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TLR9-Dependent IL-23/IL-17 Is Required for the Generation of *Stachybotrys chartarum*-Induced Hypersensitivity Pneumonitis

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Hypersensitivity pneumonitis (HP) is an inflammatory lung disease that develops following repeated exposure to inhaled particulate Ag. *Stachybotrys chartarum* is a dimorphic fungus that has been implicated in a number of respiratory illnesses, including HP. In this study, we have developed a murine model of *S. chartarum*-induced HP that reproduces pathology observed in human HP, and we have hypothesized that TLR9-mediated IL-23 and IL-17 responses are required for the development and progression of HP (8, 9). For example, athymic nude mice that lack T cells are protected from the development of HP in response to inhaled antigenic challenge. In rodent models of HP, there is a shift in the T cell population to CD8+ predominance by 24–48 h after challenge with Ag (9–11). In humans with HP, there is an increase in the number of both CD4+ and CD8+ T cells in bronchoalveolar lavage fluid, lung interstitium, or both (12–15). Adoptively transferred, sensitized CD4+ Th1 cells can cause HP in healthy animals (10). Mechanisms that promote T cell accumulation or activation have not been thoroughly defined.

TLRs are a family of type I transmembrane receptors that respond to pathogen-associated molecular patterns expressed by a diverse group of infectious microorganisms, resulting in the activation of the host’s immune system (16–19). The role of TLRs in the generation of HP is incompletely defined. Given the composition of fungal cell wall components (20, 21), the most relevant TLRs are TLR4, which binds to and is activated by LPS and fungal mannans, and TLR2, which recognizes fungal β-glycans and zymosan (22, 23). TLR9 is of particular interest because it recognizes fungal DNA, and TLR9 has been shown to drive type 1 responses to both microbial and nonmicrobial Ags (24).

Historically, granulomatous tissue responses that were once believed to be solely Th1 mediated have more recently been shown to require IL-17 (25–27). Accordingly, several groups have shown that the gene deletion or in vivo neutralization of IL-17 in an experimental model of HP driven by repeated *Saccharopolyspora rectivirga*-Ag challenges results in protection from HP, indicating that IL-17 and Th17 cells are major driving factors (28, 29). IL-17 drives a proinflammatory immune response by inducing chemokine and chemoattractant production from resident immune and stromal cells (30, 31). Subsequently, neutrophils and other immune cells are recruited, thereby intensifying the inflammatory response (32). Th17...
differentiation requires the presence of IL-6 and TGF-β, whereas expansion and growth of Th17 cells is regulated by IL-23 (33, 34). The exact signaling mechanisms that lead to Th17 differentiation during immune responses, such as HP, are unclear.

In our recent study, we found that TLR9 was necessary for the generation of HP in a murine model using sensitization and challenge in response to S. chartarum. In this study, we investigated the novel role of TLR9 in driving IL-17 responses in experimental HP using a murine model, which involves repeated i.p. sensitization and intratracheal (i.t.) challenge of mice with the S. chartarum. Our study indicates that IL-17 is an important cytokine mediator of S. chartarum-induced HP, and that the production of IL-17 in this model requires TLR9-dependent IL-23 expression from DCs.

Materials and Methods

Reagents

Murine recombinant cytokines were purchased from R&D Systems (Minneapolis, MN). Polyclonal antimurine cytokine antiserum used in ELISA or neutralization experiments were produced by immunization of rabbits with recombinant murine cytokines in multiple intradermal sites with complete Freund adjuvant. ELISA for quantization of murine IFN-γ was purchased from PBL Biomedical Laboratories.

Animals

Specific pathogen-free (SPF) BALB/c mice were purchased from Jackson Laboratories. Breeding pairs of TLR9−/− mice generated by S. Akira (Osaka University, Osaka, Japan) were obtained from Celye Pharmaceutical Group, and a colony was established at the University of Michigan (Ann Arbor, MI). These mice were generated on a BALB/c background (more than five backcrosses), are phenotypically normal in the unaffected state, and reproduce without difficulty. All mouse strains were housed in SPF conditions within the animal care facility (Unit for Laboratory Animal Medicine) until the day of sacrifice.

