Interleukin-35 reduces inflammation in acute lung injury through inhibiting TLR4/NF-κB signaling pathways

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Abstract. Acute lung injury (ALI) in children is a complex disease that is accompanied by an inflammatory response. The pathogenesis of ALI in children is not yet well understood. Mice with ALI exhibit inflammation of the lungs and decreased expression of interleukin (IL)-35. To investigate whether the function of IL-35 affects lipopolysaccharide (LPS)-induced ALI, IL-35 was overexpressed in cells. Enzyme-linked immunosorbent assays indicated decreased levels of IL-6 and tumor necrosis factor-α in LPS-induced and agomir-IL-35-treated murine RAW264.7 macrophages. Finally, toll-like receptor 4 (TLR4)/NF-κB signaling pathways were analyzed. The expression of TLR4, NF-κB p65 and NF-κB p50 were decreased, as was the degradation of NF-κB inhibitor-α, in LPS-induced and agomir-IL-35-treated murine RAW264.7 macrophages. The results of the present study demonstrated that IL-35 may exhibit a protective role in ALI by modulating the TLR4/NF-κB signaling pathways.

Introduction

Pulmonary inflammation occurs in severe disorders, including in acute lung injury (ALI), where it results in diffuse alveolar damage, which can lead to hypoxemia and respiratory failure (1). ALI is estimated to occur, with a high incidence rate, in the pediatric population worldwide (2). The genetic and environmental factors associated with ALI in children have been previously reported (3). However, an effective and specific treatment for pediatric ALI is not currently available.

In recent years, accumulating evidence has indicated that inflammation serves an important role in ALI in children (4). Pediatric ALI is characterized by the infiltration of inflammatory cells, including mast cells, eosinophils, lymphocytes, basophils and monocytes. An increased production of interleukin (IL)-1, IL-6 or tumor necrosis factor (TNF)-α by inflammatory macrophages has been previously reported in children with ALI (5). As an important transmembrane pattern-recognition receptor of the innate immune system, toll-like receptor 4 (TLR4) was identified in a variety of inflammatory conditions, including pneumonia (6). The activation of TLR4 was associated with the expression of pro-inflammatory cytokines and the activation of NF-κB signaling pathways (7). Furthermore, the expression of TLR4 was positively correlated with TNF-α and IL-6 expression (8).

Recently, as a novel member of the IL-12 family, IL-35 has been identified to be associated with pulmonary disease (9). IL-35 is a heterodimer that is composed of Epstein-Barr (EB) virus-induced gene 3 and p35; the structure is homologous to that of IL-12 due to the similar β chains of EB12 (10). High expression of IL-35 was indicated in non-stimulated mouse T regulatory cells, and the expression of IL-35 was upregulated in human non-T cells, including microvascular endothelia cells, aortic smooth muscle cells and epithelial cells, following stimulation with TNF-α (11). The expression of IL-35, at the protein and mRNA levels, was decreased in individuals with allergic asthma (12). *In vivo*, the high expression of IL-35 was reported to decrease the number of inflammatory cells and the levels of IL-4, IL-5, IL-13 and IL-17 in a mouse model of asthma (13). However, the precise mechanism by which IL-35 regulates ALI in children is unclear. In the present study, the expression of IL-35 was investigated *in vivo* and *in vitro*. The potential mechanisms and signaling pathways associated with the expression of IL-35 were studied. The results of the present study demonstrated that IL-35 expression was downregulated *in vivo* and *in vitro*, and resulted in the activation of the TLR4/NF-κB signaling pathways. The present study provides a theoretical foundation for the targeting inflammation in the pathogenesis of ALI in children.

