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A deficient TLR2 signaling promotes airway mucin production in *Mycoplasma pneumoniae*-infected allergic mice

Qun Wu, Richard J. Martin, John G. Rino, Samithamby Jeyaseelan, Rachel Breed, and Hong Wei Chu

Department of Medicine, National Jewish Medical and Research Center, Denver, Colorado

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Wu Q, Martin RJ, Rino JG, Jeyaseelan S, Breed R, Chu HW. A deficient TLR2 signaling promotes airway mucin production in *Mycoplasma pneumoniae*-infected allergic mice. *Am J Physiol Lung Cell Mol Physiol* 292: L1064–L1072, 2007. First published December 28, 2006; doi:10.1152/ajplung.00301.2006.—The original hygiene hypothesis suggests that early childhood respiratory infections preceding allergen exposure may decrease the prevalence of allergic diseases. We have recently demonstrated that *Mycoplasma pneumoniae* infection preceding allergen exposure reduced allergic responses in mice. However, the molecular mechanisms underlying the protective role of *M. pneumoniae* in allergic responses, particularly airway mucin production, remain unclear. Wild-type and Toll-like receptor 2 (TLR2)-deficient mice with a respiratory *M. pneumoniae* infection preceding allergen (ovalbumin) challenge were utilized to determine the regulatory role of TLR2-IFN- γ signaling pathway in airway mucin expression. Furthermore, air-liquid interface cultures of mouse primary tracheal epithelial cells were performed to examine the effects of IFN- γ on mucin expression. In wild-type mice, *M. pneumoniae* infection preceding allergen challenge significantly reduced airway mucins but increased IFN- γ . In sharp contrast, in TLR2-deficient mice, *M. pneumoniae* preceding allergen challenge resulted in increased mucin protein without a noticeable change of IFN- γ . In cultured mouse primary tracheal epithelial cells, IFN- γ was shown to directly inhibit mucin expression in a dose-dependent manner. Our study demonstrates for the first time that a respiratory *M. pneumoniae* infection preceding allergen challenge reduces airway epithelial mucin expression in part through TLR2-IFN- γ signaling pathway. A bacterial infection in asthmatic subjects with weakened TLR2-IFN- γ signaling may result in an exaggerated airway mucin production.

asthma; mucus; interferon- γ ; Toll-like receptor 2

AIRWAY INFECTIONS have been shown to modulate allergic responses in human asthmatics and animals. Both pro- and anti-allergic responses have been reported in allergic hosts with an infection depending on the timing of an infection relative to allergen exposure (3, 4, 10, 18). The original hygiene hypothesis suggests that early childhood respiratory infections preceding allergen exposure may decrease the prevalence of allergic diseases. Recent research efforts from various groups have focused on a protective role of infectious pathogens, especially noninfectious components derived from pathogens in asthma (2). Previous studies in animal models have shown that viral infection or bacterial DNA (e.g., CpG) could ameliorate allergic responses such as airway mucus production and eosinophilic inflammation (8, 28, 38). Our human studies have identified airway *Mycoplasma pneumoniae* (Mp) as a potential contributor to chronic asthma (26). However, it appears that timing of Mp infection and allergen exposure is critical. Our

recent experiments in mouse models suggested that Mp infection preceding allergen challenges reduced allergic responses. In contrast, if the infection occurred after allergen challenges, allergic responses were enhanced (10). Presently, the molecular and immunologic mechanisms underlying the protective role of Mp in allergic responses remain unclear.

Airway mucus overproduction is a prominent feature of asthma pathology and could be a significant factor in asthma exacerbations or even death (1). The main component of mucus secretion is mucins synthesized by the mucosal epithelial cells. Among 21 identified mucins, MUC5AC appears to be the predominant secreted gel-forming mucin in airways of asthmatics as well as normal subjects (27). Airway epithelial cells provide both a barrier to infections and an active defense mechanism against invading microbes. Toll-like receptors (TLRs) are pattern-recognition receptors expressed on many cell types, such as airway epithelial cells, to recognize microorganisms and subsequently induce signals responsible for the activation of genes involved in effective host immunity, especially those of proinflammatory cytokines (e.g., IFN- γ , TNF- α , IL-6) (20). The role of TLR2 signaling in asthma or allergic inflammation remains unclear. Several allergic disease-related single nucleotide polymorphisms in TLR2 coding regions have been identified in human subjects and have been linked to a diminished cell response to TLR2 agonists. Interestingly, farmers' children carrying the TLR2 – 16934A allele were shown to have three times higher morbidity from asthma than those carrying the TLR2 – 16934T allele (6, 14). These epidemiologic and genetic studies suggest that an abnormal TLR2 signaling may contribute to asthma pathogenesis. In previous studies in our laboratory (11), TLR2 signaling pathway was shown to be critical for Mp-induced airway mucin expression in allergen-naïve mice. It also has been reported that TLR2 signaling pathway contributes to IFN- γ production (15). IFN- γ , a Th1-type cytokine involved in airway inflammation, plays an important role in cellular immunity against intracellular microbes (i.e., mycobacteria) (5, 15, 23, 34, 35). Delivery of IFN- γ to the airways of allergic mice could prevent airway hyperresponsiveness, reduce mucous goblet cell metaplasia, and downregulate Th2-type inflammation including Th2 cytokine production and airway eosinophilia (17, 22, 24, 33). IFN- γ also has been shown to reduce transforming growth factor (TGF)- β 2 production in cultured normal human bronchial epithelial cells (36). Moreover, our group has found that TGF- β 2 directly induced mucin expression in cultured human primary airway epithelial cells (9). However, the exact role of TLR2 activation and subsequent IFN- γ and TGF- β 2 produc-

Address for reprint requests and other correspondence: H. W. Chu, Dept. of Medicine, National Jewish Medical and Research Center, 1400 Jackson St., Rm. D104, Denver, CO 80206 (e-mail: chuww@njc.org).

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tion in the interplay of respiratory infection and allergic inflammation to regulate airway mucin expression in allergic hosts has not been well studied.

