A Murine IL-4 Receptor Antagonist That Inhibits IL-4- and IL-13-Induced Responses Prevents Antigen-Induced Airway Eosinophilia and Airway Hyperresponsiveness

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A Murine IL-4 Receptor Antagonist That Inhibits IL-4- and IL-13-Induced Responses Prevents Antigen-Induced Airway Eosinophilia and Airway Hyperresponsiveness


The closely related Th2 cytokines, IL-4 and IL-13, share many biological functions that are considered important in the development of allergic airway inflammation and airway hyperresponsiveness (AHR). The overlap of their functions results from the IL-4R α-chain forming an important functional signaling component of both the IL-4 and IL-13 receptors. Mutations in the C terminus region of the IL-4 protein produce IL-4 mutants that bind to the IL-4R α-chain with high affinity, but do not induce cellular responses. A murine IL-4 mutant (C118 deletion) protein (IL-4R antagonist) inhibited IL-4- and IL-13-induced STAT6 phosphorylation as well as IL-4- and IL-13-induced IgE production in vitro. Administration of murine IL-4R antagonist during allergen (OVA) challenge inhibited the development of allergic airway eosinophilia and AHR in mice previously sensitized with OVA. The inhibitory effect on airway eosinophilia and AHR was associated with reduced levels of IL-4, IL-5, and IL-13 in the bronchoalveolar lavage fluid as well as reduced serum levels of OVA-IgE. These observations demonstrate the therapeutic potential of IL-4 mutant protein receptor antagonists that inhibit both IL-4 and IL-13 in the treatment of allergic asthma.

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Clinical and experimental investigations have identified CD4+ Th2 cells and the production of specific cytokines (particularly IL-4, IL-5, IL-10, and IL-13) as crucial in orchestrating the allergic inflammatory response leading to airway hyperresponsiveness (AHR) (1–9). IL-5 is known to regulate growth, differentiation, activation, and survival of eosinophils and appears to be essential in the development of allergic airway eosinophilia and AHR in mice (10–12). IL-4 is critical for the commitment of T cells to the CD4+ Th2 phenotype, and IL-13, which shares several biological responses with IL-4, may also be important to the development of allergic airway eosinophilia and AHR (4, 13–15). Although not necessary for or even capable of inducing Th2 development, IL-13 plays a regulatory role in Th2 activation (16). IL-4 or IL-13 is required for IgE isotype switching in B cells (17, 18). In addition, both IL-4 and IL-13 up-regulate the expression of MHC class II and the low-affinity IgE receptor (CD23, FceRII) on B cells and monocytes, enhancing Ag presentation as well as regulating the function of macrophages (18–20). Importantly, IL-4 and IL-13 increase the expression of VCAM-1 on endothelial cells, facilitating the preferential recruitment of eosinophils (and T cells) to the airway tissues (21, 22). IL-4 and IL-13 may also increase airway mucus secretion, which can exacerbate airway responsiveness (23, 24).

Using neutralizing Ab to IL-4 administered during sensitization or mice deficient in IL-4, the development of airway eosinophilia, AHR, and increases in serum IgE seen following sensitization and allergen provocation are reduced or abolished (25–27). However, administration of Ab to IL-4 during the secondary allergen challenge only partially reduces the response, suggesting alternate mechanisms or even a sequential requirement for IL-4, then IL-13 (25, 26, 28). More recently, inhibition of airway eosinophilia and AHR using a soluble IL-13R fusion protein that specifically binds and neutralizes IL-13 has been shown (29, 30). Moreover, administration of IL-13 or overexpression of IL-13 in the airways can induce airway eosinophilia, mucus production, and AHR to various degrees (24, 29, 30). Cumulatively, the data suggest that IL-4 and IL-13 can play both overlapping and independent roles in allergic responses in the lung and that targeting either cytokine alone in previously sensitized hosts may have limited efficacy.

