

The Journal of Immunology

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J. Immunol. 2009;182:6540-6549

doi:10.4049/jimmunol.0900013

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IL-17A-Expressing T Cells Are Essential for Bacterial Clearance in a Murine Model of Hypersensitivity Pneumonitis¹

Philip L. Simonian,^{2*} Christina L. Roark,[†] Fabian Wehrmann,^{*} Allison M. Lanham,^{*} Willi K. Born,[†] Rebecca L. O'Brien,[†] and Andrew P. Fontenot^{*†}

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 V γ 6/V δ 1⁺ $\gamma\delta$ 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 V γ 6/V δ 1⁺ $\gamma\delta$ T cells. To further investigate the role of V γ 6/V δ 1⁺ $\gamma\delta$ T cells in *B. subtilis*-induced lung fibrosis, we exposed transgenic V γ 6/V δ 1 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 V γ 6/V δ 1⁺ T cells, a compensatory increase in IL-17A expression by CD4⁺ T cells was seen in the absence of $\gamma\delta$ T cells that resulted in similar levels of IL-17A in the lungs of TCR δ ^{-/-} and wild-type C57BL/6 mice. In combination, our data suggest an important role for IL-17A-expressing T lymphocytes, both $\gamma\delta$ and $\alpha\beta$ 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.

Hypersensitivity pneumonitis (HP)³ is an inflammatory 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 bacteria) and certain small m.w. volatile and nonvolatile chemical compounds. 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. 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). Although these mononuclear infiltrates contain large numbers of CD4⁺ and CD8⁺ T cells that are

important in the pathogenesis of HP, $\gamma\delta$ T cells have also been described in the lungs of patients with HP and other granulomatous diseases such as sarcoidosis, but their function remains unknown in these lung disorders (8–10).

$\gamma\delta$ T cells are a unique subset of lymphocytes whose function is poorly understood. However, these cells have been implicated in the regulation of the immune response generated against microbial pathogens and allergens (11). The fact that $\gamma\delta$ T cells are found in the subepithelium of alveolar and nonalveolar regions of the lung (12) suggests that they play an important role in the immune response directed against inhaled particles such as microbial pathogens (13). For example, mice intratracheally infected with *Cryptococcus neoformans* develop a transient increase in $\gamma\delta$ T cells in the lung that peaks 3–6 days after infection. In the absence of $\gamma\delta$ T cells, *C. neoformans* is cleared more rapidly from the lung, suggesting that $\gamma\delta$ T cells may down-regulate the Th1-mediated immune response initiated by *C. neoformans* at the expense of microbial persistence (14). Similar to pulmonary infection with *C. neoformans*, mice infected with *Pneumocystis carinii* or *Bordetella pertussis* have a transient increase in $\gamma\delta$ T cells in the lung and enhanced clearance of these microbial pathogens in the absence of $\gamma\delta$ T lymphocytes (15, 16). These data suggest that $\gamma\delta$ T cells in these models of lung infection down-regulate the immune response generated against the inhaled pathogen, which may prevent tissue damage. Conversely, in the absence of $\gamma\delta$ T cells, mice infected with *Nocardia asteroides* have a delay in microbial clearance and increased mortality (17, 18). The reason for these contradictory observations is not clear, but all of these studies suggest that $\gamma\delta$ T cells are important in pulmonary infections caused by a variety of inhaled microorganisms.

We developed a murine model of HP whereby mice repeatedly exposed to the ubiquitous microorganism *Bacillus subtilis* develop mononuclear infiltrates in a peribronchovascular distribution and pulmonary fibrosis similar to the human disease (19). Interestingly,

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Received for publication January 5, 2009. Accepted for publication March 11, 2009.

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¹ This work is supported by National Institutes of Health Grants HL62410 and ES011810 (to A.P.F.) and HL89766 (to P.L.S.).

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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 $V\gamma 6/V\delta 1^+$ TCR. In the absence of $V\gamma 6/V\delta 1^+$ T cells, mice develop increased numbers of $CD4^+$ and $CD8^+$ T cells and accelerated pulmonary fibrosis, suggesting that these $V\gamma 6/V\delta 1^+$ T lymphocytes protect against collagen deposition caused by chronic exposure to *B. subtilis*. In this report, we show that transgenic $V\gamma 6/V\delta 1$ mice repeatedly exposed to *B. subtilis* develop large expansions of $V\gamma 6/V\delta 1^+$ $\gamma\delta$ T cells in lung that associate with reduced numbers of $CD4^+$ 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 $V\gamma 6/V\delta 1^+$ T cells. In the absence of IL-17 receptor signaling, IL-17 $\alpha^{-/-}$ 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 $CD4^+$ 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 $V\gamma 6/V\delta 1$ (R. L. O'Brien) (20, 21), C57BL/6, TCR $\delta^{-/-}$ (The Jackson Laboratory), and IL-17 $\alpha^{-/-}$ mice (Amgen) were treated with 30 μ 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. A *Limulus* amebocyte assay (Sigma-Aldrich) was performed to confirm that the *B. subtilis* preparation and sterile PBS contained <20 μ g of endotoxin per milliliter. Mice were lightly anesthetized with isoflurane to allow inhalation of either *B. subtilis* or sterile PBS. These studies were approved by the Animal Care and Use Committee at the University of Colorado Denver (Aurora, CO).

Preparation of mononuclear cells from lung homogenates

Mice at each time point were sacrificed 24 h after the last treatment with *B. subtilis* as previously described (19). 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 at 37°C, 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.

Flow cytometry and immunofluorescence analysis

T lymphocytes isolated from the lungs of mice treated with either *B. subtilis* or sterile PBS were surface stained with mAbs directed against CD3, CD4, CD8, and C δ (BD Biosciences). Because $V\gamma 6/V\delta 1^+$ $\gamma\delta$ T cells from transgenic $V\gamma 6/V\delta 1$ mice express CD8 α , $CD3^+CD8^+$ T cells were identified by gating on C δ^- and NK1.1 $^-$ cells and analyzed for expression of $CD3^+CD8^+$ T cells. For intracellular cytokine staining, total lung cells were cultured at 1×10^6 cells/ml in complete medium containing 10 μ g/ml brefeldin A (Sigma-Aldrich) at 37°C for 4 h. After activation, the cells were washed and stained with mAbs directed against CD3, CD4, and CD8. $\gamma\delta$ T cells were identified by staining with anti-C δ mAb (BD Biosciences)

followed by fixation in 1% paraformaldehyde overnight. $\gamma\delta$ T cells that express the $V\gamma 6^+$ TCR were identified using a mAb, 17D1, that recognizes the $V\gamma 6^-$ TCR after staining with anti-C δ mAb (21). Fixed cells were permeabilized for 10 min at room temperature in 0.5% saponin and stained with mAbs directed against IL-17A (eBioscience) 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 FACS Aria cytometer (BD Immunocytometry Systems). The data files were analyzed using FloJo software (Tree Star).

