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TLR2-Mediated Production of IL-27 and Chemokines by Respiratory Epithelial Cells Promotes Bleomycin-Induced Pulmonary Fibrosis in Mice

Hye Sung Kim,*† Heonjeong Go,* Shizuo Akira,‡ and Doo Hyun Chung*†

Idiopathic pulmonary fibrosis is a fatal disease characterized by progressive destruction of the lung parenchyma. The fibrosis, which is initiated by inflammatory consequence to lung tissue injury, induces the massive production of collagen in the alveolar septa and ultimately destroys pulmonary function (1, 2). During pulmonary fibrosis, various immune cells infiltrate the lung tissues, which have a complicated cytokine network that regulates inflammation and fibrosis (3). Th1-type cytokines, such as IFN-γ, prevent fibroblast activation and pulmonary fibrosis (4, 5), whereas Th2-type cytokines, such as IL-4 and IL-13, promote these processes (6, 7). Mice deficient in myeloid differentiation factor 88 (MyD88), an adaptor protein in the signal transduction pathway mediated by IL-1 and TLRs, exhibit a profound defect in the activation of Ag-specific Th1 immune responses, but not in Th2 immune responses, suggesting that TLR signaling has a critical role in balancing Th1/Th2 responses (8).

The TLRs are a family of conserved innate immune receptors that function in the first line of defense against pathogens by recognizing microorganisms-associated molecular pattern (9). These receptors also sense tissue injury by recognizing damage-associated molecular patterns of endogenous ligands generated during inflammation and cell damage (10). Therefore, TLRs has been proposed to regulate pulmonary fibrosis by sensing alveolar epithelial cell damage. Consistent with this suggestion, several studies have shown that TLRs expressed in lung tissues regulate pulmonary fibrosis (11–14).

TLR2, a TLR expressed on the surface of various types of cells, recognizes conserved structure motif, including lipoteichoic acid (LTA), found on Gram-positive bacteria (15). Stimulation of respiratory epithelial (RE) cells with TNF-α, IFN-γ, or corticosteroid significantly increases the expression of TLR2 on the membranes of these cells (16–18), suggesting that TLR2 expression on RE cells might be regulated by tissue damage–mediated alteration to the cytokine milieu of the lungs. However, the functional role of TLR2 on RE cells has not been investigated in pulmonary fibrosis. Recently, Yang et al. (11) demonstrated that bleomycin-induced inflammation and fibrosis are attenuated in TLR2-deficient mice and in wild type (WT) mice treated with TLR2-neutralizing mAb, suggesting that TLR2 promotes bleomycin-induced pulmonary fibrosis (BIPF). However, in their comparison of the immune response phenotypes of WT and TLR2-targeted mice during BIPF, they did not examine the mechanistic details of TLR2-mediated regulation of BIPF.

In this study, we sought to identify the lung cell type with a critical role in TLR2-mediated regulation of bleomycin-induced tissue injury and to examine the mechanism by which TLR2 promotes pulmonary fibrosis. Our results demonstrate that TLR2-mediated signaling induces the production of IL-27 and chemokines by RE cells, rather than immune cells, and that these effects enhance BIPF by inhibiting IL-17 production and stimulating the recruitment of inflammatory cells into the lungs.
Materials and Methods

**Mice**

C57BL/6 (B6) mice were purchased from the Orient Company (Seoul, Korea) and Jackson Laboratory (Bar Harbor, ME). TLR2^{-/-} mice were a gift from Shizuo Akira (Osaka University, Osaka, Japan). IL-17A^{-/-} mice (C57BL/6 background) were obtained from Y. Iwakura (University of Tokyo, Tokyo, Japan). Mice were bred and maintained in a specific pathogen-free environment at the Clinical Research Institute of Seoul National University Hospital (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International) or the Center of Animal Resource Development of Seoul National University College of Medicine. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute of Seoul National University Hospital.

**Induction of pulmonary fibrosis using bleomycin**

BIPF was induced by the instillation of a 50-μl bolus of bleomycin (Nippon Kayaku, Tokyo, Japan) into the trachea of young mice (8–10 wk old). For experiments using non-bone marrow (BM)-chimeric mice and for survival studies with BM-chimeric mice, bleomycin was dissolved in sterile PBS to produce a total dose of 2 mg bleomycin per kilogram body weight. For other experiments using BM-chimeric mice, the bleomycin dose was 1 mg/kg body weight. Mice were anesthetized with 2,2,2-trifluoroethanol (Sigma-Aldrich, St. Louis, MO) before dosing with bleomycin. Seven, 14, or 21 d after the administration, the bleomycin were sacrificed, and the bronchoalveolar lavage fluid (BALF) and lung tissues were examined.

**Bronchoalveolar lavage**

After the mice were sacrificed, a 1-ml syringe containing 1 ml cold PBS was inserted into the exposed trachea. The PBS was injected, aspirated back into the syringe, and then after 3 cycles. This process was then repeated four times, and the five individual samples were pooled. After the pooled samples were centrifuged at 2000 rpm for 10 min at 4˚C, the supernatant fractions were used for cytokine measurements. The pellets were reserved for the determination of total cell numbers and for flow cytometric analysis.

