Interleukin-17–mediated Immunopathogenesis in Experimental Hypersensitivity Pneumonitis

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Rationale: T cells play a critical role in the development of Saccharopolyspora rectivirgula–induced hypersensitivity pneumonitis (HP) but little is known about the role of IL-17A in this disease.

Objectives: We examined the role of IL-17A in a murine model of S. rectivirgula antigen (SR-Ag)-induced HP.

Methods: Experimental HP was induced by oropharyngeal instillation of SR-Ag in wild-type and IL-17 gene-deficient mice.

Measurements and Main Results: SR-Ag–induced murine HP was characterized by increased transcript levels of IFN-γ and IL-12p35 compared with saline-treated control mice. Furthermore, mice with HP showed increased IL-17 in lung homogenates, bronchoalveolar lavage fluid, and ex-vivo lung cultures compared with control mice. Flow cytometric analysis of SR-Ag–challenged lungs revealed increased Th17 and CD11c+ cells. The role of IL-17 in SR-induced HP was examined in IL-17 deficient (IL-17−/−) and in wild-type (IL-17+/+) mice immunodepleted of IL-17. Histological examination of IL-17+/+ mice challenged with SR-Ag revealed reduced inflammatory cell infiltration, decreased CD11c+ cells, and reduced levels of inflammatory mediators such as IL-12p70, CCL3, and CXCL9 compared with similarly treated IL-17+/+ mice. Anti–IL-17 antibody treatment of IL-17−/− mice with HP resulted in reduced inflammation and a lower percentage of CD11c+ cells compared with IgG-treated IL-17+/+ mice with HP.

Conclusions: SR-Ag–induced IL-17 plays a pivotal role in the immunopathology of HP and targeting IL-17 is an attractive therapeutic option for this disease.

Keywords: Th17; CD11c+ cells; IL-17 knock-out mice; immunoneutralization of IL-17

Hypersensitivity pneumonitis (HP) or extrinsic allergic alveolitis is a T-cell–driven pulmonary disorder caused by repeated exposure to organic and inorganic antigens, including bacterial (for example, Mycobacterium immunogenum), fungal (for example, Trichosporon cutaneum), animal (bird feathers and droppings), or low molecular weight chemicals (for example, isocyanates) (1). Although only 5 to 15% of individuals exposed to these types of antigens develop HP, the incidence of HP is rising for reasons that are not entirely clear (2). Farmer’s lung is a type of HP resulting from the repeated inhalation of antigens from a thermophilic actinomycete Saccharopolyspora rectivirgula, which grows in hot and humid conditions on hay. Farmer’s lung can present itself in three clinical forms: acute, subacute, and chronic with the chronic form of HP often progressing toward a fatal end-stage fibrotic disease (3). Diagnosis of HP remains clinically challenging because of the wide variety of causative agents, frequency of exposure, and a variable clinical course associated with this disease (1).

The most commonly used experimental model for HP involves the repeated intratracheal or intranasal administration of S. rectivirgula antigen (SR-Ag) into C57Bl/6 mice. Bronchoalveolar lavage fluid (BALF) from SR-Ag–challenged mice reveals copius quantities of neutrophils. At more distal times, the inflammatory response in this model is characterized by lymphocytic peribronchial and perivascular granulomatous lesions and diffuse interstitial mononuclear infiltrates (4). Both CD4+ and CD8+ T cells have been shown to be recruited to the lungs of mice exposed to SR-Ag (5, 6), but the exact mechanism through which innate (i.e., neutrophils) and adaptive immune cells (i.e., lymphocytes) are activated and recruited during HP has yet to be elucidated. It is known that neutrophils and T cells produce IFN-γ, a cytokine important for development of HP (7). Several groups have reported evidence for a critical role of Th1 immune response in development of HP. Th1 mediators such as IFN-γ, IL-12, and CCL3 have been shown to be present in lungs of mice challenged with SR-Ag (8, 9) and IFN-γ gene–deficient mice are protected from development of HP (10). Adoptively transferred, sensitized CD4+ Th1 cells can cause HP in healthy animals (11). Finally, mice overexpressing GATA-3, a transcription factor required for Th2 differentiation, are protected from HP because of the suppression of Th1 responses (11).

