total picograms of each analyte were calculated based on the volume of nasal fluid collected. For comparing analyte levels or *SA* survival between groups, paired t-test, unpaired non-parametric Mann Whitney test, or Wilcoxon rank test were performed according to the data distribution. For the competitive WT/ SpA inoculation studies, *SA* carriage patterns were displayed with Kaplan-Meier survival curves and compared using log-rank (Mantel-Cox) test and median survival time calculations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

SA	*Staphylococcus aureus*
CA-*SA*	community-acquired *SA*
SpA	Staphylococcal protein A
CFU	colony forming unit
NEC	nasal epithelial cells

IL interleukin

SE *Staphylococcus epidermidis*

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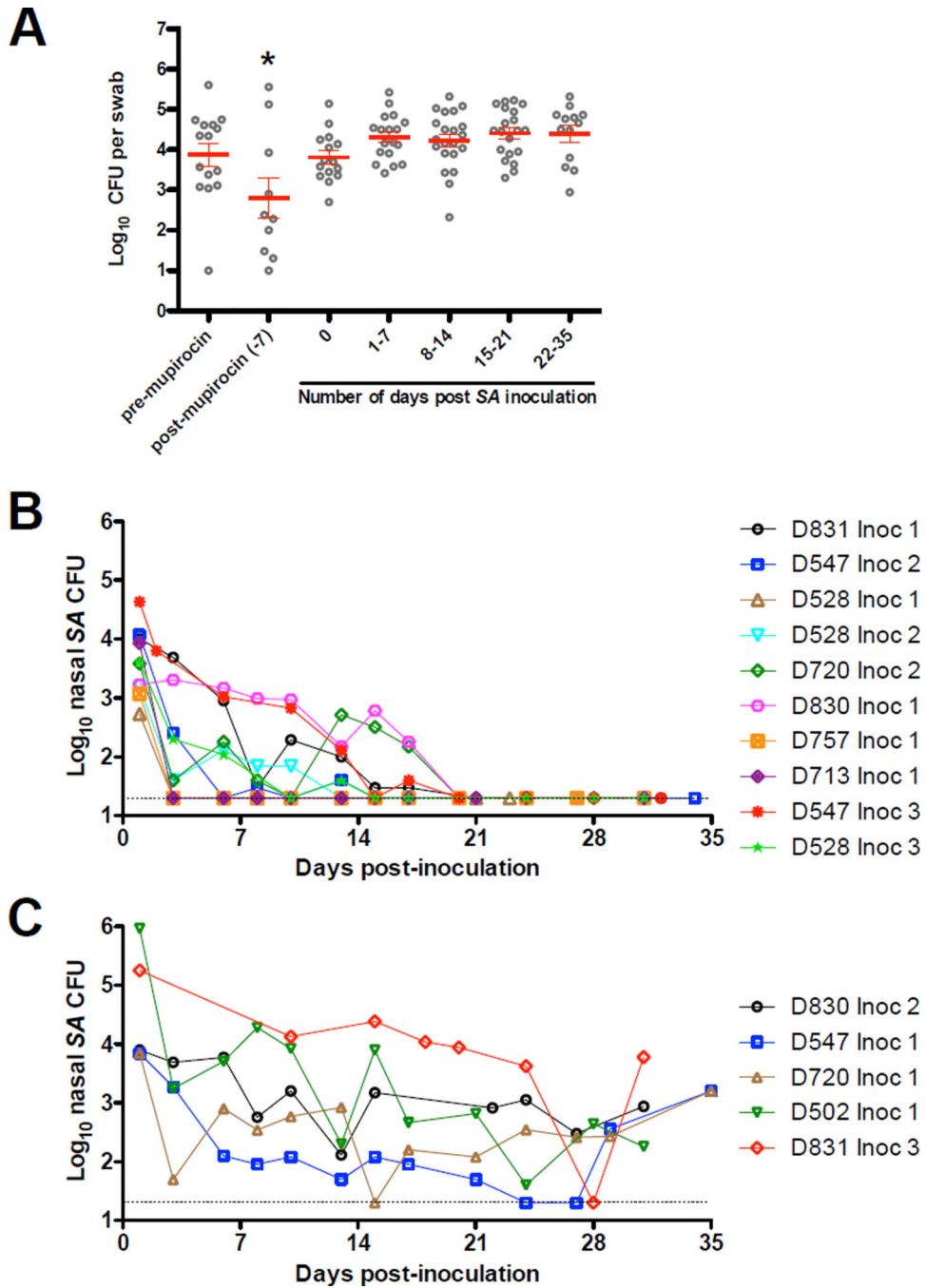
Figure 1. Autologous nasal *SA* inoculation revealed distinct carriage patterns in healthy humans
Nasal *SA* carriers were treated for 5 days with topical mupirocin to clear *SA*. Two weeks
after the last mupirocin application, subjects were intranasally inoculated on two consecutive
days with their own previously isolated nasal *SA*. Left and right nostril swabs were
performed pre-mupirocin, one week post-mupirocin (day -7), at inoculation (days 0–1), and
at 2–4 day intervals post-inoculation. Commensal and *SA* colony forming units (CFU)
enumerated from left and right nostrils were averaged. In total, 15 independent inoculation
studies were performed on eight participants (subjects and inoculation ("Inoc") numbers

