

NK Cells Contribute to Intracellular Bacterial Infection-Mediated Inhibition of Allergic Responses¹

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To experimentally examine the hygiene hypothesis, here we studied the effect of chlamydial infection on the development of allergic responses induced by OVA and the involvement of NK cells in this process using a mouse model of airway inflammation. We found that prior *Chlamydia muridarum* infection can inhibit airway eosinophilic inflammation and mucus production induced by allergen sensitization and challenge. The inhibition was correlated with an alteration of allergen-driven cytokine-producing patterns of T cells. We demonstrated that NK cells were activated following chlamydial infection, showing both cell expansion and cytokine secretion. The *in vivo* depletion of NK cells using anti-NK Ab before OVA sensitization and challenge partially abolished the inhibitory effect of chlamydial infection, which was associated with a partial restoration of Th2 cytokine production. In contrast, the adoptive transfer of NK cells that were isolated from infected mice showed a significant inhibitory effect on allergic responses, similar to that observed in natural infection. The data suggest that the innate immune cells such as NK cells may play an important role in infection-mediated inhibition of allergic responses. *The Journal of Immunology*, 2008, 180: 4621–4628.

The hygiene hypothesis proposes that microbial infection or microbial products may alter the host's immune system, thus modulating the development of allergic diseases (1, 2). Several groups including our own have used experimental approaches to test this provocative hypothesis. It has been demonstrated that certain intracellular bacterial infection or bacterial products are capable of inhibiting airway eosinophilic inflammation, mucus overproduction, allergen-specific IgE production, airway hyperresponsiveness, and airway remodeling (3–8). Alteration of cytokine patterns and induction of immune suppressive or regulatory T lymphocytes appear to be important mechanisms for infection-mediated inhibition of allergy (9–11). However, the potential involvement of innate immune cells, such as NK cells, in the process remains unexplored. Because the role of the innate immune system in modulating adaptive immune responses has been unraveled in numerous studies (12), we hypothesized that the innate immune responses caused by infections play an important role in shaping the outcomes of T cell responses to allergens.

NK cell is an important component of innate immune system and is the first line of host defenses against intracellular infection, including *Chlamydia*. It has been shown in murine genital tract infection models that *C. muridarum* infection can activate NK cells

which may produce cytokines and recognize/lyse infected cells that showed decreased expression of classical and nonclassical MHC molecules on the cell surface (13). Although the nature of NK cell responses following respiratory tract chlamydial infection has yet to be examined, it is pertinent to hypothesize that NK cells are involved in the host defense against chlamydial lung infection.

In our previous studies, we have shown that *C. muridarum* infection can inhibit *de novo* allergic responses induced by ragweed exposure (6–8). The inhibition was associated with a switch of allergen-driven cytokine production patterns from Th2 dominance to Th1 dominance in the mice. In the present study, using a mouse asthma model induced by OVA, we further studied the effect of chlamydial infection in the development of allergic responses. Moreover, we investigated the kinetics of NK cell responses following respiratory tract chlamydial infection and examined the role of NK cells in influencing the allergic responses induced by OVA exposure. The experiments using NK cell depletion and NK cell adoptive transfer approaches showed that infection-induced NK cell activity plays an important role in the infection-mediated inhibition of allergic responses.

Materials and Methods

Mice

Breeding pairs of J α 18 knockout (KO)³ mice that had a deficiency in NK T cells (NKT-KO mice) with B6 background were purchased from The Jackson Laboratory. The knockout mice and female C57BL/6 mice (7–10 wk old) were bred and maintained at the specific-pathogen-free animal care facility at University of Manitoba (Winnipeg, Manitoba, Canada). Animals were used in accordance with the guidelines issued by the Canadian Council on Animal Care.

Organism

The mouse pneumonitis biovar of *C. trachomatis* (MoPn), more recently called *C. muridarum*, was used as an infectious agent in this study. The

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³ Abbreviations used in this paper: KO, knockout; MoPn, mouse pneumonitis biovar of *C. trachomatis*; i.n., intranasal(ly); IFU, inclusion-forming unit; BAL, bronchoalveolar lavage; PAS, periodic acid-Schiff; HMI, histological mucus index; DC, dendritic cell.

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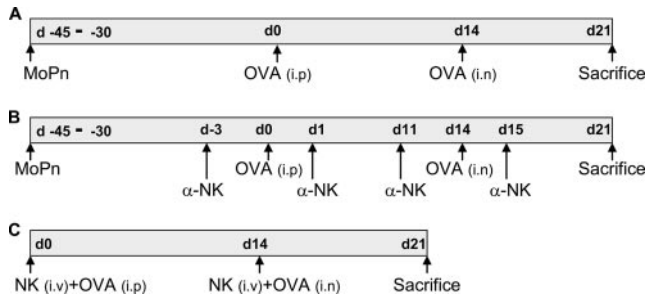


FIGURE 1. Diagram of experimental protocols. *A*, General protocol of mice infection and allergen sensitization and challenge. *B*, Timeline of anti-NK1.1 Ab (α -NK) treatment in the NK depletion experiments. *C*, Timeline of NK cell transfer and allergen treatment in the recipient mice in the cell transfer experiments.

procedures for MoPn growth, harvesting, and elementary body purification were as previously described (14).

Reagents

Complete RPMI 1640 medium was prepared as previously described (15). Fluorescence-conjugated anti-CD3e, -CD4, and -NK1.1 Abs and matched isotype controls were purchased from BD Pharmingen. Allophycocyanin-conjugated anti-IFN- γ Ab and isotype control were purchased from eBioscience. Paired Abs for ELISA analysis of IFN- γ , IL-4, IL-5, and IL-12 were purchased from BD Pharmingen. Paired Abs for IL-13 ELISA were purchased from R&D Systems. Anti-NK1.1 mAb for in vivo injection was produced by hybridoma PK136 and purified by ammonium sulfate precipitation, followed by dialysis and spectrophotometry. Anti-NK (DX5) microbeads were purchased from Miltenyi Biotec.

