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Ulrike Binsker, … , Alexandria J. Hammond, Jeffrey N. Weiser

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Immune exclusion by naturally-acquired secretory IgA to the pneumococcal pilus-1

Ulrike Binsker[1], John A. Lees[1], Alexandria J. Hammond[1], Jeffrey N. Weiser[1]

[1]Department of Microbiology, New York University School of Medicine, New York, NY, 10016, USA

Corresponding author

Prof. Dr. 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, NY, 10016, USA

Phone: +1 212 263 1080

E-Mail: Jeffrey.Weiser@nyulangone.org

Conflict of interest statement

The authors have declared that no conflict of interest exists.

Abstract

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 *S. 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 immunoglobulin A (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 prior to 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 non-piliated 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 semi-permeable 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 two 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 proteinaceous components present in the mucus harbor anti-bacterial 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% to 65% in children and 5% to 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 comprised 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 nasal secretions (13). Furthermore, Spn expresses multiple exo- and endoglycosidases able to degrade O-and N-linked glycans of mucosal proteins (14-16). Mucus components, including lactoferrin, secretory component, secretory immunoglobulin A (sIgA), as well as mucins have been shown to be substrates of Spn glycosidases (14, 17, 18). Potential changes in the mucus integrity and its protective function by Spn glycosidases might contribute to the movement of the 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 the effectiveness 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. Furthermore, 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 a 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 provide a demonstration of host defense mediated by mucosal antigen-specific sIgA (referred to as immune exclusion) (22, 23).

Results

Pneumococci interact with human nasal mucus via mucosal proteins

Colonizing Spn are found predominantly within the glycocalyx, 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 by using a solid-phase assay with BSA as blocking reagent. Spn adhered to hNF more readily compared to bovine submaxillary mucus, which has been recently used in a similar approach (13) (Fig. 1A). Adherence to either source of mucus was higher than controls with BSA alone. As a control for the functionality of the assay, we demonstrate that adherence of an isogenic capsule-deficient mutant to hNF was significantly increased as previously described for bovine submaxillary mucus (Fig. 1B) (13).

Next, we considered whether Spn interactions with hNF involved binding to host carbohydrate moieties and/or protein(s). hNF was pretreated with either sodium periodate ($NaIO_4$) to oxidize oligosaccharides or trypsin to degrade protein. Even high concentrations of sodium periodate did not affect the binding of Spn to hNF (Fig. 1C). In contrast, incubation of hNF with increasing concentrations of trypsin reduced the adherence of Spn in a concentration-dependent manner (Fig. 1D). Inhibition of trypsin activity restored adherence levels equivalent to those without added protease.

Three of the most abundant proteins in hNF include lactoferrin, sIgA, and lysozyme (Fig. 2A, B, C) (24). The pneumococcal surface protein A (PspA) and the pilus-1 have been shown to bind to purified human lactoferrin (25, 26). In addition, pneumococcal surface protein C (PspC/CbpA) interacts specifically with secretory component of human sIgA (27, 28). Furthermore, a recent study suggests the interaction of the 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 (Fig. 2D, dot plots in Fig. S1). 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 when compared to the isogenic parental strain. The *pspC*-deletion mutant also showed a significant impaired binding of mucosal sIgA, however, 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 the pilus-1 are immunogenic in humans and antibodies to 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 wild type 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

The minority of clinical *S. pneumoniae* isolates expresses the 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 using primers within *rrgB* (Fig. S2). To examine a contribution of the pilus-1 in Spn adherence to hNF, we used, in addition to the TIGR4 strain, two piliated (types 9V and 19F) and two non-piliated (types 6A and 23F) clinical isolates. Over a time period of 5h, adherence levels of the pilus-expressing strains were similar to the TIGR4 reference strain, while adherence of the non-piliated strains was significantly lower compared to TIGR4 (Fig. 3A). Detachment was quantified as a further measure of the strength of interaction between Spn and hNF. In comparison to piliated strains, the non-piliated strains

detach significantly more readily from the nasal mucus (Fig. 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 knock-in mutant of the 23F strain. Again, adherence to hNF correlated with pilus expression over genetic background (Fig. 3C, D, E, F). In addition, the *rrgA* and *rrgB* genes of the 9V and 19F strain were sequenced and both belong to the common clades which include TIGR4 (Fig. S3/4). Overall, adherence to hNF of the mutants without the pilus-1 islet was reduced 4 to14-fold. Furthermore, adherence of the piliated strain was reduced to the levels of the non-piliated mutant by pretreatment of hNF with trypsin (Fig S5). In contrast to pilus-1, we were unable to detect a role for PspA or PspC in adherence to immobilized hNF (Fig. 3C, F) despite the role of these Spn proteins in binding factors in human nasal secretions (Fig. 2D, dot plots in Fig. S1). Our findings show that the pilus-1 is the major Spn surface structure mediating binding of *S. pneumoniae* to normal human nasal mucus from adults.

Next, we determined which specific component of the pilus is involved in binding to human mucus. The pilus-1 is composed of three 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 one or two pilus-1 components. Adherence of the *rrgA*-deficient strain was slightly but significantly reduced in comparison to WT (Fig. 4A). The *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 the single *rrgB*-deletion mutant (Fig. 4A). Additionally, recruitment of sIgA from soluble hNF by each of the mutants directly correlated with their capability to adhere to immobilized mucus (Fig. 4B, dot plots in Fig. S6). 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 pre-treated with either anti-RrgA or anti-RrgB antisera adhered significantly less to hNF compared to controls (Fig. 4C). Blocking

of RrgB resulted in a greater inhibitory effect and consequently lower levels of Spn adherence compared to the blocking of RrgA. As expected, the pre-treatment of the pilus-1 deficient mutant with the RrgA- and RrgB-specific antibodies had no effect on the adherence to human nasal fluid (Fig. 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 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. sIgA bound in a concentration-dependent fashion to the surface of the TIGR4 as well as to the isogenic *pilus-1*- and *pspC*-deficient mutants (Fig. 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 valency of the molecule, monomeric divalent serum IgA was used in a 2-fold molar ratio. Interestingly, recruitment of serum IgA by the non-piliated mutant was lower in comparison to the binding of sIgA. However, when the 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 non-piliated 23F parental strain and its isogenic pilus-1-knock in mutant. When pilus-1 is expressed, Spn bound higher amounts of human sIgA (Fig. 5B). Surprisingly, the 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 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_{α}-portion to Spn was maintained after cleavage with recombinant IgA1-protease (Fig. 5C,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 (Fig. 5C, 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, two 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 (Fig. 5F,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 human nasal fluid.

As well as assessing the binding of Spn to pooled hNF, we tested pilus-1-mediated Spn adherence to hNF from six individual healthy adult donors. Spn adhered to all tested hNF samples in a pilus-1-dependent manner (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 6D).

Human nasal fluid agglutinates pilus-expressing *S. pneumoniae*

Next, we examined whether hNF is able to agglutinate pilus-expressing bacteria. Indeed, we could visualize the formation of immune complexes of piliated Spn but not the pilus-deficient mutant (Fig. 7A). The pilus-deficient strain appeared mostly as diplococci and were 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 in a pilus-dependent manner (Fig. 7B).

Nasal mucus forms a highly heterogeneous network in which the mucins form heterotypic complexes with other mucosal proteins such as sIgA (42). We therefore wanted to check whether the composition and integrity of mucus are necessary for Spn binding, or if human sIgA alone mediates Spn binding. We centrifuged hNF to sediment large mucus particles and performed the solid-phase assay with the resulting supernatant. We observed that WT Spn adherence is reduced to a level comparable to that of the pilus-1-deficient mutant. (Fig 7C). The supernatant of centrifuged hNF still contains sIgA able to agglutinate piliated Spn. However, as shown in Fig. 6D, sIgA alone does not mediate Spn adherence. Our results suggest that the interaction between mucus particles and Spn, which has been agglutinated by sIgA, is necessary for Spn binding to hNF.

