Chlamydia muridarum Infection Subverts Dendritic Cell Function to Promote Th2 Immunity and Airways Hyperreactivity

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There is strong epidemiological evidence that *Chlamydia* infection can lead to exacerbation of asthma. However, the mechanism(s) whereby chlamydial infection, which normally elicits a strong Th type 1 (Th1) immune response, can exacerbate asthma, a disease characterized by dominant Th type 2 (Th2) immune responses, remains unclear. In the present study, we show that *Chlamydia muridarum* infection of murine bone marrow-derived dendritic cells (BMDC) modulates the phenotype, cytokine secretion profile, and Ag-presenting capability of these BMDC. *Chlamydia*-infected BMDC express lower levels of CD80 and increased CD86 compared with noninfected BMDC. When infected with *Chlamydia*, BMDC secrete increased TNF-α, IL-6, IL-10, IL-12, and IL-13. OVA peptide-pulsed infected BMDC induced significant proliferation of transgenic CD4$^+$ DO11.10 (D10) T cells, strongly inhibited IFN-γ secretion by D10 cells, and promoted a Th2 phenotype. Intratracheal transfer of infected, but not control noninfected, OVA peptide-pulsed BMDC to naive BALB/c mice, which had been i.v. infused with naive D10 T cells, resulted in increased levels of IL-10 and IL-13 in bronchoalveolar lavage fluid. Recipients of these infected BMDC showed significant increases in airways resistance and decreased airways compliance compared with mice that had received noninfected BMDC, indicative of the development of airways hyperreactivity. Collectively, these data suggest that *Chlamydia* infection of DCs allows the pathogen to deviate the induced immune response from a protective Th1 to a nonprotective Th2 response that could permit ongoing chronic infection. In the setting of allergic airways inflammation, this infection may then contribute to exacerbation of the asthmatic phenotype.   *The Journal of Immunology,* 2008, 180: 2225–2232.

C hlamydia are obligate intracellular Gram-negative bacteria and three species are known human pathogens. *Chlamydia trachomatis* is the causative agent of genital tract and ocular infections (1). *Chlamydophila pneumoniae* infects the respiratory tract and is responsible for 10–20% of community-acquired pneumonia (2–4). *Chlamydophila psittaci* is primarily a pathogen of psittacine birds but infection may lead to atypical pneumonia or pyrexia in man (5). *Chlamydia* are primarily pathogens of epithelial cells, however they can infect a range of cell types including smooth muscle cells, vascular endothelial cells, and immune cells, such as macrophages and dendritic cells (DCs)[2] (6–9). Resolution of infection in the mouse requires a cell-mediated immune response driven by IFN-γ-secreting CD4$^+$ Th type 1 (Th1) cells. An inability to mount an adequate *Chlamydia*-specific

Th1 response can often lead to persistence of the bacteria and associated immunopathology in the host. The central role of IFN-γ in clearance is evidenced by prolonged infections that occur in IFN-γ, and IFN-γ receptor-, deficient mice (10, 11). Notably, DC-derived IL-12 is essential for the generation of this Th1 response (12, 13).

Allergic asthma is a chronic inflammatory disease of the airways that results in reversible airflow obstruction, airways hyperreactivity (AHR), mucous overproduction, Ag-specific IgE production, and the development of airway-specific lesions (collectively termed remodeling of the airways). The inflammatory response is complex but CD4$^+$ Th type 2 (Th2) cells are a predominant feature of cellular infiltrates in the airways (14). Furthermore, Th2 cytokines, which include IL-4, IL-5, IL-10, and IL-13, induce many of the hallmark features of asthma. In particular, IL-13 is the central regulator of the effector phase of the asthmatic response due to its capacity to induce AHR, mucous hypersecretion, inflammation, and many of the lesions associated with airway wall remodeling (15–17). It is well recognized that DCs play a central role in the initial priming of Th2 responses during allergic sensitization. Recent investigations have demonstrated that DCs are also essential for the maintenance of many of the cardinal features of asthma through local reactivation of allergen-specific Th2 cells in the airways (18, 19).

Although allergens are common triggers of asthma, the role of respiratory tract infections in predisposition and exacerbations is becoming increasingly recognized and is a common cause of the difficulty in managing steroid-insensitive asthma. Emerging epidemiological and clinical evidence has strongly associated *C. pneumoniae* lung infection as a trigger for the development of asthma and as a common cause of exacerbations of this disorder

(20–23). The mechanism(s) whereby infection with *C. pneumoniae*, a bacterium associated with a Th1 response, can exacerbate or trigger asthma, a disease that is often characterized by a Th2 phenotype, remains unclear. The observation that *Chlamydia* can infect many different cell types, including immune cells, suggests that this mechanism may also be linked to the processes that trigger infection-induced asthma. As DCs are pivotal in regulating both Th1 and Th2 immune responses, we hypothesized that a *Chlamydia* infection of these APCs may subvert their ability to elicit protective Th1 immunity and instead promote Th2 responses that may amplify pre-existing allergic inflammation. This mechanism could operate to induce exacerbations of asthma and also perpetuate *Chlamydia* survival and persistent infection.

Materials and Methods

Reagents

Biotinylated mouse $CD4^+$ T lymphocyte enrichment mixture, IMag Streptavidin Particles Plus-direct magnet (DM), BD IMagnet, anti-CD45R/B220 DM beads, and anti-$CD8\alpha$ DM beads were all obtained from BD Biosciences. The OVA peptide (m.w. = 1773.94), consisting of aa 323–339 (ISQAVHAAHAEINEAGR) of the OVA protein was synthesized at the Biomolecular Resource Facility, John Curtin School of Medical Research, Canberra, Australia. Recombinant murine (rm) GM-CSF was produced in yeast using an expression construct provided by Dr. Tracey Wilson, Walter and Eliza Hall Institute, Melbourne, Australia. Recombinant mouse IL-4 was obtained from PeproTech (rmIL-4). All Abs were purchased from BD Pharmingen.

