Cross-protective mucosal immunity mediated by memory Th17 cells against *Streptococcus pneumoniae* lung infection

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Pneumonia caused by *Streptococcus pneumoniae* (*Sp*) remains a leading cause of serious illness and death worldwide. Immunization with conjugated pneumococcal vaccine has lowered the colonization rate and consequently invasive diseases by inducing serotype-specific antibodies. However, many of the current pneumonia cases result from infection by serotype strains not included in the vaccine. In this study, we asked if cross-protection against lung infection by heterologous strains can be induced, and investigated the underlying immune mechanism. We found that immune mice recovered from a prior infection were protected against heterologous *Sp* strains in the pneumonia challenge model, as evident by accelerated bacterial clearance, reduced pathology, and apoptosis of lung epithelial cells. *Sp* infection in the lung induced strong T-helper type 17 (Th17) responses at the lung mucosal site. Transfer of CD4⁺ T cells from immune mice provided heterologous protection against pneumonia, and this protection was abrogated by interleukin-17A (IL-17A) blockade. Transfer of memory CD4⁺ T cells from IL-17A-knockout mice failed to provide protection. These results indicate that memory Th17 cells had a key role in providing protection against pneumonia in a serotype-independent manner and suggest the feasibility of developing a broadly protective vaccine against bacterial pneumonia by targeting mucosal Th17 T cells.

INTRODUCTION

Streptococcus pneumoniae (*Sp*) is a Gram-positive bacterium that is frequently found asymptotically colonizing the mucosal surface of the human nasopharynx. Colonization rates are as high as 50% in infants and 5–10% in healthy adult populations.¹ High colonization rates in young children are the primary source of pneumococcal transmission through direct contact or by aerosols.² This ability of *Sp* to establish asymptomatic colonization is an important mechanism that the organism uses to keep itself widely distributed in humans.^{2–4} Although the interactions between *Sp* and humans in the upper respiratory tract (RT) are benign, certain conditions (such as influenza virus infections) can alter the balance of the host–*Sp* interaction, leading to the progression of *Sp* infections into deep tissues and the development of various diseases, including otitis

media, pneumonia, sepsis, and meningitis.^{5–7} *Sp* is the most frequent cause of secondary bacterial pneumonia following influenza virus infection that often leads to severe diseases requiring hospitalization and resulting in high mortality.^{6,7}

Sp bacteria are encapsulated by polysaccharide layers surrounding their outer cell walls. The polysaccharide capsule has a significant role in the virulence of the organism, functioning to reduce clearance by mucosal secretions, prevent bacterial uptake by phagocytes, and decrease the binding of complement to the bacterial surface.^{8,9} The polysaccharide capsule is also a major target of antibody responses, and to date over 90 distinct serotypes of *Sp* have been identified that are characterized by structural and compositional variations in their capsules. Current pneumococcal-conjugated vaccine (PCV) is based on capsular polysaccharides conjugated to a

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protein carrier and its introduction has resulted in marked reductions in rates of nasopharyngeal colonization and consequently invasive pneumococcal diseases. However, PCV-induced immunity is antibody-based and highly serotype-specific, and only protective against 13 serotype strains whose capsular polysaccharide types are included in PCV.^{10,11} In recent years, there is increased prevalence of pneumococcal diseases caused by serotypes that are not included in PCV—a phenomenon termed serotype replacement that is leading to a decline of PCV efficacy in clinics.^{12,13} In addition, the high cost of PCV due to complex manufacturing processes limits its use in developing countries. Thus, there is an urgent, global need for an alternative pneumococcal vaccine that is affordable and can provide broad protection against various circulating serotype strains.^{2,14–17}

