Low CD4 T Cell Immunity to Pneumolysin Is Associated with Nasopharyngeal Carriage of Pneumococci in Children

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Background. Recent studies in mice have suggested that T cell immunity may be protective against pneumococcal infection.

Methods. CD4 T cell proliferative responses to the pneumococcal proteins pneumolysin (Ply), Ply toxoid (F433), and choline-binding protein A were investigated in peripheral blood mononuclear cells (PBMCs) and adenoidal mononuclear cells (MNCs) obtained from children undergoing adenoidectomy.

Results. Ply and F433 induce significant proliferation of CD4 T cells in both PBMCs and adenoidal MNCs, and both memory and naive phenotypes of CD4 T cells proliferated after stimulation. In PBMCs, CD4 T cell proliferation induced by Ply and F433, which was associated with increased production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α, was significantly lower in children who were culture positive for pneumococcus than in those who were culture negative for pneumococcus (P<.05). Between groups, no such difference was observed in adenoidal MNC CD4 T cell proliferation, which was associated with production of IFN-γ and interleukin (IL)-10. The CD4 T cell proliferation induced by Ply and F433 was inhibited by antibodies to Toll-like receptor 4.

Conclusion. These data suggest that Ply induces CD4 T cell proliferative responses with production of IFN-γ and TNF-α in PBMCs or of IFN-γ and IL-10 in adenoidal MNCs, which may be important in modulating pneumococcal carriage in children.
ies alone, because the protection can occur before the development of measurable systemic anticapsular antibodies [9]. We recently noted that serum antibody levels to Ply and CbpA were higher in children with no detectable pneumococcal carriage than in children who were culture positive for pneumococcus, suggesting that these antibodies are associated with protection against carriage [10]. However, recent studies in mice have suggested that T cell immunity may also provide an antibody-independent protection against pneumococcal colonization [11, 12]. Other evidence supporting a role for CD4 T cell immunity against pneumococcal infection includes the observation that major histocompatibility complex II–deficient mice, which are deficient in CD4 T cells, are more susceptible to pneumococcal pneumonia and septicemia [13] and that induction of CD4 T cell migration and activation by pneumococci is important for the development of early protective immunity against infection [13].

To investigate whether there is an additional or complementary role for CD4 T cell activation by Ply and CbpA in the development of protection against pneumococcal colonization in humans, we investigated T cell proliferative responses and cytokine production to Ply and CbpA in peripheral blood mononuclear cells (PBMCs) and adenoidal mononuclear cells (MNCs) in relation to nasopharyngeal carriage in children.

**MATERIALS AND METHODS**

**Subjects and samples.** Peripheral blood and adenoid tissue samples were obtained from otherwise healthy children aged 3–10 years (median age, 5 years) who were undergoing adenoidectomy for adenoidal hypertrophy at either Bristol Royal Hospital for Children or Southmead Hospital in Bristol, United Kingdom. Criteria for exclusion from the study included previous immunization against pneumococcus, receipt of antibiotics or steroids within 2 weeks of surgery, and the presence of an immunodeficiency or any serious infection. Nasopharyngeal swab specimens were obtained on the day that the operation was performed, and they were cultured for pneumococcus as described elsewhere [10]. In brief, swabs were placed in tubes containing skim milk–tryptone-glucose-glycerin broth, and they were stored at −70°C until bacterial culture was performed by inoculating 50 μL of the broth onto a blood agar plate by use of a standard inoculating technique [14]. After incubation was performed overnight at 37°C (in an atmosphere of 5% CO₂), pneumococcal colonies were identified on the basis of typical morphologic findings, α-hemolysis, and subculture with optochin disk testing. The pneumococcal load was assessed semiquantitatively by grading growth density on a scale from + to ++++, as described elsewhere [14]. The study was approved by the South Bristol local research ethics committee, and written, informed consent was obtained from all subjects.

**Protein antigens.** Recombinant Ply, a Ply toxoid (F433), and recombinant CbpA proteins were used as antigen stimulants. The 3 proteins were expressed in and purified from *Escherichia coli* expressing the respective genes [15, 16]. The original source of each of the genes was the encapsulated type 2 pneumococcal strain D39. F433 is a detoxified mutant of Ply with a Trp433-Phe mutation, which reduces cytolytic activity but retains antigenicity [17]. Purified tetanus toxoid (National Institute for Biological Standards and Control) was used as a positive control for antigen stimulation. The endotoxin levels of the recombinant proteins were <0.01 IU/μg of protein, as determined by limulus assay (BioWhittaker). An optimal dosage for each protein was chosen for stimulation at which no detectable cell toxicity was observed, by use of both trypan blue staining and/or flow cytometric analysis after propidium iodide staining (data not shown).

