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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2010/12/03/jimmunol.1001963.DC1

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CD8⁺ Cells Enhance Resistance to Pulmonary Serotype 3 Streptococcus pneumoniae Infection in Mice

Sarah E. Weber,* Haijun Tian, † and Liise-anne Pirofski* †

Despite the success of the pneumococcal conjugate vaccine, pneumococcal pneumonia remains a significant clinical problem, and there is still much to learn about natural resistance and cellular immunity to pneumococci. We investigated the role of T lymphocytes in resistance to serotype (ST) 3 Streptococcus pneumoniae in an intranasal infection model in C57BL/6 (wild-type [Wt]) and CD8⁺ (CD8⁻/⁻) and CD4⁺ (MHC class II⁻/⁻)-deficient mice. CD8⁻/⁻ mice exhibited significantly more bacterial dissemination and lung inflammation and a significantly more lethal phenotype than Wt mice. However, there was no difference in the bacterial dissemination, lung inflammation, or survival of Wt and MHC class II⁻/⁻ mice. Perforin (Pfn)⁻/⁻ and IFN-γ⁻/⁻ mice, which were used to dissect the role of CD8⁺ T cells in our model, also exhibited a more lethal survival phenotype than Wt mice. Comparison of lung chemokine/cytokine levels by Luminescence and cellular recruitment by FACS in Wt mice and knockout strains revealed that CD8⁻/⁻ and IFN-γ⁻/⁻ mice, which had the most lethal survival phenotype, had more CD4⁺IL-17⁺ T (Th17) cells, IL-17, neutrophil chemoattractants, and lung neutrophils, and fewer regulatory T cells than Wt mice. CD4⁺ T cell depletion improved the survival of ST-infected CD8⁻/⁻ mice, and survival studies in Th17-deficient mice revealed that the Th17 response was dispensable for ST3 resistance in our model. Taken together, these findings demonstrate that CD8⁺ cells are required, but CD4⁺ T cells are dispensable for resistance to ST3 pneumonia in mice and suggest a previously unsuspected role for CD8⁺ cells in modulating the inflammatory response to ST3. The Journal of Immunology, 2011, 186: 432–442.

Use of the seven-valent Streptococcus pneumoniae capsular polysaccharide conjugate vaccine led to a decrease in invasive pneumococcal disease with included serotypes (STs) in children (1) and adults as a result of herd immunity (2). Nonetheless, there are still important roadblocks to achieving universal prevention of pneumococcal disease. For example, immunocompromised individuals remain at higher risk for disease (2), the emergence of nonvaccine STs is a significant concern (3), and there is uncertainty as to whether the current (unconjugated) polysaccharide vaccine that is used in adults prevents pneumonia (4). Among non–seven-valent Streptococcus pneumoniae capsular polysaccharide conjugate vaccine STs, ST3 is an important cause of pneumococcal disease that has a higher mortality rate than other STs (5). ST3 has emerged as a cause of severe pneumonia and empyema in children (6) and investigational pneumococcal conjugate vaccines, which contained an ST3 moiety, failed to protect vaccinated children against ST3 pneumonia (7). Hence, there is a need to gain a better understanding of host factors that bear upon immunity to ST3 pneumonia, such as the role of T cells in resistance to disease.

CD8⁺ T cells are known to contribute to host defense against microbes through IFN-γ production and/or cytotoxic effects mediated by secretion of perforin and granzyme. The role of CD8⁺ T cells in host defense has been studied most extensively for intracellular pathogens, such as Mycobacterium tuberculosis and Listeria monocytogenes (8). However, a role for CD8⁺ T cells in resistance to fungi, including Cryptococcus neoformans and Pneumocystis (9, 10), and some extracellular bacteria (11) has also been established. In addition, CD8⁺ T cells were shown to be required for resistance to ST3 pulmonary infection in immune (ST3 pneumococcal capsular polysaccharide-immunized) mice (12), but to our knowledge, the role of CD8⁺ T cells in natural resistance to pneumococcus in naïve hosts has not been investigated previously.

The role of CD4⁺ T cells in immunity to experimental pneumococcal infection has been studied in colonization and pneumonia models. CD4⁺ T cells were required for resistance to nasopharyngeal colonization with ST 6B, 7F, 14, and 23 (13–15) and bacterial clearance in an ST2 pneumonia model in a study comparing MHC class II-deficient (MHCII⁻/⁻) and wild-type (Wt) mice (16). The role that CD4⁺ T cells play in resistance to colonization has been linked to neutrophil recruitment and enhancement of bacterial clearance by CD4⁺ Th17 cells (14, 16, 17). However, the IL-17 response has also been linked to detrimental inflammation, albeit in other models (18, 19). In this article, we investigated the role of CD8⁺ and CD4⁺ T cells in resistance to intranasal (i.n.) infection with ST3 in naïve mice. Our results show that CD4⁺ T cells were dispensable, but CD8⁺ cells were required for resistance to lethal challenge with ST3, which was associated with a reduced inflammatory response in the lungs and less bacterial dissemination in CD8⁺ T cell sufficient compared with CD8⁺-deficient mice.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: DC, dendritic cell; i.n., intranasal; KC, keratinocyte-derived chemokine; MHCII⁻/⁻, MHC class II-deficient; ND, not detectable; Pfn⁻/⁻, perforin-deficient; ST, serotype; Treg, regulatory T cell; TSB, tryptic soy broth; Wt, wild-type.