**Histologic examination and immunohistochemistry**

To examine lung tissues for histologic alterations, whole lungs were fixed in 10% formalin, embedded in paraffin, and sectioned onto slides. The slides were stained with H&E and scored for the extent of pathology on a scale of 0 to 5, where 0 was defined as no lung abnormality, and 1, 2, 3, 4, and 5 were defined as the presence of inflammation involving 10%, 10–30%, 30–50%, 50–80%, or >80% of the lungs, respectively.

For immunohistochemistry, the lung sections were deparaffinized with xylene for 20 min and thoroughly dehydrated with serially diluted ethanol. Immunohistochemical staining was performed using an Envision kit (Dako, Ely, U.K.) containing a peroxidase-conjugated anti-mouse Ig polymer. mAbs against TLR2 (1:100 dilution; NovusBio, Littleton, CO) were used as primary Ab. To visualize the reaction, diaminobenzidine was used for 5 min.

**Treatment of mice with TLR2 agonists in vivo**

Five micrograms of LTA (Sigma-Aldrich) or Pam3csk4 (Invivogen, San Diego, CA) was injected i.p. into the mice immediately after bleomycin instillation.

**Treatment of cultured cells with TLR2 agonists, recombinant IL-27, MyD88-inhibitory peptide, and concanavalin A**

For in vitro experiments, cells in culture were treated with 1 μg LTA or 100 ng Pam3csk4 for 24 h. MyD88 signaling was inhibited by the addition of an MyD88-inhibitory peptide or control peptide (both from Invivogen) into the culture medium at 20 μM. Cells in BALF obtained from mice sacrificed 7 d after bleomycin injection were incubated with concanavalin A (1 μg/ml) or recombinant mouse IL-27 (100 ng/ml; Prospeck-Tany, Rehovot, Israel), or both, for 24 h.

**Quantitation of hydroxyproline in the lungs**

Whole lungs were obtained 21 d after bleomycin injection, homogenized in 2 ml PBS, and dried in a vacuum dryer for 6 h. The dried powder for each lung was mixed with 1 ml 6 N HCl, incubated overnight at 110˚C, and filtered through a 45-μm syringe filter (Millipore, Bedford, MA). A 50-μl aliquot of the filtered sample was mixed with 50 μl citrate-acetate buffer and 1 ml chloramine T solution for 15 min at room temperature. Next, 1 ml Ehrlich’s solution was added, and the mixture was incubated for 15 min at 65˚C to allow the conversion of pyrrole to a red chromogen. This chromogen was detected spectrophotometrically at 550 nm used as a measure of the amount of hydroxyproline in the lung. All reagent used in the preparation of citrate-acetate buffer prepared as 5 g citric acid, 1.2 ml glacial acetic acid, 7.24 g sodium acetate, 3.4 g NaOH, and 98 ml distilled water and Ehrlich’s solution prepared as 4.5 g p-dimethlamino-benzaldehyde, 18.6 ml n-propanol, and 7.8 ml 70% perchloric acid were purchased from Sigma-Aldrich, with the exception of n-propanol, which was purchased from Junsei Chemical (Tokyo, Japan).

**Real-time PCR quantitation of cytokine expression**

Total RNA was isolated from mouse lungs using an RNaseasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA yield and purity were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The total RNA was reverse-transcribed into cDNA and amplified with MMLV-RT Taq polymerase (Promega, Madison, WI). Gene-specific PCR was performed using primers and probes from Applied Biosystems (Foster City, CA) and followed using an Applied Biosystems 7500 Sequence Detection System. The results for each cytokine were normalized to that for GAPDH expression in the sample. The following TaqMan assay reagents from Applied Biosystems were used: for GAPDH, 4352339E; for TLR2, Mm00442346_m1; for IL-6, Mm99999064_m1; for IL-17, Mm00439619_m1; for IL-23, Mm00518984_m1; for IRF-2, Mm0461164_m1; for MIP-1α, Mm9999057_m1; for MCP-1, Mm_99999056_m1; and for IL-10, Mm0045235_m1; and for RANTES, Mm_01302428_m1.

**ELISAs**

To quantify cytokines and chemokines, ELISAs were performed using kits from BD Pharmingen (for MCP-1, IL-4, IFN-γ, IL-10, and TGF-β; San Diego, CA) and R&D Systems (for all others; Minneapolis, MN) according to the manufacturer’s instructions. Colorimetric reactions were neutralized using 3 N HCl, and the sample absorbance at 450 and 570 nm was measured on a Biotek Powerwave HT 96/384 Microplate reader (BioTek Instruments, Colmar, France).

