

ORIGINAL RESEARCH ARTICLE

NLRC5 negatively regulates LTA-induced inflammation via TLR2/NF- κ B and participates in TLR2-mediated allergic airway inflammation

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Funding information

Research Project of Anhui Province, Grant/Award Number: 10021303028; Key Lab of Geriatric Molecular Medicine of Anhui Province, Grant/Award Number: 1206c0805028

Abstract

NLRC5, the largest member of the Nod-like receptor (NLR) family, has been reported to play a pivotal role in regulating inflammatory responses. Recent evidence suggests that NLRC5 participates in Toll-like receptor (TLR) signaling pathways and negatively modulates nuclear factor- κ B (NF- κ B) activation. In this study, we investigated the interaction between NLRC5 and TLR2 in the NF- κ B inflammatory signaling pathway and the involvement of NLRC5 in TLR2-mediated allergic airway inflammation. We knocked down TLR2 and NLRC5, respectively in the RAW264.7 macrophage cell line by small interfering RNA (siRNA) and then stimulated the knockdown cells with lipoteichoic acid (LTA). In comparison with the negative siRNA group, the level of NLRC5 expression was lower in the TLR2 siRNA group, with a reduction in the NF- κ B-related inflammatory response. Conversely, in the NLRC5 knockdown cells, after LTA-treated, the level of TLR2 expression did not change but the expression levels of both NF- κ B pp65 and NLRP3 increased remarkably. Thus, we hypothesize that NLRC5 participates in the LTA-induced inflammatory signaling pathway and regulates the inflammation via TLR2/NF- κ B. Similarly, in subsequent in vivo experiments, we demonstrated that the expression level of NLRC5 was significantly increased in the ovalbumin-induced allergic airway inflammation. However, this effect disappeared in TLR2-deficient (TLR2^{-/-}) mice and was accompanied by reduced levels of NF- κ B expression and airway inflammation. In conclusion, NLRC5 negatively regulates LTA-induced inflammatory response via a TLR2/NF- κ B pathway in macrophages and also participates in TLR2-mediated allergic airway inflammation.

KEYWORDS

allergic airway inflammation, macrophage, negative regulation, NLRC5, TLR2

1 | INTRODUCTION

Innate immunity provides an immediate response, which is nonspecific and generalized to injury or infection. The response is mediated by germline-encoded pattern recognition receptors (PRRs) (Brubaker, Bonham, Zanoni, & Kagan, 2015), such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectin receptors, RIG-I-like receptors, and AIM2-like receptors (Meunier & Broz, 2017). NLRs represent a family of intracellular PRRs that play a role in host defense against

relevant pathogen-associated molecular patterns (PAMPs). NLRC5 is a newly found protein of the NLR family, containing an atypical N-terminal caspase activation and recruitment domain, centrally located nucleotide-binding domain, and 27 C-terminal leucine-rich repeats, which is capable of regulating inflammatory responses and cell death (Qiu et al., 2017; Rodriguez et al., 2016). Both human and mouse NLRC5 are highly expressed in lungs, spleen, and thymus (Cui et al., 2010). Numerous studies have demonstrated that NLRC5 acts as a regulator of homeostasis in innate immunity. Cui et al. (2010) found that in vitro, NLRC5

can negatively modulate the TLR4-mediated nuclear factor- κ B (NF- κ B) activation and Type I interferon production. Moreover, NLRC5 expression in T cells is required to protect them from natural killer (NK)-cell-mediated elimination during inflammation and an NLRC5 deficiency provides a manner in which to fine-tune NK cell tolerance (Ludigs et al., 2016). In addition, NLRC5 has been shown to improve myocardial damage, as well as reverse hepatic fibrosis by negatively regulating inflammation (X. Liu et al., 2016; Ma & Xie, 2017). However, there is very little known about whether NLRC5 is involved in TLR2-mediated inflammatory responses and the underlying mechanism of NLRC5 to regulate the inflammation.

TLRs are Type I membrane-bound proteins, which recognize and defend against invading microorganisms (Y. Liu, Yin, Zhao, & Lu, 2014). Of which, TLR2 is a key innate immune receptor that is primarily expressed in immune cells and airway epithelial cells (Gopalakrishnan & Salgame, 2016). To date, studies have reported that TLR2 induces a proinflammatory response within immune cells. Lugade, Bogner, Murphy, and Thanavala (2011) demonstrated that the levels of both immune cell infiltration and proinflammatory cytokine secretion were reduced in the lungs of TLR2^{-/-} mice exposed to non-typeable *Haemophilus influenzae*. Fei, Jifeng, Tiantian, Yi, and Linghui (2017) revealed that the downregulation of the TLR2 signaling cascade in alveolar macrophages may be a protective effect to inhibit ischemia-reperfusion lung injury-induced inflammatory response. Moreover, another study reported that peptidoglycan-associated lipoprotein-induced the production of proinflammatory cytokines via TLR2 in RAW264.7 cells (Ihalin, Eneslatt, & Asikainen, 2018).

Allergic airway disease is based on chronic or nonresolving airway inflammation, which involves various inflammatory cells and factors. Recently, innate immune responses were suggested to play an important role in allergic airway inflammation (Castillo, Zheng, & Yang, 2018). Moreover, our previous study has suggested that TLR2 plays a pivotal role in allergic airway inflammation by regulating the phosphoinositide-3-kinase/protein kinase B signaling pathway in mice (Jiang et al., 2017). Another report indicated that TLR2 deletion can also reduce mucous cell metaplasia and bronchial fibrosis in mice after being challenged by ovalbumin (OVA; Li et al., 2014). Meanwhile, some evidence has also shown that TLR-induced NF- κ B signaling is enhanced in NLRC5 deficient mice (Tong et al., 2012). But to date, whether NLRC5 participates in TLR2-mediated allergic airway inflammation was unknown.

In this study, we aimed to explore what is the interaction between NLRC5 and TLR2 in TLR2-mediated inflammatory responses, how NLRC5 regulates inflammation through the TLR2/NF- κ B signaling pathway, and whether NLRC5 involves in TLR2-mediated allergic airway inflammation.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

The mouse macrophage cell line RAW264.7 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St.

Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY), 100 U/ml penicillin (Sigma-Aldrich), and 100 μ g/ml streptomycin (Sigma-Aldrich) at 37°C in humidified atmosphere of 5% CO₂. RAW264.7 cells were pretreated, respectively with 40 nM TLR2 small interfering RNA (siRNA) or NLRC5 siRNA for 48 hr and then stimulated with 200 ng/ml lipoteichoic acid (LTA). Dimethyl sulfoxide (DMSO) was used as vehicle control.

2.2 | RNA interference

siRNAs targeting TLR2 and NLRC5 were purchased from Shanghai Gene Pharma Corporation. The TLR2 siRNA sequence was (sense: 5'-GGAACAGAGUGGCAACAGUTT-3' and antisense: 5'-ACUGUUGC-CACUCUGUUCCTT-3'); NLRC5 siRNA (sense: 5'-UCAAGUUAUUUCCUGAGUUCUGGDTDT-3' and antisense: 5'-CCAGAACUCAGGAAUUAACUUGADTDT-3'); negative siRNA (sense: 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense: 5'-ACGUGACAGUUCGCGAGAATT-3'). Cells were transfected with siRNAs by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Knockdown efficiency was determined by western blot analysis. Three independent transfection experiments were performed.

2.3 | Immunocytochemistry

RAW264.7 cells were seeded onto coverslips in a 12-well plate. Cells adhered to coverslips overnight at 37°C in a humidified atmosphere of 5% CO₂. The cells were treated as described above, formalin-fixed, permeabilized (0.3% Triton X-100; Sigma-Aldrich), and blocked (5% donkey serum; Sigma-Aldrich) at 37°C for 30 min. Primary antibodies used include: rabbit anti-NLRC5 (Abcam, Cambridge, MA), rabbit anti-NF- κ B p65 (phosphorylated NF- κ B p65 subunit; Cell Signaling Technology, Beverly, MA), and mouse anti-NF- κ B p65 (Cell Signaling Technology). All primary antibodies were used at a 1:200 dilutions. These antibodies were added followed by fluorescein isothiocyanate (FITC) antirabbit (Jackson Immuno Research Lab, West Grove, PA) or FITC antimouse (Jackson Immuno Research Lab) secondary antibody at a 1:200 dilutions. The cells were then counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). The coverslips were mounted and observed by using a Zeiss LMS 880 (Carl Zeiss, Jena, Germany) confocal microscope. The overall immunofluorescence intensity of P-p65 and p65 was divided by the cell number contained from the same image quantified by Imaris 9.2.1 to calculate the averaged-immunofluorescence intensity and relative fluorescence intensity per RAW264.7 cell.

