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TLR2 Signaling Is Critical for *Mycoplasma pneumoniae*-Induced Airway Mucin Expression[1]

Hong Wei Chu, Samithamby Jeyaseelan, John G. Rino, Dennis R. Voelker, Rachel B. Wexler, Krista Campbell, Ronald J. Harbeck, and Richard J. Martin[2]

Excessive airway mucin production contributes to airway obstruction in lung diseases such as asthma and chronic obstructive pulmonary disease. Respiratory infections, such as atypical bacterium *Mycoplasma pneumoniae* (Mp), have been proposed to worsen asthma and chronic obstructive pulmonary disease in part through increasing mucin. However, the molecular mechanisms involved in infection-induced airway mucin overexpression remain to be determined. TLRs have been recently shown to be a critical component in host innate immune response to infections. TLR2 signaling has been proposed to be involved in inflammatory cell activation by mycoplasma-derived lipoproteins. In this study, we show that TLR2 signaling is critical in Mp-induced airway mucin expression in mice and human lung epithelial cells. Respiratory Mp infection in BALB/c mice activated TLR2 signaling and increased airway mucin. A TLR2-neutralizing Ab significantly reduced mucin expression in Mp-infected BALB/c mice. Furthermore, Mp-induced airway mucin was abolished in TLR2 gene-deficient C57BL/6 mice. Additionally, Mp was shown to increase human lung A549 epithelial cell mucin expression, which was inhibited by the overexpression of a human TLR2 dominant-negative mutant. These results clearly demonstrate that respiratory Mp infection increases airway mucin expression, which is dependent on the activation of TLR2 signaling. *The Journal of Immunology,* 2005, 174: 5713–5719.

T oll-like receptors are important pattern recognition receptors in the host innate defense against invading pathogens. Signaling through the TLRs leads to transcription and translation of a variety of cytokines/mediators (1). TLR2 is particularly involved in signal transduction of cellular responses to lipoproteins/lipopeptides, Gram-positive bacteria, and mycobacterial wall constituents (2).

Mycoplasma pneumoniae (Mp),[3] an atypical bacterium, is one of the common causes of community-acquired pneumonia (3). Previous studies have linked this pathogen to asthma (4–7). Lipoproteins and/or lipopeptides from mycoplasmas have been demonstrated to initiate the host innate immune response predominantly through the TLR2 signaling pathways. Three forms of lipoprotein/peptide have been identified in the mycoplasma species. These include macrophage-activating lipopeptide 2 (MALP-2), p48, and M161Ag (8, 9). All three lipoproteins/peptides can bind to TLR2 and share similar immunomodulatory effects. Murine models of intratracheal instillation of MALP-2 from *Mycoplasma fermentans* resulted in lung neutrophilic and lymphocytic inflammation (10). When macrophages from TLR2-deficient mice were stimulated with MALP-2, the activation of NF-κB and subsequent production of proinflammatory cytokines (e.g., TNF-α, IL-8, and MCP-1)

were abrogated, thus suggesting an essential role of TLR2 signaling in mycoplasma-induced inflammatory response (11).

Overexpression of mucus, or its major component mucin, is a significant contributor to airway obstruction in chronic lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) (12–14). Although atypical bacterium infection has been proposed to play a role in lung diseases with mucin overexpression (15), it remains to be determined whether and how the infection induces airway mucin expression. In this study, we hypothesized that activation of TLR2 signaling by Mp infection is critical in the induction of airway mucin expression. We have demonstrated that respiratory Mp infection in mice results in activation of TLR2 signaling, which is essential to the induction of airway mucin expression following the infection.

Materials and Methods

Animals

All experimental animals used in this study were covered by a protocol approved by our Institutional Animal Care and Use Committee. Wild-type BALB/c and C57BL/6 mice (8–10 wk old) were obtained from The Jackson Laboratory. TLR2 gene-deficient (TLR2$^{-/-}$) mice were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan). These mice were inbred from 129/SV × C57BL/6 and backcrossed with C57BL/6 mice for eight generations (16). All the mice were quarantined for 4 wk before the experiment and bled to establish that they were virus and *Mycoplasma pulmonis* free.

Mp preparation

Mp (strain FH, ATCC 15531) was grown in SP-4 broth for 72 h at 37°C, spun at 10,000 × g for 20 min, and resuspended in saline to yield ~1 × 10^8 CFU/50 μl (17).

Mp inoculations in wild-type BALB/c mice

On day 0, mice were inoculated with either Mp or saline (control). Before the inoculation, all mice were i.p. anesthetized with Avertin (ethanol) at 0.25 g/kg. Mice in the infected group were inoculated intranasally with 50 μl of Mp at ~1 × 10^8 CFU. A 50-μl inoculation of saline was similarly given to the mice in the control groups. Mice were sacrificed on 4 h, days 1 and 3 after Mp or saline and examined to determine the activation and/or expression levels of TLR2 signaling and airway mucins.