**Isolation of epithelial cells from mouse lung**

For isolation of alveolar RE cells, lungs were perfused via the pulmonary circulation with sterile PBS, lavaged three times with PBS, and then filled (2 ml per lung) via the airway with RPMI 1640 with 2.5% FBS (HyClone Laboratories, Logan, UT), 80 U elastase (Worthington Biochemical, Lorne Diagnostics; Reading, U.K.), and 0.05 mg/ml trypsin (Sigma-Aldrich). After 20 min incubation at 37˚C, lungs were homogenized in a 7 ml homogenizer (Wheaton Scientific, Millville, NJ). After the homogenate was centrifuged at 2000 rpm, the supernatant fraction containing the cell suspension was layered on top of a sterile, discontinuous Percoll (GE Healthcare, Little Chalfont, U.K.) gradient and centrifuged for 20 min at 1˚C and 1500 rpm. The cells at the interface between Percoll layers were removed and cultured in plates coated with anti-Fc receptor mAb (BD Pharmingen) at 37˚C for 20 min. Next, the nonadherent cells were removed, centrifuged, and resuspended in DMEM containing with 10% FBS and 1% penicillin-streptomycin (Life Technologies BRL, Paisley, U.K.). To determine the purity of the isolated RE cells, the cells were stained with PE-conjugated anti-mouse pan-cytokeratin mAb (Abcam, Cambridge, U.K.) and fluorescein isothiocyanate-conjugated anti-mouse FcγRIII/II receptor mAb (BD Pharmingen). Flow cytometric analysis of the stained cells established the purity of the RE cell preparation as >90%.

**Western blot analysis**

Mice were sacrificed 7 d after bleomycin administration with or without LTA or Pam3csk4 injection. To estimate the level of IRF1, IRF3, and IRF7 gene transcription, the proteins were eluted from the cells using extraction reagent (GuenDepot, Barker, TX) and analyzed by Western blotting, as described previously (19), with rabbit anti-mouse IFN regulatory factors (IRFs) 1, 3, or 7 (all from Cell Signaling Technology, Beverly, MA) or an mAb against β-actin (control; Sigma-Aldrich). Appropriate HRP-conjugated secondary Abs were then applied, and the detected proteins were visualized using an LAS-4000 Mini imaging system (FujiFilm, Tokyo, Japan).

**Intracellular staining and flow cytometric analysis**

For flow cytometric analysis, cells were incubated with an mAb for 15 min on ice to block FcγRI/III, washed, and stained in a 150-μl reaction mixture containing 0.2% paraformaldehyde, 2% mAb mixture containing mAbs specific for TLR2, F4/80, Gr-1(Ly6C^+Ly6G^-), CD11c, CD4, CD8, and CD8-TCR (BD Biosciences or eBioscience, San Diego, CA). CD14 tetramers were obtained from the Tetramer Core Facility of the National Institutes of...
Health (Bethesda, MD) and used for detection of NKT cells. After the cells were incubated with the Abs for 15 min at 4 °C, they were washed twice with PBS and analyzed by flow cytometry on a BD FACSCalibur instrument (Becton Dickinson, Mountain View, CA).

For intracellular cytokine staining, cells were collected from BALF obtained 7 d after bleomycin administration, incubated with concanavalin A (1 μg/ml; Sigma-Aldrich) for 24 h at 37 °C, surface-stained with mAbs specific for CD4, CD25, or IFN-γ, fixed, and permeabilized with Cytofix/Cytoperm (BD Biosciences or Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Anti-mouse IL-17 and anti-mouse Foxp3 Abs were purchased from BD Biosciences and Miltenyi Biotec, respectively.

Generation of BM-chimeric mice
Chimeric mice were generated by adoptive transfer of donor BM cells into irradiated recipient WT or TLR2−/− mice. BM cells were obtained by flushing the tibias and femurs of donor mice and then depleted of CD4+ and CD8+ T cells using a magnetic bead separation kit (Miltenyi Biotec) according to the manufacturer’s protocol. T cell-depleted BM cells (1 × 10^6 cells per mouse) were injected into the tail veins of recipient mice pretreated with a lethal dose (800 Gy) of radiation. Bleomycin was administered to the chimeric mice 8–12 wk after BM cell transplantation.

Neutralization of IL-17 and IL-27 in vivo and injection of chemokine mixture
To neutralize IL-17 and IL-27 in vivo, mice were injected i.p. with anti-mouse IL-17 mAb (100 μg per mouse) or anti–IL-27 mAb (200 μg per mouse), respectively, immediately after bleomycin administration. Monoclonal rat IgG2a and goat IgG were used as negative controls for anti–IL-17 and anti–IL-27 mAbs, rat IgG2a, and goat IgG were obtained from R&D Systems. For the functional effects of chemokines in vivo, mice were injected i.p. with a mixture of recombinant mouse MIP-1α, MCP-1, and IP-10 (100 μg per mouse; all from R&D Systems) after bleomycin administration.

Depletion of CD4+CD25+ regulatory T cells in mice
To deplete CD4+CD25+Foxp3+ regulatory T (Treg) cells, WT and TLR2−/− mice were injected i.v. with 100 μg anti-CD25 mAb (3C7) or rat IgG2c,κ isotype control (both from BD Pharmingen, San Diego, CA) 48 h before bleomycin administration.

Statistical analysis
Statistical analysis was performed using the Prism 4.0 program (GraphPad Software, San Diego, CA). Unpaired t tests were used for two-group comparisons. Paired one-way ANOVA tests were used for multigroup comparisons. Differences were considered statistically significant at p < 0.05.

Results
BIPF is attenuated in TLR2-deficient mice
To investigate whether TLR2 is involved in the development of pulmonary fibrosis, we examined the effects of intratracheally instilled bleomycin on pulmonary inflammation and fibrosis in WT and TLR2−/− mice. Kinetic analysis of BALF revealed that the number of cells in the lungs of WT mice reached a peak 1 wk after bleomycin administration and then gradually decreased, and it was greater than the number of cells in TLR2−/− mouse lungs (Fig. 1A).

