IL-27 enhances innate immunity of human pulmonary fibroblasts and epithelial cells through upregulation of TLR4 expression.

Yufeng Su\textsuperscript{a,b,1}, Hua Yao\textsuperscript{b,1}, Hong Wang\textsuperscript{b}, Fang Xu\textsuperscript{c}, Dagen Li\textsuperscript{b}, Dairong Li\textsuperscript{d}, Xuemei Zhang\textsuperscript{b}, Yibing Yin\textsuperscript{b}, Ju Cao\textsuperscript{a,*}

\textsuperscript{a}Department of Laboratory Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China.

\textsuperscript{b}Key Laboratory of Diagnostic Medicine designated by the Ministry of Education, Chongqing Medical University, Chongqing, China.

\textsuperscript{c}Department of Emergency and Intensive care unit, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China.

\textsuperscript{d}Department of Respiratory Diseases, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China.

\textsuperscript{1}Both Yufeng Su and Hua Yao contributed equally to this work.

*Address for reprint requests and other correspondence: J. Cao, Department of Laboratory Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing Medical University, Yuzhong District, Chongqing, China.

E-Mail: caoju723@163.com

Tel: 86-89012513; Fax: 86-23-89012513

Running title: TLR4 expression in lung tissue cells by IL-27
Abstract

Lung tissue cells play an active role in the pathogenesis of pulmonary inflammatory diseases by releasing a variety of cytokines and chemokines. However, how lung tissue cells respond to microbial stimuli during pulmonary infections remains unclear. In this study, we found that patients with community-acquired pneumonia displayed increased IL-27 levels in bronchoalveolar lavage fluid and serum. We subsequently examined the immunopathological mechanisms for the activation of primary human lung fibroblasts and bronchial epithelial cells by IL-27. We demonstrated that IL-27 priming enhanced LPS-induced production of IL-6 and IL-8 from lung fibroblasts and bronchial epithelia cells via up-regulating TLR4 expression. IL-27 up-regulated TLR4 expression in lung fibroblasts through activation of Janus-activated kinase (JAK) and Jun N-terminal kinase (JNK) signaling pathways, and inhibition of the JAK pathway could partially decrease IL-27-induced TLR4 expression, while inhibition of JNK pathway could completely suppress IL-27-induced TLR4 expression. Our data suggest that IL-27 modulates innate immunity of lung tissue cells through upregulating TLR4 expression during pulmonary infections.

Key words: IL-27, human lung fibroblasts, epithelial cells, TLR4, innate immunity
**Introduction**

Lower respiratory tract infections, particularly pneumonia, are an important cause of morbidity and mortality in humans (3). Lung tissue cells as well as a number of inflammatory cells are major cellular players of innate immunity against respiratory infections (1). Among these cells, lung fibroblasts are abundant in connective tissue as cellular communication–bridging interstitium and vasculature in the lung (2). Lung fibroblasts are known to function as sentinel or effector cells during airway host response, regulating the activities of other immune cells by producing inflammatory cytokines and chemokines (2, 13). Knowledge of the regulation of immune response in lung fibroblasts is thus important for an understanding of the pathogenesis of pulmonary infectious diseases.

The important role of lung fibroblasts in pulmonary defense to eliminate inhaled pathogens relies on the signals generated from membrane-bound and cytosolic pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) (3). TLRs have been found to be a pivotal family of receptors for recognition of structural components that are unique to pathogens, such as lipopolysaccharide (LPS), lipoteichoic acid, flagellin, and microbial DNA (22). Upon ligand binding, TLRs induce the production of inflammatory mediators and, ultimately, clearance of infection (3, 22). TLRs are expressed on a wide variety of cell types, including fibroblasts (4). Understanding the mechanisms underlying the regulation of TLR expression is critical to design therapeutic and/or prevention strategies against microbial infections in the lungs.
IL-27 is a heterodimeric cytokine bridging innate and adaptive immunity (32). IL-27 could induce a range of inflammatory events involved in immune defense in the lung through T-cell cytokine receptor (TCCR)/IL-27 receptor alpha (WSX-1) and glycoprotein 130 (gp130) (12). In prior studies, we have observed that IL-27 expression was increased in a variety of pulmonary infectious diseases (6, 8, 9, 11, 31), and IL-27 could directly activate airway epithelial cells or lung fibroblasts to modulate the expression of IL-6, CXCL10 and intercellular adhesion molecule 1 (ICAM-1) (7, 9, 11). In this study, we hypothesized that IL-27 could modulate TLR signaling in lung tissue cells, thereby regulating the ability of immune system to respond to inhaled pathogens in the lung. Our results showed a novel mechanism by which IL-27 primes the TLR4 signaling in human lung tissue cells.