**Cell isolation and culture.** Adenoids were processed, and MNCs were isolated according to methods described elsewhere [10, 18]. PBMCs were also isolated using Ficoll (Amersham Biosciences) according to the manufacturer’s instructions. Cells were washed in PBS and were resuspended at 4 × 10^6 cells/mL in RPMI 1640 culture medium containing 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, as well as 10% fetal bovine serum (FBS; Sigma). Cells were cultured in 96-well culture plates (Corning). In some experiments, cell culture supernatants were collected and stored at −70°C until they were assayed for cytokines.

**Depletion of T cell subsets from adenoidal MNCs.** In some experiments, cellular depletion of CD45RO⁺ (i.e., memory phenotype) cells from adenoidal MNCs was performed using magnetic cell sorting (MACS) according to the manufacturer’s instructions, before cells were cultured with stimulants. This procedure was not performed for PBMCs, because there generally were not enough cells available from patients’ blood samples to allow for both experiments to be performed using undepleted and depleted cells. In brief, adenoidal MNCs were washed in PBS with 1% bovine serum albumin (PBS/BSA) and were incubated with anti-human CD45RO microbeads (Miltenyi Biotec) at 4°C for 15 min. The cells were washed and passed through a column on a magnetic cell separator. The CD45RO⁺ cell–depleted MNCs were collected and resuspended in PBS/BSA. Depletion consistently yielded RO⁻ cells that were >99% pure (data not shown).

**Analysis of cellular proliferation.** Carboxyfluorescein diacetate 5,6 succinimidyl ester (CFSE; Molecular Probes) was used to label adenoidal MNCs or PBMCs before culture, allowing for tracking of cell division. In brief, cells stained with CFSE (5 mmol/L) in PBS were incubated at 37°C for 8 min, followed by the addition of cold culture medium to quench the reaction. The labeled cells were cultured with or without antigen for a predetermined period. In some experiments, cells were preincubated with blocking antibodies to Toll-like receptor.
CD4+CD45RA+ T cells (naive phenotype) predominated in PBMCs (78%–89% of all CD4+ T cells). No differences were noted between adenoidal MNCs and PBMCs.

A total of 38 patients were recruited into the study, and pneumococcal carriage rates were assessed in undepleted MNCs and PBMCs after antigen stimulation with Ply, the toxoid F433, and CbpA (figure 1A). These responses were dose dependent (figure 1B). At concentrations >0.1 μg/mL (for Ply) and 1 μg/mL (for F433), there were decreases in proliferation associated with cytolytic effects, as confirmed by propidium iodide staining and flow cytometric analysis (data not shown). The pro-proliferative effects of Ply, F433, and CbpA were abrogated by prior treatment with proteinase K followed by boiling, but not by coinubcation with polymyxin B (data not shown).

CD4 T cell proliferation induced by Ply in both memory and naive phenotypes. To determine whether the proliferated CD4 T cells originate from memory or naive phenotypes, cellular depletion of CD45RO+ cells from adenoidal MNCs was performed. Ply and F433 induced significant proliferative responses (detectable at day 4) in undepleted adenoidal MNCs and weaker cellular proliferative responses (detectable from day 6) in MNCs that contained naive T (RO) cells only (figure 2A and 2B). Figure 2C shows the CD4 T cell proliferation in naive (RO) cells, compared with a stronger response noted in undepleted MNCs (containing memory T cells), at day 8 after stimulation. Although CbpA induced CD4 T cell proliferation in undepleted MNCs, no significant proliferation was shown in CD45RO− (naive phenotype) cells.