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, embedded in paraffin, 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 μ 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- β 1 (R&D Systems), IL-22 (R&D Systems), IL-17A (eBioscience), IFN- γ (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 pepsin/10 mg of tissue, and incubated for 24 h at 4°C. After centrifugation at high speed for 10 min, 100 μ l of each supernatant was mixed with 900 μ 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 OD₅₄₀ 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 cytocentrifuge 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 homogenizing whole lung or spleen samples in 500 μ 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.

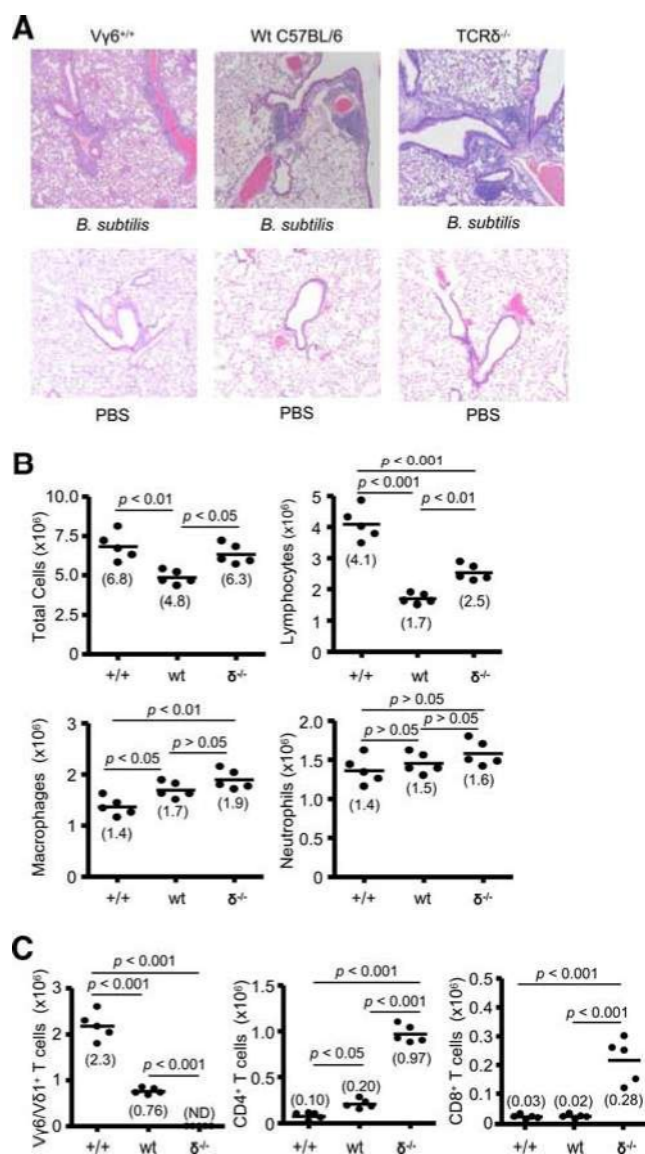


FIGURE 1. Transgenic $V\gamma 6^{+/+}$ mice repeatedly exposed to *B. subtilis* develop mononuclear infiltrates. **A**, Representative H&E of lungs from transgenic $V\gamma 6^{+/+}$, wt C57BL/6, and $TCR\delta^{-/-}$ mice repeatedly exposed to inhaled *B. subtilis* or PBS for 4 consecutive weeks is shown. $V\gamma 6^{+/+}$ mice develop mononuclear infiltrates in both the peribronchovascular and the interstitial spaces. Data shown represent at least five mice from at least two separate experiments (original magnification $\times 100$). **B**, Total and differential cell counts were performed on total lung homogenates from $V\gamma 6^{+/+}$ ($+/+$), wt C57BL/6 (wt), and $TCR\delta^{-/-}$ ($\delta^{-/-}$) mice repeatedly treated with inhaled *B. subtilis* for 4 consecutive weeks. **C**, $V\gamma 6/V\delta 1^{+}$, $CD4^{+}$, and $CD8^{+}$ T cell counts were performed on total lung homogenates from $V\gamma 6^{+/+}$ ($+/+$), wt C57BL/6 (wt), and $TCR\delta^{-/-}$ ($\delta^{-/-}$) mice repeatedly treated with inhaled *B. subtilis* for 4 consecutive weeks. Data were compiled from at least two separate experiments.

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). $p < 0.05$ was considered statistically significant.

Results

Altered composition of mononuclear cell infiltrates after *B. subtilis* inhalation in transgenic $V\gamma 6/V\delta 1$ mice

To further investigate the potential regulatory role of $\gamma\delta$ T cells in lung inflammation and fibrosis, we repeatedly exposed homozy-

