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Targeting TLR2 Attenuates Pulmonary Inflammation and Fibrosis by Reversion of Suppressive Immune Microenvironment

Hong-Zhen Yang,² Bing Cui,² Han-Zhi Liu, Zhi-Rong Chen, Hui-Min Yan, Fang Hua, and Zhuo-Wei Hu³

Pulmonary fibrosis is a consequence of chronic lung injury and is associated with a high mortality. Despite the pathogenesis of pulmonary fibrosis remaining as an enigma, immune responses play a critical role in the deregulation of wound healing process after lung injury, which leads to fibrosis. Accumulating evidence argues the rationales for current treatments of pulmonary fibrosis using immunosuppressive agents such as corticosteroids. In this study, we report that bleomycin (BLM), a well-known fibrogenic agent functioning as a TLR2 agonist, induced the maturation of dendritic cells and release of cytokines. The BLM activation of TLR2 mediated a time-dependent alteration of immune responses in the lung. These responses resulted in an increase in the tissue-infiltrating proinflammatory cells and cytokines in the early period initially following BLM exposure and an increase in the tissue-infiltrating suppressive immune cells and factors during the later period following BLM exposure. TLR2 deficiency, however, reduced pulmonary inflammation, injury, and subsequently attenuated pulmonary fibrosis. Targeting TLR2 by a TLR2-neutralizing Ab not only markedly decreased animal death but also protected animals from the development of pulmonary fibrosis and reversed the established pulmonary fibrosis through regulating BLM-induced immunosuppressive microenvironments. Our studies suggest that TLR2 is a promising target for the development of therapeutic agents against pulmonary fibrosis and that eliminating immunosuppressive cells and factors via immunostimulants is a novel strategy for fibro-proliferative diseases. Moreover, combining BLM with an anti-TLR2 Ab or TLR2 antagonist for cancer therapy will improve the BLM therapeutic profile by enhancing anti-cancer efficacy and reducing systemic inflammation and pulmonary fibrosis. The Journal of Immunology, 2009, 182: 692–702.

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4 Abbreviations used in this paper: PAMP, pathogen-associated molecular patterns; BALF, bronchoalveolar lavage fluid; BLM, bleomycin; DAMP, damage-AMP; DCs, dendritic cells; IOD, integrated OD; mDC, marrow-derived DC; pDC, plasmacytoid DC; Treg, regulatory T cell; WT, wide type; MMP, matrix metallopeptidase.
Obviously, these observations strongly argue the rationale for current treatments of pulmonary fibrosis using a group of anti-inflammatory agents and immunosuppressive agents, including corticosteroids and cytotoxic agents (7). Indeed, a great number of studies indicate that suppressing the immune response is unlikely to be a good approach to treat the fibrotic tissue damage that occurs during chronic inflammation (8). Alternatively, strategies directed toward eliminating immunosuppressive cells and cytokines might prove highly beneficial in the context of tissue fibrosis during chronic inflammation, as their elimination would presumably diminish the concomitant chronic inflammatory and fibrotic mechanisms (9). For example, we recently found that either the vaccine bacillus Calmette-Guérin or adjuvant administration of TLR4 agonist can protect experimental hypertensive mice from pressure overload-induced cardiovascular fibrosis via regulation of cardiovascular immune microenvironment (10).

Based on Razonable’s recent work (11) and our recent findings that bleomycin (BLM) is an agonist of TLR2, which mediates BLM-induced inflammation and lung injury, we tested the hypothesis that TLR2 not only mediates BLM-induced systemic inflammation and lung injury but also mediates BLM-induced suppressive immune microenvironments, which is critical to the development of pulmonary fibrosis. We found that TLR2 deficiency or targeting TLR2 not only prevents BLM-induced inflammation, but that it also protects from and reverses progressive pulmonary fibrosis through a reversion of the immunosuppressive microenvironments in the BLM-caused fibrotic tissue. Our results demonstrate that TLR2 is a promising target for the prevention and treatment of pulmonary fibrosis and that targeting immunosuppressive microenvironments using immunomodulators such as TLR2 antagonist is a novel therapeutic strategy for the life-threatening illness of pulmonary fibrosis.

Materials and Methods

Animals and reagents

Male C57BL/6J mice (17 ± 1 g, 6–8 wk) were obtained from the Vital River Laboratory Animal Technology. TLR2−/− and corresponding wild-type (WT) mice were purchased from The Jackson Laboratory. Ultra-pure Es-LPS (from Escherichia coli) and Pam3Cys were obtained from InvivoGen, FITC-, PE-, or PE-cy5-conjugated anti-mouse CD11c, MHCII, CD40, CD80, CD86, TLR2 Abs (mAb), and Stat3 inhibitory peptide were purchased from e Bioscience. The neutralizing TLR2 mAb was purchased from R&D System. The ELISA kits for IFN-γ and TGF-β1 were purchased from e Bioscience. The ELISA kit for HMGB1 was purchased from Adlteram Diagnostic Laboratories. The BLM was purchased from Nippon Kayaku. Smad 3 inhibitor (SIS3) was from Merck-Calbiochem. All other materials were purchased from standard commercial resources.