IL-4 exerts its biological effects by binding to the 140-kDa IL-4R α-chain that complexes with the 64-kDa common γ-chain of the type I cytokine receptor family. Formation of the receptor heterodimer initiates signal transduction by activation of STAT6 (via the IL-4R α-chain), resulting in the gene transcription that is critical for many of the IL-4-induced effects associated with the allergic response (31). The IL-4R α-chain also forms the functional signaling component of the IL-13R heterodimer (IL-13R α1-chain/IL-4R α-chain) and largely explains the overlap of IL-4 and IL-13 biological functions (15, 31).

Mutagenesis studies have identified the regions of IL-4 important for interaction with the IL-4R α-chain. Importantly, mutations of arginine 121, tyrosine 124, and serine 125 in the C terminus region of the human protein result in IL-4 mutants that have the same capacity to bind to the IL-4R α-chain as native IL-4, but have no signaling activity and are antagonists of both human IL-4- and IL-13-induced responses (32–35). Similarly, mutation of amino acids.
acids glutamine 116 and tyrosine 119 to aspartic acid in murine IL-4 produces a mutant protein (Q116D/Y119D) that binds with high affinity to the IL-4R α-chain without inducing signal transduction (36). This mutant was shown to inhibit IL-4-induced cell proliferation and differentiation in vitro and also inhibited IgE production when administered during the primary sensitization of mice (36, 37). Thus, the development of such IL-4 mutant proteins that bind to the IL-4R α-chain and prevent formation of the biologically active IL-4/IL-13R complexes may have better therapeutic benefit in the treatment of allergic airway disease than either antagonists of cytokine or receptor alone. In the present study we have used a (C118 deletion) truncated protein (murine IL-4R antagonist (ml-4Ra)) in which the terminal amino acids from position 119 onward are deleted. In this mutant the glutamine in position 116 is still intact, and this mutant is therefore analogous to the single mutant human IL-4R antagonist (Y124D). The effects of mIL-4Ra on IL-4- and IL-13-induced STAT6 phosphorylation and IgE production in vitro were assessed. Moreover, we evaluated the effect of the mIL-4Ra (administered after allergen sensitization, but at the time of challenge) on the development of allergic airway eosinophilia and AHR in vivo.

Materials and Methods

Animals

Female BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained on OVA-free diets. All experimental animals used in this study were under a protocol approved by the institutional animal care and use committee of the National Jewish Medical and Research Center.

Construction and expression of murine IL-4 mutant

Clone p2A-E3 containing mouse IL-4 cDNA was obtained from American Type Culture Collection (Manassas, VA). Δ118/mouse IL-4 cDNA was synthesized by PCR amplification with a 5′ oligonucleotide containing a BamHI site, and a 3′ oligonucleotide incorporating a stop codon at position 119 and an XbaI site. PCR fragments were digested with BamHI and XbaI, subcloned into the baculovirus transfer vector pVL1393 (PharMingen, San Diego, CA), and mutations were confirmed by dideoxy sequencing. DNAs were transfected into insect cells (Sp9), and morphology-positive plaques were picked, expressed in Sp9 cells, and screened by Western blot with rabbit anti-mouse IL-4 Ab (R&D Systems, Minneapolis, MN) followed by 125I-labeled protein A. The highest expressing clone was expanded for protein production.

Protein purification

The Δ118-containing Sp9 culture supernatant was applied to an anti-IL-4 (11B11) affinity column equilibrated in NaHCO, (pH 8.3). The bound protein was eluted with acetic acid (pH 3.0), and the pH was adjusted to 7.0 with Tris. Fractions containing the Δ118/mouse IL-4 were located by SDS-PAGE, and Δ118-containing fractions were pooled and lyophilized to dryness.

