

Aeroallergen-induced Eosinophilic Inflammation, Lung Damage, and Airways Hyperreactivity in Mice Can Occur Independently of IL-4 and Allergen-specific Immunoglobulins

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Abstract

In this investigation we have used a mouse model containing certain phenotypic characteristics consistent with asthma and IL-4- and CD40-deficient mice to establish the role of this cytokine and allergen-specific immunoglobulins in the initiation of airways hyperreactivity and morphological changes to the airways in responses to aeroallergen challenge. Sensitization and aerosol challenge of mice with ovalbumin resulted in a severe airways inflammatory response which directly correlated with the induction of extensive airways damage and airways hyperreactivity to β -methacholine. Inflammatory infiltrates were primarily characterized by the presence of CD4⁺ T cells and eosinophils. In IL-4-deficient mice, the recruitment of airways eosinophils was impaired, but not abolished in response to aeroallergen. Moreover, the characteristic airways damage and hyperreactivity normally resulting from allergen inhalation were not attenuated. Induction of these structural and functional changes to the airways occurred in the absence of ovalbumin-specific IgE and IgG₁, but IgG_{2a} and IgG₃ were detected in the sera of IL-4-deficient mice. CD4⁺ T cells isolated from both wild-type and IL-4-deficient mice given ovalbumin produced significant levels of IL-5 after in vitro stimulation. Treatment of IL-4-deficient mice with anti-IL-5 mAb before aeroallergen challenge abolished blood and airways eosinophilia, lung damage, and airways hyperreactivity. These results indicate that IL-4 is not essential for the development of IL-5-producing CD4⁺ T cells or for the induction of eosinophilic inflammation and airways damage and hyperreactivity. In response to sensitization and aerosol challenge, CD40-deficient mice did not produce ovalbumin-specific IgE, IgG isotypes, or IgA, and airways inflammation and hyperreactivity were not attenuated. Our results suggest that allergic airways disease can occur via pathways which operate independently of IL-4 and allergen-specific immunoglobu-

lins. Activation of these pathways is intimately associated with IL-5 and eosinophilic inflammation. Such pathways may play a substantive role in the etiology of asthma. (*J. Clin. Invest.* 1997. 99:1329–1339.) Key words: asthma • interleukin 4 and 5 • IgE • eosinophils • airways hyperreactivity

Introduction

Asthma is a chronic disease of the airways which is clinically characterized by airways obstruction, enhanced bronchial responsiveness to spasmogens (airways hyperreactivity), and airways inflammation (1). The underlying cellular and molecular mechanisms predisposing to airways hyperreactivity and morphological changes to the airways in asthmatics are unknown. The asthmatic response may be regulated by IgE-dependent release of inflammatory mediators from activated mast cells (2–4). Similarly, after accumulation at sites of inflammation, activated eosinophils may degranulate to release cationic proteins and lipid mediators which have the potential to cause local tissue damage and airways hyperreactivity (3–5). Eosinophils and mast cells also secrete cytokines which can exacerbate the inflammatory response by autocrine or paracrine mechanisms (6–12).

Clinical and experimental investigations suggest that allergen-specific CD4⁺ Th2-type cells and the cytokines IL-4 and IL-5 play central roles in initiating and sustaining an asthmatic response by regulating the recruitment and/or activation of airways mast cells and eosinophils (13, 14). IL-4 is a critical factor for the regulation of T cell commitment to the CD4⁺ Th2 phenotype and plays an essential role in IgE isotype switching in B cells (15–18). IL-4 also has potential roles in the maintenance of the inflammatory response to recall antigens and in regulating the production of the eosinophil-specific chemoattractant, eotaxin (15, 19, 20). By contrast, IL-5 regulates the growth, differentiation, and activation of eosinophils (21–23) and provides an essential signal for the recruitment of this leukocyte to the lung during allergic inflammation (24–26). IL-4 and IL-5 may also regulate eosinophil trafficking by activating adhesion systems at the vascular endothelium (27, 28).

The key roles of IL-4 and IL-5 in the development and maintenance of allergic airways inflammation have identified these molecules as important targets for pharmacological modification of the inflammatory response (29). Furthermore, both cytokines have been implicated in the development of airways hyperreactivity to spasmogens after antigen inhalation (25, 29–33). However, there is conflicting evidence about the comparative importance of each cytokine in the molecular mechanism underlying aeroallergen-induced airways hyperreactivity (25, 30, 33, 34). Recently, we have demonstrated an obligatory role for IL-5 in the induction of airways damage and hyperreactiv-

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ity during allergic inflammation in a mouse asthma model (25). In IL-5-deficient (IL-5^{-/-})¹ mice, eosinophilia, airways damage, and airways hyperreactivity normally resulting from aeroallergen challenge were abolished (25). This and other investigations support the hypothesis that airways hyperreactivity and damage to the airways may be directly associated with increased levels of eosinophils and their products in the lung (5, 6, 30–32).

The role of IL-4 in the onset of allergic airways inflammation has also been investigated in IL-4-deficient (IL-4^{-/-}) mice (34). Eosinophil accumulation, but not neutrophil or lymphocyte infiltration, was significantly reduced in aeroallergen challenged IL-4^{-/-} mice in comparison to wild-type mice. However, in this model, aeroallergen challenge did not induce pathological changes in airways tissues, and airways reactivity to spasmogens was not examined. Neutralization of IL-4 by mAb administered during the period of systemic immunization (but not during aeroallergen challenge) reduced airways hyperreactivity to acetylcholine and the accumulation of pulmonary eosinophils in response to antigen inhalation (30). In contrast, pretreatment of mice with anti-IL-5 mAb inhibited airways eosinophilia, but did not inhibit airways hyperreactivity (30). These investigations suggested an essential role for IL-4, and not IL-5 or eosinophils, in the development of aeroallergen-induced airways hyperreactivity.

Recently, an essential requirement for IgE in the development of eosinophil-mediated airways hyperreactivity in response to antigen inhalation was proposed (33). IgE-dependent mechanisms may also be important in the induction of cytokine production by CD4⁺ Th2-type cells and in the subsequent infiltration of eosinophils into the lung in response to aeroallergen challenge (35). IgE production and immunoglobulin class switching are critically regulated by the recognition of allergen-specific CD4⁺ Th2-type cells and B cells through the MHC-II/TCR-CD3 complex and the subsequent interaction of the costimulatory molecules IL-4 and CD40 ligand with their receptors (36). IgE and other allergen-specific immunoglobulins produced after isotype switching are thought to play a critical role in the allergic cascade by activating mast cells (2–4, 9–11). Eosinophils also express Fc receptors for IgG, IgA, and low and high affinity receptors for IgE, which mediate degranulation *in vitro* (7, 8, 12). However, the precise role that IgE and allergen-specific immunoglobulins play in the development of airways hyperreactivity and the morphological changes to the lung during eosinophilic inflammation is unknown.

The effectiveness of blocking the actions of IL-4, IL-5, or allergen-specific immunoglobulins as therapeutic approaches to the treatment of asthma will depend on establishing the role of these molecules in the events which regulate the development of allergen-induced morphological changes to the airways and airways hyperreactivity. In this investigation, we have used a mouse model of asthma (25), IL-4^{-/-} mice (18) and CD40-deficient (CD40^{-/-}) mice (36), to define the role of this cytokine and antigen-specific immunoglobulins in the development of aeroallergen-induced airways damage and hyperreactivity. Aeroallergen-induced airways hyperreactivity or

changes in airways structure were not attenuated in IL-4^{-/-} mice or CD40^{-/-} mice. However, pretreatment of IL-4^{-/-} mice with anti-IL-5 mAb before aeroallergen challenge abolished eosinophilia and the development of airways damage and hyperreactivity. These results suggest that activation of inflammatory cells by IL-4 and allergen-specific immunoglobulins is not essential for regulating eosinophil-derived airways hyperreactivity and lung damage during allergic airways inflammation. Furthermore, pathways which operate independently of these inflammatory molecules are critically linked to the production of IL-5 and eosinophilic inflammation and are central to the pathogenesis of allergic airways disease.

