

IL-12 Contributes to Allergen-Induced Airway Inflammation in Experimental Asthma¹

Isabelle Meyts,*[¶] Peter W. Hellings,* Greet Hens,* Bart M. Vanaudenaerde,[†] Bert Verbinnen,* Hubertine Heremans,[‡] Patrick Matthys,[‡] Dominique M. Bullens,* Lut Overbergh,[§] Chantal Mathieu,[§] Kris De Boeck,[¶] and Jan L. Ceuppens^{2,*||}

Lack of sufficient IL-12 production has been suggested to be one of the basic underlying mechanisms in atopy, but a potential role of IL-12 in established allergic airway disease remains unclear. We took advantage of a mouse model of experimental asthma to study the role of IL-12 during the development of bronchial inflammation. Administration of anti-IL-12p35 or anti-IL-12p40 mAb to previously OVA-sensitized BALB/c mice concomitantly with exposure to nebulized OVA, abolished both the development of bronchial hyperresponsiveness to metacholine as well as the eosinophilia in bronchoalveolar lavage fluid and peripheral blood. Anti-IL-12 treatment reduced CD4⁺ T cell numbers and IL-4, IL-5, and IL-13 levels in the bronchoalveolar lavage fluid and the mRNA expression of IL-10, eotaxin, RANTES, MCP-1, and VCAM-1 in the lung. Anti-IL-12p35 treatment failed to show these effects in IFN- γ knockout mice pointing to the essential role of IFN- γ in IL-12-induced effects. Neutralization of IL-12 during the sensitization process aggravated the subsequent development of allergic airway inflammation. These data together with recent information on the role of dendritic cells in both the sensitization and effector phase of allergic respiratory diseases demonstrate a dual role of IL-12. Whereas IL-12 counteracts Th2 sensitization, it contributes to full-blown allergic airway disease upon airway allergen exposure in the postsensitization phase, with enhanced recruitment of CD4⁺ T cells and eosinophils and with up-regulation of Th2 cytokines, chemokines, and VCAM-1. IFN- γ -producing cells or cells dependent on IFN- γ activity, play a major role in this unexpected proinflammatory effect of IL-12 in allergic airway disease. *The Journal of Immunology*, 2006, 177: 6460–6470.

Asthma is a chronic respiratory disorder characterized by episodes of reversible airway obstruction, airway hyperresponsiveness (AHR),³ IgE production, increased mucus secretion, and an airway infiltrate consisting mainly of eosinophilic granulocytes, mast cells, and lymphocytes (1). Relatively recent insights indicate that CD4⁺ T cells are the initiators and regulators of the asthmatic response and that Th2 cells are the predominant T cell population in the asthmatic airway (2, 3). Also, transfer experiments provided strong evidence that Th2 cells play a fundamental role in the pathogenesis of asthma (4–6). Consequently, the feasibility of down-regulating pathological Th2 re-

sponses by enhancing Th1 development has received a lot of attention.

The cytokine IL-12 has an essential role in Th1 development and has therefore also been considered as a potential therapy for asthma. IL-12 is a heterodimeric cytokine, produced by activated monocytes, macrophages, neutrophils, and dendritic cells (DCs). Biologically active IL-12 is composed of a covalently linked 35-kDa L chain (p35) and a 40-kDa H chain (p40) (7). The p40 subunit of IL-12 is shared by IL-23, a cytokine with similar but also distinct actions compared with IL-12 (8). IL-12 promotes Th1 responses and stimulates activated T cells and NK cells to maximally produce IFN- γ , whereas it inhibits the development of IL-4-producing Th2 cells in response to *Dermatophagoides pteronyssinus* and *Leishmania major* (9–11). In addition, IL-12 markedly suppresses the IL-4-induced IgE production by human PBMC in vitro (11, 12). These studies thus suggest that IL-12, through its capability of inducing Th1 cells, might be capable of down-regulating pathological Th2 responses. Additional evidence for this hypothesis comes from murine models of experimental asthma. Systemic administration of IL-12 has been reported to abolish the airway hyperreactivity and pulmonary eosinophilia in a mouse model of allergic asthma (13, 14). Also, intranasal treatment with IL-12 was effective in abrogating the phenotype of experimental asthma (15). Finally, mucosal gene transfer of IL-12 in the lung via a vaccinia virus vector inhibited local Th2 cytokine production and prevented the development of airway hyperreactivity in a mouse model of OVA-induced asthma (16). Keane-Myers et al. (17) suggested that the endogenous IL-12 production is essential for resistance to allergen-induced AHR in C3H mice. Unfortunately, the only interventional study on IL-12 in human asthma patients showed that administration of IL-12 significantly decreased peripheral blood and sputum eosinophil counts without any significant effect on

*Laboratory of Experimental Immunology and [†]Laboratory of Pneumology, University Hospitals, Faculty of Medicine, Katholieke Universiteit Leuven, [‡]Laboratory of Immunobiology, Rega Institute, Faculty of Medicine, Katholieke Universiteit Leuven, [§]Laboratory of Experimental Medicine and Endocrinology, Faculty of Medicine, Katholieke Universiteit Leuven, and [¶]Department of Pediatrics and ^{||}Department of Internal Medicine, University Hospitals, Faculty of Medicine, Katholieke Universiteit Leuven, Leuven, Belgium

Received for publication September 1, 2005. Accepted for publication August 11, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by grants of the Fonds Wetenschappelijk Onderzoek (FWO) Vlaanderen G.0255.05 and of the research council of the Katholieke Universiteit Leuven. I.M. and G.H. are recipients of fellowships from the FWO, and P.M., D.M.B., and P.W.H. are postdoctoral fellows of the FWO. B.V. is a recipient of a fellowship of the Innovatie door Wetenschap en Technologie.

² Address correspondence and reprint requests to Dr. Jan L. Ceuppens, Laboratory of Experimental Immunology, Onderwijs en Navorsing, Herestraat 49, B-3000 Leuven, Belgium. E-mail address: Jan.Ceuppens@uz.kuleuven.ac.be

³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; DC, dendritic cell; KO, knockout; MCh, metacholine; PenH, enhanced pause; BAL, bronchoalveolar lavage; sVCAM-1, soluble VCAM-1.

AHR (18). Also contrary to expected was the finding that endogenous IL-12 is required for the elaboration of eosinophilic inflammation, because IL-12 knockout (KO) mice have significantly lower airway eosinophil counts following OVA sensitization and challenge (19).

Taken together, the role of endogenous IL-12 in established asthma remains at present largely unknown. Therefore, we investigated the effect of neutralization of IL-12 in a biphasic model of allergic airway inflammation, induced by OVA sensitization and airway exposure and characterized by production of allergen-specific IgE, bronchial eosinophilic inflammation, and AHR (20, 21). The effects of either anti-IL-12p35mAb or anti-IL-12p40 mAb administration were studied. Furthermore, the use of anti-p35 mAb enabled us to specifically target IL-12, whereas anti-p40 mAb targets both IL-12 and IL-23.

Materials and Methods

Sensitization and challenges with OVA and anti-IL-12 mAb treatment schedule

Male BALB/c mice that were 6–8 wk old were obtained from Harlan. IFN- γ KO (BALB/c background) mice were purchased from JAX mice (The Jackson Laboratory). The mice were kept in conventional pathogen-free housing and were maintained on OVA-free diets. The experimental procedures were approved by the Ethical Committee of Animal Experiments of the University of Leuven.

The protocol for inducing experimental asthma is biphasic (i.p. sensitization followed by airway challenge through nebulization) and has been described previously (20, 21). Accordingly treated mice exhibit features reminiscent of human asthma, e.g., production of allergen-specific IgE, eosinophilic airway inflammation, and airway hyperreactivity. In brief, groups of mice ($n = 6$) were sensitized by i.p. injection of 10 μ g of OVA in 0.5 ml of pyrogen-free saline on alternate days from days 1 to 13. The mice were then challenged via the airways with OVA, 1% in saline, by nebulization during 5 min from days 33 to 40 (PARI TurboBOY). Analyses were performed on day 41, i.e., 24 h after the final airway challenge.

To study the effect of blocking IL-12 during the airway challenge phase, rat anti-murine anti-IL-12p35 mAb (C18.2) or rat anti-murine anti-IL-12p40 mAb (C17.8) was administered i.p. (250 μ g in 250 μ l of PBS) on days 33 and 37, 2 h before the allergen challenges (treatment during challenge: treatment protocol I). In a separate kinetic experiment, analyses were performed at 8, 24, and 48 h after the final airway challenge. In a second series of experiments, to study the effect of neutralizing IL-12 during sensitization, the same dose of anti-IL-12p40 mAb was administered on days 0, 4, 8, and 12 (treatment during sensitization: treatment protocol II) (Fig. 1).

Measurement of airway responsiveness to metacholine (MCh)

On day 41, 24 h after the last airway challenge, the airway responsiveness to inhaled MCh was measured in unrestrained animals, using whole-body plethysmography (Buxco; EMKA Technologies) (20, 22). Mice were challenged during 1 min with aerosolized MCh in increasing concentrations (0, 2.5, 5, 10, 20, 50 mg/ml), and enhanced pause (PenH)—a dimensionless parameter of bronchial tone—was recorded and averaged for 3 min after each nebulization. The airway tone is further expressed as the mean PenH.

Bronchoalveolar lavage (BAL)

BAL was performed in anesthetized mice using five aliquots of 1 ml of 5% BSA in PBS. The samples were kept on ice until further processing. The first fraction (on average, 0.8 ml) was centrifuged at $1400 \times g$ for 5 min, and the supernatant was stored at -70°C until analyzed for cytokines. The pellet of this aliquot was added to that of the subsequent aliquots (on average, 3.5 ml). After centrifugation ($1400 \times g$; 5 min), the cells were resuspended for cell counts (Türk's solution; hematocytometer). Cytospin preparations were stained with May-Grünwald-Giemsa. Differential cell counts were performed using morphological criteria.

