ORIGINAL ARTICLE

Density and Duration of Pneumococcal Carriage Is Maintained by Transforming Growth Factor β1 and T Regulatory Cells

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Abstract

Rationale: Nasopharyngeal carriage of *Streptococcus pneumoniae* is a prerequisite for invasive disease, but the majority of carriage episodes are asymptomatic and self-resolving. Interactions determining the development of carriage versus invasive disease are poorly understood but will influence the effectiveness of vaccines or therapeutics that disrupt nasal colonization.

Objectives: We sought to elucidate immunological mechanisms underlying noninvasive pneumococcal nasopharyngeal carriage.

Methods: Pneumococcal interactions with human nasopharyngeal and bronchial fibroblasts and epithelial cells were investigated *in vitro*. A murine model of nasopharyngeal carriage and an experimental human pneumococcal challenge model were used to characterize immune responses in the airways during carriage.

Measurements and Main Results: We describe the previously unknown immunological basis of noninvasive carriage and highlight mechanisms whose perturbation may lead to invasive disease. We identify the induction of active transforming growth factor (TGF)-β1 by *S. pneumoniae* in human host cells and highlight the key role for TGF-β1 and T regulatory cells in the establishment and maintenance of nasopharyngeal carriage in mice and humans. We identify the ability of pneumococci to drive TGF-β1 production from nasopharyngeal cells *in vivo* and show that an immune tolerance profile, characterized by elevated TGF-β1 and high nasopharyngeal T regulatory cell numbers, is crucial for prolonged carriage of pneumococci. Blockade of TGF-β1 signaling prevents prolonged carriage and leads to clearance of pneumococci from the nasopharynx.

Conclusions: These data explain the mechanisms by which *S. pneumoniae* colonize the human nasopharynx without inducing damaging host inflammation and provide insight into the role of bacterial and host constituents that allow and maintain carriage.

Keywords: *Streptococcus pneumoniae*; nasopharynx; immune tolerance; T-cell regulation; host–pathogen interactions

At a Glance Commentary

Scientific Knowledge on the Subject: The immunological mechanisms underpinning nasopharyngeal carriage with *Streptococcus pneumoniae* are not fully understood but are crucial to deciphering why and how carriage may lead to invasive disease.

What This Study Adds to the Field: *S. pneumoniae* induces an immunoregulatory response in the naso-oropharynx, characterized by high levels of transforming growth factor β1 and T regulatory cell infiltration, which is conducive to long-term noninvasive carriage and beneficial to both pathogen and host. The effects of vaccination strategies on immune responses in carriage must be reconsidered.

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Streptococcus pneumoniae (the pneumococcus) is an important human pathogen that accounts for significant morbidity and mortality worldwide, causing a range of invasive diseases, including pneumonia, meningitis, and sepsis. The pneumococcus is also a commensal of the human nasopharynx, and colonization of this important ecological niche is thought to be a prerequisite for invasive disease (1, 2). Where carriage rates are highest, risk of invasive pneumococcal disease is also high (3, 4). However, the actual incidence of progression from carriage to invasive disease (the attack rate) is thought to be less than 1 in 10,000 (5), suggesting that the occurrence of invasive disease is the exception rather than the norm. The contributory factors that lead to invasive disease in the minority of such carriage events are not known, but the host immune system and its interactions with colonizing pneumococci is certainly one aspect (3).

Transforming growth factor (TGF)-β1 has a crucial immunosuppressive role in innate and adaptive immunity (6). TGF-β1 activity limits proinflammatory responses and promotes tissue healing and remodeling after damage (6), and activation can occur in response to damage to the epithelial layer (7). We have demonstrated the importance of TGF-β1 in invasive pneumococcal infections, where inhibition of TGF-β1 markedly increased susceptibility to invasive pneumonia in normally disease-resistant mice (8). The importance of TGF-β1 in the context of pneumococcal infection of the lung relates to its ability to drive T regulatory cell differentiation and expansion, and the correlation of high TGF-β1 levels and T regulatory cell numbers with reduced lung apoptosis suggests that immune regulation plays a key role in preventing bacterial dissemination by limiting inflammatory damage to the lung epithelial barrier (8).

Correlative evidence suggests T regulatory cells also play a role in nasopharyngeal colonization: children with pneumococcus positive nasopharyngeal swabs contained higher proportions of T regulatory cells in adenoidal tissue than children with pneumococcus negative cultures (9). We sought to elucidate the host–pathogen interactions underpinning asymptomatic nasopharyngeal carriage. Identifying the immune phenotype in the carrier state will aid our understanding of how this equilibrium is disturbed in invasive disease.

Some of the results of these studies have been previously reported in the form of an abstract (10).

Methods

Ethics Statement

The UK Home Office and University of Liverpool ethics committee approved the study. Animal experiments were performed at the University of Liverpool. Ethical approval for human experimental pneumococcal challenge was obtained from the National Health Service Research Ethics Committee (08/H1001/52 and 11/NW/0592).

Mice

Female MF1 mice (9–14 wk of age) (Charles River, Margate, UK) were acclimatized for 1 week before use.

Bacteria

Mouse-virulent serotype 2 *Streptococcus pneumoniae* strain D39 (NCTC 7466) or its isogenic pneumolysin (Ply)-deficient mutant PLN-A were used throughout the experiment. Infectious doses were prepared as described previously (11). Samples from experimental human pneumococcal challenge were taken from patients challenged with serotype 6B pneumococci (12).

Pneumolysin

Recombinant Ply was expressed in *Escherichia coli* and purified as previously described (13). Additional details are provided in the online supplement.