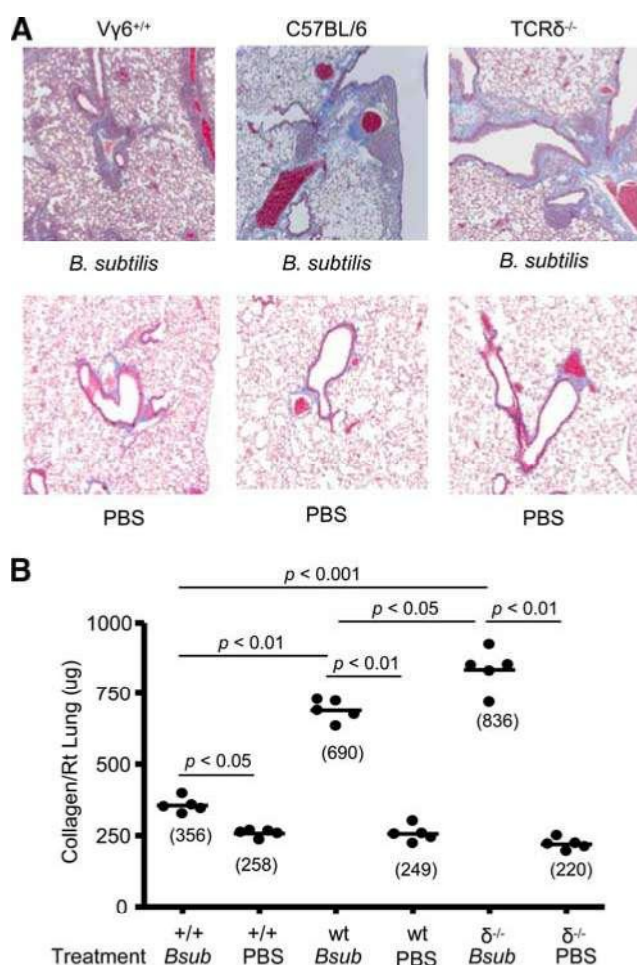


FIGURE 2. Transgenic $V\gamma 6^{+/+}$ mice repeatedly exposed to *B. subtilis* develop less lung fibrosis. **A**, Representative trichrome staining of lungs from transgenic $V\gamma 6^{+/+}$, wt C57BL/6, and $TCR\delta^{-/-}$ mice repeatedly treated with inhaled *B. subtilis* or PBS for 4 consecutive weeks is shown. Data shown represent at least five mice from at least two separate experiments (original magnification $\times 100$). **B**, Quantification of collagen content using Sirius red colorimetric assay in the lungs of individual transgenic $V\gamma 6^{+/+}$ ($+/+$), wt C57BL/6 (wt), and $TCR\delta^{-/-}$ ($\delta^{-/-}$) mice treated with *B. subtilis* (Bsub) or PBS for 4 consecutive weeks. Data were compiled from at least two separate experiments.

gous $V\gamma 6/V\delta 1$ transgenic ($V\gamma 6^{+/+}$) mice to *B. subtilis* by nasal inhalation for 4 consecutive weeks. Nearly all $\gamma\delta$ T cells from $V\gamma 6^{+/+}$ mice expressed the canonical $V\gamma 6/V\delta 1$ TCR (20, 21). $V\gamma 6^{+/+}$ 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 *B. subtilis*-treated $V\gamma 6^{+/+}$ mice ($p < 0.01$) (Fig. 1B). In addition, a significantly increased number of lung lymphocytes was seen in $V\gamma 6^{+/+}$ mice compared with either wt C57BL/6 mice ($p < 0.001$) or $TCR\delta^{-/-}$ mice ($p < 0.001$) (Fig. 1B), with a 3-fold increase in $V\gamma 6/V\delta 1^{+}$ $\gamma\delta$ T cells compared with wt C57BL/6 mice ($p < 0.001$) (Fig. 1C). Interestingly, $CD4^{+}$ T cells were decreased by 50% in the lungs of $V\gamma 6^{+/+}$ mice compared with wt C57BL/6 mice ($p < 0.05$), but were markedly expanded in $TCR\delta^{-/-}$ mice ($p < 0.001$) (Fig. 1C). $CD8^{+}$ T cells were not significantly different between $V\gamma 6^{+/+}$ and wt C57BL/6 mice but were increased in $TCR\delta^{-/-}$ mice in response to chronic treatment with *B. subtilis* (Fig. 1C). Macrophage numbers were also decreased in $V\gamma 6^{+/+}$ mice compared

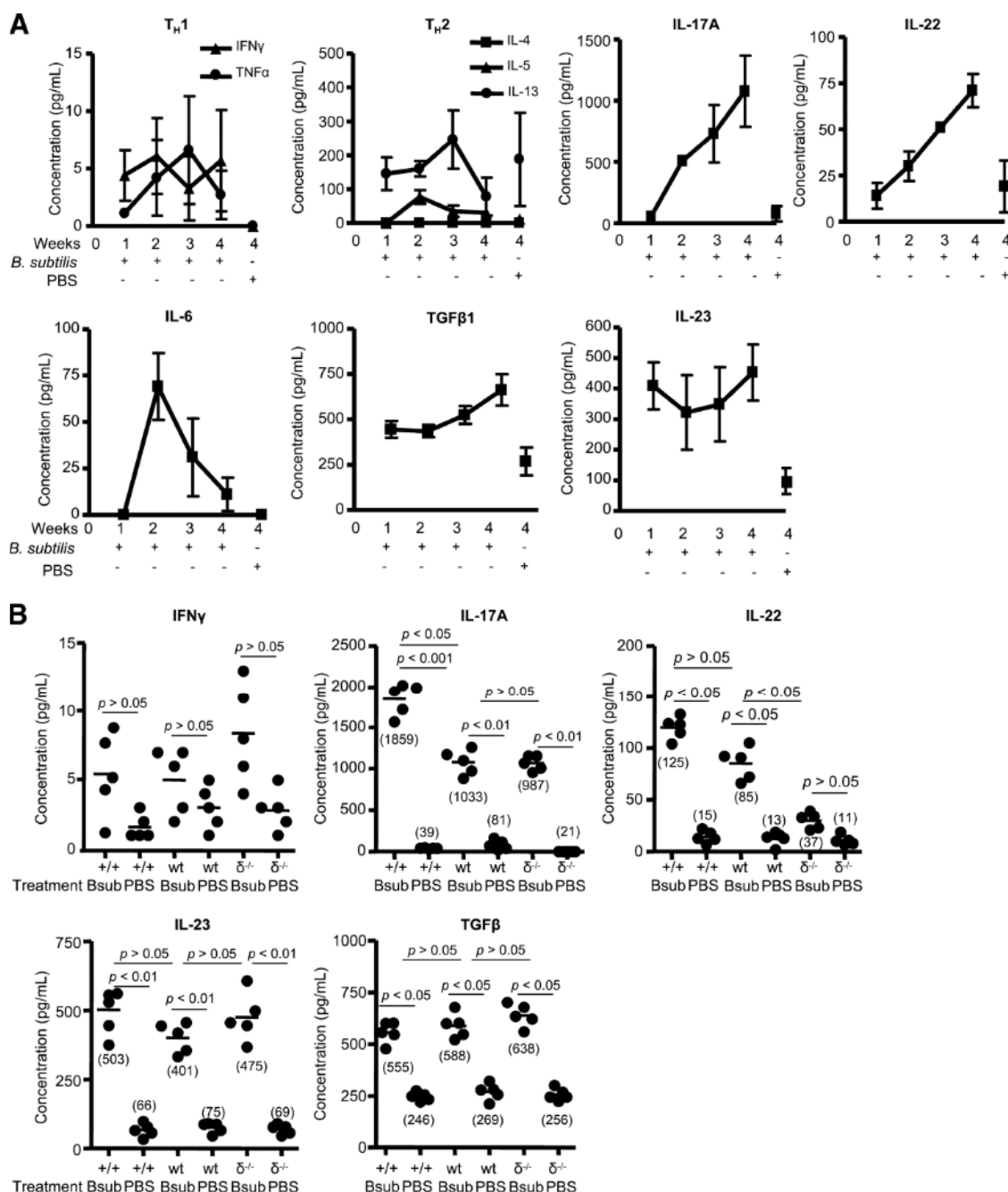


FIGURE 3. Analysis of Th1, Th2, and Th17 cytokines from the lungs of wt C57BL/6 mice treated with *B. subtilis*. **A**, Cytokines from supernatants of total lung homogenates were analyzed from wt C57BL/6 mice treated with *B. subtilis* for 1, 2, 3, and 4 wk compared with control mice treated in an identical fashion with sterile PBS for 4 wk. Data represent the mean $\pm$ SD of five mice at each time point treated with *B. subtilis* or sterile PBS from at least two separate experiments. **B**, Cytokines from supernatants of total lung homogenates were analyzed by ELISA from $V\gamma 6^{+/+}$ ($+/+$), wt C57BL/6 (wt), and $TCR\delta^{-/-}$ ($\delta^{-/-}$) mice treated with either *B. subtilis* (Bsub) or PBS for 4 wk. Data represent the mean $\pm$ SD of five mice treated with *B. subtilis* or sterile PBS from at least two separate experiments.

