Th17-Polarized Immune Response in a Murine Model of Hypersensitivity Pneumonitis and Lung Fibrosis[1]

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Abstract

Hypersensitivity pneumonitis is an environmental 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. Using a well-established murine model of hypersensitivity pneumonitis, we repeatedly exposed C57BL/6 mice to *Saccharopolyspora rectivirgula* to investigate whether T cells are required for lung fibrosis. In the absence of $\alpha\beta$ T cells, TCR$\beta^{-/-}$ mice exposed to *S. rectivirgula* for 4 wk had markedly decreased mononuclear infiltrates and collagen deposition in the lung compared with wild-type C57BL/6 mice. In contrast to CD8$^+$ T cells, adoptive transfer of CD4$^+$ T cells reconstituted the *S. rectivirgula*-induced inflammatory and fibrotic response, suggesting that the CD4$^+$ T cell represents the critical $\alpha\beta$ T cell subset. Cytokine analysis of lung homogenates at various time points after *S. rectivirgula* exposure failed to identify a predominant Th1 or Th2 phenotype. Conversely, IL-17 was found in the lung at increasing concentrations with continued exposure to *S. rectivirgula*. Intracellular cytokine staining revealed that 14% of CD4$^+$ T cells from the lung of mice treated with *S. rectivirgula* expressed IL-17A. In the absence of IL-17 receptor signaling, Il17ra$^{-/-}$ mice had significantly decreased lung inflammation and fibrosis compared with wild-type C57BL/6 mice. These data are the first to demonstrate an important role for Th17-polarized CD4$^+$ T lymphocytes in the immune response directed against *S. rectivirgula* in this murine model of hypersensitivity pneumonitis and pulmonary fibrosis.

Hypersensitivity pneumonitis (HP)[3] is an environmental lung disease that results from repeated inhalation of aerosolized Ags (1). The etiologic agents are composed of a wide variety of organic particles (e.g., mammalian and avian proteins, fungi, and thermophilic bacteria) and certain small molecular mass volatile and nonvolatile chemical compounds. A classic example of HP is Farmer's lung, which is caused by the thermophilic actinomycete *Saccharopolyspora rectivirgula*. This disorder occurs in genetically susceptible individuals who are repeatedly exposed to moldy hay. HP occurs in several clinical forms (e.g., acute, subacute, and chronic), depending on the nature of the Ag, the quantity and duration of exposure, and host/environment interactions (1). The acute form of disease is typically nonprogressive, with spontaneous resolution after cessation of Ag exposure. The subacute and chronic forms of disease result

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

from continued low-level exposure to inhaled Ags. In the chronic subset of patients, pulmonary fibrosis occurs in up to 41% of cases, resulting in irreversible pulmonary dysfunction and right heart failure (2–5). Lung fibrosis has been shown to be an independent predictor of mortality in these patients, with a 5-year mortality of 27% and a median survival of 13 years (6). Histopathologically, this disorder is characterized by a diffuse mononuclear cell infiltration of the lung with poorly formed granulomas located near the bronchovascular bundle (7).

In a well-described murine model of HP, mice treated with *S. rectivirgula* for 3 days each week by nasal inhalation develop mononuclear infiltrates in a peribronchovascular distribution that approximates the human disease (8–11). The mononuclear infiltrates are predominantly composed of alveolar macrophages and T cells. These T cells have been shown to be important in the immunopathogenesis of this disease, as athymic nude mice that lack T cells develop significantly less severe HP (10). The role of T cells in the development of pulmonary fibrosis, however, is unknown. Evidence from the bleomycin model of lung fibrosis and other models of solid organ fibrosis suggests that type 2 cytokines promote fibrosis through stimulation of fibroblast secretion of extracellular matrix proteins, types I and III collagen, and fibronectin, while the production of type 1 cytokines such as IFN-γ attenuates these fibrotic diseases (12–15). Interestingly, Th1-biased C57BL/6 mice are more susceptible to HP than are Th2-biased DBA/2 mice after repeated exposure to *S. rectivirgula* (16). Additionally, mice deficient in IFN-γ do not develop significant mononuclear infiltrates consistent with HP after repeated exposure to *S. rectivirgula* (17), while administration of type 2 cytokines such as IL-4 and IL-10 modulates the severity of HP (18,19). Although these data suggest that HP induced by *S. rectivirgula* is a Th1-mediated disease, the cellular source of IFN-γ is unclear, with a recent study suggesting that neutrophil secretion of IFN-γ in the absence of T cell IFN-γ production was sufficient for granuloma formation in response to *S. rectivirgula* (20). Although mice chronically exposed to *S. rectivirgula* have type 1 cytokines in their lungs, these mice have also been shown to develop pulmonary fibrosis (21). This paradox remains unresolved. Some reports suggest that a relative decrease in type 1 cytokines (22) may allow the development of pulmonary fibrosis, while other investigators have hypothesized that a transition from a predominantly Th1 to Th2 cytokine milieu occurs in the lung with repeated exposure to an inhaled Ag, promoting the development of lung fibrosis (23).

In this report, we show that *αβ*-expressing T cells are essential for the development of the peribronchovascular mononuclear infiltrates and resultant pulmonary fibrosis that occur in response to *S. rectivirgula*. In contrast to CD8$^+$ T cells, lung CD4$^+$ T cells represent the critical *αβ* T cell subset, expressing an activated effector phenotype and a polarized Th17 cytokine response. After exposure of Il17ra$^{-/-}$ mice to *S. rectivirgula,* a significantly decreased T cell alveolitis was seen, with an associated decrease in collagen deposition. Taken together, these data are the first to show the importance of a Th17-polarized immune response in *S. rectivirgula*-induced lung inflammation and the resultant lung fibrosis.

Materials and Methods

Treatment of mice

Six- to 8-wk-old female C57BL/6, TCR$\beta^{-/-}$, and TCR$\beta^{-/-}\delta^{-/-}$ mice (The Jackson Laboratory) and B6.129-*Il17ra^{tm1Koll}* (Il17ra$^{-/-}$) mice (Amgen) were treated with 30 μl (150 μg) of *S. rectivirgula* (American Type Tissue Collection catalogue no. 29034 and a generous gift from Dr. Gary Hunninghake, University of Iowa) or sterile PBS on 3 consecutive days each week for up to 12 consecutive weeks by nasal inhalation. *S. rectivirgula* was prepared by growing the microorganism in tryptic soy broth at 55°C with constant agitation. The *S. rectivirgula* culture was centrifuged, resuspended in sterile PBS, and quantified by Lowry method (Sigma-Aldrich) before administration to mice by nasal inhalation. A *Limulus* amebocyte assay (Sigma-Aldrich) was performed to confirm that the *S. rectivirgula* preparation and sterile PBS

contained <20 μg endotoxin/ml. Mice were lightly anesthetized with isoflurane to allow inhalation of either *S. rectivirgula* or sterile PBS. These studies were approved by the Animal Care and Use Committee at the University of Colorado Denver.

Preparation of mononuclear cells from lung homogenates

Mice at each time point were sacrificed 24 h after the last treatment with *S. rectivirgula* as previously described (24). Briefly, the chest cavity was opened using sterile surgical dissection, and the inferior vena cava and abdominal aorta were clamped. The left atrium was opened by incision, and the right ventricle was infused with at least 2 ml of sterile PBS to remove any residual blood from the pulmonary vasculature. The heart and lungs were removed en bloc, and the heart, thymus, and lymph nodes were dissected away from the lungs. The right lung was removed, snap-frozen in liquid nitrogen, and stored at −80°C for collagen quantification. The left lung was cut into small pieces and placed in RPMI 1640 containing 5% FBS, collagenase (Sigma-Aldrich), and DNase (Boehringer Mannheim). After 30 min of collagenase digestion in a 37°C water bath, lungs were further disrupted by aspiration through an 18-gauge needle. The collagenase-digested lungs were layered on top of Ficoll (Accurate Chemical & Scientific) and centrifuged at 1200 rpm for 30 min at room temperature. The interface between the medium and Ficoll was removed and washed twice with RPMI 1640 containing 5% FBS. Total cell and differential cell counts were performed on C57BL/6 and Il17ra$^{-/-}$ mice as previously described (24).