Preparation of pulmonary fibrosis model

The mice were anesthetized with 50 mg/kg i.p. pentobarbital (Merck). Using an insulin syringe, 50 μl of LPS-free saline or clinical grade LPS (3.0 μg/kg) was directly injected into the trachea as previously described (12). The neutralizing anti-TLR2 or isotype-matched Ab (2 μg/mouse) in 200 μl saline was injected i.v. 1 day before, or on days 7 and 14 after BLM instillation. Mice were then sacrificed by excessive anesthesia for the collection of single-cell suspensions, bronchoalveolar lavage fluid (BALF), and lungs at different times. The lungs were excised and fixed or frozen for morphological evaluation or for the measurement of hydroxyproline content. The lung index was determined by lung weight (mg/body weight g).

Culture and maintenance of dendritic cells (DCs)

Mouse immature DCs (CRL-11904; ATCC) were maintained in α-MEM, supplemented with 20% FBS (BioWhittaker), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.05 mM 2-ME, 100 U/ml penicillin and streptomycin, and 5 ng/ml mouse recombinant GM-CSF as previously described (13). To evaluate the regulatory effects of BLM on the phenotype and activity of DCs, immature DCs were cultured in 6-well plate in a density of 5 × 10^5 cell/ml and BLM (20 μg/ml) was added. To investigate the role of TLR2 in BLM-induced DC maturation, anti-TLR2 or isotype-matched Ab (10 μg/ml) was added 1 h before BLM addition. The neutralizing effects of anti-TLR2 Ab were previously proven (14).

Preparation of lung single-cell suspensions

Single-cell suspensions were prepared from murine lungs as previously described (7) with minor modification. Briefly, lungs were inflated with disperse II, allowed to collapse, and then placed in 1 ml of dispase II, while gently agitating at room temperature for 45 min. Lungs were minced to ~1-mm pieces and resuspended in 2 ml of dispase II containing collagenase IV (2 μg/ml) and DNase (50 μg/ml). Digested lungs were resuspended in DMEM supplemented with 10% FBS and sequentially filtered through 200-μm filters. The expression of various surface molecules and the number of immune cells, such as marrow-derived DCs (mDCs), plasmacytoid DCs (pDCs), M1 type of macrophages (M1 cells), and Treg cells were then analyzed using the lung single-cell suspensions.

Flow cytometry

Surface molecule expression of lung cells and cultured DCs were analyzed using multicolor flow cytometry as described previously (15). In brief, lung cells and cultured DCs were first harvested, washed, and suspended in cold PBS containing 5% FBS and 0.02% NaN3. The cells were then incubated with a mixture of rat and mouse IgG (1:1) to reduce nonspecific binding followed by serial incubations with saturating concentrations of FITC-conjugated mAb, PE-conjugated mAb, and/or PE-cy5-conjugated mAb for 1 h at 4°C. Isotype-matched mAb were used in control samples. After incubation, at least 20,000 stained cells were analyzed using CellQuest software (BD Biosciences). In addition, the levels of various cytokines, such as IL-6, IL-10, IL-13, and TGF-β1, were determined by an intracellular staining method as previously described (8). To prevent cytokine secretion, Monensin (1.7 μg/ml) was added for the final 4 h. The cells were then fixed (2% paraformaldehyde), permeabilized (0.5% saponin or methanol), and stained with FITC-, PE-, or PE-cy5-conjugated mAb or isotype-matched mAb. The fluorescence data was collected and analyzed as described above.

ELISA for HMGB1 and cytokines in BALF

The concentrations of HMGB1, IFN-γ, and TGF-β1 in BALF were detected using ELISA kits in accordance with the manufacturer’s instructions.

Morphological evaluation of lung sections

At the end of the experiment, the lungs were rapidly excised, fixed with 4% paraformaldehyde, and embedded in paraffin for histo-pathological examination. Tissue sections (5-μm thick) were prepared and stained with HE or Masson’s Trichrome. The grades of pulmonary inflammation and fibrosis were analyzed by a professional researcher of pathology, who was blinded for groups. Additionally, expressions of IFN-γ, TGF-β1, IL-13, MCP-1, and the phosphorlyative activity of Stat3 or Smad3 in the lungs were stained with corresponding Abs and were semiquantitatively assessed according to the methods described (9). The average integrated OD (IOD) of the collagen deposition was determined by Image-Pro Plus image analysis software (Media Cybernetics) in 10 randomly chosen regions per tissue sample at a magnification of ×200.

Measurement of lung hydroxyproline

Collagen deposition was determined by assaying total hydroxyproline content of the lung according to revised Reddy GK’s method (16). In brief, the total lungs were hydrolyzed with the 2.5 N NaOH at 120°C 0.1% KOH for 40 min. After neutralization with hydrochloric acid, the hydrolyzates were diluted with distilled water. Hydroxyproline in hydrolyzates was assessed calorimetrically at 550 nm with p-dimethylaminobenzaldehyde. Results were represented as μg per lung.