The development of mouse models of *Sp* colonization and invasive disease has aided in the identification of bacterial factors involved in *Sp* pathogenesis and host immune mechanisms of protection. Asymptomatic colonization (a carrier state) can be established by a concentrated bacterial inoculum applied in a small (10 μ l) volume to the nares of unanesthetized mice. In this model of defined nasal infection, bacteria remain detectable in nasal lavage fluid for several weeks without progressing into invasive diseases.^{18,19} Invasive *Sp* infections can be experimentally induced by the intranasal administration of a high volume bacterial inoculum (30–50 μ l) to anesthetized mice (direct lung infection model). Dosage in this manner bypasses the initial colonization step and introduces the organism directly into the lower RT, whereby pneumonia rapidly develops.²⁰ Prior colonization can induce protection against subsequent direct lung challenge with the same serotype strains (homologous), and the homologous protection against pneumonia is dependent on antibodies^{21,22} and T-helper type 17 (Th17) CD4⁺ cells.²² However, prior colonization induces minimal or partial protection against pneumonia by different serotype strains (heterologous).²³ Repeated immunizations with killed whole-cell vaccines or conserved proteins with potent experimental adjuvants (pertussis toxin) confer protection against lung infections by different serotype strains.^{24–29} The mechanisms of cross-protection against pneumonia remain controversial and undefined. Depletion of B cells, CD4⁺, or CD8⁺ T cells after immunization did not abrogate protection.^{25,27} On the other hand, immunization of μ MT mice with conserved *Sp* proteins/adjuvant failed to protect against pneumonia induced by heterologous strains, suggesting a role for B cells/antibodies.²⁹

To study the immune mechanism of cross-protection against pneumonia, we first tested approaches to induce the highest level of protection using the mouse model of direct lung infection.^{21,23,30} We found that a sublethal prior lung infection provided the best protection against pneumonia from subsequent direct lung infection by a heterologous *Sp* strain, whereas colonization or immunization with heat-killed *Sp* provided little or partial protection. We further investigated the immune mechanism of cross-protection induced by a prior

sublethal lung infection. Our results showed that Th17 cells, not antibodies, from immune mice contributed to the bacterial clearance in the lung following challenge with several different serotype strains.

RESULTS

Prior lung infection induces cross-protection in the murine pneumonia model

To test if cross-protection against pneumonia can be induced, we immunized B6 mice by colonization, lung infection, or intrapulmonary immunization with heat-killed *Sp* and asked if these mice are protected against subsequent direct lung infection by a heterologous *Sp* strain. For colonization, mice were inoculated intranasally with 1×10^7 colony-forming units (CFU) of P1121 (serotype 23F) in 10 μ l without anesthesia, which resulted in colonization of the upper RT.³¹ For direct lung infections, anesthetized mice were inoculated intranasally with 1×10^7 CFU of P1121 in 30 μ l, which resulted in direct infection of the lower RT and acute bacterial pneumonia. Similarly, 1×10^7 CFU of heat-killed P1121 were introduced intranasally to the lung of B6 mice in 30 μ l under anesthesia (intrapulmonary immunization). After 30 days, these mice were challenged with a lethal dose (2×10^7 CFU) of *Sp* strain TIGR4 (T4, serotype 4) by direct lung infection that resulted in acute pneumonia and mortality in naïve mice. Mice previously colonized with P1121 all succumbed to T4 challenge, although they survived for 1–2 days longer than the unimmunized mice. Immunization with heat-killed P1121 provided partial protection against T4 challenge, with 50% survival. Mice that resolved a prior lung infection by P1121 were 90% protected against subsequent challenge with the heterologous T4 strain (**Figure 1a**). These results show that prior live lung infection induces the highest level of protective immunity against pneumonia by a heterologous strain.

To further characterize heterologous protection induced by prior lung infection, we challenged mice with a lower dose of T4 (5×10^6 CFU) that caused morbidity but not death, allowing us to assess morbidity, bacterial load, and lung pathology at different time-points after the challenge. Mice resolved a prior P1121 lung infection (P1121 immune) and control mice lost body weight at a similar rate during the first two days after the T4 challenge. However, P1121-immune mice regained body weight much faster than control mice and fully recovered to their original body weight around days 7–9 postinfection (**Figure 1b**). Bacterial loads in the lung and bronchoalveolar lavage fluid (BALF) were determined at days 0.5, 1, 2, 3, 5, and 7 after T4 challenge. By day 1 after T4 challenge, there were already significantly fewer bacteria (~ 100 -fold) in the lungs and BALF of P1121-immune mice compared with unimmunized control mice. P1121-immune mice cleared bacteria between 3 and 5 days after infection, which was faster than unimmunized mice that did not clear till day 7 (**Figure 1c,d**). Histology showed that P1121-immune mice had moderate lung pathology with fewer inflammatory cells and less apoptosis of bronchial epithelial cells compared with control mice (**Figure 1e**). Taken together, these results of