Intratracheal administration of S. chartarum conidia

Mice were injected with ketamine and xylazine i.p. After adequate anesthesia, mice were restrained in the supine position. The anterior aspect of the neck was exposed, and an incision was made to visualize the trachea. A 26-gauge needle was advanced endotracheally, and 30 μl normal saline containing S. chartarum conidia was injected. The animals were then allowed to recover, and the wound was closed with surgical staples. No adverse effects were encountered with this procedure.

Intranasal administration of S. chartarum conidia

Animals were lightly anesthetized with ketamine and xylazine. After adequate anesthesia, 10–20 μl of a solution containing S. chartarum conidia was placed in the nares of mice until the solution was inhaled.

Intraperitoneal administration of S. chartarum conidia

Mice were injected i.p. with 50 μl of a solution containing S. chartarum conidia.

IL-23 reconstitution

Murine recombinant IL-23 was purchased from R&D Systems (Minneapolis, MN). Mice were anesthetized, and 2 μg rmIL-23 was administered i.t. at the same time as i.t. challenge with S. chartarum spore.

Removal of various organs at the time of necropsy

Mice were euthanized at various intervals after S. chartarum sensitization and challenge by inhalation of carbon dioxide. Mice were then exsanguinated and the organs (e.g., lungs and spleens) were removed.

Splenocyte isolation

Splenic single-cell suspensions were prepared by homogenization through a 70-μm cell strainer (BD Biosciences). Splenic CD4+ and CD8+ T lymphocytes were positively selected using anti-mouse–conjugated MidiMACS beads, according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA).

Total lung leukocyte preparation

Lungs were removed from euthanized animals, and leukocytes were prepared as previously described. Lungs were minced with scissors to a fine slurry in 15 ml lung digestion buffer (RPMI 1640, 5% FCS, 1 ng/ml collagenase [Boehringer Mannheim Biochemical], 30 μg/ml DNAase (Sigma, St. Louis, MO). Lung slurries were enzymatically digested for 30 min at 37°C. The total lung cell suspension was pelleted, resuspended, and spun through a 20% Percoll gradient to enrich for leukocytes prior to further analysis.

Multiparameter flow cytometric analyses

Total lung leukocytes were isolated as described above. Lymphocyte subsets were analyzed by first gating on CD45 positive “lymphocyte sized” leukocytes, and then examined for lymphocyte-associated markers. Abs (PharMingen and Caltag) used for phenotyping include T cell markers (anti-CD4, anti-CD8, anti-CD69, B cells [anti-CD19], pan-NK cells [anti-DX5] yô T cells [anti-yô TCR]). Cells were collected on a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) using Cellquest software (Becton Dickinson). Analyses of data were performed using the Cellquest software package.

Cytokine ELISA

Lung homogenate, DC supernatants, or DC-splenocyte coculture supernatants were collected, and cytokine levels were determined by a sandwich ELISA method as described previously (35).

Generation of bone marrow–derived DCs

Bone marrow was harvested from wild type (WT) and mutant mice and was seeded in tissue culture flasks in RPMI 1640 based complete media with 10 ng/ml murine rGM-CSF. Loosely adherent cells collected after 6–7 d and incubated with anti-CD11c Ab coupled to magnetic beads (Miltenyi Biotech). Cells were purified using positive selection for CD11c+ cells by using the cell suspension through a magnetic column. CD11c+ DCs were plated overnight and resuspended in fresh media the following day.

