

Chronic and Repeated *Chlamydomphila pneumoniae* Lung Infection can Result in Increasing IL-4 Gene Expression and Thickness of Airway Subepithelial Basement Membrane in Mice

Chiung-Zuei Chen,¹ Bei-Chang Yang,² Tsun-Mei Lin,³ Cheng-Hung Lee,¹ Tzuen-Ren Hsiue^{1*}

Background/Purpose: *Chlamydomphila pneumoniae* infection has been associated with several pulmonary and cardiac diseases. However, it has not been explored for its ability to activate the same immunopathologic mechanisms of asthma, namely, a predominant Th2 immune response and structural changes that are termed airway remodeling. This study evaluated immune responses in the lung and airway pathology of BALB/c mice with chronic and repeated *C. pneumoniae* infections.

Methods: Mice were inoculated intranasally with 5×10^6 inclusion-forming units of *C. pneumoniae* TWAR strain, and re-inoculated at 14 and 42 days after the primary inoculation. Cytokine gene expression in bronchoalveolar lavage (BAL) cells was analyzed by RT-PCR on day 70. Airway pathology was also evaluated by morphometric measurements.

Results: A significant increase of interleukin (IL)-4 mRNA was detected in BAL cells in infected mice, and a significant increase in subepithelial basement membrane thickness of the airways was also noted in infected mice as compared with control mice ($8.95 \pm 0.28 \mu\text{m}$ vs. $5.54 \pm 0.22 \mu\text{m}$, $p < 0.0001$). We further analyzed the correlation between IL-4 cytokine expression and the increased subepithelial basement membrane thickness of airways in infected mice. We found that mice with increased IL-4 mRNA expression had significant increases in the thickness of subepithelial basement membrane as compared with mice without increased IL-4 mRNA expression ($9.87 \pm 0.51 \mu\text{m}$ vs. $6.49 \pm 0.52 \mu\text{m}$, $p < 0.0001$).

Conclusion: It is believed that our results demonstrated for the first time that chronic and repeated infections with *C. pneumoniae* increased IL-4 gene expression and thickness of airway subepithelial basement membrane in mice. [J Formos Med Assoc 2009;108(1):45–52]

Key Words: airway remodeling, *Chlamydomphila pneumoniae*, interleukin-4

Chlamydomphila pneumoniae is a common cause of obstructive pulmonary disease and ischemic heart respiratory infections. Infection with this obligate disease.^{1–3} Hahn and colleagues were the first respiratory intracellular pathogen may remain chronic to report an association between infection with several serious disorders such as asthma, chronic *C. pneumoniae* and the development of asthma.⁴ Since then, increasing evidence has suggested that

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¹Department of Internal Medicine, National Cheng Kung University Hospital, ²Department of Microbiology and Immunology, and ³Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University Medical College, Tainan, Taiwan.

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*Correspondence to: Dr Tzuen-Ren Hsiue, Department of Internal Medicine, National Cheng Kung University Hospital, 138 Sheng-Li Road, Tainan 704, Taiwan.
E-mail: hsiue@mail.ncku.edu.tw

persistent lung infection caused by *C. pneumoniae* is associated with the initiation, exacerbation and promotion of asthma.⁵⁻⁷ However, a causal relationship between *C. pneumoniae* infection and asthma remains to be established.

