T Cell Memory Response to Pneumococcal Protein Antigens in an Area of High Pneumococcal Carriage and Disease

Marianne W. Mureithi,†,*, Adam Finn,† Martin O. Ota,† Qibo Zhang,†,*, Victoria Davenport,‖,*, Timothy J. Mitchell,‖
Neil A. Williams,‖ Richard A. Adegbola,‖ and Robert S. Heyderman†,‡

†Bacterial Diseases Programme, Medical Research Council Laboratories, Banjul, Gambia; ‡Department of Cellular and Molecular Medicine, School of Medical Sciences, University of Bristol, Bristol, and ‖Division of Infection & Immunity, University of Glasgow, Glasgow, United Kingdom; ‡Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Blantyre, Malawi

Background. Streptococcus pneumoniae is a leading cause of vaccine-preventable disease worldwide. Pneumococcal protein antigens are currently under study as components of potential vaccines that offer protection against multiple serotypes. We have therefore characterized T cell pneumococcal immunity acquired through asymptomatic carriage.

Methods. Peripheral blood mononuclear cells from 40 healthy Gambian adults were stimulated with supernatants derived from S. pneumoniae strain (D39), 2 isogenic mutant strains lacking either pneumolysin or choline binding protein A, and recombinant pneumolysin. Immune responses were measured by cellular proliferation and by interleukin-10 (IL-10) and interferon-γ (IFN-γ) enzyme-linked immunosorbent spot and bioplex cytokine assays. Nasopharyngeal swabs were cultured to determine carriage rates.

Results. S. pneumoniae nasopharyngeal carriage was detected in 60% of individuals. Both effector and resting (or central) CD4+ T cell memory were frequently present to a range of pneumococcal antigens. However, the level of the effector memory response did not relate to current nasopharyngeal carriage. Pneumolysin was not immunodominant in these T cell responses but induced a distinct proinflammatory profile (high IFN-γ, IL-12[p40], and L-17 levels and low IL-10 and IL-13 levels).

Conclusions. In this population, T cell–mediated immunological memory potentially capable of pathogen clearance and immune surveillance is common but is not associated with the absolute interruption of pneumococcal carriage. How this naturally acquired immune memory influences pneumococcal vaccine efficacy remains to be determined.

Invasive bacterial diseases caused by pathogens that normally colonize the upper respiratory tract are most common in young children, elderly persons, and immunocompromised individuals [1]. An estimated 1.6 million deaths associated with pneumococcal infection occur each year, and they largely involve children, rather than healthy adults, and predominantly occur in resource-poor countries [2]. The relative infrequency of Streptococcus pneumoniae–associated cases of pneumonia, meningitis, and sepsis among healthy adults, even in these settings where the childhood incidence is high, may be due to the progressive acquisition of immune memory through multiple nasopharyngeal colonization events that commence early in life [3].

Classically, serum antibodies to pneumococcal capsular polysaccharide and, possibly, noncapsular protein antigens have been thought to provide protection against disease [4]. However, more recently it has been
suggested that although antibody has an important role, T cells may also be crucial for the development of natural immunity [5]. Murine challenge models exploiting B cell, T cell, β2-microglobulin, and major histocompatibility complex (MHC) class II knockout animals suggest that immunity to pneumococcal colonization and infection is dependent on CD4+ T cells but not on antibody production [6–8].

In humans, the incidence of invasive pneumococcal disease among human immunodeficiency virus (HIV)–positive patients is 20 times that among HIV-negative patients, even in the early phases of HIV infection [9, 10]. Mucosal CD4+ T cell immunity to pneumococcal protein antigens has been demonstrated in children and appears to modulate colonization [11]. Whether naturally acquired T cell memory persists into adulthood and protects against carriage and invasive disease remains to be determined.

It has been proposed that exposure to microbial antigens leads to the induction of effector T cells that home to peripheral tissues, resulting in pathogen elimination, but that have limited proliferative capacity. This immunity is consolidated by the generation of long-term resting (or central) memory T cells that have limited effector function but offer both immune surveillance and proliferative capacity and, thus, sustained protection [12]. Each population of immune cells has distinct cell surface markers and homing patterns; effector memory cells have lost the constitutive expression of CCR7 and frequently express CD45RO. Central memory cells are thought to express CCR7 and CD45RO ubiquitously [13–15]. What determines the evolution of these memory populations and their ontology/lineage is currently unclear. However, it has been proposed that T cell receptor signal strength is a major factor in determining T cell differentiation [16]. To investigate naturally acquired immunity to pneumococcus, it is important to determine whether persistent antigen exposure that occurs at the mucosal surface during colonization results in the predominance of a particular memory subset and whether different antigens result in different immune phenotypes.