2.4 | Animals

Female C57BL/6 mice at 6–8 weeks of age (Shanghai Laboratory Animal Center) and TLR2^{-/-} mice on C57BL/6 background at the same age (a generous gift from Dr ZG Tian, Institute of Immunology, School of Life Sciences, University of Science and Technology of

China) were maintained in an animal facility under specific pathogen-free conditions. All animal experiments were carried out in accordance with the guidelines set forth by the Animal Care and Use Committee of the University of Science and Technology of China.

2.5 | Experiment protocols and treatment

All mice were divided into four groups ($n = 6$ per group): wild-type (WT) control, WT-OVA, TLR2^{-/-} control, and TLR2^{-/-} OVA. Model mice were sensitized with an intraperitoneal injection of 10 μ g OVA (Sigma-Aldrich) complexed with 1 mg aluminum potassium sulfate (Sangon Biotech, Shanghai, China) on Day 0. The mice were then challenged daily for 30 min by aerosol inhalation with 1% aerosolized-OVA on days 14–20. The control mice were sensitized with saline instead and challenged with aerosolized-saline. One day after the last challenge, mice were killed. The left lungs were used for bronchoalveolar lavage, and the right lungs were used to perform histopathological and western blot analysis.

2.6 | Bronchoalveolar lavage fluid and differential cell counts

The mice were killed and bronchoalveolar lavage was performed three times with the left lung with 0.5 ml of 4°C phosphate-buffered saline (PBS). The bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 700g at 4°C for 5 min. The cell pellets in BALF were resuspended in 0.2 ml PBS. Total cell numbers were counted with a hemocytometer. The remainder of the cells were stained with Wright Stain solution (Sigma-Aldrich) and were used for differential cell counting. At least 200 cells were counted in each mouse sample.

2.7 | Enzyme-linked immunosorbent assay

The levels of interleukin 6 (IL-6), tumor necrosis factor α (TNF- α), interferon γ (IFN- γ), and IL-13 in the BALF were measured by specific enzyme-linked immunosorbent assay (ELISA) kits (Cusabio, Wuhan, China). The detection limit of IL-6, TNF- α , IFN- γ , and IL-13 was 0.39, 3.9, 3.9, and 7.8 pg/ml, respectively. These detection limits were from the manufacturer's specifications, the protocols of this assay were based on the manufacturer's specifications.

2.8 | Histological analysis of the lung tissue

The right lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and the middle lobe of the lungs was sectioned (5 μ m). Tissue sections were stained with hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS) staining. These tissues were observed separately to analyze the inflammatory cell infiltration and mucus production. The quantitative analysis of peribronchial inflammation in H&E-stained lung slices was performed by assessing the number of rings of inflammatory cells around the bronchial (Koltsida et al., 2013). PAS-positive cell numbers divided by the perimeter of the basement membrane in

the airway was assessed for quantitative analysis of goblet cell hyperplasia and mucus production in lung tissue (Camateros et al., 2007).

2.9 | Western blot analysis

The cells were harvested and lysed in radioimmunoprecipitation assay buffer mixed with protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor (PhosSTOP; Roche). The lung tissues were homogenized with a homogenizer. The protein concentration was determined by a Bicinchoninic acid Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat milk for 1 hr and then incubated with primary antibodies overnight at 4°C. The primary antibodies included: anti-NLRC5 (Abcam), anti-NF- κ B p65, anti-NF- κ B pp65, anti-NLRP3, anti-ASC (Cell Signaling Technology), anti-TLR2 (Millipore), and anti-GAPDH (KANGCHEN Biotech, Shanghai, China). The bound antibodies were detected with horseradish peroxidase-conjugated antirabbit immunoglobulin G (IgG; Promega, Madison, WI) and polyclonal rabbit antimouse IgG (Dako, Copenhagen, Denmark). All blots were detected with enhanced chemiluminescence (Thermo Fisher Scientific).

2.10 | Statistical analysis

All data were analyzed by using the SPSS 19 and were expressed as the mean $\pm$ standard error of mean of triplicate samples. Data were representative of at least three separate experiments. Some groups were analyzed using the independent sample *t* test. One-way analysis of variance with a post hoc Bonferroni's test was used for other groups to distinguish differences. A value of $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | LTA induces an inflammatory response and NLRC5 activation in RAW264.7 cells

To verify if NLRC5 participates in the TLR2-mediated inflammatory response, we designed in vitro cell culture experiments. Recent reports have demonstrated LTA as a major virulence factor causing an inflammatory response via TLR2 (Kang, Sim, Yun, & Han, 2016). Thus, we stimulated RAW264.7 cells with 200 ng/ml of LTA at five different time points. After LTA exposure, the inflammatory response was evaluated by detecting the expression level of NF- κ B pp65. As shown in Figure 1a,b NF- κ B pp65 was increased in a time-dependent manner and peaked at 12 hr. In addition, we found that the protein expression of NLRC5 began to increase at the 4-hr time point and also peaked at 12 hr (Figure 1a,c).

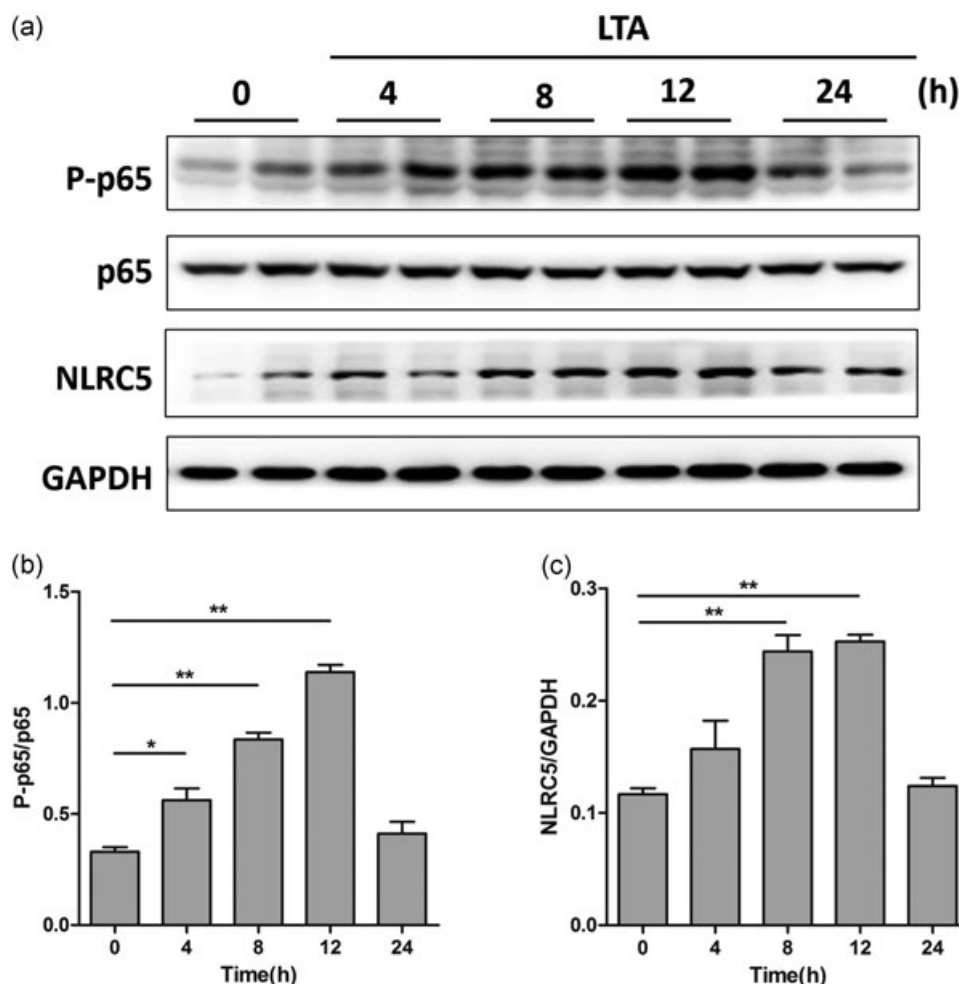


FIGURE 1 LTA (lipoteichoic acid) induced an inflammatory response and activated NLRC5 in RAW264.7 cells. (a) Western blot analysis of the expression of P-p65, p65, NLRC5, GAPDH in RAW264.7 cell lysates treated with 200 ng/ml LTA at the indicated time points. (b,c) The relative levels of P-p65 and NLRC5 in RAW264.7 cell treated with LTA at the indicated time points. The results are expressed as means $\pm$ SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ were determined with the independent sample t test. NLRC5: Nod-like receptor C5; SEM: standard error of mean

3.2 | LTA-induced activation of NLRC5 is TLR2-dependent

We were interested in determining if the activation of NLRC5 was dependent upon TLR2 during LTA-induced inflammatory responses. Thus, RAW264.7 cells were transfected with TLR2 siRNA or negative siRNA. The efficacy of the knockdown was approximately 60% (Figure S1A and S1C). The experimental groups were then exposed to LTA for 12 hr whereas DMSO was applied as a vehicle control. As we expected, after LTA stimulation the level of NLRC5 expression was lower in TLR2 siRNA group compared with the negative siRNA group (Figure 2d,e). Furthermore, fluorescence imaging clearly revealed that knockdown of TLR2 by siRNA attenuated LTA-induced NF- κ B p65 phosphorylation (Figure 2a,c). Western blot analysis showed similar results (Figure 2d,f). In addition, the activation of NLRP3 and apoptosis-associated speck-like protein containing a pyrin domain and caspase activation and recruitment domain (ASC) as also reduced in TLR2 siRNA groups (Figure 2d,g,h).