Methods

Induction of allergic airways inflammation. Mice (IL-4^{-/-} mice (18) [kindly provided by Manfred Kopf, Basel Institute of Immunology, Basel, Switzerland] or CD40^{-/-} mice (36) both on a 129Sv × C57BL/6 background, 6–10 wk of age) were sensitized by intraperitoneal injection with 50 µg ovalbumin (OVA)/1 mg Alhydrogel (CSL Ltd., Parkville, Australia) in 0.9% sterile saline on days 0 and 12. Nonsensitized mice received 1 mg of Alhydrogel in 0.9% saline. On day 24, the appropriate groups of mice were exposed to an aerosol of OVA (10 mg/ml) in 0.9% saline (nonsensitized mice received saline only) for 30 min three times (1-h intervals), and then every second day thereafter, for 8 d. The aerosol was generated at 6 liters/min by a nebulizer which produced a mean particle diameter of 3.9 µm into a closed chamber of 800 cm³. 24 h after the last aeroallergen challenge, mice were killed by cervical dislocation or airways hyperreactivity was measured. Lung responses of IL-4^{-/-} mice or CD40^{-/-} mice were compared with control littermates (IL-4^{+/+} or CD40^{+/+}) which were simultaneously treated. Mice were treated according to Australian National University Animal Welfare guidelines and were housed in a specific pathogen-free facility.

Pretreatment of mice with anti-IL-5 mAb (TRFK-5) or anti-CD4⁺ mAb (GK1.5). Mice were sensitized (as described above) and injected intraperitoneally with anti-IL-5 mAb (2 mg/mouse, days 23 and 27), anti-CD4⁺ mAb (2 mg/mouse, day 22), or isotype control antibody (2 mg βGL113/mouse) in sterile 0.9% saline. On day 24, the appropriate groups of mice were aerosolized with OVA as described above.

Measurement of airways hyperreactivity. Airways constriction was measured with a bronchospasm transducer (Ugo Basil 7020; Ugo Basile, Varese, Italy) which was coupled to a Lab Mac/8 analysis station (AdInstruments, Sydney, Australia). Changes in respiratory overflow volume were determined during cumulative intravenous administration of β-methacholine as described previously (25). The increase in respiratory overflow volume provoked by β-methacholine is represented as a percentage of the maximal overflow volume (100%) obtained by totally occluding the tracheal cannula.

Characterization of lung morphology and leukocytes in blood, tissue, and bronchoalveolar lavage fluid (BALF). Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways were fixed in 10% phosphate-buffered formalin, sectioned, and stained with May-Grunwald-Giemsa solution or hematoxylin-eosin. Leukocytes in the blood, BALF, and lung were identified by morphological criteria and quantified as described previously (25). Eosinophil numbers were quantified in peribronchial regions by counting 10 fields of view (per lung section) immediately below (within 2 mm) the respiratory epithelium.

Preparation of CD4⁺ T cells. Red blood cell lysed splenocytes (~1 × 10⁸) were suspended in 90 µl of HBSS/5% FCS with anti-CD4 mAb (L3T4) coated microbeads and incubated for 20 min at 6–12°C. After centrifugation, the supernatant was discarded and the cells were resuspended in 1 ml of PBS/0.5% BSA. Bead-bound CD4⁺ T cells were isolated using high-gradient magnetic MiniMACS separa-

1. *Abbreviations used in this paper:* BALF, bronchoalveolar lavage fluid; CD40^{-/-}, CD40-deficient; IL-4^{-/-}, IL-4-deficient; IL-5^{-/-}, IL-5-deficient; OVA, ovalbumin.