Our research question was, does a deficient TLR2 signaling contribute to airway mucin overproduction in allergic hosts with an infection? To address this question, we utilized a mouse model in which *M. pneumoniae* infection preceded the allergen challenge, because this model has implications in attenuating the allergic responses. Furthermore, TLR2-deficient mice were utilized to examine the role of TLR2 activation and IFN- γ production in airway mucin expression. Our results indicated that 1) in allergen-naïve wild-type mice, lung IFN- γ was upregulated by Mp, which disappeared in TLR2^{-/-} mice; 2) in allergen-challenged wild-type mice, Mp preceding allergen challenges significantly reduced airway mucins coupling with increased IFN- γ production and decreased TGF- β 2 production; 3) in contrast to wild-type mice, Mp infection preceding allergen challenges in TLR2^{-/-} mice failed to reduce airway mucins but instead increased mucin protein in airways and presented an enhanced TGF- β 2 production; and 4) in air-liquid interface cultures of mouse primary tracheal epithelial cells, IFN- γ was shown to directly inhibit mucin expression and TGF- β 2 secretion in a dose-dependent manner.

MATERIALS AND METHODS

Mice

All experimental animals used in this study were covered by a protocol approved by our Institutional Animal Care and Use Committee. Female wild-type C57BL/6 mice (TLR2^{+/+}, 8–10 wk old) were obtained from Harlan Sprague Dawley (Indianapolis, IN). TLR2 gene-deficient (TLR2^{-/-}) mice were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan). These mice were inbred from 129/SV $\times$ C57BL/6 and backcrossed with C57BL/6 mice for eight generations (39). All the mice were quarantined for 4 wk before the experiments and bled to establish that they were virus and *M. pulmonis* free.

Mp Preparation

Mp (strain FH, ATCC 15531) was prepared in sterile saline to yield $\sim 1 \times 10^8$ colony-forming units (cfu)/50 μ l.

Animal Models

Mp model with allergen exposure. Wild-type or TLR2^{-/-} mice were intraperitoneally sensitized twice in a span of 14 days by injection of 20 μ g of ovalbumin (OVA; Sigma, St. Louis, MO) emulsified in 2.25 mg of aluminum hydroxide (Imject Alum; Pierce, Rockford, IL). Twelve days after the last OVA sensitization, mice were inoculated intranasally with 50 μ l of Mp at 1×10^8 cfu or 50 μ l of saline (control). Two days after Mp or saline treatment, mice were

placed in a Plexiglas chamber and challenged with 1% aerosolized OVA for 20 min using the ultrasonic nebulizer (De Vilbiss, Heston, UK). The OVA challenges were given once daily for 3 consecutive days. Mice were killed 5 days after the last OVA challenge to examine mucin, IFN- γ , and TGF- β 2 expression (Fig. 1).

To examine a role of IFN- γ in mucin production, we performed an additional experiment by treating allergic mice with or without exogenous recombinant mouse IFN- γ protein (R&D Systems, Minneapolis, MN) before the OVA challenges. The dose and duration of IFN- γ administration were chosen on the basis of results obtained from experiments in Fig. 1. and the previous study (33). OVA-sensitized wild-type mice were intranasally instilled with 50 μ l of saline or 100 ng of IFN- γ in saline once daily on days 0–2. OVA-sensitized TLR2^{-/-} mice were similarly inoculated intranasally with Mp (10^8 cfu/mouse) or Mp (10^8 cfu/mouse) combined with 100 ng of IFN- γ in saline on day 0 and were then repeatedly instilled with 50 μ l of saline (for Mp group) or 100 ng of IFN- γ in saline (for Mp + IFN- γ group) once daily on days 1 and 2. On day 2, the saline/IFN- γ treatment was given 2 h before the OVA challenge. Mice were killed 5 days after the last OVA challenge and examined for mucin expression.

Mp model without allergen exposure. Allergen-naïve wild-type and TLR2^{-/-} mice were inoculated intranasally with 50 μ l of Mp at 1×10^8 cfu or 50 μ l of saline (control) and killed on days 1, 3, and 9 after treatment for examination of IFN- γ production and Mp load in lung tissues.

Bronchoalveolar Lavage and Lung Tissue Processing

The lung was lavaged with 1 ml of saline. Cell-free bronchoalveolar lavage (BAL) fluid were stored at -80°C for cytokine analysis. The left lung lobe was fixed in 4% paraformaldehyde, embedded in paraffin, and cut at 4- μ m thickness for mucin Alcian blue/periodic acid-Schiff (AB/PAS) staining. Ten microliters of minced lung tissue from the right middle lung lobe (total size of $3 \times 3 \times 3$ mm) were placed onto mycoplasma pleuropneumonia-like organism (PPLO) culture plates (Remel, Lenexa, KS) and incubated at 37°C , 5% CO_2 for 7 days when colony-forming units were counted. The remaining tissue was used for total RNA extraction.

Mucin AB/PAS Staining and Quantification in Airways

The general mucins in lung tissues were identified by AB/PAS staining. Medium-sized airways, defined by an airway epithelial basement membrane perimeter of 600–900 μ m (maximal diameter/minimum diameter ≤ 2), were examined for airway mucins. The area of mucins in airway epithelium was measured using a NIH Scion Image program (National Institutes of Health, Bethesda, MD). At least five complete airways per mouse were examined. The results were expressed as airway mucin area/total airway epithelium area (percentage) (11).

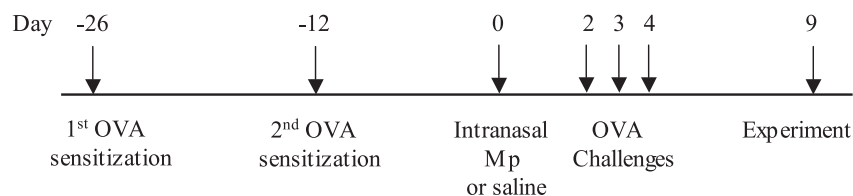


Fig. 1. Ovalbumin (OVA) sensitization, *Mycoplasma pneumoniae* (Mp) infection, and OVA challenge protocol. Wild-type (WT) or Toll-like receptor 2-deficient (TLR2^{-/-}) mice were sensitized twice in a span of 14 days by intraperitoneal injection of 20 μ g of OVA emulsified in aluminum hydroxide. After 12 days, mice were inoculated intranasally with 50 μ l of Mp at 1×10^8 colony-forming units (cfu) or saline. After 2 additional days, mice were challenged with 1% aerosolized OVA once daily for 3 consecutive days. After 5 days of last OVA challenge, mice were killed to examine mucin, IFN- γ , and transforming growth factor (TGF)- β 2 expression. $n = 6$ –8/group.