Figure 1 Cross-protection against pneumonia induced by prior colonization, lung infection, or intrapulmonary immunization with heat-killed *Sp*. Mice were colonized with P1121 (colonization), infected with a sublethal dose of P1121 (live *Sp*), or immunized with heat-killed P1121 (HK *Sp*), and 30 days later were challenged by direct lung infection with a (a) lethal or (b–d) sublethal dose of T4. (a) Survival rates; (b) body weight loss; bacterial loads in (c) lung homogenate and (d) bronchoalveolar lavage fluid (BALF) on different days after T4 challenge; (e) representative TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) (green) and DAPI (4',6-diamidino-2-phenylindole; blue) staining of lung section at indicated days after T4 challenge of P1121 immune (P1121-T4) and control (PBS-T4) mice. Data are mean $\pm$ s.e.m. from at least three independent experiments with > 10 mice in each group. Bar = 200 μ m. LOD, limit of detection. A full color version of this figure is available at the *Mucosal Immunology* journal online.

reduced morbidity and mortality, lower bacterial loads, and less lung pathology clearly show that prior lung infection induces protective immunity against subsequent lung infection by a heterologous strain.

CD4⁺ T cells mount a robust Th17 primary and recall responses localized in the lung mucosa following *Sp* infection

To understand the immune mechanisms of cross-protection against pneumonia, we examined the primary responses in T4-infected mice (T4) as well as recall responses in P1121-immune mice challenged with T4 (P1121-T4). Compared with naïve mice, T4-infected mice had much higher percentages and

numbers of CD4⁺ T cells in the lungs. The P1121-T4 mice had even higher percentages and numbers of CD4⁺ T cells in the lungs than T4-infected mice (Figure 2a,b). Most of CD4⁺ T cells in the lungs of T4-infected and P1121-T4 mice had an activated CD44^{high} phenotype (data not shown). On the other hand, lung CD8⁺ T-cell population was not significantly increased in T4 and P1121-T4 mice compared with naïve mice (Figure 2a,b). Furthermore, no significant differences were observed in both CD4⁺ and CD8⁺ T cells in the spleen of naïve, T4, and P1121-T4 groups (Supplementary Figure S1A,B online). These results show that *Sp* lung infection induces a strong CD4⁺ T-cell response localized in the lung mucosa, and immune mice mount an even stronger CD4⁺

Figure 2 CD4⁺ T cells mount a rapid and robust T-helper type 17 (Th17) recall responses in the lung. (a) Percentage and (b) absolute number of pulmonary CD4 and CD8 T cells in naïve mice, mice infected with T4 only (T4), or P1121-immune mice challenged with T4 (P1121-T4) on day 7 after T4. Interleukin-17A (IL-17A) and interferon-γ (IFN-γ) production by CD4⁺ and CD8⁺ T cells after stimulation with heat-killed T4 as (c) visualized by FACS and (d) calculated as the number of CD4⁺IL-17A⁺, CD4⁺IFN-γ⁺, and CD8⁺IFN-γ⁺ per lung.

T-cell response, presumably due to recall responses by memory CD4⁺ T cells.

To characterize the function of T cells responding to *Sp* infection, we analyzed cytokine expression by intracellular cytokine staining following *in vitro* stimulation with heat-killed T4. A high percentage (~11%) of CD4⁺ T cells from lungs of T4-infected mice expressed interleukin-17A (IL-17A), whereas a small percentage of them (~2%) produced interferon-γ (IFN-γ) (Figure 2c,d). In the spleen, few CD4⁺ T cells expressed either IL-17A (~0.3%) or IFN-γ (~0.5%) (Supplementary Figure S1C,D). Lung CD8⁺ T cells expressed only IFN-γ (~4% in T4-infected mice; Figure 2) but no IL-17A (data not shown).

Very low levels of IFN-γ⁺CD8⁺ T cells were detected in the spleen of naïve and T4-infected mice (Supplementary Figure S1C). Thus, the primary T-cell response to *Sp* infection of the lung is a strong CD4⁺ response localized in the lung and consisted of mostly IL-17A-secreting CD4⁺ T cells with a small population of IFN-γ⁺CD4⁺ T cells.