Asthma is a chronic inflammatory disease of the lung, characterized by airway hyperresponsiveness (AHR), and structural changes in the airway walls. Such airway remodeling includes thickening of the subepithelial basement membrane, goblet cell hyperplasia with mucus hypersecretion, enlargement of the bronchial smooth muscle mass and growth of new vessels. Currently available data suggest that an important mechanism for AHR and airway remodeling in asthma is the action of Th2 cytokines.⁸⁻¹¹ However, repeated infection with *C. pneumoniae* elicits interferon- γ (IFN- γ) production in mice, which plays an important role in the clearance and pathogenesis of *C. pneumoniae*.¹² In addition to IFN- γ , previous studies have reported that *C. pneumoniae*-infected monocytes produce interleukin (IL)-10,¹³ which is a powerful inhibitor of IFN- γ and an important immunosuppressive regulator of cell-mediated immunity and T-cell differentiation.¹⁴ In addition to IL-10, Burian et al reported that IL-4 has a role in host defense during acute *C. pneumoniae* infection.¹⁵ A Th2-predominant immune response would be expected if there is an association between chronic infection with *C. pneumoniae* and asthma, but a role for Th2 immune responses in chronic *C. pneumoniae* infection has not been established. IL-4, IL-5 and IL-13 are important Th2 cytokines that play a central role in bronchial asthma.¹⁶⁻¹⁸

In the present study, we analyzed immune responses, including gene expression of IFN- γ , IL-4, IL-5 and IL-13, and the associated airway remodeling in mice with chronic and persistent *C. pneumoniae* infection. Our results demonstrated that chronic and repeated *C. pneumoniae* lung infection increased IL-4 gene expression. Moreover, the increased expression of IL-4 mRNA correlated positively with the increased thickness of subepithelial basement membrane of airways in infected mice. These results revealed that chronic and repeated lung infections with *C. pneumoniae* induced Th2

cytokine expression and airway remodeling similar to the inflammatory responses in human asthma.

Methods

Mice

Specific pathogen-free, 6–8-week-old male BALB/c mice were obtained from the Laboratory Animal Center, National Cheng Kung University, Taiwan. The mice were housed in a micro-isolated cage and provided with sterile food and water *ad libitum*. All procedures were approved by the Animal Research Ethics Board at National Cheng Kung University.

C. pneumoniae strain and inoculum preparation

TWAR ATCC strain AR 183 was used for inoculation of mice. The organism was grown in HL cells and purified by density gradient centrifugation using diatrizoate meglumine (Hypaque-76; Winthrop-Breon Laboratories, New York, NY, USA). The inoculum preparation was resuspended in sucrose phosphate glutamic acid (SPG) medium to provide a final inoculum of 1×10^7 inclusion-forming units (IFU)/mL. These preparations were frozen at -70°C until use.

Inoculation of mice

Mice were sedated by subcutaneous injection of a mixture of ketamine and xylazine, and were inoculated intranasally with 5×10^6 IFU *C. pneumoniae* suspended in 0.5 mL SPG medium. Mice were re-inoculated at 14 days and 42 days after the primary inoculation. Control mice received 0.5 mL sterile SPG medium, during primary inoculation and re-inoculation. A total of 24 mice (experimental mice) received intranasal inoculation of *C. pneumoniae* suspension, and six control mice received inoculation of SPG medium.

Bronchoalveolar lavage (BAL)

BAL was obtained at 70 days after primary inoculation. Two separate doses of 1 mL sterile endotoxin-free saline were injected into the lung via the

trachea of each mouse. Approximately 1.8 mL washing solution was recovered consistently. The recovered BAL fluid volume was measured and placed immediately on ice. Cells were sedimented by centrifugation at 200g for 10 minutes at 4°C. The supernatants were stored at -70°C until assay.

Histologic examination of lung

Mice were killed by an overdose of pentobarbital and the whole lung and trachea were removed, and the left lung was resected for culture of *C. pneumoniae* immediately at day 70. Two milliliters of formalin fixative was instilled intratracheally until the surface of the right lung was smooth. After fixation for 2 days, four paraffin-embedded 5-mm thick sections of the right lung were stained with hematoxylin and eosin (H&E), and then examined by microscopy.