denoted to the right of panels B and C). A) Non-*SA* CFU pre- and post-mupirocin and at subsequent visit intervals. * indicates $P<0.05$ versus pre-mupirocin. Error bars indicate mean ±SEM. B) *SA* CFU for 10 inoculation studies in which nasal *SA* was cleared by day 20 for all subjects. C) *SA* CFU for 5 studies in which nasal *SA* was not cleared within one month. Dotted horizontal lines in (B) and (C) indicate the limit of detection.
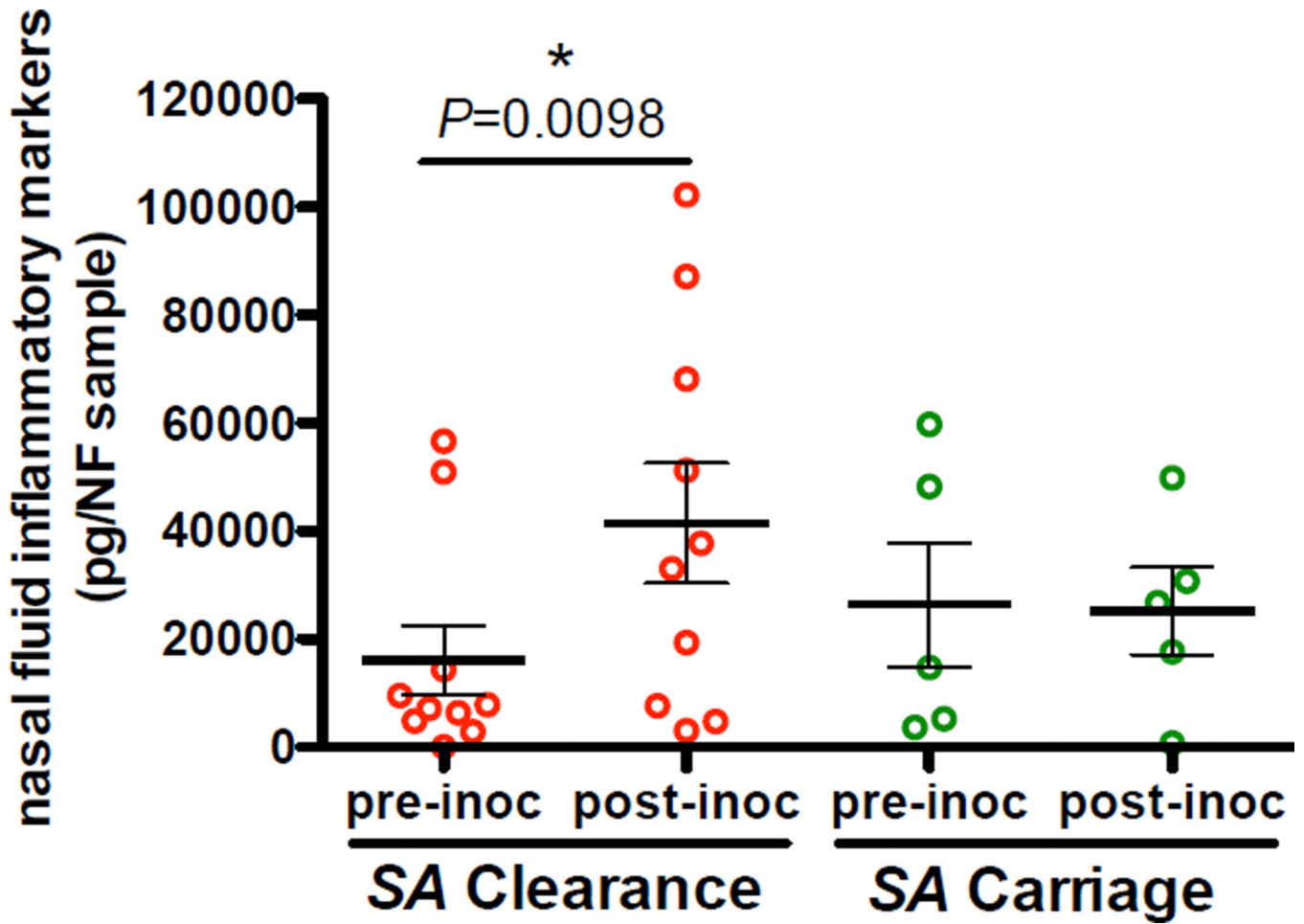
Figure 2. Aggregate nasal mucosal response to *SA* inoculation associates with clearance
Collected nasal fluids from the autologous inoculation studies were analyzed by multi-plex bead array (Bio-Plex). Assay data (pg/mL) was normalized to the volume of each collected nasal fluid. Cumulative expression levels of all 16 detectable analytes preinoculation (day -7) and post-inoculation (day 2) for 15 nasal inoculation studies are presented. Red circles indicate the 10 participants who cleared nasal *SA.* Green circles represent the 5 participants in which nasal *SA* carriage persisted during the month of follow-up. Error bars indicate mean±SEM.
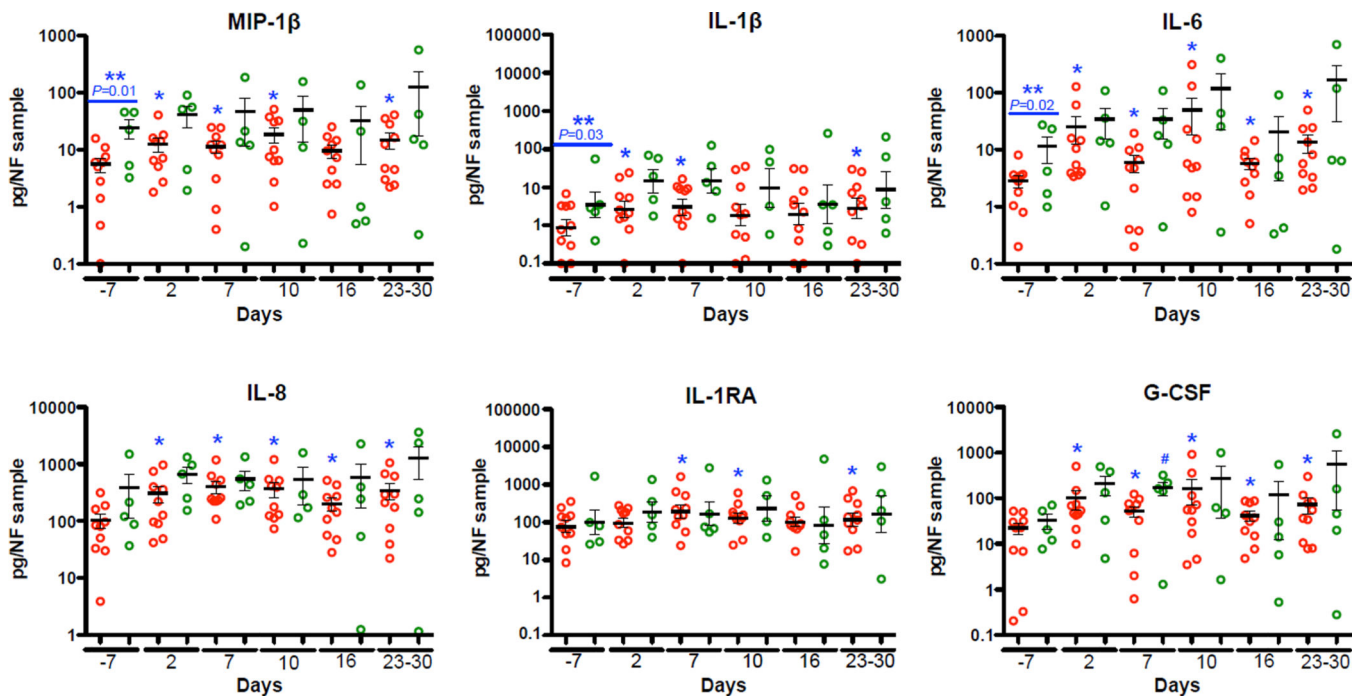
Figure 3. Induction of pro-inflammatory cytokines, chemokines, and growth factors correlates with nasal *SA* clearance

