Toll-like receptor 4 promotes fibrosis in bleomycin-induced lung injury in mice

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Genet. Mol. Res. 14 (4): 17391-17398 (2015)
Received August 9, 2015
Accepted October 14, 2015
Published December 21, 2015
DOI http://dx.doi.org/10.4238/2015.December.21.8

ABSTRACT. The specific role of Toll-like receptor 4 (TLR4) in bleomycin-induced lung fibrosis of mice, a model of human idiopathic pulmonary fibrosis, has not been characterized. We injected bleomycin intratracheally into TLR4 knockout ($TLR4^{-/-}$) and wild-type (WT) mice. Twenty-one days after injection, mice were sacrificed and their lungs were harvested for pathological, hydroxyproline, mRNA expression, and collagen I analyses. Body weight changes and mortality were observed. Light microscopy showed that lung fibrosis was minimal in $TLR4^{-/-}$ compared to that in WT mice on day 21 after bleomycin instillation. The Ashcroft score was significantly lower in $TLR4^{-/-}$ than in WT mice (3.667 ± 0.730 *vs* 4.945 ± 0.880, $P < 0.05$). Hydroxyproline content was significantly lower in $TLR4^{-/-}$ than in WT mice on day 21 after bleomycin injection (0.281 ± 0.022 *vs* 0.371 ± 0.047, $P < 0.05$). Compared to WT mice, bleomycin-treated $TLR4^{-/-}$ mice expressed significantly lower type I collagen mRNA levels (mesenchymal marker; 11.069 ± 2.627 *vs* 4.589 ± 1.440, $P < 0.05$). Collagen I was significantly lower in $TLR4^{-/-}$ than in WT mice (0.838 ± 0.352 *vs* 2.427 ± 0.551, $P < 0.05$).

Bleomycin-treated TLR4$^{-/-}$ mice had a significantly lower mortality rate on day 21 than WT mice (33 *vs* 75%, $P < 0.05$). Body weight reduction was lower in TLR4$^{-/-}$ mice than in WT mice; this difference was not statistically significant (-3.735 ± 5.276 *vs* -6.698 ± 3.218, $P > 0.05$). Thus, bleomycin-induced pulmonary fibrosis is TLR4-dependent and TLR4 promoted fibrosis in bleomycin-challenged mice.

Key words: Bleomycin; Idiopathic pulmonary fibrosis; Lung fibrosis; Toll-like receptor

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease of unknown causes and has poor prognosis; it is characterized by fibrosis-induced destruction of the lung parenchyma. The pathogenesis of IPF is not completely understood. It is widely accepted that IPF begins with injury to pulmonary epithelial and endothelial cells, which is followed by dysregulated repair in damaged tissues (Margaritopoulos et al., 2012). The immune system is also involved in lung fibrosis. Toll-like receptors (TLRs) are a family of conserved innate immune receptors. Matrix-derived endogenous ligands such as hyaluronan and biglycan, released during tissue injury, are recognized by TLRs (Jiang et al., 2005; Schaefer et al., 2005). TLR-matrix interactions are involved in non-infectious diseases (Schaefer et al., 2005). Interactions between the host matrix component hyaluronan and TLR4/2 provide signals that initiate inflammatory responses, maintain epithelial cell integrity, and promote recovery from acute lung injury (Jiang et al., 2005). TLR4 can sense tissue injury by recognizing endogenous damage-associated molecules. Therefore, TLR4 is thought to regulate pulmonary fibrosis by sensing alveolar epithelial cell damage. In the lungs of bleomycin-treated mice, infiltration of TLR4+ cells was found to be significantly enhanced, and the TLR4 signal pathway was markedly upregulated, indicating that the TLR4 signaling pathway was activated. However, blocking TLR4 enhanced the production of interleukin-17 and transforming growth factor-β1, which promoted lung fibrosis (Liu et al., 2010). Thus, the specific role of TLR4 in bleomycin-induced lung fibrosis, a model of human IPF, remains unclear.

MATERIAL AND METHODS

Animals

TLR4-knockout C57BL/10ScNJ (TLR4$^{-/-}$) mice and corresponding wild-type (WT) mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Mice were bred and maintained in a specific pathogen-free environment at the Capital Medical University. Male WT and TLR4$^{-/-}$ mice, 7-8 weeks old and weighing 22-25 g, were used in all experiments. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Capital Medical University (Beijing, China).

