Successful infection by mucosal pathogens requires overcoming the mucus barrier. To better understand this key step, we performed a survey of the interactions between human respiratory mucus and the human pathogen *Streptococcus pneumoniae*. Pneumococcal adherence to adult human nasal fluid was seen only by isolates expressing pilus-1. Robust binding was independent of pilus-1 adhesive properties but required Fab-dependent recognition of RrgB, the pilus shaft protein, by naturally acquired secretory IgA (sIgA). Pilus-1 binding by specific sIgA led to bacterial agglutination, but adherence required interaction of agglutinated pneumococci and entrapment in mucus particles. To test the effect of these interactions in vivo, pneumococci were preincubated with human sIgA before intranasal challenge in a mouse model of colonization. sIgA treatment resulted in rapid immune exclusion of pilus-expressing pneumococci. Our findings predict that immune exclusion would select for nonpiliated isolates in individuals who acquired RrgB-specific sIgA from prior episodes of colonization with piliated strains. Accordingly, genomic data comparing isolates carried by mothers and their children showed that mothers are less likely to be colonized with pilus-expressing strains. Our study provides a specific example of immune exclusion involving naturally acquired antibody in the human host, a major factor driving pneumococcal adaptation.
Immune exclusion by naturally acquired secretory IgA against pneumococcal pilus-1

Ulrike Binsker, John A. Lees, Alexandria J. Hammond, and Jeffrey N. Weiser

Department of Microbiology, New York University School of Medicine, New York, New York, USA.

Successful infection by mucosal pathogens requires overcoming the mucus barrier. To better understand this key step, we performed a survey of the interactions between human respiratory mucus and the human pathogen Streptococcus pneumoniae. Pneumococcal adherence to adult human nasal fluid was seen only by isolates expressing pilus-1. Robust binding was independent of pilus-1 adhesive properties but required Fab-dependent recognition of RrgB, the pilus shaft protein, by naturally acquired secretory IgA (sIgA). Pilus-1 binding by specific sIgA led to bacterial agglutination, but adherence required interaction of agglutinated pneumococci and entrapment in mucus particles. To test the effect of these interactions in vivo, pneumococci were preincubated with human sIgA before intranasal challenge in a mouse model of colonization. sIgA treatment resulted in rapid immune exclusion of pilus-expressing pneumococci. Our findings predict that immune exclusion would select for nonpiliated isolates in individuals who acquired RrgB-specific sIgA from prior episodes of colonization with piliated strains. Accordingly, genomic data comparing isolates carried by mothers and their children showed that mothers are less likely to be colonized with pilus-expressing strains. Our study provides a specific example of immune exclusion involving naturally acquired antibody in the human host, a major factor driving pneumococcal adaptation.

Introduction
Airway mucus plays a critical role in host defense by providing a physicochemical barrier that protects the underlying respiratory epithelium from inhaled particulate matter, including infectious agents (1, 2). The mucus forms a semipermeable network, which allows the transport of water, nutrients, and gases, and facilitates mucosal defense involving innate and adaptive immunity (3). Moreover, the adhesive properties of mucus trap bacteria as well as other particles, promoting their mechanical clearance through mucociliary movements (1). The mucus is divided into 2 layers: an inner periciliary layer, which is relatively impenetrable to microbes, and an outer loose layer providing a niche for commensal bacteria that is continuously removed by ciliary activity (4). The nasal mucus is composed of 95% water, high-molecular weight glycoproteins (so called mucins, which confer the viscous properties of the mucus), lipids, proteins, and inorganic salts (5). Nearly all of the proteaceous components present in the mucus harbor antibacterial activity (6–8). Given the importance of the mucus layer in maintaining airway homeostasis, there is little mechanistic understanding of bacterial-mucus interactions.

Streptococcus pneumoniae (Spn, the pneumococcus) is an opportunistic pathogen of the human upper respiratory tract with colonization rates of 25%–65% in children and 5%–10% in the adult population (9, 10). Carriage is usually asymptomatic; however, under certain circumstances the pneumococcus gains access to normally sterile sites, leading to invasive infections such as otitis media, pneumonia, sepsis, and meningitis (11). Successful colonization requires penetration through the mucus barrier protecting the respiratory epithelium. Spn has evolved several strategies to overcome the mucus layer and to evade mucociliary clearance. Most strains are surrounded by a thick capsule composed of negatively charged polysaccharide that repels anionic mucins and other mucus glycoproteins (12). The amount of capsule and its serotype affect binding to mucus, which is inversely correlated with persistence during early colonization. Capsule-dependent release from mucus entrapment also allows for bacterial shedding and host-to-host transmission following contact with nasalsecretions (13). Furthermore, Spn expresses multiple exo- and endoglycosidases able to degrade O- and N-linked glycans of mucosalproteins (14–16). Mucus components, including lactoferrin, secretorycomponent, secretory immunoglobulin A (sIgA), and mucins, have been shown to be substrates of Spn glycosidases (14, 17, 18). Potential changes in the integrity and protective function of mucus by Spn glycosidases might contribute to the movement ofthe bacterium through the mucus layer. Additionally, cleaved carbohydrates serve as a carbon source in the normally nutrient-poor environment of the nasopharynx (19). Spn also alters the mucus composition via its major toxin pneumolysin, which triggers the upregulation of Muc5AC, a prominent secretory mucin in the airways (20). This excessive mucus production could overwhelm theeffectiveness of mucociliary flow and increase nasal discharge, allowing for pneumococcal transmission (21).

Herein, we evaluated the interactions of Spn with respiratory mucus. We identified bacterial components and mucus factors involved in binding of Spn and impacting colonization. Since Spn is a human-specific organism, we focused on its interaction with human nasal secretions. We found that the pneumococcal pilus-1 is the major determinant of Spn binding to human mucus. Fur-
Results

**Pneumococci interact with human nasal mucus via mucosal proteins.** Colonizing Spn are found predominantly within the glyocalyx, the mucus layer overlaying the epithelial surface (12). We established an in vitro assay to study Spn interactions with human mucus, considering both attachment and detachment. The association of encapsulated Spn (isolate TIGR4) with immobilized pooled human nasal fluid (hNF) collected from healthy adults was quantified using a solid-phase assay with BSA as blocking reagent. Spn adhered to hNF more readily compared with bovine submaxillary mucus (BM) (Figure 1A). Adherence to either source of mucus was higher in the presence of 0.1% BSA for 2 hours at 30°C. Bound bacteria were determined by resuspension with 0.001% Triton X-100 following plating on TS agar plates supplemented with 200 μg/mL streptomycin.

We provide a demonstration of host defense mediated by mucosal antigen-specific sIgA (referred to as immune exclusion) (22, 23). We show that naturally acquired sIgA mediates pilus-dependent agglutination, facilitating binding to mucus, and that this interaction inhibits the establishment of colonization in a murine model. Our study provides mechanistic insight into the interactions of Spn with mucus and may explain the low abundance of pilus-1 among clinical pneumococcal isolates, particularly after childhood exposure when pilus-specific sIgA has accumulated. Furthermore, we show that adherence of TIGR4 and TIGR4Δcps to hNF is highest in the presence of 0.1% BSA and incubation with 2 × 10^4 Spn TIGR4. Immobilized hNF was incubated with increasing concentrations of trypsin with or without protease inhibitor (PI) for 30 minutes at 37°C followed by incubation of 0.1% BSA and 2 × 10^4 Spn TIGR4 in 100 μL DMEM for 2 hours at 30°C. Experiments were performed in duplicate, and mean values of 3 independent experiments are shown with error bars corresponding to SD. **P < 0.01, ****P < 0.0001 by 2-tailed unpaired t test, n = 6 (A, B, and D), or 1-way ANOVA followed by Dunnett’s multiple comparison, n = 6 (C).