Immunization

The protocols for mouse treatment in this study are shown in Fig. 1. As a general experimental protocol (Fig. 1A), C57BL/6 mice were intranasally (i.n.) infected with 1×10^3 inclusion-forming units (IFU) MoPn, or treated with PBS. Thirty to 45 days after infection, mice were sensitized i.p. with 2 μ g of OVA (ICN Biomedicals) in 2 mg of Al(OH)₃ adjuvant (alum). Two weeks postsensitization, mice were challenged with 50 μ g of OVA (40 μ l) intranasally. Mice were sacrificed 7 days after challenge, and samples were collected for further analysis. For the experiments involving NK cell depletion (Fig. 1B), mice were injected i.p. with 0.2 mg of anti-NK1.1 mAb (PK-136) at 3 days before and 1 day after sensitization and challenge. The efficiency of NK cell depletion by this protocol was >96%. Purified isotype control Abs for anti-NK1.1 in vivo treatment were anti-severe acute respiratory syndrome coronavirus mAb (F26G3) and anti-Nipah virus mAb (F45G5) (both were mouse IgG2a κ) provided by Dr. X. Yuan (National Microbiology Laboratories of Canada, Winnipeg, Manitoba, Canada). Treatment with these isotype controls had no significant effect on NK population.

Isolation and adoptive transfer of NK cells

NKT-KO mice or C57BL/6 mice were i.n. infected with 1×10^3 IFU MoPn; 3–5 days after infection, spleens from either infected or naive mice were aseptically collected and NK cells were isolated using anti-NK (DX5) microbeads and a MACS-positive selection column (Miltenyi Biotec) according to manufacturer's instructions. The purity of the isolated NK1.1⁺ NK cells was ~90% based on flow cytometric analysis. For adoptive transfer, isolated NK cells from wild-type C57BL/6 or NKT-KO mice were first washed in protein-free PBS and 2×10^6 cells were injected into the tail vein of the syngeneic recipient mice. Naive C57BL/6 mice were sensitized and challenged with OVA as described in the immunization schedule. Immediately before sensitization and before challenge, recipient mice received NK cells isolated from either infected mice or naive mice. An experimental schema is shown in Fig. 1C. Control mice were treated with OVA in the same manner without NK cell transfer. Mice were sacrificed at 7 days post-OVA challenge and samples were collected for further analysis.

Bronchoalveolar lavage (BAL) and leukocyte differentials

The mouse trachea was cannulated and the lungs were washed twice with 1 ml of PBS. The BAL fluids were centrifuged immediately. The cells were

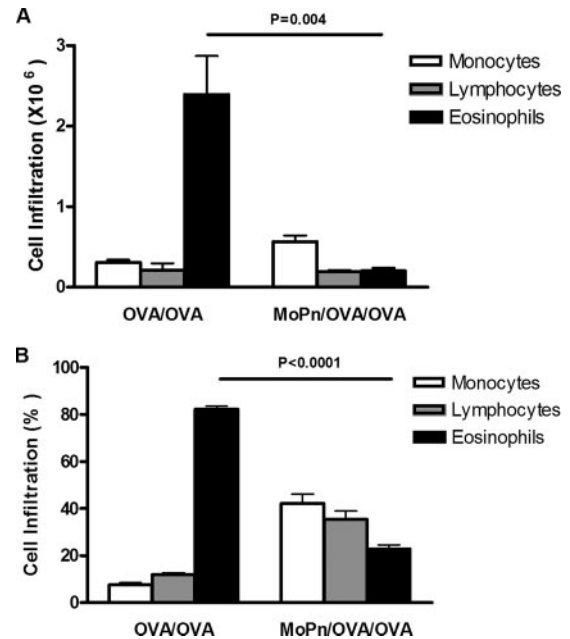


FIGURE 2. Prior chlamydial infection inhibited the increase of eosinophils in BALs induced by OVA sensitization and challenge. C57BL/6 mice were i.n. infected with 1×10^3 IFU MoPn or sham treated with PBS. At the time range of 30–45 days postinfection, the mice were sensitized once with an i.p. injection of 2 μ g of OVA in 2 mg of Al(OH)₃ adjuvant (alum). Two weeks postsensitization, mice were i.n. challenged with 50 μ g of OVA (40 μ l). Seven days after challenge, mice were sacrificed and the lungs were washed twice with 1 ml of PBS by tracheal cannulation. The cells in the BAL fluids were examined by differential cell counting. Pooled data from six independent experiments with similar results (four mice in each group in each experiment) are shown as mean $\pm$ SEM. *A*, Absolute number of infiltrating cells; *B*, percentage of different cells composed of the total infiltrating cells.

resuspended for cell counting and BAL smears. For leukocyte differential, the BAL smear slides were air-dried, fixed, and stained with the Hema 3 Stain Set (Fisher Scientific). The numbers of monocytes, lymphocytes, neutrophils, and eosinophils per 200 cells were counted based on cellular morphology and staining characteristics. Blood smears from mice were prepared and stained by the same reagent for blood leukocyte differentials.

