Host and Bacterial Factors Contributing to the Clearance of Colonization by *Streptococcus pneumoniae* in a Murine Model

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Nasopharyngeal colonization is the first step in the interaction between *Streptococcus pneumoniae* (the pneumococcus) and its human host. Factors that contribute to clearance of colonization are likely to affect the spread of the pneumococcus and the rate of pneumococcal disease in the population. To identify host and bacterial factors contributing to this process, we examined the time course of colonization using genetically modified mice and pneumococci. Severe combined immunodeficient mice remained persistently colonized (>6 weeks). Major histocompatibility complex II-deficient mice, but not μMT mice, were unable to clear colonization and showed a diminished T helper 1 response. Thus, CD4+ T cells, rather than the generation of specific antibody, appear to be required for effective Th1-mediated clearance. In addition, the microbial pattern recognition receptor toll-like receptor 2 (TLR2), but not TLR4, was necessary for efficient clearance of colonization. In contrast, no role of complement component 3, inducible nitric oxide synthetase, interleukin 12 (IL-12), or IL-4 could be demonstrated. Expression of the pneumococcal toxin pneumolysin enhanced acute localized inflammatory responses and promoted clearance of colonization in a TLR4-independent manner. We conclude that both innate and CD4+ T-cell-mediated immunity and proinflammatory bacterial factors, rather than a humoral adaptive immune response, are important for clearance of *S. pneumoniae* from the murine nasopharynx.

*Streptococcus pneumoniae* is an important human pathogen, causing diseases ranging from respiratory tract infections such as otitis media, sinusitis, and pneumonia to invasive infections such as meningitis and sepsis. Morbidity and mortality rates remain high, both in the developing world and in countries with access to health care (64). Although effective antibiotics and vaccines exist, emerging antibiotic resistance and replacement with non-vaccine-type pneumococci emphasize the need for new treatment options and a better understanding of pathogenesis (1, 32).

The pneumococcus colonizes the nasopharynx as the initial step in its pathogenesis (15, 18). Human nasopharyngeal carriage is also the major reservoir of pneumococci and the source of horizontal spread of this pathogen within the community (18, 20). Factors that contribute to clearance of colonization are likely to affect the frequency of transmission of the pneumococcus and the overall incidence of pneumococcal disease in the population. Both host and bacterial factors that contribute to clearance remain incompletely characterized. Colonization is generally cleared 4 to 8 weeks after a new strain is acquired, but the length of carriage is highly variable both between individuals and among different serotypes (20, 33). It has been widely assumed that specific antibodies induced in response to carriage mediate the clearance of pneumococcal colonization. This assumption was based on surveys that showed that carriage rates decrease from more than 50% in infants to 5 to 10% in adults and correlate with rising levels of both mucosal and serum antibodies to pneumococcal surface polysaccharides (20, 58, 59, 67). In addition, antibodies were thought to be important in the clearance of colonization because of decreased rates of colonization in vaccinated populations (11, 12, 30, 39).

An experimental human carriage study reported by McCool et al. raised questions about whether or not antibodies induced during carriage contributed to clearance of bacteria from the human nasopharynx (40). In that study, healthy adults became colonized with a minimally passaged type 23F clinical isolate. Colonized subjects developed a serum and mucosal antibody response to the serotype-determining capsular polysaccharide and to a major antigen, pneumococcal surface protein A (PspA). However, this rise in antibody levels did not correlate temporally with the loss of carriage, suggesting that the role of antibody in the clearance of preestablished colonization may be limited (40, 41).

To further explore the mechanisms contributing to natural carriage and clearance of carriage, a mouse model of pneumococcal colonization was developed with a strain derived from the same clinical isolate that was used in the experimental human carriage study (42). As with carriage events in humans, colonization of mice was self-limited and there was no evidence of lower respiratory tract infection or invasive disease. Carriage induced a mucosal and serum antibody response to pneumococci and PspA, but individual mice did not demonstrate a correlation between the density of colonization and amounts of serum or mucosal antibodies. In addition, the role of antibody in the clearance of carriage was examined in mice with a genetic defect in humoral immunity. These mice were
not affected in the density or duration of colonization, demon-
strating that antibody may not be a requirement for efficient
clearance of pneumococcal colonization (42).

