Toll-like Receptor 9 Activation Is a Key Mechanism for the Maintenance of Chronic Lung Inflammation

Toshihiro Ito¹, Matthew Schaller¹, Tracy Raymond¹, Amrita D. Joshi¹, Ana L. Coelho¹, Fabiani G. Frantz¹, William F. Carson IV¹, Cory M. Hogaboam¹, Nicholas W. Lukacs¹, Theodore J. Standiford², Sem H. Phan¹, Stephen W. Chensue^{1,3}, and Steven L. Kunkel¹

¹Department of Pathology, University of Michigan Medical School, Ann Arbor; ²Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Michigan Medical Center, Ann Arbor; and ³Department of Pathology and Laboratory Medicine, Veterans Affairs Ann Arbor Healthcare System, Ann Arbor, Michigan

Rationale: Accumulating evidence supports the hypothesis that the continuous host response to a persistent challenge can polarize the cytokine environment toward a Th2 cytokine phenotype, but the mechanisms responsible for this skewing are not clear.

Objectives: We investigated the role of Toll-like receptor 9 (TLR9) in a Th2-driven pulmonary granulomatous response initiated via the embolization of *Schistosoma mansoni* eggs to the lungs of mice.

Methods: Mice were intravenously injected with *S. mansoni* eggs. Histological and flow cytometric analysis, cytokine measurement, adoptive transfer of bone marrow (BM)-derived dendritic cells (DCs), and *in vitro* T-cell treatments with antigen-presenting cells were examined.

Measurements and Main Results: In comparison to wild-type mice, TLR9^{-/-} mice showed increased pulmonary granuloma size, augmented collagen deposition, increased Th2 cytokine phenotype, and impaired accumulation of DCs. BM-derived DCs, but not macrophages, recovered from animals with developed Th2-type lung granulomas promoted the production of type 2 cytokines from CD4⁺ T cells. BM-derived DCs from TLR9^{-/-} mice induced impaired Th1 cytokine and enhanced Th2 cytokine production by T cells, compared with DCs from WT mice. Macrophages from TLR9^{-/-} mice expressed a significantly higher alternatively activated (M2) phenotype characterized by increased "found in inflammatory zone-1" (FIZZ1) and arginase-1 expression. The adoptive transfer of BM-derived DCs from syngeneic WT mice into TLR9^{-/-} mice restored the granuloma phenotype seen in WT mice.

Conclusions: These studies suggest that TLR9 plays an important mechanistic role in the maintenance of the pulmonary granulomatous response.

Keywords: granuloma; pulmonary fibrosis; innate immunity; dendritic cell; macrophage

The granulomatous response is a complex host defense mechanism that has evolved to provide containment of infectious and/or environmental agents (1, 2). Although this reaction is designed to be protective, the associated tissue injury is often responsible for a profound degree of pathology. Although many of the mechanisms that sustain the development of the granuloma are enigmatic, it is accepted that the maintenance of this inflammatory process is dependent upon dynamic interactions

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

A number of studies have suggested that the innate immune response not only supports acute inflammation but also maintains the chronic inflammatory response. However, the mechanistic role of the innate immune response in the development of antigen-dependent chronic lung inflammation has not been well studied.

What This Study Adds to the Field

We demonstrate that the initiation and maintenance of chronic lung inflammation induced in Toll-like receptor 9-knockout mice is associated with an augmented granulomatous lung response, increased collagen deposition, skewed Th2 cytokine phenotype, impaired dendritic cell function, and activation of alternatively activated macrophages characterized by the production of "found in inflammatory zone-1" (FIZZ1).

between an inciting agent, inflammatory mediators, various immune and inflammatory cells, and structural cells of the involved tissue (3). The best studied of the host-dependent processes during granuloma development is the adaptive immune response, which is characterized by specific immune cell populations expressing a defining phenotype of inflammatory mediators (3–8). Recent data suggest that the innate immune response initiated by the activation of specific toll-like receptors can contribute mechanistically to maintaining the intensity and chronicity of the lung pathology associated with the developing granuloma (9–11).

We have used an experimental system of granuloma development via the embolization of *Schistosoma mansoni* eggs to the lungs, which release highly antigenic glycoproteins, referred to as *Schistosoma* egg antigen (SEA), that promote a dominant Th2 response (11). In its chronic phase, this inflammatory response promotes a dominant Th2 cytokine-driven response characterized by IL-4, IL-5, and IL-13 that involves the recruitment and activation of eosinophils, alternatively activated (M2) macrophages, dendritic cells (DCs), CD4⁺ Th2 cells, and the development of fibrotic granulomas (12, 13). Although a significant body of data exists on the cytokine phenotype and cellular composition of developing lung granulomas, little is known about the mechanistic contribution of TLRs to the maintenance and fibrotic aspect of this inflammatory response.

