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AJP - Lung 289:125-133, 2005. First published Mar 18, 2005; doi:10.1152/ajplung.00167.2004

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Mycoplasma pneumoniae infection increases airway collagen deposition in a murine model of allergic airway inflammation

Hong Wei Chu, John G. Rino, Rachel B. Wexler, Krista Campbell, Ronald J. Harbeck, and Richard J. Martin

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Submitted 10 May 2004; accepted in final form 11 March 2005

Chu, Hong Wei, John G. Rino, Rachel B. Wexler, Krista Campbell, Ronald J. Harbeck, and Richard J. Martin. *Mycoplasma pneumoniae* infection increases airway collagen deposition in a murine model of allergic airway inflammation. *Am J Physiol Lung Cell Mol Physiol* 289: L125–L133, 2005. First published March 18, 2005; doi:10.1152/ajplung.00167.2004.—*Mycoplasma pneumoniae* (Mp) has been linked to chronic asthma. Airway remodeling (e.g., airway collagen deposition or fibrosis) is one of the pathological features of chronic asthma. However, the effects of respiratory Mp infection on airway fibrosis in asthma remain unclear. In the present study, we hypothesized that respiratory Mp infection may increase the airway collagen deposition in a murine model of allergic airway inflammation in part through upregulation of transforming growth factor (TGF)- β 1. Double (2 wk apart) inoculations of Mp or saline (control) were given to mice with or without previous allergen (ovalbumin) challenges. On days 14 and 42 after the last Mp or saline, lung tissue and bronchoalveolar lavage (BAL) fluid were collected for analyses of collagen and TGF- β 1 at protein and mRNA levels. In allergen-naïve mice, Mp did not alter airway wall collagen. In allergen-challenged mice, Mp infections did not change airway wall collagen deposition on day 14 but increased the airway collagen on day 42; this increase was accompanied by increased TGF- β 1 protein in the airway wall and reduced TGF- β 1 protein release from the lung tissue into BAL fluid. Our results suggest that Mp infections could modulate airway collagen deposition in a murine model of allergic airway inflammation with TGF- β 1 involved in the collagen deposition process.

transforming growth factor- β 1

RESPIRATORY INFECTION WITH atypical bacteria (e.g., mycoplasma and chlamydia) has drawn increasing attention in clinical asthma research (2, 11, 15, 18, 27). Our previous investigation showed that nearly 40% of chronic stable asthma patients had evidence of airway infection with *Mycoplasma pneumoniae* (Mp) (17). In a double-blind, placebo-controlled trial, antibiotic treatment in those asthmatics with Mp significantly improved pulmonary function (16). These observations suggest a close link between chronic asthma and chronic airway Mp infection. To further investigate the role of airway Mp infection, we have reported that airway inflammation and bronchial hyperresponsiveness increased in a mouse model with Mp infection (17).

Airway remodeling is an important feature of asthma pathobiology (22). Several structural changes have been identified in the airway remodeling process, which include subepithelial fibrosis and smooth muscle and mucous gland hypertrophy/

hyperplasia. Airway subepithelial fibrosis has been documented in asthmatics with different disease severity (5). Although the mechanisms of this pathological finding remain to be determined, profibrotic mediator transforming growth factor (TGF)- β 1 has been proposed to play a pivotal role in airway collagen deposition or fibrosis (20, 26).

The effects of respiratory infection on airway remodeling (e.g., airway fibrosis) in asthma have not been well defined. A clinical study suggested that diffuse interstitial fibrosis occurred following Mp pneumonia (24). Respiratory infection with a nonhuman pathogen *Mycoplasma pulmonis* alone (no allergen) has been demonstrated to induce airway fibrosis in rats from 1 to 3 mo after the infection (19). However, there is no systematic study to address whether human pathogen Mp could induce airway fibrosis in asthma.

In the present study, we hypothesized that respiratory Mp infection in a murine model of allergic airway inflammation will induce airway fibrosis that may be associated with increased expression of TGF- β 1 by Mp infection. Therefore, collagen and TGF- β 1 at both mRNA and protein levels were evaluated in the lungs of mice that were infected with Mp in the presence and absence of previous allergen exposure.

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. Female BALB/c mice (4 wk old) were obtained from Jackson Laboratories (Bar Harbor, ME). They were quarantined for 4 wk before the experiment and bled to establish that they were virus and *M. 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 $\sim 1 \times 10^8$ colony-forming units (cfu)/50 μ l (17).

Mp Inoculations

Mice were inoculated with either Mp or saline (control). Before the inoculation, all the mice were intraperitoneally anesthetized with 2,2,2-tribromoethanol (Aldrich, Milwaukee, WI) at 0.25 g/kg body wt. Mice in the infected group were inoculated intranasally with 50 μ l of Mp at $\sim 1 \times 10^8$ cfu. A 50- μ l inoculation of saline was similarly given to the mice in the control groups. After the inoculation, mice were placed at a 45-degree angle on their bedding for 5 min to allow

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Mp to be evenly distributed throughout the lung and also to prevent suffocation.

Animal Models

Allergen-Mp model. This model (see Fig. 1A) was designed to elucidate the effects of Mp infections on airway collagen deposition in mice with experimental allergic asthma induced by ovalbumin (OVA). Mice were intraperitoneally sensitized twice in a span of 14 days by injection of 20 μ g of OVA emulsified in 2.25 mg of aluminum hydroxide (AlmImject; Pierce, Rockford, IL). Fourteen days after the last OVA sensitization, mice were placed in a Plexiglas chamber and challenged with 1% aerosolized OVA for 20 min with an ultrasonic nebulizer (De Vilbiss). The OVA challenges were given once daily for 3 days (6). Two days after the last OVA, mice received the first Mp infection or saline (control) inoculation. To mimic the condition of human asthmatic patients who may be exposed to mycoplasma more than one time or have a chronic mycoplasma infective process, we inoculated the mice with a second Mp or saline after 14 days of the first Mp or saline. Fourteen and 42 days after the second Mp or saline inoculation (28 and 56 days after the first Mp or saline inoculation), mice were killed for lung inflammation, collagen, and TGF- β 1 measurement.