Three of the most abundant proteins in hNF are lactoferrin, sIgA, and lysozyme (Figure 2, A–C, and ref. 24). Pneumococcal surface protein A (PspA) and pilus-1 have been shown to bind to purified human lactoferrin (25, 26). In addition, pneumococcal surface protein C (PspC/CbpA) interacts specifically with the secretory component of human sIgA (27, 28). Furthermore, a recent study suggests the interaction of pilus-1 with the extracellular domain of polymeric immunoglobulin receptor (pIgR) that is identical to secretory component (29). To assess the relevance of these interactions in human nasal secretions, we incubated whole TIGR4 or defined mutants lacking these surface proteins with soluble hNF and detected bound lactoferrin or sIgA by flow cytometry. Our data confirmed that PspA is the major protein responsible for lactoferrin recruitment to the Spn surface (Figure 2D), and dot plots in Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI32005DS1). The mutant lacking the entire pathogenicity islet expressing pilus-1 did not display impaired binding of mucosal lactoferrin. However, lack of pilus-1 resulted in a 72% reduction in binding of sIgA in comparison with the isogenic parental strain. The pspC-deletion mutant also showed a significantly impaired binding of mucosal sIgA, though to a lesser extent than the pilus-deficient mutant. Loss of PspA did not alter the acquisition of sIgA to the bacterial surface. PspA, PspC, and pilus-1 are immunogenic in humans, and antibodies against these surface factors, which likely result from prior exposure during colonization episodes, are common (30–34). Therefore, we also analyzed the binding of IgG in hNF to Spn and found no significant difference between WT Spn and the protein-deficient mutants. Overall, the results demonstrate a role for interactions between bacterial factors and host mucosal proteins: PspA–lactoferrin and pilus-1/PspC–sIgA.

**Pilus-1 is the major factor interacting with human mucus.** A minority of clinical Spn isolates express pilus-1 (35–37). We screened clinical isolates for pilus expression by immunoblotting and confirmed the presence or absence of the pilus-1 pathogenicity islet by PCR.
Next, we determined which specific component of the pilus is involved in binding to human mucus. Pilus-1 is composed of 3 subunits: the tip RrgA, which is also described as adhesin; the shaft RrgB; and the ancillary protein RrgC (26, 37–40). We performed solid-phase assays and flow cytometry using pneumococcal mutants, which lacked 1 or 2 pilus-1 components. Adherence of the \textit{rrgA}-deficient strain was slightly but significantly reduced in comparison with WT (Figure 4A). The \textit{rrgB}-deletion mutant was most impaired in adherence to hNF. The absence of the pilus-1 subunit RrgC did not impact Spn adherence, and loss of both RrgB and RrgC resulted in levels comparable to those of the single \textit{rrgB}-deletion mutant (Figure 4A). Additionally, recruitment of sIgA from soluble hNF by each of the mutants directly correlated with their ability to adhere to immobilized mucus (Figure 4B, and dot plots in Supplemental Figure 6). To further verify a contribution of RrgA and RrgB in binding to hNF, we performed an inhibition assay using specific antibodies against these pilus-1 components. WT Spn pretreated with either anti-RrgA or anti-RrgB antisera adhered significantly less to hNF compared with controls (Figure 4C). Blocking of RrgB resulted in a greater inhibitory effect and consequently lower levels of Spn adherence compared with blocking of RrgA. As expected, the pretreatment of the pilus-1-deficient mutant with the RrgA- and RrgB-specific antibodies had no effect on adherence to hNF (Figure 4C). Together, these findings suggest that the pilus-1 shaft-forming subunit RrgB is the main Spn factor bound by human mucus, with a small contribution from the tip component RrgA.

Pneumococcal pilus-1 is a major determinant responsible for human sIgA recruitment. Based on the impaired sIgA binding of the using primers within \textit{rrgB} (Supplemental Figure 2). To examine a contribution of pilus-1 in Spn adherence to hNF, we used, in addition to the TIGR4 strain, 2 piliated (types 9V and 19F) and 2 non-piliated (types 6A and 23F) clinical isolates. Over a time period of 5 hours, adherence levels of the pilus-expressing strains were similar to those of the TIGR4 reference strain, while adherence of the non-piliated strains was significantly lower in comparison with TIGR4 (Figure 3A). Detachment was quantified as a further measure of the strength of interaction between Spn and hNF. In comparison with piliated strains, the nonpiliated strains detached significantly more readily from the nasal mucus (Figure 3B). To further confirm the role of pilus-1 in Spn binding to immobilized hNF, we used pilus-1-deficient constructs of the TIGR4, 9V, and 19F strains and a pilus-1 islet–knockin mutant of the 23F strain. Again, adherence to hNF correlated with pilus expression over genetic background (Figure 3, C–F). In addition, the \textit{rrgA} and \textit{rrgB} genes of the 9V and 19F strain were sequenced, and both belong to the common clades that include TIGR4 (Supplemental Figures 3 and 4). Overall, adherence to hNF of the mutants without the pilus-1 islet was reduced 4- to 14-fold. Furthermore, adherence of the pilated strain was reduced to the levels of the nonpiliated mutant by pretreatment of hNF with trypsin (Supplemental Figure 5). In contrast to pilus-1, we were unable to detect a role for PspA or PspC in adherence to immobilized hNF (Figure 3, C and F) despite the role of these Spn proteins in binding factors in human nasal secretions (Figure 2D, and dot plots in Supplemental Figure 1). Our findings show that pilus-1 is the major Spn surface structure mediating binding of Spn to normal human nasal mucus from adults.
is not specific to secretory component, suggesting that binding is mediated by the Fab portion and is independent of the secretory component or the J chain only present in sIgA. We confirmed this interpretation by performing binding studies with cleaved sIgA using WT Spn and the pspC-deficient mutant as control. Binding of the sIgA Fab portion to Spn was maintained after cleavage with recombinant IgA1 protease (Figure 5, C and D). In contrast, acquisition of the heavy chain comprising the Fc portion, the secretory component, and the J chain was significantly reduced after protease treatment (Figure 5, C and E). The residual binding of the heavy chain was mediated entirely by PspC. These findings reveal an immunodominant antigen-antibody interaction between pneumococcal pilus-1 and naturally acquired human sIgA.