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Materials and Methods

Bacteria and pneumococcal infection model

Three ST3 Streptococcus pneumoniae strains were used: 1) WU2 (provided by S. Hollingshead, University of Alabama, Birmingham, AL), 2) 6303 (American Type Culture Collection, Manassas, VA), and 3) A66.1 (A66) (provided by D. Briles, University of Alabama). S. pneumoniae strains 6308 (ST8) and D39 (ST2) (both American Type Culture Collection) were also used for survival studies. Pneumococci were grown in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) to midlog phase at 37˚C in 5% CO2 as described previously (12). Aliquots were frozen in TSB-10% glycerol at −80˚C for use as needed. For infection studies in mice, aliquots of pneumococci were thawed immediately before use and diluted to contain the desired amount of bacteria in TSB. Upon challenge, mice were anesthetized with isoflurane and inoculated i.n. with pneumococci by age-matched mice at a dose of 40 µl TSB/mouse, 20 µl to each nare. An i.a. control was performed from Wt mice with mAb 2.43 (Bio X Cell), and CD8+ cell-deficient mice (B6.129S2-Ifnγnull/J; The Jackson Laboratory, Bar Harbor, ME). C57BL/6 mice were obtained from the National Cancer Institute (Charles River Laboratories, Wilmington, MA) and used as Wt controls. All mice were maintained in the Institute for Animal Studies of the Albert Einstein College of Medicine (AECOM) (Bronx, NY). C57BL/6 mice were obtained from the National Cancer Institute (Charles River Laboratories, Wilmington, MA) and used as Wt controls. All mice were maintained in the Institute for Animal Studies of AECOM and given unrestricted access to food and water. All mouse experiments were conducted with prior approval from the Animal Care and Use Committee of AECOM, following established guidelines.

Adaptive transfer studies

Peripheral (superficial inguinal, cervical, brachial, and axillary) and mesenteric lymph nodes and the spleen were collected from naive Wt mice. Tissues were minced for single-cell suspensions and counted. A negative CD8+ T cell selection was performed using magnetic beads according to the manufacturer’s recommended protocol (Miltenyi Biotec, Auburn, CA). The lymph nodes and the spleen were homogenized in HBSS without calcium, magnesium, or phenol red, and mixed tissues were put through 70-µm nylon mesh, and placed in a 37˚C incubator for 30 min. After incubation, minced tissues were put through 70-µm nylon mesh, and adhered to slides. Staining with fluorescent Abs to confirm that the CD4+ or CD8+ cells were removed aseptically and homogenized in HBSS without calcium, magnesium, or phenol red. Both the blood and lung homogenate samples were serially diluted in TSB and plated onto trypticase soy agar with 5% sheep’s blood plates (BD Biosciences). The plates were incubated for 18 h at 37˚C in 5% CO2, and then, the number of CFUs was counted. A 48-h postinfection time was analyzed with four to seven mice per group and repeated for WU2.

Histopathology

Mice were anesthetized and killed by cervical dislocation 48 h after infection with A66 and 24 and 36 h after infection with WU2 and 6303, respectively, after which their lungs were inflated with 4% formalin (Fisher Scientific, Fairlawn, NJ), fixed for at least 48 h in situ, removed, embedded in paraffin, cut into 5-µm-thick sections, and adhered to slides. Staining was then performed with H&E (Polyscience, Bayshore, NY). Sections were obtained from three mice per group for each of the mouse strains, except for MHCII−/− mice, for which n = 2. H&E sections were evaluated based on the distribution and severity of pneumonia, vasculitis, and overall tissue damage. Scores ranging from 0 to 5 were assigned, whereby 0, no finding (i.e., pneumonia, vasculitis, or tissue damage); 1, minimal; 2, moderate; 4, marked; and 5, severe. A moderate score for the pneumonia classification was defined as affecting 25% of the lobe, and moderate vasculitis was defined as focal areas of dense cellular infiltration around veins or blood vessels. Moderate overall tissue damage was characterized by a large, acute area (~200 by 4000 µm) of infiltrating cells, with some appearing necrotic in the perivascular and peribronchiolar space.