Mp model without allergen exposure. This model (Fig. 1B) was designed to elucidate the effects of Mp alone on airway collagen and TGF- β 1. These mice received two Mp infections or saline inoculations in a span of 14 days. Fourteen and 42 days after the second Mp or saline inoculation (28 and 56 days after the first Mp or saline inoculation), mice were killed for lung inflammation, collagen, and TGF- β 1 measurement.

Bronchoalveolar Lavage and Lung Tissue Processing

Bronchoalveolar lavage (BAL) was performed using 1 ml of saline in all mice. The BAL sample (300 μ l) was analyzed for the total and differential leukocyte counts, mycoplasma culture, and polymerase chain reaction (PCR) for *M. pneumoniae*. The remaining BAL was centrifuged, and the supernatant was collected and stored at -80°C for TGF- β 1 protein measurement.

After BAL, the lungs were removed and excised. The middle lobe of the right lung was utilized for RNA extraction and *M. pneumoniae* culture. The remaining lung was fixed in 4% paraformaldehyde, embedded in paraffin, and cut at 4 μ m for routine histopathological analysis, collagen, and TGF- β 1 staining.

Analysis of Lung Tissue Inflammation

To evaluate general inflammation in mouse lung tissue including airways, we evaluated hematoxylin and eosin-stained tissue sections in a double-blinded fashion under a light microscope using a histopathological inflammatory scoring system as described previously

in mouse models of *M. pneumoniae* infection (8). A final score per mouse on a scale of 0 to 26 (least to most severe) was obtained on the assessment of quantity and quality of peribronchiolar and peribronchial inflammatory infiltrates, luminal exudates, perivascular infiltrates, and parenchymal pneumonia.

Mp Culture and PCR

To determine whether live Mp existed in BAL and lung tissue samples, we performed mycoplasma culture as previously described (4). In brief, 100 μ l of BAL fluid with cells or minced lung tissue (approximate total size, $5 \times 5 \times 5$ mm) were placed into 900- μ l SP-4 broth tubes, which were then placed in a 37°C incubator without CO_2 for 4 wk. At the end of the culture, any tube with color change (from pink to yellow) was recorded as positive. Similarly, 10 μ l of BAL fluid with cells or minced lung tissue were placed onto mycoplasma pleuropneumonia-like organism (PPLO) culture plates, which were then placed in a 37°C incubator with CO_2 for 4 wk. At the end of the culture, any PPLO plate with mycoplasma growth was recorded as positive.

TaqMan real-time PCR was able to detect Mp-specific P1 adhesin gene from both live and dead organisms and thus was performed to further confirm the specificity of culture results and at the same time to increase the sensitivity of Mp detection (4).

Measurement of Airway Hyperresponsiveness

As previously reported (6), airway resistance ($\text{cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}$) was measured to indicate airway hyperresponsiveness (AHR) at baseline, after saline control, and after each subsequent doubled methacholine dose from 1.6 to 100 mg/ml. The log of the dose of aerosolized methacholine required for a 200% increase of airway resistance over baseline (LogPC_{200} , mg/ml) was calculated from each mouse to represent the degree of AHR. Lower LogPC_{200} values represent greater airway resistance or AHR (6).

Evaluation of Airway Collagen

Airway wall collagen. The general or collective collagen (all types of collagen, e.g., types I, III, V) in the lung tissue was identified by Sirius red staining as previously described (5). Medium-sized airways, defined by an epithelial basement membrane perimeter of 600–900 μ m (maximal diameter/minimum diameter ≤ 2), were evaluated for airway wall collagen deposition. The area of collagen in the airway wall was measured with an NIH Scion image program. The results were expressed as airway wall collagen area (μm^2)/length of bronchial epithelial basement membrane (μ m), which indicates the thickness of collagen deposition in the airway wall (21). At least five complete airways per mouse were randomly selected and evaluated. 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.

Procollagen type I ($\alpha 1$) mRNA levels in the lung tissue. Expression of procollagen type I($\alpha 1$) [$\alpha 1(\text{I})$ -procollagen] mRNA in mouse lung tissue was determined by reverse transcription (RT), followed by real-time quantitative PCR. Lung tissue total RNA was extracted using TRIzol reagent (GIBCO-BRL, Rockville, MD). To remove any potentially contaminated genomic DNA from the total RNA, the extracted total RNA samples were then treated with RNase-free DNase I (Ambion, Austin, TX) before RT-PCR was performed. RT 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 $\alpha 1(\text{I})$ -procollagen primers and probe (GenBank accession number: M17491) were designed using Primer Express software (Applied Biosystems). Forward primer: 5'-TTCCCGGTCAGAGAGGAGAA-3'; reverse primer: 5'-CCGCGTTCACCACTTGATC-3'; probe 5'-CCCCTCTGGTGAA-CCTGGCAAACAA-3'. Real-time PCR was performed on the ABI

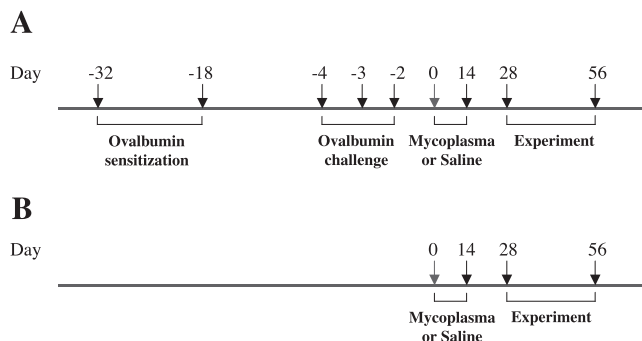


Fig. 1. Mouse models. A: allergen exposure followed by double *Mycoplasma pneumoniae* (Mp) infections or saline; $n = 5$ or 6 mice/group. B: double Mp infections or saline without previous allergen exposure; $n = 5$ or 6 mice/group.



Prism 7700 sequence detection system (Applied Biosystems). The 50- μ l PCR reaction contained 60 ng 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 α 1(I)-procollagen. No-template (no cDNA) controls and RT (–) samples (total RNA samples) were also included as controls for PCR of either α 1(I)-procollagen or 18S rRNA, and no PCR signal was found in these control samples. The threshold cycle (CT) was recorded for each sample to reflect the mRNA expression levels. The comparative CT method was used to demonstrate the relative expression level of α 1(I)-procollagen mRNA as previously reported (4).