Mouse Primary Tracheal Epithelial Cell Air-Liquid Interface Culture

Air-liquid interface (ALI) cultures of mouse primary tracheal epithelial cells were performed to study the direct interactions among IFN- γ , MUC5AC, and TGF- β 2, thus extending our *in vivo* findings. All the materials used in mouse tracheal epithelial cell cultures were obtained from Sigma unless specifically indicated. Tracheas from naive C57BL/6 mice were cut longitudinally and digested with ice-cold DMEM (GIBCO, Grand Island, NY) supplemented with 0.1% protease solution and amphotericin (50 μ g/ml) at 4°C for 6 h. The released cells from tracheas were plated on a collagen-coated 12-well transwell plates (Corning Costar, Corning, NY) at $\sim 4 \times 10^4$ cells in 500 μ l of DMEM/BEBM (1:1) supplemented with insulin (0.4 μ g/ml), transferrin (5 μ g/ml), hydrocortisone (0.5 μ g/ml), epinephrine (0.5 μ g/ml), bovine hypothalamus extract (52 μ g/ml), gentamicin/amphotericin (50 mg/ml and 50 μ g/ml) from BEGM SingleQuots (Cambrex BioScience, Walkersville, MD); bovine serum albumin (0.5 μ g/ml), ethanolamine (50 mM), MgCl₂ (3.0 mM), MgSO₄ (4.0 mM), CaCl₂ (1.0 mM), retinoic acid (30 ng/ml), mouse epidermal growth factor (15 ng/ml; BD Biosciences, San Jose, CA). One and one-half milliliters of complete culture medium were added to the basolateral side of transwells. These cells were transferred to an ALI culture after 7 days in an immersed cultured condition. Culture media were refreshed every other day for 7 additional days to induce epithelial mucociliary differentiation. Cells were then treated with PBS or IFN- γ (0.1, 1, and 10 ng/ml; R&D Systems) for 9 days or culture media for 4 days, followed by TGF- β 2 (0.01, 0.1, and 1 ng/ml; R&D Systems) for 5 days to match the exposure duration after Mp in our animal model (Fig. 2). Cytokines or PBS was added to both apical and basolateral sides. At the end of culture, apical supernatants (brought up to 200 μ l in PBS) were harvested for MUC5AC dot-blot assay, basolateral supernatants were saved for TGF- β 2 protein measurement, and cells were lysed for RNA extraction.

Dot Blot for MUC5AC Protein in Mouse Primary Tracheal Epithelial Cells

We utilized dot-blot analysis to measure the released levels of MUC5AC protein in apical supernatants as previously reported (9). Fifty microliters of each sample were loaded into a Bio-Dot micro-filtration apparatus (Bio-Rad Laboratories, Hercules, CA) in duplicate and blotted to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in Tris buffer (pH 7.6) with 0.1% Tween 20 and then incubated with anti-MUC5AC (45M1) monoclonal mouse anti-human antibody (Lab Vision, Fremont, CA) overnight at 4°C. After washes, the membrane was incubated with anti-mouse IgG conjugated with a horseradish peroxidase and detected using the ECL Western blotting detection reagents (Amersham Biosciences, Amersham, UK). The film was scanned, and the intensity of the MUC5AC protein signal was measured by densitometry to represent the protein secretion levels.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed as previously described (10). Briefly, total RNA of lung tissue or cell lysates was

extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Ambion, Austin, TX). Reverse transcription was performed using 1 μ g of total RNA and random hexamers in a 50- μ l reaction according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The mouse MUC5AC (GenBank accession no. L42292) and TGF- β 2 (GenBank accession no. NM_009367) primers and probe were designed using Primer Express software (Applied Biosystems) and listed as follows: MUC5AC primers and probe: forward primer, 5'-AGAGGAGCGGAGAGAGACTCTGT-3'; reverse primer, 5'-CTCCATCTCTCTCTCAGGGTAGTTCT-3'; probe, 5'-CGAGAGGAGATCACACAGTTGCAATGCA-3'; and TGF- β 2 primers and probe: forward primer, 5'-CCACCTCCCCTCCGAAAA-3'; reverse primer, 5'-AGACATCAAAGCGGACGAT-TCT-3'; probe, 5'-CATCCCGCCCACTTTCTACAGACCCTAC-3'. Real-time PCR was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems). The 50- μ l PCR contained 30 ng of cDNA, 100 nM probe, 100 nM primers, and other components from the TaqMan RT-PCR kit. Housekeeping gene 18S rRNA was evaluated as internal control. 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 interest of genes.

ELISA

The antibodies for a sandwich ELISA of IFN- γ and TGF- β 2 were purchased from R&D Systems (Minneapolis, MN). The detection limit is 2 pg/ml.

Statistical Analysis

Normally distributed data are presented as means $\pm$ SE and were compared using Student's *t*-test between the relevant groups. Non-normally distributed data are expressed as medians with interquartile (25–75%) ranges and were compared using the Wilcoxon rank-sum test. A value of *P* < 0.05 was considered significant.

RESULTS

Mp Suppresses Mucin Expression in Allergic Wild-Type Mice But Not TLR2^{-/-} Mice

We examined the general mucin protein and specific MUC5AC mRNA expression in airway tissues. In wild-type mice, Mp infection preceding the OVA challenges significantly reduced airway mucin protein production (*P* < 0.05) as well as MUC5AC transcripts (*P* < 0.05) compared with saline-treated allergic wild-type mice (Fig. 3, A–D). In sharp contrast, in Mp-infected allergic TLR2^{-/-} mice, airway epithelial mucin protein levels were significantly increased compared with those in saline-treated allergic TLR2^{-/-} mice as well as Mp-infected allergic wild-type mice (Fig. 3, A, E, and F). However, Mp preceding the OVA challenge did not increase the levels of MUC5AC mRNA in allergic TLR2^{-/-} mice (Fig. 3B).