Healthy participants were nasally inoculated with autologous *SA* as described in Fig.1, and nasal fluids were collected at the indicated days pre- (day -7) or post-inoculation and analyzed by multi-plex bead array as described in Fig. 2. Red and green circles indicate clearance and carriage groups, respectively. ** indicates baseline differences between the clearance and carriage groups, with *P* values displayed in the graphs. * indicates *P*<0.05 versus baseline (day -7) for the clearance group. # indicates *P*<0.05 versus baseline (day -7) for the carriage group. Error bars indicate mean±SEM.
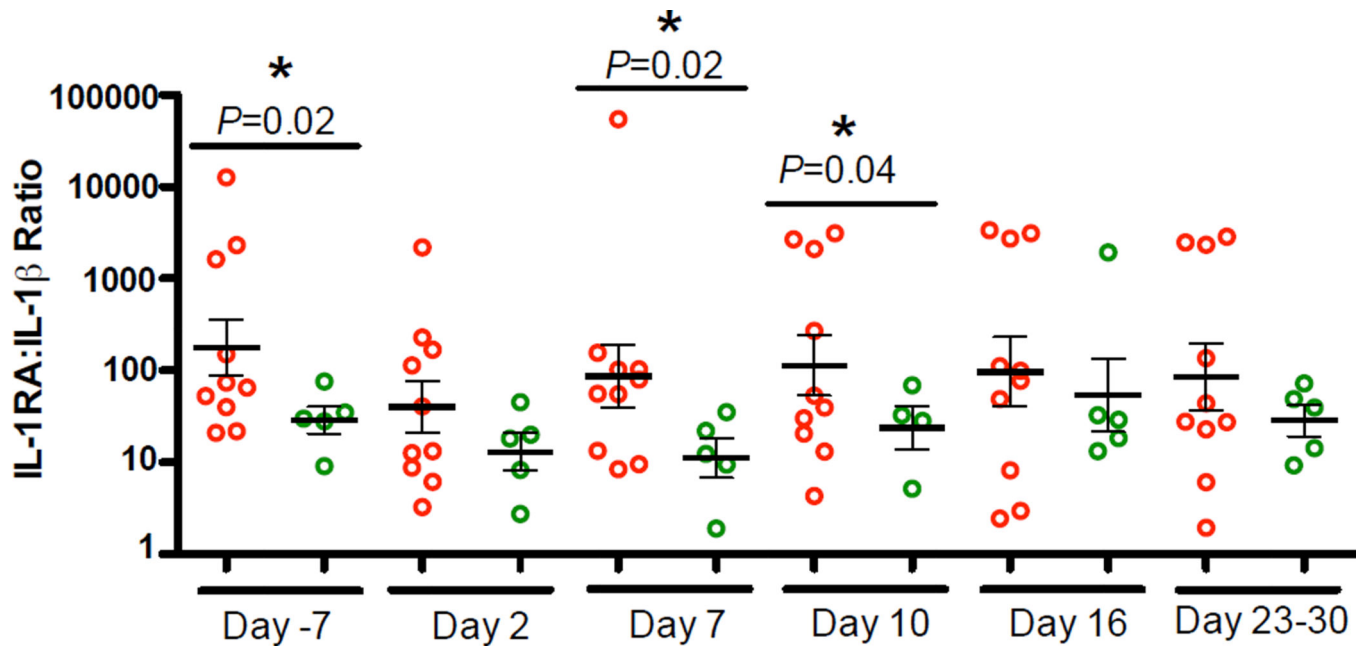
Figure 4. Decreased IL-1RA:IL-1β ratio in the nasal mucosa of participants who failed to clear nasal *SA*