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, agglutination of 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 (i.n.) challenged with equal numbers of the piliated TIGR4 and its isogenic non-piliated mutant. Before administration, bacteria were pre-incubated 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 h and 22 h post-infection. In the absence of human sIgA, WT and the non-piliated mutant established equal levels of colonization (CI approximately equal to 1) (Fig. 8A and C). This indicated a minimal effect of pilus in early colonization in the murine model. When pre-incubated with human sIgA, which contains naturally-acquired antibody to pilus-1, pilus-expressing Spn were impaired in establishing colonization relative to the non-piliated mutant at 4 and 22 h post-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 (Fig. 8A). Additionally, sIgA was pre-treated with recombinant IgA1-protease to eliminate its multivalent binding. Protease cleavage, which was confirmed by Western analysis (Fig. 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_{α}-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 (Fig 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*, (Fig. 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 *S. pneumoniae*, 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 when compared to carriage in children from the same population.

To test this hypothesis, we analyzed genomic data produced from *S. pneumoniae* 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 adult. We excluded infants under six months of age because of maternal antibody. In a naive association, we find adults are less likely to be colonized by pilus-expressing strains, which is significant at the genome-wide level (Table 1 – OR = 2.23; $p = 2.0 \times 10^{-13}$). 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).

Discussion

We examined the interactions of a mucosal pathogen with mucus-containing human secretions. Our results first confirmed the binding of 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 are a major driving force of pneumococcal population dynamics (55). Our work gives an example of mechanism for this important form of selection, whereby immune exclusion of piliated strains mediated by sIgA would increase as population prevalence of pilus-1 increases.

Naturally-acquired immunoglobulin to other surface structures, including the main surface antigen capsular polysaccharide, is common in adults (30, 34, 54), but based on our

findings apparently does not contribute in a significant manner to the association of Spn with human nasal mucus. More than 70% of sIgA binding to Spn was attributable to pilus-1 (Figs. 2A, 5A). In contrast to other Spn surface components, the 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 sIgA against RrgB>>RrgA that had been generated through exposure during prior carriage events.

Mucosal sIgA in human nasal secretions consist of up to 90% of the subclass IgA1. Like many successful respiratory pathogens, *S. pneumoniae* expresses a protease with specificity for the hinge region of IgA1 to subvert the protective functions of sIgA1. Previous studies have shown that Spn cleaves anti-capsule IgA1/sIgA1 thereby abrogating the agglutinating and opsonophagocytic properties of sIgA1 (49, 56, 57) The long protrusion of the pilus could explain why there was no detectible effect of the Spn IgA1-protease in limiting immune exclusion. The protease is cell surface-associated where it might not access sIgA1 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 the host defense by protecting the underlying epithelium from invading pathogens. The protective function of sIgA is often attributed to blocking 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 non-expressing strains were equivalent, suggesting a human-specific benefit. Therefore, anti-pilus-1 sIgA does not act by impeding an adhesive function but rather through its agglutinating activity. Secretory IgA, which is polymeric (quadrivalent), is more effective at agglutinating piliated Spn compared to 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 anti-capsular 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 mucins via the mucin-like hinge region of IgA1 and/or though the hydrophilic secretory component (62). In this regard, secretory component ensures the appropriate localization of sIgA within the mucus and thereby contributes to sIgA-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 sIgA (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 *S. pneumoniae* by natural secretory immunoglobulin. Our observations also raise the question 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 sIgA/sIgM. Spn binding of secretory component attached to sIgA via PspC might limit Fab-dependent recognition that leads to agglutination and clearance.

S. pneumoniae colonization is predominantly observed 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 due to the presence of mucosal host defense molecules such as sIgA. In contrast to other immunoglobulins, sIgA 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 to 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 to 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 and 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 piliated strains in children suggests a colonization benefit conferred by pilus-1 in naive hosts that is diminished later in life, presumable due to 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 piliated 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 natural-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.

Material and Methods

Ethics statement

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

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 IgA (I4036), 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 goat anti-human IgA antibody (A-3063), FITC-conjugated goat anti-human IgG (F3512), goat anti-human kappa light chain antibody (K-0628), FITC-conjugated goat anti-rabbit IgG (F1262), 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 received from Amresco. Polyclonal rabbit anti-human lysozyme antibody (A0099) was purchased from Dako. FITC-labeled goat anti-human IgA1 (A18782) was obtained from Invitrogen. Allophycocyanin (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 (NBT/BCIP) solution, 4% paraformaldehyde solution (in PBS), and DMEM medium were received 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. Recombinant IgA-protease from *Neisseria gonorrhoeae* was obtained from MoBiTec. Bradford Reagent was received from Bio-Rad. 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% CO_2.

Recombinant *Escherichia coli* M15 strains were grown on LB agar plates supplemented with 100 µg/ml ampicillin 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,TIGR4, deficient for the genes *rrgA*, *rrgB*, *rrgC*, *rrgBC*, or *cbpA* were obtained by generating a PCR product containing a previously described insertion of the “sweet Janus” cassette (66). Deletion of *rrgA* and *rrgB* were 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 (TIGR4Δ*pilus-1*) followed by selection on TS agar plates supplemented with 2 µg/ml chloramphenicol and two rounds of back transformation. A pilus-insertion mutant of serotype 23F was obtained by

transformation of genomic DNA of strain P2535 (TIGR4Δ*rrgA::*Janus) followed by selection on TS agar plates containing 250 µg/ml kanamycin followed by two 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 the pilus-1 of the serotype 23F pneumococci was confirmed via dot blot as described above. The *pspA*-deficient mutants were obtained by transformation of chromosomal DNA from TIGR4Δ*pspA* (collection from Marc Lipsitch) or 23FΔ*pspA* (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).

In vitro proteolytic cleavage of human IgA1

Human sIgA and human nasal secretions were proteolytically cleaved with recombinant IgA-protease from *Neisseria gonorrhoeae*. The digestion occurred in reaction buffer (50mM Tris, 100 mM NaCl, 1mM EDTA, pH 7.5) for 20 h at 37°C with and enzyme to protein ratio of 1:50 (w/w). Cleavage of IgA was confirmed by western blot and anti-human IgA conjugated to alkaline phosphotase.

Human nasal secretions-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). To generate homogeneous samples, hNF samples were sonicated for 1 s with an amplitude of 10. In brief, nasal mucus or protease-treated mucus (10 µg/cavity) was immobilized in PBS in a 96-well flat-bottom plate (Sarstedt) followed by centrifugation at 250 × g for 3 min and incubation overnight at 37°C. The plates were gently washed three times with DMEM medium, and the wells were blocked with 0.1% BSA/DMEM for 2 h at room temperature. Spn cultures were grown to mid-log phase

(OD_{620} = 0.6) and diluted in DMEM. After washing the plate with DMEM a bacterial suspension of 2 x 10^4 bacteria in 100 µl was applied to the wells by centrifugation at 250 × g for 3 min at RT. Interaction with hNF was allowed for at least 2 h and up to 5 h at 30°C and 5% CO_2. Unbound bacteria were recovered by collecting the supernatant and gently washing the wells 19 times each with 100 µl DMEM. Adherent bacteria were lifted by adding 100 µl of 0.001% Triton X-100/PBS for 15 min at 30°C and 5% CO_2 followed by vigorous mixing. Unbound and adherent pneumococci were quantified by plating in triplicate on TS agar plates supplemented with appropriate antibiotics and incubated overnight at 30°C and 5% CO_2.

For protein cleavage and carbohydrate oxidation immobilized human mucus was treated for 30 min with 50 µg/ml and 100 µg/ml of trypsin (with and without protease inhibitor), or 100 mM sodium periodate ($NaIO_4$) in 50 mM sodium acetate buffer (pH 4.5), followed by blocking with 0.1% BSA/DMEM as described. Mild periodate concentrations (1 mM $NaIO_4$) leads to selective oxidation of terminal sialic acid whereas higher concentration of $NaIO_4$ (up to 100 mM) oxidizes vicinal hydroxyl groups of internal monosaccharides (70-72).

In inhibition assays pneumococci were pre-incubated with 5 µg/ml of anti-RrgA, anti-RrgB, and control serum, or increasing concentrations of sIgA (10-50 µg/ml), or serum IgA in a 2-fold molar ratio (related to 50 µg/ml sIgA) for 30 min at 37°C and 5% CO_2.