Infection of Mice

Animals were infected intranasally with 1×10^5 colony-forming units (CFU) (low-dose) *S. pneumoniae* D39 or PLN-A or with 1×10^7 CFU (high-dose) D39 in 10 μl PBS as previously described (14). Mice were culled by cervical dislocation, and nasopharynx and lungs were prepared for assessment of bacterial CFU or leukocytes. A viable count of bacteria in murine tissue was determined at prechosen intervals as previously described (14). Colonization for 48 hours or more was considered a carriage event. Additional details are provided in the online supplement.

P17 Treatment of Mice

Animals were infected intranasally with 1×10^5 CFU *S. pneumoniae* D39 and then immediately administered 500 μg of the peptide TGF-β1 inhibitor P17 (15) or PBS by intraperitoneal injection. Further doses of P17 or PBS were given at 1, 3, and 4 days after infection. This treatment regime was chosen to inhibit infection-induced TGF-β1 production without appreciably altering homeostatic TGF-β1 levels in the nasopharynx.

Experimental Human Pneumococcal Challenge Model and Nasal Wash

Healthy adults with no respiratory disease and no natural carriage were recruited. Pneumococci were instilled in 100 μl saline into each naris. Nasal wash was performed at 48 hours after inoculation (12, 16). The presence of carriage was assessed by serial dilution of nasal wash onto blood agar. Additional details are provided in the online supplement.

Flow Cytometry

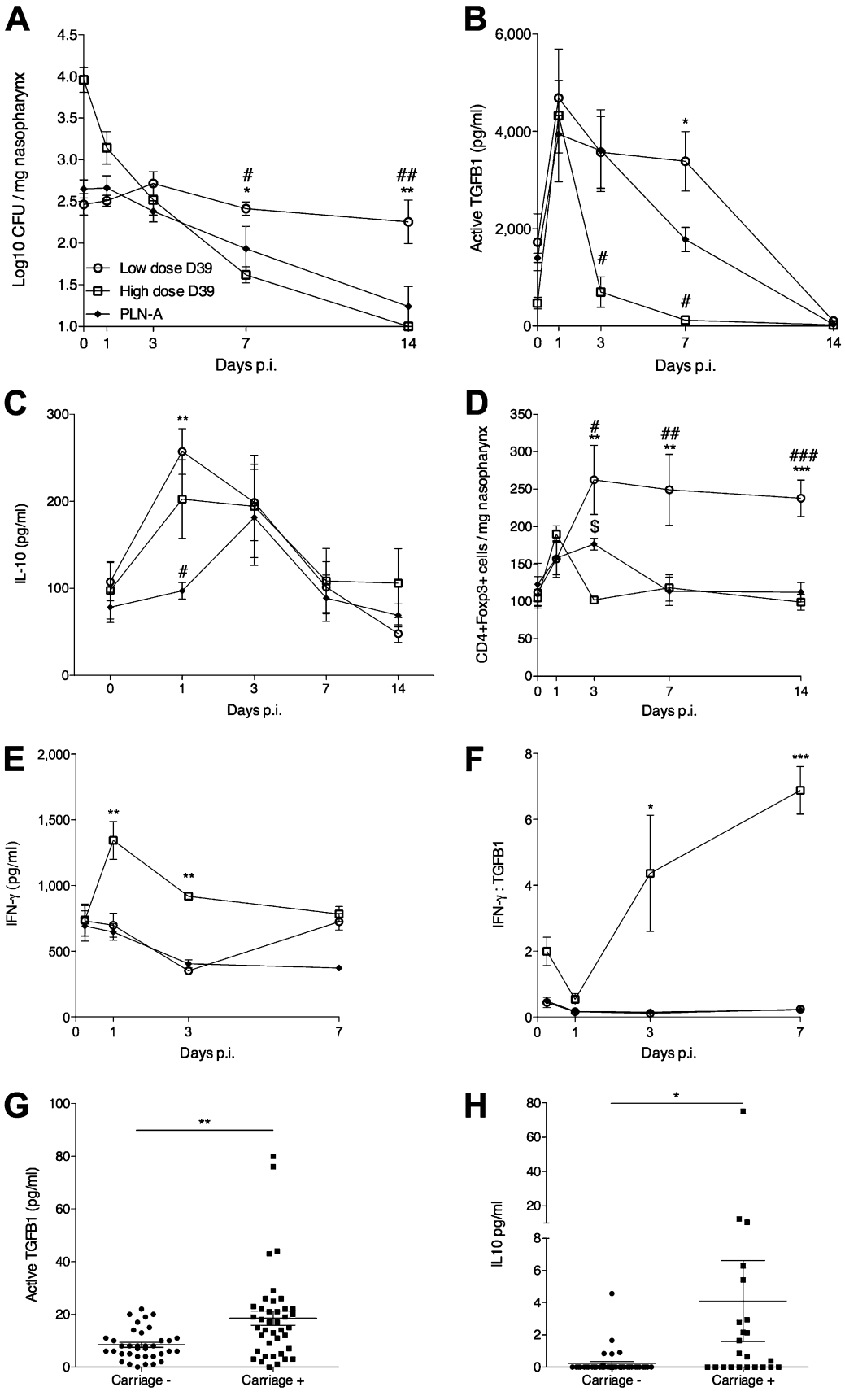
Tissue cell suspensions were stained for surface markers or transcription factors as previously described (8). Samples were analyzed using a FACScalibur flow cytometer (Becton Dickinson, Oxford, UK) running CellQuest acquisition and analyzed using FlowJo (version 8.8.3; Tree Star, Ashland, OR). Additional details are provided in the online supplement.