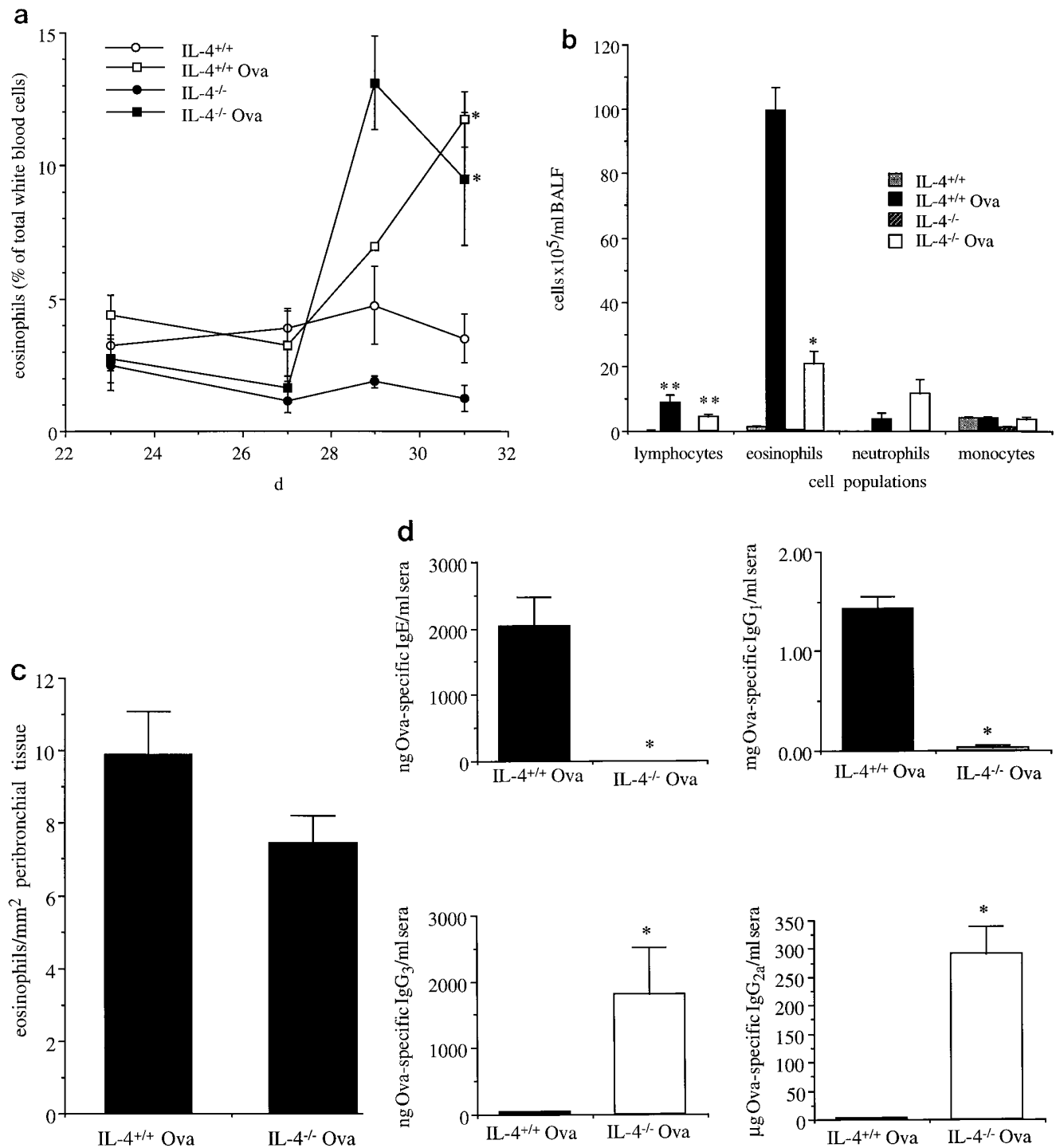


Figure 1. Characterization of inflammatory cells and OVA-specific immunoglobulins in IL-4^{+/+} and IL-4^{-/-} mice. (a) Eosinophil numbers in blood, (b) inflammatory cell numbers in BALF, (c) eosinophil numbers in peribronchial tissue, and (d) serum OVA-specific IgE and IgG isotype levels in nonsensitized or aeroallergen-challenged sensitized IL-4^{+/+} and IL-4^{-/-} mice. Serum titers for OVA-specific immunoglobulins were determined by ELISA (see Methods). Leukocytes in the blood and BALF were identified by morphological criteria and quantified as described previously (25). Eosinophil numbers were quantified in peribronchial regions by counting 10 fields of view (per lung section) immediately below (within 2 mm) the respiratory epithelium. Eosinophil levels in the blood were determined from days 23–31 as indicated. All other data were obtained at day 31. Data represent the mean \pm SEM for groups of four to six mice. The significance of differences between experimental groups was analyzed using the unpaired Student's *t* test. Differences were considered significant if $P < 0.05$. (a) $*P < 0.01$ compared with IL-4^{+/+} or IL-4^{-/-}, (b) $*P < 0.01$ compared with IL-4^{+/+} OVA, (c) no significant difference between IL-4^{+/+} OVA and IL-4^{-/-} OVA, and (d) $*P < 0.001$ compared with IL-4^{+/+} OVA and $**P < 0.001$ compared with IL-4^{+/+} or IL-4^{-/-}.

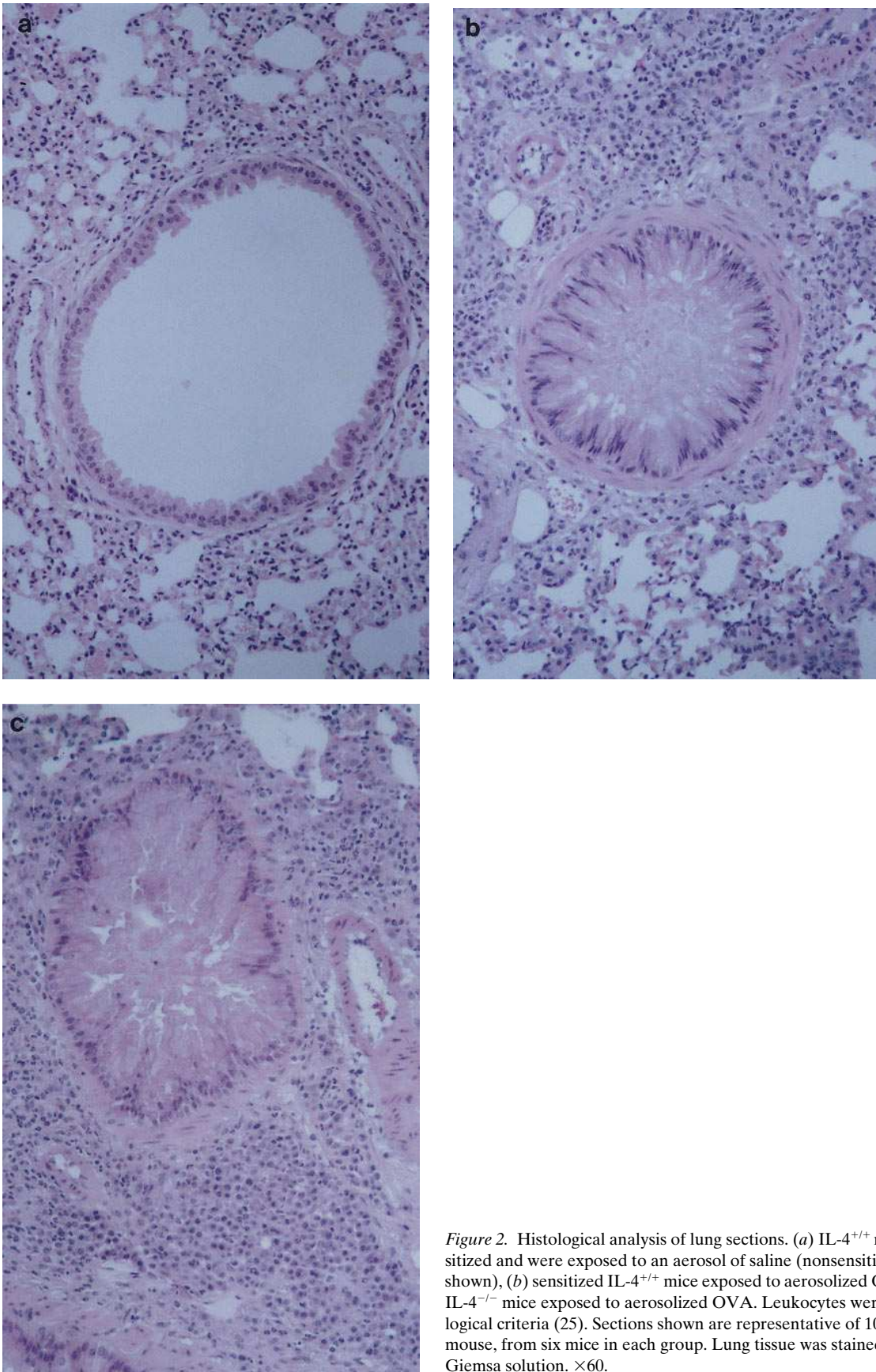


Figure 2. Histological analysis of lung sections. (a) IL-4^{+/+} mice that were not sensitized and were exposed to an aerosol of saline (nonsensitized IL-4^{-/-} mice not shown), (b) sensitized IL-4^{+/+} mice exposed to aerosolized OVA, and (c) sensitized IL-4^{-/-} mice exposed to aerosolized OVA. Leukocytes were identified by morphological criteria (25). Sections shown are representative of 10 sections of lung per mouse, from six mice in each group. Lung tissue was stained with May-Grunwald-Giemsa solution. $\times 60$.

tion columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), washed, and resuspended in MLC/10% FCS. The purity of the CD4⁺ T cells obtained by this method was consistently 90–95% by FACScan[®] analysis.

Culture conditions. To induce cytokine production, CD4⁺ T cells were resuspended in MLC/10% FCS (~10⁶ cells/well) and stimulated for 72 h with immobilized anti-CD3 mAb in flat-bottom, 96-well plates (100 μl/well). Cell-free culture supernatants were collected and stored in aliquots at –70°C until analysis.