TLR2-neutralizing Ab treatment in wild-type BALB/c mice

To examine whether TLR2 signaling blockade would reduce Mp-induced airway mucin expression, a blocking experiment with a TLR2-neutralizing Ab (TLR2Ab) was performed in the following four groups of mice. TLR2Ab is a goat polyclonal Ab raised against an epitope mapping within an extracellular domain of TLR2 of mouse origin (sc-16237; Santa Cruz Biotechnology). In our previous cell culture experiments, this TLR2Ab was shown to markedly inhibit Mp-induced TNF-α production by Raw 264.7 cells (a mouse macrophage cell line) (our unpublished observations). Group 1, TLR2Ab (5 μg in 50 μl of saline per mouse) + saline (control); group 2, a goat IgG (5 μg in 50 μl of saline per mouse; control for TLR2Ab) + Mp; group 3, TLR2Ab (5 μg in 50 μl of saline per mouse) + Mp (10^8 CFU/mouse); and group 4, TLR2Ab pretreated with a specific TLR2 blocking peptide (BP; Santa Cruz Biotechnology) that was used to generate the TLR2Ab (control for TLR2Ab specificity) + Mp (10^8 CFU/ mouse). On day 0, at 2 h before infection or saline, mice were treated intranasally with TLR2Ab, goat IgG, or TLR2Ab pretreated with BP, which were repeated once daily on days 1 and 2 postinfection or saline. On day 3, lung tissue was collected for determination of mucin expression.

Mp inoculations in wild-type and TLR2$^{-/-}$ C57BL/6 mice

TLR2$^{-/-}$ C57BL/6 mice were used to further confirm an essential role of TLR2 in Mp-induced mucin expression. As our preliminary data in wild-type BALB/c mice demonstrated an increase of mucin mRNA and/or protein expression on days 1 and 3 after Mp, we focused TLR2$^{-/-}$ mouse experiments on days 1 and 3 after the infection. Four groups of mice were examined. Group 1, wild-type C57BL/6 mice with an intranasal saline inoculation; group 2, wild-type C57BL/6 mice with an intranasal Mp inoculation (10^8 CFU/mouse); group 3, TLR2$^{-/-}$ mice with an intranasal Mp inoculation (10^8 CFU/mouse); group 4, TLR2$^{-/-}$ mice with an intranasal saline inoculation.

Lung tissue processing

Lungs were removed and excised. The middle lobe of the right lung was processed for total RNA extraction. The remaining right lung was processed for Western blot analysis and NF-κB detection. The left lung tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and cut at 4 μm thickness for mucin and TLR2 immunostaining.

Real-time quantitative RT-PCR for TLR2 and mucin MUC5AC mRNA

Real-time quantitative RT-PCR was performed as previously described (17). Briefly, total RNA of lung tissue was extracted using TRIzol reagent (Invitrogen Life Technologies) and treated with DNase I. Reverse transcription was performed using 1 μg of total RNA and random hexamers in a 50-μl reaction according to the manufacturer's protocol (Applied Biosystems). The mouse TLR2 and MUC5AC primers and probes were designed using the Primer Express software (Applied Biosystems) and listed as below. TLR2 (GenBank accession number, AF124741) primers and probe: forward primer, 5′-AAGGCATTAAGTCTCCGGAATTATC-3′; reverse primer, 5′-TCGCTTAAGTGAAGAGTCAGGTGAT-3′; probe, 5′-TCCCAAAGTCTAAAGTCGATCCGCGAC-3′. MUC5AC (GenBank accession number, L42292) primers and probe: forward primer, 5′-AGAG GAGCGGAGAGAGACTCTGT-3′; reverse primer, 5′-CTCCATCTCTC TCTCAGGGTAGTTCT-3′; probe, 5′-CGAGAGGAGATCACACAGTT GCAATGCA-3′. Real-time PCR was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems). The 50-μl PCR contained 60 ng of cDNA, 100 nM fluogenic probe, and 200 nM primers and other components from the TaqMan RT-PCR kit. Housekeeping gene 18S rRNA was evaluated using the same PCR protocol as TLR2 and MUC5AC. The specificity of PCR for target genes was verified by no signal in no-template controls or reverse transcription ($-$) samples. The threshold cycle was recorded for each sample to reflect the mRNA expression levels. The comparative threshold cycle method was used to demonstrate the relative expression level of TLR2 and MUC5AC mRNA as previously reported (17).

Detection of lung tissue NF-κB activity

A TransAM ELISA-based assay NF-κB kit (Active Motif) was used to detect the activation of the p65 (Rel A) of NF-κB in mouse lung tissue (18). NF-κB specific oligonucleotide was immobilized to a 96-well plate. Lung tissue nuclear extract (20 μg) was added to the plate and incubated for 1 h at room temperature. After washes, a primary Ab identifying activated p65 was added and incubated for another hour. An anti-IgG HRP conjugate was then added to the plate, and the color was developed according to the manufacturer's instruction. OD value at 450 nm was measured on a plate reader.