Statistics

Data was represented as mean ± SE. Statistical analysis was performed with one-way ANOVA, in which α value was set at 0.05, followed by Tukey-Kramer’s or Dunnnett’s post hoc multiple comparison test. The survival rates were analyzed by the Kaplan-Meier method.

Results

TLR2 mediated the BLM-stimulated maturation and function of DCs

Using an in vitro DC functional screening system, we investigated the effects of BLM on DC maturation and function. The precursor
FIGURE 1. BLM induced functional maturation of DCs via activation of TLR2. Immature DCs were pretreated with a neutralizing anti-TLR2 or IgG Ab (10 μg/ml) 1 h before treatment with BLM (20 μg/ml) or Pam3Cys (100 ng/ml). After 24 h, the cells were collected. The expression of the DC surface molecules, including CD11c and MHC class II (A), CD11c and MHC class I (B), CD40 and CD80 (C), and CD40 and CD86 (D), were analyzed using a multicolor flow cytometry. E–G, BLM treatment significantly enhanced the release of IL-6 (E) and IL-12 (G) but not IL-10 from DCs. The levels of IL-6, IL-10, and IL-12 were determined by a standard intracellular flow cytometry. Data were representative of dotplots or overlapped histograms from five independent experiments with identical results.
DCs were exposed to BLM (20 μg/ml), or TLR2 specific agonist Pam3Cys (100 ng/ml), or pg-LPS (data not shown). The morphological phenotypes, such as CD11c, MHC, and costimulatory molecules, were analyzed by flow cytometry. The TLR2 agonist Pam3Cys significantly stimulated the maturation of DCs by enhancing the surface expression of CD11c, MHC class I, MHC

FIGURE 2. TLR2 deficiency prevented the time-dependent alterations of pulmonary immune responses induced by BLM. BLM (3 U/kg) was instillated intratracheally to TLR2-deficient and WT mice. The mice were sacrificed on days 0, 1, 3, 7, 14, 21, and 28. The single-cell lung suspensions were prepared and flow cytometry was used to determine the alterations of pulmonary immune cells or surface molecules, including CD11c (A), MHCII (B), CD40 (C), CD80 (D), CD86 (E), CD11b F4/80 CD206 M1 cells (F), CD11b F4/80 CD206 M2 cells (G), PDCA- pDC (H), and FoxP3 CD4 CD25 Tregs (I). J–K, The time-dependent effects of BLM on HMGB1 expression in the lung (J) and BALF (K). The expression of HMGB1 in a lung was determined by the immunohistochemistry method and the IOD of each section for HMGB1 was analyzed by an Image-Pro Plus image analysis software. The level of HMGB1 in BALF was determined by a HMGB1-specific ELISA kit. Data was presented as mean ± SE (n = 12). L and M, TLR2 deficiency reversed the effects of BLM in the enhancement of TGF-β1 production and in the reduction of IFN-γ production in BALF. The levels of TGF-β1 (L) and IFN-γ (M) in BALF of BLM-treated mice were determined by the specific ELISA kits. N, BLM-caused time-dependent alterations in the properties of immune responses and in the degree of pulmonary fibrosis. The relative collagen deposition was semiquantitatively analyzed by Image-Pro Plus image analysis software. The suppressive immune cells (percentage of Tregs plus M2 cells) and proinflammatory cells (percentage of M1 cells plus CD11c mDCs) in lungs of BLM-treated mice were determined by flow cytometry. Data was presented as mean ± SE (n = 18). *, p < 0.05, ***, p < 0.01, ###, p < 0.001 vs WT mice on day 0; #, p < 0.05, ##, p < 0.01, ###, p < 0.001 vs TLR2-deficient mice on day 0; $, p < 0.05, $$, p < 0.01, $$$, p < 0.001, vs WT mice on corresponding time point.
class II, CD40, CD80, and CD86. BLM treatment not only significantly enhanced the percentage of CD11c\(^{+}\)MHCII\(^{+}\) DCs (\(p < 0.001\), Fig. 1A) and CD11c\(^{+}\)MHCII\(^{+}\) DCs (\(p < 0.001\), Fig. 1B), but also up-regulated the expression of CD40, CD80, and CD86 (\(p < 0.001\), Fig. 1, C and D). However, this BLM-induced expression of surface molecules was markedly abrogated by a TLR2-neutralizing Ab (Fig. 1, A–D). Moreover, BLM treatment significantly increased the levels of IL-6 (\(p < 0.01\), Fig. 1E) and IL-12 (\(p < 0.001\), Fig. 1G) but did not change the level of IL-10 (\(p > 0.05\), Fig. 1F). Treatment of these cells with Pam3Cys also significantly stimulated the release of IL-6 and IL-10 but not IL-12 (Fig. 1, E–G). Blocking TLR2 evidently attenuated BLM-stimulated release of IL-6 and IL-12 (\(p < 0.01\), Fig. 1E; \(p < 0.001\), Fig. 1G). These results indicated that BLM is a specific agonist of TLR2 in DCs.