The IL-1RA:IL-1β ratio was calculated for each nasal fluid that was collected on the indicated days during the nasal inoculation studies. * indicates differences between the clearance (red circles) and carriage (green circles) groups, with *P* values displayed in the graph. Error bars indicate mean±SEM.
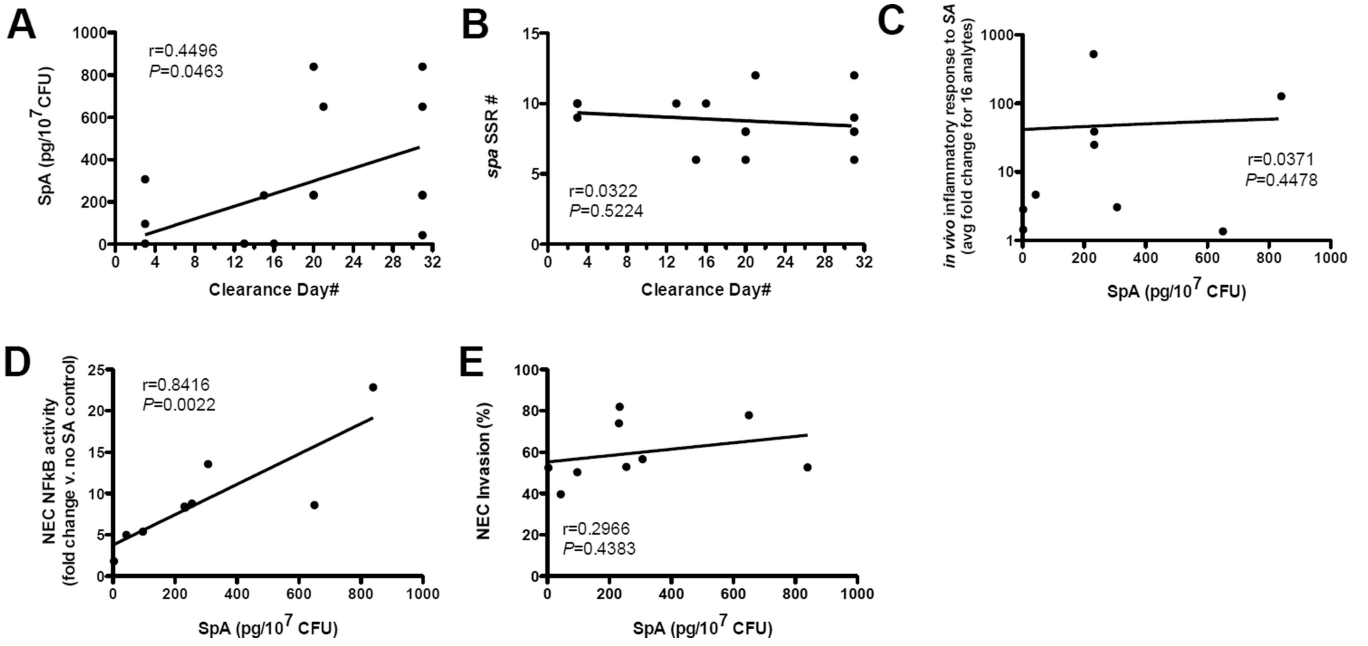
Figure 5. *SA* SpA levels correlate with nasal carriage duration and nasal epithelial cell NFkB activity