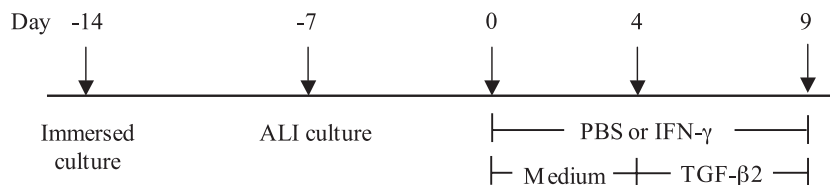


Fig. 2. Experimental timeline for mouse primary tracheal epithelial cell air-liquid interface (ALI) culture. Tracheal epithelial cells of naive C57BL/6 WT mice were transferred to ALI culture after 7 days in an immersed cultured condition. After 7 additional days to induce epithelial mucociliary differentiation, cells were treated as indicated. At the end of the culture, apical supernatants were harvested for MUC5AC dot-blot assay, basolateral supernatants were saved for TGF- β 2 protein measurement, and cells were lysed for RNA extraction.

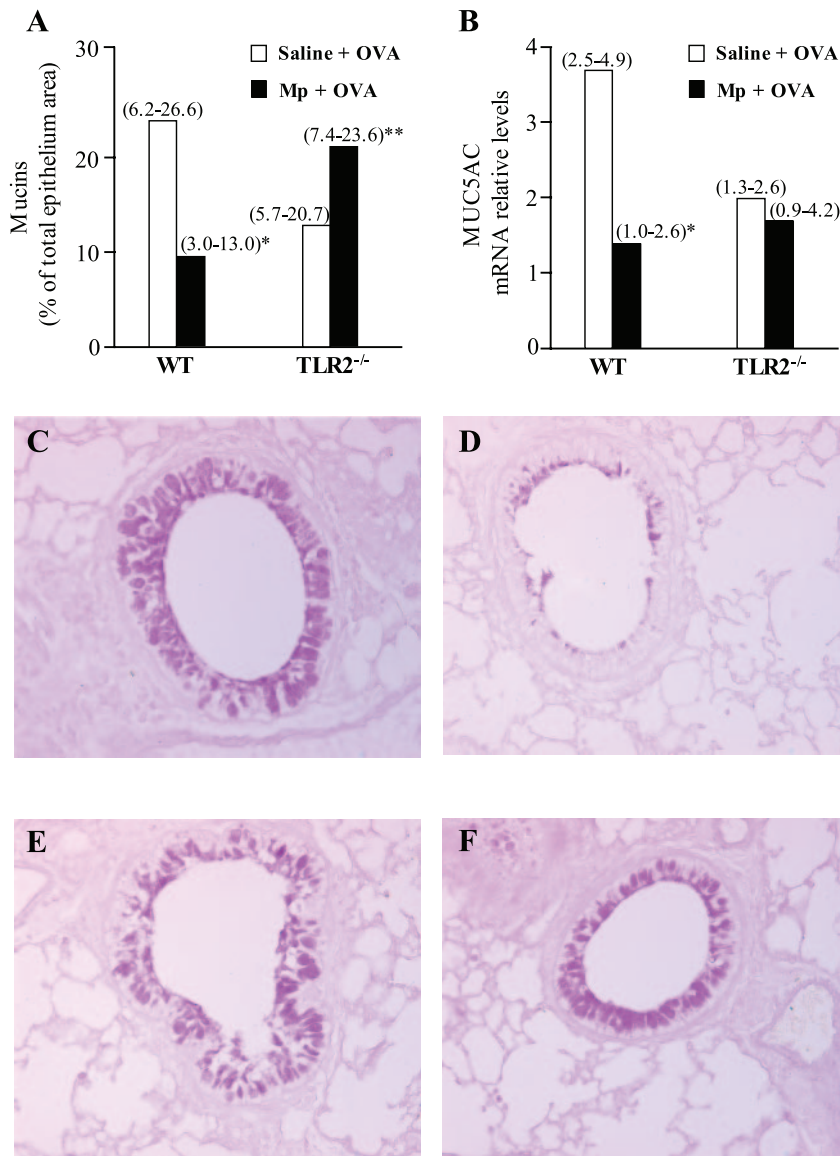


Fig. 3. Lung mucin expression in allergic WT and TLR2^{-/-} mice. **A:** general mucins in medium-sized airways were identified by mucin Alcian blue/periodic acid-Schiff (AB/PAS) staining and expressed as airway mucin area per total airway epithelium area (percentage). **B:** quantitative real-time RT-PCR for lung tissue MUC5AC mRNA levels. Data are expressed as medians (25–75% range); $n = 6–8$ /group. * $P < 0.05$ compared with saline-treated WT mice. ** $P < 0.05$ compared with Mp-treated WT mice. **C–F:** representative microphotographs of airway mucins (deep purple) in lung tissues (×200 magnification): saline-treated WT mice (**C**), Mp-infected WT mice (**D**), saline-treated TLR2^{-/-} mice (**E**), and Mp-infected TLR2^{-/-} mice (**F**).

Mp-Induced Upregulation of IFN- γ is Dependent on TLR2 Signaling

Allergen-naïve wild-type and TLR2^{-/-} mice were inoculated with saline (control) or Mp (10^8 cfu/mouse) and killed on days 1, 3, and 9 after treatment. As shown in Fig. 4A, Mp increased lung IFN- γ production in a TLR2-dependent manner. Mp post day 1 significantly increased IFN- γ protein levels in BAL fluid of wild-type mice (11-fold) but not TLR2^{-/-} mice (<2-fold, $P < 0.05$). IFN- γ protein levels were very low in wild-type mice on day 3 and undetectable in TLR2^{-/-} mice on day 3 as well as in any Mp-infected mice on day 9. Furthermore, lung tissue Mp culture confirmed that TLR2^{-/-} mice had a higher Mp load than wild-type mice on day 1 following Mp infection ($P < 0.05$). Mp load was decreased on day 3 in both TLR2^{-/-} mice and wild-type mice ($P = 0.94$) and was undetectable on day 9 in any Mp-infected mice (Fig. 4B).