**TLR2 mediated the time-dependent alteration of BLM-stimulated immune responses**

To determine the relationship between TLR2 and the immune response properties of “BLM lungs,” the time-dependent in vivo effects of BLM on tissue-infiltrating immune cells and cell surface molecule expression were examined in TLR2-deficient and WT mice. In the WT mice, intratracheal administration of BLM resulted in a significant increase in expression of CD11c (\(p < 0.01\), Fig. 2A) and MHC class II (\(p < 0.01\), Fig. 2A) on day 1, but was shortly followed by a time-dependent decrease from day 1 to 28. TLR2 deficiency, however, noticeably dampened the BLM-induced expression of CD11c (\(p < 0.01\), Fig. 2A) and MHC class II (\(p < 0.01\), Fig. 2B). Additionally, the expression of CD40 was significantly up-regulated from days 1 (10.3 ± 1.71\%, \(p > 0.05\)) to 7 (15.3 ± 1.54\%, \(p < 0.01\)) and down-regulated from days 7 to 28 (10.9 ± 0.48\%, \(p < 0.05\)) in the WT mice but did not change in the TLR2-deficient mice (Fig. 2C). The expression of CD80 on lung cells was inhibited on day 7 after BLM treatment (\(p < 0.05\)) in the WT mice but not in the TLR2-deficient mice (Fig. 2D). Interestingly, the expression of CD86 was significantly up-regulated from days 1 to 7 after BLM administration in WT mice, but TLR2 deficiency almost completely inhibited the BLM-induced expression of CD86 throughout the experimental period (\(p < 0.001\), Fig. 2E). The M1 cells (CD11b\(^{+}\)F4/80\(^{+}\)CD206\(^{−}\) cells) were increased from 4.45 ± 0.37\% on day 0 to 13.0 ± 2.25\% (\(p < 0.01\)) on day 3, and were sustained at 9.32 ± 1.23\% (\(p > 0.05\)) on days 28 in the BLM-treated WT mice. TLR2 deficiency, however, resulted in decreased tissue-infiltrating M1 macrophages throughout the length of the experiment (Fig. 2F). Notably, the administration of BLM did not change the number of tissue-infiltrating suppressive immune cells, including M2 cells (CD11b\(^{+}\)F4/80\(^{−}\)CD206\(^{+}\) cells) were increased from 4.45 ± 0.37\% on day 0 to 13.0 ± 2.25\% (\(p < 0.01\)) on day 3, and were sustained at 9.32 ± 1.23\% (\(p > 0.05\)) on days 28 in the BLM-treated WT mice. TLR2 deficiency, however, resulted in decreased tissue-infiltrating M1 macrophages throughout the length of the experiment (Fig. 2F). Notably, the administration of BLM did not change the number of tissue-infiltrating suppressive immune cells, including M2 cells (CD11b\(^{+}\)F4/80\(^{−}\)CD206\(^{+}\) cells) (Fig. 2G), pDCs (PDCA-1\(^{+}\) cells, Fig. 2H), and FoxP3\(^{+}\) Tregs (Fig. 2I) during the early stages of exposure (days 1 and 3). However, as the BLM-induced inflammatory responses declined, these suppressive cells significantly increased from days 7 to 28. TLR2 deficiency markedly blocked the BLM-induced infiltration of pDCs, M2 cell, and FoxP3\(^{+}\) Tregs from the days 7 to 28 (Fig. 2, G–I). These results indicate that BLM initiated the recruitment of proinflammatory cells at the early
stages of BLM treatment (days 1 to 7) and promoted an infiltration of the suppressive immune cells into the lungs at later stages of BLM treatment (days 7 to 28) in a TLR2 activity-dependent manner.

HMGB1 is a DAMPs molecule and plays a critical role in the development of inflammation (17). We found that BLM significantly up-regulated the expression of HMGB1 over 10-fold by day 3 \((p < 0.001)\) and remained at a higher level than sham animals by 3-fold on days 28 post-BLM treatment \((p < 0.001)\) (Fig. 2F). The extracellular level of HMGB1 in BALF was also remarkably enhanced from days 1 to 28 following BLM treatment (Fig. 2K). In contrast, TLR2 deficiency significantly blocked the BLM-stimulated accumulation and release of HMGB1 (Fig. 2, J and K). BLM administration resulted in a TGF-\(\beta\)1 production peak on days 7 and sustained a higher level of TGF-\(\beta\)1 in the BALF from days 1 to 28 after BLM treatment (Fig. 2L), whereas IFN-\(\gamma\) production peaked on day 1 and was markedly inhibited in the BALF between days 5 and 28 post-BLM treatment (Fig. 2M). TLR2 deficiency significantly attenuated BLM-enhanced TGF-\(\beta\)1 production by 3-fold on days 28 post-BLM treatment \((p < 0.001)\) (Fig. 2J). BLM administration caused a persistent apoptosis in the lungs from days 1 to 28 post-BLM treatment, while TLR2 deficiency significantly protected from the BLM-induced apoptosis (Fig. 3).