In a separate series of experiments, FACS analysis was performed on the BAL fluid lymphocytes. To this purpose, BAL was performed using five aliquots of 1 ml of Ca^{2+} - and Mg^{2+} -free PBS to which 0.1 mM EDTA was added. After centrifugation, the pellet was treated with ammonium chloride lysis buffer to lyse RBC. Staining was performed using PE-conjugated anti-mCD3 mAb, PerCP-conjugated anti-mCD4, FITC-conjugated anti-CD8, anti-CD19, and anti-CD49b mAb. BAL fluid lymphocyte subtypes were identified based on staining with Abs for the different cell types:

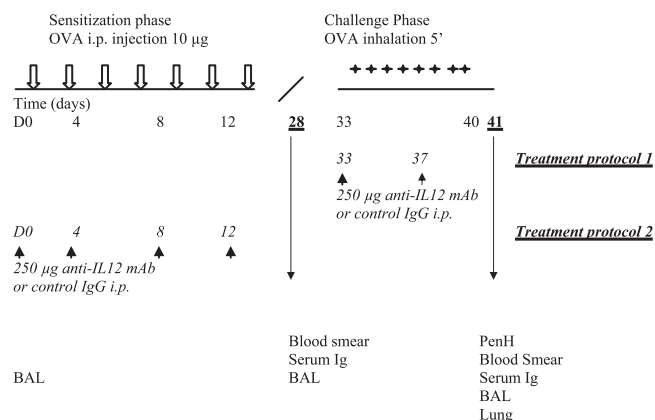


FIGURE 1. Protocol for inducing experimental asthma and anti-IL-12 mAb treatment. BALB/c mice, 6–8 wk old, were sensitized to OVA by seven i.p. injections of OVA (10 μ g/500 μ l) on alternate days from days 1 to 13 (arrows). Challenges consisted of inhalation of nebulized OVA (10 mg/ml) during 5' daily on days 33–40 (stars). Blockade of IL-12 during the challenge phase (treatment protocol 1) was accomplished by administration of 250 μ g of anti-IL-12p35 or anti-IL-12p40 mAb i.p. on days 33 (2 h before the first airway challenge) and day 37. Blockade of IL-12 during allergen priming (treatment protocol 2) was obtained by i.p. injection of the same dose of anti-IL-12 mAbs on days 0, 4, 8, and 12. Control mice received the same dose of control IgG. Analyses were performed on day 41, i.e., 24 h after the last airway challenge, unless indicated otherwise. An additional blood sample was obtained after completion of the sensitization on day 28.

$\text{CD}3^+\text{CD}4^+$ T cells, $\text{CD}3^+\text{CD}8^+$ T cells, $\text{CD}3^-\text{CD}19^+$ cells (B cells), $\text{CD}3^+\text{CD}49b^+$ cells (population of NKT cells), and $\text{CD}3^-\text{CD}49b^+$ cells on 10,000 events within the lymphocyte gate of the forward scatter/side scatter plots. Cells were counted with the FACSscan (BD Biosciences), and data were analyzed with CellQuest Software (BD Biosciences). Absolute numbers of lymphocyte subsets were calculated by multiplying the percentages of subsets with the absolute numbers of lymphocytes in the BAL fluid as determined by morphological criteria.

Lung histology

After BAL sampling, the right lung was removed and fixed in 6% neutral-buffered formalin. Paraffin-embedded sections were made and stained with H&E. Peribronchial and perivascular inflammation was assessed using light microscopy under $\times 20$ – 100 magnifications.

Real-time quantitative PCR on lung tissue

Approximately 100 mg of lung tissue was dissected, snap-frozen in liquid nitrogen, and stored at -80°C . Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen). A constant amount of 2 μ g of RNA was reverse transcribed using the Ready-to-Go T-Primed First-Strand kit (Amersham Biosciences). Real-time quantitative RT-PCR was performed for murine IL-4, IL-5, IFN- γ , IL-10, MCP-1, eotaxin, RANTES, VCAM-1, and ICAM-1. The primers and probes for the murine cytokines and chemokines were based on the published sequences (23). For eotaxin and VCAM-1, the primers and probes were designed with Primer Express. Eotaxin primer and probe sequences were as follows: forward primer, TGT CTC CCT CCA CCA TGC A; reverse primer, GAT CTT CTT ACT GGT CATGAT AAA GCA; TaqMan probe, CTC ACC CAG GCT CCA TCC CAA CTT C. VCAM-1 primers and probes were as follows: forward primer, GGA AGC CGG TCA CAG TCA A; reverse primer, TGT CTG TTC ATG AGC TGG TCA C; and TaqMan probe, TGG CTC CAG ACA TTT ACC CAG TTT ACA GGC.

Mouse β -actin was used as housekeeping gene. Real-time quantitative PCR was performed with Universal TaqMan PCR MasterMix (Applied Biosystems) in the ABI Prism 7700 Sequence detector (Applied Biosystems) using the following conditions: 10 min at 94°C , followed by a total of 45 two-temperature cycles (15 s at 94°C and 1 min at 60°C). Each PCR amplification was performed in duplicate wells and quantified by using a standard curve protocol. Water was used as a negative control.

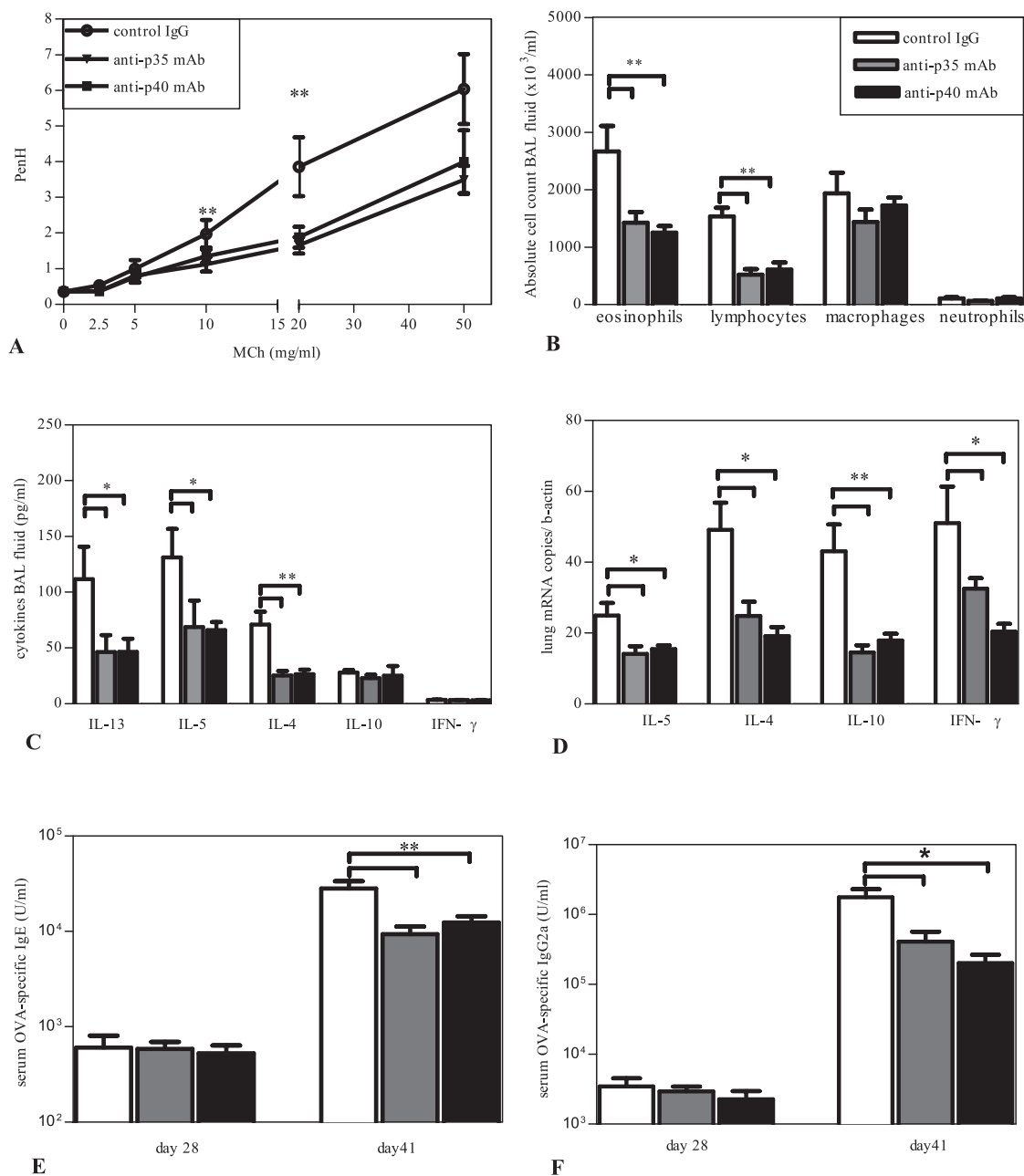


FIGURE 2. Blockade of endogenous IL-12 during the challenge phase attenuates airway reactivity and Th2-type airway inflammation. All mice ($n = 6$ per group) were sensitized and challenged as outlined in Fig. 1. They were treated i.p. with either anti-IL-12p40 or anti-IL-12p35 mAb (treatment protocol 1) or control IgG on days 33 and 37 of the protocol. Analyses were performed on day 41, i.e., 24 h after the final airway challenge. **A**, Bronchial hyperresponsiveness to aerosolized MCh in increasing concentrations was determined by measuring PenH values (whole-body plethysmography). **B**, Absolute cell numbers in the BAL fluid. Cell types were identified on May-Grünwald-Giemsa-stained cytospin preparations. **C**, Protein levels of IL-13, IL-4, IL-5, IL-10, and IFN- γ in BAL fluid supernatant determined by sandwich ELISA. **D**, mRNA levels for IL-5, IL-4, IL-10, and IFN- γ mRNA in lung tissue using real-time quantitative RT-PCR. Represented are IL-5 and IL-4 copy numbers/ β -actin $\times 10^3$ and IL-10 and IFN- γ copy numbers/ β -actin $\times 10^4$. **E**, Levels of OVA-specific IgE in serum obtained at day 28 and day 41 as measured by ELISA. **F**, Levels of OVA-specific IgG2a in serum obtained at day 28 and day 41 as measured by ELISA. Represented are mean and SEM. *, $p < 0.05$ compared with mice treated with control IgG; **, $p < 0.01$ compared with mice treated with control IgG. One representative of three independent experiments is shown, except for Fig. 3D showing pooled data of three independent experiments (total of 18 mice) for each condition.

