

Early-life chlamydial lung infection enhances allergic airways disease through age-dependent differences in immunopathology

Jay C. Horvat, PhD,^{a*} Malcolm R. Starkey, BBioMedSci(Hon),^{a*} Richard Y. Kim, BBioMedSci(Hon),^a Simon Phipps, PhD,^a Peter G. Gibson, MBBS,^a Kenneth W. Beagley, PhD,^b Paul S. Foster, PhD,^a and Philip M. Hansbro, PhD^a *Newcastle and Kelvin Grove, Australia*

Background: Asthma typically originates in early-life, and the impact of infection during immunologic maturation is a critical factor in disease pathogenesis. The progression of aberrant T_H2 cell responses and disease development has been attributed to a lack of infections. However, exposure to specific pathogens such as *Chlamydia* may alter immunologic programming and predispose to asthma.

Objective: To investigate the effects of chlamydial infection at different ages on allergic airways disease in later life.

Methods: Neonatal, infant, or adult BALB/c mice were infected and 6 weeks later were sensitized and subsequently challenged with ovalbumin. Hallmark features of allergic airways disease were compared with uninfected allergic and nonallergic controls.

Results: Early-life (neonatal and infant) but not adult chlamydial infection enhanced the development of hallmark features of asthma in ovalbumin-induced allergic airways disease. Notably early-life infection increased mucus-secreting cell numbers, IL-13 expression, and airway hyperresponsiveness. Neonatal infection attenuated eosinophil influx and ovalbumin-specific T_H2 cytokine release and numbers of activated myeloid dendritic cells (DCs) in lymph nodes. By contrast, infant infection augmented features of allergic inflammation with increased airway eosinophils, T_H2 cytokine, and DC responses. Both neonatal and infant infection increased systemic DC-induced IL-13 release from CD4⁺ T cells. The timing of infection had

significant effects on lung structure because neonatal but not infant or adult infection induced increases in alveolar diameter. **Conclusion:** Early-life respiratory chlamydial infections modulate immune responses, alter lung function and structure, and enhance the severity of allergic airways disease in later life. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Asthma, Chlamydia, immunologic programming, lung structure, dendritic cell, IL-13, infant, neonate, lung function

Asthma is a chronic inflammatory condition of the airways with recurring exacerbations of disease that is underpinned by aberrant T_H2-cell responses to environmental antigens.^{1,2} The hallmark features of asthma are promoted by effector functions of the T_H2 cytokines, IL-4, IL-5, and IL-13.³ In particular, IL-13 is a key regulator of mucus-secreting cell hyperplasia, airway hyperresponsiveness (AHR), and remodeling of the airways, which lead to bronchial obstruction and limitations in airflow.⁴

Asthma is most common in children, and the majority of adult asthma originates in childhood,⁵⁻⁷ indicating that early-life events are important in asthma pathogenesis. The inflammatory basis of asthma suggests that altered immunologic programming in early life by specific infections or the lack of exposure to infection may play a critical role in the induction and progression of disease. However, the role and nature of infections and mechanisms that are involved remain unknown. Some studies show inverse associations between T_H1-inducing infections during early life and asthma prevalence.⁸ However, respiratory tract infection with *Chlamydomydia pneumoniae* is a notable exception and is increasingly linked with the development and exacerbation of asthma in children and adults.⁹

Respiratory infections with *C pneumoniae* are common and usually asymptomatic but are responsible for as many as 22% of all cases of community-acquired pneumonia requiring hospitalization.^{10,11} Protection and resolution are mediated by strong T_H1 immune responses,^{12,13} but the mechanisms of how T_H1-inducing chlamydial lung infections are associated with T_H2-mediated asthma remain unknown. The age of infection may be critical because the phenotype of the immune system may be molded by early-life infection.

We have previously shown that neonatal chlamydial lung infection in mice induced mixed T_H1/T_H2 immune responses and increased mucus-secreting cell numbers and AHR in allergic airways disease in later life.¹⁴ However, the underlying mechanisms

From ^athe Centre for Asthma and Respiratory Disease and Hunter Medical Research Institute, University of Newcastle, and ^bthe Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove.

*These authors contributed equally to this work.

Supported by grants from the National Health and Medical Research Foundation of Australia (project grants 401238 and 569219), the Asthma Foundation of NSW, the Rebecca Cooper Medical Research Foundation, the University of Newcastle project grants and Brawn Post-doctoral Fellowship, the Hunter Medical Research Institute, and the Australian Research Council (0559210).

Disclosure of potential conflict of interest: K. W. Beagley has received research support from NHMRC Australia and ARC Australia and is a councilor for the Society for Mucosal Immunology. The rest of the authors have declared that they have no conflict of interest.

Received for publication April 5, 2009; revised October 16, 2009; accepted for publication October 19, 2009.

Reprint requests: Philip M. Hansbro, PhD, Discipline of Immunology & Microbiology, Level 3, David Maddison Clinical Sciences Building, corner of King and Watt Streets, Newcastle, Australia 2300. E-mail: Phil.Hansbro@newcastle.edu.au.

0091-6749/\$36.00

© 2010 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2009.10.018

Abbreviations used

AHR: Airway hyperresponsiveness
DC: Dendritic cell
mDC: Myeloid dendritic cell
MLN: Mediastinal lymph node
Treg: Regulatory T

for the generation of immune phenotype and enhanced disease was not elucidated. Respiratory tract infections in early life may alter the nature of DC:T-cell interactions to antigenic exposure, which promote T-cell responses and drive the development and progression of asthma.¹⁵⁻¹⁸ Early-life infection may also lead to impaired lung function and irreversible damage to pulmonary structure because the lungs, unlike most other organs, continue to mature during the first 2 years of life.¹⁹ Thus, early-life lung infections may permanently affect pulmonary immunity, function, and structure.²⁰

Understanding of the relationship between early-life infection and its impact on the immune system and the expression of asthma is limited. In this investigation we examined the effect of infection at different periods of life (neonatal, infant, and adult) on the subsequent expression of hallmark features of allergic airways disease in later life.

METHODS

Additional details are described in this article's [Methods](#) section in the Online Repository at www.jacionline.org.

Experimental models

Neonatal (24 hours), infant (3 weeks), and adult (6 weeks) BALB/c mice were infected intranasally with *Chlamydia muridarum* (400 [neonates] or 100 [infants/adults] inclusion-forming units, ATCC VR-123, in 5 μ L [neonates] or 30 μ L [infants/adults] sucrose phosphate glutamate buffer). After 45 days, mice were sensitized and challenged with ovalbumin in the T_H2-inducing adjuvant, alum ([Fig 1](#)), as previously described.^{14,21} Animals were euthanized 24 hours after the final ovalbumin challenge, and allergic airways disease was assessed. Controls were infected and nonallergic (Neo, Inf, and Ad), uninfected and allergic (Ova), or uninfected and nonallergic (Sham). All experiments were approved by the University of Newcastle animal ethics committee.

Airway inflammation

Lungs were perfused, inflated, fixed, embedded, sectioned, and stained. Eosinophil and mucus-secreting cell numbers were enumerated in inflamed airways.¹⁴

Lung function

Lung function, in terms of AHR, was measured in anaesthetized mice using whole-body plethysmography by determination of average peak airway resistance and dynamic compliance in response to increasing doses of methacholine (Sigma, Castle Hill, Australia).¹⁴

IL-13 mRNA expression in lung tissue

Total RNA was extracted from whole lung tissue by using TRIzol (Invitrogen, Mount Waverley, Australia) and reverse-transcribed by using Superscript III and random hexamer primers (Invitrogen). Relative abundance of cytokine cDNA was determined compared with the reference gene

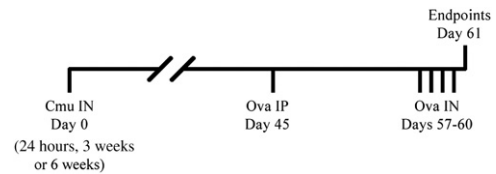


FIG 1. Study protocols. Neonatal, infant, or adult mice were infected intranasally (IN) with *C muridarum* (Cmu) and 45 days later sensitized intraperitoneally (IP) and challenged IN with ovalbumin (Ova) to induce allergic airways disease. Key features of allergic airways disease were characterized 1 day after the final Ova challenge on day 61. Day 0, Infection at <24 hours (neonates), 3 weeks (infants), or 6 weeks (adults) of age.

hypoxanthine-guanine phosphoribosyltransferase by real-time PCR by using an ABIPrism7000 Sequence Detection System (Applied Biosystems, Scoresby, Australia).²² Primers used are shown in this article's [Table E1](#) in the Online Repository at www.jacionline.org.