Western blot analysis

Lung tissue was homogenized in Western lysis buffer with protease inhibitors, and boiled for 5 min. Fifty micrograms of protein lysate was electrophoresed on 10% SDS-PAGE, transferred onto nitrocellulose membrane, blocked with 5% nonfat milk in Tris buffer (pH 7.6) with 0.1% Tween 20, then incubated with a primary Ab (e.g., TLR2, MyD88, or β-actin) overnight at 4°C. After washes, the membranes were incubated with an anti-IgG conjugated with a HRP and detected by using the chemiluminescence system (19).

Mucin and TLR2 staining

The general mucin in the lung tissue was identified by Alcian Blue/periodic acid Schiff staining. Medium-sized airways, defined by an epithelial basement membrane perimeter of 600–900 μm (maximal diameter/minimum diameter $\leq$2), were examined for airway mucin. The area of mucin in airway epithelium was measured using a NIH Scion image program (National Institutes of Health). The results were expressed as airway mucin area/total airway epithelium area (percentage). At least five complete airways per mouse were examined. The coefficient of variation for two to three repeated measurements by the same observer or between two different observers was <7%. The observers were blinded to the treatments of mice.

To localize TLR2 on the lung tissue, immunofluorescent staining of TLR2 protein was performed on deparaffinized lung tissue sections using a rabbit anti-mouse TLR2 Ab (1 μg/ml; Santa Cruz Biotechnology) or an irrelevant rabbit IgG (1 μg/ml) as a negative control. The immunofluorescent staining procedure has been previously described (19).

Lung epithelial cell culture and transient transfection

A549 cells (a human lung adenocarcinoma cell line) were used to determine the direct effects of Mp on epithelial mucin expression since mouse primary airway epithelial cells are difficult for the transfection assay and there are no mouse lung epithelial cell lines available for mucin study. Cells were cultured in 24-well plates in triplicate at 8 × 10^4 cells per well, infected with Mp at 50 CFU/cell, and incubated for 48 h. Cells from one well were processed for cell cytospin slides to immunostain MUC5AC protein. A total of 500 cells (both MUC5AC positive and negative) were counted to determine the percentage of MUC5AC protein expression levels using the following formula: [(number of MUC5AC positive cells/500) × 100]. MUC5AC mRNA was examined in the remaining cells using real-time RT-PCR.

To determine the effects of TLR2 blockade on epithelial cell NF-κB and mucin expression, transient transfection was performed in A549 cells. Cells were seeded into 24-well plates at 8 × 10^4 cells per well, allowed to grow to 70–80% of confluence, and were then treated in triplicate with a mixture of LipofectAMINE 2000, 0.2 μg of an expression vector bearing a human TLR2 dominant-negative mutant (a generous gift from Dr. D. Underhill, Institute for Systems Biology, Seattle, WA) and NF-κB-luciferase. As a control, cells were transfected with a mixture of LipofectAMINE 2000, 0.2 μg of an empty vector, and NF-κB-luciferase (gifts from Dr. J. Park, University of Colorado Health Sciences Center, Denver, CO). After 48 h of transfection, cells were infected with Mp at 50 CFU/cell and incubated for another 48 h. Cells were then harvested for NF-κB luciferase activity assay and MUC5AC protein immunostaining.

Effects of Mp-derived lipoproteins on mucin expression by A549 cells

To determine whether purified Mp TLR2 ligands (i.e., Mp-derived lipoproteins) would also be able to stimulate mucin expression by lung epithelial cells, A549 cell culture was similarly performed as described above. Cells were incubated for 48 h in the absence or presence of Mp-derived lipoproteins at 0.1, 0.5, and 2.5 μg/ml. The details of purification of Mp-derived lipoproteins have been previously described (20). At 48 h, cells were harvested to determine mucin MUC5AC protein expression using immunocytochemistry.

Statistical analysis

If the data were normally distributed, they were presented as means ± SEM and compared between the groups using the ANOVA. When the data were not normally distributed, the data were expressed as medians with interquartile (25–75%) ranges and the comparisons between the groups were performed using the Wilcoxon rank-sum test. A two-tailed p value <0.05 is considered statistically significant.