Morphometry measurements

The thickness of the subepithelial basement membrane layer of the airways was evaluated by measuring the basement membrane thickness beneath the bronchial epithelium with H&E stain. We applied design-based stereology to biopsy analysis of the thickness beneath the bronchial epithelium by using an integrated system that includes a microscope, automated microscope stage, video camera, and computer equipped with stereology software. Images of the airways under a microscope at 10× and 20× magnification were captured in the computer by an investigator who was blind to the tissue codes for the study specimen. The thickness of the subepithelial basement membrane at four different quadrants for each airway cross section was examined, and the mean of these four measurements was calculated. At least 14 airways were examined in each mouse, and the mean number of airways examined was 18 (range, 14–25). All fields of lung tissue were evaluated for morphometric analysis.

Isolation of *C. pneumoniae* in lung tissue

Left lung tissues were homogenized and tested for the presence of viable *C. pneumoniae* by inoculation in HL cells. Cells were grown in a 24-well

cell culture plate. Each sample was inoculated into three wells and centrifuged at 1500 rpm for 60 minutes and subsequently incubated with fresh medium that contained 0.6 mg/L cycloheximide. After 3 days, one of the three wells was fixed with methanol and stained by FITC-conjugated TT401. The other two wells were harvested and passed to HL cells. The specimens were regarded as negative if inclusions were not detected within three passages. In each culture series, *C. pneumoniae* was included in parallel as a positive control.

Semiquantitative RT-PCR

BAL cells were collected and after washing twice in ice-cold PBS, the cell number was adjusted to 10⁶/mL. Total cellular RNA was extracted by the RNeasy total RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was converted to cDNA with Strata Script H-reverse transcriptase (Stratagene, La Jolla, CA, USA) and oligo dT as a primer. The generated cDNA was subjected to 35 cycles of PCR amplification by a DNA Thermal Cycler (Hybrid Omnigene, UK). Gene-specific primer pairs (sense and antisense, respectively) were as follows: IL-4: 5'-ATGGGTCTCAACCCCCAGCTAGT-3' and 5'-GCTCTTTAGGCTTTCCAGGAAGTC-3'; IL-5: 5'-GCACAGTGGTGAAGAGACC-3' and 5'-TTAACTGACTACAGGACATAAATA-3'; IL-13: 5'-AGACCA-GACTCCCCTGTGCA-3' and 5'-TGCACAGGGGAG-TCTGGTCT-3'; IFN- γ : 5'-TGAACGCTACACACTG-CATCTTGG-3' and 5'-CGACTCCTTTTCCGCTTC-CTGAG-3'; and β -actin: 5'-TGGAATCCTGTGG-CATCCATGAAAC-3' and 5'-TAAAACGCAGCTC-AGTAACAGTCCG-3'. For PCR amplification, a 100- μ L reaction contained 1–3 μ L cDNA mixture, 100 pmol for each primer, 2 mM of each dNTP, 1.5 nM of MgCl₂, and 2.5 U of Taq DNA polymerase. Thirty-five amplification cycles were performed, consisting of denaturation, 94°C for 30 seconds; annealing, 60°C for 45 seconds; and extension, 72°C for 1 minute. PCR products were fractionated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light.

Statistical analysis

Results were expressed as mean ± standard error of the mean for the measurements of thickness of subepithelial basement membrane. Differences between infected and control groups were analyzed by Student's two-tailed *t* test. Multiple groups were analyzed by ANOVA test. Differences were defined as significant if *p* was < 0.05.

Results

Models of chronic and repeated *C. pneumoniae* lung infections in mice

Four *C. pneumoniae*-inoculated mice died before day 70, and only 20 infected and six control mice underwent the further experiments and were analyzed. On day 70 after primary inoculation, a chronic type of inflammation with perivascular and peribronchial lymphocyte infiltration in the lung was observed in all *C. pneumoniae*-inoculated mice (Figure 1). Isolation of *C. pneumoniae* from

lung tissues was positive in all of the 20 *C. pneumoniae*-inoculated mice, and all infected mice had a positive PCR test for *C. pneumoniae* DNA (Table 1). No isolation of *C. pneumoniae* and negative PCR test for *C. pneumoniae* DNA were found for all six control mice.