Evaluation of TGF- β 1

TGF- β 1 protein measurement in BAL fluid. Levels of total TGF- β 1 protein in BAL fluid were measured using a TGF- β 1 sandwich ELISA kit (detection range, 15.6–2,000 pg/ml) from R&D Diagnostics (Minneapolis, MN) (7). All BAL fluid samples were acidified to activate latent TGF- β 1 so that total TGF- β 1 (both latent and active) levels were detected. In a preliminary study, the detection of total TGF- β 1 in BAL fluid from 12 mice of different groups was independently repeated two or three times with a <10% variation between different measurements.

TGF- β 1 protein in the lung tissue. Immunohistochemistry was performed on lung tissue sections to localize TGF- β 1 using a rabbit polyclonal TGF- β 1 antibody or a rabbit IgG (negative control) [Santa Cruz Biotechnology, Santa Cruz, CA] (5). TGF- β 1 expression was found intracellularly (e.g., macrophages, epithelial cells) and extracellularly. Because the focus of this study was on airway wall collagen deposition and TGF- β 1 expression, we evaluated the deposition of TGF- β 1 in the wall of medium-sized airways (defined as epithelial basement membrane perimeter 600–900 μ m with maximal diameter/minimum diameter ratio $\leq$ 2). TGF- β 1 staining in the airway wall was evaluated by using a semiquantitative approach. A score of 0 to 3 was given to the TGF- β 1 staining in the airway wall with 0 for no staining, 1 for weak, 2 for moderate, and 3 for strong staining (9). At least five airways per mouse were evaluated, and the score was averaged for each mouse.

TGF- β 1 mRNA in the lung tissue. Mouse lung tissue TGF- β 1 mRNA expression was detected by real-time quantitative RT-PCR as described for mouse lung α 1(I)-procollagen mRNA. The following are the sequences for mouse TGF- β 1 mRNA gene (GenBank acces-

sion number: NM_011577). Forward primer: 5'-GAGCCCGAAGC-GGACTACTA-3'; reverse primer: 5'-GGTTTTCATAGATGGCG-TTGTT-3'; probe 5'-CACCCGCGTGCTAATGGTGGACC-3'.

Statistics

If the data were normally distributed, they are presented as means $\pm$ SE and compared between the groups by analysis of variance and Bonferroni multiple-comparison procedure. When the data were not normally distributed, the data are expressed as medians with interquartile (25–75%) ranges, and the comparisons between the groups were performed by the Wilcoxon rank-sum test. Correlations were performed using Spearman's rank correlation coefficients. A two-tailed *P* value <0.05 is considered statistically significant.

RESULTS

Inflammatory Response in the Lung Tissue and BAL

As shown in Table 1, in allergen-challenged mice, lung tissue (including airways) inflammation score was significantly higher in Mp-infected mice on both days 14 and 42 after the second infection than their saline controls. However, in allergen-naïve mice, lung inflammation was only increased on day 14 in Mp-infected mice.

In allergen-challenged mice, Mp infection resulted in an increase of BAL total leukocyte count on day 14, but not day 42, after the second infection. Among the leukocytes, the percentage of eosinophils and lymphocytes was significantly higher in Mp-infected mice on day 14. Consistent with the lung tissue inflammation, Mp infection in allergen-naïve increased BAL total leukocyte count and lymphocyte differential count on day 14. Additionally, in allergen-naïve mice on day 42, the percentage of BAL lymphocytes was higher in Mp-infected mice than in the saline control mice.

Mp Culture and PCR

Live Mp was found in the lungs (lung tissue and/or BAL cells) of five of six (83.3%) and one of six (16.7%) mice with allergen challenges on days 14 and 42 after the second Mp

Table 1. Inflammatory indexes in lung tissue and BAL

Inflammatory Indexes	Day 14 Post-Sa or Mp		Day 42 Post-Sa or Mp	
	Sa	Mp	Sa	Mp
Lung tissue inflammation score				
Sa or Mp alone	1.2 (1.0–2.3)	5.1 (2.9–5.9)*	1.2 (0.5–2.3)	2.3 (1.9–3.1)
OVA + Sa or Mp	5.4 (5.1–6.7)	11.0 (10.1–11.4)*	4.8 (3.7–5.5)	7.4 (7.0–8.0)*
BAL total leukocytes, $\times 10^4$ /ml				
Sa or Mp alone	2.8 (1.8–9.8)	10.5 (7.3–12.1)*	9.0 (2.3–10.0)	5.3 (4.8–7.0)
OVA + Sa or Mp	6.0 (4.0–6.5)	12.8 (9.8–16.0)*	4.5 (2.8–5.0)	4.5 (3.5–8.5)
BAL neutrophils, %				
Sa or Mp alone	0.4 (0.3–0.9)	0.6 (0.2–1.2)	0.2 (0.1–1.2)	0.6 (0.2–1.3)
OVA + Sa or Mp	0 (0–1.5)	0.4 (0.2–1.0)	0.8 (0.2–2.6)	0.7 (0.2–1.6)
BAL eosinophils, %				
Sa or Mp alone	0	0	0	0
OVA + Sa or Mp	0 (0–0.3)	0.7 (0.4–1.0)*	0 (0–0.3)	0.2 (0.1–0.3)
BAL lymphocytes, %				
Sa or Mp alone	2.6 (2.0–3.4)	11.8 (6.9–14.8)*	1.2 (0.8–2.5)	10.4 (4.0–13.1)*
OVA + Sa or Mp	25.6 (16.5–44.0)	38.4 (30.1–49.7)*	6.0 (4.6–9.9)	12.3 (6.3–18.2)
BAL macrophages, %				
Sa or Mp alone	97.0 (96.2–97.0)	86.6 (84.5–92.7)*	98.8 (96.3–99.0)	88.6 (85.8–95.8)*
OVA + Sa or Mp	73.4 (55.8–81.4)	59.9 (49.0–67.8)*	93.2 (88.7–93.7)	87.2 (80.0–92.8)

Data are expressed as medians (25%–75% range). Sa, saline; Mp, *Mycoplasma pneumoniae*; OVA, ovalbumin; BAL, bronchoalveolar lavage. **P* < 0.05, Mp vs. Sa mice.



infection, respectively. In contrast, no live or even dead Mp was found in the lungs of allergen-naïve mice at 14 and 42 days after the second Mp infection. No Mp was detected in saline control (sham infection) mice with or without allergen challenge.