Flow cytometric analysis

The binding of soluble hNF, as well as purified sIgA, human serum IgA, lactoferrin, and human serum IgG to viable pneumococci was analyzed by using flow cytometry. In brief, 5 x 10^6 bacteria were incubated with either 50 µg/ml human nasal secretions, increasing concentrations of human sIgA (0 – 25 µg/ml), serum IgA in a molar ratio of 1:1 and 1:2 compared to 25 µg/ml sIgA, or increasing concentrations of lactoferrin (0 – 1.0 µg/ml) and serum IgG (0- 17.9 0 µg/ml) for 30 min at 37 °C and 5% CO_2 in 96-well plates (U-bottom; Greiner Bio-One). Likewise, Spn were also incubated with 12.5 µg/ml of proteolytically cleaved sIgA. After washing twice with 1%

BSA/PBS, binding of human proteins was detected using either FITC-labeled goat anti-human IgA1 (1:250), rabbit anti-human lactoferrin IgG (1:500), or FITC-conjugated goat anti-human IgG (1:250) followed by secondary APC-labeled anti-rabbit IgG (1:100). Antibody incubations occurred in 0.1 % BSA/PBS for 45 min at 4°C. Finally, bacteria were washed and fixed with 2% paraformaldehyde in PBS at RT for 1 h and fluorescence intensity was measured using a FACS LSR II flow cytometer and FACSDiva software 8.0.1 (Becton Dickinson) for data acquisition and analysis with FlowJo software 10.3. A gating region was set to exclude bacterial aggregates and debris. The results of human protein binding to pneumococci are shown as the percentage of labeled bacteria.

Heterologous expression and purification of recombinant RrgB protein

Purification of recombinant RrgB protein used in this study has previously been described (73). His_6-tagged protein was expressed in *E. coli* M15 after induction with 1 mM isopropyl β-d-thiogalactopyranoside for 4 h 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® purifier system according to the manufacturer's instructions (GE Healthcare). Protein was dialyzed (30 kDa molecular weight cut off) against PBS (pH 7.4) at 4°C using centrifugal filters (Millipore). Determination of the protein concentration was performed using the Bradford reagent and protein purity was confirmed by western blot.

ELISA

To determine anti-RrgB IgA titers in hNF, colostrum sIgA, 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 three 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 h at room temperature. Afterwards the washed wells were

incubated with 200 µg/ml of hNF, 25 µg/ml sIgA or serum IgA in PBS for 1 h 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 (Bar Harbor, ME), and bred and housed in a conventional animal facility as described (74). During colonization, all mice appeared healthy and did not experience any weight loss in comparison to uninfected controls. Spn strains were grown to an OD_{620} = 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 pre-incubated with either human sIgA (final concentration 0.9 mg/ml in PSB), human serum IgA, or PBS as control for 30 min at RT. Five-week old adult mice were inoculated intranasally without anesthesia with 15 µl containing 1.5 – 2 x 10^5 CFU of the mixed pneumococcal strains. At 4 h or 22 h post pneumococcal challenge, mice were euthanized using CO_2 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 h followed by the incubation with a primary rabbit anti-RrgB antibody (1:200000 in 0.1% BSA/0.01% Tween 20/PBS) overnight at 4°C. The membrane was washed three 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 h at room temperature. The colorimetric visualization of the pilus-expressing pneumococcal colonies occurred by use of NBT/BCIP as substrate. To determine the quantity of the non-piliated colonies, number of pilus-expressing colonies were subtracted from the total number of colonies. The competitive indices 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 duplicates from at least two independent colonization experiments to ensure reproducibility.

Agglutination assay and microscopic visualization

For agglutination, 5 x 10^4 bacteria were incubated with 10 µl of undiluted hNF or purified sIgA (which contained same titer of anti-RrgB IgA) for 2 h at 37°C and 5% CO_2. Samples were placed onto glass slides and immobilized via heat fixation. To visualize mucus, samples were incubated with 3% acetic acid followed by the incubation with alcian blue (in 3% acetic acid, pH 2.5) for 30 min. After washing in water for 10 min, 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 for brightness and contrast.

SDS-PAGE and Western blot analysis

Western blots were performed to detect proteinaceous components in hNF. 1 µg of purified lactoferrin, secretory IgA, or lysozyme, as well as pooled hNF were separated under denaturing conditions using a 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 the membrane with 2% BSA/PBS for at least 3 h and washing 3

times with PBS, 0.05% Tween 20, proteins were detected with a polyclonal rabbit anti-human lactoferrin antibody (1:10000), alkaline phosphatase-coupled goat anti-human IgA antibody (1:20000), or a rabbit anti-human lysozyme antibody (1:10000) overnight at 4°C followed by the incubation with an alkaline phosphatase-coupled goat anti-rabbit IgG (1:5000) for 1 h at RT. All antibody incubations occurred in 0.1% BSA/0.01% Tween 20/PBS. Protein bands were visualized after washing the membrane three times with 0.05% Tween 20/PBS using NBT/BCIP as substrate.

Association between pilus and age in a human population

We used genomic data from the Maela refugee camp (75). This consists of around 600 children and their mothers sampled every month from birth to two years of age, where 3000 randomly selected positive swabs have associated genomic data available (53). We gave each sample a binary outcome, mothers being positive and infants being negative; infants under six 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 one of the three *rrgB* alleles (COGs 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, which have been previously defined using a hidden Markov model (76).

We then used a generalized linear mixed model with a Bernoulli error structure and logit link function to test for an association between pilus presence and age. Pilus 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 a linear mixed model (LMM) with the genetic kinship between isolates as random effects, as in genome-

wide association studies (77, 78). We used the pyseer package (v1.2.0) in LMM mode, with the kinship/covariance matrix calculated from a neighbor-joining tree of all genome sequences in the cohort (53, 79).

Statistical analyses

Statistical analysis was performed using GraphPad Prism (version 7.01, Inc., SanDiego, CA). 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 one-way Anova with either Sidak's or Dunnett´s multiple comparison test. A p-value <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, 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: corresponding author and oversaw the project conception and design, data interpretation, and manuscript preparation, funding acquisition.

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Data availability

Data and code to perform the association between pilus and age can be found on github: https://github.com/johnlees/pilus-age

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Figures

Figure 1

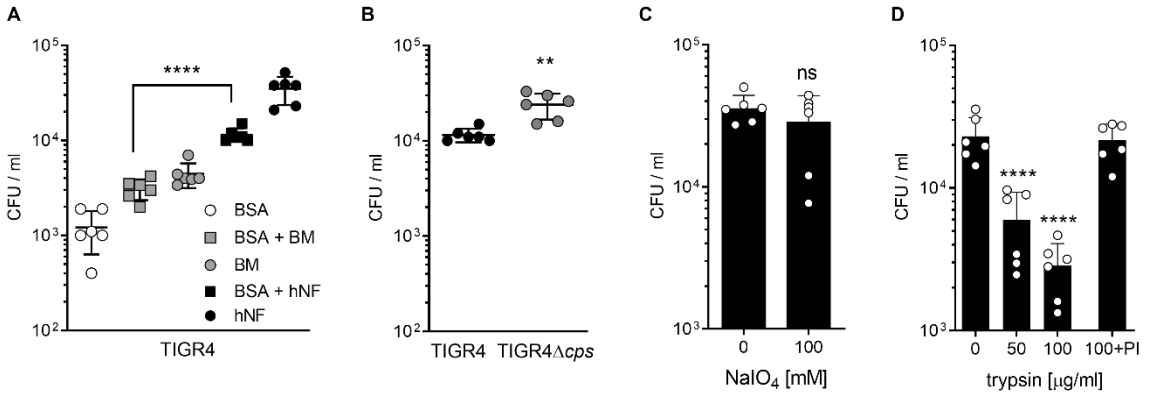

Figure 1: Mucosal protein-mediated binding of *S. pneumoniae* to human nasal fluid.
(A-D) Adherence of Spn TIGR4 to human nasal fluid (hNF) was analyzed in a solid-phase assay. **(A)** Bacteria (1 x 10^4) in 100 µl DMEM were incubated with 10 µg immobilized bovine submaxillary mucus (BM) or hNF in presence or absence of 0.1 % BSA for 2 h at 30°C. Bound bacteria were determined by resuspending with 0.001% Triton X-100 following plating on TS agar plates supplemented with 200 µg/ml streptomycin. **(B)** Adherence of TIGR4 and TIGR4Δ*cps* (each 1 x 10^4/ 100 µl) to hNF. **(C)** Treatment of immobilized hNF with 100 mM $NaIO_4$ in 50 mM sodium acetate buffer for 30 min at 4°C in the dark followed by blocking with 0.1% BSA and incubation with 2 x 10^4 Spn TIGR4. **(D)** Immobilized hNF was incubated with increasing concentrations of trypsin with or without protease inhibitor (PI) for 30 min at 37°C followed by the incubation of 0.1% BSA and 2 x 10^4 Spn TIGR4 in 100 µl DMEM for 2 h at 30°C. Experiments were performed in duplicates and mean values of three independent experiments are shown with error bars corresponding to S.D. **,p<0.01; ****,p<0.0001 by two-tailed unpaired t-test, *n* = 6 **(A, B, D)** or 1-way ANOVA followed by Dunnett´s multiple comparison, *n* = 6 **(C)**.

