



Expression of toll-like receptor 2 and 4 is increased in the respiratory epithelial cells of chronic idiopathic interstitial pneumonia patients



Heunjeong Go ^{a,c}, Jaemoon Koh ^a, Hye Sung Kim ^{a,d},
Yoon Kyung Jeon ^{a,**}, Doo Hyun Chung ^{a,b,d,*}

^a Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Republic of Korea

^b Ischemic/Hypoxia Institute, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Republic of Korea

^c Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro, 43-gil, Songpa-gu, Seoul 138-736, Republic of Korea

^d Department of Biomedical Sciences, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Republic of Korea

Received 30 July 2013; accepted 16 December 2013

Available online 22 January 2014

KEYWORDS

TLR2;
TLR4;
UIP;
NSIP;
Type II pneumocyte;
Bronchial epithelial cell

Summary

Background: Idiopathic interstitial pneumonia (IIP) is characterized by chronic interstitial inflammation and fibrosis. Although mounting evidence has suggested that toll-like receptor (TLR) 2 and TLR4 are involved in the pathogenesis of non-infectious lung injury *in vitro* and in mouse models, their roles in human IIP remain unknown.

Methods: To address this issue, we investigated the expression patterns of TLR2 and TLR4 by immunohistochemistry in resected lung tissues from patients with usual interstitial pneumonia (UIP) or nonspecific interstitial pneumonia (NSIP).

Results: Type II pneumocytes, bronchial epithelial cells (BECs), and alveolar macrophages accounted for the majority of TLR2- and TLR4-expressing cells in both UIP and NSIP. The numbers of TLR2 and TLR4-positive respiratory epithelial (RE) cells, including type II pneumocytes and BECs, were significantly greater in UIP and NSIP than in the control. In particular, the numbers of TLR2-positive RE cells were much greater in UIP than in NSIP. The intensities of TLR2 and

* Corresponding author. Department of Pathology and Biomedical Sciences, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Republic of Korea. Tel.: +82 2 740 8915; fax: +82 2 743 5530.

** Corresponding author. Department of Pathology, Seoul National University Hospital, 101 Daehak-ro, Jongno-gu, Seoul 110-799, Republic of Korea. Tel.: +82 2 2072 1347; fax: +82 2 743 5530.

E-mail addresses: junarplus@chol.com, ykjeon@snu.ac.kr (Y.K. Jeon), doohyun@snu.ac.kr (D.H. Chung).

TLR4 expression in type II pneumocytes were also significantly stronger in UIP and NSIP than in the control. A comparison of the TLR expression patterns between the fibroblastic and fibrotic areas in UIP indicated that the numbers TLR2 and TLR4-positive RE cells were similar in fibroblastic areas, whereas the TLR2-positive RE cells outnumbered the TLR4-positive RE cells in the fibrotic areas.

Conclusions: This study demonstrates that RE cells over-express TLR2 and TLR4 in the lungs of IIP patients. These findings suggest that high expression of TLRs may contribute to the pathogenesis of human IIP.

© 2014 Elsevier Ltd. All rights reserved.

Introduction

Chronic diffuse or interstitial lung disease (ILD) includes a spectrum of non-infectious inflammatory conditions that typically evolves over weeks, months, or years [1]. Idiopathic interstitial pneumonia (IIP) is a subtype of ILD characterized by interstitial inflammation and a variable degree of fibrosis. Usual interstitial pneumonia (UIP) and nonspecific interstitial pneumonia (NSIP) are the major histologic patterns in human IIP. The most unique and diagnostic characteristics of UIP are heterogeneous appearances at a low magnification. Thus, areas of fibrosis with scarring and honeycomb changes are interspersed with areas of less-affected or nearly normal parenchyma [2]. In addition to the advanced fibrotic areas, scattered fibroblastic foci of proliferating fibroblasts or myofibroblasts are observed in UIP. In contrast, NSIP is characterized by the spatially and temporally homogeneous involvement of the pulmonary parenchyma with inflammatory cell infiltration and/or fibrosis in the interstitium [3]. In general, UIP is associated with the idiopathic pulmonary fibrosis (IPF) that is characterized by the worst prognosis, including the progressive destruction of the lung and the decline of pulmonary function [4]. Several hypotheses have been suggested to explain the mechanisms of pulmonary fibrosis, for example, the loss of integrity of the alveolar-capillary barrier basement membrane; cytokines, such as TGF- β ; continuous stimulus of antigen; epithelial-to-mesenchymal transition; and recruitment of bone marrow-derived fibrogenic progenitor cells [5]. However, the pathogenesis of human IPF remains unknown.

Toll-like receptors (TLRs) are a family of pattern-recognition receptors that trigger innate immune responses that are involved in the first line of host defense against microbial infection. These receptors also modulate adaptive immune responses. In addition to sensing the pathogen-associated molecular patterns (PAMP), TLRs also recognize various host-derived endogenous ligands [i.e., damage-associated molecular patterns (DAMP)] associated with tissue injury. TLRs are, thus, involved in the regulation of inflammatory or reparative processes and the remodeling of tissue during non-infectious tissue injury. TLRs are expressed in non-hematopoietic cells, including endothelial and epithelial cells, as well as immune cells [6]. Their expression patterns and functions vary as a function of the cell types, tissues, and forms of infectious or non-infectious stimuli [7,8]. Among TLRs, TLR2 and TLR4 have been demonstrated to play crucial roles in regulating non-infectious lung injury, although the results have been

inconsistent [7–12]. TLR2 and TLR4 on alveolar macrophage and lung epithelial cells recognize hyaluronan, an extracellular matrix degradation product generated during lung tissue injury. A study using both TLR2- and TLR4-deficient mice suggested that these receptors promote inflammatory response and recovery from acute lung injury [7,12]. TLR4 was reported by others to promote the resolution of inflammation and fibrosis after acute or chronic lung injury [10]. In contrast to TLR4, blocking TLR2 attenuated bleomycin-induced pulmonary fibrosis, which suggested that TLR2-mediated signals contributed to pulmonary fibrosis [11]. Moreover, we recently demonstrated that TLR2, expressed by respiratory epithelial (RE) cells rather than immune cells, promoted bleomycin-induced pulmonary fibrosis (BIPF) by increasing the producing IL-27 and chemokines by these cells [9]. These findings suggest that TLRs expressed on RE cells may play a crucial role in the regulation of human IIP. Therefore, a comprehensive investigation of the expression patterns of TLRs in the lung tissues from IIP patients should be helpful for understanding the TLR-mediated regulation of IIP. The cellular expression of TLRs in chronic human IIP has not previously been described, although studies using cell lines, bronchoalveolar lavage (BAL) fluid, or animal tissues have been reported. TLR2 expression was elevated in BAL fluid from IPF patients compared to control [13]. However, there have been no reports of the TLR expression patterns in individual cell types in lung tissues from patients with IPF. Therefore, we investigated the expression patterns of TLR2 and TLR4 using surgically resected lung tissues from UIP or NSIP patients in comparison with control tissues.