In addition, we analyzed the contribution of pilus-1 in Spn binding of purified human lactoferrin and pooled serum IgG, 2 other components of nasal secretions. As shown in Figure 2D, pilus-1 is not involved in recruitment of lactoferrin or IgG to the Spn surface (Figure 5, F and G). Our findings confirm PspA as the main surface protein responsible for binding of lactoferrin.

sIgA is not the only factor required for pneumococcal adherence to hNF. As well as assessing the binding of Spn to pooled hNF, we tested pilus-1-mediated Spn adherence to hNF from 6 individual healthy adult donors. Spn adhered to all tested hNF samples

Figure 3. Pilus-1–mediated adherence of Spn to hNF. (A and B) Adherence to and detachment from hNF using pilus-1–expressing (T4 [TIGR4], 9V, 19F) and nonpiliated (6A, 23F) clinical isolates. (A) Adherence of Spn (2 × 10^6 per 100 μL) was observed over a time period of 5 hours; n = 6–12. (B) Detachment (unbound bacteria recovered after 19 washing steps) is calculated as the percentage of the total CFU per well after 2 hours of incubation; n = 6–12. (C–F) Adherence of WT Spn and isogenic mutants after 2 hours of incubation with immobilized hNF; n = 6–11. Experiments were performed in duplicate, and values of at least 3 independent experiments are shown in box-and-whiskers columns including minimal and maximal value (A and B) or as bar graphs ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by 1-way ANOVA followed by Sidák’s multiple comparison vs. T4 (A) or by Dunnett’s multiple comparison (B, C, and F) or 2-tailed unpaired t test (D and E) vs. T4 or WT.

pilus-1-deficient mutant when incubated with hNF, we analyzed the acquisition of human sIgA (purified from pooled colostrum) to the Spn surface using flow cytometry. Because of the binding of PspC to the human secretory component of sIgA, a pspC-deficient mutant was used as control. Secretory IgA bound in a concentration-dependent fashion to the surface of the TIGR4 as well as to the isogenic pilus-1– and pspC-deficient mutants (Figure 5A). Deletion of the pilus-1 islet decreased sIgA acquisition by up to 78%, whereas the lack of PspC reduced binding of sIgA only up to 38%. To decipher whether binding of this immunoglobulin isotype is exclusive for sIgA, we also analyzed binding of pooled human serum IgA. Based on the dimeric structure of sIgA and the resulting tetrameric valence of the molecule, monomeric divalent serum IgA was used in a 2-fold molar ratio. Interestingly, recruitment of serum IgA by the nonpiliated mutant was lower in comparison with the binding of sIgA. However, when pilus-1 is expressed, the binding of serum IgA to the bacterial cell surface is similar to that of sIgA. This observation was confirmed using the nonpiliated 23F parental strain and its isogenic pilus-1–knockin mutant. When pilus-1 was expressed, Spn bound higher amounts of human sIgA (Figure 5B). Surprisingly, pilus-1 seems to be the main pneumococcal determinant capable of binding large amounts of human sIgA. Furthermore, due to the recruitment of serum IgA, binding
in a pilus-1-dependent manner (Figure 6A). We analyzed further whether all hNF samples contain anti-pilus sIgA and verified the presence of anti-RrgB sIgA within the hNF from individual adults (Figure 6B). Using purified sIgA as competitor for Spn binding to hNF showed that adherence of WT Spn, but not the pilus-1-deficient mutant, was inhibited (Figure 6C). This effect was greater for sIgA than an equivalent titer of serum IgA. Due to the inhibitory effect of sIgA, these findings suggest that the sIgA–pilus-1 interaction plays the main role in Spn binding to hNF. Furthermore, we immobilized purified sIgA and BSA (same protein concentration as hNF) and performed the solid-phase assay. WT Spn did not adhere to pilus-1-specific sIgA, suggesting that sIgA alone is not sufficient to mediate adherence (Figure 6D).

Human nasal fluid agglutinates pilus-expressing Spn. Next, we examined whether hNF is able to agglutinate pilus-expressing bacteria. Indeed, we could visualize the formation of immune complexes of pilated Spn but not the pilus-deficient mutant (Figure 7A). The pilus-deficient strain appeared mostly as diplococci and was distributed uniformly in the hNF. In contrast, the pilus-expressing bacteria were more often found associated with mucus particles, in which they form longer chains (threading, an early stage of agglutination) and aggregates (41). Likewise, purified human sIgA (same anti-RrgB titer as hNF) agglutinated Spn (Figure 6A), and performed the solid-phase assay. Each bacteria (2 × 10⁴ per 100 μL DMEM) were incubated with 10 μg hNF in the presence of 0.1% BSA for 2 hours at 30°C. Detection of bound bacteria was analyzed as described in A. n = 6. Results of at least 3 independent experiments are illustrated as mean values ± SD. *P < 0.05, ****P < 0.0001 vs. TIGR4 by 1-way ANOVA followed by Dunnett’s multiple comparison. (C) Inhibition adherence assay using pilus-1-specific antisera. Bacteria were each pretreated with 5 μg/mL of rabbit control serum or anti-RrgA or anti-RrgB antiserum before incubation with immobilized hNF for 2 hours at 30°C. Detection of bound bacteria was analyzed as described in A. n = 6. Results of at least 3 independent experiments are illustrated as mean values ± SD. **P < 0.01, ****P < 0.0001 vs. without by 1-way ANOVA followed by Šidák’s multiple comparison.

Secretory IgA inhibits acquisition of colonization by pilus-expressing pneumococci. A variety of functions of mucosal sIgA have been proposed, including neutralizing released bacterial factors, agglutinating bacteria, and inhibiting bacterial attachment to the epithelium (22, 23, 43–47). Given the importance of sIgA in protection of the respiratory epithelium, we tested whether human sIgA targeting the type 1 pilus could prevent the establishment of Spn colonization in vivo. Using a mouse model of competitive infection, adult mice were intranasally challenged with equal numbers of the pilated TIGR4 and its isogenic nonpiliated mutant. Before administration, bacteria were preincubated with either a physiologically relevant concentration of human sIgA or PBS as control. Because of the potential of sIgA to disrupt early events in colonization, we determined the competitive index (CI) as early as 4 and 22 hours after infection. In the absence of human sIgA, WT and the nonpiliated mutant established equal levels of colonization (CI approximately equal to 1) (Figure 8, A and C). This indicated a minimal effect of pilus in early colonization in the murine model. When preincubated with human sIgA, which contains naturally acquired antibody against pilus-1, pilus-expressing Spn were
impaired in establishing colonization relative to the nonpiliated mutant at 4 and 22 hours after challenge. The experiment was then repeated using human serum IgA (at a titer to pilus-1 equivalent to sIgA). Serum IgA did not select against the piliated strain, indicating that structural differences in the characteristics of secretory immunoglobulin are needed for this effect (Figure 8A). Additionally, sIgA was pretreated with recombinant IgA1 protease to eliminate its multivalent binding. Protease cleavage, which was confirmed by Western analysis (Figure 8B), was sufficient to eliminate selection against the piliated strain. Since the protease is specific for sIgA1, this result also suggested that any contribution of sIgA2 is secondary. Overall, these findings suggest that naturally acquired sIgA specific to the pneumococcal pilus-1 is able to inhibit colonization acquisition.

