

IL-4-independent pathways exacerbate methacholine-induced airway hyperreactivity during mycoplasma respiratory disease

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Background: Mycoplasma respiratory infections are associated with wheezing and exacerbation of airway hyperresponsiveness (AHR) in asthmatic patients. IL-4 is a key cytokine in the development of AHR and airway reconstruction in asthmatic patients and might be an important component in exacerbation of AHR during pulmonary mycoplasma infection.

Objective: This study evaluates the effect of IL-4 on the severity of methacholine-induced AHR associated with mycoplasma pulmonary mycoplasma infection.

Methods: BALB/c and IL-4 knockout (KO) mice were infected with *Mycoplasma pulmonis*, and their enhanced pause scores were monitored before and after methacholine inhalation with whole-body plethysmography.

Results: IL-4 KO mice showed no difference in histopathology of the lungs before or after *Mycoplasma pulmonis* infection when compared with BALB/c mice. There was an increase in airway obstruction from days 7 to 21 after infection in both strains of mice, but there was no strain difference in airway resistance-associated mycoplasma disease. However, IL-4 KO mice had significantly higher methacholine-induced AHR after *M. pulmonis* infection when compared with BALB/c mice. There was no difference in AHR between uninfected IL-4 KO and control mice.

Conclusion: In contrast to our hypothesis, IL-4-independent pathways exacerbate methacholine-induced AHR and promote airway obstruction during the pathogenesis of mycoplasma respiratory disease. (J Allergy Clin Immunol 2004;114:645-9.)

Key words: IL-4, Mycoplasma, airway hyperresponsiveness

Since the 1970s, epidemiologic data show a correlation between asthma exacerbations and respiratory infections in human subjects. The physiologic and inflammatory

Abbreviations used

AHR: Airway hyperresponsiveness

OVA: Ovalbumin

Penh: Enhanced pause

mechanisms leading to increased airway hyperresponsiveness (AHR) in asthmatic patients with respiratory infections are not fully understood. Evidence suggests that allergic asthma is associated with T_H2 responses, in which IL-4-mediated inflammation causes AHR.¹ Studies in both human subjects and mice demonstrate the pathogenic importance of IL-4 in the development of AHR in allergic asthmatic patients.^{1,2} In fact, studies with IL-4 knockout (KO) mice demonstrate an attenuation of an allergic model of AHR compared with wild-type mice, yet few studies have examined the effect of IL-4 in an infectious model of AHR.¹ IL-4 is the key cytokine that leads to a T_H2 environment in the lung and to the development of asthma and asthma-induced AHR, and therefore IL-4 production might contribute to infection-mediated AHR, increasing the severity of asthma.

Mycoplasma pneumoniae is a common cause of both upper and lower respiratory tract infections in human subjects and is associated with the exacerbation of asthma. *M. pneumoniae* can be isolated from the respiratory tract of up to 25% of asthmatic patients experiencing acute exacerbations and is suggested as a possible contributing factor to the development of asthma in human subjects.³ In support, *M. pneumoniae* infection in mice after sensitization and challenge with ovalbumin (OVA) increases AHR.^{4,5} The mechanisms of mycoplasma-mediated AHR are unknown. However, mycoplasma infections initiate both T_H1- and T_H2-mediated adaptive immune responses in the lungs.⁶ Thus a likely hypothesis is that mycoplasma infections might lead to increased IL-4 production from mycoplasma-specific T_H2 cells, which thereby enhances the IL-4-mediated AHR in an asthmatic patient.

To examine the relationship of IL-4 and mycoplasma-mediated AHR, we took advantage of a well-characterized murine model of mycoplasma-induced respiratory disease. *Mycoplasma pulmonis* causes a natural murine respiratory disease with high morbidity and low mortality. Importantly, *M. pulmonis* infection in mice is similar to

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M pneumoniae infection in human subjects in terms of both the nature of histologic inflammation generated and the chronic nature of the disease.⁷ This murine model allows us to study a natural host-pathogen interaction that is similar to that of *M pneumoniae* in human subjects and its effects on AHR. In the present study IL-4 KO mice were used to identify the effect of IL-4 on methacholine-induced AHR after Mycoplasma infection.

