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IL-12-Dependent Vascular Cell Adhesion Molecule-1 Expression Contributes to Airway Eosinophilic Inflammation in a Mouse Model of Asthma-Like Reaction[1]

Shuhe Wang, Yijun Fan, Xiaobing Han, Jie Yang, Laura Bilenki, and Xi Yang[2]

Bronchial-alveolar eosinophilic inflammation is among the characteristic pathological changes in asthma, which has been shown to be correlated with type 2 cytokine and chemokine production. Exogenous IL-12 has been found to be inhibitory for pulmonary eosinophilia in reported studies. Using a murine asthma-like model induced by OVA, we found in the present study that IL-12 gene knockout (KO) mice showed substantially reduced airway recruitment of eosinophils compared with wild-type control mice following OVA sensitization/challenge, although the levels of circulating eosinophils were comparable in these two groups of mice. Cytokine analysis showed Ag-driven Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-10, and IL-13) cytokine production by CD4 T cells from local draining lymph nodes and spleen. Similarly, local eotaxin production was comparable in wild-type and IL-12 KO mice. In contrast, immunohistochemical analysis showed that the expression of VCAM-1 on the lung endothelium of IL-12 KO mice was dramatically less than that in wild-type mice. Furthermore, administration of rIL-12 at the stage of sensitization and challenge with OVA restored airway eosinophilia and VCAM-1 expression in IL-12 KO mice. The results suggest that endogenous IL-12 contributes to the recruitment of eosinophils into airways observed in asthma, possibly via enhancement of the expression of VCAM-1 on local vascular endothelial cells. ***The Journal of Immunology*****, 2001, 166: 2741–2749.**

Airway eosinophilia is central in the pathogenesis of atopic asthma. It has been demonstrated that the degree of eosinophilia in airways correlates with the level of bronchial hyper-responsiveness and clinical asthma symptoms (1–7). Eosinophils can release numerous mediators preformed and stored in cytoplasmic granules during an asthmatic reaction, including leukotrienes, cationic proteins, platelet-activating factor, and PGs (PGE_1 and PGE_2). Recent studies show that allergen-specific CD4 T cells from atopic individuals are skewed toward a Th2 cytokine profile, which plays an important role in the development and maintenance of allergic responses (8–12). In contrast, Th1 cytokines, especially IFN-γ, are inhibitory for allergic responses via the suppression of Th2 cell development and expansion. In addition, eotaxin, a CC chemokine produced by bronchial and alveolar epithelial cells, plays an important role in the chemotaxis and migration of eosinophils in vivo and in vitro (13–16). Eotaxin gene knockout (KO)[3] mice show diminished eosinophilia and neutralization of endogenous eotaxin with Abs reduces the eosinophil chemoattractant activity of bronchoalveolar lavage (BAL) from asthmatic individuals (17–19).

The migration of inflammatory cells into airways is dependent on the interaction between inflammatory cells and vascular endothelial cells via adhesion molecules. In particular, in vivo and in vitro experiments have shown the extreme importance of the interaction between VCAM-1 and very late activation Ag 4 in the adhesion of eosinophils to vascular endothelial cells and their transendothelial migration (20–26). Proinflammatory cytokines such as TNF-α and IL-1 are able to induce VCAM-1 expression on cultured endothelial cells (27, 28). Furthermore, proteins released by eosinophil degranulation can selectively induce VCAM-1 expression by vascular endothelial cells (29).

Functional IL-12 is a heterodimer (p70) composed of two disulfide-linked chains of 35 (p35) and 40 (p40) kDa. The particular role of IL-12 in allergic responses remains unclear. Because IL-12 can enhance IFN-γ production and is critical for Th1 cytokine responses, IL-12 has been considered an inhibitory factor for allergic responses and Th2 cytokine production (30–32). Indeed, local delivery of large doses of exogenous rIL-12 abolished pulmonary eosinophilia induced by single or repeated intratracheal challenge with SRBC Ag, which correlated with an increase in IFN-γ and a decrease in IL-4 and IL-5 production (33). Moreover, i.p. injection of rIL-12 inhibited serum IgE and airway eosinophilia induced by ragweed sensitization/challenge (34). Furthermore, local IL-12 gene transfer using adenoviral vector (35) or vaccinia virus (36) abrogated airway eosinophilia elicited by OVA in mouse asthma models. In addition, we recently showed that *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) infection inhibited pulmonary eosinophilia induced by OVA sensitization/challenge, which is correlated with an increase in IL-12 and IFN-γ production and a decrease in IL-5 production (37). However, all these studies examined the role of IL-12 in a cytokine excessive fashion. No data have been reported regarding the alteration in eosinophilic inflammation in individuals with an endogenous deficiency of IL-12.

To more directly examine the role played by IL-12 in atopic allergy, especially in eosinophilic inflammation, we studied here

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[3] Abbreviations used in this paper: KO, gene knockout; BCG, bacillus Calmette-Guérin; alum, $Al(OH)_3$; BAL, bronchoalveolar lavage; H&E, hematoxylin and eosin.