Spn expresses a cell surface–anchored protease (Iga, ZmpA) with specificity for human IgA1, the predominant immunoglobulin in nasal secretions (48, 49). Cleavage of human IgA1 in the hinge region generates monovalent Fab fragments, eliminating its capacity to agglutinate its target (50, 51). This would predict that the IgA1 protease would limit agglutination-dependent adherence. When tested in the solid-phase assay with hNF, however, the protease-deficient mutant showed slightly decreased rather than increased adherence (Figure 8D). This observation correlates with a previous report showing that IgA1-mediated adherence of Spn to epithelial cells is enhanced by the enzyme (52). In addition, absence of the IgA1 protease had no effect on immune exclusion by human sIgA in the competition assay in vivo (Figure 8E).

Pilus-expressing pneumococci preferentially colonize children. Given our results in vitro and in mice, we hypothesized that human adults, who have had more cumulative exposure to Spn, will have higher levels of mucosal anti-pilus sIgA. As a result, piliated strains may be preferentially excluded during the establishment of adult carriage, and thereby be less commonly found in comparison with carriage in children from the same population.

To test this hypothesis, we analyzed genomic data produced from Spn isolates taken from asymptomatic carriage episodes in unvaccinated mothers and their children (53). We tested for an association between the presence of pilus in the colonizing strain and whether the host was an infant or an adult. We excluded infants under 6 months of age because of maternal antibody. In a naive association, we found adults were less likely to be colonized by pilus-expressing strains, which was significant at the genome-

Figure 5. Fab-mediated binding of sIgA to pneumococcal type 1 pilus. (A and B) Concentration-dependent binding of soluble purified sIgA to Spn. Acquisition of sIgA and serum IgA (2-fold molar ratio compared with 25 μg/mL sIgA) to type 4 Spn and isogenic mutants (A) or type 23F Spn and isogenic pilus-1–knockin mutant (B) was measured by flow cytometry using a specific FITC-labeled goat anti-human IgA1 antibody. The percentage binding of at least 3 independent experiments is shown as mean values with error bars ± SD. ****P < 0.0001 vs. WT by 2-way ANOVA followed by Tukey’s multiple comparison, n = 6–8. (C) Schematic model of cleaved sIgA treated with recombinant IgA1 protease (dashed line), generating Fab fragments (binding analyzed in D) and Fc fragments with bound secretory component (binding analyzed in E). (D and E) Flow cytometric analysis of sIgA binding following cleavage with recombinant IgA1 protease. Binding of sIgA light chain (D) or secretory component (E) was analyzed using an anti–human κ-light chain antibody (D) or a monoclonal anti–secretory component antibody (E). Results of 3 independent experiments are illustrated as mean values with error bars ± SD. *P < 0.05, ****P < 0.001 by 1-way ANOVA followed by Tukey’s multiple comparison, n = 6. (F and G) Concentration-dependent binding of soluble human lactoferrin and serum IgG to type 4 Spn and isogenic mutants. Results of 3 independent experiments are shown as mean values with error bars ± SD; n = 6.
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**RESEARCH ARTICLE**

**Discussion**

We examined the interactions of a mucosal pathogen with mucus-containing human secretions. Our results first confirmed the binding of the Spn surface proteins PspA and PspC to the mucus components lactoferrin and secretory component, respectively (25, 27). The dominant interaction of Spn with hNF from adults, however, involved the binding of naturally acquired specific sIgA to a single antigen, the main pilus-1 subunit, RrgB. Binding of sIgA to Spn expressing pilus-1 promoted agglutination and the association of bacterial aggregates (immune complexes) with mucus particles. In a mouse model of upper respiratory tract colonization, pretreatment with human sIgA enhanced rapid pilus-1–dependent Spn clearance. Thus, our findings provide an antigen-specific demonstration of mucosal defense with natural antibody via immune exclusion; the consecutive events of agglutination, mucus entrapment, and removal of pathogens by mucociliary activities (22).

It was unexpected that a single antigen could account for the majority of Spn adherence to mucus-containing secretions. Pilus-1 is one of many immunogenic surface components but is expressed by only a minority of Spn isolates (less than 20% in adults from the population we analyzed) (31, 33, 35). This suggests that the pilus locus is subject to negative frequency-dependent selection, where the population-wide fitness advantage is higher the rarer the locus. It has been shown that negative frequency-dependent selection on genes present in only a minority of the population is a major driving force of pneumococcal population dynamics (55). Our work gives

Figure 6. Secretory IgA is necessary but not sufficient for pneumococcal adherence to hNF. (A) Adherence of Spn TIGR4 and isogenic pilus-1–deficient mutant to hNF from 6 individual donors was assessed in a solid-phase assay. Bacteria (2 × 10^4 per 100 μL DMEM) were incubated with 10 μg immobilized hNF in the presence of 0.1% BSA for 2 hours at 30°C. n = 6. (B) Anti-RrgB IgA was determined using an ELISA. Recombinant purified RrgB protein was immobilized in a microtiter plate (Immulon 2HB, Thermo Fisher Scientific) and, after blocking, incubated with 200 μg/mL hNF, or 25 μg/mL sIgA and serum IgA, respectively. Binding of RrgB-specific IgA was detected using a biotin-labeled anti-human IgA and peroxidase-coupled streptavidin. The values of control wells without hNF or sIgA were subtracted from each measured value. Results are illustrated as mean values ± SD of 2 independent experiments; n = 4. (C) Inhibition adherence assay using purified sIgA in increasing concentrations or purified serum IgA in a 2-fold molar ratio (compared with 50 μg/mL sIgA). Bacteria were pretreated with either sIgA or serum IgA for 30 minutes at 37°C before incubation with immobilized pooled hNF for 2 hours at 30°C. n = 6. (D) Binding of WT Spn to immobilized sIgA or BSA. Secretory IgA and BSA (each 10 μg) were immobilized overnight followed by blocking with 0.1% BSA and incubation with 2 × 10^3 per 100 μL bacteria for 2 hours at 30°C. n = 6. (A–C) Experiments were performed in duplicate, and mean values of 3 independent experiments are shown with error bars corresponding to SD. **P < 0.01, ****P < 0.0001 by 1-way ANOVA followed by Šidák’s multiple comparison (C).

wide level (Table 1; OR = 2.23; P = 2.0 × 10^-13). When controlling for the genotypic background of the colonizing strain, as in a genome-wide association study, we found no significant association (P = 0.50). Given the strong association between pilus presence and specific strains, this latter result is unsurprising (54).

In a mouse model of upper respiratory tract colonization, pretreatment with human sIgA enhanced rapid pilus-1–dependent Spn clearance. Thus, our findings provide an antigen-specific demonstration of mucosal defense with natural antibody via immune exclusion; the consecutive events of agglutination, mucus entrapment, and removal of pathogens by mucociliary activities (22).
Secretory IgA is the most abundant immunoglobulin on mucosal surfaces and plays a critical role in the first line of host defense by protecting the underlying epithelium from invading pathogens. The protective function of sIgA is often attributed to its blocking of adherence to cellular receptors or neutralization of secreted toxins, enzymes, or virulence factors via Fab-mediated binding (22). In our mouse model, however, there was no apparent contribution of pilus-1 to adherence, since early colonization of pilus-expressing and that of non–pilus-expressing strains were equivalent, suggesting a human-specific benefit. Therefore, anti–pilus-1 sIgA does not act by impeding an adhesive function but rather acts through its agglutinating activity. Secretory IgA, which is polymeric (quadrivalent), is more effective at agglutinating piliated Spn compared with serum IgA, which is primarily monomeric (divalent). Several reports document the protective role of secretory polymeric immunoglobulin against mucosal pathogens (58–60). The importance of the agglutination function of antcapsular human immunoglobulin in blocking establishment of colonization has previously been documented in a mouse model of carriage following systemic administration of high levels of purified type-specific antibody, and in experimental human pneumococcal carriage following immunization with pneumococcal conjugate vaccine (56, 61). In the current study involving naturally acquired mucosal antibody, we demonstrate that sIgA-mucus interaction is necessary for efficient binding to hNF and subsequent removal of agglutinated bacteria. There is evidence that sIgA specifically interacts with the cell surface, where it might not access slgA1 bound to the extended pilus (48).

an example of mechanism for this important form of selection, whereby immune exclusion of piliated strains mediated by slgA would increase as population prevalence of pilus-1 increases.