METHODS

Mice

Viral- and mycoplasma-free BALB/c and IL-4 (BALB/c-IL4^{tm2Nnt}) KO mice were obtained from the Jackson Laboratories.⁸ Mice were housed in sterile microisolator cages supplied with sterile bedding and sterile food and water ad libitum. Female mice were used between 8 and 12 weeks of age. Before experimental infection, mice were anesthetized with an intramuscular injection of ketamine-xylazine.

Mycoplasma

The UAB CT strain of *M pulmonis* was used in all experiments. Stock cultures were grown, as previously described,⁹ in mycoplasma medium and frozen in 1-mL aliquots at -80°C . For inoculation, thawed aliquots were diluted to 10^5 colony-forming units/20 μL . Nasal pulmonary inoculations of 20 μL of diluted *M pulmonis* were given for experimental infections.

Plethysmography

Whole-body unrestrained plethysmography (Buxco, Troy, NY) was used to monitor the respiratory dynamics of mice in a quantitative manner before and after methacholine exposure. Before methacholine exposure, previously inoculated mice were allowed to acclimate to the chamber, and then plethysmography readings were recorded to establish baseline values. Next the mice were exposed to aerosolized methacholine (50 mg/mL); after exposure, plethysmography readings were recorded again. Enhanced pause (Penh) is a dimensionless value that represents a function of the ratio of peak expiratory flow to peak inspiratory flow and a function of the timing of expiration. Penh correlates with pulmonary airflow resistance or obstruction.

Penh, as measured by means of plethysmography, was previously validated in animal models of AHR.¹⁰ Baseline and methacholine challenge Penh readings were evaluated at days 7, 14, and 21 after infection.

Assessment of gross lesions and histopathology

Lungs were removed, and each lobe was examined by 2 observers for the presence of gross lesions. The percentage of each lobe with gross lesions was recorded. The gross lesion scores were weighted by the percentage that each lobe contributes to the total lung weight in arriving at the gross lesion index for lungs.¹¹

Lungs were fixed in alcohol formalin (4% glacial acetic acid [Fisher Scientific, Pittsburgh, Pa], 6% formaldehyde solution [Fisher Scientific], 40% deionize water, and 50% of 95% ethanol). Tissues were embedded in paraffin, sectioned at a thickness of 5 μm , and stained with hematoxylin and eosin for light microscopy. Each lung lobe was sectioned separately, randomly coded, and subjectively scored for lesion severity (scale of 0-4) on the basis of the characteristic lesions of murine respiratory mycoplasmosis, as described previously.¹² Scores refer to (1) peribronchial and vascular lymphoid hyperplasia or infiltration (lymphoid), (2) mixed neutrophilic and histiocytic exudate in alveoli (alveolitis), (3) neutrophilic exudate in airway lumina (neutrophilic exudate), and (4) hyperplasia

of airway mucosal epithelium (epithelial hyperplasia).¹² A score for each lesion was weighted according to the percentage each lobe contributes to the total lung weight in arriving at a total lesion score for each set of lungs. For each of the 4 lesions, a lesion index was calculated by dividing the observed lesion score by the maximum lesion score possible. Thus the maximum lesion index possible for any lesion was 1.0.

Statistical analysis

Data were evaluated by using ANOVA, followed by the Fisher protected least-square differences multigroup comparison. These analyses were performed with the StatView (SAS Institute, Cary, NC) computer program. A *P* value of .05 or less was considered statistically significant.

RESULTS

Control and IL-4 KO mice had increased airway obstruction after infection with *M pulmonis*. To determine whether IL-4 had an effect on pulmonary airway resistance and obstruction, control and IL-4 KO mice were experimentally infected with *M pulmonis* or sham inoculated, and then baseline Penh scores were monitored on days 7, 14, and 21 after infection by using whole-body unrestrained plethysmography.

M pulmonis infection leads to a time-dependent increase in baseline Penh values in both control and IL-4 KO mice (Fig 1, A). At day 7, there were no differences seen in Penh values between strains. However, by day 14 after infection, a significant increase in baseline Penh scores was detected in both strains of mice used and remained increased through day 21 after infection. There was no difference between infected control and IL-4 KO mice in terms of airway obstruction.