MATERIALS AND METHODS

Human studies

All community-acquired pneumonia (CAP) patients 18 years of age or older who were hospitalized in the intensive care unit (ICU) of The First Affiliated Hospital of Chongqing Medical University were prospectively enrolled in the study if there was a clinical suspicion of infectious pneumonia, defined by a new and persistent infiltrate on chest radiography associated with at least one of the following: fever (temperature ≥ 38.0°C) or hypothermia (temperature < 35.0°C), cough, pleuritic chest pain, dyspnea, and altered breath sounds on auscultation (14, 20). Healthy volunteers without lung diseases who underwent broncho-alveolar lavage (BAL) via
bronchoscopy and donated serum served as control subjects. The data collected included patient demographics, underlying diseases or conditions, illness severity scores including Acute Physiological and Chronic Health Evaluation (APACHE) II, laboratory data including microbiological tests, length of ICU stay, and outcome. In addition, 7 patients with CAP were recruited to collect serial serum samples after effective treatment. The delay between BALF and blood sampling and the beginning of laboratory procedures was always < 2 h, and BALF and serum samples were stored at -80°C until use. This protocol was approved by the Clinical Research Ethics Committee of Chongqing Medical University, and informed consent was obtained from all participants according to the Declaration of Helsinki.

Reagents

Recombinant human IL-27 was purchased from R&D Systems. Ultra-purified lipopolysaccharide (LPS) from Escherichia coli K12 strain without any contamination by lipoprotein. Mouse anti-phospho-Jun N-terminal kinase (JNK), anti-phospho-Janus kinase (JAK) 2, anti-JNK, and anti-JAK2 mAb were purchased from Cell Signaling Technology Corp (Beverly, MA). IκB-α phosphorylation inhibitor BAY11-7082, extracellular signal-regulated kinase (ERK) inhibitor U0126, JNK inhibitor SP600125, p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, phosphatidylinositol 3-OH kinase (PI3K) inhibitor LY294002 and JAK inhibitor AG490 were purchased from Calbiochem Corp. Fluticasone propionate were purchased from GlaxoSmithKline. In all studies, the concentration of dimethyl sulfoxide (DMSO) was 0.1 % (vol/vol).
Human lung fibroblasts culture

Primary human lung fibroblasts (HLF) were purchased from ScienCell Research Laboratories and cultured in fibroblast cell growth medium as described previously (11,18). Human lung fibroblasts were incubated in medium alone or in the presence of recombinant IL-27 for 16 h. Cells were then washed and resuspended in fresh medium containing 1% serum, and subsequently stimulated by LPS or bacteria. In some assays, human lung fibroblasts were pre-treated with signaling molecule inhibitors for 1 h, followed by stimulation with IL-27 and LPS.

Bacterial strains

Gram-positive *S. pneumoniae* TIGR4 (serotype 4) and Gram-negative *P. aeruginosa* (ATCC27853) were purchased from American Type Culture Collection (Manassas, VA, USA), and they were cultured as described previously (5, 8). For cell culture stimulation studies, bacteria were harvested by centrifugation and resuspended in cell culture medium at the indicated concentration without antibiotics. Cell viability was validated by microscopy and measurement LDH release into the supernatant.