Ovalbumin-specific mediastinal lymph node T-cell cytokine release

Mediastinal lymph node (MLN) cells (5×10^6 cells) were isolated, restimulated with ovalbumin (200 μ g/mL; Sigma), and cultured for 6 days in Gibco RPMI-1640 (Invitrogen) containing 10% FCS, 20 mmol/L HEPES, 10 μ g/mL penicillin/streptomycin, 2 mmol/L L-glutamine, and 50 μ mol/L 2-mercaptoethanol. IFN- γ , IL-5, IL-13, and IL-10 concentrations in culture supernatants were determined by ELISA.

Flow-cytometric analysis of MLN cells

Single-cell suspensions of MLN cells (1×10^6) were stained for surface markers.^{23,24} Cells were analyzed by flow cytometry (FACSCanto; BD Biosciences, San Jose, Calif). Regulatory T (Treg) cells were characterized as CD4⁺CD25⁺forkhead box protein (Foxp) 3⁺,²⁴ myeloid DCs (mDCs) were characterized as CD11c⁺CD11b^{hi}B220⁺,²³ and maturation and activation of mDCs was assessed by determination of MHC II expression. All antibodies were from BD Biosciences except MHC II (eBioscience, San Diego, Calif).

Systemic DC-induced CD4⁺ T-cell IL-13 release

DCs (5×10^4 cells) were isolated from spleens, pulsed with ovalbumin-peptide (50 μ g/mL), and cocultured with ovalbumin-peptide-specific CD4⁺ T cells (DO11.10, 3×10^5 cells).²⁵ DC:T-cell coculture supernatants were collected and assessed for IL-13 release by ELISA.

Lung structure

Average alveolar diameter was evaluated in infected, nonallergic lungs 9 weeks after infection by determination of the mean linear intercept.²⁶ This is the same time after infection that allergic airways disease was determined in infected, allergic groups. The development of ovalbumin-induced allergic airways disease induces substantial inflammation in the lung that does not allow accurate analysis of alveolar size because of a lack of definition between cellular infiltrates and parenchymal structures. Forty random images of hematoxylin and eosin-stained lung sections were captured per lung ($\times 40$ magnification). The first 10 images that did not contain airways and/or blood vessels were overlaid with an 11-horizonal line template. Intercepts of alveolar walls with lines were enumerated and the alveolar diameter calculated by dividing the total length of the 11 lines by the average number of intercepts per lung section.

Statistics

Results are representative of 2 or 3 independent experiments. Each experimental iteration consisted of at least 4 mice. Results are presented as mean $\pm$

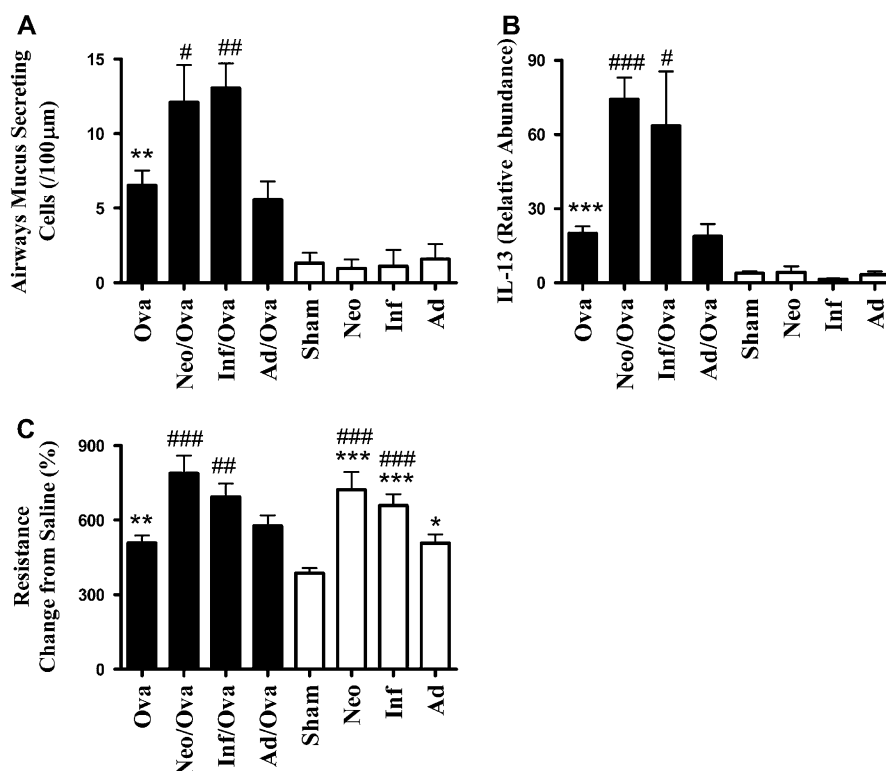


FIG 2. Early-life infection increases airway mucus-secreting cells, IL-13 expression in lung tissue, and AHR in allergic airways disease in later life. **A**, Mucus-secreting cells surrounding the airway lumen. **B**, Relative abundance of IL-13 mRNA in lung tissue. **C**, AHR in terms of averaged peak airways resistance at the maximal methacholine dose (50 mg/mL) administered. For the entire dose response curves, see Fig E4. Black bars represent allergic groups, whereas white bars represent nonallergic groups. Results are representative of 2 or 3 independent experiments, and combined results are presented as means $\pm$ SEMs where $n \geq 8$ individual mice. #Significant differences compared with uninfected, allergic (Ova) control; *significant differences compared with uninfected, nonallergic (Sham) control. #/ $P < .05$; ##/ $P < .01$; ###/ $P < .001$. Group abbreviations: Ova, uninfected, allergic groups; Neo/Ova, Inf/Ova, and Ad/Ova, neonatal, infant, and adult infected, allergic groups, respectively; Sham, uninfected, nonallergic groups; Neo, Inf, and Ad, neonatal, infant, and adult infected, nonallergic groups, respectively. Uninfected controls (Ova and Sham) were 6 weeks old at the time of sensitization.

SEM, which were determined by combining results from each independent experiment. Therefore, each result is representative of at least 8 individual mice. The Mann-Whitney test for 2 independent samples was used for nonparametric tests. Between-group comparisons of airways resistance and compliance were performed by using 1-way repeated-measures ANOVA. Analyses were conducted by using the intercooled Stata 9.1 statistical package (Stata Corp, College Station, Texas) or Graph Pad Prism 4 (GraphPad Software, La Jolla, Calif).

RESULTS

For some results, see this article's Figs E1 to E4 in the Online Repository at www.jacionline.org.

We have previously shown that chlamydial numbers and histopathology in the lung during neonatal and adult infections peak between 10 and 15 days after inoculation, with bacterial clearance achieved by 21 days and pulmonary inflammation largely resolved by 45 days (ie, time of ovalbumin sensitization in this study; Fig 1).¹⁴ In preliminary studies we demonstrated that infection of infants with *C muridarum* elicited similar profiles of infection, inflammation, and pathological responses compared with infection of neonates and adults (Fig E1). We have also shown that the age at the time of sensitization (6 weeks, 9 weeks,

or 12 weeks old) does not affect key features of ovalbumin-induced allergic airways disease (Figs E2 and E3). Therefore, any changes in allergic airways disease induced by infection are determined by the age of infection and not the age of sensitization. To simplify the presentation of the results, only single ovalbumin and sham control groups (sensitized at 6 weeks) are presented in some of the figures. We have also shown that uninfected, sham-sensitized control groups do not differ in any features of pulmonary immune responses or physiology compared with uninfected groups that have not been sham-sensitized.¹⁴

Early-life infection enhances mucus-secreting cell numbers and IL-13 mRNA expression in the lung and AHR during allergic airways disease

First we assessed the effect of infection at different stages of life on the development of hallmark features of asthma according to the experimental protocols in Fig 1. The induction of allergic airways disease in the absence of infection (uninfected, allergic groups; Ova) resulted in significantly increased airways mucus-secreting cell numbers, augmented IL-13 mRNA expression in