**TLR2 mediated BLM-induced pulmonary inflammation, injury, and fibrosis**

We further investigated the role of TLR2 in BLM-induced pulmonary inflammation and fibrosis using the TLR2-deficient and corresponding WT mice. BLM exposure (3 U/kg) resulted in a 60% death rate \((p < 0.001,\) Fig. 4A) and significantly increased pulmonary inflammation and fibrosis \((p < 0.01,\) Fig. 4, B–E) in WT mice. TLR2 deficiency clearly decreased this BLM-induced animal death (the death rate 13.2%, \(p < 0.001)\) and significantly decreased the BLM-increased lung index \((p < 0.01,\) Fig. 4B), pulmonary inflammatory score \((p < 0.01,\) Fig. 4, C and E), and collagen deposition \((p < 0.01,\) Fig. 4, C and D). Moreover, TLR2 deficiency significantly decreased the infiltrating FoxP3\(^+\) Tregs from 18.3 ± 3.85% to 5.22 ± 4.5% \((p < 0.05,\) Fig. 4F), pDCs from 18.1 ± 2.25% to 15.5 ± 2.1% \((p < 0.05,\) Fig. 4G), M2 cells from 9.82 ± 1.05% to 5.73 ± 1.50% \((p < 0.05,\) Fig. 4H), and M1 cells from 6.28 ± 1.26% to 3.90 ± 0.90% \((p < 0.05,\) Fig. 4I).

These results indicate that TLR2 activity has a critical role in the BLM induction of pulmonary inflammation and fibrosis and that targeting TLR2 is a potential strategy for the prevention and treatment of BLM-induced pulmonary inflammation and fibrosis. Indeed, treatment of animals with a TLR2-neutralizing Ab beginning from the day 0, 7, or 14 after BLM administration markedly improved BLM-induced animal survival (Fig. 5A and 6, A and B). Importantly, the TLR2-neutralizing Ab not only prevented BLM-induced animal death at the early stage of BLM administration (Fig. 5A) but also therapeutically attenuated BLM-induced animal death at the later stages following BLM administration (Figs. 5A and 6, A and B). The preventive effects of anti-TLR2 Ab on BLM-induced animal death was not only associated with the inhibition of BLM-stimulated pulmonary inflammation and injury \((p < 0.05,\) Fig. 5, B, C, and E) but also related to the attenuation of BLM-induced pulmonary fibrosis (Fig. 5, C, D, F, and G). The TLR2 Ab’s reduction of pulmonary fibrosis was associated with a reduced collagen deposition \((p < 0.05,\) Fig. 5, C and D), a decreased lung index \((p < 0.05,\) Fig. 5F), a reduced hydroxyproline content \((p < 0.05,\) Fig. 5G), and a down-regulated expression of HMGB1 in the lungs \((p < 0.001,\) Fig. 5H). Moreover, treatment of animals with the anti-TLR2 Ab beginning on days 7 or 14 after BLM administration significantly decreased the tissue-infiltrating inflammatory cells and inflammation score (Fig. 6, C and D), reduced the collagen deposition (Fig. 6, C and D) and hydroxyproline content (Fig. 6F) in the lung, and improved the lung index (Fig. 6G). These results suggest that targeting TLR2 could attenuate pulmonary inflammation and reverse the established pulmonary fibrosis.

**Targeting TLR2 reversed the BLM-induced immunosuppressive microenvironment**

To determine the mechanism of TLR2 inhibition in the attenuation of pulmonary fibrosis, we investigated the effects of targeting
TLR2 on the regulation of a BLM-induced immunosuppressive microenvironment in the lungs. Inhibition of TLR2 was found to decrease the BLM-enhanced infiltration of anti-inflammatory cells, including FoxP3+ Tregs ($p < 0.01$, Fig. 7A), pDCs ($p < 0.01$, Fig. 7A), and M2 cells ($p < 0.01$, Fig. 7B) and increased the infiltration of proinflammatory cells including CD11c+ mDCs ($p < 0.01$, Fig. 7C) and M1 cells ($p < 0.05$, Fig. 7E). The anti-TLR2 Ab decreased the number of FoxP3+ Tregs while increasing the number of mDCs.

The Th1/Th2 balance is a critical factor in the determination of immune microenvironment. Although BLM instillation did not affect the level of Th1 cytokine IFN-$\gamma$ ($p > 0.05$, Fig. 7F), BLM significantly polarized the immune paradigm toward Th2 by enhancing in vivo production of the Th2 cytokines, such as TGF-$\beta$1 ($p < 0.01$, Fig. 7G) and IL-13 ($p < 0.05$, Fig. 7H). Blockage of