Human bronchial epithelial cell culture

Normal human bronchial epithelial cells were purchased from ScienCell Research Laboratories and cultured in bronchial epithelial cell growth medium as described previously (9), and bronchial epithelial cells were stimulated as human lung fibroblasts described above.

Endotoxin-free solutions

Cell culture medium was purchased from Gibco Invitrogen Corporation (CA) free of
detectable LPS (<0.1EU/ml). No solution contained detectable LPS, as determined by
the Limulus amoebocyte lyase assay (sensitivity limit 12 pg/ml; Biowhittaker, Inc.,
MD).

**PCR analysis**

Total cellular RNA was extracted from cells and organs with RNeasy columns
(QIAGEN). Quantitative real-time PCR analysis for TLR4 was performed using
specific Quantitect Primer/Probe assays (QIAGEN). The gene for GAPDH was
amplified as an endogenous reference. Quantification was determined using both a
standard curve and comparative $\Delta\Delta$CT methods.

**Enzyme-linked Immunosorbent Assay (ELISA)**

The concentration of human IL-27 was quantitated by ELISA reagents from
Biolegend Systems according to the manufacturer’s instructions.

**Flow cytometric analysis**

Fibroblasts (2x10^5 cells) were harvested and resuspended with cold PBS. After
blocking and washing, cells were incubated with the FITC-conjugated mouse
anti-human TLR4 monoclonal antibody (BD Pharmingen, or Miltenyi Biotec), or
FITC conjugated mouse IgG isotype at 4 °C in dark for 45 min. Expression of TLR4
of 5,000 viable cells was then quantitatively analyzed by flow cytometry in terms of
mean fluorescence intensity (MFI).

**Immunofluorescence microscopy**

Fibroblasts were cultured in the presence or absence of IL-27 in 12-well plates. After
stimulation, 4% (vol/vol) formaldehyde/phosphate buffered saline (PBS) was used to
fix the cells for 15 minutes. After the blocking step, primary anti-TLR4 monoclonal
antibody (ebioscience) was added and incubated for 1 hour. Finally, the second
antibody (Invitogen) or 4′,6-diamidino-2-phenylindole (for staining nucleus) was
added and incubated for 1 hour. The samples were visualized under confocal
microscopy (Carl Zeiss LSM 510 Meta DuoScan, Carl Zeiss Micro Imaging GmbH,
Germany).

Western blot analysis

Cells (1 x 10^6) were washed with ice-cold PBS and lysed in 0.2 ml lysis buffer (20
mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 mM EGTA,
0.05% 2-mercaptoethanol, 1 x protease inhibitors). Cell debris was removed by
centrifugation at 14,000 x g for 15 min, and the supernatant was boiled in Laemmli
sample buffer (Bio-Rad Laboratory, Hercules, CA) for 5 min. An equal amount of
proteins was subjected to sodium dodecyl sulfate-10% polyacrylamide gel
electrophoresis before blotting onto a PVDF membrane (Amersham and Pharmacia
Biotech). The membrane was blocked with 5% skimmed milk in Tris-buffered saline
with 0.05% Tween 20 for 1 h at room temperature, and probed with anti-human
phospho-JNK and total JNK, anti-human phospho-JAK2 and total JAK2 at 4°C
overnight. After washing, membrane was incubated with corresponding secondary
donkey anti-rabbit or sheep antimouse antibodies coupled to horseradish peroxidase
(Amersham and Pharmacia Biotech) for 1 h at room temperature. Antibody–antigen
complexes were then detected using ECL chemiluminescent detection system.

Statistical analysis
All data were expressed as either mean ± standard deviation or median values and interquartile ranges. Differences between groups were assessed by Mann-Whitney U test, one-way analysis of variance or unpaired t-test, and a P value < 0.05 was considered significantly different. When ANOVA indicated a significant difference, a Bonferroni post hoc test was then used to assess the difference between groups. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, version 10.1.4 (SPSS Inc., Chicago, IL).