Materials and methods

Patient selection

We selected 65 patients who underwent lung wedge resections under the clinical and radiological suspicion of IPF or NSIP at Seoul National University Hospital between 2005 and 2011. Among them, 36 cases were histologically confirmed to be UIP, and 29 cases were diagnosed as NSIP based on the diagnostic guidelines [1,3,14]. In addition, normal or mildly inflamed lung parenchyma was obtained from 18 patients who underwent lobectomy for primary pulmonary adenocarcinomas, and control tissues were taken from regions at least 5 cm away from the tumors. The clinical data were obtained from the medical record, and are summarized in Table 1. This study followed the World

Table 1 Clinical features of the study subjects.

	Control	NSIP	UIP
No. of patients	18	29	36
Age, years (mean \pm S.D.)	65.6 \pm 9.3	54.6 \pm 11.5	61.2 \pm 12.3
Sex, M/F	9/9	8/21	18/18
PCO ₂ (mean \pm S.D.)	40.7 \pm 4.1	46.0 \pm 7.0	40.2 \pm 8.0
pO ₂ (mean \pm S.D.)	122.3 \pm 44.7	104.6 \pm 38.1	113.5 \pm 50.2
FEV ₁ (mean \pm S.D.)	2.3 \pm 0.6	1.9 \pm 0.8	2.0 \pm 0.7
%VC (mean \pm S.D.)	71.4 \pm 8.7	84.4 \pm 6.6	79.5 \pm 10.4
FEF ₂₅₋₇₅ (mean \pm S.D.)	1.8 \pm 0.8	2.4 \pm 1.1	2.3 \pm 1.0
%DLCO (mean \pm S.D.)	100.2 \pm 19.4	53.6 \pm 19.1	61.1 \pm 22.2

NSIP, Nonspecific interstitial pneumonia; UIP, usual interstitial pneumonia; FEV₁, forced expiratory volume in 1 s; VC, vital capacity; FEF₂₅₋₇₅, forced expiratory flow from 25 to 75% of the vital capacity; DLCO, carbon monoxide diffusion in the lung; S.D., standard deviation.

Medical Association declaration of Helsinki and was approved by the institutional review board of Seoul National University Hospital (IRB No.: H-1104-061-358).

Histologic examination and immunohistochemistry

Formalin-fixed and paraffin-embedded (FFPE) tissue blocks were used for histological and immunohistochemical examinations. Serial 4 μ m-thick sections were cut from each block and processed for hematoxylin & eosin (H&E) staining and immunohistochemistry. Two pathologists (HG and DHC) reviewed the H&E and immunostained slides of all cases independently or together for consensus.

For immunohistochemistry, sections were deparaffinized with xylene for 20 min, rehydrated with serially diluted ethanol, and immunostained using antibodies against TLR2 (1:500, rabbit polyclonal, Cat No. PAB11280, Abnova, Taipei city, Taiwan) and TLR4 (1:1000, rabbit polyclonal, Cat No. PAB15432, Abnova) as primary antibodies. Detection was performed using an Envision kit (Dako, Ely, U.K.) containing a peroxidase-conjugated anti-mouse Ig polymer. The sections were then incubated with diaminobenzidine for 5 min to visualize the reaction.

For the quantitative analysis of the immunohistochemical results, the immunostained slides were scanned using the AperioScanscope Cs (Aperio Technologies, Vista, CA) with a 20 \times objective. Using virtual microscope software (ImageScope V12.0.0.5039, Aperio Technologies), 10 fields (total area; 966,042 μ m²) were randomly selected and captured in each case. The numbers of TLR2- and TLR4-positive cells, types of TLR2- and TLR4-expressing cells, and intensity of immunostaining were independently evaluated by two pathologists (HG and JK). The identification of BECs, type II or type I pneumocytes was performed on a morphological basis [1,15]. The mean numbers and standard deviations (S.D.) of TLR2- and TLR4-positive cells were calculated from values from 10 fields. The intensity of the TLR2 and TLR4 expression was scored from absent (0) to strong (3+).

Statistical analysis

To compare the numbers of TLR2- and TLR4-positive cells and the intensity of TLR2 and TLR4 expression between these groups, the Kruskal–Wallis test was used. Statistical significance was defined as a value of $p < 0.05$. Post-hoc comparisons were performed by using Bonferroni-corrected Mann–Whitney U tests; in this case, a p -value of <0.017 was considered statistically significant. Paired Student's t -test was performed to compare the number of TLR2- and TLR4-positive cells between the fibroblastic and fibrotic areas in each case. All statistical tests were two-sided. The statistical analyses were conducted using the IBM-SPSS Statistics software package (version 19.0; IBM Corporation, Armonk, NY, U.S.A.).

Results

Expression patterns of TLR2 and TLR4 in human lung tissues

In the control tissues, TLR2 expression was observed in RE cells, alveolar macrophages, and immune cells, including lymphocytes and neutrophils. Among the RE cells, TLR2 was expressed in the cytoplasm of bronchial epithelial cells (BECs) and type II pneumocytes (Fig. 1A and C). In contrast, TLR2 expression was not observed in peribronchial muscles, fibroblasts, endothelial cells, or type I pneumocytes. Like TLR2, TLR4 was also expressed in BECs, type II pneumocytes, alveolar macrophages, and immune cells (Fig. 1B and D). Of note, the staining intensity of TLR4 in the type II pneumocytes was variable. Specifically, TLR4 expression in the type II pneumocyte was generally weak, but increased in some cases where inflammatory cell infiltration was prominent (Fig. 1E).

In chronic IIP, i.e., UIP and NSIP, the types of TLR2- and TLR4-expressing cells were basically similar to those of the control tissues. BECs, Type II pneumocytes, and alveolar macrophages comprised the majority of TLR2- and TLR4-expressing cells in chronic IIP. However, the frequencies and intensities of TLR2 and TLR4 expression in these cells of chronic IIP tissues were different from the control tissues (Figs. 2 and 3A–F). Metaplastic ciliated columnar epithelial cells, considered to have originated from BEC and found in chronic IIP, also expressed TLR2 and TLR4, whereas the hyperplastic peribronchial muscular tissues and endothelial cells did not. A few fibroblasts or myofibroblasts in the fibroblastic foci and fibrotic scar areas of the tissues from chronic IIP patients expressed TLR2 (Fig. 3C and D), but not TLR4 (Fig. 3E and F).