FIGURE 1. TLR2 expression in wild-type BALB/c mouse lung tissue. *A,* TLR2 mRNA expression was examined by quantitative real-time RT-PCR and expressed as relative level using the comparative cycle of threshold method. Data expressed as medians (25–75% range). $n = 6–8$ mice per group. *B,* TLR2 protein Western blot analysis. Raw 264.7 mouse macrophages infected with Mp at 50 CFU/cell serve as a positive control. β-Actin immunoblot onto the TLR2 Ab-stripped membrane was performed to confirm equal protein loading. *C,* TLR2 protein immunofluorescent staining. The green and blue colors indicate TLR2 and cell nuclei, respectively. On day 1, saline treated BALB/c mouse lung showing very weak TLR2 staining (*a*), while on day 1, Mp infected BALB/c mouse lung (*b*) showing strong TLR2 staining on bronchial epithelial cells (white arrows) and alveolar macrophages (red arrows). The specificity of TLR Ab staining was confirmed by no fluorescent staining in infected BALB/c mouse lung (day 1) incubated with an irrelevant goat IgG (*c*). On day 1 after Mp infection, wild-type (*d*), but not TLR2$^{-/-}$ C57BL/6 mice (*e*), demonstrated airway epithelial TLR2 staining (white arrow). Original magnification, ×200
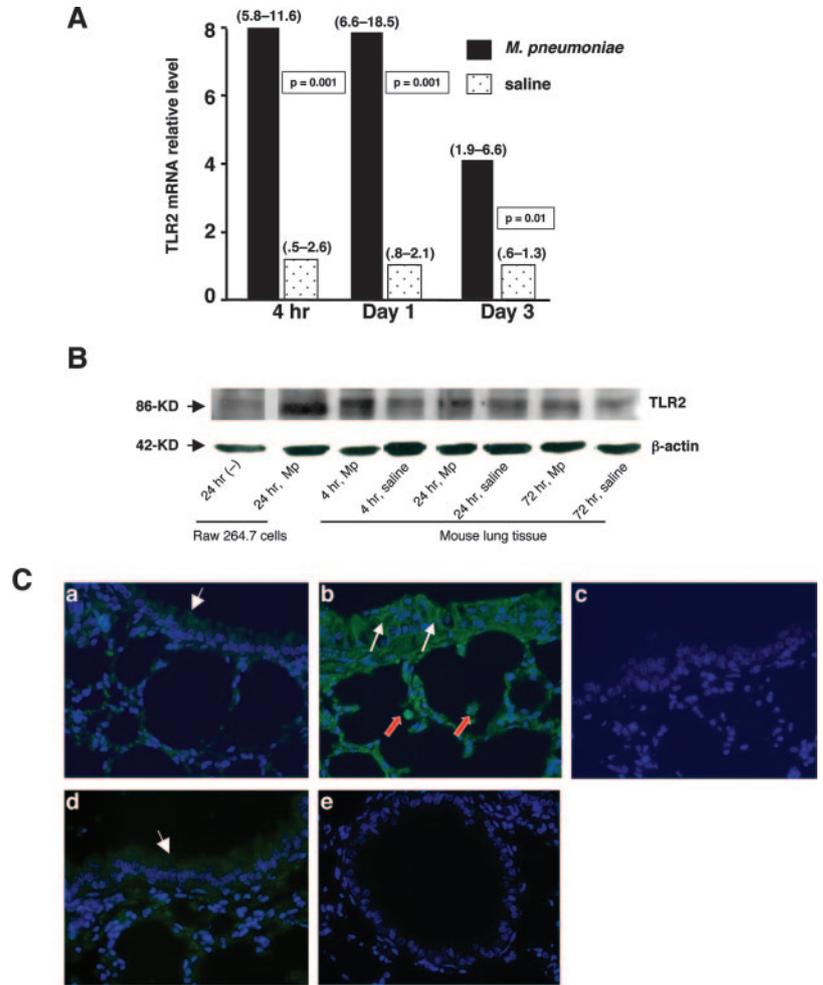
Results

Respiratory Mp infection in wild-type BALB/c mice activates TLR2 signaling

We first tested whether in vivo lung Mp infection in BALB/c mice increased the activation levels of TLR2 signaling. To accomplish this, several key TLR2 signaling components including TLR2, adaptor protein MyD88, and transcription factor NF-κB were examined.

After Mp infection, TLR2 mRNA expression was significantly increased at all the time points examined (Fig. 1*A*), especially at the earlier time points (4 h and day 1). Western blot analysis demonstrated a similar increase of TLR2 protein in the lungs (Fig. 1*B*). Airway epithelial cells and alveolar macrophages were identified as the predominant types of cells expressing TLR2 protein by immunofluorescent staining (Fig. 1*C*).

MyD88 protein expression as detected by Western blot did not appear to be increased in Mp-infected mice as compared with saline control mice. However, the recruitment of MyD88 to TLR2 was increased especially on day 1 as detected by MyD88 Western blot in lung lysate samples coimmunoprecipitated with a TLR2 Ab (Fig. 2*A*).

