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Cross Protective Mucosal Immunity Mediated by Memory Th17 Cells against *Streptococcus pneumoniae* Lung Infection

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Abstract

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 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 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 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 played 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.

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DISCLOSURE

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Keywords

pneumonia; *Streptococcus pneumoniae*; Th17; heterologous protection; vaccine

INTRODUCTION

Streptococcus pneumoniae (*Sp*) is a Gram-positive bacterium that is frequently found asymptomatically 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 which the organism uses to keep itself widely distributed in humans. [2-4] While 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 plays 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 protein carrier and its introduction has resulted in dramatic reductions in rates of nasopharyngeal colonization and consequently invasive pneumococcal diseases. However, PCV-induced immunity is antibody-based and highly serotype-specific, 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 μl) volume to the nares of un-anesthetized mice. In this model of defined nasal infection, bacteria remain detectible in nasal lavage fluid for several weeks without progressing into invasive diseases. [18, 19] Invasive *Sp* infections can be experimentally induced by the intranasal (i.n.) administration

of a high volume bacterial inoculum (30-50 μl) to anesthetized mice (direct lung infection model). Dosage in this manner bypasses the initial colonization step and introduces the organism directly into the lower respiratory tract, 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 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 μMT 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 sub-lethal prior lung infection provided the best protection against pneumonia from subsequent direct lung infection by a heterologous *Sp* strain, while 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 sub-lethal 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 i.n. with 1×10^7 colony-forming units (CFU) of P1121 (serotype 23F) in 10 μl without anesthesia which resulted in colonization of the upper respiratory tract. [31] For direct lung infections, anesthetized mice were inoculated i.n. with 1×10^7 CFU of P1121 in 30 μl which resulted in direct infection of the lower respiratory tract and acute bacterial pneumonia. Similarly, 1×10^7 CFU of heat killed P1121 were introduced i.n. to the lung of B6 mice in 30 μl under anesthesia (intrapulmonary immunization). 30 days later, 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 (Fig. 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 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 day 7-9 post infection (Fig. 1B). Bacterial loads in the lung and bronchoalveolar lavage fluid (BALF) were determined at day 0.5, 1, 2, 3, 5, and 7 post 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 to unimmunized control mice. P1121-immune mice cleared bacteria between 3-5 day post infection, faster than unimmunized mice which did not clear till day 7 (Fig. 1C, D). Histology showed that P1121-immune mice had moderate lung pathology with fewer inflammatory cells and less apoptosis of bronchial epithelial cells compared to control mice (Fig. 1E). Together, these results of 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 to 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 (Fig. 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 to naïve mice (Fig 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 (Fig S1A, B). 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 $CD4^+$ 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 ICS following *in vitro* stimulation with heat-killed T4. A high percentage (~11%) of $CD4^+$ T cells from lungs of T4-infected mice expressed IL-17A, while a small percentage of them (~2%) produced IFN-γ (Fig 2C, D). In the spleen, few $CD4^+$ T cells expressed either IL-17A (~0.3%) or IFN-γ (~0.5%) (Fig S1C, D). Lung $CD8^+$ T cells expressed only IFN-γ (~4% in T4-infected mice, Fig. 2) but no IL-17A (data not shown). Very low levels IFN-γ^+ $CD8^+$ T cells were detected in spleen of naïve and T4 infected mice (Fig. 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 to T4-infected mice (~11% $CD4^+$). This represented a 6-fold increase in the total number of IL-$17A^+$ $CD4^+$ T cells in the lungs of the P1121-T4 mice over T4-infected mice (Fig. 2D), indicating a strong Th17 recall response by memory $CD4^+$ 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 (~0.7% $CD4^+$) compared to T4-infected mice (~2.2% $CD4^+$). Similarly, there were less IFN-γ^+ $CD8^+$ T cells in the lungs of P1121-T4 mice (~1.7% $CD8^+$) than in T4-infected mice (~3.8% $CD8^+$, Fig. 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. Together, these data suggest that Th17 recall response by memory $CD4^+$ T cells may play 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-fold and 186-fold fewer bacterial CFU in the lungs and BALF, respectively, compared to mice that received naïve T (Tn) cells from unimmunized mice (Fig 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 naive mice (Fig. 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 day 0, 2, and 7 after T4 challenge (Fig. 3B). On day 0, ~44% of donor T cells were $CD4^+$ while ~56% being $CD8^+$. Following the T4 challenge, $CD4^+$ T cell population expanded greatly to make up ~64% and ~91% of the donor population on day 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 (~49% on day 7), while very few of them produced IFN-γ^+ (~2% on day 7). In contrast to $CD4^+$ T cells, donor $CD8^+$ T cell population did not expand much in the lung (Fig 3B), and very few IFN-γ-producing $CD8^+$ T cells were detected on day 2 or 7 post T4 challenge (Fig S3A, B). In addition, no recall responses by donor $CD4^+$ or $CD8^+$ T cells were evident in the spleen (Fig 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$^+$ 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, while isotype control IgG had no effect on protection (Fig 4A). Increased level of IL-17A and IFN-γ were detected in BALF on day 2 after T4 challenge in mice that received CD4$^+$ Tm, as compared to 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 (Fig 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 (Fig 4D). Donor CD4$^+$/IL-17A$^{-/-}$ cells did not produce IL-17A as expected (Fig. 4F), but they became activated and expanded to levels similar to donor CD4$^+$ T cells from immune B6 mice (Fig. 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. 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* stains 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 to 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 (Fig 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 to ~2% from unimmunized mice infected with these strains (Fig 5B). In contract, 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 (Fig 5C). P1121 immune sera had very low levels of cross-reactive IgG against BAA659, 700671 or 700676, which might account for the limited protection from P1121 immune sera (Fig 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 respiratory tract using the model of defined nasal infection. While 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. Though 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 play 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 respiratory tract. 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 respiratory tract. 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 sub-lethal lung infection. Our results showed that prior lung infection induced the highest level of protection, better than prior colonization and killed *Sp* immunization (Fig 1). Our results further revealed robust primary and recall Th17 responses localized in the lung mucosa in response to *Sp* lung infection, while only weak Th17 responses were induced by prior colonization, or heat inactivated *Sp* immunization (Fig 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 didn't cause lethal pneumonia in mice (data not shown), there was significant reduction (100-1000 fold) of bacterial loads in the lungs by memory $CD4^+$ T cells against all 4 serotype strains tested. 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. have identified two new pneumococcal proteins (SP2108 and SP0148) that induce Th17 responses and protection against colonization. [32] 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 (PsaA), 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-$\gamma^{-/-}$ 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] Up-regulation of *ifng* 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 re-infection. 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 to unimmunized mice infected with T4 (Fig. S5). Furthermore, *in vivo* blockade of IL-17A abrogated protective immunity conferred by adoptively transferred immune $CD4^+$ T cells (Fig. 4), while *in vivo* blockade of IFN-γ did not (Fig. S6). Together, these results indicate that Th1 and IFN-γ play a minimal role in protective immunity conferred by memory $CD4^+$ T cells against re-infection, though IFN-γ plays 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 respiratory tract while Th17 cells attenuating infection in the lungs.