The relationship between TLR2 and TLR4 expressions in chronic IIP was different according to the pathologic patterns. In NSIP patients, the numbers of TLR2-expressing epithelial cells were positively correlated with those of TLR4-expressing epithelial cells as follows: for type II pneumocyte with Spearman's correlation coefficient (r) of 0.657 ($p < 0.001$); for BEC with r of 0.392 ($p = 0.036$); for RE cells with r of 0.471 ($p = 0.010$). On the other hand, in UIP cases, the numbers of TLR2-positive type II pneumocytes and RE cells showed no relationship with those of TLR4-positive type II pneumocytes and RE cells [$r = 0.276$].

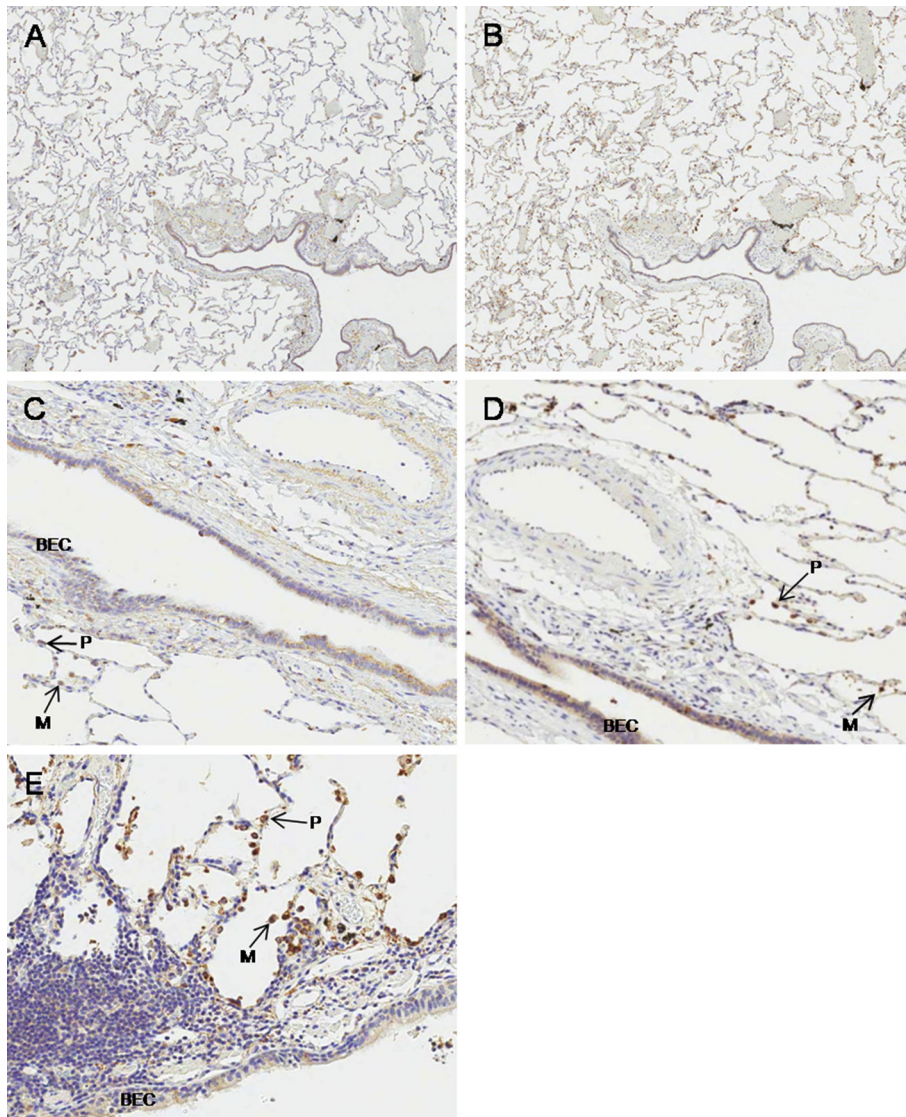


Figure 1 Expression of TLR2 and TLR4 in control lung tissues. TLR2 (A and C) and TLR4 (B and D) are expressed in bronchial epithelial cells ("BEC"), type II pneumocytes ("P"), and alveolar macrophages ("M"). (E) TLR4 expression is increased in type II pneumocytes in a case showing inflammatory cell infiltration. (A and B, $\times 40$ and C–E, $\times 200$).

($p = 0.104$) and $r = 0.282$ ($p = 0.096$), respectively], although the numbers of TLR2-positive BECs were positively correlated with those of TLR4-positive BECs ($r = 0.346$ and $p = 0.039$).

Different expression patterns of TLR2 in respiratory epithelial cells of human chronic IIP

Although immune cells other than alveolar macrophages also expressed TLR2 and TLR4 in chronic IIP, the degree of immune cell infiltration in chronic IIP was variable. Moreover, the expression intensity of TLR2 and TLR4 in the immune cells, including alveolar macrophages of the IIP tissues, was similar to that of the control tissues. These results led us to compare the TLR2 and TLR4 expression in RE cells in the control, NSIP, and UIP lung tissues.

The numbers and expression intensities of TLR2-positive RE cells are displayed in Fig. 4A–C and summarized in Table

2. In addition to the epithelial cells lining the bronchus or bronchioles, metaplastic columnar ciliated epithelial cells in honeycomb areas were also included in BECs. The mean numbers of TLR2-expressing RE cells, i.e., type II pneumocyte plus BEC, were significantly higher in chronic IIP than in the control tissues (NSIP vs. control, $p < 0.001$; UIP vs. control, $p < 0.001$). Furthermore, the numbers of TLR2-expressing RE cells were significantly higher in UIP than NSIP ($p < 0.001$) (Fig. 4A and Table 2). The analysis of the numbers of TLR2-positive RE cells showed that the numbers of TLR2-positive type II pneumocytes were significantly higher in either NSIP or UIP compared to control ($p < 0.001$, both). The numbers of TLR2-expressing type II pneumocytes were slightly higher in the UIP tissues than in those from the NSIP patients, but the difference was not statistically significant. On the other hand, the numbers of TLR2-positive BEC were significantly higher in UIP than in NSIP ($p < 0.001$) or control ($p < 0.001$) (Fig. 4B and Table 2). In