RESULTS

Patients with CAP displayed elevated lung and serum IL-27 levels

To obtain a first insight into IL-27 expression during pneumonia, we measured IL-27 levels in BALF or serum from CAP patients requiring treatment in the ICU, and from control volunteers without lung diseases (Table 1). *Streptococcus pneumoniae* (n=3) was the most common isolated bacteria, followed by *Haemophilus influenzae* (n=2), *Acinetobacter baumannii* (n=2), *Pseudomonas aeruginosa* (n=2) and *Klebsiella pneumonia* (n=2). Patients with CAP showed significantly increased levels of serum IL-27 compared with controls (Figure 1A). 11 BALF samples were randomly collected from these patients, and patients with CAP also showed significantly elevated BALF IL-27 levels compared with controls (Figure 1B). Correlation analyses revealed that the serum levels of IL-27 were significantly correlated with their corresponding BALF levels in these patients (Figure 1C). There was no significant difference in IL-27 levels between patients with Gram-positive bacteria infection and patients with Gram-negative bacteria infection (data not shown). 7 patients with CAP
were recruited after effective treatment, and serum IL-27 levels decreased in all 7 patients with CAP by week 8 (Figure 1D).

**IL-27 enhanced LPS-induced proinflammatory cytokine release in lung fibroblasts**

Previously it was reported that IL-27 could directly activate human lung fibroblasts to expression chemokine CXCL10 (11). Here we sought to investigate whether IL-27 could modulate TLR signaling events in the context of lung infections. Primary human lung fibroblasts were preincubated (primed) with IL-27 for 16 h, culture medium was replaced, and cells were subsequently treated with the TLR4 ligand LPS for 8 h. Since IL-27 alone could not induce the production of IL-6 and IL-8 in human lung fibroblasts as observed in our previous studies (11), we performed ELISA for IL-6 and IL-8 as a readout for LPS responsiveness. We found a significant increase in LPS-induced production of IL-6 and IL-8 following IL-27 pretreatment, compared with release levels with IL-27 or LPS treatment alone (Figure 2A).

To further characterize the inflammatory activation of human lung fibroblasts mediated by IL-27 upon direct exposure to bacteria, we infected human lung fibroblasts by Gram-positive *S.pneumoniae* or Gram-negative *P. aeruginosa* with an infection dose of $10^6$ cfu/ml according to our previous studies (5, 8). No significant cytotoxicity was observed at 6 h after *S.pneumoniae* or *P. aeruginosa* infection in comparison to the unstimulated controls following IL-27 pretreatment (data not shown). Interestingly, IL-27 pretreatment could significantly augment IL-6 production induced by Gram-negative *P. aeruginosa* but not by Gram-positive *S.pneumoniae* (Figure 2B).
IL-27 induced TLR4 expression in human lung fibroblasts

Cytokine-mediated regulation of TLR expression in human lung fibroblasts remains poorly understood. Therefore, we asked whether IL-27–mediated priming ability on LPS responses in primary human lung fibroblasts could be consequent to IL-27 upregulating TLR4 expression. We observed that IL-27 could induce TLR4 mRNA expression in human lung fibroblasts as early as 6 h (Figure 3A). Cell surface protein expression of TLR4 was also significantly increased after IL-27 stimulation when compared with medium control, as confirmed by flow cytometry (Figure 3B). Furthermore, immunofluorescence microscopy showed increased TLR4 expression at the cell surface of human lung fibroblasts primed with IL-27, compared with cells cultured in medium alone (Figure 3C). Together, these results demonstrate that IL-27 has a direct role in inducing TLR4 expression at the mRNA and protein level in primary human lung fibroblasts.

