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Epidemiology And Innate Immune Monocyte Function Of Staphylococcus Aureus Carriers And Non-Carriers In A Medical School Community: A Pilot Study

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Abstract

Epidemiology and innate immune monocyte function of *Staphylococcus aureus* carriers and non-carriers in a medical school community: A pilot study

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Purpose: The purpose of this study was to examine host risk factors and measure innate immune function in order to assess their potential associations with carriage of *Staphylococcus aureus (S. aureus)*. Nearly a third of individuals worldwide are carriers of *S. aureus*. These bacteria usually exist as harmless commensal organisms of the skin or mucosa, most frequently in the anterior nares. Some people are persistently colonized with *S. aureus* while other people are intermittently or never colonized. If *S. aureus* gains access to underlying tissues or to the bloodstream, it can cause serious life-threatening infections, and colonization with *S. aureus* is a known risk factor for *S. aureus* infection. Several factors have been associated with carriage such as diabetes, a compromised immune system, obesity, eczema, and smoking tobacco. In addition, microbial genetics and host defense mechanisms play a role in both colonization and infection. In this study, we aimed to identify potential host factors related to *S. aureus* carriage.

Methods: Using serial nasal swabs over a 3-5 month period, we identified two cohorts within our medical school community: those who were persistently colonized and those who were never colonized with *S. aureus*. We assessed for risk factors for colonization by administering questionnaires. We collected blood samples from a subset of individuals within each cohort and isolated peripheral blood monocyte cells (PBMCs)

in order perform quantitative innate immune function experiments. Toll-like-receptors (TLRs) are an integral part of the innate immune system. They are present on nasal epithelial cells as well as immune cells such as PBMCs. We stimulated host PBMCs with known TLR ligands and measured secretion of cytokines interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-) by Enzyme-Linked ImmunoSorbent Assays (ELISAs).

Results: We enrolled 190 volunteer subjects from our medical school community and 25% were carriers of *S. aureus* after a single swab. Among those who completed all swabs, we identified 33 (33%) who were persistently colonized and 69 (68%) who were persistently not colonized. We did not identify any host risk factors significantly associated with persistent colonization. We noted a decreased secretion of both IL-8 and TNF-α by PBMCs from persistent carriers. IL-8 secretion was significantly diminished after stimulation with FSL-1, LTA, Agr (+) *S. aureus*, and Agr (-) *S. aureus*, as shown by non-parametric two-sided t-test analysis ($P < 0.05$). Although there was an observed decrease in TNF-α secretion by PBMCs from persistent carriers, none of the differences reached statistical significance.

Conclusion: One third of subjects who completed all swabs were found to be persistent carriers; and thus persistently at increased risk of *S. aureus* infection. We found a trend in which persistent carriers had a diminished innate immune response evidenced by less IL-8 and TNF-α secretion following TLR stimulation when compared to non-carriers. In particular, we noted decreased IL-8 secretion after stimulation with ligands known to have lipoprotein properties, suggesting a possible underlying dysfunction in TLR-2. Further investigation into the significance of our findings is warranted.

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Introduction

Staphylococcus aureus: benign and pathogenic

Staphylococcus aureus (S. aureus) usually is a harmless commensal bacterium of the skin and mucosa of humans and some animals (1). It has a predilection for human colonization because of its enhanced capacity to extract iron nutrients from human hemoglobin in comparison to the hemoglobin of other animals, such as the mouse, making humans an excellent source of both benign and pathogenic organisms (2). The most frequent site of colonization, or carriage, is the anterior nare. The anterior nares contain keratinized squamous epithelium and hair follicles but are devoid of cilia and subepithelial glands (3). *S. aureus* binds preferentially to the keratinized epithelial cells (4) and depends on surface mucin secreted by the host for colonization (5). Other anatomical sites for *S. aureus* colonization are the perineum, gastrointestinal tract, axillae, vagina, pharynx, or damaged skin surfaces (6). Although *S. aureus* is known to colonize several locations, the relevance of extra-nasal carriage is not well known (1).

When there is a breach of the epithelial or mucosal barrier, staphylococci gain access to adjoining tissues or the bloodstream and can potentiate infection. Both host and bacterial factors affect the progression of an infection because there is a complex interplay between *S. aureus* virulence determinants and host defense mechanisms (7). As a species within the genus Staphylococcus, *S. aureus* is the most human-pathogenic and does not require a predisposing condition or an immunosuppressive setting in which to proliferate. In other words, infected patients can be generally healthy at baseline. *S. aureus* can cause both mild and life-threatening infections ranging from folliculitis and furunculosis to bacteremia, sepsis, deep tissue abscesses, pneumonia, osteomyelitis, and

infective endocarditis. The presence of a foreign material, such as an indwelling catheter, increases susceptibility to infection in an immunocompetent host (7). *S. aureus* ranks second as the cause of nosocomial blood infections, and such infections increase morbidity, mortality, length of stay, and health care costs (1). The growing threat of resistant organisms adds additional complications. The associated clinical and social costs are estimated at $6–9 billion per year in the United States alone, making *S. aureus* a pressing health care issue (8).

S. aureus carriage and infection are interconnected

The link between carriage and infection was first noted in 1931. Subsequently, there has been considerable effort to better understand the relationship between carriage of *S. aureus* and *S. aureus* infection. It is now widely accepted that colonization with *S. aureus* is linked to an increased risk of infection, including life-threatening ones (9) and that the strain that establishes colonization is often the same one that causes infection in the host (5). Persistent carriers are known to have higher loads of *S. aureus*, and hence are at particular risk for infection (6).

The association of carriage and infection is important in the hospitalized setting where there are resistant bacteria, such as methicillin-resistant *S. aureus* (MRSA), which are more challenging and costly to treat. Such challenges have influenced formation of hospital policies across the world. Healthcare workers have been found to be a source and means of transmission or cross-infection between patients (10). Identification of asymptomatic carriers with active surveillance and placing them under contact precautions are standard practices. In some countries, such precautions are mandated

since colonized patients have been found to be the chief source of *S. aureus* within hospitals (11).

Those who are colonized with *S. aureus* have a threefold increase in the risk for nosocomial infection (1). Von Eiff et al. has shown a particularly increased risk of infection for certain colonized populations, such as post-surgical, peritoneal dialysis, and hemodialysis patients; and they have noted that catheter related infections are the most common cause of *S. aureus* blood infections (5). Once carriers are identified, treating with nasal mupirocin leads to a decrease in nosocomial infections in some populations (12) and elimination from the nares and other parts of the body such as the hands (13). Unfortunately, eradication has not been definitively shown to affect infection rates (6), and such treatments may allow for recolonization of the epithelium by the same or a different strain (14) or even make colonization more permissible (15).

Identifying *S. aureus*

Most studies of *S. aureus* colonization of humans obtain swab samples from the anterior nares for culture, which are then evaluated for *S. aureus*. *S. aureus* was first discovered by surgeon Sir Alexander Ogston in 1880 in Aberdeen, Scotland after he examined pus from post-operative wound infections under a microscope. The organisms he saw appeared like a cluster of grapes and he called his finding "staphyle," which is Greek for "a bunch of grapes." *S. aureus* bacteria appear as gram-positive cocci situated in clusters or pairs. In 1884, scientist Rosenbach grew the same bacteria from pus and observed its yellow-orange pigment and he called it "aureus," which is Latin for "gold"(1,16). These fundamental properties that were observed over a hundred years ago

are still considered defining features of *S. aureus*. In addition to having a distinct shape and color, *S. aureus* is among the population of bacteria that can induce hemolysis. Hemolysis is the capacity to break down red blood cells, which *S. aureus* uses to extract iron, a vital nutrient (2). The microscopic and macroscopic appearances of *S. aureus* and its hemolytic property serve as laboratory indicators of its presence and can be used in devising a method for identification.

Studies have identified three phenotypes of carriage: persistent, intermittent, and non-carriers (17). Nouwen et al. found that the positive predictive value of two consecutive positive swab cultures was 79% in determining persistent carriage status, but the best model for determining persistent carriage was two positive swab cultures plus quantitative evaluation of the *S. aureus* culture (positive predictive value 93.6%). They found that one negative screening virtually excluded the persistent carriage phenotype, and no person whose first two cultures were positive was found to be of the non-carrier phenotype. In order to distinguish non-carriers from intermittent carriers, their findings suggest that at least seven cultures are necessary (18). In order to develop a reliable system for identification of different carrier phenotypes, one must carefully consider how many swabs to obtain and how each cohort is distinguished.

Prevalence of *S. aureus* carriage

Over two billion individuals worldwide are colonized with *S. aureus* (19). The National Health and Nutrition Examination Survey (NHANES) has been administered continuously since 1999 to a nationally representative sample of civilian and non-institutionalized people in the United States. A single swab test along with survey data

regarding *S. aureus* nasal carriage has been collected since 2001. Gorwitz et al. investigated the NHANES data from 2001-2004 and found that the prevalence of colonization decreased from 32.4% to 28.6% over that time period, and colonization of MRSA increased from 0.8% to 1.5%. It is unclear, however, if such changes represent trends or short term modulations (9).

Colonization is influenced by environmental factors such as age, health, economic status, and country of residency - colonization is higher in developed countries. In the United States, carriage of *S. aureus* ranges from 26-32% of people at any given time, and nearly 20% of people are persistently colonized (1). In developed countries, approximately 20% of people are persistent carriers of *S. aureus*, 30% are intermittent carriers, and 50% are non-carriers (9) (20).

Persistent carriers have particular characteristics that impact their risk for serious infections. Those who carry MRSA are most often persistent carriers (21). Persistent carriers have higher loads of *S. aureus* (21), and usually carry a single strain, while intermittent carriers can carry different strains over time suggesting a different mechanism of colonization for those two phenotypes (22). Children, especially infants, have high persistent carrier rates, but values level off in adulthood as people change their carrier state between the ages of ten and twenty (6).

Factors associated with *S. aureus* carriage

There are various medical conditions shown to promote increased *S. aureus* carriage rates, including diabetes; obesity; end stage renal disease; hemodialysis; peritoneal dialysis; HIV infection; compromised immune system; IV drug use; eczema;

psoriasis; history of smoking, and history of stroke (6) (1) (9) (17) (23). Analysis of the NHANES data from 2001-2004 identified an association between MRSA colonization and recent healthcare exposure, diabetes, old age, and poverty (9). There has been no relation found between carriage and seasonality, temperature, or humidity (6). Some activities have been associated with increased carriage rates, such as river rafting; football; and pig farming. The most common mode of transmission is hand-to-hand contact (6), and, not surprisingly, nasal carriage is strongly associated with hand carriage. Interestingly, most mothers carry the same strain as her children, revealing the significance of transmission within cohort populations.

The host immune system employs various barrier defense strategies against *S. aureus* including mechanical clearance and antimicrobial secretions (24). Important components of nasal secretions that mediate inflammation are defensins, lysozymes, lactoferrin, and mucin (25). Carriage is increased in hosts whose nasal secretions are deficient in antimicrobial activity (26). The nasal mucosa also releases complement and cytokines (27). Molecules such as C-reactive protein (CRP) and cytokines such as tumor necrosis factor alpha (TNF-α), Interleukin-8 (IL-8), Interleukin-6 (IL-6), and Interleukin-1β (IL-1β) have been identified as host mediators during *S. aureus* colonization (28). Although it is not known, one might surmise that host deficiencies in these immunologic molecules would likely facilitate the carrier phenotype.

Persistent carriers and persistent non-carriers who were inoculated with identical mixtures of *S. aureus* had differing colonization results suggesting the importance of genetic host factors (14). It has been postulated that the presence of the histocompatibility antigen HLA-DR3 may be a predisposition to nosocomial *S. aureus* infections (29).