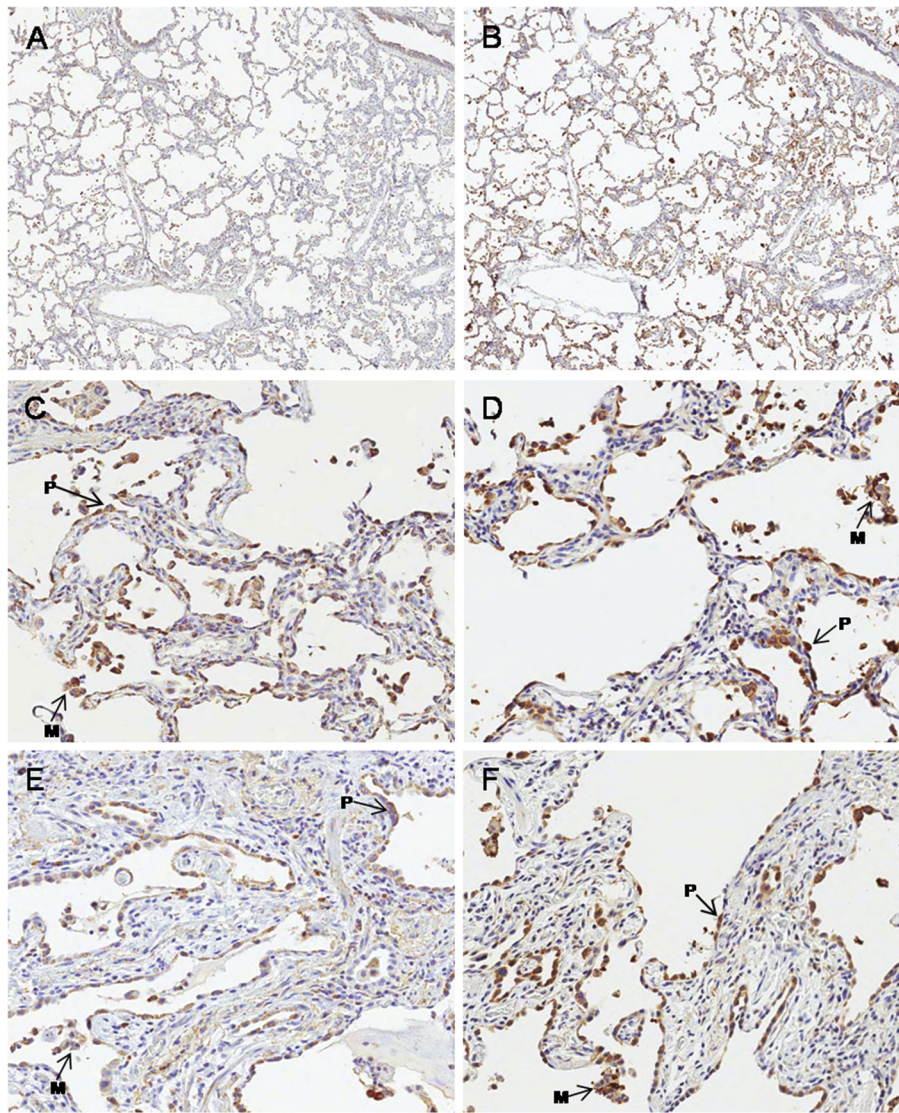


Figure 2 Expression of TLR2 and TLR4 in nonspecific interstitial pneumonia. TLR2 (A and C) and TLR4 (B and D) were over-expressed in respiratory epithelial cells in nonspecific interstitial pneumonia (NSIP), cellular type. TLR2 (E) and TLR4 (F) were also over-expressed by respiratory epithelial cells in NSIP, fibrosing type. (A and B, $\times 40$ and C–F $\times 200$. "P", type II pneumocyte; "M", alveolar macrophage).

chronic IIP, the average intensity of the TLR2-positive RE cells was moderate (Figs. 1C, 2C and 2E, 3C and 3D). The intensity of TLR2 expression in type II pneumocytes was not different between NSIP and UIP (Fig. 4C and Table 2). Furthermore, the intensity of TLR2 expression in BECs and alveolar macrophages was similar between control, NSIP, and UIP cases. However, the intensity of the TLR2 staining of type II pneumocytes was much stronger in NSIP or UIP than that in control ($p < 0.001$, both).

Different expression patterns of TLR4 in respiratory epithelial cells of human chronic IIP

The numbers and the expression intensity of TLR4-positive RE cells are displayed in Fig. 4D–F and summarized in Table 3. The mean numbers of TLR4-expressing RE cells, i.e., type II pneumocyte plus BECs, were significantly higher in

chronic IIP than in control (NSIP vs. control, $p < 0.001$; NSIP vs. control, $p < 0.001$). However, the numbers of TLR4-positive RE cells were not significantly greater in UIP than NSIP, which contrasted with the frequent TLR2 expression in RE cells of UIP compared to NSIP (Fig. 4D and Table 3). In terms of RE cell types, the numbers of either TLR4-positive type II pneumocytes or BECs were significantly higher in the NSIP and UIP tissues than in the control ($p < 0.001$). In contrast, the numbers of TLR4-expressing type II pneumocytes were approximately the same for the UIP and NSIP samples, similar to the results for TLR2. The numbers of TLR4-positive BECs were greater in the UIP than on the NSIP tissues ($p = 0.003$) (Fig. 4E and Table 3). Most of the type II pneumocytes in the chronic IIP tissues showed moderate intensity of TLR4 expression, which was much stronger than that of the control tissues (NSIP vs. control, $p < 0.001$; UIP vs. control, $p < 0.001$) (Figs. 1D, E, 2D, 2F, 3E and 3F).

