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Chlamydid Respiratory Infection during Allergen Sensitization Drives Neutrophilic Allergic Airways Disease

Jay C. Horvat,* Malcolm R. Starkey,* Richard Y. Kim,* Kenneth W. Beagley,*,† Julie A. Preston,* Peter G. Gibson,* Paul S. Foster,* and Philip M. Hansbro*

Neutrophilic asthma is a prevalent, yet recently described phenotype of asthma. It is characterized by neutrophilic rather than eosinophilic airway inflammation and airways hyperresponsiveness (AHR) and may have an infectious origin. Chlamydid respiratory infections are associated with asthma, but how these Th1-inducing bacteria influence Th2-mediated asthma remains unknown. The effects of chlamydid infection on the development of asthma were investigated using a BALB/c mouse model of OVA-induced allergic airways disease (AAD). The effects of current and resolved Chlamydia muridarum infection during OVA sensitization on AAD were assessed and compared with uninfected and nonsensitized controls. Current, but not resolved, infection attenuated hallmark features of AAD: pulmonary eosinophil influx, T cell production of IL-5, mucus-secreting cell hyperplasia, and AHR. Current infection also induced robust OVA-driven neutrophilic inflammation and IFN-γ release from T cells. The phenotype of suppressed but persistent Th2 responses in association with enhanced neutrophilia is reminiscent of neutrophilic asthma. This phenotype was also characterized by increased pulmonary IL-12 and IL-17 expression and activation of APCs, as well as by reduced thymus- and activation-regulated chemokine and altered APC activation. Inhibition of pulmonary neutrophil influx during infection blocked OVA-induced neutrophilic inflammation and T cell IFN-γ production and reversed the suppressive effects on mucus-secreting cell hyperplasia and AHR during AAD. These changes correlated with decreased IL-12 and IL-17 expression, increased thymus- and activation-regulated chemokine and altered APC activation. Blocking IFN-γ and IL-17 during OVA challenge had no effect. Thus, active chlamydid respiratory infection during sensitization enhances subsequent neutrophilic inflammation and Th1/Th17 responses during allergen exposure and may have a role in the pathogenesis of neutrophilic asthma. The Journal of Immunology, 2010, 184: 4159–4169.

Asthma is characterized by allergic airways inflammation and recurrent episodes of wheezing, breathlessness, and cough (1, 2). Aberrant CD4+ Th2 responses to environmental Ags play pivotal roles in the development of disease (3, 4). Upon exposure to specific stimuli, affected individuals typically mount strong Th2 responses with increased levels of IL-4, IL-5, and IL-13 that promote the hallmark features of asthma, which include mucus-secreting cell (MSC) hyperplasia, eosinophil influx, and airways hyperresponsiveness (AHR) (5–7).

Respiratory chlamydid infection is associated with the development of asthma in children and adults (reviewed in Ref. 8). However, chlamydial infections typically induce potent Th1-type immune responses (9–11), and it remains unknown how these infections influence Th2-mediated asthma. The association may be driven by chlamydial infection-induced alterations in the inflammatory profile and phenotype of asthma, such as a switch from eosinophil-dominated inflammation to that of neutrophilia in the allergic lung.

Indeed, different inflammatory subtypes of asthma that may be related to infection have recently been recognized. A proportion of asthmatics (~25%; neutrophilic asthmatics) have strong neutrophilic inflammation when stable or during asthmatic episodes (12–16). Neutrophilic asthmatics also have reduced eosinophilic inflammation and AHR (15, 16). Furthermore, increased numbers of neutrophils in the sputum of asthmatics correlate with increased Th1 and reduced Th2 responses in the airways (17). Neutrophilic inflammation in asthma has been associated with viral respiratory infection (18) and correlates with increased activation of innate immune factors, namely, increased IL-8 and TLR-2 and TLR-4 expression in sputum (16). Neutrophil levels are also elevated in the sputum of exacerbating asthmatics with evidence of chlamydid respiratory infection, compared with uninfected individuals (18, 19). Furthermore, chlamydial infection of mice induces a robust pulmonary neutrophil influx that is mediated by the TLR-dependent production of keratinocyte chemokine (KC) and macrophage inflammatory protein-2 (MIP-2), the mouse orthologs of human IL-8 (20, 21). Taken together, these observations suggest that chlamydial infection may be linked to asthma with a neutrophilic phenotype.