Polymorphisms of various genes have also been liked to nasal carriage, such as the Fc fragment of IgG, the human glucocorticoid receptor (30), polymorphic variations in the vitamin D receptor gene in patients with type I diabetes (31), IL-4 (C542T genotype), and the human complement cascade activator serine protease C1 inhibitor (C1INH V480M) (15). Emont et al. has shown that the IL-4 C-allele is associated with nasal carriage, and they observed decreased host production of IL-4 and mucin. Some CRP gene haplotypes may be associated with resistance to colonization (23). Gonzalez-Zorn et al. have identified CFTR and TLR-2 as genetic host factors that may protect the system against carriage of *S. aureus* in animal models (32), and one might postulate that a defect in these genes may diminish the protection. Despite many varied investigations, a significant association between carriage and a particular genetic host factor has yet to be identified.

Bacteria factors also play a role in colonization in concert with host factors. It has been suggested that there may be a bacteria-induced local immunosuppression because nasal fluid from carrier hosts is defective in killing carrier strains compared to non-carrier strains (24). Host IL-1 is secreted as an immunologic mechanism to avoid colonization, but it seems that are strains of *S. aureus* that are less susceptible to IL-1 and can maintain a competitive advantage in their microenvironment by evading the host immune response (24). Nitric oxide (NO) is a broad-spectrum host mechanism for resistance to microorganisms since it disrupts oxygenation, but *S. aureus* employs its own strategies to combat those of the host. *S. aureus* can persist and replicate using hypoxic/anaerobic metabolism. Unlike for any other staphylococcal species, lactate dehydrogenase (LDH) is induced by NO in *S. aureus,* providing a mechanism for survival. If this genetic mechanism for survival against NO is disrupted, *S. aureus* is less virulent (19).

Colonizing and invasive strains of Methicillin-Susceptible *S. aureus* (MSSA) and Methicillin-Resistant *S. aureus* (MRSA) have been be categorized through multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, multilocus sequence typing (MLST), and amplified fragment length polymorphism (AFLP). They can be categorized into five distinct clusters: CC8, CC30, CC5, CC22, CC45; or three major and two minor clusters: I, II (CC30), III (CC45), IVa, IVb. Clusters II and III (CC30 and CC45) account for nearly half of all carriage isolates and have evolved to colonize humans particularly well. There does not seem to be clear distinctions between colonizing and pathogenic organism between the clusters. Acquisition of the mecA regulon, which bestows methicillin resistance, can occur in all cluster populations. Any *S. aureus* genotype has the capacity to colonize and transform into a human pathogen. Thus, it is hypothesized that microbial accessory genes, apart from the core genome, may affect the pathogenic potential of *S. aureus* (1). Agr, a *S. aureus* accessory regulator virulence gene, is functional in the majority of *S. aureus* bacteria, but it is not a necessary virulence gene since agr-defective *S. aureus* mutants maintain the capacity for infection. It has been shown that 9% of host carriers are actually harboring an agr-defective organism (33). Further investigation into microbial accessory genes is warranted in order to determine which aspects of microbial genetics play a role in colonization and infection.

Colonization is a dynamic process that involves competition between different microorganisms within the context of the host immune response. An epidemiologic study with healthy human volunteers has shown that colonization with a species of *S. epidermidis* that secretes serine protease Esp will lead to elimination of *S. aureus* nasal colonization. This study identifies a particular circumstance in which the local microbiome plays a significant role affecting the presence of certain bacteria (34). In

children, there is an inverse correlation between the colonization of *S. aureus* and the colonization of *Streptococcus pneumoniae (S. pneumoniae)*, and it has been shown that he presence of *S. aureus* or *S. pneumoniae* can facilitate the invasion of a *Haemophilus influenza* population (35), indicating the importance of inter-organism dynamics (36). When a resident bacteria population occupies a niche, other bacteria do not seem to have the capacity to replace the resident population. Margolis et al. have studied the ecology of nasal colonization in a neonatal rat model and have shown that bacteria in the nasal epithelium reach a steady state within forty-eight hours regardless of inoculum dose, which lasts for at least three days. They observed that *S. aureus* strains required the host to have no other *S. aureus* bacteria present in order to colonize effectively (1). It is thought, therefore, that any disruption of the local microbiome, such as with antibiotic use or nasal sprays, may significantly impact *S. aureus* colonization.

The innate immune system and *S. aureus*

Carriage elicits an immune response that can be identified by seroconversion after colonization (37). The manners in which *S. aureus* evokes a host immune response remain topics of continued research. The primary cellular host defense against *S. aureus* infection are polymorphonuclear leukocytes (2), but the innate immune system plays an important role early in exposure. The innate immune system has an integral role at the interface between the host and its external environment. Pattern recognition receptors (PRRs) are expressed on the surface of effector immune cells and other cell types that are first to encounter pathogens, such as surface epithelia. PRRs induce endogenous signals allowing for effective combat against microbial invaders. PRRs have evolved to

distinguish pathogenic organisms, and one set of receptors for pathogen-associated molecular patterns (PAMPs) are Toll-Like-Receptors (TLRs) (38).

There have been eleven human and thirteen mouse TLRs identified. Each TLR identifies a conserved feature of a pathogenic organism. For example, TLR-4 recognizes LPS on gram-negative bacteria and TLR-9 recognizes bacterial DNA (39). Each TLR is a type I transmembrane receptor possessing an extracellular leucine-rich repeat and a cytoplasmic Toll/IL-1 receptor homology domain. When a ligand binds a TLR, the adaptor molecule MyD88 is recruited to the Toll/IL-1 receptor domains, which confers a series of intracellular signaling. MyD88 is essential for TLR signaling, and its downstream effect is activation of transcription factor NFkB, which permits the transactivation of proinflammatory cytokine genes (40).

Gram-positive bacteria, such as *S. aureus*, are recognized by TLR-2, but the nature of the TLR-2 PAMP was under debate until recently. It was thought that peptidoglycan (PGN) was a potential TLR-2 ligand, but instead it was found that PGN binds Nod2/Nod1 receptors. Other PAMPs such as LPS, LTA, lipomannans, and lipoarabinomannans have been associated with TLR-2 stimulation (39), but such a variety of ligands seemed dubious given the pattern principles underlying TLRs (41). It has been shown that the lipoprotein fraction isolated from *S. aureus* specifically activates TLR-2 (42) and that lipoprotein is necessary to invoke a cytokine response (35) (43) (41). Bacterial variants that lack lipoproteins have been found to evade immune recognition and cause particularly lethal infections (44). Adequate detection and response by the host innate immune system is paramount in keeping colonizing bacteria at bay, and it appears that lipoproteins are vital for TLR-2 recognition.

Bacterial lipoproteins can be diacylated or triacylated. Synthetic lipoproteins, such as Pam3Cys and Pam2CSK4, have been used experimentally to mimic the pro-inflammatory response to lipoproteins. These experiments led to a longstanding model that triacylated lipopeptides from gram-negative bacteria stimulate TLR2/TLR1 heteromers and that diacylated lipopeptides from gram-positive bacteria stimulate TLR2/TLR6 heteromers (45). Tawaratsumida et al. have since isolated the activating TLR-2 lipoproteins identifying several molecules including diacylated lipoproteins, quinol oxidases, and ATP-binding cassette transporters, such as the iron regulated ATP-binding cassette, SitC. (46).

It turns out that TLR-2 senses a pattern of abundant bacterial ABC transporter substrate-binding lipoproteins (41) as well as buried triayclated lipoproteins on the bacterial cell wall (47). *S. aureus* infection in mice that are deficient in TLR-2 or MyD88 results in increased mortality, disease severity, bacterial burden, and impaired cytokine production, suggesting the importance of an intact TLR pathway in the fight against *S. aureus* (48). Strict mouse models of *S. aureus* infection have limitations, however, and future studies in transgenic mouse models may lead to more correlative findings regarding human TLR-2 and its impact on the host immune response to *S. aureus* colonization and infection (2).

In vitro experiments have shown that TLR-2 is upregulated by exposure to *S. aureus* (49). Incubation with escalating doses of *S. aureus* results in a dose dependent increase of pro-inflammatory cytokines TNF-α and IL-8 (49); IL-8 secretion is TLR-2 dependent in human keratinocytes (50). Expression of TLR-2 is delayed by up to four hours by carrier strains of *S. aureus* compared to non-carrier strains (3) resulting in a

possible local immunosuppressive mechanism for colonization. In their studies examining the immunomodulatory factors of atopic eczema, Mempel showed that TLR-2 expression is distributed unevenly within the layers of the skin with increased density in the basal epidermis, suggesting a mechanism for atopic dermatitis in which colonization remains benign until the barrier is disturbed. Once bacteria are exposed to the high concentration of TLR-2 in the deeper skin layers, the immune response is exacerbated (50). In vitro experiments with human cells have offered some insights into the relationship between *S. aureus* and TLR-2, but many of the secrets regarding *S. aureus* and its complex dance with the host immune system remain unknown.

Specific Aims

There are environmental, medical, and genetic host factors that affect *Staphylococcus aureus* colonization. We are interested in looking at two populations of *S. aureus* carriers: persistently colonized and persistently not colonized individuals. Identification of the two target populations of *S. aureus* carriers who represent the extreme phenotypes of colonization provides an opportunity for comparison. Certain environmental factors that occur in our healthy population may be more represented in those who are persistently colonized, such as children in day care, hospital exposure, and public gym exposure. Such factors may play a role because of the skin-to-skin transmission of *S. aureus.* Medical factors such as diabetes and current tobacco use also may be over-represented in our persistent carrier group. Other studies have demonstrated this association, possibly due to the effect of chronic systemic inflammation of the immune system's ability to clear colonizing organisms. It is also possible that the function of the innate immune system may be impaired in those who are persistently colonized. Faced with *S. aureus* in the anterior nares, an impaired immune system may not effectively clear the organism. Epithelial cells expressing Toll-Like-Receptors (TLRs) in the anterior nares serve as innate immune system defenses triggering an immunologic cascade. Many other cell types, such as monocytes, express TLRs as a surveillance mechanism for invading or colonizing organisms. Since experiments with epithelial cells are less common and technically more complicated, peripheral blood monocyte cells (PBMCs) are a reasonable alternative for immunologic testing, and this was our avenue of exploration. If there were dysfunction in a particular TLR pathway, we would expect to observe a decrease in pro-inflammatory cytokine production, specifically Interleukin-8 (IL-8) and tumor necrosis

factor alpha (TNF-α), after stimulation with corresponding TLR pathogen-associated molecular patterns (PAMPs). We aim to study the PBMC function as it relates to TLR stimulation. If the function of the innate immune system is a significant factor in colonization status, we may see a difference in cytokine response between our two cohort groups.

1) Identify a persistent *S. aureus* carrier group and a persistent non-carrier group comprised of healthcare workers and students within this institution's community.

2) Examine questionnaire data from the identified groups and perform statistical analysis to assess for risk factors associated with persistent non-carriers or persistent carriers.

3) Isolate peripheral blood monocyte cells (PBMCs) from the identified groups and assess innate immune system function by stimulating with TLR ligands and measuring secretion of IL-8 and TNF-α in order to determine if there is a difference in innate immune function between the identified groups.

Materials and Methods

Recruitment

Protocol for this study and its associated documents was approved by the local Institutional Review Board and Human Investigation Committee (HIC #0805003891). Methods for recruiting human subjects for consent and participation included the following: informative postings on bulletin boards around our institution's medical school campus; e-mail solicitation of members of our institution's medical community; informative meetings organized for specific medical community populations; and personal encounters with members of the community. Informed consent was obtained for each participant. The personal information of all subjects was stored in a password protected, secured computer. Enrollment in this study was by volunteer only; individuals were not compensated for their participation.

