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IL-17A-Expressing T Cells Are Essential for Bacterial Clearance in a Murine Model of Hypersensitivity Pneumonitis

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Hypersensitivity pneumonitis (HP) is an inflammatory lung disease characterized by a diffuse mononuclear cell infiltrate in the lung that can progress to pulmonary fibrosis with chronic exposure to an inhaled Ag. We previously reported that C57BL/6 mice repeatedly exposed to the ubiquitous microorganism Bacillus subtilis develop mononuclear infiltrates in the lung that contain Vγ6/Vδ1+ γδ T cells. In the absence of this T cell subset, mice treated with B. subtilis had significantly increased collagen deposition in the lung, suggesting a regulatory role for Vγ6/Vδ1+ γδ T cells. To further investigate the role of Vγ6/Vδ1+ γδ T cells in B. subtilis-induced lung fibrosis, we exposed transgenic Vγ6/Vδ1 mice to this microorganism and found decreased collagen content in the lung compared with wild-type C57BL/6 mice. Cytokine analysis of lung homogenates from wild-type C57BL/6 mice demonstrated increased IL-17A concentrations with repeated exposure to B. subtilis. In the absence of IL-17 receptor signaling, IL-17ra−/− mice had delayed clearance of B. subtilis with increased lung inflammation and fibrosis. Although IL-17A was predominantly expressed by Vγ6/Vδ1+ T cells, a compensatory increase in IL-17A expression by CD4+ T cells was seen in the absence of γδ T cells that resulted in similar levels of IL-17A in the lungs of TCRδ−/− and wild-type C57BL/6 mice. In combination, our data suggest an important role for IL-17A-expressing T lymphocytes, both γδ and αβ T cells, in eliminating this microorganism that prevents excessive inflammation and eventual lung fibrosis in this murine model of B. subtilis-induced hypersensitivity pneumonitis. The Journal of Immunology, 2009, 182: 6540–6549.

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3 Abbreviations used in this paper: HP, hypersensitivity pneumonitis; wt, wild type.

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these mononuclear infiltrates contain large numbers of \( \gamma \delta \) T cells that express the V\textsubscript{66}/V\textsubscript{61} TCR. In the absence of V\textsubscript{66}/V\textsubscript{61} T cells, mice develop increased numbers of CD\textsuperscript{4} and CD\textsuperscript{8} T cells and accelerated pulmonary fibrosis, suggesting that these V\textsubscript{66}/V\textsubscript{61} T lymphocytes protect against collagen deposition caused by chronic exposure to B. subtilis. In this report, we show that transgenic V\textsubscript{66}/V\textsubscript{61} mice repeatedly exposed to B. subtilis develop large expansions of V\textsubscript{66}/V\textsubscript{61} \( \gamma \delta \) T cells in lung that associate with reduced numbers of CD\textsuperscript{4} T cells and macrophages and less lung fibrosis. Although HP is considered a Th1-mediated disease, we found increased levels of IL-17A that was predominantly expressed by V\textsubscript{66}/V\textsubscript{61} T cells. In the absence of IL-17 receptor signaling, IL-17ra\textsuperscript{−/−} mice developed increased lung inflammation and collagen deposition compared with wild-type (wt) C57BL/6 mice, with a marked delay in clearance of B. subtilis. Interestingly, in mice deficient in \( \gamma \delta \) T cells, TCR \( \delta \)−/− mice had similar levels of IL-17A compared to those of wt C57BL/6 mice, with a compensatory increased expression of IL-17A by CD4\textsuperscript{+} T cells. Taken together, these data further our understanding of the role of this \( \gamma \delta \) T cell subset and its secreted cytokines in the immune response directed against B. subtilis.