Mice

Male BALB/c mice (6–8 wk old) were obtained from the central animal house, the University of Newcastle (Callaghan, Australia). DO11.10 mice on a $Rag2^{-/-}$ background expressing a transgenic TCR that recognizes only the $OVA_{323-339}$ peptide in the context of I-A^d, and IL-13$^{-/-}$ mice (IL-13 KO, a gift from Dr. A. Mackenzie, Cambridge University, Cambridge, United Kingdom) on a BALB/c background were obtained from the John Curtin School of Medical Research. All studies were approved by and conducted in accordance with guidelines set out by the University of Newcastle Animal Care and Ethics Committee.

DC culture and purification

DC were generated based on a method previously described (24) with several modifications. In brief, femurs and tibias of naive adult male wild-type BALB/c or IL-13 knockout mice were collected and bone marrow flushed out with growth medium (RPMI 1640, 5×10^{-5} M 2-ME, 10% heat-inactivated FCS, 2 mM L-glutamine, 20 mM HEPES, 100 μg/ml penicillin, and 100 μg/ml streptomycin). RBCs were lysed and cells were washed through a 70-μm nylon cell strainer with RPMI 1640 media. Magnetic nanoparticle-labeled anti-$CD8\alpha$ and anti-CD45R/B220 mAbs were used to deplete cells according to the manufacturer's instructions. The negative cell fraction was resuspended at 1×10^6 cells/ml and supplemented with 10 ng/ml of both rmGM-CSF and rmIL-4. On day 3 and 6 of culture, 75% of the medium was removed and replaced with fresh growth medium resupplemented with cytokines. On day 8, the nonadherent and semiadherent cells were harvested. DC purity was increased by CD11c positive selection using incubation with a biotin-conjugated anti-mouse CD11c Ab and streptavidin-conjugated DM magnetic nanoparticles. The DCs were plated out in *Chlamydia* media (RPMI 1640, 10% heat-inactivated FCS, 2 mM L-glutamine, 20 mM HEPES, 5 μg/ml gentamicin, and 100 μg/ml streptomycin sulfate) at 4×10^5 cells/ml.

Infection assay

Chlamydia muridarum (ATCC VR-123, formerly the Mouse Pneumonitis strain, Cmu) was obtained from the American Type Culture Collection. Stock cultures were prepared as previously described (25) and stored at −80°C. Cmu was added to DCs at a 2:1 multiplicity of infection. The cultures were then incubated for 36 h at 37°C in 5% CO_2. After 24 h, *Escherichia coli* LPS (Sigma-Aldrich) at 1 μg/ml was added where appropriate for 12 h. DCs were harvested and analyzed by flow cytometry and supernatants collected for cytokine analysis. To examine DC infection by fluorescence microscopy, 10-mm diameter glass coverslips were placed into the wells of culture plates before cell seeding. Similarly, to assess the viability of infection after the 36-h incubation with Cmu, the DCs were harvested and washed to remove any extracellular Cmu. The cells were then lysed by sonication followed by vortexing to release any intracellular Cmu. The cell lysate was centrifuged to remove cell debris from the supernatant, which was then added to McCoy cells, plated on 10 mm diameter glass coverslips, and incubated for 3 h at 37°C in 5% CO_2. Media was removed and replaced with fresh media containing 1 mg/ml cyclohexamide and the cells were incubated for a further 48 h or until active infection could be visualized.

Fluorescence microscopy

After infection with Cmu, coverslips were washed to remove any cell debris, and cells fixed with methanol for 10 min. The cells were stained with FITC-conjugated anti-*Chlamydia*-specific LPS (Cellabs) for 30 min at 37°C followed by washing. Cells were photographed using an Olympus fluorescence microscope (model BX51), digital camera (Olympus DP70), and DP software (Olympus). The percentage of infected cells was quantitated by counting a total of 200 cells per slide. Infected cells were defined as those containing large FITC-positive granular inclusions.

Flow cytometry

Cells were washed three times in PBS plus 2% FCS and incubated with anti-mouse CD16/32 Fc receptor block at 1 mg/1×10^6 cells for 10 min on ice. Cells (1×10^6) were then incubated with the relevant Ab on ice for 20 min (CD80-biotin, CD86-biotin, I-A^d-PE, and CD11c-PE) and washed twice. For biotinylated Abs, a streptavidin-conjugated fluorochrome (streptavidin-allophycocyanin) was added at 4°C for 30 min. Cells were fixed in 2% formaldehyde (Polysciences) in PBS and analyzed on a FACSCanto (BD Biosciences).

Ag presentation and T cell proliferation assay

DCs were exposed to 5 μg/ml OVA peptide for 6 h to allow uptake. DCs were then washed twice to remove nonacquired OVA peptide. $CD4^+$ T cells were purified from spleens of naive adult male D011.10 mice on a $Rag2^{-/-}$ background. Spleens were excised and tissue matrix was digested with collagenase (1 mg/ml). The splenic material was then homogenized with RBC lysis and cells were resuspended in media. Mixed splenocytes were plated out in six-well plates at $5–10 \times 10^6$ cells/ml and incubated for 2 h at 37°C 5% CO_2, to deplete adherent leukocytes. Nonadherent cells were collected, washed, and $CD4^+$ T cells were enriched by negative selection using the biotinylated mouse CD4 T lymphocyte enrichment mixture and magnetic nanoparticles, according to manufacturer's instructions. $CD4^+$ T cell purity was readily assessed to be >95%. Purified T cells were stained with CFSE (Molecular Probes) as previously described (26). Cells were then washed twice, resuspended in *Chlamydia* media, and added to DCs at a DC:T cell ratio of 1:4. For cytokine analysis, T cells were not labeled with CFSE. Cells were then centrifuged (1500 rpm for 2 min at room temperature) to initiate immediate DC and T cell contact. Culture was continued for 4 days at 37°C in 5% CO_2. Supernatants were collected and nonadherent cells were harvested for detection of proliferation by CFSE fluorescence.