Nevertheless, in an allergic mouse model, even after 9 days of Mp infection, IFN- γ protein production was still dependent

on TLR2 signaling. IFN- γ protein levels were significantly increased in Mp-infected allergic wild-type mice compared with saline-treated allergic wild-type mice ($P < 0.05$), which was also much higher than that of Mp-infected allergic TLR2^{-/-} mice ($P < 0.05$). However, Mp inoculation did not increase IFN- γ protein levels in BAL fluid of allergic TLR2^{-/-} mice (Fig. 5A). Moreover, Mp culture confirmed that all infected mice were positive for mycoplasma. Interestingly, allergic TLR2^{-/-} mice demonstrated higher levels of lung tissue Mp load than allergic wild-type mice even after 9 days of Mp infection ($P < 0.05$) (Fig. 5B).

IFN- γ Rescues Mp-Infected TLR2^{-/-} Allergic Mice From Airway Mucin Overproduction

The observation that high IFN- γ levels were coupled with reduced airway mucins in Mp-infected allergic wild-type mice led us to hypothesize that IFN- γ might be crucial to mucin downregulation. To test our hypothesis, we performed an IFN- γ rescue experiment to determine whether a deficient

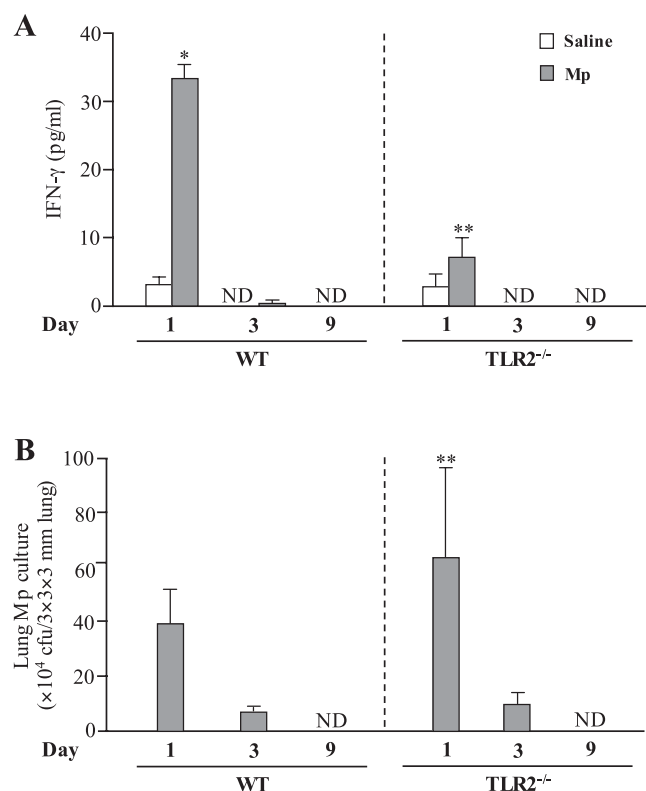


Fig. 4. IFN- γ production and lung Mp load in allergen-naïve mice. A: IFN- γ protein levels in bronchoalveolar lavage (BAL) fluid. B: Mp culture of lung tissues. Data are expressed as means $\pm$ SE; $n = 6-8$ /group. * $P < 0.05$ compared with saline-treated WT mice on day 1. ** $P < 0.05$ compared with Mp-treated WT mice on day 1. ND, not detectable.

IFN- γ production in Mp-infected allergic TLR2^{-/-} mice might be responsible for an increased airway mucin expression. As shown in Fig. 6, compared with saline treatment, instillation of exogenous IFN- γ in allergic wild-type mice without Mp significantly decreased airway mucin protein levels, which were similar to those in Mp-infected allergic wild-type mice. Furthermore, instillation of exogenous IFN- γ in Mp-infected allergic TLR2^{-/-} mice significantly decreased airway mucin protein levels compared with Mp-infected allergic TLR2^{-/-} mice without IFN- γ instillation ($P < 0.05$, Fig. 7).

IFN- γ Directly Inhibits Mucin Production in Cultured Mouse Primary Airway Epithelial Cells

Although our current study and previous studies in mouse allergic asthma models suggest a role of IFN- γ in suppressing airway mucin production (12, 33), such direct in vitro evidence was surprisingly not available. To determine whether IFN- γ had a direct inhibitory effect on mouse airway mucin expression, we performed ALI cultures using allergen-naïve wild-type C57BL/6 mouse primary tracheal epithelial cells.

In our pilot study ($n = 3$ /group), we infected allergen-naïve wild-type and TLR2^{-/-} mouse tracheal epithelial cells with Mp (10 cfu/cell) or PBS (control) for 9 days under ALI culture conditions. Compared with the control, Mp was shown to significantly upregulate (up to 8-fold) MUC5AC mRNA levels in wild-type tracheal epithelial cells but not in those TLR2^{-/-} tracheal epithelial cells. These data were consistent with our

previous in vivo study in allergen-naïve and Mp-infected C57BL/6 mice (11), thus confirming the validity of our mouse tracheal epithelial ALI culture system.

In the present study, we found that after 9 days of ALI culture, mucin MUC5AC mRNA and protein were easily detectable in cultured mouse tracheal epithelial cells even at the baseline (PBS treatment). A 9-day treatment of IFN- γ at doses of 0.1–10 ng/ml did not cause cell death or detachment from the transwell membrane. IFN- γ significantly inhibited MUC5AC transcripts in a dose-response manner ($P < 0.05$, Fig. 8A). Released MUC5AC protein levels from the apical surface also were significantly reduced by IFN- γ at all three doses ($P < 0.05$, Fig. 8B). These data support the suggestion that IFN- γ has a direct inhibitory effect on airway epithelial mucin expression.