Materials and Methods

Treatment of mice

Eight-week-old transgenic V\textsubscript{66}/V\textsubscript{61} (R. L. O’Brien) (20, 21), C57BL/6, TCR\textsuperscript{δ−/−} (The Jackson Laboratory), and IL-17ra\textsuperscript{−/−} mice (Amen) were treated with 30 \( \mu \)l (5 million CFU) of Bacillus subtilis (American Type Culture Collection no. 21332) or sterile PBS on 3 consecutive days each week for up to 4 consecutive weeks by nasal inhalation. All mice have been backcrossed onto a C57BL/6 background for at least 10 generations. B. subtilis was prepared as previously described (19). Briefly, a single colony of B. subtilis was grown in tryptic soy broth at 37°C with constant agitation into log phase, centrifuged, and resuspended in sterile PBS before administration to mice by nasal inhalation.

Histology

All mice were sacrificed 24 h after their last exposure to B. subtilis or sterile PBS. The lungs were removed and infused with 10% formalin, and stained with H&E (Dako) or Masson’s trichrome (Sigma-Aldrich) stain following the manufacturer’s instructions.

Cytokine analysis

Total lung homogenates were prepared by homogenizing whole lung samples in 500 \( \mu \)l of sterile PBS from C57BL/6 mice treated with either B. subtilis or sterile PBS for 1, 2, 3, and 4 consecutive weeks. Sterile PBS was infused through the pulmonary vasculature by right heart puncture as described above before preparation of lung homogenates. One hundred microliters of supernatant from each lung homogenate was analyzed for cytokines using the Protein Multiplex Immunoassay kit (BioSource International) per the manufacturer’s protocol. Briefly, Multiplex beads were loaded onto a Millipore MultiScreen BV 96-well filter plate followed by diluting each sample 1/2 with assay diluent. Serial dilutions of cytokine standards were prepared in parallel and added to the plate. After 2 h of incubation on a plate shaker (600 revolutions/minute) in the dark at room temperature, the samples were washed and biotinylated anti-mouse multi-cytokine reporter was added to each well. After incubation on a plate shaker at room temperature for 1 h, the plate was washed and PE-conjugated streptavidin was added directly to each well. The plate was incubated for 30 min, washed, and transferred to the Bio-Plex Luminex 100 XYP instrument for analysis. Cytokine concentrations were calculated using Bio-Plex Manager 3.0 software with a five parameter curve-fitting algorithm applied for standard curve calculations. Activated TGF-\( \beta \) (R&D Systems), IL-22 (R&D Systems), IL-17A (eBioscience), IFN-\( \gamma \) (eBioscience), and IL-23 (eBioscience) in supernatants from whole lung homogenates were quantified by ELISA according to the manufacturer’s instructions.

Collagen quantification

The collagen content of the right lung from mice treated with either B. subtilis or sterile PBS was determined using Sirius red staining (19). Each lung sample was thawed at 4°C in sterile PBS supplemented with protease inhibitors (Sigma-Aldrich), homogenized in 5 ml of 0.5 M acetic acid containing 1 mg of pepstatin/10 mg of tissue, and incubated for 24 h at 4°C. After centrifugation at high speed for 10 min, 100 \( \mu \)l of each supernatant was mixed with 900 \( \mu \)l of Sirius red dye reagent, allowed to incubate at room temperature for 30 min, and then centrifuged at high speed for 10 min. After aspiration of the supernatant, the pellet containing the complex of soluble collagen and Sirius red dye reagent was resuspended in 0.5M NaOH, and the OD\textsubscript{540} was measured using a spectrophotometer. The collagen content in micrograms was calculated from a standard curve generated using known concentrations of collagen per the manufacturer’s instructions.

Total and differential cell counts

Total and differential cell counts were performed on collagenase-digested lung before purification of mononuclear cells over Ficoll as previously described (19). Briefly, single-cell suspensions of total lung cells were transferred to a glass slide using a cytospinette apparatus and stained with Wright-Giemsa stain (VWR) per manufacturer’s instructions. Epithelial cells were not included in the total cell count while RBCs were removed by RBC lysis. Differential cell counts were performed by counting at least 200 cells under high power field.