IL-4 KO mice had increased AHR after *M pulmonis* infection. To determine whether IL-4 had an effect on AHR during mycoplasma respiratory disease, control and IL-4 KO mice were experimentally infected with *M pulmonis* or sham inoculated, and Penh scores after methacholine exposure were monitored on days 7, 14, and 21 after infection by means of whole-body unrestrained plethysmography.

M pulmonis-infected IL-4 KO mice had a significant increase in Penh scores after methacholine exposure over sham-inoculated IL-4 KO mice and both uninfected and infected control mice (Fig 1, B), demonstrating an increase in methacholine-induced AHR in IL-4 KO mice infected with *M pulmonis*. However, there was no significant difference between infected and uninfected control mice in their methacholine-induced AHR.

There were also differences in mucus secretion from nasal passages between BALB/c and IL-4 KO mice. All IL-4 KO mice, control or infected, had little visible mucus accumulation on their muzzles after methacholine treatment, whereas infected and control BALB/c mice each consistently had significant mucus secretion from the nares ($n = 7$ for each group at each time point).

IL-4 KO mice and BALB/c mice did not differ in disease histopathology at day 21 after infection (Fig 2). To

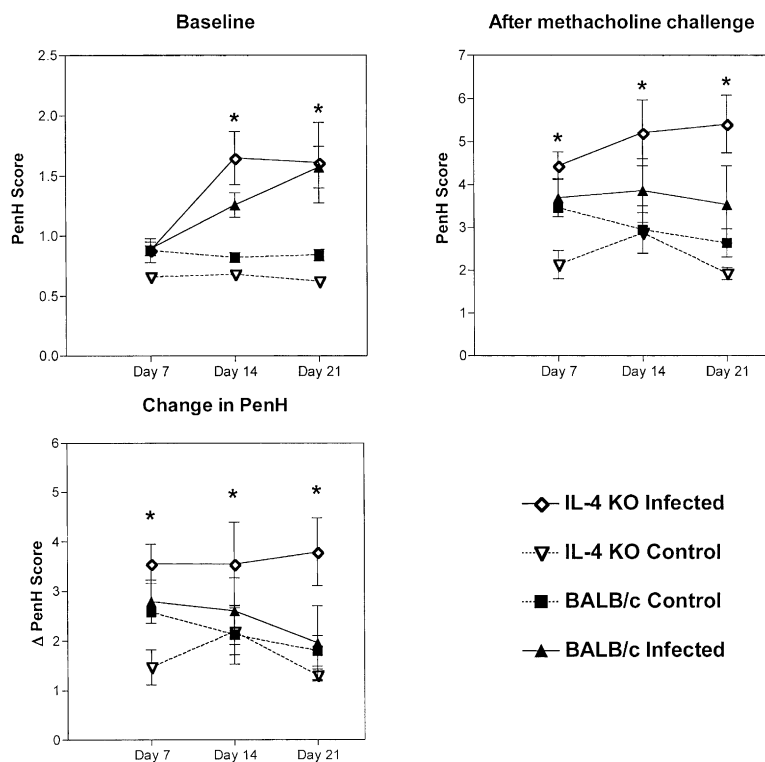


FIG 1. Airway obstruction and AHR scores of BALB/c and IL-4 KO mice. At days 0, 7, 14, and 21 after infection, breathing was monitored in BALB/c and IL-4 KO mice by means of unrestrained whole-body plethysmography. **A**, Penh scores before methacholine inhalation (*Baseline*); **B**, Penh scores after methacholine challenge; and **C**, differences between Penh scores before and after methacholine inhalation (*Change in Penh*). Points and error bars represent means $\pm$ SE ($n = 7$), and the asterisk denotes statistical difference ($P \leq .05$) from uninfected mice.

determine whether the loss of IL-4 affected disease pathology at day 21 after infection, lungs were removed after plethysmography at day 21 after infection. Lungs were analyzed for percentage of gross lesions and fixed for histopathology and scored for lesions severity.

At day 21 after infection, IL-4 KO mice did not significantly differ in gross lesion severity from BALB/c mice. Furthermore, there were no characteristic differences in types of histopathologic lesions or severity of lesions seen between both strains of mice after 21 days of infection.