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an asthma-like reaction in IL-12 KO mice induced by OVA sensitization/challenge. We surprisingly found that, compared with wild-type mice, which showed predominant eosinophilic infiltration into airways, IL-12 KO mice exhibited dominant mononuclear cell inflammation in airways following OVA exposure. Comparison of cytokine production by CD4 T cells from draining lymph nodes and spleen showed comparable Th2-related (IL-4, IL-5, IL-10, and IL-13) and Th1-related (IFN-γ) cytokine production in IL-12 KO and wild-type mice following Ag-specific in vitro restimulation. Moreover, local eotaxin production was comparable between the two types of mice. Interestingly, it was observed that IL-12 KO mice exhibited dramatically less VCAM-1 expression on pulmonary vascular endothelial cells compared with wild-type mice following OVA sensitization and challenge. Confirmatory experiments supplementing rIL-12 to IL-12 KO mice in the early (sensitization) and late stages (challenge) of OVA exposure showed substantially increased bronchial eosinophilia in parallel with enhanced pulmonary VCAM-1 expression. Taken together, the present study suggests that endogenous IL-12 plays an important role in promoting bronchial eosinophilic inflammatory reaction, at least partially via enhancing VCAM-1 expression.

Materials and Methods

Animals and immunization

Female homozygous IL-12 p40 KO mice (C57BL/6-IL12<tm1Cgn>) were purchased from The Jackson Laboratory (Bar Harbor, ME) and IL-12 p35 KO mice (C57BL/6 background) were bred at the University of Manitoba using breeding pairs purchased from The Jackson Laboratory. Most of the IL-12 KO mice used in this study were p40 KO mice unless specified otherwise. Age- and sex-matched wild-type C57BL/6 mice were purchased from Charles River Canada (St. Constant, Canada). Animals were used in accordance with the guidelines issued by Canadian Council on Animal Care. Mice were initially sensitized i.p. with 2 μg of OVA (ICN Biomedicals, Montreal, Canada) in 2 mg of $Al(OH)_3$ adjuvant (alum). Two weeks after sensitization, mice were challenged intranasally with 50–100 μg of OVA (40 μl) and were sacrificed on various days (days 2–10) following the challenge.

BAL and cell counting

After euthanasia, the trachea of the mice were cannulated, and the lungs were washed twice with 1 ml of PBS. BAL fluids were collected for cell count and eotaxin testing. The fluids were counted for total BAL cells and then spun down. The supernatants were collected to test local eotaxin production. Cell pellets were resuspended with saline, and slides were prepared for differential cell counting. Cells on the slides were stained with the Leukostat Stain Kit (Fisher Scientific, Ontario, Canada) for leukocyte differential. The numbers of monocytes, neutrophils, lymphocytes, and eosinophils in a total of 200 cells were counted in each slide based on morphology and staining characteristics.

Histopathological analysis

Lung tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, stained by hematoxylin-eosin (H&E), and examined for pathological changes under light microscopy as previously described (38). For immunohistochemical staining of VCAM-1, lung tissues were snap-frozen in liquid N_2 and kept at -80°C until sectioning. Sections were mounted onto slides and fixed by 100% acetone. An Envison system kit (Dako, Carpinteria, CA) was used for tissue staining and color development. Purified rat anti-mouse VCAM-1 Ab and isotype-matched control Abs (PharMingen, San Diego, CA) were left on slides for 1 h at room temperature and successively washed using wash buffer. The sections were then incubated with secondary Ab (rabbit anti-rat Ab) conjugated with HRP and developed with 3-amino-9-ethylcarbazol chromogen. The intensity of VCAM-1 expression on the pulmonary vascular endothelium was classified according to the criteria defined by Brisco et al. (39). A score of 0 represents absent staining or faint staining of an occasional vessel only, 1+ is faint staining of several vessels, 2+ is moderate intensity staining of most vessels, and 3+ is intense staining of most vessels. The sections were examined by two observers in a blind manner, and the average of the two determinations for each section was used for calculation of VCAM-1 expression.

Cytokine and chemokine analysis

For examination of cytokine production patterns, splenocytes and cells from draining lymph nodes (mediastinal lymph nodes) were cultured as previously described (38, 40). Briefly, single-cell suspensions were prepared and cultured at 7.5×10^6 cells/ml (2 ml/well) for spleen cells and 5×10^6 cells/ml (1 ml/well) for lymph node cells with OVA (1 mg/ml) in the presence or the absence of anti-CD4 mAb (YTS 191.1) at 5 μg/ml. Culture supernatants were harvested at 72 h for measurement of various cytokines using ELISA. Purified (capture) and biotinylated (detection) Abs purchased from PharMingen were used for the ELISAs for IL-4, IL-5, IFN-γ, and IL-12 as previously described (37). IL-13 and eotaxin were determined by ELISAs using paired Abs purchased from R&D Systems (Minneapolis, MI). Cytokines in BALs were also determined by the aforementioned ELISAs.

To study the effect of endogenous IL-12 on IFN-γ production induced by intracellular bacterial infection, mice were infected i.v. with *M. bovis* BCG and examined for cytokine production as previously described (37). Mice were infected with BCG (1×10^5 CFU) and sacrificed 3 wk following infection. Lymph node cells were cultured at 5×10^6 cells/ml with dead BCG stimulation. IFN-γ production in the 72-h culture supernatants was determined by ELISA.