Other work has shown that Chlamydia muridarum infection prior to the induction of allergic airways disease (AAD) in mice suppresses pulmonary eosinophil influx, MSC hypersecretion, and allergen-specific Th2 cytokine responses, suggesting that infection may protect against Th2-mediated inflammation during AAD (22,

*Centre for Asthma and Respiratory Disease and Hunter Medical Research Institute, The University of Newcastle, Newcastle, New South Wales; and †Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland, Australia

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Address correspondence and reprint requests to Dr. Philip M. Hansbro, Centre for Asthma and Respiratory Disease and Hunter Medical Research Institute, Level 3, David Maddison Clinical Sciences Building, Newcastle 2300, Australia. E-mail address: Philip.Hansbro@newcastle.edu.au

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Abbreviations used in this paper: AAD, allergic airways disease; AHR, airways hyperresponsiveness; AL, airway lumen; BALF, bronchoalveolar lavage fluid; BM, basement membrane; DC, dendritic cell; i.n., intranasal; KC, keratinocyte chemokine; mDC, myeloid dendritic cell; MIP-2, macrophage inflammatory protein-2; MLN, mediastinal lymph node; MSC, mucus-secreting cell; pDC, plasmacytoid dendritic cell; TARC, thymus- and activation-regulated chemokine.

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23). However, we have demonstrated that a *C. muridarum* lung infection in early life results in the generation of both allergen-specific Th2 and Th1 responses. Although the generation of this mixed phenotype results in a suppression of Th2 cytokine production and eosinophil influx into the airways, the severity of MSC hyperplasia and AHR is increased and inflammation prolonged in later life (9). Clinical studies have also found that respiratory infection may be related to strong IFN-γ and Th1-type responses in moderate to severe asthmatics (24–27). Thus, *Chlamydia*-induced Th1 responses in the asthmatic lung may enhance or modify disease, although Th2-mediated inflammation may be suppressed. Indeed, the presence of both allergen-specific Th1 and Th2 cells in the airways enhance disease in a model of OVA-induced asthma (28).

In this study, we investigated whether *Chlamydia*-induced neutrophilic and Th1 responses during allergen sensitization may subsequently enhance or modify AAD, despite reducing Th2-mediated inflammation. Because immune responses vary during chlamydial lung infection (11, 29), the timing of infection relative to allergen exposure may be important in determining the effects on AAD. Thus, we employed murine models of resolved or current chlamydial lung infection and OVA-induced AAD to examine the effect on neutrophils, Th1 responses, and Th2-mediated AAD. We demonstrate that current chlamydial infection induces the development of AAD with a phenotype reminiscent of neutrophilic asthma.

**Materials and Methods**

**Experimental models**

Six-week-old female BALB/c mice were used throughout. We previously shown that bacterial recovery (using both real-time PCR and tissue culture) and histopathological status of chlamydial pulmonary infection in adult mice peaks between 10 and 15 d postinoculation. Clearance of infection and the majority of inflammation occur within 21 and 45 d, respectively (9). So that infections were either resolved or at their peak during OVA exposure, mice were infected 45 (D-45) or 7 (D-7) days before OVA sensitization (Fig. 1A). Mice were infected via the intranasal (i.n.) route with 100 inclusion-forming units *C. muridarum* (30 μl, sucrose-phosphate-glutamate buffer). Mice were sensitized to OVA (50 μg [Sigma-Aldrich, Castle Hill, Australia], 1 mg Rehydrogel [Reheis, Berkeley Heights, NJ], and 200 μl 0.9% w/v sterile saline) on day 0 by i.p. injection and were challenged i.n. with OVA 12–15 d later (D-45/OVA and D-7/OVA) (9). One day later, mice were euthanized and AAD was characterized. Controls were: uninfected and allergic (OVA); 2) uninfected, nonallergic (Sal); or 3) infected, sham-sensitized (D-45/Sal and D-7/Sal) groups. *B*. *Controls were infected with* *C. muridarum* 7 d prior to OVA sensitization (day 0) and were treated with anti-KC and anti–MIP-2 mAbs (αKCMIP2) on 3, 5, 7, and 9 d postinfection (D-4, -2, 0, and 2, αKCMIP2/D-7/OVA). AAD was induced by i.n. OVA challenges on days 12–15 postinfection. The effect of treatment on pulmonary and systemic leukocyte numbers was assessed at the peak of infection (day 3) and before OVA challenge (day 12). Chlamydial numbers in lung tissue were determined at day 3. AAD was assessed 24 h after the final challenge. Controls were 1) uninfected, isotype-treated, allergic (Iso/OVA); 2) uninfected, nonallergic (Sal); or 3) infected and sham-sensitized (D-45/Sal and D-7/Sal) groups. *C*. Groups were infected with *C. muridarum* 7 d prior to OVA sensitization (day 0) and were treated with anti-KC and anti–MIP-2 mAbs (αKCMIP2/OV A); and 3) infected, isotype-treated, allergic (Iso/D-7) groups. *D*. Groups were infected i.n. with *C. muridarum* 7 d prior to OVA sensitization (day 0) and were treated with anti–IFN-γ mAb (αIFN-γ) on days 11, 13, and 15 or anti–IL-17 mAb (αIL-17) on days 11 and 13 during OVA challenge (αIFN-γ/D-7/OVA or αIL-17/D-7/OVA, respectively). The effect of treatment on BALF leukocyte numbers was assessed 24 h after the final challenge. Controls were 1) uninfected, isotype-treated, allergic (Iso/OVA); 2) uninfected, Ab-treated, allergic (αKCMIP2/OVA); and 3) infected, isotype-treated, allergic (Iso/D-7) groups.