Cytokine assays. IFN γ and IL-4 concentrations were determined by ELISA. Briefly, round-bottom immunoassay plates were coated with rat anti-mouse IFN γ mAb (5 μg/ml, RA-6A2) and rat anti-mouse IL-4 mAb (5 μg/ml, 11B11). Plates were blocked with 5% FCS/PBS for 1 h at 37°C and incubated with serial dilutions of cultured supernatants or standard murine IFN γ or IL-4. Murine IFN γ and IL-4 were detected with a rabbit anti-mouse IFN γ antiserum and biotinylated rat anti-mouse IL-4 (5 μg/ml), respectively. For the IFN γ ELISA, plates were incubated with peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at 37°C and washed, and ABTS substrate solution (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was added. For the murine IL-4 ELISA, plates were incubated with streptavidin-conjugated alkaline phosphatase (Amersham International plc, Buckinghamshire, United Kingdom) for 1 h at 37°C and washed, and alkaline phosphatase substrate solution (Sigma Chemical Co., St. Louis, MO) was added. Reactions were stopped with 1 M citric acid. Plates were read by a Microplate reader (Bio-Tek Instruments Inc., Winooski, VT) at 490 nm with reference to 405 nm. The sensitivity of the ELISA system was 0.5 ng/ml of IFN γ and 0.5 ng/ml for IL-4. IL-5 concentrations in cultured supernatants were determined by bioassay using the BCL₁ cell line and murine recombinant IL-5 as the standard. The reagents used in cytokine assays were kindly provided by Drs. G. Karupiah, B. Charlton, and I.G. Young (John Curtin School of Medical Research).

Determination of OVA-specific antibody titers by ELISA. Briefly, flat-bottom 96-well microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with OVA (100 μg/100 μl/well in NaHCO₃ buffer, pH 9.6) and incubated overnight at 4°C. Plates were blocked with 3% skim milk powder/PBS for 1 h at 37°C. After incubation with dilutions of serum samples and standard murine IgG₁, IgG_{2a}, IgG₃, and IgE (Sigma Chemical Co.) for 1.5 h at 37°C, OVA-specific Igs were detected with biotinylated goat anti-mouse IgG₁, IgG_{2a}, IgG₃, and IgE (BioSource International, Camarillo, CA). Plates were incubated with streptavidin-conjugated alkaline phosphatase (Amersham International plc) for 1 h at 37°C and alkaline phosphatase substrate solution (Sigma Chemical Co.) was added. Reactions were stopped with 0.1 M citric acid and the plates were read at 405 nm in a Microplate reader (Bio-Tek Instruments Inc.).

Results

Characterization of allergic airways inflammation and airways dysfunction in IL-4^{-/-} mice. The role of IL-4 in the accumulation of inflammatory cells and the development of lung damage and airways hyperreactivity in response to aeroallergen challenge was analyzed in IL-4^{+/+} mice and IL-4^{-/-} mice. Aerosol challenge of mice with OVA induced a significant increase in eosinophils in the blood (Fig. 1 a) and BALF (Fig. 1 b), in comparison with mice given aerosolized saline (Fig. 1). Lymphocytes, but not neutrophils or monocytes, were also elevated in BALF after aeroallergen challenge (Fig. 1 b). OVA aerosolization of sensitized IL-4^{-/-} mice resulted in reduced levels (approximately sixfold) of eosinophils in the BALF, while blood and peribronchial numbers of eosinophils (Fig. 1 c), and BALF lymphocyte numbers, were comparable with that observed in IL-4^{+/+} mice (Fig. 1, a–c). OVA-specific IgE

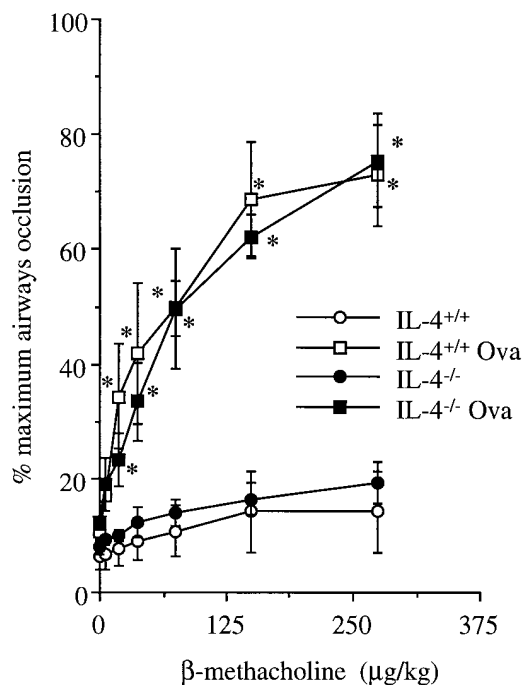


Figure 3. Measurement of airways hyperreactivity to β -methacholine in nonsensitized and aeroallergen challenged (OVA), sensitized IL-4^{+/+} and IL-4^{-/-} mice. Airways constriction was measured by determining changes in respiratory overflow volume during cumulative intravenous administration of β -methacholine and is represented as a percentage of the maximal overflow volume (100%) obtained by totally occluding the tracheal cannula. Data represent the mean \pm SEM for groups of four to six mice. The significance of differences between experimental groups was analyzed using the unpaired Student's *t* test. Differences were considered significant if *P* < 0.05. **P* < 0.001 compared with IL-4^{+/+} or IL-4^{-/-}. Airways hyperreactivity was measured on day 31.

and IgG₁ were detected in the sera from IL-4^{+/+} mice, but not in sera from IL-4^{-/-} mice after aeroallergen challenge (Fig. 1 d). However, sensitized IL-4^{-/-} mice produced IgG₃ and IgG_{2a} (Fig. 1 d).

In both IL-4^{+/+} mice and IL-4^{-/-} mice given OVA, eosinophilic inflammation was widespread, with dense cellular peribronchial and perivascular infiltration (Fig. 2, b and c). Lung tissue after aeroallergen challenge was characterized by gross alterations in the structural integrity of the airway walls (bronchi and bronchioli), epithelial cell shedding, microvascular leakage (results not shown), and extensive mucosal edema. Increased tissue cellularity and particulate exudates were also observed in the airways lumina and alveolar septa (Fig. 2, b and c). Furthermore, aeroallergen-induced inflammation and airway damage were directly associated with the induction of airways hyperreactivity to β -methacholine in both IL-4^{+/+} mice and IL-4^{-/-} mice (Fig. 3).

The role of IL-5 in regulating allergic airways inflammation and airways dysfunction in IL-4^{-/-} mice. Many inflammatory cells and mediators have the potential to augment the inflammatory response and induce airways hyperreactivity. To determine the contribution of IL-5 and eosinophilic inflammation to the induction of lung damage and airways hyperreactivity in IL-4^{-/-} mice, animals were pretreated with anti-IL-5 mAb before aeroallergen challenge. In marked contrast to the results