Antibody-independent immunity to pneumococcal coloni-
zation of mice was recently demonstrated by Malley et al. (36). In
addition, they showed that intranasal immunization by live
pneumococci or by a killed, nonencapsulated whole-cell vac-
cine protected antibody-deficient mice, but not CD4\(^+\) T-cell
deficient mice, against intranasal challenge (36).

In this study we investigated the role of CD4\(^+\) T cells (includ-
ing both Th1 helper type 1 and 2 [Th1 and Th2] responses),
interleukins 12 and 4 (IL-12 and IL-4), nitric oxide synthetase
2 (NOS2), complement component 3 (C3), and toll-like recep-
tors 2 and 4 (TLR2 and TLR4) in the ability of mice to clear
pneumolysin-induced acute inflammation during its
expression reverting of strain P633, the minimally passaged type 23F isolate
of S. pneumoniae (45, 66). In the absence of appropriate mutants defi-
cient in cell wall or lipoteichoic acid, we investigated the role of
pneumolysin in the clearance of colonization. Pneumolysin is a pore
forming cytotoxin produced by all pneumococci and an
important virulence determinant (5, 16) that has been pro-
duced by the immune system through its interaction with
TLR4 (35). Additionally, it has been shown that pneumo-
lysin is capable of attracting CD4\(^+\) T cells (23, 24). We dem-
strate that pneumolysin induces acute inflammation during
carrying and promotes more-efficient clearance of colonization in
a TLR4-independent manner.

**MATERIALS AND METHODS**

**Mice.** The following strains of mice were obtained from Jackson Laboratories (Bar Harbor, Maine): C57BL/6J (wild type), B6.129P2-Il4 tm1Cgn/J (IL-4
knockout), B6.129S1-Il12btm1Jm (IL-12b
ds mutant), C57BL/6 TLR2
deficient, C57BL/6 TLR4 (35). Additionally, it has been shown that pneumo-
lysin is capable of attracting CD4\(^+\) T cells (23, 24). We dem-
strate that pneumolysin induces acute inflammation during
carrying and promotes more-efficient clearance of colonization in
a TLR4-independent manner.

**RESULTS**

**Role of innate immunity in clearance of pneumococcal colon-
ization.** The roles of TLR2 and TLR4 were investigated by
comparing the colonization of TLR2−/− mice, TLR4-deficient mice, and parental strains challenged intranasally with strain P1121. At weeks 3 and 4 postinoculation, TLR2−/− mice were colonized at significantly (P = 0.008 and P = 0.009, respectively) higher levels than parental mice (Fig. 1A). Eight weeks following the inoculation, no pneumococci were found in upper respiratory lavage fluids of either parental or TLR2−/− mice. By this time point, however, four of six TLR2−/− mice, but no parental mice, had become colonized with other neomycin-resistant alpha-hemolytic streptococci. Based on analysis of 16S rRNA PCR products and comparison to sequence databases, these were most similar to a distantly related streptococcal species found in nonhuman hosts, *Streptococcus acedominimus*.

In contrast to TLR2, the expression of functional TLR4 did not contribute to the clearance of colonization with P1121, since no difference was observed in the rate of clearance between TLR4-deficient and TLR4-sufficient mice (Fig. 1B). Both C3−/− mice and NOS2−/− mice cleared colonization with P1121 at the same rate as wild-type mice (data not shown). We were unable to fully examine the role of neutrophils in this process because of the prolonged period of neutropenia required.

Role of adaptive immunity in clearance of pneumococcal colonization. C57BL/6 SCID mice were colonized for a significantly longer period, and at a higher level, than parental C57BL/6 mice (P = 0.004 at week 6; P = 0.001 at week 8) (Fig. 2). Because we previously documented that mature B cells are not necessary for the clearance of colonization, we investigated the role of CD4+ T cells (42). MHC-II−/− mice, which are functionally Th deficient and also have significantly decreased CD4 T-cell counts, showed a persistence of colonization (>6 weeks) similar to that of SCID mice. C57BL/6 MHC-II−/− mice had significantly higher levels of colonization at 3 and 6 weeks postinoculation than the parental C57BL/6 mice (P = 0.005 and P = 0.002, respectively) (Fig. 2).