Our previous studies of mucosal immunity in humans have pointed to the potential importance pneumococcal proteins pneumolysin (Ply) and choline binding protein—A (CbpA). We have shown that the presence of immune responsiveness to both antigens—specifically, B cell responses to CbpA [4] and T cell responses to Ply [11]—seems to correlate with the absence of carriage in children. Here, we study the immune response to these pneumococcal antigens in the blood to characterize natural immunity in a Gambian population in which the prevalence of pneumococcal carriage is high during early life (70%–90% of children aged <5 years) and adulthood [3, 17]. We demonstrate robust proliferation of T cells, consisting of both effector and long-term resting memory T cells, in response to a range of pneumococcal antigens in healthy adults. The level of effector T cell memory was not related to current nasopharyngeal carriage. Our data indicate that immunological priming presumably acquired after mucosal colonization results in T cell–mediated immunological memory. This immunity is potentially capable of pathogen clearance and immune surveillance but is not associated with the absolute interruption of pneumococcal carriage.

**SUBJECTS, MATERIALS, AND METHODS**

**Study population and samples.** A total of 40 healthy subjects were recruited from the West African country of Gambia, of whom 20 were from an urban setting and 20 were from a rural setting. The urban population was recruited from healthy blood donors at the GAMBLOOD national blood service. The rural population was recruited from Sibanor villages in Western Region. Venous blood and nasopharyngeal swab specimens were obtained after written consent was taken. The study was approved by the Medical Research Council Scientific Coordinating Committee and the Joint Gambian Government Ethics Committee, number 1020. All enrolled subjects had venous blood collected in heparinized tubes and processed soon after collection. Sufficient levels of blood were not available to perform all assays for all subjects. Swabs of nasopharyngeal specimens were inoculated into a vial of skim milk-tryptone-glucose-glycerol (STGG) transport medium.

**Microbiological investigation.** Nasopharyngeal swab specimens in STGG media were thawed and plated on gentamicin blood agar for the isolation of *S. pneumoniae*. Pneumococci were identified by morphologic characteristics and optochin susceptibility. Serotyping was performed with capsular and factor-typing sera (Staten Serum Institut), using antibody-coated latex agglutination [18].

**Antigens.** Pneumococcal culture supernatants were prepared from a standard encapsulated type 2 (D39) *S. pneumoniae* strain, an isogenic choline binding protein A–deficient mutant (CbpA−), and an isogenic pneumolysin–deficient mutant (Ply−); kindly provided by James Paton) [19]. The protein concentration of the concentrated pneumococcal culture supernatant (pneumoCCS) was determined using the Bio-Rad protein assay (Bio-Rad). Discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western immunoblotting demonstrated that the mutant supernatants lacked CbpA and Ply, respectively (results not shown). The concentrated culture supernatants were then heated at 56°C for 30 min to reduce toxic effects of pneumococcal proteins and used at a final concentration of 8 μg/mL. Recombinant Ply (rPly) was obtained from proteins expressed and purified from *Escherichia coli* expressing the gene and used at 1 μg/mL. The original source of the gene was the encapsulated type 2 pneumococcal strain D39 (NCTC7466) [20, 21].

Inactive trivalent split-virion influenza vaccine (Fluarix 2002/
2003 vaccine) was obtained from Aventis-Pasteur. M. tuberculosis purified protein derivative (PPD RT49) was obtained from Statens Serum Institut. Phytohemaglutinin (PHA) and soluble keyhole limpet hemocyanin were obtained from Sigma-Aldrich.

**Cell culture and immunodepletions by MACS sorting.** Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by Ficoll density gradient centrifugation and resuspended in complete RPMI 1640 medium supplemented with penicillin (100 U/mL), L-glutamine (4 mmol/L), HEPES, (10 mmol/L; Sigma-Aldrich), and 5% of heat-inactivated human AB serum. Cell depletion experiments for CD45RO+ and CCR7+ T cells were performed by negative selection, using MACS microbeads and magnetically-assisted cell sorting columns according to the manufacturer’s instructions (Miltenyi) as previously described [22–24]. Purity and efficiency of cellular depletions was assayed by flow cytometry and found to be >90% (data not shown).