Subset analysis of BALF obtained 7 d after bleomycin administration revealed that the numbers of T, B, NK, and NKT cells, F4/80+ macrophages, CD11c+ dendritic cells, and Gr-1+ granulocytes were all higher in BALF from WT mice than in BALF from TLR2−/− mice (Fig. 1B). Histologic examination of WT mouse lungs 7 d after bleomycin injection demonstrated multifocal fibrotic pulmonary lesions with thickened alveolar septa, collapsed alveolar spaces, and massive infiltration of immune cells. In contrast, inflammation and structural alteration in the lungs were decreased in TLR2−/− mice (Fig. 1C, 1D). The amount of

**FIGURE 1.** TLR2-mediated signals promote BIPF. Lung and BALF were obtained from WT or TLR2−/− mice 7, 14, and 21 d after the intratracheal instillation of bleomycin (BLM) or PBS (vehicle control) and analyzed for signs of BIPF. A, BALF obtained from WT and TLR2−/− mice on days 7, 14, and 21, and the number of cells in the BALF was determined. B, Subset analysis of BALF cells obtained from WT and TLR2−/− mice on day 7. C, Histologic examination of WT and TLR2−/− mouse lungs removed on day 7. H&E-stained paraffin sections are shown. Original magnification ×100. D, The extent of inflammation and structural alteration in the pulmonary lesion was scored. E, Hydroxyproline content of lungs removed from WT and TLR2−/− mice on day 21. F and G, Effect of TLR2 agonists on BALF cell numbers (F) and hydroxyproline content (G) in WT and TLR2−/− mice. Mice were injected i.p. with LTA or Pam3csk4 before BLM or PBS administration. F, BALF cell numbers were estimated on days 7, 14, and 21. G, The hydroxyproline content was determined on day 21. A–G, Data shown represent means ± SEM for one experiment (n = 4 per group in A, B, F, and G; n = 3 per group in C and D; n = 6 per group in E) of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant.
hydroxyproline, a collagen component, was significantly lower in TLR2−/− mouse lungs than in WT mouse lungs 21 d after bleomycin injection (Fig. 1E). Furthermore, treatment of mice with TLR2 agonists, such as LTA or Pam3csk4, during BIPF increased the number of cells and the hydroxyproline level in the lungs of WT mice (Fig. 1F, 1G) but not those of TLR2−/− mice. Thus, BIPF is attenuated in TLR2-deficient mice compared with WT mice, indicating that TLR2 promotes BIPF.

TLR2 expression on nonhematopoietic cells, rather than immune cells, of the lung contributes to BIPF

To examine the pattern of TLR2 expression in the lungs, RE cells from the lungs (Fig. 2A, 2B) and immune cells from the BALF of WT mice (Fig. 2C, 2D) with BIPF were examined. Real-time PCR analysis revealed that the TLR2 mRNA transcript levels in RE and BALF immune cells gradually increased after bleomycin administration, peaked at 7 d, and then gradually decreased (Fig. 2A, 2D). In flow cytometric analysis, TLR2 expression was detected on both RE and immune cells of lungs from mice with BIPF (Fig. 2B, 2E). Of the BALF immune cells, notable levels of TLR2 were detected on the surfaces of F4/80+ macrophages, Gr-1+CD11b+ cells, CD11c+ dendritic cells, and B220+ B cells but not on CD4+, CD8+, γδ, or α-GalCer/CD1d tetramer+ NKT cells (Fig. 2E). In immunohistochemical analysis, no cellular elements of the lung from WT mice expressed TLR2 except bronchial epithelial cells before bleomycin injection (Fig. 2C). However, TLR2 expression was upregulated in bronchial epithelial and immune cells of the lungs from WT mice during BIPF, whereas fibroblasts, myofibroblasts, and endothelial cells showed minimal expression of TLR2 (Fig. 2C). Moreover, a small number of alveolar epithelial cells expressed TLR2 during BIPF. In contrast, TLR2 was barely detected in the lungs of TLR2−/− mice during BIPF.

To determine which of the TLR2-expressing lung cell types function in TLR2-mediated stimulation of BIPF, we generated BM chimeric mice by irradiating WT and TLR2−/− mice and reconstituting their hematopoietic cells using the BM of WT or TLR2−/− mice. In the BIPF model, the levels of hydroxyproline and TGF-β in the lungs of WT mice reconstituted with WT or TLR2-deficient
BM (WT→WT and TLR2<sup>−/−</sup>→WT chimeras) were higher than those of TLR2<sup>−/−</sup> mice treated with WT or TLR2-deficient BM (WT→TLR2<sup>−/−</sup> and TLR2<sup>−/−</sup>→TLR2<sup>−/−</sup> chimeras; Fig. 2F, 2G). Whereas high-dose administration of bleomycin killed half of the WT→WT and TLR2<sup>−/−</sup>→WT chimeric mice within 8 d, all WT→TLR2<sup>−/−</sup> and TLR2<sup>−/−</sup>→TLR2<sup>−/−</sup> chimeric mice remained alive at 8 d (Fig. 2H). These findings indicate that TLR2 expression on nonhematopoietic cells, rather than immune cells, of the lungs exacerbates BIPF.