Airway Wall Collagen Deposition

As shown in Fig. 2, in allergen-naïve mice, Mp infections did not significantly alter airway wall collagen deposition. In allergen-challenged mice, airway collagen deposition in infected mice 14 days after the second infection was similar to that in saline control mice. However, the chronic infection (day 42 after the second infection) in allergen-challenged mice significantly increased the airway wall collagen deposition (Figs. 2 and 3). In addition, regardless of infection status, airway wall collagen thickness in allergen-challenged mice was greater than that in allergen-naïve mice ($P < 0.05$).

$\alpha 1(I)$ -Procollagen mRNA Levels in the Lung Tissue

The levels of $\alpha 1(I)$ -procollagen mRNA expression were evaluated in the lung tissue to determine the transcriptional activity of collagen.

Like the aforementioned airway wall collagen protein data, Mp infections did not significantly change lung $\alpha 1(I)$ -procollagen mRNA expression levels in allergen-naïve mice (Fig. 4). Similarly, lung $\alpha 1(I)$ -procollagen mRNA expression levels were not significantly different between allergen-challenged mice 14 days after Mp or saline. However, lung $\alpha 1(I)$ -procollagen mRNA expression levels tended to be higher ($P = 0.09$) 42 days after the second Mp infection in allergen-challenged mice compared with allergen-challenged saline control mice (Fig. 4).

Airway Wall TGF- $\beta 1$ Protein Expression

TGF- $\beta 1$ protein expression in the airway wall was evaluated to elucidate the potential mechanisms by which Mp modified the airway collagen deposition.

Although many types of cells (e.g., epithelial cells, macrophages) in the lung were found to express TGF- $\beta 1$, we focused on the evaluation of extracellular TGF- $\beta 1$ protein in the airway

wall since extracellular matrix TGF- $\beta 1$ has been proposed to contribute to the fibrotic process (21).

In allergen-naïve mice, Mp infections reduced airway wall TGF- $\beta 1$ staining 14 days, but not 42 days after the second Mp infection (Fig. 5). In allergen-challenged mice, compared with the control mice, Mp infections significantly increased airway wall TGF- $\beta 1$ staining 42 days, but not 14 days, after the second infection (Figs. 5 and 6), which was consistent with the airway wall collagen deposition data.

Lung Tissue TGF- $\beta 1$ mRNA Expression

Lung tissue TGF- $\beta 1$ mRNA was evaluated to further determine the effects of Mp infection on TGF- $\beta 1$ expression (Table 2).

In allergen-naïve mice, Mp infections on day 42, but not on day 14, significantly increased TGF- $\beta 1$ mRNA expression. In allergen-challenged mice, however, Mp infections on both 14 and 42 days postinfection significantly increased TGF- $\beta 1$ mRNA expression levels compared with the control groups.

TGF- $\beta 1$ Protein Levels in BAL Fluid

TGF- $\beta 1$ protein levels in BAL fluid were measured to evaluate the release of TGF- $\beta 1$ protein from the lung tissue (Table 3).

In allergen-naïve mice, Mp infections on both days 14 and 42 significantly increased TGF- $\beta 1$ protein levels. In contrast, in allergen-challenged mice, Mp infections on both 14 and 42 days postinfection significantly decreased TGF- $\beta 1$ protein levels in BAL fluid.

Correlations Between Collagen, TGF- $\beta 1$, and AHR

Correlations between airway wall collagen deposition and TGF- $\beta 1$ are demonstrated in Table 4. Airway wall TGF- $\beta 1$ protein expression significantly correlated with airway wall collagen thickness in allergen-challenged, but not in allergen-naïve, mice. Interestingly, there was a significant inverse correlation between airway wall TGF- $\beta 1$ protein expression and BAL fluid TGF- $\beta 1$ protein levels in both allergen-naïve and -challenged mice. In addition, in allergen-challenged mice, BAL fluid TGF- $\beta 1$ protein levels tended to inversely correlate

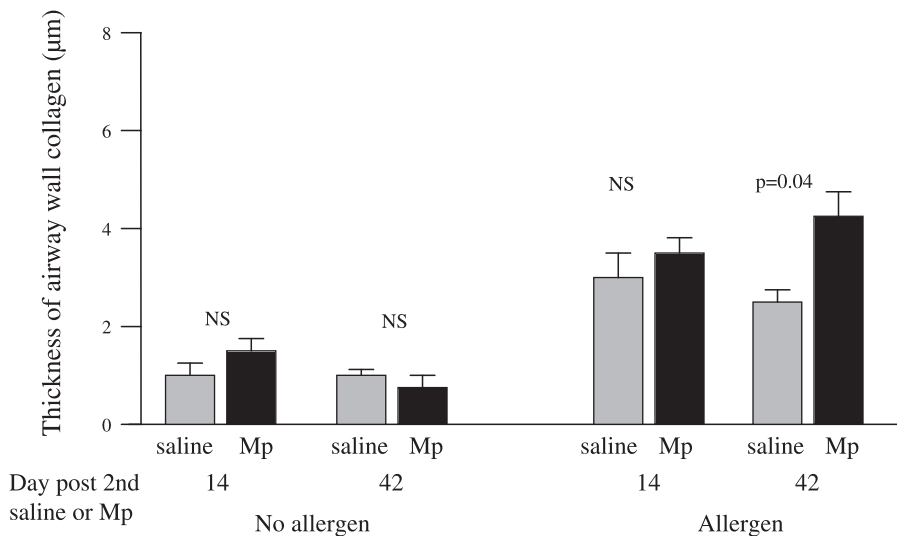


Fig. 2. Thickness of airway collagen deposition in allergen-naïve and allergen-challenged mice with double Mp infections or saline (control); $n = 5$ or 6 mice/group. NS, not significant.