Recent clinical analysis suggests that in addition to Th1 factors, IL-17 and IL-17–associated transcripts are increased in clinical HP, but little is known about the role of this cytokine in disease progression (12). In this study, we show that IL-17 A (here on referred to as IL-17) is generated during SR-Ag–induced murine HP. A higher percentage of Th17 cells was present in the lungs of mice exposed to SR-Ag compared with saline-challenged mice, indicating that one source of IL-17 in this disease is Th17 cells. Genetic deletion of IL-17 or antibody-mediated immunodepletion resulted in decreased cell infiltration, lower production of chemokines and cytokines in the lungs of SR-Ag challenged...
mice, and protection against the disease. Together, our data suggest that IL-17 is of critical importance to the development and severity of HP.

METHODS

Mice

Six to 8-week-old C57BL/6 mice were bought from Taconic Farms, Inc. (Hudson, NY) or Jackson Laboratories (Bar Harbor, ME). C57BL/6 mice were used in this study because their Th1 bias makes them more susceptible to HP than DBA/2 mice, which have a Th2 bias (8). IL-17−/− mice were generated as described in detail by Nakae and colleagues (13) and were a kind gift from Dr. Harry T. Mobley (University of Michigan Medical School). Mice were housed under specific pathogen free (SPF) conditions in the University Laboratory Animal Medicine (ULAM) facility at the University of Michigan Medical School. All experiments were approved by the Animal Care Committee at the University of Michigan.

S. rectivirgula–induced HP Model

S. rectivirgula (ATCC number 29,034) was purchased from American Type Culture Collection (Manassas, VA). To prepare S. rectivirgula Ag (SR-Ag), bacteria were grown in Trypticase Soy Broth at 55°C for 4 days in a shaker. The bacterial cells were then spun down, and the resulting pellet was washed with endotoxin-free water three times. Next, bacterial cells were lysed by repeated freeze-thaw and then lyophilized. The lyophilized pellet (SR-Ag) was reconstituted in saline at 5 mg/ml and stored at −80°C until further use. Oropharyngeal aspiration into lungs, as described by Lakatos and colleagues (14), was used to instill 20 µg of SR-Ag per mouse for 3 consecutive days per week for 3 weeks.

To neutralize IL-17, 250 µg of rabbit anti-IL-17 polyclonal antibody (kind gift of Pam Lincoln and Dr. Steven L. Kunkel, University of Michigan) was injected intraperitoneally into each mouse every other day starting in the second week of SR-Ag exposure. A total of five anti-IL-17 or control IgG injections were administered per mouse. The timeline for SR-Ag and anti-IL-17 administration is shown in Figure 1.

Real-time Quantitative Polymerase Chain Reaction Analysis and Detection of Soluble Cytokine and Chemokine Levels

At Days 1, 4, 8, and 16 after the last challenge, lung lobes were excised, flash frozen in liquid nitrogen, and stored at −80°C. For isolation of RNA, one right lung lobe was thawed on ice. TRIzol (Invitrogen Life Technologies, Carlsbad, CA) was used for RNA extraction according to manufacturer’s instructions. cDNA was converted into cDNA using Murine Moloney Leukemia Virus Reverse Transcriptase (Invitrogen, Life Technologies) and then analyzed by quantitative polymerase chain reaction analysis using the ABI PRISM 7700 detection system (Applied Biosystems, Foster City, CA). Pre-mixed primer/probe reagents were bought from Applied Biosystems to detect IFN-γ. IL-4, IL-5, IL-13, IL-17, and IFN-706 AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE VOL 179 2009 Basel, Switzerland protease inhibitor and 0.1% Triton-100). Fifty frozen lung lobe from each mouse was thawed on ice and homogenized in 100 µl of TRIzol, then stored at −80°C until further analysis by mutiplex immuno-bead assay.

Statistical Analysis

Three to five mice were used per group per time-point in each experiment. Student’s t test or one-way analysis of variance and Student’s Newman-Keuls post test were used to determine statistical significance. P < 0.05 were considered statistically significant.