**Proliferation assays.** Thymidine proliferation assay was performed as previously described [23, 24]. Briefly, fresh PBMCs were cultured in the presence of antigen or medium alone as background for up to 8 days. Proliferation was assayed by incorporation of 0.4 mCi [3H] thymidine (Amersham) during the final 16 h of incubation, and thymidine incorporation was quantified using a 1450 microbeta liquid scintillation counter (Wallac). Results were expressed as counts per minute. To identify the dividing T cell population, PBMCs were labeled with 3 μmol/L carboxyfluorescein diacetate 5, 6 succinimidyl ester (CFSE) (Molecular probes) as previously described [25]. Labeled cells were then cultured with antigen as described above for 7 days, stained with anti–CD4-APC and anti–CD8-PE (BD), and assessed by flow cytometry (FACSCalibur). Analysis was undertaken using FlowJo software (Treestar). Antigen-specific T cell proliferation was expressed as the percentage of proliferated CD4+ or CD8+ T cells.

**Ex vivo enzyme-linked immunosorbent spot (ELISPOT) assay.** To assess effector T cell responses, freshly isolated PBMCs were assayed for cells producing interferon-γ (IFN-γ) and interleukin-10 (IL-10), using an ELISPOT assay [26]. Nitrocellulose-backed 96-well MAIP S45 plates (Millipore) were coated overnight at 4°C with monoclonal antibody to either IFN-γ (concentration, 10 μg/mL) in sodium bicarbonate buffer or IL-10 (concentration, 5 μg/mL) in PBS. Plates were washed and blocked for 1 h with 10% fetal calf serum, for IFN-γ detection, and autologous plasma in RPMI, for IL-10 detection. PBMCs were seeded at 3 × 10^5 cells/well and incubated with or without antigen for 18 h. Spot-forming cells (SFCs) were then detected using 1 μg/mL of biotinylated anti-human IFN-γ antibody (MABTECH) or biotinylated anti-human IL-10 (BD) for 2 h at room temperature. Thereafter, plates were developed using a chromogenic alkaline phosphatase substrate kit (Bio-Rad). SFCs were quantified with an automated ELISPOT reader (AID), and data were expressed as SFCs per million PBMCs.

**Cultured IFN-γ ELISPOT.** To measure long-term resting (ie, central) memory, 1 × 10^6 PBMCs were cultured in 48-well plates (Nunc) with or without antigen for 10 days. On day 5 of the incubation, 500 μL of cell culture supernatant was removed for cytokine analysis, and cell cultures were enriched by replacing this with 500 μL of RPMI, 10% AB serum, and 40 IU/mL IL-2 (Biotest). On day 10, the cells were washed, counted, and resuspended in complete RPMI with 5% AB human serum before analysis by ELISPOT as described above.

**Cytokine profiles in culture supernatants.** Cell culture supernatants were analyzed for IL-10, IL-12p40, IL-13, IL-17, IL-18, TNF-α, and IFN-γ, using the human bioplex cytokine assay (Bio-Rad) according to the manufacturer’s instructions. Beads were read on the Bio-Plex 100 suspension array system (Applied Cytometry), and data were analyzed using Bio-Plex Manager software, version 3.0.

**Statistical analysis.** Statistical analyses and graphical presentation was done using Graphpad Prism 5 (Graphpad). Normally distributed data were analyzed using parametric tests. Nonnormally distributed data were analyzed using the Mann-Whitney U test. Results are given as means with standard deviations (for parametric tests) or as medians with ranges (for nonparametric tests). Differences after comparisons were considered statistically significant if they yielded P values of <.05.

**RESULTS**

**Subjects demographic data and nasopharyngeal carriage status.** A total of 40 healthy adults (median age, 36 years [range, 19–50 years], were recruited into the study. Twenty participants were urban-dwelling adult blood-bank donors (median age, 36 years [range, 19–49 years]); all were men. The remaining participants, of whom 10 were women, were from a rural area (median age, 40 years [range, 19–50 years]). Nasopharyngeal carriage of S. pneumoniae was detected in 8 (40%) of 20 urban dwellers and in 16 (80%) of 20 nonurban dwellers (P = .01, by the Fisher exact test). As in previous studies in Gambia, there was no difference in the carriage rate between men and women [3, 11].