MATERIALS AND METHODS

Animals

Female C57BL/6 and B6-Ly5.2/Cr (CD45.1) mice (6-8 wks old) were purchased from National Cancer Institute (Fredericksburg, MD). IL-17A deficient (IL-17 KO) mice were originally developed by Dr. Yoichiro Iwakura [40] and provided by Dr. David Artis (Weill Cornell Medical College). All animal experiments were performed in accordance with The University of Pennsylvania Institutional Animal Care and Use Committee protocols.

Pathogens and infections

S. pneumoniae (*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 (Dept. of Pathology and Laboratory Medicine, University of Pennsylvania). All *Sp* strains were grown in tryptic soy broth or agar plates as described. [42] For lung infections, mice were anaesthetized by intraperitoneal (i.p.) injections with 100 μl Ketamine/ Xylazine (100 mg/ 3.8 mg/ kg) and inoculated with 30 μl of *Sp* suspensions (10^6-10^7 CFU) i.n. For colonization, unanesthetized mice were inoculated i.n. with 10 μl 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 μl of serial dilutions were plated in triplicate. The limit of detection (LOD) for bacteria in lavage or lung homogenate was 10 CFU/ml.

Flow cytometry and Luminex Assay

Lymphocytes from the lungs and spleens were isolated and stained as previously described. [44, 45] For intracellular staining, cells were stimulated with heat killed bacteria (65°C for 30 min) at indicated multiplicity of infection (MOI) for 16 hrs with Golgi Plug/ Stop added at the last 4 hrs, 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, Germany). 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 ($3\text{-}5 \times 10^6$) were transferred i.v. into naive mice one day prior to *Sp* challenge.