Histology

Lungs were harvested 3 d after stimulation and challenge with S. chartarum and were inflated and fixed in 10% formalin. The fixed lung lobe was embedded in paraffin; 5-μm sections were then stained with H&E. Images were captured using Olympus BX40 microscope and IP Lab Spectrum software (Signal Analytics, Vienna, VA).

mRNA extraction and real-time (TaqMan) quantitative PCR

Total RNA from cells was isolated per the manufacturer’s protocol for the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA amounts were determined by spectrometric analysis at 260 nm. All primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). Levels of mRNA were determined by real-time quantitative RT-PCR analysis using an ABI PRISM 7700 Sequence Detection System (ABI/Perkin Elmer, Foster City, CA).

Retroviral transduction of DCs

Expression vectors in pGFP-V-RS plasmid producing short-hairpin RNA (shRNA) against murine IL-23 mRNA were purchased from Origener Technologies. All shRNA constructs were prepared in the murine stem cell virus–based pLMP retroviral vectors. Supernatants from transfected cells were collected at 48 h after transfection. Supernatants were added to DCs for stable knockdown of IL-23 verified by real-time PCR. The sequences of the shRNA that are generated from these vectors are as follows (only the sense strands are shown): sh-miL-23, 5’-CAGAGCAGTAATAATGCTA-3’; Transfected cells were seeded at 1 × 106 × 12-well plates. Cells were stimulated and lysed for mRNA extraction as described.

Statistical analyses

Statistical significance was determined using the unpaired, two-tailed Student t test and nonparametric Mann–Whitney U test. Calculations were performed using InStat for Macintosh (GraphPad Software, San Diego, CA). In all cases, a p value < 0.05 was considered significant.

Results

Increased IL-17 mRNA and protein levels in whole lung

We have developed a murine model of HP using the fungus S. chartarum, which has been associated with a variety of respiratory diseases, including sick building syndrome and black mold disease. This model is particularly attractive because S. chartarum is a clinically relevant cause of lung disease and is relatively
understudied. In this model, BALB/c mice (WT) were sensitized with i.p. injections of $10^6$ S. chartarum spores on days $-14$ and $-7$, and then administered $10^6$ fungal spores i.t., then lungs removed at day 2 and day 5. As we have shown previously, WT mice had increased mononuclear cell infiltrates that occurred in a peribronchial distribution (Fig. 1A, arrow), with the presence of giant cells and evidence of loose granuloma formation. By comparison, reduced peribronchial inflammation was observed in sensitized TLR9$^{-/-}$ mice challenged i.t. with S. chartarum, as well as a reduction in the total number of CD4$^+$ (Fig. 1B) and CD8$^+$ T (Fig. 1C) cells in mutant mice. Moreover, S. chartarum sensitization and challenge in WT mice resulted in a substantial increase in the percentage of CD4$^+$ T cells expressing the activation marker CD69, whereas this increase in CD69 expression was blunted in CD4$^+$ T cells from TLR9$^{-/-}$ mice (Fig. 1D, 1E). There were no differences in number of CD69 expression in CD8$^+$ T cells between the two groups (data not shown).

IL-17 has previously been causally linked to hypersensitivity responses in a murine S. rectivirgula–Ag-induced HP model (29). To determine whether protection against the development of HP in TLR9$^{-/-}$ mice was attributable to differences in the expression of IL-17, WT and TLR9$^{-/-}$ mice were sensitized and i.t. challenged with S. chartarum. Next, IL-17 mRNA and protein levels measured in lung homogenates. In WT animals, sensitization and subsequent challenge with S. chartarum induced a vigorous time-dependent expression of IL-17 mRNA (Fig. 2A) and protein (Fig. 2B) in whole lung. By comparison, mRNA IL-17 mRNA and protein levels were substantially diminished in the lungs of TLR9-deficient mice after sensitization and challenge with S. chartarum (37 and 48% decrease, respectively).