Induction of pulmonary fibrosis using bleomycin (BLM)

Mice were treated with a single intratracheal instillation of 0.9% saline or 3.5 mg/kg body weight saline containing BLM sulfate in 40-μL total volume and were sacrificed after 21 days by

pentobarbitone overdose. The thorax was opened, the whole lung was removed, and the right lung was frozen in liquid nitrogen and stored at -80°C until analysis of hydroxyproline content or isolation of RNA and protein. The left lung was fixed in 10% paraformaldehyde for histolopathological analysis.

Histolopathological analysis for fibrosis

The left lung was inflated and fixed in 10% paraformaldehyde, embedded in paraffin, and cut into 5-μm thick sections. Sections were stained with hematoxylin and eosin or Masson trichrome. All sections were studied using light microscopy. The severity of fibrosis was semi-quantitatively assessed according to the method proposed by Ashcroft et al. (1988). Briefly, the grade of lung fibrosis was scored on a scale from 0 to 8 by examining randomly chosen sections, with six fields per sample at a magnification of 100X. Criteria for grading lung fibrosis were as follows: grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to lung architecture; grade 5, increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of structure and large fibrous areas; grade 8, total fibrous obliteration of fields.

RNA isolation and real-time reverse transcription-polymerase chain reaction (PCR)

Total RNA was isolated from mouse lung tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol. The concentration and purity of RNA were evaluated by spectrometry at 260 and 280 nm. All RNA samples were stored at -80°C until processing for reverse transcription. Reverse transcription was conducted for 60 min at 37°C using the cDNA synthesis kit as per manufacturer instructions (Tiangen Biotech Co., Ltd., Beijing, China). Quantitative RT-PCR was performed using an ABI 7500 Real Time PCR System Sequence system (Applied Biosystems, Foster City, CA, USA). We used the SYBR-Green PCR Master Mix and reverse transcription-PCR kit, as per manufacturer instructions (Tiangen Biotech Co., Ltd.). The following primers were used: β-actin, forward, 5'-CTTTGACATCCGTAAAGACC-3', reverse, 5'-AACAGTCCGCCTAGAAGCAC-3'; collagen I, forward, 5'-GAGCCTGAGTCAGCAGATTG-3', reverse, 5'-AATCCATCGGTCATGCTCT-3'. The amplification conditions were as follows: 15 min at 95°C and 50 cycles of 10 s at 95°C, 20 s at 55°C, and 32 s at 72°C. For relative quantification, we calculated the n-fold differential expression using the $2^{-\Delta\Delta Ct}$ method (Ct indicates the threshold cycle of PCR amplification at which the product was first detected by fluorescence), which compares the amount of target gene amplification, normalized to mouse β-actin as an endogenous reference.

Western blotting analysis

Frozen lung tissues were homogenized in modified RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; and 1 mg/mL of each aprotinin, leupeptin, and pepstatin; 1 mM Na_3VO_4; 1 mM NaF). The total protein concentration from each sample was measured using the bicinchoninic acid method. Next, 30 μg/laneprotein samples were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 4% stacking gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and incubated overnight with primary rabbit antibodies to collagen I (1:250; Abcam, Cambridge, UK) at 4°C,

followed by incubation with infrared-labeled secondary antibodies for 1 h at room temperature. Glyceraldehyde 3-phosphate dehydrogenase was also blotted on the membrane as an internal control. Quantification and radiography were conducted using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). The density of each band is reported as the percentage of glyceraldehyde 3-phosphate dehydrogenase density.

Measurement of hydroxyproline content

Hydroxyproline content was measured spectrophotometrically to quantify lung collagen accumulation as described previously (Sisson et al., 2010). Briefly, lung tissues were washed in phosphate-buffered saline, weighed, minced, and diluted in 1 mL phosphate-buffered saline. Samples were then hydrolyzed in 12 N HCl at 110°C overnight. Each 5-µL sample was mixed with an equal volume of citrate-acetate buffer and 100 µL chloramines-T solution. The mixture was incubated for 20 min at room temperature. Ehrlich's solution was then added and the samples were incubated at 65°C for 18 min. Absorbance was measured at 550 nm using hydroxyproline as a standard. The results are reported as hydroxyproline content per milligram lung tissue.

Statistical analysis

Data are reported as means ± SD (standard deviation). The SPSS17.0 software was used for data analysis (SPSS, Inc., Chicago, IL, USA). Independent-sample *t*-testing or nonparametric tests were used to analyze differences between groups. The mortality rates among groups were compared using Kaplan-Meier statistics. $P < 0.05$ was considered to be statistically significant.

