Protection from the acquisition of *Staphylococcus aureus* nasal carriage by cross-reactive antibody to a pneumococcal dehydrogenase

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Nasal colonization by *Staphylococcus aureus* is the major risk factor for disease and transmission. Epidemiological studies have reported a reduced risk of *S. aureus* carriage in immunocompetent but not in immunocompromised children colonized by *Streptococcus pneumoniae*. We investigate the hypothesis that the immune response to pneumococcal colonization affects *S. aureus* colonization. We demonstrate that pneumococcal colonization in mice inhibits subsequent *S. aureus* acquisition in an antibody-dependent manner and elicits antibody that cross-reacts with *S. aureus*. We identify the staphylococcal target of cross-reactive antibody as 1-pyrroline-5-carboxylate dehydrogenase (P5CDH), and the homologous immunogen in *S. pneumoniae* as SP_1119, both of which are conserved dehydrogenases. These antigens are necessary and sufficient to inhibit the acquisition of *S. aureus* colonization in a mouse model. Our findings demonstrate that immune-mediated cross-reactivity between *S. pneumoniae* and *S. aureus* protects against *S. aureus* nasal acquisition and thus reveal a paradigm for identifying protective antigens against *S. aureus*.

pneumococcus | methicillin-resistant *S. aureus* | vaccine

The Gram-positive bacterial pathogen *Staphylococcus aureus* is responsible for significant morbidity, mortality, and excess healthcare costs worldwide. The management of *S. aureus* disease has become increasingly difficult because of the rising prevalence of methicillin-resistant *S. aureus* (MRSA), which can account for 60% of *S. aureus* infections in hospital and community settings (1, 2). Given the limited treatment options for MRSA infection, novel preventative approaches are needed to protect against *S. aureus* infection and transmission.

A predominant risk factor for *S. aureus* infection and transmission is asymptomatic colonization of the anterior nares (3). Eighty percent of *S. aureus* invasive infections in humans are caused by the host's colonizing strain (4). However, the specific host and bacterial determinants of *S. aureus* nasal carriage are not well understood (5). In children, significantly reduced *S. aureus* colonization rates have been associated with carriage of another member of the upper respiratory tract flora, *Streptococcus pneumoniae* (6–14). These large and geographically diverse cohorts have demonstrated reproducibly that colonization with *S. pneumoniae* reduces the risk of *S. aureus* carriage by approximately half. This interference phenomenon has been reported for both vaccine and nonvaccine serotypes of *S. pneumoniae* (13). Moreover, pneumococcal vaccination, which reduces *S. pneumoniae* carriage, has been associated with an increased incidence of *S. aureus*-induced otitis media in children (15).

The etiology of this interference phenomenon between *S. pneumoniae* and *S. aureus* colonization is unknown. Although in vitro studies have demonstrated that hydrogen peroxide secreted by *S. pneumoniae* is bactericidal to *S. aureus* in coculture (16–18), neither hydrogen peroxide secretion by *S. pneumoniae* nor hydrogen peroxide sensitivity of *S. aureus* is predictive of cocolonization patterns in vivo (19–21). Moreover, any direct competitive effect in vivo is unlikely, because *S. aureus* is found primarily in the anterior nares (5), whereas *S. pneumoniae* colonizes the nasopharynx (22). Instead, we and others (21) have hypothesized that an immunological mechanism may be involved, because the antagonistic effect of pneumococcal colonization on *S. aureus* carriage is observed in HIV-negative but not immunocompromised HIV-positive individuals within the same cohort (8, 9, 23). To date, the only study that has addressed the role of the immune system measured antibody titers to 17 predetermined pneumococcal proteins and found no correlation with *S. aureus* carriage in 57 infants (24). Therefore, a comprehensive examination of this hypothesis without preselection of candidate antigens has not yet been performed.

Here we investigate whether the host immune response to *S. pneumoniae* carriage can influence *S. aureus* colonization in vivo. We demonstrate that antibodies elicited during pneumococcal colonization in a mouse model cross-react with *S. aureus,* leading to a reduction in *S. aureus* nasal colonization. We identify the staphylococcal target of cross-reactive antibody and the homologous immunogen in *S. pneumoniae* and confirm that these antigens are necessary and sufficient to limit the acquisition of *S. aureus* nasal colonization in vivo.