Figure 2

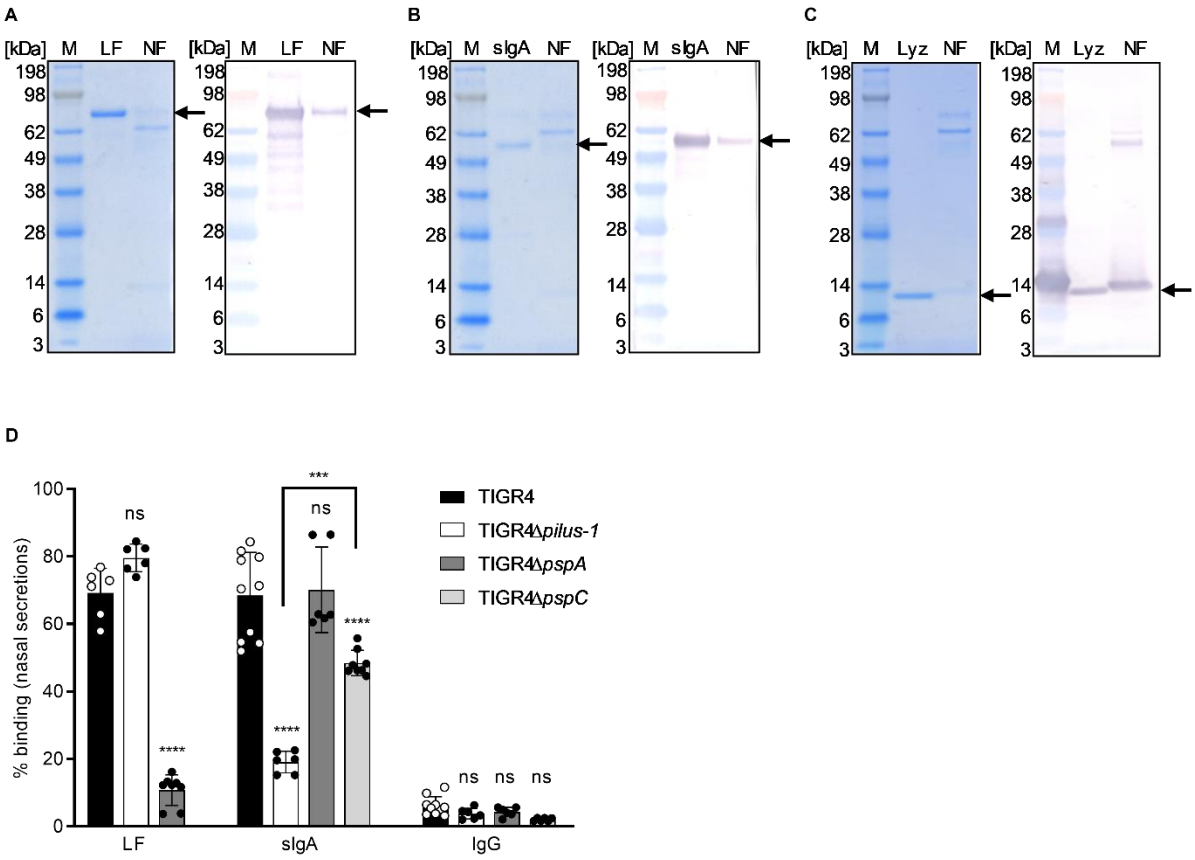

Figure 2: Major pneumococcal surface proteins interacting with human mucus proteins. **(A-C)** Gradient SDS-PAGE (4-12%) and Western blot of purified mucosal proteins lactoferrin (LF), sIgA, lysozyme (Lyz), and human nasal fluid (each 1 µg). Proteins in hNF were detected using a specific polyclonal rabbit anti-human lactoferrin antibody **(A)**, an alkaline phosphatase-coupled goat anti-human IgA antibody **(B)**, or a rabbit anti-human lysozyme antibody **(C)**. Protein band at 62 kDa likely represents albumin. Lane M, SeeBlue Plus2 Pre-Stained Standard. **(D)** Acquisition of soluble LF, sIgA, and IgG from human nasal fluid by Spn. Bacteria (5 x 10^6 CFU/ 50 µl) were incubated with 50 µg/ml of hNF. Binding of bacteria-associated LF, sIgA, and IgG was analyzed by flow cytometry. The percentage binding of at least three independent experiments is shown as mean values with error bars ± S.D. ***,p<0.001; ****,p<0.0001 by 1-way ANOVA followed by Tukey´s multiple comparison, *n* = 6 to 10.