Lung cytokine and chemokine levels

Chemokine and cytokine levels in the lungs were determined in Wt mice and the knockout strains in the naive state and 24 and 48 h after infection with A66. The chemokines and cytokines that were analyzed are those that have been previously described in mouse models of pneumococcal pneumonia (23–25). Mice were anesthetized with isoflurane and killed by cervical dislocation, after which their lungs were removed aseptically, homogenized in HBSS without calcium, magnesium, or phenol red, and centrifuged at 3000 × g for 30 min. The supernatants were extracted and immediately frozen at −20˚C. Before use, the supernatants were thawed and spun at 10,000 × g for 15 min to remove any further cellular debris. Beadlyte mouse 21-plex (Upstate, Temecula, CA) and Milliplex mouse 32-plex (Millipore, Billerica, MA) cytokine detection systems were used according to the manufacturer’s instructions to identify cytokines and chemokines that changed postinfection. The plates were read on the Luminex 200. Data were analyzed by converting median fluorescence intensities of each cytokine into picograms per milliliter concentrations using Beadview software (Upstate).

For detection of TGF-β1, ELISA Duoset kit (R&D Systems, Minneapolis, MN) was used, according to the manufacturer’s supplied protocol. Absorptions were measured on a microplate reader (Tecan, Durham, NC). Two separate experiments with five mice per group were performed for each time examined.

Flow cytometry

The phenotypes of cells in the lungs were determined by flow cytometry in Wt mice and the knockout strains in the naive state and 24 and 48 h after infection with A66. Mice were anesthetized and killed, after which their lungs were perfused immediately with 10 ml PBS and excised. Excised lungs were then rinsed in PBS, minced with razor blades in petri dishes containing 10 ml digestion buffer (10% FBS, 1 mg/ml collagenase A [Calbiochem, La Jolla, CA], and 0.025 mg/ml DNase I [Roche, Basel, Switzerland]), and placed in a 37˚C incubator for 30 min. After incubation, minced tissues were put through 70-µm mesh strainers and centrifuged at 1200 rpm for 7 min, the supernatant was removed, and the cells were lysed in 5 ml 0.17 M NH4Cl (Sigma-Aldrich, St. Louis, MO) buffer (pH 7.2) for 7 min and centrifuged. The pelleted cells were washed in PBS and centrifuged again, and the pellets were resuspended in staining buffer, 1% PBS, and centrifuged at 3000 × g for 30 min. After staining, cells were stained with combinations of the following Abs: CD3 Alexa Fluor 647 (BD Pharmingen, San Diego, CA), CD8α PE (BioLegend, San Diego, CA), CD8α FITC (BD Pharmingen), and CD4 PE-Cy5 (eBioscience). To identify neutrophils (all BD Pharmingen), CD11c PE-Cy7 to identify dendritic cells (DCs), F4/80 allopoxycyanin to identify macrophages (all eBioscience), and DX5 Biotin to identify NK and NKT cells followed by streptavidin PE-Cy7 (both BioLegend, San Diego, CA).
The cells were incubated with the Abs for 15 min, after which the samples were washed and resuspended in staining buffer. DAPI was added immediately before samples were collected on an LSRII (BD Biosciences, San Jose, CA). Data were analyzed with FlowJo (Tree Star, Ashland, OR) by double gating on live cells in the forward and side scatter and DAPI negative. The positive populations of each marker were then gated. Two separate experiments were performed with three mice per group for each time examined.

**Intracellular staining**

Lungs were prepared from Wt mice and the knockout mouse strains 48 h after infection with A66 in the same manner as for flow cytometry. A total of $10^7$ cells were resuspended in 1 ml 10% FBS RPMI 1640 medium in the presence of leukocyte activation mixture with GolgiPlug (BD Pharmingen) in a 24-well tissue culture plate and incubated at 37°C for 4 h. Cells were stained following the BD Pharmingen-recommended protocol for intracellular staining. Briefly, cellular FcRs were blocked with CD16/32, CD3 Alexa Fluor 647 (both BD Pharmingen), CD4 PE-Cy5, and CD6 Alexa Fluor 750 (both eBioscience) were added to the cells and incubated for 15 min. The cells were washed, and live/dead violet dye (Invitrogen, Carlsbad, CA) was added for viability, after which the cells were washed and membranes fixed/permeabilized using BD cytofix/cytoperm solution, and IL-17A PE or isotype control (BD Pharmingen) was added for 30 min.

For regulatory T cell (Treg) staining, $10^6$ cells were stained with CD3 PE-Cy5, CD6 Alexa Fluor 750, CD25 allophycocyanin (all eBioscience), and CD4 FITC (BD Pharmingen), washed, incubated with live/dead violet dye, washed, and permeabilized using Foxp3 staining set (eBioscience). Foxp3 PE or isotype control (eBioscience) was added for 30 min.