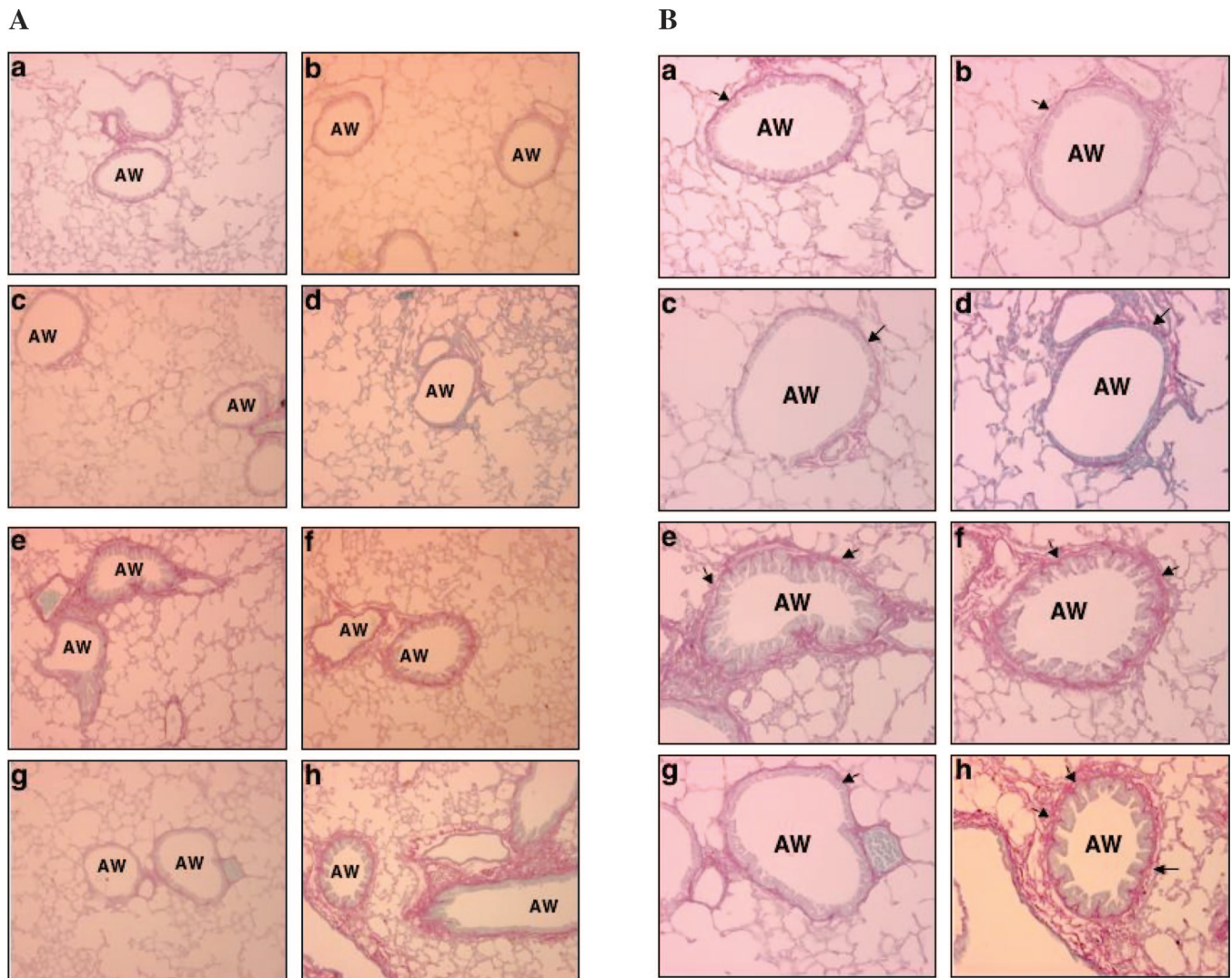


Fig. 3. Representative photomicrographs of airway collagen Sirius red staining. The quantitative data are shown in Fig. 2. **A**: lower-magnification ($\times 100$) view of airway collagen staining. *a–d*: Allergen-naïve mouse lung tissues. *a*: Day 14 saline; *b*: day 14 Mp; *c*: day 42 saline; *d*: day 42 Mp. *e–h*: Allergen-challenged mouse lung tissues. *e*: Day 14 saline; *f*: day 14 Mp; *g*: day 42 saline; *h*: day 42 Mp. **B**: higher-magnification ($\times 200$) view of airway collagen staining from *a–h* of **A**. AW, airway. Arrows indicate airway collagen deposition.

with airway wall collagen thickness. Therefore, our statistical correlation analyses suggest that in allergen-challenged mice, airway wall, but not BAL fluid TGF- β 1 protein levels correlated with airway wall collagen deposition.

Although the role of airway wall collagen deposition or fibrosis in AHR remains to be controversial, an attempt was made to correlate AHR with airway wall collagen thickness in allergen-challenged mice. First, no significant correlations were found between airway wall collagen thickness and AHR ($\rho = -0.03$, $P = 0.90$). Second, AHR as indicated by LogPC₂₀₀ was not significantly different ($P = 0.43$) between Mp-infected and noninfected mice with allergen challenges at either 14 and 42 days postinfection or saline (Table 5). It would be more informative if our present study could demonstrate an increased AHR in allergen-challenged mice (with or without infection) compared with allergen-naïve mice (with or without infection). However, because an unsolvable mechanical problem with the mouse physiology equipment occurred during the experiments for allergen-naïve mice, we were unable to collect

AHR data in these mice. Nevertheless, studies from other groups have clearly demonstrated an increase of AHR in allergen (OVA)-challenged mice compared with allergen-naïve mice (10).