RESULTS

Development of BLM-induced lung fibrosis was less severe in TLR4$^{-/-}$ mice

Histological evaluation of hematoxylin and eosin-stained sections showed that BLM instillation produced a significant increase of fibrosis in the lung as compared to saline solution instillation (Figure 1A). TLR4$^{-/-}$ mice that underwent bleomycin tracheal instillation showed a lower increase in lung fibrosis compared to bleomycin-treated WT mice (Figure 1A). This was confirmed by histological grading of lung fibrosis according to the criteria of Ashcroft et al. (1988), conducted using Masson's trichrome-stained slides. The results showed a lower degree of pulmonary fibrosis in the TLR4$^{-/-}$ group than in the WT group on 21 days after BLM intratracheal injection ($N = 6$, $P < 0.05$, Figure 1B). Compared with WT mice, the hydroxyproline content of the right lung on day 21 was lower in TLR4$^{-/-}$ mice after the intratracheal injection of bleomycin (WT mice *vs* TLR4$^{-/-}$ mice: 0.371 ± 0.047 µg/mg *vs* 0.281 ± 0.022 µg/mg, $N = 6$, $P < 0.05$; Figure 1C). Thus, the degree of lung fibrosis was lower in TLR4$^{-/-}$ mice than in WT mice on day 21 after bleomycin intratracheal injection.

BLM-treated TLR4$^{-/-}$ mice had significantly lower mRNA expression levels and protein levels of collagen I in the lungs than WT mice

Twenty-one days after BLM intratracheal injection, significant increases in mRNA expression levels and protein of collagen I compared to saline solution instillation were observed in the lung (Figure 2A and B). In bleomycin-treated TLR4$^{-/-}$ mice, a significant decrease in collagen I

mRNA expression was observed compared to WT mice (11.069 ± 2.627 *vs* 4.589 ± 1.440, P < 0.05; Figure 2A). Similarly, protein expression profiling by western blotting demonstrated significantly decreased levels of collagen I following BLM challenge in TLR4$^{-/-}$ mice compared to WT mice (0.838 ± 0.352 *vs* 2.427 ± 0.551, P < 0.05, Figure 2B and C).

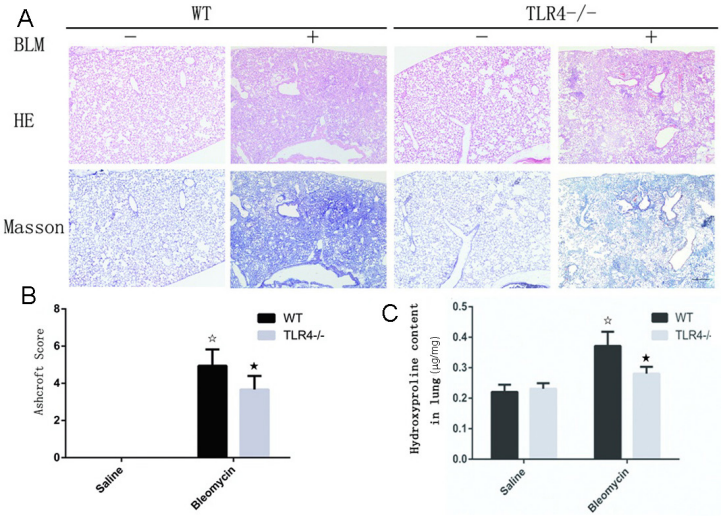

Figure 1. Changes in lung histopathology, Ashcroft score, and hydroxyproline content on day 21 in WT and TLR4$^{-/-}$ mice exposed to saline or bleomycin (BLM). **A.** Lung histopathological changes. Representative lung sections stained with hematoxylin and eosin or Masson's trichrome. Scale bars = 200 μm. **B.** Ashcroft score of lung fibrosis. Data are reported as means ± SD from 6 mice for each group. Open star = P< 0.05 *vs* saline-treated WT mice. Filled star = P < 0.05 *vs* BLM-treated WT mice. **C.** Hydroxyproline content in lungs. Data are reported as means ± SD (N = 6 for each group). Open star = P < 0.05 *vs* saline-treated WT mice. Filled star = P < 0.05 *vs* BLM-treated WT mice.