**T cell proliferative responses to pneumococcal antigens.** PBMCs obtained from 6 sequentially recruited urban subjects and cultured in the presence of pneumococcal strains derived from either the WT pneumococcal strain (D39), Ply− and CbpA− D39 mutants, or rPly showed proliferative responses that peaked above background on day 7 (figure 1A). No early proliferative response typical of mitogenic activity was seen with any of the pneumococcal antigens. In comparison, peak responses to influenza virus antigen, which typically gives a secondary recall response in this assay [23], were detected on day 6 or 7 after
stimulation. Proliferative responses to keyhole limpet hemocyanin, which typically gives a primary response in this assay [24], were more modest, reaching a peak on day 8 or later after stimulation. To compare the antigen specificity of this T cell memory, collated day 7 peak proliferative responses in 6 subjects were examined (figure 1B). Although rPly induced a robust proliferative response suggestive of antigen-specific memory (figure 1C), there was no difference between the overall proliferative response to the pneumoCCS derived from the WT strain and the Ply− isogenic mutant.

Analysis of PBMCs from a further 6 adults from the urban population revealed that these proliferative responses involved both CD4+ and CD8+ T cell subsets, with CD4+ predominating in the CFSE assay (figure 2A). These findings were collated and are displayed in figure 2B.

**Ex vivo IFN-γ responses to pneumococcal protein antigens.** To assess whether the T cell proliferative responses were associated with memory cells of an effector phenotype, response to pneumococcal antigens was measured by ex vivo ELISPOT analysis in all subjects from the urban and rural populations.

All antigens were found to stimulate IFN-γ secretion after overnight stimulation (figure 3A and 3B). Again, rPly induced a response suggestive of antigen-specific memory, but there was no difference in the overall proliferative response to the pneumoCCS derived from the WT strain and the Ply− isogenic mutant. As shown in figure 3B, effector IFN-γ responses to WT pneumoCCS and rPly among adults from the rural setting were higher than those among their urban counterparts (113 vs 76 SFCs per million PBMCs for WT pneumoCCS [P = .02] and 108 vs 56 SFCs per million PBMCs for rPly [P = .04]), whereas the effector responses to the Ply− pneumoCCS and the control influenza virus antigen were similar in both groups. Detectable nasopharyngeal carriage from both the urban and village cohorts at the time of blood sampling was not a significant direct determinant of these effector responses (figure 3C).

**IFN-γ ex vivo responses to pneumococcal antigens mediated by CD45RO+ and not CCR7+ T cells.** Having demonstrated memory to a range of pneumococcal antigens by using the IFN-γ release assay, we evaluated PBMCs from 5 sequentially
Figure 2. Proliferative capacity of pneumococcal antigen–specific CD4+ and CD8+ T cells in response to a wild-type Streptococcus pneumoniae strain (WT), an isogenic pneumolysin (Ply)–deficient mutant (Ply−), and recombinant Ply (rPly). A, Flow cytometric data demonstrating CD4+ and CD8+ T cell proliferative responses to pneumococcal antigens, to phytohemagglutinin (PHA), and in unstimulated cells. The percentage indicated in the upper-left quadrant represents the cell population that proliferated over the 7-day culture period. B, Collated data from 6 adults from an urban setting, showing CD4+ and CD8+ T cell proliferative responses to pneumococcal antigens.
recruited urban subjects to confirm whether these cells had an effector memory phenotype. Ex vivo IFN-γ responses to pneumococcal antigens did not significantly change after CCR7+ depletion ($P = .74$) (figure 4A) but decreased significantly after CD45RO+ depletion ($P = .03$) (figure 4B). Thus, these IFN-γ effector pneumococcal responses have a CD4+CD45RO+CCR7+ effector memory phenotype.

**IFN-γ memory responses after 10-day cultured ELISPOT.**

To examine the possibility that the generation of effector memory is associated with the induction of long-term resting memory, PBMCs from all 20 sequentially recruited urban subjects were cultured for 10 days in the presence of pneumococcal antigens and IL-2 (added on day 5 of culture) before identification of IFN-γ SFCs by an ELISPOT assay (ie, cultured ELISPOT test). A higher number of antigen-specific IFN-γ-producing memory cells were detected by the cultured ELISPOT test than by the ex vivo overnight assay (figure 5). The cultured ELISPOT test revealed a bimodal distribution of both high and a low responding population. As before, rPly gave a robust memory response, but in contrast to effector memory, WT pneumoCCS gave a significantly greater IFN-γ response, compared with Ply+ pneumoCCS ($P = .03$).