Because NF-κB activation has been proposed as a key event of TLR signaling cascade, we next examined whether levels of lung tissue NF-κB activation increased after Mp infection. As shown in Fig. 2*B*, activated p65 NF-κB levels were significantly higher in lung tissue nuclear extracts of infected mice than those in saline control mice. The temporal pattern of NF-κB activation levels was almost identical to that of TLR2 mRNA expression levels. These
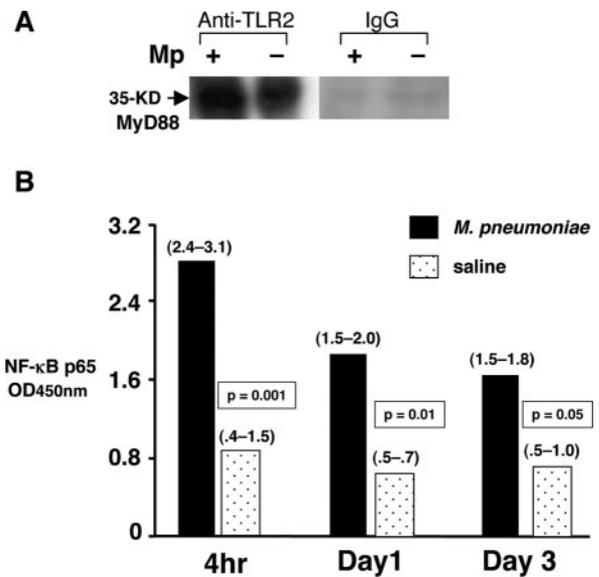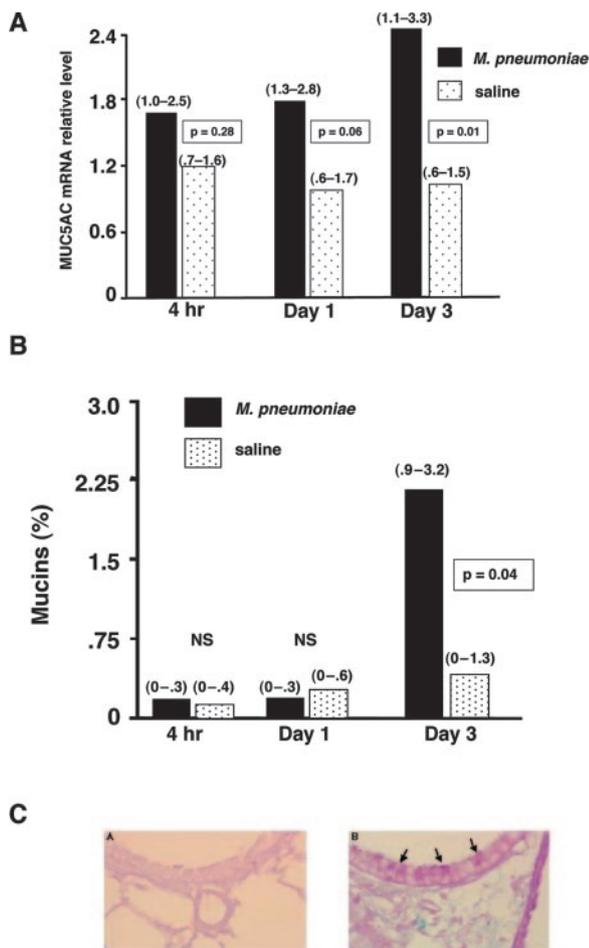

FIGURE 2. *A,* Mp infection increases recruitment of adaptor protein MyD88 to TLR2. Lung tissue lysates (400 μg) from BALB/c mice after 24 h of Mp infection (Mp +) or saline inoculation (Mp −) were immunoprecipitated with a goat anti-mouse TLR2 Ab or goat IgG control Ab. Coprecipitating MyD88 was detected using Western blot analysis. *B,* NF-κB p65 activation levels were measured in BALB/c mouse lung tissue nuclear extracts by an ELISA-based NF-κB p65 assay kit. OD value at 450 nm was used to represent the NF-κB p65 activation levels. $n = 6–8$ mice per group.

data demonstrate that Mp infection can rapidly activate TLR2 signaling in the lung.

Respiratory Mp infection in wild-type BALB/c mice increases airway mucin expression

To determine whether Mp-induced TLR2 signaling activation in BALB/c mice is accompanied by airway mucin expression, lung tissues from infected and control mice were examined for mucin expression at both mRNA and protein levels.