In the present study, we investigated the contribution of TLR9 to the initiation and maintenance of a Th2-dependent lung granulomatous response. We demonstrate that TLR9-deficient mice develop significantly larger granulomas with

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Correspondence and requests for reprints should be addressed to Steven L. Kunkel, Ph.D., Immunology Program, Department of Pathology, University of Michigan Medical School 4071 BSRB, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200. E-mail: slkunkel@umich.edu

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augmented collagen deposition, associated with a selective abrogation in IFN- γ (Th1 cytokine) production, an enhanced Th2 (IL-4, IL-5, and IL-13) cytokine profile, and an increased M2 macrophage phenotype (FIZZ1 and Arginase-1) in the lung. Our *in vivo* experiments demonstrate functional relevance for

found in inflammatory zone-1 (FIZZ1) because the intranasal administration of recombinant FIZZ1 enhanced collagen deposition in the Th2-dependent lung granulomas. We also found that antigen-pulsed, bone marrow (BM)-derived DCs, but not BM-derived macrophages, from naive mice promoted Th1 and

Figure 1. Toll-like receptor 9 (TLR9)^{-/-} mice showed larger pulmonary granulomas and enhanced collagen deposition. All panels compare parameters between wild-type (WT) and TLR9^{-/-} mice. (A) Lung tissues were histologically analyzed by Masson's Trichrome staining at Day 4, 8, 16, and 30 after initiation of lung granuloma. Original magnification: $\times 40$ or $\times 400$. (B) Kinetic analysis of the development of lung granulomas using morphometric analysis of the evolving lung lesions in WT and TLR9^{-/-} mice. Dotted line represents mean granuloma cross-sectional area (μm^2) $\pm$ SEM. * $P < 0.05$; ** $P < 0.001$. (C) The percentage of collagen in each granuloma was quantified using IP lab software. * $P < 0.05$; ** $P < 0.01$. (D) Quantitative real-time PCR (TaqMan) was performed to measure the transcript level of α -SMA in whole lungs. * $P < 0.05$; ** $P < 0.01$. (E) Confocal immunofluorescent examination of pulmonary granulomas showed increased α -SMA⁺ cells (green) in TLR9^{-/-} mice. Shown are representative sections from one mouse of four per group. Original magnification: $\times 400$. (A–D) Data shown are mean $\pm$ SEM and are from a representative example of three independent experiments; $n = 4$ or 5 mice per group.

Th2 effector activation of CD4⁺ T cells. Our results further show that the adoptive transfer of BM-derived DCs isolated from wild-type (WT) mice, but not from TLR9^{-/-} mice, can restore the granulomatous response in TLR9^{-/-} to a WT phenotype. Taken together, our results indicate that TLR9 plays a key role in the initiation and maintenance of Th2-type pulmonary granulomatous inflammation.

METHODS

Mice

Male WT mice and male DO11.10 mice, both on a BALB/c background, were purchased from Jackson Laboratories (Bar Harbor, ME). Male BALB/c mice lacking the TLR9 gene (TLR9^{-/-}) were provided by T.J. Standiford. All mice were used for experiments at 8 to 12 weeks of age. These mice were maintained under specific pathogen-free conditions and provided with food and water *ad libitum* in the University Laboratory Animal Medicine facility at the University of Michigan Medical School. All animal protocols were approved by the University Laboratory Animal Medicine facility.

Antibodies

Rat mAbs specific for mouse CD11b (M1/70), CD11c (HL3), CD16/32 (2.4G2), CD45 (30-F11), and CD45R/B220 (RA3-6B2) were purchased from BD PharMingen (San Diego, CA). Rat anti-F4/80 (CI: A3-1) mAb was purchased from Serotec (Raleigh, CA).

Th2-driven Lung Granuloma Model

Mice were intravenously injected with 5,000 viable *S. mansoni* eggs via the tail vein. Live *S. mansoni* eggs were purified from the livers of *S. mansoni* cercariae-infected Swiss-Webster mice, which were kindly provided by Dr. Fred Lewis (Biomedical Research Laboratory, Rockville, MD). Mice were killed 4, 8, 16, or 30 days after intravenous injection. In some experiments, mice were treated with 5 μg recombinant FIZZ-1 (Peprtech Inc., Rocky Hill, NJ) or phosphate-buffered saline (PBS) intranasally on Days 9, 11, 13, and 15 of egg challenge and killed on Day 16.

Histological and Immunofluorescent Examination

Individual excised lung lobes were inflated and fixed with 10% buffered formalin for morphometric analysis. The areas of the granulomas were measured in a blinded fashion on H&E-stained sections of paraffin-embedded lungs using IP Lab Spectrum software, and morphometric analysis of collagen present in Masson's Trichrome-stained lung sections was performed using light microscopy and the same software as described previously (11). For immunofluorescent analysis, lungs were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. Cryostat sections (7- μm) were fixed in ice-cold acetone and incubated with primary antibodies, followed by appropriate Alexa-labeled secondary reagents (Invitrogen Corporation, Carlsbad, CA). The sections were analyzed by Zeiss LSM 510 confocal microscope system (Carl Zeiss, Inc., Thornwood, NY).