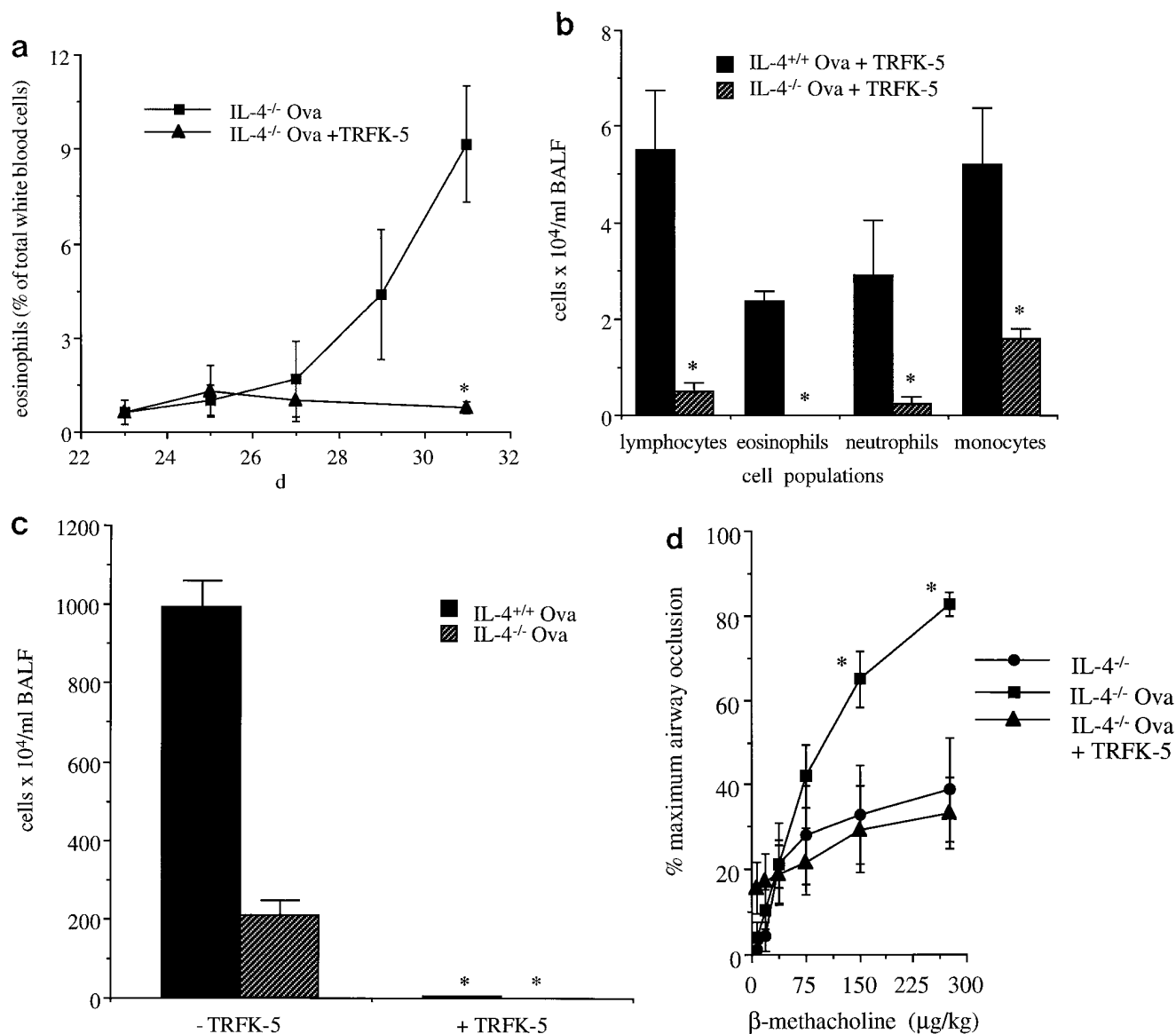


Figure 4. Effect of anti-IL-5 mAb on (a) eosinophil numbers in the blood of IL-4^{-/-} mice, (b) inflammatory cells in BALF of IL-4^{+/+} and IL-4^{-/-} mice, (c) eosinophil numbers in the BALF of IL-4^{+/+} OVA and IL-4^{-/-} OVA mice, (d) airways hyperreactivity to β-methacholine in IL-4^{-/-} mice, and (e) lung section of sensitized IL-4^{-/-} mouse exposed to aerosolized OVA after pretreatment with anti-IL-5 mAb. Corresponding data for cellular infiltrates in all mice in the absence of anti-IL-5 mAb are shown in Fig 1 b. Mice were sensitized (as described in Methods) and injected intraperitoneally with anti-IL-5 mAb (2 mg/mouse, days 23 and 27) or isotype control antibody (2 mg bGL113/mouse) (results not shown) in sterile 0.9% saline. On day 24, the appropriate groups of mice were aerosolized with OVA. Lung section shown ×300. Eosinophil levels in the blood were determined from days 23–31 as indicated. All other data were obtained at day 31. Data represent the mean ± SEM for groups of six mice. The significance of differences between experimental groups was analyzed using the unpaired Student's *t* test. Differences were considered significant if *P* < 0.05. (a) **P* < 0.001 compared with IL-4^{-/-} OVA, (b) **P* < 0.001 compared with IL-4^{+/+} OVA + TRFK-5, (c) **P* < 0.001 compared with IL-4^{+/+} OVA and IL-4^{-/-} OVA in the absence of TRFK-5, and (d) **P* < 0.001 compared with IL-4^{-/-} or IL-4^{-/-} OVA + TRFK-5.

with IL-4^{-/-} mice, OVA aerosolization of mice pretreated with IL-5 mAb induced no blood or airways eosinophilia (Fig. 4, a–c), no airways hyperreactivity to β-methacholine (Fig. 4 d), and minimal morphological changes in airways structure (Fig. 4 e). Lung histology and eosinophil numbers in the blood and BALF of these animals resembled those of mice that were not actively sensitized (Figs. 1 and 2 a). Lymphocyte numbers were also significantly reduced in the BALF by anti-IL-5 mAb treatment (Fig. 4 b). Eosinophilic inflammation was also signifi-

cantly attenuated in IL-4^{+/+} mice treated with anti-IL-5 mAb (Fig. 4 c) and this effect directly correlated in a reduction of cellular infiltrates into the lung (Fig. 4 b, corresponding data for cellular infiltrates in all mice in the absence of anti-IL-5 mAb are shown in Fig. 1 b) and the abolition of airways hyperreactivity (results not shown). Administration of isotype control antibody did not result in inhibition of the inflammatory response (result not shown). Depletion of CD4⁺ T cells by pretreatment of mice with GK1.5 mAb before OVA aerosoliza-

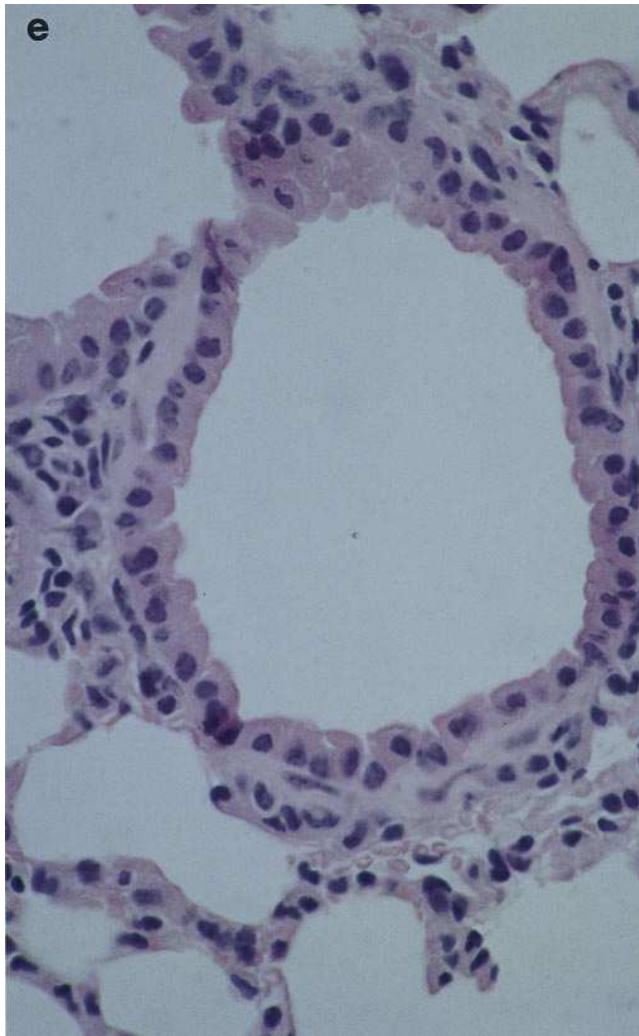


Figure 4 (Continued)

tion completely abrogated the development of hyperreactivity and morphological changes to the airways and the production of Th2-type cytokines (results not shown).