**TLR2-mediated signals in RE cells suppress the production of IL-17, a BIPF inhibitor**

To explore the mechanisms by which TLR2-mediated signaling promotes BIPF, we evaluated the levels of various cytokines in the BALF of WT and TLR2<sup>−/−</sup> mice 7 d after bleomycin administration (Fig. 3A). Relative to WT mice, the TLR2<sup>−/−</sup> mice exhibited a higher level of IL-17, a lower level of TGF-β, and similar levels of IL-4, IL-10, and IFN-γ. Moreover, injection of the mice with LTA or Pam3csk4 increased the TGF-β level and decreased the IL-17 level in the BALF of WT mice but not in that of TLR2<sup>−/−</sup> mice (Fig. 3B). Consistent with the IL-17 results, the percentages of IL-17–producing CD4<sup>+</sup> and γδ T cells in the BALF were higher in TLR2<sup>−/−</sup> mice with BIPF than in WT mice with BIPF. LTA or Pam3csk4 treatment of WT mice reduced these percentages even further (Fig. 3C).

Because these results suggested that IL-17 might be involved in TLR2-mediated regulation of BIPF, we i.p. injected WT and TLR2<sup>−/−</sup> mice with an anti–IL-17 mAb. This blockade of IL-17 significantly increased the lung levels of hydroxyproline and TGF-β in TLR2<sup>−/−</sup> mice, but not in WT mice, as compared with injection of a control Ab (Fig. 3D). Furthermore, the lung hydroxyproline and BALF TGF-β levels during BIPF were higher in IL-17A<sup>−/−</sup> mice than in WT mice (Fig. 3D). These findings indicate that IL-17 production in the lungs suppresses TLR2-mediated promotion of BIPF.

When we analyzed IL-17 expression levels in the BALF of BM-chimeric mice with BIPF, we found that they were lower in WT→WT and TLR2<sup>−/−</sup>→WT chimeras than in WT→TLR2<sup>−/−</sup> and TLR2<sup>−/−</sup>→TLR2<sup>−/−</sup> chimeras (Fig. 3E), indicating that IL-17 production in the lungs during BIPF is regulated by TLR2 expression on nonhematopoietic cells, rather than immune cells. Our findings suggest that signaling mediated by TLR2 on pulmonary nonhematopoietic cells promotes BIPF by inhibiting IL-17 production by CD4<sup>+</sup> and γδ T cells in the lungs.

**TLR2-mediated production of IL-27 by RE cells inhibits IL-17 production, enhancing BIPF**

To explore how TLR2-mediated signaling in nonhematopoietic cells of the lungs inhibits IL-17 production during BIPF, we evaluated the transcription levels of IL-17–related cytokines in the

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**FIGURE 3.** TLR2 signaling mediated by RE cells regulates the production of IL-17 that inhibits BIPF. A, BALF was obtained from WT or TLR2<sup>−/−</sup> mice 7 d after bleomycin (BLM) administration and analyzed for levels of cytokines including IL-17 and TGF-β using ELISA. B, Same as A except that mice were pretreated with LTA or Pam3csk4 before BLM administration. C, Percentages of IL-17–producing CD4<sup>+</sup> and γδ T cells in the BALF of WT and TLR2<sup>−/−</sup> mice pretreated or not pretreated with LTA or Pam3csk4 before BLM administration. Numbers shown indicate the percentages of IL-17-positive cells as indicated by intracellular staining. D, Effect of blockade of IL-17 on TGF-β and hydroxyproline levels in the BALF of WT or TLR2<sup>−/−</sup> mice with BIPF. BALF was analyzed for the levels of TGF-β levels in BALF on day 7 using ELISA and for the amount of hydroxyproline in the lungs on day 21 in WT and TLR2<sup>−/−</sup> mice injected with control Ab or anti–IL-17 mAb, and IL-17A<sup>−/−</sup> mice. E, BM-chimeric mice were generated and treated with BLM as described above. IL-17 mRNA and protein levels in whole lungs and BALF on day 7 were estimated using real-time PCR and ELISA, respectively. Data shown represent mean ± SEM for each group (n = 4 per group in A, B, D; n = 3 per group in C; n = 5 per group in E) for one representative experiment of three or four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant.
lungs of WT and TLR2\(^{-/-}\) mice with BIPF. Levels of IL-27 and IL-6 mRNA were lower in the lungs of TLR2\(^{-/-}\) mice than in those of WT mice, whereas the levels of IL-23 mRNA were similar in the two groups (Fig. 4A). Although IL-6 is known to induce Th17 differentiation (20), it appears to exert a proinflammatory effect on TLR2-mediated immune regulation, rather than an inductive effect on IL-17 production, based on lower levels of IL-6 in the lungs of TLR2\(^{-/-}\) mice. Because IL-27 is known to inhibit Th17 differentiation during various immune responses, we hypothesize that TLR2-mediated IL-27 production by nonhematopoietic lung cells inhibits IL-17 production during BIPF. Consistent with this hypothesis, IL-27 levels in BALF during BIPF were higher in WT→WT and TLR2\(^{-/-}\)→WT chimeras than in WT→TLR2\(^{-/-}\) and TLR2\(^{-/-}\)→TLR2\(^{-/-}\) chimeras (Fig. 4B).