**FIGURE 5.** Targeting TLR2 prevented BLM-induced pulmonary injury, inflammation and fibrosis. BLM (3 U/kg) was given to mice as described in Fig. 2. A, The anti-TLR2 Ab increased the survival rate of BLM-instilled mice ($n = 30$). B, Blockade of TLR2 activity markedly inhibited BLM-induced acute pulmonary injury. The TLR2-neutralizing or isotype-matched control Ab was administered i.v. on day 0 after BLM administration and the mice were sacrificed on days 7. The lungs were rapidly excised, fixed, and stained with H&E. The acute pulmonary injury was evaluated by a professional researcher of pathology who was blinded for the groups. C–H, Inhibition of TLR2 activity attenuated BLM-induced pulmonary inflammation and fibrosis. The TLR2-neutralizing or isotype-matched control Ab was administered i.v. on days 0, 7, and 14 after BLM administration. The anti-TLR2 Ab markedly blocked BLM-induced inflammatory responses (C and E, H&E staining) and prevented the BLM-induced pulmonary fibrotic reactions, including improving fibrotic pathological changes (C, Masson staining), decreasing collagen deposition (D), lung indexes (F), and hydroxyproline content (G) ($n \geq 20$). H, Targeting TLR2 significantly reduced the production of HMGB1. The lung sections were stained with an anti-HMGB1 Ab. The IOD of each section was analyzed by Image-Pro Plus image analysis software. Data was presented as mean $\pm$ SE ($n = 12$). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared with BLM treated mice; $$, $p < 0.01$, $$$, $p < 0.001$ compared with sham mice.

**FIGURE 6.** Blockade of TLR2 reversed BLM-induced pulmonary inflammation and fibrosis. BLM was given to mice as indication. The TLR2-neutralizing or isotype-matched Ab ($200 \mu g/kg$) was administered i.v. on days 7 and 14 (anti-TLR2_7) or days 14 and 21 (anti-TLR2_14). The mice were sacrificed on days 35 after BLM administration. A and B, Treatment with the anti-TLR2 Ab beginning from day 7 or 14 decreased the BLM-caused mice death. The survival rate was analyzed by the Kaplan-Meier method. A, The survival rate of mice from days 0 to 35 after BLM treatment. B, The survival rate of mice from days 14 to 35 after BLM treatment. C–E, Blockade of TLR2 attenuated BLM-induced pulmonary inflammation and fibrosis. The anti-TLR2 Ab markedly blocked BLM-induced inflammatory responses (C and E) and reversed BLM-induced collagen deposition (C and D). F, Blockade of TLR2 decreased the hydroxyproline content in the lung of BLM-treated mice. G, Blockade of TLR2 reduced the lung indexes of BLM-treated mice. Data was presented as mean $\pm$ SE ($n = 12$). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared with BLM treated mice; $$, $p < 0.01$, $$$, $p < 0.001$ compared with sham mice.
TLR2 significantly elevated the level of IFN-γ by more than 2-fold ($p < 0.01$, Fig. 7F) and reduced the levels of TGF-β1 ($p < 0.05$, Fig. 7G) and IL-13 ($p < 0.05$, Fig. 7H). Additionally, anti-TLR2 Abs also reduced the BLM-induced expression of MCP-1, a critical chemokine responsible for the recruitment of immune cells to the tissue ($p < 0.01$, Fig. 7I).