Reverse Transcription and Real-time Quantitative PCR Analysis

Total RNA was isolated from whole lungs or cultured cells by using TRIzol (Invitrogen) according to the manufacturer's instructions.

Briefly, a total of 2.0 μg of RNA was reverse-transcribed to cDNA, and real-time quantitative PCR analysis was performed by using an ABI 7700 sequence detector system (PE Applied Biosystems, Foster City, CA).

Protein Analysis of Cytokine

Murine cytokine levels were measured in 50- μl samples from whole lung homogenates using a Bio-plex bead-based cytokine assay (Bio-Rad Laboratories, Hercules, CA). FIZZ1 protein levels were measured by ELISA as described previously (10). The cytokine levels in lung homogenates were normalized to the protein (in milligrams) using the Bradford assay. Cells from draining lymph nodes were plated at a concentration of $5 \times 10^6/\text{ml}$ onto a 96-well plate and restimulated with purified SEA at 10 $\mu\text{g}/\text{ml}$. Supernatants were harvested 48 hours after stimulation and analyzed by Bio-plex assay.

Flow Cytometry

Flow cytometric analyses of lung cells were performed as previously described (14). Briefly, whole lungs were dispersed in RPMI-1640 containing 0.2% collagenase (Sigma-Aldrich, St. Louis, MO) and 5% fetal bovine serum (Atlas, Fort Collins, CO) at 37°C for 45 minutes to obtain a single-cell suspension. The cells were stained with indicated antibodies after 10 minutes of preincubation with CD16/CD32 Abs (Fc block) and fixed overnight with 4% formalin. Cells were analyzed using a Cytomics FC-500 (Beckman Coulter, Miami, FL), and data were analyzed by FlowJo software (TreeStar, Inc., Ashland, OR).

Generation of BM-derived DCs

For generation of BM-derived DCs, after depletion of erythrocytes with lysis buffer, BM cells were seeded in T-150 tissue culture flasks at 10^6 cells/ml in RPMI 1640-based complete media with GM-CSF 20 ng/ml (R&D Systems, Minneapolis, MN). Six days later, loosely adherent cells were collected and incubated with anti-CD11c coupled to magnetic beads for positive selection of conventional DCs from the GM-CSF cultures using a magnetic column (>95% purity) (Miltenyi Biotec, Auburn, CA). For generation of BM-derived macrophages, BM cells were cultured in L929 cell-conditioned medium as described previously (14). In some experiments, after 2 hours of stimulation with SEA (10 $\mu\text{g}/\text{ml}$) at 37°C and 5% CO₂, BM-derived DCs were administered intratracheally.

In Vitro T-Cell Treatments

BM-derived DCs or macrophages were plated on 96-well plates at a cell density of 4×10^4 cells/well and incubated overnight. DCs or macrophages were then pulsed with SEA (10 $\mu\text{g}/\text{ml}$) for 2 hours and washed before T cells were added. Sixteen days after intravenous injection of 5,000 *S. mansoni* eggs, CD4⁺ T cells from lungs, draining lymph nodes, or spleens were isolated using a magnetic bead column (>95% purity). These cells (2×10^5 cells/well) were exposed to SEA-pulsed, BM-derived DCs or macrophages on 96-well plates at an antigen-presenting:CD4⁺ cell ratio of 1:5 (lungs) or of 1:20 (draining lymph nodes and spleens). Supernatants were harvested 48 hours later for analysis in a Bio-plex (Bio-Rad Laboratories) bead-based cytokine assay.

Statistical Analysis

Two-tailed Student's *t* test was used to compare groups. Values of $P < 0.05$ were considered statistically significant.

RESULTS

The Chronicity of the Pulmonary Granulomatous Response Is Augmented in TLR9^{-/-} Mice

Recent data suggest that mechanisms that drive the innate immune response, such as activation of the TLR system, serve as a novel bridge to facilitate the acquired immune response (14, 15). To further explore this notion, we determined whether TLR9 is involved in the development of the Th2-type pulmonary granulomatous response. We examined histological lung sections from WT and TLR9^{-/-} mice at Days 4, 8, 16, and 30 after initiation of the primary Th2 response. Histological examination of lung tissue revealed that the granulomas were similar in size in WT and TLR9^{-/-} mice for the first 4 days after challenge (Figure

1A). However, at Day 8, Masson's Trichrome-stained lung sections revealed that TLR9^{-/-} mice exhibited significantly larger granulomas, as determined by morphometric analysis when compared with WT mice (Figure 1B). Associated with the enhanced granuloma size is the appearance of a significant amount of matrix deposition starting at Day 16 in the TLR9^{-/-} mice (Figure 1C). There are neither pathologic nor abnormal features in naive TLR9^{-/-} mice; their lung tissues appear normal. In addition, granulomatous lungs of TLR9^{-/-} mice expressed significantly higher levels of α -SMA mRNA expression (Figure 1D) compared with WT mice. These results were further supported by confocal immunofluorescent analysis, which showed an increase in α -SMA⁺ cells associated with the local granuloma in TLR9^{-/-} mice (Figure 1E).