Colony counts

Whole lung samples were isolated from mice treated with B. subtilis for 4 consecutive weeks at different time points after the last administration of the microorganism as described above. Colony counts were performed by homogenating whole lung or spleen samples in 500 \( \mu \)l of sterile PBS using a tissue homogenizer. Serial dilutions of the entire lung homogenate were plated on tryptic soy broth agar plates and grown at 37°C for 24 h.
To further investigate the potential regulatory role of V6 T cells in lung inflammation and fibrosis, we repeatedly exposed homozygous Vγ6/V81 transgenic (Vγ6+/+) mice to B. subtilis by nasal inhalation for 4 consecutive weeks. Nearly all γδ T cells from Vγ6+/+ mice expressed the canonical Vγ6/V81 TCR (20, 21). Vγ6+/+ mice developed peribronchovascular mononuclear cell infiltrates after B. subtilis exposure, whereas no cellular infiltrates were seen in the lungs of PBS-treated control mice (Fig. 1A). As compared with wt C57BL/6 mice treated in an identical fashion, a 1.4-fold increase in total lung cell numbers was seen in Vγ6+/+ mice compared with either wt C57BL/6 mice (p < 0.001) or TCRδ−/− mice (p < 0.001) (Fig. 1B). In addition, a significantly increased number of lung lymphocytes was seen in Vγ6+/+ mice compared with either wt C57BL/6 mice (p < 0.001) or TCRδ−/− mice (p < 0.001) (Fig. 1C). Interestingly, CD4+ T cells were decreased by 50% in the lungs of Vγ6+/+ mice compared with wt C57BL/6 mice (p < 0.05), but were markedly expanded in TCRδ−/− mice (p < 0.001) (Fig. 1C). CD8+ T cells were not significantly different between Vγ6+/+ and wt C57BL/6 mice but were increased in TCRδ−/− mice in response to chronic treatment with B. subtilis (Fig. 1C). Macrophage numbers were also decreased in Vγ6+/+ mice compared...
with wt C57BL/6 (p < 0.05) and TCRδ−/− mice (p < 0.01) (Fig. 1B). Similar numbers of neutrophils were seen in each group of mice after 4 consecutive weeks of B. subtilis exposure (Fig. 1B). Taken together, these data document a strong response of the transgenic Vγ6/Vδ1 T cells to treatment with B. subtilis and the inhibitory effect this T cell subset has on expansion of other cell types such as CD4+ T lymphocytes and macrophages.

**Vγ6/Vδ1 transgenic mice have attenuated lung fibrosis after B. subtilis exposure**

We have previously shown that in the absence of Vγ6/Vδ1 T cells, lung fibrosis is accelerated in mice chronically exposed to B. subtilis (19). To determine whether γδ T cells prevent collagen deposition in the lung, we repeatedly treated Vγ6+/- mice with B. subtilis for 4 consecutive weeks. As shown in Fig. 2A, Vγ6+/- mice repeatedly exposed to B. subtilis showed significantly decreased collagen deposition in the peribronchovascular space by Masson’s trichrome staining compared with both wt C57BL/6 (wt), and TCRδ−/− (δ−/−) mice treated with either B. subtilis (Bsub) or PBS for 4 wk. Data represent the mean ± SD of five mice treated with B. subtilis or sterile PBS from at least two separate experiments.
T cells (TCRδ−/− mice) (p < 0.001) (Fig. 2B). Interestingly, Vγ6+/− mice had increased collagen deposition in the lung compared with PBS-treated control animals (mean ± SD; 356 ± 26 pg/ml vs 258 ± 12 pg/ml; p < 0.05) (Fig. 2B), suggesting that Vγ6Vδ1+ γδ T cells can significantly attenuate but not completely abrogate B. subtilis-induced lung fibrosis.