Results

Pneumococcal Colonization in Mice Reduces Subsequent *S. aureus* Carriage in an Antibody-Dependent Manner. To recapitulate the observed interference between *S. pneumoniae* and *S. aureus* colonization, we developed a mouse model of *S. aureus* nasal colonization using strain 502A, a clinical isolate known for superior nasal colonization in humans (25). Unlike previously described models of *S. aureus* nasal carriage, which are highly variable, nasal colonization by 502A is established reproducibly in naive C57BL/6 mice with higher and less variable densities than seen with other strains (Fig. S1 *A* and *B*). 502A colonization was achieved with a dose of 10^5 cfu, but levels were highest and most reproducible at day 1 postinoculation with a dose of 10^8 cfu (Fig. S1 *C* and *D*). For all further experiments, we chose to use these latter conditions, which reproducibly model the first step in colonization—nasal acquisition—but do not model the long-term human carrier state. Therefore, our studies with this model focus on the initial establishment of *S. aureus* nasal colonization rather than on persistent carriage. Under these conditions, the levels of *S. aureus* detected in our model are comparable to those recovered from experimentally colonized humans (26).

We next combined our 502A acquisition model with an established murine model of pneumococcal nasopharyngeal colonization that has colonization dynamics and immune responses similar to those observed in humans, including a robust antibody response to

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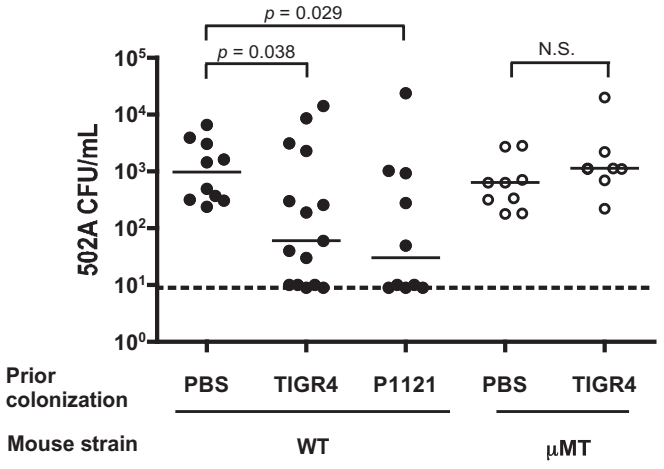

Fig. 1. Pneumococcal colonization in mice reduces subsequent *S. aureus* carriage in an antibody-dependent manner. Colonization density of *S. aureus* 502A in C57BL/6 wild-type (closed circles) or antibody-deficient μMT mice (open circles) 5 wk after prior colonization with *S. pneumoniae* TIGR4, *S. pneumoniae* P1121, or sham (PBS) colonization. 502A carriage levels were assessed in lavages of the upper respiratory tract at day 1 postchallenge. Horizontal solid lines indicate median values; dotted line indicates limit of detection, N.S., not significant.

a diversity of pneumococcal antigens (27, 28). After colonizing mice with *S. pneumoniae* and allowing 5 wk for complete pneumococcal clearance, we challenged mice intranasally with *S. aureus* 502A. Compared with mock-colonized (PBS) controls, mice previously colonized with *S. pneumoniae* TIGR4 had significantly reduced levels of *S. aureus* 502A carriage (Fig. 1, closed circles), similar to observations made in children. The protective effect of prior pneumococcal colonization was not dependent on pneumococcal strain or serotype, because similar reductions in 502A colonization were seen following prior colonization with *S. pneumoniae* P1121 (Fig. 1, closed circles).

Because the effect of prior pneumococcal colonization was observed at a time when pneumococci no longer can be detected in the nasopharynx, we hypothesized that the reduction in *S. aureus* levels might be caused by the presence of anti-pneumococcal antibody. To test this hypothesis, we repeated the dual-species colonization experiment in antibody-deficient μMT mice and found no significant difference in 502A colonization levels between mice colonized previously with *S. pneumoniae* and mock colonized controls (Fig. 1, open circles). These data suggest that antibody is necessary for the protective effect of pneumococcal colonization on *S. aureus* colonization.

Pneumococcal Colonization Elicits Antibody That Cross-Reacts with *S. aureus*. We next investigated whether the antibody response elicited by pneumococcal colonization was capable of recognizing *S. aureus*. Mice colonized with *S. pneumoniae* developed significantly increased levels of IgG that bound to the surface of live *S. aureus*, compared with levels in precolonization sera ($P = 0.043$ for five mice) (Fig. 2*A*). In Western blots of staphylococcal whole-cell lysates, sera from mice postcolonization with *S. pneumoniae* cross-reacted with a single prominent band of about 55 kD (Fig. 2*B*, *Right* two panels, arrow). In contrast, blots using sera after mock colonization with PBS resembled background levels of sera before colonization (Fig. 2*B*, *Left* two panels). After further separation by 2D gel electrophoresis and Western blot, the staphylococcal target of antibody induced by pneumococcal colonization was isolated for mass spectrometric analysis. Only two proteins, dihydrolipoamide dehydrogenase (DLDH, YP_499592) and 1-pyrroline-5-carboxylate dehydrogenase (P5CDH, YP_501325), were present at this position in equal abundance as determined by empirical protein-abundance index scores. For each staphylococcal protein, one closely homologous protein was identified in the *S. pneumoniae* TIGR4 genome by tBLASTn. The homologous loci in the TIGR4 genome are *sp_1161* (E value = $1e^{-56}$) and *sp_1119* (E value = $6e^{-68}$), respectively, and both encode putative but uncharacterized dehydrogenases which we refer to hereafter as "SP_1161" and "SP_1119."