Chronic and repeated *C. pneumoniae* lung infections result in increasing gene expression of IL-4

We used RT-PCR to measure the gene expression of IL-4, IL-5, IL-13 and IFN-γ in BAL fluid cells. Repeated inoculations with *C. pneumoniae* resulted in increased gene expression of IL-4 (Figure 2). Twelve of 20 (60%) infected mice had increased expression of IL-4 mRNA in BAL cells. No IL-4 mRNA expression was detected in control mice. In contrast to IL-4, the level of IL-13 and IFN-γ mRNA in BAL fluid cells did not differ between the two groups (Table 2). No IL-5 mRNA expression was detected in infected or control mice.

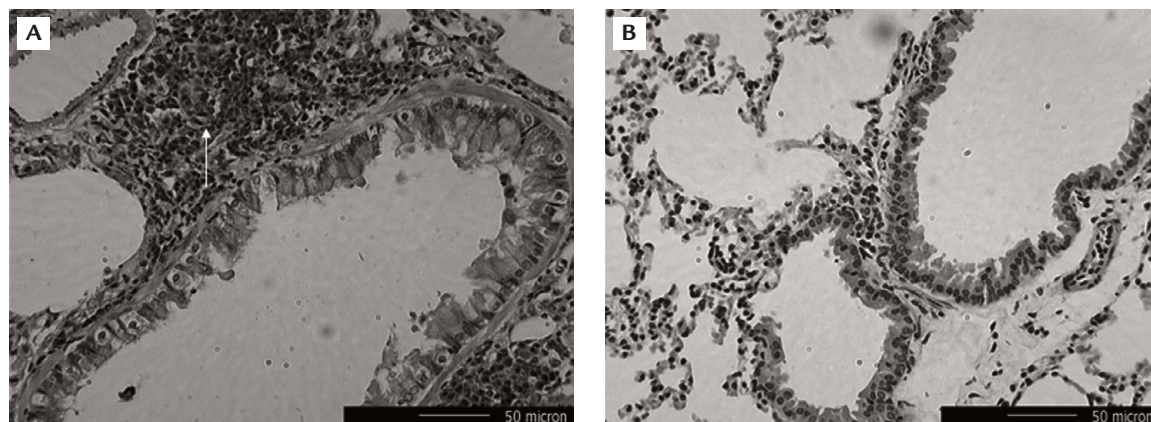


Figure 1. Histologic examination of lungs (hematoxylin & eosin). (A) Diffuse perivascular and peribronchial lymphoid reaction with accumulation of foci of lymphocytes in the lung tissue of *C. pneumoniae*-infected mice (arrow). Also shows increased thickness of subepithelial basement membrane layer in the airways of *C. pneumoniae*-infected mice. (B) Almost complete absence of pathologic change in the lung tissue of control mice. Bar = 50 μm.

Table 1. Isolation of *C. pneumoniae* in lung tissue and PCR test for *C. pneumoniae* in bronchoalveolar lavage fluid in infected and control mice

	Ratio of positive isolation of <i>C. pneumoniae</i> in lung tissue	Ratio of positive PCR test for <i>C. pneumoniae</i> in BAL fluid
<i>C. pneumoniae</i> inoculated mice	20/20 (100%)	20/20 (100%)
Control mice	0/6 (0%)	0/6 (0%)

Chronic and repeated *C. pneumoniae* lung infections result in increased thickness of subepithelial basement membrane