In vivo IL-17 or IFN-γ neutralization was achieved as previously described with slight modifications. [48-50] Mice received 180 μg i.p. and 20 μg i.n. of anti-IL-17 (Clone 17F3) or IFN-γ antibody (clone XMG1.2) on days −1, 0, 1 following *Sp* challenge. Neutralizing efficiency was verified using Luminex for BALF and ELISA for serum on days 2 and 7 post 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 (ANOVA) 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 ≤ 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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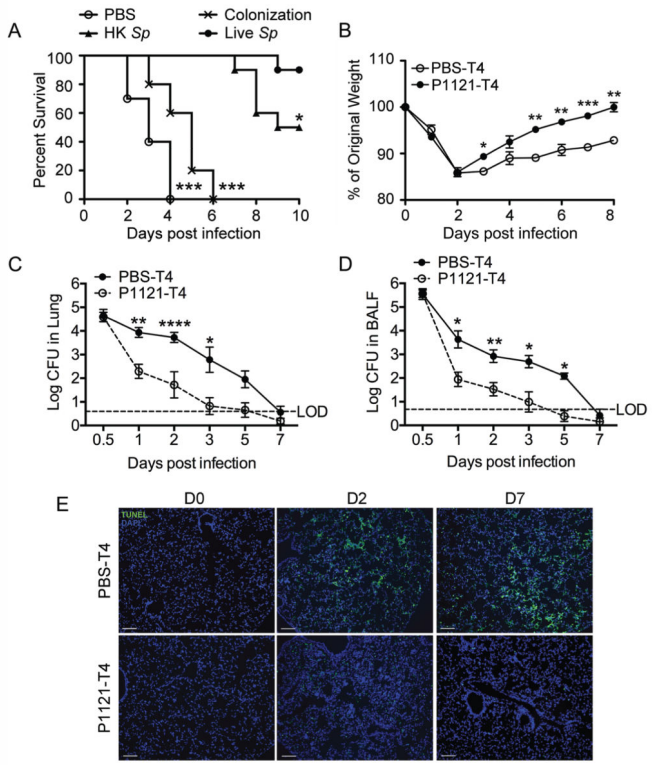

Fig 1. Cross protection against pneumonia induced by prior colonization, lung infection, or intrapulmonary immunization with heat-killed *Sp*

Mice were colonized with P1121 (colonization), infected with a sub-lethal 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 lethal (A) or sub-lethal (B-D) dose of T4. (A) Survival rates; (B) body weight loss; Bacterial loads in (C) lung homogenate and (D) BALF on different days post T4 challenge; (E) Representative TUNEL (green) and DAPI (blue) staining of lung section at indicated days post T4 challenge of P1121 immune (P1121-T4) and control (PBS-T4) mice. Data are mean ± SEM from at least three independent experiments with >10 mice in each group. LOD: limit of detection. Scale bar = 200 μM.

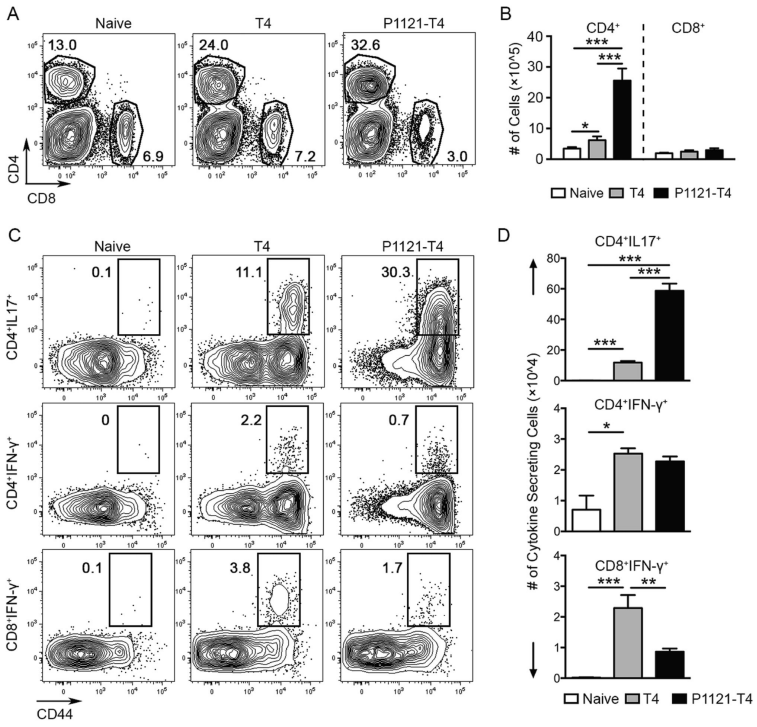

Fig 2. CD4$^+$ T cells mount a rapid and robust Th17 recall responses in the lung

(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. IL-17A and 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.