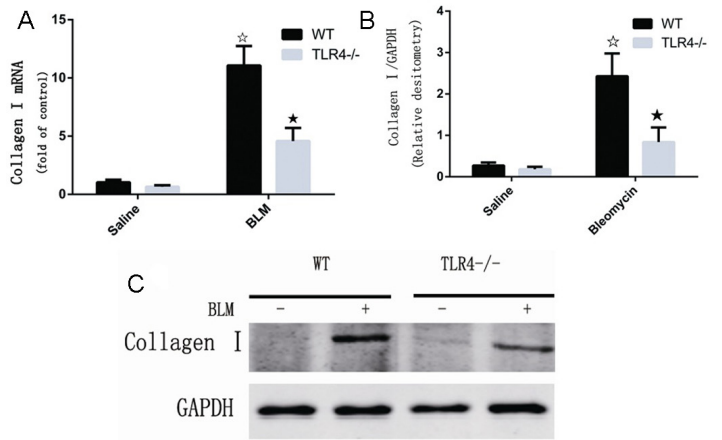

Figure 2. Collagen I mRNA expression level and protein production on day 21 after bleomycin (BLM) or saline intratracheal injection in WT and TLR4$^{-/-}$ mice. **A.** Quantitative TLR4 mRNA expression relative to β-actin mRNA. Data are reported as means ± SD. Open star = P< 0.05 *vs* saline-treated WT mice. Filled star = P < 0.05 *vs* BLM-treated WT mice. **C.** and **B.** Gel photograph and densitometric analysis of collagen I expression represented as a percentage of glyceraldehyde 3-phosphate dehydrogenase in WT and TLR4$^{-/-}$ mice exposed to saline or BLM. Data are reported as means ± SD. Open star = P < 0.05 *vs* saline-treated WT mice. Filled star = P < 0.05 *vs* BLM-treated WT mice.

Compared to WT mice, TLR4$^{-/-}$ mice showed significant change in survival rate but not body weight after BLM intratracheal injection

All saline-treated control mice survived for 21 days. Following bleomycin injection, mortality was substantial, but differed between WT mice and TLR4$^{-/-}$ mice (Figure 3A). After intratracheal injection of bleomycin, 33% of WT mice survived to 21 days, while 75% of TLR4$^{-/-}$ mice survived to 21 days. The mortality of bleomycin-treated WT mice was significantly higher than TLR4$^{-/-}$ mice ($P < 0.05$). Intratracheal instillation of 3.5 mg/kg bleomycin was associated with a significant decrease in body weight. In bleomycin-treated WT mice, the decrease in body weight was more severe than in bleomycin-treated TLR4$^{-/-}$ mice (Figure 3B), but the difference was not significant. These results indicate that TLR4 deficiency protects mice from death, but not from body weight loss induced by bleomycin.

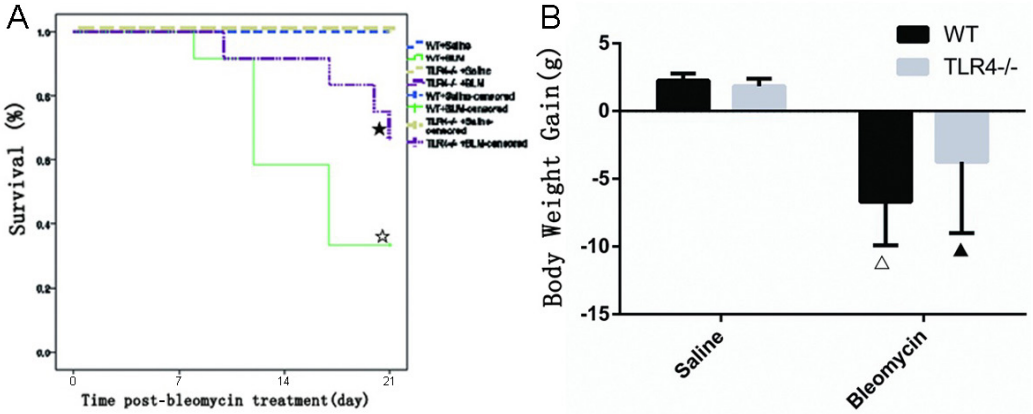

Figure 3. Changes in body weight and mortality on day 21 in WT and TLR4$^{-/-}$ mice exposed to saline or bleomycin (BLM). **A.** Survival rate was measured after tracheal instillation (N = 12 for each group). Open star = $P < 0.05$ *vs* saline-treated WT mice. Filled star = $P < 0.05$ *vs* BLM-treated WT mice. **B.** Body weight was recorded immediately before BLM administration and day 21 after BLM administration. Data are reported as means ± SD from 12 mice for each group. Open star = $P < 0.05$ *vs* saline-treated WT mice. Filled star = $P > 0.05$ *vs* BLM-treated WT mice.