with wt C57BL/6 ($p < 0.05$) and $TCR\delta^{-/-}$ 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 $V\gamma 6/V\delta 1^{+}$ $\gamma\delta$ 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.

V\gamma 6/V\delta 1 transgenic mice have attenuated lung fibrosis after *B. subtilis* exposure

We have previously shown that in the absence of $V\gamma 6/V\delta 1^{+}$ T cells, lung fibrosis is accelerated in mice chronically exposed to *B.*

subtilis (19). To determine whether $\gamma\delta$ T cells prevent collagen deposition in the lung, we repeatedly treated $V\gamma 6^{+/+}$ mice with *B. subtilis* for 4 consecutive weeks. As shown in Fig. 2A, $V\gamma 6^{+/+}$ 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 and $TCR\delta^{-/-}$ mice treated in an identical fashion. Using the more sensitive Sirius red colorimetric assay for collagen deposition, *B. subtilis*-treated $V\gamma 6^{+/+}$ mice developed a 1.9-fold decrease in collagen content in the lung compared with wt C57BL/6 mice ($p < 0.01$) and a 2.3-fold decrease compared with mice deficient in $\gamma\delta$

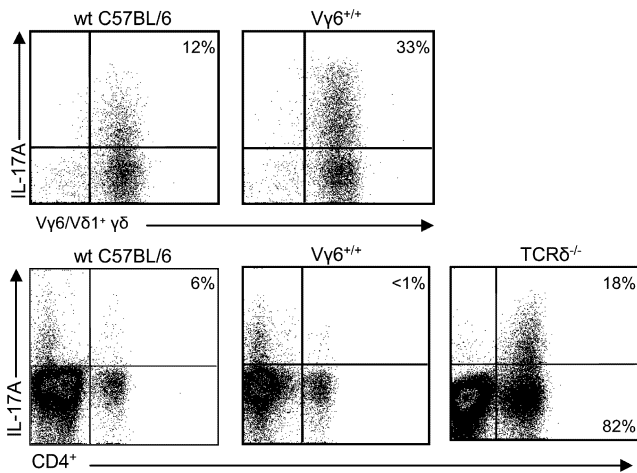


FIGURE 4. IL-17A expression by $V\gamma 6/V\delta 1^+$ and $CD4^+$ T cells. Representative density plots of intracellular IL-17A expression in $V\gamma 6/V\delta 1^+$ or $CD4^+$ T cells from $V\gamma 6^{+/+}$, wt C57BL/6, or $TCR\delta^{-/-}$ treated with *B. subtilis* for 4 wk are shown. $V\gamma 6/V\delta 1^+$ 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 $V\gamma 6/V\delta 1^+$ 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 ($TCR\delta^{-/-}$ mice) ($p < 0.001$) (Fig. 2B). Interestingly, $V\gamma 6^{+/+}$ mice had increased collagen deposition in the lung compared with PBS-treated control animals (mean $\pm$ SD; 356 ± 26 pg/ml vs 258 ± 12 pg/ml; $p < 0.05$) (Fig. 2B), suggesting that $V\gamma 6/V\delta 1^+$ $\gamma\delta$ 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

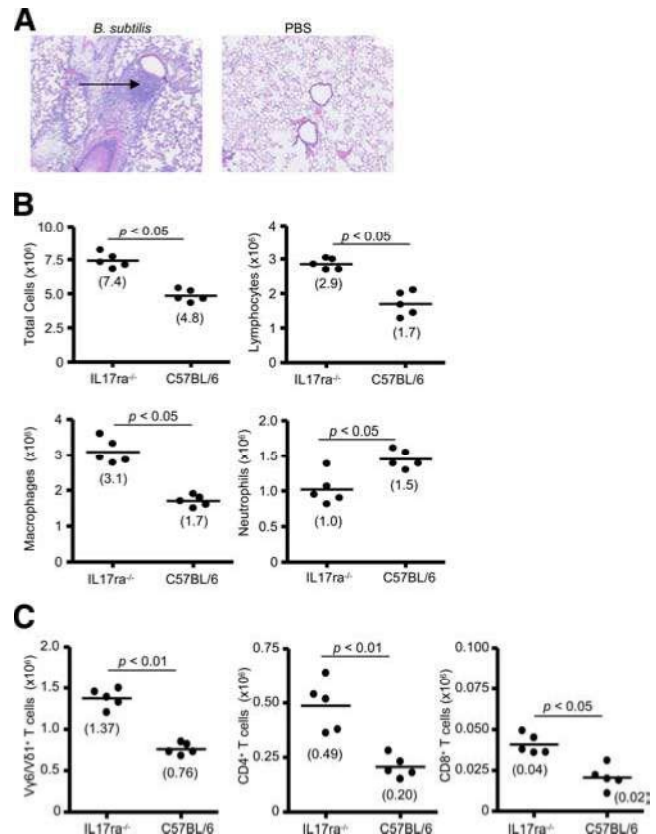


FIGURE 5. $IL17ra^{-/-}$ mice develop increased mononuclear infiltrates in response to *B. subtilis*. A, Representative H&E staining of lungs from $IL17ra^{-/-}$ mice repeatedly treated with inhaled *B. subtilis* for 4 consecutive weeks shows increased mononuclear infiltrates compared with PBS-treated controls. Data shown for H&E represent at least four mice from at least two separate experiments (original magnification $\times 100$). Arrow denotes a mononuclear infiltrate in a peribronchovascular distribution. B, Total cell and lymphocyte, macrophage, and neutrophil counts from the lungs of *B. subtilis*-treated $IL17ra^{-/-}$ or wt C57BL/6 mice repeatedly treated with inhaled *B. subtilis* for 4 consecutive weeks are shown. C, $V\gamma 6/V\delta 1^+$, $CD4^+$, and $CD8^+$ T cell counts from the lung of *B. subtilis*-treated $IL17ra^{-/-}$ or wt C57BL/6 mice repeatedly treated with inhaled *B. subtilis* for 4 consecutive weeks are depicted. Data are representative of at least five individual mice treated with *B. subtilis* from at least two separate experiments.