Identifying target groups

At enrollment, subjects completed a questionnaire and provided a nasal swab sample. The subject repeated the questionnaire and nasal swab in 4-6 weeks increments for a total of four encounters over approximately 3-5 months. Follow-up appointments were scheduled using contact information provided by the participant. The presence or absence of *S. aureus* from the nasal swab was determined for each subject after each encounter. Questionnaire data were collated. Subjects were noted to be positive (presence of *S. aureus*) or negative (absence of *S. aureus*). A pattern was identified for each subject after the second encounter in which a subject had either the same or a different result compared to the prior swab. Those subjects who had different results were excluded from

the study. Patterns were re-assessed after each encounter for each subject. Those subjects who maintained a pattern of either all positive or all negative results continued to be included in the study. After four encounters were completed, subjects were contacted to schedule a fifth appointment at their convenience to donate approximately 60mL of blood anonymously, offer two nasal swabs, and repeat the questionnaire. After preliminary analysis of results from blood sample studies, it was noted that some samples were extreme outliers when compared to others. The subjects associated with these samples were identified and contacted to schedule a sixth appointment at their convenience to donate another blood sample, nasal swab, and repeat questionnaire.

Questionnaire

The questionnaire was developed and formatted by this author and Barbara Kazmierczak M.D., Ph.D. The questionnaire included questions about demographics and risk factors thought to be associated with *S. aureus* colonization or infection. It also inquired about settings in which subjects were in contact with hospitalized patients. The same questionnaire was administered at each subject encounter. The demographics section was usually completed only once. The data from the questionnaires were put into a digital spreadsheet using Microsoft® Access and Microsoft® Excel. (See Supplemental Material for example questionnaire).

Nasal swab

Nasal swabs were obtained using sterile Starswab II™ (Starplex Scientific Inc.; Ontario, Canada). Each packaged unit contained two swabs attached to a plastic top and a capped sheath in which to put the swabbed sample. A member of the study team collected each sample. Holding the capped end, the two swabs were inserted into the anterior portion of one nostril and were rotated against the walls of the inner nose four times around. The swabs were then inserted into the anterior portion of the other nostril and rotated in the same fashion. The swabs were then placed in the labeled sheath for transport. Samples were kept at room temperature. At the fifth encounter, an additional swab was collected for frozen storage: the anterior nares were swabbed as described above, and swabs were cut in a sterile fashion into a sterile tube. These samples were stored at -20 degrees Celsius.

Plate culture

Nasal swabs were plated onto Mannitol Salt Agar (MSA) (Remel; Lenexa, KS) and Blood Agar: TSA with 5% Sheep Blood (BSA) (Remel; Lenexa KS) within 12-14 hours after collection. Swabs were removed from the sheath and spread onto one-third of each plate. A sterile loop was used to spread the sample into the second third of the plate. The second portion was then spread to the third portion of the plate with a sterile loop. Plates were incubated at 37 degrees Celsius for 24-48 hours or 30 degrees Celsius for 72 hours. Observations of color, scent, and hemolysis of were recorded for each plate. Swab samples and culture plates were discarded into the appropriate biohazard containers.

Identifying *Staphylococcus aureus*

Colonies that appeared bright yellow on the MSA plate and colonies that appeared golden/yellow-gray on the BSA plate were suggestive of *S. aureus*. The golden BSA colonies were evaluated using the Staphaurex* test (Remel; Lenexa, KS) according to the manufacturer's instructions, which has positive predictive value of 99.6% and negative predictive value of 99.8% for identification of *S. aureus*. Yellow colonies on the MSA plate were not evaluated directly by Staphaurex*. When identified, a few of the yellow MSA colonies were sub-cultured from the MSA plate onto a fresh BSA plate with a sterile loop and were incubated as described above. Golden colonies from the sub-cultured BSA plates were then evaluated by Staphaurex* as described above. Once a sample revealed the presence of *S. aureus*, further evaluation of other plate samples was discontinued. When there was suspicion for the presence of *S. aureus* but the Staphaurex* test was negative, further work-up was pursued. For some samples, a catalase test was administered with 3% hydrogen peroxide. If bubbles formed, the sample was noted to be catalase positive with the possibility of *S. aureus* upon further testing. If no bubbles formed, sample was noted to be catalase negative and further testing was not done. For some samples, Gram Stain (Sigma Diagnostics, Inc.; St. Louis, MO) was performed on suspected samples followed by a Coagulase Plasma test (Remel; Lenexa, KS) of the identified gram positive cocci colonies. Plates with a negative Staphaurex* test, a very low suspicion for *S. aureus*, or a negative Coagulase Plasma test were noted to be negative for *S. aureus*. Each positive *S. aureus* sample was cultured in liquid LB media and stored at -80 degrees Celsius in a 30% concentration of 50% glycerol. The plate processing and identification of *S. aureus* was performed by this author and other

members of the Kazmierczak lab. (See Supplemental Material Figure 1 for schematic of microbial analysis).

Blood Draw

Using standard phlebotomy techniques, a 21 gauge Safety-Lok™ (BD Vacutainer®; Franklin Lakes, NJ) needle with plastic applicator (BD Vacutainer®; Franklin Lakes, NJ) was used to collect approximately 60mL of blood into 6-7 blood collection tubes containing 143 USP units sodium heparin (BD Vacutainer®; Franklin Lakes, NJ). Samples were processed for peripheral blood monocyte cells (PBMCs) within two hours of collection. The blood draws were performed by this author or Barbara Kazmierczak M.D., Ph.D.

Peripheral Blood Monocyte Cell (PBMC) Isolation

Blood was mixed 1:1 with Gibco® PBS (Invitrogen; Grand Island, NY) and separated into 30mL aliquots. Approximately 20-30 mL of the sample was layered carefully on top of 10.0mL Ficoll-PagueTM PLUS (GE Healthcare Biosciences Piscataway, NJ) in as many conical tubes as necessary. Samples were centrifuged (Sorvall® Legend RT) at 1800 rpm for 20 minutes at room temperature with the brake off. Glass pasteur pipettes were used to collect the middle, opaque layer containing mononuclear leukocytes (MNLs). The collected sample was transferred to a conical containing 10mL Gibco® RPMI Medium 1640 (Invitrogen; Grand Island, NY). Additional RPMI was added for a total volume 40mL per tube. Samples were centrifuged at 1300rpm for 10 minutes at 4 degrees Celsius. The supernatant was discarded. The pellet was washed twice with 15mL

chilled RPMI at 1300rpm for 10 minutes at 4 degrees Celsius. For some samples, there appeared to be an increased amount of red blood cell contaminants. For those samples, 1mL red blood cell lysing buffer (Sigma® Aldrich; Steinheim, Germany) was added between the two washes and incubated for 5 minutes. 15ml of RPMI was then added to neutralize the reaction. This was followed by the second wash. After two washes, the pellet was re-suspended in 2mL warm culture medium, which consisted of Gibco® RPMI Medium 1640 (Invitrogen; Grand Island, NY) with 20% human serum (Lonza Group Ltd.; Basel, Switzerland) and 1% Penicillin/Streptomycin. Cells were counted and PBMC concentration was determined for each sample. PBMC isolation and stimulation experiments were performed by this author and other members of the Kazmierczak lab.

Stimulation of PBMCs

The PBMC isolate was diluted to a concentration of 2 x 10^6 cells/mL by adding the appropriate volume of culture media. 10^6 cells were added to each well of a 24-well plate (Costar® 3526 Corning Inc.; Corning, NY). Plates were cultured at 37 degrees Celsius in 5% CO_2 for 2 hours. Plates were washed twice with 0.5mL/well RPMI in order to remove non-adherent cells. Monocytes remained adherent, and cells were confirmed by microscopy. Ligand preparations were added 0.5mL/well in concordance with our experiment template. Control wells contained only culture media. The following ligands were used: Muramyl dipeptide (MDP) (InvivoGen; San Diego, CA) 10ug/mL, which is a minimal bioactive peptidoglycan motif NOD 2 ligand; Ultra pure E. coli K12 LPS (InvivoGen; San Diego, CA) 100ng/mL, which is a purified lipopolysaccharide from *E. coli* K12 strain and TLR-4 ligand; Purified LTA-SA (InvivoGen; San Diego, CA)

1ug/mL, which is a purified lipoteichoic acid from *S. aureus* and TLR-2 ligand; ST-FLA (InvivoGen; San Diego, CA) 1ug/mL, which is a purified Flagellin from *S. typhimurium* and TLR-5 ligand; Pam3CSK4 (InvivoGen; San Diego, CA) 1ug/mL, which is a synthetic bacterial lipoprotein and TLR-2/TLR-1 ligand;. FSL-1 (InvivoGen; San Diego, CA) 0.1ug/mL, which is a synthetic diacylated lipoprotein and TLR-2/TLR-6 ligand; Agr(+) *S. aureus* thawed preparation (Barbara Kazmierczak M.D., Ph.D lab; New Haven, CT) 10^7cells/mL; Agr(-) *S. aureus* thawed preparation (Barbara Kazmierczak M.D., Ph.D lab; New Haven, CT) 10^7cells/mL. Plates were incubated at 37 degrees Celsius in 5% CO_2. Supernatants were collected in a sterile fashion and stored at -80 degrees Celsius. Some wells were re-stimulated with an aforementioned ligand according to our experiment protocol such that cells initially stimulated with LPS or MDP were re-stimulated with LPS or LTA. Plates were incubated for 24 hours at 37 degrees Celsius in 5% CO_2. Supernatants were collected in a sterile fashion and stored at -80 degrees Celsius.

Enzyme-Linked ImmunoSorbent Assays (ELISAs)

Supernatants that were collected from the PBMC stimulation experiments were used to perform ELISAs specific for detecting human interleukin-8 (IL-8). Human Interleukin-8 ELISA Ready-SET-Go! Kit (eBioscience; San Diego, CA) and Human TNF-α ELISA MAX™ Set Standard kit (BioLegend, Inc.; San Diego, CA) were used according to the manufacturer's instructions. Samples for the IL-8 ELISAs were diluted 1:500. Samples for the TNF-α ELISAs were diluted 1:50 for Agr(+), Agr(-), Flagellin, and LPS samples and diluted 1:10 for all other samples. Nicole Jackson (Yale College '11) and Carla

Weibel aided in the troubleshooting and protocol development of both the IL-8 and TNF-α ELISAs as well aiding in acquisition of data from the supernatant samples. This author and other members of the Kazmierczak lab also performed ELISA experiments on samples using the developed protocols.

Statistical methods

Chi squared or Fisher's exact test was used to test for significant associations for categorical variables obtained from the questionnaire. Two-tailed t-test was used in comparing mean age for each group. In the instances where there was no response for a given categorical variable on the questionnaire, the missing data was omitted from the analysis if its frequency was ≤ 3%. Duplicate ELISA data was averaged for each subject and corrected by subtracting the control value (media only well). Two-tailed Mann-Whitney test was used to test for significant differences in cytokine levels between carriers and non-carriers for each PBMC stimulation scenario. One-way ANOVA Kruskall-Wallis test with Dunn's comparison post-test was also used to test for significance in the IL-8 dataset. GraphPad Prism® 5 software package was used for statistical calculations. A value $P < 0.05$ was considered statistically significant.

Results

Prevalence: Nasal carriage of *S. aureus*

A total of 190 volunteer subjects enrolled in our study and donated a nasal swab for culture. After the first nasal swab, 48 (25%) subjects were carriers of *S. aureus* while 142 (75%) were not (Figure 1). As swabs were collected, subjects were deliberately not followed if their colonization status changed. After the fourth swab was obtained, we identified 102 individuals who either remained culture positive for each swab or culture negative for each swab, and we designated these cohorts as persistently colonized or persistently not colonized, respectively. Of the 102 individuals who donated four swabs, 33 (33%) were persistently colonized and 69 (68%) were persistently not colonized. A total of 21 subjects switched colonization statuses during the 3-5 month period in which the first four swabs were collected. A change from non-carrier to carrier was most common (data not shown). The blood draw occurred several months to a year after the four swabs were obtained. We lost 43 individuals to follow-up and there were 3 failed blood draw attempts. Of the 102 subjects whose colonization status was identified, 21 persistent carriers donated blood, and 35 persistent non-carriers donated blood. Interestingly, 3 subjects changed their colonization status by the time the blood draw occurred as evidence by the fifth culture swab obtained during the blood donation. One subject switched from carrier to non-carrier while two subjects switched from non-carrier to carrier. These individuals were excluded from cytokine analysis.