Flow cytometry and immunofluorescence analysis

T lymphocytes isolated from the lungs of C57BL/6 mice treated with either *S. rectivirgula* or sterile PBS were surface stained with mAbs directed against CD3, CD4, CD8, CD44, CD45RB, CD27, CD62L, and CD69 (BD Biosciences). For intracellular cytokine staining, total lung cells were cultured at 1×10^6 cells/ml in complete media containing 10 μg/ml brefeldin A (Sigma-Aldrich), 50 ng/ml PMA (Sigma-Aldrich), and 1 μg/ml ionomycin (Sigma-Aldrich) or *S. rectivirgula* (10 μg/ml) at 37°C for 4 h. After activation, the cells were washed and stained with mAbs directed against CD3, CD4, and CD8 followed by fixation in 1% paraformaldehyde overnight. Fixed cells were permeabilized for 10 min at room temperature in 0.5% saponin and stained with mAbs directed against IL-17A (eBio-science), IL-17F (eBioscience), IL-22 (R&D Systems), and isotype controls for 30 min at 4°C. Electronic compensation was performed with Ab capture beads (BD Biosciences) stained separately with individual mAbs used in the test samples. The lymphocyte population was identified using forward and 90° light scatter patterns, and fluorescence intensity was analyzed using a FACSAria cytometer (BD Immunocytometry Systems) (25,26). The data files were analyzed using FloJo software (Tree Star).

Adoptive transfer experiments

CD4$^+$ and CD8$^+$ T cells were isolated from spleens of untreated C57BL/6 mice and purified by MACS (Miltenyi Biotec) using negative selection for either CD4$^+$ or CD8$^+$ T lymphocytes. Flow cytometry confirmed >95% purity for each T cell population before adoptive transfer of 5×10^5 cells/100 μl of sterile PBS by tail vein injection into TCRβ$^{-/-}$δ$^{-/-}$ mice. Briefly, total splenocytes were obtained by pushing spleens through a 70-μm pore size mesh (Falcon) with subsequent resuspension in RBC lysis buffer (eBioscience). Cells were resuspended in buffer and biotinylated Ab cocktail, mixed, and incubated at 4°C for 10 min. Anti-biotin microbeads were added per the manufacturer's instructions, mixed, and incubated for 15 min at 4°C. The cells were washed and applied to the column per the manufacturer's instructions. The effluent that contained the unlabeled CD4$^+$ or CD8$^+$ T cells was collected and passed over a second column to further enrich each T cell population. Cell counts for CD4$^+$ and CD8$^+$ T lymphocytes were determined by a Coulter counter (Beckman Coulter).

Histology

C57BL/6, TCR$\beta^{-/-}$, TCR$\beta^{-/-}\delta^{-/-}$, and Il17ra$^{-/-}$ mice were sacrificed 24 h after their last exposure to *S. rectivirgula*. The lungs were removed and infused with 10% formalin, embedded in paraffin, and stained with H&E or Masson trichrome following the manufacturer's instructions.

Cytokine analysis

Total lung homogenates were prepared by homogenizing whole-lung samples in 500 μl of sterile PBS from C57BL/6 and TCR$\beta^{-/-}\delta^{-/-}$ mice treated with either *S. rectivirgula* for 1, 2, 3, and 4 consecutive weeks or sterile PBS for 4 wk. The 100 μl of supernatant from each lung homogenate was analyzed for 20 cytokines and chemokines using the protein multiplex immunoassay kit (BioSource International) as per the manufacturer's protocol. Briefly, multiplex beads were loaded onto a Millipore MultiScreen BV 96-well filter plate followed by each sample diluted 1/2 with assay diluent. Serial dilutions of cytokine standards were prepared in parallel and added to the plate. After a 2-h incubation on a plate shaker (600 revolutions/ min) in the dark at room temperature, the samples were washed and biotinylated anti-mouse multicytokine 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 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-β1 in supernatants from whole-lung homogenates was quantified by ELISA according to the manufacturer's instructions (R&D Systems). IL-17A, IL-23 (eBioscience), IL-17F, and IL-22 (R&D Systems) from whole-lung homogenates were quantified by ELISA according to the manufacturers' instructions.

Collagen quantification

The collagen content of the right lung from the various mouse strains treated with either *S. rectivirgula* or sterile PBS was determined using Sirius red staining (24). Sirius red stain allows quantification of types I–V mammalian collagen. 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 pepsin/10 mg of tissue, and incubated for 24 h at 4°C with constant stirring. After centrifugation at high speed for 10 min, 100 μl of each supernatant was mixed with 1 ml 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 0.5 M NaOH, and the OD 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.

Statistical analysis

A Mann-Whitney *U* analysis and one-way ANOVA with a Bonferroni's multiple comparison test were used to determine whether there were significant differences between the treatment groups at each time point (Prism 4; GraphPad Software). A *p* value of <0.05 was considered statistically significant.

Results

TCRβ$^{-/-}$ mice repeatedly exposed to *S. rectivirgula* do not develop severe pulmonary fibrosis

To define the role of $\alpha\beta$ T cells in the development of pulmonary fibrosis, TCR$\beta^{-/-}$ mice (i.e., mice deficient in CD4$^+$ and CD8$^+$ T cells) were repeatedly exposed to *S. rectivirgula* for 4 wk.

As shown in Fig. 1, *A* and *B*, TCR$\beta^{-/-}$ mice do not develop mononuclear cell infiltrates or obvious collagen deposition in a peribronchovascular distribution by H&E and Masson trichrome staining, respectively, in the absence of CD4$^+$ and CD8$^+$ T cells. Using the more sensitive Sirius red colorimetric assay, *S. rectivirgula*-treated TCR$\beta^{-/-}$ mice developed a 1.5-fold increase in collagen content in the lung compared with PBS-treated control animals ($p < 0.01$) but significantly less collagen deposition compared with wild-type (wt) C57BL/6 mice exposed to *S. rectivirgula* in an identical fashion ($p < 0.001$) (Fig. 1*C*). Lungs of TCR$\beta^{-/-}$ mice, however, contain a resident $\gamma\delta$ T cell population (Vγ1$^+$ and Vγ4$^+$) (27). Interestingly, after 4 wk of exposure to *S. rectivirgula*, TCR$\beta^{-/-}$ mice develop an expanded number of $\gamma\delta$ T cells in the lung that includes Vγ6$^+$ $\gamma\delta$ T cells compared with PBS-treated TCR$\beta^{-/-}$ mice ($1.21 \pm 0.31 \times 10^5$ vs $0.51 \pm 0.23 \times 10^5$). However, no difference in collagen content was seen in the lung of TCR$\beta^{-/-}$ mice compared with mice deficient in both $\alpha\beta$ and $\gamma\delta$ T cells (TCR$\beta^{-/-}\delta^{-/-}$ mice) treated with *S. rectivirgula* for 4 wk (Fig. 2*C* and data not shown), suggesting that $\gamma\delta$ T cells most likely do not have a significant role in attenuating *S. rectivirgula*-induced pulmonary fibrosis. Conversely, these findings strongly suggest that $\alpha\beta$ T cells are essential for the development of lung fibrosis in response to *S. rectivirgula*. Additionally, the slight increase in collagen deposition in response to *S. rectivirgula* in the absence of $\alpha\beta$ T cells raises the possibility of a T cell-independent mechanism of lung fibrosis in this model.

