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Soluble IL-4 Receptor Inhibits Airway Inflammation Following Allergen Challenge in a Mouse Model of Asthma[1]

William R. Henderson, Jr.,[2]* Emil Y. Chi,[†] and Charles R. Maliszewski[‡]

In vitro and in vivo studies, in both animal models and human asthmatics, have implicated IL-4 as an important inflammatory mediator in asthma. In a murine asthma model, we examined the anti-inflammatory activities of soluble IL-4R (sIL-4R). In this model, mice sensitized to OVA by i.p. and intranasal (i.n.) routes are challenged with the allergen by i.n. administration. The OVA challenge elicits an eosinophil infiltration into the lungs, with widespread mucus occlusion of the airways, and results in bronchial hyperreactivity. sIL-4R (0.1–100 μg) was administered by either i.n. or i.p. routes before OVA challenge in OVA-sensitized mice. Both blood and bronchoalveolar lavage fluid levels of sIL-4R were significantly elevated compared with controls by i.n. delivery of 100 μg sIL-4R; i.p. delivery of 100 μg sIL-4R only raised blood levels of sIL-4R. The i.n. administration of 100 μg sIL-4R before allergen challenge significantly reduced late phase pulmonary inflammation, blocking airway eosinophil infiltration, VCAM-1 expression, and mucus hypersecretion. In contrast, i.p. delivery of 100 μg sIL-4R inhibited only the influx of eosinophils into the lungs, but not airway mucus release. Furthermore, sIL-4R treatment by either i.n. or i.p. routes did not reduce airway hyperreactivity in response to methacholine challenge. Thus, elevating airway levels of sIL-4R through the administration of exogenous sIL-4R is effective in blocking the late phase pulmonary inflammation that occurs in this murine allergen-challenge asthma model. These results suggest that sIL-4R may have beneficial anti-inflammatory effects in asthmatic patients. *The Journal of Immunology,* **2000, 164: 1086–1095.**

Asthma is characterized by a complex inflammatory response of pulmonary eosinophilia, edema, mucus hypersecretion, bronchial epithelial injury, and airway hyperreactivity (AHR).[3] Inhaled allergen challenge provokes an immediate airway hypersensitivity reaction, an early airway response, that is frequently followed several hours later by a delayed airway reaction, a late phase airway response (LAR). During the LAR, an influx of eosinophils, lymphocytes, and macrophages throughout the airway wall and into the bronchial fluid occurs. After recovery from the LAR, there is an increase in acquired AHR to agents such as methacholine that may persist for several days. CD4^{+} Th and CD8^{+} T cytotoxic (Tc) appear essential for the chronic inflammation of asthma (1). These lymphocytes infiltrate the airways of asthmatic subjects, with increased numbers recruited to the lungs by Ag challenge (1). CD4^{+} and CD8^{+} T cells in asthmatic patients exhibit a type 2 cytokine phenotype (Th2 and Tc2, respectively) with secretion of IL-4, IL-5, and IL-13 (1–6).

IL-4, a 20-kDa glycoprotein, has numerous biologic activities relevant to the mediation of allergic inflammation (7). IL-4 stimulates proliferation of mast cells, anti-Ig-activated B cells, and T cells. IL-4 also induces Ia and FcϵRII expression on monocytes and lymphocytes, and class switching/secretion of IgE and IgG1 by B cells. Sib-pair analysis of individuals from Amish families provides evidence for linkage of the IL-4 gene in 5q31.1 with a gene controlling total serum IgE concentrations (8). In bronchial biopsies from both atopic and nonatopic asthmatics compared with nonasthmatic controls, an increase in the number of cells expressing IL-4 and IL-5 mRNA and protein is observed (9). In these asthmatic lung tissues, the majority of the IL-4 and IL-5 mRNA is expressed in CD3^{+} T cells (CD4^{+} > CD8^{+} T cells), with the remainder expressed in mast cells and eosinophils (4). By immunocytochemistry, most of the IL-4 and IL-5 protein colocalizes with mast cells and eosinophils in the bronchial tissue from the atopic and nonatopic asthmatics (4).

The biologic actions of IL-4 are mediated by its binding to its receptor, IL-4R (molecular mass, 139 kDa in the mouse), expressed on diverse cells. IL-4R is composed of IL-4Rα and γc subunits or IL-4Rα and IL-13Rα subunits (10–14). Bronchial biopsies from atopic asthmatics compared with atopic control subjects exhibit increased expression of IL-4Rα mRNA and protein in the epithelium, subepithelium, and endothelial cell layer (15). IL-4Rα mRNA expression is also demonstrated in CD3^{+} T cells and mast cells in lung tissue from atopic patients with asthma (15). A naturally occurring soluble form of IL-4R (sIL-4R; molecular mass, 39 kDa in the mouse) is secreted and inhibits the biologic actions of IL-4. rIL-4R contains only the extracellular portion of IL-4R and lacks the transmembrane and intracellular domains. In vitro, sIL-4R blocks B cell binding of IL-4, B cell proliferation, and IgE and IgG1 secretion (16). In vivo, sIL-4R inhibits IgE production by up to 85% in anti-IgD-treated mice (17), suggesting that sIL-4R may be useful in the treatment of IgE-mediated inflammatory diseases. A previous study demonstrated that sIL-4R blocked IgE production and AHR when administered at the time of sensitization (18). To test the therapeutic potential of sIL-4R in asthma, we examined the effect of exogenously administered sIL-4R in a murine model of allergen-induced asthma (19, 20).

[3] Abbreviations used in this paper: AHR, airway hyperreactivity; alum, aluminum potassium sulfate; AP, alkaline phosphatase; BAL, bronchoalveolar lavage; C_{dyn}, dynamic compliance; G_L, conductance; H, human; i.n., intranasal; LAR, late airway response; M, mouse; MSA, mouse serum albumin; s, soluble; Tc, T cytotoxic.

Insights into the mechanisms of airway inflammation and hyperreactivity in asthma have come from investigations of the LAR in animal models. Sensitization to a variety of allergens and subsequent airway challenge with the allergen have been shown to produce typical features of the LAR in a number of species including: mouse, rat, guinea pig, and nonhuman primate. We have developed a protocol for administration of OVA as a model allergen to induce late phase allergen-specific pulmonary disease in mice (19–21). Our protocol includes i.p. immunization of mice with OVA in alum adjuvant on days 1 and 14, and single intranasal (i.n.) doses of OVA on days 14, 25, 26, and 27. On day 28, OVA-treated mice display a disease similar to allergen-induced human asthma including: 1) increased levels of total and OVA-specific IgE in the blood, 2) increased release of leukotrienes B_4 and C_4 in BAL fluid, 3) a striking eosinophil influx into BAL fluid and lung tissue, 4) airway mucus hypersecretion, 5) increased expression of Th2 and Tc2 cytokines (IL-4, IL-5, and IL-13) and decreased expression of Th1 cytokines (IL-2 and IFN-γ) in bronchial lymph node tissue, and 6) AHR, as assessed by a significantly greater decrease in airway conductance and dynamic compliance in response to methacholine compared with control mice. Employing this model, we found that sIL-4R treatment before OVA challenge in OVA-immunized mice inhibits mucus hypersecretion and eosinophil influx into the lungs, but not AHR, to methacholine following allergen challenge.

Materials and Methods

Special reagents

Recombinant sIL-4R mouse (M) was prepared as previously described (17). For a negative control protein, a 5 mg sample of sIL-4R(M) (10 mg/ml) in PBS was inactivated by acidification to pH 3 in 1 N HCl and heating for 4 h at 100°C. After cooling in ice water to room temperature, the samples were neutralized to pH 6–7 with 2 M NaOH. Inactivation of the sIL-4R(M) sample was confirmed using an in vitro assay that measures inhibition of IL-4-induced B cell proliferation (16). Recombinant sIL-4R human (H), which does not exhibit cross-species binding with mouse IL-4, and mouse serum albumin (MSA; Sigma, St. Louis, MO) were used as additional negative controls.