Candidate Antigen Is SP_1119 in *S. pneumoniae* and Its *S. aureus* Homolog, P5CDH. Each candidate antigen was cloned, recombinantly expressed, purified, and used to generate specific antisera. IgG to P5CDH and DLDH bound to the surface of live *S. aureus*, indicating that these proteins are antibody accessible (Fig. 3*A*). In contrast, incubation of *S. pneumoniae* TIGR4 with antisera specific to the pneumococcal proteins did not result in surface IgG binding (Fig. 3*B*). However, elimination of the antiopsonic capsular polysaccharide in TIGR4*cps* facilitated surface binding by anti-SP_1119 and anti-SP_1161 IgG, suggesting that these antigens are surface associated but masked by capsule (Fig. 3*C*).

We investigated whether antibodies raised against each candidate protein could cross-react with the heterologous species. When *S. aureus* was incubated with antisera to the pneumococcal proteins, we observed cross-reactive binding with anti-SP_1119 but not with anti-SP_1161 IgG (Fig. 3*A*). Similarly, antisera to the staphylococcal homolog of SP_1119, P5CDH, bound to the surface of unencapsulated *S. pneumoniae*, but antisera to DLDH did not (Fig. 3*C*). Together, these data suggest that antisera to the homologous pair P5CDH and SP_1119, but not to DLDH and SP_1161, cross-react with the surface of the heterologous species.

For SP_1119 to induce cross-reactive antibody in vivo, it must be immunogenic during pneumococcal colonization. We investigated whether pneumococcal colonization in mice elicited antibodies to SP_1119 by Western blot and ELISA. By Western blot we observed an increase in antibody binding to both SP_1119 and P5CDH in sera of mice after pneumococcal colonization as compared with sera from mice before pneumococcal colonization (Fig. 4*A*). No increase in binding was observed in mock-colonized animals (Fig. 4*A*). Similarly, by ELISA, mice colonized with TIGR4 had significantly elevated IgG titers to SP_1119, whereas mock-colonized control mice did not (Fig. 4*B*). There was a significant intraindividual correlation between elevated IgG tiers to SP_1119 and P5CDH, indicating that animals with a robust response to SP_1119 mounted commensurate responses to P5CDH (Fig. 4*C*, solid squares).

Because the clinical negative association between pneumococcal and *S. aureus* colonization appears to be independent of *S. aureus* strain, we reasoned that any target of cross-reactive antibody must be well conserved. In all publically available whole *S. aureus* genomes

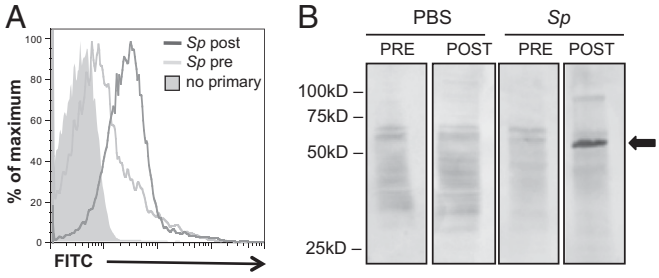

Fig. 2. Pneumococcal colonization elicits serum antibody that cross-reacts with *S. aureus*. Sera were collected from C57BL/6 mice before (pre) and after (post) intranasal colonization with *S. pneumoniae* TIGR4. (*A*) Representative histogram of flow cytometric detection of antibody binding to *S. aureus* 8325–4*spa* incubated with mouse sera taken before (light gray line) and after (dark gray line) pneumococcal colonization. Control (no primary antibody) is shaded gray. (*B*) Western blot of *S. aureus* 8325–4*spa* lysates incubated with mouse sera taken before (PRE) and after (POST) pneumococcal (*Sp*) or sham (PBS) colonization. Data are representative of >10 biological replicates. Arrow indicates candidate target of cross-reactive antibody.