B. subtilis exposure induces a Th17-polarized immune response in the lung

Although HP is considered a Th1-mediated disease (22), lung fibrosis is seen in both humans and murine models of this disease (6), suggesting that other cytokines may be important for the development of B. subtilis-induced disease. We analyzed the cytokine profile from homogenized lungs of mice treated with B. subtilis and PBS for 1, 2, 3, and 4 wk. Because there was no statistically significant difference in cytokine levels in PBS-treated control animals after 1, 2, 3, or 4 wk of treatment, only the 4-wk time point is shown. As seen in Fig. 3A, very low levels of both IFN-γ and TNF-α were detected in the lungs of mice treated with B. subtilis at each time point compared with controls. IL-5, a Th2 cytokine, was elevated in the lungs of mice after 2 wk of treatment with B. subtilis, but IL-5 levels were not statistically significant at any other time points compared with PBS-treated control mice. IL-13 was also found in the lungs of mice treated with B. subtilis, but the levels were not significantly different from controls, whereas IL-4 was below the detection limit of the assay in both the B. subtilis-treated and control animals (Fig. 3A).

As opposed to type 1 and 2 cytokines, we found increasing amounts of IL-17A in the lungs of mice with repeated exposure to B. subtilis, with a 14-fold increase in IL-17A in the lung of B. subtilis-treated mice compared with control animals (p < 0.001) after 4 wk of treatment (Fig. 3A). IL-22 was also elevated in the lungs of B. subtilis-treated C57BL/6 mice with a 3.7-fold increase in IL-22 (p < 0.01) compared with PBS-treated control mice after 4 wk of treatment (Fig. 3A). In addition, IL-17A and IL-22 were not detected in the spleens of the same animals (data not shown), suggesting a compartmentalized Th17 immune response directed against B. subtilis in the lung. IL-6, a cytokine important for the commitment of Th17 cells, increased rapidly after the second week of exposure but steadily declined with continued exposure to B. subtilis, suggesting that commitment to the Th17 lineage occurs in the early stages of exposure to this microorganism (Fig. 3A). TGF-β1, also important in the differentiation of Th17 cells (24), was 2.6-fold higher in the lung of B. subtilis-exposed mice compared with PBS-treated control mice after 4 wk of treatment (Fig. 3A). In addition, IL-23, essential for expansion and maintenance of committed Th17 cells, was elevated in the lungs of C57BL/6 mice with repeated exposure to B. subtilis for 4 wk compared with control animals (4.6-fold) (Fig. 3A).

Because increasing numbers of Vγ6Vδ1+ T cells correlated with decreased lung fibrosis, we next compared Th1, Th2, and Th17 cytokines from lung homogenates of Vγ6+/+, wt C57BL/6, and TCRδ−/− mice after 4 wk of treatment with either B. subtilis or PBS. Low levels of IFN-γ and TNF-α were detected in the lungs of Vγ6+/+, wt C57BL/6, and TCRδ−/− mice but were not significantly different from controls after 4 wk of treatment (Fig. 3B and data not shown). For Th2 cytokines, IL-4 was below the
detection limit of the assay while IL-5 and IL-13 levels were similar to those of PBS-treated control animals (data not shown). Conversely, levels of the Th17-associated cytokines IL-6, IL-23, and TGF-β1 were all increased in the lungs of Vγ6/Vδ1−/− and TCRδ−/− mice treated with B. subtilis compared with B. subtilis for 4 wk. Data represent the mean ± SD of five mice treated with B. subtilis or sterile PBS from at least two separate experiments. B. Representative density plots of intracellular IL-17A expression in Vγ6/Vδ1+ or CD4+ T cells from IL17ra−/− mice treated with B. subtilis for 4 wk. Vγ6/Vδ1+ and CD4+ T cells were isolated from the lungs of individual mice and placed in medium with brefeldin A for 4 h. The percentage of Vγ6/Vδ1+ or CD4+ T cells expressing IL-17A is shown in the upper right quadrant of each density plot. Data are representative of at least five individual mice treated with B. subtilis from at least two separate experiments.