Animals

Female BALB/c mice (6–8 wk of age; obtained from D&K, Seattle, WA, and The Jackson Laboratory, Bar Harbor, ME) were used. All animal study protocols were approved by the University of Washington Animal Care Committee.

Allergen induction of eosinophil infiltration

To induce eosinophil infiltration into murine airway tissue, mice were sensitized and later challenged with OVA (Pierce, Rockford, IL) as the allergen. Mice were immunized with OVA (100 μg) complexed with aluminum potassium sulfate (alum) in a 0.2 ml vol, administered by i.p. injection on days 0 and 14, as previously described (19). On days 14, 25, 26, and 27, mice were anesthetized with 0.2–0.3 ml of ketamine (6.5 mg/ml) and xylazine (0.44 mg/ml) diluted in normal saline. The OVA and sIL-4R/OVA groups all received 100 μg OVA in 0.05 ml normal saline by the i.n. route on day 14, and 50 μg of OVA in 0.05 ml normal saline by the i.n. route on days 25, 26, and 27. The control group received normal saline with alum by the i.p. route on days 0 and 14, and 0.05 ml of normal saline by the i.n. route on days 14, 25, 26, and 27.

Administration of sIL-4R

The sIL-4R/OVA group received a sIL-4R dosage of 0.1–100 μg/mouse/day. The sIL-4R dose was administered by either i.n. or i.p. injection 30 min before challenge with OVA on day 25. Prior data indicate that i.n. administration of small particles (i.e., 1% solution of carbon particles, 0.027–0.050 μm diameter) in mice results in the uniform distribution of the particles throughout the upper and lower lungs reaching to the alveoli (E. Y. C., unpublished observations). Previous pharmacokinetic studies indicated that the plasma $t_{1/2}$ of sIL-4R in the mouse is 4.6 h after i.p. administration (22). Therefore, to increase sIL-4R levels above endogenous levels during the course of the allergen challenge, mice received additional sIL-4R dosages on days 26 and 27, by the i.n. or i.p. route, 30 min before OVA treatment.

Pulmonary function testing

On day 28, ~24 h following the last i.n. administration of either normal saline or OVA, pulmonary mechanics in response to an i.v. infusion of methacholine were evaluated in the mice by a plethysmographic method (19), modified from previously described methods (23, 24). After pentobarbital anesthesia (70–90 mg/kg by the i.p. route), the jugular vein was cannulated, the trachea was intubated and connected to a Harvard ventilator, and the thorax was opened by a thoracotomy, as previously described (19). The animal was placed in a supine position in one compartment (0.25 ml dead space) of a two-chamber whole body plethysmograph. The following minute ventilation maintained normal arterial blood gases: tidal volume = 0.2 ml/20 g, frequency 120 breaths/min, and positive end expiratory pressure of 2.5–3 cm H_2O (25). Copper mesh in the plethysmograph served as a heat sink for rapid gas compression during each tidal breath. Because the plethysmograph is a closed system, the change in box pressure (P_{box}) of the first chamber (measured by a sensitive transducer (± 0.7 cm H_2O)) represented the change in lung volume ($\Delta vol = P_{box}$) of the mouse; P_{box} is equivalent to lung pleural pressure. Ambient pressure swings and temperature increases in the first chamber were offset as previously described (19). Pressure movements at the opening of the tracheal tube (P_{aw}) were measured by another transducer, referenced to P_{box} to determine transpulmonary pressure ($P_{tp} = P_{aw} - P_{box}$). After initial inflation to a P_{aw} of 30–35 cm H_2O, the lungs were inflated at least once 1–2 min before each measurement to prevent partial collapse. An analogue-to-digital data acquisition system (Strawberry Tree) was employed to sample P_{tp} and P_{box} at 5-ms intervals, with a smoothing function employed to dampen background noise, as previously described (19). The change in volume from P_{box}:point-to-point/5 ms was used to calculate flow.

Data from seven consecutive breaths collected three times during the first 10 min were used to calculate basal pulmonary function. Methacholine was then infused by hand delivery into the jugular vein over 10 s at a concentration of 120 μg/kg, after 10 min of ventilation. Resistance (R), lung conductance ($G_L = 1/R$), and dynamic compliance (C_{dyn}) determined for both the control period and during the peak response to methacholine challenge. R was calculated as the difference in P_{tp} and airflow at midtidal volume on inflation and deflation. Tracheal tube resistance (0.63 cm $H_2O \times ml^{-1} \times s$) was subtracted from all airway resistance measurements. C_{dyn} was calculated as the change in tidal volume (Vt) divided by the difference between P_{tp} at end inspiration and end expiration when flow is zero ($C_{dyn} = \Delta Vt/\Delta P_{tp}$). At the completion of pulmonary function testing, each mouse was exsanguinated by cardiac puncture; plasma samples were collected from all mice in each experimental group and stored at -70°C until assay of sIL-4R.

Bronchoalveolar lavage

After tying off the left lung at the mainstem bronchus, the right lung was lavaged three times with 0.4 ml of normal saline. The three BAL fluid samples collected from each animal were pooled, and the number of cells in a 0.05-ml aliquot was determined using a hemocytometer. The remaining sample was centrifuged at 4°C for 10 min at 200 $\times$ g, and the supernatant was stored at -70°C until assay of sIL-4R levels. The cell pellet was resuspended in normal saline containing 10% BSA, and BAL fluid cell smears were made on glass slides. To stain eosinophils, dried slides were stained with Discombe's diluting fluid (0.05% aqueous eosin and 5% (v/v) acetone in distilled water) for 5–8 min, rinsed with water for 0.5 min, and counterstained with 0.07% methylene blue for 2 min.

Lung histology

The trachea and left lung (upper and lower lobes) were collected and fixed in Carnoy's solution at 20°C for ~15 h. After embedding in paraffin, the tissues were cut into 5-μm sections. For each mouse, 10 airway sections randomly distributed throughout the left lung were assessed for the severity of the cellular inflammatory response and mucus occlusion by morphometric analysis (19), by individuals blinded to the protocol design. The intensity of the cellular infiltration around pulmonary blood vessels and airways was assessed on a semiquantitative scale ranging from 0–4$^+$.

Eosinophils were stained in the lung tissue with Discombe's Solution, as described above. The number of eosinophils per unit airway area (2200 μm) (2) was determined by morphometric analysis, as previously described (19, 26). Airway mucus (i.e., mucin and sulfated mucosubstances) was identified by the following staining methods: methylene blue, hematoxylin/eosin, and Alcian blue, as previously described (19). Occlusion of the airway diameter by mucus was assessed on a semiquantitative scale ranging from 0–4$^+$ (19). Each airway section was assigned a score for airway

FIGURE 1. Blood and BAL fluid levels of sIL-4R after i.n. and i.p. administration of sIL-4R. A total of 0.1–100 μg of sIL-4R(M) (sIL-4R(M)/OVA) was administered i.n. or i.p. 30 min before OVA on days 25, 26, and 27, and blood and BAL fluid were obtained 24 h later on day 28 for assay of sIL-4R(M), as described in *Materials and Methods*. As controls, 100 μg of heat-inactivated sIL-4R(M) (inactive sIL-4R(M)/OVA), sIL-4R(H) (sIL-4R(H)/OVA), or MSA (MSA/OVA) was delivered i.n. or 100 μg of sIL-4R(H) was given i.p. on days 25, 26, and 27 in place of sIL-4R(M) before OVA challenge. Values between experimental groups were compared using a Student's two-tailed *t* test. *p* value, i.n. or i.p. sIL-4R(M)/OVA vs OVA.
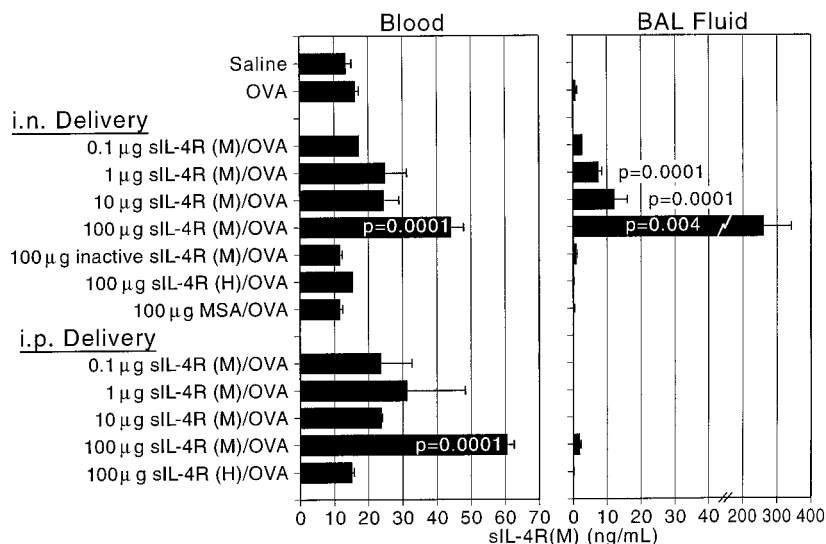
diameter occlusion by mucus based on the following criteria: 0, 0–10% occlusion; 1, 10–30% occlusion; 2, 30–60% occlusion; 3, 60–90% occlusion; 4, 90–100% occlusion (19).