3.3 | NLRC5 negatively regulates the activation of NF- κ B in the inflammatory response via TLR2

Because TLR2 mediated both the inflammatory response and NLRC5 activation, we were interested in understanding how NLRC5 regulated the TLR2-mediated inflammatory signaling pathway. There has been evidence that the deletion of the NLRC5 gene can strongly enhance NF- κ B activation induced by TLR3, TLR4, TLR7, and TLR9 ligands (Tong et al., 2012). Whether NLRC5 has the same protective effect upon TLR2 ligand stimulation is still unknown. To further investigate the function of NLRC5, RAW264.7 cells were transfected with NLRC5 siRNA or negative siRNA and then stimulated with LTA. Transfection efficiency was shown in Figure S1B and S1D. Interestingly, western blot analysis showed that the level of TLR2 expression did not change, but the phosphorylation level of NF- κ B p65 had increased remarkably in LTA-treated cells in the absence of NLRC5 (Figure 3d-f). In addition, the expression of inflammasome-related proteins, such as NLRP3, significantly increased, whereas ASC revealed a significant reduction (Figure 3d,g,h). To further confirm this, the levels of NF- κ B p65 and NF- κ B pp65 were examined by

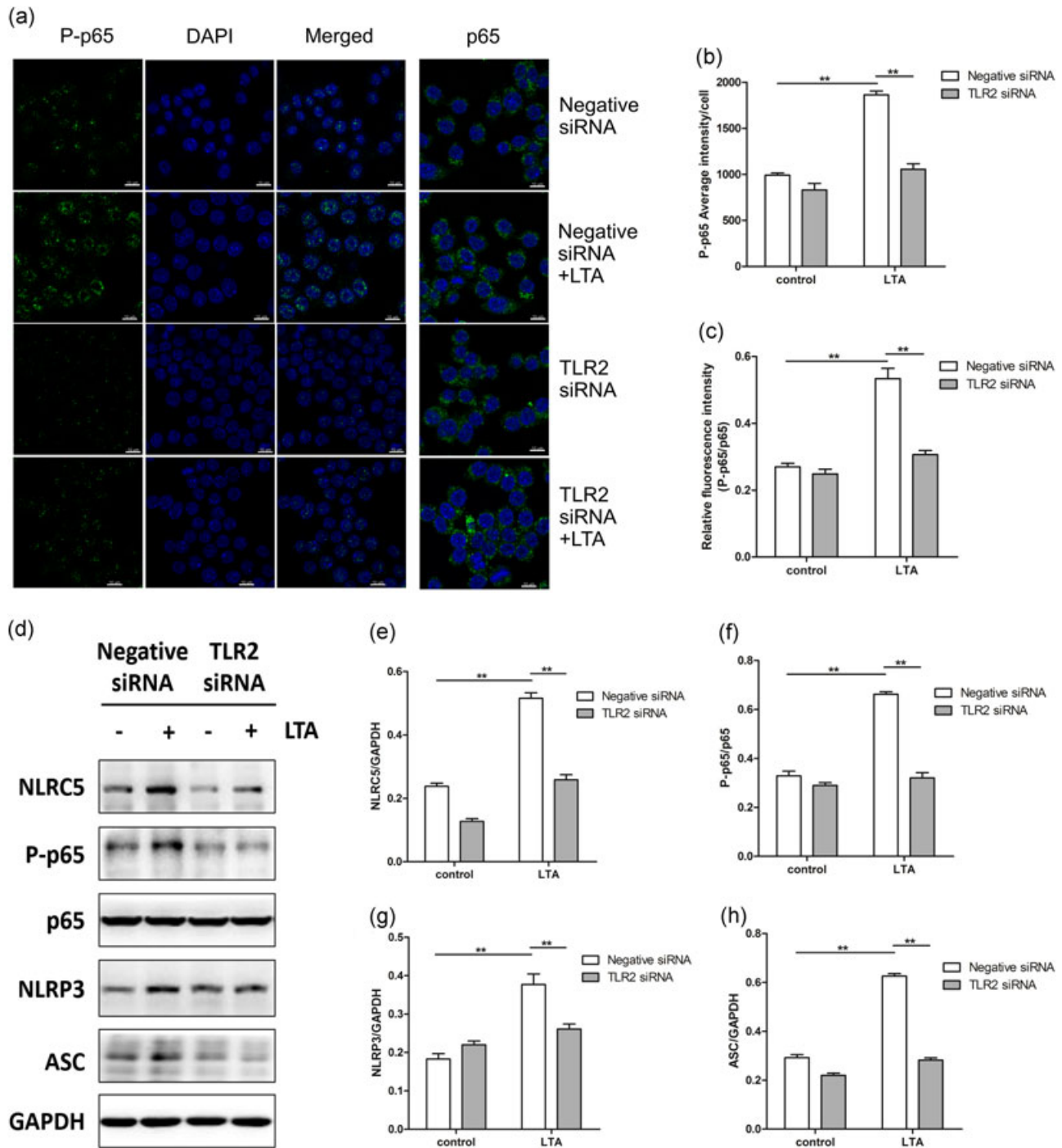


FIGURE 2 Effect of TLR2 on the changes of NLRC5 protein expression and LTA-stimulated inflammatory response in RAW264.7 cells. (a) Immunocytochemistry was used to analyze the expression of P-p65 and p65 in LTA-stimulated RAW264.7 cells after transfection of negative siRNA or TLR2 siRNA. (b) The averaged-immunofluorescence intensity of P-p65 per RAW264.7 cell. (c) The relative fluorescence intensity of P-p65 per RAW264.7 cell. (d) RAW264.7 cells were transfected with negative siRNA or TLR2 siRNA and stimulated with 200 ng/ml LTA or DMSO for 12 hr, then western blot analysis of the expression of NLRC5, P-p65, p65, NLRP3, ASC, and GAPDH in the RAW264.7 cell lysates. (e–h) The relative levels of NLRC5, P-p65, NLRP3, and ASC in the RAW264.7 cell lysates. Scale bars = 10 μ m. The results are expressed as means $\pm$ SEM of three independent experiments. ** p < 0.01 was determined with one-way ANOVA with a post hoc Bonferroni's test. ASC: apoptosis-associated speck-like protein containing a pyrin domain and caspase activation and recruitment domain; DAPI: 4',6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; LTA: lipoteichoic acid; NLRC5: Nod-like receptor C5; SEM: standard error of mean; siRNA: small interfering RNA; TLR2: Toll-like receptor 2 [Color figure can be viewed at wileyonlinelibrary.com]