All cells were washed and resuspended in staining buffer, and the samples were run on an LSRII (BD Biosciences), and the data were analyzed with FlowJo (Tree Star). The experiment was performed twice, with three mice per group.

**Statistical analyses**

Data were analyzed using Prism (GraphPad, San Diego, CA). Differences in survival rates were analyzed by the Kaplan Meier plot and log-rank test. Analysis of CFU data was done using Mann-Whitney U test or Kruskal-Wallis test with Dunn’s postcomparison test. Cytokine/chemokine and cellular phenotyping analysis was performed using Student’s t-test; $p < 0.05$ was considered statistically significant.

**Results**

The absence of CD8+ cells rendered mice more susceptible to death after pulmonary challenge with ST3.

The inoculum for each ST3 strain was determined by preliminary LD$_{50}$ experiments in C57BL/6 (Wt) and CD8-/- mice. These experiments revealed that CD8-/- mice were more likely to die of the same inoculum than Wt mice (data not shown). On the basis of this observation, inocula that resulted in death in CD8-/- mice at a time when the majority of Wt mice were alive were selected for additional study. On the basis of this criterion, Wt and CD8-/- mice were infected with $2 \times 10^7$ CFU WU2 (Fig. 1A), $3 \times 10^4$ CFU 6303 (Fig. 1B), or $2 \times 10^5$ CFU A66 (Fig. 1C). For all ST3 infections, the majority of CD8-/- mice died by day 4. WU2- and A66-infected Wt mice each survived significantly longer than similarly infected CD8-/- mice (p < 0.001 and p < 0.003, respectively), and 6303-infected mice exhibited a trend toward reduced survival.

Mice were also infected with ST2 and ST8 to investigate the ST specificity of the effect observed with ST3. ST2 (D39)-infected CD8-/- manifested longer survival than Wt mice (p < 0.02; Supplemental Fig. 1A), and the survival of ST8 (6308)-infected CD8-/- and Wt mice was statistically similar (Supplemental Fig. 1B).

**Lung bacterial burdens differed based on ST3 strain, but CD8-/- mice exhibited earlier and more bacteremia irrespective of ST3 strain**

On the basis of when the first deaths of CD8-/- mice were expected (Fig. 1A–C), lung and blood CFUs were determined 24, 36, or 48 h postinfection, respectively, for WU2-, 6303-, and A66-infected CD8-/- and Wt mice. Compared with Wt mice, WU2-infected CD8-/- mice had an average of 1 log fewer (p < 0.05; Fig. 1D). 6303-infected CD8-/- mice had significantly more (p < 0.05; Fig. 1E), and A66-infected CD8-/- mice had statistically comparable lung CFUs (Fig. 1F).

Blood CFUs were 4 logs higher among WU2- and A66-infected CD8-/- than Wt mice at both times examined (p < 0.05 for both strains; Fig. 1D, 1F). CD8-/- 6303-infected mice had blood CFUs that were 6 logs higher than those of Wt mice (Fig. 1E), but this difference was not statistically significant.

Given that each ST3 strain used in this study was more lethal in CD8-/- than Wt mice, we chose to use A66 to further dissect the role of CD8+ cells in resistance to ST3 in our model. A66 has been used extensively in pneumococcal research and, as confirmed by our histopathological studies (see below), induces pneumonia and sepsis in mice (26).

**Transfer of CD8+ T cells to CD8-/- mice was associated with better survival after challenge with A66**

When naive CD8+ T cells were transferred into CD8-/- mice prior to infection with A66, reconstituted mice had a survival advantage over control CD8-/- mice (p < 0.03; Fig. 2A). However, the survival of the reconstituted mice was less than that of Wt mice.

Depletion of CD8+ cells from Wt mice was associated with reduced survival after challenge with A66.

The majority of CD8+ T cells express both the CD8α- and CD8β-chain (27). Mice were depleted of CD8α+ cells, CD8β+ cells, or
both prior to i.n. infection with A66 (Fig. 2B). There was no significant survival difference between CD8α−/− cell-depleted (CD8α−, CD8β−, or CD8α+/CD8β+) and CD8−/− mice. The survival of CD8α−/−, CD8β−/−, and CD8α+/CD8β−–depleted mice was shorter than that of WT and control mAb–treated WT mice, but the differences did not reach statistical significance.

**Depletion of CD4+ cells from CD8−/− mice was associated with better survival after infection with A66**

In light of our finding that ST3–infected CD8−/− mice exhibited a lethal survival phenotype and data from other groups that 1) CD4+ T cells were required for clearance of ST2 from the lungs (16) and 2) CD8+ T cells can modulate CD4+ T cell–mediated inflammation (28, 29), we depleted CD4+ cells from CD8−/− mice prior to infection with A66 and compared their survival with that of CD8−/− mice (Fig. 2C). CD4+ T cell–depleted CD8−/− mice survived significantly longer than control CD8−/− mice treated with a control mAb or PBS (p < 0.02), although their survival was less than that of WT mice (p < 0.04).