Association of CD4 T cell proliferation and nasopharyngeal carriage. To investigate whether there is any association between CD4 T cell immunity and current nasal carriage, CD4 T cell proliferation was compared between children with detectable nasal colonization and those without such colonization. Figure 3A shows that the CD4 T cell proliferation indices in PBMCs after stimulation with Ply or F433 were significantly higher in children who were culture negative than in those who were culture positive for pneumococci. Among culture-positive patients, there was a statistically significant inverse relationship between CD4 T cell proliferation indices and pneumococcal density in culture ($r = -0.64; P < .05$). This effect of carriage status on CD4 proliferation was independent of age ($P > .05$, general linear model of ANOVA). No such difference was found after CbpA stimulation. In contrast, in adenoidal MNCs, there was no such difference between culture-negative and -positive children in CD4 T cell proliferation after either Ply, F433, or CbpA stimulation (figure 3B). However, when memory T cells were depleted from adenoidal MNCs, a higher CD4 T cell proliferation index was shown in the RO− cells from culture-negative patients than in the RO+ cells from patients who were culture positive after stimulation with Ply or F433 as seen in PBMCs (figure 3C). There were no differences in CD4 T cell proliferation in either PBMCs or adenoidal MNCs after stim-
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Figure 1. A, Cellular proliferation analyzed by carboxyfluorescein diacetate 5,6 succinimidyl ester (CFSE) at day 7 (when cell proliferation became significant for all responding subjects and on the linear part of the proliferative curve [see figure 2A]) after stimulation with recombinant protein antigens (pneumolysin [Ply], 0.05 μg/mL; Ply toxoid [F433], 0.5 μg/mL; and choline-binding protein A [CbpA], 1 μg/mL). Fluorescence-activated cell sorter dot plots show percentages of proliferated CD4 T cells in adenoidal mononuclear cells (Ad MNCs) (top) and peripheral blood mononuclear cells (PBMCs) (bottom). B, Dose-response curve of CD4 T cellular proliferation upon stimulation by Ply (circles), F433 (squares), and CbpA (triangles) at day 7 in Ad MNCs (black symbols) and PBMCs (white symbols). Both panels show representative data from 1 of 6 experiments.

ulation with tetanus toxoid between culture-negative and culture-positive children (figure 3A–3C).

**IFN-γ and TNF-α production associated with CD4 T cell proliferation in PBMCs.** To identify the cytokines associated with Ply-induced T cell proliferation and with protection against carriage, concentrations of IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were measured in cell culture supernatants after antigen stimulation and were compared with such concentrations in unstimulated controls. In PBMCs, production of IFN-γ and TNF-α was shown to be associated with CD4 T cell proliferation after stimulation with Ply (figure 4A and 4B). Similar associations were also shown after F433 stimulation (\( r = 0.69 \) and \( r = 0.71 \) for IFN-γ and TNF-α, respectively; \( P < .05 \)). The concentrations of IFN-γ and TNF-α in cell culture supernatants at day 3 were higher in patients who were culture negative for pneumococcus than in patients who were culture positive for pneumococcus after stimulation by Ply and F433 (figure 4C). For the other cytokines measured, apart from a minor increase in IL-5 after Ply stimulation, no significant changes in concentrations of IL-2, IL-4, and IL-10 were found in PBMCs after stimulation by any antigen (data not shown). In adenoidal MNCs, increases in concentrations of IFN-γ and IL-10 (figure 4D), but not in concentrations of IL-2, IL-4, IL-5, and TNF-α (data not shown), compared with those noted in unstimulated controls, were seen after stimulation by Ply and F433 and, to a lesser extent, by CbpA. IFN-γ and IL-10 concentrations in culture-positive patients tended to be higher than those in culture-negative patients, although the differences were not significant (\( P > .05 \)) (figure 4D).

**Induction of CD4 T cell proliferation by Ply and its association with TLR4.** To study whether TLR2 or TLR4 is involved in the induction of CD4 T cell proliferation by Ply,
Figure 2.  

A, Time course of CD4+ T cell proliferation after stimulation with pneumolysin (Ply; 0.05 μg/mL), Ply toxoid (F433; 0.5 μg/mL), and choline-binding protein A (CbpA; 1 μg/mL) in adenoidal (Ad) mononuclear cells (MNCs) (black symbols) and memory T cell–depleted (RO−) cells (white symbols).

B, Cellular proliferation shown by carboxyfluorescein diacetate 5,6 succinimidyl ester (CFSE) staining at day 8 after stimulation with Ply, F433, and CbpA in memory (CD45RO+) T cell–depleted Ad MNCs. Some proliferated cells have changed phenotype from RA+ to RA− after stimulation. A and B, Representative data from 1 of 6 experiments.