Materials and methods

Animal model. All experiments in the present study were performed in accordance with the Guidelines on Animal Experiments from The Committee of Medical Ethics, The National Health Department of China. The animal experiments were approved by the Institutional Animal Care and Use Committee of Jilin University (Changchun, China) (KT201902017). The ALI mouse model was set up according

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to a previous study (14). The mice (6-8 weeks old; 25-35 g) were housed in isolator cages and received food and water *ad libitum*. The laboratory temperature was 24±1°C, and relative humidity was 40-80% with a 12-h light/dark cycle. A total of 20 normal male BALB/c juvenile mice were randomly divided into two groups (n=10/group): Control group and the model group. The model group was treated with 0.5 mg/kg lipopolysaccharide (LPS) and the control group was given an equivalent amount of 0.9% NaCl solution with 8 h.

Histology. For euthanasia, the mouse home cage was placed in a 22-l transparent polycarbonate euthanasia chamber (Shanghai Yuyan Instruments Co., Ltd.). The euthanasia chamber was covered with an acrylic lid with ports for gas inlet and outlet. The chamber air was replaced with CO_2 at 30%/min. Death was confirmed when the blood pressure and heart rate had reached 0, according to telemetry.

CO$_2$ treatment ensured that the animals were anaesthetized prior to euthanasia and all efforts were made to minimize their suffering. The mice were sacrificed and the lungs were exposed and removed. In a series of experimental steps, lung samples were sectioned into 4-6 μm slices, fixed in 4% formalin for 12 h at room temperature and embedded in paraffin for histological analysis. Hematoxylin and eosin staining was performed at room temperature (Hematoxylin for 3-5 min and eosin for 1 min) and was used to assess morphology and inflammation. The results were detected by light microscopy with x100 magnification.

Cell culture. Murine RAW264.7 macrophages (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were plated in culture dishes and cultured in DMEM (high glucose; Hyclone; GE Healthcare Life Sciences) with 10% FBS (Hyclone; GE Healthcare Life Sciences) and 1% antibiotic solution at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced every 2 days and cells were subcultured at 37°C in a humidified atmosphere of 5% CO_2 or subjected to subsequent experimental procedures when the culture reached a confluence of 70-80%.

Cloning of IL-35 into a lentiviral vector and transfection. Murine IL-35 (mIL-35) cDNA from murine RAW264.7 macrophages was obtained using the TIANscript RT kits according to the manufacturer's instructions (Tiangen Biotech Co., Ltd.) and amplified using the following primers: Forward, 5'-CGCGGATCCCTGAGATCACCGGTAGGAGG-3'; and reverse, 5'-TCCCCCCGGGGAGCTAGCTTTAGGCGG-3'. PrimeSTAR HS DNA Polymerase (Takara Bio, Inc.) was used for the PCR. The reaction conditions were as follows: A total of 30 cycles of pre-denaturation at 98°C for 30 sec, denaturation at 98°C for 10 sec, annealing at 50°C for 15 sec, extension at 72°C for 1 min followed by a final extension at 72°C for 7 min. PCR products were recovered using the Agarose gel DNA Recovery kit (Tiangen Biotech Co., Ltd). The recovered PCR products were digested using restriction endonuclease (Takara Bio, Inc.) and subcloned into a lentiviral vector-pMD2.G (Beijing Huayueyang Co., Ltd.) by T4 DNA ligase (Takara Bio, Inc.) to be used as an IL-35 agomir (agomir-IL-35). The empty lentiviral vector was used as a control (agomir-NC). Following 24 h, the murine RAW264.7 macrophages were transfected using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) with agomir-IL-35 or agomir-NC. Murine RAW264.7 macrophages were plated on 24-well plate at 500 μl/well at density of $5x10^4$-$10x10^4$ cells/ml in medium and propagated to 80% confluency at the time of transfection. In this present study, the Opti-MEM reduced serum medium (Thermo Fisher Scientific, Inc.) contained 500 ng agomir-IL-35 or agomir-NC. Agomir-IL-35 or agomir-NC was complexed with 2 μl of Lipofectamine 3000 with 1.5 μl of P3000 as described in the manufacturer's protocol, in the Opti-MEM reduced serum medium. The medium containing the lentivirus was replaced with complete medium 24 h after transfection. Following treatment with 1 μg/ml LPS for 24 h, the murine RAW264.7 macrophages were collected and used for subsequent experiments. The murine RAW264.7 macrophages were divided into five groups: Control (non-LPS-induced cells), agomir-NC, LPS, agomir-LPS, agomir-IL-35 + LPS and agomir-IL-35.