In vivo adoptive transfer experiments

On day 0, DO11.10 $Rag2^{-/-}$ $CD4^+$ T cells were purified as above and resuspended in PBS before 2×10^6 cells/200 μl were transferred i.v. via the tail vein into adult male BALB/c mice. DCs were cultured and infected as described. After 36 h of infection, DCs were pulsed with 5 μg/ml OVA peptide overnight. On day 1, infected or noninfected DCs were washed twice to remove free peptide, resuspended in PBS and transferred intratracheally at 3×10^5 cells/50 μl to each mouse. On day 4, mice were sacrificed and airway function was assessed. Bronchoalveolar lavage fluid (BALF) was also collected by administering 1 ml HBSS to the trachea and extracting fluid and cells.

Assessment of airway function

AHR was measured as previously described (27). In brief, assessment was performed on anesthetized, mechanically ventilated mice with a plethysmograph (Buxco) by measuring changes in lung resistance and compliance in response to increasing doses of inhaled methacholine.

Cytokine analysis

The concentration of TNF-α in supernatants was determined by ELISA according to the manufacturer's instructions (mouse Duoset TNF-α kit; R&D Systems). A custom-designed 7 cytokine (IL-4, −5, −6, −10, −12(p70), −13, and IFN-γ) multiplex analysis kit was purchased from Bio-Rad and the assay performed according to the manufacturer's instructions. Plates were analyzed for fluorescence using a Bio-Rad bioplex reader.
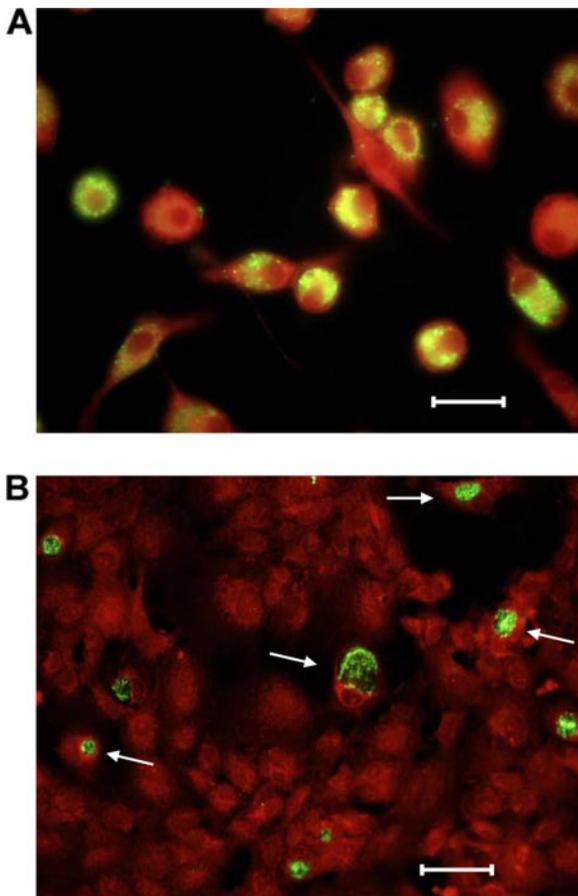
FIGURE 1. Cmu forms a viable infection within murine DCs. BMDC generated by culture in GM-CSF and IL-4 were exposed to Cmu for 36 h. Cell monolayers were then stained with FITC-conjugated anti-*Chlamydia* LPS to detect the presence of inclusion bodies within BMDC. *A*, A typical field is shown. To demonstrate that infection of BMDC resulted in viable Cmu progeny, infected BMDC were washed, lysed, and cell lysates plated on McCoy cells. *B*, Inclusions characteristic of a typical Cmu infection were observed and indicated with arrows. Scale bars represent 5 μm (*A*) and 25 μm (*B*). Green (FITC) represents Cmu inclusion bodies and red (rhodamine) represents a general cellular stain. Fluorescent micrographs are from one experiment representative of four independent experiments.

Statistical analysis

Data presented are the means $\pm$ SEM. A nonparametric Mann-Whitney *U* test (two-tailed) was used to establish statistical significance between any two separate groups with different means. For AHR data, an ANOVA was used to analyze the difference between groups. A significant difference between any two groups was considered to exist when the *p* value was <0.05. Prism software version 4 was used for all calculations.

Results

Cmu infects murine BMDC

To determine whether murine DCs could be infected with Cmu, BMDC were exposed to Cmu at a multiplicity of infection of 2:1. After 36 h, BMDC were fixed and stained with FITC anti-*Chlamydia* LPS. Fig. 1*A* shows that BMDC stained positive for chlamydial inclusions (60% of BMDC; data not shown), which were notably granular in appearance, indicating replicating bacteria (infection with Cmu). To confirm that this was a viable infection rather than phagocytic uptake by the DC, BMDC were harvested from primary cultures, washed to remove any extracellular Cmu, and then lysed to release intracellular Cmu. The cell lysate was then plated on the epithelial McCoy cell line. Cmu isolated

from infected BMDC were able to infect and form sizeable inclusions within McCoy cells (Fig. 1*B*). Infectivity of Cmu in McCoy cells was reduced following growth in BMDC ($1.5 \times 10^4 \pm 0.3 \times 10^4$ inclusion forming units per million DC), however it is possible this may be increased at later time points. At the time points studied, DC cell death postinfection with *Chlamydia* was negligible as determined by staining with trypan blue. This is a novel observation and indicates that Cmu can readily form viable infections within murine DCs.