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- $\beta 1$, 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 $V\gamma 6/V\delta 1^+$ T cells correlated with decreased lung fibrosis, we next compared Th1, Th2, and Th17 cytokines from lung homogenates of $V\gamma 6^{+/+}$, wt C57BL/6, and $TCR\delta^{-/-}$ 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 $V\gamma 6^{+/+}$, wt C57BL/6, and $TCR\delta^{-/-}$ 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

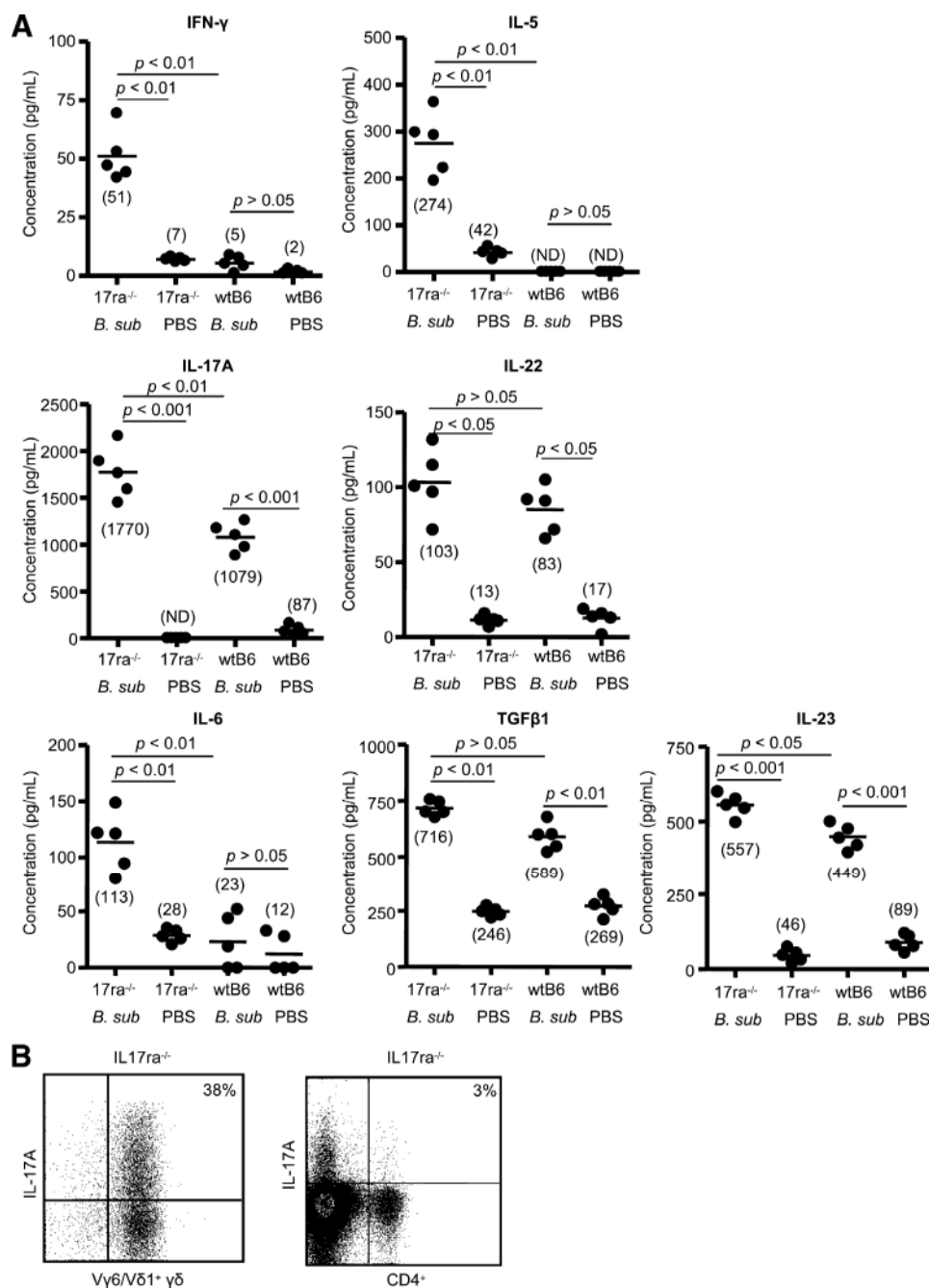


FIGURE 6. Increased inflammatory cytokines in the lung of IL17ra^{-/-} mice treated with *B. subtilis*. **A**, Cytokines from supernatants of total lung homogenates were analyzed from IL17ra^{-/-} or wt C57BL/6 mice treated with *B. subtilis* (*B. sub*) or PBS for 4 wk. Data represent the mean $\pm$ 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 V γ 6/V δ 1⁺ or CD4⁺ T cells from IL17ra^{-/-} mice treated with *B. subtilis* for 4 wk. V γ 6/V δ 1⁺ 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 V γ 6/V δ 1⁺ 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.

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- β 1 were all increased in the lungs of V γ 6^{+/+} and TCR δ ^{-/-} mice treated with *B. subtilis* compared with control animals, but they were similar to those in wt C57BL/6 mice treated in an identical fashion (Fig. 3B and data not shown). Interestingly, IL-17A levels were further increased in the lungs of *B. subtilis*-treated V γ 6^{+/+} mice compared with both wt C57BL/6 and TCR δ ^{-/-} mice with similar concentrations of IL-17A in TCR δ ^{-/-} and wt C57BL/6 mice (Fig. 3B). In addition, IL-22 levels were increased in V γ 6^{+/+} mice compared with wt C57BL/6 mice, whereas in the absence of $\gamma\delta$ T cells, TCR δ ^{-/-} mice had significantly decreased concentrations of IL-22 in the lung compared with both V γ 6^{+/+} and wt C57BL/6 mice (Fig. 3B). These data indicate that a Th17-polarized immune response predominates in the lungs of animals repeatedly exposed to *B. subtilis*.