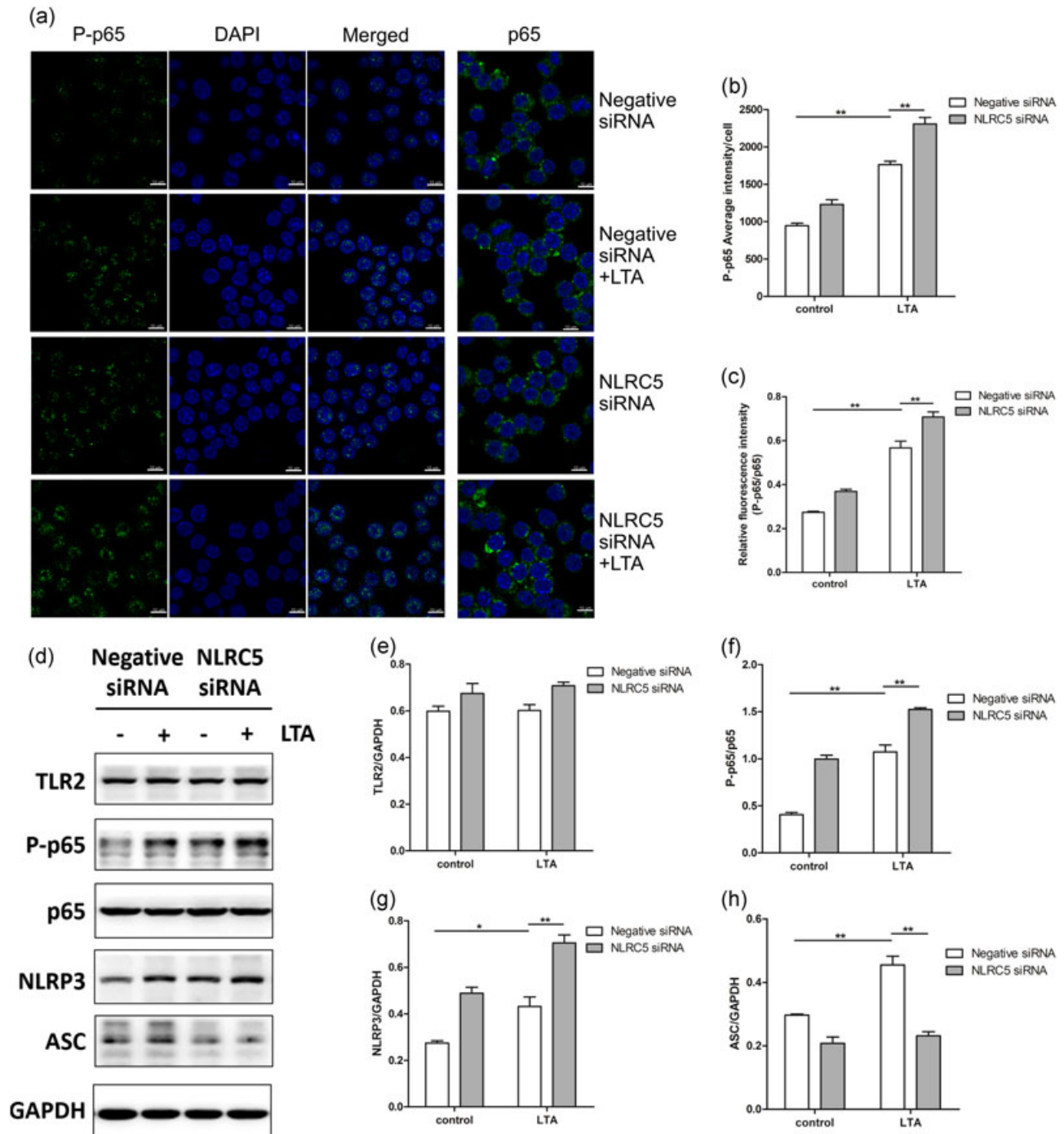


FIGURE 3 The role of NLRC5 in the changes of TLR2 protein expression and LTA-stimulated inflammatory response in RAW264.7 cells. (a) Immunocytochemistry was used to analyze the expression of P-p65 and p65 in LTA-stimulated RAW264.7 cells after transfection of negative siRNA or NLRC5 siRNA. (b) The averaged-immunofluorescence intensity of P-p65 per cell. (c) The relative fluorescence intensity of P-p65 per RAW264.7 cell. (d) RAW264.7 cells were transfected with negative siRNA or NLRC5 siRNA and stimulated with 200 ng/ml LTA or DMSO for 12 hr, then western blot analysis of the expression of TLR2, P-p65, p65, NLRP3, ASC, and GAPDH in the RAW264.7 cell lysates. (e-h) The relative levels of TLR2, P-p65, NLRP3, and ASC in the RAW264.7 cell lysates. Scale bars = 10 μ m. The results are expressed as means $\pm$ SEM of three independent experiments. * p < 0.05, ** p < 0.01 were determined with one-way ANOVA with a post hoc Bonferroni's test. ANOVA: analysis of variance; ASC: apoptosis-associated speck-like protein containing a pyrin domain and caspase activation and recruitment domain; DAPI: 4',6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; LTA: lipoteichoic acid; NLRC5: Nod-like receptor C5; SEM: standard error of mean; siRNA: small interfering RNA; TLR2: Toll-like receptor 2 [Color figure can be viewed at wileyonlinelibrary.com]

immunocytochemistry. The results from fluorescence imaging (Figure 3a–c) were consistent with the western blot analysis.

3.4 | OVA-induced mice allergic airway inflammation was accompanied by the increased expression of TLR2 and NLRC5

Numerous experiments have shown that ovalbumin-induced experimental allergic asthma is TLR2-dependent (Jiang et al., 2017; Li et al.,

2014). Because it was shown that NLRC5 and TLR2 interact in vitro, we wanted to know whether NLRC5 participates in the TLR2-mediated allergic airway inflammation. We performed in vivo experiments as illustrated in Figure 4a. In comparison with the healthy mice, a significant increase of total cell numbers in BALF was found in OVA-challenged WT mice. Meanwhile, inflammatory cells, such as neutrophils, monocytes, eosinophils, and lymphocytes in the BALF were remarkably increased (Figure 4b). Histopathology analysis showed a narrowing of the bronchial cavity, remarkable