In order to determine if chronic and repeated *C. pneumoniae* lung infection resulted in increased thickness of the subepithelial basement membrane layer of the airways, morphometric analysis was carried out. There was a significant increase in the thickness of the basement membrane layer, present in the airways of infected mice after chronic and repeated *C. pneumoniae* lung infections (Figure 1). The thickness of the subepithelial basement membrane layer of the airways increased 1.6-fold in the infected as compared with control mice ($8.95 \pm 0.28 \mu\text{m}$ vs. $5.54 \pm 0.22 \mu\text{m}$, $p < 0.0001$) (Figure 3A). Mice with increasing IL-4 mRNA expression showed a positive correlation

with the increased thickness of subepithelial basement membrane in infected mice. The thickness of the subepithelial basement membrane in infected mice with increasing IL-4 mRNA and no IL-4 mRNA was $9.87 \pm 0.51 \mu\text{m}$ and $6.49 \pm 0.52 \mu\text{m}$, respectively ($p < 0.0001$) (Figure 3B). Neither increasing IFN- γ mRNA nor IL-13 mRNA revealed a correlation with the thickness of the subepithelial basement membrane in infected mice.

Discussion

The cause of intrinsic asthma is not clear, and persistent *C. pneumoniae* lung infection is speculated to be associated with initiation or exacerbation of adult intrinsic asthma. In this study, we developed a mouse model of chronic and repeated *C. pneumoniae* lung infection, and their pathologic changes with perivascular and peribronchial lymphocyte infiltration, which were similar to those of previous studies of chronic *C. pneumoniae* infection.^{19–22} We demonstrated that chronic and repeated *C. pneumoniae* lung infection in mice increased IL-4 mRNA expression, and this was correlated positively with the increased thickness of the basement membrane of the airways. These findings had some similarities with the increased IL-4 gene expression and airway remodeling in clinical asthma.

Increased production of IFN- γ and IL-4 have been observed in a mouse model of acute *C. pneumoniae* lung infection¹⁵ and prolonged ovalbumin challenge in sensitized mice.²³ Halme et al also reported interesting preliminary findings

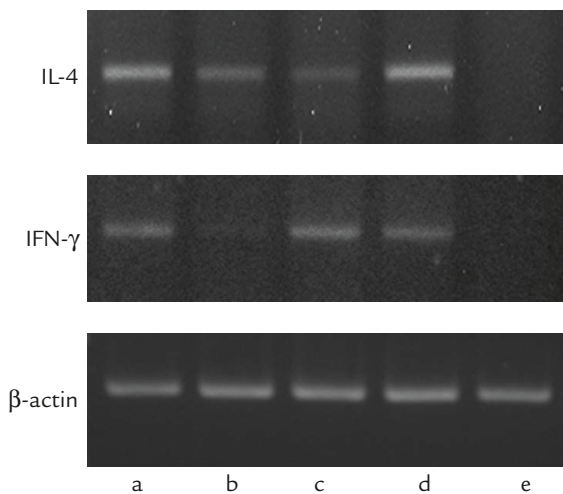


Figure 2. Representative gel shows increased expression of IL-4 mRNA in bronchoalveolar lavage cells in the first four samples (a–d) detected by RT-PCR. The first four samples were collected from infected mice and the last sample (e) was from control mice. No IL-4 mRNA expression was noted in control mice.

Table 2. RT-PCR analysis of cytokine gene expression in bronchoalveolar lavage cells in infected and control mice

	Mice with <i>C. pneumoniae</i> infection (n = 20)	Control mice (n = 6)
Ratio of IL-4 mRNA expression	12/20 (60%*)	0/6 (0%)
Ratio of IL-13 mRNA expression	10/20 (50%)	4/6 (66%)
Ratio of IFN- γ mRNA expression	10/20 (50%)	4/6 (66%)
Ratio of IL-5 mRNA expression	0/20 (0%)	0/6 (0%)

* $p < 0.01$ vs. control mice.