In the P1121-T4 mice, there was an even stronger Th17 response (~30% CD4⁺) in the lung compared with T4-infected mice (~11% CD4⁺). This represented a sixfold increase in the total number of IL-17A⁺ CD4⁺ T cells in the lungs of the P1121-T4 mice over T4-infected mice (Figure 2d), indicating a strong Th17 recall response by memory CD4⁺

Figure 3 Transfer of *Streptococcus pneumoniae* (*Sp*)-specific memory T cells provides cross-protection against pneumonia. Lung and spleen T cells from naïve (Tn) or P1121 immune (Tm) were adoptively transferred into congenic (Ly5.2) B6 mice, which were then challenged with T4. **(a)** Bacterial loads in lung homogenate and bronchoalveolar lavage fluid (BALF) on day 2 after challenge. **(b)** Percentages and **(c)** number of donor (Ly5.1⁺) CD4 and CD8 T cells recovered from the lung at indicated days after T4 infection. **(d)** Percentage and **(e)** number per lung of interleukin-17A (IL-17A) and interferon- γ (IFN- γ)-producing donor CD4⁺ T cells after stimulation with heat-killed T4.

T cells. Surprisingly, there was minimal Th1 memory recall response. In fact, IFN- γ ⁺ CD4⁺ T cells in the lung of P1121-T4 mice were lower ($\sim 0.7\%$ CD4⁺) compared with T4-infected mice ($\sim 2.2\%$ CD4⁺). Similarly, there were less IFN- γ ⁺ CD8⁺ T cells in the lungs of P1121-T4 mice ($\sim 1.7\%$ CD8⁺) than in T4-infected mice ($\sim 3.8\%$ CD8⁺; **Figure 2c,d**).

The above results show that a primary T4 infection induces a rapid and robust CD4⁺ T-cell response in the lungs that consists of predominantly Th17 but also Th1 cells. In P1121-immune mice, the recall response to T4 challenge is exclusively by Th17 CD4⁺ T cells with minimal contribution by Th1 CD4⁺ or CD8⁺ T cells. Taken together, these data suggest that Th17 recall response by memory CD4⁺ T cells may have an important role in cross-protection against invasive pneumococcal disease by a different serotype strain.

***Sp*-specific memory T cells transfer acquired immunity against heterologous *Sp* pneumonia**

To investigate the role of immune cells in protection against heterologous *Sp* pneumonia, CD3⁺ (T cells) and CD3[−] (non-T cells) lymphocytes from the spleens and lungs of P1121-immune and naïve mice were transferred into congenic (Ly5.2) B6 mice, which were then challenged with a heterologous T4 strain. Mice that received memory T (Tm) cells from P1121-immune mice had 107- and 186-fold fewer bacterial CFU in the lungs and BALF, respectively, compared with mice that received naïve T (Tn) cells from unimmunized mice (**Figure 3a**). Mice that received non-T cells from immune mice were not protected, with similar numbers of CFU both in the lungs and BALF compared with mice that received non-T cells from naïve mice (**Supplementary Figure S2**). These results show that CD3⁺ T cells from P1121-immune

mice provide cross-protection against lung infection with a heterologous T4 strain.

The recall response and cytokine production by donor (Ly5.1) memory CD4⁺ and CD8⁺ T cells were examined on days 0, 2, and 7 after T4 challenge (**Figure 3b**). On day 0, $\sim 44\%$ of donor T cells were CD4⁺, while $\sim 56\%$ being CD8⁺. Following the T4 challenge, CD4⁺ T-cell population expanded greatly to make up $\sim 64\%$ and $\sim 91\%$ of the donor population on days 2 and 7, respectively. The total number of donor CD4⁺ T cells increased by 12.7-fold from $0.4 \pm 0.7 \times 10^4$ /lung on day 0 to $6.2 \pm 7.5 \times 10^4$ /lung on day 7. Many of the responding donor CD4⁺ T cells expressed IL-17A ($\sim 49\%$ on day 7), while very few of them produced IFN- γ ⁺ ($\sim 2\%$ on day 7). In contrast to CD4⁺ T cells, donor CD8⁺ T-cell population did not expand much in the lung (**Figure 3b**), and very few IFN- γ -producing CD8⁺ T cells were detected on day 2 or 7 after T4 challenge (**Supplementary Figure S3A,B**). In addition, no recall responses by donor CD4⁺ or CD8⁺ T cells were evident in the spleen (**Supplementary Figure S3C,D**).