Figure 2. Toll-like receptor 9 (TLR9)^{-/-} mice showed impaired Th1 cytokine and enhanced Th2 cytokine levels. All panels compare parameters between wild-type (WT) and TLR9^{-/-} mice. (A) Protein levels of IFN- γ , IL-12(p70), tumor necrosis factor (TNF)- α , IL-4, IL-5, and IL-13 measured in whole lungs using a luminex system. Data shown are mean $\pm$ SEM and are from a representative example of three independent experiments. Each time point indicates at least four or five mice per group. * P < 0.05; ** P < 0.01. (B) Whole cells from the draining lymph nodes of WT or TLR9^{-/-} mice at Day 16 after *Schistosoma mansoni* egg challenge were restimulated *in vitro* with *Schistosoma* egg antigen for 48 hours. Cytokine levels of IFN- γ , IL-4, IL-5, and IL-13 were measured using a luminex system. * P < 0.05. Data shown are mean $\pm$ SEM and are from a representative example of three independent experiments.

Lungs from TLR9^{-/-} Mice Demonstrate Increased Th2 and Decreased Th1 Cytokines during Pulmonary Granuloma Formation

To help elucidate the mechanism underlying the changes in pulmonary granuloma size and collagen deposition in TLR9^{-/-} mice versus WT mice, we examined the cytokine profile in the granulomatous lungs. Whole lungs from TLR9^{-/-} mice had significantly lower protein levels of Th1-dependent cytokines (e.g., IFN- γ and IL-12p70) and tumor necrosis factor (TNF)- α , whereas protein levels of cytokines that promote the Th2-biased immune response (IL-4, IL-5, and IL-13) were significantly increased in TLR9^{-/-} mice as compared with WT mice (Figure 2A). Further mRNA analysis confirmed lower expression of Th1 type cytokines (IFN- γ , IL-12p35, and TNF- α) (Figure E1, in the online supplement). IL-12p40 mRNA expression was below detection levels of our assay (data not shown). We also characterized the production of Th1 and Th2 cytokines from cells recovered from draining lymph nodes of granulomatous mice after *in vitro* rechallenge with SEA. The data illustrate that there were considerable decreases in IFN- γ production from the lymph node-derived cells from the TLR9^{-/-} mice compared with WT mice, whereas the Th2 cytokines IL-4, IL-5, and IL-13 were significantly up-regulated in TLR9^{-/-} mice compared with WT mice (Figure 2B).

We evaluated M1 and M2 macrophage markers in the developing granulomas from WT and TLR9^{-/-} mice. The expression level of M2 markers FIZZ1 (mRNA and protein; Figures 3A and 3B) and arginase-1 (mRNA; Figure 3C) were significantly increased in TLR9^{-/-} mice compared with WT mice. In contrast, iNOS, a M1 macrophage marker, was significantly less in TLR9^{-/-} mice at Days 16 and 30 (Figure 3D).

Granulomatous Lungs from TLR9^{-/-} Mice Display Reduced Numbers of DCs and Increased Number of Macrophages

To further characterize the changes in cell phenotype between WT and TLR9^{-/-} mice during granuloma formation, we assessed the numbers of antigen-presenting cells (APCs) in granulomatous lungs by examining CD45⁺ leukocytes. Compared with WT mice, the number of myeloid DCs (CD11b⁺CD11c⁺) was significantly decreased in TLR9^{-/-} mice at Day 16 (Figure 4A). There was no significant difference in the number of plasmacytoid DCs (B220⁺CD11c⁺) (data not shown). In contrast, the number of F4/80⁺ macrophages was significantly increased in TLR9^{-/-} mice at Day 16 (Figure 4B). These results were supported by confocal immunofluorescent analysis, which showed fewer CD11c⁺-staining cells (Fig. 4C, red arrow) and an increase in F4/80⁺ cells (Fig. 4C, green arrow) associated with the local lung granulomas in TLR9^{-/-} mice. To

Figure 3. Toll-like receptor 9 (TLR9)^{-/-} mice showed decreased M1 markers and increased M2 markers during *S. mansoni* egg challenge. All panels compare parameters between wild-type (WT) and TLR9^{-/-} mice. The gene expression level of (A) FIZZ1, (C) arginase-1 and (D) iNOS, and (B) the protein level of FIZZ1 in the whole lungs at Days 0, 4, 8, 16, 30 are shown. **P* < 0.05; ***P* < 0.01. Data shown are mean $\pm$ SEM and are from a representative example of three independent experiments; *n* = 4 or 5 mice per group.