Immunocytochemistry

Paraffin sections of lung tissue were deparaffinized, hydrated, and washed in PBS. To localize VCAM-1, the sections were incubated at room temperature for 30 min with rat anti-mouse VCAM-1 mAb (PharMingen, San Diego, CA) diluted 1/40 in PBS. Control sections were treated with PBS and rat IgG. After rinsing in PBS, the sections were incubated for 30 min with goat anti-rat IgG (Vector Laboratories, Burlingame, CA). After rinsing in PBS, the sections were incubated for 60 min with alkaline phosphatase (AP) solution of Vectastain ABC-AP standard kit (Vector Laboratories) in PBS. The sections were rinsed in PBS and incubated for 30 min with the AP solution of Vector Red-AP substrate kit in Tris-HCl buffer, pH 8.2, containing 1% levamisol (Vector Laboratories). The AP reaction products stain pink. The sections were rinsed in distilled water, counterstained with 0.4% methylene blue in 70% ethanol and 0.01% NaOH, dehydrated in a series of ethanol concentrations up to 100%, cleared in xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Assay of sIL-4R levels in plasma and BAL fluid

Levels of sIL-4R(M) in the plasma and BAL fluid were determined by a capture ELISA protocol based on two anti-IL-4R mAbs, M1 and M2, as previously described (27).

Specific Ab levels

Total and OVA-specific IgE levels in the blood were determined by ELISA. For the total IgE ELISA, the plates were coated with 100 μl of 1 μg/ml rat anti-mouse IgE (Serotec, Kidlington, Oxford, U.K.) in PBS overnight at 4°C. The plates were washed six times with PBS/Tween and blocked with 150 μl of 10% FBS in PBS for 1 h at room temperature. The plates were washed again six times with PBS. Samples were titrated in 10% FBS in PBS with dilutions beginning at 1/20 and standards started at 1 μg/ml; the final volume per well was 100 μl. After washing six times with PBS/Tween, the plates were incubated with rat anti-mouse IgE heavy chain biotin Ab (100 μl/well; Serotec) diluted 1/5000 in 50% goat sera (Life Technologies, Gaithersburg, MD) in PBS for 1 h at room temperature. The plates were washed 10 times with PBS/Tween. After addition of 100 μl of 1:1000 streptavidin:HRP in 50% goat sera in PBS, the plates were incubated for 1 h at room temperature. The plates were washed 10 times with PBS/Tween, developed with 1:1 substrate of 100 μl tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and read at 650 nm or stopped with 50 μl/well of 2 N H_2SO_4 and read at 450 nm. Data were analyzed on the Δ Soft program (Molecular Devices, Sunnyvale, CA). The protocol for determination of OVA-specific IgE was the same as for total IgE ELISA, except that the first step was performed with 100 μg/ml of OVA in PBS.

Statistical analysis

The data are presented as the mean ± SE of the mean. A Student's two-tailed *t* test was used to compare data for BAL fluid eosinophil counts and airway mucus between the different experimental groups. For the evaluation of pulmonary mechanics, a Fisher PLSD test was used to compare values for G_L and C_{dyn} between experimental groups. Differences were considered statistically significant for *p* values <0.05.
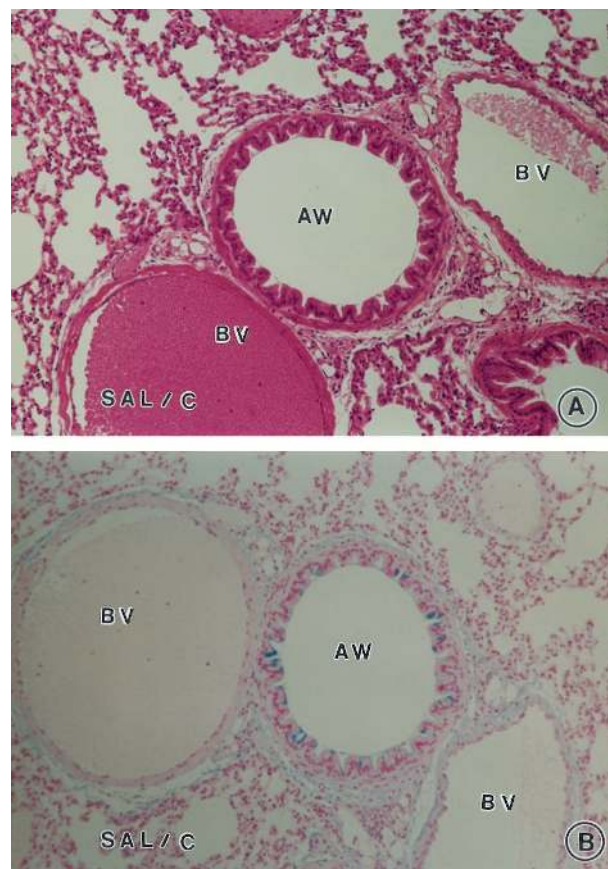