Thus, adoptive transfer of memory T cells from P1121-immune mice conferred cross-protection against pneumonia. Donor memory CD4⁺ but not CD8⁺ T cells mounted a strong recall response in the lungs, and the responding donor CD4⁺ T cells consisted of mostly Th17 and very few Th1 effector cells.

Cross-protection against pneumonia by memory CD4⁺ T cells is dependent on IL-17A

Our results have thus far shown a strong Th17 recall response by memory CD4⁺ T cells in immune mice correlating with cross-protection. We next investigated the role of memory CD4⁺ T cells and IL-17A in cross-protection against pneumonia by adoptive transfer of purified immune CD4⁺

Figure 4 Memory CD4⁺ T cells transfer heterologous protection against pneumonia via interleukin-17 (IL-17)-mediated mechanism. (a–c) Purified CD4⁺ T cells from naïve (CD4⁺ Tn) or P1121 immune (CD4⁺ Tm) were transferred into congenic (Ly5.2) B6 mice, which were then challenged with T4. One group of mice that received CD4⁺ Tm were also treated with IL-17-neutralizing antibody (CD4⁺ Tm + αIL-17) or isotype control antibody (CD4⁺ Tm + isogG). (a) Bacterial loads in lung homogenate, (b) IL-17 and (c) interferon-γ (IFN-γ) in bronchoalveolar lavage fluid (BALF) on day 2 after T4 challenge. (d–f) Purified CD4 T cells from naïve (CD4⁺ Tn), P1121-immune (CD4⁺ Tm), and P1121-immune IL-17 knockout (KO) mice (CD4⁺ Tm/IL-17^{-/-}) were transferred into congenic (Ly5.2) B6 mice, which were then challenged with T4. (d) Bacterial loads in lung homogenate on day 2 after T4 challenge. (e) Percentage of donor CD4 cells in lung, and (f) IFN-γ and IL-17A production by CD4 T cells on day 7 after T4 challenge. IgG, immunoglobulin G; Tm, memory T; Tn, naïve T.

T cells and *in vivo* blockade of IL-17A. Purified CD4⁺ T cells from naïve or P1121-immune mice were adoptively transferred to congenic Ly5.2-naïve recipient mice that were then challenged with T4. On day 2 after T4 challenge, mice that received memory CD4⁺ T (CD4⁺ Tm) cells from P1121-immune mice had 224-fold fewer bacteria in the lung (1.58×10^5 CFU) than mice that received naïve CD4⁺ T (CD4⁺ Tn) cells (7×10^2 CFU). Blockade of IL-17A by anti-IL-17A-neutralizing antibodies abrogated the protective effect of donor CD4⁺ T cells, whereas isotype control immunoglobulin G had no effect on protection (Figure 4a). Increased levels of IL-17A and IFN-γ were detected in BALF on day 2 after T4 challenge in mice that received CD4⁺ Tm, as compared with mice that received CD4⁺ Tn. Treatment with anti-IL-17A-neutralizing antibodies decreased the level of IL-17A in the BALF but not IFN-γ as expected (Figure 4b,c).

To further evaluate a protective role for IL-17A specifically produced by memory CD4⁺ T cells, we purified CD4⁺ T cells from IL-17A^{-/-} mice that have resolved a prior P1121 infection, and adoptively transferred them into congenic naïve Ly5.2 mice. CD4⁺ T cells from P1121 immune IL-17A^{-/-} mice (CD4⁺ Tm/IL-17A^{-/-}) failed to mediate cross-protection; bacterial CFU in mice that received CD4⁺ Tm/IL-17A^{-/-} cells were similar to those in mice that received CD4⁺ Tn, while mice that received CD4⁺ Tm had less CFU (Figure 4d). Donor CD4⁺/IL-17A^{-/-} cells did not produce IL-17A as expected (Figure 4f), but they became activated and

expanded to levels similar to donor CD4⁺ T cells from immune B6 mice (Figure 4e). Thus, memory CD4⁺ T cells deficient in IL-17A failed to mediate cross-protection and this failure was not due to any defects in activation or expansion of donor CD4⁺/IL-17A^{-/-} cells in adoptive hosts. Taken together, these results show that cross-protection by memory CD4⁺ T cells is dependent on its ability to produce IL-17A.