FIGURE 1: Experimental design and subject count during course of this study

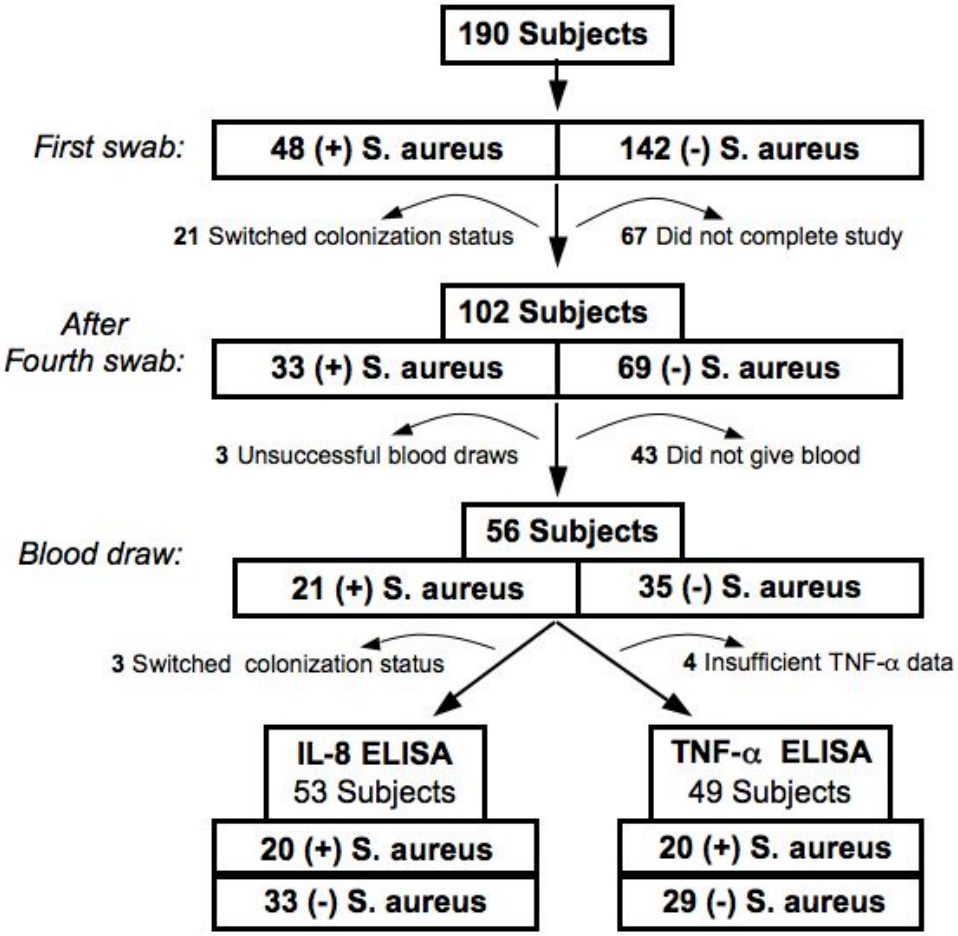


TABLE 1: Characterization of cohort populations after the first nasal swab

	Non-carriers N = 142 (75%)	**Carriers** N = 48 (25%)	P value
Gender			
Male	63 (44%)	25 (52%)	0.4039[a]
Female	79 (56%)	23 (48%)	
Ethnicity			
Hispanic	8 (6%)	1 (2%)	0.5033[b]
Non-Hispanic	116 (82%)	39 (81%)	
No Response	18 (12%)	8 (17%)	
Race			
White	95 (67%)	37 (77%)	0.2831[b]
Black	6 (4%)	1 (2%)	
Asian	29 (20%)	10 (21%)	
Mixed	5 (4%)	0 (0%)	
Not Response	7 (5%)	0 (0%)	
Age (years)			
Mean	31	29	0.6194 [c #]
Age (years)			
Range	22 -71	23- 68	
Median	27	27	

a, Fisher exact test; b, Chi-squared test; c, Unpaired t-test
#, 1-2% of data in a group not available and was excluded in analysis.

Characterization of cohort groups after one and four nasal swabs

The 190 individuals who enrolled in the study were predominantly of non-Hispanic ethnicity and white race. Asian was the second most represented race while black and mixed were least represented. The median age for both groups was 27 years and the range of ages for each group was similar. Carriers and non-carriers identified after the first nasal swab were well matched for gender, ethnicity, race, and age (Table 1). Within each group, the gender breakdown was nearly half female and half male. There were no notable differences in demographics between the two carrier groups. The 102 individuals who completed four swabs were representative of the larger cohort that initially enrolled, with a predominance of non-Hispanic white individuals and predominance of Asian subjects within the non-white population. The median age for each group was similar to that of the initial cohort. There were no notable differences between persistent carriers and persistent non-carriers with respect to gender, ethnicity, race, and age (Table 2).

Potential risk factors for nasal carriage of *S. aureus*

The volunteer subjects in this study completed a questionnaire at each encounter. The questionnaire collected information related to demographics, past medical history, use of certain medications, and exposures potentially associated with *S. aureus* carriage. Since our cohort populations were composed of healthy adults from the institution's medical community, we did not include questions regarding more serious medical issues that have been associated with carriage, such as HIV, hemodialysis, and intravenous drug use. Many of the volunteers were recruited in the clinical setting; thus we included questions regarding patient and hospital exposures as potential factors associated with *S. aureus*

TABLE 2: Characterization of cohort populations after the fourth nasal swab

	Not colonized N = 69 (68%)	**Colonized** N = 33 (32%)	P value
Gender			0.4009 [a]
Male	29 (42%)	17 (52%)	
Female	40 (58%)	16 (48%)	
Ethnicity			0.5417 [b]
Hispanic	4 (6%)	1 (3%)	
Non-Hispanic	57 (83%)	30 (91%)	
No Response	8 (11%)	2 (6%)	
Race			0.4111 [b]
White	44 (65%)	26 (79%)	
Black	3 (4%)	1 (3%)	
Asian	16 (23%)	6 (18%)	
Mixed	5 (7%)	0 (0%)	
No Response	1 (1%)	0 (0%)	
Age (years)			0.5046 [c #]
Mean	31	30	
Age (years)			
Range	23 - 63	24 - 69	
Median	28	27	

a, Fisher exact test; b, Chi-squared test; c, Unpaired t-test
#, 1-2% of data in a group not available and was excluded in analysis.

TABLE 3: Potential risk factors associated with *S. aureus* colonization of cohort populations after the first nasal swab

	Non-carriers N = 142 (75%)	**Carriers** N = 48 (25%)	P value
Diabetes present			
Yes	0 (0%)	0 (0%)	1.0[a]
No	142 (100%)	47 (98%)	
No Response	0 (0%)	1 (2%)	
Asthma present			
Yes	11 (8%)	5 (10%)	0.5559[a]
No	131 (92%)	43 (90%)	
Use tobacco			
Yes	8 (6%)	2 (4%)	1.0[a]
No	134 (94%)	46 (96%)	
Seasonal allergies			
Yes - active	20 (14%)	5 (10%)	0.7078[b #]
Yes - not active	40 (28%)	16 (33%)	
No allergies	81 (57%)	27 (57%)	
No Response	1 (1%)	0 (0%)	
Active eczema			
Yes	13 (9%)	7 (15%)	0.2871[a]
No	129 (91%)	41 (85%)	
Use of nasal steroids			
Yes	9 (6%)	2 (4%)	0.733[a]
No	133 (94%)	46 (96%)	
Cold/URI within 10 days			
Yes	9 (6%)	3 (6%)	1.0[a]
No	133 (94%)	45 (94%)	
Use of systemic steroids			
Yes	1 (1%)	1 (2%)	0.4337[b #]
Occasionally	3 (2%)	0 (0%)	
No	138 (97%)	46 (96%)	
No Response	0 (0%)	1 (2%)	
Antibiotics within 6 months			
Yes	28 (20%)	7 (15%)	0.5215[a]
No	114 (80%)	41(85%)	
Antibiotic ointment within 2 months			
Yes	23 (16%)	5 (10%)	0.4788[a #]
No	117 (82%)	42 (88%)	
No Response	2 (2%)	1 (2%)	
Use of any nasal spray within 1 month			
Yes	11 (8%)	3 (6%)	1.0[a #]
No	128 (90%)	45 (94%)	
No Response	3 (2%)	0 (0%)	
Hospitalization within 6 months			
Yes	2 (2%)	0 (0%)	1.0[a #]
No	139 (97%)	48 (100%)	
No Response	1 (1%)	0 (0%)	
S. aureus infection within 6 months			
Yes	0 (0%)	1 (2%)	0.1266[b]
No	134 (94%)	47 (98%)	
Not sure	3 (2%)	0 (0%)	

No Response	5 (4%)	0 (0%)	
Children in daycare			
Yes	5 (4%)	5 (10%)	0.127[a #]
No	135 (94%)	43 (90%)	
No Response	2 (2%)	0 (0%)	
Last use of public gym			
>2 months	35 (25%)	8 (17%)	0.204[b #]
1-2 months	12 (8%)	5 (10%)	
2-4 weeks	11 (8%)	10 (21%)	
This week	49 (35%)	14 (29%)	
Today	5 (3%)	1 (2%)	
Never use	28 (20%)	10 (21%)	
No Response	2 (1%)	0	
Visit to hospital within 2 weeks			
Yes	92 (65%)	37 (77%)	0.2061[a #]
No	46 (32%)	11 (23%)	
No Response	4 (3%)	0	
Interact with patients as part of studies or job			
Yes	114 (80%)	41 (85%)	0.6618[a #]
No	26 (18%)	7 (15%)	
No Response	2 (2%)	0 (0%)	
Type of patient interaction			
Talk, no contact	6 (4%)	0 (0%)	0.2766[b #]
Occasional contact	8 (6%)	1 (2%)	
Repeated contact	101 (71%)	40 (83%)	
NA	25 (18%)	7 (15%)	
No Response	2 (1%)	0 (0%)	
Type of patient Interaction, binary analysis			
Repeated contact	101 (71%)	40 (83%)	0.1755 [a #]
Not repeated contact	39 (28%)	8 (17%)	
No Response	2 (1%)	0 (0%)	

a, Fisher exact test; b, Chi-squared test; c, Unpaired t-test
#, 1-2% of data in a group not available and was excluded in analysis.

colonization. Initial carrier status was identified after a single swab. There were no statistically significant findings differentiating initial carriers and non-carriers (Table 3). After the fourth swab, we determined which subjects were persistently colonized and which were persistently not colonized. There were no statistically significant findings differentiating colonized from not colonized (Table 4). There was a notable difference, however, in the type of patient interaction between the two groups, more apparent in our final cohort comparison. Those who were persistently culture positive for *S. aureus* had an increased proportion of repeated contact with patients as opposed to no contact, just talking, or occasional contact with patients. Comparing carriers and non-carriers of *S. aureus* after a single swab, 83% versus 71% had repeated contact with patients at the initial swab, respectively, and 85% versus 68% had repeated contact with patients as assessed after the fourth swab, respectively.