DISCUSSION

IPF is a progressive and fatal disease characterized by fibrosis-induced destruction of the lung parenchyma. The pathogenesis of the disease is poorly understood. However, it is widely accepted that IPF is caused by an unknown insult to the lung that leads to alveolar epithelial cell injury and subsequent dysregulated repair (Hübner et al., 2008). During pulmonary fibrosis, various immune cells infiltrate the lung tissues, which participate in a complicated cytokine network that regulates inflammation and fibrosis (Lukacs et al., 2001; Marchal-Sommé et al., 2006, 2007). These previous studies suggested that the immune system is involved in lung fibrosis.

TLRs represent a conserved family of innate immune recognition receptors that play roles not only in the regulation of innate and adaptive immune responses (Akira and Takeda, 2004), but also in non-infectious inflammatory diseases (Kiechl et al., 2002; Zhai et al., 2004; Tsung et al., 2005). To date, more than 10 mammalian TLRs have been identified (Akira and Takeda, 2004), of which TLR4 was the first to be described. TLR4 is expressed in a wide range

of cells in the lung, such as alveolar macrophages, dendritic cells, and pulmonary epithelium and endothelium cells (Andonegui et al., 2003; Hoppstadter et al., 2010). Previous studies suggested that TLR4 participates in the pathogenesis of non-infectious lung injury. TLR4 recognizes diesel exhaust particles (Inoue et al., 2006) and pollutant ozone (Kleeberger et al., 2010), expressed in smoke-induced lung injury (Karimi et al., 2006), and is required for hemorrhage-induced lung tumor necrosis factor-α production, neutrophil accumulation, and protein permeability (Barsness et al., 2004), supporting that TLR4 accelerates the progress of non-infectious lung disease. However, TLR4 maintained appropriate levels of anti-apoptotic responses during oxidant stress, and thus played a protective role in oxidant-mediated lung injury (Zhang et al., 2005). Similarly, in TLR2$^{-/-}$ TLR4$^{-/-}$ mice with bleomycin-induced lung injury, increased tissue injury and epithelial cell apoptosis were observed. Thus, TLR4 appears to improve epithelial cell repair, which is important in tissue injury recovery.

On the contrary, the roles of TLR4 in renal fibrosis and hepatic fibrosis are promoting fibrosis. In a model of hepatic fibrogenesis (Seki et al., 2007), TLR4 enhanced transforming growth factor-β signaling and myofibroblast activation, suggesting that TLR4 bridges proinflammatory and profibrogenic signals in the liver tissue. In murine kidneys after unilateral ureteral obstruction, several endogenous ligands were strongly upregulated, which may activate TLR4. TLR4 promotes fibrosis but attenuates tubular damage in progressive renal injury, suggesting that TLR4 signaling may be a therapeutic target for preventing renal fibrosis (Pulskens et al., 2010). The role of TLR4 in pulmonary fibrosis is not well-understood. Because mechanistic studies of lung fibrogenesis are difficult to carry out in humans, intratracheal instillation of the anti-tumor agent BLM is commonly conducted in animal models for pulmonary fibrosis.

In this study, TLR4$^{-/-}$ mice were injected intratracheally with bleomycin and evaluated for the development of pulmonary fibrosis to determine whether TLR4 was required for the induction of bleomycin-induced pulmonary fibrosis. After bleomycin administration, TLR4$^{-/-}$ mice exhibited a reduced degree of lung fibrosis compared with WT mice, as shown by the reduction in mortality rate, histological evidence of lung fibrosis, mRNA expression levels of collagen I and protein content in lung tissue. Our previous study showed that bleomycin-induced pulmonary fibrosis was TLR4-dependent, as TLR4$^{-/-}$ mice developed minimal fibrosis compared with WT mice. TLR4 promoted fibrosis in bleomycin-challenged mice. The mechanism of how TLR4 participate in the pathogenesis of IPF requires further analysis. We hypothesize that in the pathogenesis of IPF, an unknown insult first leads to alveolar epithelial cell injury, causing the release of endogenous factors or a damage-associated molecular pattern (such as hyaluronan). Next, the host response to damage-associated molecular pattern occurs through interactions with pattern recognition receptors (i.e., TLR4). Then, inflammatory cells are recruited and the extracellular matrix accumulates as a consequence of chronic epithelial injury and failure of repair because of aberrant epithelial-mesenchymal interactions (Thannickal, 2012). The several immune and nonimmune cell types in the damaged tissue contribute to the chronic inflammatory response and tissue remodeling through secretion of growth factors, cytokines, and chemokines and activation of extracellular matrix-synthesizing processes (Meneghin and Hogaboam, 2007). Finally, pulmonary fibrosis develops. Further investigation is needed to confirm these findings not only in humans but also in *in vitro* experiments and to assess the biological significance of TLR4 in the development of IPF.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (Grant #81070046).

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