Naturally acquired immunoglobulin against other surface structures, including the main surface antigen capsular polysaccharide, is common in adults (30, 34, 54) but, on the basis of our findings, apparently does not contribute in a significant manner to the association of Spn with human nasal mucus. More than 70% of slgA binding to Spn was attributable to pilus-1 (Figure 2A and Figure 5A). In contrast to other Spn surface components, pilus-1 protrudes up to 1 μm from the cell surface, where it is no longer shielded by the thick layer of capsular polysaccharide (26). Thus, the physical properties of pilus-1 may allow for binding of slgA against RrgB>>RrgA that was generated through exposure during prior carriage events.

Up to 90% of mucosal slgA in human nasal secretions is of the subclass IgA1. Like many successful respiratory pathogens, Spn expresses a protease with specificity for the hinge region of IgA1 to subvert the protective functions of slgA1. Previous studies have shown that Spn cleaves anti-capsule IgA1/sIgA1, thereby abrogating the agglutinating and opsonophagocytic properties of slgA1 (49, 56, 57). The long protrusion of the pilus could explain why there was no detectable effect of the Spn IgA1 protease in limiting immune exclusion. The protease is associated with the cell surface, where it might not access slgA1 bound to the extended pilus (48).

Secretory IgA is the most abundant immunoglobulin on mucosal surfaces and plays a critical role in the first line of host defense by protecting the underlying epithelium from invading pathogens. The protective function of slgA is often attributed to its blocking of adherence to cellular receptors or neutralization of secreted toxins, enzymes, or virulence factors via Fab-mediated binding (22). In our mouse model, however, there was no apparent contribution of pilus-1 to adherence, since early colonization of pilus-expressing and that of non–pilus-expressing strains were equivalent, suggesting a human-specific benefit. Therefore, anti–pilus-1 slgA does not act by impeding an adhesive function but rather acts through its agglutinating activity. Secretory IgA, which is polymeric (quadrivalent), is more effective at agglutinating piliated Spn compared with serum IgA, which is primarily monomeric (divalent). Several reports document the protective role of secretory polymeric immunoglobulin against mucosal pathogens (58–60). The importance of the agglutination function of antcapsular human immunoglobulin in blocking establishment of colonization has previously been documented in a mouse model of carriage following systemic administration of high levels of purified type-specific antibody, and in experimental human pneumococcal carriage following immunization with pneumococcal conjugate vaccine (56, 61). In the current study involving naturally acquired mucosal antibody, we demonstrate that slgA-mucus interaction is necessary for efficient binding to hNF and subsequent removal of agglutinated bacteria. There is evidence that slgA specifically interacts with the cell surface, where it might not access slgA1 bound to the extended pilus (48).
with mucins via the mucin-like hinge region of IgA1 and/or through the hydrophilic secretory component (62). In this regard, secretory component ensures the appropriate localization of slgA within the mucus and thereby contributes to slgA-mediated immune exclusion. This has been shown in the case of Shigella flexneri infection of the murine gut mucosa using a monoclonal antibody modified to resemble slgA (58). Alternatively, larger particles generated by agglutination together with their physical association with viscous mucus could be more efficiently swept away and cleared from the nasal surfaces by the mechanics of normal mucociliary flow.

Our study demonstrates protection against Spn by natural secretory immunoglobulin. Our observations also raise the question of whether PspC–secretory component binding is beneficial for the bacterium or aids in mucus-mediated host defense. Secretory component is present in the mucus in free form or bound to slgA/slgM. Spn binding of secretory component attached to slgA via PspC might limit Fab-dependent recognition that leads to agglutination and clearance.

Spn colonization is observed predominantly in children younger than 5 years of age, in contrast to much lower carriage rates in healthy adults (9, 10). It is apparent that the upper respiratory tract of adults is a less advantageous niche for Spn, perhaps because of the presence of mucosal host defense molecules such as slgA. In contrast to other immunoglobulins, slgA production starts late during childhood and reaches adult levels in saliva around 7 years of age (63). The natural development of adaptive immunity against immunogenic pneumococcal proteins reaches its maximum at 3–5 years of age, although these observations are...
based on serum IgG levels (64). Likewise, anti-pilus antibodies are commonly found in humans, and reach maximum levels around 10–15 years of age (31). Previous studies have tested for an association between pilus-expressing isolates and age of the carriers in vaccinated populations, using either pilus-specific PCR or whole-genome sequencing to perform genotyping, but have found conflicting results (33, 35, 65). Our results from a large unvaccinated mother-infant cohort showed that pilus-expressing pneumococci were more commonly found in colonized children (after maternally derived immunoglobulin wanes), when antigen-specific mucosal sIgA levels would be low. Indeed, the higher prevalence of pilated strains in children suggests a colonization benefit conferred by pilus-1 in naive hosts that is diminished later in life, presumable as a result of higher levels of specific mucosal sIgA. This effect itself could contribute to higher carriage rates in young children. However, the lack of independent association when controlling for genetic background means we cannot determine whether this effect is causally driven by the pilus itself or other genetic features on the background of pilated strains. Our experimental studies, however, were carried out with isogenic strains that control for the effect of strain background.

In summary, our study suggests that augmenting mucosal immunity, particularly against the pilus-1 shaft component RrgB, might accelerate the protection of children, who have the highest carriage rates and burden of disease due to Spn. Additionally, we demonstrate how naturally acquired antibody enhances bacteria-mucus interactions that promote mucosal defense. Finally, we show how these effects of mucosal antibody may drive pathogen adaptation in the natural host among different populations.

Methods

Ethics statement. All animal experiments in this study followed the guidelines outlined by the National Science Foundation Animal Welfare Act and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee (IACUC) at the New York University School of Medicine oversees the welfare, well-being, and proper care and use of all animals. It approved the protocols used in this study.