Adoptive transfer of CD4$^+$ T cells into TCR$\beta^{-/-}$ $\delta^{-/-}$ mice reconstitutes the *S. rectivirgula*-induced inflammatory and fibrotic response

For adoptive transfer experiments, we chose TCR$\beta^{-/-}\delta^{-/-}$ mice (i.e., mice deficient in both $\alpha\beta$- and $\gamma\delta$-expressing T cells) as the recipient animal to eliminate any potential effects due to $\gamma\delta$ T cells. To determine whether CD4$^+$ and/or CD8$^+$ T cells are required for the immune response due to *S. rectivirgula*, TCR$\beta^{-/-}\delta^{-/-}$ mice were reconstituted with either 5×10^5 CD4$^+$ or CD8$^+$ T cells by tail vein injection and subsequently treated with *S. rectivirgula* by nasal inhalation. As shown in Fig. 2, mice receiving adoptively transferred CD4$^+$ T cells developed peribronchovascular mononuclear infiltrates upon exposure to inhaled *S. rectivirgula* compared with control TCR$\beta^{-/-}\delta^{-/-}$ mice reconstituted with CD4$^+$ T lymphocytes and repeatedly exposed to inhaled sterile PBS. Although CD4$^+$ T cells were found in the lungs and spleens of all mice reconstituted with CD4$^+$ T lymphocytes, there were much higher numbers of CD4$^+$ T cells in the lungs of CD4$^+$-reconstituted TCR$\beta^{-/-}\delta^{-/-}$ mice exposed repeatedly to *S. rectivirgula* compared with similar mice treated with inhaled PBS. For example, $1.8 \pm 0.3 \times 10^6$ CD4$^+$ T cells were recovered from the lungs of CD4$^+$ T cell-reconstituted TCR$\beta^{-/-}\delta^{-/-}$ mice after *S. rectivirgula* treatment compared with $0.11 \pm 0.02 \times 10^6$ CD4$^+$ T cells from the lungs of CD4$^+$ T cell-reconstituted TCR$\beta^{-/-}\delta^{-/-}$ mice after PBS exposure. Conversely, TCR$\beta^{-/-}\delta^{-/-}$ mice reconstituted with CD8$^+$ T cells did not show a significant accumulation of cells in the lung after subsequent exposure to either *S. rectivirgula* or PBS by nasal inhalation (Fig. 2*A* and data not shown). These data indicate that CD4$^+$ T cells preferentially accumulate in the lung and expand in response to chronic inhalation of *S. rectivirgula*, suggesting their importance in the *S. rectivirgula*-induced immune response.

As opposed to mice reconstituted with CD8$^+$ T cells, the adoptive transfer of CD4$^+$ T cells into TCR$\beta^{-/-}\delta^{-/-}$ mice also resulted in lung fibrosis as detected by Masson trichrome (Fig. 2*B*). Additionally, quantification of collagen deposition by Sirius red colorimetric assay showed a 2.7-fold increase in collagen content in the lungs of mice reconstituted with CD4$^+$ T cells when repeatedly treated with *S. rectivirgula* compared with CD4$^+$ T cell-reconstituted control mice treated with PBS by nasal inhalation ($p < 0.001$) (Fig. 2*C*). TCR$\beta^{-/-}\delta^{-/-}$ mice reconstituted with CD8$^+$ T cells also had a slight increase in collagen content in the lung when chronically exposed to *S. rectivirgula* compared with CD8$^+$ T cell-reconstituted control mice. However, similar levels of collagen deposition were seen in TCR$\beta^{-/-}\delta^{-/-}$ mice given sterile PBS by tail vein injection upon repeated treatment with *S. rectivirgula* (Fig. 2*C*). Therefore, CD8$^+$ T cells

most likely do not have a significant role in the development of pulmonary fibrosis. Taken together, these data suggest that CD4$^+$ T cells represent the critical $\alpha\beta$-expressing T cell subset that promotes both lung inflammation and collagen deposition in this murine model of HP.

CD4$^+$ T cells that accumulate in the lung in response to *S. rectivirgula* express an activated effector phenotype

Next, we analyzed the phenotypic markers expressed by CD4$^+$ T cells in the lung and spleen of mice after repeated *S. rectivirgula* exposure. As compared with splenic CD4$^+$ T cells, CD4$^+$ T lymphocytes in lung were characterized by a significantly increased expression of CD44, a memory cell marker, and CD69, a marker of recent activation (Fig. 3*A*). Additionally, lung CD4$^+$ T cells had down-regulated the expression of CD45RB, CD27, and CD62L, consistent with an effector T cell phenotype. Adoptively transferred CD4$^+$ T cells that accumulated in the lung of TCR$\beta^{-/-}\delta^{-/-}$ mice in response to *S. rectivirgula* exposure also showed an increased expression of CD44 and CD69 and decreased CD45RB, CD27, and CD62L expression compared with CD4$^+$ T cells recovered from the spleen (Fig. 3*B*). CD4$^+$ T cells that accumulated in the spleen of TCR$\beta^{-/-}\delta^{-/-}$ mice treated with PBS expressed a similar phenotype compared with CD4$^+$ T cells in the spleen of TCR$\beta^{-/-}\delta^{-/-}$ mice that were repeatedly exposed to *S. rectivirgula* after CD4$^+$ T cell reconstitution (data not shown). These findings show that the recruitment of activated effector CD4$^+$ T cells to the lung in response to *S. rectivirgula* was not due to homeostatic expansion, thus confirming the critical role of this $\alpha\beta$ T cell subset in the immune and fibrotic response directed against *S. rectivirgula*.

S. rectivirgula exposure induces a Th17-polarized immune response in the lung

Although HP induced by *S. rectivirgula* is thought to be a Th1-mediated disease (16,17), we and others (21) have shown that mice repeatedly exposed to this microorganism develop pulmonary fibrosis. To determine whether specific cytokines correlated with the onset of lung fibrosis, we analyzed the cytokine profile from homogenized lung of mice treated with *S. rectivirgula* for 1, 2, 3, and 4 wk. As seen in Fig. 4*A*, IFN-γ levels were increased in the lungs of mice treated with *S. rectivirgula* at each time point compared with control animals but near the limit of detection of the cytokine bead assay (1 pg/ml) while TNF-α was below the detection limit of the assay. Interestingly, the Th2 cytokine IL-5 was elevated in the lungs of mice treated with *S. rectivirgula* compared with PBS-treated control mice. IL-13 was found in the lungs of mice treated with *S. rectivirgula* but levels were not significantly different than controls, whereas IL-4 was below the limit of detection of the assay in both the *S. rectivirgula*-treated and control animals (Fig. 4*A*).

Interestingly, we found increasing amounts of IL-17 in the lungs of mice upon repeated exposure to *S. rectivirgula*. For example, at 3 and 4 wk, 227 ± 86 pg/ml and 527 ± 87 pg/ml, respectively, were detected in the lung of *S. rectivirgula*-treated mice (Fig. 4). Additionally, IL-17 was not detected in the spleens of the same animals (data not shown), suggesting a compartmentalized immune response directed against *S. rectivirgula* in the lung. Importantly, increased IL-17 secretion was also seen in the lung of CD4$^+$ T cell-reconstituted TCR$\beta^{-/-}\delta^{-/-}$ mice that were subsequently exposed to *S. rectivirgula* compared with PBS-treated controls (207 ± 68 vs 38 ± 28 pg/ml; $p < 0.05$) (Fig. 4*B*). IL-6 and TGF-β1, cytokines important in the differentiation of Th17 cells (28), were also elevated in the lung of *S. rectivirgula*-exposed mice compared with PBS-treated control mice (Fig. 4*A*). IL-17 was not present in the lungs of CD8$^+$ T cell-reconstituted TCR$\beta^{-/-}\delta^{-/-}$ mice that were subsequently treated with *S. rectivirgula* (Fig. 4*B*). These data indicate that with repeated exposure to *S. rectivirgula*, a Th17-polarized immune response predominates, which likely inhibits T cell IFN-γ production.