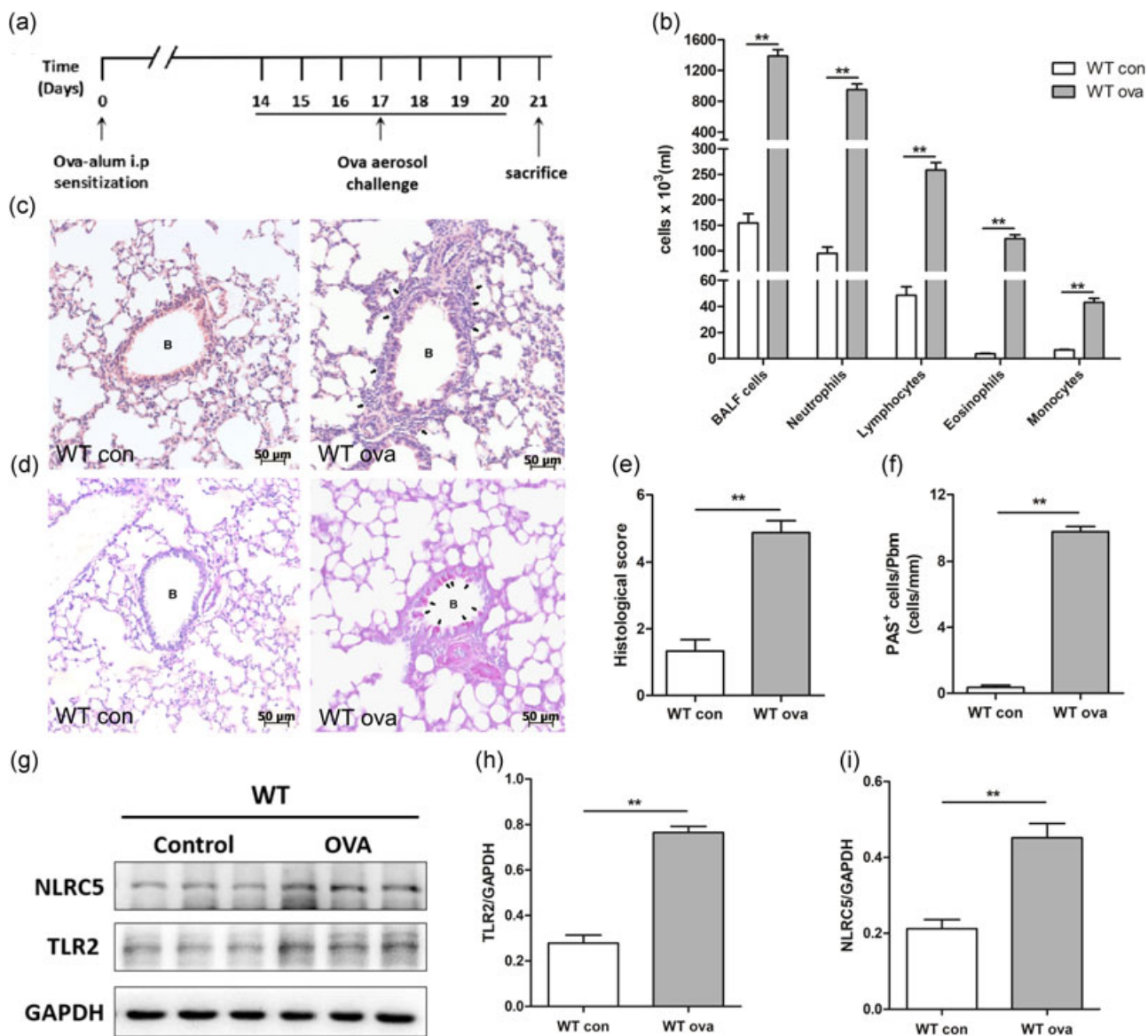


FIGURE 4 The levels of TLR2, NLRC5, and airway inflammation changed in OVA-induced allergic mice. (a) Study protocol to establish allergic airway diseases mice model. (b) The total and differential cell counts in BALF samples. (c) Representative HE-stained of lung sections from control and OVA-induced mice. Scale bars = 50 μ m. (d) Representative PAS-stained of lung sections from control and OVA-induced mice. Scale bars = 50 μ m. (e) Histological score of airway inflammatory of control and OVA-induced mice. (f) Quantification of PAS⁺ cells in the airway of control and OVA-induced mice. (g) The expression of TLR2 and NLRC5 in lung tissues of control and OVA-induced mice was detected by western blot. (h,i) The relative levels of TLR2 and NLRC5 in the lung tissues. The results are expressed as the means $\pm$ SEM of six mice per group. ** $p < 0.01$ was determined with the independent sample t test. B: bronchus; BALF: bronchoalveolar lavage fluid; HE: hematoxylin and eosin; NLRC5: Nod-like receptor C5; OVA: ovalbumin; SEM: standard error of mean; PAS: periodic acid-Schiff; TLR2: Toll-like receptor 2; WT: wild-type [Color figure can be viewed at wileyonlinelibrary.com]

inflammatory infiltrates (Figure 4c,e), and mucus production (Figure 4d,f) in the allergic group of mice. To determine how TLR2 and NLRC5 expression changes during allergic airway inflammation, TLR2 expression was analyzed by western blot analysis, which showed a notable increase of TLR2 expression in the lung of WT-OVA mice compared with that in control mice (Figure 4g,h). Moreover, a similar increase in the expression of NLRC5 was observed as well (Figure 4g,i).

3.5 | TLR2 contributes to the allergic airway inflammation and activation of NLRC5 in mice

We deleted the single TLR2 gene in C57BL/6 mice and induced allergic airway inflammation in WT and TLR2^{-/-} mice. As expected, OVA-challenged TLR2^{-/-} mice showed a significant decrease in the bronchial cavity inflammatory cell infiltrate (Figure 5a,c) mucous production (Figure 5b,d) compared with that of OVA-challenged WT mice. In view of the histopathology findings, we detected inflammatory cells and Th1/Th2 cytokines in the BALF. Our results showed that TLR2^{-/-} mice had remarkably lower levels of total cells, neutrophils, eosinophils, monocytes, and lymphocytes compared with those from OVA-challenged WT mice (Figure 6a–e). Similarly, through ELISA analysis, OVA challenge increased the levels of IL-6, IL-13, and TNF- α , which were notably attenuated in TLR2^{-/-} mice, whereas IFN- γ had increased in TLR2^{-/-} groups (Figure 6f–i). To explore the changes in the expression of inflammatory proteins, western blot analysis was used to detect the levels of NF- κ B pp65, NLRP3, and ASC—all of which are known to play various roles during inflammation. As shown in Figure 7a–d, after OVA stimulation, the expression levels of NF- κ B pp65, NLRP3, and ASC were increased significantly in WT mice, whereas such increase was reduced in

TLR2^{-/-} mice. It is noteworthy to mention that the level of NLRC5 was increased in WT mice but the increase decreased in TLR2^{-/-} mice (Figure 7a,e). Our results demonstrate that the level of allergic airway inflammation was decreased in TLR2^{-/-} mice. Moreover, our results show that TLR2 contributes to the activation of NLRC5 in murine allergic airway inflammation. Thus, we conclude that NLRC5 participates in TLR2-mediated allergic airway inflammation.

4 | DISCUSSION

In the present in vitro experiments, we found that LTA, which is recognized by TLR2, resulted in the initiation of the immune response and activated NLRC5 protein, whereas such an effect was lost upon the silencing of TLR2. In addition, knockdown of the NLRC5 gene enhanced the LTA-induced inflammatory response by increasing the protein expression of NF- κ B pp65 and NLRP3, but it had no effect on the expression of TLR2. Our in vivo experimental results showed that the deletion of a single TLR2 gene in mice effectively inhibited allergic airway inflammation and concomitantly reduced the protein expression of NF- κ B pp65, NLRP3, and ASC. Interestingly, the expression of NLRC5 was also remarkably increased in the OVA-challenged WT mice, whereas such an increase did not occur in TLR2-deficient mice. These findings show for the first time that NLRC5 is a downstream signaling molecule of TLR2 and negatively regulates inflammatory signaling pathways through TLR2/NF- κ B. Moreover, NLRC5 participates in the TLR2-mediated allergic airway inflammation.

Inflammatory signaling pathways are regulated at multiple levels by distinct mechanisms. TLRs are important members of the PRR superfamily and can serve as therapeutic targets to regulate the

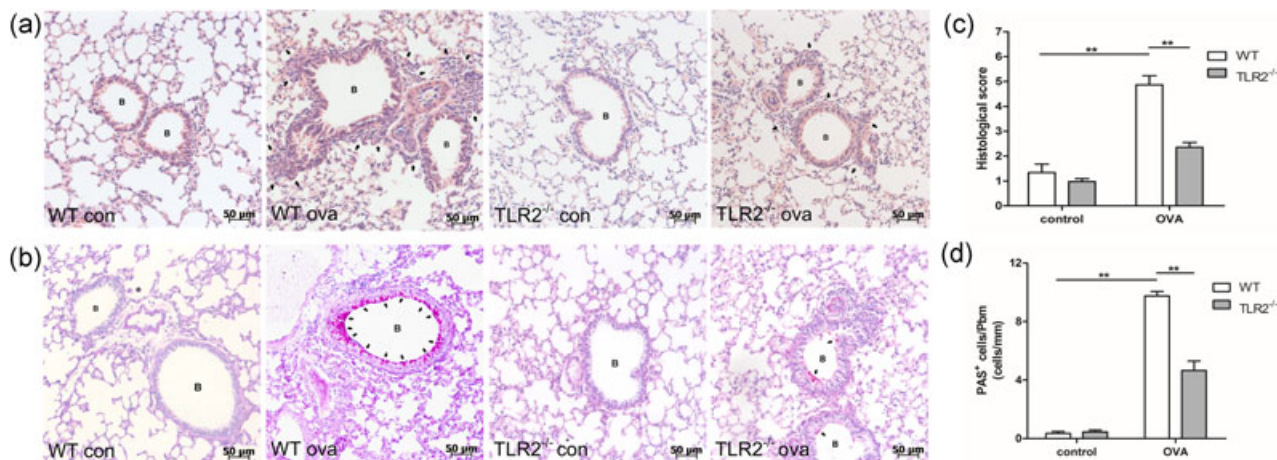


FIGURE 5 The levels of inflammatory cell infiltration and goblet cell hyperplasia were significantly reduced in TLR2^{-/-} mice.

(a) Representative HE-stained of lung sections from WT and TLR2^{-/-} mice treated with OVA or control. (b) Representative PAS-stained of lung sections from WT and TLR2^{-/-} mice treated with OVA or control. (c) istological score of airway inflammatory of WT and TLR2^{-/-} mice treated with OVA or control. (d) Quantification of PAS+cells in the airway of WT and TLR2^{-/-} mice treated with OVA or control. Scale bars = 50 μm. Data are expressed as the means ± SEM of six mice per group. ***p* < 0.01 was determined with one-way ANOVA with a post hoc Bonferroni's test. ANOVA: analysis of variance; B: bronchus; HE: hematoxylin and eosin; NLRC5: Nod-like receptor C5; OVA: ovalbumin; SEM: standard error of mean; PAS: periodic acid-Schiff; TLR2: Toll-like receptor 2; WT: wild-type [Color figure can be viewed at wileyonlinelibrary.com]