IFN- γ Downregulates Mucin Expression Partly By Inhibiting TGF- β 2 Production

To determine the potential downstream target of Mp-induced IFN- γ in downregulating airway mucin expression, we further examined TGF- β 2 protein levels in BAL fluid. Unlike IFN- γ , total (activated and nonactivated) TGF- β 2 protein in BAL fluid of allergic wild-type mice was downregulated (36% decrease) by Mp infection compared with saline treatment. In contrast, in allergic TLR2^{-/-} mice, Mp infection resulted in a 21% increase of TGF- β 2 protein compared with saline treatment, which was significantly higher than that in Mp-treated allergic wild-type mice ($P < 0.05$, Fig. 9A). Although Mp did

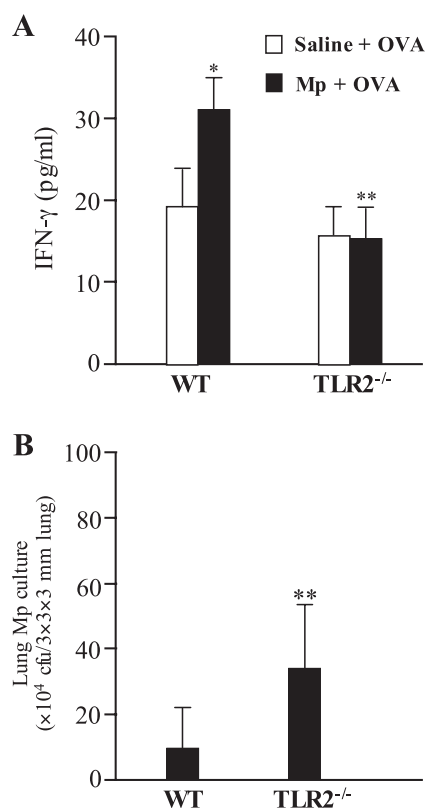


Fig. 5. IFN- γ production and lung Mp load in allergic mice. A: IFN- γ protein levels in BAL fluid. B: Mp culture of lung tissues. Data are expressed as means $\pm$ SE; $n = 6-8$ /group. * $P < 0.05$ compared with saline-treated WT mice. ** $P < 0.05$ compared with Mp-treated WT mice.

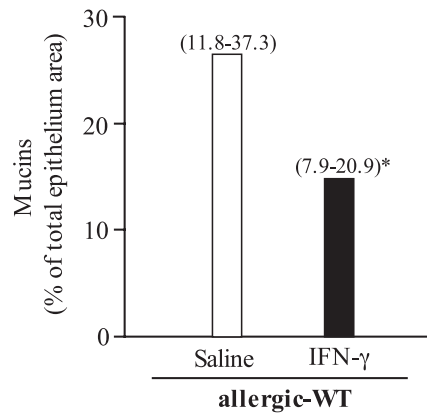


Fig. 6. Intranasal instillation of IFN- γ before OVA challenges in allergic WT mice decreases mucin production. OVA-allergic mouse model was the same as illustrated in Fig. 1. Mice were treated with saline or recombinant mouse IFN- γ protein. General mucins in medium-sized airways were identified by AB/PAS staining and expressed as airway mucin area per total airway epithelium area (percentage). Data are expressed as medians (25–75% range); $n = 6$ –9/group. * $P < 0.05$ compared with saline-treated mice.

not affect lung TGF- β 2 mRNA levels in allergic wild-type mice, it increased lung TGF- β 2 mRNA levels (3.2-fold) in allergic TLR2 $^{-/-}$ mice compared with Mp-infected allergic wild-type mice ($P < 0.05$, Fig. 9B).

Furthermore, in mouse primary tracheal epithelial cell cultures, IFN- γ directly inhibited epithelial TGF- β 2 production in a dose-dependent manner (Fig. 9C). A physiological dose (0.01 ng/ml) of TGF- β 2 marginally increased MUC5AC mRNA (up to 2.1-fold, $P > 0.05$) and protein levels (up to 1.7-fold, $P > 0.05$) compared with PBS control in mouse trachea epithelial cells.

DISCUSSION

Our current study has demonstrated that a respiratory Mp infection preceding allergen challenge reduced airway epithelial mucin expression, which occurred in part through activation of TLR2-IFN- γ signaling pathway in an allergic murine

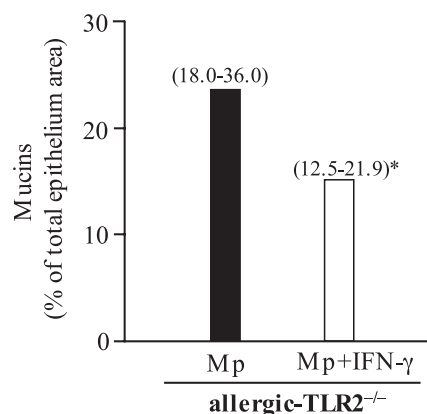


Fig. 7. Intranasal instillation of IFN- γ before OVA challenges rescues Mp-infected allergic TLR2 $^{-/-}$ mice from mucin overproduction. OVA-allergic mouse model was the same as illustrated in Fig. 1. Mice were treated with Mp or Mp combined with recombinant mouse IFN- γ protein. General mucins in medium-sized airways were identified by AB/PAS staining and expressed as airway mucin area per total airway epithelium area (percentage). Data are expressed as medians (25–75% range); $n = 6$ –9/group. * $P < 0.05$ compared with Mp-treated TLR2 $^{-/-}$ mice.

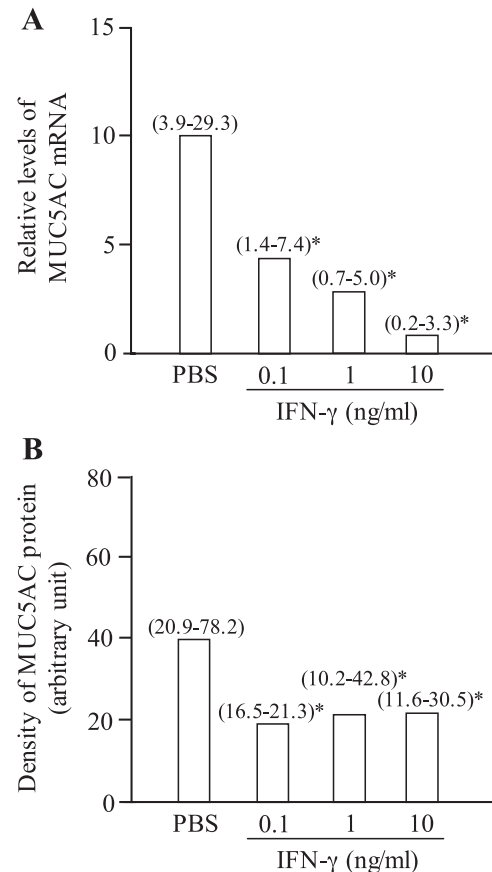


Fig. 8. IFN- γ directly inhibits MUC5AC expression in mouse primary tracheal epithelial cells. A: relative levels of MUC5AC mRNA by quantitative real-time RT-PCR. B: density of MUC5AC protein signal in apical supernatants by dot-blot analysis. Data are from 3 independent experiments performed in triplicate and are expressed as medians (25–75% range). * $P < 0.05$ compared with PBS-treated cells.