Effects of signaling molecule inhibitors on TLR4 expression in human lung fibroblasts activated by IL-27

To decipher the molecular mechanism involved in IL-27–induced TLR4 expression in human lung fibroblasts, we inhibited different signaling pathways and assayed for IL-27-induced TLR4 expression. The effective doses of signaling molecule inhibitors with significant inhibitory effects on specific signaling pathways in human lung fibroblasts have been described in our previous studies (10, 11, 15). We then used the optimal concentrations of JAK inhibitor AG490 (5 μM), NF-κB inhibitor BAY11-7082 (1 μM), PI3K inhibitor LY294002 (5 μM), ERK inhibitor U0126 (10
μM), p38 MAPK inhibitor SB203580 (20 μM), and JNK inhibitor SP600125 (5 μM) with significant inhibitory effects, and without any cellular toxicity. As shown in Figure 4, AG490 could partially but significantly inhibit IL-27-induced TLR4 mRNA (Figure 4A) and surface protein expression (Figure 4B), while SP600125 could completely suppress IL-27-induced TLR4 expression to the basal levels. However, other inhibitors including BAY11-7082, LY294002, SB203580, and U10126 did not exert any significant effect on IL-27-induced TLR4 expression in human lung fibroblasts. Accordingly, SP600125 could significantly down-regulated LPS-induced increase of IL-6 and IL-8 following IL-27 pretreatment (Figure 4C). AG490 could also decrease increased IL-6 and IL-8 following IL-27 pretreatment, however, it did not reach statistical significance.

IL-27 activated the activities of JAK and JNK in human lung fibroblasts

Figure 5 showed that IL-27 could rapidly induce the phosphorylation of JAK2 and JNK at 10 min. Phosphorylation of JAK2 and JNK was detected after 30 min of IL-27 treatment and sustained to 1 h. AG490 (5 μM) and SP600125 (5 μM) could significantly suppress the phosphorylation of JAK2 and JNK after 30 min of IL-27 activation, respectively.

Effects of IL-27 on TLR4 expression in human bronchial epithelial cells

Besides to lung fibroblasts, lung epithelial cells are also known to participate in airway inflammatory responses and play important roles in the pathogenesis of pulmonary infectious diseases (9), we further investigated the impact of IL-27 on TLR4 expression in human bronchial epithelial cells. IL-27 could activate bronchial
epithelial cells to significantly upregulate their mRNA and surface protein expression of TLR4 (Figure 6A). Although IL-27 could not induce bronchial epithelial cells to produce IL-6 and IL-8, it could significantly increase LPS-induced production of IL-6 and IL-8 (Figure 6B). These results also revealed a priming ability of IL-27 on LPS responses in human bronchial epithelial cells via up-regulating TLR4 expression.

DISCUSSION

Lung tissue cells, such as fibroblasts and epithelial cells, play an important role in the response to tissue injury, contributing to cytokine and chemokine release and the development of pulmonary fibrosis (21, 29). However, little is known about how lung tissue cells respond to microbial stimuli in pathogenesis of pulmonary infectious diseases. In this report, we demonstrated that IL-27 could enhance LPS-induced proinflammatory cytokine and chemokine production via up-regulation of TLR4 expression in human lung fibroblasts and bronchial epithelial cells. Furthermore, activation of the JAK and JNK pathway was responsible for IL-27-induced TLR4 expression in human lung fibroblasts. To our knowledge, this study is the first to show a role for IL-27 in the induction of TLR expression in lung tissue cells. Our work adds to the proinflammatory reactions of IL-27 and describes new mechanisms by which IL-27 can regulate innate immune responses and microbial-driven inflammation in the lung.