Th17-polarized CD4$^+$ T cells in the lung differentially express IL-17A and IL-22

To further characterize Th17 cytokines present in the lung of C57BL/6 mice treated with *S. rectivirgula*, we performed cytokine analysis for IL-17A, IL-17F, IL-22, and IL-23 on total lung homogenates obtained after 4 wk of treatment. As shown in Fig. 5*A*, IL-17A and IL-22 were elevated in the lung of *S. rectivirgula*-treated C57BL/6 mice compared with PBS-treated control mice at 4 wk, with a 3.6-fold increase in IL-17A ($p < 0.01$) and a 4.8-fold increase in IL-22 ($p < 0.01$). In mice deficient in $\gamma\delta$ T cells (TCR$\delta^{-/-}$), levels of IL-17A in lung homogenates were not significantly different than those in wt C57BL/6 mice treated with *S. rectivirgula* for 4 wk, suggesting that $\gamma\delta$ T cells do not contribute significantly to IL-17A levels in wt C57BL/6 mice (data not shown). There was no difference in the level of IL-17F between *S. rectivirgula*- and PBS-treated animals. IL-23 was also increased in the lung of *S. rectivirgula*-treated animals compared with controls (6.2-fold; $p < 0.01$) (Fig. 5*A*).

To determine the frequency of IL-17-expressing CD4$^+$ T cells in the lung of *S. rectivirgula*-treated C57BL/6 mice at 4 wk, we performed intracellular cytokine staining on lung CD4$^+$ T cells for the expression of IL-17A, IL-17F, and IL-22. As seen in Fig. 5*B*, ~12% of ex vivo CD4$^+$ T cells from the lung of *S. rectivirgula*-treated C57BL/6 mice expressed IL-17A, regardless of whether the cells were unstimulated or stimulated with either PMA/ionomycin or *S. rectivirgula* Ag, suggesting that the secretion of IL-17A in response to *S. rectivirgula* exposure is occurring in an Ag-nonspecific manner. The vast majority of IL-17 expressed was IL-17A, with minimal, if any, IL-17F detected (Fig. 5*B*). Interestingly, differential expression of IL-17A and IL-22 was seen on lung CD4$^+$ T cells after 4 wk of *S. rectivirgula* exposure (Fig. 5*C*). CD8$^+$ T cells did not express IL-17A or IL-22 (data not shown). Consistent with previously published reports (20), we only detected intracellular IFN-γ expression in CD4$^+$ T cells stimulated with PMA/ionomycin at earlier time points but not after 4 wk of treatment with *S. rectivirgula* (data not shown).

CD4$^+$ T cells and lung fibrosis are decreased in the absence of IL-17 receptor signaling

To further define the role of IL-17A, we repeatedly exposed IL-17A receptor-deficient mice (Il17ra$^{-/-}$) to *S. rectivirgula* for 4 wk. As shown in Fig. 6*A*, Il17ra$^{-/-}$ mice had decreased mononuclear infiltrates on H&E staining compared with wt C57BL/6 mice treated in an identical fashion (shown in Fig. 1*A*). Il17ra$^{-/-}$ mice had a decreased number of total lung cells ($1.6 \pm 0.18 \times 10^6$ vs $2.7 \pm 0.28 \times 10^6$; $p < 0.01$) and macrophages ($0.85 \pm 0.21 \times 10^6$ vs $1.7 \pm 0.16 \times 10^6$; $p < 0.01$) in the lung compared with wt C57BL/6 mice (Fig. 6*B*). In contrast, neutrophil counts were not significantly different in the lung of Il17ra$^{-/-}$ compared with wt C57BL/6 mice (Fig. 6*B*). Lymphocytes ($0.96 \pm 0.19 \times 10^6$ vs $0.71 \pm 0.10 \times 10^6$; $p < 0.05$) and CD4$^+$ T cells ($0.38 \pm 0.06 \times 10^6$ vs $0.22 \pm 0.07 \times 10^6$; $p < 0.05$) were also significantly decreased in the lung of Il17ra$^{-/-}$ compared with *S. rectivirgula*-treated wt C57BL/6 mice. Numbers of CD8$^+$ T cells also declined in the lung of Il17ra$^{-/-}$ compared with *S. rectivirgula*-treated wt C57BL/6 mice without a significant difference in B cell numbers (Fig. 6*B* and data not shown).

To determine whether IL-17 receptor signaling is necessary for the development of lung fibrosis in response to *S. rectivirgula*, we analyzed the lungs from Il17ra$^{-/-}$ mice for collagen deposition after 4 wk of treatment. As shown in Fig. 7, there was a 1.4-fold decline in collagen content in the lung of Il17ra$^{-/-}$ mice compared with *S. rectivirgula*-treated wt C57BL/6 mice ($p < 0.05$). These data suggest, for the first time, that IL-17 receptor signaling is important for both lung inflammation and fibrosis in this murine model of HP.

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 is poorly understood. Using a murine model of Farmer's lung, we demonstrate that repeated inhalation of the thermoactinomycete *S. rectivirgula* results in mononuclear infiltrates and collagen deposition in a peribronchovascular distribution in the lung consistent with earlier reports (16–21). In the absence of $CD4^+$ and $CD8^+$ T lymphocytes, mononuclear infiltrates and collagen deposition are markedly reduced, indicating that $\alpha\beta$ T cells are essential for lung fibrosis. Adoptive transfer experiments show that $CD4^+$ T cells represent the critical $\alpha\beta$ T cell subset necessary for the development of severe pulmonary fibrosis. With chronic exposure to *S. rectivirgula*, $CD4^+$ T cells were not Th1 polarized but were rather Th17 polarized with differential expression of IL-17A and IL-22. In the absence of IL-17 receptor signaling, inflammation and lung fibrosis were both diminished. Thus, these studies establish an important role for $CD4^+$ Th17 cells in the immune response directed against *S. rectivirgula* and for the subsequent development of lung fibrosis.

Numerous studies suggest that IFN-γ is important for granulomatous inflammation in this murine model of HP (17,20) as well as being protective against collagen deposition in other models of lung fibrosis (22). Additionally, type 1 cytokines have been shown to attenuate collagen deposition while type 2 cytokines promote collagen deposition in numerous models of solid organ fibrosis (15). Despite the fact that HP is considered a Th1-mediated disease (29), it is well established that pulmonary fibrosis develops in both humans and murine models of this disease (6,21). This paradox, however, remains unresolved. Consistent with these observations, we found very low levels of IFN-γ in the lung and did not find expression of IFN-γ by T cells after the first 2 wk of exposure to *S. rectivirgula* (P.L.S. and A.P.F., unpublished observation). Additionally, recent studies have shown that the cell responsible for IFN-γ secretion in response to *S. rectivirgula* is the neutrophil and not the T cell (20). Therefore, IFN-γ expression by neutrophils and not T lymphocytes may be all that is necessary for granuloma formation in response to *S. rectivirgula* exposure. Although numerous reports suggest that type 2 cytokines modulate the severity of HP in this model (18), we did not find increased levels of IL-4 and IL-13 in the lung of *S. rectivirgula*-treated mice compared with controls, with the exception of IL-5. 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 as a direct mediator of pulmonary fibrosis (30).

The absence of significant type 1 and 2 cytokine expression in the lungs of mice treated with *S. rectivirgula* raised the possibility that Th17 cytokines were involved in the immune response directed against *S. rectivirgula*. With the emergence of Th17 cytokines, diseases that where once considered Th1 mediated have been, in fact, shown to be Th17 mediated (31). For example, in a murine model of multiple sclerosis, mice deficient in IL-17 had delayed onset of experimental autoimmune encephalitis with markedly diminished severity (28). Expression of IL-17 has also been detected in the sera and target tissues of patients with various autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus as well as asthma (25,26,32). In this regard, the predominant cytokine identified in the lungs of mice treated with *S. rectivirgula* was IL-17. IL-17-secreting T cells belong to a unique subset of $CD4^+$ helper T lymphocytes, Th17 cells, which are characterized by the production of IL-17A and IL-17F as well as IL-21, IL-22, TNF-α, IL-6, and GM-CSF but not IL-4 or IFN-γ. In this model, we found that $CD4^+$ T cells expressed IL-17A and IL-22 but not IL-4, IFN-γ, TNF-α, or IL-17F. Interestingly, 12% of $CD4^+$ T cells expressed IL-17A ex vivo without further antigenic stimulation, which most likely occurs as a result of recent exposure to the *S. rectivirgula* microorganism. In contrast, in a murine model of pathogen-mediated arthritis, only 0.9% of $CD4^+$ T cells isolated from the inflamed joint expressed IL-17 ex vivo and only up to 3.9% with Ag stimulation, although IL-17 is required for disease development (33). Additionally, we did not find expression of IL-17F by $CD4^+$ T cells, and IL-17F levels in the lung were not different by ELISA between *S. rectivirgula*- and PBS-treated mice at 4 wk,

suggesting that in this model redundant functions of IL-17A and IL-17F may not be required. Taken together, our findings suggest that CD4$^+$ T cells differentiate toward a Th17 phenotype and thus inhibit the development of a Th1 response early in the course of disease.