RESULTS

Cytokine Transcript Expression Analysis of SR-Ag–Challenged Lungs

A recent microarray analysis of surgical lung biopsies from patients with HP revealed a number of transcripts indicative of altered Th17 activity in this disease (12). To determine whether similar changes characterized experimental HP, we analyzed the expression of key Th1, Th2, and Th17 cytokine genes at 1, 4, and 8 days after the final SR-Ag challenge in lungs of mice (see Figure 1 for scheme of model). As shown in Figure 2, transcript

Figure 1. Schematic representation of a timeline for oropharyngeal instillation of S. rectivirgula antigen (SR-Ag) and intraperitoneal administration of anti-IL-17 antibody. Mice were challenged with 20 µg SR-Ag per mouse for 3 consecutive days per week for 3 weeks. In some experiments, 250 µg of anti-IL-17 antibody was injected intraperitoneally every other day starting at the second week of SR-Ag exposure. Bronchoalveolar lavage (BAL), lungs, and lymph nodes, were harvested at Days 1, 4, and 8 after the last SR-Ag challenge.
expression of Th1 cytokines, IFN-γ (15) and IL-12p35 (8) was up-regulated in SR-Ag–challenged mice compared with saline-challenged mice at 1 and 4 days after the last exposure. Interestingly, we found Th2 cytokines were also up-regulated in mice treated with SR-Ag. These mice showed a tenfold higher IL-4 and IL-10, at Day 1 after the last challenge compared with saline-challenged mice. IL-4 expression in SR-Ag-instilled mice decreased after Day 1 whereas IL-10 expression remained unchanged. To determine whether Th17 cells contributed to the HP pathology, we analyzed expression of IL-21, a cytokine...
involved in differentiation toward Th17 cells (16–18). SR-challenged mice showed increased levels of IL-21 transcripts at all timepoints examined compared with saline-challenged mice. IL-23 plays a critical role in expansion and maintenance of Th17 cells (19, 20). As shown in Figure 2, IL-23 p19 expression was increased in lungs of mice at Day 4 and Day 8 after the last SR-Ag instillation compared with saline-challenged mice. Consistent with IL-21 and IL-23 p19 gene expression analysis, IL-17 transcript levels were elevated in lungs of SR-Ag–challenged mice at Days 1, 4, and 8 compared with saline-challenged mice (Figure 2). In summary, these data suggest that, similar to previous clinical observations, transcript levels of IL-17 and Th17-associated cytokines were upregulated in experimental SR-Ag–induced HP.

After the initial neutrophil infiltration in HP lungs, it is likely that antigen-presentation cells process antigens and direct the T-cell differentiation toward Th17 by production of mediators such as IL-23. Data presented here shows up-regulation of IL-23 p19 in whole lung samples and bone marrow–derived dendritic cells (BMDCs) (Figure 2 and Figure E1 in the online supplement). Moreover, transcripts of toll-like receptor 2 (TLR2), toll-like receptor 6 (TLR6), and dectin-1 were up-regulated in lungs of mice challenged with SR-Ag compared with control mice (Figure E2). TLR2, TLR6, and dectin-1 are pathogen recognition receptors that sense pathogen-associated molecular patterns and play a pivotal role in innate and adaptive immune response. Signaling via dectin-1 and TLR2 results in the up-regulation of IL-23 (21, 22). Thus, a model can be proposed in which antigen-presenting cells recognize SR-Ag via pathogen recognition receptors such as dectin-1 and TLR2, up-regulate IL-23, and, in presence of other signals, direct a Th17 response.

### Soluble Cytokine Levels in Lungs of SR-Ag–Challenged Mice

Next we analyzed lung homogenates by multiplex immuno-bead assay for the presence of soluble cytokines in the lungs of saline-instilled and SR-Ag–instilled mice. As shown in Figure 3A,
protein levels of IFN-γ, IL-12p70, and IL-13 showed a noticeable trend toward increased levels over time that did not reach statistical significance and were similar in saline- and SR-Ag–challenged mice at Days 1, 4, and 8 after last SR-Ag challenge. One explanation for the failure to detect higher levels of IFN-γ and IL-12 in SR-Ag–challenged mice could be that the peak of protein production for these cytokines occurred on days not analyzed in this study. In contrast, levels of IL-4 and IL-5 were significantly higher in SR-Ag–challenged mice compared with saline-challenged mice at 1 day after the last exposure. In line with the gene expression data, IL-17 was significantly elevated in lungs of SR-Ag–challenged mice compared with saline-challenged mice at 1 and 4 days after the last challenge. Furthermore, bronchoalveolar lavage (BAL) collected 1 day after the last challenge contained significantly higher IL-5 and IL-17 levels compared with saline-challenged mice (Figure 3B). Analysis of soluble cytokine levels at 16 days after the last challenge revealed that IL-17 remained significantly elevated in SR-Ag–challenged mice compared with saline-challenged mice (Figure E3). In summary, the gene and protein expression data demonstrate that HP involves an increased and sustained Th17 immune response.