Histopathological and immunohistochemical analysis

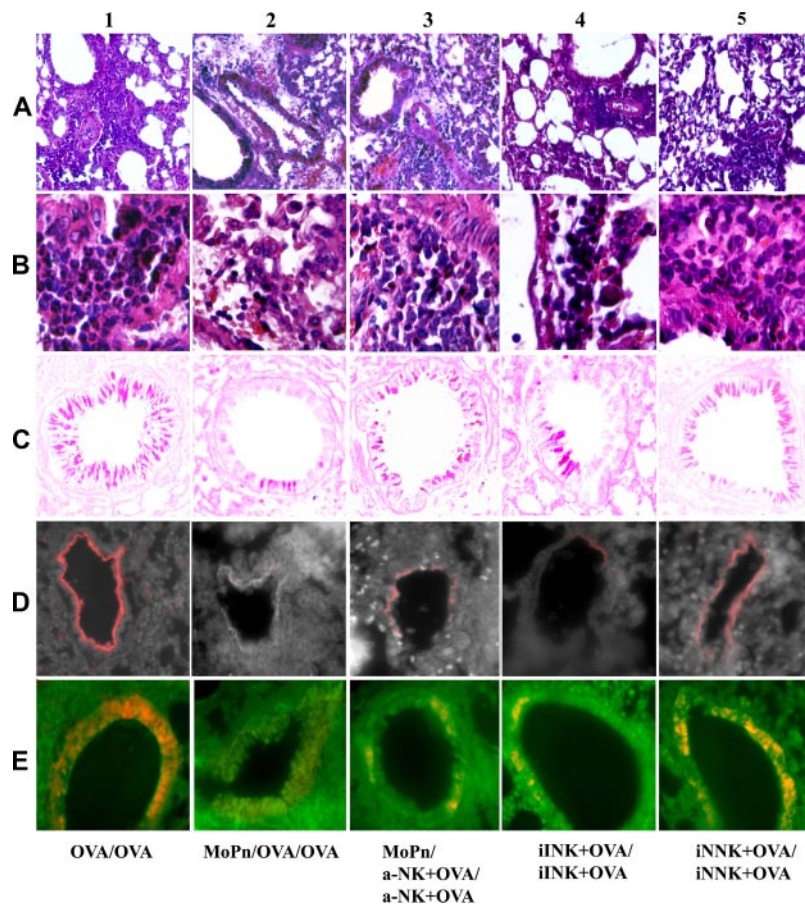
Lungs were collected and fixed with 10% formalin, embedded, sectioned, and stained by H&E. Slides were examined for pathological changes under light microscopy. Mucus and mucus-containing goblet cells within bronchial epithelium were analyzed by a periodic acid-Schiff (PAS) staining kit (Sigma-Aldrich). The histological mucus index (HMI) was measured by Image-Pro Plus software (Media Cybernetics) as described (15).

For analysis of VCAM-1 and eotaxin expression, lung tissues were snap-frozen in liquid nitrogen when the mice were killed and stored at -80°C until sectioning. Frozen sections (10 μM) were placed on slides and fixed by 99.6% acetone. For VCAM-1 staining, slides were incubated with rat anti-mouse VCAM-1 (clone 429), or isotype-matched control Abs, followed by biotin-conjugated anti-rat IgG (BD Pharmingen), developed by Texas Red (Vector Laboratories). For eotaxin staining, slides were incubated with purified goat anti-mouse eotaxin Ab that was purchased from R&D Systems followed by PE-conjugated anti-goat IgG secondary Ab purchased from Jackson ImmunoResearch Laboratories. Epithelial cells were stained with anti-pan-cytokeratin (B311.1) (Calbiochem), followed by FITC-conjugated anti-mouse IgG secondary Ab (Sigma-Aldrich). The blocking reagent was purchased from DakoCytomation. IL-5R expression on BM cells was determined as previously described (15).

Spleen and local lymph node cell culture and cytokine analysis

Spleen and draining (mediastinum) lymph node cells were cultured as previously described (8). Briefly, spleen cells were cultured at 7.5×10^6 cells/ml without or with OVA (1 mg/ml), lymph node cells were cultured at 5×10^6 cells/ml. Culture supernatants were collected at 72 or 120 h for

FIGURE 3. Prior chlamydial infection inhibited allergic inflammation in lung tissues induced by OVA sensitization and challenge and NK cells contribute significantly to the infection-mediated inhibition of allergy. Lung tissues from uninfected mice (OVA/OVA), mice with prior MoPn infection (MoPn/OVA/OVA), MoPn-infected mice with anti-NK1.1 treatment (MoPn/anti-NK+OVA/anti-NK+OVA), mice receiving NK cells that were isolated from infected mice (iINK+OVA/iINK+OVA), and mice receiving NK cells that were isolated from naive mice (iNNK+OVA/iNNK+OVA) were analyzed following OVA sensitization and challenge. *A*, The lung tissues were fixed with formalin and the sections (5 μ M) were stained with H&E and examined under microscopy (magnification $\times 200$). *B*, A higher power ($\times 1000$) of *A*. *C*, The lung sections were stained with a PAS staining kit for mucus-producing goblet cells in the airway. *D*, Frozen sections of the lung were stained with anti-VCAM-1 Ab followed by biotin-conjugated secondary Ab and developed with Texas Red. The red color indicates VCAM-1 expression. *E*, The sections were stained with anti-eotaxin and -epithelial Ab (B311.1) followed by PE-conjugated and FITC-conjugated secondary Abs, respectively, as described in *Materials and Methods*. The orange color represents eotaxin on bronchial epithelial cells.



cytokine measurement. All cytokine determinations were done by two mAb sandwich ELISA.

Statistical analysis

Results are expressed as mean $\pm$ SEM. The unpaired Student *t* test was used for comparison of the data between two experiment groups. The ANOVA Newman-Keuls multiple comparison test was used to determine statistical significance among multiple groups (see Figs. 5 and 6). A *p* value of <0.05 was considered significant.