C, CD4+ T cell proliferation indices (mean ± SD) after stimulation at day 8 in RO− MNCs, compared with Ad MNCs (n = 10). *P < .01, compared with medium control.
Figure 3. CD4 T cell proliferation indices (mean + SD) measured at day 7 after stimulation with recombinant pneumolysin (Ply; 0.05 μg/mL), Ply toxoid (F433; 0.5 μg/mL), choline-binding protein A (CbpA; 1 μg/mL), and tetanus toxoid (TT; 1 Lf/mL) in peripheral blood mononuclear cells (PBMCs) (A) and adenoidal mononuclear cells (MNCs) (B) from children who were either culture positive (+) or negative (−) for pneumococci in nasal swab specimens. C, Cell proliferation in memory T cell–depleted (RO−) MNCs measured at day 8 after stimulation (n = 6 and n = 12 for culture-positive and culture-negative patients, respectively). *P < .05, compared with medium control; †P < .05, compared with culture-positive patients.

Association of CD4 T cell proliferation and serum antibodies to Ply and CbpA. To determine whether peripheral CD4 T cell responses are correlated with serum antibody levels, antigen-specific IgG and IgM antibodies to Ply and CbpA were measured in serum. There was a moderate correlation between CD4 T cell proliferation indices to Ply and F433 and anti-Ply IgG antibody levels (r = 0.31 and r = 0.34, respectively; n = 38; P < .05), but not anti-Ply IgM antibody levels (P > .05). There was no correlation between CD4 T cell proliferation and anti-CbpA IgG or IgM antibody levels (P > .05; n = 38).

DISCUSSION

In the present study, we showed that the pneumococcal proteins Ply and F433 and, to a lesser extent, CbpA induce CD4 T cell proliferation in both PBMCs and adenoidal MNCs from children. Upon stimulation by Ply or F433, the CD4 T cell proliferation index in PBMCs was significantly higher in children who were culture negative than in children who carried pneumococci in their nasopharynx. To our knowledge, this is the first report of T cell immunity to pneumococci in children and of any association between natural T cell immunity and carriage. It suggests that natural CD4 T cell immunity to pneumococcal protein antigens may modulate nasopharyngeal carriage, although it remains unclear whether this immunity can prevent new colonization or help clear existing carriage. The inverse relationship between a low CD4 T cell proliferation index (in response to stimulation by Ply and F433) and carriage found in PBMCs was not observed in adenoidal MNCs. One possible explanation for this apparent difference is that, in children who carried pneumoccus, antigen-specific (e.g., Ply+) memory CD4 T cells in the circulation (peripheral blood) may migrate to the site of infection (nasopharynx) and become sequestered in nearby lymphoid tissue, such as adenoids. Thus, fewer antigen-specific memory T cells would remain in peripheral blood, resulting in diminished memory T cell responses after stimulation in vitro. It has been previously reported that during acute pneumococcal infection, there is a transient loss of T cells, followed by the reappearance of these cells after treatment and clinical improvement [19, 20]. However, the...
Figure 4. Association between CD4 T cell proliferation and production of interferon (IFN)–γ and tumor necrosis factor (TNF)–α in peripheral blood mononuclear cells (PBMCs) after stimulation with pneumolysin (Ply; 0.05 μg/mL) (A and C); and association of cytokine production in PBMCs (B) and in adenoidal mononuclear cells (MNCs) (D) with patients’ carriage status. * , compared with culture-positive patients ( ). CbpA, choline-binding protein A; IL, interleukin; F433, pneumolysin toxoid.

children who carried pneumococcus in the present study did not seem to have a loss of CD4+ T cells from peripheral blood, because there was no difference in the percentage of CD4+ T cells in PBMCs between these subjects and subjects who were culture negative. In this context, colonized subjects did not have acute invasive infection. There appeared to be differences between CD4 T cell activation by Ply and CbpA, and our previous results showed that both Ply- and CbpA-specific antibodies were associated with absence of carriage [10]. The reasons why CbpA seems to induce mainly antibody-mediated immunity and Ply induces both CD4 T cell– and antibody-mediated immunity are currently unknown, but they could be explained, in part, by the apparent activating effects of Ply on antigen-presenting cells (authors’ unpublished observations).

CD45RA+ naive T cells predominated in PBMCs, whereas CD45RO+ memory T cells predominated in adenoidal MNCs. Using MACS cell separation, we assessed naive T cell proliferation in the latter cell populations, which showed the same pattern of lower responses to Ply and F433 in culture-positive children (figure 3C) as seen in peripheral blood (figure 3A). This observation raises the possibility that the differences in PBMCs observed between culture-positive and culture-negative children could be caused, at least in part, by differences in naive cell responses to these antigens. The ability of Ply to induce primary as well as memory CD4 T cell responses may be important for cellular immunity against pneumococcal carriage and also may have implications for the use of this antigen in pneumococcal vaccines intended for use in young children.