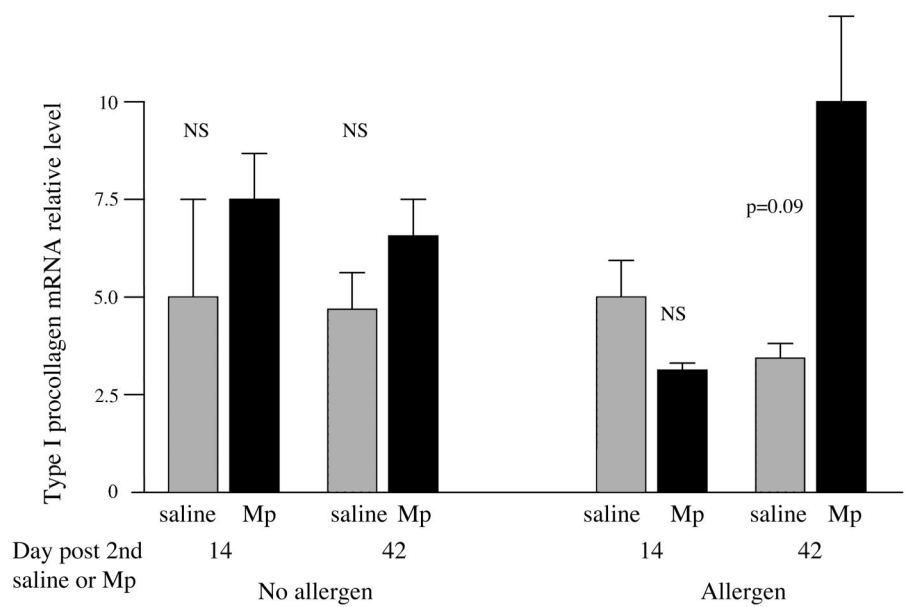
DISCUSSION

We have determined the effects of double Mp infections separated by 2 wk on airway collagen deposition and TGF- β 1 expression in mice with and without previous allergen challenges. The present study demonstrated that Mp infections increased airway collagen and TGF- β 1 deposition in allergen challenged- but not allergen-naïve mice. These results suggest that multiple chronic Mp infections in an existing allergic setting may cause airway fibrosis.

Although airway remodeling such as fibrosis usually occurs as a result of airway inflammation, the inflammatory process may not always cause an abnormal airway repair such as fibrosis. We have previously shown that a single Mp infection



Fig. 4. Lung tissue type I procollagen [$\alpha 1(I)$ procollagen] mRNA relative expression levels in allergen-naïve and allergen-challenged mice with double Mp infections or saline (control); $n = 5$ or 6 mice/group.



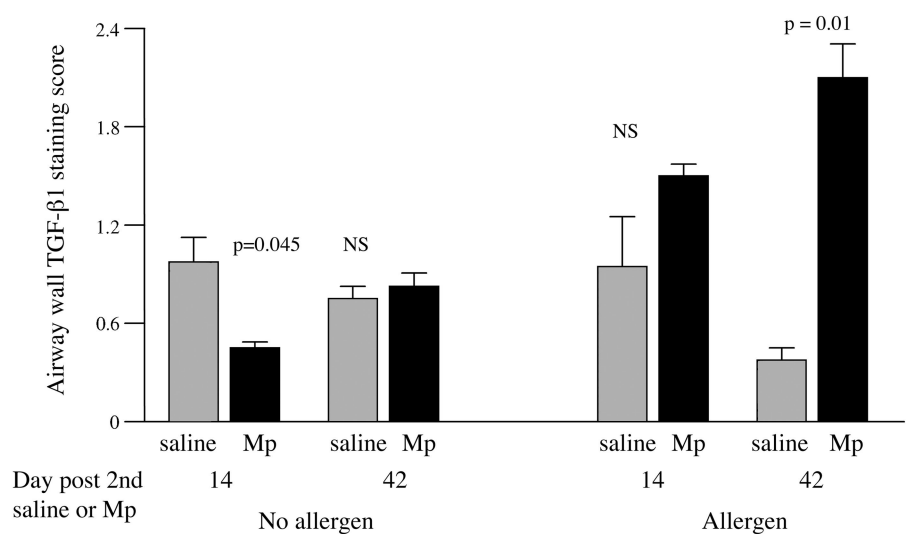
(no allergen) increased airway inflammation, but not collagen deposition in mice (17). In the present study, double Mp infections in allergen-naïve mice also did not alter airway collagen deposition. However, in allergen-challenged mice, a recurrent or chronic Mp infectious process increased airway collagen deposition, which was linked to an enhanced lung, but not BAL, inflammation. Our data suggest that a pre-existing allergic inflammatory milieu or a Th2 background may be required for the human pathogen *M. pneumoniae* to induce airway fibrosis in mice. Our results did not support an early study in which a rodent, but not human pathogen, *M. pulmonis*, alone was shown to induce airway fibrosis in rats (19). This discrepancy may be explained by the differences in host (mouse vs. rat) genetic background, and mycoplasma species used between the two different studies (*M. pneumoniae* vs. *M. pulmonis*).

Our findings that multiple chronic *M. pneumoniae* infections induced airway fibrosis only in an allergic milieu may have some clinical implications. In clinical settings, asthmatic sub-

jects can be repeatedly or chronically infected with Mp after allergen sensitization, which may contribute to airway subepithelial fibrosis. We have previously demonstrated that Mp infection following, but not before, an allergen challenge amplified inflammatory and AHR (6). Additionally, in chronic asthmatic subjects who were positive for atypical bacteria in their airways had approximately a sixfold increase in tissue mast cells compared with those asthmatic subjects who were negative for the bacteria (18).

The mechanism by which Mp regulates airway collagen remains unknown. As chronic airway inflammation has been proposed to contribute to airway collagen deposition or fibrosis in asthma, we examined the general inflammation in the mouse lung including airways. Increased airway collagen deposition in allergen-challenged and Mp-infected mice was indeed accompanied by an exaggerated airway inflammation that appeared to link to a persistent lung Mp infection in an allergic milieu. Although the molecular mechanisms involved in collagen deposition are very complex, in the present study, we

Fig. 5. Semiquantitative analyses of airway wall transforming growth factor (TGF)- $\beta 1$ protein deposition in allergen-naïve and allergen-challenged mice with double Mp infections or saline (control); $n = 5$ or 6 mice/group.