IL-27 is a pleiotropic cytokine involved in a variety of inflammatory diseases. IL-27 is mainly produced by antigen-presenting cells (APC), such as dendritic cells (DC) and macrophages, after stimulation by toll-like receptor ligands or infectious
agents (17, 32). IL-27 exhibits proinflammatory effects by stimulating monocytes, mast cells, epithelial cells, fibroblasts and keratinocytes to produce cytokines and chemokines (7, 9, 11, 22, 26). On the other hand, IL-27 has antiinflammatory functions by suppressing the development of Th1, Th2, Th17 cell subsets and promoting IL-10 production under various pathophysiologic conditions (16, 24, 32). Previous studies have demonstrated that IL-27 was up-regulated in airway inflammatory diseases, including asthma (11, 30), chronic obstructive pulmonary disease (COPD) (9, 10), pulmonary tuberculosis (PTB) (9), and influenza (6, 20). In this study, we further reported elevated serum IL-27 levels in newly admitted CAP patients. IL-27 levels were notably enhanced at the primary site of infection, as detected in BALF, corresponding to increased levels in the abdominal cavity and serum during sepsis (8, 25). Recently, it has been suggested that IL-27 serves as a sepsis diagnostic biomarker (27, 28). Since early diagnosis of pneumonia is very challenging (14), a large prospective study of different pneumonia patients was required to evaluate the value and accuracy of a rapid test for IL-27 in the BALF or serum of patients to diagnose bacterial, or viral fungal pneumonia.

Having observed that pneumonia was associated with increased local and systemic release of IL-27 in human patients, we investigated whether IL-27 could modulate immune response of lung tissue cells. Our previous studies have demonstrated that IL-27 could directly stimulate primary human lung fibroblasts to produce chemokine CXCL10 (9, 11). Here we found a striking priming ability of IL-27 on LPS responses in primary human lung fibroblasts and bronchial epithelial cells by enhancing
LPS-induced production of IL-6 and IL-8, and direct exposure to bacteria demonstrated that IL-27 could augment cytokine production from lung fibroblasts induced by Gram-negative *P. aeruginosa* but not by Gram-positive *S.pneumoniae*. Most importantly, IL-27 was shown to upregulate TLR4 expression in human lung fibroblasts and bronchial epithelial cells. However, IL-27 did not regulate gene expression of LPS binding protein and CD14 involved in LPS binding as well as TLR2 (data not shown). TLR4, one of the well-characterized TLRs, senses LPS of bacteria. At least 2 separate signaling pathways exist for LPS ligation of TLR4, one of which is dependent on the adaptor protein MyD88 and another that is MyD88 independent (3, 17). Ligation of TLR4 leads to up-regulation of proinflammatory cytokines and chemokines as well as adhesion molecules important for recruitment and activation of phagocytes, contributing to pulmonary immunity against external pathogens. Earlier studies have shown that IL-27 itself was induced by various bacterial, viral, and host-derived inflammatory stimuli (16, 32). Results from this study support a role for IL-27 in a positive feedback loop during pulmonary infectious diseases: LPS induces IL-27 production from APC and then IL-27 upregulates TLR4 expression in lung tissues cells, such as fibroblasts and bronchial epithelial cells, which augments inflammatory reactions and immune responses in the lung. Our current study also expanded data from Guzzo and colleagues, who showed that IL-27 primed the LPS response in primary human monocytes (15). Therefore, IL-27-TLR4 axis may play an important regulatory role in pulmonary inflammation and immunity. Further studies using human lung tissues from control and pneumonia patients
showing TLR4 expression on fibroblasts and epithelial cells in vivo would further strengthen our present findings in vitro.

Our previous study has demonstrated that IL-27 induced CXCL10 production in primary lung fibroblasts via activation of PI3K–Akt and p38 MAPK signaling pathways (11). Here we found that IL-27 upregulated TLR4 expression via activation of JAK and JNK signaling pathways in human lung fibroblasts. The above discrepancy of the intracellular signaling mechanisms suggests that CXCL10 and TLR4 expression induced by IL-27 in primary human lung fibroblasts is differentially regulated by PI3K–Akt, p38 MAPK, JAK-signal transducer and activator of transcription factor and JNK pathways. In another study, IL-27 up-regulated TLR4 expression in primary human monocytes in a JAK- and NF-κB–dependent manner (15). The above discrepancy of the intracellular signaling mechanisms of IL-27 for the induction of TLR4 expression suggests that different signaling pathways selectively regulate TLR4 expression in a cell type-specific manner.

In conclusion, the present report is the first demonstration that IL-27 augments LPS responses by increasing TLR4 expression in human lung tissue cells. This might provide a novel mechanism of IL-27-mediated innate immunity during pulmonary infections, which might be promising for the prevention, treatment, or both of pulmonary infectious diseases.