Chemicals, reagents, and antibodies. Pooled or individual human nasal secretion samples from adult volunteers were purchased from LeeBio. Secretory IgA from human colostrum (I1010), human serum IgG (I2511), human lactoferrin, bovine serum albumin (BSA), alkaline phosphatase–coupled goat anti-rabbit IgG (A3687), rabbit anti–human lactoferrin antibody (L-3262), alkaline phosphatase–coupled goat anti-human IgA antibody (A-3063), FITC-conjugated goat anti–human IgG (A3687), goat anti-human IgA coupled with biotin (B-1015), and Alcian blue solution were obtained from Sigma-Aldrich. Rabbit anti–pneumococcus type 4 serum (16747) was obtained from Statens Serum Institut. Tween-20 and Triton X-100 were obtained from Amresco. Polyclonal rabbit anti–human lysozyme antibody (A0099) was purchased from Dako. FITC-labeled goat anti-human IgA1 (A1872) was obtained from Invitrogen. Allophycocyanin-conjugated (APC-conjugated) goat anti-rabbit IgG (A10931), APC-conjugated anti-mouse IgG (A865), HRP-coupled streptavidin (21130), 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) solution, 4% parafomaldehyde solution (in PBS), and DMEM were obtained from Thermo Fisher Scientific. Mouse monoclonal anti–secretory component antibody (LS-C45754) was purchased from LSBio. FITC-conjugated mouse anti-goat antibody (sc-2356) was purchased from Santa Cruz Biotechnology. Recombinant IgA protease from Neisseria gonorrhoeae was obtained from Mobitec. Bradford reagent was obtained from BioRad. Protease inhibitor cocktail tablets were purchased from Roche.

Bacterial cultivation. Spn strains used in this study are listed in Table 2. Pneumococci were grown statically in tryptic soy (TS) broth (BD) or in semisynthetic medium (C+Y, pH 6.8) at 37°C to an optical density of 0.6 at 620 nm (OD 620) for all in vitro experiments, or until an optical density of 1.0 for in vivo colonization of mice. Alternatively, pneumococcal strains were cultivated on TS agar plates supplemented with 100 μL of catalase (30,000 U/mL; Worthington Biomedical) and appropriate antibiotics (200 μg/mL streptomycin, 250 μg/mL kanamycin, or 2 μg/mL chloramphenicol) overnight at 37°C and 5% CO2.

Recombinant E. coli M15 strains were grown on Luria-Bertani (LB) agar plates supplemented with 100 μg/mLampicillin at 30°C or in liquid LB medium to mid-log phase (OD 600 = 0.8) at 200 rpm on an environmental shaker.

Bacterial strains and mutant construction. The primers used for the construction of Spn mutants are summarized in Table 3. In-frame and unmarked deletion mutants of the pneumococcal serotype 4 strain, TIGRA4, deficient for the genes rrgA, rrgB, rrgC, rrgBC, or cbpA were obtained by generation of a PCR product containing a previously described insertion of the “sweet Janus” cassette (66). Deletion of rrgA and rrgB was confirmed by dot blots with immunological detection using anti-RrgA and anti-RrgB sera. Serotype 9 and 19F pneumococci lacking the rlrA pathogenicity island (pilus-1) were constructed by transformation with genomic DNA isolated from strain P2454 (TIGRA4(pilus-1)) followed by selection on TS agar plates supplemented with 2 μg/mL chloramphenicol and 2 rounds of back-transformation. A pilus-insertion mutant of serotype 23F was obtained by transformation of genomic DNA of strain P2355 (TIGRA4(rrgA:Janus)) followed by selection on TS agar plates containing 250 μg/mL kanamycin followed by 2 rounds of back-transformation. The Janus insertion within the rrgA gene was replaced with a PCR product of the pilus-1 islet with selection for streptomycin resistance and screening for acquisition of kanamycin-sensitivity. The expression of pilus-1 of the serotype 23F pneumococci was confirmed via dot blot as described above. The ppaA-deficient mutants were obtained by transformation of chromosomal DNA from TIGRA4(ppaA) (collection from Marc Lipsitch, Harvard School of Public Health, Boston, MA, USA) or 23F3(ppaA) (67) into P2406 and P2499, respectively, followed by selection on 200 μg/mL spectinomycin and streptomycin. The iga-deletion mutant was generated by transformation of chromosomal DNA from a previously described construct into P2499 (68).

Table 1. Proportion of pilated strains in human infants and mothers, based on asymptomatic carriage episodes in an unvaccinated host

<table>
<thead>
<tr>
<th></th>
<th>Nonpiliated</th>
<th>Piliated</th>
<th>% Piliated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants (6–24 months)</td>
<td>1058</td>
<td>598</td>
<td>56.5</td>
</tr>
<tr>
<td>Mothers</td>
<td>438</td>
<td>79</td>
<td>18.0</td>
</tr>
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</table>
**Table 2. Bacterial strains and plasmids used in the study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Sero- and genotype</th>
<th>Antibiotic resistance</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pneumoniae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2406 (TIGR4)</td>
<td>4, clinical isolate</td>
<td>Strep’</td>
<td>Ref. 80</td>
</tr>
<tr>
<td>P2422</td>
<td>TIGR4ΔpsB</td>
<td>Kan’</td>
<td>Ref. 80</td>
</tr>
<tr>
<td>P2554</td>
<td>TIGR4Δπilus-1 (Δπilus-1::Cm)</td>
<td>Strep’, Cmr’</td>
<td>Ref. 80</td>
</tr>
<tr>
<td>P2542</td>
<td>TIGR4ΔπilA</td>
<td>Strep’</td>
<td>This study</td>
</tr>
<tr>
<td>P2592</td>
<td>TIGR4ΔrrgB</td>
<td>Strep’</td>
<td>This study</td>
</tr>
<tr>
<td>P2593</td>
<td>TIGR4ΔrrgC</td>
<td>Strep’</td>
<td>This study</td>
</tr>
<tr>
<td>P2594</td>
<td>TIGR4ΔrrgBC</td>
<td>Strep’</td>
<td>This study</td>
</tr>
<tr>
<td>P2502</td>
<td>TIGR4ΔπapA</td>
<td>Kan’</td>
<td>This study</td>
</tr>
<tr>
<td>P2583</td>
<td>TIGR4ΔπapC</td>
<td>Kan’</td>
<td>This study</td>
</tr>
<tr>
<td>P2615</td>
<td>TIGR4ΔπapA</td>
<td>Strep’, Spec’</td>
<td>This study</td>
</tr>
<tr>
<td>P2618</td>
<td>9V, clinical blood isolate</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>P2569</td>
<td>9VΔπilus-1 (Δπilus-1::Cm)</td>
<td>Cmr’</td>
<td>This study</td>
</tr>
<tr>
<td>P1680</td>
<td>19F, clinical isolate</td>
<td>Strength</td>
<td>This study</td>
</tr>
<tr>
<td>P2572</td>
<td>19FΔπilus-1 (Δπilus-1::Cm)</td>
<td>Cmr’</td>
<td>This study</td>
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<tr>
<td>P2499</td>
<td>23F, clinical isolate</td>
<td>Strep’</td>
<td>Ref. 81</td>
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<tr>
<td>P2588</td>
<td>23FΔπilus-1 (πilus-1 insertion)</td>
<td>Strep’</td>
<td>This study</td>
</tr>
<tr>
<td>P2617</td>
<td>23FΔπilus-1Δlα3α</td>
<td>Strep’, Kan’</td>
<td>This study</td>
</tr>
<tr>
<td>P2618</td>
<td>23FΔα3α3α (pgpA–Spec)</td>
<td>Strep’, Spec’</td>
<td>This study</td>
</tr>
<tr>
<td>P2625</td>
<td>23FΔα3α3α</td>
<td>Kan’</td>
<td>This study</td>
</tr>
<tr>
<td>P1547</td>
<td>6A, clinical isolate</td>
<td></td>
<td>Ref. 68</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M15 (pREP4)</td>
<td>F, β280ΔlacMIS, thi, lac’, mtl’, rec’</td>
<td>Kan’</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pOE30-rrgB</td>
<td>His’ tagged WT rrgB gene</td>
<td>Ampr</td>
<td>Ref. 73</td>
</tr>
</tbody>
</table>

**In vitro proteolytic cleavage of human IgA1.** Human slgA and human nasal secretions were proteolytically cleaved with recombinant IgA protease from *N. gonorrhoeae*. The digestion occurred in reaction buffer (50 mM Tris, 100 mM NaCl, 0.1 mM EDTA, pH 7.5) for 20 hours at 37°C with an enzyme/protein ratio of 1:50 (wt/wt). Cleavage of IgA was confirmed by Western blot and anti-human IgA conjugated to alkaline phosphatase.