*Cmu infection induces unique phenotypic changes in murine BMDC*

Following infection with Cmu, BMDC were stained with anti-MHC class II, anti-CD80, and anti-CD86 and analyzed by flow cytometry. Because DC generated in vitro by culture with GM-CSF and IL-4 are not fully matured, BMDC were also activated in culture with LPS (*Escherichia coli*) and included as positive controls. MHC class II surface expression was increased by exposure to LPS. In contrast, expression by infected BMDC did not significantly differ from that of noninfected BMDC, indicating Cmu infection failed to up-regulate this DC maturation marker (Fig. 2*A*). Interestingly, Cmu infection of BMDC reduced the number of cells positively expressing the costimulatory marker CD80 compared with noninfected BMDC, while as expected, LPS increased the number of cells expressing this costimulatory molecule (Fig. 2*B*). By contrast, CD86 expression was significantly increased ($p < 0.05$) on both infected and LPS-stimulated BMDC as compared with noninfected controls (Fig. 2*C*). These data reveal that in comparison to LPS stimulation (MHC class II$^+$, CD80^{high}, CD86^{high}) Cmu induces a distinct DC phenotype characterized by MHC class II$^{+/-}$, CD80^{low}, and CD86^{high} levels of expression.

Cmu infection alters the cytokine secretion profile of murine BMDC

To determine whether Cmu infection altered the DC cytokine secretion profile, supernatants from control noninfected, Cmu-infected, and LPS-stimulated BMDC were collected after 36 h and analyzed by a multiplex bead assay. This experiment was also performed for TLR-2 agonist peptidoglycan (PGN)-stimulated BMDC. Infected BMDC secreted significantly more IL-12, TNF-α, IL-6, IL-10, and IL-13 (Fig. 3, *A–E*) than noninfected BMDC. With the exception of IL-10 and TNF-α, cytokine levels were approximately equivalent between infected and LPS-stimulated BMDC (Fig. 3, *A–E*), and likewise for PGN-stimulated BMDC (data not shown). TNF-α was produced by infected BMDC at <75% of the level induced by LPS stimulation (Fig. 3*B*); this was despite the large dose of Cmu administered. Similarly, TNF-α was lower in cultures of PGN-stimulated BMDC compared with LPS-stimulated BMDC (data not shown). Interestingly, IL-10 was ~3-fold higher in cultures of infected BMDC compared with both LPS-stimulated (Fig. 3*D*) and PGN-stimulated BMDC (data not shown), suggesting an important role for this Th1-inhibiting cytokine. Furthermore, very high levels of IL-6, a regulatory T cell inhibitory cytokine and activator of Th2 immunity, were detected from Cmu-infected BMDC.

Chlamydia-*infected BMDC induce T cell proliferation and a Th2 phenotype in D10 cells*

To evaluate the potential of Cmu-infected BMDC to polarize T cells to a specific phenotype, infected or noninfected BMDC were pulsed with the dominant antigenic peptide (OVA$_{323-339}$ peptide) of the archetypal allergen OVA. The cells were washed and then
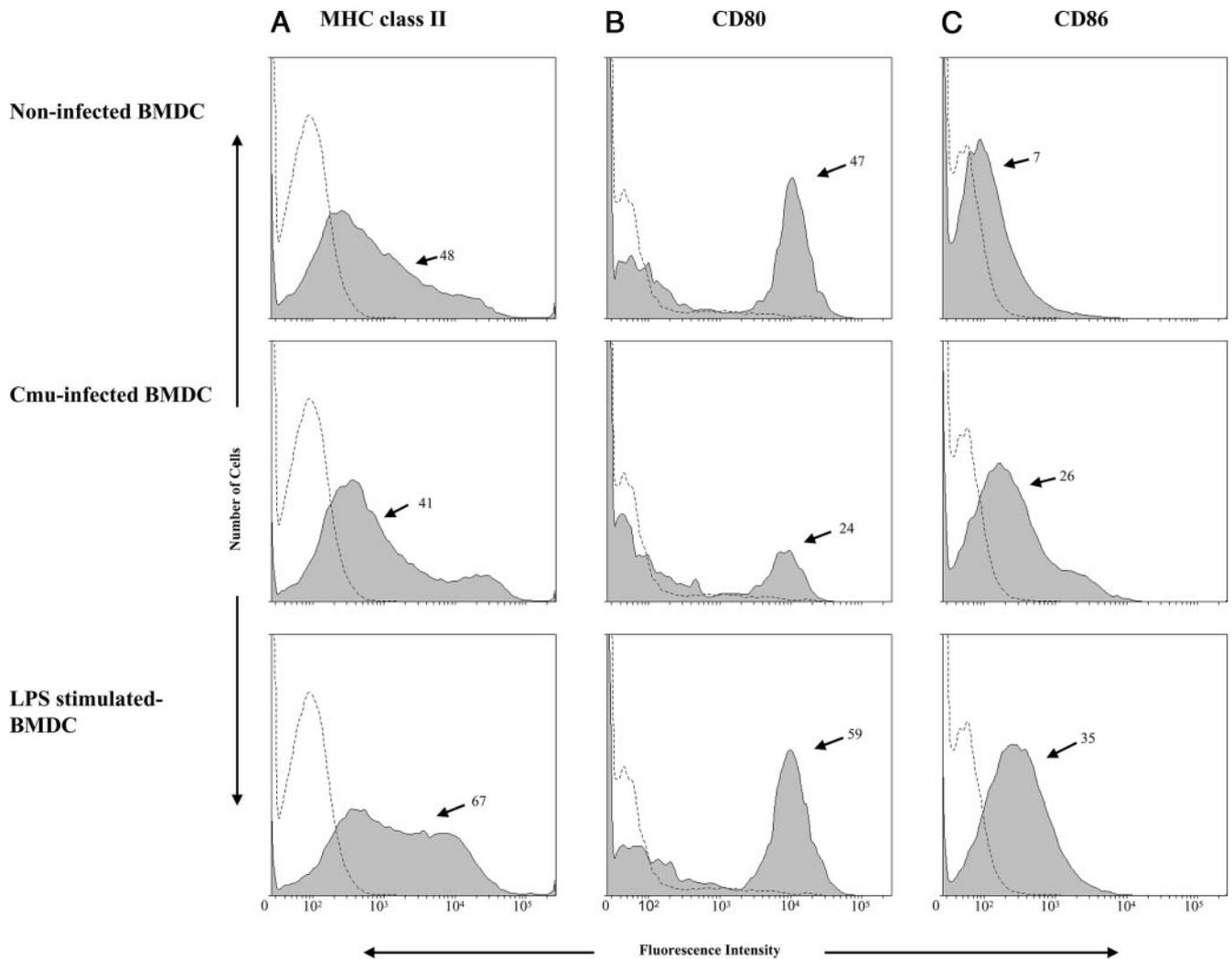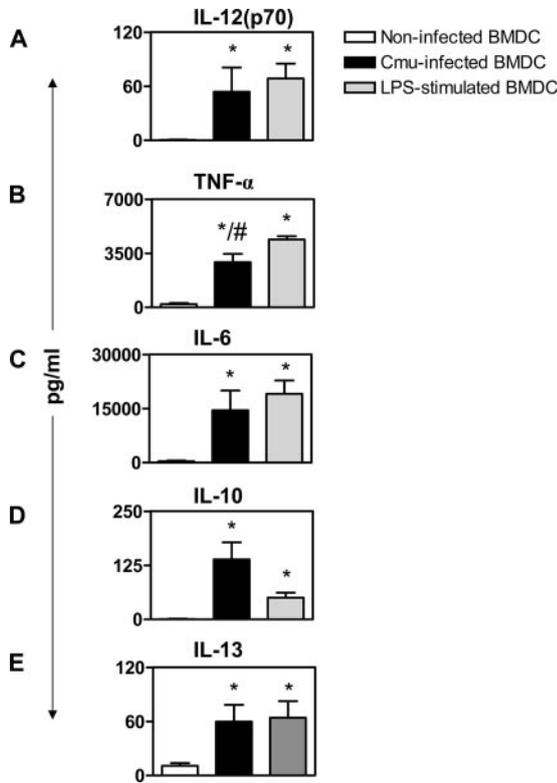
FIGURE 2. *Chlamydia* infection of BMDC prevents up-regulation of MHC class II and CD80 but increases CD86 expression. Cmu-infected BMDC (shaded), noninfected control BMDC (shaded), or noninfected BMDC cultured for 24 h then stimulated with LPS for the final 12 h (shaded) were each stained with anti-MHC class II (*A*), anti-CD80 (*B*), and anti-CD86 (*C*) and analyzed by flow cytometry. The dashed line (unshaded area) represents the isotype control. Values depicted reflect the percentage of cells expressing the relevant surface molecule. Results are representative of four independent experiments.