Expression of IL-17A by V γ 6/V δ 1⁺ $\gamma\delta$ 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 $\gamma\delta$, 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 V γ 6/V δ 1⁺ T cells express IL-17A compared with CD4⁺ T cells in both V γ 6^{+/+} and wt C57BL/6 mice while CD8⁺ T cells did not express IL-17A (data not shown). Interestingly, in the absence of $\gamma\delta$ T cells, CD4⁺ T cells isolated from the lungs of *B. subtilis*-treated TCR δ ^{-/-} mice had increased expression of IL-17A (Fig. 4), which may account for the similar levels of IL-17A in the lungs of wt C57BL/6 and TCR δ ^{-/-} mice (Fig. 3B). Therefore, in the absence of V γ 6/V δ 1⁺ T cells there is not only a 5-fold increase in the number of CD4⁺ T lymphocytes but also a 13-fold increase in the number of CD4⁺

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 V γ 6/V δ 1⁺ 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 *B. subtilis*-treated IL-17ra^{-/-} mice compared with wt C57BL/6 mice treated with *B. subtilis* (Fig. 6A). Although there was a trend toward higher levels of IL-22 and TGF- β 1 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). V γ 6/V δ 1⁺ $\gamma\delta$ 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 V γ 6/V δ 1⁺ T cells (V γ 4^{-/-}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, V γ 6^{+/+}, 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 $\pm$ 62 μ g vs 690 $\pm$ 38 μ g; $p < 0.05$) 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.

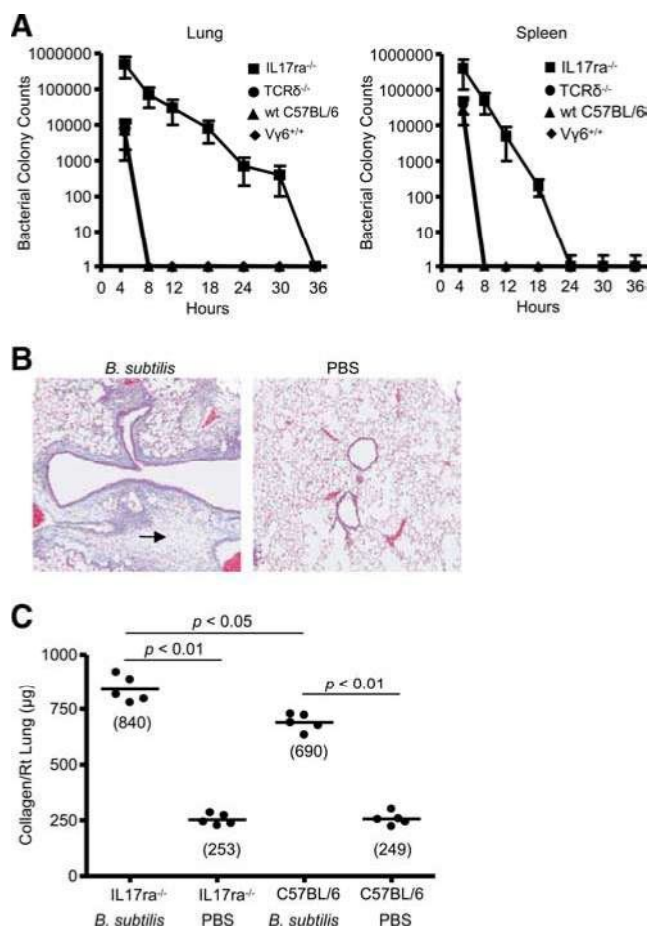


FIGURE 7. IL17ra^{-/-} mice develop increased lung fibrosis in response to persistent *B. subtilis* infection. **A**, *B. subtilis* colony counts from lung homogenates of individual IL17ra^{-/-}, TCR δ ^{-/-}, wt C57BL/6, and V γ 6^{+/+} mice. All mice were treated with *B. subtilis* for 3 consecutive days each week for 4 consecutive weeks and analyzed for the persistence of *B. subtilis* in the lung and spleen at different time points after last exposure to *B. subtilis*. Data represent the mean $\pm$ SD colony counts from at least four mice at each time point treated with *B. subtilis* from at least two separate experiments. **B**, Representative Masson's trichrome staining of lungs from IL17ra^{-/-} mice repeatedly treated with inhaled *B. subtilis* for 4 consecutive weeks shows collagen deposition in a peribronchovascular distribution. Arrow denotes collagen deposition in a peribronchovascular distribution. Data shown for Masson's trichrome represent at least four mice from at least two separate experiments (original magnification $\times 100$). **C**, Quantification of collagen content using Sirius red colorimetric assay shows increased collagen deposition in the lungs of individual IL17ra^{-/-} compared with wt C57BL/6 mice treated with *B. subtilis* or PBS for 4 consecutive weeks. Data compiled from at least two separate experiments.

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 V γ 6/V δ 1⁺ $\gamma\delta$ T cells (19). In the absence of $\gamma\delta$ T cells, V γ 4^{-/-}6^{-/-} mice had increased numbers of CD4⁺ and CD8⁺ T lymphocytes with accelerated lung fibrosis upon repeated exposure to inhaled *B. subtilis*, suggesting that V γ 6/V δ 1⁺ T cells protect against lung fibrosis by limiting the expansion of $\alpha\beta$ T

cells. In the present study, we build on these studies using V γ 6/V δ 1 transgenic mice. With chronic exposure to *B. subtilis*, V γ 6/V δ 1 transgenic mice have further increased numbers of V γ 6/V δ 1⁺ 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 Th17 cytokine IL-17A, which was predominantly expressed by V γ 6/V δ 1⁺ T cells. In the absence of IL-17 signaling, *B. subtilis* persisted in the lung, resulting in increased inflammation and fibrosis and suggesting an important role for IL-17A in eliminating *B. subtilis*.

To investigate the mechanisms by which $\gamma\delta$ T lymphocytes attenuate *B. subtilis*-induced lung inflammation and fibrosis, we initially analyzed lung homogenates for type 1 and 2 cytokines. In a number of models of solid organ fibrosis, Th1 and Th2 cytokines differentially regulate organ fibrosis (26). Numerous studies suggest that the type 1 cytokine IFN- γ is important for the granulomatous inflammation seen in HP (17, 20) as well as protective against collagen deposition in the bleomycin-induced model of lung fibrosis (27). Therefore, we hypothesized that $\gamma\delta$ T cells prevent fibrotic lung disease through the expression of IFN- γ . We found, however, very low levels of IFN- γ in the lung and did not find significant expression of IFN- γ by $\gamma\delta$, CD4⁺, or CD8⁺ 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 the type 2 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 expressed by CD4⁺ T cells in mucosal immunity against lung pathogens (29), whereas IL-17 expression by $\gamma\delta$ T cells is important in pulmonary tuberculosis (23), collagen-induced arthritis (30), and *Listeria monocytogenes*-induced liver disease (31). In this regard, the predominant cytokine identified in the lungs of wt C57BL/6 mice treated with *B. subtilis* was IL-17A, which was expressed predominantly by V γ 6/V δ 1⁺ T cells. The fact that Th17 cytokines were identified 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, V γ 6^{+/+}, and TCR δ ^{-/-} mice, indicating that IL-17A signaling is important for neutrophil recruitment to the lung in