To determine whether the CD4+ T-cell-dependent clearance of colonization correlates with Th1 or Th2 responses, we characterized specific serum IgG generated in response to colonizing pneumococci. The predominant IgG isotypes against PspA of P1121 were IgG2b and IgG3, subtypes typically generated in Th1 responses. MHC-II−/− mice generated smaller total amounts of IgG antibody against PspA (P = 0.02) (Fig. 3A) than wild-type mice. This was largely attributable to significantly reduced levels of IgG3 antibodies (P = 0.007) (Fig. 3). To further address the question whether a Th1 or Th2 response is important in the clearance of colonization, IL-12−/− and IL-4−/− mice were inoculated with P1121. No differences in the clearance of colonization between C57BL/6 and IL-12−/− or IL-4−/− mice were detected (data not shown).

**Role of pneumolysin in clearance of pneumococcal colonization.** To investigate whether pneumolysin had an effect on the duration of colonization, we inoculated mice of two different genetic backgrounds (C57BL/6 and C3H) and compared their colonization with P1121 versus the ply-negative mutant of P1121. In C57BL/6 mice, a significant difference between strains (P = 0.03) (Fig. 4A) was observed by 3 weeks postinoculation. The density of pneumococci in mice inoculated with the P1121 ply-negative mutant was significantly higher than that in mice inoculated with P1121. In the C3H genetic background, the clearance of P1121 and the P1121 ply-negative mutant was assessed after 4 and 8 weeks, because C3H mice tend to clear colonization more slowly than mice of the C57BL/6 background (Fig. 1B). Like the C57BL/6 mice, C3H mice inoculated with the P1121 ply-negative mutant had significantly higher (P = 0.004) (Fig. 4B) levels of colonization than C3H mice inoculated with P1121 at 8 weeks postinoculation. Differences between pneumolysin-expressing and non-pneumolysin-expressing strains were observed in both TLR4-deficient (C3H/HeJ) and TLR4-sufficient (C3H/HeOuJ) hosts (data not shown). Likewise, a ply-negative mutant of a mouse virulent type 6A clinical isolate showed enhanced colonization.

![Comparison of the density of colonization in C57BL/6 (filled diamonds) versus C57BL/6 TLR2−/− (open squares) mice (A) or in C3H/HeOuJ (TLR4-sufficient) parental (filled diamonds) versus C3H/HeJ (TLR4-deficient) (open squares) mice (B) over time. The density of pneumococci in upper respiratory tract lavage specimens is expressed as the mean log CFU/ml ± the standard error of the mean. n = 5 to 15 mice per group per time point. *, P < 0.05.](http://iai.asm.org/DownloadedFrom/)
compared to its isogenic $p^{+}$ parent in C57BL/6 mice at 20 days postinoculation (mean density of colonization, $2.6 \times 10^{4}$ versus $5.3 \times 10^{2}$ CFU/ml lavage fluid [$P < 0.01$]).

More-efficient clearance of the pneumolysin-expressing strain correlated with enhanced acute inflammatory responses during initial colonization. Mice inoculated with P1121 secreted more MIP-2 ($P < 0.03$) (Fig. 5A), a mouse chemokine that exhibits potent neutrophil chemotactic activity, in upper respiratory tract lavage fluid (13). In animals with elevated MIP-2 levels, a moderate influx of neutrophils into the nasal cavity adjacent to the turbinates was also observed (Fig. 5B). This influx of cells was noted at 24 h postinoculation and only in those mice challenged with P1121, not in those challenged with the P1121 $p^{+}$-negative mutant. This suggested that pneumolysin-expressing pneumococci stimulate a mild local suppurative rhinitis in mice (46). At later time points when mice were still colonized, no leukocytes (neutrophils or lymphocytes) were seen in nasal spaces or tissues of mice challenged with either pneumolysin-expressing or non-pneumolysin-expressing pneumococci (data not shown).