**IFN-γ−/− and Pfn−/− mice exhibited increased susceptibility to infection with A66 but CD4+ T cell–deficient mice did not**

To examine the function of CD8+ cells in our model, we determined the survival and bacterial burden in the blood and lungs of IFN-γ−/− and Pfn−/− mice. The survival of IFN-γ−/− (Fig. 3A) and Pfn−/− mice (Fig. 3B) was significantly reduced compared with that of WT mice (p < 0.04 and p < 0.01, respectively). Lung and blood CFUs were higher in IFN-γ−/− than WT mice (p < 0.02 for both; Fig. 3D), and lung CFUs were higher in Pfn−/− than WT mice (p < 0.03), with a trend toward higher blood CFUs 48 h postinfection (Fig. 3E).

Given that CD4+ T cell depletion resulted in an improvement in the survival of CD8−/− mice, we examined the role of CD4+ T cells in our model using MHCII−/− mice. The survival of A66–infected MHCII−/− mice was statistically comparable to WT mice (Fig. 3C). There were fewer lung CFUs in MHCII−/− than WT mice 48 h postinfection (p < 0.05; Fig. 3F) and neither strain exhibited blood CFUs.

**CD8−/− and IFN-γ−/− mice exhibited more lung pathology than WT, Pfn−/−, and MHCII−/− mice after infection with A66**

Among the groups of mice that we studied in this experiment, three of WT and CD8−/−, two of three IFN-γ−/− and Pfn−/−, and one of two MHCII−/− mice exhibited histopathological findings. These differences could reflect experimental or technical variables such as sectioning. Histopathological examination of A66–infected WT (Fig. 4A), CD8−/− (Fig. 4B), IFN-γ−/− (Fig. 4C), Pfn−/− (Fig. 4D), and MHCII−/− (Fig. 4E) mice revealed differences in the presence and degree of pneumonia, vasculitis, and inflammation (Table I). WT mice had mild, localized areas of pneumonia and inflammation, whereas CD8−/− mice had extensive focal areas of cellular infiltration with necrotic cells, perivascular inflammation, and areas of moderate vasculitis and neutrophilic infiltrates (see inset, Fig. 4B). One of three IFN-γ−/− mice exhibited moderate suppurative vasculitis and peribronchiolar inflammation, whereas Pfn−/− mice with pathology exhibited minimal findings, predominantly perivascular inflammation, as did MHCII−/− mice.

Compared with WT mice (Supplemental Fig. 2A), histopathological sections from WU2–infected CD8−/− mice (Supplemental Fig. 2B) exhibited marked areas of cellular infiltration and multifocal inflammation (Supplemental Table I). WT 6303–infected mice showed no evidence of vasculitis, inflammation, or pneumonia (Supplemental Fig. 2C), whereas CD8−/− 6303–infected mice had evidence of pneumonia but less infiltration and inflammation than those infected with A66 or WU2 (Supplemental Fig. 2D, Supplemental Table I).

**Absent CD8 cell function and an absence of CD8+ but not CD4+ T cells was associated with higher levels of inflammatory mediators in the lungs after infection with A66**

Cytokines and chemokines were quantified in the lungs after infection with A66. To examine the function of CD8+ cells in our model, we determined the presence and degree of pneumonia, vasculitis, and inflammation. These differences could reflect experimental or technical variables such as sectioning. Histopathological examination of A66–infected WT (Fig. 4A), CD8−/− (Fig. 4B), IFN-γ−/− (Fig. 4C), Pfn−/− (Fig. 4D), and MHCII−/− (Fig. 4E) mice revealed differences in the presence and degree of pneumonia, vasculitis, and inflammation (Table I). WT mice had mild, localized areas of pneumonia and inflammation, whereas CD8−/− mice had extensive focal areas of cellular infiltration with necrotic cells, perivascular inflammation, and areas of moderate vasculitis and neutrophilic infiltrates (see inset, Fig. 4B). One of three IFN-γ−/− mice exhibited moderate suppurative vasculitis and peribronchiolar inflammation, whereas Pfn−/− mice with pathology exhibited minimal findings, predominantly perivascular inflammation, as did MHCII−/− mice.

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Twenty-four hours postinfection, there were no significant differences in any of the mediators examined between CD8−/− or IFN-γ−/− and Wt mice. Compared with Wt mice, Pfn−/− mice had lower levels of IL-17 (p < 0.01) and IFN-γ (p < 0.04), and MHCII−/− mice had lower levels of IFN-γ (p < 0.04) and TGF-β1 (p < 0.02).