SA strains were prepared as for the nasal inoculations and then assayed for SpA content by ELISA. A) Mean SpA values (measured from 3 individual frozen stocks of each strain, shown in Table 1) correlated positively with the number of days that *SA* strains survived in their host during the autologous inoculation studies. B) *spa* polymorphic X region short sequence repeat number did not correlate with carriage duration. C) SpA level did not correlate with the average fold induction of 16 inflammatory markers two days post-inoculation *in vivo*. SpA protein level correlated strongly with the *in vitro* induction of NFkB activity in cultured nasal epithelial cells (NEC) (D), but not with the ability to invade NEC *in vitro* (E). Correlation coefficients (Pearson r), *P* values, and linear regression lines are shown in each graph.
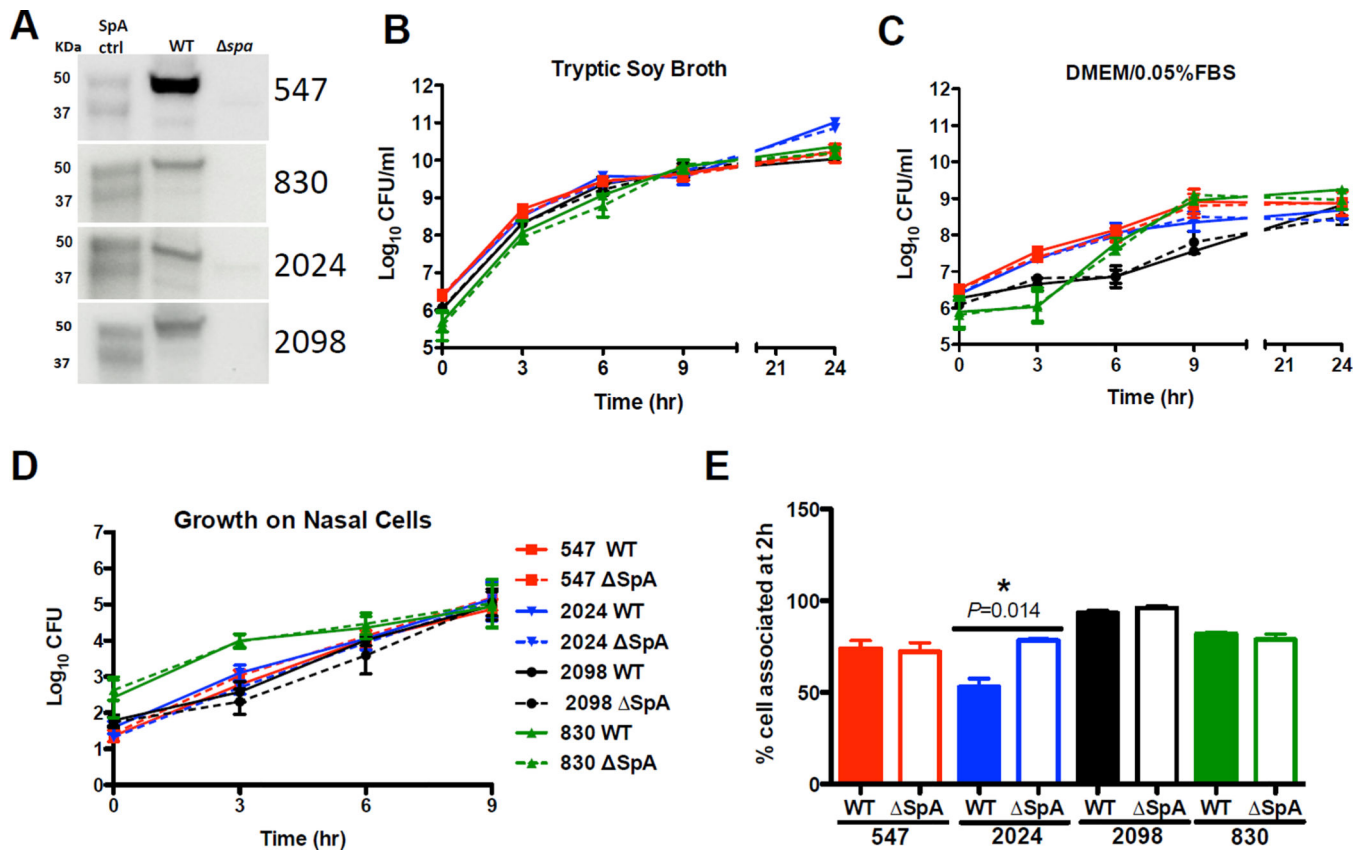
Figure 6. Knockout of SpA does not alter growth kinetics or adhesion to NEC
A) Western blot analysis of SpA expression in wild-type (WT) and SpA-knockout (ΔSpA) strains, compared to recombinant SpA (control, left lane). Growth kinetics of WT (solid lines) and ΔSpA (dotted lines) strains in nutrient rich media (B), lower nutrient cell culture media (C), and on the surface of NEC (D, with color scheme for B–D indicated); n=3–4 for each strain in each assay. E) WT and ΔSpA strains were applied to NEC (MOI=5) for 2hr, then evaluated for adhesion/entry; n=4 for each strain. B–E) Error bars indicate mean±SEM.
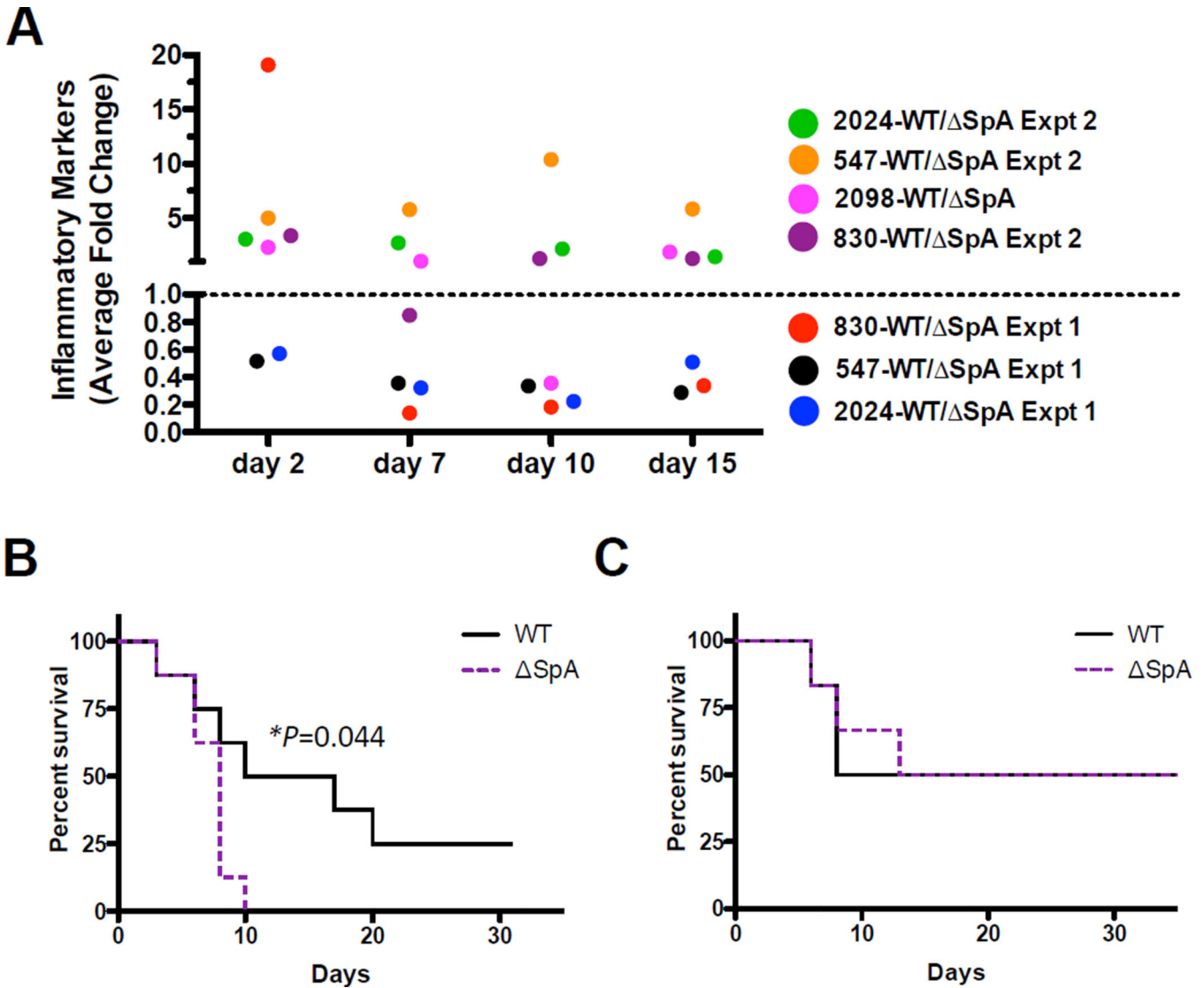
Figure 7. Participants mounting a mucosal response to inoculated *SA* cleared ΔSpA variants faster than WT strains