The difference in BALF IL-27 levels between WT and TLR2\(^{-/-}\) mice during BIPF was enhanced by pretreatment of mice with LTA or Pam3csk4; this treatment further increased the amount of IL-27 in the BALF of WT mice but not TLR2\(^{-/-}\) mice (Fig. 4C). Furthermore, pretreatment with LTA or Pam3csk4 enhanced the expression of IFN regulatory factors (IRFs) 1, 3, and 7 in the lungs of WT mice, but not in those of TLR2\(^{-/-}\) mice (Fig. 4D). The pattern of TLR2 expression we observed in the lungs suggested that RE cells might be a major subset of the nonhematopoietic cells responsible for the production of IL-27 during BIPF. Bleomycin stimulation of purified RE cells from WT mice enhanced their production of IL-27, which was further increased by the addition of LTA or Pam3csk4 to the culture medium (Fig. 4E). This effect was decreased by the addition of a MyD88-inhibitor peptide to the culture medium, as compared with a control peptide (Fig. 4E). However, bleomycin and TLR2-agonist treatment of RE cells purified from TLR2\(^{-/-}\) mice did not alter TLR2-mediated IL-27 production in these cells. These findings suggest that RE cells produce IL-27 during BIPF in a manner dependent on MyD88 and the IRF complexes.

Next, to investigate whether TLR2-mediated induction of IL-27 production by RE cells inhibits IL-17 production by T cells, we measured IL-17 levels in the supernatant fractions of cocultures of RE and lung immune cells. Upon stimulation with LTA and bleomycin, the amount of IL-17 in the supernatant fractions from cocultures of WT RE cells and WT or TLR2-deficient immune cells was lower than that from cocultures of TLR2\(^{-/-}\) RE cells and WT or TLR2-deficient immune cells (Fig. 4F). This difference was enhanced by the addition of a neutralizing anti–IL-27 mAb to the cocultures of WT RE cells and TLR2-deficient immune cells (Fig. 4G). The difference in BALF IL-27 levels between WT and TLR2\(^{-/-}\) mice during BIPF was enhanced by pretreatment of mice with LTA or Pam3csk4; this treatment further increased the amount of IL-27 in the BALF of WT mice but not TLR2\(^{-/-}\) mice (Fig. 4C). Furthermore, pretreatment with LTA or Pam3csk4 enhanced the expression of IFN regulatory factors (IRFs) 1, 3, and 7 in the lungs of WT mice, but not in those of TLR2\(^{-/-}\) mice (Fig. 4D).
cultures; blockade of IL-27 decreased the IL-17 level in cocultures with WT but not TLR2-deficient RE cells (Fig. 4F). Furthermore, the injection of anti–IL-27 mAb into WT mice increased the amount of IL-17 in BALF and the percentages of IL-17–producing CD4+ and γδ T cells in the lungs, whereas it reduced the lung levels of hydroxyproline and TGF-β (Fig. 4G, 4H). In contrast, injection of anti–IL-27 mAb into TLR2−/− mice only minimally affected these parameters. Alternatively, recombinant mouse IL-27 reduced both the BALF IL-17 level and the percentages of IL-17–producing CD4+ and γδ T cells in cultured BALF immune cells from TLR2−/− mice, whereas it did not significantly alter these parameters in WT mice (Fig. 4I). These findings suggest that TLR2-mediated IL-27 production by RE cells inhibits IL-17 production in the lungs, thereby enhancing BIPF.

TLR2 signaling-induced production of chemokines by RE cells also promotes BIPF by recruiting immune cells into the lungs

Fewer immune cells were found in the lungs of TLR2−/− mice with BIPF than in WT mice with BIPF (Fig. 1A, 1B), suggesting that fewer immune cells were recruited into the lungs of TLR2−/− mice during BIPF. Therefore, we evaluated the mRNA expression levels or various chemokines in the lungs of WT and TLR2−/− mice during BIPF. During BIPF, the levels of MCP-1, IP-10, and MIP-1α mRNA were higher in lungs of WT mice than in those of TLR2−/− mice, whereas the level of RANTES mRNA in the lungs was similar in the two groups (Fig. 5A). Similarly, MCP-1, IP-10, and MIP-1α levels were higher in the lungs of WT→WT and TLR2−/−→WT chimeric mice than those of WT→TLR2−/− and TLR2−/−→TLR2−/− chimeric mice during BIPF (Fig. 5B). These findings suggest that TLR2 signaling mediated by non-hematopoietic cells, rather than immune cells, of the lungs induces MCP-1, IP-10, and MIP-1α production during BIPF. Stimulation of WT RE cells, but not TLR2−/− RE cells, with bleomycin and LTA or Pam3csk4 increased levels of MCP-1, IP-10, and MIP-1α, and this effect was suppressed by MyD88 inhibition (Fig. 5C). Injection of a mixture of recombinant mouse MCP-1, IP-10, and MIP-1α into TLR2−/− mice, but not WT mice, increased the hydroxyproline content in the lungs during BIPF (Fig. 6A, 6B), whereas it only minimally affected IL-27 and IL-17 levels in the lungs of WT or TLR2−/− mice (Fig. 6B, 6C). However, this chemokine mixture increased immune cell numbers in both WT and into TLR2−/− mice (Fig. 6A). These findings indicate that