**Human nasal secretion-binding assay.** Adherence of different pneumococcal strains to human nasal fluid (hNF) was assessed in a solid-phase binding assay as previously described (13, 69). His6-tagged protein was expressed in *E. coli* Eβ-β-d-thiogalactopyranoside for 4 hours at 30°C. Overexpressed RrgB protein was purified from *E. coli* lysates under native conditions via immobilized metal affinity chromatography using a HisTrap column and the ÄKTA purification system according to the manufacturer’s instructions (GE Healthcare). Protein was dialyzed (30 kDa molecular weight cutoff) against PBS (pH 7.4) at 4°C using centrifugal filters (Millipore). Determination of the protein concentration was performed using Bradford reagent, and protein purity was confirmed by Western blot.

**Flow cytometric analysis.** The binding of soluble hNF as well as purified slgA, human serum IgA, lactoferrin, and human serum IgG to viable pneumococci was analyzed by using flow cytometry. In brief, 5 × 10^6 bacteria were incubated with either 50 μg/mL human nasal secretions, increasing concentrations of human slgA (0–25 μg/mL), serum IgA in a 2-fold molar ratio (in relation to 50 μg/mL slgA), for 30 minutes at 37°C and 5% CO₂.

Inhibition assays, pneumococci were preincubated with 5 μg/mL of anti-RrgA, anti-RrgB, and control serum, or increasing concentrations of slgA (10–50 μg/mL), or serum IgA in a 2-fold molar ratio (in relation to 50 μg/mL slgA), for 30 minutes at 37°C and 5% CO₂.

Protein was dialyzed (30 kDa molecular weight cutoff) against PBS (pH 7.4) at 4°C using centrifugal filters (Millipore). Determination of the protein concentration was performed using Bradford reagent, and protein purity was confirmed by Western blot.
### Table 3. Primers used in the study

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
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<tbody>
<tr>
<td>rrgA: Janus-cassette</td>
<td>rrgA upstream forward</td>
<td>CGTGTATACAGATTGAATTGAAAGTACCTATGTAAC</td>
</tr>
<tr>
<td>rrgA::Janus-cassette rrgA</td>
<td>rrgA upstream reverse</td>
<td>CATTACATTATCTTACAGAATTGAAAGTACCTATGTAAC</td>
</tr>
<tr>
<td>rrgA: Janus forward</td>
<td>rrgA downstream forward</td>
<td>GGAAATAAACCATGTTTTCCGTTTGATTTTTAATGGATAATGTG</td>
</tr>
<tr>
<td>rrgA: Janus reverse</td>
<td>rrgA downstream reverse</td>
<td>GTACTCTTTAAAAGTATCCTTGCAAAG</td>
</tr>
<tr>
<td>rrgB: Janus-cassette</td>
<td>rrgB upstream forward</td>
<td>GGAAATAAACCATGTTTTCCGTTTGATTTTTAATGGATAATGTG</td>
</tr>
<tr>
<td>rrgB::Janus-cassette rrgB</td>
<td>rrgB upstream reverse</td>
<td>CATTACATTATCTTACAGAATTGAAAGTACCTATGTAAC</td>
</tr>
<tr>
<td>rrgB: Janus forward</td>
<td>rrgB downstream forward</td>
<td>GGAAATAAACCATGTTTTCCGTTTGATTTTTAATGGATAATGTG</td>
</tr>
<tr>
<td>rrgB: Janus reverse</td>
<td>rrgB downstream reverse</td>
<td>GTACTCTTTAAAAGTATCCTTGCAAAG</td>
</tr>
<tr>
<td>rrgBC: Janus-cassette</td>
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<tr>
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<td>rrgBC downstream forward</td>
<td>GGAAATAAACCATGTTTTCCGTTTGATTTTTAATGGATAATGTG</td>
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<tr>
<td>rrgBC: Janus reverse</td>
<td>rrgBC downstream reverse</td>
<td>GTACTCTTTAAAAGTATCCTTGCAAAG</td>
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<tr>
<td>pspC: Janus-cassette</td>
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<tr>
<td>pspC: Janus forward</td>
<td>pspC downstream forward</td>
<td>GGAAATAAACCATGTTTTCCGTTTGATTTTTAATGGATAATGTG</td>
</tr>
<tr>
<td>pspC: Janus reverse</td>
<td>pspC downstream reverse</td>
<td>GTACTCTTTAAAAGTATCCTTGCAAAG</td>
</tr>
</tbody>
</table>

**Presence of rlrA pathogenicity island**
- **RrgB_seq_forw**: CGAAAACTTGCAGAAAAAACGTATTATTGTC
- **RrgB_seq_rev1**: GCTTTGGAGTATTCCCGTGATCTGG

**Absence of rlrA pathogenicity island**
- **Pilus1-neg_Forw**: CGCCTTGGATGCATTGAGC
- **Pilus1-neg_Rev**: GTATTACAAGATATTATTTCACC
ELISA. To determine anti-RrgB IgA titers in hNF, colostrum slgA, and serum IgA, wells of microtiter plates (96-well, Immulon 2HB plate, Thermo Fisher Scientific) were coated with 0.1 μg/well recombinant RrgB in PBS (pH 7.4) overnight at 4°C. The plates were washed 3 times with washing buffer (PBS, 0.05% Tween-20), and blocked with blocking buffer (PBS, 0.1% Tween-20 supplemented with 1% BSA) for 1 hour at room temperature. Afterward the washed wells were incubated with 200 μg/mL hNF, 25 μg/mL slgA, and 25 μg/mL serum IgA in PBS for 1 hour at 37°C. Bound anti-RrgB IgA was measured using a goat anti-human IgA coupled with biotin and HRP-coupled streptavidin. O-Phenylenediamine dihydrochloride was used as HRP substrate, and color reaction was measured at an absorbance of 492 nm using a spectraMax M3 reader (Molecular Devices). The values of control wells without IgA were subtracted from each measured value.