Memory CD4⁺ T cells provide broad protection against pneumonia by different serotype strains

We further investigated if memory CD4⁺ T cells can provide broad protection against many different serotype strains prevalent in clinics. CD4⁺ T cells from naïve (CD4⁺ Tn) and P1121-immune (CD4⁺ Tm) mice were transferred to naïve mice, which were then challenged with 10^7 CFU of the homologous *Sp* strain P1121 (serotype 23F) or heterologous *Sp* strains including BAA659 (serotype 6A), 700671 (serotype 9V), and 700676 (serotype 14). Mice that received CD4⁺ Tm cells had significantly fewer bacteria in the lungs compared with mice that received CD4⁺ Tn after challenge with homologous P1121 or heterologous strains, indicating that memory CD4⁺ T cells provide broad protection against lung infection by different serotype *Sp* strains (Figure 5a). Consistent with broad protection, we observed strong Th17 recall responses to various serotype strains in P1121 immune mice; ~20% of lung CD4⁺ T cells from P1121-immune mice expressed IL-17A on day 7 after heterologous challenge, compared with ~2% from

Figure 5 *Streptococcus pneumoniae* (*Sp*)-specific memory CD4 T cells provide broad protection against lung infection by different clinic *Sp* isolates. (a and b) CD4 T cells from naïve (CD4⁺Tn, filled circle) or P1121-immunized mice (CD4⁺ Tm, open circle) were transferred into naïve B6 mice, which were then challenged with different *Sp* serotype strains. (a) Bacterial loads in lung homogenate on day 2 after challenge. (b) Interleukin-17A (IL-17A) production by CD4⁺ T cells following stimulation with heat-killed P1121, BAA659, 700671, and 700676. (c and d) Mice received sera from naïve (naïve sera) or P1121-immunized (P1121-immune sera) were infected with different *Sp* clinic isolates. (c) Bacterial loads in lung homogenate on day 2 after challenge. (d) Serum immunoglobulin G (IgG) against different *Sp* strains (whole bacteria) from P1121-immune mice (filled symbols), and against P1121 from naïve mice (open circle). Tm, memory T; Tn, naïve T.

unimmunized mice infected with these strains (Figure 5b). In contrast, transfer of P1121-immune sera protected against homologous P1121 challenge but resulted in no significant reduction of bacteria in the lung of mice challenged with heterologous strains (Figure 5c). P1121-immune sera had very low levels of crossreactive immunoglobulin G against BAA659, 700671, or 700676, which might account for the limited protection from P1121-immune sera (Figure 5d). Thus, CD4⁺ T cells but not sera from P1121-immune mice conferred broad protection against pneumonia caused by various serotype *Sp* strains.

DISCUSSION

Extensive studies of *Sp*-host interactions have focused on *Sp* colonization of the upper RT using the model of defined nasal infection. Although colonization is asymptomatic, it induces a rapid infiltration of neutrophils, followed by recruitment of monocytes/macrophages that is sustained until *Sp* colonization is cleared within 1–2 months. Although serotype-specific antibodies are generated, natural clearance of *Sp* colonization is not dependent on antibodies, but instead is reliant on

monocytes/macrophages and Th17 CD4⁺ T cells.¹⁹ On the other hand, capsule-specific antibodies induced by PCV have an important role in reducing the carriage rate over time in a serotype-specific manner.¹⁷ It is not known whether this is the result of immune-mediated protection against colonization in vaccine recipients or the result of reduced circulation of these serotypes through herd immunity. Introduction of PCV has led to a substantial decrease in pneumonia caused by *Sp*, which is thought to result from blockage of the initial step of colonization at the upper RT. It is not known if antibodies induced by PCV actually provide protective immunity in the lungs once *Sp* has gained entry and established infection in the lungs. In hosts that have weakened natural airway defenses such as following IAV infection, *Sp* may gain access and infect lungs directly resulting in rapid pneumonia without prior colonization of the upper RT. In this case, serotype-specific antibody induced by PCV may not be effective in preventing establishment of infection, and protection may require immune mechanisms other than capsule-specific antibodies that can attenuate infection in the lungs. Furthermore, increasing cases of *Sp* pneumonia are caused by replacement strains whose

capsular polysaccharides are not included in PCV. Thus, understanding immune mechanisms of protection against *Sp* in the lungs is critical for developing effective vaccines that are broadly protective against bacterial pneumonia.