**Antigen Restimulation in Ex Vivo Lung Cultures**

To specifically study antigen recall response by lung cells, we cultured dispersed lung cells in the presence of SR-Ag for 48 hours and then analyzed protein levels in cell-free culture supernatants. We focused on lung cells at 4 days after the last exposure because it was at this time-point that we consistently observed maximal histologically apparent inflammation. Dispersed lung cell cultures from SR-Ag–challenged mice contained significantly higher levels of IL-13 and IL-5 when compared with saline controls (Figure 4, open bars). Restimulation in vitro with SR-Ag promoted the release of IFN-γ, IL-4, IL-13, IL-5, and IL-17, and levels of these cytokines were significantly increased compared with cultures of lung cells derived from SR-Ag–challenged mice which were not restimulated. Antigen challenge of lung cells derived from saline-challenged mice did not promote cytokine release at levels different from cells cultured in media alone. These data show that effector T cells from lungs of mice undergoing HP release high levels of Th1, Th2 and Th17 cytokines in an antigen-dependent manner.

**Histopathological Analysis of Lungs**

The histopathology in SR-Ag–challenged lungs is shown in Figure 5. Hematoxylin-eosin–stained histological sections of lungs obtained at 4 days after the last SR-Ag challenge were characterized by the presence of extensive mononuclear cell infiltration around airways (Figure 5B) and the vasculature (Figure 5D) in contrast to the airways (Figure 5A) and
vasculature (Figure 5C) in saline-challenged mice. Next, we analyzed the phenotype of immune cells present in lungs and lymph nodes of saline- and SR-Ag–challenged mice by flow cytometry. Several studies have reported an increased CD4\(^+\):CD8\(^+\) ratio (23, 24) in SR-Ag–induced HP. Consistent with these previous reports, we observed that SR-Ag–challenged mice had a significantly higher CD4:CD8 ratio (Figure 6A). To examine antigen presenting cells such as dendritic cells and macrophages, dispersed lung cells were stained with fluorophore-conjugated anti-CD11b and anti-CD11c. As shown in Figure 6B, SR-Ag–challenged mice had significantly increased CD11b\(^+\) and CD11c\(^+\) cells compared with saline-challenged mice. Because gene and protein analysis of whole-lung homogenate and ex vivo lung cultures demonstrated the enhanced presence of IL-17, we analyzed whether Th17 cell numbers were present in the lungs of SR-Ag–challenged mice. Flow cytometric analysis of CD4\(^+\) cells revealed a relatively small but significantly higher population of IL-17\(^+\) cells in SR-Ag–challenged mice compared with saline-challenged mice (Figure 7A). Interestingly, when mediastinal lymph nodes were analyzed, SR-Ag–challenged mice showed lower percentage of Th17 cells compared with saline-challenged mice (Figure 7B). This decrease might reflect the recruitment of Th17 cells into the lungs from lymph nodes during SR-Ag–driven inflammation. We next tested whether stimulation of lung cells with SR-Ag, anti-CD3, anti-CD28, PMA, and ionomycin result in an increase in Th17 cell numbers in vitro. As shown in Figure 7C, this stimulation increased the percentage of Th17 cells but not of CD8\(^+\) IL-17\(^+\) cells. Taken together, our data suggest that repeated exposure to SR-Ag results in increased recruitment of antigen-presenting cells such as dendritic cells and macrophages. Furthermore, Th17 cells are more abundant and readily activated in the lungs of mice undergoing SR-Ag-induced HP.