cultured with CFSE-labeled naive CD4$^+$ OVA peptide-specific DO11.10 T (D10) cells. After 4 days, 60–70% of D10 cells cocultured with infected BMDC had undergone (3) three cell divisions in contrast to only 10–12% of T cells activated by noninfected BMDC (Fig. 4*A*). Conversely, Cmu infection of BMDC produced a significantly lower proportion of static or poorly proliferative (0–2 divisions) D10 cells compared with the coculture of noninfected BMDC with D10 cells. These data clearly demonstrate that Cmu infection of BMDC stimulates significantly greater allergen-specific T cell proliferation. BMDC exposed to LPS and OVA and then cultured with T cells did not produce any significant increase in proliferation compared with noninfected BMDC controls (data not shown). This strongly suggests that the enhanced proliferation was specific to *Chlamydia* infection and not simply a result of maturation of the BMDC. The T cell cytokine production was determined after 4 days of coculture with infected or noninfected, OVA peptide-pulsed BMDC. Infected and noninfected BMDC cultured alone were included as controls. In addition, nonpeptide-pulsed unstimulated BMDC cultured with D10 cells were also used as controls and produced very low levels of all cytokines (Fig. 4, *B–D*). Not only did Cmu-infected BMDC secrete significantly more TNF-α, IL-10, and IL-13 as compared with noninfected con-

trols (as shown in Fig. 3), but they also drove D10 cells to secrete additional levels of these cytokines (Fig. 4, *B–D*). D10 cells cultured with infected BMDC produced much higher levels of TNF-α, and the Th2 cytokines IL-10 and IL-13, compared with D10 cells cultured with noninfected BMDC. At baseline, activation of peptide-specific D10 cells by BMDC pulsed with OVA peptide resulted in IFN-γ secretion. Notably, while D10 cells activated by noninfected BMDC produced substantial levels of IFN-γ, Cmu infection of BMDC significantly attenuated this response (Fig. 4*E*). In fact infection of BMDCs elicited a near total reduction (>20-fold) in the secretion of the prototypical Th1 cytokine, IFN-γ, by D10 cells. Collectively these results suggest that chlamydial infection of DCs promotes the proliferation of allergen-specific T cells to a bystander Ag, and this is associated with commitment to a Th2 phenotype and suppression of Th1 responses.

Adoptive transfer of Chlamydia-*infected BMDC plus D10 cells causes airways hyperreactivity in naive BALB/c mice*

Next, we determined whether chlamydial infection of BMDC could induce AHR, a critical clinical feature of infection-induced exacerbations of asthma. Naive BALB/c mice were i.v. infused

FIGURE 3. *Chlamydia* infection of BMDC increases the secretion of a diverse array of cytokines including Th2-inducers. Cmu-infected (black) and noninfected BMDC (unshaded) were cultured for 36 h while noninfected BMDC were cultured for 24 h then stimulated with LPS for the final 12 h (gray). Cytokines in BMDC supernatants were determined by multiplex bead array system, IL-12(p70) (*A*), TNF-α (*B*), IL-6 (*C*), IL-10 (*D*), and IL-13 (*E*). All values are concentrations of pg/ml. ∗, $p < 0.05$ compared with noninfected BMDC; #, $p < 0.05$ compared with LPS-stimulated BMDC. Data represent mean ± SEM.