Figure 4. Impaired migration of dendritic cells (DCs) in Toll-like receptor 9 (TLR9)^{−/−} mice during pulmonary granuloma formation. Fluorescence-activated cell sorter analysis of (A) lung DCs and (B) macrophages isolated from *S. mansoni* egg-challenged mice. **P* < 0.05 compared with wild-type (WT) mice. (C) Confocal immunofluorescent examination of pulmonary granulomas showed fewer CD11c⁺ cells (red arrows) and increased F4/80⁺ cells (green arrows) in TLR9^{−/−} mice. Shown are representative sections from one mouse of four per group. Original magnification: ×400. (D) Quantitative real-time PCR (TaqMan) was performed to measure the transcript levels of chemokines in whole lungs at Day 16 after the initiation of lung granuloma. **P* < 0.05; ***P* < 0.01. Data shown indicate mean ± SEM and are from a representative example of three independent experiments; n = 4 mice per group.

further determine the reason why the number of DCs was reduced in TLR9^{−/−} mice, we investigated chemokine expression during granuloma formation. The mRNA expression levels of CCL20 and CCL22 were significantly decreased in TLR9^{−/−} mice (Figure 4D). These chemokines play a crucial role in the recruitment of immature DCs into inflammatory lesions (16, 17). Moreover, the mRNA expression levels of CXCL9, CXCL10, and CXCL11, which support the migration of Th1 cells (18), were significantly reduced in TLR9^{−/−} mice. In contrast, the expression level of CCL17, which is released from M2 macrophages and supports the migration of Th2 cells (19), was significantly increased in TLR9^{−/−} mice.

BM-derived DCs but Not Macrophages Promote the Expression of Th1 and Th2 Cytokines from T Cells during a Th2-induced Immune Response

Because there is a significant difference in Th1/Th2 cytokine balance between WT and TLR9^{−/−} mice during pulmonary

granuloma formation, we assessed whether these APCs (DCs and macrophages) could promote CD4⁺ T effector cell expression upon pulsing with SEA using a coculture cytokine elicitation assay. DCs, but not macrophages, were capable of inducing Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4, IL-5, and IL-13) expression from the CD4⁺ cells (Figure 5). IFN-γ production was significantly less when the DCs were derived from TLR9^{−/−} mice, whereas IL-4, IL-5, and IL-13 production was significantly increased using DCs recovered from TLR9^{−/−} mice.

BM-derived DCs from Mice with *S. mansoni* Egg-induced Granulomas Induce Enhanced Th2-Shifted Response from Lung CD4⁺ T Cells

To further investigate whether the Th2 cytokine polarization evoked by *S. mansoni* eggs was successfully supported by DCs, we performed an *in vitro* cytokine expression assay using lung CD4⁺ T cells recovered from granulomatous lungs. The CD4⁺ T cells were coincubated with SEA-pulsed, BM-derived DCs

Figure 5. Bone marrow (BM)-derived dendritic cells (DCs) but not bone marrow (BM)-derived macrophages promote the differentiation of Th1 and Th2 cells from T cells restimulated with *Schistosoma* egg antigen. CD4⁺ T cells were isolated from spleens of *S. mansoni* egg-challenged wild-type (WT) mice and restimulated with DCs or macrophages isolated from WT or Toll-like receptor 9 (TLR9)^{-/-} mice, as indicated. Cytokine levels of (A) IFN-γ, (B) IL-4, (C) IL-5, and (D) IL-13 were measured using a luminex system. **P* < 0.05. Data shown indicate mean ± SEM and are from a representative example of three independent experiments; n = 4 mice per group. Mφ = macrophage.

from WT or TLR9^{-/-} mice recovered from naive or granulomatous mice. In the presence of DCs derived from mice with pulmonary granulomas, lung CD4⁺ T cells (also from granulomatous lungs) demonstrated reduced IFN-γ production and increased IL-4, IL-5, and IL-13 production (Figure 6). The APC function of DCs from the TLR9^{-/-} mice, as compared with WT

DCs, caused a decrease in Th1 cytokine (IFN-γ) production and an increase in Th2 cytokine (IL-4, IL-5, and IL-13) production. These responses were also seen when using CD4⁺ T cells from draining lymph nodes (Figure E2). To determine whether these responses were antigen specific, we evaluated the production of the above cytokines by splenic CD4⁺ T cells from DO11.10