FIGURE 2. Lung histopathology of control mice. The lung sections of saline sham-sensitized/challenged control mice (SAL/C) were stained with hematoxylin/eosin (*A*) or with Alcian blue (*B*), and examined by light microscopy. *A*, In the saline-treated control mice, the airways (AW) and pulmonary blood vessels (BV) are of normal appearance. Inflammatory cells are absent in the bronchiolar and alveolar parenchyma, and mucus release is not present in the airway lumen. *B*, Airway mucus cells are identified by Alcian blue staining. Mucus secretion in the airways is not observed in the saline-treated mice.
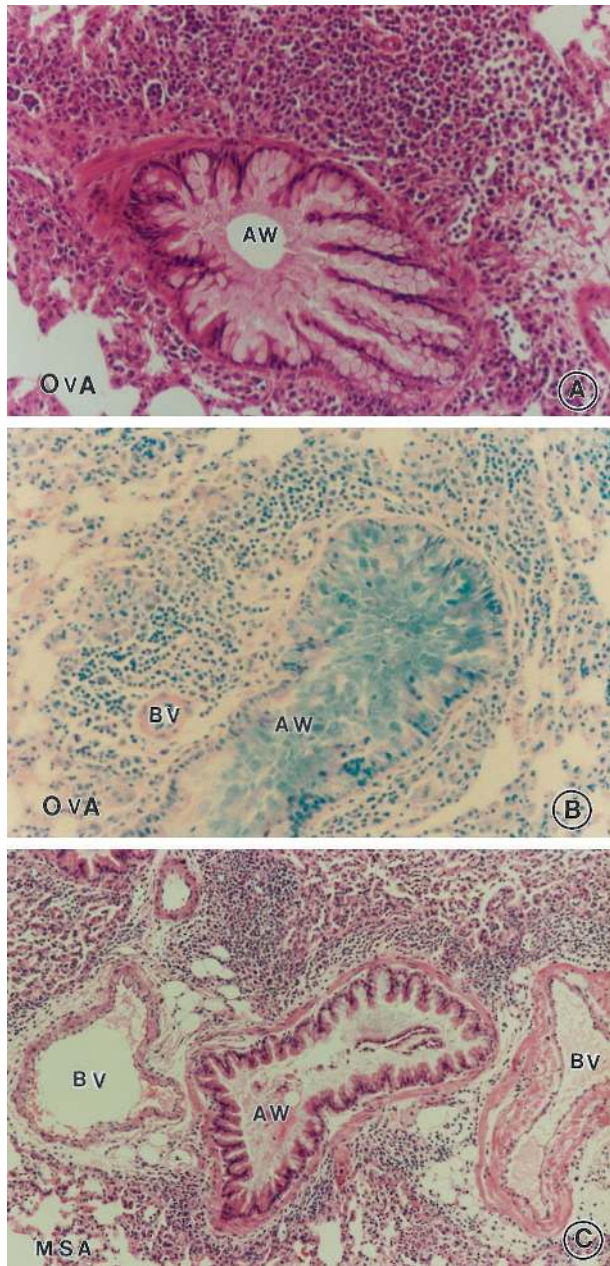
FIGURE 3. Histologic evidence of airway inflammation in OVA-treated mice. Lung tissue was obtained from OVA-immunized/challenged mice in the absence (*A*, *B*; OVA) or presence of MSA (*C*; MSA) treatment (100 μg i.n. on days 25, 26, and 27, 30 min before OVA treatment). Lung sections were stained with hematoxylin/eosin (*A*, *C*) or with methylene blue (*B*), and examined by light microscopy, as described in *Materials and Methods*. *A*, The interstitial space surrounding the airway (AW) is characterized by a dense, mixed cellular infiltrate of eosinophils and mononuclear cells. Marked narrowing of the airway lumen by secretions is observed. *B*, Mucus occlusion of the airway lumen of the OVA-sensitized/challenged mice is identified by methylene blue staining. Massive infiltration of the interstitial tissue by eosinophils and other inflammatory cells around blood vessels (BV) and airways (AW) is noted. *C*, MSA treatment before OVA challenge on days 25, 26, and 27 did not affect the airway cellular inflammatory response, edema, and mucus occlusion observed on day 28.

FIGURE 4. sIL-4R(M) inhibits airway inflammation in OVA-treated mice. Lung tissue was obtained from OVA-immunized/challenged mice in the presence of sIL-4R(M) (100 μg i.n.) treatment (M/IL-4R). Lung sections were stained in hematoxylin/eosin (*A*, *C*) or with Alcian blue (*B*), and examined by light microscopy, as described in *Materials and Methods*. *A*, Reduction in the cellular infiltration surrounding the airways (AW) and blood vessels (BV) is observed. Scant mucus release is noted in the airways after OVA challenge in the sIL-4R(M)-treated mice. *B*, Compared with OVA treatment alone (*A* and *B*), MSA/OVA (*C*), or sIL-4R(H)/OVA (Fig. 7) treatment, airway mucus secretion is markedly reduced in the sIL-4R(M)-treated animals. *C*, At higher magnification, little cellular infiltration is found surrounding the airways.

Results

sIL-4R levels in the plasma and BAL fluid

From previous pharmacokinetic studies (22), it was known that the elimination $t_{1/2}$ of sIL-4R in the mouse is 4.6 h after i.p. admin-
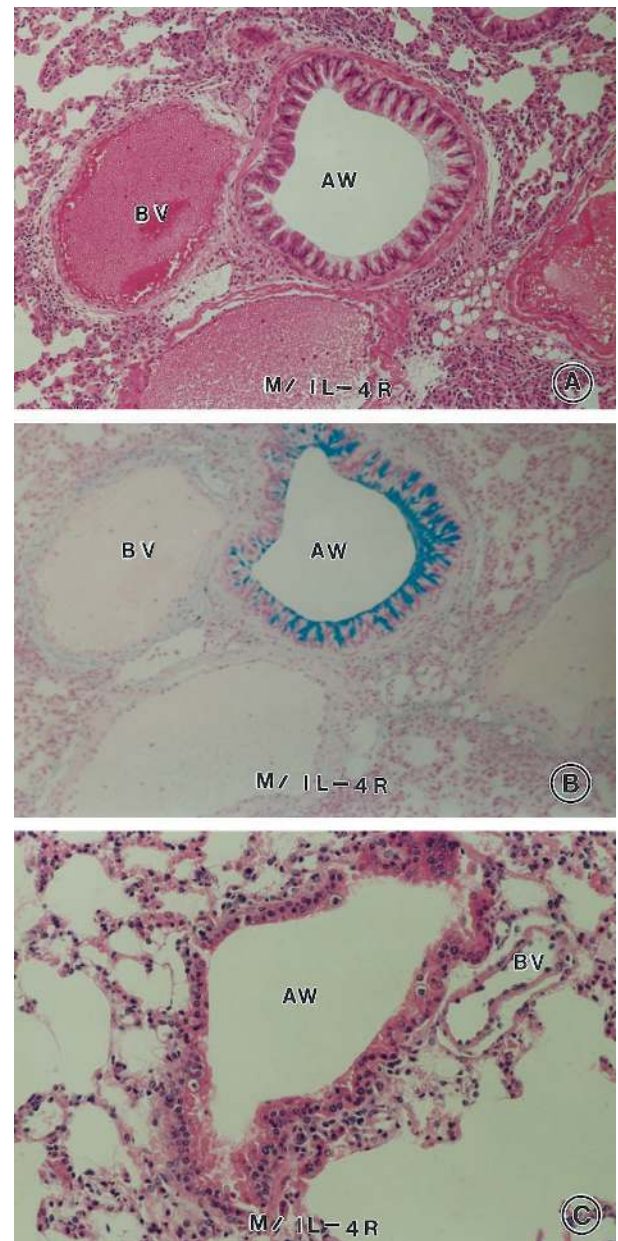
istration. sIL-4R(M) doses from 0.1–100 μg were administered i.n. or i.p. 30 min before OVA challenge on days 25, 26, and 27. On day 28, plasma and BAL fluid samples were collected 24 h following administration of the final sIL-4R dosage and assayed for sIL-4R levels (Fig. 1). The levels of sIL-4R(M) were similar in blood from saline control and OVA-sensitized/challenged mice (13.6 ± 1.5 ng/ml (*n* = 7) and 16.2 ± 1.3 ng/ml (*n* = 9), respectively); sIL-4(M) levels were <1 ng/ml in the BAL fluid from
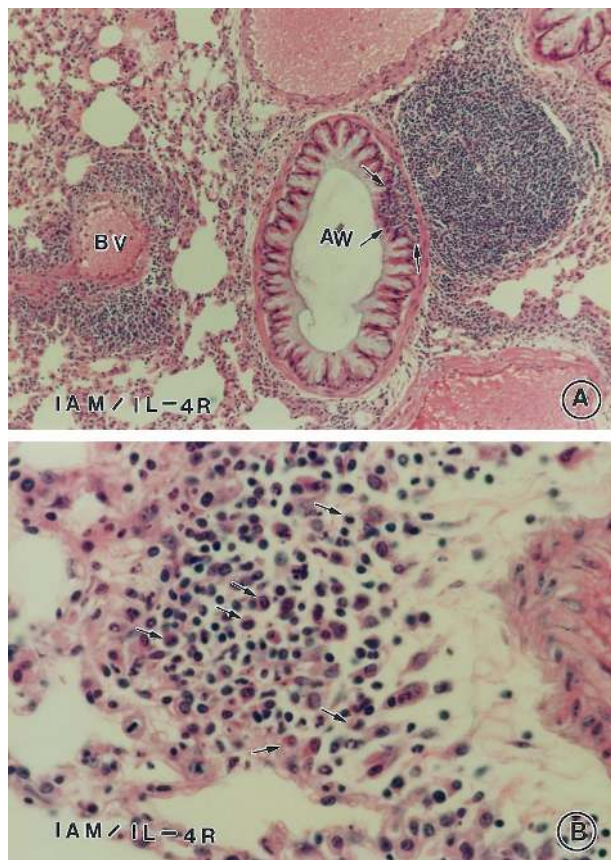
FIGURE 5. Inactivated sIL-4R(M) fails to inhibit airway inflammation in OVA-treated mice. Lung tissue was obtained from OVA-immunized/challenged mice in the presence of heat-inactivated sIL-4R(M) (100 μg i.n.) treatment (IAM/IL-4R). Lung sections were stained in hematoxylin/eosin and examined by light microscopy, as described in *Materials and Methods*. *A*, Inactivated sIL-4(M) treatment does not diminish the cellular inflammatory response around airways (AW) or blood vessels (BV) in the lungs. Cellular infiltration (arrows) into the epithelial cell layer of the airways is observed. Mucus cell hypertrophy and airway lumenal narrowing by secreted mucus are present. *B*, At higher magnification, the intense mixed cellular influx of eosinophils (arrows) and other inflammatory cells into the lung interstitium is evident.