Ab analysis

OVA-specific IgE, IgG1, and IgG2a Abs were measured using ELISAs. Biotinylated anti-IgE Ab were purchased from PharMingen. Biotinylated goat anti-mouse IgG1 or goat anti-mouse IgG2a Abs were purchased from Southern Biotechnology Associates (Birmingham, AL). Sera were determined for OVA-specific IgG1 and IgG2a using ELISAs as previously described (41). For detection of OVA-specific IgE, sera were incubated twice with a 50% slurry of protein G-Sepharose (Pharmacia) in PBS before the

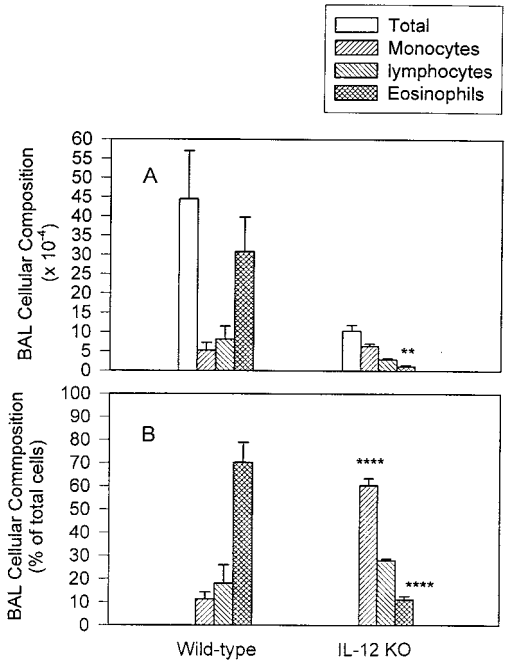

FIGURE 1. IL-12 KO mice show a significant reduction in BAL of eosinophils following OVA sensitization and challenge compared with wild-type mice. Mice (four mice per group) were sensitized i.p. with OVA (2 μg) in alum and were challenged with OVA (50 μg) on day 15 following sensitization. The mice were sacrificed on day 7 after intranasal challenge, and BAL fluids were collected by cannulating the trachea of mice and washing the lungs twice with 1 ml of PBS. The components of infiltrating cells in BAL fluids were examined by differential cell counts using the Fisher Leukostat Stain Kit. The figure shows the absolute number of each infiltrating cellular population (*A*) and the proportion of each cellular component composing of the total BAL cells (*B*). Data are shown as the mean ± SD of each group. One representative experiment of five independent experiments is shown. **, $p < 0.01$; ****, $p < 0.0001$ (IL-12 KO mice compared with that in wild-type mice).

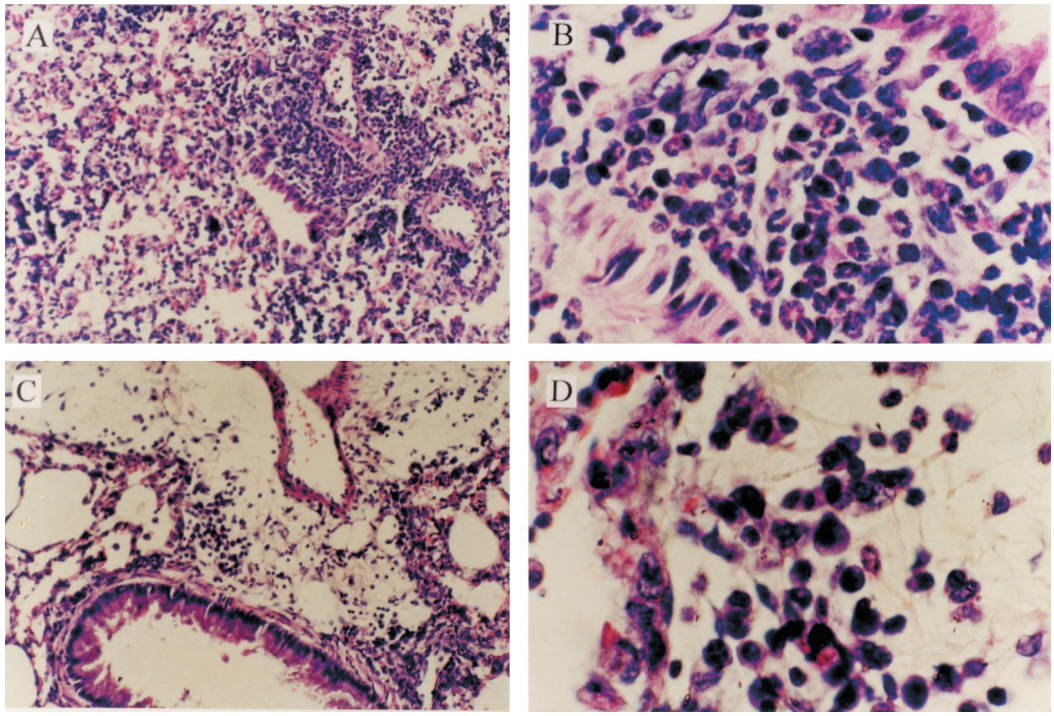

FIGURE 2. Dramatic reduction of peribronchial eosinophilic inflammation in IL-12 KO mice following OVA sensitization/challenge compared with that in wild-type control mice. Mice were sensitized and challenged with OVA as described in Fig. 1. Lung tissues were routinely fixed, sectioned, and stained with H&E. Sections were photographed at low (×100; *A* and *C*) and high (×1000; *B* and *D*) magnification. *A* and *B*, OVA-sensitized and challenged wild-type mice. *C* and *D*, OVA-sensitized and challenged IL-12 KO mice. A total of 20 mice in each group were analyzed, and representative findings are shown.

ELISA. This treatment allows the removal of about 95% of total IgG1 without affecting the concentration of IgE.

Statistical analysis

Ab titers were $\log_{10}$ transformed and analyzed by unpaired Student's *t* test. Cytokine and chemokine levels and differential BAL cell counts in different groups were analyzed by unpaired Student's *t* test.