Blood eosinophilia and serum levels of OVA-specific IgE and IgG2a

On day 28, a small blood sample (<0.5 ml) was taken through the tail vein in mice anesthetized with ketamine-xylazine. On day 41, after the measurement of airway responsiveness, the mice received a lethal dose of pentobarbital and a retro-orbital bleed was performed. Blood smears were made, and sera were frozen at -80°C until further analysis. The blood smears were stained with May-Grünwald-Giemsa and differential cell counts were performed.

The levels of OVA-specific IgE and IgG2a were determined by ELISA. For OVA-specific IgE, 96-well plates (Nunc) were coated overnight with $2 \mu\text{g/ml}$ rat anti-mouse IgE (BD Pharmingen) in pH 9.5 sodium carbonate buffer. After a blocking step (gelatin, 0.2% in PBS), samples were incubated and OVA and peroxidase-conjugated anti-OVA rabbit IgG ($240 \text{ ng}/100 \mu\text{l}$; Rockland) were added, incubated, and washed in sequential steps. 3,3',5,5'-Tetramethylbenzidine substrate reagent (BD Pharmingen) and $50 \mu\text{l}$ of $2 \text{ N H}_2\text{SO}_4$ was added to stop the reaction. The OD_{450} was measured. For OVA-specific IgG2a, the plates were coated overnight with OVA (10

$\mu\text{g/ml}$ PBS) and residual binding sites were blocked. In sequential steps, the samples were added, followed by biotin-labeled anti-mouse IgG2a (BD Pharmingen) and peroxidase-conjugated streptavidin, after which the substrate step and stop reaction were performed as described above. The OD_{450} was measured. A standard curve was calculated from serial dilutions of a reference serum pool of OVA-sensitized BALB/c mice. The undiluted serum was assigned a value of 1,000 experimental units of OVA-specific IgE per ml and 10,000 experimental units of OVA-specific IgG2a per milliliter (EU/ml).

ELISA

Murine IL-4, IL-10, IFN- γ (Biosource International), IL-5, IL-12 (BD Biosciences), soluble VCAM-1 (sVCAM-1), and IL-13 (R&D Systems) were measured by ELISA using paired matched Abs. The lower limit of detection for IL-4, IL-10, IL-5, IL-12, and IL-13 was 10 pg/ml; for sVCAM-1, it was 7.5 pg/ml; for IFN- γ , it was 20 pg/ml.

mAbs and reagents

OVA (grade V), BSA, and MCh were purchased from Sigma-Aldrich. The LPS concentration in the OVA preparation was 0.49 $\mu\text{g/mg}$ OVA (LAL test; Cambrex). Neutralizing anti-IL-12p40 mAb (C17.8, rat IgG) and anti-IL-12p35 mAb (C18.2, rat IgG) were provided by G. Trinchieri (Laboratory for Immunological Research, Schering-Plough Research Institute, Dardilly, France). Control rat IgG was obtained from Rockland. PE-conjugated anti-mCD3 mAb (clone 145-2c11), PerCP-conjugated anti-mCD4 (L3T4 RM4-J), FITC-conjugated anti-mCD8a (Ly-2 53-6.7), anti-mCD19 (1D3), and anti-mCD49b (DX5) were purchased from BD Biosciences. Türk's solution and ammonium chloride were purchased from Merck. Ketamine and xylazine were from Parke-Davis and Roche, respectively.

Statistical analysis

The Mann-Whitney U test was used for statistical analysis. A value of $p < 0.05$ was considered statistically significant. The results are expressed as mean $\pm$ SEM, unless stated otherwise.

Results

In vivo blockade of endogenous IL-12 during the challenge phase attenuates airway reactivity and Th2-type airway inflammation

We studied the role of endogenous IL-12 during the challenge phase, in which OVA-sensitized mice are exposed to OVA and develop allergic airway inflammation. All mice were sensitized to OVA i.p. and challenged via aerosol containing OVA. In a pilot experiment, it was determined that IL-12 levels in the BAL fluid increase significantly in OVA-sensitized mice at (day 28, 11.64 ± 1.16 pg/ml) compared with naive mice (day 0, 2.18 ± 0.29 pg/ml; $p < 0.01$). After the challenge phase, there is a further significant increase in IL-12 values (day 41, 92.22 ± 14.14 pg/ml; $p < 0.01$). Either anti-IL-12p35 or anti-IL-12p40 mAb were administered i.p. on days 33 and 37, with daily airway challenges from days 33 to 40. Both anti-IL-12 mAb significantly decreased the AHR, as reflected by a shift of the PenH/Mch dose-response curve to the right ($p < 0.01$; Fig. 2A). This was accompanied by significantly lower eosinophil and lymphocyte counts in the BAL fluid (Fig. 2B). Moreover, total cell counts in BAL fluid were profoundly reduced in the anti-IL-12 mAb-treated mice ($p < 0.01$; $32 \pm 2.5 \times 10^5/\text{ml}$ in anti-p35-treated mice and $40 \pm 3.2 \times 10^5/\text{ml}$ in anti-p40-treated mice vs $61 \pm 6.6 \times 10^5/\text{ml}$ in mice treated with control rat IgG). A similar decrease in eosinophil percentages was also observed on day 41 in the peripheral blood: 13 ± 1 and $12 \pm 2\%$ in anti-p35 and anti-p40 mAb-treated mice, respectively, vs $20 \pm 2\%$ in mice treated with control rat IgG; $p < 0.01$). Histological analysis revealed reduced density of perivascular and peribronchial inflammatory infiltrates after blockade of IL-12 (Fig. 3).

The levels of BAL fluid IL-13, IL-4, and IL-5 were significantly lower in mice treated with either anti-IL-12 blocking Ab (Fig. 2C). IFN- γ levels were low in all groups. Decreased cytokine production was further confirmed at the mRNA level by real-time RT-PCR in lung tissue. For comparison of mRNA levels in lung tissue,

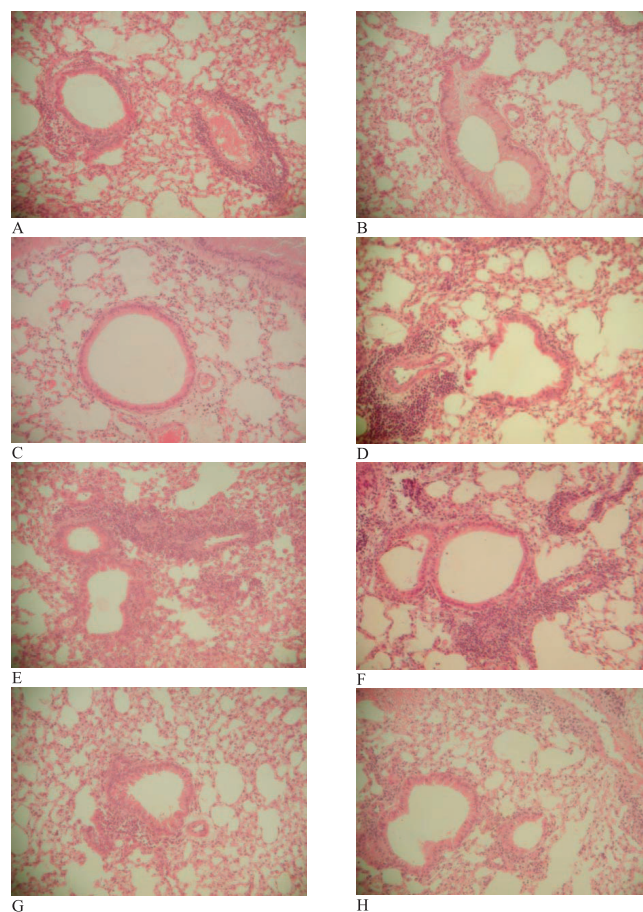


FIGURE 3. Histologic sections of inflamed lungs. Mice were sensitized and challenged as outlined in Fig. 1. Lungs were removed for histologic examination on day 41 of the protocol after BAL was performed. Bronchial sections of mice treated during challenge (treatment protocol 1) with control IgG (A), anti-IL-12p40 mAb (B), or anti-IL-12p35 mAb (C) were stained with H&E for qualitative evaluation of peribronchial and perivascular inflammation. Similar sections are shown of mice treated during sensitization (treatment protocol 2) with control IgG (D), anti-IL-12p40 mAb (E), and anti-IL-12 p35 mAb (F). G and H represent lung sections of IFN- γ KO mice treated with control IgG (G) or anti-IL-12p35 mAb (H) during challenge.

the significance was calculated from three times six individual mice per experiment (18 individual mice). Tissues were never pooled. Thus, IL-4, IL-5, IL-10, and IFN- γ mRNA levels were significantly lower in the anti-IL-12-treated groups compared with the control ($p < 0.05$). For the IL-10 mRNA expression, the difference reached significance in each separate experiment ($p = 0.01$) (Fig. 2D). OVA-specific IgE levels at completion of the challenge phase on day 41 proved to be significantly lower in the mice treated with anti-IL-12 vs those treated with control IgG (Fig. 2E). OVA-specific IgG2a levels were also significantly lower in the anti-IL-12p35- and anti-IL-12p40-treated mice (Fig. 2F) after completion of the challenge phase. Of note, none of the investigated parameters differed significantly between the mice treated with the anti-p35 vs those treated with anti-p40 blocking mAb.