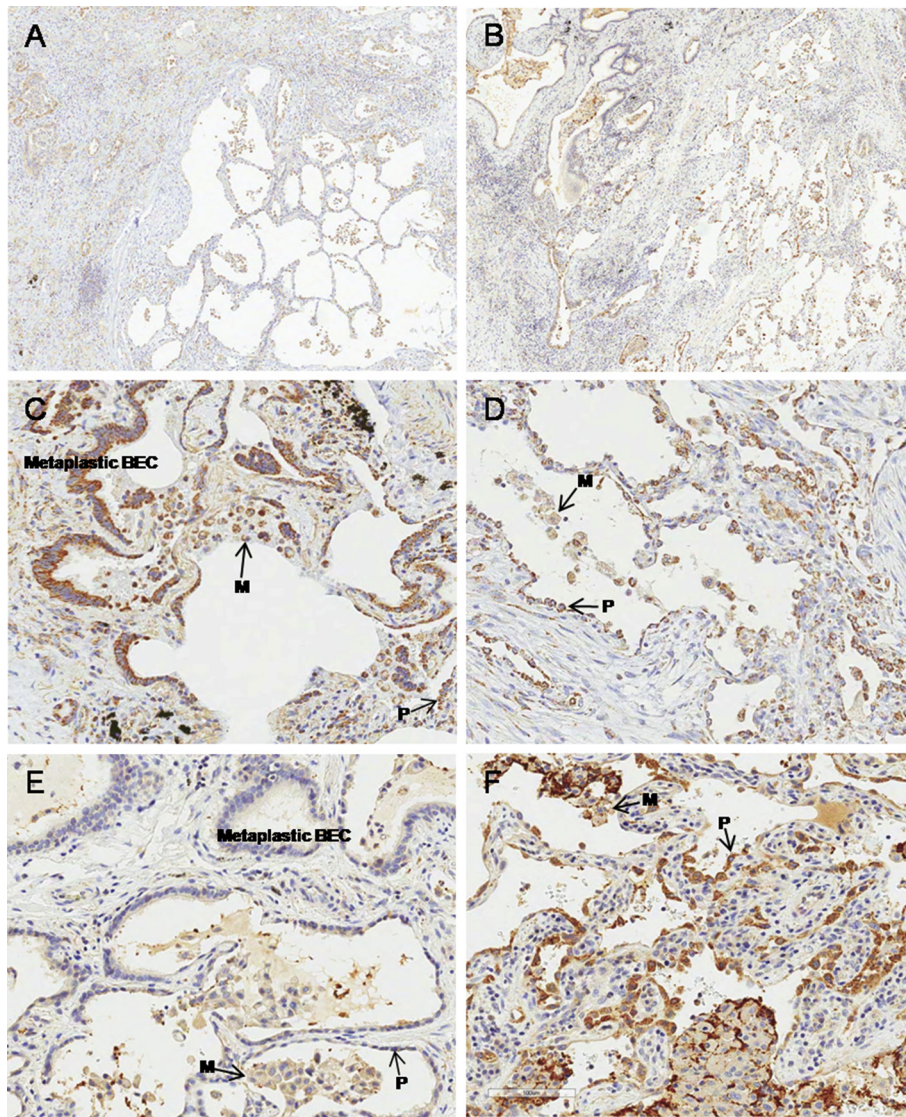


Figure 3 Expression of TLR2 and TLR4 in usual interstitial pneumonia. TLR2 (A) and TLR4 (B) were over-expressed in respiratory epithelial cells in usual interstitial pneumonia (UIP). TLR2 expression was increased in a fibrotic area of UIP (C) compared to a fibroblastic area (D) in a representative case. TLR4 expression was down-regulated in the fibrotic areas (E) relative to fibroblastic areas (F) in a case of UIP. (A and B, $\times 40$ and C–F, $\times 200$. “BEC”, bronchial epithelial cell; “P”, type II pneumocyte; “M”, alveolar macrophage).

However, there was no difference in the TLR4 intensity of type II pneumocytes between NSIP and UIP (Fig. 4F and Table 3). Similar to the expression patterns of TLR4 in control tissues, BECs expressed TLR4 at variable intensities from negative to strong, even within a single category of disease. Thus, no significant difference was observed for TLR4 expression intensity between control and chronic IIP. The expression intensity of TLR4 in alveolar macrophages was generally weak and comparable in control, NSIP, and UIP cases.

Differences in TLR2 and TLR4 expression patterns between the fibroblastic and fibrotic areas in human UIP

Whereas interstitial inflammation and/or fibrosis were temporally and spatially uniform in NSIP, temporally

heterogeneous fibrosis due to tissue injury is typically progressive and irreversible in UIP. In the resected lung tissues of UIP cases, fibroblastic areas composed of proliferating fibroblasts or myofibroblasts and advanced fibrotic areas are frequently found, suggesting that fibrosis is actively ongoing and progressive in the lung of UIP patients.

Higher numbers of TLR2-positive RE cells, but not TLR4-positive cells, in UIP compared to NSIP led us to hypothesize that TLR2 might contribute more than TLR4 to fibrosis in human chronic IIP. To provide more evidence for this, we compared the TLR2 and TLR4 expression between the fibroblastic and fibrotic areas in UIP. Fig. 3 shows representative images demonstrating that the RE cells in the honeycomb areas of UIP frequently showed increased TLR2 expression compared to fibroblastic areas (Fig. 3C and D). On the other hand, TLR4 expression in the RE cells in the fibrotic areas was weaker, and the majority of metaplastic