Results

IL-12 KO mice show decreased bronchial and pulmonary eosinophilia following OVA challenge

Our kinetics study showed that the recruitment of inflammatory cells, including eosinophils, into airways was apparent 2 days following intranasal challenge with OVA in OVA alum-sensitized mice and peaked at 6–8 days, followed by a gradual decline (38). For most experiments in this study we chose the time of peak cell infiltration into the lungs to analyze the role of IL-12 in allergen-induced pulmonary inflammation using IL-12 p40 KO mice. The results showed that the cellular components in the BALs following allergen-specific intranasal challenge were dramatically different between OVA-sensitized IL-12 KO and wild-type mice (Fig. 1). Wild-type mice showed predominant eosinophil infiltration (~60–90% of total BAL cells were eosinophils), while IL-12 KO mice showed dominant mononuclear cell recruitment. Eosinophils in the BALs of IL-12 KO mice comprised 10–20% of the total infiltrating cells, and the absolute number of eosinophils in BALs of IL-12 KO mice was 10- to 100-fold less than that in wild-type control mice. Histopathologic analysis (H&E staining) of the lung sections showed massive eosinophil infiltration in peribronchial and perivascular areas of wild-type mice, while the inflammation in the same areas of IL-12 KO mice was much milder, and the infiltrating cells were mainly lymphocytes and monocytes/macrophages (Fig. 2). Similar differences between IL-12 KO and wild-type mice in airway inflammation were observed on days 3 and 10 following intranasal challenge with OVA (data not shown). The results indicate that IL-12 KO mice are generally intact in mounting a pulmonary inflammatory reaction, but are selectively deficient in recruiting eosinophils into the lung.

Table I. *Differential leukocyte counts of peripheral blood in wild-type and IL-12 KO mice*

Group	Mice	Treatment	Lymphocytes (%)	Neutrophils (%)	Monocytes (%)	Eosinophils (%)
A	WT[a]	None	79.3 ± 4.1	11.1 ± 1.1	9.3 ± 4.6	0.2 ± 0.2
B	WT	OVA	75.3 ± 4.2	9.0 ± 3.0	8.7 ± 1.2	7.0 ± 1.0
C	IL-12 KO	None	74.1 ± 3.3	11.4 ± 2.3	14.3 ± 2.0	0.3 ± 0.1
D	IL-12 KO	OVA	69.7 ± 1.5	11.3 ± 1.2	13.2 ± 3.8	5.8 ± 1.3
p	(A vs B)		>0.05	>0.05	>0.05	<0.01
p	(C vs D)		>0.05	>0.05	>0.05	<0.01
p	(B vs D)		>0.05	>0.05	>0.05	>0.05

[a] WT, Wild type.

Table II. *Cytokine production by cells from draining lymph nodes and spleen in wild-type and IL-12 KO mice*

Cytokine Production	Lymph Nodes			Spleen		
	WT[a]	IL-12 KO	*p*	WT	IL-12 KO	*p*
IL-5 (pg/ml)	6894.7 (1451.0)	6693 (1532.0)	0.91	3174.0 (666.0)	3662 (651.0)	0.61
IL-4 (pg/ml)	164.5 (75.0)	232.0 (77.1)	0.50	97.9 (29.0)	90.5 (22.1)	0.84
IL-13 (pg/ml)	4238.0 (1053.0)	6204.1 (1130.5)	0.25	168.0 (31.2)	123.0 (55.0)	0.91
IL-10 (pg/ml)	168.0 (31.2)	123.0 (55.0)	0.90	505.0 (179.0)	437.0 (196.1)	0.80
IFNγ (U/ml)	22.6 (7.2)	26.4 (5.5)	0.63	23.6 (9.9)	32.6 (12.0)	0.56
TNFα (pg/ml)	123.1 (55.1)	148.2 (23.2)	0.85	752.1 (322.2)	409.2 (111.3)	0.30

[a] WT, Wild type.

One potential possibility for the deficiency of IL-12 KO mice in recruiting eosinophils into airways is that these mice may fail to promptly generate eosinophils from bone marrow following allergen exposure and thus lack circulating eosinophils to migrate into the lung. We therefore examined the leukocyte components in the peripheral blood of wild-type and IL-12 KO mice. As shown in Table I, both wild-type and IL-12 KO mice showed a significant increase (20- to 60-fold higher than naive mice) in eosinophils in the peripheral blood following OVA sensitization/challenge. However, in contrast to the sharp differences in airway eosinophilia between wild-type and IL-12 KO mice, the levels of eosinophils in the peripheral blood of these two types of mice were virtually identical following OVA sensitization/challenge (Table I). The results suggest that the reduction of airway eosinophilia in IL-12 KO mice is not due to a deficiency in systemic eosinophilic response, but, rather, it is probably caused by a failure of circulating eosinophils to migrate into airways.

IL-12 KO mice show similar patterns of Th1 and Th2 cytokine production and Ab responses as wild-type mice following OVA challenge

Because IL-12 is critical in the development of Th1 cells, and Th1 cytokines have been shown to be cross-inhibitory for Th2 development, we examined Th1 and Th2 cytokine production by CD4 T cells from the draining lymph nodes and spleen of IL-12 KO and wild-type mice following OVA sensitization/challenge. To our surprise, the stereotypic Th1 cytokine, IFN-γ, produced by local (draining lymph nodes) and systemic (splenic) lymphocytes of IL-12 KO mice was virtually identical with that in wild-type mice (Table II). Similarly, Th2 cytokines, IL-4, IL-5, and IL-13, were produced at similar levels in IL-12 KO and wild-type mice. Similar cytokine production patterns were observed in experiments analyzing cytokine production on days 3 and 10 following intranasal challenge with allergen (data not shown). In both wild-type and IL-12 KO mice, OVA-driven IL-4, IL-5, IL-13, and IFN-γ production by splenocytes and local lymph node cells was virtually blocked (>80%) by anti-CD4 mAb (data not shown), suggesting that CD4 T cells are the predominant cell type responsible for the measured cytokine production. In addition, analysis of cytokine levels in BALs also showed comparable IL-5 and IFN-γ production in wild-type and IL-12 KO mice following OVA sensitization/challenge (data not shown). The results argue against the hypothesis that the reduction in eosinophil recruitment into airways observed in IL-12 KO mice is caused by changes in Th1 and/or Th2 cytokine patterns in IL-12 KO mice following allergen exposure.