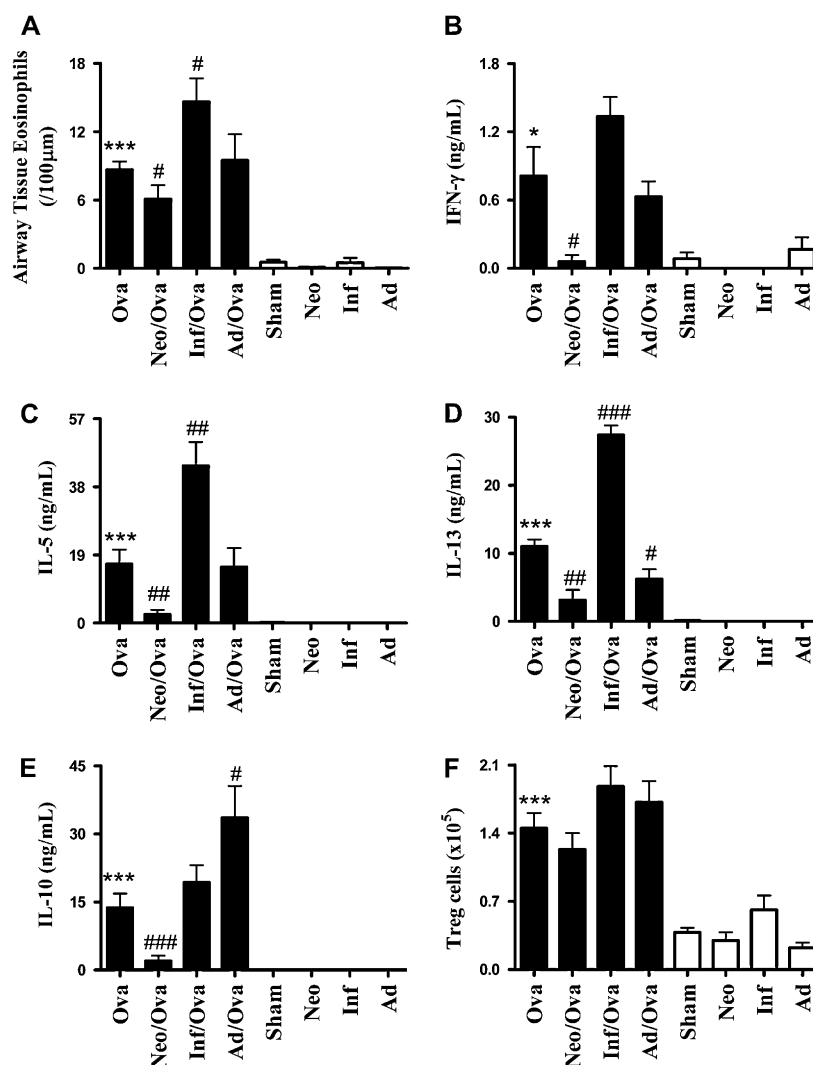


FIG 3. Early-life infection differentially affects pulmonary eosinophil influx and ovalbumin-specific cytokine responses in MLNs during allergic airways disease in later life. **A**, Eosinophil numbers within 100 μ m of airway basement membrane. Ovalbumin-specific IFN- γ (**B**), IL-5 (**C**), IL-13 (**D**), and IL-10 (**E**) release in MLN culture supernatants. **F**, CD4⁺CD25⁺forkhead box protein 3⁺ Treg cell numbers in MLNs. Black bars represent allergic groups, whereas white bars represent nonallergic groups. Results are representative of 2 or 3 independent experiments, and combined results are presented as means $\pm$ SEMs where $n \geq 8$ individual mice. #Significant differences compared with uninfected, allergic (Ova) control; *significant differences compared with uninfected nonallergic (Sham) control. ## $P < .05$; ### $P < .01$; #### $P < .001$. Group abbreviations: Ova, uninfected, allergic groups; Neo/Ova, Inf/Ova, and Ad/Ova, neonatal, infant, and adult infected, allergic groups, respectively; Sham, uninfected, nonallergic groups; Neo, Inf, and Ad, neonatal, infant, and adult infected, nonallergic groups, respectively. Uninfected controls (Ova and Sham) were 6 weeks old at the time of sensitization.

lung tissue, and reduced lung function in terms of increased AHR (increased airways resistance and decreased compliance) compared with uninfected, nonallergic (Sham) controls (Fig 2, A-C; Figs E2-E4).

Importantly, both neonatal (Neo/Ova) and infant (Inf/Ova) infections significantly increased mucus-secreting cell numbers and IL-13 mRNA expression in lung tissue and reduced lung function compared with uninfected, allergic (Ova) controls (Fig 2, A-C; Fig E4). By contrast, adult infection (Ad/Ova) had no effect on these features of allergic airways disease.

Interestingly, infection at all ages in the absence of allergic airways disease (Neo, Inf, and Ad) resulted in reduced lung

function compared with uninfected, nonallergic (Sham) controls (Fig 2, C; Fig E4). The effects were more severe after early-life infection, with neonatal and infant infections resulting in increased airways resistance and decreased dynamic compliance, whereas an adult infection resulted in increased resistance only (Fig 2, C; Fig E4).

Notably there was no difference in airways resistance between infected, allergic, and infected groups without allergy that were infected as neonates or infants. However, allergic groups that had been infected in early life (Neo/Ova and Inf/Ova) had significantly reduced dynamic compliance compared with infected, nonallergic (Neo and Inf) controls (Fig E4).

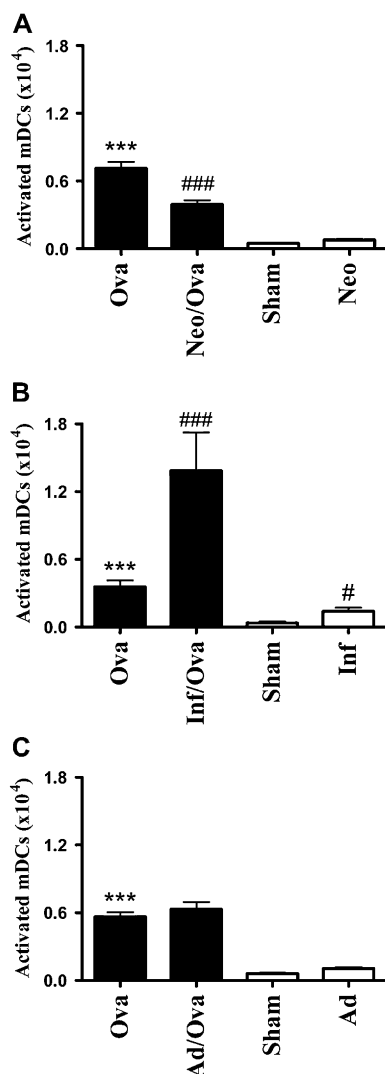


FIG 4. Early-life infection alters the numbers of activated mDCs in MLNs during allergic airways disease in later life. **A-C**, Number of activated (MHC II⁺) CD11c⁺CD11b⁺B220⁺ mDCs in MLNs in neonatal, infant, and adult infected groups, respectively. Black bars represent allergic groups, whereas white bars represent nonallergic groups. Results are representative of 2 or 3 independent experiments, and combined results are presented as means $\pm$ SEMs where $n \geq 8$ individual mice. #Significant differences compared with uninfected, allergic (Ova) control; *significant differences compared with uninfected, nonallergic (Sham) control. # $P < .05$; ###/**** $P < .001$. Group abbreviations: Ova, uninfected, allergic groups; Neo/Ova, Inf/Ova, and Ad/Ova, neonatal, infant, and adult infected, allergic groups, respectively; Sham, uninfected, nonallergic groups; Neo, Inf, and Ad, neonatal, infant, and adult infected, nonallergic groups, respectively. All controls are age-matched.

Age of infection differentially affects pulmonary eosinophil numbers during allergic airways disease

Eosinophils are pivotal in allergic responses and have been associated with mucus accumulation and AHR.^{27,28} Therefore, we investigated the effect of chlamydial infection at different ages on pulmonary eosinophil numbers.

Uninfected, allergic (Ova) groups had significantly greater numbers of eosinophils associated with airway basement membrane than uninfected, nonallergic (Sham) controls (Fig 3, A). Interestingly, neonatal infection (Neo/Ova) decreased whereas an infant infection (Inf/Ova) increased eosinophil

numbers compared with uninfected, allergic (Ova) controls (Fig 3, A). Adult infection (Ad/Ova) had no effect on eosinophil numbers (Fig 3, A).

Age of infection differentially affects ovalbumin-specific MLN T-cell cytokine release during allergic airways disease

Enhanced mucus hypersecretion, IL-13 expression, and AHR during allergic airways disease may be driven by antigen-specific T_H2 cytokine release from T_H2 cells in the lung draining MLNs. Therefore, we examined whether infection enhanced antigen-specific cytokine release from MLN T cells. Ovalbumin-specific release of inflammatory T_H1 (IFN- γ) and T_H2 (IL-5 and IL-13) and anti-inflammatory (IL-10) cytokines from MLN T cells was assessed.

Uninfected, allergic (Ova) groups had increased levels of ovalbumin-specific IFN- γ , IL-5, IL-13, and IL-10 release from MLN T cells compared with uninfected, nonallergic (Sham) controls (Fig 3, B-E; Fig E3). Interestingly, neonatal infection (Neo/Ova) suppressed ovalbumin-specific IFN- γ , IL-5, IL-13, and IL-10 release compared with uninfected, allergic (Ova) controls (Fig 3, B-E). By contrast, infant infection (Inf/Ova) increased the release of T_H2 cytokines IL-5 and IL-13 (Fig 3, C and D) but had no significant effect on IFN- γ or IL-10 (Fig 3, B and E). Therefore, although early-life infections enhance mucus-secreting cell hyperplasia, pulmonary IL-13 expression and AHR in allergic airways disease in later life, neonatal, and infant infections have differential effects on MLN T-cell cytokine release. Adult infection (Ad/Ova) had no effect on IFN- γ or IL-5 but reduced IL-13 (Fig 3, B-E). Although IL-10 release is increased compared with the uninfected, allergic (Ova) control in this figure, there is no increase compared with age-matched controls (Fig E3, D).