Cell Culture

The nasopharyngeal cell line Detroit 562 (NE), immortalized human bronchial epithelial cells (J.W. Shay, University of Texas, Dallas, TX) (17), primary human bronchial fibroblasts (C.A. Feghali-Bostwick, University of Pittsburgh School of Medicine, Pittsburgh, PA) (18), and human nasopharyngeal fibroblasts (Institut d'Investigacions Biomèdiques August Pi I Sunyer and Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias, Barcelona, Spain) (19) were cultured as described in the online supplement. Cells were grown to confluence before addition of Ply or live or heat-killed D39. After 24 hours, culture supernatants were collected and stored at −80°C.

MTT Assay

Cell toxicity was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium


A
Log10 CFU / mg nasopharynx
Low dose D39
High dose D39
PLN-A
Days p.i.
B
Active TGFB1 (pg/ml)
Days p.i.
C
IL-10 (pg/ml)
Days p.i.
D
CD4+Foxp3+ cells / mg nasopharynx
Days p.i.
E
IFN-γ (pg/ml)
Days p.i.
F
IFN-γ : TGFB1
Days p.i.
G
Active TGFB1 (pg/ml)
Carriage -
Carriage +
H
IL10 pg/ml
Carriage -
Carriage +

bromide (MTT) assay. Before the assay, culture supernatants were centrifuged at 13,000 × *g* for 2 minutes, and the supernatant was passed through a 0.2-μm filter. Subsequently, 20 μl MTT (5 mg/ml) (Sigma Aldrich, Gillingham, UK) in culture media was added to cells and incubated for 1 hour. Medium was removed, and 100 μl of dimethyl sulfoxide (Sigma Aldrich) was added for 30 minutes. Absorbance was read at 550 nm. Values are expressed as % MTT absorbance of medium control.

Identification of Cytokines

Active TGF-β1 within supernatants was determined using luciferase-reporting transformed mink lung epithelial cells (D.B. Rifkin, Langone Medical Center and School of Medicine, New York University, New York) as previously described (20). Additional details are provided in the online supplement.

Statistics

Data were analyzed in GraphPad Prism using a two-tailed unpaired Student's *t* test or one- or two-way ANOVA with post-tests. Results with *P* values < 0.05 were considered significant. Data represent mean ± SEM unless otherwise indicated.

Results

Elevated *In Vivo* Levels of TGF-β1 and T Regulatory Cell Numbers in the Nasopharynx Are Associated with Prolonged Pneumococcal Carriage in Murine and Human Models of Respiratory Challenge

Low-density colonization of pneumococci (~500 pneumococci per mg of nasopharyngeal tissue or 5,000 per ml recovered nasal homogenate) induces prolonged carriage in mice (Figure 1A), characterized by a lack of inflammation and no progression to invasive disease (14). In contrast, high-density pneumococcal colonization (~10,000 pneumococci per mg of nasopharyngeal tissue or 100,000 per ml homogenate) induces transient carriage that is cleared within 2 weeks of infection (Figure 1A). Even with high-density colonization, pneumococci do not establish infection in the lungs, and disease signs do not materialize (data not shown). Similarly, intranasal infection with an isogenic Ply-deficient mutant of *S. pneumoniae* D39 (PLN-A), at the same low dose that establishes prolonged carriage when wild-type (WT) D39 are used, fails to induce carriage but leads to transient colonization (14) (Figure 1A). We hypothesized that failure to carry high densities of WT pneumococci or a lower density of its Ply-deficient mutant might be the result of an altered balance of pro- and anti-inflammatory cytokine production in the upper airways as compared with low-density WT carriage, which promotes conditions that favor stable carriage. To explore this, levels of active TGF-β1, a major immunoregulatory cytokine, were measured in nasopharyngeal tissue samples taken over the first 2 weeks of low-density colonization with WT or PLN-A or high-density colonization with WT (Figure 1B). All infections induced rapid elevation of TGF-β1 in the nasopharynx within 24 hours (Figure 1B). However, TGF-β1 returned to preinfection levels in high-density WT (by Day 3) or PLN-A (by Day 7) colonization, despite the continuing presence of bacteria in the nasopharynx, but elevated TGF-β1 levels were maintained in low-density WT colonization, only declining in the second week of carriage (Figure 1B). Thus, sustained elevation of TGF-β1 levels in the nasopharynx is associated with prolonged pneumococcal carriage, and maintenance of TGF-β1 depends upon colonization density and Ply. IL-10, another major immunomodulatory cytokine, was elevated in the nasopharynx of low-density WT, high-density WT, and PLN-A colonized mice between Days 1 and 3 after infection, but no significant differences were observed between groups.

We have previously shown that TGF-β1–induced T regulatory cell activity has a critical role in the outcome of pneumococcal pneumonia (8). To examine whether nasopharyngeal TGF-β1 drives T regulatory cell responses during carriage, we assessed CD4+Foxp3+ T regulatory cell numbers in murine nasopharyngeal homogenates after low- or high-density colonization with WT or PLN-A pneumococci. Elevated T regulatory cell numbers were observed at 3, 7, and 14 days after low-density WT colonization but not after PLN-A or high-density WT colonization (Figure 1D).