Expression of IL-17A by Vγ6/Vδ1+ γδ T cells and conditionally by CD4+ T cells

Because IL-17A was the predominant cytokine identified in response to B. subtilis and has been shown to be expressed by numerous cell types including γδ, CD4+, and CD8+ T cells (24), we analyzed these T lymphocyte populations in the lungs of mice for IL-17A. As shown in Fig. 4, a higher percentage of Vγ6/Vδ1+ T cells expressed IL-17A compared with CD4+ T cells from IL17ra−/− mice treated with B. subtilis for 4 wk. Vγ6/Vδ1+ and CD4+ T cells were isolated from the lungs of individual mice and placed in medium with brefeldin A for 4 h. The percentage of Vγ6/Vδ1+ or CD4+ T cells expressing IL-17A is shown in the upper right quadrant of each density plot. Data are representative of at least five individual mice treated with B. subtilis from at least two separate experiments.
T cells expressing IL-17A, suggesting a crucial role for IL-17A in the immune response directed against B. subtilis.

**IL17ra−/− mice have increased inflammation**

To further define the role of IL-17A in the immune response to repeated exposure to B. subtilis, we treated IL-17ra−/− mice with B. subtilis for 4 wk. In the absence of IL-17A receptor signaling, B. subtilis-treated IL-17ra−/− mice had increased lung inflammation by H&E (Fig. 5A) with a 1.5-fold increase in total cell numbers (p < 0.05), a 1.7-fold increase in lymphocytes (p < 0.05), and 1.9-fold expansion of macrophages (p < 0.05) compared with the lungs of wt C57BL/6 mice treated in an identical fashion (Fig. 5B). A decreased number of neutrophils in the lungs of IL-17ra−/− compared with wt C57BL/6 mice (p < 0.05) was also seen (Fig. 5B). Consistent with an expanded number of total lymphocytes, there was a significant increase in Vγ6/Vδ1+ T cells in the lungs of IL-17ra−/− compared with wt C57BL/6 mice (p < 0.01) after 4 wk of treatment with B. subtilis (Fig. 5C). In addition, CD4+ (p < 0.01) and CD8+ (p < 0.05) T cells were also expanded in the lungs of IL-17ra−/− compared with the lungs of wt C57BL/6 mice (Fig. 5C).

In the absence of IL-17A receptor signaling, B. subtilis-treated IL-17ra−/− mice also had increased levels of inflammatory cytokines. As shown in Fig. 6A, IFN-γ and IL-5 were increased in the lungs of B. subtilis-treated mice compared with PBS-treated controls as well as wt C57BL/6 mice treated with either B. subtilis or PBS. IL-6, IL-17A, and IL-23 were also elevated in the lungs of IL-17ra−/− treated with wt C57BL/6 mice (p < 0.01) for 4 wk of treatment with B. subtilis (Fig. 6B). Although there was a trend toward higher levels of IL-22 and TGF-β1 levels in the lungs of B. subtilis-treated IL-17ra−/− mice compared with wt C57BL/6 mice, the differences were not statistically significant (Fig. 6A). Vγ6/Vδ1+ γδ T cells remained the cell type predominantly responsible for IL-17A expression in IL-17ra−/− mice treated with B. subtilis for 4 wk (Fig. 6B).

**IL17ra−/− mice have increased lung fibrosis with bacterial persistence in the lung**

We previously reported that wt C57BL/6 and mice deficient in Vγ6/Vδ1+ T cells (Vγ4−/− 6−/− mice) clear B. subtilis from the lung with similar efficiency (18, 19). To determine whether IL-17A receptor signaling is necessary for bacterial clearance, we performed colony counts on whole lung homogenates at different time points after inhalation of the microorganism. As shown in Fig. 7A, Vγ6+/+, wt C57BL/6, and TCR δ−/− mice were able to clear B. subtilis from the lung and spleen 8 h after exposure. In contrast, B. subtilis persisted in the lungs of IL-17ra−/− mice up to 30 h after inhalation of B. subtilis.