Measurement of IL-6 and TNF-α activity in murine RAW264.7 macrophages. The cellular activities of IL-6 and TNF-α were determined using ELISAs kits (IL-6, cat. no. 431304; TNF-α, cat. no. 430907) according to the manufacturers' instructions (BioLegend, Inc.). All samples were assayed six times.

Western blotting. Nuclear and cytosolic proteins were extracted using Nuclear and Cytoplasmic Protein Extraction kit (cat. no. P0028; Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Protein concentrations were quantified using the bicinchoninic acid (BCA) assay kit (cat. no. P0011; Beyotime Institute of Biotechnology). A total of 50 μg of each sample was used for 10% SDS-PAGE and, the proteins were transferred onto PVDF membranes using the wet transfer method. Membranes were blocked in 5% milk for 1 h at room temperature. Subsequently, the proteins were probed with the diluted primary antibodies anti-IL-35 (cat. no. ab133751), -TLR-4 (cat. no. ab13556), -NF-κB p65 (cat. no. ab207297), -NF-κB p50 (cat. no. ab14059), -NF-κB inhibitor-α (IκBα) (cat. no. ab7217), -β-actin (cat. no. ab8226) and -histone H3 (cat. no. ab1791; all 1:1,000; all from Abcam), at 4°C overnight. Following three 8 min PBST washes, the membranes were incubated with goat-anti-rabbit IgG secondary antibody (cat. no. TA130015; 1:5,000; OriGene Technologies, Inc.), goat-anti-mouse IgG secondary antibody (cat. no. ab205719; 1:5,000; Abcam) or goat anti-chicken IgY secondary antibody (cat. no. ab6877; 1:5,000; Abcam) for 1 h at room temperature. An enhanced chemiluminescence reagent (cat. no. WBKLS0500; Pierce; Thermo Fisher Scientific, Inc.) was used to visualize the protein bands. ImageJ version 1.38 (National Institutes of Health) software was used for densitometry analysis of the appropriate lanes.