SA mutants lacking SpA protein (ΔSpA) were created for strains 2024, 547, 2098, and 830. Seven competitive inoculation experiments were performed by intranasally administering equivalent amounts of WT and ΔSpA *SA* to each nostril of the donor-matched (autologous) human host. Individual experiments (participant number and inoculation experiment ("Expt") number) are indicated in panel A. Collected nasal fluids were analyzed by multiplex bead array and the fold change for each of the 16 detectable analytes, relative to baseline, were averaged and plotted for each participant at the indicated timepoints (A). Dotted line marks the threshold between elevated (>1-fold) and decreased (<1-fold) analyte levels compared to baseline. Based on the corresponding nasal *SA* CFU values, survival curves (WT versus ΔSpA) were generated for four studies (green, orange, pink, purple in panel A) that demonstrated a positive inflammatory response to *SA* inoculation (B) and the three studies (red, black, blue in panel A) exhibiting no induction of inflammatory mediators in response to *SA* inoculation (C).
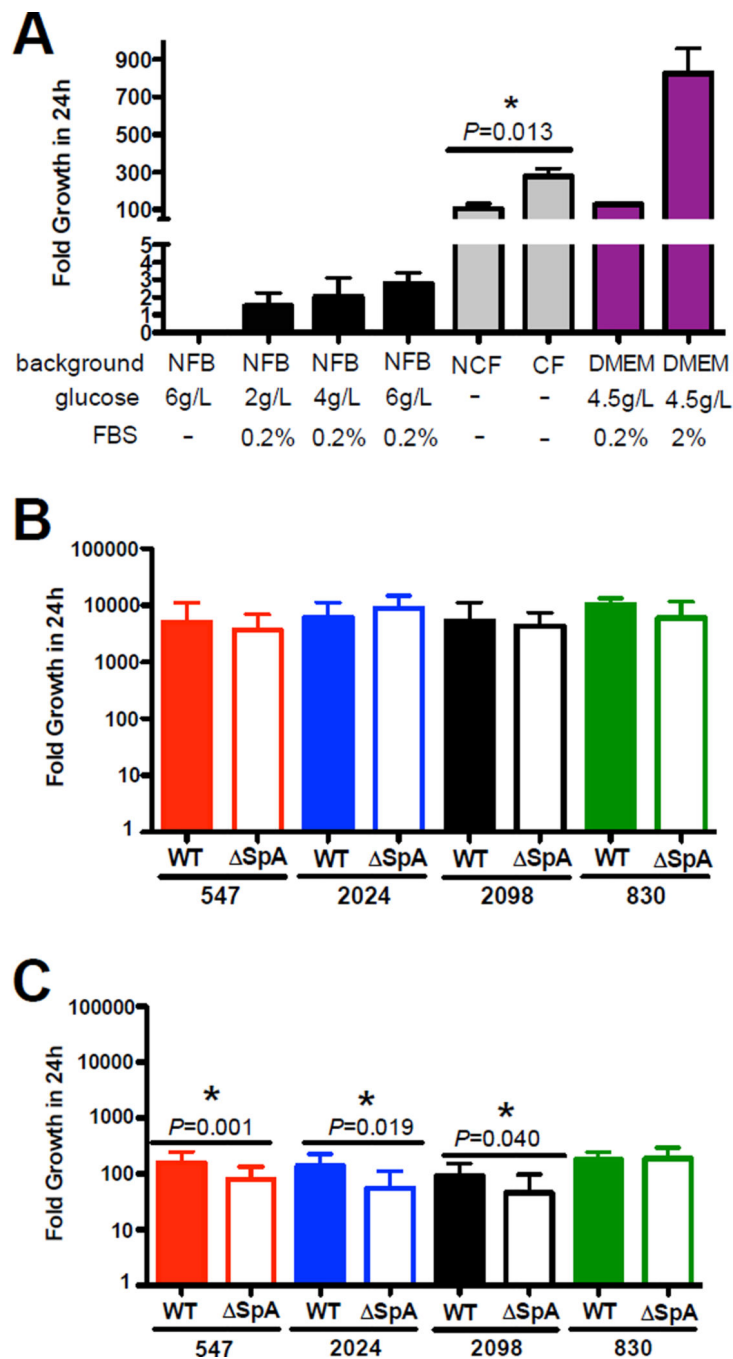
Figure 8. Attenuated growth of ΔSpA versus WT *SA* in nasal fluid