control and OVA-treated mice. After i.n. or i.p. delivery of 0.1–10 μg sIL-4R(M) before OVA on days 25 to 27, sIL-4R(M) blood levels were increased <2-fold on day 28. At 100 μg of sIL-4R(M), i.n. delivery increased blood sIL-4R(M) levels by 2.7-fold (44 ± 3.8 ng/ml, $n = 8$, $p = 0.0001$ vs OVA) and i.p. delivery by 3.7-fold (60.5 ± 2, $n = 2$, $p = 0.0001$ vs OVA). Whereas i.n. administration of sIL-4R(M) at 100 μg dose increased BAL fluid levels of sIL-4R(M) 283-fold (255 ± 81.6 ng/ml, $n = 16$, $p = 0.004$ vs OVA), i.p. delivery of 100 μg sIL-4R(M) did not significantly increase BAL fluid levels of sIL-4R(M). Pretreatment with 100 μg doses of heat-inactivated sIL-4R(M), sIL-4R(H), or MSA by i.n. administration or 100 μg of i.p. sIL-4R(H) did not affect sIL-4R(M) levels of OVA-treated mice.

Effect of sIL-4R(M) on IgE

Blood was obtained on day 28 in the OVA-sensitized/challenged mice. Treatment with sIL-4R(M) 30 min before OVA challenge on days 25, 26, and 27 did not significantly affect the circulating levels of either total IgE or OVA-specific IgE in the OVA-treated mice (data not shown).
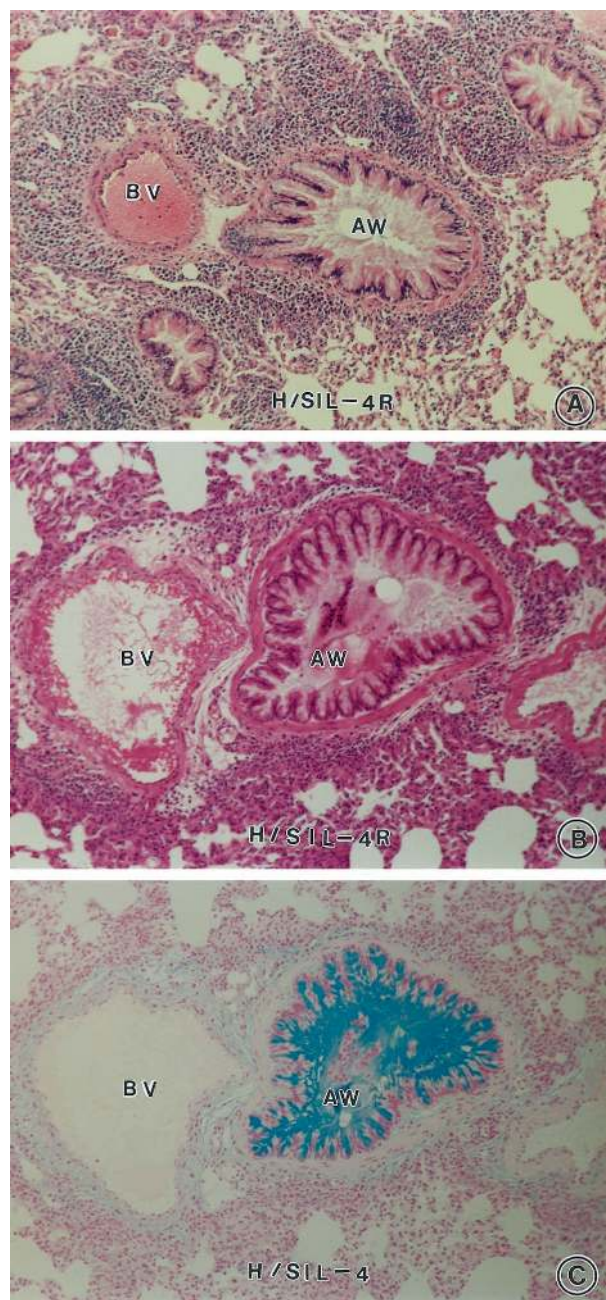

FIGURE 6. Lack of effect of sIL-4R(H) on airway inflammation in OVA-treated mice. Lung tissue was obtained from OVA-immunized/challenged mice in the presence of sIL-4R(H) (100 μg i.n.) treatment (H/sIL-4R). Lung sections were stained in hematoxylin/eosin (*A*, *B*) or with Alcian blue (*C*), and examined by light microscopy, as described in *Materials and Methods*. An intense cellular infiltration around pulmonary blood vessels (BV) and medium sized airways (AW) is observed. Eosinophils are the predominant inflammatory cell in the lung interstitial tissue. *B*, A marked cellular inflammatory response surrounding large airways is also noted in the sIL-4R(H)/OVA-treated mice. *C*, Alcian blue staining of neutral and acidic mucosubstances is shown. Mucus cell hypertrophy is seen in the sIL-4R(H)/OVA-treated mice. Large accumulation of mucus in the airway lumen is observed.

Effect of sIL-4R(M) on recruitment of inflammatory cells into the lungs

Twenty-four hours following the final i.n. OVA challenge in animals from each experimental group, BAL was performed on the right lung, and left lung tissue was obtained to assess the effect of

FIGURE 7. sIL-4R(M) reduces the cellular infiltration of airways and pulmonary blood vessels and mucus occlusion of airways in OVA-treated mice. Lung tissue was obtained from OVA-treated mice (*n* = 6) in the absence (OVA) or presence on days 25, 26, and 27 of i.n. treatment with 100 μg of sIL-4R (sIL-4R(M)), heat-inactivated sIL-4R(M) (inactive sIL-4R(M)/OVA), sIL-4R(H) (sIL-4R(H)/OVA), and MSA (MSA/OVA), or i.p. treatment with 100 μg of sIL-4R(M) (sIL-4R(M)/OVA) or sIL-4R(H) (sIL-4R(H)/OVA). The infiltration by eosinophils and other inflammatory cells around airways and pulmonary blood vessels (*A*), the number of eosinophils per unit area (2,200 μm²) (*B*), and occlusion of airway diameter by mucus (0–4⁺ scale) (*C*) were determined by morphometric analysis, as described in *Materials and Methods. p* value, sIL-4R(M)/OVA vs OVA.
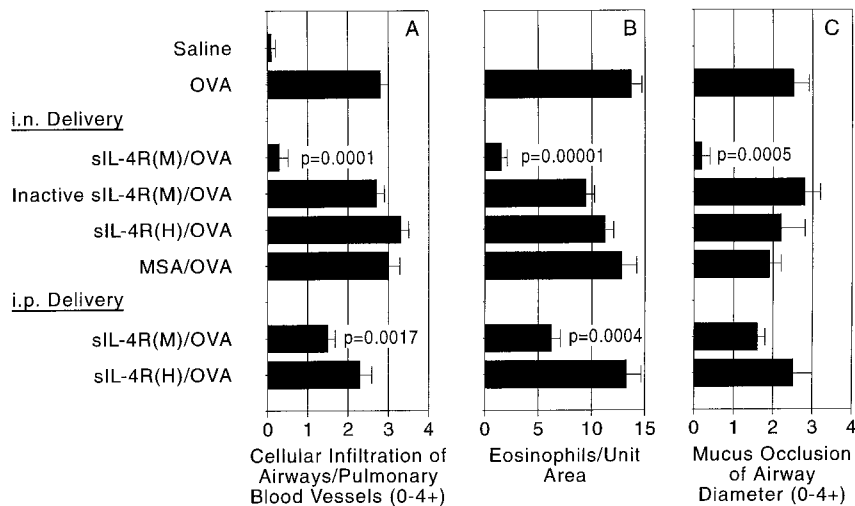
sIL-4R on airway inflammation. By light microscopy, compared with saline controls (Fig. 2, *A* and *B*) a marked influx of eosinophils and mononuclear cells into the lungs around blood vessels and airways was observed in OVA-sensitized/challenged mice (Fig. 3, *A* and *B*). Administration of 100 μg of sIL-4R(M) i.n. before OVA challenge on days 25, 26, and 27 inhibited the cellular infiltration surrounding airways and pulmonary blood vessels (Fig. 4, *A*–*C*). In contrast, i.n. pretreatment with 100 μg of MSA (Fig. 3*C*), heat-inactivated sIL-4R(M) (Fig. 5, *A* and *B*), and sIL-4R(H) (Fig. 6, *A*–*C*), failed to affect the cellular inflammatory response in OVA-treated mice.