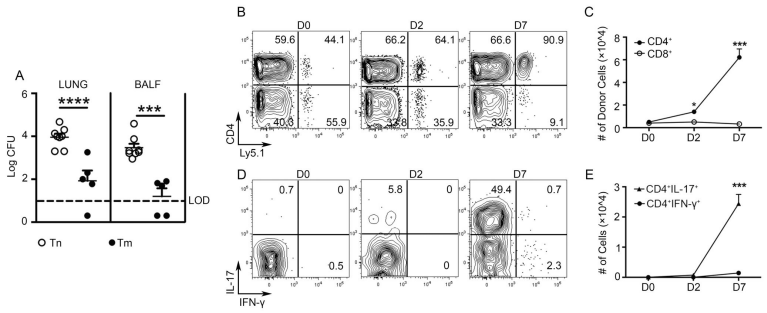

Fig. 3. Transfer of *Sp*-specific memory T cells provides cross-protection against pneumonia

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 BALF on day 2-post challenge. (B) Percentages and (C) number of donor ($Ly5.1^+$) CD4 and CD8 T cells recovered from the lung at indicated days post T4 infection. (D) Percentage and (E) number per lung of IL-17A and IFN-γ producing donor $CD4^+$ T cells after stimulation with heat killed T4.

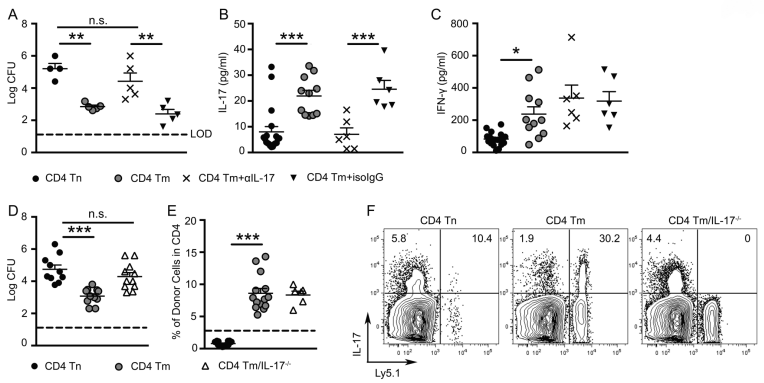

Fig. 4. Memory CD4$^+$ T cells transfer heterologous protection against pneumonia via IL-17 mediated mechanism

(**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 received CD4$^+$Tm were also treated with IL-17 neutralizing antibody (CD4$^+$Tm +αIL-17) or isotype control antibody (CD4$^+$Tm+isoIgG). (A) Bacterial loads in lung homogenate, (B) IL-17 and (C) IFN-γ in BALF on day 2 post T4 challenge. **(D-F)** Purified CD4 T cells from naïve (CD4$^+$Tn), P1121 immune (CD4$^+$Tm), and P1121 immune IL-17KO 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 post T4 challenge**;** (E) % of donor CD4 cells in lung, and (F) IFN-γ and IL-17A production by CD4 T cells on day 7 post T4 challenge.

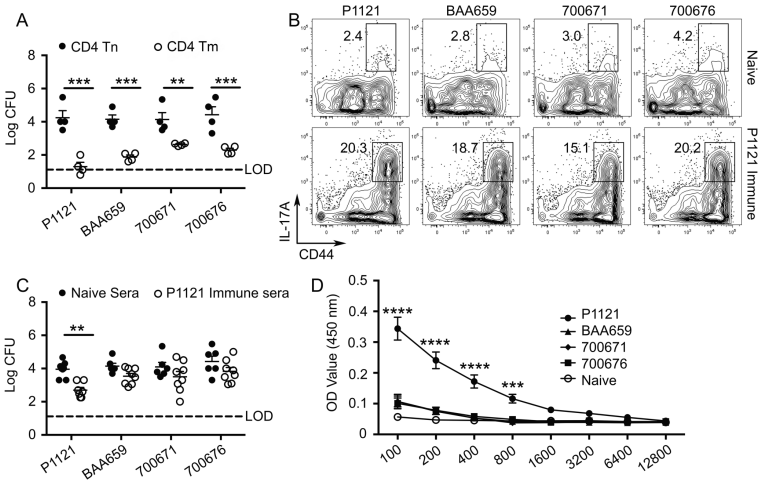

Fig. 5. *Sp*-specific memory CD4 T cells provide broad protection against lung infection by different clinic *Sp* isolates

(**A-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 post challenge. (B) IL-17A production by $CD4^+$ T cells following stimulation with heat killed P1121, BAA659, 700671, 700676. (**C-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 post challenge. (D) Serum IgG against different *Sp* strains (whole bacteria) from naïve (filled circle) and P1121 immune mice (open circle).