To elucidate the effect of the deficiency of endogenous IL-12 on Ab responses to allergen, we examined OVA-specific IgE, IgG1, and IgG2a responses in IL-12 KO and wild-type mice (Fig. 3). The levels of OVA-specific Ab responses of all three isotypes in IL-12 KO mice were comparable to those measured in wild-type mice. In particular, the levels of OVA-specific IgE, an Ab isotype critical for allergic reactions, were virtually the same between IL-12 KO and wild-type mice. The results were consistent with the findings regarding the cytokine production in this model, which showed comparable levels of IgE- and IgG1–related Th2 cytokine (IL-4, IL-5, and IL-13) and IgG2a-related Th1 cytokine (IFN-γ) between IL-12 KO and wild-type mice.

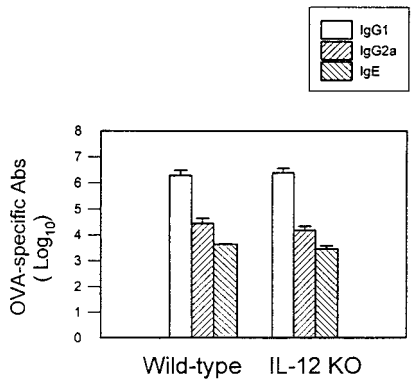

FIGURE 3. Comparable OVA-specific Ab production in IL-12 KO and wild-type mice following OVA sensitization/challenge. Mice were sensitized and challenged with OVA as described in Fig. 1. Mice were bled on day 7 after intranasal challenge with OVA, and serum OVA-specific IgE, IgG1, and IgG2a Abs in individual mice were determined using ELISA. ELISA titers were transformed to $\log_{10}$ and are presented as the mean $\pm$ SEM. Pooled data from three independent experiments with similar results are shown.

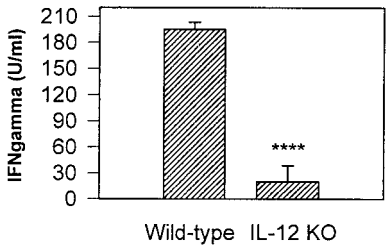

FIGURE 4. Dramatic reduction in IFN-γ production in IL-12 KO mice compared with that in wild-type mice following intracellular bacterial infection. Mice (four mice per group) were infected i.v. with *M. bovis* BCG (1×10^5 CFU) and sacrificed on day 23 postinfection. Draining lymph node cells were cultured at 5×10^6 cells/ml with dead BCG organism restimulation. Culture supernatants were harvested at 72 h and tested for IFN-γ production using ELISA. Data are presented as the mean $\pm$ SD of each group. ***, $p < 0.001$, IFN-γ production in IL-12 KO mice compared with that in wild-type mice.

IL-12 KO mice show decreased Th1 cytokine production following intracellular bacterial infection

The finding that IL-12 KO mice show levels of IFN-γ production similar to those in wild-type mice was inconsistent with the numerous reports that show impaired Th1 responses caused by deficiency of endogenous IL-12 and increased Th1 responses following in vivo delivery of exogenous rIL-12 (32, 42–45). We hypothesize that this may be due to the fact that OVA mainly induce Th2 responses; thus, the involvement of IL-12 in cytokine response might not be as important as that shown in models of Th1-dominant responses such as intracellular bacterial infection. To test this hypothesis, we examined IFN-γ production in IL-12 KO mice infected with intracellular bacteria, *M. bovis* BCG, which have been demonstrated to predominantly induce Th1 responses. As shown in Fig. 4, in contrast to the similarity in IFN-γ production observed in IL-12 KO and wild-type mice following OVA exposure, IFN-γ production in IL-12 KO mice was significantly lower than that in wild-type mice following intracellular bacterial infection. Of note, the levels of IFN-γ production in wild-type mice following BCG infection were remarkably higher than those in OVA-sensitized/challenged mice. The results suggest that IL-12 may play a more important role in the development of IFN-γ responses in models with a predominant Th1 reaction than in models with a predominant Th2 reaction.

IL-12 KO mice show deficiency in VCAM-1 expression on airway vascular endothelium following OVA challenge

The fact that the levels of circulating eosinophils in IL-12 KO mice were comparable to those in wild-type mice suggests that the reduction of airway eosinophilic inflammation in IL-12 KO mice is caused by an alteration in recruiting mechanisms rather than a deficiency of eosinophil development. Because eotaxin is an important chemotactic factor for eosinophils to migrate into the lungs, we examined eotaxin levels in the BALs of IL-12 KO mice. The results showed that eotaxin production in the BALs of IL-12 KO mice was comparable to that in wild-type mice, suggesting that the decrease in eosinophilic inflammation was not due to a lack of local eotaxin production (Fig. 5).