Cell preparation for in vitro studies

The peritoneum of naive mice was lavaged twice with 5 ml of ice-cold HBSS. Samples were pooled and kept on ice until used. Spleens and peri-bronchial lymph nodes (PBLN) were harvested from mice, and mononuclear cells were purified by passing the tissue through stainless steel mesh, followed by density gradient centrifugation (Organon Teknika, Durham, NC). Lungs were perfused with warmed (37°C) HBSS (10% FCS and 0.6 mM EDTA) via the right ventricle at a rate of 4 ml/min for 4 min. Lungs were removed and cut into pieces. Four milliliters of HBSS containing 175 U/ml collagenase (type IA; Sigma, St. Louis, MO) and 10% FCS was added to the minced lungs and incubated for 60 min in an orbital shaker at 37°C. The digested lungs were washed with a sterile 20-gauge needle and filtered through 45- and 15-μm filters, followed by density gradient centrifugation.

STAT6 phosphorylation

The phosphorylation status of STAT6 was determined by Western blot analysis. Briefly, 107 cells (peritoneal macrophages or spleen mononuclear cells from naive mice) were preincubated with or without mIL-4Ra (5 μg/ml) for 10 min on ice, followed by stimulation with IL-4 (10 ng/ml) or IL-13 (50 or 200 ng/ml) for 10 min at 37°C. The cells were lysed, and the proteins were precipitated with anti-STAT6 Ab (Santa Cruz Biotecnology, Santa Cruz, CA). Following SDS-PAGE and transfer to nitrocellulose membranes, anti-phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) or anti-STAT6 immunoblotting was performed.

IgE production

Spleen cells from naive mice were washed three times in PBS and resuspended in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM glutamine, and 50 μM 2-ME. Cells were cultured in 24-well plates (1 × 106 cells/well), and stimulated with anti-CD40 (clone 3/23, 10 ng/ml) and either murine IL-4 (15 ng/ml, 404-ML; R&D Systems) or murine IL-13 (30 ng/ml, 413-ML, R&D Systems). Simultaneous cultures were performed in the presence of mIL-4Ra (3 and 10 μg/ml) or human IL-4Ra (10 μg/ml). Fourteen days later, culture supernatants were collected and frozen for analysis of total IgE.

Sensitization and airway challenge

Groups of mice (four mice per group per experiment), 10–12 wk of age, were sensitized by i.p. injection of 20 μg of OVA (grade V; Sigma) emulsified in 25 mg of aluminum hydroxide (AlumMxject; Pierce, Rockford, IL) in a total volume of 100 μl on days 1 and 14. Mice were challenged (20 min) with the aerosols with OVA (1% in saline) for 3 days (days 28–30) using ultrasonic nebulization (AeroSonic ultrasonic nebulizer; DeVilbiss, Sommerset, PA). Control mice groups received OVA challenge alone. Forty-eight hours after the last OVA challenge (day 32) AHR was assessed, and tissues were obtained for further analysis.

Administration of murine/human IL-4Ra

Murine IL-4Ra (0.5 mg/kg) was administered s.c. 1 h before and 4 h after OVA challenge on days 28–30. The dose of mIL-4Ra was chosen based on preliminary lung function dose-response studies. The half-life (t1/2) of mIL-4Ra was previously measured at 0.83 h (our unpublished observations). As a control, a human IL-4 double mutant (hIL-4Ra; R121D/Y124D) was administered to one group of animals in the same fashion.

Determination of airway responsiveness

Airway responsiveness to inhaled methacholine (MCh) in conscious, spontaneously breathing animals was measured by barometric plethysmography (Burco, Troy, NY) as previously described (38). Mice were challenged with aerosolized saline or MCh (6–100 mg/ml) for 3 min, and readings were taken and averaged for 3 min following each nebulization. Enhanced pause (Penh) was expressed as the fold increase above saline challenge baseline values.

Bronchoalveolar lavage

Immediately after assessment of AHR, lungs were lavaged via the tracheal tube with HBSS (once, 1 ml, 37°C). Total leukocyte numbers were measured (Coulter counter; Coulter, Hialeah, FL). Differential cell counts were performed by counting at least 300 cells on cytocentrifuged preparations (Cytospin 2; Cytospin, Shandon, Runcorn, U.K.), stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA), and differentiated by standard hematological procedures.