Results

Prior MoPn infection inhibited airway eosinophilia and mucus overproduction induced by OVA exposure

Our previous studies have shown that MoPn infection is normally cleared by C57BL/6 mice within 20–25 days following intranasal infection (16, 17). To study the effect of a resolved infection on the development of allergic responses, we chose 30–45 days postinfection as the starting point for OVA sensitization, and applied i.n. challenge 14 days later. Airway inflammation was examined by differential counting of BAL cells and histological analysis. As a baseline, the total number of cells in the BAL of the naive mice and the mice at 30 days postinfection was $<10^4$, among which most were epithelial cells without notable eosinophils. In sharp contrast, after OVA sensitization and challenge, as shown in Fig. 2*A*, mice without prior MoPn infection exhibited significantly higher infiltration of inflammatory cells ($>2 \times 10^6$ cells), among which 82% were eosinophils. However, mice with prior MoPn infection showed much less cellular infiltration, the total cell number in BAL was markedly reduced, and only 23% of BAL cells were eosinophils ($\sim 2 \times 10^5$ cells) (Fig. 2*B*). The severity of airway inflammation was confirmed by histological analysis of the lung (Fig. 3). Mice without prior infection showed massive eosin-

ophilia in the bronchial submucosa, alveolar and perivascular sheaths, with a few lymphocytes and monocytes (Fig. 3, *A1* and *B1*). At the same time, mice with prior infection developed less cellular infiltration, mainly containing monocytes and lymphocytes around the small bronchi, bronchioles, and blood vessels in the lung with very few eosinophils (Fig. 3, *A2* and *B2*). The staining of mucus-containing goblet cells also showed that the mice with prior MoPn infection had less mucus secretion and epithelial hyperplasia compared with mice with OVA treatment only (Fig. 3, *C2* vs *C1*). The results indicate that prior MoPn infection is able to inhibit airway eosinophilic inflammation and mucus overproduction induced by allergen sensitization and challenge.

Prior MoPn infection inhibited the expression of VCAM-1 and eotaxin in the lung following OVA challenge

Because the migration of eosinophils to the airway is largely dependent on the interaction of VCAM-1 and VLA-4 and because eotaxin is chemokine which can selectively attract eosinophils, we further analyzed the expression of VCAM-1 and eotaxin in the lung of the OVA-sensitized and i.n.-challenged mice with or without prior MoPn infection. The results showed that the expression of VCAM-1 and eotaxin in the lung was significantly lower in the infected mice than controls (Fig. 3, *D2* vs *D1* and *E2* vs *E1*).

Prior MoPn infection inhibited Th2 cytokine production and increased Th1 cytokine production following OVA challenge

To examine the effect of MoPn infection on allergen-specific T cell activation, we compared Th1 (IFN- γ) and Th2 (IL-4, IL-5, and IL-13) cytokine production in mice with OVA sensitization and challenge with or without prior MoPn infection. Spleen cells and draining lymph node cells were restimulated with OVA to test

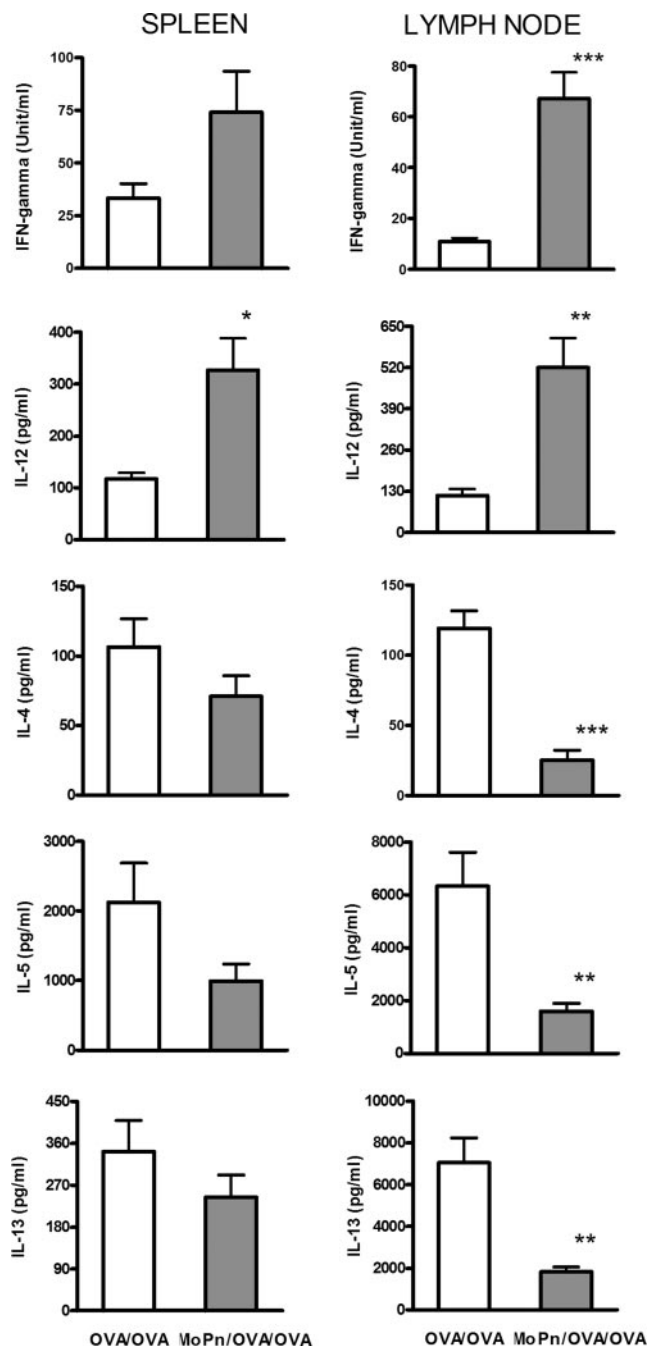


FIGURE 4. Prior chlamydial infection altered the pattern of allergen-driven cytokine production by splenic and draining lymph node lymphocytes. Mice were treated as described in Fig. 1. At 7 days post-OVA challenge, spleen cells and lymph node cells were cultured in the presence or absence of OVA. Cytokines in the culture supernatants were measured by ELISA. Levels for cytokines (except IL-12) in the culture wells without OVA stimulation were marginal, thus the figure only shows the results with OVA stimulation. IL-12 levels in the unstimulated wells were similar to those with OVA stimulation. Pooled data from four independent experiments (four mice per group in each experiment) are presented as mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

allergen-driven cytokine profile. The mice with prior MoPn infection showed dramatically decreased levels of allergen-driven Th2 cytokine production, namely IL-4, IL-5, and IL-13 (Fig. 4). In contrast, the production of OVA-driven IFN- γ and the Th1-promoting cytokine (IL-12) in mice with prior infection was significantly higher than in those without infection. Notably, this cyto-