To study the cell and cytokine kinetics, OVA-sensitized and -challenged mice were treated with either anti-IL-12p35 mAb or control IgG during challenge, and BAL fluid was obtained at 8, 24, and 48 h after the last airway challenge. Already at 8 h after the last airway challenge, numbers of eosinophils and lymphocytes were significantly lower in the anti-IL-12p35-treated mice (Fig. 4A).

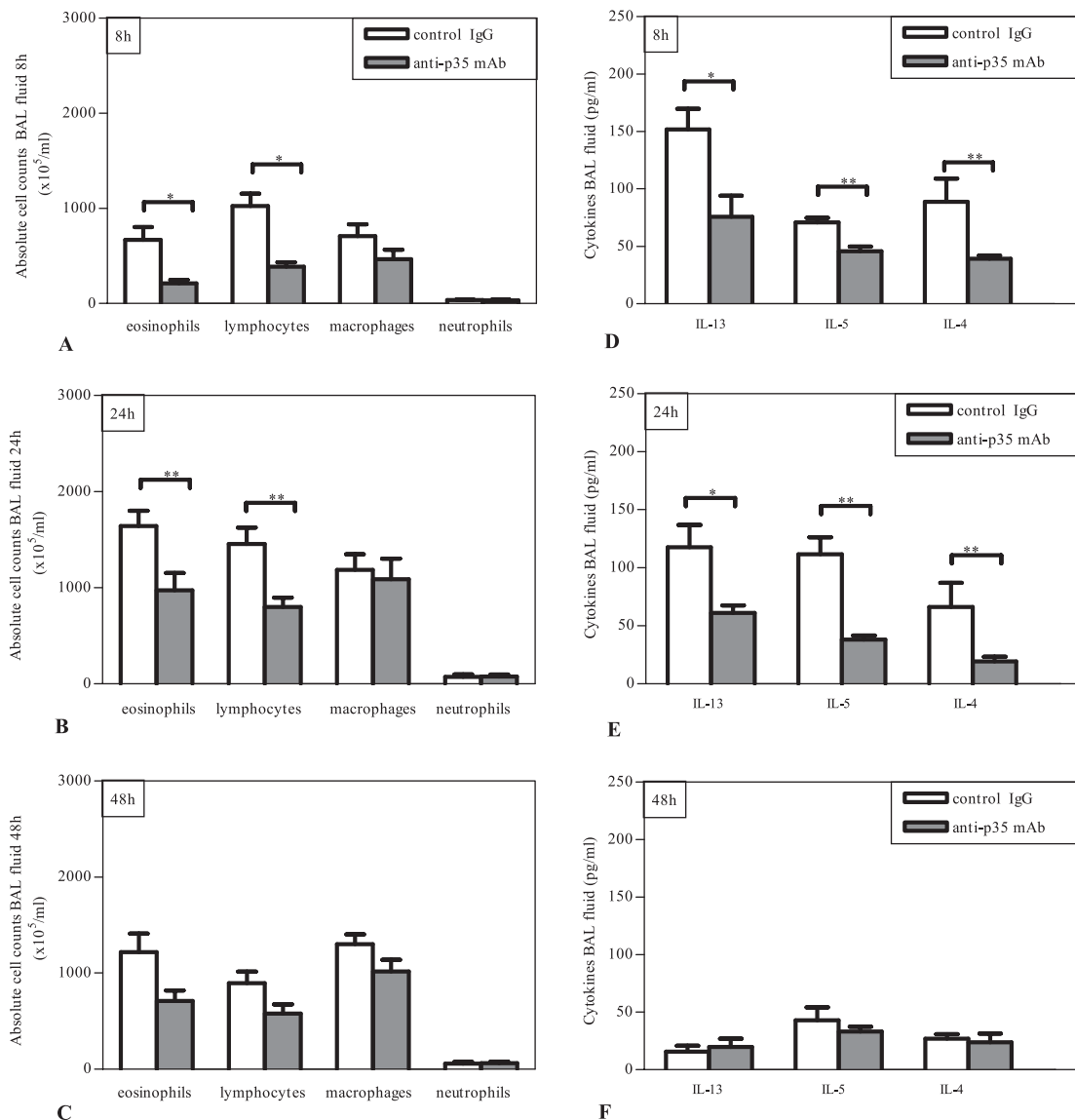


FIGURE 4. Kinetic analysis of the effect of blockade of endogenous IL-12 during the challenge phase on BAL fluid eosinophil and lymphocyte counts and on Th2 cytokines. All mice ($n = 6$ per group) were sensitized and challenged with OVA as outlined in Fig. 1. They were treated i.p. with anti-IL-12p35 mAb (treatment protocol 1) or control IgG on days 33 and 37 of the protocol. BAL fluid was obtained and analyses were performed at 8 h (A–D), 24 h (B–E), and 48 h (C–F) after the last airway challenge. Represented are the absolute cell numbers in the BAL fluid (A–C) (cell types were determined by May-Grünwald-Giemsa) and protein levels of IL-13, IL-4, and IL-5 in BAL fluid supernatant sandwich ELISA (D–F). *, $p < 0.05$ compared with mice treated with control IgG; **, $p < 0.01$ compared with mice treated with control IgG.

The differences persisted at 24 h and slowly waned after 48 h, at which time point only trends could be observed (Fig. 4, B and C). The same was true for the levels of IL-13, IL-5, and IL-4 in the BAL fluid (Fig. 4, D–F).

To study whether a specific subset of lymphocytes was reduced by the anti-IL-12 treatment, flow cytometry analysis was performed on BAL fluid samples of mice treated with anti-IL-12p35 mAb or control IgG. The percentage and absolute number of CD3⁺CD4⁺ cells proved to be significantly lower in the mice treated with anti-IL-12p35 mAb, whereas no differences were found for percentage or absolute numbers of CD3⁺CD19⁺ cells, CD3⁺CD49b⁺, CD3⁺CD49b⁺, and CD3⁺CD4⁺CD8⁺CD49b⁺ cells (Fig. 5). The absolute number but not the percentage of CD3⁺CD8⁺ cells was significantly lower in the anti-p35-treated group.

In vivo blocking of IL-12 during sensitization exacerbates AHR and Th2-type airway inflammation

Because the above results on reduced allergic airway inflammation were unexpected, we then aimed to evaluate the effects of blocking IL-12 during sensitization. Anti-IL-12p35 mAb, anti-IL-12p40 mAb, or rat IgG were administered i.p. on days 0, 4, 8, and 12, and i.p. sensitization was performed from day 1 until day 13. The mice were then exposed to aerosolized OVA from day 33 until day 40. The anti-IL-12 treatment at the time of sensitization had the opposite (yet expected) result on the outcome of allergic airway inflammation compared with administration during challenge. Anti-IL-12 mAb indeed increased AHR as reflected by a shift of the PenH/Mch dose-response curve to the left in anti-IL-12-treated mice compared with the mice treated with rat IgG (Fig. 6A). In the

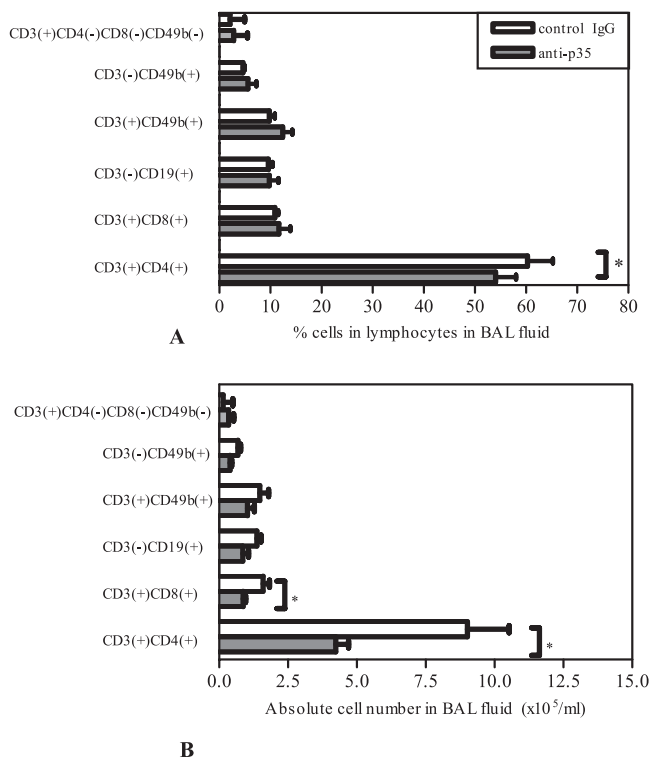


FIGURE 5. Effect of blockade of endogenous IL-12 during the challenge phase on lymphocyte subsets in the BAL fluid. All mice ($n = 6$ per group) were sensitized and challenged as outlined in Fig. 1. They were treated i.p. with anti-IL-12p35 mAb (treatment protocol 1) or control IgG on days 33 and 37 of the protocol. BAL fluid was obtained and analyses were performed at 24 h after the last airway challenge. BAL fluid lymphocyte subsets were identified based on staining with the following combinations of Abs: CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, CD3⁻CD19⁺ B cells, CD3⁺CD49b⁺ NKT cells, CD3⁺CD4⁻CD8⁻CD49b⁻ double-negative T cells, and CD3⁻CD49b⁺ cells. A total of 10,000 events was acquired within the lymphocyte gate of the forward- and side-scatter plots on FACS analysis. Mean $\pm$ SEM percentages (A) or absolute cell numbers (B) of the different lymphocyte subsets are represented. *, $p < 0.05$ compared with mice treated with control IgG.