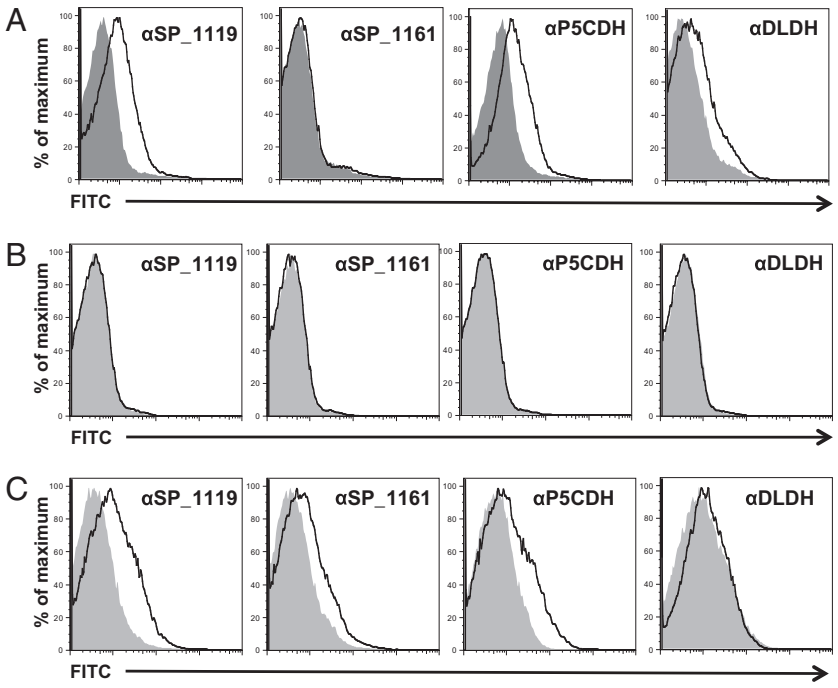

Fig. 3. Antisera to P5CDH and SP_1119, but not to DLDH and SP_1161, cross-react with the heterologous species. Flow cytometric detection of IgG binding to the surface of *S. aureus* 8325–4*spa* (*A*), *S. pneumoniae* TIGR4 (*B*), and *S. pneumoniae* TIGR4*cps* (*C*) after incubation with rabbit antisera raised to purified recombinant P5CDH, DLDH, SP_1119, or SP_1161 as indicated. Gray shaded area, preimmune rabbit sera; black line, immune rabbit sera.

($n > 12$), the amino acid sequence for P5CDH is at least 98% identical. We confirmed this widespread conservation by Western blot using a selection of methicillin-sensitive and methicillin-resistant *S. aureus* strains including the epidemic clinical isolate USA300. P5CDH was detected in all the strains tested but not in the unmarked, in-frame P5CDH deletion mutant (502A*rocA*), which was used as a negative control (Fig. 4*D*). Similarly, SP_1119 is broadly conserved across pneumococci with at least 99% amino acid identity in all the publically available whole *S. pneumoniae* genomes ($n > 35$).

SP_1119 and P5CDH Are Necessary to Reduce *S. aureus* Carriage in a Mouse Model. We deleted the locus *sp_1119* from *S. pneumoniae* TIGR4 to assess whether SP_1119 is necessary for the protective effect of pneumococcal colonization on subsequent *S. aureus* carriage. Although mice previously colonized with wild-type TIGR4 had significantly reduced levels of 502A carriage, mice previously colonized with TIGR4*sp_1119* did not differ from mock (PBS)-colonized controls in 502A colonization density (Fig. 5*A*). Colonization with both the wild-type and mutant resulted in significant increases in antibody titers to whole pneumococci compared with PBS controls (Fig. S2), indicating that the lack of protection against 502A was not caused by an overall deficiency in the antibody response to the mutant. We confirmed by ELISA that animals colonized with TIGR4*sp_1119* did not mount antibodies to SP_1119, and animals colonized with wild-type TIGR4 had significantly higher anti-SP_1119 titers than those seen in PBS-inoculated controls (Fig. 5*B*). Following colonization with TIGR4*sp_1119*, cross-reactive titers to P5CDH were not significantly higher than those in PBS-inoculated controls and no longer correlated with intraindividual titers to SP_1119 (Fig. 4*C*, open symbols). The requirement of SP_1119 for cross-reactivity was supported by flow cytometry using a TIGR4*sp_1119cps* double mutant, demonstrating that deletion of SP_1119 abrogates binding by P5CDH antisera (Fig. 5*C*). Similarly, the protective effect of previous colonization with wild-type *S. pneumoniae* TIGR4 was lost when animals were challenged with 502A*rocA*, which lacks P5CDH (Fig. 5*D*). These results