**Chlamydial infection**

Pulmonary *Chlamydia* numbers were determined by real-time PCR of DNA extracted from lung homogenates (9).

**Local and systemic leukocytes**

Cytospins were prepared from 2 ml bronchoalveolar lavage fluid (BALF), and blood smears were prepared from whole blood (9). Cytospins and blood smears were stained with May-Grunwald-Giemsa, and differential leukocyte counts were determined using morphological criteria (<200 cells by light microscopy [×40]) (9). All samples were coded and counts performed in a blinded fashion.

**Histopathological findings and airway inflammation**

Lungs were perfused (0.9% saline) and fixed by inflation with formalin (1.5 ml, 10% buffered formalin: Sigma-Aldrich). The trachea was tied off, and the entire heart-lung block was removed from the thoracic cavity and immersed in buffered formalin. The left lung was excised and embedded in paraffin, sectioned (4-μm), and stained using hematoxylin and eosin (H&E), periodic acid-Schiff (for MSCs). Average eosinophil and MSC counts in 10 × 100-μm fields adjacent to the basement membrane (eosinophils) and within the airway lumen (MSCs) were determined using light microscopy (9). All samples were coded and counts performed in a blinded fashion.

**T cell cytokines**

Cytokine release from lung draining mediastinal lymph node (MLN) cells was determined by ELISA (9). Cells (5 × 10^5) were cultured in RPMI (1 ml, 10% FBS, 20 mM HEPES, 10 μg/ml penicillin/streptomycin, 2 mM l-glutamine, and 50 μM 2-ME) and restimulated with OVA (200 μg/ml) for 6 d. Concentrations of IFN-γ, IL-5, and IL-13 were determined in supernatants using mouse IFN-γ and IL-5 (BD Biosciences, North Ryde, Australia) and IL-13 (R&D Systems, Gymea, Australia) Ab combinations according to the manufacturers’ instructions (9).

**Lung function**

Lung function was measured using whole-body, invasive plethysmography in restrained and anesthetized animals (9). Mice were anesthetized with ketamine/xylazine (80–100 mg/kg and 10 mg/kg, respectively; Troy
Lung tissue cells

Single-cell suspensions of collagenase-digested lungs (1 × 10^6 cells) were stained for surface markers (31). Cells were characterized as follows: neutrophils, CD11c<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup>, low-moderate forward scatter and moderate-high side scatter; plasmacytoid dendritic cells (pDC), CD11c<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup>, moderate forward scatter and low-moderate side scatter; and myeloid dendritic cells (mDC), CD11c<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup>, moderate forward scatter and low-moderate side scatter. Maturation and activation of dendritic cells (DCs) were assessed by determination of MHC II expression. The functional capacity of pulmonary APCs (CD11c<sup>+</sup>) was also assessed by analyzing expression of the costimulatory markers CD80 and CD86.

Depletion of IFN-γ and IL-17

Groups were infected with *C. muridarum* 7 d prior to OVA sensitization. AAD was induced by i.n. OV A challenges on days 12–15 postsensitization. To assess the role of IFN-γ and IL-17 in infection-induced changes during AAD, mice were infected i.p. with a combination of anti–KC and anti–MIP-2 (18 μg and 4 μg, respectively [αKCMIP2, R&D Systems], in 200 μl PBS) or corresponding isotype control Abs (18 μg and 4 μg, Rat IgG2a and IgG2b, respectively [Iso; R&D Systems]). Treatment occurred 3, 5, 7, and 9 d postinfection. On day 7, mice were sensitized and subsequently challenged with OVA (Fig. 1B). One day later, mice were euthanized and AAD was characterized. Controls were uninfected and treated with αKCMIP2 or isotype control Abs.