IL-17 is a proinflammatory cytokine that is thought to increase inflammation by recruiting cells, particularly neutrophils, to the site of infection to assist in pathogen clearance (34,35). Consistent with these reports, mice deficient in IL-17 receptor signaling (Il17ra$^{-/-}$) had less inflammation, by H&E staining, with decreased numbers of macrophages and lymphocytes, including CD4$^+$ and CD8$^+$ T cells. Interestingly, the number of neutrophils was not significantly different in the absence of IL-17 receptor signaling, suggesting that neutrophil recruitment to the lung is not affected by IL-17 receptor signaling in this model. Neutrophil numbers, however, are not markedly elevated in the lung of mice repeatedly exposed to *S. rectivirgula* even in the presence of IL-17 receptor signaling. Therefore, IL-17A must serve other distinct functions in this model of HP. Il17ra$^{-/-}$ mice repeatedly treated with *S. rectivirgula* not only had diminished inflammation but also less lung fibrosis compared with wt C57BL/6 mice. These data suggest that IL-17 receptor signaling plays a role in the development of pulmonary fibrosis. Histologic analysis of lungs from transgenic mice with forced IL-17 expression showed focal mononuclear cell infiltrates containing CD4$^+$ T lymphocytes as well as collagen deposition in the subepithelium of bronchioles adjacent to these lymphocytic aggregates (36). These data suggest that CD4$^+$ T cells promote collagen deposition in the lung through expression of IL-17. The role of IL-17 in lung fibrosis, however, may occur indirectly through activation or recruitment of other inflammatory cells to the lung. Myofibroblasts are a primary collagen-producing cell when activated and are present in the lung of *S. rectivirgula*-treated C57BL/6 mice (P.S.L. and A.P.F, unpublished observation). Myofibroblasts can be generated from a variety of cell types, including resident mesenchymal cells, epithelial and endothelial cells, and circulating fibrocytes derived from bone marrow stem cells (15). IL-17 has been shown to stimulate secretion of IL-6, IL-8, and MCP-1 in human colonic subepithelial myofibroblasts (37). Therefore, it is plausible that IL-17A activates subepithelial myofibroblasts in the lung, resulting in increased collagen deposition in the lung. Alternatively, TGF-*β* and IL-6 are necessary for the differentiation of naive CD4$^+$ T lymphocytes into Th17 cells in mice via the transcription factor ROR*γ*t (38–42). TGF-*β* has also been shown to be a critical cytokine for the development of pulmonary fibrosis (15,43) and is elevated in the lung in response to *S. rectivirgula*. Therefore, TGF-*β* may promote differentiation of CD4$^+$ T cells into Th17 cells with the adverse consequence of promoting collagen deposition in the lung in response to chronic exposure to *S. rectivirgula*.

A small percentage of lung CD4$^+$ Th17 cells also expressed IL-22. Interestingly, we found that the CD4$^+$ T cells that express IL-17A differentially express IL-22, indicating the existence of two distinct subpopulations of CD4$^+$ Th17 cells with potentially differing functions. IL-22 has been shown to be an effector cytokine of the Th17 lineage that acts synergistically with IL-17A or IL-17F to induce expression of antimicrobial peptides on keratinocytes (44). More recently, IL-22 has been shown to be important for mucosal host defense against *Klebsiella pneumonia* in the lung (45). 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 (45). 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 (46). These data suggest that CD4$^+$ T cells that express IL-17A and IL-22 are most likely important for mucosal immunity against extracellular microorganisms but may promote pulmonary fibrosis through activation or recruitment of other cell types to the lung in response to an inhaled microorganism.

In summary, we show that activated CD4$^+$ T cells that express IL-17A and IL-22 are essential for the development of lung inflammation and fibrosis induced by repeated inhalation of *S.*

rectivirgula. Although the mechanism by which these CD4$^+$ Th17 cells promote pulmonary fibrosis is not completely understood, these data suggest that CD4$^+$ T cells that express IL-17A and IL-22 are most likely important for mucosal immunity against *S. rectivirgula*, with the adverse effect of pulmonary fibrosis due to recruitment of other cell types or the necessity of TGF-*β* for the development of the Th17 lineage. Studies are ongoing to determine the mechanism by which CD4$^+$ T cells promote the development of pulmonary fibrosis and the exact roles of IL-17A and IL-22 in this *S. rectivirgula*-induced model of HP and pulmonary fibrosis.