Statistical analysis. Experimental results are presented as the mean ± SEM. Using the SSPS 17.0 (SPSS, Inc.) statistical analysis software for data analysis, two groups were analyzed using independent Student's t-test, whereas multiple groups were compared using a one-way ANOVA test with Bonferroni. $P < 0.05$ was considered to indicate a statistically significant difference.
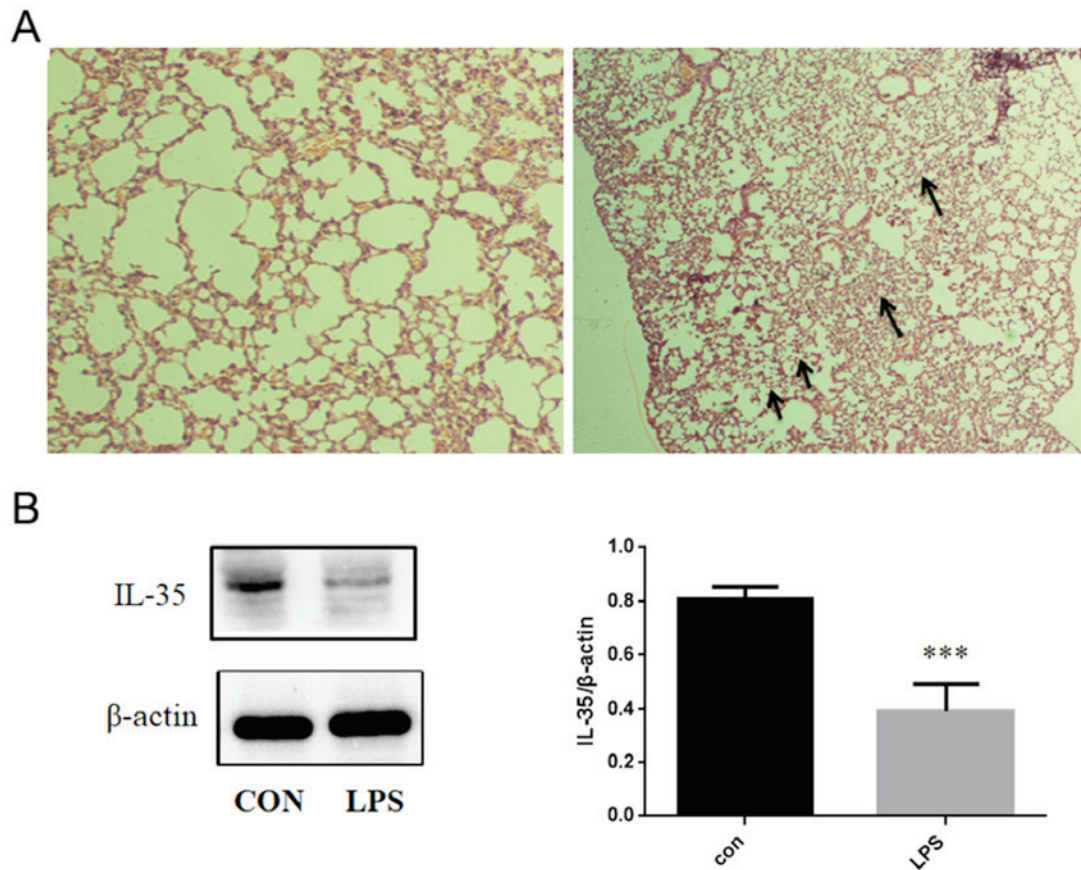
Figure 1. Expression levels of IL-35 were decreased in mice treated with LPS. (A) Lung tissue of mice was analyzed using hematoxylin and eosin staining in control (left) and LPS (right; x100). (B) Expression level of IL-35 was detected by western blotting. All data are presented as mean ± SEM (n=6, per group). ***P<0.001 vs. CON. CON, control; IL-35, interleukin-35; LPS, lipopolysaccharide.
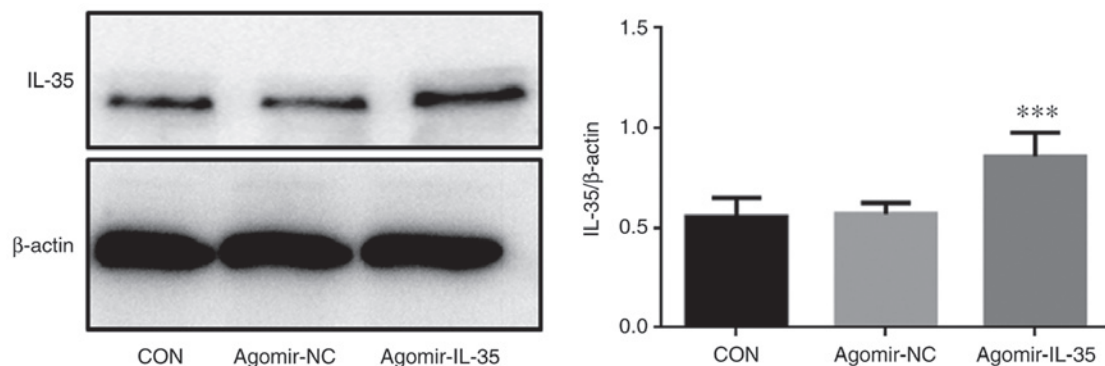

Figure 2. IL-35 was upregulated in transfected RAW264.7 macrophages. All data are presented as the mean ± SEM (n=3, per group). ***P<0.001 vs. CON. CON, control; NC, negative control; IL-35, interleukin-35.