PBMC secretion of IL-8 after stimulation with a single ligand

A total of 56 subjects donated blood samples for analysis, 21 of which were persistently colonized and 35 were persistently not colonized. Three subjects were excluded from cytokine analysis because of a switch in colonization status. After exclusion, the analyzed population included 20 samples from persistently carriers and 33 samples from persistently non-carriers. Peripheral blood monocyte cells (PBMCs) were isolated from each subject's blood sample and plated 10^6 cells/well. Cells were stimulated overnight with one of several ligands: a synthetic bacterial lipoprotein (PAM3CSK), a diacylated lipoprotein (FSL-1), flagellin, lipopolysaccharide (LPS), muramyl dipeptide

TABLE 4: Potential risk factors associated with *S. aureus* colonization of cohort populations after the fourth nasal swab

	Not colonized N = 69 (68%)	Colonized N = 33 (32%)	P value
Diabetes present			
Yes	0 (0%)	0 (0%)	1.0[a]
No	69 (100%)	33 (100%)	
Asthma present			
Yes	6 (9%)	4 (12%)	0.7237[a]
No	63 (91%)	29 (88%)	
Use tobacco			
Yes	3 (4%)	2 (6%)	0.6575[a]
No	66 (96%)	31 (98%)	
Seasonal allergies			
Yes-active	5 (7%)	3 (9%)	0.9257[b]
Yes-not active	25 (36%)	11 (33%)	
No	39 (57%)	19 (58%)	
Active eczema			
Yes	8 (12%)	4 (12%)	1.0[a]
No	61 (88%)	29 (88%)	
Use of nasal steroids			
Yes	3 (4%)	4 (12%)	0.7237[a]
No	66 (96%)	29 (88%)	
Cold/URI within 10 days			
Yes	7 (10%)	3 (9%)	1.0[a]
No	62 (90%)	30 (91%)	
Use of systemic steroids			
Yes	0 (0%)	0 (0%)	1.0[a]
Occasionally	2 (3%)	1 (3%)	
No	67 (97%)	32 (97%)	
Antibiotics within 6 months			
Yes	11 (16%)	4 (12%)	0.7686[a]
No	58 (84%)	29 (88%)	
Antibiotic ointment within 2 months			
Yes	7 (10%)	5 (15%)	0.5179[a]
No	62 (90%)	28 (85%)	
Use of any nasal spray within 1 month			
Yes	5 (7%)	3 (9%)	0.7114[a]
No	64 (93%)	30 (91%)	
Hospitalization within 6 months			
Yes	0 (0%)	0 (0%)	1.0[a]
No	69 (100%)	33 (100%)	
S. aureus infection within 6 months			
Yes	1 (1.5%)	0 (0%)	0.6873[b]
No	62 (90%)	31 (94%)	
Not sure	1 (1.5%)	1 (2%)	
No Response	5 (7%)	1 (2%)	
Children in Daycare			
Yes	4 (6%)	2 (6%)	1.0[a #]
No	63 (91%)	31 (98%)	
No Response	2 (3%)	0 (0%)	
Last public gym use			

>2months	14 (20%)	4 (12%)	0.6565 [b]
1-2 months	4 (6%)	1 (3%)	
2-4 weeks	8 (12%)	7 (21%)	
This week	27 (39%)	11 (33%)	
Today	2 (3%)	1 (3%)	
Never use	14 (20%)	9 (28%)	
Visit to hospital within 2 weeks			
Yes	47 (68%)	22 (67%)	1.0 [a]
No	22 (32%)	11 (33%)	
Interact with patients as part of studies or job			
Yes	52 (75%)	27 (82%)	0.6141 [a]
No	17 (25%)	6 (18%)	
Type of patient interaction			
Talk, no contact	3 (4%)	0 (0%)	0.2434 [b]
Occasional contact	2 (3%)	0 (0%)	
Repeated contact	47 (68%)	28 (85%)	
NA	17 (25%)	5 (15%)	
Type of patient Interaction, binary analysis			
Repeated contact	47 (68%)	28 (85%)	0.0944 [a]
Not repeated contact	22 (32%)	5 (15%)	

a, Fisher exact test; b, Chi-squared test; c, Unpaired t-test
#, 1-2% of data in a group not available and was excluded in analysis.

TABLE 5: List of ligands for stimulation of PBMCs
Mechanism of action for each ligand (except S. aureus) was obtained from the InvivoGen product information included with the purchase of the ligand.

Ligand	Innate immune system receptor
PAM3CSK: synthetic lipoprotein	TLR-2/TLR-1
FSL-1: synthetic diacylated lipoprotein	TLR-2/TLR-6
Flagellin: purified from *S. typhimurium*	TLR-5
Lipopolysaccharide (LPS): from *E. coli*	TLR-4
Muramyl dipeptide (MDP): peptidoglycan motif	NOD 2
Lipoteichoic acid (LTA): purified from *S. aureus*	TLR-2
S. aureus Agr (+): wild type accessory regulator gene	TLR-2/TLR-6
S. aureus Agr (-): mutant accessory regulator gene	TLR-2/TLR-6

(MDP), lipoteichoic acid (LTA), *S. aureus* with Agr (+) gene, and *S. aureus with* Agr (-) mutation (Table 5). Supernatants were collected for IL-8 ELISA. PBMCs from persistent carriers secreted less IL-8 after stimulation with each ligand compared to those cells from persistent non-carriers (Figure 2). After stimulation with FSL-1, median IL-8 secretion was 41 ng/ml versus 61 ng/ml for colonized and not colonized samples respectively, $p < 0.05$ (Figure 2b). Similarly, median IL-8 secretion after stimulation with LTA (40 ng/ml versus 58 ng/ml), Agr (+) (22 ng/ml versus 60 ng/ml), and Agr (-) (29 ng/ml versus 53 ng/ml) revealed a significant difference ($p < 0.05$) between persistent carrier and persistent non-carrier samples, respectively (Figure 2b, f-h). The diminished secretion of IL-8 in response to PAM3CSK and flagellin approached statistical significance (Figure 2a, c). With a more strict analysis of variance using a one-way ANOVA Kruskall-Wallis test with Dunn's Multiple Comparisons Post-test nullified any suspected difference between the cohorts for any of the TLR experiments (Table 6).

IL-8 secretion after PBMC sensitization

PBMCs from each subject's blood sample were plated 10^6 cells/well and stimulated with ligands as described above. Some wells that were initially stimulated with LPS or MDP overnight were stimulated overnight for a second time with either LPS or LTA. Supernatants were collected after the second stimulation. Cells from persistent carriers and non-carriers that were initially stimulated with LPS had equal median secretion (0 ng/ml) after the second stimulation (Figure 3a, b), but the mean secretion revealed a pattern in which cells from persistent carriers secreted more IL-8 after re-stimulation compared to cells from non-carrier (Figure 3c, d). Cells from persistent carriers that were

FIGURE 2: IL-8 secretion by PBMCs after stimulation with a single ligand
10^6 PBMCs from each subject were stimulated with an individual ligand: synthetic bacterial lipoprotein (PAM 3CSK) (a), diacylated lipoprotein (FSL-1) (b), Flagellin (c), lipopolysaccharide (LPS) (d), muramyl dipeptide (MDP) (e), lipoteichoic acid (LTA) (f), *S. aureus* Agr (+) (g), *S. aureus* Agr (-) (h). Supernatants were collected after overnight incubation and IL-8 ELISA was performed. Data represents quantitative box-plot analysis (median, minimum, maximum value, 25% and 75% percentile). Comparison for significance with two-tailed Mann-Whitney test. (*, denotes statistical significance, $p < 0.05$).

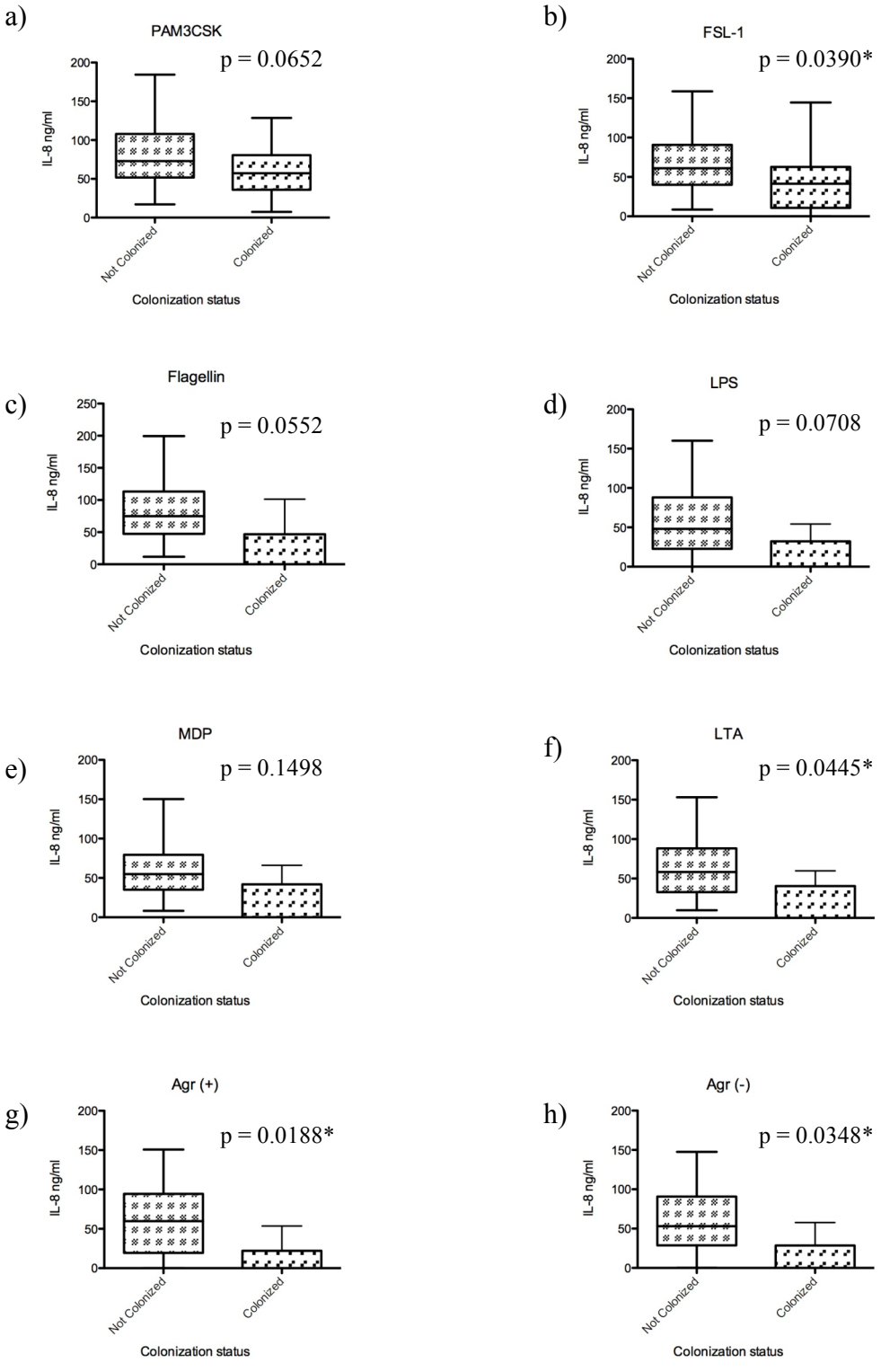
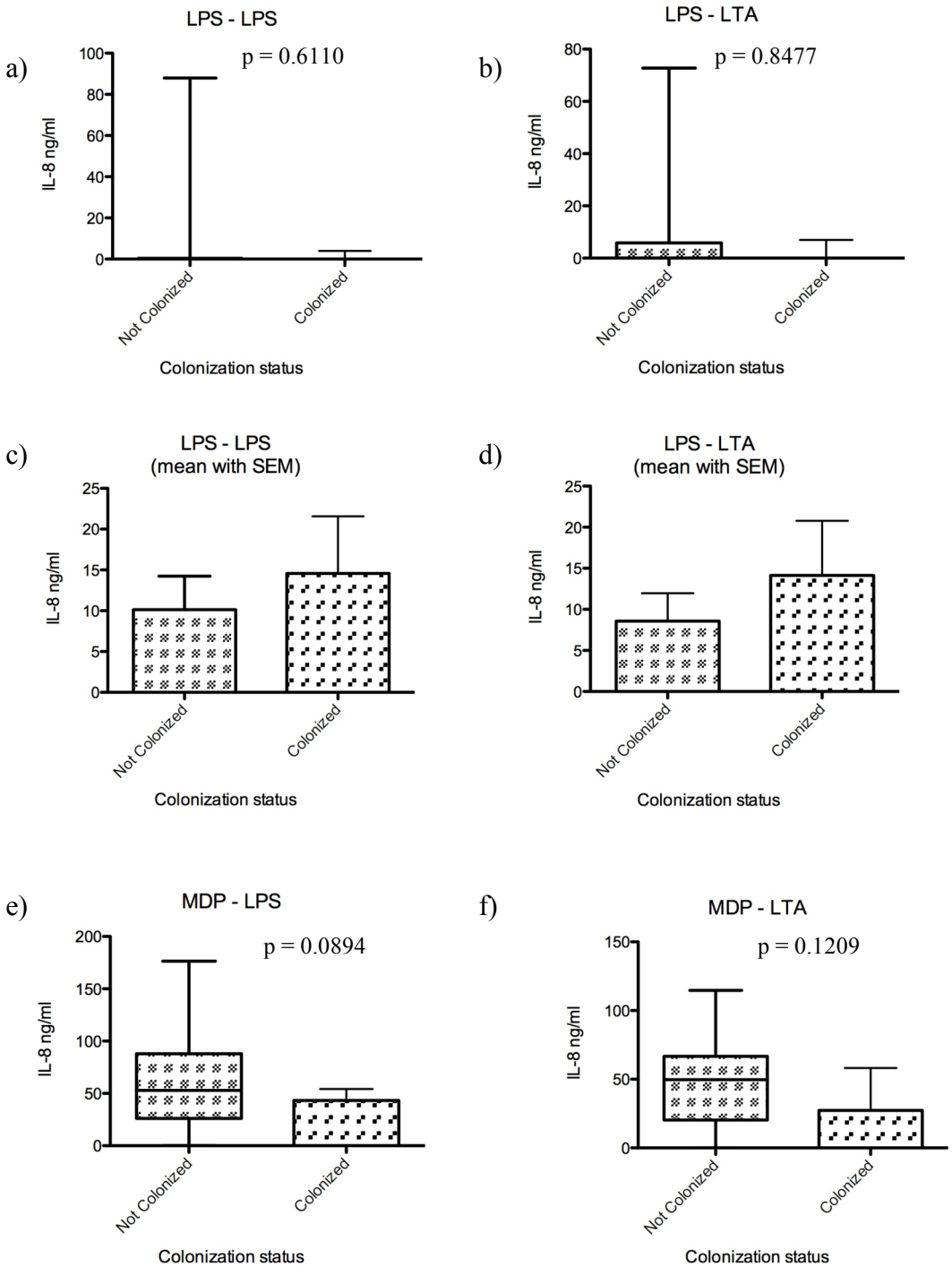