Because VCAM-1 is the most important adhesion molecule in regulating eosinophil migration from peripheral blood to extravascular and alveolar areas in the lung, we examined the expression of this adhesion molecule in the lung vessels of OVA-sensitized/challenged IL-12 KO and wild-type mice. VCAM-1 expression on the endothelium of pulmonary vessels of sensitized wild-type mice was readily detectable on day 2 following intranasal challenge with OVA, increasing to a peak at 6–8 days postchallenge and remaining at high levels for at least 2 wk. The kinetics of VCAM-1 expression is parallel with the kinetics of eosinophil recruitment into the lung (data not shown). As shown in Fig. 6, significant expression of VCAM-1 was observed in wild-type mice on day 6 following intranasal challenge with OVA, while very faint or undetectable VCAM-1 expression was seen in identically sensitized/challenged IL-12 KO mice. Scores of VCAM-1 staining in pulmonary blood vessels based on Briscoe's method (38) also showed significantly less VCAM-1 expression in IL-12 KO mice (Fig. 7). The results indicate that IL-12 deficiency prevents the expression of VCAM-1 on pulmonary blood vessels, thus probably blocking the recruitment of eosinophils into airways following local allergen challenge.

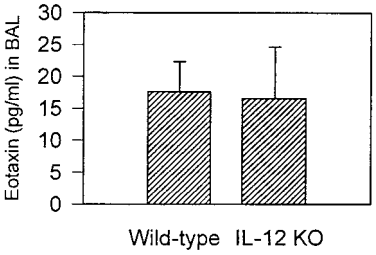

FIGURE 5. Comparable eotaxin production in BALs in IL-12 KO and wild-type mice following OVA sensitization/challenge. Mice were treated as described in Fig. 1 and were sacrificed on day 7 following intranasal OVA challenge. The BAL fluids were spun down, and supernatants were for tested for eotaxin using ELISA. Paired Abs were purchased from R&D Systems, and the manufacturer's instructions were followed. The sensitivity of the ELISA is 5 pg/ml. Data are presented as the mean ± SD. Data from one representative experiment of four independent experiments are presented.

p35 KO mice also show reduction in airway eosinophilia and VCAM-1 expression

To confirm the association between IL-12 deficiency and the reduction of both pulmonary vascular VCAM-1 expression and airway eosinophilia, we repeated the experiments using IL-12 p35 KO mice. As shown in Table III, similar to what was observed in p40 KO mice, p35 KO mice showed a dramatic decrease in airway eosinophilia (10% eosinophils in BALs of IL-12 p35 KO mice vs 80% eosinophils in BALs of wild-type mice). Similarly, VCAM-1 expression on pulmonary blood vessels was significantly less than that in wild-type mice (VCAM-1 score in wild-type mice was 1.67 vs 0.17 in p35 KO mice; $p < 0.001$). Cytokine analysis showed that allergen-driven Th1 (IFN-γ) and Th2 cytokine (IL-4, IL-5, and IL-13) production patterns were comparable between IL-12 p35 KO and wild-type mice following OVA sensitization and intranasal challenge (data not shown).

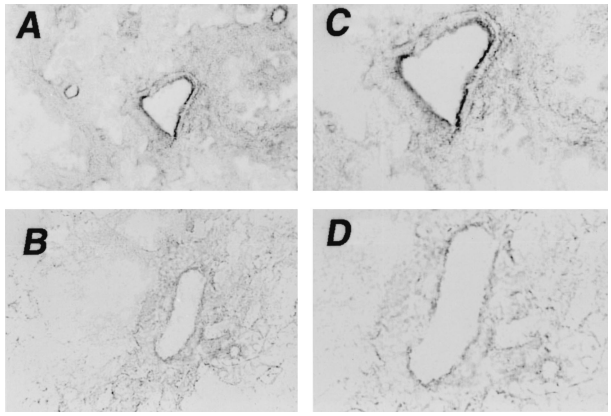

FIGURE 6. Marked reduction of VCAM-1 expression on airway vascular endothelium in IL-12 KO mice compared with wild-type mice following OVA sensitization/challenge. Mice were treated with OVA as described in Fig. 1 and sacrificed on day 7 postchallenge. Lung tissues were snap-frozen in liquid N_2, and sections were stained with purified rat anti-mouse VCAM-1 Ab for 1 h at room temperature. The sections were then incubated with secondary (rabbit anti-rat) Ab conjugated with HRP and developed with 3-amino-9-ethylcarbazole (AEC). An isotype-matched control showed negative staining. *A* and *C*, Wild-type mice; *B* and *D*, IL-12 KO mice. *A* and *B*, Photographed at low magnification (×100); *C* and *D*, photographed at high power magnification (×400). No measurable VCAM-1 expression was observed in the lung sections of naive IL-12 KO and wild-type mice. A total of 20 mice in each group were examined, and representative findings are shown.

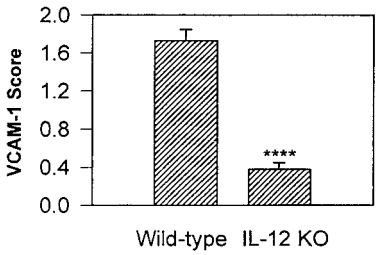

FIGURE 7. VCAM-1 expression on airway vascular endothelium of IL-12 KO and wild-type mice presented as the intensity score. The intensity of VCAM-1 expression on the lung sections of the mice described in Fig. 6 was classified according to the criteria defined by Briscoe et al. (38). A score of 0 represents absent staining or faint staining of an occasional vessel only; 1+ represents faint staining of several vessels; 2+ represents moderate intensity staining of most vessels, and 3+ means intense staining of most vessels. The sections were examined by two observers in a blind manner, and the average of the two determinations for each section was used for calculation of VCAM-1 expression. Ten sections in each lung were examined. Data from four independent experiments (a total of 20 mice/group) were pooled and are presented as the mean + SEM. **, $p < 0.01$, IL-12 KO vs wild-type mice.