Overall, levels of cytokines and chemokines were higher than 24 h postinfection in each mouse strain examined. Compared with Wt mice, levels of MIP-1β (p < 0.01), KC (p < 0.04), MIP-2 (p < 0.03), and IL-17 (p < 0.02) were higher and levels of TGF-β1 (p < 0.01) were lower in CD8−/− mice. Similar to CD8−/− mice, levels of MIP-1β (p < 0.02), KC (p < 0.01), MIP-2 (p < 0.01), and IL-17 (p < 0.02) were higher in IFN-γ−/− than Wt mice, as was IL-6 (p < 0.01). There were no statistical differences in the levels of any of the mediators examined between Pfn−/− and Wt mice. MHCII−/− mice had lower levels of IFN-γ (p < 0.03) than Wt mice.

**Susceptible mouse strains exhibited more neutrophil recruitment to the lungs than Wt mice after infection with A66**

There were differences in the distribution of cell types in the lungs of the mouse strains in the naive state (Fig. 6). Compared with Wt mice, CD8−/− mice had a higher percentage of B cells (37 versus 10%; p < 0.01) and an absent population of CD8+ T cells (0 versus 10%; p < 0.01); Pfn−/− mice had a higher percentage of CD4+ T cells (6 versus 2%; p < 0.02) and a lower percentage of macrophages (16 versus 26%; p < 0.04); and MHCII−/− mice had a larger population of CD8+ T cells (15 versus 10%; p < 0.01). The percentage of lung DCs, neutrophils, and NK and NKT cells in each of the mouse strains was comparable in the naive state.

At 48 h postinfection, there were significant differences in one or more of the following populations: CD4+ T cells, CD8+ T cells, NK cells, or neutrophils between the susceptible mouse strains and Wt mice (Fig. 6). CD8−/− mice had no detectable CD8+ T cells (0 versus 9%; p < 0.01) and a higher percentage of neutrophils (41 versus 15%, p < 0.01) but a lower percentage of CD4+ and MHCII−/− mice had a higher percentage of neutrophils (41 versus 15%, p < 0.01) but a lower percentage of CD4+ T cells (6 and 7 versus 9 and 11%, respectively; p < 0.02 for both), Pfn−/− mice had a smaller percentage of NK cells (8 versus 13%; p < 0.01) and MHCII−/− mice had a higher percentage of CD8+ T cells (17 versus 9%; p < 0.04) and a lower percentage of CD4+ T cells (2 versus 11%; p < 0.01). There was no significant difference in the percentages of macrophages, DC, NKT, or B cells between any of the knockout strains and Wt mice.

**IL-17 and IL-23 are not required for resistance to infection with A66**

To explore the role of the Th17 response in our model, we compared the survival and blood and lung CFUs of ST3 A66-infected IL-23p19−/−, IL-17RA−/−, and Wt mice. There was no statistical difference in the survival of IL-23p19−/− or IL-17RA−/− and Wt.
mice (Fig. 7A, 7C). Compared with Wt mice, IL-23p19−/− mice had significantly fewer lung CFUs 48 h postinfection (p < 0.01; Fig. 7B). There were no differences in lung CFUs between IL-17RA−/− and Wt mice (Fig. 7D) or in blood CFUs between any of the strains 48 h postinfection.

Susceptible mouse strains had higher levels of CD4+ IL-17 (Th17) cells and fewer Tregs after infection with A66

CD8−/− and IFN-γ−/− mice had significantly higher levels of IL-17–producing CD4+ T cells than Wt mice (p < 0.04 for both; Fig. 8A). The percentage of IL-17–producing cells corresponded with total IL-17A levels in the lungs 48 h postinfection: MHCII−/− mice had the least, Pfn−/− mice had more than Wt, and CD8−/− and IFN-γ−/− mice had the most. Intracellular staining of cells isolated from lung tissue 48 h postinfection revealed that Wt mice had a higher percentage of Tregs (CD4+CD25+Foxp3+) than CD8−/− mice (p = 0.05; Fig. 8B), whereas the values obtained with CD8−/−, IFN-γ−/−, Pfn−/−, and MHCII−/− mice were numerically similar to that of CD8−/− mice, they were not significantly different from that of Wt mice.

### Table I. Scoring of lung sections from ST3 (A66)-infected Wt and knockout mice

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Scoring refers to the sections shown in Fig. 4. The scoring scale is as follows: 0, no finding (pneumonia, vasculitis or inflammation); 1, minimal; 2, mild; 3, moderate; 4, marked; and 5, severe. Scores were averaged for each group (n = 2–3).
Discussion