To determine whether IL-17ra−/− mice developed accelerated lung fibrosis due to increased inflammation, we analyzed collagen content in the lung using both Masson’s trichrome staining and a Sirius red colorimetric assay. As shown in Fig. 7B, IL-17ra−/− mice developed collagen deposition in a peribronchovascular distribution compared with PBS-treated control animals. By Sirius red colorimetric assay, IL-17ra−/− mice developed a statistically significant increase in collagen content in the lung compared with wt C57BL/6 mice (840 ± 62 µg vs 690 ± 38 µg; p < 0.05) treated in an identical fashion with B. subtilis for 4 wk (Fig. 7C). Taken together, these data suggest that in the absence of IL-17A signaling, IL-17ra−/− mice fail to efficiently eliminate B. subtilis from the lung, which results in increased inflammation and accelerated fibrosis.

**Discussion**

Hypersensitivity pneumonitis is an inflammatory lung disease caused by repeated inhalation of a variety of environmental Ags that can progress to lung fibrosis with chronic exposure to the inhaled Ag. Although T cells are important in the pathogenesis of HP, their role in the development of pulmonary fibrosis remains poorly understood (25). We previously reported that C57BL/6 mice repeatedly exposed to B. subtilis develop lung fibrosis with increased numbers of CD4+ and CD8+ T cells in the lung and a large expansion of Vγ6/Vδ1+ γδ T cells (19). In the absence of γδ T cells, Vγ4−/− 6−/− mice had increased numbers of CD4+ and CD8+ T lymphocytes with accelerated lung fibrosis upon repeated exposure to inhaled B. subtilis, suggesting that Vγ6/Vδ1+ T cells protect against lung fibrosis by limiting the expansion of αβ T
cells. In the present study, we build on these studies using Vγ6/Vδ1 transgenic mice. With chronic exposure to B. subtilis, Vγ6/Vδ1 transgenic mice have further increased numbers of Vγ6/Vδ1+ T cells in the lung with fewer CD4+ T cells and macrophages and less lung fibrosis compared with wt C57BL/6 mice. Although HP is considered a Th1-mediated disease, we found increasing levels of the Th1 cytokine IFN-γ, yet decreased lung fibrosis compared with wt C57BL/6 mice. Although HP is considered a Th1-mediated disease, we found increasing levels of IFN-γ/H9253, CD4+ T cells at any of several time points examined (P. L. Simonian and A. P. Fontenot, unpublished observation). In contrast to type 1 cytokines, type 2 cytokines have been reported to promote lung fibrosis (26). We found increased levels of Th1 cytokine IL-5 compared with PBS-treated control mice. IL-5 has been shown to exacerbate bleomycin-induced lung fibrosis, but IL-5−/− mice showed no impairment in fibrosis, suggesting that IL-5 may act as an amplifier rather than a direct mediator of pulmonary fibrosis (28). In addition, we did not find increased levels of other type 2 cytokines such as IL-4 and IL-13 compared with controls. These data suggest that in this model of B. subtilis-induced HP, type 2 cytokines play at most a minor role in promoting lung fibrosis.