Results

IL-35 is downregulated in mice treated with LPS. In the present study, LPS was used to induce an ALI model. The lungs from LPS-treated mice revealed the infiltration of neutrophils, thickening of the alveolar wall, edema and hemorrhage (Fig. 1A). The expression of IL-35 was determined using western blotting following the different treatments. The expression of IL-35 in mice treated with LPS was significantly lower compared with the control group (Fig. 1B).

IL-35 expression is upregulated in murine RAW264.7 macrophages. Following treatment with 1 µg/ml LPS for 24 h, the murine RAW264.7 macrophages were transfected with agomir-IL35 or agomir-NC. Western blotting indicated upregulated expression of IL-35 in agomir-IL-35 murine RAW264.7 macrophages (Fig. 2).

Expression levels of IL-6 and TNF-α are downregulated in murine RAW264.7 macrophages transfected with agomir-IL-35. The levels of IL-6 and TNF-α were evaluated

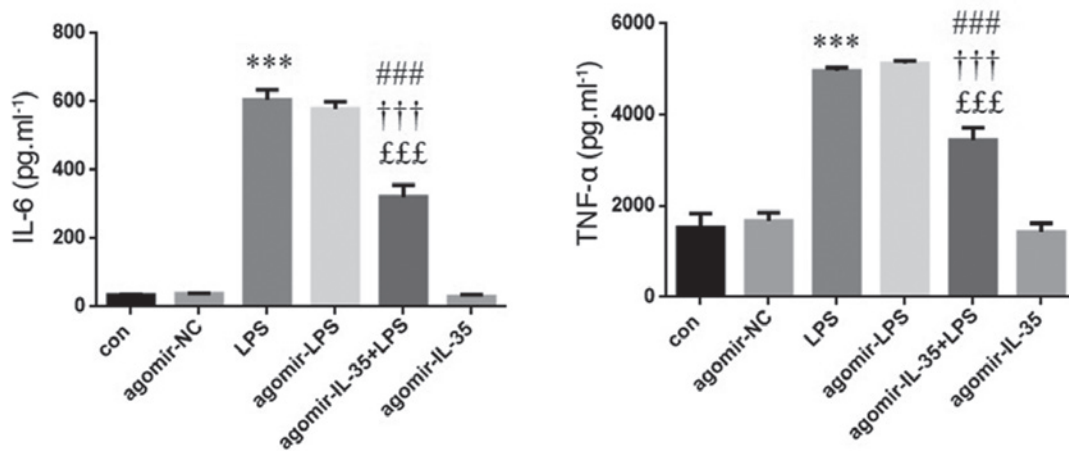Figure 3. Agomir-IL-35 in murine RAW264.7 macrophages prevents LPS-induced inflammation. All data are shown as mean ± standard error of mean (n=6, per group). ***P<0.001 vs. control; ###P<0.001 vs. LPS; †††P<0.001 vs. agomir-NC; £££P<0.001 vs. agomir-LPS. IL-35, interleukin-35; LPS, lipopolysaccharide; con, control; NC, negative control; TNF, tumor necrosis factor.

using ELISA. The levels of IL-6 and TNF-α were increased in murine RAW264.7 macrophages following treatment with LPS compared to control, whereas IL-6 and TNF-α expression was decreased in agomir-IL-35 + LPS-treated murine RAW264.7 macrophages compared to LPS or agomir-LPS (Fig. 3).