Deletion of IL-17 Gene Results in Decreased Inflammation

To directly examine the role of IL-17 in development of immunopathology during SR-Ag–induced HP, IL17\(^{-/-}\) mice were analyzed in our experimental HP model. Histological examination of lung sections obtained 4 days after the last challenge revealed reduced inflammation in the IL17\(^{-/-}\) group compared with IL17\(^{+/+}\) group (Figures 8A, 8B, and Table E1). Dampened inflammation in IL17\(^{-/-}\) mice was also reflected in reduced cell counts from lung, LN and BAL of the gene-deficient mice compared with their wild type counterparts (Figures 8C, 8D, and 8E). Although there was no difference in the CD4\(^+\):CD8\(^+\) ratio between IL-17\(^{+/+}\) and IL-17\(^{-/-}\) mice (Figure 9A), there was a significantly lower percentage of Cd11c\(^+\) cells in IL-17\(^{-/-}\) lung samples compared with IL-17\(^{+/+}\) lung samples (Figure 9B). Analysis of soluble protein levels in the lungs of IL17\(^{+/+}\) and IL17\(^{-/-}\) mice via Bioplex revealed that IL-12, CCL3, and CCL4 were significantly lower in IL17\(^{-/-}\) mice compared with IL17\(^{+/+}\) mice (Figure 9C).
CXCL9 were significantly lower in these mice compared with IL-17+/− mice (Figure 9C). Notably, IL-10 was not detected in IL-17−/− mice. In summary, IL-17 gene-deficient mice revealed that IL-17 plays a critical role in the immune response against SR-Ag.

Use of IL-17 Antibody as a Therapeutic Agent in SR-Ag–induced HP

Immunoneutralization of IL-17 has effectively attenuated disease in several experimental models of disease, including allergen-induced contact hypersensitivity responses and TNF-induced lethal shock (25, 26). An antibody directed against IL-17 was assessed for its effectiveness in protection against SR-Ag–induced HP. Mice either received five doses of purified anti–IL-17 antibody or control IgG antibody after the initial SR-Ag stimulation and challenge (Figure 1). Histological examination of lungs 4 days after the last challenge showed a decreased inflammatory response in mice that received the anti–IL-17 antibody (Figures 10A–10D, Table E2). To verify the efficacy of the antibody in neutralizing IL-17, we measured IL-17 protein levels in lungs of mice. As shown in Figure 10E, the antibody therapy significantly decreased IL-17 levels in SR-Ag–challenged mice compared with mice that received the IgG. Flow cytometric analysis revealed that neutralizing IL-17 did not alter the CD4:CD8 ratio (Figure 10F) but this antibody therapy did significantly reduce the percentage of CD11c+ cells in SR-Ag–challenged mice (Figure 10G). In conclusion, therapeutically targeting IL-17 via a neutralizing antibody was effective in decreasing disease severity in SR-Ag–induced HP.

Effect of IL-17 Deficiency on IFN-γ Protein Expression

An intriguing question raised by our study is whether the role of IL-17 in murine HP is independent of IFN-γ. Khader and

Figure 7. Th17 cells in lung and lymph nodes 4 days after the last antigen challenge. (A) Dispersed lung and (B) lymph node cells were cultured for 4 hours in the presence of GolgiPlug. The cells were stained with anti-CD4 antibodies and then permeabilized to stain intracellular IL-17. Dot plot shows side scatter (SSLog) and IL-17 positive staining cells in the lymphocyte gate that were further gated for CD4 positive staining. Bar graphs show percentage of IL-17+ cells in CD4 gate of 50,000 cells in (E) lung and (F) lymph node cells. Solid bars represent SR-Ag; open bars represent saline. (C) Flow cytometric analysis of stimulated lung cells shows increased Th17 cells in SR-Ag challenged mice. Dot plot shows CD4 and IL-17 staining of cells gated on lymphocyte gate from a total of 10,000 cells. (f) Bar graph shows percentage of CD4+IL-17+ and CD8+IL17+ cells in the lymphocyte gate. Data shown are mean ± SE from experiment using three to five mice per group. Student’s t test was used to determine significances; **P < 0.01.
colleagues have demonstrated that the depletion of IL-17 in mice vaccinated with a peptide derived from Mycobacterium tuberculosis protein and then challenged with the same peptide results in decreased IFN-γ-producing cells suggesting that IL-17 is critical for protective immunity via IFN-γ (27). We analyzed IFN-γ levels in lungs of mice by multiplex immuno-bead assay and observed that IL-17−/− mice exhibited higher levels of IFN-γ in whole lung homogenates compared with IL-17+/+ mice (Figure 11A). The immuno-neutralization of IL-17 in wild-type mice with HP resulted in actually modestly higher levels of IFN-γ in lungs of mice compared with mice that received control antibody (Figure 11B). Together, these data demonstrate that in the context of SR-Ag–induced HP, IL-17 is not required for IFN-γ expression. Also, our study suggests that the protective effect observed following IL-17 deletion or immuno-neutralization was independent of IFN-γ, but further studies are required to address this novel hypothesis.