TABLE 6: IL-8 secretion analysis using one-way ANOVA (Kruskall-Wallis) with Dunn's Multiple Comparison Test

Ligand	Comparison	Difference in rank sum	P < 0.05?
PAM3CSK	Not colonized vs. Colonized	78.77	No
FSL-1	Not colonized vs. Colonized	94.77	No
Flagellin	Not colonized vs. Colonized	104.7	No
LPS	Not colonized vs. Colonized	94.10	No
MDP	Not colonized vs. Colonized	57.78	No
Agr (+)	Not colonized vs. Colonized	121.2	No
Agr (-)	Not colonized vs. Colonized	104.6	No
LTA	Not colonized vs. Colonized	84.24	No
LPS – LPS	Not colonized vs. Colonized	-21.36	No
LPS – LTA	Not colonized vs. Colonized	-24.25	No
MDP – LPS	Not colonized vs. Colonized	81.64	No
MDP – LTA	Not colonized vs. Colonized	63.60	No

FIGURE 3: IL-8 secretion by PBMCs after sensitization

10^6 PBMCs from each subject were stimulated overnight with either lipopolysaccharide (LPS) (a and b) or muramyl dipeptide (MDP) (e and f). Supernatants were discarded and cells were re-stimulated with either LPS (a and e) or lipoteichoic acid (LTA) (b and f). Supernatants were collected after overnight incubation and IL-8 ELISA was performed. A, b, e, f data represent quantitative box-plot analysis (median, minimum, maximum value, 25% and 75% percentile); c and d represent mean with SEM.



initially stimulated with MDP secreted less IL-8 after the second stimulation compared to cells from non-carriers (Figure 3d, e). This difference approached significance. It seems that LPS and MDP have opposing effects on persistent carrier PBMCs in regards to IL-8 secretion when used to prime the cells. Priming with LPS led to an increased IL-8 response for persistent carriers, and priming with MDP led to a diminished IL-8 response for persistent carriers after re-stimulation compared to non-carriers.

PBMC secretion of TNF-α after stimulation with a single ligand

Of the 53 subjects who donated blood and met criteria for IL-8 cytokine analysis, four samples did not produce adequate TNF-α secretion for analysis. Thus 49 samples were included in the TNF-α analysis. PBMCs from persistently colonized individuals secreted less TNF-α after stimulation with each ligand compared to those cells from not colonized individuals, except with LPS stimulation in which the two groups secreted a similar amount (Figure 4). None of the observed differences were statistically significant, but a trend toward significance was noted with PAM3CSK, FSL-1, LTA, Agr (+), and Agr (-) (Figure 4a-c, f-h).

TNF-α secretion after PBMC sensitization

The sensitization experiments were preformed as described above and TNF-α was measured after re-stimulation. Cells from persistent carriers that were primed with LPS secreted less TNF-α after re-stimulation compared to cells from non-carriers (Figure 5a, b). This difference was statistically significant for re-stimulation with LTA after LPS priming. Cells from persistent carriers and non-carriers that were primed with MDP secreted similar amounts of TNF-α after re-stimulation with LPS or LTA (Figure 5c, d).

Taking both the IL-8 and TNF-α datasets into consideration, it appears that priming cells with LPS had an opposite effect on carrier cells depending on which cytokine was measured. For persistent carriers, LPS priming invoked an increased IL-8 but a decreased TNF-α response compared to non-carriers.

FIGURE 4: TNF-α secretion by PBMCs after stimulation with a single ligand
10^6 PBMCs from each subject were stimulated with an individual ligand: synthetic bacterial lipoprotein (PAM 3CSK) (a), diacylated lipoprotein (FSL-1) (b), Flagellin (c), lipopolysaccharide (LPS) (d), muramyl dipeptide (MDP) (e), lipoteichoic acid (LTA) (f), *S. aureus* Agr (+) (g), *S. aureus* Agr (-) (h). Supernatants were collected after overnight incubation and TNF-α ELISA was performed. Data represents quantitative box-plot analysis (median, minimum, maximum value, 25% and 75% percentile). Comparison for significance with two-tailed Mann-Whitney test. (*, denotes statistical significance).

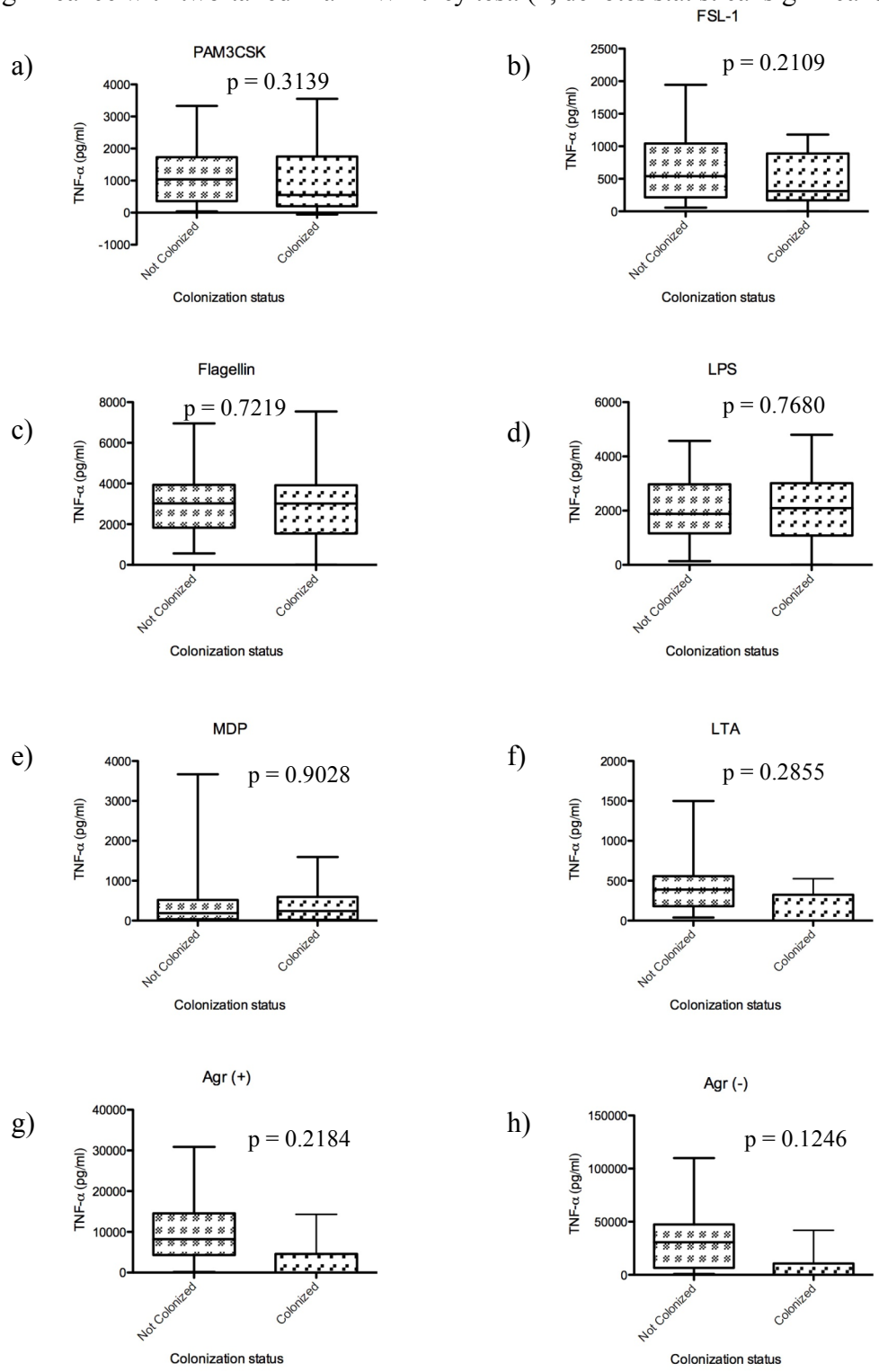
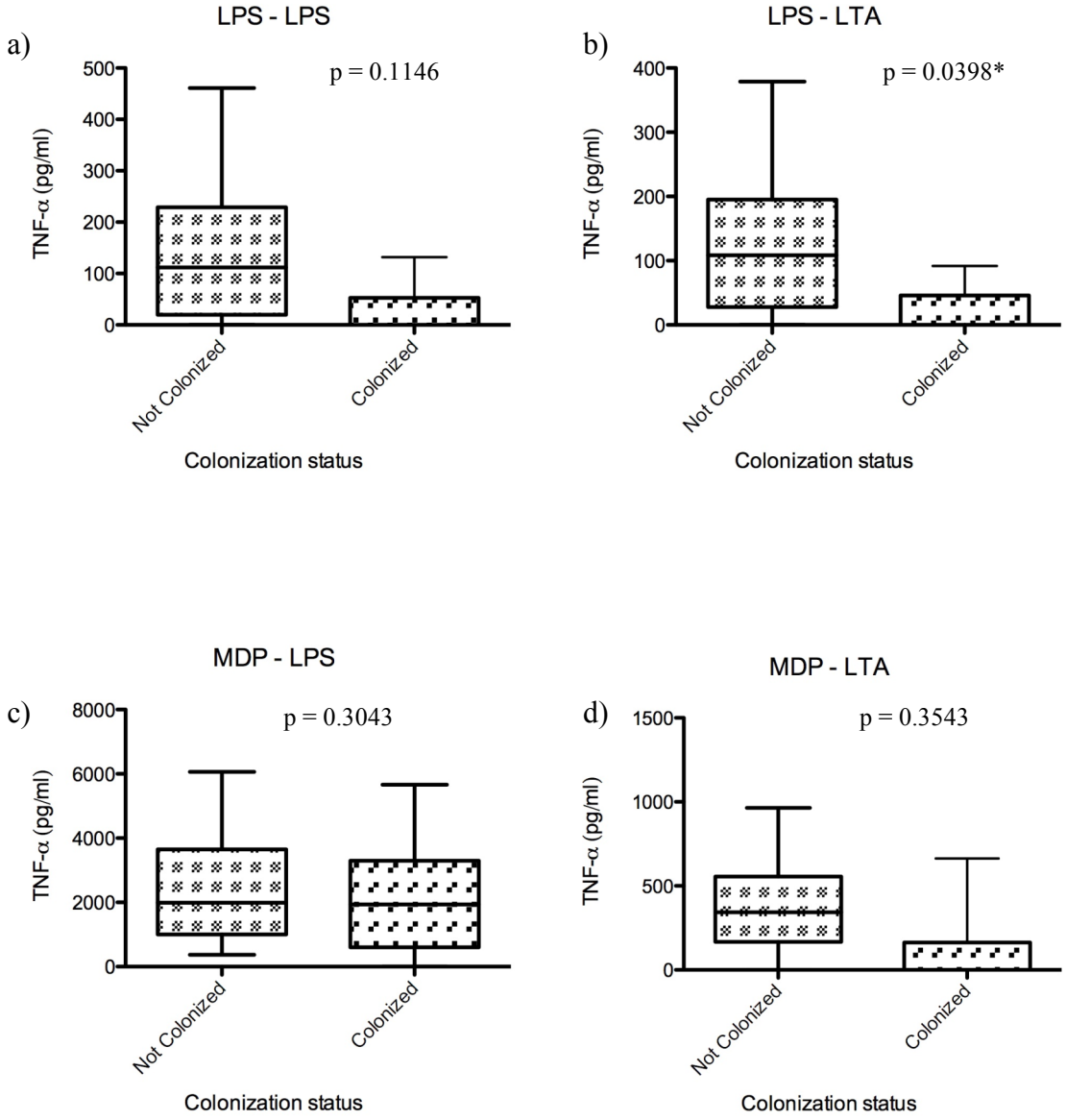