In this study, we used the murine pneumonia model where *Sp* is directly introduced into the lungs while bypassing the initial colonization step,^{21,23,30} and focused on immune mechanisms of cross-protection against lung infection. We tested the possibility of inducing heterologous protection against pneumonia by prior colonization, immunization with heat-inactivated *Sp* and prior sublethal lung infection. Our results showed that prior lung infection induced the highest level of protection, better than prior colonization and killed *Sp* immunization (**Figure 1**). Our results further revealed robust primary and recall Th17 responses localized in the lung mucosa in response to *Sp* lung infection, whereas only weak Th17 responses were induced by prior colonization, or heat-inactivated *Sp* immunization (**Supplementary Figure S4**). Furthermore, our results showed that transfer of purified memory CD4⁺ T cells protected against pneumonia in an IL-17-dependent manner. We also tested the ability of memory CD4⁺ T cells to provide broad protection against clinical strains of different serotypes. While these clinical strains did not cause lethal pneumonia in mice (data not shown), there was significant reduction (100–1,000-fold) of bacterial loads in the lungs by memory CD4⁺ T cells against all four serotype strains tested. Taken together, these results show that induction of localized Th17 response in the lungs by mucosal immunization is critical for cross-protection against *Sp* pneumonia.

Our results suggest that conserved *Sp* protein antigens might be vaccine candidates to offer broad protection by inducing strong memory Th17 response in the lungs. Moffitt *et al.*³² have identified two new pneumococcal proteins (SP2108 and SP0148) that induce Th17 responses and protection against colonization. It remains to be determined if these antigens can induce protective immunity against lung infection. Other conserved *Sp* proteins, such as pneumococcal surface antigen A, pneumolysin, pneumolysoid PdT, and histidine triad proteins have been used with potent adjuvants as subunit vaccines that protect mice against pneumonia.^{24,26,27,29,33} Although these studies have not directly tested the role of memory Th17 cells in protection against pneumonia, these findings together with our results support the notion that Th17-inducing conserved *Sp* protein antigens might be vaccine candidates capable of conferring broad protection by inducing strong memory Th17 responses in the lungs.

Following a primary *Sp* infection, the CD4⁺ T-cell response consists of a mix of weak Th1 and dominant Th17 responses, and a weak CD8⁺ T-cell response in the lungs. Surprisingly, the recall response in immune mice was exclusively by Th17 CD4⁺ T cells with minimal contribution by Th1 CD4⁺ or CD8⁺ T cells. Previous studies have shown that IFN- γ ^{-/-} mice quickly succumb to *Sp* lung infection, indicating an important role for IFN- γ in controlling a primary lung infection.³⁴ NKT cells and neutrophils are the major source of IFN- γ for the early control of a primary lung infection.^{35–38} Upregulation of *ifn*

gene expression in the lung tissue is observed in *Sp*-immune mice challenged with the homologous strains, but the source and requirement of IFN- γ has not been identified.³⁹ Our studies specifically examined the role of IFN- γ produced by memory T cells in protection against reinfection. Our results showed that the levels of IFN- γ -secreting CD4⁺ and CD8⁺ T cells in immune mice challenged with T4 (P1121-T4) were similar at day 2, and even lower at day 7 compared with unimmunized mice infected with T4 (**Supplementary Figure S5**). Furthermore, *in vivo* blockade of IL-17A abrogated protective immunity conferred by adoptively transferred immune CD4⁺ T cells (**Figure 4**), while *in vivo* blockade of IFN- γ did not (**Supplementary Figure S6**). Taken together, these results indicate that Th1 and IFN- γ have a minimal role in protective immunity conferred by memory CD4⁺ T cells against reinfection, although IFN- γ has an important role in early control of primary infections.^{34–38} Th17 and Th1 responses are known to inter-regulate each other, and it remains to be determined if Th1 recall response is suppressed by a strong Th17 response or by other factors in this setting. Our data clearly demonstrated a critical role for Th17 cells, but it remains possible that the “balance” of Th17 and Th1 cells elicited following vaccination may be critical for generating effective protective immunity that promotes bacterial clearance and reduced immunopathology.