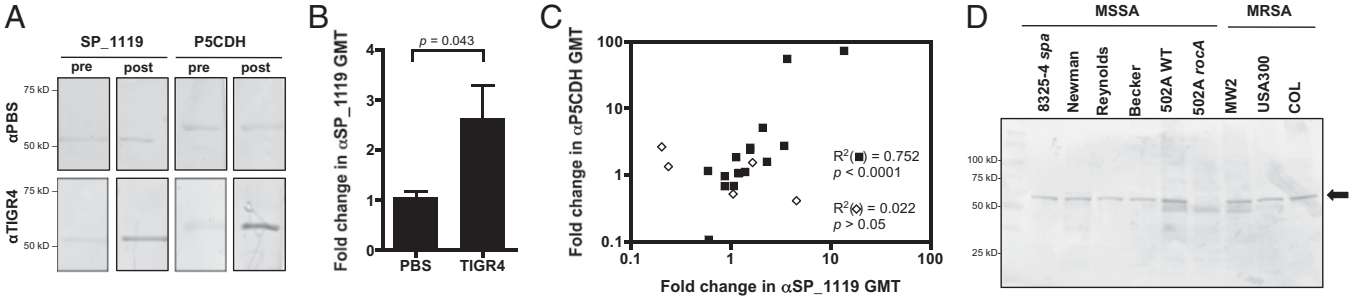

Fig. 4. SP_1119 is immunogenic during *S. pneumoniae* colonization. (*A*) Western blot of purified recombinant SP_1119 or P5CDH incubated with mouse sera before (pre) and after (after) pneumococcal (TIGR4) or sham (PBS) colonization. (*B*) Quantification by ELISA of the increase in serum IgG titers to SP_1119 after pneumococcal (TIGR4) or sham (PBS) colonization. $n = 10$ mice per group. (*C*) Correlation between fold increase in serum IgG titers to SP_1119 and P5CDH in mice colonized with *S. pneumoniae* TIGR4 (closed squares) or *S. pneumoniae* TIGR4*sp_1119* (open diamonds). (*D*) Detection of P5CDH (arrow) by specific anti-P5CDH sera in a Western blot of whole-cell lysates of the *S. aureus* strains indicated. MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*.

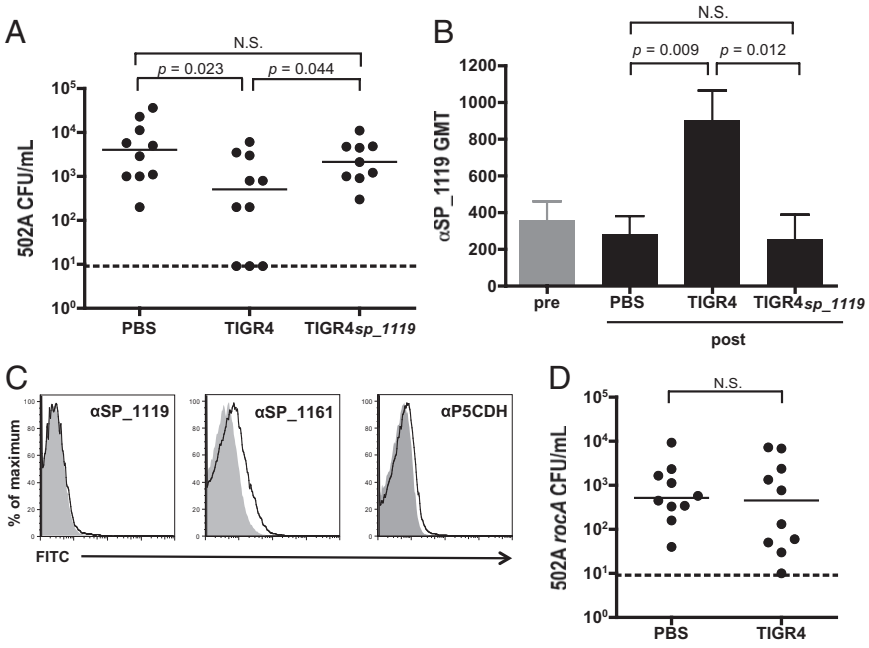

Fig. 5. Deletion of SP_1119 or P5CDH from whole bacteria abrogates protective effect of prior pneumococcal colonization on *S. aureus* 502A carriage. (*A*) Colonization density of *S. aureus* 502A in C57BL/6 wild-type mice 5 wk after prior colonization with *S. pneumoniae* TIGR4, *S. pneumoniae* TIGR4*sp_1119* or sham treatment (PBS). 502A carriage levels were assessed in upper respiratory tract lavages at day 1 postchallenge. Horizontal lines indicate median values. N. S., not significant. (*B*) Detection of SP_1119-specific IgG titers in mouse sera before (pre, gray bar) and after (post, black bars) colonization with *S. pneumoniae* TIGR4, *S. pneumoniae* TIGR4*sp_1119* or sham treatment (PBS). (*C*) Flow cytometric detection of antibody binding to the surface of *S. pneumoniae* TIGR4*sp_1119cps* following incubation with rabbit antisera specific to SP_1119, SP_1161, and P5CDH as indicated. Gray shaded area, preimmune rabbit sera; black line, immune rabbit sera. (*D*) Colonization density of *S. aureus* 502A*rocA* in C57BL/6 mice 5 wk after prior colonization with *S. pneumoniae* TIGR4 or sham (PBS) inoculation. 502A*rocA* carriage levels were assessed in lavages of the upper respiratory tract at day 1 postchallenge. Horizontal solid lines indicate median values; dotted line indicates limit of detection. N.S., not significant.