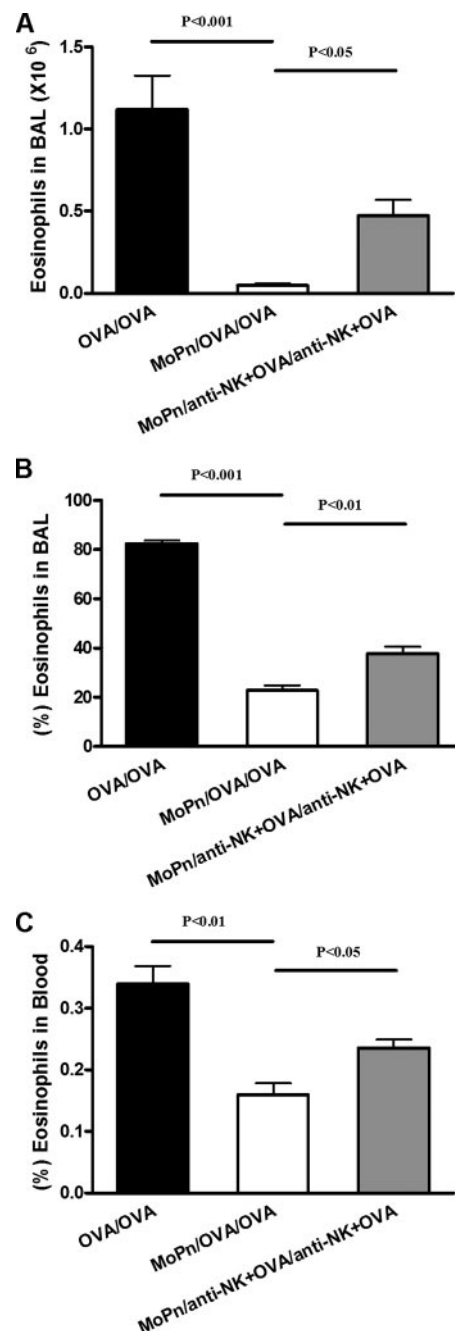


FIGURE 5. In vivo depletion of NK cells partially abolished the inhibitory effect of MoPn infection on local and systemic eosinophilic inflammation. Mice were infected i.n. with 1×10^3 IFU MoPn or treated with PBS. Thirty to 45 days later, mice were sensitized i.p. with OVA ($2 \mu\text{g}$) in $\text{Al}(\text{OH})_3$. Two weeks postsensitization, mice were i.n. challenged with $50 \mu\text{g}$ of OVA ($40 \mu\text{l}$). Three days before and 1 day after the sensitization and challenge with OVA, one group of infected mice was injected i.p. with anti-NK1.1 Ab. Mice were sacrificed at 7 days after OVA challenge. The experimental schema is shown in Fig. 1B. The absolute number (A) and percentage (B) of eosinophils in total BAL cells and the percentage of eosinophils in the peripheral blood (C) of the different groups of mice were examined. Pooled data from four independent experiments (four mice in each group in each experiment) are shown as mean $\pm$ SEM.

kine pattern switch was more apparent in the culture of local draining lymph node cells than that of spleen cells (Fig. 4). These results suggest that prior respiratory tract chlamydial infection is

Table I. The effect of adoptively transferred NK cells on the development of allergen-induced local and systemic eosinophilia^a

	OVA/OVA	OVA+iNK/OVA+iNK	OVA+iNKK/OVA+iNKK
% Eosinophils of blood leukocytes	0.701 ± 0.095	0.280 ± 0.104 ^b	0.570 ± 0.115
% Eosinophils in BAL	79.24 ± 5.652	43.81 ± 13.14 ^b	77.24 ± 4.702
% IL-5Rα ⁺ cells in bone marrow	28.51 ± 4.732	10.16 ± 2.522 ^b	22.90 ± 3.424

^a NK cells were isolated from spleens of infected or naive mice as described in *Materials and Methods*. Cell transfer was performed twice, one at 2 h before OVA sensitization and the other at 2 h before OVA challenge. Seven days after OVA challenge, different groups of mice were examined for eosinophilia in peripheral blood, in the lung (BAL) and eosinophil precursors (IL-5Rα⁺) in the bone marrow as described (15). Pooled data from three independent experiments (four mice per group in each experiment) are presented as mean ± SEM.

^b Value of $p < 0.05$ (OVA/OVA group vs OVA+iNK/OVA+iNK group).

capable of switching the subsequent allergen-driven cytokine-producing pattern from Th2 dominance to Th1 dominance.

In vivo depletion of NK cells partially abolished the inhibitory effect of MoPn infection on the development of allergic responses, which correlated with increased VCAM-1 and eotaxin expression

To study the role of NK cells in MoPn-mediated inhibition of allergic responses to OVA, we depleted NK cells from one group of mice that had recovered from prior MoPn infection, by administration of anti-NK1.1 Ab before and after OVA sensitization and challenge. As shown in Fig. 5, the NK-depleted, MoPn-infected mice showed significantly higher levels of OVA-induced local and systemic eosinophilia than the infected mice without NK cell depletion, although the eosinophilia in NK-depleted, MoPn-infected mice was still lower than the OVA sensitized/challenged control mice without infection. The treatment of the mice with isotype control Abs (mouse IgG2a, κ) had no significant effect on the inhibitory effect of MoPn infection on OVA-induced airway inflammation (data not shown). The analysis of mucus production also showed a partial reverse of OVA-induced airway mucus overproduction in the NK-depleted mice (Fig. 3, C2 vs C3). Moreover, the anti-NK1.1-treated, MoPn-infected mice showed significantly higher levels of VCAM-1 and eotaxin than the infected mice without NK cell depletion (Fig. 3, D3 vs D2 and E3 vs E2), although their levels were still lower than those in mice without infection (Fig. 3, D3 vs D1 and E3 vs E1). The results indicate that NK cells play a significant role in infection-mediated inhibition of airway eosinophilia and mucus overproduction caused by allergen exposure.