Figure 6. Bone marrow (BM)-derived dendritic cells (DCs) from *S. mansoni* egg-challenged mice promoted an enhanced Th2 response. CD4⁺ T cells were isolated from lungs of *S. mansoni* egg-challenged wild-type (WT) mice and restimulated with DCs or macrophages isolated from naive or *Schistosoma* egg antigen (SEA)-stimulated WT or Toll-like receptor 9 (TLR9)^{-/-} mice, as indicated. Cytokine levels of (A) IFN-γ, (B) IL-4, (C) IL-5, and (D) IL-13 were measured using a luminex system. **P* < 0.05 compared with naive DCs; #*P* < 0.05 compared with WT mice. Data shown indicate mean ± SEM and are from a representative example of three independent experiments; n = 4 mice per group.

mice, which have an OVA-specific TCR. These cells were cocultured with BM-derived DCs from WT or TLR9^{-/-} mice recovered from mice with developing Th2 lung granulomas or from naive mice. No significant differences in the production of Th1 and Th2 cytokines were seen in T cells cocultured with BM-derived DCs under any of the described experimental conditions after stimulation with only OVA peptide (Figure E3). These results underscore the specificity of the response to the original sensitizing antigen, SEA, and the importance of DC-derived TLR9 activation in eliciting the Th1 phenotype during T-cell activation events.

Reconstitution of the WT Pulmonary Granulomatous Response in TLR9^{-/-} Mice after Adoptive Transfer of WT Syngeneic BM-derived DCs

Studies contained in this article have demonstrated an impairment of DC accumulation and effector cell function in TLR9^{-/-} mice after *S. mansoni* egg challenge. To determine whether these DC defects contributed meaningfully to larger granuloma and impaired cytokine balance during pulmonary granuloma formation in TLR9-deficient mice, we adoptively transferred BM-derived DCs recovered from WT mice into TLR9^{-/-} mice and assessed histological appearance and cytokine production. For these experiments, we administered BM-derived DCs intra-

tracheally, an approach that has previously been shown to stimulate intrapulmonary immunity in other model systems in which the inflammatory response was localized to the lung (20). BM-derived DCs (1×10^6 cells) obtained from WT mice or TLR9^{-/-} mice were administered intratracheally just after *S. mansoni* egg challenge. Lungs were harvested 16 days later, and histological assessment was performed. Granulomatous lungs administered BM-derived DCs from WT mice histologically and morphometrically demonstrated a reduced granuloma size (Figures 7A and 7B) and a reduction in collagen deposition (Figure 7C) compared with animals receiving BM-derived DCs from TLR9^{-/-} mice. The intratracheal delivery of WT DCs into TLR9^{-/-} mice resulted in a marked reduction in Th2 cytokines (IL-4, IL-5, and IL-13) production (Figures 8A–8C), but these difference were not seen when TLR9^{-/-} DCs were administered. Moreover, the production of Th1 and Th2 cytokines from draining lymph nodes of treated mice was assessed after *in vitro* rechallenge with SEA. There was a significant increase in IFN- γ production (Figure E4A), whereas the Th2 cytokines, IL-4 and IL-5, were significantly decreased in the lymph nodes of mice that received DCs isolated from WT mice compared with mice given DCs from TLR9^{-/-} mice (Figures E4B and E4C). There were no significant differences in the production of IL-13 between these two groups, although these levels were significantly reduced compared with the control

Figure 7. Effect of intratracheal administration of bone marrow (BM)-derived dendritic cells (DCs) from wild-type (WT) or Toll-like receptor 9 (TLR9)^{-/-} mice on granuloma formation in TLR9^{-/-} mice with *S. mansoni* egg. (A) Histological appearance of pulmonary granulomas from TLR9^{-/-} mice treated with phosphate-buffered saline (PBS), WT-DCs, or TLR9^{-/-}-DCs at Day 16 after granuloma development. Arrows point out collagen deposition. H&E staining and Masson's Trichrome staining. Original magnification: $\times 40$ and $\times 400$. Experiments were performed three times with similar results. (B) Morphometric analysis of pulmonary granuloma size from mice treated with PBS, WT-DCs, or TLR9^{-/-}-DCs after 16 days of lung granuloma development. $**P < 0.01$. (C) The percentage of collagen in each granuloma was quantified using IP lab software. $**P < 0.01$. Data shown indicate mean $\pm$ SEM and are from a representative example of two independent experiments; $n = 4$ to 5 mice per group. TR = Masson's Trichrome staining.

Figure 8. The intratracheal delivery of wild-type-dendritic cells (WT-DCs) into Toll-like receptor 9 (TLR9)^{-/-} mice resulted in a marked reduction in Th2 cytokines and M2 phenotype. Protein level of (A) IL-4, (B) IL-5, (C) IL-13, and (D) found in inflammatory zone-1 (FIZZ1) was measured in whole lungs using a luminex system or ELISA system. (E) FIZZ1 mRNA assessed by quantitative real-time PCR (TaqMan). **P* < 0.05; ***P* < 0.01. Data shown indicate mean ± SEM and are from a representative example of two independent experiments; *n* = 4 or 5 mice per group.