Age of infection does not affect MLN Treg cell numbers during allergic airways disease

Differential release of T_H1 and T_H2 cytokines from MLN T cells in neonatal and infant infected, allergic groups cannot be explained by alterations in the levels of the anti-inflammatory cytokine IL-10. Because Treg cells regulate cytokine release in MLNs,²⁴ we investigated whether the differential effects of neonatal and infant infection on T-cell cytokine release correlated with altered Treg cell numbers in MLNs. Uninfected, allergic (Ova) groups had significantly increased Treg cell numbers in the MLNs compared with nonallergic (Sham) controls (Fig 3, F). Infection, regardless of age, did not alter Treg cell numbers in MLNs compared with uninfected, allergic (Ova) controls (Fig 3, F).

Age of infection differentially affects the number of activated mDCs in MLNs

Dendritic cells are pivotal inducers of T-cell responses, and increased numbers of activated DCs are associated with increased inflammatory responses.^{29,30} mDCs have a more mature phenotype with increased costimulatory molecule and MHC II expression than plasmacytoid DCs, induce T_H2 cytokine release, and promote allergic airways disease.³¹⁻³³ MHC II surface expression is a marker of DC activation and enhanced antigen presentation.^{29,30} Therefore, we investigated whether

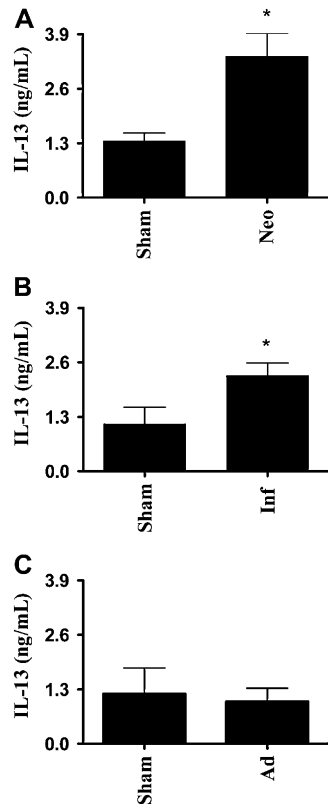


FIG 5. Early-life infection alters systemic DC function in later life. **A-C**, Systemic ovalbumin-peptide pulsed DC-induced IL-13 release by ovalbumin-peptide-specific CD4⁺ T cells; DCs were from infected nonallergic neonatal, infant, and adult groups, respectively. Results are representative of 2 or 3 independent experiments and combined results are presented as means $\pm$ SEMs where $n \geq 8$ individual mice. *Significance compared with uninfected, nonallergic (Sham) control. Group abbreviations: Neo, Inf, and Ad, neonate, infant, and adult infected, nonallergic groups, respectively; Sham, uninfected, nonallergic groups. All controls are age-matched.

differences in MLN T-cell cytokine release from different groups may be driven by infection-induced alterations in the number of activated (MHC-II⁺) mDCs.

Uninfected, allergic (Ova) groups had significantly increased numbers of activated mDCs in MLNs compared with nonallergic (Sham) controls (Fig 4, A-C). Interestingly, neonatal infection (Neo/Ova) significantly reduced the number of activated mDCs compared with uninfected, allergic (Ova) controls (Fig 4, A). By contrast, infant infection (Inf/Ova) substantially increased the number of activated mDCs (Fig 4, B). Adult infection (Ad/Ova) had no effect (Fig 4, C).

Age of infection differentially affects systemic DC-induced IL-13 release from CD4⁺ T cells

Although early-life infections had differential effects on the influx of eosinophils into the airways and ovalbumin-specific T-cell cytokine and DC responses in the MLNs during allergic airways disease, both neonatal and infant infections augmented IL-13 expression in the lung. To identify the potential common source of IL-13 in the lung, we investigated whether infection altered systemic DC-induced release of IL-13 from T cells. DCs from infected, nonallergic rather than infected, allergic groups

were used. DCs from infected, nonallergic groups give a better indication of the effect of infection on DC function. The effect of infection on DCs from infected, allergic groups may be masked by the effects of ovalbumin sensitization and challenge on DC phenotype.

Significantly, systemic DCs from both neonatal and infant (Neo and Inf) but not adult (Ad) infected, nonallergic groups induced an increase in IL-13 release from ovalbumin-peptide-specific CD4⁺ T cells compared with DCs from uninfected (Sham) controls (Fig 5, A-C).

Infection results in altered lung structure in later life

Because lung function was reduced in all infected, nonallergic groups compared with uninfected, nonallergic controls, lungs were examined for the presence of remodeling of the airways or altered structure.

Infection at any age did not induce any detectable changes in the thickness of airways-associated basement membrane, epithelial cell, or smooth muscle layers compared with uninfected controls (not shown). However, neonatal infection induced striking changes in parenchymal structure with substantial increases in alveolar size (Fig 6, A and B). Neonatal infection resulted in a $\approx 50\%$ increase in average alveolar diameter compared with uninfected (Sham) controls (Fig 6, B). Infant and adult infections did not significantly affect alveolar diameter (Fig 6, B).

DISCUSSION

We have demonstrated that early-life chlamydial infection enhances hallmark features of allergic airways disease that correlates with alterations in immune responses and lung structure. Neonatal and infant but not adult infection increased mucus-secreting cell numbers and IL-13 expression in the lung and reduced lung function during allergic airways disease in later life. The age of infection is crucial in determining the nature of the effects. Neonatal infection reduced eosinophil influx into the airways and ovalbumin-specific T_H2 cytokine release and the number of activated mDC in MLNs. By contrast, infant infection enhanced features of allergic inflammation with increased eosinophil numbers and enhanced T_H2 cytokine and DC responses. Both neonatal and infant infection increased systemic DC-induced IL-13 release from CD4⁺ T cells. The timing of infection had significant effects on lung structure because neonatal but not infant or adult infection induced substantial increases in alveolar size.

These studies used the natural mouse pathogen *C muridarum*, which was originally isolated from a mouse with respiratory infection.^{34,35} The time-course, immunologic, and histopathological progression of *C muridarum* infection of mice closely resembles that observed with *C pneumoniae* infection in human beings.^{9,14} Therefore, *C muridarum* is the organism of choice for investigating natural host-bacteria-allergen interactions in mice. By using *C muridarum*, we show that early-life (neonatal and infant) infections augment IL-13 expression in lung tissue, which correlates with increased mucus-secreting cell numbers and AHR.^{36,37} IL-13 is known to promote increased immune responses to otherwise innocuous inhaled allergens, T_H2 responses, eosinophilic inflammation, mucus hypersecretion, and AHR.^{4,38-40} These results suggest that early-life infection

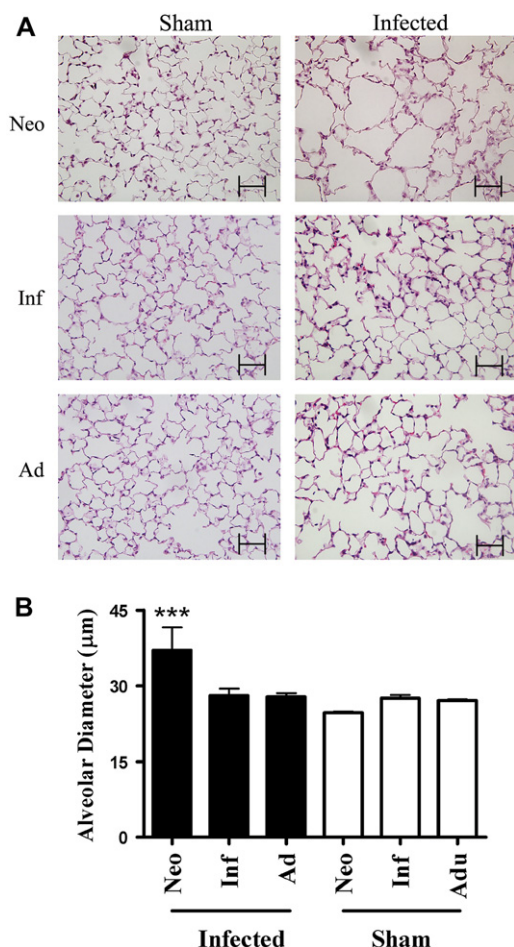


FIG 6. Neonatal infection increases alveolar size in later life. **A**, Photomicrographs of representative lung sections 9 weeks after infection ($\times 40$ magnification; scale bars represent 50 μm). **B**, Alveolar diameter of hematoxylin and eosin-stained lung sections. Black bars represent infected groups, whereas white bars represent uninfected groups. Results are representative of 2 or 3 independent experiments, and combined results are presented as means $\pm$ SEMs where $n \geq 8$ individual mice. *Significance compared with uninfected, nonallergic (Sham) control. *** $P < .001$. Group abbreviations: Neo, Inf, and Ad, neonatal, infant, and adult groups, respectively. Sham indicates uninfected control groups.

promotes pulmonary IL-13 responses to allergens that may drive increased asthma severity in later life.