Adoptive transfer of NK cells that were isolated from infected mice inhibited the development of allergic responses induced by OVA exposure

To further study the role of NK cells in modulating allergic reactions in vivo, we analyzed the effect of adoptively transferred NK cells on the development of allergic responses. NK cells were isolated from either infected or naive mice, and then adoptively transferred, respectively, to syngeneic naive mice for two times, one at 2 h before OVA sensitization and the other at 2 h before OVA challenge. As shown in Fig. 3, the recipients of NK cell from infected mice (iNK), compared with control mice, showed a significant decrease of eosinophilic inflammation, mucus overproduction, as well as VCAM-1 and eotaxin expression in the lung (Fig. 3, column 4 vs column 1). However, the transfer of NK cells isolated from naive mice (iNKK) had no significant effect on the development of these allergic reactions (Fig. 3, column 1 vs column 5). Furthermore, the transfer of iNK cells, but not the transfer of iNKK, not only inhibited the increase of circulating and infiltrating eosinophils, but remarkably suppressed the production of IL-5Rα⁺ eosinophil precursors in the bone marrow (Table I). The

results suggest that adoptive transfer of NK cells that were isolated from infected mice had a significant inhibitory effect on the development of allergic reaction. These observations, together with the data from NK cell depletion experiments, indicate that NK

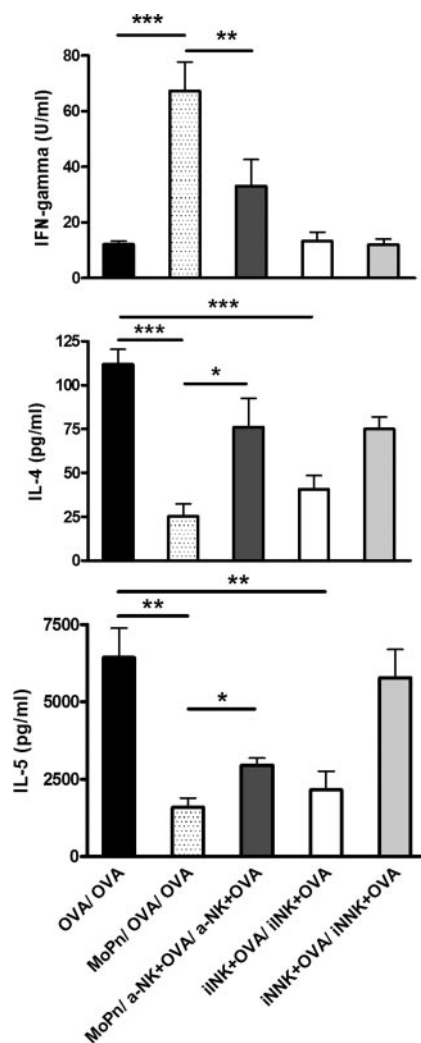


FIGURE 6. NK cells contributed significantly to the alteration of allergen-driven cytokine production by draining lymph node lymphocytes caused by MoPn infection. Mice were treated as described in Fig. 2. At 7 days following i.n. OVA challenge, mediastinum lymph node cells were cultured in the presence of OVA. Cytokines in the supernatants were measured by ELISA. Pooled data from four independent experiments (four mice per group in each experiment) are presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

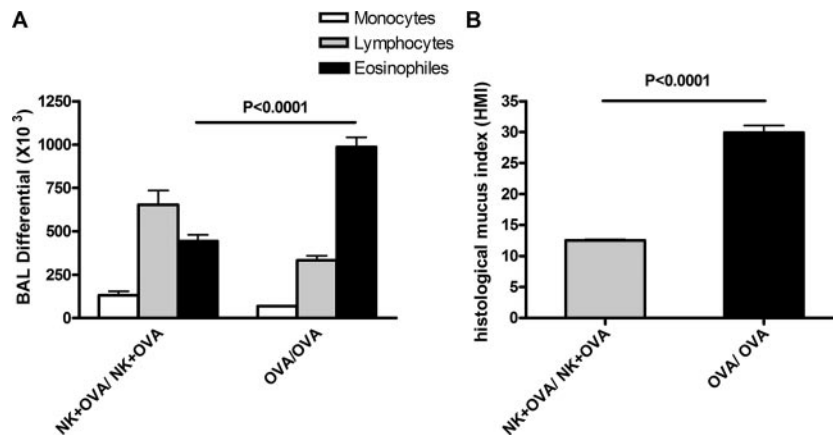


FIGURE 7. Adoptively transferred NK cells from NKT-KO mice showed inhibitory effect on the development of allergen-induced airway eosinophilia and bronchial mucus overproduction. NK cells were isolated from spleens of infected NKT-KO mice as described in *Materials and Methods*. Mice airway inflammation was induced by our standard protocol: i.p. sensitization with OVA (2 μ g, alum) followed by i.n. challenge (50 μ g) 2 wk later (14, 15). Cell transfer was performed twice, once 2 h before OVA sensitization and once 2 h before OVA challenge. Seven days after OVA challenge, different groups of mice (eight mice per group) were examined for eosinophil infiltration in the lung (BAL) and bronchial mucus production. **A**, The cells in the BAL fluids were examined by differential cell counting. **B**, Mucus and mucus-containing goblet cells within the bronchial epithelium were analyzed by a PAS staining kit (Sigma-Aldrich). The HMI was measured by Image-Pro Plus software (Media Cybernetics) and the result was indicated as: HMI = area of mucus-containing goblet cells (μ m²)/area of epithelial cells (μ m²) $\times$ 100%. Data are presented as mean $\pm$ SEM.

cells activated by MoPn infection play an important role in infection-mediated inhibition of allergic responses.