The results of this study demonstrate a surprising, previously unsuspected role for CD8+ cells in protection against ST3 pneumonia in mice. The ST3 stains WU2 and A66 were significantly more lethal in CD8^-/- than Wt mice, with a similar trend for 6303. Given that A66 has been used extensively in pneumococcal pneumonia models (23, 26), we used this strain to conduct further studies of the role of CD8+ T cells in our model. To our knowledge, there are no other direct studies of the role of CD8+ T cells in pneumococcal resistance in naive hosts. CD8+, but not CD4+, T cells were required for protection against ST3 (WU2) in immune (ST3 pneumococcal capsular polysaccharide-immunized) mice (12). CD4+ T cells were required for bacterial clearance in an ST2 infection model (16), but neither survival nor CD8+ T cells were evaluated. Hence, our data provide, to our knowledge, the first direct evidence that CD8+ T cells enhance resistance to ST3 pneumonia and dissemination in naive mice. Although this phenomenon could be ST specific, as CD8+ T cells were dispensable for resistance to ST2 and ST8 in our model (Supplemental Fig. 1), the requirement for CD8+ T cells could also be a function of mouse genetics, inoculum, and/or the nature of the inflammatory response, rather than ST per se. Nonetheless, the ability of CD8+ T cells to enhance resistance to ST3 is potentially important clinically, given that ST3 is independently associated with mortality (5), and to date, ST3-containing conjugate vaccines have failed to protect against ST3 (7).

CD8+ T cells are the most likely CD8+ cell type responsible for the resistance phenotype in our model, given the comparable survival of A66-infected CD8a^-/- and CD8B^-/-depleted Wt mice, and that the survival of A66-infected CD8^-/- mice was improved.
by adoptive transfer of CD8<sup>+</sup> T cells. The precise mechanism by which CD8<sup>+</sup> T cells mediate resistance in our model remains under investigation. However, our findings that CD4<sup>+</sup> T cell-deficient MHCII<sup>−/−</sup> mice had a resistant survival phenotype and CD4<sup>+</sup> T cell depletion prolonged the survival of A66-infected CD8<sup>−/−</sup> mice indicate that CD4<sup>+</sup> T cells were dispensable, if not detrimental, for survival. Hence, one function of CD8<sup>+</sup> T cells in our model could be to dampen CD4<sup>+</sup> T cell-mediated inflammation. CD4<sup>+</sup> T cells, via their secreted products, are known to promote the inflammatory response to pneumococcus in the lungs by recruiting neutrophils and enhancing bacterial clearance (14, 16, 17). However, high levels of chemokines and IL-6 enhance lethality in models of pneumococcal pneumonia (24, 30), and modulation of the inflammatory response is crucial for resistance to pneumococcal infection in mice (26, 30, 31). Interestingly, although lung CFUs of A66-infected CD8<sup>−/−</sup> and Wt mice were similar, their survival, inflammatory response, and blood CFUs differed markedly. In addition, levels of CD4<sup>+</sup>Th17 cells, Tregs, IL-17, IFN-γ, and neutrophils were significantly higher in CD8<sup>−/−</sup> mice, which had more inflammatory pathology. Hence, Wt mice exhibited control of the inflammatory response, which could reflect modulation of CD4<sup>+</sup> T cell-mediated inflammation by CD8<sup>+</sup> T cells, as described in other models (28, 29, 32, 33).

We used mice deficient in IFN-γ and perforin, secreted products of CD8<sup>+</sup> cells, to further dissect the role of CD8<sup>+</sup> T cells in resistance to ST3 in our model. ST3 infection was more lethal in both IFN-γ<sup>−/−</sup> and Pfn<sup>−/−</sup> than Wt mice, but Pfn<sup>−/−</sup> mice exhibited a less lethal phenotype. Consistent with the latter, Pfn<sup>−/−</sup> and Wt mice had similar levels of lung CD8<sup>+</sup> T and CD4<sup>+</sup> Th17 cells, neutrophil recruiting chemokines and neutrophils, and minimal lung pathology, whereas CD8<sup>−/−</sup> and IFN-γ<sup>−/−</sup> mice had higher levels of the aforementioned factors than Wt mice. CD8<sup>−/−</sup> and IFN-γ<sup>−/−</sup> mice also had the lowest, whereas Pfn<sup>−/−</sup> mice had the highest levels of B cells 48 h postinfection. B cells can function as immunomodulators (34, 35), and their Ab products have been implicated in innate clearance of ST3 (36). Compared with Wt mice, Pfn<sup>−/−</sup> and IFN-γ<sup>−/−</sup> mice had significantly more lung CFUs, but those of Pfn<sup>−/−</sup> mice were lower. Therefore, although perforin could contribute, IFN-γ appears to be more important for ST3 clearance among the CD4<sup>+</sup> T cell-sufficient mouse strains in our model. We realize that the concept that perforin contributes to host defense against ST3 is unconventional and that more work is needed to validate this finding. Nonetheless, our results suggest a previously unsuspected role for perforin in resistance to ST3 and corroborate similar data obtained with other extracellular pathogens (10, 11).