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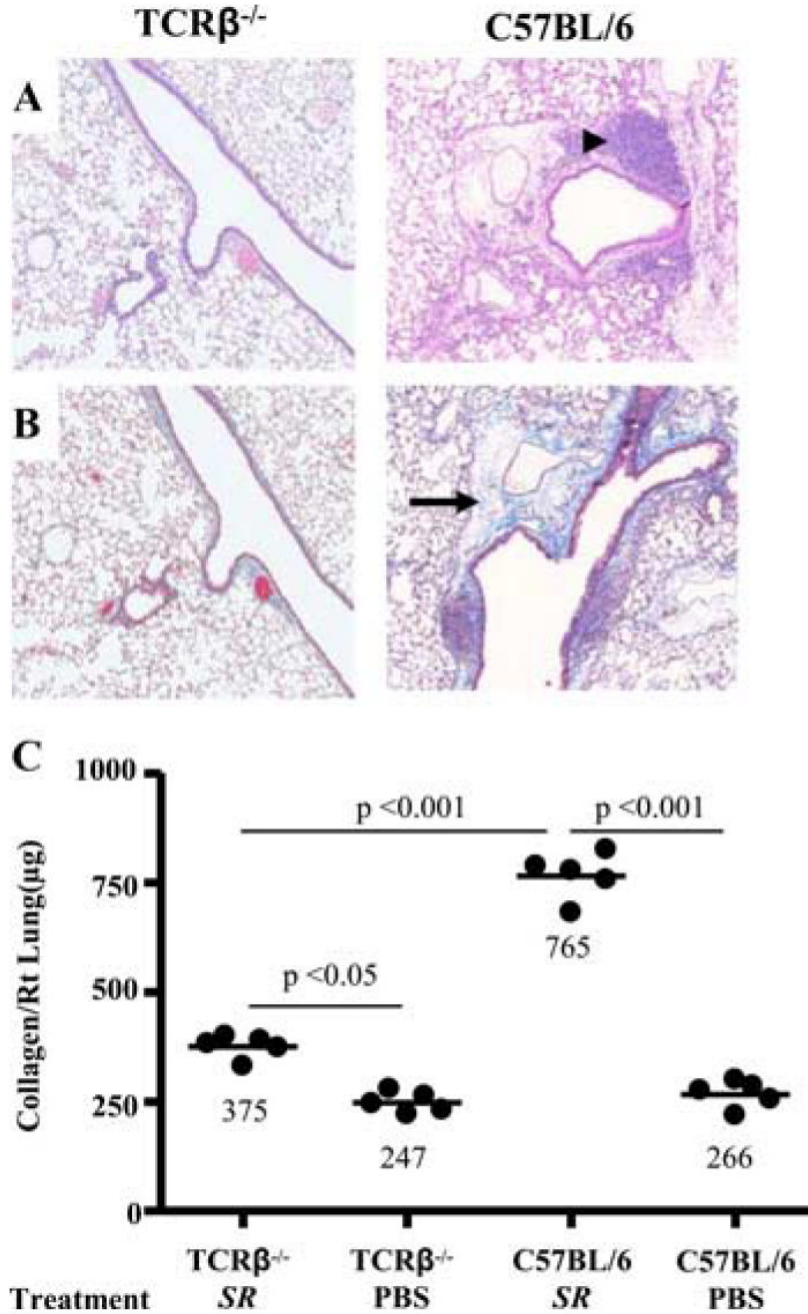
FIGURE 1.
TCR$\beta^{-/-}$ mice do not develop mononuclear infiltrates or severe pulmonary fibrosis. Representative H&E (*A*) and Masson trichrome (*B*) staining of lungs from TCR$\beta^{-/-}$ and C57BL/6 mice repeatedly treated with *S. rectivirgula* for 4 consecutive weeks is shown. Data shown represent at least four mice from at least two separate experiments for H&E and Masson trichrome staining (×40). Arrowhead denotes mononuclear infiltrates in the peribronchovascular space. Arrow denotes collagen deposition. *C*, Quantification of collagen content using Sirius red by colorimetric assay in the lungs of individual TCR$\beta^{-/-}$ and C57BL/6 mice treated with *S. rectivirgula* (*SR*) or sterile PBS for 4 consecutive weeks. Data compiled from at least two separate experiments.
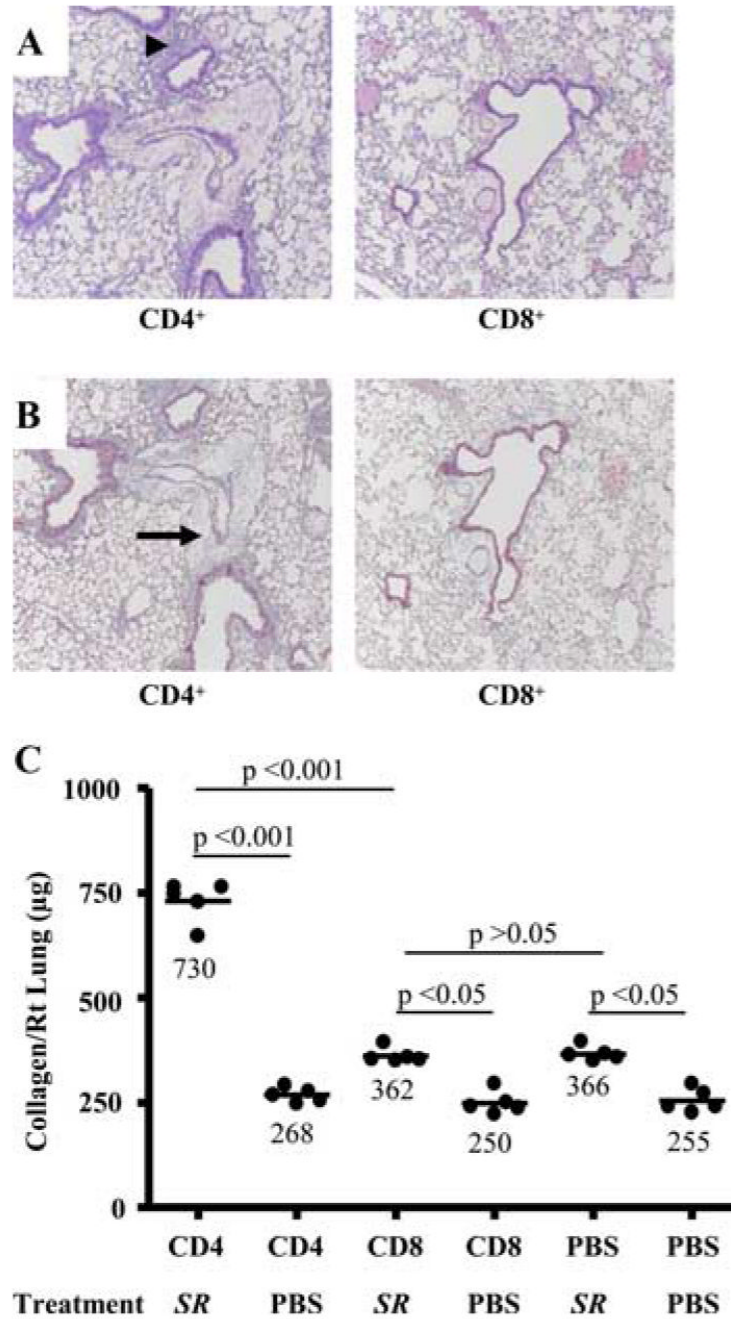
FIGURE 2.
TCR$\beta^{-/-}\delta^{-/-}$ mice reconstituted with CD4$^+$ T cells develop mononuclear infiltrates and pulmonary fibrosis in a peribronchovascular distribution after treatment with *S. rectivirgula.* *A*, Representative H&E staining of lungs from TCR$\beta^{-/-}\delta^{-/-}$ mice reconstituted with either CD4$^+$ or CD8$^+$ T cells followed by intranasal treatment with *S. rectivirgula* for 4 consecutive weeks is shown. Arrowhead denotes mononuclear infiltrates in the peribronchovascular space. *B*, Representative Masson trichrome staining of lungs from TCR$\beta^{-/-}\delta^{-/-}$ mice reconstituted with either CD4$^+$ or CD8$^+$ T cells followed by intranasal treatment with *S. rectivirgula* for 4 consecutive weeks. Arrow denotes collagen deposition in a peribronchovascular distribution. Data represent at least four mice from at least two separate experiments for H&E and Masson

trichrome staining (×40). *C*, Quantification of collagen content using Sirius red by colorimetric assay in the lungs of individual TCR$\beta^{-/-}\delta^{-/-}$ reconstituted with CD4$^+$, CD8$^+$, or sterile PBS by tail vein injection followed by repeated intranasal treatment with *S. rectivirgula* (*SR*) or sterile PBS for 4 consecutive weeks. Data compiled from at least two separate experiments.
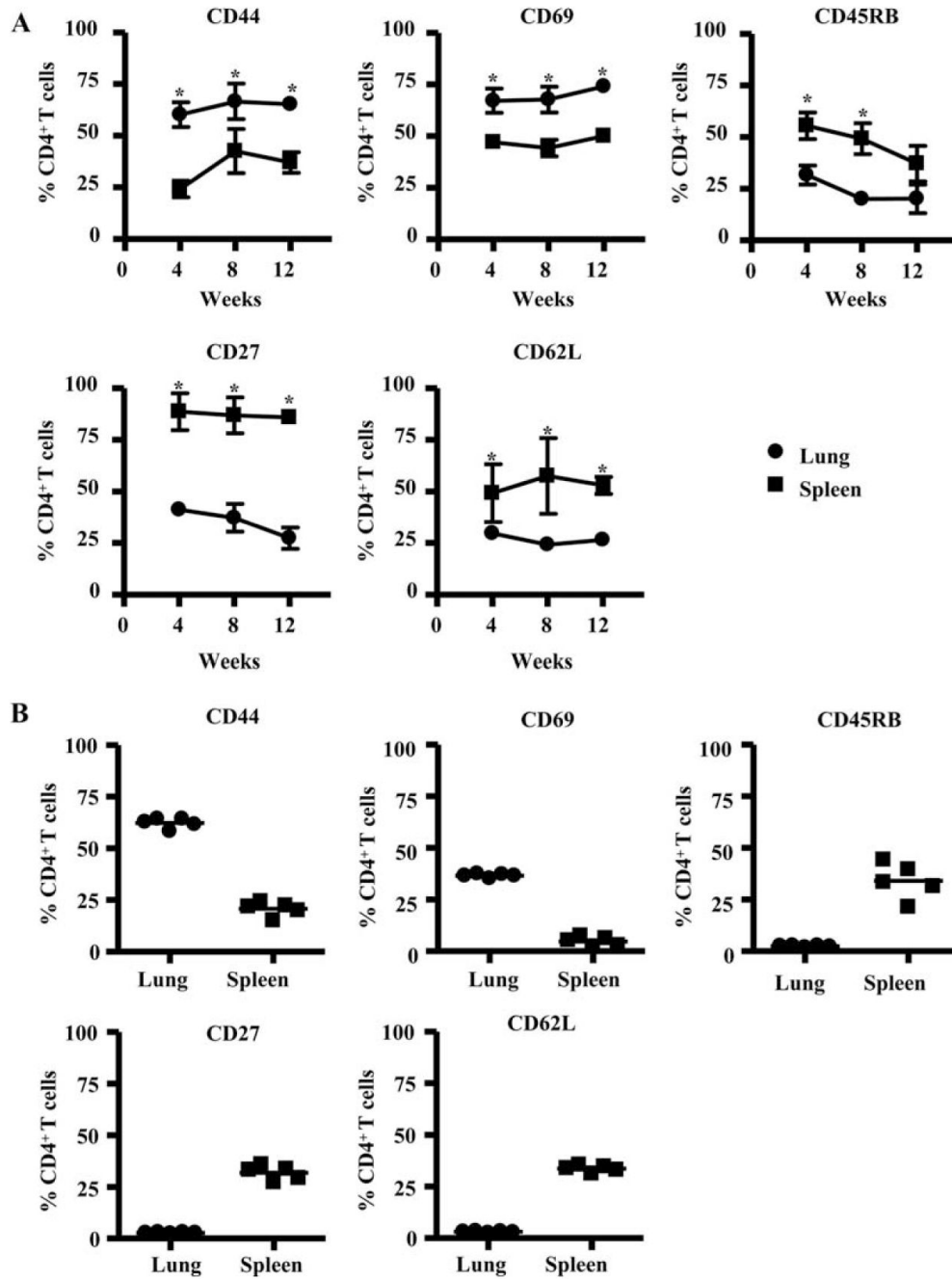
FIGURE 3.
CD4^{+} T cells that accumulate in the lung in response to *S. rectivirgula* express a highly activated phenotype. *A*, Expression of CD44, CD69, CD45RB, CD27, and CD62L on CD4^{+} T cells isolated from the lungs and spleens of C57BL/6 mice treated with *S. rectivirgula* for 4, 8, and 12 consecutive weeks is shown. Data represent the means ± SD of five mice at each time point treated with *S. rectivirgula* from at least two separate experiments. *, Denotes a statistically significant ($p < 0.05$) difference in the percentage of CD4^{+} T cells that express each surface marker from the lung compared with spleen of C57BL/6 mice treated with *S. rectivirgula* at each time point. *B*, Surface expression of CD44, CD69, CD45RB, CD27, and CD62L on CD4^{+} T cells isolated from the lungs and spleens of TCR$\beta^{-/-}\delta^{-/-}$ mice reconstituted with