Figure 3

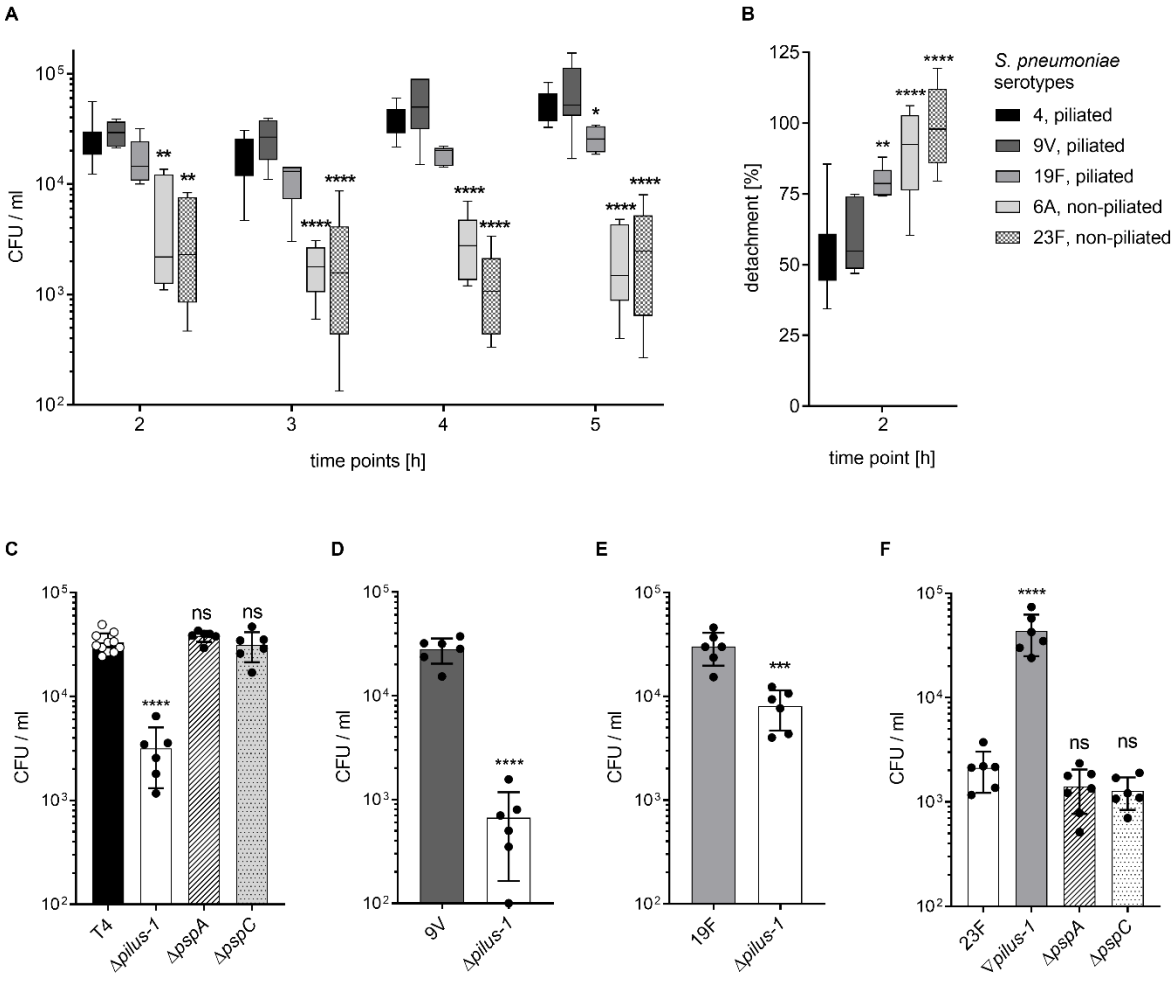

Figure 3: Pilus-1-mediated adherence of *S. pneumoniae* to human nasal fluid.
(A and B) Adherence to and detachment from hNF using pilus-1-expressing [T4 (TIGR4), 9V, 19F] and non-piliated (6A, 23F) clinical isolates. **(A)** Adherence of Spn (2 x 10^4/ 100 µl) was observed over a time period of 5 h. *n* = 6 to 12 **(B)** Detachment (unbound bacteria recovered after 19 washing steps) is calculated as the percentage of the total CFU per well after 2 h incubation. *n* = 6 to 12 **(C-F)** Adherence of wild type Spn and isogenic mutants after 2 h incubation with immobilized hNF. *n* = 6 to 11. Experiments were performed in duplicates and values of at least three independent experiments are shown in box and whiskers columns including minimal and maximal value **(A and B)** or as bar graphs ± S.D. *,p<0.05; **,p<0.01; ***,p<0.001; ****,p<0.0001 by 1-way ANOVA followed by Sidak´s multiple comparison vs. T4 **(A)** or Dunnett´s multiple comparison **(B,C,F)**, or two-tailed unpaired t-test **(D, E)** vs. T4 or wild type.

Figure 4

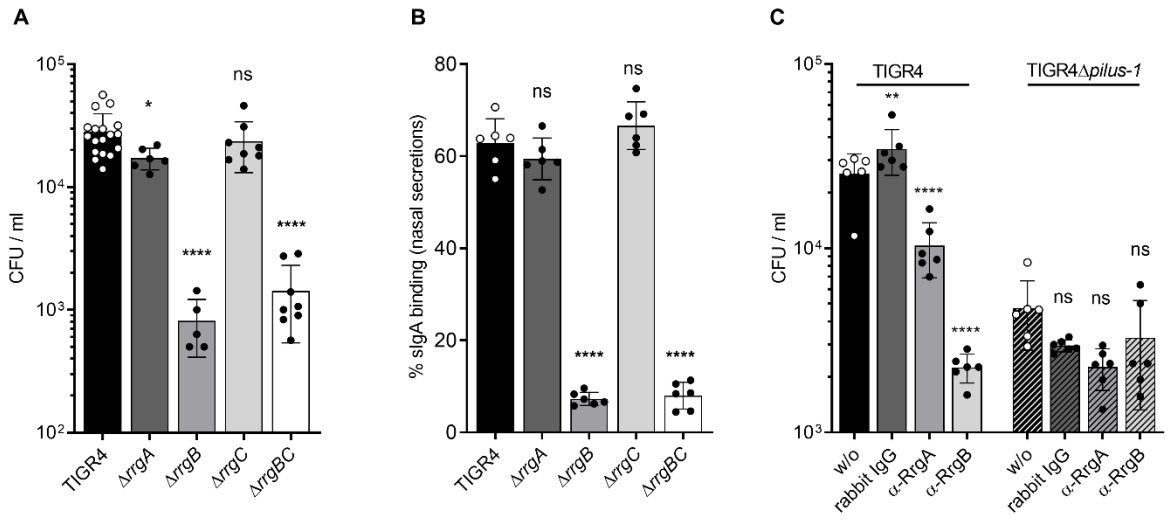

Figure 4: Pilus-1 component RrgB mediates pneumococcal adherence and binding to sIgA. (A) Adherence of WT Spn an isogenic mutants, deficient for one or two pilus-1 components to hNF was assessed in a solid-phase assay. Each bacteria (1 x 10^4 / 100 µl DMEM) were incubated with 10 µg hNF in presence of 0.1 % BSA for 2 h at 30°C. Bound bacteria were determined by resuspending with 0.001% Triton X-100 following plating on TS agar plates supplemented with 200 µg/ml streptomycin. *n* = 5 to 18 **(B)** Flow cytometric analysis of sIgA-binding from soluble hNF by WT Spn and isogenic mutants. Bacteria (5 x 10^6 CFU/ 50 µl) were incubated with 50 µg/ml of hNF. Binding of surface associated sIgA was analyzed using a FITC-labeled goat anti-human IgA1 antibody and is shown as the percentage binding. *n* = 6 **(A and B)** Results of at least three independent experiments are illustrated as mean values ± S.D. *,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 pre-treated with each 5 µg/ml of rabbit control serum, anti-RrgA, or anti-RrgB antiserum before incubation with immobilized hNF for 2 h at 30°C. Detection of bound bacteria was analyzed as described in **(A)**. *n* = 6. Results of three independent experiments are illustrated as mean values ± S.D. **,p<0.01; ****,p<0.0001 vs. w/o by 1-way ANOVA followed by Sidak´s multiple comparison.