DISCUSSION

Hypersensitivity pneumonitis is a T-cell–mediated alveolitis with a marked Th1 response. Repeated inhalation of large amounts of antigen leads to nonnecrotizing lymphocytic granulomas around the airways and vasculature. Several studies have pointed to a major role for T cells in the pathology of HP, but little is known about the precise nature of the T-cell response. Previous studies have shown that lymphocytes in the BAL of patients with acute HP are of a Th1 phenotype, but how these cells contribute to the course of HP is poorly understood (28). A recent microarray analysis of biopsies from patients with HP suggested that IL-17 and Th17 cells might have a role in HP. Selman and colleagues studied gene expression in lungs of patients with HP and found that genes involved in TH17 differentiation, such as IL-17 receptor C, IL-6 receptor, and IL-21 receptor, were up-regulated (12). In another
study, BAL from patients with HP showed significantly higher IL-6 protein levels compared with normal BAL (29). From the present study it was apparent that IL-17 is prominently expressed in experimental SR-Ag–induced HP. Importantly, the targeting of IL-17 provided a major therapeutic effect.

Previous studies on HP have reported severe disease progression associated with Th1 phenotype and disease attenuation upon Th2 skewing. Results presented in this study demonstrate that, in addition to Th1, mediators of Th2 responses are highly expressed in the lungs of C57Bl/6 mice with HP. Our data is consistent with another study in which it was shown that IL4 and IL-13 are expressed by splenocytes obtained from mice undergoing HP, and IL-13 transcripts are increased in whole lung samples (30). Our data showed that transcripts of IL-4 and IL-10 were up-regulated in lung samples from mice challenged with SR-Ag. In addition, significantly higher amounts of IL-4 and IL-5 protein were produced in lungs from mice exposed to SR-Ag compared with saline-challenged mice. Moreover, IL-5 was also detected in BALF of mice exposed to SR-Ag. A study reporting phenotypic analysis of T cells from BALF showed Th2 skewing in patients with chronic HP but not in patients with subacute HP (31). It is possible that the unregulated expression of Th2 mediators might lead to fibrotic response during chronic HP. For example, IL-4 is a chemotactic factor for fibroblasts and induces fibroblast proliferation. In addition, IL-4 induces production of profibrotic mediators such as TGF-β, MCP-1, and

Figure 9. (A) CD4:CD8 ratio at Day 4 after the last challenge obtained from IL-17+/+ (open bars) and IL-17−/− mice (solid bars) determined by flow cytometric analysis of dispensed lung cells. (B) Presence of CD11c+ cells is significantly reduced in IL-17−/− mice (solid bars) challenged with S. rectirigula antigen (SR-Ag) compared with similarly treated IL-17+/+ mice (solid bars). Data are shown as mean ± SE, with n = 3–5 for each group. Student’s t test was used to analyze significance. Significant differences are shown as *P < 0.05, **P < 0.01. (C) Protein expression data from whole lung samples of SR-Ag or saline-exposed mice 4 days after the last challenge. Fifty µl of cell-free homogenate was analyzed by multiplex immuno-bead assay. Open bars = IL-17+/+ mice; solid bars = IL-17−/− mice. Data are shown as mean ± SE, with n = 3–5 for each group. Student’s t test was used to analyze significance. Significant differences are shown as *P < 0.05, **P < 0.01.
Figure 10. H&E staining of lung sections at Day 4 after the last exposure from mice challenged with (A, C) S. rectirugula antigen (SR-Ag) + IgG and (B, D) SR-Ag + anti-IL-17. Reduced inflammation can be seen in anti-IL-17 injected mice at 100× and 200× magnification. (E) Efficacy of immunoneutralization was tested by multiplex immuno-bead assay for IL-17. (F) Flow cytometric analysis of dispersed lung cells shows anti-IL-17 did not alter CD4:CD8 ratio in lungs of mice 4 days after the last challenge. (G) Anti-17 antibody decreased the percentage of Cd11c+ cells in the antigen presenting cells APC gate. Data are shown as mean ± SE, with n = 3–5 for each group. One-way analysis of variance followed by Student’s Newman-Keuls post-test was used to determine significance. Significant differences are shown as *P < 0.05, **P < 0.01.
C10. Extracellular matrix proteins such as collagen, fibronectin, and tenasin are also induced by IL-4 (32). IL-5 has been shown to have a major role in lung remodeling (33). As shown in Figure E4, we analyzed lung fibroblasts for their expression of IL-5. Fibroblasts were grown from lungs of mice at Day 16 after the last exposure, replated in triplicate wells, and challenged with TLR2 agonist, Pam3cys, to model restimulation of TLR2. Fibroblasts derived from lungs of SR-Ag–challenged mice showed significantly elevated IL-5 transcript and protein expression compared with saline-challenged mice. It is currently unknown why some patients with chronic HP progress toward extensive fibrosis, fail to respond to steroid treatment, and have poor prognosis. The factors that contribute to this excessive Th2 response are the focus of investigation in several laboratories including our own.