GRANTS

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DISCLOSURES
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

REFERENCES


**Figure legend**

**Figure 1** Human community-acquired pneumonia (CAP) resulted in an increase of IL-27 in lungs and serum. (A) ELISA analysis for IL-27 levels in serum from control patients and patients with CAP. Each dot represents a measurement within an individual patient, with horizontal lines showing medians. (B) ELISA analysis for IL-27 levels in bronchoalveolar lavage fluid (BALF) from control patients and patients admitted to the intensive care unit with CAP. (C) The serum IL-27 correlated with the corresponding BALF IL-27 in CAP patients. (D) 7 patients with CAP were also recruited after 8 weeks of treatment to investigate BALF concentrations of IL-27. *** P < 0.001, when compared between groups denoted by horizontal lines, Mann–Whitney U test.

**Figure 2.** IL-27 pretreatment augmented LPS-induced production of IL-6 and IL-8 from lung fibroblasts. (A) Primary human lung fibroblasts were incubated in medium alone or in the presence of IL-27 (50 ng/ml) for 16 h. After 16 h, cells were washed and resuspended in fresh medium containing LPS (10 ng/ml) for an additional 8 h, and supernatants were collected for ELISA. (B) Human lung fibroblasts were incubated in medium alone or in the presence of IL-27 (50 ng/ml) for 16 h. After 16 h, cells were washed and resuspended in fresh medium and then infected with *S. pneumoniae* (10⁶ cfu/ml) or *P. aeruginosa* (10⁶ cfu/ml) for an additional 6 h, and supernatants were then collected for ELISA. Results are expressed as the mean ± SD of three independent experiments. *** P < 0.001, when compared between groups denoted by horizontal lines, one-way ANOVA with Bonferroni post hoc test.
Figure 3. Gene and protein expression of TLR4 in primary human lung fibroblasts activated by IL-27. (A) TLR4 mRNA levels determined using quantitative RT-PCR in primary human lung fibroblasts activated by IL-27 (50 ng/ml) for 0–24 h. (B) TLR4 expression on the surface of human lung fibroblasts activated by IL-27 for 24 h determined by flow cytometry. Surface expression of TLR4 on 5,000 cells was analyzed by flow cytometry as MFI. Results are expressed as mean ± SD of three independent experiments. *P <0.05, ***P <0.001, when compared between groups denoted by horizontal lines. (C) IL-27 induced enhanced TLR4 membrane expression by immunofluorescence microscopy. Cells were harvested in PBS-0.1% azide and stained for TLR4 (red fluorescence).

Figure 4. Effects of different signalling molecule inhibitors on IL-27-induced TLR4 expression. Primary human lung fibroblasts were pre-treated with AG490 (5 μM; AG), BAY11-7082 (1 μM; BAY), LY294002 (5 μM; LY), SB203580 (20 μM; LY), SP600125 (5 μM; SP), or U0126 (10 μM; U) for 1 hr, followed by incubation with or without IL-27 (50 ng/ml). DMSO (0.1%) was used as the vehicle control. (A) mRNA expression of TLR4 determined by quantitative RT-PCR after IL-27 stimulation for further 6 h. (B) Surface expression of TLR4 determined by flow cytometry after IL-27 stimulation for further 24 h. (C) IL-6 and IL-8 production after LPS (10 ng/ml) stimulation for an additional 8 h following IL-27 (50 ng/ml) pretreatment for 16 h. Results are expressed as the mean ± SD of three independent experiments. *P <0.05, ***P <0.001, when compared between groups denoted by horizontal lines.