Figure 5

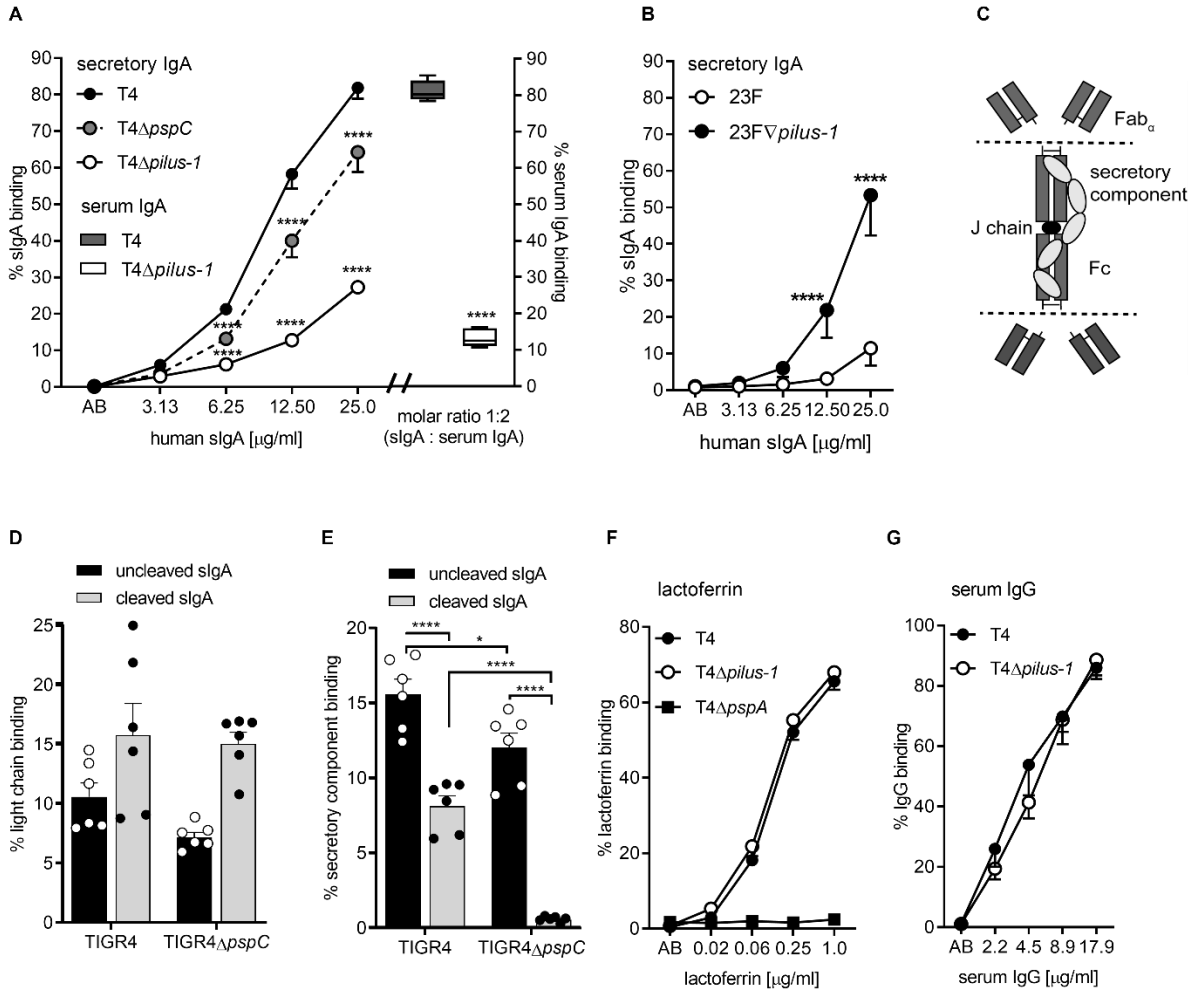

Figure 5: Fab-mediated binding of sIgA to pneumococcal type-1 pilus.
(A and B) Concentration-dependent binding of soluble purified sIgA to Spn. Acquisition of sIgA and serum IgA (2-fold molar ratio compared to 25 µg/ml sIgA) to type 4 Spn and isogenic mutants **(A)**, or type 23F Spn and isogenic pilus-1-knock in mutant **(B)** was measured by flow cytometry using a specific FITC-labeled goat anti-human IgA1 antibody. The percentage binding of at least three independent experiments is shown as mean values with error bars ± S.D. ***,p<0.001; ****,p<0.0001 vs. wild type by 2-way ANOVA followed by Tukey´s multiple comparison. *n* = 6 to 8. **(C)** Schematic model of cleaved sIgA treated with recombinant IgA1-protease (dashed line), generating Fab$_\alpha$-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 kappa light chain antibody **(D)** or a monoclonal anti-secretory component antibody **(E)**. Results of three independent experiments are illustrated as mean values with error bars ± S.D. *,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 three independent experiments are shown as mean values with error bars ± S.D. *n* = 6.

Figure 6

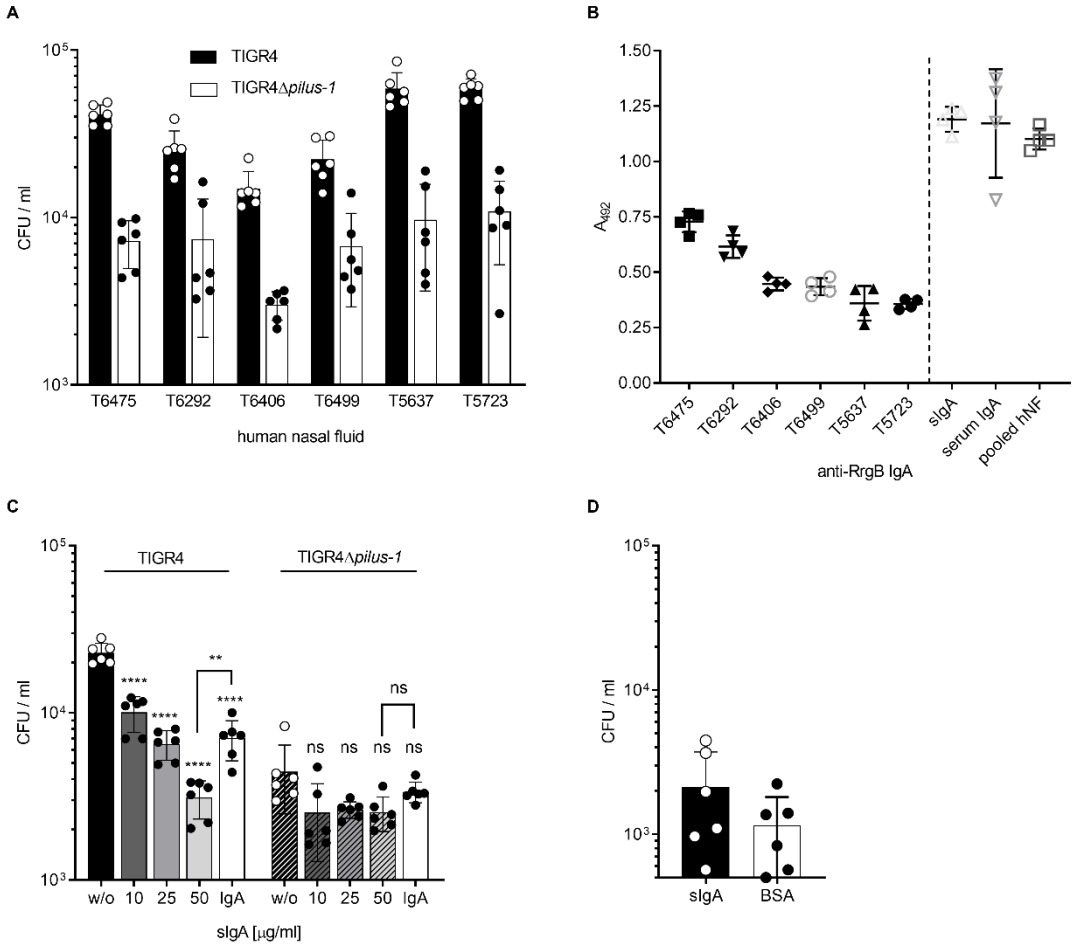

Figure 6: Secretory IgA is necessary but not sufficient for pneumococcal adherence to human nasal fluid. (A) Adherence of Spn TIGR4 and isogenic *pilus-1*-deficient mutant to six hNF from individual donors was assessed in a solid-phase assay. Bacteria (1 x 10^4/100 µl DMEM) were incubated with 10 µg immobilized hNF in presence of 0.1 % BSA for 2 h 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), 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 ± S.D. of two 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 to 50 µg/ml sIgA). Bacteria were pre-treated with either sIgA or serum IgA for 30 min at 37°C before incubation with immobilized pooled hNF for 2 h at 30°C. *n* = 6. **(D)** Binding of wild type 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 1 x 10^4/100 µl bacteria for 2h at 30°C. *n* = 6. **(A-C)** Experiments were performed in duplicates and mean values of three independent experiments are shown with error bars corresponding to S.D. **,p<0.01; ****,p<0.0001 by 1-way ANOVA followed by Sidak´s multiple comparison **(C)**.