The number of eosinophils in the cellular infiltrates in the lung interstitium (13.7 eosinophils/2200 μm² airway area) of the OVA-treated mice was determined by morphometric analysis (Fig. 7, *A* and *B*). Pretreatment with i.n. sIL-4R(M) reduced the number of eosinophils in the pulmonary tissue of OVA-treated mice by 89% (*p* = 0.0001, i.n. sIL-4R(M)/OVA vs OVA). MSA, heat-inactivated sIL-4R(M), and sIL-4R(H) pretreatment did not alter this infiltration of the airways by eosinophils (Fig. 7*B*). Administration of 100 μg of sIL-4R(M) by the i.p. route also reduced the cellular infiltrate by eosinophils and other inflammatory cells in the lungs (Fig. 7, *A* and *B*), but to a lesser extent than by i.n. administration.

OVA-treated mice had a 6.5-fold increase in total cells recovered in BAL fluid compared with the saline group (Fig. 8*A*; saline group vs OVA group, *p* = 0.0001). Pretreatment with i.n. delivery of 100 μg of sIL-4R(M) significantly reduced the total number of cells in the BAL fluid of OVA-sensitized/challenged mice (*p* = 0.046, i.n. sIL-4R(M)/OVA vs OVA group). The mean number of eosinophils in BAL fluid collected from the saline group was 1.3 × 10³ cells (Fig. 8*B*). After OVA treatment, the number of eosinophils in the BAL fluid from the OVA group increased 22.7-fold to 3 × 10⁵ (saline group vs OVA group, *p* = 0.0001). Pretreatment with i.p. and i.n. 100 μg dosing of sIL-4R(M) reduced eosinophil infiltration into the BAL fluid by 73.3% (*p* = 0.0009, i.p. sIL-4R(M)/OVA vs OVA) and by 80% (*p* = 0.0003, i.n. sIL-4R(M)(100 μg)/OVA vs OVA), respectively (Fig. 8*B*).

By immunocytochemistry, OVA-treated mice had a marked increase in VCAM-1 expression in endothelial cells of large and small pulmonary blood vessels of OVA-treated mice (Fig. 9*B*) compared with saline-treated controls (Fig. 9*A*). VCAM-1 expression was also noted in inflammatory cells recruited to the lung interstitium of OVA-sensitized/challenged mice (Fig. 9*A*). Pretreatment on days 25, 26, and 27 with 100 μg of sIL-4R(M) reduced VCAM-1 expression in the endothelium of the lung blood

vessels and in the interstitium surrounding the airways of the OVA-treated mice (Fig. 9*C*).

Effect of sIL-4R(M) on mucus accumulation in the lungs

Mucus hypersecretion and occlusion of the airways was a prominent histopathologic feature of the OVA-sensitized/challenged mice (Fig. 3, *A* and *B*). Mucus hypersecretion and occlusion of airway diameter were significantly inhibited by pretreatment with 100 μg of sIL-4R(M) in the OVA-treated mice (Figs. 4*A*, *B*, and *C*, and 7*C*). Mucus release in OVA-treated animals was unaffected by i.n. administration of 100 μg of MSA (Figs. 3*C* and 7*C*), heat-inactivated sIL-4R(M) (Figs. 5*A* and 7*C*), and sIL-4R(H) (Figs. 6*A*, *B*, and *C*, and 7*C*). In contrast to i.n. delivery, i.p. treatment with 100 μg of sIL-4R(M) did not significantly reduce mucus occlusion of airway diameter in OVA-treated mice (Fig. 7*C*).

Effect of sIL-4R(M) on allergen-induced AHR to methacholine

Airway reactivity was evaluated on day 28, which was 24 h following the third i.n. challenge with OVA. In the OVA group, AHR
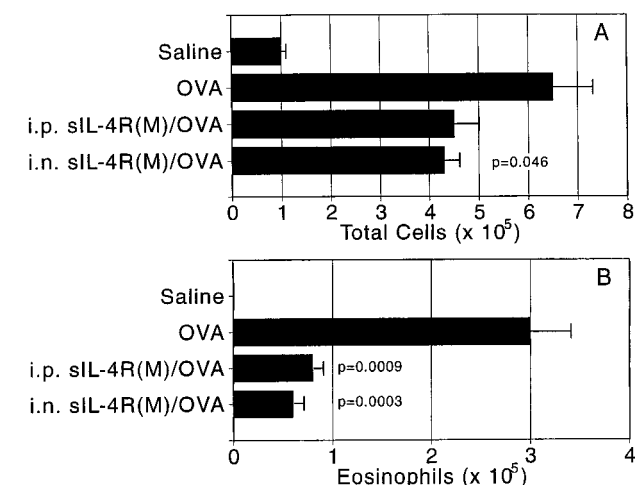
FIGURE 8. sIL-4R(M) reduces the number of eosinophils in the BAL fluid following OVA challenge. BAL fluid was obtained from saline-treated mice (Saline; *n* = 19), OVA-sensitized/challenged mice (OVA; *n* = 15), OVA-sensitized/challenged mice pretreated with sIL-4R(M) at a dosage 100 μg given either i.p. (i.p. sIL-4R(M)/OVA; *n* = 9) or i.n. (i.n. sIL-4R(M)/OVA; *n* = 6). The number of total cells (*A*) and eosinophils (*B*) per total BAL fluid collected from each group are presented as the mean ± SE. *p* value, i.p. or i.n. sIL-4R(M) vs OVA.