As shown in Fig. 3A, as compared with saline control, Mp infection increased mucin MUC5AC mRNA expression starting on day 1 and being the highest on day 3 with the three examined time points. However, MUC5AC mRNA levels were not increased at 4 h postinfection. To examine whether an up-regulated mucin gene expression by Mp would result in an increase of mucin protein, general mucin staining was performed on lung tissues. As shown in Fig. 3, B and C, airway mucin protein levels were increased on day 3, but not at 4 h and on day 1 after the infection. Within the infected mice, mucin protein, but not MUC5AC mRNA levels,
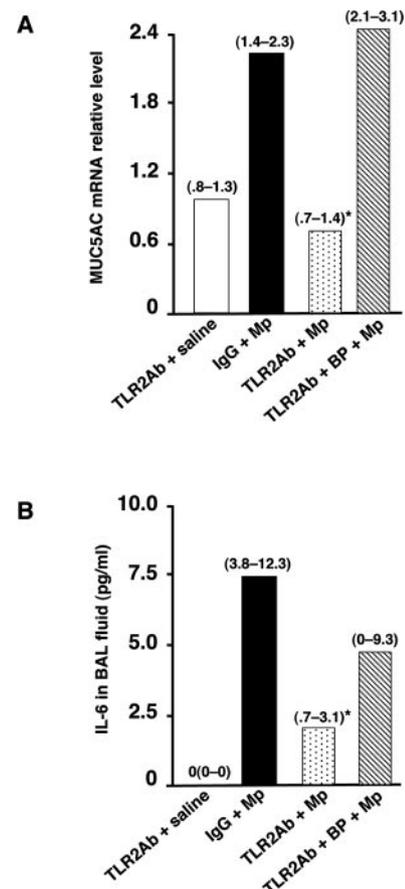
were significantly higher ($p < 0.05$) on day 3 than at 4 h and on day 1 postinfection.

These results suggest that respiratory Mp infection increases airway mucin expression at both transcriptional and translational levels. An increase of TLR2 activation appears to precede mucin mRNA and then the protein expression.

TLR2-neutralizing Ab blocks Mp-induced airway mucin expression in wild-type BALB/c mice

As shown in Fig. 4A, a TLR2Ab significantly ($p = 0.02$) reduced Mp-induced lung MUC5AC mRNA expression to the control level (TLR2Ab + saline). TLR2Ab pretreated with a specific TLR2 BP failed to reduce Mp-induced MUC5AC mRNA expression. Consistent with the MUC5AC mRNA data, mucin protein levels in airway epithelium were also significantly decreased (data not shown).

To further investigate the effects of TLR2Ab on TLR2 signaling pathways, proinflammatory cytokine IL-6 protein was measured in bronchoalveolar lavage fluid because IL-6 has been shown to be one of the cytokines up-regulated by the activation of TLR2 signaling (16). Similar to MUC5AC mRNA data, TLR2Ab also significantly ($p = 0.005$) decreased Mp-induced IL-6 protein levels (Fig. 4B).

FIGURE 3. Mucin expression in wild-type BALB/c mouse lung tissue. A, Mucin MUC5AC mRNA expression was examined by quantitative real-time RT-PCR and expressed as relative level. Data expressed as medians (25–75% range). $n = 6$–8 mice per group. B, General mucin protein expression in the medium-sized airway epithelium. The morphometric results were expressed as a percentage of airway epithelium occupied by mucin protein. Data expressed as medians (25–75% range). $n = 6$–8 mice per group. C, Representative photomicrographs of airway general mucin protein Alcian Blue/periodic acid Schiff staining. As compared with the saline control (A), Mp infection (B) increased airway epithelial mucin (arrows) on day 3 postinfection. Original magnification, ×400

FIGURE 4. An anti-mouse TLR2-neutralizing Ab (TLR2Ab) significantly reduced Mp-induced lung tissue mucin MUC5AC mRNA expression (A) and bronchoalveolar lavage fluid IL-6 protein levels (B) on day 3 after infection. Data are expressed as medians (25–75% range). $n = 5$–6 mice per group. BP of TLR2-neutralizing Ab. ∗, $p < 0.05$, TLR2Ab + Mp group vs IgG + Mp and TLR2Ab + BP + Mp groups in A. ∗, $p < 0.05$, TLR2Ab + Mp group vs IgG + Mp group in B.
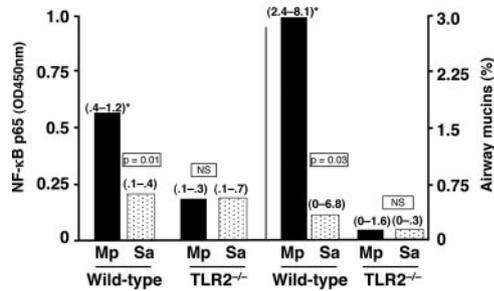
FIGURE 5. In wild-type C57BL/6 mice, Mp infection increased NF-κB p65 activity and airway mucin protein levels on day 3 postinfection. However, in TLR2$^{-/-}$ mice, Mp infection failed to increase NF-κB p65 activity and airway mucin protein levels on day 3 postinfection. n = 5–6 mice per group. Sa, Saline.

Lack of Mp-induced airway mucin expression in TLR2$^{-/-}$ mice

In wild-type C57BL/6 mice, levels of lung TLR2 mRNA expression and NF-κB activity, but not mucin expression (protein or mRNA), were increased on day 1 in the infected group as compared with the noninfected saline control group. Interestingly, on day 3 after the infection, TLR2 mRNA expression levels were similar between the two groups, but lung NF-κB activity along with airway mucin protein levels were significantly increased in infected mice (Fig. 5). These results suggest that, like BALB/c mice, wild-type C57BL/6 mice also demonstrated activation of TLR2 signaling and an increase of mucin expression after Mp infection.