**FIGURE 7.** Targeting TLR2 regulated immune microenvironment in the lungs. The mice were sacrificed four weeks after BLM treatment. The lung tissues were fixed for immunohistochemistry analysis or digested to obtain a single cell suspension for analysis of immune cells by flow cytometry. A–E, Neutralization of TLR2 activity markedly decreased the tissue-infiltrating FoxP3$^+$ Tregs (A), PDCA$^+$ pDCs (B), M1 (D), and M2 macrophages (E), and increased the tissue-infiltrating CD11c$^+$ mDCs (C). Data was the representative result of two independent animal experiments ($n = 8$ group/experiment). The lung tissue sections were stained with the respective Abs including anti-IFN-γ (F), TGF-β (G), IL-13 (H), or MCP-1 Ab (I). The IOD of cytokine or chemokine staining in each section was analyzed by Image-Pro Plus image analysis software. Data was mean ± SE ($n = 8$ group/experiment). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs BLM-treated mouse; #, $p < 0.05$, ##, $p < 0.01$, ###, $p < 0.001$ vs sham mice.
Blockage of TLR2 inhibited BLM-increased activity and function of Stat3 and Smad3. The mice were treated as indication and sacrificed 28 days after BLM treatment. The lung tissues were isolated and fixed for immunohistochemistry analysis. The lung tissue sections were stained with the anti-phosphorylative Stat3 or Smad3 Ab. The IOD of each section was analyzed by Image-Pro Plus image analysis software. A and B, The TLR2-neutralizing Ab inhibited BLM-induced phosphorylation of transcription factor Stat3 (A) and Smad3 (B). Data was mean ± SE (n = 15). *, p < 0.05, **, p < 0.01, ###, p < 0.001 vs BLM-treated mice; #, p < 0.05, ##, p < 0.01, ###, p < 0.001 vs sham mice. C, Blockage of TLR2 significantly inhibited the translocation activity of smad3 induced by BLM. The arrows showed the active smad3. D, Inhibition of Stat3 and Smad 3 activity attenuated IL-6 production in the BLM-treated DCs. The precursor DCs were treated with Stat3 I (3 μM) or SIS3 (10 μM) 1 h before BLM (20 μg/ml) exposure. The IL-6 production was determined by standard intracellular flow cytometry. The illustration was the representative overlapped histograms of three independent experiments. E, The schematic diagram illustrates the mechanisms that targeting TLR2 attenuates BLM-induced pulmonary inflammation, injury, and fibrosis. BLM induces acute inflammatory response and acute lung injury via TLR2-dependent or independent manner. The TH1-type response contributes to resolution of the lung. Meanwhile, the tissue injury and acute inflammation stimulate the release of DAMPs, e.g., HMGB1 from the damaged tissue, which are subsequently recognized by pattern recognition receptors (e.g., TLR2), and induce the TH2-predominant chronic inflammatory responses. Moreover, BLM activation of TLR2 stimulates transcript factors Stat3 and Smad3 that produce inhibitory cytokines and chemokines to recur suppressive immune cells (e.g., Tregs, M2 cells, and pDCs) and the release of suppressive cytokines (e.g., IL-6 and TGF-β1). This results in the formation of immunosuppressive microenvironment to promote the deposition of collagens and fibrogenesis.
Previous work indicates that the expression of suppressive cytokines (such as IL-6 and TGF-β1) and chemokines (MCP-1) are controlled by the transcription factors Stat3 and Smad3 (18). We found that BLM instillation significantly enhanced phosphorylation of Stat3 and Smad3 in vivo ($p < 0.01$, Fig. 8, A and B) and that blocking TLR2 evidently inhibited this BLM-stimulated phosphorylation of Stat3 ($p < 0.01$, Fig. 8A) and Smad 3 ($p < 0.01$, Fig. 8B). In addition, blocking TLR2 reduced the nucleus translocation of Smad3, which is normally induced by BLM administration (Fig. 8C). Interestingly, inhibiting the activity of either Stat3 with specific Stat3 I or Smad 3 with SIS3 (a Smad3 inhibitor) significantly reduced the in vitro production of IL-6 by BLM-stimulated DCs ($p < 0.01$, Fig. 8D).

Discussion

Accumulated evidence suggests that inflammation disassociates from tissue fibrosis and that ongoing inflammation is needed to reverse established and progressive tissue fibrosis (19). Indeed, tissue fibrosis might be a consequence of the actions of unique cells, such as the extracellular matrix component-synthesizing myofibroblast, and immunosuppressive cells, such as immature DCs, M2 macrophage, and Tregs, which produces inhibitory cytokines and chemokines (2). Many types of fibrotic tissue share the common feature of a demonstrable skewing of the cytokine response toward a Th2-type cytokine and chemokine profile. This leads to the accumulation and activation of fibroblasts derived from resident cells, bone marrow-derived cells, and M2 cells or epithelial-mesenchymal transition, which leads to recurring infiltration of immunosuppressive cells into the damaged tissue (2). Thus, the immunosuppressive cells, cytokines, and chemokines favor a tissue microenvironment for fibrotic progression. TLRs are expressed in the respiratory epithelial cells and many types of immune cells including mast cells, DCs, T cells, B cells, endothelium, fibroblasts, and vascular smooth muscle (20, 21). Following PAMPs or DAMPs binding, TLRs or non-TLRs are able to regulate the function of these cells and skew specific immune responses toward the Th1, Th2, Th17 or Tregs phenotype according to the antigenic stimulation involved (22). In the family of TLRs, TLR2 is a unique member that mediates a Th1 response on one hand (23) and TH2-biased response on the other (24). For instance, it can be activated by injury through a vitamin D-dependent mechanism and is also involved in Th2-biased immune responses (24–26). Thus, targeting TLR2 signaling provides substantial new opportunities for the prevention and treatment of chronic lung diseases (11, 20, 27).

Our current study demonstrates that TLR2 plays a critical role in the BLM-induced inflammatory and fibrotic responses (Fig. 8E).

During the early exposure to BLM, a strong Th1-dominant inflammatory response is caused by activation of TLR2 (Figs. 1G and 2, F and M). This BLM-induced inflammatory response can be induced either by the interactions between T cells and BLM-pulsed DCs or by BLM effects independent of APCs. In addition, the IFN-γ-producing Th1-cell response triggered by BLM stimulates the acute phase reactions that cause tissue damage, cell death, and apoptosis. Tissue injury can enhance the TLR2-mediated functions via a Vitamin D dependent mechanism (25) or via the release of DAMPs, such as HMGB1, heat shock protein 60, and hyaluronan, all of which have been identified as endogenous ligands of TLR2 (28). For instance, HMGB1 is a critical NF that mediates the response to infection, injury, and inflammation (17). TLR2-mediated HMGB1 is closely associated to the inflammation induced by BLM. In contrast, an anti-inflammatory Th2 response may be established by a direct interaction between TLR2 and BLM or an indirect interaction between TLR2 and BLM-induced apoptotic cells (29–31). The TLR2-mediated Th2 responses, induced by IL-4 as well as IL-5 and IL-13, are strongly linked with the process of fibrogenesis by up-regulating the transcription and expression of several genes involved in wound healing and fibrosis, such as procollagen I, procollagen III, matrix metalloproteinase (MMP) 2, and MMP9 in the lungs of BLM-treated mice (32). The Th2 responses also function as a double-edged sword, facilitating wound healing while simultaneously contributing to tissue remodeling by reducing the ratio of tissue inhibitor of metalloproteinase 2/MMP2. Thus, TLR2 activation induces an immunosuppressive tissue microenvironment by increasing the infiltration of immunosuppressive cells and up-regulating suppressive cytokines during the later stage of BLM exposure, which contributes to the pathogenesis of BLM-induced fibrosis (Fig. 8E).