Histochemistry

Lungs were fixed by immersion (1 ml) and immersion in 10% Formalin. Cells containing eosinophilic major basic protein (MBP) were identified by immunohistochemical staining as previously described using rabbit anti-mouse MBP (provided by J. J. Lee, Mayo Clinic, Scottsdale, AZ) (39). The slides were examined in a blinded fashion with a Nikon microscope (Ni-El, Melville, NY) equipped with a fluorescence filter system. Numbers of peribronchial eosinophils in the tissues were evaluated using IPLab software (Signal Analytics, Vienna, VA) for the Macintosh, counting six to eight different fields per animal.

For detection of mucus-containing cells in Formalin-fixed airway tissue, sections (10 μm) were cut and stained with periodic acid-Schiff (PAS), hematoxylin, and eosin. The number of mucus-containing cells per millimeter of basement membrane was determined. In addition, epithelial cells were scored according to their mucus content (<75% of the cytoplasm PAS positive, 0; >75% of the cytoplasm PAS positive, 1).
Cytokine levels in the bronchoalveolar lavage fluid (BALF) were measured by ELISA. Briefly, 96-well plates (Immuno 2; Dynatech, Chantilly, VA) were coated with anti-IFN-γ (R4-6A2), anti-IL-4 (11B11), or anti-IL-5 (TRFK-5; all from PharMingen) and blocked with PBS/10% FCS overnight, and samples were added. Biotinylated anti-IFN-γ (XMG 1.2), anti-IL-4 (BVD6-24G2), and anti-IL-5 (TRFK-5) were used as detection Abs (all from PharMingen), and the reaction was amplified with avidin-HRP (Sigma). Cytokine levels were determined by comparison with the known cytokine standards (PharMingen). The limit of detection for each cytokine was 1.5 pg/ml. Similarly, IL-13 levels were determined using a quantitative colorimetric sandwich ELISA kit (Quantikine M mouse IL-13 ELISA, R&D Systems). The limit of detection was 4 pg/ml. IL-13 levels were calculated by comparison with known mouse IgE standards (all from PharMingen), and the reaction was amplified with avidin-HRP (Sigma). The limit of detection was 100 pg/ml for total IgE.

**Measurement of total and OVA-specific Ab**

Serum levels of total IgE and OVA-specific IgE and IgG2a were measured by ELISA. Briefly, 96-well plates (Immuno 2; Dynatech) were coated with either OVA (5 μg/ml) or purified anti-IgE (02111D; PharMingen). After addition of serum samples, a biotinylated anti-IgE Ab (02122D; PharMingen) was used as detecting Ab, and the reaction was amplified with avidin-HRP (Sigma). IgG2a was detected using alkaline phosphatase-labeled anti-IgG2a (02013 E; PharMingen). The OVA-specific Ab titer of the samples was related to pooled standards that were generated in the laboratory and expressed as ELISA units (EU) per milliliter. Total IgE levels were calculated by comparison with known mouse IgE standards (PharMingen). The limit of detection was 100 pg/ml for total IgE.

**FACS analysis**

Spleen mononuclear cells, PBLN cells, or lung digest cells from mice receiving OVA challenge alone, OVA-sensitized/challenged mice, and OVA-sensitized/challenged mice pretreated with mIL-4Ra were isolated as described above. The cells were washed three times in PBS and plated in 96-well round-bottom plates at 400,000 cells/well. Following preincubation with mouse serum, cells were incubated with FITC-conjugated anti-CD3 (clone 145-2C11), anti-B220 (clone RA3-6B2), anti-CD23 (clone B3(B4)), PE-conjugated anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7; PharMingen), or isotype controls in staining buffer (PBS, 2% FCS, and 0.1% sodium azide) for 30 min on ice. After washing, cells were examined (10,000 gated events were analyzed) using an EPICS XL analyzer (Coulter). Results are expressed as the percentage of cells expressing a given surface marker.