Supplement of rIL-12 to IL-12 KO mice increases airway eosinophilia and VCAM-1 expression

To further confirm the role of IL-12 in airway eosinophilia and VCAM-1 expression, we administered rIL-12 to IL-12 KO mice in both stages of sensitization and intranasal challenge with OVA. As expected, the supplement of rIL-12 to IL-12 KO mice significantly increased the airway eosinophilia of these mice (Fig. 8). The percentage of eosinophils in the BALs of IL-12 KO mice increased from <20% to nearly 60% ($p < 0.001$). Parallel with the increase in airway eosinophilia, the expression of VCAM-1 on pulmonary vessels was also significantly enhanced. The VCAM-1 expression score in rIL-12-treated IL-12 KO mice was 1.17, while the score in IL-12 KO mice without rIL-12 treatment was 0.17 ($p < 0.05$). This amount of IL-12 supplement did not have a significant impact on Ag-driven Th1 and Th2 cytokine production and OVA-specific Ab responses and local (BAL) eotaxin levels (data not shown). The data further confirmed that IL-12 plays a critical role in vascular expression of VCAM-1 and airway eosinophilia during allergenic inflammation.

Discussion

The most striking finding in this study is that mice deficient in endogenous IL-12 show significantly reduced (10- to 100-fold) airway eosinophilic inflammation induced by allergen, which is correlated with significantly impaired VCAM-1 expression on pulmonary vascular endothelium. The correlation between IL-12 deficiency and the reduction of airway eosinophilia appears to be a causal relationship rather than a confounding defect in IL-12 KO mice, because both p40 KO and p35 KO mice show the same phenomenon, and more importantly, our confirmatory experiments delivering rIL-12 in vivo restored airway eosinophilia in IL-12 KO mice following allergen exposure. The present findings in IL-12 KO mice contrast with the observations from studies involving in vivo delivery of exogenous IL-12 or delivery of IL-12 gene by recombinant viral vectors to wild-type animals, which showed that IL-12 inhibits IL-5 production and eosinophilic inflammation (34–36). The reason for the discrepancy remains unclear. This study using KO mice examines the function of endogenous IL-12 and therefore is arguably more representative of the role of IL-12 in physiological conditions. However, the findings involving exogenously delivered rIL-12 may reflect the effect mediated by excessive, thus nonphysiological, levels of IL-12. Moreover, administration of exogenous IL-12 mainly tests the immediate effect of this cytokine on cell development and allergic responses, whereas the study using KO mice examines the effect of chronic IL-12 deficiency on the allergic reaction. It is also possible that low levels of IL-12 are required for the recruitment of eosinophils into airways, while medium or high levels (even within the physiological range) of IL-12 are inhibitory for this process. Interestingly, in a parasite infection model, administration of rIL-12 appeared to enhance eosinophilic inflammation in corneas, in line with our finding that IL-12 in certain conditions may promote eosinophilia (46). It should be noted, however, that the expression of chemokine genes, including the gene for eotaxin, was increased following IL-12 treatment in that study. Although we had a similar finding of enhanced eosinophilia in IL-12 KO mice following IL-12 treatment, we failed to find an alteration in eotaxin production in the present study. The reason for this discrepancy was unclear, but apparently the two models are different in terms of pathogenic agents (allergen vs parasite), location (lung vs cornea), and animals (gene knockout vs wild-type mice). In addition, that study only measured chemokine gene expression, not eotaxin protein production, and the level of VCAM-1 expression was not examined in that study.

Another interesting finding in the present study is that IL-12 KO mice did not show parallel alterations in Th2 cytokine and IgE responses with the reduction of airway eosinophilia in this model system. Although IL-12 KO mice exhibited decreased pulmonary eosinophilic responses in comparison with wild-type control mice, Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-10, and IL-13) cytokine and IgE responses between these two groups of mice were comparable following OVA sensitization/challenge. The comparable Th2 cytokine, especially IL-5, production between IL-12 KO and wild-type mice is consistent with the similarity in the levels of circulating eosinophils between these two groups of mice both before and after allergen exposure (Table I). In addition to enhancing eosinophil infiltration, IL-5 affects eosinophil maturation, degranulation, and survival (47, 48). Studies have shown that in vivo IL-5 deficiency caused by Ab neutralization or knockout of the gene prevents both circulating and tissue eosinophilia induced by helminth infection or soluble allergen (49–51). The similarity in circulating eosinophilia between wild-type and IL-12 KO mice following OVA sensitization/challenge indicates that the mechanism

Table III. *IL-12 p35 KO mice also show decreased airway eosinophilia*

Group	Mice	Total BAL Cells ($\times 10^5$)	Eosinophils		Monocytes		Lymphocytes	
			$\times 10^5$	%	$\times 10^5$	%	$\times 10^5$	%
A	WT[a]	29.4 ± 6.6	25.1 ± 6.5	84.0 ± 3.46	2.2 ± 0.2	8.5 ± 1.7	1.9 ± 0.8	7.0 ± 2.8
B	IL-12P35KO	18.9 ± 3.5	2.9 ± 1.8	14.9 ± 7.4	6.4 ± 2.3	32.9 ± 7.1	9.6 ± 1.1	51.3 ± 12.5
p	(A vs B)	>0.05	<0.01	<0.001	<0.05	<0.001	>0.05	>0.05

[a] WT, Wild type.