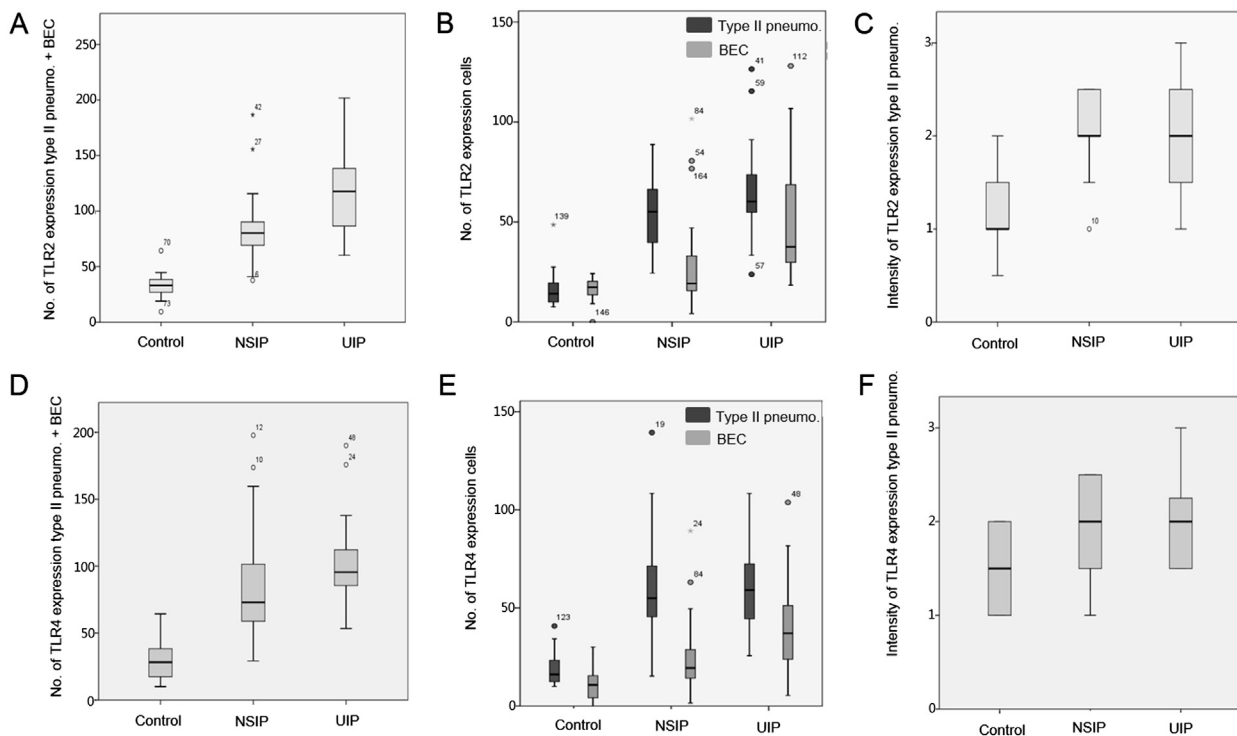


Figure 4 Numbers of TLR2- and TLR4-expressing respiratory epithelial cells and their expression intensity in human chronic idiopathic interstitial pneumonia. (A) The numbers of TLR2-positive respiratory epithelial cells, including type II pneumocytes and bronchial epithelial cells (BECs), were much higher in nonspecific interstitial pneumonia (NSIP) and usual interstitial pneumonia (UIP) compared to control. (B) The numbers of TLR2-positive type II pneumocytes or BECs are displayed in control, NSIP, and UIP. (C) The intensity of TLR2 expression in type II pneumocytes was stronger in NSIP and UIP than control. (D) The numbers of TLR4-positive respiratory epithelial cells including type II pneumocytes plus BECs were increased in NSIP and UIP than control. (E) The numbers of TLR4-positive type II pneumocytes or BECs are shown. (F) The intensity of TLR4 expression in type II pneumocytes was stronger in NSIP and UIP than control.

BECs of honeycomb areas exhibited faint expression of TLR4 (Fig. 3). Quantitatively, the mean numbers of type II pneumocytes and BECs expressing TLR2 or TLR4 were similar in the fibroblastic areas; however, TLR2-positive type II pneumocytes and BECs far outnumbered the TLR4-positive cells in the fibrotic areas (Table 4). Overall, the mean numbers of TLR2-positive type II pneumocytes or BECs were significantly higher in fibrotic areas compared to the fibroblastic areas, whereas the numbers of TLR4-positive cells were similar between these two areas (Fig. 5A and B). Moreover, the numbers of TLR2-expressing RE cells were markedly greater in the fibrotic areas rather than in fibroblastic areas of each case ($p < 0.001$ by paired Student's *t*-test), while the differences in the numbers of TLR4-expressing RE cells were only marginally significant between two areas (Fig. 5C and D, Supplement figs1).

Discussion

The present study demonstrated that TLR2 and TLR4 are expressed in BECs, type II pneumocytes, alveolar macrophages, and immune cells in the control lung tissues. Importantly, TLR2 and TLR4 expression in RE cells, including BECs and type II pneumocytes, was increased in human IIP. These findings suggest that the regulation of TLR2 and -4

expression in the lung tissues might be involved in the pathogenesis of chronic IIP in humans. Human RE cells, including BECs and type II alveolar epithelial cells or pneumocytes, have consistently been shown to express a variety of TLRs and their signaling molecules, which elicit different responses to microbial and/or endogenous ligands [16–20]. However, the possible roles of TLR expression or function in the context of human chronic IIP are not yet well understood. Pneumocyte hyperplasia is invariably prominent in almost all chronic IIP cases, and it has previously been suggested that proliferation of type II pneumocytes might reflect alveolar damage [21,22]. Most type II pneumocytes and BECs expressed TLR2 and TLR4 in human normal and chronic IIP lung tissues, and TLR2 expression was more polarized on the apical surface of BECs (Fig. 1C), consistent with a previous report [19]. The numbers and expression intensity of TLR2 or TLR4-expressing RE cells were much higher in UIP and NSIP than control. In particular, the numbers of TLR2-positive RE cells were significantly higher in UIP compared to NSIP; however, this was not the case for TLR4. Furthermore, TLR2 expression was maintained or significantly increased in type II pneumocytes and metaplastic BECs in the fibrotic areas of UIP compared with fibroblastic areas, whereas the TLR4 expression was similar between these two areas. In addition, a few fibroblasts/myofibroblasts in UIP tissues

Table 2 Toll-like receptor 2 (TLR2) expression in respiratory epithelial cells.

	Control	NSIP	UIP	p-Value		
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	Control vs. NSIP	Control vs. UIP	NSIP vs. UIP
Average number of TLR-2 positive RE cells (x 200)						
Type II pneumo.	16.49 (9.60)	53.24 (18.45)	64.80 (20.74)	<0.001*	<0.001*	0.042
BEC	16.70 (5.76)	28.67 (22.74)	50.67 (27.75)	0.128	<0.001*	<0.001*
Type II pneumo. + BEC	33.19 (11.40)	81.92 (31.44)	115.47 (36.14)	<0.001*	<0.001*	<0.001*
Intensity of TLR2-positive RE cells						
Type II pneumo.	1.19 (0.55)	2.00 (0.40)	1.96 (0.53)	<0.001*	<0.001*	0.824
BEC	2.00 (0.00)	1.77 (0.43)	1.92 (0.37)	0.040	0.341	0.053

NSIP, nonspecific interstitial pneumonia; UIP, usual interstitial pneumonia; RE, respiratory epithelial; pneumo., pneumocyte; BEC, bronchial epithelial cells; S.D., standard deviation. * $p < 0.017$ is considered statistically significant.

Table 3 Toll-like receptor 4 (TLR4) expression in respiratory epithelial cells.

	Control	NSIP	UIP	p-Value		
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	Control vs. NSIP	Control vs. UIP	NSIP vs. UIP
Average number TLR4-positive RE cells (x 200)						
Type II pneumo.	19.21 (8.97)	60.78 (29.38)	60.43 (21.49)	<0.001*	<0.001*	0.792
BEC	11.31 (7.67)	25.35 (18.55)	38.68 (20.62)	0.001*	<0.001*	0.003*
Type II pneumo. + BEC	30.52 (3.74)	86.14 (42.42)	99.10 (28.91)	<0.001*	<0.001*	0.029
Intensity of TLR4-positive RE cells						
Type II pneumo.	1.42 (0.40)	2.02 (0.45)	2.00 (0.40)	<0.001*	<0.001*	0.721
BEC	0.80 (0.77)	0.67 (0.62)	0.41 (0.56)	0.633	0.084	0.095

NSIP, nonspecific interstitial pneumonia; UIP, usual interstitial pneumonia; pneumo., pneumocyte; BEC, bronchial epithelial cells; S.D., standard deviation, * $p < 0.017$ is considered statistically significant.