The modulating effect of NK cells on allergic responses was associated with alteration of allergen-driven cytokine-producing patterns

To elucidate the mechanism by which NK cells from infected mice inhibit allergic responses, we examined the pattern of allergen-driven cytokine production in infected mice that were depleted of NK cells or in mice with NK cell adoptive transfer. Local draining lymph nodes were collected post-OVA intranasal challenge, and cells from different groups of mice were cultured with allergen-specific restimulation. As shown in Fig. 6, the group of infected mice with NK cell depletion (MoPn/a-NK+OVA/a-NK+OVA) exhibited reduced IFN- γ production and increased IL-4 and IL-5 production compared with the infected mice without NK cell depletion (MoPn/OVA/OVA), although their Th2 cytokine levels were still higher than the mice with OVA exposure only (OVA/OVA). In contrast, the adoptive transfer of iNK, but not iNNK, inhibited the production of Th2 (IL-4 and IL-5) cytokines. Unlike what was observed in infected mice, the transfer of NK cells from infected mice failed to enhance Th1 cytokine (IFN- γ) production (Fig. 6). The results suggest that NK cells from infected mice can modulate the allergen-driven cytokine production, especially being able to inhibit Th2 cytokine production.

NK cells that were isolated from NKT-KO mice, same as wild-type counterparts, are also capable of inhibiting allergic responses

Because both NK and NK T cells in C57BL/6 mice express the NK1.1 marker, we further used NKT KO mice as the donor of NK cells to repeat the adoptive transfer experiment to exclude the possibility that the effect observed in the experiment using wild-type C57BL/6 mice was caused by contaminated NKT cells. We used NK cells that were isolated from NKT KO mice to study the role of NK cells in modulating allergic reaction in vivo. As shown in Fig. 7, the same as what was observed with NK cells isolated from WT mice, the transfer of NK cells that were isolated from MoPn-

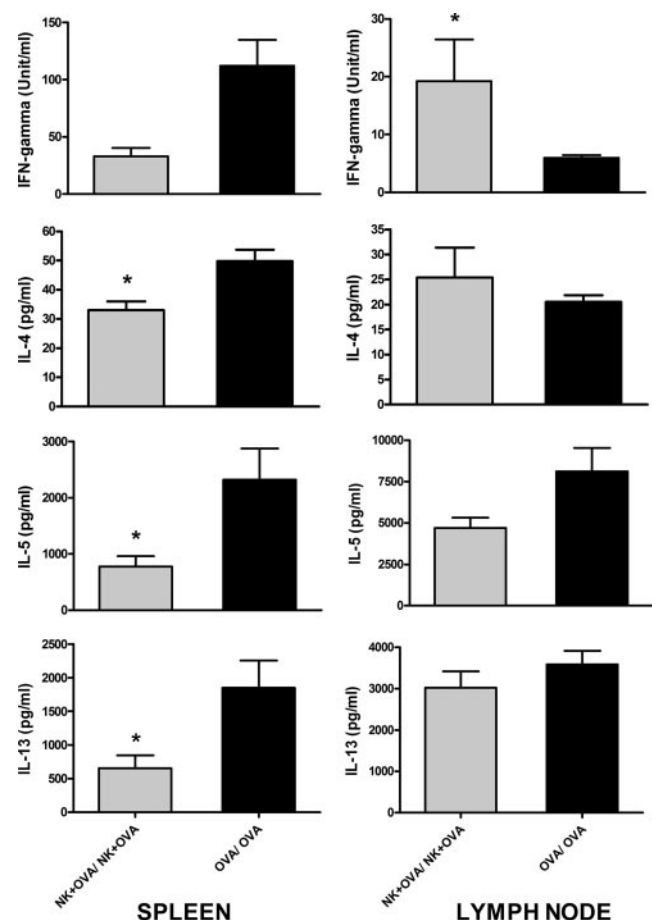


FIGURE 8. Adoptively transferred NK cells from NKT-KO mice altered the pattern of allergen-driven cytokine production by splenic and lymph node lymphocytes. Mice were treated as described in Fig. 6. At 7 days post-OVA challenge, spleen cells and lymph node cells were cultured in the presence of OVA. Cytokine levels in the culture supernatants were measured by ELISA. Data from eight mice per group are presented as mean $\pm$ SEM. *, $p < 0.05$.

infected NKT-KO mice significantly inhibited eosinophilic inflammation (Fig. 7A) and mucus overproduction (Fig. 7B) induced by allergen exposure. Moreover, the adoptive transfer of iNKT from NKT-KO mice altered the allergen-driven cytokine-producing pattern in recipient mice (Fig. 8). The change of cytokine pattern induced by NK cell transfer from NKT-KO mice was comparable with those induced by the transfer of NK cells from WT mice. The results confirmed that NK cells activated by MoPn infection play an important role in infection-mediated inhibition of allergic responses.

Discussion

Although the hygiene hypothesis has been raised and studied for more than a decade, the mechanisms by which infections modulate allergy are still unclear. In our previous studies, we have explored the potential casual relationship between infection and allergy by using different infectious agents, allergens, and different immunization schedules (6–8, 15). In these reported studies, we had consistent findings on infection-mediated inhibition of allergy, typically observing reduced airway eosinophilic inflammation and mucus production as well as decreased Th2 cytokine production. To determine which components of the immune system are contributing to the down-regulation of allergen-driven Th2 responses, we examined in the present study the role played by the NK cell, an important effector of innate immunity, in infection-mediated inhibition of allergic responses by two approaches: depletion of NK cells in vivo and adoptive transfer of NK cells. Experiments using both approaches demonstrated an important role of NK cells in infection-mediated inhibition of allergy. The study represents an important step in revealing the mechanisms by which infections modulate allergy.