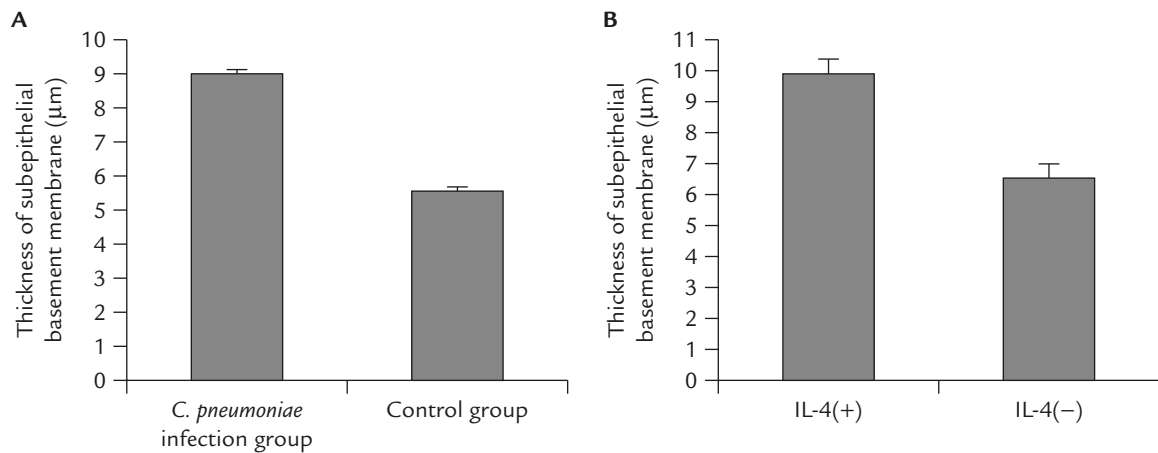


Figure 3. Morphometric analysis of thickness of subepithelial basement membrane of airway. (A) Thickness of the subepithelial basement membrane of airways was significantly increased in infected mice ($n=20$) in comparison with control mice ($n=6$). The thickness of the subepithelial basement membrane of airways increased 1.6-fold in the infected mice compared with control mice ($8.95 \pm 0.28 \mu\text{m}$ vs. $5.54 \pm 0.22 \mu\text{m}$, $p < 0.0001$). (B) Thickness of subepithelial basement membrane of airways was significantly increased in infected mice with increases of IL-4 mRNA expression ($n=12$), in comparison with infected mice without IL-4 mRNA expression ($n=8$). The thickness of subepithelial basement membrane in infected mice with and without increased IL-4 mRNA was $9.87 \pm 0.51 \mu\text{m}$ and $6.49 \pm 0.52 \mu\text{m}$, respectively ($p < 0.0001$).

that demonstrate IFN- γ and IL-4 mRNA expression in four human T-cell clones stimulated by *C. pneumoniae* antigens.²⁴ In our study, chronic and repeated *C. pneumoniae* lung infection was associated with increased IL-4 mRNA expression in BAL cells of mice. IL-4 level in BAL cells was also examined by ELISA in our study; however, an undetectable concentration was noted in infection and control groups.

McMillan and Lloyd developed a model of prolonged allergen challenge that shares many characteristics with the airway remodeling observed in human chronic asthma; increased goblet cell hyperplasia and collagen deposition during the chronic phase were observed.²³ In addition, up-regulation of IL-4 was noted in their study, which was similar to our study. We have developed a model of chronic and repeated *C. pneumoniae* lung infection, in which coexistent Th1 (IFN- γ) and Th2 (IL-4) responses were detected. IL-4 was found to be dominant in the BAL fluid cells during the chronic phase, and the increased expression of IL-4 mRNA correlated positively with the increased thickness of the subepithelial basement membrane. IL-4 has been described by several investigators to be a fibrogenic cytokine and has

been shown to regulate collagen biosynthesis in fibroblasts.^{10,11} The increased thickness of the subepithelial basement membrane in response to IL-4 is an important characteristic of airway remodeling in asthma.

We found increased expression of IL-4 mRNA in the mouse lung following repeated *C. pneumoniae* infection, but this result was different from previous studies. Penttila et al reported that a Th1-type immune response with IFN- γ production is characteristic of repeated infections, and have suggested that it plays a major role in the pathogenesis of *C. pneumoniae* infection.¹² In contrast, IL-4 immune responses were detected in our study, but no IL-4 production was detected in their study.