provide evidence that cross-protection against *S. aureus* by *S. pneumoniae* requires SP_1119 as an immunogen and P5CDH as a target.

Intranasal Immunization with SP_1119 or P5CDH Is Sufficient to Reduce *S. aureus* Colonization Levels. Because SP_1119 and P5CDH were necessary for the protective effect of pneumococcal colonization on the acquisition of *S. aureus* carriage, we investigated whether immunization with these antigens alone was sufficient to recapitulate this effect. Mice were immunized intranasally with either adjuvant alone or in combination with purified recombinant SP_1119, P5CDH, or DLDH as a control protein. Mice immunized with SP_1119 had significantly lower levels of 502A colonization than those seen in controls administered adjuvant alone (Fig. 6*A*). Immunization with P5CDH resulted in a similar reduction in 502A colonization, but immunization with the control protein DLDH did not (Fig. 6*A*). As predicted, P5CDH or SP_1119 had no protective effect after challenge with 502A*rocA*, which lacks P5CDH (Fig. 6*B*). Complementation of the *rocA* deletion (using strain 502A*rocA*::pCL55-rocA$^+$) restored expression of P5CDH (Fig. S3) and the protective effect of prior immunization with SP_1119 and P5CDH (Fig. 6*C*). Together, these data suggest that SP_1119 and P5CDH are necessary for the pneumococcal effect on *S. aureus* nasal carriage and are sufficient as mucosal immunogens to inhibit the acquisition of *S. aureus* 502A nasal carriage.

Discussion

The concept of interspecies immune-mediated cross-reactivity is as old as vaccinology itself. Indeed, the first vaccine was based on Jenner's observation of immune-mediated cross-reactivity between cowpox and smallpox. This seminal discovery was made by first identifying a naturally protected subset of the population. In that vein, we sought to investigate a subset of the population—healthy children colonized with *S. pneumoniae*—that was observed to be at reduced risk for *S. aureus* nasal carriage. This interspecies interference is one of the few epidemiological examples of protection against *S. aureus* acquisition, especially because exposure to *S. aureus* is not protective against future *S. aureus* carriage or infection in humans. Interspecies cross-reactive antibody is an important factor in natural immunity to other bacterial pathogens of the upper respiratory tract. For example, cross-reactivity between the capsular polysaccharides of certain enteric commensal *Escherichia coli* and *Haemophilus influenzae* type b has been implicated in the development of age-related natural immunity against this pathogen (29). Our study establishes that antibodies elicited in response to a specific protein during pneumococcal colonization cross-react with and inhibit *S. aureus* in vivo and thereby demonstrates the use of interspecies cross-reactivity to identify protective antigens.

Our findings implicate the antibody response to a homologous pair of putative dehydrogenases, P5CDH and SP_1119, in mediating cross-protection against *S. aureus*. SP_1119 elicits antibody to which the pneumococcus is resistant, whereas P5CDH may have limited immunogenicity during *S. aureus* colonization but still can be targeted by preexisting cross-reactive antibody. In humans, experimental colonization with *S. aureus* does not elicit antibody to P5CDH (30), although some antibody can be detected after invasive infection (31), indicating P5CDH is expressed in vivo. In addition to the identification of SP_1119 by *in silico* analysis, three lines of experimental evidence support the specific role of SP_1119 in inducing cross-protection: (*i*) antisera raised to SP_1119 cross-reacts with the surface of *S. aureus* in vitro; (*ii*) loss of SP_1119 in *S. pneumoniae* abolishes the protective effect of prior pneumococcal carriage on *S. aureus* colonization; and (*iii*) immunization with purified SP_1119 inhibits the establishment of *S. aureus* nasal carriage. The fact that SP_1119, like other protein antigens of *S. pneumoniae*, can be hidden from antibody by the antiopsonic