Enhanced expression of IL-13 in the lung after early-life infections may be the result of permanent infection-induced changes in the phenotype of local (after infant infection) or systemic (after neonatal or infant infection) DCs. We show that DCs from animals exposed to chlamydial infections in early life have a greater propensity to induce IL-13 release from T cells on presentation of allergen. A previous study by Jiao et al⁴¹ showed that exposure of neonatal mice to killed *C muridarum* induces DCs that have a suppressive effect on T-cell function and allergic inflammation in later life. However, this study by Jiao et al⁴¹ did not investigate the effects on mucus-secreting cells, IL-13 expression in the lung, or lung function. This study supports our observations that exposure to infectious agents in early life can have profound effects on DC phenotype and function and that these changes may influence immune programming in later life. However, it is likely that exposure to killed *C muridarum* may have different effects than live infection.⁴¹

Although neonatal and infant chlamydial lung infections have similar effects on mucus-secreting cell numbers, pulmonary IL-13 expression, and AHR in allergic airways disease, they have differential effects on eosinophilic inflammation and ovalbumin-specific cytokine responses. Neonatal infection reduced the infiltration of eosinophils into the airways, which correlated with reduced levels of IL-5. IL-5 induces the development, maturation, and migration of eosinophils from blood to pulmonary tissue.^{2,42} By contrast, infant infection potentially enhanced the infiltration of eosinophils into the lungs, which correlated with increased IL-5 responses. Neonatal infection suppressed, whereas an infant infection enhanced, ovalbumin-specific cytokine release from MLN T cells. The anti-inflammatory cytokine IL-10 and Treg cells have been shown to suppress T-cell cytokine release.^{43,44} However, the suppression of MLN cytokine release in the neonatally infected group did not correlate with IL-10 production or altered Treg cell numbers during allergic airways disease. T-cell cytokine release did, however, correlate with infection-induced changes in the number of activated (MHC II⁺) mDCs in MLNs. MHC II surface expression is a marker of DC activation and antigen presentation, and increased expression is associated with enhanced T-cell responses.^{29,30} We show that neonatal infection reduces the levels of activated mDCs in the MLN during allergic airways disease. The reduction in mDCs may result in reduced T-cell activation and lead to the reduced MLN cytokine release observed in this group. By contrast, infant infection increased the numbers of activated mDCs in MLNs, which may account for the augmented release of T_H2 cytokines by MLN T cells in allergic airways disease.

We demonstrate that early-life infections result in significant reductions in lung function in terms of AHR (increased resistance and decreased compliance) during allergic airways disease in later life. These observations agree with epidemiologic studies that show *C pneumoniae* infection is associated with persistent airflow limitation in subjects with adult-onset asthma compared with subjects with asthma without infection⁴⁵ and correlate with an increase in asthma severity.⁴⁶ Our data also show that infected, nonallergic groups have substantially reduced lung function compared with uninfected controls. Notably, maximal airways resistance in infected, allergic groups was not significantly increased above infected, nonallergic groups (Fig 2, C). This may occur because infection alone may induce the maximal response that cannot be significantly increased by the induction of allergic airways disease, as we have previously described.¹⁴ However, a significant additional decrease in dynamic compliance was observed after either neonatal or infant infection (Fig E4). Clinical studies have shown that a number of respiratory infections in early life result in reduced lung function in adults without asthma.^{47,48} Therefore, although early-life infection may predispose to reduced lung function in subjects with asthma, infections may also result in substantially reduced lung function in later life even in the absence of asthma.

We also show that a neonatal chlamydial lung infection induces dramatic changes in alveolar structure in later life. This novel observation provides further evidence that the age of infection may play a major role in driving infection-induced and infection-associated disease. Alveolar development occurs postnatally, and only 15% of the adult pulmonary alveolar component is present at birth.¹⁹ Therefore, the demonstration that increases in alveolar size occur only after a neonatal infection provides evidence that there is a window early in postnatal lung development during

which respiratory insult and inflammation can result in permanent and irreparable damage to the lungs in later life. The alteration in lung structure by infection may also contribute to impaired lung function. Increased alveolar diameter results in the reduction of alveolar attachments to the airway wall, which decreases airway support and elastic recoil.⁴⁹ These changes lead to airflow limitations, enhanced transpulmonary resistance, and reduced tissue compliance. Taken together, the combined effects of increased IL-13 responses and altered lung structure by neonatal infection may significantly contribute to the enhancement of allergic airways disease in later life. Significantly, neonatal infection-induced changes in lung structure may predispose to, or increase the severity of, other chronic respiratory diseases that are associated with detrimental changes in alveoli such as chronic obstructive pulmonary disease and emphysema.

It is increasingly recognized that inflammatory events during early life may predispose to asthma in later life; however, the mechanisms involved are largely unknown. Respiratory viral infection and associated wheeze during infancy are strong predictors for persistent wheeze and asthma.⁵⁰ Furthermore, *C pneumoniae* infection during childhood is increasingly associated with asthma.⁵¹⁻⁵⁴ Our studies suggest novel mechanisms of how early-life infection may predispose to enhanced asthma. We show that early-life chlamydial lung infections result in the progression of a similar worsening of features of asthma (mucus-secreting cell hyperplasia, increased IL-13 expression, and impaired lung function) in later life. This occurs even though neonatal and infant infections have differential effects on eosinophil influx into the lungs and ovalbumin-specific MLN cytokine and DC responses. We show that these changes correspond with early-life infection-induced increases in the ability of DCs to induce IL-13 release from T cells on exposure to allergen. This study suggests that the prevention or treatment of early-life pulmonary chlamydial infection and/or associated inflammatory responses may be beneficial in suppressing the development of future disease. Indeed, vaccination and treatment strategies that promote the clearance of infection and prevent inflammation may be beneficial for lung function and predisposition to asthma in the long term.

We thank Prof Rakesh Kumar (Department of Pathology, University of NSW, Sydney, Australia) for assistance in the analysis of changes in lung histology.

Clinical implications: Early-life chlamydial lung infections permanently alter immunity and lung function and structure and enhance the severity of allergic airways disease, indicating a need for strategies that target infection and its effects to suppress asthma.