**FIGURE 5.** RE cells produce chemokines in response to TLR2 signaling during BIPF. A, Seven days after the intratracheal administration of bleomycin (BLM) to WT and TLR2−/− mice, chemokine mRNA levels in the lungs were evaluated using real-time PCR. B, Same as A, except that BM-chimeric mice were used. C, Purified RE cells from WT and TLR2−/− mice were incubated with BLM in the presence or absence of LTA or Pam3csk4 for 24 h. MyD88-mediated signaling was suppressed by the addition of MyD88-inhibitory peptide to the culture medium, and levels of MIP-1α, IP-10, and MCP in the supernatant fraction were evaluated. Data represent mean ± SEM for each group (n = 4 per group in A; n = 5 per group in B; n = 3 per group in C) for one representative experiment of three or four independent experiments. *p < 0.05, **p < 0.01. ns, not significant.
TLR2-induced chemokine production by RE cells recruits immune cells into the lungs, thereby promoting BIPF through a second, additional mechanism.

**TLR2-dependent Treg cells have minimal inhibitory effect on Th17 cells in the lungs, although Treg cells attenuate BIPF**

In several in vivo studies, TLR2 has been demonstrated to control the expansion and function of Treg cells (21–23), which inhibit Th17 cells in the lungs, although Treg cells attenuate BIPF. To address this hypothesis, we measured numbers of Treg cells and levels of cytokines and hydroxyproline in the lungs of WT and TLR2−/− mice. Numbers of Treg cells in the lungs were greater in the WT mice than in the TLR2−/− mice, although the percentage of Treg cells in the lungs was higher in the TLR2−/− mice (Fig. 7A). When we depleted Treg cells in WT and TLR2−/− mice using an anti-CD25 mAb before bleomycin administration, TGF-β levels, cell numbers, and hydroxyproline levels in the lungs increased in both WT and TLR2−/− mice (Fig. 7B, 7C). In contrast, the IL-17 and IL-27 levels in BALF and the percentages of IL-17–producing T cells (C) were also evaluated. Data represent mean ± SEM for each group (n = 3 per group) for one representative experiment of three or four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant.

**Discussion**

To explore TLR2-mediated regulatory effects in BIPF, we examined the pattern of TLR2 expression in the lungs and identified the lung cell type critical to the development of BIPF. In our experiments, both RE and immune cells exhibited increased TLR2 expression during BIPF in WT mice, and pulmonary fibrosis during BIPF was attenuated in TLR2−/− mice relative to WT mice. Among RE cells, bronchial epithelial cells highly expressed TLR2 compared with alveolar epithelial cells, but other non-hematopoietic cells such as fibroblasts, myofibroblasts, and endothelial cells showed minimal expression of TLR2 during BIPF, as analyzed using immunohistochemistry. Furthermore, BIPF was enhanced by TLR2-signaling mediated by RE cell, rather than immune cells, of the lungs. Therefore, these findings suggest that bronchial epithelial cells might be a major subset of the lung in TLR2-mediated regulation of BIPF. Consistent with these results, RE cells are known to have critical roles in the development and progression of pulmonary fibrosis (25). However, two independent research groups have suggested that RE cells contribute only minimally to the TLR2-mediated promotion of pulmonary fibrosis. Yang et al. (11) have proposed that bleomycin-induced tissue injury causes release of HMGB1 protein, which is recognized by TLR2 on immune cells and induces Th2-dominant immune responses that enhance pulmonary fibrosis. However, we observed that BALF levels of Th1 and Th2 cytokines were similar in TLR2−/− mice during BIPF (Fig. 7B, 7C). In contrast, the IL-17 and IL-27 levels in BALF and the percentages of IL-17–producing T cells (C) were also evaluated. Data represent mean ± SEM for each group (n = 3 per group) for one representative experiment of three or four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant.
RE cells does not protect mice from BIPF; rather, it promotes BIPF.

IL-27 is a heterodimeric cytokine of the IL-12 family consisting of p28 and EBI3 (26). TLR3, TLR4, and TLR7/8 signaling induce the expression of EBI3 and p28 in macrophages or dendritic cells in a MyD88–, NF-κB–, IRF-1–, and IRF-3–dependent manner (27–30). In humans, IL-27 production by macrophages is also regulated by IFN-α in an autocrine manner (31). However, the administration of TLR2 agonists to human macrophages fails to stimulate IFN-α production, showing that unlike these TLRs,
TLR2 does not activate p28 gene transcription in these cells (31). Thus, TLR2-mediated signaling in immune cells appears to minimally induce IL-27 production.