Pulmonary cytokine expression

Pulmonary cytokine expression was evaluated by real-time PCR (30). Total RNA was extracted from whole-lung tissue, using TRIzol according to the manufacturer’s instructions (Invitrogen, Mount Waverley, Australia). Reverse transcription of RNA (1000 ng) was performed using Superscript III random hexamer primers (Invitrogen). Relative abundance of genes was determined by comparison with the reference gene hypoxanthine-guanine phosphoribosyltransferase, using a Prism7000 Sequence Detection System (Applied Biosystems, Scoresby, Australia). Primers used were IL-12, Fwd 5’-ACC AGT TCT TTC ATC AGG GAC ATC-3’; thymus- and activation-regulated chemokine (TARC), Fwd 5’-AGT CCA GTA GGA GAG CAC CT-3’; IL-17, Fwd 5’-TCC CCA GGA GAG GAC ATC-3’; Rev 5’-ACT GAG CTT CCC AGA TCA-3’; IFN-γ, Fwd 5’-GAT CAG GGA GAC ATC-3’; Rev 5’-CAG GGA GAG CAC CT-3’; and hypoxanthine-guanine phosphoribosyltransferase, Fwd 5’-AGG CCA GAC TTT GGT GGA TTT GAA-3’, Rev 5’-CAA CTT GCC CTC ATC TTA GCC TTT-3’.

FIGURE 2. Airway inflammatory cell influx postinfection and AAD. Experimental groups were infected 7 (D-7/OVA) or 45 (D-45/OVA) d prior to OVA sensitization and challenge, as described in Fig. 1A. Total leukocyte (A), neutrophil (B), and eosinophil (C) numbers were determined in BALF. Results are presented as mean ± SEM from *n* = 4, in duplicate, with allergic groups represented by black bars and nonallergic controls by white bars. Significant differences between groups are shown as *p* < 0.05, ***pp* < 0.01, and ****ppp* < 0.001.

Infection and AAD

To investigate the effect of current or resolved infection on the development of AAD, groups were infected 7 or 45 d before sensitization (Fig. 1A). There were no significant differences in bacterial recovery from the lungs of infected allergic groups, compared with infected, nonallergic controls (data not shown).

Current infection during sensitization alters cellular responses during AAD

The effects of infection on cellular responses during AAD were assessed. Current (D-7/OVA), but not resolved (D-45/OVA), infection during sensitization had substantial effects on immune cell influx into the lungs during OVA-induced AAD.

Although current infection had minimal effects on the influx of total leukocytes (Fig. 2A), macrophages, or lymphocytes (not shown) into the BALF during AAD, the numbers of neutrophils were significantly increased, whereas eosinophil influx was attenuated.

Results

Development of AAD

Compared with uninfected, nonallergic (Sal) groups, allergic groups without infection (OVA; Fig. 1) displayed hallmark features of Th2-driven AAD characterized by eosinophil influx into the airways (Fig. 2) and tissue (Fig. 3), airways MSC hyperplasia
compared with that in uninfected, allergic (OV A) controls (Fig. 2B, 2C). By contrast, resolved infection suppresses eosinophil numbers only in the BALF. Current, but not resolved, infection also attenuated the numbers of tissue eosinophils and MSCs in the airways (Fig. 3A, 3B). Notably, the increased recruitment of neutrophils to the airways correlated with OV A-induced mobilization of neutrophils into the blood (Fig. 3C).

Current infection during AAD (D-7/OV A) also significantly increased neutrophils in BALF and blood, compared with infection in the absence of AAD (D-7/Sal; Figs. 2B, 3C). This indicates that the influx of neutrophils during AAD occurs in response to OV A sensitization and challenge, suggesting that infection primes for the neutrophil influx during AAD.

Current infection during sensitization drives allergen-specific Th1 T cell cytokine responses during AAD

The mobilization of neutrophils into the blood during allergen challenge suggested that OV A-specific Th1 cells had been generated during sensitization. To assess the effect of infection on OV A-specific T cell cytokine responses, MLN T cells were cultured with OV A. T cells from uninfected mice with AAD (OV A) produced strong OV A-specific Th2 responses characterized by substantial increases in the production of IL-5 and IL-13, compared with T cells from uninfected, nonallergic controls (Sal; Fig. 4B, 4C). By contrast, T cells from mice that had a current infection during OV A sensitization released substantially increased levels of OV A-specific IFN-\( \gamma \), suppressed IL-5 (Fig. 4A, 4B), but unaltered IL-13 release (\( p = 0.17; \) Fig. 4C), compared with T cells from uninfected, allergic (OV A) controls. A resolved infection (D-45/OV A) had no effect on IFN-\( \gamma \) or IL-5 production but decreased IL-13 levels.