A major function of TGF-β1 and T regulatory cells is to limit the production of proinflammatory cytokines. Levels of IFN-γ were elevated in nasopharyngeal homogenates of high-density colonized WT, but not in low-density WT or PLN-A colonized mice, at Days 1 and 3 after infection (Figure 1E). Furthermore, the ratio of IFN-γ to TGF-β1 demonstrated the proinflammatory state of the nasopharynx in high-density colonized mice (Figure 1F).

The involvement of TGF-β1 signaling pathways in the establishment of carriage is not restricted to mice. Analysis of active TGF-β1 levels in nasal washes from volunteers recruited to a unique experimental human pneumococcal carriage model where healthy adults are intranasally challenged with live pneumococci (12, 16) revealed that mean

Figure 1. Prolonged nasopharyngeal carriage is associated with a regulatory cytokine and cellular environment characterized by high transforming growth factor (TGF)-β1 and T regulatory cell numbers. (*A–C*) Mice were challenged with 1×10^5 CFU D39 (low dose, *open circles*) or PLN-A (*closed diamonds*) or with 1×10^7 CFU D39 (high dose, *open squares*) in 10 μl PBS. (*A*) Colony-forming units (CFU) per mg nasopharynx. (*B*) Active TGF-β1 levels determined by the luciferase-reporting transformed mink lung epithelial cell assay. (*C*) IL-10 levels determined by ELISA from nasopharyngeal homogenates. (*D*) T regulatory cells (Foxp3+CD4+) per mg nasopharynx as determined by flow cytometry. p.i. = postinfection. Data in *A* through *D* are representative of three independent experiments with more than five mice per group per time point. For low-dose D39, asterisks represent significant difference according to two-way ANOVA from high-dose D39 ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$), and number symbols represent significant difference from PLN-A group ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$). (*E* and *F*) IFN-γ (*E*) and IFN-γ:TGF-β1 (*F*) determined by ELISA from nasopharyngeal homogenates. Data are from a single experiment with five mice per group. Asterisks represent significant difference according to two-way ANOVA from low-dose D39 ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (*G* and *H*) Active TGF-β1 (*G*) and IL-10 (*H*) in human nasal wash taken 48 hours after experimental pneumococcal challenge. Each point represents a single individual in which 6B pneumococcal carriage was detected (carriage +) or not detected (carriage −) when nasal wash was plated on blood agar. Data are from a single experiment. $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t* test).

TGF-β1 levels are higher in volunteers in whom carriage successfully establishes after pneumococcal challenge than in those who fail to develop carriage (Figure 1G). Furthermore, assessment of IL-10, another marker of immunomodulation and tolerance (21), revealed elevated levels in carriage-positive individuals but not in those who fail to carry (Figure 1H). Carriage density in volunteers tested in the study averaged 10,000 pneumococci per ml recovered nasal wash, which is comparable to that seen when long-term carriage is successfully established in mice (5,000 per ml nasopharyngeal homogenate) (Figure 1A).

These data demonstrate the increased immunoregulatory activity in the nasopharynx of mice and humans during prolonged low-density episodes of carriage.

Carriage Is Associated with Reduced Polymorphonuclear Leukocyte Infiltration and an Accumulation of Alternatively Activated Macrophages in the Nasopharynx

To assess the impact of increased immunoregulatory activity in the nasopharynx during carriage, neutrophil and macrophage numbers were assessed. High- or low-density colonization with WT or low-density colonization with PLN-A led to infiltration of neutrophils into the nasopharynx by 24 hours (Figure 2A). However, neutrophil numbers were reduced by Day 3 after infection, and this decline was significantly more rapid in low-density colonization with WT than in high-density WT or with PLN-A (Figure 2A).

Macrophage numbers increased in the nasopharynx during all infections and were significantly elevated at Days 3 and 7 after infection as compared with prechallenge levels (Figure 2B). Although macrophage numbers in nasopharynx at Day 7 after infection were comparable in all infection groups, a significantly higher proportion of macrophages from the low-density WT colonization group (69%) expressed mannose receptor than from the high-density colonization with WT (29%) or PLN-A (26%) (Figure 2C). This result suggests that alternative activation of macrophages might occur in the nasopharynx of mice during long-term carriage, and this is supported by the observation of high levels of major histocompatibility complex class II expression on the mannose receptor–expressing macrophages from low-density WT colonized mice, as compared with mannose receptor–negative