It is known that protein antigens generally induce T cell–dependent antibody responses, and we have previously shown...
that human mucosal anti-CbpA and -Ply antibody production is T cell dependent [21]. One could postulate that the observed inverse association between CD4 T cell immunity to Ply and carriage could reflect T cell support for production of antibodies in vivo. However, only a weak correlation was found between PBMC CD4 T cell proliferation and serum anti-Ply IgG antibodies, and no association with anti-CbpA IgG or IgM antibodies was found. This is compatible with the hypothesis that CD4 T cell immunity may have an antibody-independent effect against colonization. These results therefore concur with results of recent studies in mice showing that CD4 T cell immunity may protect against pneumococcal colonization [13, 22] by an antibody-independent mechanism [11, 12].

Human naive and memory T cells can generally be identified by the reciprocal expression of CD45RA or CD45RO isoforms [23, 24]. Using CD45RO+ T cell–depleted MNCs, we showed that Ply and F433 can induce CD4 T cell responses in both memory and naive phenotypes (figure 2C), whereas CbpA induced CD4 T cell responses of memory phenotypes only. The phenotypically naive T cellular response to Ply and F433 is interesting, because protein antigens usually require adjuvant help to induce primary responses, perhaps through effects on APCs (e.g., dendritic cells and macrophages) [25, 26], whereas such need for APCs in memory T cell responses is not as strict [27, 28]. Ply has been shown to induce activation of murine macrophages to secrete inflammatory cytokines TNF-α and IL-6 through TLR-4 [29]. In the present study, we showed that CD4 T cell proliferation induced by Ply is partially inhibited by antibody to TLR4, suggesting that Ply may induce T cell proliferation in part through a TLR4-dependent mechanism. Because no significant expression of TLR4 on CD4 T cells was observed, and because dendritic cells and macrophages are known to express TLR4 [30, 31], it is possible that the Ply-induced CD4 T cell response is mediated through TLR4-dependent activation of APCs.

We showed significant production of IFN-γ but negligible production of IL-4 in both PBMCs and adenoideal MNCs after stimulation with Ply and F433, suggesting a Th1-type response. In most infections, Th1-type responses have been associated with strong cell-mediated inflammatory responses, which may be favorable for pathogen elimination from the host [32]. It has been reported that pneumococcal infections are associated with increased trafficking of Th1 cells [19]. Mice lacking IFN-γ have been shown to be more susceptible to pneumococcal infection [33], and administration of IFN-γ could enhance survival of mice after pneumococcal challenge [34]. However, there appear to be differences in cytokine production between PBMCs and adenoideal MNCs after antigen stimulation. Significant induction of TNF-α was found in PBMCs but not in adenoideal MNCs, whereas the reverse was noted for IL-10. TNF-α is a proinflammatory cytokine that can promote protective immune responses against a variety of pathogens but that may also cause inflammatory host injury under certain conditions [35]. In contrast, IL-10 is a regulatory cytokine that can suppress the proliferation and differentiation of Th1 cells and, thus, can limit the potential immunopathology caused by inflammatory responses [36]. It is possible that, in the mucosal compartment exemplified by adenoideal MNCs, the production and actions of the proinflammatory cytokines IFN-γ and TNF-α are tightly controlled by IL-10. In accordance with this, we previously demonstrated inhibition by IL-10 of IFN-γ production by adenoideal cells [21]. CD4 T cell depletion from PBMCs or adenoideal MNCs showed significant reduction (75%–95%) in IFN-γ and TNF-α or IL-10 production after Ply or F433 stimulation (data not shown), suggesting that the CD4 T cell is a major cellular source of these cytokines. The induction of IFN-γ and TNF-α in PBMCs by Ply in association with T cell proliferation may play a role in protection against pneumococcal carriage in children.

The results of the present study support the hypothesis that CD4 T cellular immunity is involved in modulation of pneumococcal colonization in humans. The induction of both primary and memory CD4 T cell responses associated with Th1-type cytokine production adds further information on the nature of the immune responses to pneumococcal protein antigens. The induction of CD4 T cell responses by Ply appears to be regulated, in part, through TLR4 expressed by APCs. Novel vaccines that include conserved protein antigens, such as Ply or detoxified analogues, may induce significant CD4 T cellular immunity and reduce colonization of multiple serotypes of pneumococci.

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References