Current infection during sensitization suppresses AHR to methacholine

The effects of infection on AHR during AAD were assessed. The induction of AAD without infection (OV A) resulted in the development of AHR, characterized by increased airway resistance and decreased compliance, compared with findings in uninfected, nonallergic controls (Sal; Fig. 5). Current, but not resolved, infection suppressed AHR.

Current infection during sensitization (D-7/OV A) resulted in reduced AHR with decreased resistance and increased compliance,
compared with findings in uninfected, allergic (OVA) controls (Fig. 5). By contrast, a resolved infection (D-45/OVA) had no effect. Notably, infected, nonallergic groups, regardless of the timing of infection (D-7/Sal and D-45/Sal), had increased AHR, compared with uninfected, nonallergic (Sal) controls, indicating that infection alone altered baseline lung function.

Suppression of neutrophil influx during infection
Chlamydial lung infections induce potent neutrophil influx into the lungs (20, 32); therefore, the effects of these cellular responses to infection on AAD were investigated. First, the effectiveness of suppressing neutrophil influx during infection by treatment with αKCMIP2 to reduce the levels of the chemotactic chemokines KC and MIP-2 was assessed.

The effects of Ab treatment on leukocyte influx during infection in the absence of AAD (i.e., without sensitization or challenge with OVA) were determined. Pulmonary neutrophil, but not lymphocyte, influx was reduced in infected, Ab-treated (αKCMIP2/D-7) groups, compared with untreated (Iso/D-7) controls, 10 d post-infection (Supplemental Fig. 1A). This finding is equivalent to day 3 of the experimental model (Fig. 1B) and is the time of peak infection. Neutrophil numbers were reduced by 52% in the BALF. However, treatment had no effect on the percentage of neutrophils in the blood (Supplemental Fig. 1B). Treatment did not affect infection, with no change in the number of chlamydia recovered from the lungs (Supplemental Fig. 1C).

Suppression of neutrophil influx during infection alters cellular responses in Chlamydia-driven neutrophilic AAD
Next, the influence of neutrophil influx into the lungs during infection on AAD was assessed. This was achieved by suppressing neutrophil influx during infection and analyzing the effects on the subsequent development of OVA-induced AAD (Fig. 1B). Treatment during infection reduced neutrophil influx into the lung and blood in AAD (αKCMIP2/D-7/OVA), compared with isotype-treated (Iso/D-7/OVA) controls. Neutrophil numbers were reduced by 48% in BALF, 65% in lung tissue, and 63% in blood (Fig. 6A–C).

We investigated whether the reduction in neutrophils in AAD resulted from removing the effects of neutrophils during infection or from a direct influence of Ab treatment on neutrophil influx during the challenge phase of AAD. To find out, neutrophil numbers in lungs and blood were assessed on day 12 (D12; Fig. 1B) of the experimental protocol, when groups had been infected and sensitized with OVA but prior to OVA challenge. Ab treatment did not alter the numbers of neutrophils in lungs or blood, compared with findings in isotype-treated controls prior to OVA challenge (not shown). Therefore, the influence of treatment on neutrophil numbers during infection-induced AAD resulted from suppression of the effects of neutrophils during infection and not from the prolonged effects of Abs on neutrophil influx during the challenge phase of the model.

Taken together, these results show that suppression of neutrophil influx during infection leads to suppression of neutrophil numbers.
in the airways and blood during AAD. This occurs despite neutrophil numbers being similar between infected groups prior to OVA challenge.

The suppression of neutrophil influx during infection removed the suppressive effects of infection on MSC numbers during AAD (Fig. 6D). However, treatment did not alter the infection-induced reduction of pulmonary eosinophil numbers during AAD (Supplemental Fig. 2A, B).

**Reduction of pulmonary neutrophil influx during infection alters T cell responses in Chlamydia-driven neutrophilic AAD**

The suppression of neutrophil influx during infection reversed the effects of infection on OVA-specific cytokine release from MLNs during AAD. Ab treatment of infected and allergic (αKCMP2/D-7/OVA) groups substantially reduced OVA-specific IFN-γ release, compared with that in infected, isotype-treated (Iso/D-7/OVA) controls (Fig. 6E). Treatment also increased IL-5 release by >2-fold, compared with controls, however, these levels were still significantly lower than those in Ab- (αKCMP2/OVA) or isotype-treated, uninfected, allergic (Iso/OVA) groups (Supplemental Fig. 2C). Treatment did not affect IL-13 levels in infected, allergic (αKCMP2/D-7/OVA) groups (Supplemental Fig. 2D).