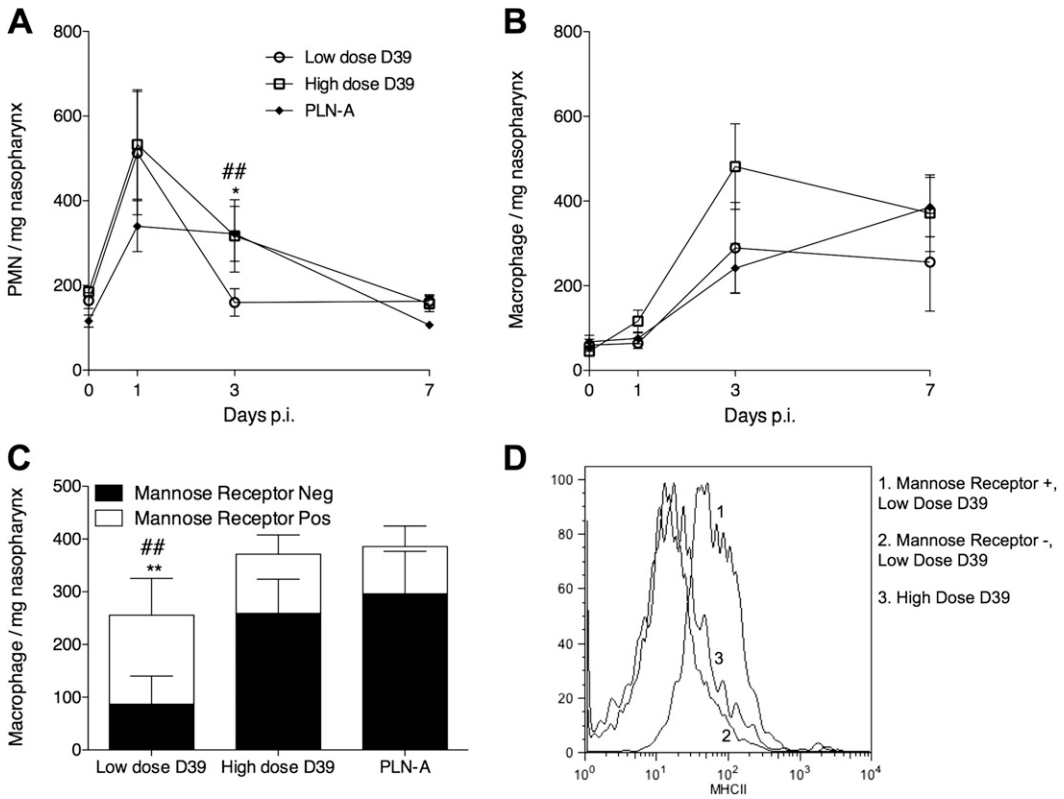


Figure 2. Carriage is associated with decreased polymorphonuclear leukocyte (PMN) infiltration of the nasopharynx and alternative activation of macrophages. Mice were challenged with 1×10^5 colony-forming units (CFU) D39 (low dose, *open circles*) or PLN-A (*closed diamonds*) or with 1×10^7 CFU D39 (high dose, *open squares*) in 10 μl PBS. Neutrophil (PMN) (*A*) and macrophage (*B*) numbers per mg nasopharynx, as determined by flow cytometry. Neutrophils are defined as $SSc^{low}CD45^{+}Gr\text{-}1^{hi}CD11b^{hi}F4/80^{int/low}$; macrophages are defined as $SSc^{hi}CD45^{+}Gr\text{-}1^{-}F4/80^{hi}$. p.i. = post infection. CD206 (mannose receptor) (*C*) and major histocompatibility complex class (MHCII) expression on macrophages (*D*) at Day 7 after infection. Data in *A* through *D* are representative of three independent experiments with more than five mice per group per time point. For low-dose D39 in *A* and *C*, asterisks represent significant difference according to two-way ANOVA from high-dose D39 (*$P < 0.05$; **$P < 0.01$), and number symbols represent significant difference from PLN-A ($^{\#\#}P < 0.01$).

macrophages or macrophages from high-density infections (Figure 2D).

Together, these data demonstrate that local immune responses are altered in the nasopharynx during long-term pneumococcal carriage. High levels of TGF-β1 and T regulatory cells, reduced neutrophil infiltration, and alternative activation of macrophages characterize this response, whereas lower TGF-β1 and T regulatory cell levels and prolonged neutrophil infiltration aligned with classical macrophage activation favor clearance of infection.

Human Nasopharyngeal Epithelial Cells and Fibroblasts Secrete Active TGF-β1 in Response to Live *S. pneumoniae* and Ply

To elucidate the mechanism by which pneumococcal colonization of the nasopharynx induced immunoregulatory responses, human nasopharyngeal fibroblasts or epithelial cells were treated with pneumococci, resulting in significant induction of active TGF-β1 (Figures 3A and 3B). TGF-β1 production was completely abrogated in assays performed with heat-killed D39 or PLN-A (Figures 3A–3D). TGF-β1 production drops off substantially at the highest D39 CFU numbers (2.5×10^6) tested in fibroblasts and to a lesser extent in epithelial cells (Figures 3A–3D), suggesting differential host responses to differing bacterial densities. This drop cannot be explained by increased cell death alone in the case of fibroblasts because they retain 100% viability even at 3×10^6 D39 CFU (Figure 3E). By contrast, the slight drop in TGF-β1 at high CFU numbers in epithelial cells (Figures 3B and 3D) may be due to the significant cell death observed at these bacterial concentrations (Figure 3F). Furthermore, consistent with the observation that Ply is required for induction of active TGF-β1 production, purified Ply was sufficient to induce TGF-β1 production from epithelial cells and fibroblasts (Figures 3G and 3H).

We have conducted preliminary investigations into the mechanistic detail of TGF-β1 induction in epithelial cells and fibroblasts of the upper and lower airways (*see* Figures E1 and E2 in the online supplement). Our findings implicate the NLRP3 inflammasome (Figure E1) and suggest the responses to pneumococci differ in the upper and lower airways (Figure E2).