**Effector IL-10 responses.** We have previously reported the predominance of IFN-γ and IL-10 production by mucosal mononuclear cells in response to pneumococcal antigens [27]. To define the nature of the immune memory described above and the regulatory effects of the pneumococcal antigens employed, an IL-10 ex vivo ELISPOT assay was established, and PBMCs from the rural population were analyzed. Both the pneumococcal antigens and PPD induced IL-10 production, but there was little IL-10 response to influenza virus antigens (figure 6A). No significant difference in the IL-10 response was observed between the different pneumococcal antigens. To assess the relationships between the proinflammatory and anti-inflammatory ex vivo responses to each antigen, an IFN-γ:IL-10 response ratio was calculated (figure 6B). This revealed an IFN-γ:IL-10 ratio for Ply+ CCS that was significantly lower than that for WT pneumoCCS ($P = .04$).
Cytokine profile of the cellular immune responses to pneumococcal antigens. To evaluate the spectrum of cytokines induced by the pneumococcal antigens, cytokines in samples from 6 sequentially recruited rural subjects were quantified in culture supernatants obtained after 5 days of PBMC culture (figure 7). All antigens induced IFN-γ, TNFα, IL-10, IL-12(p40), IL-13, and IL-17 production. Pneumolysin induced a distinct pro-inflammatory cytokine profile (high IFN-γ, IL-12[p40], IL-17 levels and low IL-10 and IL-13 levels). The differences between the IFN-γ and IL-10 ELISPOT data for the influenza virus antigens (high for IFN-γ and low for IL-10) were reconfirmed in the cell culture supernatants and were associated with the absence of TNF-α IL-18, IL-12(p40), and IL-13.

DISCUSSION

*S. pneumoniae* is highly adapted to its ecological niche in the upper respiratory tract and only rarely causes disease in healthy adults [28]. Pneumococcal proteins are major determinants of this adaptation, facilitating both colonization and invasion through host cell attachment, toxicity, and inflammation [29, 30]. Studies in animal challenge models have revealed that many of these virulence determinants are T cell–dependent antigens capable of inducing immunological memory and, thus, protection against invasive disease [5, 6, 31]. In contrast, there are few data describing pneumococcal T cell immune responsiveness in adults. We show that in an area of high pneumococcal colonization, adults have been primed and harbor peripheral blood CD4+ T cell immune responses to a range of pneumococcal proteins. This response has proliferative kinetics suggestive of T cell memory [32] and comprises both effector and resting memory [33]. As has been observed with naturally acquired immunity to other pathogens [34], there was no significant correlation between the ex vivo and the cultured ELISPOT responses to pneumococcal antigens within individuals. This suggests that the induction and maintenance of these functionally different memory populations are mediated in different ways.

Pneumococcal carriage experiments comparing antibody-deficient with CD4+ T cell–deficient mice suggest that CD4+ T cells are key to the mucosal clearance of *S. pneumoniae* [31]. Whether this is true for human mucosal T cell immunity against the pneumococcus has yet to be conclusively demonstrated. In contrast to what we have previously reported in the mucosa of UK children [4, 11], a more potent immune response in the blood was not directly associated with lower rates of carriage in this Gambian adult population. We therefore suggest that
although immunological priming by mucosal colonization induces immune responses in the blood that may prevent invasive disease, this does not entirely prevent carriage. Nonetheless, current pneumococcal carriage does not provide a complete picture of either single or multiple carriage events that have occurred in the past. When we compared a population of urban adults with a rural adult population who had a 2-fold higher rate of colonization, a significant difference in the frequency of effector memory was observed. Although this comparison has several potential confounders, we suggest that populations with a higher pneumococcal exposure have a higher frequency of effector memory and speculate that the relationship between carriage and immune memory is complex. In addition, pneumococcal capsule facilitates immune avoidance, and we and others [35] have demonstrated T-reg activity against a range of mucosal colonizers. We are therefore currently undertaking longitudinal studies of infant priming by carriage to address the balance between immunity, carriage, and disease.