A combination of PCV with vaccines inducing Th17 memory T cells in the lungs may offer the best protection against bacterial pneumonia as they act at two distinct steps and by different immune mechanisms, antibodies blocking colonization at the upper RT while Th17 cells attenuating infection in the lungs.

METHODS

Animals. Female C57BL/6 and B6-Ly5.2/Cr (CD45.1) mice (6–8 weeks old) were purchased from National Cancer Institute (Fredericksburg, MD). IL-17A-deficient (IL-17 knockout (KO)) mice were originally developed by Dr Yoichiro Iwakura⁴⁰ and provided by Dr David Artis (Weill Cornell Medical College, New York, NY). All animal experiments were performed in accordance with The University of Pennsylvania Institutional Animal Care and Use Committee protocols.

Pathogens and infections. *Sp* strain P1121 (serotype 23F)⁴¹ and TIGR4 (serotype 4) were used as described previously.^{42,43} *Sp* strain BAA659 (serotype 6A), 700671 (serotype 9V), 700676 (serotype 14) were gifts from Dr Irving Nachamkin (Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA). All *Sp* strains were grown in tryptic soy broth or agar plates as described.⁴² For lung infections, mice were anesthetized by intraperitoneal injections with 100 μ l ketamine/xylazine (100 mg/3.8 mg kg⁻¹) and inoculated with 30 μ l of *Sp* suspensions (10⁶–10⁷ CFU) intranasally. For colonization, unanesthetized mice were inoculated intranasally with 10 μ l of bacterial suspension. Mice were observed for clinical signs of morbidity by monitoring body weights daily. BALF, lung homogenates were prepared as described,²² and 10 μ l of serial dilutions were plated in triplicate. The limit of detection for bacteria in lavage or lung homogenate was 10 CFU ml⁻¹.

Flow cytometry and luminex assay. Lymphocytes from the lungs and spleens were isolated and stained as described previously.^{44,45} For intracellular staining, cells were stimulated with heat-killed bacteria

(65 °C for 30 min) at indicated multiplicity of infection for 16 h with Golgi Plug/Stop added at the last 4 h, and then stained as described.⁴⁴ Millipore Multiplex Kits (MPXMCYTO-70k-16) were used for detection of cytokines and chemokines from BALF.

Histology. Lung sections were prepared as described previously.⁴⁶ Apoptosis was analyzed using *In Situ* Cell Death Detection Kit, fluorescein (Roche, Indianapolis, IN). Sections were processed and stained by the Cardiology Histology Core and Cancer Histology Core at the University of Pennsylvania.

Adoptive transfer and *in vivo* antibody treatment. Total T cells or CD4⁺ T cells from the spleens and lungs were enriched using MACS Pan T-cell Isolation Kit II or CD4 microbeads (Miltenyi Biotec, San Diego, CA),⁴⁷ and assayed for a purity (> 95%) by flow cytometry. Purified T cells, non-T cells or CD4 T cells (3–5 × 10⁶) were transferred intravenously into naive mice one day before *Sp* challenge.

In vivo IL-17 or IFN- γ neutralization was achieved as previously described with slight modifications.^{48–50} Mice received 180 μ g intraperitoneally and 20 μ g intranasally of anti-IL-17 (clone 17F3) or IFN- γ antibody (clone XMG1.2) on days – 1, 0, and 1 following *Sp* challenge. Neutralizing efficiency was verified using Luminex for BALF and ELISA (enzyme-linked immunosorbent assay) for serum on days 2 and 7 after infection.

Statistical analyses. Unpaired, one-tailed, Student's *t*-tests were used to calculate statistical significance between two groups and one-way analysis of variance was used for comparison of multiple groups followed by Bonferroni correction unless stated otherwise. *P* values are depicted as follows: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001 and ^{ns}*P* > 0.05. A *P* value $\leq$ 0.05 was considered significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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