Figure 7

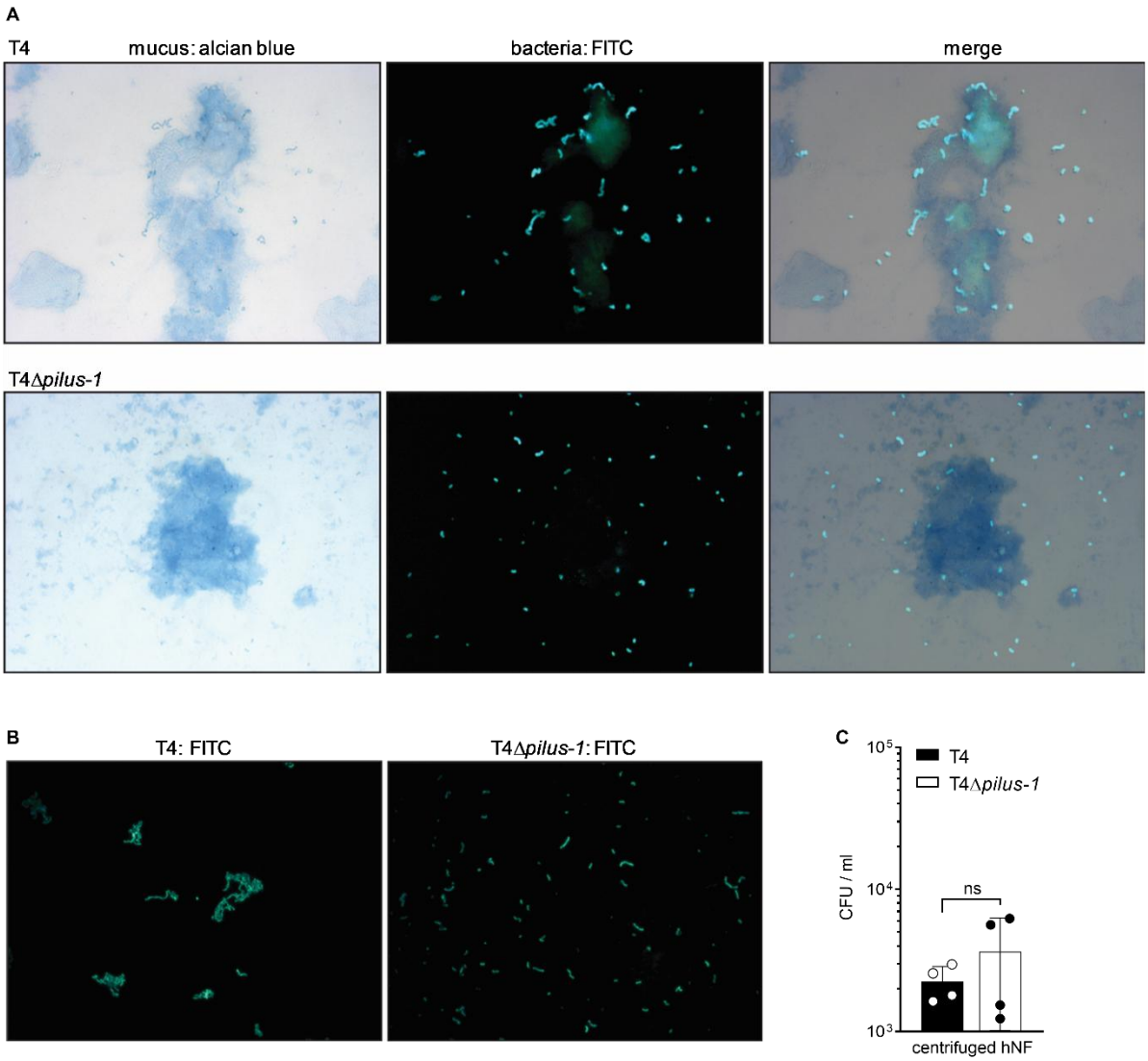

Figure 7: Human nasal fluid agglutinates pneumococci in a pilus-dependent manner.
(A and B) WT Spn and the *pilus-1*-deficient mutant were incubated with hNF **(A)** or purified human sIgA **(B)** for 2 h at 37°C and 5% CO_2. Mucus (blue) was stained with alcian blue and bacteria (green) were detected using rabbit anti-capsule antibody and secondary FITC-coupled goat anti-rabbit IgG. Agglutination was visualized by microscopy on an Axiovert 40 CFL microscope equipped with an Axiocam IC digital camera. **(C)** Adherence of Spn to supernatant of centrifuged hNF. Before immobilization, hNF was centrifuged for 5 min at 10000 xg. *n* = 4.

Figure 8

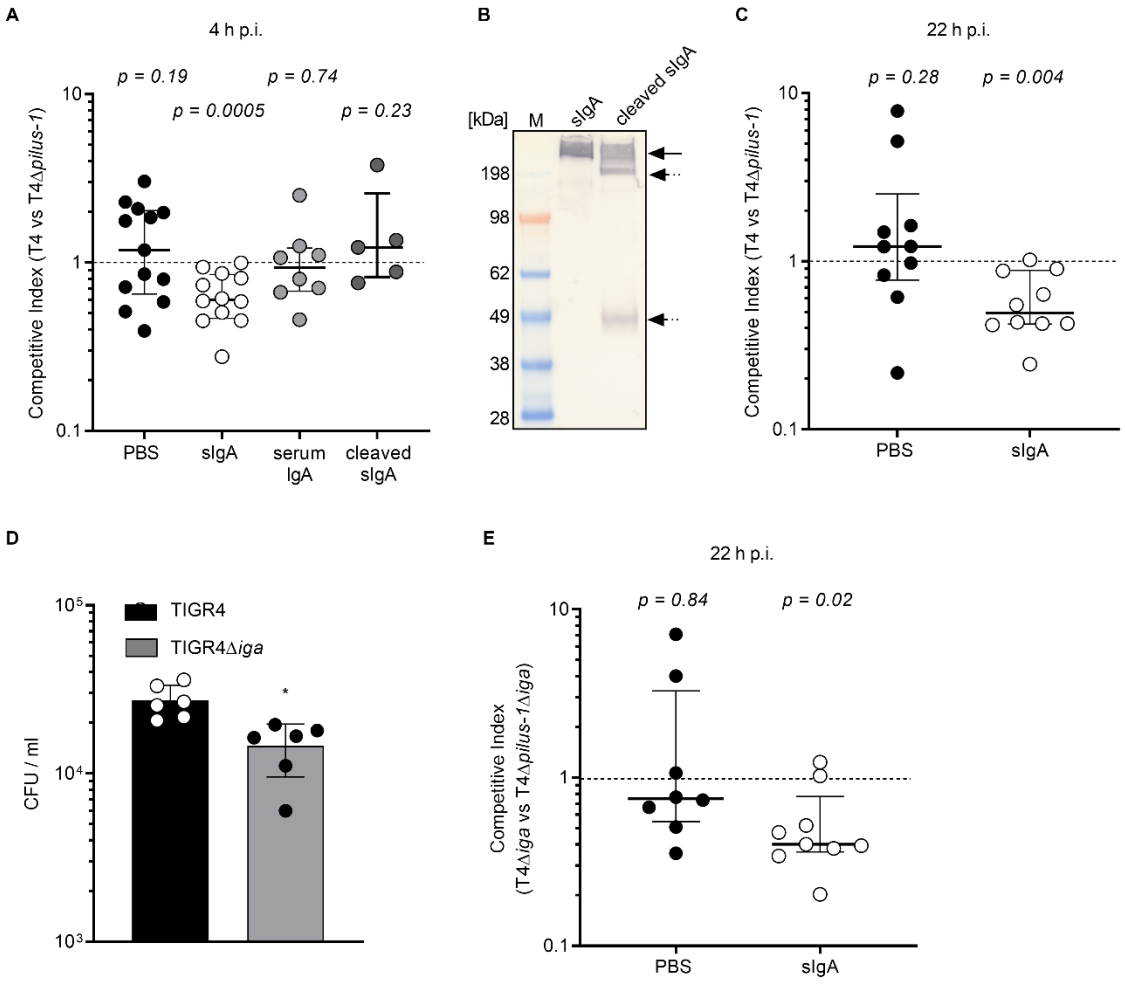

Figure 8: Immune exclusion by pilus-1 specific sIgA.
(A and B) Adult mice were intranasally (i.n.) infected with a suspension containing equal amounts of TIGR4 and isogenic *pilus-1*-deficient mutant. Before administration bacteria were either pre-incubated with sIgA, serum IgA (equal anti-RrgB titer compared to sIgA), cleaved sIgA, or PBS. Colonization density and competitive index (CI) was assessed 4 h **(A)** and 22 h **(C)** p.i. by culture of URT lavages followed by colony immunoblot using an anti-RrgB antibody. *n* = 5 to 13. **(B)** Before administration, sIgA was cleaved with recombinant IgA1-protease for 20h at 37°C. Cleavage was visualized on a denaturing and non-reducing SDS-PAGE and Western Blot. Solid arrow indicates uncleaved sIgA [400kDa] and dashed arrows indicate cleaved sIgA1 [200kDa and 50kDa]. (**D**) Adherence of Spn TIGR4 and isogenic *iga*-deficient mutant to hNF was analyzed in a solid-phase assay as described before. Results are shown as mean values of three independent experiments are shown with error bars corresponding to S.D. *,p<0.05 by two-tailed unpaired t-test. *n* = 6. **(E)** Adult mice were i.n. challenged with mutants lacking the IgA1-protease. CI was determined as described in **(C)**. *n* = 8 to 9**. (A, C, E)** Experiments were repeated twice and groups represent n = 5 –13 animals with median ± interquartile range. Dotted line represents CI = 1. Group medians were compared to a CI = 1 by Wilcoxon signed rank test and resulting p-values are indicated.

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

	Non-piliated	Piliated	[%] piliated
Infants (6-24 months)	1058	598	56.5
Mothers	438	79	18.0

Table 2: Bacterial strains and plasmids used for the study.