(PBS) group (Figure E4D). In addition, the protein and mRNA level of FIZZ1 (Figure 8D and 8E) in whole lungs was significantly decreased after intratracheal transfer of WT DCs but not in lungs of mice given DCs from TLR9^{-/-} mice.

Intranasal Administration of Recombinant FIZZ1-Enhanced Collagen Deposition during Granuloma Formation in WT and TLR9^{-/-} Mice

FIZZ1 plays an important role in the fibrotic response, and lungs from TLR9^{-/-}-deficient mice with a developing Th2-dependent pulmonary granuloma displayed enhanced FIZZ1 expression. To further investigate the distinct role of FIZZ1 *in vivo*, we performed intranasal administration of recombinant FIZZ1 into WT and TLR9^{-/-} mice during granuloma formation. Histologic and collagen analysis demonstrated that exogenous treatment with recombinant FIZZ1 in WT and TLR9^{-/-} mice significantly enhanced collagen deposition (Figures 9A and 9B). Further investigations showed that there was no significant difference in granuloma size, and the Th1 and Th2 cytokine profile in the granulomatous lungs demonstrated no significant difference between the PBS- and recombinant FIZZ1-treated groups (data not shown).

DISCUSSION

Our results demonstrate that the TLR9 signaling pathway is essential in the regulation of an antigen-dependent, Th2 cyto-

kine-driven granulomatous response. To our knowledge, the present investigation represents the first analysis of cell-mediated Th2 pulmonary granuloma formation in mice with targeted disruption of the TLR9 gene. Our studies demonstrate that the developing granulomatous response in the TLR9-deficient mice exhibits a significantly larger granuloma, augmented type 2 cytokine expression, and a higher collagen content. The altered histological findings in TLR9^{-/-} mice coincided with significantly decreased whole-lung IFN- γ , IL-12, and TNF- α levels and significantly increased whole-lung levels of IL-4, IL-5, and IL-13 during the formation and maintenance of the granuloma responses when compared with WT mice.

Although TLRs are known to provide the link between the innate and adaptive immunity (15), the relative contribution of each TLR to this process remains poorly described. The immune system can discriminate between different microbes through various pathogen-recognition receptors, such as TLRs, which are highly evolutionarily conserved (21, 22). Upon antigenic stimulation by APCs, naive CD4⁺ T cells become activated, expand, and differentiate into various effector T-cell helper subsets (termed Th1 and Th2) that are and characterized by the production of distinct cytokines and effector functions (23, 24). TLR signaling has been considered to be important in the pathogenesis of pulmonary inflammatory diseases such as lung fibrosis and bronchial asthma (25). Specifically, TLR4- and TLR5-mediated innate immunity plays an important role in cystic fibrosis lung disease (26, 27) and respiratory viral

Figure 9. Recombinant found in inflammatory zone-1 (FIZZ1) administration during granuloma formation induced enhanced collagen deposition. (A) Lung tissues were histologically analyzed by H&E and Masson's Trichrome staining at Day 16 after initiation of lung granuloma. Arrows point out collagen deposition. Original magnification: $\times 400$. (B) The percentage of collagen in each granuloma was quantified using IP lab software. $*P < 0.05$; $**P < 0.01$. Data shown indicate mean $\pm$ SEM and are from a representative example of two independent experiments; $n = 5$ mice per group. rFIZZ1 = recombinant FIZZ1. PBS = phosphate buffered saline; TLR9 = Toll-like receptor 9; WT = wild type.

infection (28). In this latter study, the recruitment of Th2 cytokine-producing cells may amplify Th2-dependent inflammation via the induction of TLR3 in the asthmatic airway (28). Furthermore, we have demonstrated that TLR3 regulates the immunopathology associated with the Th2-type pulmonary granulomatous response (11). Previous studies have shown that clinical helminth infections are associated with profound alterations in the expression and activation of various TLR on immune cells. For example, SEA directly inhibits the ability of DCs to respond to LPS (a TLR4 ligand) and CpG (a TLR9

ligand) (29, 30). In this study's we showed that SEA-pulsed DCs, but not macrophages, supported Th1/Th2 cytokine production by CD4⁺ T cells after restimulation. We further established that the adoptive transfer of BM-derived WT-DCs, but not BM-derived DCs from TLR9^{-/-} mice, administered directly into the lung markedly recapitulated the granuloma phenotype in WT mice with reduced Th2 cytokine production. TLR9^{-/-} mice did not show an alteration in other TLRs that could account for the differences seen in the development of Th2-type pulmonary granulomatous response (data not shown).