BAL fluid, significantly higher numbers of eosinophils and lymphocytes were found (Fig. 6B). Total cell counts in the BAL fluid of mice treated with anti-IL-12p35 mAb and anti-IL-12p40 mAb were significantly higher compared with the mice treated with control IgG ($p < 0.05$; $72 \pm 10 \times 10^5$ in anti-p35-treated mice and $65 \pm 9 \times 10^5$ in anti-p40-treated mice vs $43 \pm 10 \times 10^5$ in mice treated with control rat IgG). An increase in the percentage of eosinophils was noted in the peripheral blood smear: 26 ± 2 and $28 \pm 3\%$ in anti-p35 and anti-p40 mAb-treated mice, respectively, vs $16 \pm 2\%$ in mice treated with control rat IgG ($p < 0.01$). IL-13, IL-5, and IL-4 protein levels in the BAL fluid were significantly increased by either anti-IL-12p35 or anti-IL-12p40 treatment during sensitization (Fig. 6C). At the level of mRNA expression in the lung, IL-5 and IL-10 proved to be up-regulated by blocking IL-12 during sensitization in each individual experiment ($n = 6$), and when data from three independent experiments were pooled, also the increase in IL-4 mRNA expression and the decrease in mRNA expression of IFN- γ were significant in the anti-IL-12-treated mice (Fig. 6D) ($p < 0.05$). Anti-IL-12 mAb treatment resulted in significantly enhanced serum levels of OVA-specific IgE and significantly decreased serum levels of OVA-specific IgG2a at day 41 (Fig. 6, E and F). Already at day 28, after completion of the sensitization phase, a similar trend of differences in IgE and IgG2a Ab

levels was observed (Fig. 6, E and F). No differences were found between the effects of the two IL-12 blocking mAbs. It can be concluded from these experiments that anti-IL-12 given during the sensitization phase, resulted in the expected enhancement of Th2 sensitization: enhanced IgE production, aggravated airway inflammation, and higher AHR upon subsequent airway challenge.

Blocking endogenous IL-12 during challenge reduces mRNA expression of eotaxin, RANTES, MCP-1, and VCAM-1

In an attempt to explain the decreased CD4⁺ T cell and eosinophils infiltration, airway inflammation, and airway reactivity after blockade of IL-12 during airway challenge, we investigated the mRNA expression of a number of representative chemokines and adhesion molecules involved in lymphocyte and eosinophil recruitment into the lung. mRNA expression levels of eotaxin, RANTES, and MCP-1 were all significantly lower in animals treated with the anti-IL-12 mAbs during the challenge phase. Also, the mRNA expression of VCAM-1 was significantly down-regulated after anti-IL-12 mAb treatment compared with control IgG treatment (Fig. 7A). Protein levels of sVCAM-1 in the BAL fluid at 48 h after the last OVA challenge were significantly lower after anti-IL-12 mAb treatment compared with control IgG treatment (30.76 ± 3.12 vs 38.13 ± 1.59 pg/ml; $p < 0.05$). No difference was found in the ICAM-1 mRNA expression. In contrast, blockade of IL-12 during priming was accompanied by increased mRNA expression of eotaxin, MCP-1, and VCAM-1 (Fig. 7B). Of note, the increase in eotaxin and MCP-1 expression was more profound with anti-IL-12p35.

IFN- γ KO mice do not show the anti-inflammatory effects of neutralization of IL-12 during allergen challenge

Because IL-12 is the prototypic IFN- γ -inducing and Th1-promoting cytokine, we investigated whether the effects of in vivo blockade of IL-12 during the challenge phase were dependent on IFN- γ . To this purpose, IFN- γ KO mice and wild-type mice were sensitized and challenged with OVA as outlined before. Anti-IL-12p35 mAb was administered on days 33 and 37 (i.e., during challenge). Because the effects of anti-IL-12p35 and anti-p40 proved to be largely the same in all previous experiments, we did not treat mice with anti-p40 mAb. The results in control mice were in accordance with the experiments performed before. However, in IFN- γ KO mice, the airway reactivity to Mch was not influenced by the anti-IL-12p35 treatment (Fig. 8A), and no significant differences in absolute or differential cell counts in BAL were found in KO mice treated with anti-IL-12p35 (Fig. 8B). Also, IL-13, IL-4, and IL-5 levels in the BAL fluid did not differ between IFN- γ KO mice treated with anti-IL-12p35 and the IFN- γ KO mice treated with control IgG (Fig. 8C). Quantitative PCR showed similar eotaxin, RANTES, MCP-1, and VCAM-1 mRNA expression in IFN- γ KO mice treated with control Ig or anti-IL-12p35 (Fig. 8D). In IFN- γ KO mice, there was a trend toward higher protein levels of sVCAM-1 in the BAL fluid in the group treated with anti-p35 mAb (12.83 ± 0.90 vs 10.57 ± 0.66 ; $p = 0.05$) after the last allergen challenge. No differences were found in the levels of OVA-specific IgE and IgG2a between the IFN- γ KO mice treated with anti-IL-12p35 mAb or control IgG. These findings thus suggest that the attenuation of the airway reactivity and of Th2 airway inflammation induced by the blockade of IL-12 during the challenge phase is dependent on a cell that produces IFN- γ or on a cell dependent on IFN- γ for its activity.

Discussion

In the present study, we have demonstrated a differential role of endogenous IL-12 on airway inflammation in a mouse model of

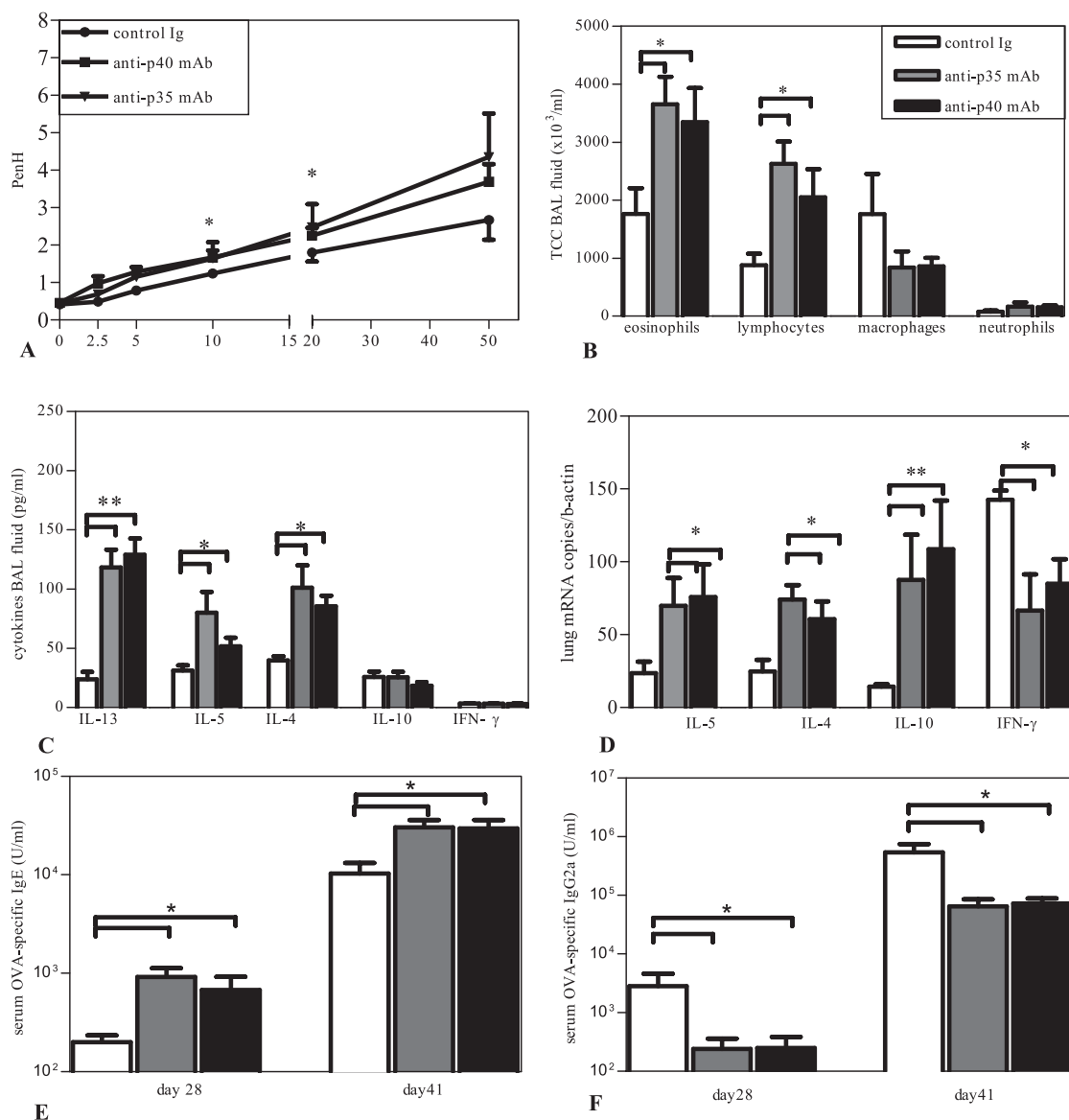


FIGURE 6. Blockade of endogenous IL-12 during sensitization exacerbates airway reactivity and Th2-type airway inflammation. All mice ($n = 6$ per group) were sensitized and challenged as outlined in Fig. 1. They were treated with either anti-IL-12p40 or anti-IL-12p35 mAb (treatment protocol 2) or control IgG on days 0, 4, 8, and 12 of the protocol. **A**, Bronchial hyperresponsiveness to aerosolized MCh in increasing concentrations was determined by measuring PenH values (whole-body plethysmography) at day 41, i.e., 24 h after the last airway challenge. **B**, Absolute numbers of leukocyte populations in the BAL fluid. BAL fluid was obtained at day 41, after PenH measurements. **C**, Protein levels of IL-13, IL-4, IL-5, IL-10, and IFN- γ in BAL fluid supernatant determined by sandwich ELISA. **D**, mRNA levels for IL-5, IL-4, IL-10, and IFN- γ mRNA in lung tissue at day 41 using real-time quantitative RT-PCR. **E**, Levels of OVA-specific IgE in serum at days 28 and 41 as measured by ELISA. **F**, Levels of OVA-specific IgG2a in serum obtained at day 28 and day 41. Represented are mean and SEM. **, $p < 0.01$ compared with mice treated with control IgG; *, $p < 0.05$ compared with mice treated with control IgG. One representative of three independent experiments is shown, except for Fig. 7D showing the pooled data of three independent experiments (total of 18 mice) for each condition.