**Reduction of pulmonary neutrophil influx during infection alters AHR in Chlamydia-driven neutrophilic AAD**

Reduced neutrophil influx into the lungs during infection completely reversed the suppressive effect of infection on AHR during AAD. Treatment (αKCMP2/D-7/OVA) significantly increased resistance ($p < 0.01$) and decreased compliance ($p = 0.06$), compared with findings in isotype-treated (Iso/D-7/OVA) controls (Fig. 7). No differences were found between treated, infected, and allergic (αKCMP2/D-7/OVA) groups and Ab- or isotype-treated, uninfected, allergic (αKCMP2/OVA and Iso/OVA) controls.

**Current infection during sensitization affects the expression of immune mediators in the lung during AAD, which are partially reversed by reduction of pulmonary neutrophil influx during infection**

The mechanisms of how infection modulates the phenotype and inflammatory profile of OVA-induced AAD were further investigated.
Chlamydial infections induce or alter the release of a variety of immune factors, including IL-12 and IL-17 and TARC, that may influence AAD. Current infection resulted in significant increases in IL-12 and IL-17 and decreased TARC expression in the lungs during AAD (Iso/D-7/OVA), compared with findings in uninfected, allergic (Iso/OVA) controls (Fig. 8A–C). Significantly, suppression of neutrophil influx into the lungs during infection partially reversed these changes. Ab-treated (αKCMIP2/D-7/OVA) groups had significantly decreased IL-12 and IL-17 and increased TARC expression in the lungs, compared with isotype-treated (Iso/D-7/OVA) controls (Fig. 8A–C).

**Current infection during sensitization increases APC activation in the lung during AAD, which is altered by reduction of pulmonary neutrophil influx during infection**

Infections may also alter the phenotype and function of APCs, which may influence how infection modulates AAD. Current infection increased the numbers of MHC II+ mDCs and costimulatory factors on APCs in lung tissue in AAD. Current infection (Iso/D-7/OVA) increased the number of viable cells that were MHC II+ mDCs and pDCs and APCs (CD11c+ cells) expressing CD80 and CD86 in the lungs during AAD, compared with findings in uninfected, allergic (Iso/OVA) controls (Fig. 8D–G).

Ab treatment during infection (αKCMIP2/D-7/OVA) increased the number of MHC II+ pDCs in the lungs during AAD over that seen in isotype-treated, infected (Iso/D-7/OVA) controls (Fig. 8D, 8E). This occurred despite treatment having no effect on the level of infection and therefore on antigenic load in the lungs. Treatment reversed the effect of infection on the number of APCs expressing CD80 during AAD (Fig. 8F) but had no effect on CD86 expression (Fig. 8G). Ab treatment in the absence of infection (αKCMIP2/OVA) did not substantially influence DCs or APCs, compared with findings in uninfected isotype-treated (Iso/D-7/OVA) controls (Fig. 8D–G).

Increased levels of IFN-γ and IL-17 during OVA challenge do not play a critical role in infection-induced neutrophilic AAD

We have shown that infection-induced changes in AAD are associated with increased IFN-γ and IL-17 responses following OVA challenge. To investigate the role of these cytokines in infection-induced neutrophilic AAD, αIFN-γ or αIL-17 mAbs were administered during OVA challenge (Fig. 1C). Infected, allergic groups that were treated with αIFN-γ (αIFN-γ/D-7/OVA) had increased numbers of neutrophils in BALF, compared with both uninfected, Ab- and isotype-treated (αIFN-γ/OVA and OVA) groups (Fig. 9). Significantly, treatment of infected, allergic groups with αIFN-γ (αIFN-γ/D-7/OVA), compared with infected, isotype-treated (D-7/OVA) controls, did not affect neutrophil numbers (Fig. 9). Similar results were observed with αIL-17 treatment (not shown). Therefore, although infection-induced changes in AAD are associated with augmented Th1/Th17 immunity, increased IFN-γ and IL-17 responses during OVA challenge do not play a critical role in driving neutrophilic inflammation in infection-induced neutrophilic AAD.

**Discussion**

In this paper, we show that chlamydial infection promotes neutrophilic inflammation and OVA-specific Th1 responses and suppresses Th2-mediated eosinophilic inflammation, resulting in a phenotype reminiscent of that of neutrophilic asthma. This phenotype was largely reversed by suppression of neutrophil influx into the lung during infection.