REFERENCES

- Jarman ER, Lamb JR. Reversal of established CD4+ type 2 T helper-mediated allergic airway inflammation and eosinophilia by therapeutic treatment with DNA vaccines limits progression towards chronic inflammation and remodelling. *Immunology* 2004;112:631-42.
- Foster PS, Yang M, Herbert C, Kumar RK. CD4(+) T-lymphocytes regulate airway remodeling and hyper-reactivity in a mouse model of chronic asthma. *Lab Invest* 2002;82:455-62.
- Foster PS, Martinez-Moczygemba M, Huston DP, Corry DB. Interleukins-4, -5, and -13: emerging therapeutic targets in allergic disease. *Pharmacol Ther* 2002;94:253-64.
- Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 2004;202:175-90.
- von Mutius E. Paediatric origins of adult lung disease. *Thorax* 2001;56:153-7.
- Piippo-Savolainen E, Remes S, Kannisto S, Korhonen K, Korppi M. Early predictors for adult asthma and lung function abnormalities in infants hospitalized for bronchiolitis: a prospective 18- to 20-year follow-up. *Allergy Asthma Proc* 2006;27:341-9.
- Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N Engl J Med* 2003;349:1414-22.
- Erb KJ. Atopic disorders: a default pathway in the absence of infection? *Immunol Today* 1999;20:317-22.
- Hansbro PM, Beagley KW, Horvat JC, Gibson PG. Role of atypical bacterial infection of the lung in predisposition/protection of asthma. *Pharmacol Ther* 2004;101:193-210.
- Blasi F. Atypical pathogens and respiratory tract infections. *Eur Respir J* 2004;24:171-82.
- Vila-Corcoles A, Ochoa-Gondar O, Rodriguez-Blanco T, Raga-Luria X, Gomez-Bertomeu F. Epidemiology of community-acquired pneumonia in older adults: a population-based study. *Respir Med* 2009;103:309-16.
- Yang X, HayGlass KT, Brunham RC. Genetically determined differences in IL-10 and IFN-gamma responses correlate with clearance of *Chlamydia trachomatis* mouse pneumonitis infection. *J Immunol* 1996;156:4338-44.
- Yang X, Gartner J, Zhu L, Wang S, Brunham RC. IL-10 Gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following *Chlamydia trachomatis* lung infection. *J Immunol* 1999;162:1010-7.
- Horvat JC, Beagley KW, Wade MA, Preston JA, Hansbro NG, Hickey DK, et al. Neonatal chlamydial infection induces mixed T-cell responses that drive allergic airway disease. *Am J Respir Crit Care Med* 2007;176:556-64.
- Brimnes MK, Bonifaz L, Steinman RM, Moran TM. Influenza virus-induced dendritic cell maturation is associated with the induction of strong T cell immunity to a coadministered, normally nonimmunogenic protein. *J Exp Med* 2003;198:133-44.
- Dahl ME, Dabbagh K, Liggitt D, Kim S, Lewis DB. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat Immunol* 2004;5:337-43.
- Yamamoto N, Suzuki S, Shirai A, Suzuki M, Nakazawa M, Nagashima Y, et al. Dendritic cells are associated with augmentation of antigen sensitization by influenza A virus infection in mice. *Eur J Immunol* 2000;30:316-26.
- Grayson MH, Cheung D, Rohlfing MM, Kitchens R, Spiegel DE, Tucker J, et al. Induction of high-affinity IgE receptor on lung dendritic cells during viral infection leads to mucous cell metaplasia. *J Exp Med* 2007;204:2759-69.
- Bush A. Asthma research: the real action is in children. *Paediatr Respir Rev* 2005;6:101-10.
- Prescott S, L. The development of respiratory inflammation in children. *Paediatr Respir Rev* 2006;7:89-96.
- Preston JA, Essilfie AT, Horvat JC, Wade MA, Beagley KW, Gibson PG, et al. Inhibition of allergic airways disease by immunomodulatory therapy with whole killed *Streptococcus pneumoniae*. *Vaccine* 2007;25:8154-62.
- Phipps S, Lam CE, Mahalingam S, Newhouse M, Ramirez R, Rosenberg HF, et al. Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. *Blood* 2007;110:1578-86.
- Snelgrove RJ, Goulding J, Didierlaurent AM, Lyonga D, Vekaria S, Edwards L, et al. A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. *Nat Immunol* 2008;9:1074-83.
- Leech MD, Benson RA, deVries A, Fitch PM, Howie SEM. Resolution of der p1-induced allergic airway inflammation is dependent on CD4+CD25+Foxp3+ regulatory cells. *J Immunol* 2007;179:7050-8.
- Kaiko GE, Phipps S, Hickey DK, Lam CE, Hansbro PM, Foster PS, et al. *Chlamydia muridarum* infection subverts dendritic cell function to promote Th2 immunity and airways hyperreactivity. *J Immunol* 2008;180:2225-32.
- Robbeson AA, Versteeg EM, Veerkamp JH, van Krieken JH, Bulten HJ, Smits HT, et al. Morphological quantification of emphysema in small human lung specimens: comparison of methods and relation with clinical data. *Mod Pathol* 2003;16:1-7.
- Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR, et al. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 2004;305:1773-6.
- Wills-Karp M, Karp CL. Biomedicine: eosinophils in asthma: remodeling a tangled tale. *Science* 2004;305:1726-9.
- Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;18:767-811.
- Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 2002;20:621-67.
- Lambrecht BN, De Veerman M, Coyle AJ, Gutierrez-Ramos J-C, Thielemans K, Pauwels RA. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest* 2000;106:551-9.

32. de Heer HJ, Hammad H, Soullie T, Hijdra D, Vos N, Willart MAM, et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* 2004;200:89-98.
33. Koya T, Kodama T, Takeda K, Miyahara N, Yang E-S, Taube C, et al. Importance of myeloid dendritic cells in persistent airway disease after repeated allergen exposure. *Am J Respir Crit Care Med* 2006;173:42-55.
34. Nigg C, Eaton MD. Isolation from normal mice of a pneumotropic virus which forms elementary bodies. *J Exp Med* 1944;79:497-510.
35. Gogolak FM. The histopathology of murine pneumonitis infection and the growth of the virus in the mouse lung. *J Infect Dis* 1953;92:254-72.
36. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258-61.
37. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261-3.
38. Pope SM, Brandt EB, Mishra A, Hogan SP, Zimmermann N, Matthaei KI, et al. IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism. *J Allergy Clin Immunol* 2001;108:594-601.
39. Venkayya R, Lam M, Willkom M, Grunig G, Corry DB, Erle DJ. The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyperresponsiveness through direct effects on resident airway cells. *Am J Respir Cell Mol Biol* 2002;26:202-8.
40. Padilla J, Daley E, Chow A, Robinson K, Parthasarathi K, McKenzie ANJ, et al. IL-13 regulates the immune response to inhaled antigens. *J Immunol* 2005;174:8097-105.
41. Jiao L, Han X, Wang S, Fan Y, Yang M, Qiu H, et al. Imprinted DC mediate the immune-educating effect of early-life microbial exposure. *Eur J Immunol* 2009;39:469-80.
42. Kips JC. Cytokines in asthma. *Eur Respir J* 2001;18:24S-33.
43. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;19:683-765.
44. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol* 2008;180:5771-7.
45. ten Brinke A, van Dissel JT, Sterk PJ, Zwinderman AH, Rabe KF, Bel EH. Persistent airflow limitation in adult-onset nonatopic asthma is associated with serologic evidence of *Chlamydia pneumoniae* infection. *J Allergy Clin Immunol* 2001;107:449-54.
46. Black PN, Scicchitano R, Jenkins CR, Blasi F, Allegra L, Wlodarczyk J, et al. Serological evidence of infection with *Chlamydia pneumoniae* is related to the severity of asthma. *Eur Respir J* 2000;15:254-9.
47. Shaheen SO, Barker DJ, Shiell AW, Crocker FJ, Wield GA, Holgate ST. The relationship between pneumonia in early childhood and impaired lung function in late adult life. *Am J Respir Crit Care Med* 1994;149:616-9.
48. Johnston IDA, Strachan DP, Anderson HR. Effect of pneumonia and whooping cough in childhood on adult lung function. *N Engl J Med* 1998;338:581-7.
49. Saetta M, Finkelstein R, Cosio MG. Morphological and cellular basis for airflow limitation in smokers. *Eur Respir J* 1994;7:1505-15.
50. Hansbro NG, Horvat JC, Wark PA, Hansbro PM. Understanding the mechanisms of viral induced asthma; new therapeutic directions. *Pharmacol Ther* 2008;117:313-53.
51. Cunningham AF, Johnston SL, Julious SA, Lampe FC, Ward ME. Chronic *Chlamydia pneumoniae* infection and asthma exacerbations in children. *Eur Respir J* 1998;11:345-9.
52. Esposito S, Blasi F, Arosio C, Fioravanti L, Fagetti L, Droghetti R, et al. Importance of acute *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* infections in children with wheezing. *Eur Respir J* 2000;16:1142-6.
53. Emre U, Roblin PM, Gelling M, Dumornay W, Rao M, Hammerschlag MR, et al. The association of *Chlamydia pneumoniae* infection and reactive airway disease in children. *Arch Pediatr Adolesc Med* 1994;148:727-32.
54. Webley WC, Salva PS, Andrzejewski C, Cirino F, West CA, Tilahun Y, et al. The bronchial lavage of pediatric patients with asthma contains infectious *Chlamydia*. *Am J Respir Crit Care Med* 2005;171:1083-8.

METHODS

Experimental models

Neonatal (24 hours), infant (3 weeks), and adult (6 weeks) BALB/c mice were infected intranasally with *C muridarum* (400 [neonates] or 100 [infants/adults] inclusion-forming units, ATCC VR-123, in 5 μ L [neonates] or 30 μ L [infants/adults] sucrose phosphate glutamate buffer). Infection resolved, and after 45 days, mice were sensitized and challenged with ovalbumin (Fig 1) as previously described.^{E1,E2} Thus, groups had a resolved neonatal, infant, or adult infection 6 weeks before the induction of allergic airways disease in later life. Animals were euthanized by sodium pentobarbital overdose (Abbott Australasia, Botany, Australia) 24 hours after the final ovalbumin challenge, and features of allergic airways disease were characterized. Controls were infected and nonallergic (Neo, Inf, and Ad), uninfected and allergic (Ova), or uninfected, and sham-sensitized (Sham [uninfected and nonallergic]). We have determined that control groups that receive no sham, sham sensitization, or sham infection do not differ in any features of pulmonary immune responses or physiology.^{E1} All experiments were approved by the University of Newcastle animal ethics committee.