response to repeated exposure to *B. subtilis*. Interestingly, V γ 6^{+/+} 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 TCR δ ^{-/-} mice. The presence of nearly identical numbers of neutrophils in V γ 6^{+/+} mice treated with *B. subtilis* compared with wt C57BL/6 and TCR δ ^{-/-} 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, V γ 6^{+/+} and TCR δ ^{-/-} 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 V γ 4^{-/-}6^{-/-} mice (19), develop accelerated lung fibrosis in response to repeated exposure to *B. subtilis*. In addition, V γ 6^{+/+} 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 V γ 6/V δ 1⁺ $\gamma\delta$ T cells may possess other mechanisms that attenuate lung inflammation and fibrosis independently of IL-17A expression. V γ 6/V δ 1⁺ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 V γ 6/V δ 1 mice compared with mice deficient in $\gamma\delta$ T cells (TCR δ ^{-/-}). Therefore, it is possible that V γ 6/V δ 1⁺ 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 V γ 6/V δ 1 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 $\gamma\delta$ T lymphocytes (19). Although a decline in the number of CD4⁺ T cells in the lung associates with less lung fibrosis in transgenic V γ 6/V δ 1 mice, V γ 6/V δ 1⁺ T cells may not directly regulate the expansion of CD4⁺ T cells in these mice. As opposed to wt C57BL/6 mice, transgenic V γ 6/V δ 1 mice have V γ 6/V δ 1⁺ 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 $\alpha\beta$ T cells are found in the lung before treatment with *B. subtilis*, transgenic V γ 6/V δ 1 mice have fewer $\alpha\beta$ 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 V γ 6/V δ 1⁺ 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 rectivirgula* 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-17 $\alpha^{-/-}$) 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, $\gamma\delta$ 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-17 $\alpha^{-/-}$ mice developed a heterogeneous expansion of $\gamma\delta$ 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 $\gamma\delta$ 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, V γ 6 $^{+/+}$ mice 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 $\gamma\delta$ T cells (TCR $\delta^{-/-}$ mice), IL-22 concentrations were 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 V γ 6/V δ 1 $^{+}$ $\gamma\delta$ 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 V γ 6/V δ 1 $^{+}$ 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-17 $\alpha^{-/-}$ mice, resulting in accelerated lung inflammation and fibrosis. These data suggest an important role for IL-17A-expressing T lymphocytes, both $\gamma\delta$ 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.