experimental asthma, depending on the phase of the allergic process. Blockade of IL-12 during the sensitization phase aggravated the typical features of allergic asthma: AHR, bronchial eosinophilia, Th2 cytokine production, and titers of allergen-specific IgE (Fig. 6). This aggravation of the allergic airway inflammation by IL-12 neutralization during sensitization is in agreement with the general belief that IL-12 is protective against excessive Th2 sensitization, as was also shown in experimental asthma (13–15, 24). However, neutralization of IL-12 at a late stage, i.e., during the course of the repeated airway challenges in previously sensitized mice, attenuated the allergen-induced phenotype of experimental asthma: AHR, Th2 cytokine production, and allergen-specific IgE were all profoundly reduced, concomitantly with a reduction of the

CD3⁺CD4⁺ lymphocytes and of eosinophils in the BAL fluid (Figs. 2, 4, and 5). These observed effects of IL-12 neutralization during the challenge phase were quite unexpected and suggest that IL-12 contributes to allergic airway inflammation in the effector phase of this model.

We also show that the anti-inflammatory effects of IL-12 blockade during the challenge phase were accompanied by attenuated expression of several cytokines (IL-4, IL-5, IL-13, IL-10), chemokines (eotaxin, RANTES, MCP-1), and VCAM-1 (Fig. 7). Blockade of IL-12 during the challenge phase in IFN- γ KO mice, did not result in any significant change neither in AHR nor in bronchial inflammation, chemokine or adhesion molecule expression in the lung (Fig. 8). These data show that the effects of IL-12 on airway

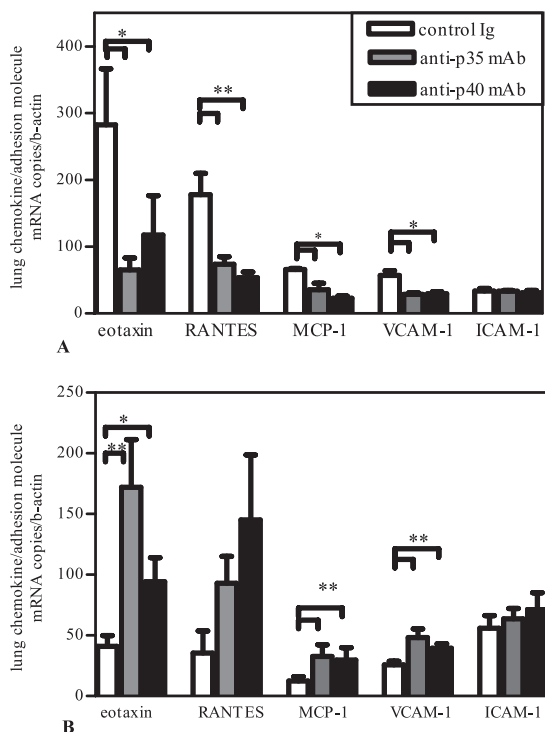


FIGURE 7. Lung mRNA expression of eotaxin, RANTES, MCP-1, VCAM-1, and ICAM-1. The mRNA levels of eotaxin, RANTES, MCP-1, VCAM-1, and ICAM-1 in lung tissue on day 41 were determined by real-time quantitative RT-PCR. All mice ($n = 6$ per group) were sensitized and challenged as outlined in Fig. 1, and lung tissue was obtained on day 41. **A**, Mice received anti-IL-12p35 or anti-IL-12p40 mAb or control Ig during the challenge phase (treatment protocol 1). **B**, Mice were treated with anti-IL-12p35 or anti-IL-12p40 mAb or control Ig during the sensitization phase (treatment protocol 2). mRNA copies/ β -actin $\times 10^4$ are shown except for MCP-1 and ICAM-1 (mRNA copies/ β -actin $\times 10^6$). Represented are mean and SEM. **, $p < 0.01$ compared with mice treated with control IgG; *, $p < 0.05$ compared with mice treated with control IgG. One representative of three independent experiments is shown.

inflammation are IFN- γ dependent either because IFN- γ is directly involved in the activity of IL-12 on allergic inflammation or because a cell type that is dependent on IFN- γ plays an essential role.

We are the first to have clearly demonstrated that blockade of IL-12 has differential effects on allergic airway inflammation depending on the timing of the blockade. Only one study by Sur et al. (25) suggested that the immunomodulatory effects of treatment with rIL-12 depend on the timing of the doses, with administration during the challenge phase having only minor influence on the airway inflammation and airway hyperreactivity. Indirectly, a more recent report by Kuipers et al. (26) on the role of IL-12 during priming favors a contributory role for IL-12 in airway inflammation. They showed that OVA-pulsed IL-12-overexpressing DCs fail to induce Th2 responses, readily observed in mice immunized with sham-transfected, OVA-pulsed DCs. However, when Th2 priming was induced using OVA/alum before intratracheal DC administration, DCs constitutively expressing IL-12 were no longer capable of preventing eosinophilic airway inflammation and even enhanced it. Of note, Wills-Karp and colleagues (17) found that anti-IL-12p40 mAb aggravated allergic airway inflammation when administered before a single airway challenge. The discrepancy between this study and the data presented here are most likely secondary to the major differences in the protocol: a single airway challenge model vs a model of repeated airway challenges.

An important point of debate is the underlying mechanism for the proinflammatory effect of IL-12 in eosinophilic inflammation taking into account that IFN- γ is apparently required for this effect. We have shown that IL-12 blockade during the challenge phase leads to decreased mRNA expression of eotaxin, RANTES (although not significant), MCP-1, and VCAM-1. The modulation of the expression of chemokines and adhesion molecules correlates with the changes in levels of Th2 cytokines in the BAL fluid. Because IL-4 and IL-13 have been identified as potent inducers of eotaxin, RANTES, MCP-1, and VCAM-1, the effect of IL-12 blockade on the expression of chemokines and VCAM-1 could be indirect (27–29). In contrast, a direct effect is also possible: in a model of parasitic keratitis, Pearlman et al. (30) have shown that IL-12 exacerbates pathology by enhancing cellular recruitment through increased expression of eotaxin, RANTES, and MCP-1. Similarly, Wang et al. (19) identified IL-12 as a potential inducer of VCAM-1 expression. Also, IFN- γ has been shown to induce expression of the named chemokines (31–33). One might speculate that, during the repeated airway challenges, IL-12 contributes directly or through an IFN- γ -dependent mechanism to enhanced expression of Th2 cytokines, eosinophil-recruiting chemokines, and VCAM-1.

A second mechanism that might explain the IL-12 effect is through an effect on Th1 cells. The decrease in IFN- γ mRNA expression and the lower levels of OVA-specific IgG2a, suggest that the allergen-induced Th1 activation was attenuated by IL-12 blockade during the challenge phase. Thus, in this chronic model of airway inflammation with repeated airway challenges, Th1 cells and Th1 cytokines might contribute in the effector phase to AHR and to airway inflammation, and this effect is then counteracted by anti-IL-12. The effects of IL-12 blockade during challenge add to the controversy on the Th1/Th2 paradigm in asthma. Allergic airway inflammation is a predominantly Th2-governed disorder, and several studies have shown that administration of Th1 cytokines like IL-12, IL-18, and IFN- γ at the time of sensitization can inhibit induction of Ag-induced Th2 responses, airway hyperreactivity, and inflammation (13, 24, 25, 34, 35). In contrast, it has now become increasingly clear that the role of Th1 cells in asthma should not be reduced to a merely counterregulatory one (6). The most convincing data for a proinflammatory role of Th1 cells in asthma originate from models of adoptive transfer of allergen-specific Th1/Th2 cells. Hansen et al. (36) transferred Th1 and Th2 cell lines, derived from OVA-specific TCR transgenic mice (D011.10) to either SCID or OVA-immunized wild-type BALB/c mice. In this model, only Th2 and Th0 lines were able to induce significant airway hyperreactivity and inflammation. However, Th1 cells were unable to attenuate the Th2-induced AHR. In accordance with these data, Li et al. (37) showed that Th1-mediated responses with influx of neutrophils coexist with Th2-induced eotaxin expression and eosinophilia at the effector stage of lung inflammation, and Randolph et al. (38) even showed a worsening of eosinophilic inflammation and increased Th2 cell recruitment to the lungs by Th1 transfer. The latter study showed that, in the absence of Th1 cells, Th2 cells did not accumulate in the airway, suggesting that Th2 cells require extra signals—potentially provided by Th1 cells—in addition to Ag for their effective recruitment to the airways (39). Furthermore, some data attribute a major role to IFN- γ in the development of AHR in experimental asthma. Hessel et al. (40) showed that anti-IFN- γ completely abolished the development of AHR in sensitized mice upon challenge with OVA. A recent study (41) demonstrated that only the blockade of IFN- γ and not of IL-5 or IL-13 dampened the AHR in experimental asthma. A reduction of the IFN- γ levels in the BAL by administration of pentoxifylline has been shown to efficiently attenuate