Chlamydial infection

Pulmonary chlamydial numbers were determined by real-time PCR of DNA extracted from lung homogenates.^{E1}

Airway inflammation

Euthanized mice were bled out by severing the aorta in the lower abdominal cavity while the heart was still beating. Lungs were perfused (0.9% saline) by puncturing the heart with a 19-gauge needle attached to a column of saline and fixed by intratracheal inflation (1.5 mL, 10% buffered formalin; Sigma, Castle Hill, Australia). The trachea was tied off, and lungs were immersed in buffered formalin. Lungs were embedded in paraffin, sectioned (4–6 μ m), and stained with chrome salt fixation (for eosinophils), periodic acid-Schiff (for mucus-secreting cells), or hematoxylin and eosin (for histopathology).^{E1} To determine airway tissue eosinophil numbers and mucus-secreting cell hyperplasia, the mean numbers of eosinophils adjacent to the basement membrane and mucus-secreting cells around airways (mean in $10 \times 100 \mu$ m fields) were determined by using light microscopy.^{E1} Histopathology was scored according to a set of custom-designed criteria.^{E1} All analyses were performed in a blind fashion.

Lung function

Mice were anesthetized (ketamine/xylazine [80–100 mg/kg and 10 mg/kg, respectively], Troy Laboratories, Smithfield, Australia) and the trachea cannulated. The cannula was connected to an inline aerosol and ventilator, which were attached to a preamplifier and computer (Buxco, Sharon, Conn) to analyze pressure and flow waveforms. Lung function, in terms of AHR, was measured in anesthetized mice using whole-body plethysmography by determination of the peak of airways resistance and dynamic compliance in response to increasing doses of nebulized methacholine (Sigma).^{E1}

IL-13 mRNA expression in lung tissue

Total RNA was extracted from whole lung tissue using TRIZOL (Invitrogen, Mount Waverley, Australia). Reverse transcription of RNA (1000 ng) was performed by using Superscript III and random hexamer primers (Invitrogen). The relative abundance of IL-13 mRNA in experimental samples was determined compared with the reference gene hypoxanthine-guanine phosphoribosyltransferase (internal control) by real-time PCR by using the ABI Prism7000 Sequence Detection System (Applied Biosystems, Scoresby, Australia).^{E3} Primers used are shown in Table E1.

Ovalbumin-specific T-cell cytokine release from MLNs

Single-cell suspensions of MLNs were prepared.^{E1} Cells were pelleted, erythrocytes lysed, and cells resuspended in complete RPMI (10% FCS, 20 mmol/L HEPES, 10 μ g/mL penicillin/streptomycin, 2 mmol/L L-glutamine,

and 50 μ mol/L 2-mercaptoethanol). Viable cell numbers were determined by Trypan blue exclusion using a hemocytometer (Neubauer, Dusseldorf, Germany) and light microscope. MLN cells (5×10^6 cells) were restimulated with ovalbumin (200 μ g/mL) and cultured for 6 days in complete RPMI. IFN- γ , IL-5, IL-13, and IL-10 concentrations in culture supernatants were determined by ELISA.^{E1}

Flow-cytometric analysis of MLN cells

Single-cell suspensions of MLNs (1×10^6 cells) were stained for surface markers as previously described.^{E4} FACS was performed by using a BD FACSCanto and results generated and assessed using BD FACSDiva software (BD Biosciences). Anti-CD11c, CD11b, B220, CD4, CD25 (BD Biosciences), and MHC II and forkhead box protein 3 (Foxp3; eBiosciences, Kensington, Australia) mAbs were used.

Regulatory T cells were characterized as CD4⁺CD25⁺Foxp3⁺, low-moderate forward scatter, and low side scatter.^{E5} Intracellular cytokine staining for Foxp3 was performed according to the manufacturer's instructions (eBioscience).

Myeloid DCs were characterized as CD11c⁺CD11b^{hi}B220⁺, moderate forward scatter, and low-moderate side scatter.^{E4} Maturation and activation of DCs was assessed by determination of MHC II expression.

These experiments are variable, and age-matched controls were run with each experiment.

DC-induced CD4⁺ T-cell IL-13 release

Single-cell homogenates of spleens were prepared, and DCs were isolated by using the BD IMag DC Enrichment Set (BD Biosciences). Negative selection was used to avoid cellular activation.

Purified DCs were cultured (5×10^4 cells, 200 μ L, complete RPMI, 16 hours, 37°C, 5% CO₂) with ovalbumin-peptide (50 μ g/mL, amino acids 323–339 of ovalbumin [Ova^{323–339}], Biomolecular Resource Facility, Australian National University, Canberra, Australia). Negative controls were cultured without peptide.

CD4⁺ T-cells were isolated from the spleens of DO11.10 recombinase-activating gene 2^{+/+} BALB/c mice, which are transgenic for the Ova^{323–339}-specific TCR, by using the BD IMag CD4 T-lymphocyte Enrichment Set (BD Biosciences). Negative selection was used to avoid cellular activation.

Ovalbumin-peptide pulsed DCs were cocultured with DO11.10 Rag2^{+/+} CD4⁺ T cells (1:7, 200 μ L, complete RPMI, 4 days, 37°C, 5% CO₂).^{E6} Supernatants were collected for assessment of IL-13 release by ELISA.

These experiments are variable, and age-matched controls were run with each experiment.

REFERENCES

- Horvat JC, Beagley KW, Wade MA, Preston JA, Hansbro NG, Hickey DK, et al. Neonatal chlamydial infection induces mixed T-Cell responses that drive allergic airway disease. *Am J Respir Crit Care Med* 2007;176:556–64.
- Preston JA, Essilfie AT, Horvat JC, Wade MA, Beagley KW, Gibson PG, et al. Inhibition of allergic airways disease by immunomodulatory therapy with whole killed *Streptococcus pneumoniae*. *Vaccine* 2007;25:8154–62.
- Phipps S, Lam CE, Mahalingam S, Newhouse M, Ramirez R, Rosenberg HF, et al. Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. *Blood* 2007;110:1578–86.
- Snelgrove RJ, Goulding J, Didierlaurent AM, Lyonga D, Vekaria S, Edwards L, et al. A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. *Nat Immunol* 2008;9:1074–83.
- Leech MD, Benson RA, deVries A, Fitch PM, Howie SEM. Resolution of Der p1-induced allergic airway inflammation is dependent on CD4⁺CD25⁺Foxp3⁺ regulatory cells. *J Immunol* 2007;179:7050–8.
- Kaiko GE, Phipps S, Hickey DK, Lam CE, Hansbro PM, Foster PS, et al. *Chlamydia muridarum* infection subverts dendritic cell function to promote Th2 immunity and airways hyperreactivity. *J Immunol* 2008;180:2225–32.

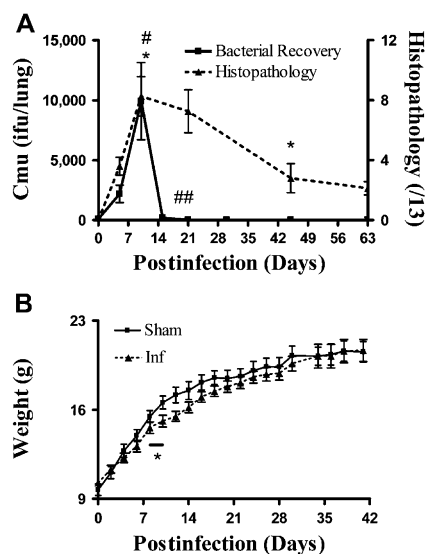


FIG E1. Infant *C muridarum* lung infection. Mice were infected at 3 weeks of age with 100 inclusion forming units (ifu) of *C muridarum* (*Cmu*), and pulmonary *Cmu* numbers and histopathology were determined over a 9-week period (**A**). Weight gain after infant (*Inf*) infection (**B**). Results are presented as means $\pm$ SEMs from $n \geq 4$. Significant differences in *Cmu* numbers between days 0 and 10 and between days 15 and 20 are shown as $\#P < .05$ and $\#\#P < .01$. Significant differences in histopathology between days 0 and 10 and days 10 and 45 are shown as $*P < .05$. Significant differences in rate of weight gain between days 8 and 10 of infected (*Inf*) compared with uninfected (*Sham*) infant mice are shown as $*P < .05$.