with 2×10^6 D10 cells 1 day before the intratracheal administration of 3×10^5 Cmu-infected or noninfected, OVA peptide-pulsed BMDC. Three days following transfer of BMDC, BALF from recipient mice was collected. IL-10 and IL-13 concentrations in BALF were significantly increased in recipients of infected BMDC and D10 cells as compared with recipients of noninfected BMDC and D10 cells (Fig. 5, *A* and *B*), or D10 cells alone (data not shown). To examine whether the increased IL-13 was DC or T cell-derived, BMDC from IL-13-deficient (IL-13$^{-/-}$) mice were used. The IL-13 levels in BAL fluid from recipients of infected vs noninfected IL-13$^{-/-}$ BMDC was not significantly different (Fig. 5*B*), demonstrating that the IL-13 was derived directly from Cmu-infected DCs themselves, consistent with our in vitro data (Fig. 3*E*). Similar to BMDCs from wild-type mice, IL-10 levels were significantly greater in recipients of Cmu-infected vs noninfected IL-13$^{-/-}$ BMDC (Fig. 5*A*). We then determined whether adoptive transfer of Cmu-infected BMDC and D10 cells affected the development of AHR in BALB/c mice, as IL-13 is a critical regulator of enhanced bronchoconstriction in mouse models of asthma. Mice were mechanically ventilated and challenged with two doses of methacholine before measurement of airways resistance and compliance. Airways resistance (Fig. 5*C*) was significantly greater in recipients of infected BMDC as compared with recipients of noninfected BMDC. Similarly, airways compliance was significantly decreased in recipients of infected BMDC compared with recipients of noninfected BMDC (Fig. 5*D*). A similar trend was also observed for mucous-secreting cells with greater numbers detected in the airways of recipients of infected vs noninfected BMDC (data not shown). Overall, these data reveal that a *Chlamydia* infection of DCs significantly increases AHR, which is associated with increased IL-13 production. Importantly, this pathway is also critical in inducing AHR in models of allergic asthma and thus identifies a common mechanism for infection-induced exacerbation of asthma.

FIGURE 4. Coculture of Ag-specific T cells with *Chlamydia*-infected BMDC creates a highly proliferative Th2 cell. Cmu-infected and noninfected BMDC were pulsed with OVA$_{323-339}$ peptide then cultured for 4 days with DO11.10 T cells. In some experiments, T cells were labeled with CFSE and T cell division was determined by a decrease of CFSE fluorescence. *A*, These data represent the percentage of cells that have undergone between 0 and 2 divisions vs 3 or more cell divisions, for unstimulated T cells (gray), noninfected (unshaded), and Cmu-infected (black) BMDC pulsed with OVA$_{323-339}$ peptide and cultured with CFSE-labeled DO11.10 T cells. ∗, $p < 0.05$ compared with unstimulated T cells; # $p < 0.05$ compared with BMDC plus OVA plus T cells. Coculture supernatants were assayed by multiplex bead array system to determine concentrations of the cytokines, TNF-α (*B*), IL-10 (*C*), IL-13 (*D*), and IFN-γ (*E*). All values are concentrations of pg/ml. Controls included unstimulated T cells (BMDC plus T cells), and Cmu-infected and noninfected BMDC pulsed with OVA peptide. ∗, $p < 0.05$ compared with BMDC plus OVA plus T cells. Data represent mean ± SEM.
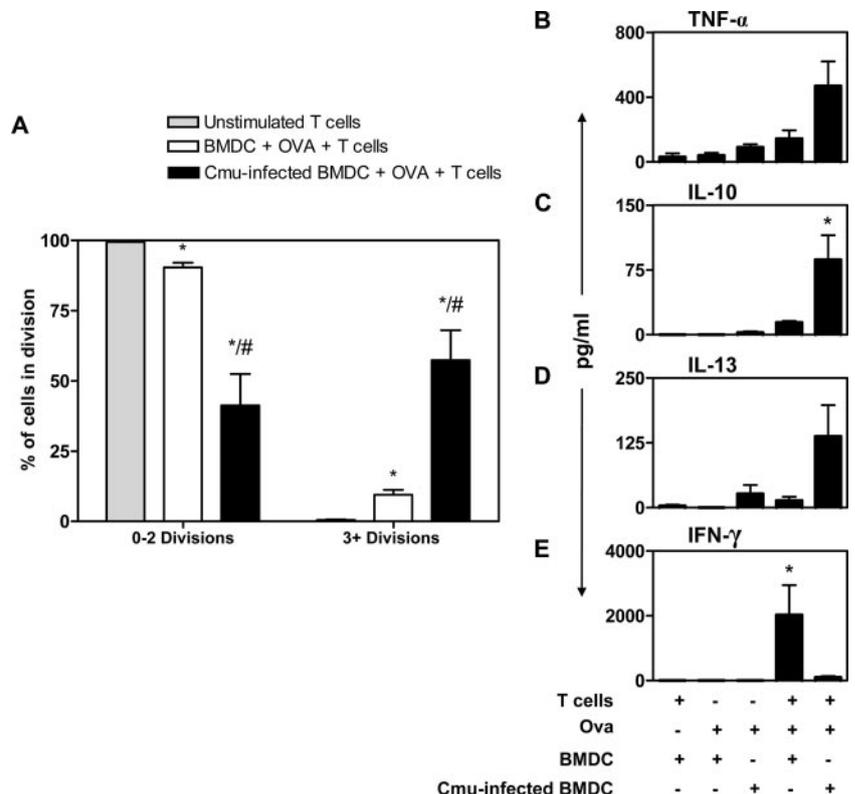
FIGURE 5. Adoptive transfer of *Chlamydia*-infected BMDC leads to DC-derived IL-13 in the BALF and increased AHR. DO11.10 T cells (2×10^6) were adoptively transferred i.v. to naive BALB/c mice on day 0. Mice received 3×10^5 OVA$_{323-339}$ peptide-pulsed BMDC (*Chlamydia* (Cmu)-infected or noninfected) intratracheally on day 1. On day 3, animals were sacrificed and the levels of IL-10 (*A*) and IL-13 (*B*) in BALF were determined. Statistical significance and *p* values are depicted. Mice were mechanically ventilated and airways resistance (*C*) and compliance (*D*) were determined by methacholine exposure for mice receiving Cmu-infected BMDC (shaded boxes) or noninfected BMDC (unshaded boxes). *, $p < 0.05$; **, $p < 0.01$ compared with noninfected BMDC control. Data represent mean ± SEM. WT; wild-type bone marrow, IL-13$^{-/-}$; IL-13 knockout bone marrow.
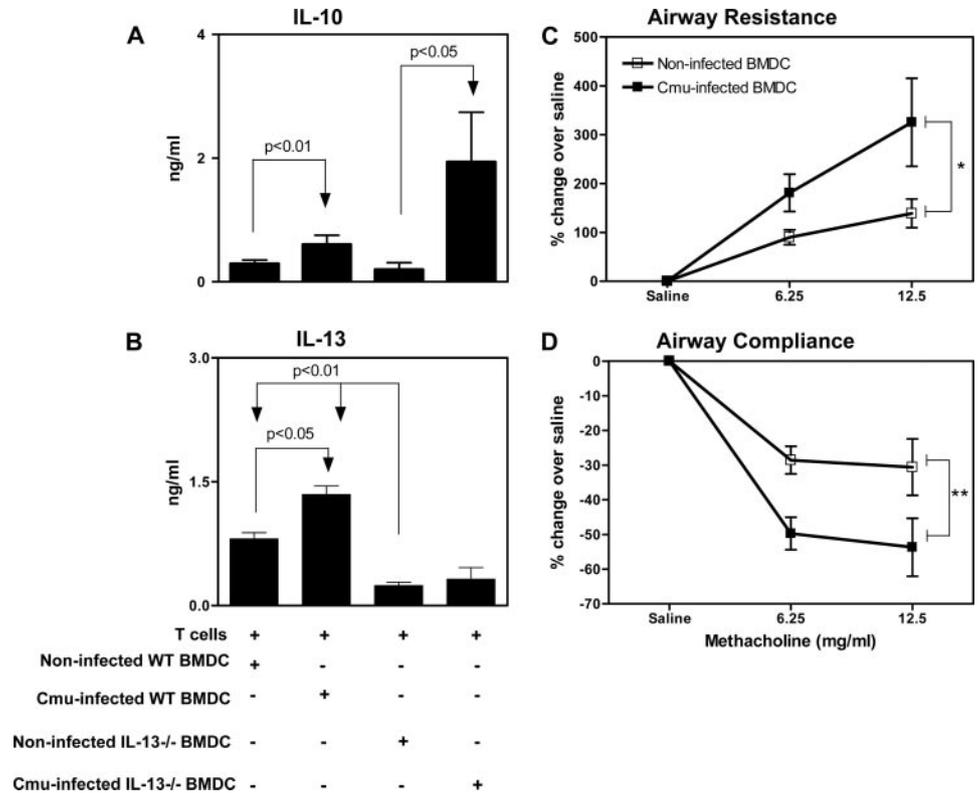
Discussion