The lethality of A66 in IFN-γ<sup>−/−</sup> mice and an early increase in lung IFN-γ (by 24 h) in Wt compared with CD8<sup>−/−</sup> mice, which had a later increase (by 48 h), are consistent with reports that IFN-γ is required for early resistance to ST3 in mice (37, 38). In contrast, MHCII<sup>−/−</sup> mice, which had a resistant survival phenotype, had minimal levels of IFN-γ, reinforcing another report that IFN-γ is dispensable for resistance to ST3 (39). These data underscore the complexity in the host response to ST3 and raise the question of how neutrophils are recruited and bacteria are cleared in MHCII<sup>−/−</sup> mice, with the caveats that MHCII<sup>−/−</sup> mice produced some IFN-γ and have some CD4<sup>+</sup> T cells, albeit without helper function (40). Lung neutrophils were required for ST3 clearance and survival in one model (23), but not another (41), whereas macrophage/monocytes were implicated in controlling bacterial dissemination in an ST3 pneumonia model (26). Given that CD8<sup>+</sup> T cells compensated for CD4<sup>+</sup> T cells in a model of fungal pathogenesis (42), the higher level of CD8<sup>+</sup> T cells in naive MHCII<sup>−/−</sup> mice could contribute to their resistant survival phenotype, perhaps by priming other cell types (e.g., macrophages or DCs) to affect bacterial clearance. However, more work is needed to identify the mediator(s) of bacterial clearance in MHCII<sup>−/−</sup> mice.

Our data show that IL-17RA<sup>−/−</sup> and IL-23p19<sup>−/−</sup> mice were as resistant to infection with A66 as Wt mice, suggesting that the Th17 response is dispensable for resistance to A66. Notably, and consistent with their higher level of neutrophils and inflammatory pathology, CD8<sup>−/−</sup> and IFN-γ<sup>−/−</sup> mice had more CD4<sup>+</sup>Th17 cells.
and higher levels of IL-17 than Wt mice 48 h postinfection. IL-17 was required for resistance to nasopharyngeal colonization with ST6B and ST23 (14, 17) and vaccine-mediated protection against ST6B (43). However, the Th17 response can also be detrimental (18, 44). Our results extend previous studies on the Th17 response to naive mice, suggesting that IL-17 could have different roles in colonization and acute infection, naive and immune hosts, and the response to different STs. We also found that CD8\(^{+}\) and IFN-\(\gamma^{+}\) mice manifested an inverse relationship between CD4\(^{+}\) Th17 cells and CD4\(^{+}\) Tregs. Although Tregs were not CD8\(^{+}\) (data not shown), CD8\(^{+}\) T cells could affect the cytokine milieu that governs the differentiation of CD4\(^{+}\) T cells into Tregs or Th17 cells.

Neither Tap\(^{−/−}\) nor \(\beta_{2}\)-microglobulin–deficient mice were more susceptible to A66 infection than Wt mice (Supplemental Fig. 3), suggests that MHCI processing is not required for the CD8\(^{+}\) response in our model, but further work is needed to rule out a role for lipoglycan-dependent MHCI processing. Although certain pneumococcal polysaccharides are recognized by CD4\(^{+}\) T cells (45), the concept that carbohydrates bind T cells continues to evolve. Nonetheless, non–Ag-specific activation of Tregs and CD8\(^{+}\) T cells, including by TLRs, has been described previously (46–48). Given that CD8\(^{+}\) T cells express TLR2 (49, 50), pneumococcal Ags bind TLR2 (51), and TLR2 was required for modulation of the inflammatory response in an ST3 pneumonia model (52), the hypothesis that CD8\(^{+}\) T cells can be activated by ST3-TLR2 binding warrants study.

In summary, our data show that the absence of CD8\(^{+}\) cells in mice results in a more lethal phenotype after i.n. infection with A66. There are some caveats to our study. First, although our results were obtained in knockout mice, CD8\(^{+}\) T depletion in Wt mice and adoptive transfer of CD8\(^{+}\) T cells to CD8\(^{−/−}\) mice corroborated the lethal survival phenotype that we observed in CD8\(^{−/−}\) knockout mice. However, how CD8\(^{+}\) T cells enhance resistance and the ST3 ligand(s) and CD8\(^{+}\) receptor(s) that induce the CD8\(^{+}\) T cell response remain under investigation, making some of our conclusions speculative. Nonetheless, to our knowledge, our findings are novel while corroborating ample evidence that immunomodulation is critical for protection against pneumococcal pneumonia. Hence, our data put forth the hypothesis that CD8\(^{+}\) T cells modulate CD4\(^{+}\) T cell-mediated and/or Th17 responses to ST3 infection in the lungs, resulting in less inflammation, damage, and dissemination. Finally, our findings also provide insights that could advance our understanding of why
conjugate vaccines have failed to protect against ST3 (7), because protection could in part lie in the ability of a vaccine to modulate inflammation.

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Disclosures

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