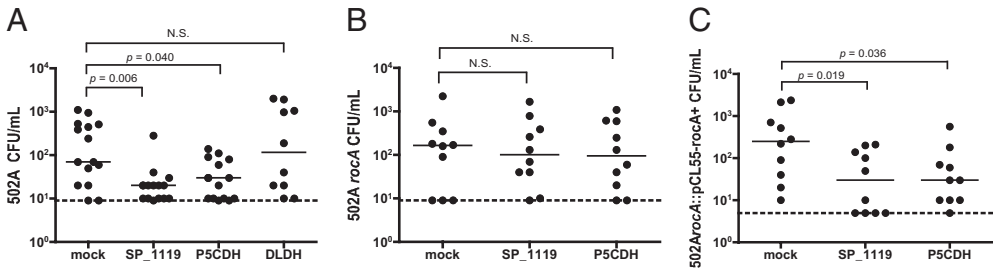

Fig. 6. Reduction in density of *S. aureus* 502A colonization following intranasal immunization with purified SP_1119 and P5CDH. Colonization density of *S. aureus* 502A in C57BL/6 mice following intranasal immunization with cholera toxin alone (mock) or in combination with recombinant antigen, SP_1119, P5CDH, or DLDH. Carriage levels of 502A (*A*), 502A*rocA* (*B*), or 502A*rocA*::pCL55-rocA^{+} (*C*) were assessed in upper respiratory tract lavages at day 1 post-challenge. N.S., not significant.

capsular polysaccharide may explain the directional negative effect of pneumococcal colonization on *S. aureus* colonization and not vice versa. Preliminary data suggest that SP_1119 is immunogenic during childhood colonization with *S. pneumoniae*, and future studies will address whether these elevated antibody titers in childhood correlate with a reduced risk of nasal carriage of *S. aureus*.

SP_1119 shares extensive overall homology with P5CDH as well as a functional classification in the aldehyde dehydrogenase superfamily (32). Both proteins are highly conserved and can be detected on the bacterial surface, adding to the growing list of anchorless surface-exposed enzymes in Gram-positive bacteria (33). We predict that cross-reactivity between these two proteins is mediated by a region(s) of conformational similarity on a surface-exposed domain(s), given the lack of an identical stretch of amino acids indicative of a common linear epitope (Fig. S4). Further investigation will be needed to define the precise region(s) responsible for inducing cross-reactivity. It would be beneficial for future studies to identify the minimal epitope(s) required for protection to minimize any undesired impact on other members of the flora or cross-reactivity with human proteins. The biological function of the proteins SP_1119 and P5CDH has not been characterized in the context of *S. pneumoniae* or *S. aureus,* respectively, and our data indicate that these proteins are not essential during in vitro growth or murine colonization. Whether these proteins affect fitness during human nasal carriage remains to be tested. However, there appears to be selective pressure for these proteins to be maintained in vivo, given their extensive conservation among genome-sequenced strains. This conservation could account for the strain-independent interference between these two species observed in children (20).

Our study required a small animal model of *S. pneumoniae* and *S. aureus* nasal colonization to evaluate our hypothesis in vivo. However, models of *S. aureus* carriage have been limited by a lack of *S. aureus* strains capable of establishing reproducible colonization. *S. aureus* 502A was used throughout the 1960s to colonize adults with furunculosis and healthy newborns deliberately to prevent acquisition of other, more virulent *S. aureus* strains during nosocomial outbreaks (25). We reasoned that 502A might be more proficient than other *S. aureus* strains at establishing colonization in mice, as appeared to be the case in humans. Indeed, the reproducibility of *S. aureus* 502A nasal acquisition in mice at day 1 postinoculation enabled the current study of *S. aureus* colonization and may be a useful tool for studying other host and bacterial determinants of the acquisition of *S. aureus* nasal carriage. Because the protective effects of our antigens were observed during the establishment of carriage, we did not test them in other animal models where disease is created artificially by circumventing the carrier state.

For many bacterial pathogens of the upper respiratory tract, antibody functions to prevent the natural acquisition of carriage (34). In humans, pneumococcal conjugate vaccine is known to induce antigen-specific serum IgG, which is transported by transcytosis across epithelial barriers where it can be detected on the mucosa and is correlated with protection from the acquisition of colonization (35). However, the role of antibody in protection against *S. aureus* has been questioned, because *S. aureus* expresses protein A (Spa) which binds Ig nonspecifically. A Spa mutant often is used in vitro, especially whenever secondary antibody-detection methods are used. It has been assumed that the effect(s) of antibody in vivo would be negated similarly by Spa, but antibody-mediated protection has been demonstrated against nasal colonization with Spa-sufficient strains (31, 36). Passive i.p. immunization with a monoclonal antibody against clumping factor B resulted in reduced nasal carriage of *S. aureus* in mice (36), indicating that systemic antibody can protect against *S. aureus* colonization regardless of Spa. Our study provides another example of antibody-dependent inhibition of nasal carriage of a Spa-sufficient strain, suggesting that the immune-evasive effect ascribed to Spa may be of limited importance during colonization.