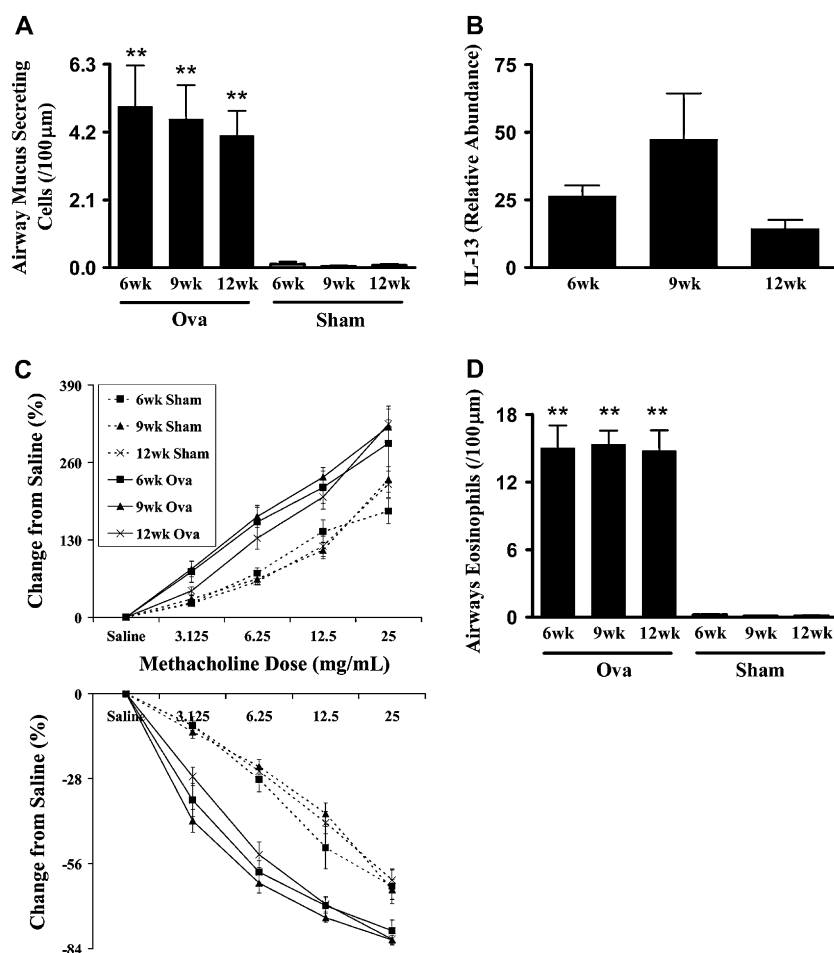


FIG E2. Age of sensitization does not affect airways mucus-secreting cell numbers, IL-13 expression in lung tissue, AHR, or eosinophil numbers in allergic airways disease. Mice were sensitized intraperitoneally and challenged intranasally with ovalbumin (*Ova*) to induce allergic airways disease in mice 6, 9, or 12 weeks old. Airways mucus-secreting cells (**A**), IL-13 expression in lung tissue (**B**), AHR in terms of averaged peak airways resistance (*upper panel*) and dynamic compliance (*lower panel*) in response to increasing doses of methacholine (**C**), and airways eosinophil numbers (**D**). Results are presented as means $\pm$ SEMs from $n \geq 4$. *Significance compared with uninfected, nonallergic (*Sham*) controls. * $P < .05$; ** $P < .01$.

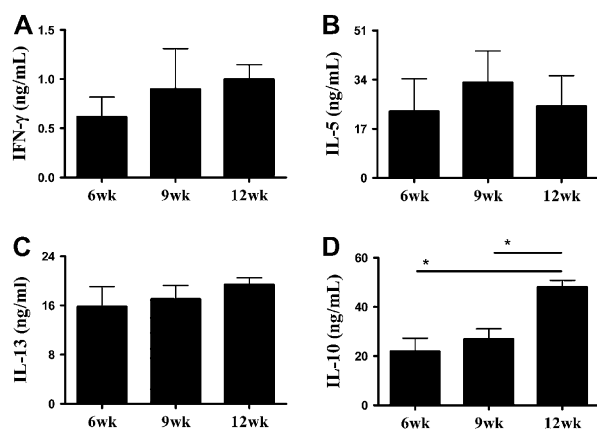


FIG E3. Age of sensitization does not affect T_H2 -cytokine responses in allergic airways disease. Mice were sensitized intraperitoneally and challenged intranasally with ovalbumin to induce allergic airways disease in mice 6, 9, or 12 weeks old. Ovalbumin-specific IFN- γ (A), IL-5 (B), IL-13 (C), and IL-10 (D) release in MLN culture supernatants were determined. Results are presented as means $\pm$ SEMs from $n \geq 4$. * $P < .05$.

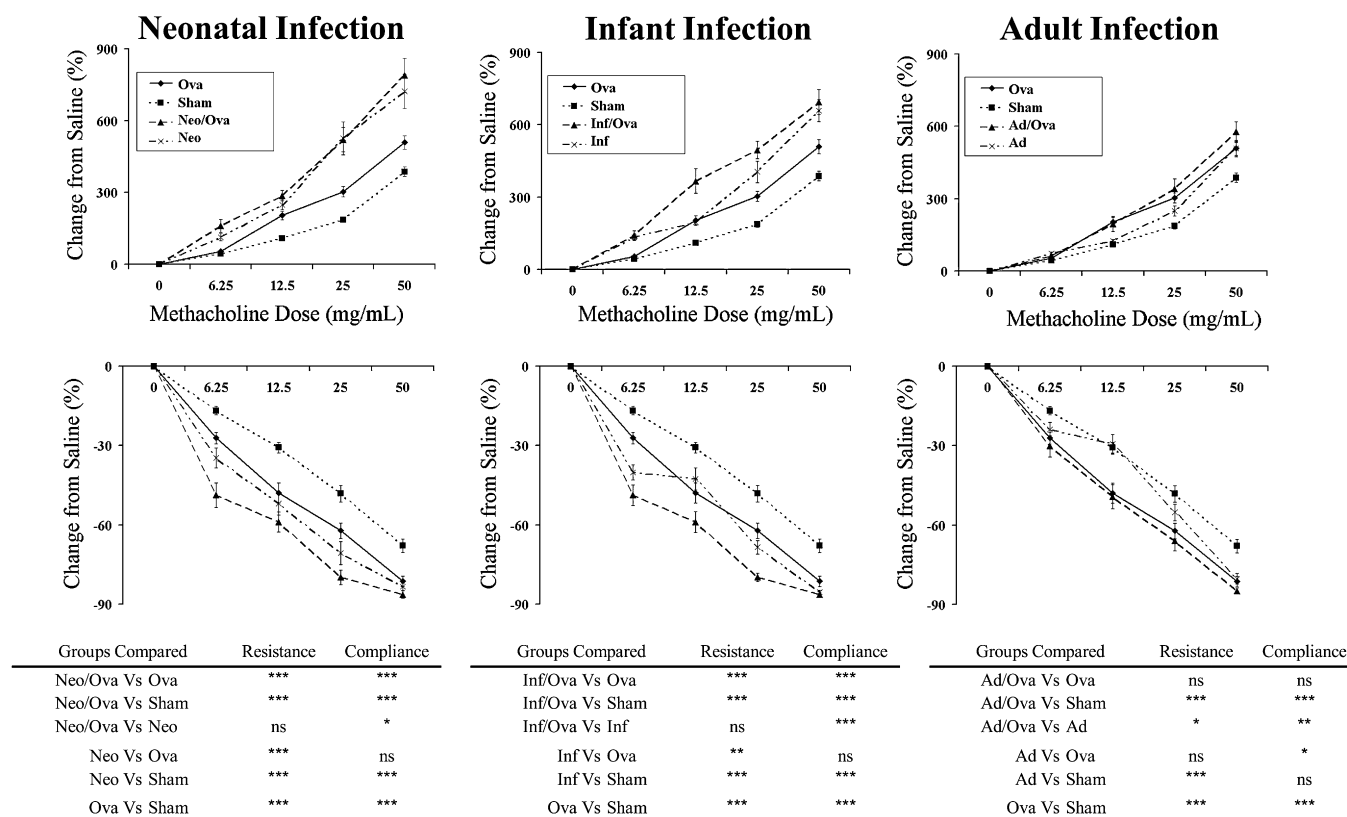


FIG E4. Early-life infection enhances AHR in allergic airways disease in later life. AHR in terms of averaged peak airways resistance (*upper panels*) and dynamic compliance (*lower panels*) in response to increasing doses of methacholine and comparison of statistical differences across whole curves. Results are representative of 2 or 3 independent experiments, and combined results are presented as means $\pm$ SEMs where $n \geq 8$ individual mice. * $P < .05$; ** $P < .01$; *** $P < .001$. Ova, Uninfected, allergic groups; Neo/Ova, Inf/Ova, and Ad/Ova, neonatal, infant, and adult infected, allergic groups, respectively; Sham, uninfected, nonallergic groups; Neo, Inf, and Ad, neonatal, infant, and adult infected, nonallergic groups, respectively. Uninfected controls (Ova and Sham) were 6 weeks old at the time of sensitization.

TABLE E1. Sequences of primers used in real-time PCR

Gene	Primers
HPRT	Forward, 5'-AGGCCAGACTTTGTTGGATTTGAA
	Reverse, 5'-CAACTTGCGCTCATCTTAGGCTTT
IL-13	Forward, 5'-AGCTGAGCAACATCACACAAGACC
	Reverse, 5'-TGGGCTACTTCGATTTTGGTATCG

HPRT, Hypoxanthine-guanine phosphoribosyltransferase.