IL-35 attenuates inflammation via the TLR4/NF-κB signaling pathways in murine RAW264.7 macrophages. The expression of TLR4, NF-κB p65 and NF-κB p50 was evaluated using western blot analysis. It was demonstrated that the expression of TLR4, and the degradation of IκBα, was increased in murine RAW264.7 macrophages following treatment with LPS compared to control (Fig. 1A). In contrast, the expression of TLR4, and the degradation of IκBα, was decreased in agomir-IL-35 + LPS-treated murine RAW264.7 macrophages compared to LPS and agomir-LPS treated RAW264.7 macrophages (Fig. 4A). The data demonstrated that the expression of IL-35 inhibited the translocation of NF-κB p65 and NF-κB p50 from the cytoplasm to the nucleus in murine RAW264.7 macrophages treated with LPS (Fig. 4B and C).

Discussion

ALI in children is one of the most common diseases in pediatric medicine worldwide (15). A study demonstrated that inflammation serves a key role in the multi-factorial pathogenesis of ALI in children (16). Additionally, IL-35 has been reported to be associated with pediatric ALI by affecting the number of inflammatory cells (9); however, the mechanism remains unclear. The present study describes a cascade of events that link TLR4/NF-κB activation to inflammation through the downregulation of IL-35 *in vivo* and *in vitro*.

In the present study, an ALI model was established using LPS. The lungs from LPS-treated mice exhibited signs of infiltration by neutrophils, thickening of alveolar walls, edema and hemorrhage. It was also demonstrated that the expression of IL-35 was downregulated in mice treated with LPS.

IL-35 has been revealed to serve an immunosuppressive role during infections, inflammation and in autoimmune diseases (17). TNF-α, which is a marker of clinical severity and airflow limitation, is primarily secreted by macrophages. A previous report indicated that the expression of TNF-α was increased in pulmonary diseases (18), and the production of IL-6 was triggered by TNF-α. To investigate the effect of IL-35 on inflammation-associated signaling cascades, IL-35 was overexpressed in murine RAW264.7 macrophages. In the present study, the levels of IL-6 and TNF-α were determined using ELISA. The levels of IL-6 and TNF-α were increased in agomir-IL-35 + LPS-treated RAW264.7 macrophages compared with agomir-NC + LPS.

Previous studies have reported that TLR4 is critical for the activation of NF-κB and the subsequent production of pro-inflammatory cytokines that are implicated in a variety of diseases (19-21). As a transcription factor, NF-κB serves vital roles in numerous processes, including inflammation, immunity and cell proliferation (22). NF-κB is an important signaling pathway in the development of a number of inflammation-mediated diseases, including pediatric bronchial asthma (23). p65 and p50 are members of the NF-κB family. In the present study, the expression of TLR4 and the degradation of IκBα were increased in murine RAW264.7 macrophages treated with LPS, whereas the expression of TLR4 and the degradation of IκBα were decreased in agomir-IL-35 + LPS murine RAW264.7 macrophages. The present study demonstrated that IL-35 inhibited the translocation of NF-κB p65 and NF-κB p50 from the cytoplasm to the nucleus in LPS-treated murine RAW264.7 macrophages. These results suggested that the upregulation of IL-35 leads to a decreased level of inflammation. Therefore, IL-35 affects inflammation through TLR4/NF-κB signaling pathways.