**Statistical analysis**

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were made using Tukey-Kramer highest significance difference test. The p values for significance were set at 0.05. Values for all measurements were expressed as the mean ± SEM.

**Results**

**Murine IL-4Ra inhibits both IL-4- and IL-13-induced phosphorylation of STAT6**

To demonstrate the effect of mIL-4Ra on known IL-4- and IL-13-induced signal transduction events in vitro, peritoneal macrophages and spleen cells from naive mice were isolated and stimulated with either IL-4 or IL-13 in the presence of the absence of the antagonist. Anti-phosphotyrosine immunoblot analysis (Fig. 1) of anti-STAT6 immunoprecipitates revealed increases in the tyrosine phosphorylation status of STAT6 when either cell population was stimulated with IL-4 or IL-13. Preincubation of cells with mIL-4Ra before cytokine stimulation resulted in a marked decrease in the phosphorylation of STAT6 in response to both cytokines. Stripping and reprobing of the immunoblot with anti-STAT6 Ab revealed comparable amounts of STAT6 in all lanes. Similar results were seen when purified T and B cells from spleen were used. Incubation of T cells with IL-4 and of B cells with IL-4 and IL-13 resulted in STAT-6 phosphorylation that was inhibited when the cells were preincubated for 10 min with mIL-4Ra (data not shown).

**Murine IL-4Ra inhibits IL-4- and IL-13-induced IgE production**

The effects of mIL-4Ra and hIL-4Ra on IL-4- and IL-13-stimulated IgE production from cultured splenocytes were investigated (Fig. 2). Supernatants from splenocytes cultured with anti-CD40 plus IL-4 or IL-13 contained increased levels of IgE compared with those from cultures containing anti-CD40 alone. These levels were significantly reduced in cultures containing mIL-4Ra. In contrast, hIL-4Ra had no significant effect on IL-4- and IL-13-stimulated IgE levels.

**AHR is reduced by mIL-4Ra**

The effect of mIL-4Ra (and the control, hIL-4Ra) on the development of AHR to inhaled MCh in OVA-sensitized/challenged mice was determined. Baseline Penh values in mice given OVA challenge alone (0.81 ± 0.05), OVA-sensitized/challenged mice receiving OVA challenge alone (0.81 ± 0.05), OVA-sensitized/challenged mice, and OVA-sensitized/challenged mice pretreated with mIL-4Ra were isolated as described above. The cells were washed three times in PBS and plated in 96-well round-bottom plates at 400,000 cells/well. Following preincubation with mouse serum, cells were incubated with FITC-conjugated anti-CD3 (clone 145-2C11), anti-B220 (clone RA3-6B2), anti-CD23 (clone B3(B4)), PE-conjugated anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7; PharMingen), or isotype controls in staining buffer (PBS, 2% FCS, and 0.1% sodium azide) for 30 min on ice. After washing, cells were examined (10,000 gated events were analyzed) using an EPICS XL analyzer (Coulter). Results are expressed as the percentage of cells expressing a given surface marker.

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![Image](http://www.jimmunol.org/)

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OVA-sensitized/challenged mice receiving mIL-4Ra (0.82 ± 0.07), and OVA-sensitized/challenged mice receiving hIL-4Ra (0.79 ± 0.06) did not differ significantly (p > 0.05).

Compared with mice given OVA challenge alone, OVA-sensitized/challenged mice developed significant AHR to inhaled MCh (Fig. 3). The administration of mIL-4Ra (during OVA challenge, but after sensitization) to OVA-sensitized/challenged mice significantly inhibited AHR (Fig. 3). In contrast, hIL-4Ra had no significant effect on the development of AHR in OVA-sensitized/challenged mice (Fig. 3).