The absence of significant type 1 and 2 cytokine expression in the lungs of mice treated with B. subtilis raised the possibility that other T cell cytokines were involved in the immune response directed against B. subtilis. Numerous studies show an important role for IL-17 in the lung by directly regulating the expansion of CD4+ T cells at any of several time points examined. Vγ6/Vδ1+ T cells are found in the lung in the absence of type 1 and 2 cytokines was not surprising. Numerous reports have recently shown that IL-6 and TGF-β are necessary for the development of T cells that express Th17 cytokines such as IL-17A and IL-22 while directly inhibiting the differentiation of Th1 and Th2 cells (24). TCR δ−/− and wt C57BL/6 mice, however, had similar levels of IL-17A in the lung associated with a compensatory increase in the number of IL-17A-expressing CD4+ T cells, suggesting that this T cell cytokine is critical for the immune response directed against B. subtilis. Numerous studies demonstrate that IL-17A is important for neutrophil recruitment to the site of inflammation and assists in pathogen clearance (24, 32). Consistent with these reports, we found a decreased number of neutrophils in the lung of B. subtilis-treated IL-17ra−/− mice compared with wt C57BL/6, Vγ6+/-, and TCRδ−/− mice, indicating that IL-17A signaling is important for neutrophil recruitment to the lung in response to repeated exposure to B. subtilis. Interestingly, Vγ6+/- mice treated with B. subtilis had further increased levels of IL-17A in the lung but a similar clearance rate of B. subtilis from the lung as compared with wt C57BL/6 and TCRδ−/− mice. The presence of nearly identical numbers of neutrophils in Vγ6+/- mice treated with B. subtilis compared with wt C57BL/6 and TCRδ−/− mice suggests that a threshold concentration of IL-17A may be required for neutrophil recruitment to lung and pathogen removal. In addition, B. subtilis persisted in the lungs of IL-17ra−/− mice compared with wt C57BL/6, Vγ6+/- and TCRδ−/− mice, suggesting that IL-17A signaling is necessary for efficient pathogen clearance, most likely through neutrophil recruitment.

Despite the fact that TCR δ−/− mice had similar levels of IL-17A, neutrophil numbers, and pathogen clearance as C57BL/6 mice, these mice, like Vγ4−/-6−/− mice, developed accelerated lung fibrosis in response to repeated exposure to B. subtilis. In addition, Vγ6+/- mice had increased levels of IL-17A with similar rates of bacterial clearance as wt C57BL/6 and TCR δ−/− mice, yet decreased lung fibrosis. Together, these findings suggest that Vγ6/Vδ1+ T cells may possess other mechanisms that attenuate lung inflammation and fibrosis independently of IL-17A expression. Vγ6/Vδ1+ T cells may regulate the immune response directed against B. subtilis by limiting the expansion of other inflammatory cell types including macrophages and CD4+ T cells, thus promoting the resolution of lung inflammation and possibly fibrosis. Numerous studies suggest that γδ T cells are important for terminating the host immune response directed against infectious agents (33, 34). In a murine model of L. monocytogenes infection, cytotoxic γδ T cells induced apoptosis of peritoneal macrophages, resulting in a return to normal macrophage homeostasis and the prevention of chronic inflammation (35). Consistent with these reports, we found decreased numbers of macrophages in the lungs of transgenic Vγ6/Vδ1 mice compared with mice deficient in γδ T cells (TCR δ−/−). Therefore, it is possible that Vγ6/Vδ1+ T cells suppress inflammation and pulmonary fibrosis by limiting the expansion and/or activation of macrophages.

We also observed a further reduction in the number of CD4+ T cells in the lungs of B. subtilis-treated transgenic Vγ6/Vδ1 mice compared with wt C57BL/6 mice treated in an identical fashion. These data are consistent with our previous report that showed increased numbers of CD4+ T cells and accelerated lung fibrosis in the absence of γδ T lymphocytes (19). Although a decline in the number of CD4+ T cells in the lung associates with less lung fibrosis in transgenic Vγ6/Vδ1 mice, Vγ6/Vδ1+ T cells may not directly regulate the expansion of CD4+ T cells in these mice. As opposed to wt C57BL/6 mice, transgenic Vγ6/Vδ1 mice have Vγ6/Vδ1+ T cells in the lung before B. subtilis exposure that may prevent the expansion of B. subtilis-specific CD4+ T cells in the lung upon repeated Ag treatment. In addition, although very few αβ T cells are found in the lung before treatment with B. subtilis, transgenic Vγ6/Vδ1 mice have fewer αβ T cells than wt C57BL/6 mice in the spleen and other immune organs. Therefore, although increasing numbers of CD4+ T cells in the lung correlate with accelerated lung fibrosis, it remains to be established whether Vγ6/Vδ1+ T cells directly regulate the expansion of CD4+ T cells in mice repeatedly exposed to B. subtilis.