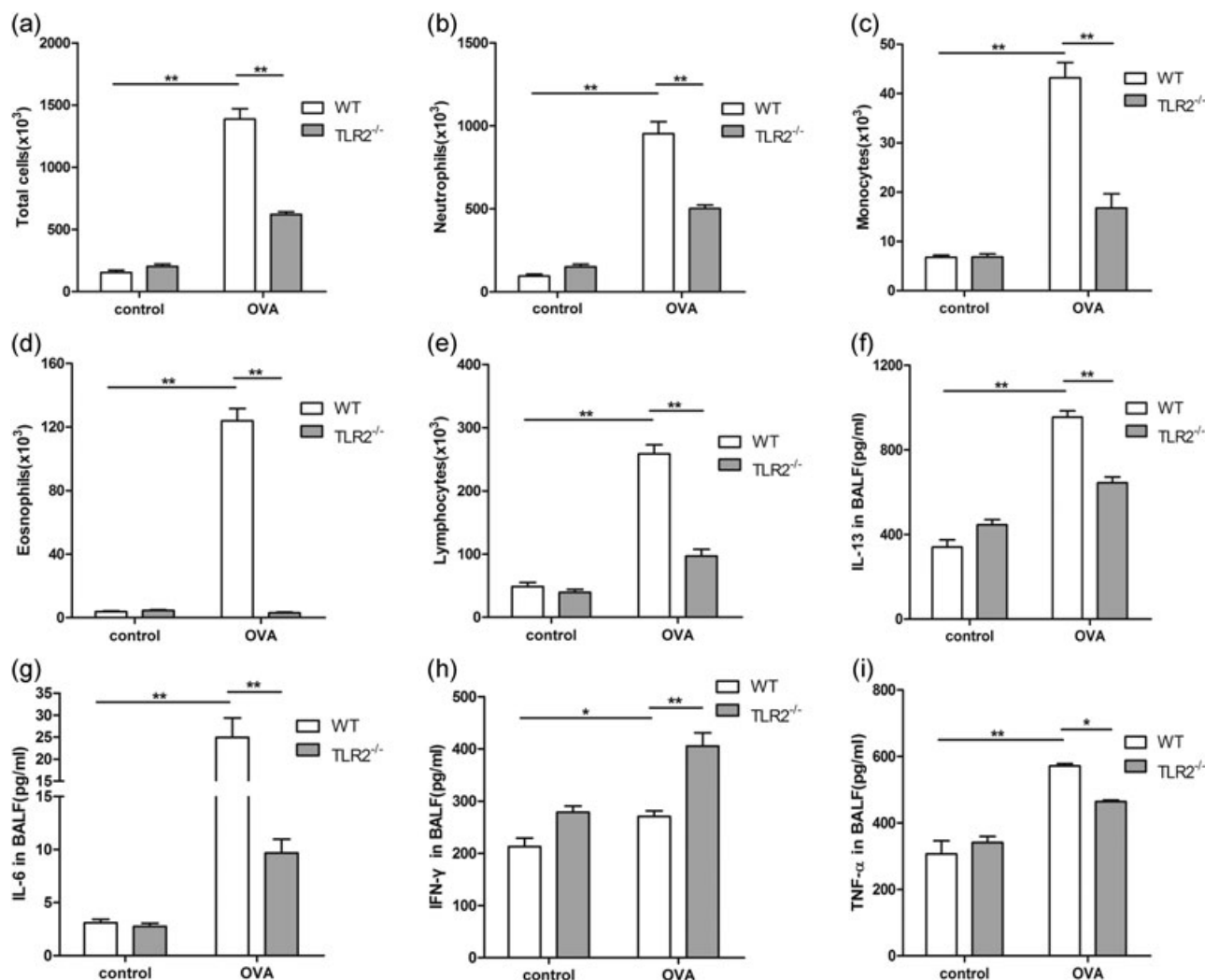


FIGURE 6 Deletion of single TLR2 gene remarkably reduced the cell numbers and cytokines in BALF after OVA treatment. (a–e) The total and differential cell counts in BALF samples. (f–i) Productions of IL-13, IL-6, IFN-γ, TNF-α in BALF samples. Data are expressed as the means ± SEM of six mice per group. * $p < 0.05$, ** $p < 0.01$ were determined with one-way ANOVA with a post hoc Bonferroni's test. ANOVA: analysis of variance; BALF: bronchoalveolar lavage fluid; IFN-γ: interferon γ; IL-6: interleukin 6; OVA: ovalbumin; SEM: standard error of mean; TLR2: toll-like receptor 2; TNF-α: tumor necrosis factor α; WT: wild-type

inflammatory response (Bezemer et al., 2012). To date, there was controversy regarding the role of TLR2 during innate immune responses. Studies have shown that TLR2 is required in order for PAMPs, such as bacterial lipoprotein and rhinovirus, to induce airway inflammation, moreover, the level of inflammation is reduced after deleting a single TLR2 gene (Han et al., 2016; Lugade et al., 2011). Recently, in vitro experiments have uncovered that LTA-induced inflammation is dependent on TLR2 (Knapp et al., 2008), and that NF-κB is involved in LTA-induced apoptosis in RAW264.7 cells (Zhao et al., 2017). Thus, consistent with our study, their results showed that phosphorylation of NF-κB was enhanced in RAW264.7 cells under LTA stimulation and that the production of NLRP3 and ASC also increased. However, these effects were abrogated with TLR2 siRNA treatment. Overall, these findings demonstrate that the TLR2 signaling pathway plays an essential role in regulating the inflammatory responses in macrophages.

NLRs are intracellular receptors that recognize microorganisms and danger-associated molecular patterns. According to the evolution and domain structure, NLRs are subdivided into four types: NLRC, NLRP, NLRA, and NLRB (Schroder & Tschopp, 2010; Ting et al., 2008). Cui et al. (2010) first discovered that the NLRC5 protein was a member of the NLR family and illustrated that NLRC5 inhibits NF-κB signaling by blocking IκB-kinase phosphorylation during LPS stimulation. This suggests that NLRC5 negatively regulates NF-κB signaling and inflammation through the TLR4 signaling pathway. However, the mechanisms by which NLRC5 negatively regulates the TLR-mediated immune responses have not been fully elucidated. Recent studies have shown that removing the polyubiquitin chains from NLRC5 can enhance the inhibition of NF-κB signaling (Meng et al., 2015). Other studies have reported that NLRC5 is likely to promote rheumatoid arthritis progression via the NF-κB signaling pathway (Y. R. Liu et al., 2017). Therefore, it is necessary to explore

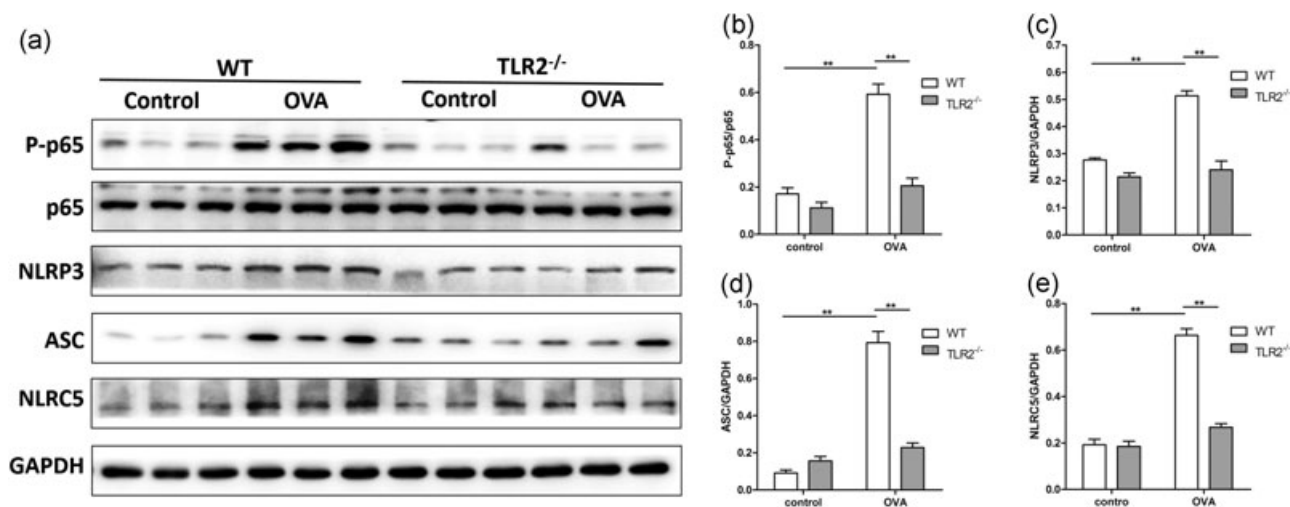


FIGURE 7 Expression levels of inflammation associated proteins were significantly reduced in TLR2^{-/-} mice. (a) Western blot analysis of the expression of P-p65, p65, NLRP3, ASC, NLRC5, and GAPDH in the lung tissues between WT and TLR2^{-/-} mice treated with OVA or control. (b–e) The relative levels of P-p65, NLRP3, ASC, and NLRC5 in the lung tissues of WT and TLR2^{-/-} mice. Data are expressed as the means ± SEM of six mice per group. ** $p < 0.01$ was determined with one-way ANOVA with a post hoc Bonferroni's test. ANOVA: analysis of variance; ASC: apoptosis-associated speck-like protein containing a pyrin domain and caspase activation and recruitment domain; OVA: ovalbumin; SEM: standard error of mean; TLR2: toll-like receptor 2; WT: wild-type

the role of NLRC5 in the TLR2-mediated NF- κ B inflammatory signaling pathway. Thus, we silenced the NLRC5 gene by using siRNA and observed an increase in the level of NF- κ B protein in comparison with the negative siRNA in LTA-stimulated RAW264.7 cells. In addition, another study indicated that NLRC5 acts on NLRP3 and promotes inflammasome activation in HEK293 cell lines in vitro during *Listeria monocytogenes* infection (Yao et al., 2012). In contrast to this finding, our results have shown that the stimulation of NLRC5-silenced macrophages with LTA improved the expression level of NLRP3, but reduced the level of ASC. This discrepancy is likely due to the diverse functions NLRs have in different cell line types (Ting, Duncan, & Lei, 2010). Furthermore, our results support the findings that the absence of NLRC5 has no impact on the transcription of NLRP3 (Davis et al., 2011), but the increased activation of NF- κ B in NLRC5-silenced groups can activate NLRP3 transcription (Koch et al., 2015). Thus, we reveal that NLRC5 negatively regulates TLR2-mediated inflammatory response in vitro.