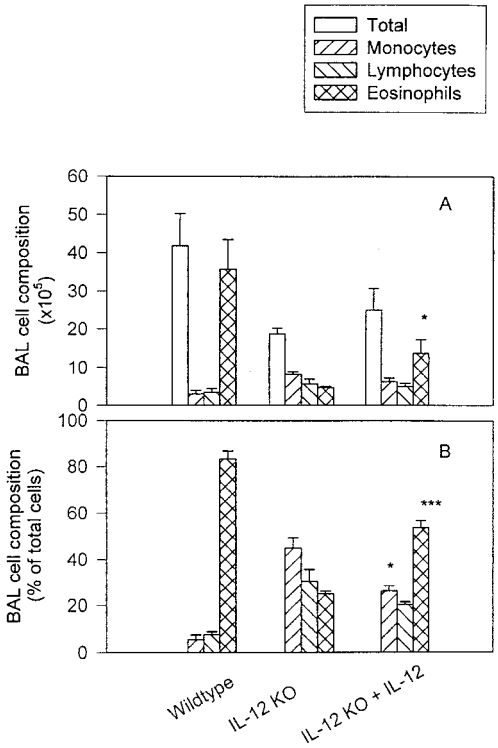

FIGURE 8. Supplementation of rIL-12 to IL-12 KO mice increases airway eosinophilia. IL-12 KO mice (four mice per group) were treated i.p. with 300 ng of rIL-12 (PeproTech, Rocky Hill, NJ) or saline 1 day before and 5 day after OVA sensitization and were given intranasally on the day of intranasal challenge with OVA. The level of endotoxin in the rIL-12 preparation was <0.05 ng/μg of rIL-12. Wild-type C57BL/6 mice were used as controls. The procedures for OVA sensitization/challenge and differential BAL cell counting in all these mice were identical with that described in Fig. 1. The figure shows the absolute cell number of each infiltrating cellular population (*A*) and the proportion of each cellular component of the total BAL cells (*B*). *, $p < 0.05$; ***, $p < 0.001$, IL-12 KO mice with supplementary rIL-12 vs IL-12 KO mice without rIL-12 supplement.

underlying the reduction of airway eosinophilia caused by IL-12 deficiency is different from that operating during IL-5 deficiency.

Our results highlight the importance of VCAM-1 in promoting circulating eosinophil migration into airways. Chemokines and adhesion molecules have been found to be critical in the migration of inflammatory cells into airways. In particular, eotaxin and VCAM-1 are most important in the recruitment of eosinophils. Our results showed that eotaxin levels in the BALs of IL-12 KO mice were comparable to those in wild-type mice, thus not accounting for the diminished airway eosinophilic inflammation in the KO mice. In contrast, we found that VCAM-1 expression on pulmonary vascular endothelium of IL-12 KO mice was significantly lower than that of wild-type mice. More importantly, it was found that IL-12 (both p40 and p35) treatment of KO mice with rIL-12 in the stages of allergen sensitization and challenge increased airway eosinophilia, which is in parallel with the enhancement of VCAM-1 expression on vascular endothelium in the lung. IL-12, therefore, appears critical for the expression of VCAM-1 expression. Indeed, some studies have demonstrated that IL-12 plays an important role in VCAM-1 expression and inflammatory infiltration (52–54). The regulatory role of IL-12 on the expression of VCAM-1 on vascular endothelium may be variable depending on the organs, tissues, and local concentrations, e.g., it is possible that low levels of IL-12 enhances VCAM-1 expression, while high levels of IL-12 inhibit VCAM-1 expression in the lung. This hypothesis is supported by our recent finding that intracellular bacterial infection, which enhances IL-12 production, inhibits VCAM-1 expression and airway eosinophilia induced by allergen (37).

Because previous studies have correlated VCAM-1 expression on lung vessels in asthma-like models with TNF-α production (55) and the synergy between TNF-α and IL-4 (56–58), we also examined TNF-α production in BALs in IL-12 KO mice and by cultured draining lymph node and spleen cells. IL-12 KO and wild-type mice showed comparable TNF-α production. Therefore, the failure in VCAM-1 expression by IL-12 KO mice did not appear to be due to a decrease in TNF-α production. However, because IL-12 is able to enhance TNF receptor expression (59), it is still possible that IL-12 deficiency prevents TNF receptor expression, consequently blocking the effect of TNF-α on VCAM-1 expression.

Because it has been demonstrated in numerous studies that IL-12 is a critical factor in the initiation and expansion of Th1-type immune responses, the finding of comparable IFN-γ production between IL-12 KO and wild-type mice is surprising. It should be noted that the level of IFN-γ production in this allergy model is much lower than that induced by intracellular bacterial infection. Because OVA is a soluble protein that mainly induces Th2 responses, the impact of IL-12 deficiency on immune responses induced by OVA may not be as obvious as that seen in intracellular bacterial infections that predominantly induce Th1 responses. In particular, LPS and CpG motifs expressed on intracellular bacteria are potent inducers of the maturation of dendritic cells, which are major IL-12 producers in vivo (60–64), while OVA lacks these components. Indeed, we have found significantly diminished IFN-γ production in IL-12 KO mice following *M. bovis* BCG infection (Fig. 4). Moreover, because IL-18 is a cytokine that promotes IFN-γ responses, it is also possible that IL-18 in IL-12 KO mice plays a role in mounting IFN-γ production in this model. In contrast, because this study measures IFN-γ production following boost challenge with allergen, we cannot exclude the possibility that IFN-γ production may be lower in IL-12 KO mice than in wild-type mice following primary immunization. Indeed, it was reported recently that IL-12 KO mice show impaired IFN-γ production by spleen cells on day 5 following sensitization with OVA (65). Therefore, it is possible that repeated exposure of soluble Ag may overcome the defect of Th1 responses in IL-12 KO mice, especially for those Ags predominantly inducing Th2 responses.

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