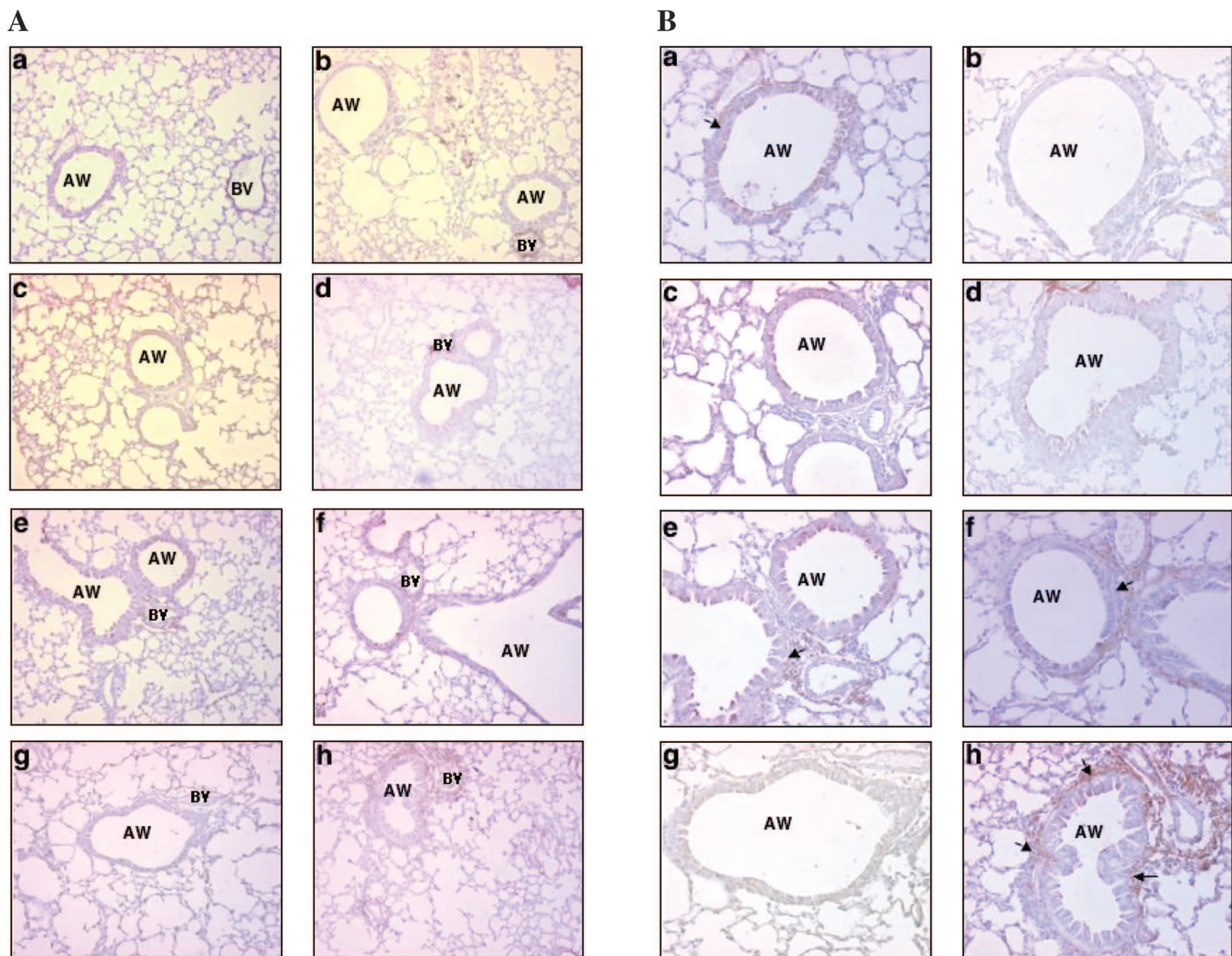


Fig. 6. Representative photomicrographs of airway TGF- β 1 staining. The quantitative data are shown in Fig. 5. A: lower-magnification ($\times 100$) view of TGF- β 1 staining. BV, blood vessel. a–d: Allergen-naïve mouse lung tissues. a: Day 14 saline; b: day 14 Mp; c: day 42 saline; d: day 42 Mp. e–h: Allergen-challenged mouse lung tissues. e: Day 14 saline; f: day 14 Mp; g: day 42 saline; h: day 42 Mp. B: higher magnification ($\times 200$) view of airway TGF- β 1 staining from a–h of A. Arrows indicate airway TGF- β 1 staining.

focused on the expression of TGF- β 1 in the lung and BAL fluid since TGF- β 1 has been shown to be one of the key fibrotic mediators (20, 26). In allergen-naïve mice, TGF- β 1 expression levels in both BAL fluid and whole lung tissue were generally higher in infected mice than control mice. However, airway wall TGF- β 1 protein deposition was not increased or even decreased in infected mice compared with the control mice, which may be in part responsible for no differences of airway wall collagen deposition between Mp-infected and noninfected allergen-naïve mice.

Unlike allergen-naïve and Mp-infected mice, TGF- β 1 protein levels in BAL fluid of allergen-challenged and Mp-infected mice were opposite to lung tissue TGF- β 1 protein and/or mRNA levels. Allergen-challenged mice without Mp infection showed significantly higher levels of TGF- β 1 protein in BAL fluid than allergen-challenged mice with Mp infection, which was reversed in the airway wall tissue. Our data suggest that in allergen-challenged mice, Mp infection induced an imbalance of TGF- β 1 between airway tissue and BAL fluid, i.e., reduction of TGF- β 1 release into the airway lumen and

Table 2. TGF- β 1 mRNA relative levels in mouse lung tissue

Groups	n	Day 14 Post-Sa or Mp		Day 42 Post-Sa or Mp	
		Sa	Mp	Sa	Mp
Sa or Mp alone	5–6	5.7 (1.6–17.9)	7.3 (3.9–15.3)	2.3 (1.4–6.6)	24.0 (21.6–25.5)*
OVA + Sa or Mp	5–6	1.7 (1.4–4.2)	7.9 (5.1–9.9)*	1.2 (0.8–2.1)	5.8 (4.2–7.1)*

Data are expressed as medians (25%–75% range). TGF, transforming growth factor. * $P < 0.05$, Mp vs. Sa mice.