There is a strong epidemiological link between infection with the intracellular bacteria *C. pneumoniae* and asthma exacerbation. A recent investigation also shows that early life infection with the mouse pneumonitis biovar Cmu exacerbates allergic asthma in a mouse model of the disease (28). However, the cellular and molecular mechanisms that underpin this association are poorly understood. In this investigation, we show that Cmu can form a viable infection within BMDC and that infection subverts DC function. This confirms a recent study demonstrating that murine DCs can be infected with the murine *Chlamydia,* Cmu (29), and importantly demonstrates that infection also alters DC function and induction of T cell responses. *Chlamydia* infection of BMDC inhibited the expression of the constitutive costimulatory marker CD80 and enhanced CD86 expression, a phenotype initially reported to promote Th2 responses (30). However, the actual effect on the polarization of T cells is perhaps more complex (31, 32). *Chlamydia* failed to up-regulate the Ag-presenting molecule MHC class II. This result complements a study from Zhong et al. (33), which showed that in cervical and airway epithelial cell lines, *Chlamydia* infection inhibits IFN-γ-inducible MHC class II expression by degrading the transcription factor upstream stimulator factor-1. This deviation of MHC class II expression combined with the lower costimulatory CD80 expression may assist the bacteria in evading recognition by the immune system. *Chlamydia*-infected BMDC spontaneously secreted high levels of IL-6 and IL-10, cytokines that promote Th2 responses, as well as the inflammatory cytokine TNF-α. PGN-stimulated BMDC produced a very similar cytokine secretion pattern to that of *Chlamydia* infection other than the respective results for IL-10. As PGN is a known agonist of both TLR2 and the NOD1 intracellular pattern recognition receptor, our results complement previous reports that indicate *Chlamydia* activates both TLR2 and NOD1 (34–37). Interestingly, IL-13 secretion, which to our knowledge, has not previously been shown to be produced by DCs in response to a pathogen, was also induced by

Chlamydia infection. Although infected BMDC also produced IL-12, which is normally associated with the induction of Th1 responses, coculture of infected, OVA peptide-pulsed BMDC with naive D10 cells inhibited the development of a Th1 phenotype through suppression of IFN-γ. In contrast, *Chlamydia*-infected BMDC promoted the expression of the Th2 cytokines IL-10 and IL-13 from D10 cells. Furthermore, *Chlamydia*-infected BMDC induced substantially greater expansion of allergen-specific T cells. Collectively, the chlamydial infection of BMDC generates an APC that favors the induction of a highly proliferative Th2 response to a bystander Ag (OVA peptide). These results were substantiated in vivo by adoptive transfer studies. Recipients of OVA peptide-pulsed, *Chlamydia*-infected BMDC and D10 cells had higher levels of IL-10 and IL-13 in the BALF as compared with recipients of noninfected BMDC. The increased production of Th2 cytokines was associated with increased airways resistance and decreased compliance (enhanced AHR) and a trend to greater numbers of mucous-secreting cells. Therefore, this study provides a novel mechanism to explain the association between *Chlamydia* lung infection and infection-induced exacerbations of asthma.