Competitive colonization in mice. C57BL/6J mice were purchased from The Jackson Laboratory, and bred and housed in a conventional animal facility as previously described (74). During colonization, all mice appeared healthy and did not experience any weight loss in comparison with uninfected controls. Spn strains were grown to an OD₆₀₀ of 1.0, washed, and diluted to the desired density in PBS. A mixture containing a 1:1 ratio of the TIGR4 strain and isogenic pilus-deficient mutant was preincubated with either human slgA (final concentration 0.9 mg/mL in PBS), human serum IgA, or PBS as control for 30 minutes at room temperature. Five-week-old adult mice were inoculated intranasally without anesthesia with 15 μL containing 1.5 × 10⁶ to 2 × 10⁷ CFU of the mixed pneumococcal strains. At 4 hours or 22 hours after pneumococcal challenge, mice were euthanized using CO₂ followed by cardiac puncture. For quantification of the colonization density, the trachea was cannulated and lavaged with 200 μL of sterile PBS, and fluid collected from the nares. The nasal lavage samples were plated in serial dilution on TS agar plates supplemented with 200 μg/mL spectinomycin. To calculate the competitive index (CI), a colony immunoblot detecting the pilus-expressing strain was performed. In brief, cultivated TS agar plates containing the total number of pneumococcal colonies were blotted on circular nitrocellulose membranes. After drying for 15 minutes, the membrane was blocked with 2% BSA/PBS for at least 3 hours followed by incubation with a primary rabbit anti-RrgB antibody (1:200,000 in 0.1% BSA/0.01% Tween-20/PBS) overnight at 4°C. The membrane was washed 3 times with 0.05% Tween-20/PBS and incubated with a secondary alkaline phosphatase–coupled anti-rabbit IgG (1:5000 in 0.1% BSA/0.01% Tween-20/PBS) for 1 hour at room temperature. The colorimetric visualization of the pilus-expressing pneumococcal colonies occurred by use of BCIP/NBT as substrate. To determine the quantity of the nonpiliated colonies, the number of pilus-expressing colonies was subtracted from the total number of colonies. The CIs were calculated as the ratio of pilus-expressing strain to pilus-deficient mutant output CFU/mL divided by the pilus-expressing strain to pilus-deficient mutant input CFU/mL. The colony immunoblots were performed at least in duplicate from at least 2 independent colonization experiments to ensure reproducibility.

Agglutination assay and microscopic visualization. For agglutination, 5 × 10⁴ bacteria were incubated with 10 μL of undiluted hNF or purified slgA (which contained the same titer of anti-RrgB IgA) for 2 hours at 37°C and 5% CO₂. Samples were placed onto glass slides and immobilized via heat fixation. To visualize mucus, samples were incubated with 3% acetic acid followed by incubation with Alcian blue (in 3% acetic acid, pH 2.5) for 30 minutes. After washing in water for 10 minutes, slides were blocked in 10% FBS at 4°C overnight. Bacteria were stained with primary rabbit anti-capsule antibody (1:200 in 0.5% FBS/PBS) and secondary FITC-coupled goat anti-rabbit IgG (1:100 in 0.5% FBS/PBS). Agglutination was visualized on an Axiovert 40 CFL microscope equipped with an AxioCam IC digital camera (Zeiss). All image analysis was performed with ZEN 2012 software, and images were processed with ImageJ 1.52a (NIH) for brightness and contrast.

SDS-PAGE and Western blot analysis. Western blots were performed to detect proteinaceous components in hNF. One microgram of purified lactoferrin, slgA, or lysozyme, as well as pooled hNF, was separated under denaturing conditions using 4%–12% Bolt Bis-Tris Plus gels (Thermo Fisher Scientific). Separated proteins were transferred on nitrocellulose membranes via a dry blotting system (iBlot 2, Thermo Fisher Scientific). Following blocking of the membrane with 2% BSA/PBS for at least 3 hours and washing 3 times with PBS/0.05% Tween-20, proteins were detected with a polyclonal rabbit anti-human lactoferrin antibody (1:10,000), alkaline phosphatase–coupled goat anti-human IgA antibody (1:20,000), or rabbit anti-human lysozyme antibody (1:10,000) overnight at 4°C followed by incubation with an alkaline phosphatase–coupled goat anti-rabbit IgG (1:5000) for 1 hour at room temperature. All antibody incubations occurred in 0.1% BSA/0.01% Tween-20/PBS. Protein bands were visualized after washing of the membrane 3 times with 0.05% Tween-20/PBS using BCIP/NBT as substrate.

Association between pilus and age in a human population. We used genomic data from the Mae La refugee camp (75). This consisted of data from around 600 children and their mothers sampled every month from birth to 2 years of age; genomic data associated with 3000 randomly selected positive swabs were available (53). We gave each sample a binary outcome, mothers being positive and infants being negative; infants under 6 months of age were excluded.

To determine pilus presence, we used a definition of the accessory genome in this population and classified any sequence containing at least 1 of the 3 rrgB alleles (cluster of orthologous genes CLS02709, CLS03842, and CLS01960) as being piliated (30, 55). To control for the same isolate being observed multiple times, we assigned a unique identifier to each individual carriage episode; these identifiers have been previously defined using a hidden Markov model (76).

We then used a generalized linear mixed model (LMM) with a Bernoulli error structure and logit link function to test for an association between pilus presence and age. Pileus presence was treated as a fixed effect, and a random intercept for each carriage episode was included. We calculated a P value for the association by using a likelihood-ratio test between this model and a nested model with the pilus term removed.

To calculate this association while also controlling for genetic background, we also used an LMM with the genetic kinship between isolates as random effects, as in genome-wide association studies (77, 78). We used the pyseer package (version 1.2.0) in LMM mode, with the kinship/covariance matrix calculated from a neighbor-joining tree of all genome sequences in the cohort (53, 79).

Data availability. Data and code to perform the association between pilus and age can be found on GitHub: https://github.com/johnlees/pilus-age/commit/d37524f52aa1d4ffadf2b1d0a2bb7cb28f7fd552 (commit ID: d37524f5).
Statistics. Statistical analysis was performed using GraphPad Prism (version 7.01, GraphPad Software Inc.). Data of in vitro experiments are reported as mean ± SD. Results from in vivo assays are shown as median with interquartile range. Unless otherwise specified, statistical analyses were performed using t test or 1-way ANOVA with either Šidák’s or Dunnett’s multiple-comparisons test. A P value less than 0.05 was considered to be statistically significant.

Study approval. The experimental protocols were approved by the IACUC of the New York University School of Medicine.

Author contributions
UB contributed to project design, performed in vitro and in vivo experiments and data analysis and interpretation, and wrote the manuscript. JAL performed population genomics, interpreted data, and contributed to the writing. AJH constructed rrgA- and pspC-deficient pneumococcal mutants. JNW oversaw the project conception and design, data interpretation, manuscript preparation, and funding acquisition.

Acknowledgments
We thank Richard Malley and Alan Basset for providing the anti-pilus-1 antibodies and the recombinant E. coli strain. We thank Nicholas Croucher for sharing data defining presence and absence of pilus genes in pneumococcal populations. This project was supported by grants from the US Public Health Service to JNW (AI038446 and AI105168).

Address correspondence to: Jeffrey N. Weiser, Department of Microbiology, New York University School of Medicine, Alexandria Center for Life Sciences – West Tower, 430 East 29th Street, New York, New York 10016, USA. Phone: 212.263.1080; Email: Jeffrey.Weiser@nyulangone.org.

36. Aguirre SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M. The presence of the pilus locus is a