expressed TLR2 but not TLR4. It is possible that the TLR2-positive fibroblastic cells in the fibroblastic foci of UIP might have been derived from epithelial cells by epithelial–mesenchymal transition [23]. Collectively, our findings indicate that TLR2 expression was much higher in UIP than NISP, particularly in advanced fibrotic areas. Consistent with our results, a study using BAL fluid immune cells showed that TLR2, but not TLR4, expression was also higher in IPF patients compared to control [13]. Thus, the results suggest that TLR2 expression may be up-regulated in RE cells and may contribute to progressive fibrosis of the lungs. In agreement with the expression patterns of TLR2 and TLR4 in the lungs, several studies showed that the TLR2 and TLR4 on human BECs and type II pneumocytes are functional, as indicated by activation of signaling pathway and production of cytokines and chemokines upon stimulation with their ligands [16,18,19]. Furthermore, TLR2 promoted bleomycin-induced immune responses and

pulmonary fibrosis, whereas TLR4 signaling attenuated pulmonary immune responses and fibrosis after lung injury [11,24]. Previously, we reported a role for a TLR2-mediated immune mechanism in BIPF by demonstrating that TLR2 signaling in RE cells promoted BIPF by inducing IL-27 and chemokine production in mice. [9] Taken together, these results suggest that TLR2 rather than TLR4 may promote chronic IIP in humans as well as in animal pulmonary fibrosis models. This hypothesis is further supported by previous observations by ourselves and others that TLR2 signaling by RE cells promotes BIPF, and the inhibition of TLR2 pathways relieves pulmonary inflammation and fibrosis in the mouse model [9,11].

Taken together, our findings suggested that TLR2 and TLR4 expressed in RE cells may be involved in the pathogenesis of human chronic IIP and that TLR2, but not TLR4, on RE cells may contribute to pulmonary fibrosis in human IPF.

Table 4 Numbers of TLR2 and TLR4-expressing respiratory epithelial cells in the fibroblastic and fibrotic areas of UIP.

Areas	Type II pneumo.			BEC		
	TLR2	TLR4	p	TLR2	TLR4	p
	Mean (S.D.)	Mean (S.D.)		Mean (S.D.)	Mean (S.D.)	
Fibroblastic	61.69 (22.88)	62.67 (24.22)	0.86	36.88 (25.41)	33.06 (24.75)	0.67
Fibrotic	78.90 (41.19)	57.48 (29.84)	0.016*	95.28 (61.28)	58.09 (63.05)	0.025*

Pneumo., pneumocyte; BEC, bronchial epithelial cells; S.D., standard deviation, * $P < 0.05$ is considered statistically significant.

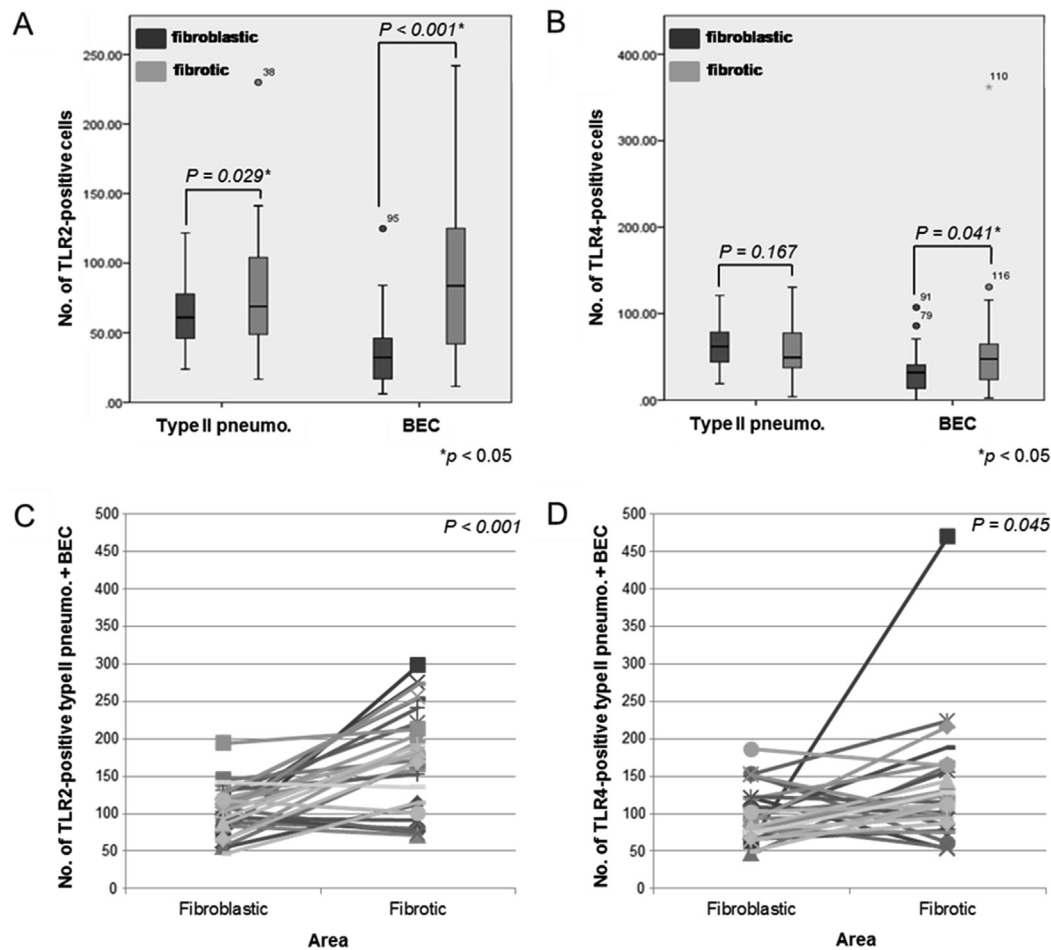


Figure 5 TLR2 and TLR4 expression patterns in respiratory epithelial cells in the fibroblastic and fibrotic areas of UIP. The mean numbers of TLR2- (A) or TLR4- (B) expressing type II pneumocytes and bronchial epithelial cells (BECs) are compared in the fibroblastic and fibrotic areas of usual interstitial pneumonia (UIP). The changes in the number of TLR2- (C) or TLR4- (D) positive respiratory epithelial cells between the fibroblastic and fibrotic areas in UIP cases are illustrated.

TLR4 recognizes hyaluronan fragments or diesel exhaust and stimulates lung inflammation [12,24,25]. The TLR4-MyD88-NF-KappaB-dependent pathway links signals to link proinflammatory and pro-fibrotic signals in hepatic fibrosis [26]. Lipopolysaccharide (LPS), a gram-negative bacterial ligand, induced pulmonary fibrosis via TLR4-mediated pathways [27,28]. In contrast, TLR4 was required for the reduction of inflammation and fibrosis after acute and chronic lung injury [10]. These results suggest that TLR4 may exert diverse effects on fibrosis, which depend on the types of target tissues and stimulating ligands. In our study, the TLR4 expression pattern appeared to be similar to TLR2 in human chronic IIP, but decreased expression was observed in UIP, particularly, in the fibrotic areas. Although the etiology or initiating stimuli in human chronic IIP are unknown, our observation raises the possibility that TLR4 may act as a regulator of pulmonary fibrosis in human chronic IIP.