Recently, it has been shown that excessive activation of the inflammasome—assembly of which is induced by NLRs—is associated with immune-mediated inflammation (Shaw, McDermott, & Kanneganti, 2011). We have shown that NLRC5 is involved in and negatively regulates TLR2-mediated inflammatory response in vitro. We were interested in determining whether NLRC5 participates in the regulation of airway inflammation in vivo. It has been revealed that down-regulation of TLR2 expression in OVA-induced allergic airway inflammation causes a reduction in host defense cytokine production (Wu et al., 2008), and our previous study reported that TLR2 plays a key role in allergic airway inflammation (Jiang et al., 2017). Thus, we developed an allergic airway inflammation mouse model. Through in vivo studies, we showed elevated expression of TLR2 that was consistent with exacerbation of airway inflammation in OVA-induced WT mice. Furthermore, in OVA-induced groups, the bronchial immune

cell infiltration, airway mucus production, as well as, total and differential inflammatory cells, Th1/Th2 proinflammatory cytokine in the BALF, and the level of NF- κ B phosphorylation were all notably attenuated following TLR2 gene deletion. Similar to these findings, we also found that the OVA-induced airway inflammation was accompanied by an increased expression of NLRC5. Interestingly, the expression of NLRC5 was significantly reduced in the OVA-induced TLR2^{-/-} mice group, which was accompanied by a decreased production of NLRP3 and ASC, and a weakening of the inflammatory immune response. The results are supported by other studies, which show that the level of NLRC5 transcription and expression can be significantly induced by TLR ligands, such as LPS, viral infection, poly (I:C), and interferons (Benko, Magalhaes, Philpott, & Girardin, 2010; Cui et al., 2010; Neerincx et al., 2010; Staehli et al., 2012). Taken together, these experimental findings suggest that NLRC5 is involved in TLR2-mediated allergic airway inflammation. We will address the role of NLRC5 in the regulation of TLR2-mediated allergic airway inflammation in future experiments.

In summary, our results show that NLRC5 participates in the TLR2-mediated inflammatory signaling pathway and functions as a negative signal to regulate the inflammatory response via the TLR2/NF- κ B pathway in macrophages. Furthermore, NLRC5 is also involved in TLR2-mediated allergic airway inflammation. These findings may provide potential immunotherapy targets in allergic airway diseases, and the negative regulatory mechanism of NLRC5 in macrophages via TLR2/NF- κ B may be an important target for therapeutic intervention as a means to protect the host from harmful pathogens.

ACKNOWLEDGMENTS

We would like to thank Dr Jiang-Ning Zhou from the University of Science and Technology of China for his technical assistance. We

would like to thank Dr Zhi-Gang Tian from the University of Science and Technology of China for providing the TLR2^{-/-} mice. This study was supported by Research Project of Anhui Province (no.10021303028), Key Lab of Geriatric Molecular Medicine of Anhui Province (1206c0805028), and Key subjects of Anhui Province

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Study conception and design: R.L.; Data acquisition: M. W., L. W., L. F. and S. L.; Data analysis or interpretation: M. W. and L. W.; Manuscript drafting: M. W. and L. F.; Critical manuscript revision: M. W. and R. L.