The discrepancy between our findings and those of Penttila et al may have several explanations. First, the type of host cells analyzed may affect the results. *C. pneumoniae* has been shown to be able to grow and multiply in several cell types, including mucosal epithelial cells, alveolar macrophages, smooth muscle cells, and endothelial cells.²⁵⁻²⁷ In our study, the cell profile in BAL fluid in the initial six infected mice was: lymphocytes, 83.5%; eosinophils, 9.6%; neutrophils, 5.6%; and macrophages, 1.3%. The study of Penttila et al

used purified mononuclear cells, which may have resulted in the loss of some important cytokine-modulating factors that are directly or indirectly contributed by other cells. A second possible explanation for the discrepancy is the difference in the methods used to analyze cytokine presentation. We measured the gene expression of BAL cells by RT-PCR, while Penttila et al used ELISA for cytokine production. ELISA may not be sufficiently sensitive to detect the small amount of cytokines present in pulmonary mononuclear cells. A third possible reason for the discrepancy is the differences in the infected doses. In our model, higher bacterial inocula were administered (5×10^6 IFU in our study vs. 1×10^6 IFU in Penttila et al's study). Cytokine response in *C. pneumoniae* infection has been shown to depend on the concentration of the bacterial inoculum, and inoculation of a higher concentration of bacteria increases the release of cytokines.²⁵ Th2-type immune response with cytokine production may have been too weak to be detected using the concentration of bacteria used by Penttila et al. A high concentration of bacteria also causes more severe inflammation and target organ damage.²⁸ In tuberculosis, the severity of lung damage determines a predominant Th1- or Th2-type response for milder or progressive disease, respectively.²⁹ Whether a similar immunologic shift occurs in repeated infections of *C. pneumoniae* should be considered. A fourth possible explanation for the discrepancy was the use of a different *C. pneumoniae* strain, which may have initiated different reactions. Penttila et al used *C. pneumoniae* isolate Kajaani 6, while *C. pneumoniae* TWAR 183 was used in this study. Studies of population antibody prevalence have shown that > 50% of adults worldwide have antibody against strain TWAR. It is important to clarify the effects of differences between strains when studying the immunopathologic mechanisms of *C. pneumoniae* infection.

IL-13 plays an important role in the pathologic changes of asthma.¹⁷ In our study, IL-13 mRNA expression in BAL cells was not increased in comparison with controls. In the study of McMillan and Lloyd, prolonged allergen challenge in mice

caused pathologic changes similar to the airway remodeling in asthma. Levels of IL-13 in BAL reduced during the chronic phase, while IL-4 and IFN- γ levels significantly increased, but the lung tissue levels of IL-13 were still elevated.²³ The level of IL-13 mRNA in the lung tissue was not measured in our study. However, the role of IL-13 in the pathology of repeated *C. pneumoniae* lung infection cannot be ruled out and needs further evaluation.

Airway remodeling is a summary term that describes structural changes in the airway in chronic asthma.³⁰ *C. pneumoniae* lung infection has not been explored for its ability to cause these structural changes. By morphometric analysis in our study, the thickness of the subepithelial basement membrane of the airways increased 1.6-fold in the infected mice in comparison with the control mice. In addition, increased IL-4 mRNA expression correlated positively with the increased thickness of the subepithelial basement membrane in infected mice.

In conclusion, we believe that we demonstrated for the first time that chronic and repeated *C. pneumoniae* respiratory infection induced Th2 immune response with increased IL-4 mRNA expression, and caused airway remodeling, with increased thickness of the subepithelial basement membrane in mice. These findings support speculation from epidemiologic studies that chronic *C. pneumoniae* infection plays some role in the initiation of intrinsic asthma. Further studies are required, such as using IL-4 knockout mice and analysis of cytokine expression in lung tissue of infected mice, to clarify the causal relationship between IL-4 gene expression and increased thickness of the subepithelial basement membrane in chronic *C. pneumoniae*-infected mice.

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