Table 3. *TGF- β 1 protein levels (pg/ml) in BAL fluid*

Groups	n	Day 14 Post-Sa or Mp		Day 42 Post-Sa or Mp	
		Sa	Mp	Sa	Mp
Sa or Mp alone	5–6	47 (45–66)	82 (59–118)*	53 (43–69)	74 (68–82)*
OVA + Sa or Mp	5–6	156 (149–185)	72 (64–84)*	219 (209–322)	81 (52–108)*

Data are expressed as medians (25%–75% range). * $P < 0.05$, Mp vs. Sa mice.

enhancement of TGF- β 1 deposition in the airway wall. Thus airway tissue, but not BAL fluid, levels of TGF- β 1 may be indicative of fibrotic process in allergen-challenged and Mp-infected mouse lung. A previous study similarly demonstrated an imbalance between plasma circulating TGF- β 1 levels and cutaneous wound tissue in a murine cutaneous wound model (23). In that study, TGF- β 1 levels in the plasma were significantly higher than those in the tissue. Further studies are needed to explain why Mp infection in the allergic lung increases TGF- β 1 tissue deposition or activation and at the same time reduces its release into the airway lumen. As TGF- β 1 regulation is a very complex process, the potential TGF- β 1 regulatory steps by Mp infection could include gene transcription, posttranscription, translation, and perhaps most importantly an activation process. It appears from our current study that Mp infection in the presence of an allergic response increases TGF- β 1 binding to the extracellular matrix in the airway wall and consequently reduces TGF- β 1 release from the airway wall into the airway lumen. Future studies are necessary to elucidate the molecular mechanisms by which Mp infection regulates TGF- β 1 expression and activation in an allergic setting. The potential molecules involved in TGF- β expression/activation may include TGF- β receptors, TGF- β signaling molecules (e.g., Smads), and others such as α v β 6-integrin, matrix metalloproteinase-9, and CD44 (21).

In the present study, there are some intriguing findings that need to be addressed and pursued in our future studies. For example, although total lung α 1(I)-procollagen mRNA expression paralleled airway collagen deposition in allergen-challenged and Mp-infected mice on day 42 after the second infection, it generally did not reflect the degree of airway wall collagen deposition. This may be in part due to the fact that

collagen protein and mRNA were examined in different lung compartments in that collagen protein and mRNA were determined in the airway wall and whole lung tissue homogenates, respectively. Therefore, collagen mRNA levels in the whole lung may not well represent the protein levels in the airway wall, which has been similarly documented in a recent publication (14). In our future studies, laser capture microdissection in combination with real-time RT-PCR will be necessary to examine collagen mRNA in the airway wall instead of the whole lung to further confirm research findings in the present study. Another intriguing finding is that increased airway collagen deposition in allergen-challenged and Mp-infected mice on day 42 after the second infection did not result in AHR. Our “unexpected” result could be explained by the very controversial role of airway fibrosis in AHR seen in both human asthmatics and animal models of allergic airway inflammation. In view of previous studies in which the presence of airway subepithelial fibrosis has not been linked to AHR in both animal and human studies (3, 12, 13), our research findings may not be surprising. Thus studies from several groups including ours are posing a challenge to the classic concept that airway subepithelial fibrosis contributes to AHR in asthma. However, it has to be emphasized that the timing from the last allergen challenge might be one of the contributors to AHR. In our previous study (6), an increased AHR was found in allergen-challenged and Mp-infected (one infection) mice at day 14 post-Mp (day 16 after the last allergen challenge). In the present study, AHR was examined in allergen-challenged and Mp-infected (two infections) mice at days 14 and 42 after the second Mp (days 30 and 58 after the last allergen challenge), and no significant differences of AHR were seen between the infected and noninfected mice. Therefore, we cannot exclude a possibility that an increased AHR might exist at an earlier time point after the second Mp (e.g., day 3) or last allergen challenge (e.g., day 19).

Airway remodeling in asthma consists of abnormalities in various airway structural components, including subepithelial fibrosis (the most studied parameter), mucus goblet cell hyperplasia/hypertrophy, smooth muscle hyperplasia/hypertrophy, angiogenesis, and others. The mechanisms underlying different remodeling components may vary. Because the focus of our

Table 4. *Statistical correlations between airway wall collagen and TGF- β 1*

	Allergen-Naïve Mice (Rho Value)	P	Allergen-Challenged Mice (Rho Value)	P
Airway wall TGF- β 1 protein vs. airway collagen thickness	−0.40	0.10	0.58	0.005
Airway wall TGF- β 1 protein vs. BAL fluid TGF- β 1 protein	−0.51	0.03	−0.55	0.008
Airway wall TGF- β 1 protein vs. lung tissue TGF- β 1 mRNA	0.14	0.58	0.39	0.07
BAL fluid TGF- β 1 protein vs. airway collagen thickness	0.13	0.59	−0.37	0.09
Lung tissue TGF- β 1 mRNA vs. BAL fluid TGF- β 1 protein	−0.01	0.98	−0.54	0.009

Table 5. *Comparison of airway hyperresponsiveness in allergen-challenged mice*

Groups	n	Day 14, LogPC ₂₀₀ , mg/ml	Day 42, LogPC ₂₀₀ , mg/ml
OVA + Sa	5–6	0.06 (0.04–0.26)*	0.19 (0.09–0.32)
OVA + Mp	5–6	0.11 (0.06–0.29)	0.40 (0.06–1.27)

*Data are expressed as medians (25%–75% range). LogPC₂₀₀, log of dose required for 200% increase of airway resistance over baseline.



current study was to determine whether Mp infection would increase airway wall collagen deposition and the potential mechanisms involved in this process, other remodeling components were not investigated. Nevertheless, in a preliminary study, we performed a morphometric analysis on medium-sized airway epithelial mucin protein expression (as determined by Alcian blue/periodic acid-Schiff staining) in allergen-challenged and Mp-infected mice on day 14 after the second infection. An upregulated airway mucin expression was observed in allergen-challenged and Mp-infected mice compared with allergen-challenged and saline control mice ($P = 0.02$). Therefore, Mp infection in allergen-challenged mice may result in remodeling of different airway tissue components.

In summary, our study suggests that mycoplasma infection can regulate airway collagen deposition. The outcome of airway collagen deposition may depend on the chronicity of infection and the interactions with the allergic response. Airway wall TGF- β 1 expression and activation may be among the regulatory mechanisms by which a recurrent or chronic mycoplasma infection modifies airway subepithelial fibrosis in asthma.

GRANTS

This work was supported by the National Heart, Lung, and Blood Institute Grant PO1 HL-073907.

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