DISCUSSION

To investigate host and bacterial factors promoting clearance of colonization, we used a previously described murine model of colonization (42). This murine model resembles human colonization when compared for the same isolate in duration of experimental carriage and enabled us to investigate the roles of different host factors by using genetically modified mice and pneumococci. Central to our study was the use of a serotype previously shown to be avirulent in mice. This allowed for examination of the duration of carriage without morbidity or mortality even in immunodeficient hosts. Our group previously showed that the role of antibody in the clearance of murine colonization is limited, but it remained unclear which arm of the immune system is responsible or whether the gradual loss of the carrier state is due to nonimmune, mechanical factors (42). CD4$^{+}$ T cells have been shown by Kadioglu et al. to contribute to protection from pneumococcal disease (23). MHC-II$^{-/-}$ mice were significantly more susceptible to pneumococcal bronchopneumonia and septicemia than their isogenic wild-type parents. Malley et al. recently demonstrated that CD4$^{+}$ T cells mediate an acquired immunity to pneumococcal colonization that is independent of the presence of antibodies (36). Our findings show that CD4$^{+}$ T cells are also
shown that TLR2 is dispensable in the innate immune response to pneumococcal pneumonia (28). However, this remained unclear colonization during a prolonged follow-up period of 6 weeks, in contrast to wild-type C57BL/6 mice. The effect of CD4+ T cells on clearance could be explained by the lack of induction of a Th1 response, which has previously been shown to have a protective role in the host response to pneumococcal disease (26, 55). We observed antibody responses in TLR2−/−, MHC-II−/−, and parental strains that consisted predominantly of IgG isotypes that are typically generated in a Th1 response. Although the levels of serum antibody against the major antigen PspA were significantly reduced in MHC-II−/− mice compared to those in parental C57BL/6 mice, the difference in antibody levels that points to the predominance of Th1 responses to pneumococcal colonization cannot explain the difference in the clearance of colonization between those mouse strains. We have previously shown that µMT mice, which are not able to generate specific antibodies, clear colonization with P1121 at the same rate as the parental C57BL/6 mice (42). Malley et al. confirmed that antibodies against pneumococci with different serotypes (6B, 7F, and 14) did not mediate protection against recolonization (36). Our study focused on the host response to colonizing pneumococci and does not address the role of immune factors, including antibody induced through vaccination, in the clearance of the carrier state.

We demonstrated that TLR2 is important in clearance of colonization, which is supportive of the hypothesis that this requires a Th1-mediated response and that signaling through TLR2 results in cytokines that stimulate a Th1 response (56). To address the role of Th1 responses further, the role of IL-12, a potent inducer of Th1 type responses, was investigated in clearance of colonization by IL-12−/− mice. However, no difference between IL-12−/− and parental mice was observed. In addition we looked at IL-4, a cytokine important in stimulation of a Th2 type response. Like IL-12, IL-4 was not found to have a crucial role in the clearance of colonization. These results do not exclude the possibility that the mechanism leading to clearance of colonization is Th1 dependent, since IL-12 is not the only inducer of a Th1 response. It has been shown that gamma interferon (IFN-γ) is also capable of stimulating CD4+ T cells toward a Th1 response and that IFN-γ is important in the host defense against pulmonary infection with Streptococcus pneumoniae (60, 65). It has also been demonstrated that nasopharyngeal colonization with D39 in IL-18-deficient mice results in a diminished clearance after 7 days (48). A major activity of

FIG. 4. Comparison of the density of colonization in C57BL/6 (A) or C3H (B) mice inoculated with P1121 (closed diamonds) or the P1121 py-negative mutant (open squares) over time. Values shown are mean log CFU/ml ± standard errors of the means. n = 10 to 15 mice per group per time point. *, P < 0.05 for comparison between P1121 and the P1121 py-negative mutant.
IL-18 is the induction of IFN-γ. The role of IL-12 in IL-12<sup>−/−</sup> mice could, therefore, have been replaced by IFN-γ. The fact that IL-12p40-deficient mice did not show a difference in clearance of colonization argues against the suggestion made by Malley et al. (36) that IL-17A-producing CD<sup>+</sup> T cells have a role in the clearance of colonization. IL-17A is released by CD<sup>+</sup> T cells together with dendritic cells, producing IL-23 through TLR-dependent pathways, and mobilizes neutrophils through granulopoiesis and chemokine induction (21). IL-12p40 associates not only with IL-12p35 but also with another molecule, p19, to form a new heterodimeric cytokine known as IL-23 (47). IL-12p40-deficient mice are therefore deficient not only in IL-12 but also in IL-23, which could result in a decreased release of IL-17A (14). It is unlikely that IL-17A-producing T cells have a role in the clearance of colonization, since we studied the effect of IL-12p40, and consequently also IL-23, in IL-12p40- and IL-23-deficient mice, and we found no difference between IL-12p40-deficient mice and wild-type mice.