FIGURE 3: TNF-α secretion by PBMCs after sensitization

10^6 PBMCs from each subject were stimulated overnight with either lipopolysaccharide (LPS) (a and b) or muramyl dipeptide (MDP) (c and d). Supernatants were discarded and cells were re-stimulated with either LPS (a and c) or lipoteichoic acid (LTA) (b and d). Supernatants were collected after overnight incubation and TNF-α ELISA was performed. Data represents quantitative box-plot analysis (median, minimum, maximum value, 25% and 75% percentile). (*, denotes statistical significance).



Discussion

Epidemiology

We examined healthy adults from our academic community. This population was amenable to volunteer in this study and maintain follow-up because of the collegial academic environment in which they work. Despite the good will of this population, there are limitations that result from their exclusive enrollment resulting in a selection bias. Those people with a particular investment in the research or academic community were preferentially targeted and enrolled, and young adults in their 20's and 30's were most agreeable to participate. The generalizability of these data, therefore, is limited because the studied population is not representative of the general, national population.

Several factors may impact the detection of *S. aureus* including aspects related to methods of sample collection and methods of culturing (11). Most studies administer nasal swabs in order to assess carriage state, but it has been reported that up to a third of colonized states can be missed if other sites of colonization are not tested (51). We chose to examine only the anterior nares for identification of *S. aureus* since it is the most common site for colonization. We cultured the swabs on agar plates rather than in liquid media following methods of prior studies. Our assessment of carrier status was based on a binary evaluation of the culture using methods that indicated that the presence or absence of *S. aureus*. We did not distinguish between samples that were floridly culture positive versus samples that were found to be positive only after several steps of work-up. It has been shown that evaluation of bacterial burden can distinguish different carrier states since a low burden could be a falsely negative swab (52). It is possible that there were false negatives in our study, but they likely would have been categorized as

intermittent carriers; the intermittent status is most difficult to distinguish from the persistently negative population. There is less likelihood of a false positive in our study since we collected five swabs and included only those who had positive cultures for each swab as persistent carriers. The positive predictive value in determining persistent carriage status with two consecutive positive swab cultures is 79% (18); thus our positive predictive value with five swabs was likely considerably higher. Nevertheless, it cannot be discounted that we did not sample all sites of colonization, and we used a binary culture analysis, both factors possibly contributing to errors in identifying our cohort groups.

A 2009 study by van Belkum et al. outlined a strict classification of persistent carriers and non-carriers in which participants were labeled persistent carriers if 80% of 5-10 swabs over 6 months were positive for *S. aureus*. They noted that allowing 1 of 5 swab samples to test negative while still categorizing a participant as persistently colonized minimizes misclassification of carriage states from culture or lab errors (20). In our study, there were subjects who would have fulfilled these criteria for persistent carriage, but we did not include these subjects in our TLR experiments. It is possible, therefore, that we unnecessarily excluded potentially revealing data from those who could be classified as persistent carriers.

In the aforementioned study, they found that when persistent carriers, intermittent carriers, and non-carriers were treated with mupiricon for eradication, only the persistent carriers re-established colonization for a long stretch of time compared to the other carriage phenotypes. It seems, therefore, that the persistent carrier phenotype is clinically most relevant. Intermittent and non-carriers share similar colonization kinetics and pose

less of a risk for infection (20). Our data maintain clinical relevance by strictly identifying the persistently colonized cohort, who is at higher risk for nosocomial infections. In order to understand the dynamics of *S. aureus* colonization, the multi-factorial mechanisms that distinguish persistent carriers and make them so susceptible to colonization warrant examination in future studies in order to extrapolate to a public health domain.

In this study, we found that 25% of our subjects were carriers of *S. aureus* following evaluation of a single nasal swab. This distribution was slightly lower than the findings from other studies in which a single swab revealed 26-31% prevalence of colonization at any given time (1). The observed decrease in prevalence, however, is in line with the finding that colonization decreased from 2001- 2004 as evidenced by the National Health and Nutrition Examination Survey (NHANES) (9).

Analysis of the NHANES revealed an association between healthcare exposure, diabetes, old age and carriage of *S. aureus* (9). In our study, we did not find any association between carriage and any risk factor. Our population was predominantly young adults and did not include any diabetic participants; thus we could not adequately evaluate for association with diabetes or old age. Regarding healthcare exposure, our data suggest a trend in which increased patient contact is associated with persistent colonization of *S. aureus*. Our study population was much smaller than that of the NHANES. The NHANES included over 9000 participants from across the United States. With larger cohort populations, it is possible that we would have revealed risk factors associated with carriage. Informal analysis of our subjects who were excluded after enrollment showed that 5 % of subjects switched their colonization status on their second

swab (data not shown). The NHANES examined culture results from a single nasal swab, providing information about a cross sectional sample rather than a targeted cohort as we did when we identified persistently colonized or not colonized subjects. Our final cohort groups were narrowed populations compared to those in the NHANES, and it is reasonable to presume that not all associations observed in the NHANES would carry over in our study.

The notion that the microbiome and local ecology of the anterior nares may affect colonization is an intriguing one - the anterior nare is not a sterile environment like the blood. Traditional theory of ecology and competition identifies organism fitness as a determining factor for occupation of a niche or habitat. Recent studies have shown the dynamics between skin and nasal flora to be more complicated than that. Resident bacterial strains seem to retain a competitive advantage, possibly because of a localized resource that serves as a limiting factor (35). Studies have also shown that recognition of microbial products from one species may activate inflammatory responses that promote clearance of another species (53). We did not perform experiments to assess for other organisms within the anterior nares of our subjects, but at the blood draw encounter, we collected an addition nasal swab that was snap frozen for future inquiries into the microbiomes of our cohort populations. We cannot, therefore, discount the unknown variable of the microbiome when considering the findings of our study

Sensitization

In our study, we examined PBMC sensitization. The notion of host sensitization has been illustrated in prior studies with gram-negative organisms in which treatment with a

non-lethal dose of LPS led to tolerance and resistance to subsequent exposures of normally lethal doses of LPS. Pre-treatment led to an attenuation of the immune response, preventing the physiologic decompensation that comes from a severe infection. Murphey et al. have examined host sensitization to cell wall elements of gram-positive bacteria. Mice were treated with a non-lethal dose of peptidoglycan (PGN) followed by challenge with an inoculum of live *S. aureus*. After challenge with *S. aureus*, pro-inflammatory cytokines TNF-α and IFN-gamma were suppressed and IL-10 was increased, which suggested a preference to a Th-2 host response after PGN priming. Clinically, they found that pre-treatment was associated with increased bacterial clearance and improved host survival after subsequent challenge. In another study, pre-treatment with pro-inflammatory cytokine IFN-gamma enhanced release of TNF-α and IL-8 in response to *S. aureus* in conjunctival epithelial cells (49). In our experiments, we did not observe significant differences between our groups when PBMCs were primed and re-stimulated. Some of our data suggests a potential pattern that LPS sensitizes carrier PBMCs more effectively resulting in a less robust TNF-α response. The opposite pattern was suggested in terms of IL-8, however, in which mean secretion from persistent carriers was greater than that by persistent non-carriers after LPS priming. Our findings regarding priming and sensitization remain inconclusive and further experiments are warranted on these topics between *S. aureus* carriers and non-carriers.

Innate immune system

Our study is limited by the fact that we did not conduct our in vitro experiments with epithelial cells, which are the cells in the anterior nares that would respond

physiologically in vivo as agents of the innate immune system in response to *S. aureus* exposure. Epithelial cells are a challenge to grow and maintain. We were interested in the function of innate immune receptors; thus we made the assumption that the function of TLRs on non-sterile epithelial cells would be comparable to the function of TLRs on sterile peripheral blood monocytes. We also assumed the PBMCs are the same at any given time point. These may be false assumptions. It is possible that any local immunologic differences are attenuated at a systemic level; thus we would be missing potential differences by not using epithelial cells. It is also possible that immune cells obtained from a sterile environment behave significantly differently from nasal epithelial cells. But by using our assumptions, we aimed to generalize our PBMC findings to what might occur physiologically in the anterior nares during the complex interaction between *S. aureus* and the innate immune system, a requisite interaction of microbial colonization and one that must be addressed.