Strain or plasmid	Sero- and genotype	Antibiotic resisance	Source or reference
Streptococcus pneumoniae			
P2406 (TIGR4)	4, clinical isolate	$Strep^r$	(80)
P2422	TIGR4Δ*cps*	Kan^r	(80)
P2454	TIGR4Δ*pilus-1* (Δ*pilus-1::Cm*)	$Strep^r$, Cm^r	(80)
P2542	TIGR4Δ*rrgA*	$Strep^r$	This study
P2592	TIGR4Δ*rrgB*	$Strep^r$	This study
P2593	TIGR4Δ*rrgC*	$Strep^r$	This study
P2594	TIGR4Δ*rrgBC*	$Strep^r$	This study
P2502	TIGR4Δ*pspA*	Kan^r	This study
P2583	TIGR4Δ*pspC*	$Strep^r$	This study
P2615	TIGR4Δ*iga*	$Strep^r$, $Spec^r$	This study
P21/ P2568	9V, clinical blood isolate		This study
P2569	9VΔ*pilus-1* (Δ*pilus-1::Cm*)	Cm^r	This study
P1860	19F, clinical isolate		This study
P2572	19FΔ*pilus-1* (Δ*pilus-1::Cm*)	Cm^r	This study
P2499	23F, clinical isolate	$Strep^r$	(81)
P2588	23F∇*pilus-1* (pilus-1 insertion)	$Strep^r$	This study
P2617	23F∇*pilus-1*Δ*iga*	$Strep^r$, Kan^r	This study
P2618	23FΔ*pspA* (*pspA::Spec*)	$Strep^r$, $Spec^r$	This study
P2625	23FΔ*pspC*	Kan^r	This study
P1547	6A, clinical isolate		(68)
Escherichia coli			
M15 (pREP4)	F-, Φ80ΔlacM15, thi, lac^-, mtl^-, $recA^+$, Kan^r	Kan^r	Qiagen
Plasmids			
pQE30-rrgB	His_6-tagged wild-type *rrgB* gene	Amp^r	(73)

Table 3: Primers used for the study.

Gene target	Primer name	Sequence (5'→3')
P2535: *rrgA::Janus-cassette*	*rrgA* upstream forward	CGTGTATACAGATTGAAAGTACCTATGAATC
	rrgA upstream reverse	CACATTATCCATTAAAAATCAAACGGAAAGCATTATTCATAGAAC
	rrgA Janus forward	GTTCTATGAATAATGCTTTCCGTTTGATTTTTAATGGATAATGTG
	rrgA Janus reverse	CATTTCTACACTTTACGGTTATGCTTTTGGACGTTTAGTACC
	rrgA downstream forward	GGTACTAAACGTCCAAAAGCATAACCGTAAAGTGTAGAAATG
	rrgA downstream reverse	GCTACTTCTTAAAAGTATCGTTCAGAAC
P2542: clean deletion *rrgA*	*rrgA* upstream forward	CGTGTATACAGATTGAAAGTACCTATGAATC
	rrgA upstream reverse	ATCATTTCTACACTTTACGGATGTTTCCGCTCCCTGTTAAGCATTATTC-ATAGAACAAG
	rrgA downstream forward	CTTGTTCTATGAATAATGCTTAACAGGGAGCGGAAACATCCGTAAAGT-GTAGAAATGAT
	rrgA downstream reverse	GCTACTTCTTAAAAGTATCGTTCAGAAC
P2589: *rrgB::Janus-cassette*	*rrgB* upstream forward	GTGCTCTATGATACGACTGAGAAAAGGATTCG
	rrgB upstream reverse	TCAAACGGATCCGATCCATTTCCTCTTTCATGATTTC
	rrgB Janus forward	GAAATCATGAAAGAGGAAATGGATCGGATCCGTTTGA
	rrgB Janus reverse	CTCTTACTTAAGCGGGCCCCTTTCCTTATGC
	rrgB downstream forward	GCATAAGGAAAGGGGCCCGCTTAAGTAAGAG
	rrgB downstream reverse	CATACTCTAGACCTTCCACTCGGAAACGAC
P2592: clean deletion *rrgB*	*rrgB* upstream forward	GTGCTCTATGATACGACTGAGAAAAGGATTCG
	rrgB upstream reverse	CTCTCTTACTTAAGCAAGTTGATCGTTGATTGATTTCATGATTTCTCC
	rrgB downstream forward	GGAGAAATCATGAAATCAATCAACGATCAACTTGCTTAAGTAAGAGAG
	rrgB downstream reverse	CATACTCTAGACCTTCCACTCGGAAACGAC
P2590: *rrgC::Janus-cassette*	*rrgC* upstream forward	GCAACGTTCGATTTGGTTAATGCTCAGACTGG
	rrgC upstream reverse	CAAACGGATCCGATCCATTTCCTCAATCATTTTCTGC
	rrgC Janus forward	GCAGAAAATGATTGAGGAAATGGATCGGATCCGTTTG
	rrgC Janus reverse	CATTGAATATCAGTTGGGCCCCTTTCCTTATGC
	rrgC downstream forward	GCATAAGGAAAGGGGCCCAACTGATATTCAATG
	rrgC downstream reverse	GGTCACATAATCATGACCTGGTACAATCAATAAATC
P2593: clean deletion *rrgC*	*rrgC* upstream forward	GCAACGTTCGATTTGGTTAATGCTCAGACTGG
	rrgC upstream reverse	CATTGAATATCAGTTATTTGGTTTGATACGACTAATCATTTTCTGCAT
	rrgC downstream forward	ATGCAGAAAATGATTAGTCGTATCAAACCAAATAACTGATATTCAATG
	rrgC downstream reverse	GGTCACATAATCATGACCTGGTACAATCAATAAATC
P2591: *rrgBC::Janus-cassette*	*rrgBC* upstream forward	GTGCTCTATGATACGACTGAGAAAAGGATTCG
	rrgBC upstream reverse	TCAAACGGATCCGATCCATTTCCTCTTTCATGATTTC
	rrgBC Janus forward	GAAATCATGAAAGAGGAAATGGATCGGATCCGTTTGA
	rrgBC Janus reverse	CATTGAATATCAGTTGGGCCCCTTTCCTTATGC
	rrgBC downstream forward	GCATAAGGAAAGGGGCCCAACTGATATTCAATG
	rrgB C downstream reverse	GGTCACATAATCATGACCTGGTACAATCAATAAATC
P2594: clean deletion *rrgB* and *rrgC*	*rrgBC* upstream forward	GTGCTCTATGATACGACTGAGAAAAGGATTCG
	rrgBC upstream reverse	CATTGAATATCAGTTATTTGGTTTGTTGATTGATTTCATGATTTCTCC
	rrgBC downstream forward	GGAGAAATCATGAAATCAATCAACAAACCAAATAACTGATATTCAATG
	rrgBC downstream reverse	GGTCACATAATCATGACCTGGTACAATCAATAAATC
P2582: *pspC::Janus-cassette*	*pspC* upstream forward	GGGCATTGGATAAGGTTTTGACAAACCTG
	pspC upstream reverse	CACATTATCCATTAAAAATCAAACGGAAAACATGTTTATTTCC
	pspC Janus forward	GGAAATAAACATGTTTTCCGTTTGATTTTTAATGGATAATGTG
	pspC Janus reverse	GTTATATTAGGTTTAGTTCCAGAGACCTGGGCCCCTTTCCTTATGC
	pspC downstream forward	GCATAAGGAAAGGGGCCCAGGTCTCTGGAACTAAACCTAATATAAC
	pspC downstream reverse	CGATATCGTCGATATCAACATGGGCTG
P2583: clean deletion *pspC*	*pspC* upstream forward	GGGCATTGGATAAGGTTTTGACAAACCTG
	pspC upstream reverse	TTAGGTTTAGTTTACCCATTCTTTTGATGCAAACATGTTTATTTCC
	pspC downstream forward	GGAAATAAACATGTTTGCATCAAAAGAATGGGTAAACTAAACCTAA
	pspC downstream reverse	CGATATCGTCGATATCAACATGGGCTG
Presence of rlrA pathogenicity island	RrgB_seq_forw	CGAAAACTTGCACAGAAAAAGGATTATTATTGTC
	RrgB_seq_rev1	GCTTTGGAGTATTCCCGTGATCTGG
Absence of rlrA pathogenicity island	Pilus1-neg_Forw	CGCCTTGGATGCATTGAGC
	Pilus1-neg_Rev	GTATTACAAGATATTATTTCACC

Changes made in the manuscript after acceptance

Changes	Page	Line
Address corresponding author	1	15-16
Study approval" section added	26	631-633
Figure legends, exact number of samples added	36-43 And supplemental figure 5	
Figures reformatted	36-43	