It has been suggested that TLR4 mediates innate immune responses to pneumococci through its interaction with pneumolysin (5, 16, 35). It has also been suggested that loss of pneumolysin correlated with significantly lower numbers of pneumococci in the nasopharynx in a murine colonization model (25). However, we could not confirm a role of TLR4 in the clearance of colonization by <i>S. pneumoniae</i>, and in contrast, in our model we found that pneumolysin promotes clearance of colonization. Our results with pneumolysin were unexpected, since most virulence determinants are thought to inhibit rather than promote clearance. Among its activities,
pneumolysin triggers inflammatory responses in the colonized mucosa (51). Although pneumococcal colonization is not known to be an inflammatory process, if similar events occur in the human host, our data suggest that a mild suppurative rhinitis may follow the initiation of colonization. This acute inflammatory process triggered, at least in part, by pneumolysin appears to promote more-rapid clearance by subsequent adaptive immune responses. Why would *S. pneumoniae* express a factor that promotes its own clearance? Other well-characterized bacterial toxins, such as cholera toxin, stimulate secretions that may contribute to transmission. Our observations are consistent with a role for pneumolysin in increasing the inflammatory response of the mucosal surface, which could in turn make transmission more efficient.

Our finding that pneumolysin promotes clearance of colonization is consistent with previously reported data from Rubins et al. (54). This study showed that colonization of mice with a pneumolysin-deficient serotype 14 strain resulted in colonization levels equal to or higher than those in mice inoculated with the pneumolysin-sufficient strain. In contrast with this study and with our data, Kadioglu et al. found that pneumolysin is essential for successful colonization with a serotype 2 strain and reported a rapid elimination of pneumolysin-deficient pneumococci (25). The latter study used a virulent serotype 2 pneumococcus that caused death within days and thus assessed significantly shorter follow-up times than our study and that of Rubins et al. It has previously been demonstrated that T cells migrate to tissues with a high density of pneumococci, especially in the setting of pneumolysin production (8, 10). Kadioglu et al. showed in an in vitro model that CD4+ T cells migrate toward pneumolysin toxin produced by in vivo-grown pneumococci. Our finding that pneumolysin promotes the clearance of colonization, therefore, is consistent with a model in which CD4+ T cells have an important role. If pneumolysin is deficient, fewer CD4+ T cells will be attracted, and clearance may be diminished. We were, however, unable to demonstrate the presence of lymphocytes in the nasopharynx during pneumococcal colonization.

There is accumulating evidence of the importance of T cells in pneumococcal disease. Patients with AIDS frequently develop severe pneumococcal infections (52). However, data on colonization rates in T-cell-deficient patients are inconsistent (50, 53). In one study where the level and duration of colonization were analyzed in relation to CD4+ T-cell counts, it was found that human immunodeficiency virus-infected subjects with lower CD4+ T-cell counts were more likely to be persistent pneumococcal carriers than non-human immunodeficiency virus-infected subjects, supporting our finding that CD4+ T cells are necessary for loss of carriage (53).

In addition to TLRs, we investigated the roles of other components of the innate immune system. NO is produced by NOS2 in human and rodent macrophages during pneumococcal infection and might have a role in intracellular killing of pneumococci following their phagocytosis (38). Complement and neutrophils are important components of innate immunity for protection against pneumococcal infections (49). However, NOS2 and complement were not found to be important in the clearance of colonization. The role of neutrophils could not be elucidated, because attempts to maintain neutropenia for the length of the time needed to show a difference between neutropenic and wild-type animals (>3 wks) were unsuccessful. Therefore, it remains unclear what factors might be induced by CD4+ cells that lead to loss of colonization. Neutrophils stimulated by IFN-γ produced in response to the Th1 response, for example, could be responsible for the actual clearance of carriage.

An inherent limitation of our study is that immunodeficient mice may harbor altered nasopharyngeal flora that may affect susceptibility to or duration of carriage of *S. pneumoniae*. This difference in flora was demonstrated for TLR2+/− and MHC-II−/− mice, which over the course of the study became carriers of another streptococcal species. On the other hand, the acquisition of another alpha-hemolytic streptococcus by these mice, but not by immunocompetent parental mice, adds to the evidence that TLR2 and CD4+ T cells contribute to host resistance to streptococcal colonization.

We conclude that both innate and CD4+ T-cell-mediated immunity, and proinflammatory bacterial factors, rather than a humoral adaptive immune response, are important for clearance of *S. pneumoniae* from the murine nasopharynx. Further studies are necessary to identify effectors of the Th1 response resulting in clearance of colonization.

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