In TLR2$^{-/-}$ mice, Mp infection did not increase NF-κB activity and mucin protein expression as compared with saline treatment on both day 1 (data no shown) and day 3 (Fig. 5), further demonstrating an essential role of TLR2 signaling in Mp-induced NF-κB activation and mucin expression. When TLR2$^{-/-}$ and wild-type C57BL/6 mice were compared, Mp infection also failed to induce NF-κB activity and airway mucin expression (p = 0.01, Fig. 5). Collectively, these data demonstrated that TLR2 signaling is crucial for Mp-induced airway mucin expression.

Mp directly induces human lung epithelial mucin expression through activating TLR2 signaling

First, we observed that a 48-h Mp infection of A549 cells upregulated a 2-fold mucin MUC5AC protein expression as compared with noninfected cells (37 vs 18%, Fig. 6). In addition, a 3-fold increase of MUC5AC mRNA expression was also found after Mp infection.

Next, transient transfection of Mp-infected A549 cells with a TLR2 dominant-negative mutant was shown to reduce NF-κB luciferase activity by 2.8-fold as compared with Mp-infected cells
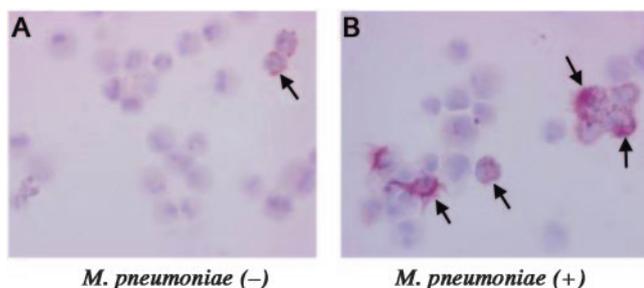
FIGURE 6. Representative photomicrographs of mucin MUC5AC protein immunostaining in A549 cells that were incubated for 48 h in the absence (*A*) or presence (*B*) of Mp. Original magnification, ×400
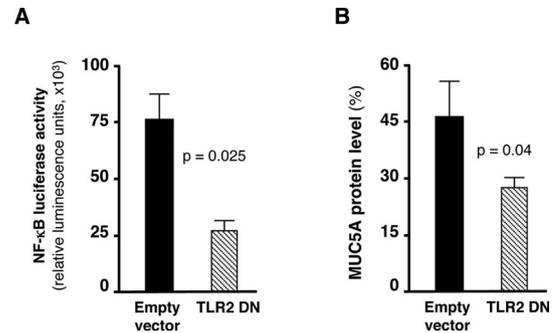
FIGURE 7. Transient transfection of a human TLR2 dominant-negative mutant significantly reduced the NF-κB luciferease activity (*A*) and mucin MUC5AC protein expression (*B*) by Mp-infected A549 lung epithelial cells. The results shown are from three different experiments.

transfected with an empty vector (Fig. 7*A*). Consistent with NF-κB luciferase activity, MUC5AC protein levels in A549 cells transfected with the TLR2 dominant-negative also decreased as compared with those in cells transfected with the empty vector (Fig. 7*B*). These data suggest that TLR2 signaling was essential in Mp-induced epithelial mucin expression and that the NF-κB pathway may be involved in mucin expression. The role of NF-κB in mucin expression was further confirmed by a 6.3-fold reduction of MUC5AC mRNA expression in Mp infected A549 cells that were pretreated with a NF-κB inhibitor (caffeic acid phenethyl ester; 10 μM) 2 h before the infection. Taken together, our cell culture studies suggest that Mp infection directly activates NF-κB and subsequently increases epithelial mucin expression in a TLR2 dependent manner.

Mp-derived lipoproteins directly increase human lung epithelial mucin expression

To determine the direct effects of purified Mp TLR2 ligands on epithelial cell mucin expression, Mp-derived lipoproteins were used to stimulate A549 lung epithelial cells. Mucin MUC5AC protein was increased by Mp-derived lipoproteins in a dose-dependent manner (Fig. 8).

Discussion

Our current study demonstrated that respiratory Mp infection in mice induced airway epithelial mucin expression, which is primarily through the innate immune mechanisms characterized by the
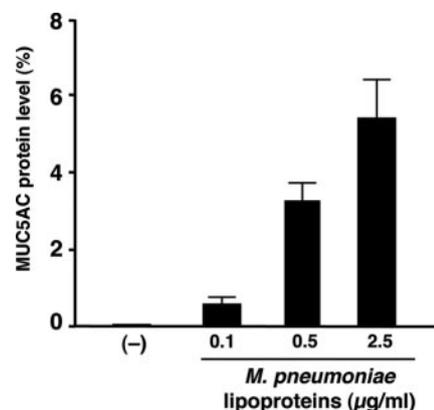
FIGURE 8. A 48-h stimulation of A549 lung epithelial cells with Mp-derived lipoproteins increased mucin MUC5AC protein expression. The results shown are from two different experiments.

activation of TLR2 signaling. Blockade of TLR2 signaling resulted in a significant decrease of Mp-induced mucin expression.