In another murine model of HP and lung fibrosis, we recently reported C57BL/6 mice repeatedly exposed to Saccharopolyspora rectivirugula in an identical fashion as B. subtilis also accumulate Th17 cytokines in the lung (36). Interestingly, we and others (37) showed that in the absence of IL-17 receptor signaling
(IL-17ra−/−) as well as in IL-17 deficient mice (IL-17−/−), inflammation and collagen deposition induced by S. rectivirgula were markedly diminished. These data suggest that in this model Th17 cytokines promote inflammation and lung fibrosis consistent with other murine models of autoimmune disease such as experimental autoimmune encephalitis (38). In the S. rectivirgula model of HP, however, γδ T cells are only found at very low numbers and do not appear to protect against lung fibrosis. In addition, CD4+ T cells were the predominant cell type expressing Th17 cytokines in response to S. rectivirgula (36). In the absence of IL-17 receptor signaling however, IL-17ra−/− mice developed a heterogeneous expansion of γδ T cells that correlated with decreased numbers of macrophages and CD4+ T cells and with lung fibrosis (P. L. Simonian and A. P. Fontenot, unpublished observation). These data further suggest that the presence of γδ T cells may attenuate lung fibrosis by limiting the expansion of inflammatory cells in the lung in response to microbial invasion, which is independent of IL-17A.

In addition to IL-17A, we also found increased levels of IL-22 in the lungs of wt C57BL/6 mice with repeated exposure to B. subtilis exposure. Interestingly, Vγ6/γδ+ T cells had further increased levels of IL-22 in the lung after repeated exposure to B. subtilis compared with wt C57BL/6 mice, whereas in the absence of γδ T cells (TCRγδ−/− mice), IL-22 concentrations are markedly decreased in the lung, which inversely correlated with the amount of B. subtilis-induced lung fibrosis. Although the function of IL-22 in this model is not known, IL-22 has been shown to act synergistically with IL-17A or IL-17F to induce expression of antimicrobial peptides on keratinocytes (39). More recently, IL-22 has been shown to be important for mucosal host defense against Klebsiella pneumoniae in the lung (40). In this model, IL-22 increased lung epithelial cell proliferation and increased transepithelial resistance to injury, whereas IL-17A was important for induction of G-CSF, neutrophil recruitment, and clearance of the microorganism (40). IL-22 has also been shown to increase expression of inflammatory cytokines but not proliferation or collagen synthesis in subepithelial myofibroblasts in inflammatory bowel disease (41). Studies are ongoing to determine the role of IL-22 in this murine model of HP and lung fibrosis.

In summary, the expansion of Vγ6/Vδ1+ γδ T cells in the lung in response to B. subtilis exposure significantly reduces lung inflammation and the resultant fibrosis. As opposed to type 1 or 2 cytokines, we found increased levels of IL-17A expressed by Vγ6/Vδ1+ T cells and, in their absence, CD4+ T cells. In the absence of IL-17A signaling, neutrophil numbers were reduced and B. subtilis persisted in the lung of IL-17ra−/− mice, resulting in accelerated lung inflammation and fibrosis. These data suggest an important role for IL-17A expressing T lymphocytes, both γδ and CD4+ T cells, in eliminating this microorganism, thus preventing excessive lung inflammation and eventual fibrosis in this murine model of HP.

Disclosures
The authors have no financial conflict of interest.

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