Immunoneutralization of IL-17 has effectively attenuated disease in several experimental models of disease, including allergen-induced contact hypersensitivity responses and S. rectivirgula–Ag-induced HP (31). To establish that IL-17 contributed meaningfully to S. chartarum–induced HP, WT mice were administered purified polyclonal rabbit anti-mouse anti-IL-17 Ab (100 μg) or control IgG 3 d prior to and the day of i.t. challenge with S. chartarum. Histologic examination of lungs 3 d after sensitization and S. chartarum challenge revealed a decreased inflammatory response in mice that received anti-IL-17 Ab (Fig. 2C) and reduced CD4$^+$ T but not CD8$^+$ T cell accumulation, as determined by flow cytometry (Fig. 2D, 2E). Moreover, there was a substantial decrease in the number of activated CD4$^+$ T cells, as measured by expression of CD69 in mice treated with the IL-17 Ab compared with control Ab–treated mice (Fig. 2F, 2G).

**FIGURE 1.** Lung IL-17 levels after S. chartarum challenge. Micrographs (original magnification ×10, H&E staining) of lung sections obtained from sensitized WT and TLR9$^{-/-}$ mice challenged with S. chartarum at day 2 (A) showing accumulation of neutrophils and mononuclear cells within the interstitial compartments, a predominantly lymphocytic infiltration within the peribronchial regions, multinucleated giant cells (arrows), and loose epithelioid granulomas within both the interstitial and peribronchial areas in S. chartarum–challenged mice. WT and TLR9$^{-/-}$ mice were sensitized and challenged with S. chartarum as described, lungs were harvested on day 3, lung collagenase digests were performed, and lymphocytes were purified. Flow cytometric analysis was performed, and the number of CD4$^+$ T cells (B), CD8$^+$ T cells (C), CD4$^+$ cells (D), and CD69$^+$ cells (E) is shown. n = 5 in each experiment; mean ± SEM of two experiments. *p < 0.01 compared with sensitized WT mice.
Decreased expression of IL-17 by TLR9−/− lung T cells in-vivo

To determine whether T cells were the cellular source of IL-17 production in response to *S. chartarum*, WT and TLR9−/− mice were sensitized and challenged with *S. chartarum*, and IL-17 mRNA (A) and protein levels by ELISA (B) were measured in lung homogenates. WT mice were administered purified polyclonal rabbit anti-mouse anti–IL-17 Ab or control IgG 3 d prior to and the day of i.t. challenge with *S. chartarum*. Histologic examination of lungs was performed 3 d after sensitization and *S. chartarum* challenge (C). H&E stain, original magnification ×10. WT and TLR9−/− mice were sensitized and challenged with *S. chartarum* and treated with either purified polyclonal rabbit anti-mouse anti–IL-17 Ab or control IgG 3 d prior to and the day of i.t. challenge with *S. chartarum*. Flow cytometric analysis was performed and the number of CD4+ T cells (D) and CD8+ T cells (E) is shown as well as CD69+ CD4+ T cells (F, G); n = 4–5 for each experiment; mean ± SEM of two experiments. *p < 0.05 compared with sensitized WT mice.

**FIGURE 3.** Recruitment of IL-17–expressing T cells in WT and TLR9−/− mice after *S. chartarum* sensitization and challenge. WT and TLR9−/− mice were sensitized and challenged with *S. chartarum* as described. Lungs were harvested at day 3; lung collagenase digests were performed, and lymphocytes were purified. Flow cytometric analysis was performed, and the percentage and number of γ6 (B, D) and CD4+ T cells (A, C) with intracellular IL-17 is shown. n = 5 in each experiment; mean ± SEM of two experiments. *p < 0.05 compared with untreated controls; #p < 0.05 compared with *S. chartarum* challenged WT mice.
Reduced IL-23 production by DCs isolated from sensitized TLR9^{−/−} mice