Airway epithelial cells serve as one of the key components in host innate immune response against invading microorganisms. At least 10 TLRs have been described. Airway epithelial cells virtually express all TLRs. Interestingly, a recent study suggests that TLR2, but not TLR4, is expressed at the apical side of airway epithelial cells (21). This specialized localization of TLR2 highlights an important role of TLR2 in mucosal defense against invading pathogens. Our current study is the first in vivo study to demonstrate that live intact Mp activates the TLR2 signaling in mouse lung tissues, especially airway epithelium, which is supported by the following findings. First, both TLR2 mRNA and protein increased after Mp infection. Second, the recruitment of adaptor protein MyD88 to TLR2 appeared to be increased. Finally, our data strongly suggest that NF-κB activity was significantly increased after Mp infection. Moreover, our in vitro lung epithelial culture experiments demonstrated that Mp directly activated TLR2 signaling.

The effects of TLR2 activation on airway mucin expression are poorly studied. A recent study has shown that *Haemophilus influenzae* up-regulates mucin MUC2 transcription in human airway epithelial cells through NF-κB activation (22). NF-κB activation alone in epithelial cells can bind to the promoters of mucin genes and directly increase mucin gene transcription. Overexpression of a human TLR2 dominant-negative mutant inhibited MUC2 induction by *H. influenzae* (22), supporting a critical role of TLR2 signaling in mucin expression in vitro. Our study is the first to provide convincing in vivo evidence for a pivotal role of TLR2 signaling in airway mucin expression in response to Mp infection. In our mouse models, Mp increases airway mucin expression at both mRNA and protein levels on days 1 and 3 postinfection, which was preceded by a significant increase in TLR2 expression and NF-κB activation. These results indicate a potential causal role of TLR2 signaling in airway mucin expression. Indeed, such a causal role of TLR2 signaling in mucin expression was supported by the following findings in the present study: 1) a TLR2-neutralizing Ab significantly reduced airway mucin expression induced by a respiratory Mp infection; 2) Mp infection in TLR2-deficient mice failed to induce airway mucin expression; and 3) Mp infection in vitro directly increased lung epithelial mucin expression, which was attenuated by the overexpression of a TLR2 dominant-negative mutant. Our results suggest that innate immune response initiated by TLR2 binding to its ligands (e.g., Mp) could contribute to airway mucin expression in respiratory diseases characterized by lung bacterial infection with airway mucin overexpression. These lung diseases may include, but not limited to, asthma and COPD in which airway mucin overexpression and infection (e.g., Mp) have been strongly proposed to contribute to the disease process (3, 23). Studies are on the way in our laboratory to determine the role of TLR2 signaling in animal models of asthma and COPD including emphysema.

Although we have examined the role of TLR2 signaling in mucin expression both in vivo and in vitro, the contribution of recruited lung inflammatory cells to TLR2 activation and consequent increase in mucin expression has not been specifically addressed in this current study. It is possible that recruited inflammatory cells may also indirectly increase epithelial mucin expression through the production of inflammatory mediators. For example, neutrophils, one of the predominant types of inflammatory cells recruited to the lung after Mp infection in our studies, are able to produce several inflammatory mediators (e.g., TNF-α, TGF-α, and elastase) that are involved in mucin up-regulation (24). Therefore, in vivo airway mucin expression may be regulated under a variety of

complex mechanisms. Future studies are warranted to address the relative contribution of lung resident (i.e., airway epithelial cells) and inflammatory cells (i.e., neutrophils) to Mp-induced airway mucin expression.

Findings from our current study have broad implications in the discovery of novel therapeutic approaches to preventing or curing airway mucin expression or goblet cell hyperplasia associated with chronic lung diseases (e.g., asthma, COPD, and cystic fibrosis) that inflict millions of people worldwide. Although some of the downstream intracellular molecules of TLR2 signaling pathway may also be the therapeutic target for Mp-induced airway mucin expression, those molecules are relatively nonspecific to TLR2 ligands. Furthermore, targeting these molecules needs highly cell permeable compounds. Therefore, targeting TLR2 might be the utmost approach to block Mp-induced airway mucin expression.

In conclusion, our present study highlights that innate immune recognition of Mp by TLR2 is a critical step governing the airway mucin expression. Unraveling the mechanisms by which innate immunity regulates mucin expression will significantly improve our understanding of mucin regulatory mechanisms and will help to develop novel therapeutic strategies to control several devastating lung diseases in humans.

Disclosures

The authors have no financial conflict of interest.

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