Characterization of cytokines produced by CD4⁺ T cells in IL-4^{-/-} mice. Cytokine profiles of CD4⁺ T cells were determined to identify a cellular source of IL-5. The production of IL-4, IL-5, and IFN γ by CD4⁺ T cells isolated from spleens was measured 72 h after stimulation with anti-CD3 (Table I). Cells from IL-4^{+/+} mice aerosolized with OVA produced increased levels of all cytokines in comparison with nonsensitized controls. CD4⁺ T cells from IL-4^{-/-} mice produced high levels of IFN γ , but no detectable levels of IL-5. Increased production of IFN γ by CD4⁺ T cells from IL-4^{-/-} mice has been reported previously and may reflect the role of this cytokine in downregulating IFN γ production (18). After sensitization and aeroallergen challenge, CD4⁺ T cells from IL-4^{-/-} mice produced significant amounts of IL-5 (albeit less than cells from IL-4^{+/+} mice) (Table I).

The role of CD40 in regulating allergic airways inflammation and airways dysfunction. Airways damage and hyperreactivity occurred in the absence of detectable OVA-specific IgE and IgG₁ in IL-4^{-/-} mice. To determine the contribution of other allergen-specific immunoglobulins in the development

Table I. Profiles of Cytokine Production by Anti-CD3-stimulated CD4⁺ T Cells Purified from the Spleen of Control and OVA-immunized IL-4^{+/+} and IL-4^{-/-} Mice

Treatment	Cytokines		
	IL-4	IFN γ	IL-5
	ng/ml	ng/ml	U/ml
IL-4 ^{+/+}	< 0.5	121.7	< 0.1
IL-4 ^{+/+} Ova	6.1	358.4	15.1
IL-4 ^{-/-}	< 0.5	1500.0	< 0.1
IL-4 ^{-/-} Ova	< 0.5	751.0	8.4

Limits of detection for IFN γ ELISA is < 0.05 ng/ml, IL-5 bioassay was 0.1 U/ml, and IL-4 ELISA was < 0.5 ng/ml. Data are shown as the mean of three separate cultures with $n = 4$ mice per group.

of these structural and functional changes to the airways, CD40^{-/-} mice were used in the aeroallergen-induced allergic airways model. CD40^{-/-} mice are specifically affected in T cell-dependent immunoglobulin class switching and germinal center formation (36). No primary or secondary anti-2,4-dinitrophenol (DNP)-conjugated OVA antibody responses, of all immunoglobulin isotypes (IgA, IgE, IgG-[1, 2a, 2b, or 3]) (except for the IgM class after immunization) were mounted against this T cell-dependent antigen in CD40^{-/-} mice (36). Aeroallergen challenge of sensitized CD40^{+/+} mice and CD40^{-/-} mice resulted in a severe inflammatory response characterized by elevated levels of blood and BALF eosinophils (Fig. 5, *a* and *b*). Lymphocyte numbers were also significantly elevated in the BALF (result not shown). In contrast to CD40^{+/+} mice, CD40^{-/-} mice did not produce OVA-specific IgE, IgG isotypes, or IgA (Fig. 5 *c*). Antibodies were detected only at background levels in aeroallergen-challenged sensitized CD40^{-/-} mice. In mice which were not sensitized, OVA-specific antibodies were not detected (results not shown). Airway eosinophilic inflammation directly correlated with the induction of airways hyperreactivity to β -methacholine in both CD40^{+/+} mice and CD40^{-/-} mice (Fig. 6 *a*). Moreover, in the absence of immunoglobulin isotypes, aeroallergen-induced airways hyperreactivity and lung damage were not attenuated (Fig. 6, *a* and *b*).

Discussion

Clinical and experimental observations have identified IL-4 and IL-5 as key molecules in the regulation of the effector function of mast cells and eosinophils which underlies the development of allergic airways inflammation. However, there is conflicting evidence about the comparative importance of these cytokines and cells in the events which initiate the structural and functional changes to the airways in response to aeroallergens (25, 30). In this investigation we have shown in a murine model of allergic airways inflammation that IL-4 and allergen-specific IgE, IgG isotypes, and IgA are not essential for the development of aeroallergen-induced lung damage and airways hyperreactivity. In IL-4^{-/-} mice, the recruitment of eosinophils into the airways, but not to the blood or peribronchial regions, was impaired in response to aeroallergen challenge, supporting previous investigations that this cytokine,

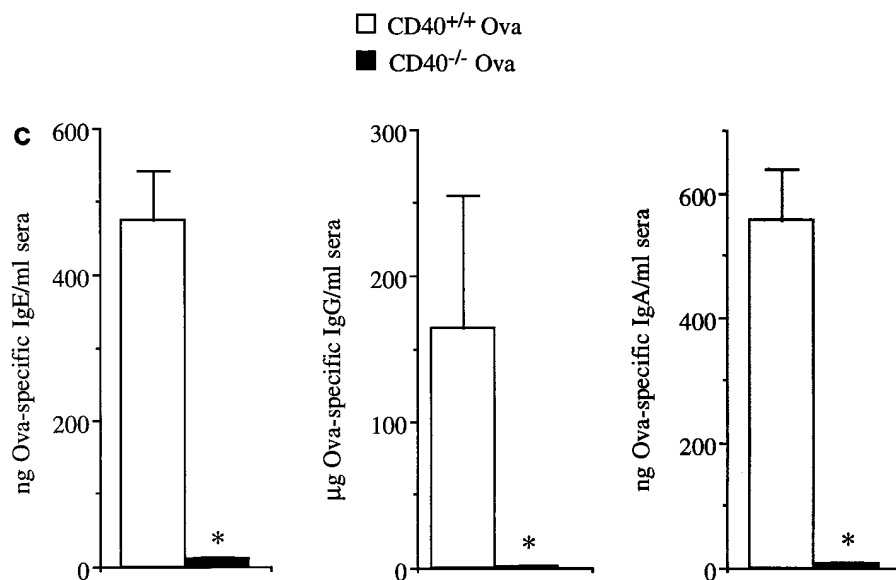
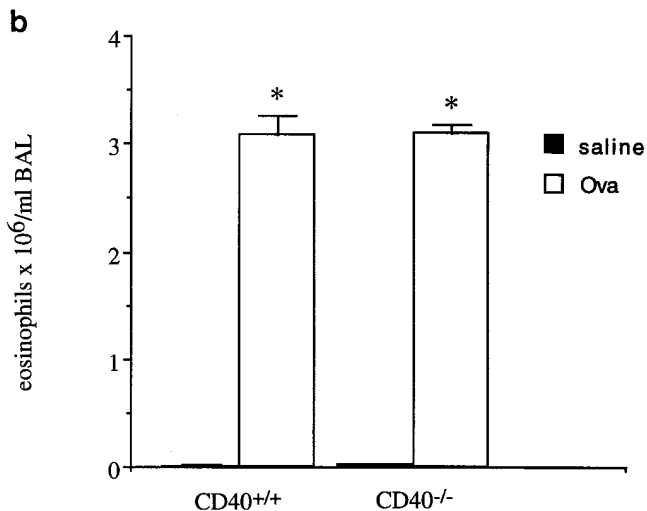
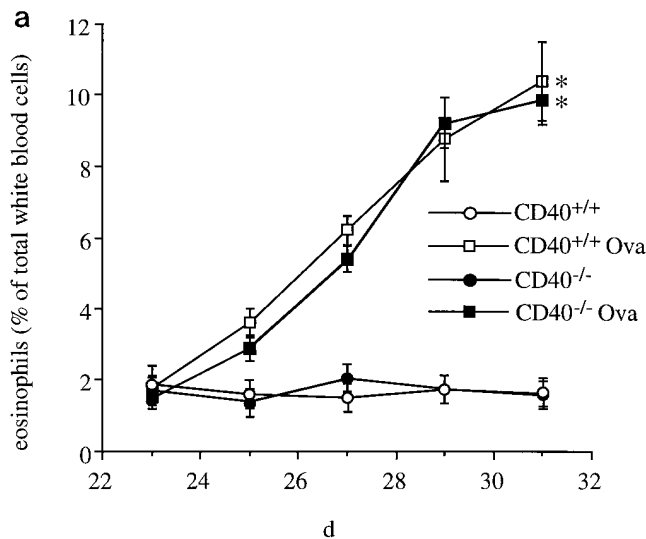