These studies employed the natural mouse pathogen *C. muridarum*, which was originally isolated from a mouse with respiratory infection (33, 34). The time course, as well as the immunological and histopathological progression, of *C. muridarum* infection of mice closely resembles that observed with *Chlamydophila pneumoniae* infection in humans (8, 9). Therefore, *C. muridarum* is the organism of choice for investigating natural host–bacteria–allergen interactions in mice.
The current and previous studies have reported that a resolved chlamydial infection during AAD either has no effect or suppresses key features of asthma in AAD (22, 23). Although the effects of resolved infection investigated in previous studies resulted in a suppression of Th2, and an increase in Th1 responses, the authors did not investigate the effects of an ongoing infection or demonstrate an increase in neutrophilic inflammation or the effects of infection on AHR. We have extended these studies to show that, although an active infection suppressed Th2 responses, infection should not be considered protective; rather, it changes
the phenotype of AAD to one underpinned by neutrophilic inflammation that is associated with allergen-specific Th1 (IFN-\(\gamma\)) responses. Neutrophilic inflammation plays an important role during acute exacerbations of asthma, and respiratory pathogens may be implicated in these events (18, 19, 35–37). More than one-third of asthmatics presenting with acute severe asthma exacerbations had elevated levels of \textit{Chlamydophila pneumoniae}-specific IgG or IgA, indicating an acute or reactivated infection (19). These subjects exhibited more intense neutrophilic inflammation, compared with acute exacerbators without infection. Our results show that AHR in groups with current infection in AAD was reduced to the same level as that induced by infection alone, but lung function was impaired in both groups, compared with uninfected, nonallergic controls. This observation suggests that infection suppresses AHR that is associated with AAD; however, infection alone impairs lung function. Clinical studies show that increased \textit{Chlamydophila pneumoniae} Ab levels are associated with persistent airflow limitation in adult-onset, non-asthmatic asthmatics (38). Therefore, infection may result in abnormal lung function in the absence of Th2-mediated allergic responses in humans. \textit{Chlamydia} driven neutrophilic asthma may also be a more protracted or chronic disease (39). Infection may result in a perpetuating feedback loop in which the neutrophilic phenotype is reinforced and prolonged, resulting in the induction of chronic disease. \textit{Chlamydia} is able to infect and grow in neutrophils (21, 40), and infected neutrophils have delayed apoptosis and secrete greater amounts of IL-8 (40). This characteristic may promote further neutrophil influx, resulting in the persistence of neutrophils within the airways during infection (40) and neutrophil-dominated asthma.

The switch toward a neutrophil-dominated phenotype correlated with increased pulmonary expression of IL-12 and IL-17, which may contribute to the suppression of Th2 responses during AAD (41). IL-12 is associated with Th1 polarization (42), whereas IL-17 is important in driving neutrophilic inflammation (43, 44). Recently, increased levels of IL-17 were detected in the airways of asthmatic patients and correlated with elevated neutrophil numbers in sputum and more severe disease (45). Taken together, our observations may help explain the clinical link between \textit{Chlamydia} and asthma. Infection may promote neutrophilic inflammation that is associated with Th1 and IL-17 responses, rather than Th2-dominated, eosinophilic responses, while maintaining some of the clinical features of asthma, such as impaired lung function. Th1 and Th17 responses have been previously shown to be associated with neutrophilic inflammation in mouse models of AAD; however, these studies used adoptive transfer of in vitro polarized Th1 and Th17 cells to demonstrate the link (46, 47). In this study, we show that chlamydial infection may drive Th1- and Th17-biased immune responses against allergens during AAD.

Neutrophilic asthma is difficult to treat, as patients are resistant to corticosteroids, which are the mainstay of asthma therapy (48, 49). Steroid treatment also decreases neutrophil apoptosis and increases neutrophil numbers in serum and tissue (50–52). Therefore, treatment may increase neutrophil numbers and perpetuate neutrophilia in the lung. Steroid treatment also promotes chlamydial infection in vitro and reactivates persistent lung infection in vivo (53, 54), and asthmatic patients on high, as opposed to low, doses of inhaled steroids are more likely to have evidence of \textit{Chlamydophila pneumoniae} infection (55). Most importantly, asthmatics with evidence of infection are more resistant to steroid treatment than are asthmatics without infection (56). These observations suggest that treatment may result in increased susceptibility to acute infection or reactivation of persistent chlamydial lung infection and that infection induces a phenotype of asthma that is more resistant to treatment. Therefore, alternative therapies may need to be considered when treating asthmatics with evidence of \textit{Chlamydia} driven neutrophilic inflammation.