Together, our results provide evidence of defective DC responses in TLR9^{-/-} mice. The potential therapeutic effect of unmethylated CpG DNA for experimental chronic lung disease is under investigation in our laboratory. Further studies are in progress to assess the potential role of other TLRs in the development of the Th2-type granulomatous response in the progression of inflammatory airway disease and exacerbation of pulmonary fibrosis.

Further evidence of a strongly skewed Th2 response in the granulomas developing in the TLR9^{-/-} mice was the presence of M2 macrophages. They express unique markers such as FIZZ1, which are not expressed by M1 macrophages (31). It has been proposed that the *in vivo* induction of FIZZ1 in macrophages depends on IL-4 (32). Additionally, arginase production is significantly increased in M2-polarized macrophages. This enzyme blocks iNOS activity by a variety of mechanisms, including competing for the arginase substrate that is required for NO production and needed for the successful killing of invading pathogens (33). We have demonstrated in this study that the lungs of TLR9^{-/-} mice showed decreased iNOS expression and increased expression of FIZZ1 and arginase-1, which is characteristic of M2 skewing. FIZZ1 directly induces fibroblast differentiation and increases expression of α -SMA and has an antiapoptotic effect on mouse lung fibroblasts, which can affect the survival of the myofibroblast in the context of the pulmonary fibrotic response (32, 34, 35). Our data establish that FIZZ1 is an important molecule during the development of a Th2-dependent pulmonary granuloma. Recent evidence suggests that *in vivo* gene transfer of FIZZ1 in the chronic hypoxia model of pulmonary hypertension enhanced the vascular remodeling, including thickening and muscularization of small arterioles (36). This report agrees with our findings showing that FIZZ1 administration *in vivo* during the initiation of the Th2 granuloma significantly increases collagen deposition. Determining the mechanism may reveal a potential clinical target for granuloma disease and for pulmonary hypertension.

A number of studies have shown that TGF- β promotes collagen synthesis, fibroblast proliferation, and transdifferentiation into myofibroblasts (37, 38). In our Th2-driven granuloma model, there is no significant difference in TGF- β levels between WT and TLR9^{-/-} mice (data not shown). IL-13 can stimulate fibroblast collagen production independently of TGF β (39, 40) and can stimulate production of FIZZ1 (32). We believe that enhanced collagen deposition in TLR9^{-/-} mice is TGF- β independent, whereas IL-13 is a key cytokine in this experimental system that contributes to the fibrotic changes found in the developing granulomas.

Chemokines constitute a family of structurally related chemotactic cytokines that direct the migration of leukocytes throughout the body under physiological and inflammatory conditions (41). CCL20 and CCL22 play a role in the recruitment of immature DCs and their precursors to sites of potential antigen entry (16, 17). The lower expression of CCL20 and CCL22 during granuloma development in TLR9-deficient lungs may contribute to the decreased DC numbers observed during pulmonary granuloma formation. In addition, lungs from TLR9^{-/-} mice were found to express lower levels of CXCL9, CXCL10, and CXCL11, which are all ligands for CXCR3 on Th1 cells (18). These chemokine-related data might explain the impaired DC migration and decreased Th1 cytokine production in TLR9-deficient lungs during the evolution of the Th2-dependent pulmonary granuloma. Using confocal laser microscopy, we demonstrated a larger difference in DC and macrophage populations in the granulomas. However, the percentage difference of DCs and macrophages in our flow

data using whole lung tissue appears small, which may be due to the fact that the confocal data used intact lung lesions, whereas the flow data used entire lung tissue.

CXCL10 has been shown to be produced by M1 macrophages, whereas CCL17 has been shown to be produced by M2 macrophages (42). CCL17 is an effector molecule that inhibits M1 macrophage generation and Th1 cytokine (IFN- γ) production (19). The above published reports agree with our findings that CCL17 expression was enhanced in TLR9^{-/-} mice compared with WT mice as the Th2 granuloma develops.

In summary, we present a comprehensive *in vivo* analysis of TLR9 participation in a granuloma model induced by Th2-skewing antigens. TLR9 deficiency resulted in an accelerated granulomatous response, a decreased Th1 profile, and an increased Th2 cytokine profile during pulmonary granuloma formation. This increased Th2 cytokine environment induced a M2 macrophage phenotype characterized by the overexpression of FIZZ1, an M2 marker protein. FIZZ1 was demonstrated to possess an important function because the administration of recombinant FIZZ1 *in vivo* induced enhanced collagen deposition in granulomas developing in TLR9^{-/-} and WT mice. Furthermore, the transfer of WT BM-derived DCs inhibited much of the augmented inflammatory response during granuloma formation in the TLR9^{-/-} mice. This study supports the concept that a more clear understanding of chronic lung inflammation maintained by a Th2 response may provide mechanistic information to support a strategy to treat a number of enigmatic chronic lung diseases.

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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