Figure 5. Characterization of inflammatory cells and OVA-specific immunoglobulins from CD40^{+/+} and CD40^{-/-} mice. Eosinophil numbers in (a) blood and (b) BALF, and (c) serum OVA-specific IgE, IgG, and IgA levels in nonsensitized or in aeroallergen-challenged sensitized CD40^{+/+} and CD40^{-/-} mice. Serum titers for OVA-specific immunoglobulins were determined by ELISA (see Methods). Eosinophil levels in the blood were determined from days 23–31 as indicated. All other data were obtained at day 31. Antibodies were detected only at background levels in aeroallergen challenged (OVA) sensitized CD40^{-/-} mice. In the absence of sensitization, antigen-specific antibodies were not detected (results not shown). Data represent the mean \pm SEM for groups of five mice. The significance of differences between experimental groups was analyzed using the unpaired Student's *t* test. Differences were considered significant if $P < 0.05$. (a) $*P < 0.001$ compared with CD40^{+/+} or CD40^{-/-}, (b) $*P < 0.001$ compared with CD40^{+/+} or CD40^{-/-} saline treatment, and (c) $*P < 0.001$ compared with CD40^{+/+} OVA.

IgE, and CD4⁺ Th2-type cells are important for the recruitment of eosinophils to the airways during allergic inflammation (18, 33, 34, 37). Eosinophil recruitment to the airways in response to eotaxin may also be impaired in the absence of IL-4 (20, 27).

However, our findings are in marked contrast to the suggestion that activation of IL-4-dependent pathways is critical for the regulation of allergic airways dysfunction (30). The severity of allergic disease was not attenuated in IL-4^{-/-} mice. Moreover, the characteristic airways damage and hyperreactivity, induced by aeroallergen challenge, were only inhibited in IL-4^{-/-} mice after pretreatment with anti-IL-5 mAb and abolition of blood and airways eosinophilia. These results confirm observations in IL-5^{-/-} mice implicating IL-5 and eosinophilic inflammation as important mediators in the patho-

genesis of allergic airways disease (25). Furthermore, our results (including anti-CD4⁺ mAb depletion data [results not shown]) suggest that IL-4 is not required for the development of CD4⁺ T cells which regulate eosinophilia and the induction of pathological changes in airways tissue and airways hyperreactivity during allergic disease. These results are in contrast to investigations in BALB/c mice using neutralizing mAbs that suggested IL-4, and not IL-5 or eosinophils, was required to generate airways hyperreactivity to inhaled antigens (30). Structural and functional changes to airways in response to aeroallergens are thought to be primarily mediated by mast cells and eosinophils. The relative contribution of these two mechanisms to the induction of allergic airways dysfunction in various strains of mice may account for the differences observed in these studies (38). However, it should be noted that

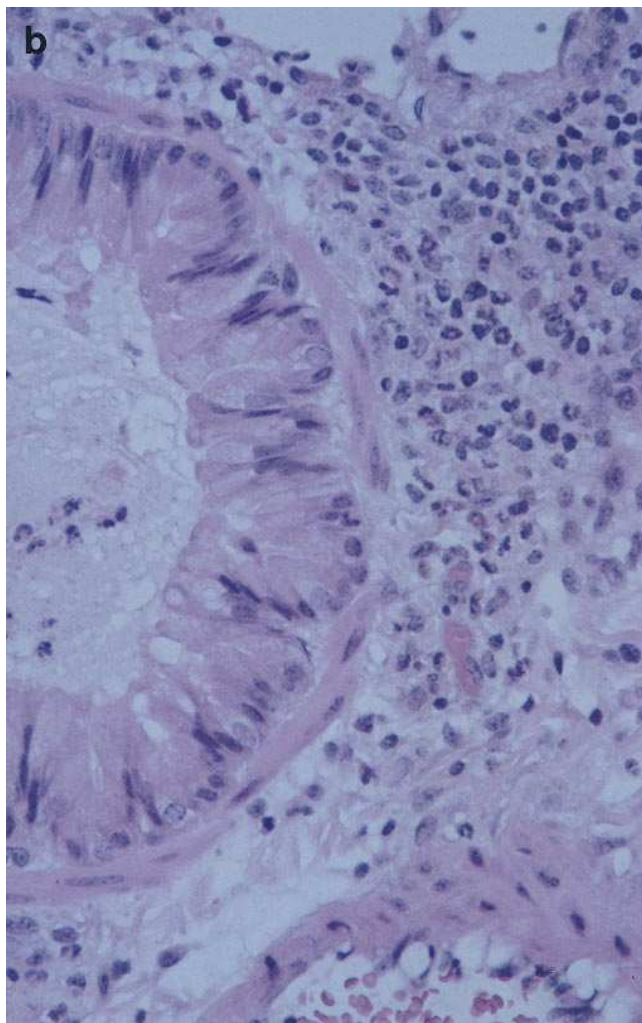
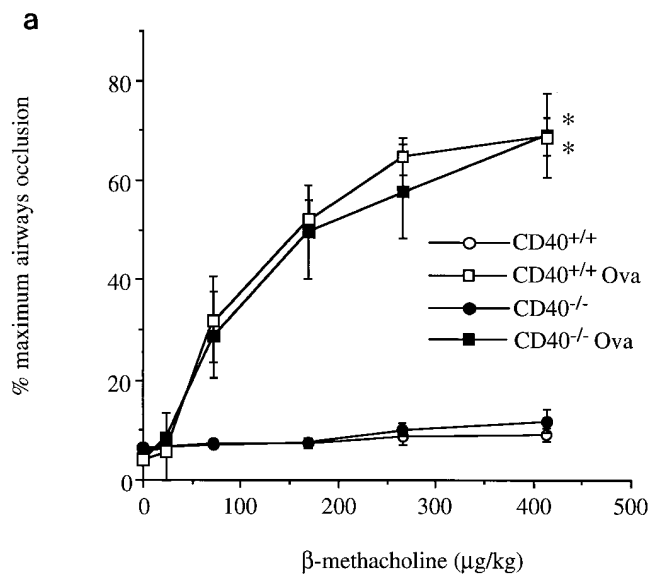


Figure 6. Analysis of (a) airways hyperreactivity to β -methacholine in nonsensitized and aeroallergen challenged (OVA), sensitized CD40^{+/+} and CD40^{-/-} mice and (b) histological sections of OVA aerosolized sensitized CD40^{-/-} mice. Airways constriction was measured by determining changes in respiratory overflow volume during cumulative intravenous administration of β -methacholine and is represented as a percentage of the maximal overflow volume (100%) ob-

like BALB/c mice, the 129Sv \times C57BL/6 cross used in our investigation expressed full-length transcripts of the mast cell protease 7 gene (result not shown). The absence of this protease has been suggested to predispose C57BL/6 mice to resistance of mast cell-mediated airways hyperreactivity (38, 39). In any event, aeroallergen-induced airways dysfunction can occur independently of IL-4.