The role of NK cells in regulating acquired T cell responses has been demonstrated recently in various infection models (18–20). In mice infected with murine CMV, virus-responsive Ly49H⁺ NK cells are necessary for maintenance of CD8 α ⁺ dendritic cells (DCs) in the spleen, which is essential for the resistance to murine CMV infection (21). In a *Bordetella pertussis* infection model, depletion of NK cells in mice resulted in a lethal infection, associated with reduction of the Ag-specific Th1 response but enhancement of the Th2 response (22). Although the immunoregulatory role of NK cells in infection is manifested and supported by plentiful evidence, studies have not yet been performed to examine the ability of infection-activated NK cells to regulate T cell response in allergy. In this study, for the first time, we showed that the depletion or transfer of infection-activated NK cells did influence the outcome of the allergen-specific T cell response, suggesting a role played by NK cells in infection-mediated inhibition of allergic responses.

The mechanisms by which NK cells modulate T cell responses to allergen are yet to be investigated. Potentially, there are at least three regulatory pathways which might be used by activated NK cells to inhibit the development of the Th2 response to allergens. First, the cytokines, particularly IFN- γ , secreted by activated NK cells may contribute to the creation of a microenvironment that promotes the development of Th1 cells and/or prevents the naive T cells from differentiating into the Th2 direction. We have found that chlamydial infection can activate NK cells which produce IFN- γ . Even at 30 days after infection, the percentage of IFN- γ -positive NK cells is significantly higher than that in naive mice (L. Jiao, X. Han, X. Yang, unpublished data). NK has been shown to enhance Th1 polarization by facilitating IL-12 production and inducing T-bet expression (23, 24). Martin-Fontecha et al. (25) showed that NK cells provide an early source of IFN- γ that is necessary for Th1 polarization. The inhibitory effect of IFN- γ and

Th1 cells on the development of the Th2 response has been observed in numerous reported studies (26, 27). In the present study, we have demonstrated that the depletion of NK cells in infected mice results in decreased allergen-specific Th1 cytokine production but increased Th2 cytokine production, which is in line with the thought that IFN- γ production by NK cells promotes the Th1 response, thus inhibiting the Th2 response to allergen. Interestingly, the adoptive transfer of IFN- γ -producing NK cells to naive mice failed to induce higher allergen-specific Th1 cytokine production although it did inhibit Th2 cytokine production and allergic reactions, suggesting that NK cells may also use other mechanisms to inhibit allergen-driven Th2 responses.

The second potential mechanism is that NK cells directly interact with T cells or inflammatory cells to inhibit allergic responses. There are emerging data suggesting that activated NK cells may communicate directly with T cells by a process involving cognate cell-cell interactions (28). It was found that activated human NK cells expressed the surface molecules of MHC class II, B7, and OX40L, suggesting their potential to interact with T cell markers such as TCR, CD28, and OX40 thus influencing T cell proliferation, cytokine production, and immune memory (29). Moreover, an interaction has been documented to occur between 2B4 on NK cells and CD48 on T cells. It was found that the expression of 2B4 on NK cells was related to proliferation of T cells (30).

Lastly, and even more likely, activated NK cells may modulate allergen-specific T cell responses via interacting with the DC, a key player in initiating and shaping adaptive immune responses upon allergen exposure. It has become evident recently that the reciprocal interaction between NK cells and DCs is crucial in determining the outcome of immune responses (31, 32). Activated NK cells can release cytokines that influence DC maturation/differentiation and subsequent migration to lymph nodes and function on T cell priming and differentiation. In addition, NK cells can also mediate the elimination of certain DCs by cell contact-dependent interactions (33–35). We recently found that chlamydial infection can induce different subtypes of DCs (36) and that DCs from *Chlamydia*-infected mice play an important role in infection-mediated inhibition of allergy (14, 15). Indeed, we found that the CD8 α ⁺ DC subset induced by chlamydial infection is more powerful in inducing Th1 responses (36) and that in vivo depletion of NK cells from MoPn-infected mice results in decreased IL-12 production by DCs (data not shown). Given the evidence that NK cells are necessary for the maintenance of the CD8 α ⁺ DC subset (21), it is possible that the activated NK cells in our model may stimulate CD8 α ⁺ DCs, thus modulating allergen-specific T cell responses.

From the present results, it is apparent that several factors are very important in infection-mediated inhibition of allergic responses. First, the activation by infection is critical in determining the inhibitory effect of NK cells, because the transfer of NK cells that were isolated from naive mice failed to show a significant modulating effect on the development of allergic inflammation and Th2 responses (Figs. 3 and 6). Notably, a previous study showed that allergen-activated NK cells can contribute to allergic airway inflammation (37) and another study showed that depletion of NK1.1 cells decreased the allergen-induced pulmonary eosinophil and CD3 T cell infiltration and blunted Th2 cytokine production in BAL (38), although the involvement of NKT cells that can promote allergic responses (39, 40) in these studies cannot be excluded. Second, the timing of NK activation is an important factor that influences the inhibitory effect of NK cells on allergic reactions. We observed that transfer of infection-activated NK cells after sensitization, but before challenge, could not inhibit airway allergic responses (data not shown). This is consistent with the finding by Broide et al. (41) who showed that depletion of NK cells

during the allergen challenge stage did not affect the ability of immunostimulatory DNA sequences to inhibit allergic eosinophilic inflammation and airway hyperreactivity. The results suggest that infection-activated NK cells may regulate the initial stage of Ag presentation to T cells and/or the differentiation of naive T cells into effector cells, but not the secondary response of allergen-specific T cells. Third, NK cells, not NKT cells, are responsible for the inhibition of allergic responses. We recently report that NKT cells can be activated by *Chlamydia* infection (42, 43). In particular, MoPn infection activates NKT cells with predominant IL-4 production that promote Th2 instead of Th1 responses (42, 43). By using NK cells that isolated from NKT KO mice, we exclude the involvement of NKT cells in the inhibitory process. Lastly, combined with our recent reports which showed the importance of DCs in infection-mediated inhibition of allergic responses (14, 15), the present study indicates that the innate immune system, including the function of NK cells, is an important mechanism underlying the infection-mediated inhibition of allergy.

Disclosures

The authors have no financial conflict of interest.

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