IL-17 has multiple effects on immune and nonimmune cells (34). It up-regulates the expression of proinflammatory cytokines and chemokines such as IL-6, IL-8, TNF-α, CXCL9, and CXCL10. IL-17 has been shown to be involved in activation and expansion of neutrophils. Furthermore, IL-17 induces the expression of acute phase response genes and antimicrobial genes. To our knowledge, this is the first demonstration of the role of IL-17 in a SR-Ag–induced model of HP. In the present study, IL-17 was detected in lung homogenates, BALF, and ex vivo lung cultures from mice undergoing HP. Previous studies suggested that IL-17 might have an important role in patients with HP, but the contribution of IL-17 was unknown (12). Flow cytometric analyses presented herein indicate that Th17 cells are responsible for some of the IL-17 measured in HP samples. The lower percentage of Th17 cells suggests that an alternative source of IL-17 contributes to the overall IL-17 levels. γδ T cells, CD8+ T cells, neutrophils, natural killer cells, and macrophages have been reported to express IL-17 (35, 36). However, when we analyzed CD8+ T cells, we did not find a significant population of these cells that express IL-17. γδ T cells are critical innate immunity cells capable of rapidly releasing IL-17 in response to pathogens. Recent studies highlight the importance of γδ T cells as producers of IL-17 during Escherichia coli, Mycobacterium tuberculosis, and Listeria monocytogenes infection (37–39). Whether IL-17 produced during HP is released by γδ T cells, natural killer T cells, or macrophages, in addition to TH17 cells, remains to be elucidated (40–42).

Several studies have reported a higher ratio of CD4/CD8 cells in HP lungs. More recently, it was shown that patients undergoing subacute HP had a lower CD4/CD8 ratio whereas, patients with chronic HP had an increased CD4/CD8 ratio (31). In the present study, the CD4/CD8 ratio did not reflect disease severity. In IL-17+/−, IL-17−/− and anti-IL-17–treated wild-type mice, the ratio remained unchanged, although disease severity varied significantly. More consistent with disease progression was cell surface marker CD11c. Our data showed that in the absence of IL-17, SR-Ag–challenged mice had fewer CD11c+ cells. CD11c is generally expressed by pulmonary macrophages and dendritic cells (43). The reduction in CD11c cells might be explained by our finding that the lungs of SR-Ag–challenged IL-17−/− mice contained significantly less CCL3, a chemokine necessary for macrophage/dendritic cell recruitment (44, 45). Histological examination and cell counts in lung, lymph node, and BALF revealed that IL17−/− had reduced inflammation, which could indirectly affect monocyte and dendritic cell infiltration into lungs. Evidence for a direct effect of IL-17 on dendritic cell maturation comes from experiments in which bone marrow–derived dendritic progenitors were grown in the presence of IL-17, which induced phenotypic and functional maturation of dendritic cells (46). Therefore, a more direct effect of IL-17 on CD11c+ cells cannot be ruled out and further investigation of this finding is warranted.

The major finding from the present study was derived from studies directed at the deletion of or neutralization of IL-17 during experimental HP. IL-17 was found to exert a clear pathogenic role in HP disease progression. Although initial IL-17 production might be necessary for neutrophil activation and expansion, persistence of IL-17 might have deleterious effects and lead to chronicity. In the present study, we effectively neutralized IL-17 by using a polyclonal antibody against IL-17. Mice that were exposed to SR-Ag and received anti-IL-17 were protected from HP. These findings concur with other studies in which Th1-associated diseases such as psoriasis, ozone exposure, or pneumococcal colonization were markedly attenuated upon diminishing IL-17 activity (47). These data underscore the important role of IL-17 during HP progression and suggest a therapy for relief from this disease. Further studies are needed to examine the role of IL-17 in the various stages of HP and the effect of IL-17 neutralization during these stages.

**Conflict of Interest Statement:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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