Figure 5. Effects of IL-27 on activation of the Janus-activated kinase (JAK) and Jun
N-terminal kinase (JNK) signaling pathways in human lung fibroblasts. (A) Cells (5 x 10^6 cells) were treated with or without IL-27 (50 ng/ml) for the indicated incubation times. Total cellular proteins were extracted for the detection of total and phosphorylated signaling proteins by Western blot analysis. Experiments were performed in three independent experiments with essentially identical results, and representative blots are shown. Densitometry quantification of blots from three independent experiments was shown in histograms on the right. Phospho-JAK2 or phospho-JNK expression was normalized to β-actin for each sample, and expression was graphed as fold change above cells at 0 min. *P < 0.05 when compared with human lung fibroblasts activated by IL-27 at 0 min. (B) Cells were pre-treated with or without AG490 (5 μM; AG) and SP600125 (5 μM; SP) for 1 h, followed by incubation for a further 30 min with or without IL-27 (50 ng/ml). Total cellular proteins were extracted for the detection of total and phosphorylated signaling proteins by Western blot analysis. Experiments were performed in three independent experiments with essentially identical results, and representative blots are shown. Densitometry quantification of blots from three independent experiments was shown in histograms on the right. Phospho-JAK2 or phospho-JNK expression was normalized to β-actin for each sample, and expression was graphed as fold change above medium control. *P < 0.05 when compared with medium control.

Figure 6. Effects of IL-27 on TLR4 expression in human bronchial epithelial cells. (A) (A) TLR4 mRNA levels determined using quantitative RT-PCR in human bronchial epithelial cells activated by IL-27 (50 ng/ml) for 6 h and TLR4 expression on the
surface of human bronchial epithelial cells activated by IL-27 for 24 h determined by flow cytometry. (B) Human bronchial epithelial cells were incubated in medium alone or in the presence of IL-27 (50 ng/ml) for 16 h. After 16 h, cells were washed and resuspended in fresh medium containing LPS (10 ng/ml) for an additional 8 h, and then supernatants were collected for determination of IL-6 and IL-8 by ELISA. Results are expressed as mean ± SD of three independent experiments. *P<0.05, ***P<0.001, when compared between groups denoted by horizontal lines.
The image shows bar graphs comparing the levels of IL-6 and IL-8 in different conditions:

- **IL-27 + LPS**
- **IL-27 + LPS + DMBO**
- **IL-27 + LPS + AG**
- **IL-27 + LPS + SP**

The graphs indicate that the IL-6 levels are significantly reduced when DMBO is present, while IL-8 levels remain relatively constant across all conditions.
Figure 5

A

Phospho-JNK
Total JNK
Phospho-JAK2
Total JAK2
β-actin

0 10 20 30 60

Time (min)

Phospho-JNK relative expression (fold increased over 0 min)

Phospho-JAK2 relative expression (fold increased over 0 min)
Figure 6

A

![Graph showing TLR4 mRNA/GAPDH levels with comparisons between Medium and IL-27 treatments.](image)

![Graph showing TLR4 MFI with comparisons between IgG CTL, Medium, and IL-27 treatments.](image)

B

![Graph showing IL-6 levels with comparisons between Medium, IL-27, LPS, and IL-27 + LPS treatments.](image)

![Graph showing IL-8 levels with comparisons between Medium, IL-27, LPS, and IL-27 + LPS treatments.](image)
Table 1 Demographic and clinical characteristics, and microbiology results of patients with community-acquired pneumonia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Pneumonia patients (n=19)</th>
<th>Controls (n=16)</th>
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<td>Age (y)</td>
<td>61 ± 15</td>
<td>55 ± 16</td>
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<td>Male/female gender, No.</td>
<td>8/11</td>
<td>10/6</td>
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<td>APACHE II Score</td>
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<tr>
<td>28-day mortality (%)</td>
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Microbiology

- *Streptococcus pneumonia* 3
- *Mycoplasma pneumoniae* 1
- *Haemophilus influenza* 2
- *Acinetobacter baumannii* 2
- *Staphylococcus aureus* 1
- *Pseudomonas aeruginosa* 2
- *Klebsiella pneumonia* 2
- *Legionella pneumophila* 1
- No micro-organism identified 5

APACHE II: Acute Physiology and Chronic Health Evaluation II.