model. Mucus hypersecretion is a common pathophysiological manifestation associated with various lung diseases including asthma, chronic obstructive pulmonary diseases (COPD), and cystic fibrosis and also is an important contributor to airway obstruction that leads to mortality. Unraveling the mechanisms underlying this problem is crucial to the development of novel therapeutic approaches. Our previous study demonstrated that respiratory Mp infection increased airway mucin expression in allergen-naïve mice, which was dependent on the activation of TLR2 signaling (11). The present study has extended our previous findings by determining whether TLR2 signaling also may modify airway mucins in an allergic mouse model. Mp infection preceding the OVA challenge strongly reduced airway mucin expression in wild-type (TLR2 sufficient) allergic mice, which supported results from other groups using other TLR2 agonists (e.g., Pam3CSK4) to ameliorate an established airway allergic inflammation (29). In contrast to wild-type mice, Mp infection in TLR2 $^{-/-}$ allergic mice failed to suppress airway mucin but instead increased mucin protein in airway epithelia. Our results suggest that the protective effects of Mp exposure preceding allergen challenges in allergic lungs may rely on TLR2 activation through TLR2 binding to its ligands (e.g., Mp and/or Mp-derived lipoproteins). Superficially, it appears confusing that TLR2 activation could either increase or

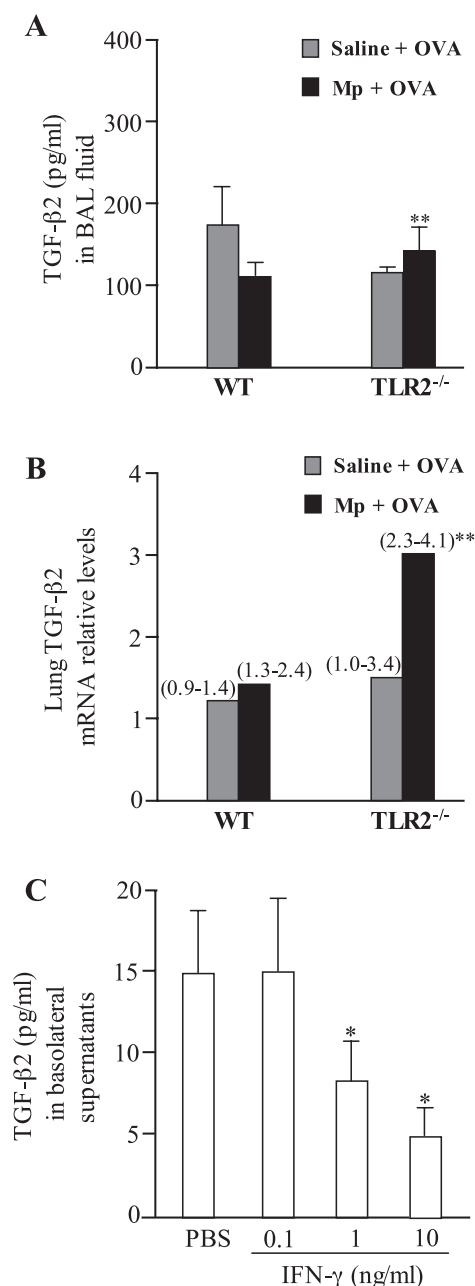


Fig. 9. TGF- β 2 expression in allergic mice and its downregulation by IFN- γ in cultured mouse trachea epithelial cells. A: TGF- β 2 protein levels determined by ELISA in BAL fluid of allergic WT and TLR2^{-/-} mice. Data are expressed as means $\pm$ SE. B: lung tissue TGF- β 2 mRNA relative levels by quantitative real-time RT-PCR. Data are expressed as medians (25–75% range); $n = 6$ –8/group. ** $P < 0.05$ compared with Mp-treated WT mice. C: TGF- β 2 protein levels by ELISA in basolateral supernatants of cultured mouse primary tracheal epithelial cells. Data are from 3 independent experiments performed in triplicate and expressed as medians (25–75% range). * $P < 0.05$ compared with PBS-treated cells.

decrease airway mucin expression. We believe that these two seemingly contradictory stories are unified in the context of nonallergic vs. allergic settings and just reveal the complexity of TLR2 signaling in regulating mucin expression. In our previous study of nonallergic mice, airway mucin induction may have resulted from the direct TLR2 activation by Mp infection in airway epithelium (11). However, in our present

study of allergic mice, OVA challenges markedly induced mucin protein expression, since mucin protein levels in airway epithelium of OVA-challenged mice in the absence or presence of prior Mp infection were much greater than those in OVA-naive Mp-infected wild-type mice. Therefore, a direct role of Mp on airway epithelial TLR2 activation and subsequent mucin regulation in these Mp-infected allergic mice seems minimal, since we found that Mp-induced mucin expression disappeared 7 days postinfection in OVA-naive mice (unpublished data). Thus it appears that interactions between TLR2 activation and allergic responses play a major role in determining the effects of Mp-induced TLR2 activation on mucins in allergic mice.

However, how TLR2 activation regulates mucin expression in an allergic setting remains unclear. Signaling through the TLRs leads to transcription and translation of a variety of cytokines/mediators that may potentially be relevant to mucin regulation (31). It has been reported that primed Th1 cell transfer into the respiratory tract of allergen-challenged mice failed to induce mucins, which was most likely due to the inhibitory effect of IFN- γ (12). So far, the role of TLR2 activation in airway mucin regulation in the context of IFN- γ production has not been well studied. In the present study, our results suggest that TLR2 signaling is critical to the in vivo IFN- γ production in response to Mp in that IFN- γ was induced by Mp during the early phase of infection in allergen-naive wild-type mice but not in TLR2^{-/-} mice. One of the potential mechanisms for a lack of IFN- γ induction by Mp in TLR2^{-/-} mice may involve IL-12. In our preliminary studies, induction of IL-12p70 protein by Mp was significantly diminished in bone marrow-derived dendritic cells from TLR2^{-/-} mice compared with that from wild-type mice (unpublished data). IL-12 has been shown to promote Th1 responses by stimulating activated T cells and NK cells to produce IFN- γ , thereby inhibiting the development of IL-4-producing Th2 cells (25, 32). These results suggest that Mp induces IFN- γ production in part through induction of IL-12 in a TLR2-dependent manner.