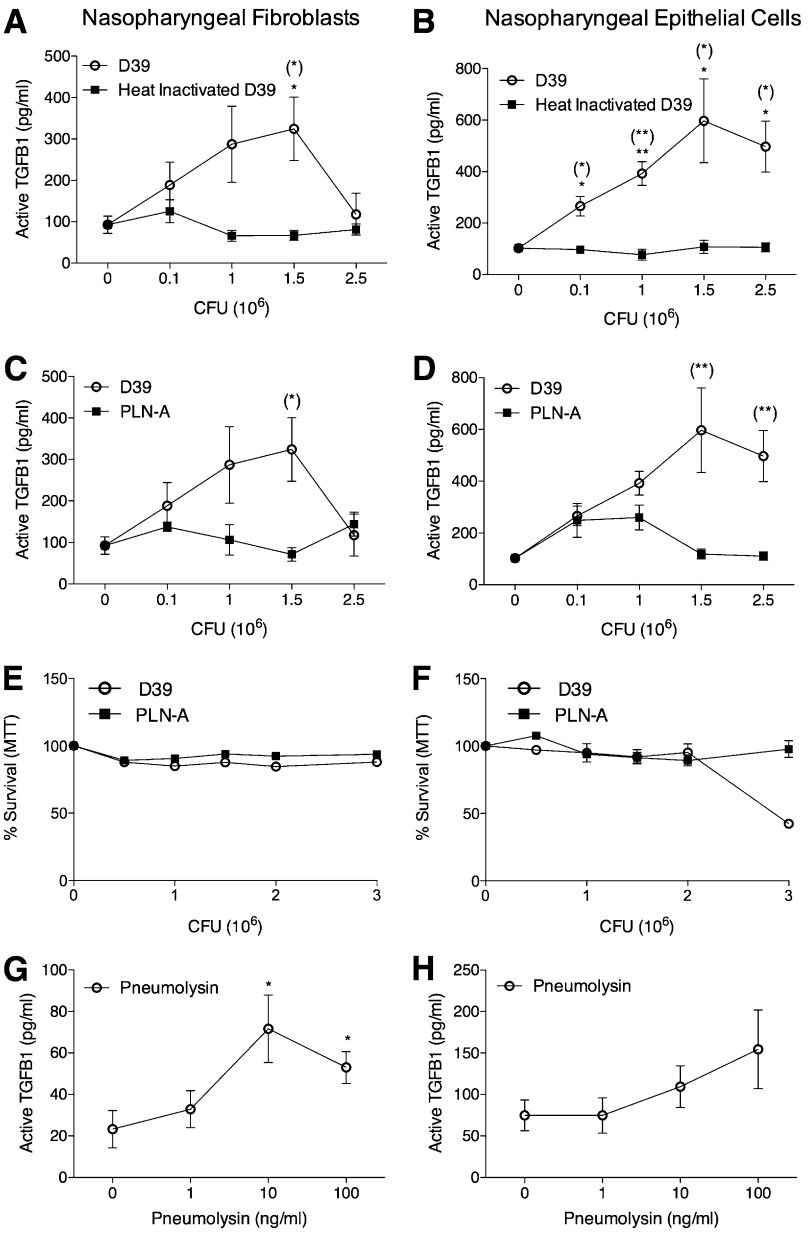


Figure 3. Pneumococci and pneumolysin induce active transforming growth factor (TGF)-β1 production in nasopharyngeal fibroblasts and epithelial cells. Active TGF-β1 determined from nasopharyngeal fibroblast (*A*, *C*, *E*, *G*) and nasopharyngeal epithelial cell (*B*, *D*, *F*, *H*) supernatants by luciferase-reporting transformed mink lung epithelial cell assay after stimulation with D39, heat-inactivated D39, pneumolysin (Ply)-deficient *S. pneumoniae* (PLN-A), or recombinant endotoxin free Ply. Survival of D39 and PLN-A stimulated nasopharyngeal fibroblasts (*E*) and nasopharyngeal epithelial cells (*F*) determined by MTT assay. Medium alone was used as control. *Open circles* = D39; *closed squares* = heat-inactivated D39 (*A* and *B*) or PLN-A (*C–F*). Asterisks in parentheses denote significant active TGF-β1 induction induced by D39 over heat-inactivated D39 (*A* and *B*) or PLN-A (*C* and *D*) ($^{(*)}P < 0.05$; $^{(**)}P < 0.01$). Asterisks denote significant active TGF-β1 induction induced by D39 over untreated cells ($^{*}P < 0.05$; $^{**}P < 0.01$). Analysis is from two-way (*A–D*) or one-way (*G*) ANOVA.

TGF-β1 Blockade Prevents Prolonged Carriage of Pneumococci in the Nasopharynx

To determine whether immunomodulatory responses are essential for prolonged pneumococcal carriage, we administered the P17 peptide inhibitor of TGF-β1 (8). Administration of P17 to mice 1 hour before intranasal administration of a colonizing low dose of pneumococci, and