DCs are required for the development of Ag-specific type 1 cytokine responses, particularly IFN-γ production by T cells (36). To determine whether the reduced granulomatous inflammation and T cell–derived IL-17 production observed in TLR9^{−/−} mice was secondary to altered DC IL-23 production, WT and TLR9^{−/−} mice were sensitized (i.p. and intranasally) as described previously and bone marrow was harvested on day 0. Bone marrow cells were cultured for 6 d in GM-CSF. DCs purified by CD11c⁺ magnetic beads separation on day 6 and stimulated with S. chartarum spores in a ratio of 1:10 (DC:S. chartarum spore). Spontaneous ex vivo production of IL-23 measured by ELISA (Fig. 4B) and IL-23 p19 mRNA expression was determined with real-time PCR (Fig. 4A). As shown in Fig. 4A, incubation of sensitized WT DC with S. chartarum resulted in considerable induction of IL-23 p19 mRNA and IL-23 protein. The expression of IL-23 p19 mRNA and IL-23 protein was substantially attenuated in bone marrow–derived dendritic cells isolated from TLR9^{−/−} mice (p < 0.05).

IL-23p19 shRNA silences IL-23 expression and blocks DC-stimulated IL-17 production by T cells

To assess the contribution of IL-23 production from S. chartarum–sensitized DCs to the expression of IL-17 from T cells, bone marrow was isolated from sensitized WT mice on day 0, and DCs were matured for 6 d in GM-CSF. DCs were then incubated with either control or IL-23 p19 shRNA to extinguish DC-derived IL-23 expression. Transfection efficiency of primary DC was high, with >90% of cells expressing GFP (Fig. 5A). Stimulation with S. chartarum resulted in strong expression of IL-23 p19 mRNA expression from sensitized WT DCs incubated with control shRNA (Fig. 5B). By comparison, treatment with IL-23 p19 shRNA nearly completely silenced IL-23 p19 expression from S. chartarum–challenged DCs. Knockdown was specific for IL-23 expression.
23p19, because this treatment had no effect on DC-derived IL-12 p40 mRNA expression (Fig. 5C). To assess whether DC-derived IL-23 was required for *S. chartarum*-induced IL-17 production from T cells, WT DCs incubated with control or IL-23 p19 shRNA were cocultured with splenic T cells harvested from naïve WT mice for 18 h. T cells cocultured with WT DCs treated with control small interfering RNA produced large quantities of IL-17 (22-fold increase over unstimulated cells). The induction of IL-17 was dependent on DC-derived IL-23, because IL-17 expression was nearly completely abolished when cocultured with IL-23 shRNA–treated DCs (Fig. 5D). Collectively, these findings suggest that the Ag-specific induction of IL-17 by T cells in response to *S. chartarum* is dependent on DC-derived IL-23.

**Administration of IL-23 to TLR9−/− mice recapitulates HP phenotype**

Having observed diminished IL-23 production by DCs and in whole lung of TLR9−/− mice after *S. chartarum* sensitization and challenge, we next evaluated whether reconstitution of IL-23 would restore a granulomatous hypersensitivity response in TLR9−/− mice. WT and TLR9−/− mice were sensitized and challenged as previously described, with some animals receiving rmIL-23 (2 μg) i.t. at the time of i.t. *S. chartarum* challenge (day 0). Lungs were harvested 3 d after challenge. As shown in Fig. 6, WT mice displayed evidence of granulomatous inflammation, as manifest by accumulation of CD4+ T cells (Fig. 6B), which was considerably diminished in TLR9−/− mice. Interestingly, administration of rmIL-23 to TLR9−/− mice resulted in histopathology (Fig. 6A) and CD4+ accumulation (Fig. 6B) indistinguishable from *S. chartarum*–challenged WT mice. Moreover, treatment of mutant mice with IL-23 significantly increased the percentage of CD4+ T cells expressing CD69 to levels observed in WT animals (Fig. 6C, 6D). This finding suggests that the defect in granulomatous responses to *S. chartarum* observed in TLR9−/− mice is largely attributable to defects in the production of IL-23.