FIGURE 9. VCAM-1 expression by immunocytochemistry is reduced in lungs of OVA-treated mice by sIL-4R(M). Lung tissue was obtained from saline-treated mice (*A*) and OVA-treated mice in 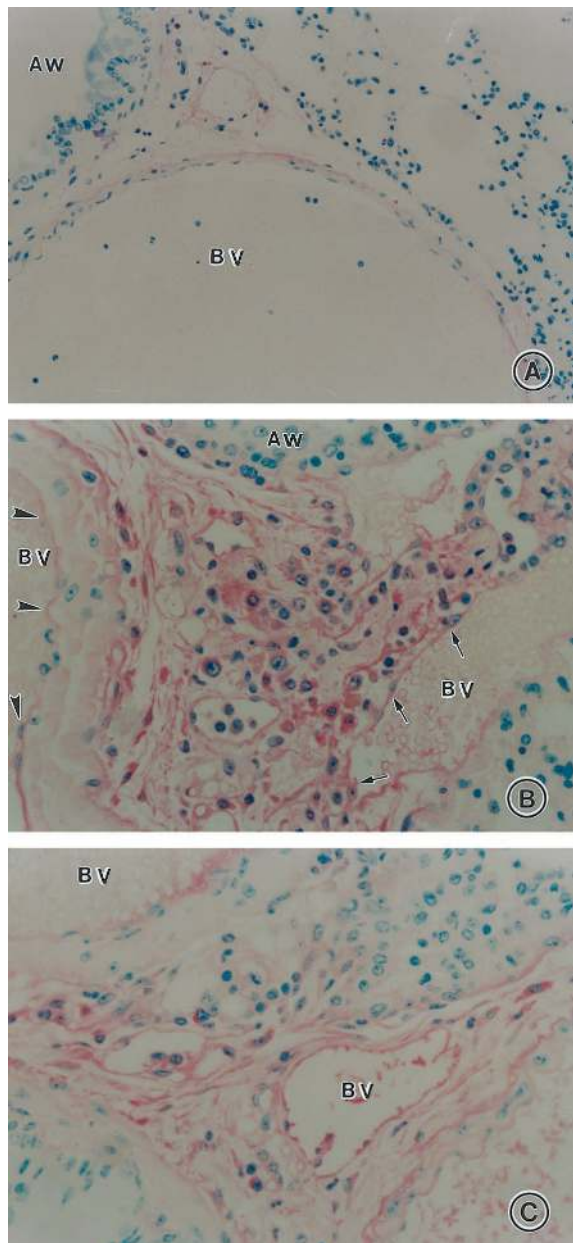the absence (*B*) or presence (*C*) of i.n. treatment with 100 μg of sIL-4R(M). Lung sections underwent immunocytochemistry for localization of VCAM-1, as described in *Materials and Methods*, and representative fields are shown. *A*, In saline-treated mice, negligible VCAM-1 expression is observed in the endothelium of small and large blood vessels (BV) surrounding the airways (AW). ×120. *B*, In OVA-treated mice, VCAM-1 immunocytochemical localization, as indicated by the prominent pink AP reaction products, is found in endothelial cells of both small (arrows) and large (arrowheads) blood vessels (BV). VCAM-1 is also expressed in inflammatory cells infiltrating the interstitium around the airway (AW). ×240. *C*, Decreased VCAM-1 expression is observed in the lung interstitium and blood vessel (BV) endothelial cells of OVA-treated mice receiving sIL-4R(M). ×240. AP reaction products were not present in lung sections from OVA-treated mice when the rat anti-mouse VCAM-1 mAb was omitted from the staining protocol (not shown).

was seen following challenge with methacholine, with a significant decrease in both G_L and C_{dyn} compared with the saline group (Table I; saline group vs OVA group, $p < 0.05$ for both G_L and

C_{dyn}). At a dose of 100 μg administered i.p. or i.n. before OVA on days 25, 26, and 27, sIL-4R(M) did not reduce bronchial hyper-responsiveness to methacholine in the OVA-sensitized and challenged mice. Similarly, pretreatment with 100 μg i.n. of sIL-4R(H) failed to alter the methacholine-induced AHR observed in OVA-treated mice (Table I).

Discussion

These studies were designed to determine whether the administration of sIL-4R reduced airway inflammation and hyperreactivity in a murine model of allergen-induced asthma. In this model, mice sensitized to OVA were exposed to i.n. OVA challenge for 3 consecutive days. This allergen challenge leads to airway hyperresponsiveness to methacholine, extensive eosinophil infiltration of the lung, and occlusion of the airways by mucus hypersecretion. Blood levels of sIL-4R(M) greater than 40 ng/ml were achieved 24 h following the final i.n. and i.p. administration of 100 μg of sIL-4R(M). However, only i.n. dosing with 100 μg of sIL-4R(M) significantly raised sIL-4R(M) levels in the BAL fluid of OVA-treated animals when measured 24 h after the last treatment with sIL-4R(M).

Goblet cell hyperplasia and mucus obstruction of the airways are key features of chronic asthma (28, 29). IL-4 is important in regulation of airway mucus release. Transgenic mice overexpressing IL-4 have increased MUC5AC, but not MUC2 mucin gene expression (30). These transgenic mice have up to a 10-fold increase in Alcian blue and periodic acid-Schiff (PAS)-positive mucus glycoprotein materals in their BAL fluid compared with transgene-negative controls (30). In our murine model, a marked increase in airway mucus is observed on day 22; by days 24 to 25, airway epithelial cells are extensively replaced by mucus-producing goblet cells (31). Our data indicate that blockade of IL-4 beginning on day 25 by i.n. delivery of sIL-4R inhibits airway mucus hypersecretion after airway OVA challenge in mice previously sensitized to this allergen. These data suggest an ongoing role for IL-4 in the maintenance of airway mucus glycoprotein secretion in the allergic airways. The failure of i.p. delivery of sIL-4R(M) to significantly reduce airway obstruction by mucus indicates that increasing the airway levels of sIL-4R is critical for its inhibitory action on mucus secretion.

Like CD4$^+$ and CD8$^+$ T cells, eosinophils are a prominent cell type in the LAR and in the chronic inflammation of asthma. Eosinophilia is noted in sputum, lavage, and biopsy samples, and eosinophils are often activated, as evidenced by morphologic changes and the identification of increased concentrations of eosinophil products, such as cationic protein and major basic protein (32). IL-4 is a critical mediator of airway eosinophilia. IL-4 increases human bronchial tissue expression of VCAM-1 (CD106) (33), which binds to the integrin heterodimer VLA-4 ($\alpha_4\beta_1$; CD49d/CD29). VLA-4 is expressed on eosinophils and all other circulating leukocytes except neutrophils, and is important for the selective influx of eosinophils and lymphocytes into the airways in asthma (21, 34). Of interest, we observed expression of VCAM-1 in inflammatory cells infiltrating the lung interstitium of OVA-treated mice. Prior reports have noted VCAM-1 expression in vascular dendritic cells present in human atherosclerotic plaques (35) and in lymphocytes and monocytes invading the renal interstitium of diabetic KKAy mice (36).

In murine asthma models using IL-4-deficient (IL-4$^{-/-}$) mice, IL-4 has been found important for both eosinophil (37) and Th2 cell (38) recruitment to the lungs, and Th2 cell production of IL-4 is necessary for the development of airway eosinophilia (39). We found that pretreatment of OVA-sensitized/challenged mice with

Table I. *Pulmonary mechanics to methacholine in OVA-treated mice: effect of sIL-4R*[a]

	Basal		Methacholine (120 μg/Kg)			
	G_L (ml/s $\times$ cm H_2O)	C_{dyn} (ml/cm H_2O)	G_L (ml/s $\times$ cm H_2O)	G_L (% basal)	C_{dyn} (ml/s $\times$ cm H_2O)	C_{dyn} (% basal)
Saline	1.16 ± 0.07	0.027 ± 0.001	0.47 ± 0.02	42 ± 3	0.017 ± 0.001	63 ± 2
OVA	1.09 ± 0.07	0.027 ± 0.001	0.30 ± 0.04*	27 ± 3*	0.011 ± 0.002*	42 ± 5*
i.p. sIL-4R(M)/OVA	1.14 ± 0.07	0.026 ± 0.001	0.25 ± 0.05*	24 ± 7*	0.007 ± 0.002*	28 ± 6*
i.p. sIL-4R(H)/OVA	1.00 ± 0.06	0.026 ± 0.001	0.18 ± 0.02*	18 ± 3*	0.007 ± 0.001*	26 ± 3*
i.n. sIL-4R(M)/OVA	1.08 ± 0.07	0.024 ± 0.001	0.30 ± 0.02*	28 ± 2*	0.011 ± 0.001*	43 ± 4*
i.n. sIL-4R(H)/OVA	1.02 ± 0.04	0.024 ± 0.000	0.23 ± 0.05*	22 ± 5*	0.009 ± 0.002*	40 ± 8*

[a] Data represent mean ± SE. G_L and C_{dyn} were determined in sham-sensitized and saline-challenged mice (saline, $n = 9$), and OVA-sensitized/challenged mice in the absence (OVA, $n = 8$) or presence of 100 μg of i.p. sIL-4R(M) (i.p. sIL-4R (M)/OVA; $n = 8$), i.p. sIL-4R(H) (i.p. sIL-4R (H)/OVA; $n = 6$), i.n. sIL-4R(M) (i.n. sIL-4R(M)/OVA; $n = 9$), or i.n. sIL-4R(H) (i.n. sIL-4R(H)/OVA; $n = 4$), treatment as described in *Materials and Methods*.