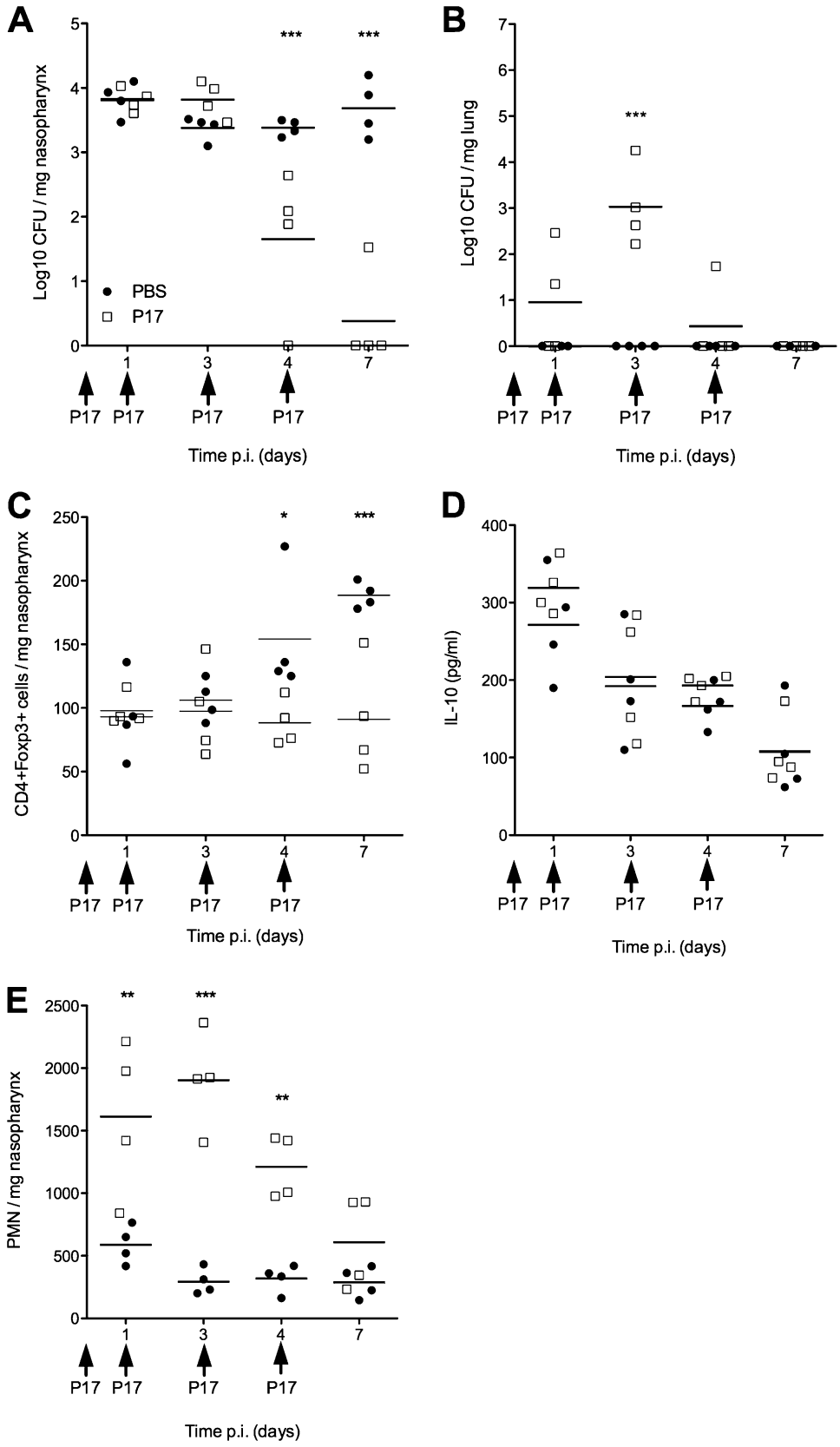


Figure 4. Impaired transforming growth factor (TGF)-β1 signaling leads to a failure to confine pneumococci to the nasopharynx and to early clearance of carriage. Mice were challenged with 1×10^5 colony-forming units (CFU) D39 and then given 500 μg P17 peptide (*open squares*) or PBS (*closed circles*)

subsequently at 1, 3, and 4 days after infection, profoundly reduced pneumococcal carriage in the nasopharynx (Figure 4A). Although initial colonization density was unaffected in P17-treated mice, by 4 days after infection mice had significantly lower bacterial numbers in the nasopharynx as compared with PBS-treated controls (1.65 $\log_{10}$ mean CFU per mg tissue vs. 3.38 $\log_{10}$ CFU per mg), and by 7 days after infection three out of four P17-treated mice had cleared carriage, whereas all control mice still had high densities of colonized bacteria in the nasopharynx. Before clearing of the infection, P17-treated mice experienced a transient translocation of pneumococci from nasopharynx to lungs between Days 1 and 4 after infection (Figure 4B). This bacterial translocation was completely absent in control animals. Blockade of TGF-β1 led to a failure to induce T regulatory cell responses in the nasopharynx (Figure 4C), although IL-10 production was unaffected (Figure 4D). TGF-β1 blockade was also associated with an exacerbated inflammatory response characterized by increased neutrophil influx of the nasopharynx (Figure 4E).

Collectively, these data highlight the key role of TGF-β1 and T regulatory cells in limiting proinflammatory responses in the nasopharynx and in allowing the persistence of pneumococcal carriage. It is likely that in different situations dysregulation of TGF-β1 signaling would lead to either pathogen clearance or pathogen dissemination to deeper tissues.

Discussion

S. pneumoniae is a pathogen of significant clinical importance, and elucidating its interactions with host immunity is crucial to our understanding of its pathogenesis. Here we identify the major immunoregulatory cytokine TGF-β1 as the key determinant of the duration of pneumococcal colonization of the upper airways. Low pneumococcal carriage density induces immunoregulatory responses characterized by sustained elevation of nasopharyngeal TGF-β1 and T regulatory cell numbers and alternative activation of macrophages. Consequently, pneumococci are maintained at low density in the nasopharynx for prolonged periods without becoming invasive. By contrast, high-density colonization induces strong inflammatory responses that supersede the TGF-β1–driven pathway and thus clear bacteria from the nasopharynx. The use of the P17 peptide (8, 15, 22) to directly inhibit TGF-β1 demonstrates the critical role of regulatory responses in mediating long-term pneumococcal carriage because mice fail to raise T regulatory cell responses in the nasopharynx and rapidly clear bacteria from the upper airways in the absence of TGF-β1 signaling.

The suppression of inflammatory responses by *S. pneumoniae*–induced TGF-β1 and T regulatory cells may be important to preserve the balance of flora and lessen tissue damage, but it is clear that regulatory responses are also essential for the prolonged carriage of pneumococci in the upper airways; thus, targeting TGF-β1 signaling may be a means to eliminate carriage, a prerequisite for invasive disease.