Moreover, we demonstrated that Mp inoculation preceding the allergen challenges in wild-type rather than TLR2^{-/-} mice increased lung IFN- γ secretion accompanied by reduction in airway mucins. Instillation of exogenous IFN- γ before the allergen challenges in allergic wild-type mice imitated the role of Mp-induced IFN- γ in downregulation of mucin production. Furthermore, in vivo instillation of exogenous IFN- γ before the allergen challenges was able to rescue Mp-infected allergic TLR2^{-/-} mice from mucin overproduction. Therefore, Mp-induced IFN- γ seems to be the downstream effector molecule of TLR2 activation in attenuating allergen-induced mucin overexpression in allergic lungs. To further elucidate the mechanisms involved in MUC5AC regulation by IFN- γ , we utilized mouse primary tracheal epithelial cells under an ALI culture, the best cell culture system that mimics in vivo epithelial cell biology. We found that IFN- γ treatment directly inhibited mucin expression at both mRNA and protein levels. Although induction of mucous goblet cell apoptosis may be one of the mechanisms by which IFN- γ reduces mucin expression in allergic airways (33), some other underlying molecular mechanisms remain elusive. Interestingly, it has been suggested that TGF- β 1 might be a downstream molecule of TLR2 signaling pathway in *Haemophilus influenza*-induced MUC2 expression by human airway epithelial cells (19). Nonetheless, our previ-

ous human studies have shown a role of TGF- β 2, but not TGF- β 1, in upregulating human airway epithelial mucin MUC5AC expression (9). Moreover, IFN- γ also has been shown to reduce TGF- β 2 production in cultured normal human bronchial epithelial cells (36). In the current study, we first found that IFN- γ and TGF- β 2 in mouse BAL fluid or lungs changed in opposite directions. Next, IFN- γ was shown to inhibit TGF- β 2 production in a dose-dependent fashion in cultured mouse tracheal epithelial cells. Last, we observed that a physiological dose of TGF- β 2 marginally upregulated MUC5AC expression in cultured mouse primary tracheal epithelial cells. Thus it appears that an alternative pathway for IFN- γ to downregulate mucins may be through inhibiting TGF- β 2. Future studies are warranted to further reveal the exact role of TLR2-IFN- γ -TGF- β 2 pathway in airway mucin regulation.

Taken together, our mouse studies have led to the following novel hypothesis: atypical bacterial infection in TLR2-insufficient or -deficient allergic hosts could exacerbate asthma pathology such as mucin overproduction. Our hypothesis is also supported by a human study in which TLR2 expression was shown to be locally impaired in nasal polyposis patients with bacterial or fungal infection (30). Thus mouse and human studies together suggest that TLR2 deficiency could exist in either an allergic setting or genetically predisposed individuals and may contribute to the uncontrolled local airway pathology and microbial burden. Specifically, we speculate that asthmatic patients with *M. pneumoniae* infection may lack or bear low levels of TLR2 expression, which may be insufficient to induce a robust local Th1-type host defense (e.g., IFN- γ) against the infection. Thus maintaining a normal TLR2 level may have therapeutic implication in attenuating airway mucin expression in asthmatics.

Our current study also yields several other interesting observations. First, in both nonallergic and allergic settings, lung Mp load is higher in TLR2 $^{-/-}$ mice than in wild-type mice. On day 9 postinfection, Mp was detectable only in allergen-challenged mice, not in allergen-naïve mice. These results suggest that 1) hosts with a deficient TLR2 signaling have an impaired Mp clearance from the lung, and 2) allergic inflammation itself also delays Mp clearance. A possibility is that allergic inflammation may dampen Mp clearance through downregulating TLR2 signaling activity. Second, in a noninfectious setting, OVA challenges in TLR2 $^{-/-}$ mice lead to less airway mucin production compared with the wild-type mice. Our results are supported by a similar previous study showing that OVA-challenged TLR4 $^{-/-}$ mice demonstrated lower Th2 responses, such as eosinophils, allergen-specific IgE levels, and Th2 cytokine production, than the wild-type mice (13). These reduced Th2 responses were attributable, at least in part, to diminished dendritic cell function, since dendritic cells from TLR4 $^{-/-}$ mice expressed lower levels of CD86, a costimulatory molecule important for Th2 responses. Although there was no report addressing the potential role of TLR2 in regulating Th2 responses in a noninfectious setting, it is conceivable that the observed reduction of mucin expression (also one of the markers of Th2 response) in our TLR2 $^{-/-}$ mice may utilize a similar mechanism that occurs in TLR4 $^{-/-}$ mice.

The mechanism involved in airway mucin regulation is very complex. Generally, Th2 cytokines such as IL-13 and IL-9 are believed to increase mucins (12, 16, 21, 37, 40). However,

controversies still exist regarding the role of Th2 cytokines in regulating mucin expression. For example, a previous study demonstrated that IL-13 did not stimulate MUC5AC or MUC5B production in human primary differentiated tracheo-bronchial epithelial cells (7). In our current study, we could not find any significant differences of lung and BAL IL-13 levels in various groups of allergic mice. Specifically, IL-13 protein levels in lung tissues were similarly decreased (20–30%) after Mp infection in both allergic wild-type and TLR2 $^{-/-}$ mice (data not shown). These results indicate that IL-13 production is TLR2 signaling independent and may not contribute to regulation of airway mucin expression in our current model.

We realize that in our current study, no human asthmatic airway tissues or epithelial cells were included to determine the role of TLR2 signaling in airway mucin production. We need to perform future human studies to confirm or refute our hypothesis that the functional level of TLR2 signaling is critical in modulating airway mucin production of asthmatics with airway bacterial infection.

In summary, our results have shown that a deficient TLR2 signaling in allergic hosts could lead to a loss of bacterial infection-induced protection of airway mucins. Mp-induced IFN- γ depends on TLR2 signaling and exerts an inhibitory effect on mucin expression in a mouse allergic asthma model. TLR2-IFN- γ signaling pathway may yield novel insights into the regulatory mechanisms of airway mucin expression in asthma associated with respiratory infections such as Mp.

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