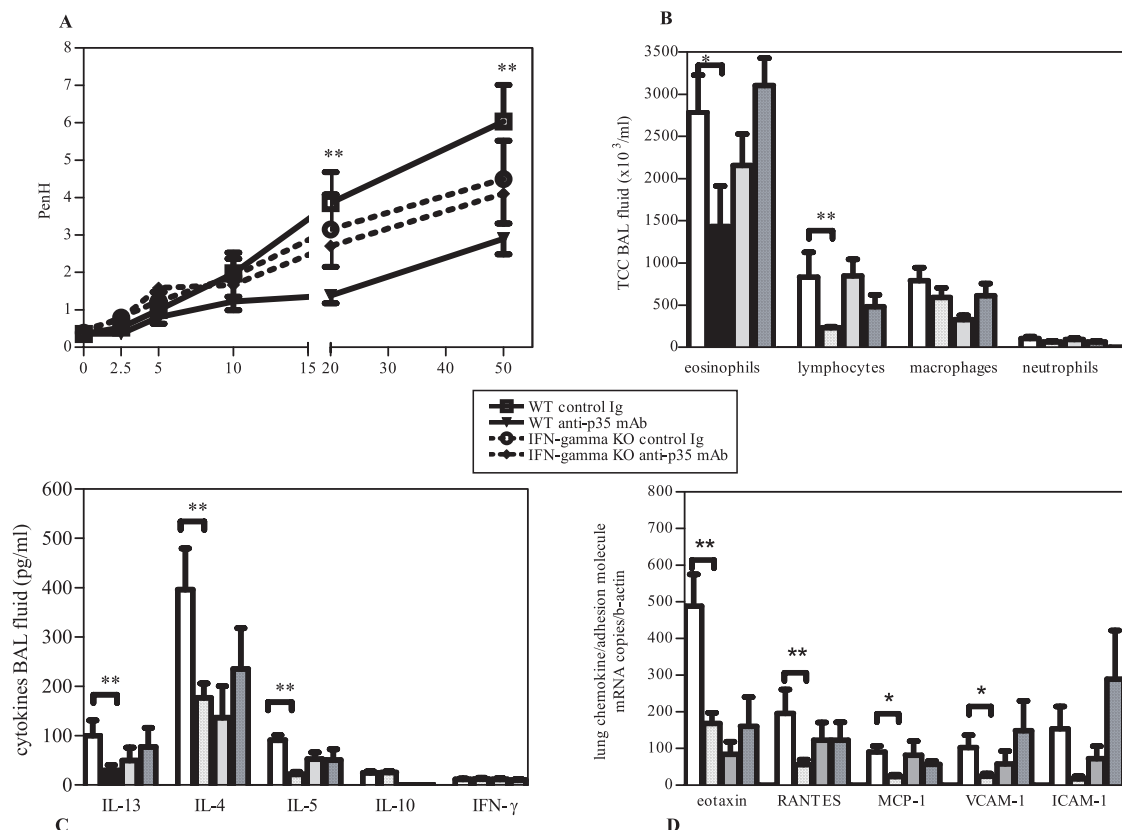


FIGURE 8. The effect of endogenous IL-12 during challenge is dependent on IFN- γ . Wild-type or IFN- γ KO BALB/c mice ($n = 6$ per group) were sensitized and challenged as outlined in Fig. 1. They received anti-IL-12p35 mAb (treatment protocol I) or control IgG on days 33 and 37 of the protocol. **A**, Bronchial hyperresponsiveness to aerosolized MCh in increasing concentrations was determined by measuring PenH values (whole-body plethysmography) at day 41, i.e., 24 h after the last airway challenge. **B**, Absolute cell numbers in the BAL fluid, obtained at day 41 after PenH measurements. **C**, Protein levels of IL-13, IL-4, IL-5, IL-10, and IFN- γ in BAL fluid supernatant determined by sandwich ELISA. **D**, The mRNA levels of eotaxin, RANTES, MCP-1, VCAM-1, and ICAM-1 in lung tissue on day 41 were determined by real-time quantitative RT-PCR. The data are expressed as mRNA copy numbers/ β -actin $\times 10^4$, except for VCAM-1 ($\times 10^3$) and ICAM-1 ($\times 10^6$). Represented are mean and SEM. **, $p < 0.01$ compared with mice treated with control IgG; *, $p < 0.05$ compared with mice treated with control IgG.

airway hyperreactivity without major effects on the expression of Th2 cytokines and eosinophilic inflammation (42). Finally, a report by Koch et al. (43) is in line with the data presented here that pulmonary IFN- γ contributes to allergic airway inflammation.

However, there are also alternative explanations for the antiallergic effect of anti-IL-12. One is that IL-12 not only contributes to Th1 cell activation but also renders them resistant to the activity of regulatory T cells (44). If this would also be the case for Th2 cells, one could explain the results of IL-12 blockade during challenge by a decreased activity of regulatory T cells. Another possibility is an effect of endogenous IL-12 on NK-T cells, which according to a report by Umetsu and colleagues (45) play an essential role in the effector phase of allergic asthma. Indeed, IL-12 has been shown to stimulate NKT cells, and blockade of IL-12 in our model may have led to reduced NKT cell activity (46, 47). Finally, the finding that depletion of CD11c⁺ (IL-12-producing) DCs during the challenge phase abrogates the characteristic features of airway inflammation is also in favor of a role for IL-12 in the effector phase of experimental asthma (48).

Lastly, our results contribute to the correct interpretation of the results of previous studies using anti-IL-12p40 mAb. Indeed, IL-12p40 was found to associate not only with IL-12p35 but also with another molecule, p19, to form a new heterodimeric cytokine, IL-23 (8). IL-23 is mainly produced by activated DCs. In contrast to IL-12, IL-23 induces strong proliferation of mouse memory (CD4⁺CD45Rb^{low}) T cells (8). IL-23p19 has no biological activity

and formation of the active heterodimer requires synthesis of both subunits within the same cell, similarly to IL-12p35. By using anti-IL-12p40 mAb to block the effects of IL-12, one also blocks the effects of IL-23. Moreover, IL-23 has been shown to play a key role in models of chronic inflammation (arthritis, encephalitis, and colitis) (49–52). If not for the similar effect of anti-p35 mAb, one could have speculated that the preferential blockade of IL-23 by anti-p40 mAb during the challenge phase in the present study led to a decreased OVA-specific memory T cell proliferation and therefore a down-regulation of OVA-induced airway inflammation in the effector phase. However, the comparison of anti-IL-12p35 and anti-IL-12p40 learns that the observed enhancement of airway inflammation in the study by Wills-Karp and colleagues (17) as well as in the present study, can be attributed to blockade of IL-12 and not to the simultaneous blockade of IL-23.

In conclusion, we have demonstrated a differential effect of blockade of IL-12 depending on the timing of the administration of anti-IL-12 in a mouse model of experimental asthma. During allergen priming, blockade of IL-12 results in enhanced Th2 inflammation and airway reactivity, as could be expected from enhanced Th2 differentiation in the absence of IL-12. However, when anti-IL-12 is given during airway challenges, this blockade of IL-12 dampened airway reactivity and Th2 inflammation, with reduced expression of eotaxin, RANTES, MCP-1, and VCAM-1 via an IFN- γ -dependent mechanism. Our results are in favor of a key role

for IL-12 and IFN- γ in the effector phase of allergic airway inflammation. Our data therefore suggest that, in the therapeutic approach of patients with allergic asthma, a combined blockade of both IL-5 and IL-12 or IFN- γ may lead to a better control of eosinophilic airway inflammation and symptoms of AHR.

Acknowledgments

We thank M. Adé, L. Coorevits, E. Dilissen, and W. Cockx for their technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

- Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17: 255–281.
- Azzawi, M., B. Bradley, P. K. Jeffery, A. J. Frew, A. J. Wardlaw, G. Knowles, B. Assoufi, J. V. Collins, S. Durham, and A. B. Kay. 1990. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am. Rev. Respir. Dis.* 142: 1407–1413.
- Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham, and A. B. Kay. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326: 298–304.
- Cohn, L., J. S. Tepper, and K. Bottomly. 1998. IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J. Immunol.* 161: 3813–3816.
- Cohn, L., R. J. Homer, N. Niu, and K. Bottomly. 1999. T helper 1 cells and interferon- γ regulate allergic airway inflammation and mucus production. *J. Exp. Med.* 190: 1309–1318.
- Huang, T. J., P. A. MacAry, P. Eynott, A. Moussavi, K. C. Daniel, P. W. Askenase, D. M. Kemeny, and K. F. Chung. 2001. Allergen-specific Th1 cells counteract effector Th2 cell-dependent bronchial hyperresponsiveness and eosinophilic inflammation partly via IFN- γ . *J. Immunol.* 166: 207–217.
- Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* 13: 251–276.
- Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, et al. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13: 715–725.
- Scharton-Kersten, T., L. C. Afonso, M. Wysocka, G. Trinchieri, and P. Scott. 1995. IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. *J. Immunol.* 154: 5320–5330.
- Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. Interleukin 12 acts directly on CD4⁺ T cells to enhance priming for interferon- γ production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* 90: 10188–10192.
- Manetti, R., P. Parronchi, M. G. Giudizi, M. P. Piccinini, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J. Exp. Med.* 177: 1199–1204.
- Kiniwa, M., M. Gately, U. Gubler, R. Chizzonite, C. Fargeas, and G. Delespesse. 1992. Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. *J. Clin. Invest.* 90: 262–266.
- Gavett, S. H., D. J. O'Hearn, M. Li, S. K. Huang, F. D. Finkelman, and M. Wills-Karp. 1995. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J. Exp. Med.* 182: 1527–1536.
- Kips, J. C., G. J. Brusselle, G. F. Joos, R. A. Peleman, J. H. Tavernier, R. R. Devos, and R. A. Pauwels. 1996. Interleukin-12 inhibits antigen-induced airway hyperresponsiveness in mice. *Am. J. Respir. Crit. Care Med.* 153: 535–539.
- Schwarze, J., E. Hamelmann, G. Cieslewicz, A. Tomkinson, A. Joetham, K. Bradley, and E. W. Gelfand. 1998. Local treatment with IL-12 is an effective inhibitor of airway hyperresponsiveness and lung eosinophilia after airway challenge in sensitized mice. *J. Allergy Clin. Immunol.* 102: 86–93.
- Hogan, S. P., P. S. Foster, X. Tan, and A. J. Ramsay. 1998. Mucosal IL-12 gene delivery inhibits allergic airways disease and restores local antiviral immunity. *Eur. J. Immunol.* 28: 413–423.
- Keane-Myers, A., M. Wysocka, G. Trinchieri, and M. Wills-Karp. 1998. Resistance to antigen-induced airway hyperresponsiveness requires endogenous production of IL-12. *J. Immunol.* 161: 919–926.
- Bryan, S. A., B. J. O'Connor, S. Matti, M. J. Leckie, V. Kanabar, J. Khan, S. J. Warrington, L. Renzetti, A. Rames, J. A. Bock, et al. 2000. Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356: 2149–2153.
- Wang, S., Y. Fan, X. Han, J. Yang, L. Bilenki, and X. Yang. 2001. IL-12-dependent vascular cell adhesion molecule-1 expression contributes to airway eosinophilic inflammation in a mouse model of asthma-like reaction. *J. Immunol.* 166: 2741–2749.
- Hellings, P. W., E. M. Hessel, J. J. Van Den Oord, A. Kasran, P. Van Hecke, and J. L. Ceuppens. 2001. Eosinophilic rhinitis accompanies the development of lower airway inflammation and hyper-reactivity in sensitized mice exposed to aerosolized allergen. *Clin. Exp. Allergy* 31: 782–790.
- Hellings, P. W., P. Vandenberghe, A. Kasran, L. Coorevits, L. Overbergh, C. Mathieu, and J. L. Ceuppens. 2002. Blockade of CTLA-4 enhances allergic sensitization and eosinophilic airway inflammation in genetically predisposed mice. *Eur. J. Immunol.* 32: 585–594.
- Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156: 766–775.
- Overbergh, L., A. Giulietti, D. Valckx, R. Decallonne, R. Bouillon, and C. Mathieu. 2003. The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *J. Biomol. Tech.* 14: 33–43.
- Tournoy, K. G., J. C. Kips, and R. A. Pauwels. 2002. Is Th1 the solution for Th2 in asthma? *Clin. Exp. Allergy* 32: 17–29.
- Sur, S., J. Lam, P. Bouchard, A. Sigounas, D. Holbert, and W. J. Metzger. 1996. Immunomodulatory effects of IL-12 on allergic lung inflammation depend on timing of doses. *J. Immunol.* 157: 4173–4180.
- Kuipers, H., C. Heirman, D. Hijdra, F. Muskens, M. Willart, S. van Meirvenne, K. Thielemans, H. C. Hoogsteden, and B. N. Lambrecht. 2004. Dendritic cells retrovirally overexpressing IL-12 induce strong Th1 responses to inhaled antigen in the lung but fail to revert established Th2 sensitization. *J. Leukocyte Biol.* 76: 1028–1038.
- Li, L., Y. Xia, A. Nguyen, Y. H. Lai, L. Feng, T. R. Mosmann, and D. Lo. 1999. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J. Immunol.* 162: 2477–2487.
- Schleimer, R. P., S. A. Sterbinsky, J. Kaiser, C. A. Bickel, D. A. Klunk, K. Tomioka, W. Newman, F. W. Lusinskas, M. A. Gimbrone, Jr., and B. W. McIntyre. 1992. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium: association with expression of VCAM-1. *J. Immunol.* 148: 1086–1092.
- Bochner, B. S., D. A. Klunk, S. A. Sterbinsky, R. L. Coffman, and R. P. Schleimer. 1995. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J. Immunol.* 154: 799–803.
- Pearlman, E., J. H. Lass, D. S. Bardenstein, E. Diaconu, F. E. Hazlett, Jr., J. Albright, A. W. Higgins, and J. W. Kazura. 1997. IL-12 exacerbates helminth-mediated corneal pathology by augmenting inflammatory cell recruitment and chemokine expression. *J. Immunol.* 158: 827–833.
- Matsukura, S., F. Kokubu, H. Kuga, M. Kawaguchi, K. Ieki, M. Odaka, S. Suzuki, S. Watanabe, H. Takeuchi, M. Adachi, et al. 2003. Differential regulation of eotaxin expression by IFN- γ in airway epithelial cells. *J. Allergy Clin. Immunol.* 111: 1337–1344.
- Casola, A., A. Henderson, T. Liu, R. P. Garofalo, and A. R. Brasier. 2002. Regulation of RANTES promoter activation in alveolar epithelial cells after cytokine stimulation. *Am. J. Physiol.* 283: L1280–L1290.
- Meyer-Hoffert, U., D. Lezcano-Meza, J. Bartels, A. R. Montes-Vizuet, J. M. Schroder, and L. M. Teran. 2003. Th2- and to a lesser extent Th1-type cytokines upregulate the production of both CXC (IL-8 and gro- α) and CC (RANTES, eotaxin, eotaxin-2, MCP-3 and MCP-4) chemokines in human airway epithelial cells. *Int. Arch. Allergy Immunol.* 131: 264–271.
- Li, X. M., R. K. Chopra, T. Y. Chou, B. H. Schofield, M. Wills-Karp, and S. K. Huang. 1996. Mucosal IFN- γ gene transfer inhibits pulmonary allergic responses in mice. *J. Immunol.* 157: 3216–3219.
- Hofstra, C. L., I. Van Ark, G. Hofman, M. Kool, F. P. Nijkamp, and A. J. Van Oosterhout. 1998. Prevention of Th2-like cell responses by coadministration of IL-12 and IL-18 is associated with inhibition of antigen-induced airway hyperresponsiveness, eosinophilia, and serum IgE levels. *J. Immunol.* 161: 5054–5060.
- Hansen, G., G. Berry, R. H. DeKruyff, and D. T. Umetsu. 1999. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 103: 175–183.
- Li, L., Y. Xia, A. Nguyen, L. Feng, and D. Lo. 1998. Th2-induced eotaxin expression and eosinophilia coexist with Th1 responses at the effector stage of lung inflammation. *J. Immunol.* 161: 3128–3135.
- Randolph, D. A., R. Stephens, C. J. Carruthers, and D. D. Chaplin. 1999. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J. Clin. Invest.* 104: 1021–1029.
- Randolph, D. A., C. J. Carruthers, S. J. Szabo, K. M. Murphy, and D. D. Chaplin. 1999. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. *J. Immunol.* 162: 2375–2383.
- Hessel, E. M., A. J. Van Oosterhout, I. Van Ark, B. Van Esch, G. Hofman, H. Van Loveren, H. F. Savelkoul, and F. P. Nijkamp. 1997. Development of airway hyperresponsiveness is dependent on interferon- γ and independent of eosinophil infiltration. *Am. J. Respir. Cell Mol. Biol.* 16: 325–334.
- Kumar, R. K., C. Herbert, D. C. Webb, L. Li, and P. S. Foster. 2004. Effects of anticytokine therapy in a mouse model of chronic asthma. *Am. J. Respir. Crit. Care Med.* 170: 1043–1048.
- Fleming, C. M., H. He, A. Ciota, D. Perkins, and P. W. Finn. 2001. Administration of pentoxifylline during allergen sensitization dissociates pulmonary allergic inflammation from airway hyperresponsiveness. *J. Immunol.* 167: 1703–1711.
- Koch, M., M. Witzenth, C. Reuter, M. Herma, H. Schutte, N. Suttorp, H. Collins, and S. H. Kaufmann. 2006. Role of local pulmonary interferon γ expression in murine allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 35: 211–219.

44. King, I. L., and B. M. Segal. 2005. Cutting edge: IL-12 induces CD4⁺. *J. Immunol.* 175: 641–645.
45. Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, T. Nakayama, M. Taniguchi, M. J. Grusby, R. H. DeKruyff, and D. T. Umetsu. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* 9: 582–588.
46. Duthie, M. S., M. Kahn, M. White, R. P. Kapur, and S. J. Kahn. 2005. Both CD1d antigen presentation and interleukin-12 are required to activate natural killer T cells during *Trypanosoma cruzi* infection. *Infect. Immun.* 73: 1890–1894.
47. Hou, R., O. Goloubeva, D. S. Neuberger, J. L. Strominger, and S. B. Wilson. 2003. Interleukin-12 and interleukin-2-induced invariant natural killer T-cell cytokine secretion and perforin expression independent of T-cell receptor activation. *Immunology* 110: 30–37.
48. Van Rijt, L. S., S. Jung, A. Kleinjan, N. Vos, M. Willart, C. Duez, H. C. Hoogsteden, and B. N. Lambrecht. 2005. In vivo depletion of lung CD11c⁺ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J. Exp. Med.* 201: 981–991.
49. Becker, C., S. Wirtz, M. Blessing, J. Pirhonen, D. Strand, O. Bechthold, J. Frick, P. R. Galle, I. Autenrieth, and M. F. Neurath. 2003. Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. *J. Clin. Invest.* 112: 693–706.
50. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.
51. Murphy, C. A., C. L. Langrish, Y. Chen, W. Blumenschein, T. McClanahan, R. A. Kastelein, J. D. Sedgwick, and D. J. Cua. 2003. Divergent pro- and anti-inflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J. Exp. Med.* 198: 1951–1957.
52. Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, et al. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421: 744–748.