Interestingly, *C. pneumoniae* has been detected in the cytoplasm of DCs in vivo from patients with atherosclerosis, and human DCs can also be infected with *C. pneumoniae* in vitro (38–40). Moreover, a study by Wittkop et al. (40) demonstrated that infectious progeny are present within DCs as much as 25 days after infection. Our studies suggest the possibility that *Chlamydia*-infected pulmonary DCs could take up allergen in the lung, migrate to lung-draining lymph nodes, and stimulate a greater Th2 response that contributes to allergic disease. Alternatively, infected DCs may reside in the respiratory epithelium and induce greater restimulation of allergen-specific Th2 cells, possibly via the secretion of IL-6 (41). Our studies uniquely demonstrate that *Chlamydia*-infected murine DCs can release the key asthmatic effector cytokine IL-13, and the enhanced secretion of this cytokine due to infection

associates with enhanced AHR and mucous secretion. This provides a mechanism by which *Chlamydia*-infected pulmonary DCs could directly exacerbate features of asthma, especially infection-associated bronchial hyperreactivity. Thus, we have identified a common etiological pathway whereby infections and allergens may converge to exacerbate asthma.

Although the mechanisms of clearance are less well defined in humans, immune protection against murine chlamydial infection requires a Th1 response, with the mechanism(s) of clearance critically dependent on the production of IFN-γ (42). DC-derived IL-12 plays an important role in the induction of this anti-chlamydial Th1 response. Administration of rIL-12 has been shown to confer protection to *Chlamydia*-infected mice, whereas IL-12-deficient mice develop reduced clearance of *Chlamydia* that is associated with severe tissue lesions due to low IFN-γ production (13, 43). We have demonstrated that *Chlamydia* infection of BMDCs suppresses a Th1 response by dramatically inhibiting the IFN-γ secretion by D10 T cells. In combination with our finding that *Chlamydia*-infected BMDC secrete IL-6, IL-10, and IL-13, which collectively induce Th2 responses and inhibit macrophage-dependent innate immunity, this could potentially offer a survival advantage for *Chlamydia* within the host. Further study is required to determine whether this may contribute to the development of persistent infections (44).

Although IFN-γ production was inhibited, IL-12 was still secreted by infected BMDCs, which implicates the involvement of other factors downstream of IL-12 in disrupting the induction of a Th1 response. IL-6 was produced by infected BMDC. Secretion of this cytokine by pulmonary DCs has been shown to suppress IL-12-mediated Th1 responses (45). Furthermore, IL-6 enhances local Th2 responses by allergen-specific T cells in the lung and promotes T cell proliferation (41). Hence, the induction of an adaptive immune response to *Chlamydia* infection, in the presence of IL-6, could favor a nonprotective Th2 response. IL-10 was also secreted by *Chlamydia*-infected BMDC and from T cells stimulated by these DC. IL-10 is a potent inhibitor of IL-12-induced Th1 differentiation. Protective anti-chlamydial Th1 cells are elicited far more effectively in IL-10 knockout mice than in wild-type mice. Igietseme et al. demonstrated this to be a consequence of the predilection of the DCs from IL-10 knockout mice to being potent initiators of a Th1 response (46, 47). Therefore, the significant IL-10 levels measured from our infected BMDCs are likely a major factor for the inhibition of the Th1 response in our T cell experiments. Studies of another inflammatory disease caused by *Chlamydia*, namely reactive arthritis, have also shown that the IL-10/IL-12 balance is critical for determining the outcome of the immune response (48). Maintenance of the Th2 cytokine pattern that drives both reactive arthritis and chlamydial persistence depended on IL-10 suppression of IL-12-mediated protective immunity. Thus, excess IL-10 can result in Th2 immunity even in the presence of IL-12. Indeed, overproduction of IL-10 is a key feature in the impaired cell-mediated response to *C. trachomatis* infection in patients with trachoma (49). Therefore, the production of IL-10 early in the innate immune response to intracellular pathogens such as *Chlamydia* may promote the establishment of persistent infections and the potential for Th2-mediated pathology.

In this study, IL-13 was produced by both *Chlamydia*-infected BMDC and from T cells stimulated by these DC. Currently, there is a paucity of data concerning the production of IL-13 by DCs, and to date only one such study exists in mice. This study demonstrated that murine DCs secrete low levels of IL-13 in response to TLR 2-stimulation by the synthetic ligand Pam3Cys (50). This stimulation of DC IL-13 production was associated with the exacerbation of allergic airways disease, which further supports our

observations. Bellinghausen et al. demonstrated that human DCs can produce IL-13 in response to allergen exposure, wherein this cytokine then acts on the T cell in a STAT6-dependent manner to contribute to the induction of Th2 cells (51). In the context of our study, IL-13 production by infected DCs may contribute to the development of nonprotective anti-chlamydial immunity by suppressing macrophage function. IL-13 is known to inhibit macrophage production of NO (52–54), IFN-γ-induced tryptophan degradation (55), and production of inflammatory cytokines, such as IL-1 (53, 56, 57). These are all important mechanisms for the resolution of chlamydial infection. Unpublished data from our laboratory also shows that IL-13-deficient mice clear a respiratory Cmu infection more rapidly than wild-type mice (our unpublished data), suggesting that IL-13 regulates the innate response to Cmu infection. By inducing the secretion of IL-13 through viable infection of the DC, *Chlamydia* may suppress the innate immune response to infection to enhance bacterial survival, while simultaneously having the deleterious effects on airway reactivity.

In summary, *Chlamydia* infection has been linked with the exacerbation of asthma. In this study, we provide a mechanism whereby the infection of DCs subverts the function of these crucial immune cells to promote Th2 immune responses that are nonprotective with regards to infection but have the potential to exacerbate preexisting allergic inflammation and induce AHR. Notably, allergen or infection both induce common effector pathways, converging on the IL-13 operated STAT6 signaling cascade that is critical for the development of enhanced bronchoconstriction (AHR). These data provide the first mechanistic explanation for the association between *Chlamydia* lung infection and exacerbation of asthma.

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