As discussed, our data reveal at least 2 populations of memory cells, CD4+CD45RO+CCR7− cells, which are effector memory phenotypes (measured by ex vivo ELISPOT analysis) implicated in pathogen clearance, and a CD4+CD45RO+CCR7+ resting memory population (measured by 10-day cultured ELISPOT), which are thought to be responsible for immune surveillance [22]. We postulate that although current or recent pneumococcal carriage resulted in the effector T cell response, multiple exposure episodes may be required to induce long-term resting memory. We suggest that these mechanisms allow for the preservation of long-lasting immune memory with a range of clonal specificities after antigen encounter. Whether this immunity is capable of both pathogen clearance and immune surveillance remains to be determined.

We provide evidence that immune memory to the pneumococcus is also differentially influenced by antigen. Ply is a potential candidate vaccine antigen primarily found in the pneumococcal cytoplasm, and immune responses against it have shown it is protective in rodent challenge models [30, 36]. We have previously shown that Ply induces CD4+ T cell proliferation in part through antigen-specific activation of APCs augmented by a TLR4-dependent mechanism [37, 38]. In this study, Ply was not immunodominant but appeared to induce distinct recall responses. We observed a proinflammatory ELISPOT profile (a significantly higher IFN-γ:IL-10 ratio with Ply-CCS than with WT) and differential effects on effector and resting memory T cell populations. Whether this due to cognate T cell receptor–mediated interactions or to the broad-ranging costimulatory effects of Ply is not certain, but the high IFN-γ and IL-12 and low IL-10 and IL-13 cytokine profile is likely to enhance both antigen presentation and microbial clearance by the innate immune system [35]. IL-17 was also induced by Ply; Th helper cells that produce IL-17 (ie, Th-17 cells) have been described in mice as a distinct subset of effector cells [39], and it is has been suggested that immunity to pneumococcal colonization is mediated by IL-17A at the mucosal surface [40, 41]. Mice lacking IFN-γ are more susceptible to infection by S. pneumoniae, and administration of IFN-γ has been shown to enhance survival after challenge with S. pneumoniae [42].

In contrast, IL-10 is a regulatory cytokine that can suppress the proliferation and differentiation of Th1 cells and, thus, limit strong immunoinflammatory responses [43]. Some sequence types of serotype 1 pneumococcal strains recovered in Africa cause considerable disease but express a variant of Ply that is less biologically active [44]. Whether such variation alters the cytokine profile and, thus, the type of long-term memory that follows pneumococcal carriage requires further investigation.

It has become widely accepted that the generation of herd immunity by limiting pneumococcal carriage at the mucosal surface and, thus, blocking person-to-person spread is key to
Figure 7. Collated secreted cytokine profiles for 6 rural-dwelling subjects in peripheral blood mononuclear cell culture supernatants stimulated with wild-type *Streptococcus pneumoniae* strain (WT), an isogenic pneumolysin (Ply)-deficient mutant (Ply⁻), recombinant Ply (rPly), influenza virus antigen (Flu), and medium (Med), as determined by bioplex cytokine assay. Data are mean error of the mean. IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor–α.

the success of novel candidate vaccines [45]. Pneumococcal capsular polysaccharide-protein conjugate vaccines have been effective in a number of different contexts and appear to provide herd immunity [46]. However, there are a number of limitations to their widespread introduction in sub-Saharan Africa. The development of the 9-valent conjugate vaccine that was successful in Gambian trials was suspended pending development of a vaccine with a wider serotype range [47]. The widely used 7-valent conjugate vaccine does not induce immunity to serotypes 1 or 5, which are important in many re-
sources-poor countries [48]. Several pneumococcal surface proteins have been identified as potential vaccine components, which could circumvent some of these drawbacks and would be synergistic to the capsular polysaccharide approach. With this increasing research focus on the development of pneumococcal protein antigen-based vaccines, particularly for use in resource-poor settings, it will be important to determine the importance of these T cell memory populations in protection against disease.

In conclusion, we demonstrate that there is immune recognition of pneumococcal proteins by CD4 T cells in healthy adults who have been previously exposed to S. pneumoniae by carriage. How this naturally acquired immune memory influences pneumococcal vaccine efficacy, particularly in populations with high rates of colonization, requires further evaluation.

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