DISCUSSION

Studies have begun to examine the role of infectious agents and the development of AHR. However, the mechanism behind exacerbated AHR caused by respiratory infections is still unknown. Studies with *M pneumoniae* clearly demonstrate that mycoplasma infections can exacerbate AHR in the OVA asthma model when the infection is introduced after sensitization to OVA.⁴ In addition, *M pneumoniae* can induce AHR independently of OVA.⁵ *M pulmonis* infection in mice creates both similar histologic inflammation and a chronic disease similar to human mycoplasma infections. The present

studies use a murine model of mycoplasma respiratory disease to help elucidate the host response to infectious agents and how these agents exacerbate AHR.

Airway obstruction increased because of *Mycoplasma*-induced disease in both IL-4 KO and wild-type mice. Both IL-4 KO and wild-type mice had airway obstruction between days 7 to 14 after mycoplasma infection, which continued through day 21. Furthermore, there was no significant difference in airway obstruction between wild-type and IL-4 KO infected mice. Interestingly, the development of airway obstruction coincides with the development of a strong T-cell response.⁶ Therefore airway obstruction, which develops after *M pulmonis* infection, is most likely adaptive and immune dependent, although independent of IL-4. Thus IL-4 is not important in the development of airway obstruction during *M pulmonis* respiratory disease.

Surprisingly, AHR during *Mycoplasma*-induced disease was also independent of IL-4. In fact, IL-4 KO mice had a greater increase in methacholine-induced AHR after *M pulmonis* infection than all other groups tested. We previously demonstrated that there was no significant difference in pulmonary disease severity between IL-4 KO and BALB/c mice through day 14,¹³ which, as shown here, continues through day 21. Furthermore, there is no significant difference in the numbers of pulmonary T cells,