TGF-β1 inhibition did not prevent establishment of pneumococcal colonization in the nasopharynx, but bacteria were gradually cleared over the first week of infection. This correlated with a failure to induce T regulatory responses in the nasopharynx in response to bacterial colonization; hence, in line with our *in vitro* human airway epithelial cell culture data, we favor a model whereby pneumococci induce airway epithelial cell TGF-β1 production, which drives induced T regulatory cell generation in the nasopharynx. These T regulatory cells limit proinflammatory responses, preventing tissue damage but also allowing persistence of carriage. In support of this model, neutrophil influx into the nasopharynx was greatly enhanced in P17-treated mice, although we cannot rule out direct or T regulatory cell–independent effects of TGF-β1 on neutrophil function, as has been reported previously (23, 24). The observation that nasopharyngeal IL-10 levels were unaffected in P17-treated mice suggests that T regulatory cells are not the exclusive source of IL-10 and that IL-10 alone is not sufficient to maintain carriage. However, the clear correlation between establishment of carriage and elevated nasal wash IL-10 in experimental human pneumococcal carriage suggests that IL-10 may play some role in the colonization process.

A consequence of the reduced T regulatory cell activity in P17-treated mice was a transient colonization of the lung by *S. pneumoniae*. We postulate that the normal role of T regulatory cells in the nasopharynx is to prevent this bacterial spread by limiting inflammatory tissue damage. A prolonged but contained infection is better for the host than an uncontrolled but shorter one.

In contrast to low-density colonization, high densities of pneumococcal colonization (a "high-risk" dose) induce only transient TGF-β1 production in the nasopharynx, with significantly reduced T regulatory cell infiltration and, instead, an inflammatory phenotype characterized by neutrophil infiltration, classical activation of macrophages, and IFN-γ production dominates. Consequently, bacteria are cleared from the nasopharynx before invasive disease can develop. We postulate that deregulation of the balance between immune tolerance and inflammatory reactivity leads to invasive disease. We have shown that Ply is a key driver of TGF-β1 and regulatory responses, and it may follow that cases of human invasive disease with pneumococci that have Ply with reduced or no hemolytic activity (such as some serotype-1 strains) (25) result from the failure to balance immune responses after infection

Our observations in the murine carriage model are supported by data obtained using a unique experimental human pneumococcal carriage model (12, 16). We show that individuals who successfully carry pneumococci in their nasopharynx by 48 hours after challenge have significantly higher levels of TGF-β1 and IL-10 in nasal wash samples than those

Figure 4. (Continued). at 0, 1, 3, and 4 days after infection. (*A*) CFU per mg nasopharynx. (*B*) CFU per mg lung. (*C*) T regulatory cells (defined as Foxp3+CD4+) per mg nasopharynx as determined by flow cytometry. (*D*) IL-10 levels in nasopharyngeal homogenates determined by ELISA. (*E*) Neutrophils (polymorphonuclear leukocytes [PMN] defined as $SSc^{low}CD45^{+}Gr\text{-}1^{hi}CD11b^{hi}F4/80^{int/low}$) per mg nasopharynx as determined by flow cytometry. Data are from a single experiment with 16 mice per treatment. Asterisks represent significant difference according to two-way ANOVA (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

who clear the challenge dose. To the best of our knowledge, this is the first demonstration in mouse and human models of nasopharyngeal colonization that regulatory cytokines play a key role in sustaining pneumococcal colonization. Carriage density in colonized human volunteers was comparable to what we observed when long-term carriage is successfully established in mice.

We have demonstrated that *S. pneumoniae* and Ply directly stimulate production of TGF-β1 from human airway epithelial cells and fibroblasts. We show that host pattern recognition receptors play an essential role in this interaction and that the dependency upon ion efflux, cathepsin B, and lysosomal degradation implicates the NLRP3 inflammasome in the signaling process. Furthermore, we have demonstrated that this occurs through a mechanism dependent upon potassium efflux, as has been demonstrated for cytokine production induced by the pore-forming toxins of *Staphylococcus aureus* (26) and *Aeromonas hydrophilia* (27).

Alternative activation of macrophages induces up-regulation of a characteristic set of surface markers, including the mannose receptor and major histocompatibility complex class II (28), and appears to be a characteristic of prolonged pneumococcal carriage in our model. Production of TGF-β1 by alternatively activated macrophages has been documented (29), and the cells have been implicated in tissue repair and remodeling (30) and in protection of the host from damaging inflammation during pathology (31, 32). In the context of pneumococcal nasopharyngeal colonization, they may limit inflammation to prevent tissue damage and thus promote prolonged carriage.

Our results highlight the delicate balance between the requirements of the host to maintain epithelial barrier integrity and prevent inflammatory tissue damage with the need to combat potentially dangerous pathogen colonization. This equilibrium needs to be considered in the design of anti-pneumococcal therapeutics and vaccines that alter the density of pneumococcal carriage and hence affect upper airways immune responses. We hypothesize that the prolonged but contained and controlled carriage mediated by T regulatory cells allows the host time to mount protective memory immune responses against the pneumococcus that may protect against invasive disease. We have previously demonstrated the potential for natural carriage to boost vaccine responses (33). Furthermore, elucidation of factors conducive to carriage or clearance may open up avenues for treatments tailored to patients, where levels of immunomodulatory cytokine in nasal wash might be used as a predictor of future responses to infection or vaccination. ■

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