Thus, the mechanisms by which targeting TLR2 attenuates BLM-induced pulmonary fibrosis may be largely attributable to a reduction of BLM-induced inflammation and a reversal of the BLM-induced immunosuppressive microenvironment. On one hand, blocking TLR2 attenuates BLM-mediated inflammatory responses and protects the lungs from the TLR2-mediated recruitment of proinflammatory cells, such as M1 cells and mDCs, into the lung tissue. On the other hand, targeting TLR2 attenuates TLR2-mediated recruitment of a large number of immunosuppressive cells (e.g., FoxP3+ Tregs, pDCs, and M2 cells) and immunosuppressive cytokines (e.g., IL-6, IL-13, and TGF-β1) in the lungs. Estes et al. (33) also found that TLR2-mediated expansion and function of Tregs induces the tissue fibrosis by producing TGF-β1 and that pDCs have a unique function in tolerance induction and development of Tregs (34). However, adaptive transfer of Tregs suppresses the efferent phase of Th1 immune response and the subsequent pulmonary interstitial fibrosis (35). Therefore, the increase in Tregs initially following BLM-exposure may contribute to resolution by inhibition of inflammatory cells. In contrast, the increase in Tregs during the later stages of BLM-exposure may result in the aggravation of pulmonary fibrosis due to the production of the suppressive cytokine TGF-β1. Misson et al. (36) reported that the development of fibrosis is associated with a shift from a M1 activation to a M2 polarization by overexpression of NO synthase-2 and arginase. Indeed, the M2 macrophages are emerging as the potential target for Th2-related diseases, such as tissue fibrosis and tumor (36, 37).

The observed reversion of the immunosuppressive microenvironment, via the targeting of TLR2, may also be the result of inhibition of transcription factors Smad3 and Stat3 (Fig. 8E). TGF-β1 is the most powerful physiological immunosuppressor in mammals (38), and the TGF-β1/Smad3 pathway is critical for damaged tissue to switch from a Th1- to Th2-predominated response. Smad3 mediates most effects of TGF-β1, including epithelial-to-mesenchymal transition and tissue fibrosis (18). Since Smad3 appears to be a key player in mediating fibrosis, independent of the agonist (BLM or TGF-β1) or cell type (fibroblasts or other mesenchymal), it offers a cleaner target to reverse tissue fibrosis in a safe predictable manner. Stat3 is initially identified as an acute phase response gene in the liver and has a pivotal role in directing inflammatory responses by inducing the gene expression of cytokines, chemokines, and adhesion molecules (39). Although prior studies suggest that Stat3 has a largely anti-inflammatory role in innate immune responses, elucidation of the role of Stat3 in biologic responses in general has been hindered by the fact that deletion of the Stat3 gene results in embryonic lethality (40). However, accumulated evidence suggests that the activation of Stat3 contributes to the establishment of an immunosuppressive tumor microenvironment (39). Also, evidence is emerging for the role of Stat3 in the pathogenesis of fibrotic diseases. For example, Ogata...
et al. (41) found that TGF-β1 is a target gene of Stat3 and that Stat3 enhances hepatic fibrosis through the up-regulation of TGF-α expression. Indeed, we recently found that TLR2 agonists significantly activate Stat3 and that blockade of the basal activity of TLR2 inhibits the constitutive activation of Stat3 and reverses immunosuppressive microenvironment in tumor tissue (unpublished data). Taken together, our studies provide further evidence to suggest that the activation of transcription factors Smad3 and Stat3 is associated with the development of BLM-established immunosuppressive microenvironment, which contributes to the BLM-induced pulmonary fibrosis (Fig. 8E).

In summary, the identification of TLR2 as a critical receptor molecule for mediating BLM-stimulated pulmonary inflammation and fibrosis by Razonable’s recent work (11) and by this study is an important observation that highlights TLR2 as a promising target for the development of therapeutic agents against BLM lung injury and many fibrol家门口diseases. Also, our studies indicate that the combination therapy of BLM with the specific anti-TLR2 Ab or TLR2 antagonists will improve the anti-cancer efficacy and reduce the life-threatening side effects of BLM.

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Disclosures

The authors have no financial conflict of interest.

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