Because an association exists between increased activation of innate immune factors and neutrophilic asthma (16), we hypothesized that the influx of neutrophils into the lungs may drive the development of neutrophilic AAD. To investigate this, the inflammatory chemokines KC and MIP-2 were inhibited during infection. Treatment resulted in ∼50% reduction in neutrophils in the lungs during infection. A lack of complete inhibition may be explained by the existence of non–CXC,2-dependent redundancies in chemotactic signaling, including C5a and MCP-1, which signals through CCR2 (57, 58). Systemic leukocyte numbers were not altered, indicating that only cellular influx into the lung, and not mobilization from the bone marrow, was affected. However, treatment during infection did suppress pulmonary and circulating neutrophil responses during subsequent AAD. This observation suggests that neutrophil accumulation during infection is necessary to prime for both local and systemic neutrophil responses during AAD. Importantly, treatment also reversed infection-driven alterations in OVA-specific IFN-\(\gamma\) levels, MSC numbers, and AHR during AAD. Treatment ceased 10 d before OVA challenge and had no significant effect on AAD in uninfected, allergic controls, demonstrating that Ab treatment had no direct or prolonged influence on AAD. Furthermore, neutrophil numbers were similar between Ab-treated and isotype control groups prior to the induction of AAD by OVA challenge. This finding shows that changes in the phenotype of AAD were related to the effects of infection. This also confirms that the suppression of neutrophil numbers by Ab treatment during infection results from a diminished ability to recruit neutrophils upon exposure to an allergen. Whether the effects of treatment result from lower numbers of neutrophils infiltrating the lungs during sensitization or from reduced de novo recruitment of neutrophils after allergen challenge remains unknown. Taken together, these data demonstrate that neutrophil influx into the lungs during infection plays an important role in driving the hallmark features of infection associated neutrophilic AAD.

The effect of treatment on pulmonary levels of IL-12, IL-17, and TARC and changes in APC activation during AAD demonstrates that the influx of neutrophils during infection is necessary to induce
a range of immune changes in Chlamydia-induced neutrophilic AAD. Treatment reduced IL-12 and IL-17 levels, which correlated with decreased Ag-specific Th1 responses and neutrophilic inflammation. Increased TARC expression posttreatment may have enhanced the chemotraction of Th2 cells (59, 60), which may explain the reinstatement of features of Th2-driven AAD (i.e., MSC hyperplasia and AHR). Increased MSC hyperplasia and AHR may also have resulted from increased pDC numbers, which promote T cell stimulation and inflammation during AAD. Finally, the correlation of increased CD80 costimulation with reduced Th1 cytokine responses supports the concept that CD80 costimulation is associated with the activation and polarization of Th1 responses (61–63).

The effect of treatment on IFN-γ responses and DC number and phenotype during AAD suggests that the influx of neutrophils into the lung during infection substantially alters the manner in which T cells are activated during AAD. Neutrophil activity may modify DC maturation and activation (64–66) and enhance the release of IL-12 and TNF-α from DCs (65), suggesting that neutrophil responses play a crucial role in influencing DCs to induce Th1 responses upon contact with T cells. Furthermore, neutrophils can present Ag to T cells and induce T cell proliferation and cytokine release (67, 68). Thus, the accumulation of neutrophils in the lungs during infection may play a key regulatory role during AAD by modulating local DC number and function, as well as DC-mediated T cell responses, or by directly interacting with T cells. Importantly, stimulation of DCs via CXCR2 does not affect DC activation or migration (69), suggesting that αKCMIP2 has no direct influence on DC number or phenotype in the lungs.

The depletion of IFN-γ or IL-17 during OVA challenge did not affect neutrophilic inflammation in Chlamydia-induced neutrophilic AAD. This finding demonstrates that neither IFN-γ nor IL-17 plays a critical mechanistic role during the challenge phase of Chlamydia-induced neutrophilic AAD. Depletion during sensitization and infection phases of the experimental models is not appropriate, as both IFN-γ and IL-17 are induced by and are required for the clearance of chlamydial respiratory infection (10, 11, 70). Thus, the absence of these factors during infection would dramatically change the profile and magnitude of infection and infection phases of the experimental models is not appropriate, as both IFN-γ and IL-17 are induced by and are required for the clearance of chlamydial respiratory infection (10, 11, 70). Thus, the absence of these factors during infection would dramatically change the profile and magnitude of infection and subsequently the responses to OVA. However, collectively, these results suggest that infection-induced neutrophilic responses alter allergic sensitization and drive the development of neutrophilic AAD that is associated with Th1/Th17 rather than Th2 responses.

Further experimental and clinical studies of the association between chlamydial infection-induced inflammatory responses and neutrophilic asthma may lead to novel treatments for individuals with this subtype of disease.

In summary, chlamydial infection may contribute to AAD by inducing neutrophil influx, which promotes a phenotype resembling that of neutrophilic asthma. These responses are associated with increased APC activation and expression of IL-12 and IL-17, suggesting that Chlamydia-induced neutrophilic AAD is associated with (but not directly regulated by) augmented Th1 and Th17 immune responses. Significantly, the influx of neutrophils in response to infection may play a novel role in driving neutrophilic AAD and associated Th1/Th17 responses.

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References