References

- Selman, M. 2003. Hypersensitivity pneumonitis. In *Interstitial Lung Disease*, 3rd Ed. M. I. Schwarz, and T. E. King, Jr., eds. B. C. Decker, Hamilton, Ontario, pp. 452–484.
- Bourke, S. J., S. W. Banham, R. Carter, P. Lynch, and G. Boyd. 1989. Longitudinal course of extrinsic allergic alveolitis in pigeon breeders. *Thorax* 44: 415–418.
- Lalancette, M., G. Carrier, M. Laviolette, S. Ferland, J. Rodrique, R. Begin, A. Cantin, and Y. Cormier. 1993. Farmer's lung: long-term outcome and lack of predictive value of bronchoalveolar lavage fibrosis factors. *Am. Rev. Respir. Dis.* 148: 216–221.
- Monkare, S., and T. Haahtela. 1987. Farmer's lung: a 5-year follow-up of eighty-six patients. *Clin. Allergy* 17: 143–151.
- Yoshizawa, Y., Y. Ohtani, H. Hayakawa, A. Sato, M. Suga, and M. Ando. 1999. Chronic hypersensitivity pneumonitis in Japan: a nationwide epidemiologic survey. *J. Allergy. Clin. Immunol.* 103: 315–320.
- Vourlekis, J. S., M. I. Schwarz, R. M. Cherniack, D. Curran-Everett, C. D. Cool, R. M. Tuder, T. E. King, Jr., and K. K. Brown. 2004. The effect of pulmonary fibrosis on survival in patients with hypersensitivity pneumonitis. *Am. J. Med.* 116: 662–668.
- Reyes, C. N., F. J. Wenzel, B. R. Lawton, and D. A. Emanuel. 1982. The pulmonary pathology of farmer's lung disease. *Chest* 81: 142–146.
- Raulf, M., V. Liebers, C. Steppert, and X. Baur. 1994. Increased $\gamma\delta$ -positive T-cells in blood and bronchoalveolar lavage of patients with sarcoidosis and hypersensitivity pneumonitis. *Eur. Respir. J.* 7: 140–147.
- Forrester, J. M., L. S. Newman, Y. Wang, T. E. King, Jr., and B. L. Kotzin. 1993. Clonal expansion of lung V δ 1 $^{+}$ T cells in pulmonary sarcoidosis. *J. Clin. Invest.* 91: 292–300.
- Trentin, L., N. Migone, R. Zambello, P. F. di Celle, F. Aina, C. Feruglio, P. Bulian, M. Masciarelli, C. Agostini, A. Cipriani, et al. 1990. Mechanisms accounting for lymphocytic alveolitis in hypersensitivity pneumonitis. *J. Immunol.* 145: 2147–2154.
- Born, W. K., N. Jin, M. K. Aydintug, J. M. Wands, J. D. French, C. L. Roark, and R. L. O'Brien. 2007. $\gamma\delta$ T lymphocytes-selectable cells within the innate system? *J. Clin. Immunol.* 27: 133–144.
- Wands, J. M., C. L. Roark, M. K. Aydintug, N. Jin, Y. S. Hahn, L. Cook, X. Yin, J. Dal Porto, M. Lahn, D. M. Hyde, et al. 2005. Distribution and leukocyte contacts of $\gamma\delta$ T cells in the lung. *J. Leukocyte Biol.* 78: 1086–1096.
- Nanno, M., T. Shiohara, H. Yamamoto, K. Kawakami, and H. Ishikawa. 2007. $\gamma\delta$ T cells: firefighters or fire boosters in the front lines of inflammatory responses. *Immunol. Rev.* 215: 103–113.
- Uezu, K., K. Kawakami, K. Miyagi, Y. Kinjo, T. Kinjo, H. Ishikawa, and A. Saito. 2004. Accumulation of $\gamma\delta$ T cells in the lungs and their regulatory roles in Th1 response and host defense against pulmonary infection with *Cryptococcus neoformans*. *J. Immunol.* 172: 7629–7634.
- Steele, C., M. Zheng, E. Young, L. Marrero, J. E. Shellito, and J. K. Kolls. 2002. Increased host resistance against *Pneumocystis carinii* pneumonia in $\gamma\delta$ T-cell-deficient mice: protective role of gamma interferon and CD8 $^{+}$ T cells. *Infect. Immun.* 70: 5208–5215.
- Zachariadis, O., J. P. Cassidy, J. Brady, and B. P. Mahon. 2006. $\gamma\delta$ T cells regulate the early inflammatory response to *Bordetella pertussis* infection in the murine respiratory tract. *Infect. Immun.* 74: 1837–1845.
- Tam, S., D. P. King, and B. L. Beaman. 2001. Increase of $\gamma\delta$ T lymphocytes in murine lungs occurs during recovery from pulmonary infection by *Nocardia asteroides*. *Infect. Immun.* 69: 6165–6171.
- King, D. P., D. M. Hyde, K. A. Jackson, D. M. Novosad, T. N. Ellis, L. Putney, M. Y. Stovall, L. S. Van Winkle, B. L. Beaman, and D. A. Ferrick. 1999. Cutting edge: protective response to pulmonary injury requires $\gamma\delta$ T lymphocytes. *J. Immunol.* 162: 5033–5036.
- Simonian, P. L., C. L. Roark, F. Diaz del Valle, B. E. Palmer, I. S. Douglas, K. Ikuta, W. K. Born, R. L. O'Brien, and A. P. Fontenot. 2006. Regulatory role of $\gamma\delta$ T cells in the recruitment of CD4 $^{+}$ and CD8 $^{+}$ T cells to lung and subsequent pulmonary fibrosis. *J. Immunol.* 177: 4436–4443.
- Sim, G. K., C. Olsson, and A. Augustin. 1995. Commitment and maintenance of the $\alpha\beta$ and $\gamma\delta$ T cell lineages. *J. Immunol.* 154: 5821–5831.
- Roark, C. L., M. K. Aydintug, J. Lewis, X. Yin, M. Lahn, Y. S. Hahn, W. K. Born, R. E. Tigelaar, and R. L. O'Brien. 2004. Subset-specific, uniform activation among V γ 6/V δ 1 $^{+}$ $\gamma\delta$ T cells elicited by inflammation. *J. Leukocyte Biol.* 75: 68–75.
- Yamasaki, H., M. Ando, W. Brazer, D. M. Center, and W. W. Cruikshank. 1999. Polarized type 1 cytokine profile in bronchoalveolar lavage T cells of patients with hypersensitivity pneumonitis. *J. Immunol.* 163: 3516–3523.
- Lockhart, E., A. M. Green, and J. L. Flynn. 2006. IL-17 production is dominated by $\gamma\delta$ T cells rather than CD4 T cells during *Mycobacterium tuberculosis* infection. *J. Immunol.* 177: 4662–4669.
- Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* 25: 821–852.
- Luzina, I. G., N. W. Todd, A. T. Iacono, and S. P. Atamas. 2008. Roles of T lymphocytes in pulmonary fibrosis. *J. Leukocyte Biol.* 83: 237–244.
- Wynn, T. A. 2008. Cellular and molecular mechanisms of fibrosis. *J. Pathol.* 214: 199–210.
- Jiang, D., J. Liang, J. Hodge, B. Lu, Z. Zhu, S. Yu, J. Fan, Y. Gao, Z. Yin, R. Homer, et al. 2004. Regulation of pulmonary fibrosis by chemokine receptor CXCR3. *J. Clin. Invest.* 114: 291–299.
- Huax, F., T. Liu, B. McGarry, M. Ullenbruch, Z. Xing, and S. H. Phan. 2003. Eosinophils and T lymphocytes possess distinct roles in bleomycin-induced lung injury and fibrosis. *J. Immunol.* 171: 5470–5481.
- Aujla, S. J., P. J. Dubin, and J. K. Kolls. 2007. Th17 cells and mucosal host defense. *Semin. Immunol.* 19: 377–382.
- Roark, C. L., J. D. French, M. A. Taylor, A. M. Bendele, W. K. Born, and R. L. O'Brien. 2007. Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing $\gamma\delta$ T cells. *J. Immunol.* 179: 5576–5583.
- Hamada, S., M. Umemura, T. Shiono, K. Tanaka, A. Yahagi, M. D. Begum, K. Oshiro, Y. Okamoto, H. Watanabe, K. Kawakami, et al. 2008. IL-17A produced by $\gamma\delta$ T cells plays a critical role in innate immunity against listeria monocytogenes infection in the liver. *J. Immunol.* 181: 3456–3463.

32. Maynard, C. L., L. E. Harrington, K. M. Janowski, J. R. Oliver, C. L. Zindl, A. Y. Rudensky, and C. T. Weaver. 2007. Regulatory T cells expressing interleukin 10 develop from Foxp3⁺ and Foxp3⁻ precursor cells in the absence of interleukin 10. *Nat. Immunol.* 8: 931–941.
33. Fu, Y. X., C. E. Roark, K. Kelly, D. Drevets, P. Campbell, R. O'Brien, and W. Born. 1994. Immune protection and control of inflammatory tissue necrosis by $\gamma\delta$ T cells. *J. Immunol.* 153: 3101–3115.
34. Carding, S. R., and P. J. Egan. 2000. The importance of $\gamma\delta$ T cells in the resolution of pathogen-induced inflammatory immune responses. *Immunol. Rev.* 173: 98–108.
35. Egan, P. J., and S. R. Carding. 2000. Downmodulation of the inflammatory response to bacterial infection by $\gamma\delta$ T cells cytotoxic for activated macrophages. *J. Exp. Med.* 191: 2145–2158.
36. Simonian, P. L., C. L. Roark, F. Wehrmann, A. K. Lanham, F. Diaz del Valle, W. K. Born, R. L. O'Brien, and A. P. Fontenot. 2009. Th17-polarized immune response in a murine model of hypersensitivity pneumonitis and lung fibrosis. *J. Immunol.* 182: 657–665.
37. Joshi, A. D., D. J. Fong, S. R. Oak, G. Trujillo, K. R. Flaherty, F. J. Martinez, and C. M. Hogaboam. IL-17 mediated immunopathogenesis in experimental hypersensitivity pneumonitis. *Am. J. Resp. Crit.Care Med.* In press.
38. Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* 177: 566–577.
39. Liang, S. C., X. Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L. A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* 203: 2271–2279.
40. Aujla, S. J., Y. R. Chan, M. Zheng, M. Fei, D. J. Askew, D. A. Pociask, T. A. Reinhart, F. McAllister, J. Edeal, K. Gaus, et al. 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat. Med.* 14: 275–281.
41. Andoh, A., S. Bamba, Y. Fujiyama, M. Brittan, and N. A. Wright. 2005. Colonic subepithelial myofibroblasts in mucosal inflammation and repair: contribution of bone marrow-derived stem cells to the gut regenerative response. *J. Gastroenterol.* 40: 1089–1099.