CD4^{+} T cells followed by intranasal treatment with *S. rectivirgula* for 4 consecutive weeks is shown. Data compiled from at least two separate experiments.
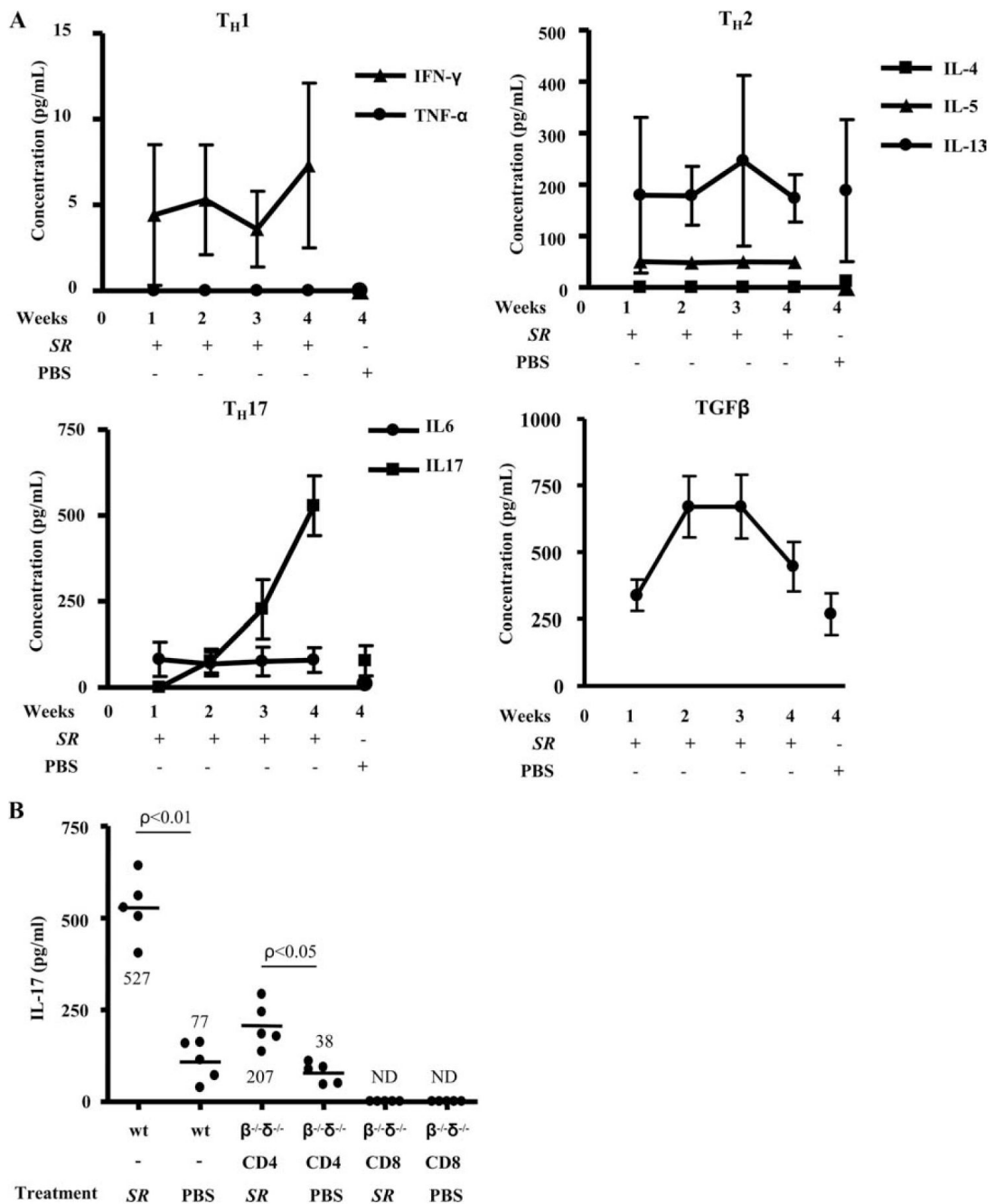
FIGURE 4.
Analysis of Th1, Th2, and Th17 cytokines from the lungs of C57BL/6 mice treated with *S. rectivirgula. A*, Cytokines from supernatants of total lung homogenates were analyzed from C57BL/6 mice treated with *S. rectivirgula* for 1, 2, 3, and 4 wk compared with control mice treated in an identical fashion with sterile PBS for 4 wk. Data represent the mean ± SD of five mice at each time point treated with *S. rectivirgula* from at least two separate experiments. *B*, Supernatants from total lung homogenates of wt C57BL/6 (wt) or TCR$\beta^{-/-}\delta^{-/-}$ mice reconstituted with either CD4$^+$ or CD8$^+$ T cells were analyzed for IL-17. TCR$\beta^{-/-}\delta^{-/-}$ mice were treated with either *S. rectivirgula* (*SR*) or sterile PBS for 4 consecutive weeks after