Nevertheless, the present study, based on immunohistochemistry in excised tissues, could not provide robust evidences for the functional roles of the TLR2 and TLR4 expressed by RE cells in human chronic IIP or IPF. Although TLR2 and TLR4 expressed by normal human RE cells were

found to be functional in previous *in vitro* studies [16,18,19], further studies are warranted to more clearly address the roles of TLR2 and TLR4 on RE cells in human chronic IIP.

In conclusion, this study demonstrates that TLR2 and TLR4 expression is increased in the RE cells of human UIP and NSIP tissues and therefore may be involved in the pathogenesis of human chronic IIP. The expression pattern of TLR2 in RE cells supports a pro-fibrotic role of TLR2 in human UIP and IPF as suggested by previous experiments using a mouse model.

Conflict of interest statement

The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

Acknowledgments

This work was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korean

government (Ministry of Education, Science and Technology no. 20120005652).

Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.rmed.2013.12.007>.

References

- [1] Fukuoka J, Leslie KO. Chronic diffuse lung diseases. In: Leslie KO, Wick MR, editors. *Practical pulmonary pathology: a diagnostic approach*. 2nd ed. Philadelphia, PA: Saunders-Elsevier; 2011. pp. 213–22.
- [2] Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, et al. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med* 2011;183(6):788–824.
- [3] Katzenstein AL, Myers JL. Nonspecific interstitial pneumonia and the other idiopathic interstitial pneumonias: classification and diagnostic criteria. *Am J Surg Pathol* 2000;24(1):1–3.
- [4] Larsen BT, Colby TV. Update for pathologists on idiopathic interstitial pneumonias. *Arch Pathol Lab Med* 2012;136(10):1234–41.
- [5] Strieter RM, Mehrad B. New mechanisms of pulmonary fibrosis. *Chest* 2009;136(5):1364–70.
- [6] Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003;21:335–76.
- [7] Jiang D, Liang J, Li Y, Noble PW. The role of toll-like receptors in non-infectious lung injury. *Cell Res* 2006;16(8):693–701.
- [8] Opitz B, van Laak V, Eitel J, Suttorp N. Innate immune recognition in infectious and noninfectious diseases of the lung. *Am J Respir Crit Care Med* 2010;181(12):1294–309.
- [9] Kim HS, Go H, Akira S, Chung DH. TLR2-mediated production of IL-27 and chemokines by respiratory epithelial cells promotes bleomycin-induced pulmonary fibrosis in mice. *J Immunol (Baltimore, Md: 1950)* 2011;187(8):4007–17.
- [10] Yang HZ, Wang JP, Mi S, Liu HZ, Cui B, Yan HM, et al. TLR4 activity is required in the resolution of pulmonary inflammation and fibrosis after acute and chronic lung injury. *Am J Pathol* 2012;180(1):275–92.
- [11] Yang HZ, Cui B, Liu HZ, Chen ZR, Yan HM, Hua F, et al. Targeting TLR2 attenuates pulmonary inflammation and fibrosis by reversion of suppressive immune microenvironment. *J Immunol (Baltimore, Md: 1950)* 2009;182(1):692–702.
- [12] Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, et al. Regulation of lung injury and repair by toll-like receptors and hyaluronan. *Nat Med* 2005;11(11):1173–9.
- [13] Samara KD, Antoniou KM, Karagiannis K, Margaritopoulos G, Lasithiotaki I, Koutala E, et al. Expression profiles of toll-like receptors in non-small cell lung cancer and idiopathic pulmonary fibrosis. *Int J Oncol* 2012;40(5):1397–404.
- [14] American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. *Am J Respir Crit Care Med* 2002;165(2):277–304.
- [15] Colby TV, Leslie KO, Yousem SA. Lungs. In: Mills SE, editor. *Histology for pathologist*. Philadelphia, PA: Lippincott Williams and Wilkins; 2007. pp. 480–1.
- [16] Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP. Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* 2004;31(3):358–64.
- [17] Droemann D, Goldmann T, Branscheid D, Clark R, Dalhoff K, Zabel P, et al. Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. *Histochem Cell Biol* 2003;119(2):103–8.
- [18] Armstrong L, Medford AR, Uppington KM, Robertson J, Witherden IR, Tetley TD, et al. Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. *Am J Respir Cell Mol Biol* 2004;31(2):241–5.
- [19] Muir A, Soong G, Sokol S, Reddy B, Gomez MI, Van Heeckeren A, et al. Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol* 2004;30(6):777–83.
- [20] Mayer AK, Muehmer M, Mages J, Gueinzus K, Hess C, Heeg K, et al. Differential recognition of TLR-dependent microbial ligands in human bronchial epithelial cells. *J Immunol (Baltimore, Md: 1950)* 2007;178(5):3134–42.
- [21] Honda T, Ota H, Yamazaki Y, Yoshizawa A, Fujimoto K, Sone S. Proliferation of type II pneumocytes in the lung biopsy specimens reflecting alveolar damage. *Respir Med* 2003;97(1):80–5.
- [22] Honda T, Uehara T, Sano K. Heterogeneous proliferation of type II pneumocytes in usual interstitial pneumonia. *Pathology* 2006;38(5):433–6.
- [23] Harada T, Nabeshima K, Hamasaki M, Uesugi N, Watanabe K, Iwasaki H. Epithelial-mesenchymal transition in human lungs with usual interstitial pneumonia: quantitative immunohistochemistry. *Pathol Int* 2010;60(1):14–21.
- [24] Inoue K, Takano H, Yanagisawa R, Hirano S, Ichinose T, Shimada A, et al. The role of toll-like receptor 4 in airway inflammation induced by diesel exhaust particles. *Arch Toxicol* 2006;80(5):275–9.
- [25] Karimi K, Sarir H, Mortaz E, Smit JJ, Hosseini H, De Kimpe SJ, et al. Toll-like receptor-4 mediates cigarette smoke-induced cytokine production by human macrophages. *Respir Res* 2006;7:66.
- [26] Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007;13(11):1324–32.
- [27] He Z, Gao Y, Deng Y, Li W, Chen Y, Xing S, et al. Lipopolysaccharide induces lung fibroblast proliferation through toll-like receptor 4 signaling and the phosphoinositide3-kinase-Akt pathway. *PLoS One* 2012;7(4):e35926.
- [28] He Z, Zhu Y, Jiang H. Inhibiting toll-like receptor 4 signaling ameliorates pulmonary fibrosis during acute lung injury induced by lipopolysaccharide: an experimental study. *Respir Res* 2009;10:126.