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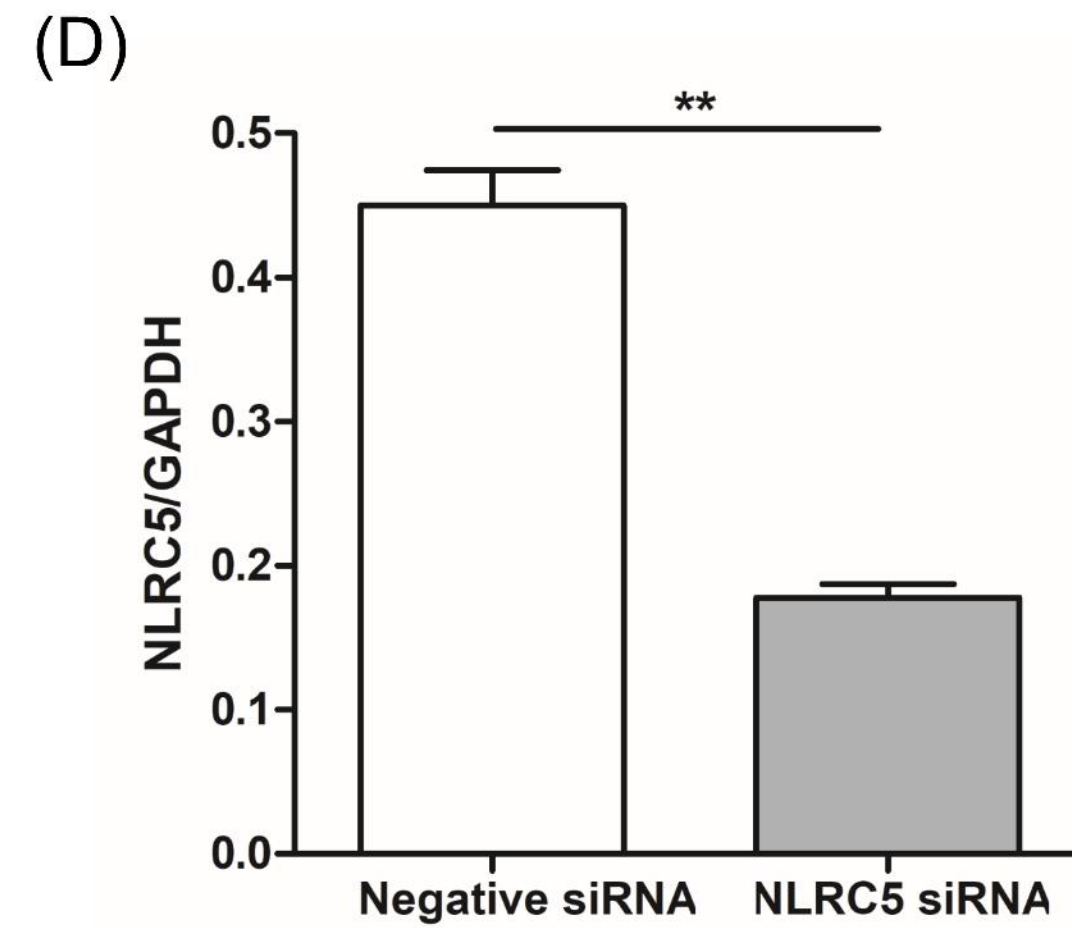
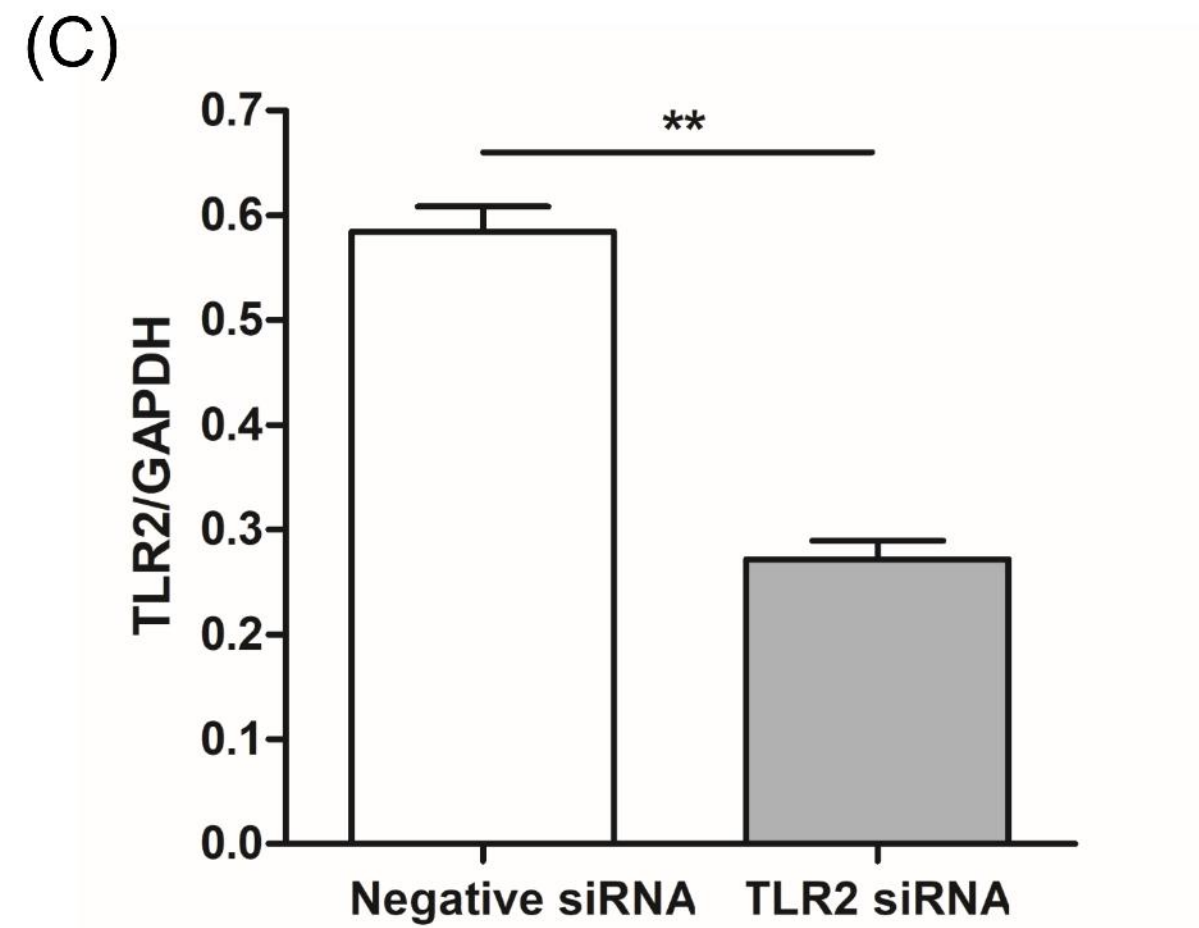
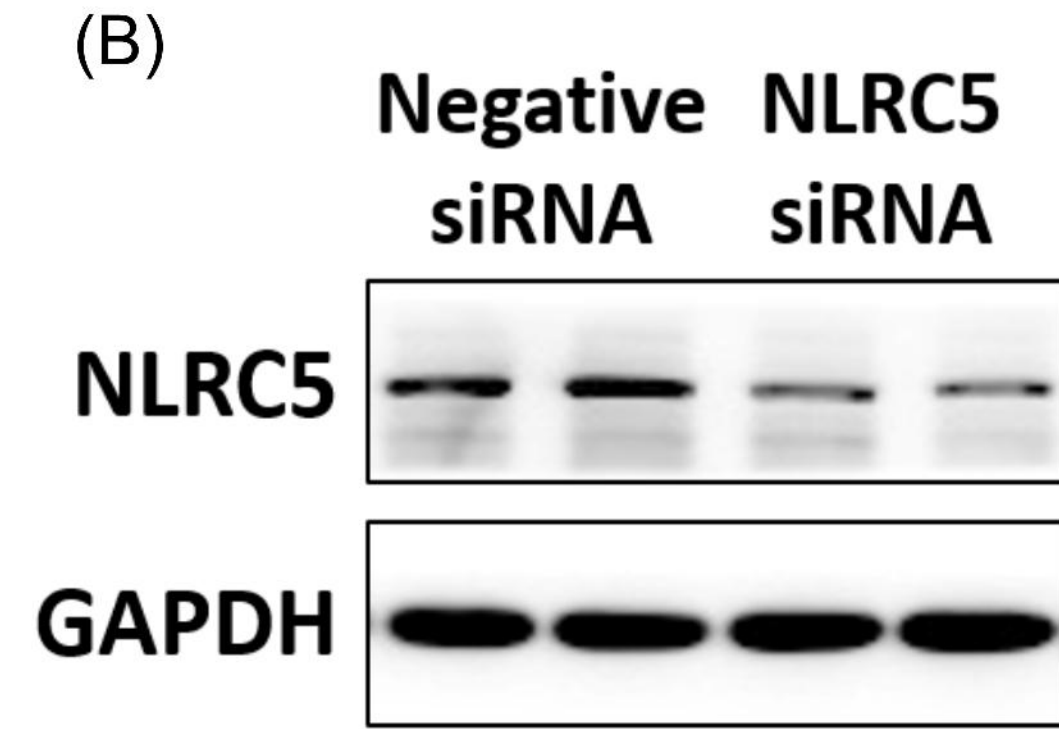
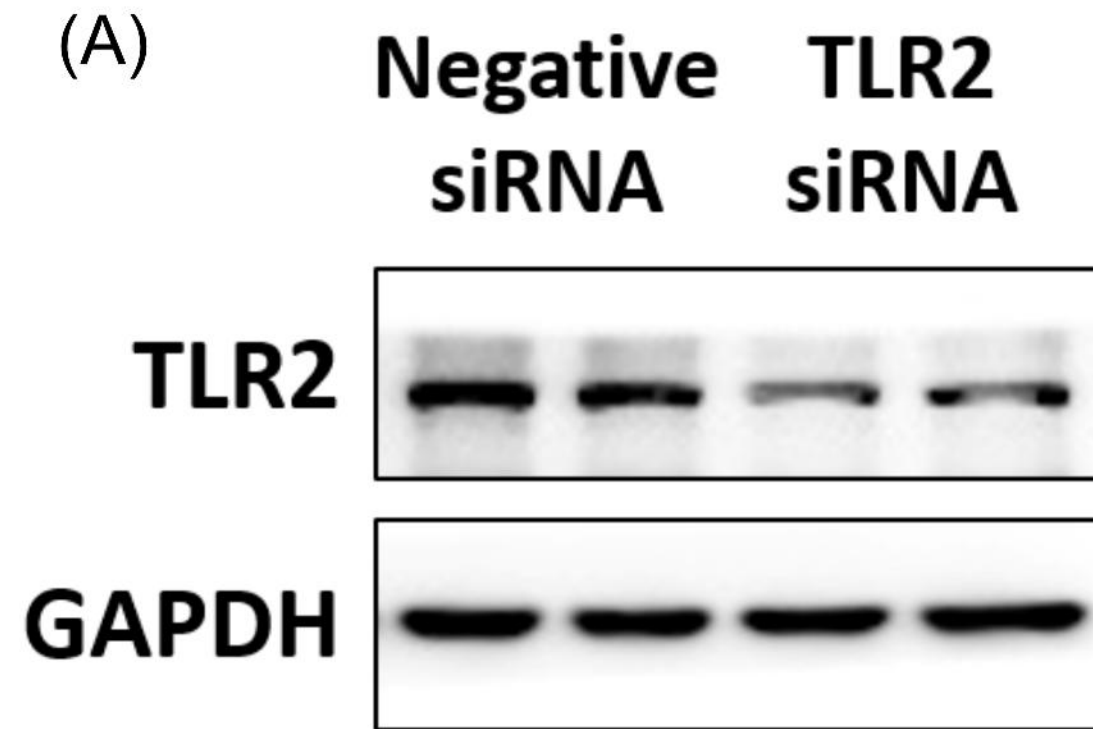
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Wang M, Wang L, Fang L, Li S, Liu R. NLR5 negatively regulates LTA-induced inflammation via TLR2/NF- κ B and participates in TLR2-mediated allergic airway inflammation. *J Cell Physiol*. 2019;1–12. <https://doi.org/10.1002/jcp.28596>



Supplemental Fig1:

The transfection efficiency of TLR2 siRNA and NLRC5 siRNA.

(A) Western blot analysis of the expression of TLR2 in RAW264.7 cell lysates.

(B) Western blot analysis of the expression of NLRC5 in RAW264.7 cell lysates.

(C) The relative level of TLR2 in RAW264.7 cell lysates.

(D) The relative level of NLRC5 in RAW264.7 cell lysates.

** $p < 0.01$ was determined with independent sample *t*-test.