Recently, IgE has been proposed to mediate IL-5 and eosinophil-dependent airways hyperreactivity and to regulate the recruitment of eosinophils to the airways in response to inhaled allergens (30, 33). The precise mechanisms underlying IgE regulation of these processes are unknown. IgE-CD23 complexes on B cells have been proposed to regulate antigen-stimulated cytokine production from CD4⁺ Th2-type cells (33). Current *in vitro* investigations also suggest that allergen-specific IgE, IgG1, IgG3 (and under some conditions, IgG2), and IgA (including secretory IgA) may trigger the release of preformed inflammatory mediators from eosinophils, which may induce airways damage and hyperreactivity (7, 8, 12). In response to sensitization and aeroallergen, CD40^{-/-} mice did not produce detectable levels of OVA-specific IgE, IgG isotypes, and IgA; however, eosinophilic inflammation, pathological changes to airways tissue, and airways hyperreactivity were not impaired. Thus, no obligatory role was found for allergen-specific immunoglobulins in regulating eosinophilic inflammation and allergic airways disease.

Aeroallergen-induced pathological changes and hyperreactivity to the airways in this model of allergic inflammation do not develop in SCID mice, in animals depleted of CD4⁺ T cells, or in mice deficient in MHC class II, indicating an essential requirement for sensitization and a functional immune system (29, 30, 32, 34, 40, 41). In asthma, while it is clear that allergen-specific immunoglobulins are important regulatory molecules of allergic responses, our results suggest that other factors produced during the inflammatory response can also induce airways damage and hyperreactivity. Furthermore, generation of these factors is intimately associated with IL-5 signaling pathways and airways eosinophilic inflammation. Recently, inhibition of leukotriene B₄ function was shown to inhibit eosinophil-derived pathology associated with a murine model of experimental allergic encephalomyelitis (42). Lipid mediators also activate eosinophils *in vitro* (41). Eosinophils may also act as antigen processing cells and activate CD4⁺ T cells, after cytokine priming at the site of inflammation (43, 44). Thus, lipid mediators and costimulatory signals derived from interactions between eosinophils and T cells may also

tained by totally occluding the tracheal cannula. Airways data represent the mean \pm SEM for groups of six mice. The significance of differences between experimental groups was analyzed using the unpaired Student's *t* test. Differences were considered significant if $P < 0.05$. (a) * $P < 0.001$ compared with CD40^{+/+} or CD40^{-/-}. Histological analysis of lung sections from sensitized CD40^{-/-} mice exposed to aerosolized OVA showed similar morphological features to those observed in CD40^{+/+} mice given OVA. Lung sections from mice that were not sensitized and were exposed to an aerosol of saline did not show morphological changes (see Fig. 2 a). Sections shown are representative of 10 sections of lung per mouse, from six mice in each group. Lung tissue was stained with May-Grunwald-Giemsa solution. Section shown $\times 141$. Histology and airways hyperreactivity were characterized on day 31.

provide an important mechanism for the induction of aeroallergen-induced airways disease.

In asthma, airways T lymphocytes are activated and are predominantly of the CD4⁺ subclass expressing cytokine mRNA profiles resembling murine Th2 cell clones or allergen-specific human T cell clones (14, 45). These cells are thought to be the primary source of cytokines which mediate allergic inflammatory responses. Depletion of CD4⁺ Th2-type cells also inhibits antigen-induced eosinophilia and airways disease in animal models of allergic inflammation (30, 40). IL-4^{-/-} mice have significantly impaired CD4⁺ Th2-type cell responses and do not develop serum IgE responses to nematode infestation (18). However, they can produce IL-5 (albeit at lower levels) in response to nematode infestation and after sensitization and aerosolized with OVA (18, 37), which may be derived from CD4⁺ T cells (nonclassical Th2-type), Th0 cells, or non-CD4⁺ cells (18). Results in this investigation clearly indicate that IL-5-producing CD4⁺ T cells can develop independently of IL-4 and secrete IL-5 when activated through the CD3-TCR complex. Furthermore, this pool of IL-5 is sufficient to provide the signal for the initiation and maintenance of aeroallergen-induced eosinophilic inflammation and the onset of airways disease. Eosinophilic inflammation has also been observed in models of parasite infestation and malaria when IL-4 was absent during the initiation of the immune response (24, 46). Thus, evidence is accumulating that CD4⁺ T cell subsets exist which do not require IL-4 for dedication into a phenotype that produces IL-5 and regulates eosinophilia. The pathway that activates aeroallergen-induced pulmonary eosinophilic inflammation in an IL-4-independent manner may function to supplement responses by classical CD4⁺ Th2-type cells.

There is increasing interest in the discordant expression of IL-4 and IL-5 by T lymphocytes in various diseases and the lineages of these cells (47, 48). Interestingly, intrinsic asthmatics show no correlation between disease and IgE production (1). Furthermore, respiratory secretions from these individuals are characterized by increased levels of IL-5 (but not IL-4) and activated CD4⁺ T cells (1, 49). Characterization of the phenotype of CD4⁺ T cells producing IL-5 in IL-4^{-/-} mice and the factors involved in their selection and activation may provide important insights into the etiology of intrinsic asthma. It will be of particular interest to determine if these CD4⁺ T cells can also produce IL-4 and provide help for IgE production.

The elemental signals derived from the site of inflammation which are critical for the onset of allergic disease are thought to be primarily regulated by IL-4, IL-5, and allergen-specific immunoglobulins. IL-4 regulates the development of CD4⁺ Th2-type cells, which elicit essential signals through IL-4 and IL-5 for the regulation of IgE production and eosinophilia, respectively. Thus, IL-4 is a key regulatory molecule of allergic disease. However, in this investigation we have shown that there is no obligatory role for IL-4 or allergen-specific IgE, IgG isotypes, or IgA in the development of aeroallergen-induced lung damage and airways hyperreactivity. In this model, allergic airways disease was dependent on IL-5 and the development of pulmonary eosinophilic inflammation. Furthermore, IL-4 was not required during antigen priming of T cells for the subsequent development of CD4⁺ T cells producing IL-5. Thus, two mechanisms exist for the immune system to regulate IL-5 production and eosinophilia in response to inhaled antigens, one involving IL-4-dependent T cells and the

other IL-4-independent T cells. IgE is a critical mediator of immune responses mediated by mast cells. This antibody has also been implicated in regulating aeroallergen-induced eosinophilic inflammation and the induction of airways disease associated with this leukocyte. However, no obligatory role was found for IgE in the regulation of airways hyperreactivity or eosinophil trafficking to the lung in response to inhaled antigen. Our results suggest that factors other than antigen-antibody complexes (IgE, IgG, and IgA) can also regulate the development of airways damage and hyperreactivity during allergic disease. The generation of these factors is dependent on an activated immune system and is intimately associated with signals elicited by IL-5 and the influx of eosinophils to the site of inflammation.

In conclusion, two pathways have been proposed to regulate allergic airways disease in asthma which centers on the effector function of mast cells and eosinophils. This investigation indicates that airways eosinophilia, extensive airways damage, and airways hyperreactivity can occur independently of IL-4 and allergen-specific immunoglobulins. While IL-4 and IgE play important roles in the regulation of allergic disease, IL-5-regulated eosinophilia and factors coupled to this pathway are also central to the development of the pathophysiology of allergic airways inflammation.

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