*, $p < 0.05$ by ANOVA: vs saline.

sIL-4R(M) at a dosage of 100 μg i.n. or i.p. significantly inhibited eosinophil infiltration into the lung interstitium and BAL fluid. Elevation of circulating levels of sIL-4R by either i.p. or i.n. delivery is sufficient to block movement of eosinophils into the lungs, indicating that eosinophil recruitment by IL-4 may be a systemic effect of this cytokine. Although a number of studies have established a key role for IL-4 in VCAM-1 expression (40–42, 42), a connection between IL-4-mediated eosinophil migration and regulation of VCAM-1 expression has not been clearly shown in asthma models. Our results demonstrate that VCAM-1 expression is reduced in allergen-challenged mice following IL-4 inhibition. These results support the hypothesis that eosinophil migration in asthma is dependent upon VCAM-1 expression, and provide an indirect mechanism whereby IL-4 may affect eosinophil accumulation in the lungs.

It has been proposed that eosinophils and their products contribute to the chronic inflammation and the development of AHR. Alternatively, they may not be causally involved in the development of AHR, but their presence may be merely an epiphenomenon of the Th2 response with elaboration of IL-5. In our model, airway reactivity was evaluated on day 28, which was 24 h following the third i.n. challenge with OVA. Mice in the OVA group demonstrated AHR when challenged with methacholine (i.e., G_L and C_{dyn} were significantly reduced compared with the control group). Despite significantly increasing BAL fluid levels of sIL-4R(M), treatment with sIL-4R(M) at a dosage of 100 μg failed to alter in vivo AHR in response to methacholine.

Renz et al. (18) examined the effect of a rsIL-4R on in vivo immediate hypersensitivity responses and AHR in mice sensitized to the airways to OVA by once weekly ultrasonic nebulizations for 4 wk. In their studies, mice received sIL-4R before initial OVA sensitization and throughout the OVA treatment period. Treatment with 150 μg of i.p. sIL-4R(M) reduced by >50% anti-OVA IgE and IgG1 Ab titers. In a dose-dependent manner (15–150 μg), sIL-4R(M) inhibited allergen-specific immediate cutaneous hypersensitivity responses in the mice. Treatment with i.p. sIL-4R(M) also prevented expansion of Vβ8.1/8.2 T cells in OVA-treated animals. Renz et al. (18) found that local administration to the lungs of sIL-4R(M) by aerosolization not only decreased the IgE/IgG1 responses to OVA, but also reduced total serum IgE levels. In vitro studies by Renz et al. (43) have also demonstrated that murine sIL-4R and dimeric sIL-4R fusion protein significantly reduce allergen-specific polyclonal IgE production by lymphocytes obtained from allergen-sensitized mice. This inhibitory effect was greatest during the first 3 days of culture and was not observed after 6 days, suggesting that sIL-4R blocks the early period of B cell maturation/IgE production (43).

In our studies, sIL-4R(M) given only before allergen challenge on days 25, 26, and 27 and not during sensitization did not reduce either total or OVA-specific IgE levels. We have previously demonstrated that mice receiving two doses of OVA by the i.p. route on days 1 and 14 have similar levels of OVA-specific IgG1 and IgE on day 28, irrespective of the number of i.n. OVA doses administered (20). In view of this robust IgE response to OVA in our model and the in vitro studies by Renz et al. (43), it is not surprising that administration of sIL-4R after allergen sensitization would not affect circulating IgE levels.

As reported by Renz et al., nebulized sIL-4R(M) given before and throughout OVA sensitization normalized airway responsiveness (as measured in vitro by electrical field stimulation of excised tracheal smooth muscle preparations) to a greater extent than i.p. administration of this protein. In contrast, administration of 100 μg sIL-4R(M) by either i.p. or i.n. routes did not alter AHR in our murine model of asthma. Corry et al. (44) found that anti-IL-4 mAb given during the systemic immunization period in a murine asthma model blocked AHR, but when administered during the allergen challenge period, had no effect on the response of the sensitized animals to acetylcholine. The results with sIL-4R and anti-IL-4 mAb treatments suggest that IL-4 generated during the sensitization period of lymphocyte priming by allergen is important for allergen-induced AHR, but that IL-4 is not required for maintenance of AHR after allergen challenge in immunized mice.

The discordance we observed between airway eosinophils and AHR has been noted in several other animal models. The presence of eosinophils clearly does not ensure AHR. Transgenic mice with IL-4 constitutively expressed in the lung develop an eosinophilic inflammatory cell infiltrate including eosinophilia but do not exhibit AHR (45), and airway administration of IL-5 produces airway eosinophilia without AHR in guinea pigs (46). AHR without eosinophilia is also observed in murine asthma models. In previous studies, we found that inhibition of leukotriene synthesis prevents allergen-induced eosinophil recruitment without affecting AHR (19). Furthermore, a CD49d mAb given by i.p. administration at doses that saturated circulating leukocytes blocked Ag-induced eosinophil accumulation in BAL fluid, but did not prevent AHR, mucus hypersecretion, or IL-4 and IL-5 release (21). Thus, the development of AHR does not appear dependent on pulmonary infiltration by eosinophils.

It remains unclear to what extent IL-4 vs IL-13 regulates the allergic diathesis. IL-13 also binds to the α-chain of the IL-4R (47, 48), and the IL-13 gene is closely linked to the IL-4 gene (49). However, IL-13 induces IL-4-independent IgE and IgG4 by human B cells (50). Because T cells lack functional IL-13R, IL-13 (unlike

IL-4) does not induce Th2 cell differentiation (51). By in situ hybridization, an increased number of cells express both IL-13 and IL-4 mRNA in endobronchial biopsies of patients with mild atopic asthma compared with normal controls (3). IL-13 and IL-4 protein levels are significantly increased 18 h after allergen challenge in patients with allergic asthma (52). A key role for IL-13 in the mediation of airway inflammation and hyperreactivity has recently been demonstrated in murine models of human asthma (53, 54). In OVA-sensitized mice, blockade of IL-13 by a sIL-13Rα2-IgGF$_c$ fusion protein that specifically binds to and neutralizes IL-13 (55) inhibits airway eosinophilia, mucus release, and AHR (53, 54). In addition, intratracheal or i.n. administration of either IL-13 or IL-4 induces airway eosinophilia, and AHR in naive animals (53, 54), but not in IL-4Rα-deficient mice, suggesting that development of the asthma phenotype in mice is dependent on signaling by either IL-4 or IL-13 through IL-4Rα.

Our results indicate that IL-4 is an important mediator in the late phase inflammatory response that occurs in this allergen-induced model of asthma. These data have therapeutic implications, in that they suggest that local antagonism of IL-4 in the lung may be beneficial even in the presence of full sensitization to an allergen. Clinical studies are currently ongoing to examine the effects of sIL-4R treatment on airway inflammation and function in patients with persistent asthma. In a phase I/II study in 25 patients with moderate persistent asthma who required inhaled corticosteroids for control of symptoms, after removal of corticosteroids, a single 1500 μg nebulization of sIL-4R stabilized lung function and decreased exhaled NO, a marker of pulmonary inflammation during the first week after sIL-4R inhalation (56). Moderation of lung cellular inflammation and mucus hypersecretion by sIL-4R in a mouse model of asthma suggests that sIL-4R may be useful in the treatment of patients with asthma.

Acknowledgments

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