reconstitution with either CD4$^+$ or CD8$^+$ T cells. Data compiled from at least two separate experiments. ND indicates not detected.
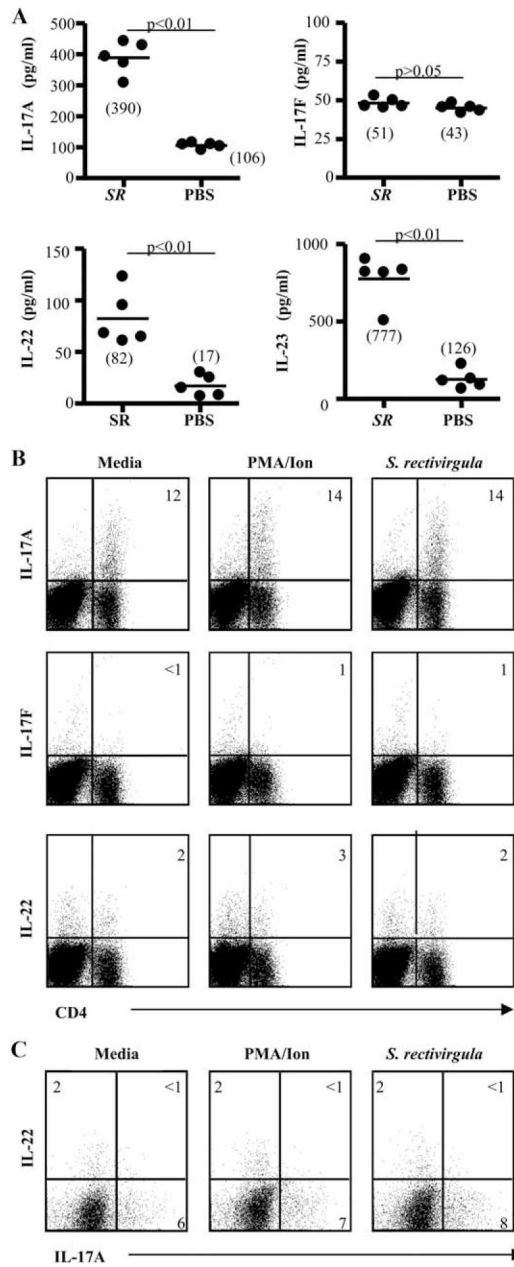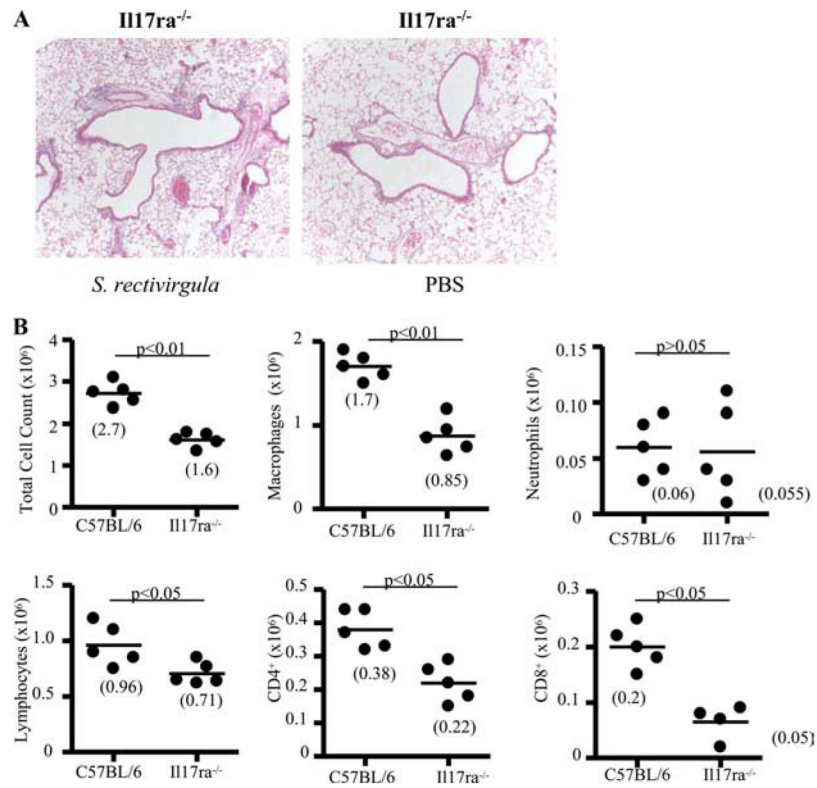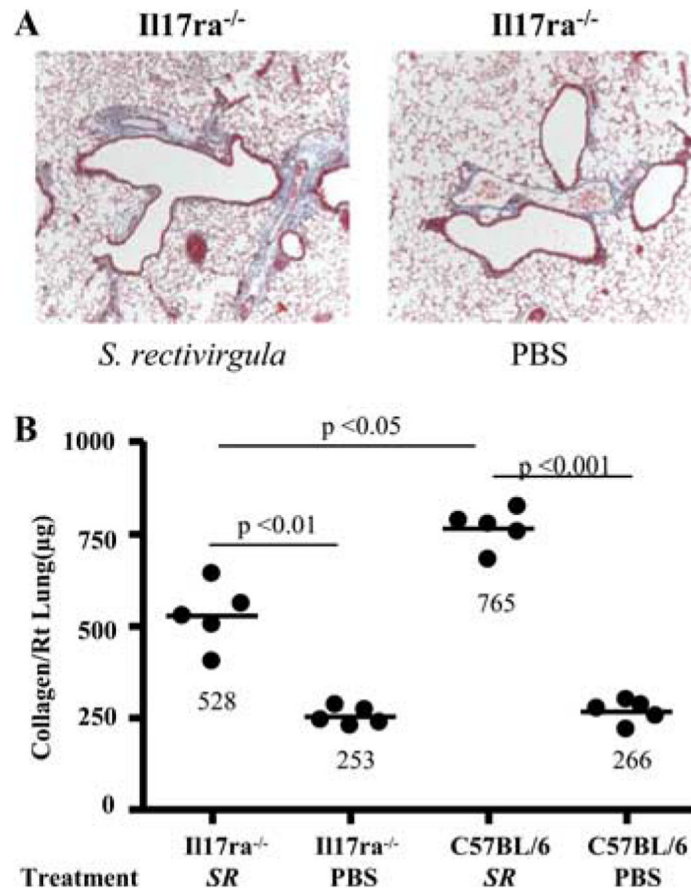
FIGURE 5.
CD4^{+} T cells that accumulate in the lung in response to *S. rectivirgula* differentially express IL-17A and IL-22. *A*, Th17 cytokines from supernatants of total lung homogenates were analyzed from C57BL/6 mice treated with *S. rectivirgula* or PBS for 4 wk. Data represent the mean ± SD of five mice at each time point treated with *S. rectivirgula* from at least two separate experiments. *B*, Representative density plots of CD4^{+} T cells expressing IL-17A, IL-17F, or IL-22 in response to medium, PMA/ionomycin, or *S. rectivirgula* (10 μg/ml) are shown. The percentage of CD4^{+} T cells expressing the respective cytokine is shown in the upper right quadrant of each density plot. *C*, Representative density plots of CD4^{+} T cells expressing IL-17A and IL-22 in response to medium, PMA/ionomycin, or *S. rectivirgula* are shown. The percentage of CD4^{+} T cells expressing each cytokine is shown in the appropriate quadrant.

Data are representative of at least five individual mice treated with *S. rectivirgula* from at least two separate experiments.

FIGURE 6.
Il17ra$^{-/-}$ mice develop less inflammation in response to *S. rectivirgula* exposure. *A*, Representative H&E staining of lungs from Il17ra$^{-/-}$ mice repeatedly treated with *S. rectivirgula* or PBS for 4 consecutive weeks is shown. Data shown represent at least four mice from at least two separate experiments (×40). *B*, Il17ra$^{-/-}$ mice treated with *S. rectivirgula* for 4 consecutive weeks have decreased numbers of total lung cells, macrophages, lymphocytes, and CD4$^+$ and CD8$^+$ T cells compared with wt C57BL/6 mice. Data shown represent at least five mice from at least two separate experiments.

FIGURE 7.
Il17ra$^{-/-}$ mice develop less pulmonary fibrosis in response to *S. rectivirgula* exposure. *A*, Representative Masson trichrome staining of lungs from Il17ra$^{-/-}$ mice repeatedly treated with *S. rectivirgula* or PBS for 4 consecutive weeks is shown. Data shown represent at least four mice from at least two separate experiments (×40). *B*, Quantification of collagen content using Sirius red by colorimetric assay in the lungs of individual Il17ra$^{-/-}$ and wt C57BL/6 mice treated with *S. rectivirgula* (*SR*) or PBS for 4 consecutive weeks. Data compiled from at least two separate experiments.