In conclusion, the present study revealed that LPS-induced inflammation contributed to ALI. IL-35 has been indicated to be an important target associated with ALI, that influences inflammation via the TLR4/NF-κB signaling pathways. Therefore, the inhibition of inflammation using IL-35 may provide a novel therapeutic strategy to prevent ALI.
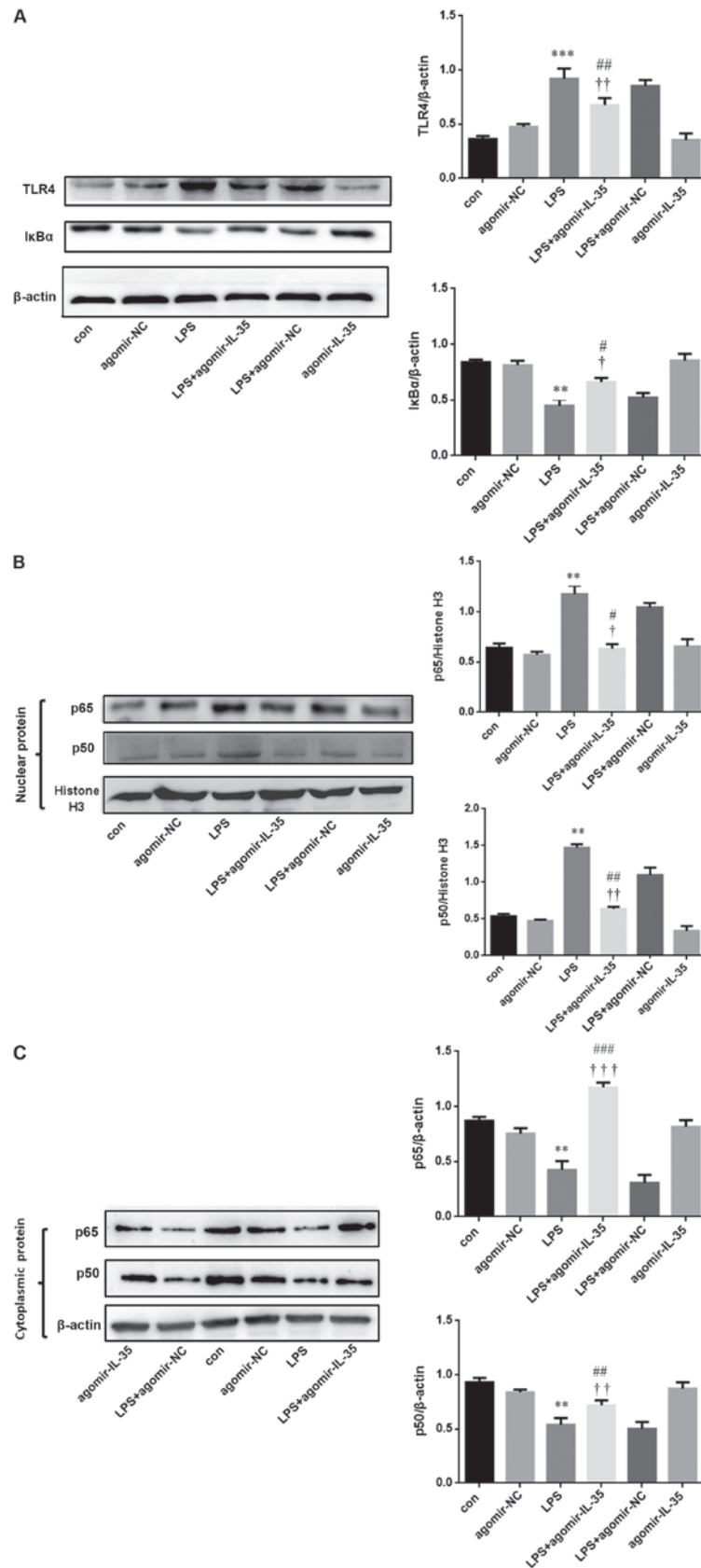
Figure 4. Murine RAW264.7 macrophage expression of TLR4/NF-κB signaling pathways TLR4 and IκBα p50 expression. (A) The expression of TLR4 and IκBα in murine RAW264.7 macrophage. (B) The expression of P65 and P50 in nuclear of murine RAW264.7 macrophage. (C) The expression of P65 and P50 in cytoplasmic of murine RAW264.7 macrophage. All data are p as the mean ± SEM (n=3, per group). $^{**}P<0.01$, $^{***}P<0.001$ vs. con. $^{#}P<0.05$, $^{##}P<0.01$, $^{###}P<0.001$ vs. LPS. $^{†}P<0.05$, $^{††}P<0.01$, $^{†††}P<0.001$ vs. agomir-NC. IL-35, interleukin-35; LPS, lipopolysaccharide; con, control; NC, negative control; TLR, toll-like receptor.

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