**Discussion**

In this study, we show for the first time, to our knowledge, the critical role of TLR9 in the generation of the Th17 response in a mouse model of *S. chartarum*-induced HP. Several pattern recognition receptors have been implicated previously in the immunopathogenesis of HP, including TLR2 and TLR6, as well as the downstream adaptor protein MyD88 in a *S. rectivirgula* mouse model of farmer’s lung disease (37, 38). We and others have identified the importance of TLR9 in the generation of a type 1 granulomatous response to Ag sensitization and challenge. Moreover, this TLR is required for granuloma formation and IFN-γ production in certain Gram-positive infection (*Propionibacterium acnes*) (39) and in response to mycobacterial Ag (40). Interestingly, many diseases thought to be entirely Th1 dependent have more recently been shown to require activation of the Th17 pathway. In this study, we found a reduced pulmonary granulomatous inflammatory response to sensitization and challenge with *S. chartarum* associated with decreased IL-17 production in TLR9−/− mice compared with their WT counterparts. Reduced IL-17 production in mutant mice was attributable, at least in part, to defective IL-23 production from DCs. These findings implicate TLR9 in mediating IL-17–driven hypersensitivity pneumonitis in response to *S. chartarum*.

Several recent reports have identified the contribution of Th17 pathway to the generation of HP. In a murine model of farmer’s

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**FIGURE 6.** Histopathology and flow cytometry after reconstitution of IL-23 in WT and TLR9−/− mice after *S. chartarum* sensitization and challenge. TLR9−/− mice were sensitized and challenged with *S. chartarum* with or without reconstitution of murine recombinant IL-23 at the time of challenge. Lungs were harvested at day 3 after challenge, and representative histology is shown in (A). H&E stain, original magnification ×10. WT and TLR9−/− mice were sensitized and challenged with *S. chartarum* and treated with either saline or rmIL-23 the day of i.t. challenge with *S. chartarum*. Flow cytometric analysis was performed, and the number of CD4+ T cells (B) and CD4+ and CD69+ T cells (C, D) is shown. n = 4 in each group; *p < 0.05 compared with WT mice; †p < 0.05 compared with TLR9−/− mice after *S. chartarum* challenge without IL-23 reconstitution.
lungs of mice exposed to *S. rectivirgula*–Ag compared with saline-challenged mice. Genetic deletion of IL-17 or Ag-mediated immunodepletion resulted in protection against the disease, as manifest by decreased cell infiltration and lower production of chemokines and cytokines in the lungs of *S. rectivirgula*–Ag-challenged mice. Similarly, IL-17 neutralization studies indicated that this cytokine was necessary for *S. chartarum*–induced lung pathology. A recent study by Fong et al. (37) has implicated TLR6 as a pivotal TLR in the production of IL-17 and the development of HP. However, IL-17 production in response to *S. rectivirgula*–Ag can occur in a fashion independent of TLR6, suggesting that other TLRs might contribute to the development of IL-17–mediated HP. Interestingly, mice deficient in TLR9 had a significantly blunted IL-17 response to sensitization and challenge with *S. chartarum* (45–47). Similarly, Daito et al. (48) have recently identified the importance of TLR9 in driving granulomatous inflammation. In the aforementioned study, TLR9<sup>−/−</sup> mice failed to mount a Th1-skewed cytokine response to *S. chartarum* Ag, as well as blunted expression of IL-23 in the lungs of TLR9<sup>−/−</sup> mice after *S. chartarum* sensitization and challenge. Importantly, intrapulmonary reconstitution of IL-23 in TLR9<sup>−/−</sup> mice recapitulated the in vivo phenotype observed in the lungs of *S. chartarum*–challenged WT mice. These findings are consistent with the hypothesis that impairment in IL-23 production by DCs is the primary defect contributing to reductions in T cell–derived IL-17 expression in TLR9-deficient mice.

In summary, this and our previous studies implicate both Th1 and Th17 pathways in the generation of HP in response to *S. chartarum*. Moreover, TLR9-mediated DC responses are critical for Ag-specific T cell responses in this model. TLR9 signaling may serve as a therapeutic target in the treatment of patients with this and other forms of granulomatous lung disease.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

TLR9-INDUCED IL-17 IN HYPERSENSITIVITY PNEUMONITIS