A) Growth of representative *SA* strains from our cohort (2024, 528-11, 713-4, 757-5, 720-7) was assessed in the presence of nasal fluid buffer (NFB) with various indicated additives, non- *SA* carrier nasal fluid (NCF), *SA* carrier nasal fluid (CF), or DMEM culture media containing 0.2–2% FBS. B) *SA* suspended in nutrient-rich DMEM/2% FBS then mixed with nasal fluid. C) *SA* suspended in glucose-free/serum-free nasal fluid buffer then mixed with nasal fluid. A–C) n=4–5 for all assay conditions. Error bars indicate mean±SEM.
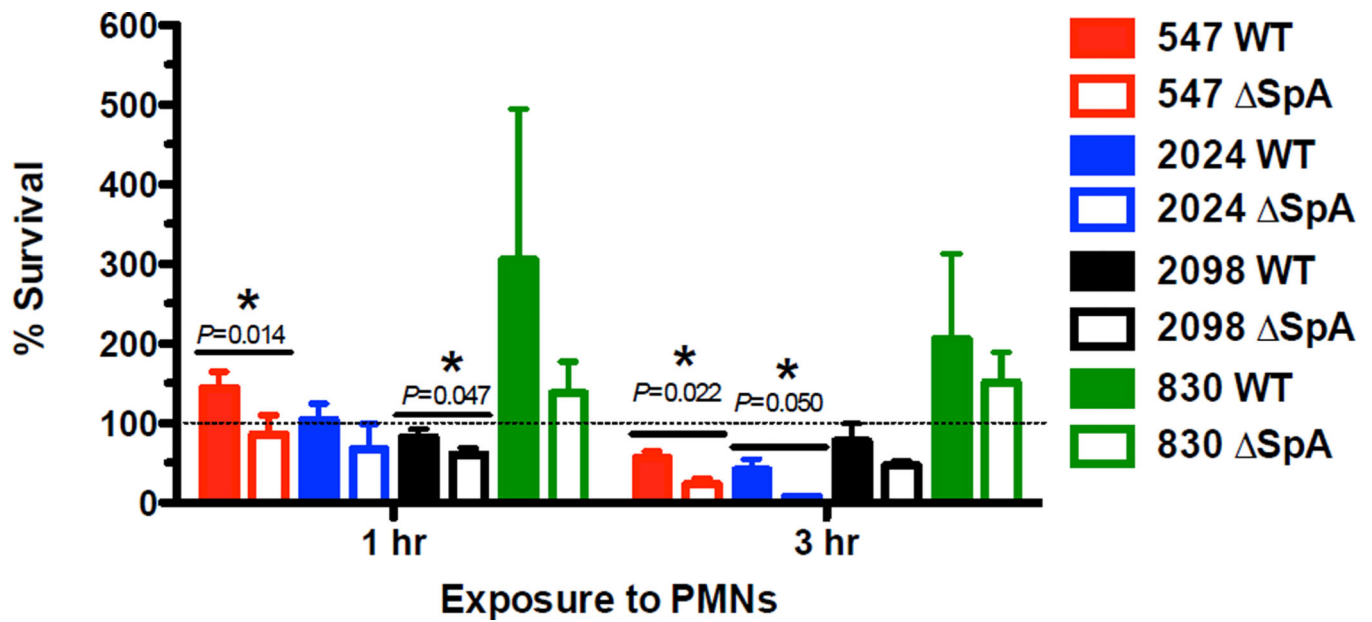
Figure 9. Decreased ΔSpA *SA* survival upon exposure to primary neutrophils
WT and ΔSpA *SA* strains were incubated alone or in the presence of primary neutrophils, and % survival was calculated relative to the neutrophil-free condition for each strain. Data represents four independent experiments, each of which used a unique donor neutrophil preparation. Dotted line at 100% represents the *SA* input. Error bars indicate mean±SEM. * indicates statistical significance between WT and ΔSpA *SA* strains, with the *P* values shown in the graph.

Table 1

Participant and *SA* strain details

Participant Designation	Gender/carriage status	*S. aureus* strain ID	MLST	*spa* type	*spa* SSR#	SpA protein (pg/10⁷ CFU)
D502	F/I	502-9	ST106	t056	9	42.81±22.13
D528	M/I	528-11	ST8	t008	10	2.32±0.69
D547	F/I	547-14	ST5	t688	6	231.02±22.77
D713	M/I	713-4	ST5	t548	9	306.74±23.17
D720	M/P	720-7	ST1657	t1001	8	839.64±342.19
D757	F/I	757-5	ST8	t008	10	96.48±45.82
D830	F/I	830	ST2233#	t12893#	8	233.27±42.86
D831	M/I	831	ST22	t852	12	650.21±43.33
D20*	M/I	2024ᵃ 2098ᵇ	ST5 ST30	t688 t12255	6 10	254.84±69.46 6501.14±827.36

I=intermittent, P=persistent,

#
previously unidentified MLST allele profile or *spa* type.

*
Participant D20 started but did not complete any wild-type autologous *SA* nasal inoculation experiments, and therefore was excluded from figures 1–5.

ᵃ,ᵇ *SA* isolates from separate nasal swab visits.