Our experiments comparing cytokine secretion by PBMCs from persistent carriers and non-carriers included analysis with non-parametric tests. The data were not consistently normally distributed; thus we chose a stricter non-parametric analysis for all data rather than a parametric approach. We stimulated PBMCs with a variety of ligands without certainty about which experiment would reveal a difference between our groups. We selected ligands that offered some probability of revealing a difference based on literature suggesting that *S. aureus* may interact with the host immune system through various pathways. The likelihood of observing a difference between our groups increased as we increased the number of ligands that we used. To take this into account and minimize false positives, we performed a one-way ANOVA Kruskall-Wallis with multiple comparison Dunn's post-test

analysis on the IL-8 data. The Mann-Whitney tests revealed statistical significance for some TLR experiments, but it proved to be marginal since the more conservative analysis with the one-way ANOVA nullified the findings. A caveat to the one-way ANOVA analysis, however, is that the chance for false negatives is increased. It is reasonable, therefore, to consider the non-parametric (Mann-Whitney) t-test comparisons as relative findings suggestive of a potential pattern for differences between our groups. If our sample size were larger, it is possible we would have detected more convincing statistical significance between our cohorts.

PBMCs from individuals who we identified as persistently colonized showed diminished secretion of IL-8 and TNF-α in response to innate immune stimulation. Our data do not identify a reason for this difference but do suggest some possible contributing factors. The statistically significant differences in the non-parametric t-test IL-8 analysis occurred with simulation by FSL-1, LTA, Agr (+) and Agr (-) strains of *S. aureus*. In addition, stimulation with these ligands approached statistical significance in regards to TNF-α secretion. These four ligands may represent an association with TLR-2. Lipoproteins are the primary ligands of TLR-2. FSL-1, *S. aureus* Agr (+) and *S. aureus* Agr (-) offer a source of lipoproteins. If TLR-2 were dysfunctional in cells from persistent carriers, it may explain the diminished cytokine response. LTA, a cell surface glycoconjugate of gram-positive bacteria is not a TLR-2 ligand, but it turns out that *S. aureus* LTA preparations contain contaminants, which serve as TLR-2 activators (54). Contaminations have resulted in several misleading conclusions about how TLR-2 functions (41). It is quite possible, therefore, that LTA itself did not contribute to the difference we observed between carriers and non-carriers; it could have been the

contaminants instead. Considering the notion that TLR-2 may be a factor in the differences in cytokine secretion, it is reassuring that there was less difference in IL-8 and TNF-α secretion between groups when cells were stimulated with MDP and LPS, known ligands of NOD2 and TLR-4, respectively.

One might expect that triacylated lipoprotein PAM3CSK would also act through TLR-2 and hence result in a significant diminution of IL-8 secretion by PBMCs from persistently colonized subjects. PAM3CSK is a synthetic lipoprotein presumed to act through the TLR-2/TLR-1 heterodimer. Recent studies have called into question the longstanding model that triacylated lipopeptides from gram-negative bacteria stimulate TLR2/TLR1 heteromers and that diacylated lipopeptides from gram-positive bacteria stimulate TLR2/TLR6 heteromers. Gram-positive *S. aureus* lipoproteins actually exist mainly in N-acylated triacyl forms rather than diacyl forms (47) and dimerization of TLR-2 is not as well understood as previously thought. Kurokawa et al. have shown that the *S. aureus* triacylated lipoprotein, SitC, is recognized by both TLR1/TLR2 and TLR2/TLR6 heteromers in mouse macrophages, but it can also induce production of IL-6 and TNF-α independently of TLR-1 and TLR-6. They also have shown that SitC acts in a TLR-2- and MyD88-dependent manner (41). It is possible, therefore, that PAM3CSK may act in a more complicated manner than previously thought. Muller et al has found that TLR-2 co-localizes intracellularly with SitC (55); thus TLR-2 may act both at the intracellular and surface levels. Intracellular TLR-2 complexes are reported to be recruited to macrophage phagosomes where they discriminate pathogens and induce pro-inflammatory signals for host defense (56). An increase in bacterial phagocytosis is associated with an enhanced cytokine response (57) (58). If PAM3CSK behaved

similarly to intracellular SitC, then it would not be affected by variations in function of surface TLR-2.

There are three well-described functional human TLR-2 genetic polymorphisms: Arg677Trp, Arg753Gln, and a microsatellite GT repeat in intron 2. Arg677Trp is not present in Caucasian patients (1). It has been correlated with lepromatous leprosy and the leprosy reversal reaction in Asian populations (59) while the Arg753Gln polymorphism has been correlated with sepsis in white populations (60). Arg753Gln also has been associated with mycobacterial infections, military tuberculosis, and pediatric urinary tract infections (UTIs) (61) (62). Children carrying the allele had a higher risk of UTI with gram positive pathogens, a higher risk of more than two previous UTIs, and a higher risk of asymptomatic UTIs (62).

When the Arg753Gln allele is inserted into human cells, it renders the cell non-responsive to triacylated or diacylated lipoproteins (60). From a clinical perspective, however, some studies have found no association between the Arg753Gln allele and the severity of *S. aureus* infection (63). This lack of association is consistent with in vitro evidence that the presence of only one wild-type TLR-2 allele is required for a full cytokine response to *S. aureus* (64). In counterpoint to those studies is a recent study examining atopy. In the recent study, over 80% of subjects with atopic dermatitis were colonized with *S. aureus*, and they had an increased prevalence of the Arg753Gln polymorphism compared to a control group, 11.5% vs. 2.5% respectively. Those with both atopic dermatitis and the polymorphism were found to have more severe atopic disease. Those with both bronchial asthma and the polymorphism were found to have increased levels of IgE. These data suggest that the TLR-2 polymorphism Arg753Gln

may increase the susceptibility to infections and chronic colonization (65). If we were to develop a hypothesis for further inquiry into our observed differences in cytokine production between persistent carriers and non-carriers, an assessment of TLR-2 polymorphisms would be a reasonable and interesting avenue of exploration.

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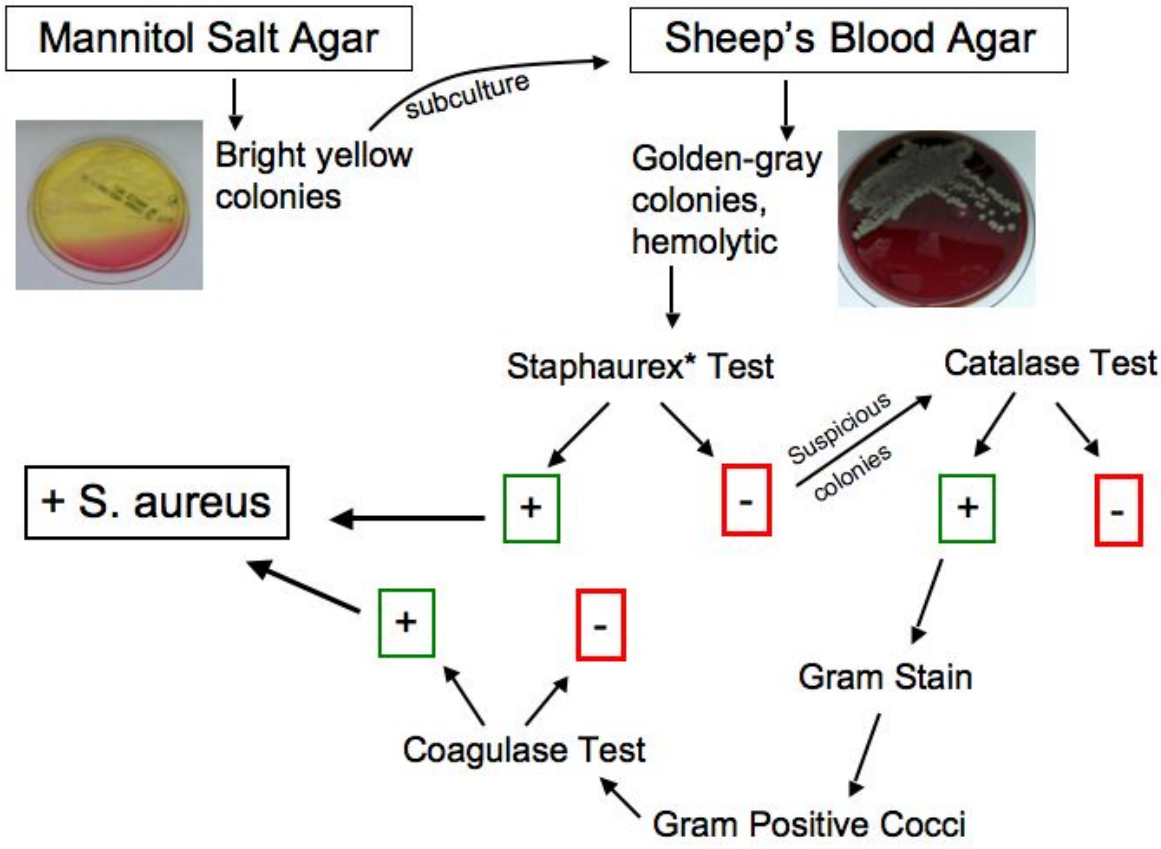
SUPPLEMENTAL FIGURE 1: **Assessing for presence of S. aureus from nasal swab culture**



SUPPLEMENTAL MATERIAL: Questionnaire administered to each subject at each encounter

I. DEMOGRAPHICS:

1. GENDER: Male Female

2. ETHNICITY: Hispanic or Latino Not Hispanic or Latino

3. RACE: American Indian/Alaska Native Asian White

Black or African American Native Hawaiian/Other Pacific Islander

4. DATE OF BIRTH (MM/DD/YYYY): ____/____/____

II. Health related risks for S. aureus colonization. Please circle Yes , No or NA (not applicable) for each question.

1. Do you have diabetes mellitus? Yes No

If you are diabetic, what has your AM fingerstick glucose been over the last week?

<110 110-180 180-250 >250 don't know NA

2. Do you have asthma? Yes No

3. Do you smoke tobacco? Yes No

4. Do you have seasonal allergies? Yes/Currently active Yes/not active No

5. Do you have active eczema or atopic dermatitis? Yes No

6. Do you use nasal steroids? Yes No

7. Did you have a cold or upper respiratory tract infection *in the last 10 days?* Yes No

8. Do you use systemic steroids, such as prednisone? Yes No Occasionally

9. If you take pulse-dose or intermittent steroids, when was your last dose? NA

Today This week Last 2-4 weeks 1-2 months ago 2-6 months >6mos

10. Have you taken antibiotics (pills or intravenous) *in the last 6 months*? Yes No

If yes, when was your most recent antibiotic dose? NA

Today This week Last 2-4 weeks 1-2 months ago 2-6 months

Do you remember the name of the most recent antibiotic? If yes, please list:_____

11. Have you used an antibiotic cream or ointment *in the last 2 months*? Yes No

When was your most recent use? NA

Today This week Last 2-4 weeks 1-2 months ago

12. Do you use medications other than prednisone that might suppress your immune system?

Yes No Not sure whether _____ counts

13. Have you used any sort of nasal spray *in the last month*? Yes No

14. Have you been hospitalized *within the last 6 months*? Yes No

If so, when: Last week Last month 2 months ago 2-6 months ago

15. Were you treated for a *S. aureus* infection *in the last 6 months*? Yes No Not sure

III. Exposure related risks for *S. aureus* colonization.

1. Do you have any children in day care? Yes No

2. When did you last use a public gym facility? NA, I never do this.

Today This week 2-4 weeks ago 1-2 months ago >2 months

3. Have you been to a hospital, nursing home, or rehab center *in the last 2 weeks*? Yes No

4. Do you interact with patients as part of your studies or job? Yes
No

If you interact with patients, please pick the description that best fits your exposure: NA

Talk, but minimal physical contact (bedside interview)

Talk, plus occasional physical contact with patient (assist with transfer, check vitals)

Talk plus repeated physical contact, i.e. patient physical exam or patient care

If you have examined patients or taken part in their care *in the last month*, please indicate in what health care setting(s)? NA

Outpatient clinic Emergency Room Inpatient Psychiatry Ward

Surgical Floor Non-surgical Inpatient Floor Intensive Care
Operating Room