Much of the public health benefit of vaccines that target mucosal pathogens of the upper respiratory tract—including *S. pneumoniae*, *Neisseria meningitidis*, and *H. influenzae* type b—is the result of herd protection based on the inhibition of carriage in children and thus reduced transmission to unvaccinated members of the population (37). Clinical studies have demonstrated repeatedly that even modest (e.g., 50%) reductions in pathogen carriage following vaccination significantly reduce the risk of transmission, so that full protection (≥90%) from invasive disease is afforded to both vaccinated and unvaccinated individuals (37). Indeed, it has been calculated that pneumococcal conjugate vaccine prevented many more cases of invasive pneumococcal disease in unvaccinated individuals than in vaccinated children (37). These findings illustrate how nonsterilizing decreases in pathogen colonization can have vast ramifications on disease incidence and population-wide protection. In our mouse model, we observed a relative reduction in *S. aureus* carriage and hypothesize that, if similar reductions in carriage were observed in humans, significant morbidity and mortality caused by *S. aureus* invasive disease could be prevented by herd immunity. Moreover, the success of our current pediatric conjugate vaccines reveals the importance of childhood colonization as a reservoir for bacterial pathogens within the population and thus the importance of designing immunizations that inhibit carriage in children. We posit that a successful vaccine against *S. aureus* may benefit from the inclusion of antigens directed at reducing the acquisition of nasal carriage, such as SP_1119 and P5CDH. Future studies will be needed to address whether these antigens can protect against *S. aureus* in humans.

Materials and Methods

Bacterial Strains and Mutants. *S. pneumoniae* was grown in tryptic soy (TS) broth at 37 °C in a nonshaking water bath. TIGR4 (a serotype 4 clinical isolate and genome-sequenced strain) and P1121 (a serotype 23F clinical isolate) were used because they colonize the murine nasopharynx efficiently (28). A TIGR4 mutant lacking *sp_1119* was constructed using overlap extension PCR (see *SI Materials and Methods* for details). *S. aureus* was grown in TS or brain-heart infusion broth at 37 °C with shaking (strains and sources are identified in *SI Materials and Methods*). An unmarked, in-frame deletion mutant of *rocA*, which encodes P5CDH, was constructed in strain 502A using

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pKOR1-*rocA* and was complemented using pCL55 (see *SI Materials and Methods* for details).

Mouse Model of Nasopharyngeal Colonization and Challenge. The murine model of pneumococcal nasopharyngeal colonization has been described previously (28) and is described in full in *SI Materials and Methods*. Mice received an intranasal dose of 10^7 cfu of *S. pneumoniae* at weeks 0 and 2 and were challenged at week 7, at which time no pneumococci remained in the nasopharynx (28). Control animals were subjected to the same protocol but were mock-colonized with PBS. Intranasal challenge of *S. aureus* consisted of 10^8 cfu. Colonization densities from nasal lavages were quantified on BBL CHROMagar Staph aureus (BD Diagnostics) 24 h postchallenge.

Identification of Candidate Antigens. The targets of cross-reactive pneumococcal antibody were identified by Western blot analysis and mass spectrometry. See *SI Materials and Methods* for further details.

Measurement of Serum Antibody Binding. Binding of total serum IgG to whole bacteria was detected by flow cytometry using a FITC-conjugated anti-mouse IgG secondary antibody. Antigen-specific serum IgG titers were quantified by ELISA. Both methods are detailed in *SI Materials and Methods*.

Recombinant Antigen Purification and Generation of Specific Antisera. The coding sequences for each of the four candidate antigens were amplified from the appropriate chromosomal DNA using primers listed in *SI Materials and Methods*. Amplicons were ligated into pET29b (Novagen) for expression of recombinant antigens in *E. coli* BL21(DE3) and purification under native conditions. As appropriate, his-tags were removed by a thrombin cleavage capture system (Novagen) and dialysis. Polyclonal rabbit sera to each purified recombinant antigen were prepared commercially.

Immunization with Purified Antigens. As previously described (38), mice were immunized intranasally with 4 μg of recombinant protein and 1 μg cholera toxin as adjuvant (List Biological Laboratories) per 20-μL dose. Control mice received adjuvant alone. Three immunizations were given at weekly intervals, followed by intranasal *S. aureus* challenge at week 5, as described above.

Statistical Analysis. Colonization density was expressed as the $\log_{10}$ cfu/mL and analyzed for statistical significance using the Mann–Whitney U test. Paired t tests were used to compare pre- vs. posttreatment groups, and linear regressions were used to assess correlations. All other comparisons were made using the unpaired t test, as appropriate. A P value of less than 0.05 was considered significant. Statistical analyses were performed using Prism 4 (GraphPad).

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