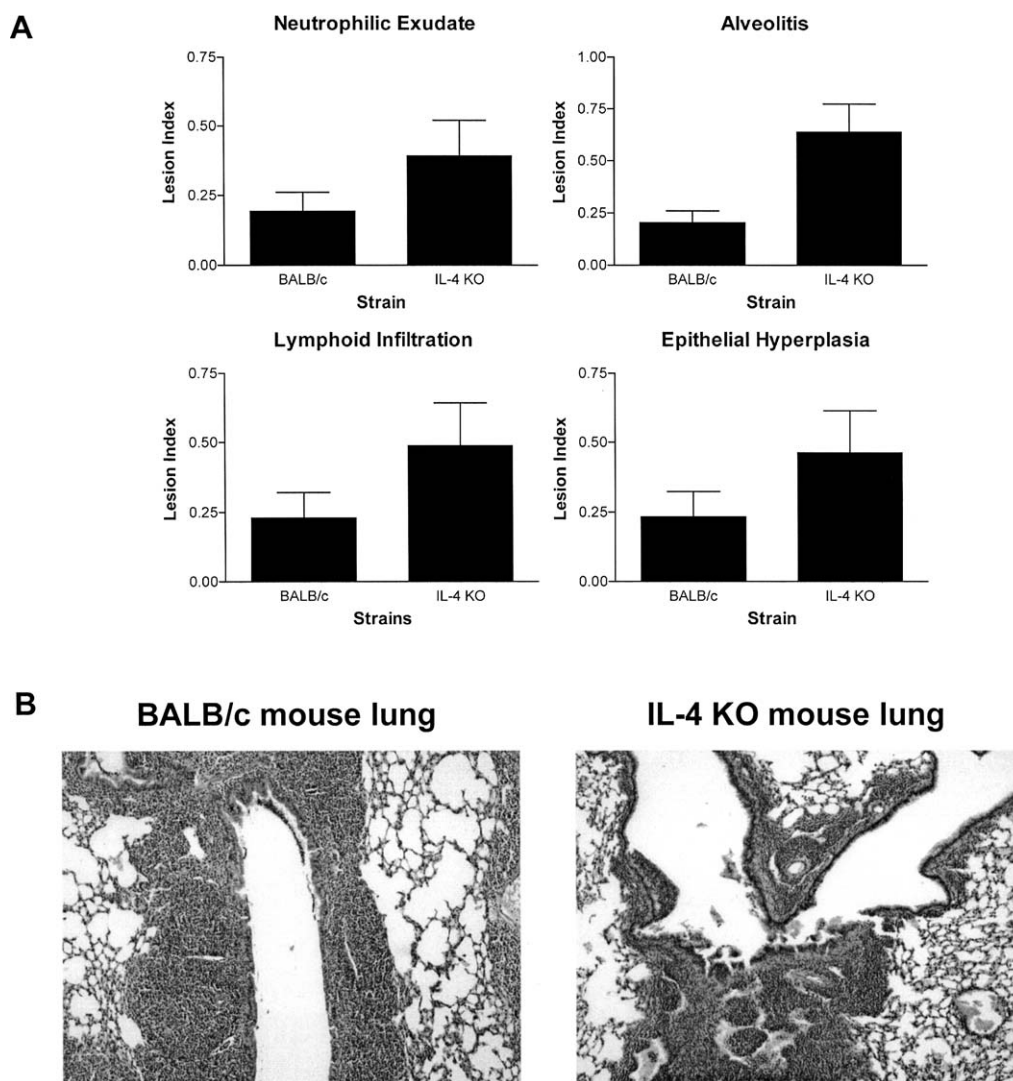


FIG 2. Histopathology of IL-4 KO and BALB/c mice 21 days after infection. **A**, Twenty-one days after infection, lungs were removed from mice and fixed. Lungs were then scored for severity of histopathologic lesions. Vertical bars and error bars represent means $\pm$ SE ($n = 6$). **B**, Images are representative of lesion severity and type seen in lungs of BALB/c and IL-4 KO mice at day 21 after infection in areas of disease. Otherwise, lungs were relatively free of lesions.

B cells, or macrophages in infected IL-4 KO mice compared with that seen in infected wild-type mice.¹³ Importantly, there is no correlation between lung disease severity and Penh scores.

Although mice breathe predominantly through their noses, and changes in the upper respiratory tract could contribute to changes in AHR, we found no significant difference in the severity of nasal passage disease between IL-4 and BALB/c mice in previous studies.¹³ An additional possibility is that increased nasal secretions in IL-4 KO mice after methacholine exposure results in an increase in airway resistance in the upper respiratory tract. However, IL-4 KO mice, naive or infected, were noted to have significantly less mucus accumulation on their muzzles after methacholine treatment than BALB/c infected and control mice. Therefore the increase in AHR in

IL-4 KO mice is not likely caused by inflammatory or secretory events in nasal passages but most likely caused by altered physiologic responses in the lower respiratory tract.

Evidence demonstrates an association between several respiratory infectious agents, notably *Mycoplasma*, in either the development or exacerbation of asthma,¹⁴ but the pathogenic mechanisms by which respiratory infections affect asthma is poorly understood. In addition, respiratory infections (eg, *M pneumoniae*) can lead to wheezing and AHR. Our studies begin to elucidate the mechanisms by which pulmonary infections can affect and exacerbate AHR. In contrast to current theories on AHR in asthma,^{1,2} we showed that IL-4-independent events can lead to increased AHR during mycoplasma infections, whereas IL-4-mediated events can dampen these responses. It is likely that other proinflammatory

cytokines (eg, IFN- γ), other T_H2 cytokines (eg, IL-9 or IL-13), or other inflammatory processes are responsible for the increased AHR in these IL-4 KO mice. In similar studies increased levels of IFN- γ were detected in the bronchoalveolar lavage fluid of BALB/c mice during *M pneumoniae*-induced pneumonia, which correlated with increased AHR.¹⁵ This coincides with recent human studies, in which T_H1 cytokines are isolated from the bronchoalveolar lavage fluid of patients experiencing acute asthmatic episodes.² Regardless of mechanism, this work clearly shows IL-4-independent pathways are contributing to AHR during mycoplasma respiratory disease, and the ability to produce IL-4 can counterbalance the processes leading to more severe methacholine-induced AHR. These studies also suggest the potential for a synergistic effect between allergen-specific IL-4-dependent responses and IL-4-independent responses against *Mycoplasma* species in the exacerbation of AHR in some asthmatic patients with mycoplasma infections.

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