In contrast, our experiments produced several pieces of evidence to suggest that TLR2 induces IL-27 production in RE cells during BIPF. First, IL-27 production in the lungs was higher in WT→WT and TLR2+/→WT chimeric mice than in WT→TLR2−/− mice and TLR2−/→TLR2+/− mice, indicating that TLR2 expression on nonhematopoietic cells of the lungs regulates IL-27 production during BIPF. Second, TLR2 ligands enhanced IL-27 production by RE cells of WT mice, but not TLR2−/− mice, in the presence and absence of bleomycin (Figs. 4E, 5C, Supplemental Fig. 1), which was in an MyD88-dependent manner. These findings suggest that TLR2 agonists modulate IL-27–mediated immune responses in the lungs even in the absence of bleomycin-induced injury. However, single intratracheal injection of TLR2 agonists into WT mice minimally modulated inflammation and cytokine environment in the lungs (data not shown), suggesting that sustained stimulation of TLR2 in RE cells for long term or additional environmental modulation such as tissue damage in the lungs might be necessary to TLR2-mediated regulation of pulmonary fibrosis. Third, blockade of IL-27 in vivo decreased the amount of hydroxyproline in the lungs of WT mice, but not of TLR2−/− mice. We have thus demonstrated for the first time that TLR2-mediated IL-27 production by RE cells promotes BIPF.

IL-27 has been demonstrated to inhibit Th17 cells in a manner dependent on the orphan nuclear receptor ROR-γt, to promote Th1 differentiation, and to enhance IL-10 production (32–34). IL-27 thus regulates various immune diseases through its dual proinflammatory and anti-inflammatory effects on immune responses (35). A role for IL-27 in pulmonary fibrosis has been suggested by the demonstration that the serum level of IL-27 is elevated in patients with systemic sclerosis and positively correlates with the extent of pulmonary and dermal fibrosis (36). We observed that levels of IL-17 and IL-27 in the lungs during BIPF were higher and lower, respectively, in TLR2−/− mice than in WT mice, suggesting that IL-27 and IL-17 production in the lungs are inversely regulated by TLR2 on RE cells during BIPF. Consistent with this suggestion, blockade of IL-27 enhanced IL-17 production in a TLR2-dependent manner in vivo and in vitro, indicating that IL-27 produced by RE cells inhibits IL-17 production during BIPF. However, the regulatory effects of TLR2 on Th17 cells have appeared to be contradictory in various studies. In pulmonary fungal infection and brain abscess, TLR2 was reported to act as a negative regulator for Th17 cells (24, 37), whereas in experimental autoimmune encephalomyelitis, skin inflammation, and tuberculosis infection, TLR2 was reported to have the opposite effect (38–40). These findings suggest that the specific effect of TLR2 on Th17 cells varies with the type of immune response and depends on the microenvironment of the target tissues. Therefore, our experiments suggest that bleomycin-induced tissue damage in the lungs might produce a microenvironment that induces TLR2 to inhibit Th17 cells by inducing IL-27 production.

In our experiments, IL-17 produced by CD4+ and γδ T cells suppressed BIPF, and IL-17 production was inhibited by the TLR2 signaling-induced production of IL-27 by RE cells. Consistent with our results, γδ T cells in the lungs of WT mice were found to predominantly produce IL-17, and the amount of hydroxyproline in the lungs increased in γδ T cell-deficient mice during BIPF (41). In contrast, in a recent study, IL-17A produced by CD4+ and γδ T cells promoted bleomycin and IL-1β–mediated pulmonary fibrosis (42). Although the functions of IL-17 in BIPF in our study appear inconsistent with this result, CD4+ and γδ T cells were the major cell subsets producing IL-17 during BIPF in both studies. Moreover, the cytokine milieu of pro- and anti-fibrogenic cytokines make critical contributions to the process of fibrosis in target tissues (43). Differences in these milieus might explain the contradictory IL-17–mediated outcomes in BIPF. Further study will be needed to define the exact function of IL-17 in pulmonary fibrosis.

Agonist stimulation of TLR2 causes RE cells to produce IL-6 and IL-8 (16, 44), suggesting that TLR2-mediated signals in RE cells regulate the immune response by regulating the production of various chemokines and cytokines. In our experiments, treatment of RE cells with TLR2 agonists induced the production of MIP-1α, MCP-1, and IP-10, stimulating the recruitment of inflammatory cells into the lungs during BIPF. Although IP-10 has been reported to exert antifibrotic effect on the lungs (45), a chemokine mixture increased the amount of hydroxyproline in the lungs of TLR2−/− mice, but not WT mice, and only minimally affected the production of IL-27 and IL-17 in the lungs of both groups of mice. These results suggest that TLR2 signaling-induced chemokines produced by RE cells promote BIPF by recruiting inflammatory cells into the lungs rather than by regulating the production of IL-27 and IL-17.

In conclusion, the results of our study demonstrate that RE cells produce IL-27 and chemokines in a TLR2-dependent manner that promotes BIPF by inhibiting IL-17 production and recruiting inflammatory cells into the lungs. We suggest that TLR2 regulates pulmonary fibrosis through two axes; RE cells/IL-27/IL-17 and RE cells/chemokines/inflammatory cells (Fig. 8). Our findings provide valuable insight needed for the design of therapeutic approaches for idiopathic pulmonary fibrosis.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental figure 1 RE cells produce IL-27 and chemokines in response to TLR2 agonists. Purified RE cells from WT and TLR2−/− mice were incubated with LTA or Pam3csk4 in the absence of bleomycin for 24 h. The levels of IL-27 and chemokines were estimated. Data shown in represent means ± SEM for each group. The results shown are representative of three or four independent experiments. (** p<0.01, * p<0.05)