**BALF eosinophil numbers are inhibited by mIL-4Ra**

The number and type of inflammatory cells in the airways of OVA-sensitized/challenged mice receiving mIL-4Ra or hIL-4Ra were assessed in BALF (Fig. 4). Total cell numbers recovered in BALF were increased in OVA-sensitized/challenged mice (828 ± 68 × 10^5 cells/ml; n = 23) compared with those in mice receiving OVA challenge alone (597 ± 71 × 10^5 cells/ml; n = 23). Administration of mIL-4Ra (573 ± 51 × 10^5 cells/ml; n = 17), but not hIL-4Ra (829 ± 88 × 10^5 cells/ml; n = 15), inhibited the increase in total cell number. The inflammatory cell types recovered in the BALF of mice that received OVA challenge alone consisted almost entirely of macrophages. In contrast, OVA-sensitized/challenged mice developed significant eosinophilic (355 ± 59 × 10^3 cells/ml; n = 23), with a smaller increase in BALF lymphocytes (Fig. 4).

Administration of mIL-4Ra significantly reduced the number of BALF eosinophils (121 ± 25 × 10^3 cells/ml; n = 17), while hIL-4Ra had no significant effect (416 ± 60 × 10^3 cells/ml; n = 15). Lymphocyte numbers were unaffected.

**Tissue eosinophilic inflammation is inhibited by mIL-4Ra**

Eosinophilic inflammation was further assessed in airway tissues using an Ab directed against MBP. Mice that received OVA challenge alone demonstrated few MBP^+ eosinophils (Fig. 5, a and e). OVA sensitization and challenge of mice resulted in a significant increase in the number of tissue MBP^+ eosinophils (Fig. 5, b and e). Administration of mIL-4Ra, but not hIL-4Ra, during OVA challenge significantly reduced the number of tissue MBP^+ eosinophils (Fig. 5, c–e). These results are consistent with our findings in BALF and confirm the inhibitory activity of mIL-4Ra, but not hIL-4Ra, on the development of allergic eosinophilic inflammation.

**Mucus hyperproduction in the airway epithelium is reduced by mIL-4Ra**

Lung sections were stained to identify mucus-containing cells in the airway epithelium. The number of epithelial cells per millimeter of basement membrane in the large airways of mice receiving OVA challenge alone was 158 ± 6 cells/mm, but these airways were almost devoid of cells staining positively for mucus (Fig. 6, a and d). The number of epithelial cells per millimeter of basement membrane in the large airways of OVA-sensitized/challenged mice was slightly elevated at 174 ± 4 cells/mm. However, of these epithelial cells, 141 ± 5 cells/mm stained positively for mucus (Fig. 6, b and d). Moreover, of those cells staining positively for mucus, 111 ± 5 were scored 1 (>75% of cytoplasm mucus-positive; Fig. 6e). In OVA-sensitized/challenged mice treated with mIL-4Ra, the number of cells per millimeter basement membrane was similar to that in the untreated OVA-sensitized/challenged animals. In contrast, only 99 ± 4 cells/mm stained positively for mucus (Fig. 6, c and d), and of these, only 38 ± 2 cells/mm were scored 1 (Fig. 6e).

**Reduced cytokine levels in BALF following mIL-4Ra administration**

The levels of IL-4, IL-5, IL-13, and IFN-γ in BALF were measured. Low levels of IL-4, IL-5, and IFN-γ (but not IL-13), were present in mice that received OVA challenge alone (Figs. 7). Following OVA sensitization/challenge, significantly (p < 0.05) elevated levels of IL-4, IL-5, and IL-13, but not IFN-γ, were present.
in BALF (Fig. 7). Administration of mIL-4Ra significantly \((p < 0.05)\) inhibited the elevations in BALF IL-4, IL-5, and IL-13 levels, but had no significant effect on IFN-\(\gamma\) levels. Administration of hIL-4Ra had no significant effect \((p > 0.05)\) on BALF cytokine levels (data not shown).

Murine IL-4Ra inhibits the development of OVA-specific IgE

Serum OVA-specific IgE and IgG2a levels were measured following OVA challenge. Mice receiving OVA challenge alone had no detectable OVA-specific IgE or IgG2a \((n = 12)\). Serum levels of OVA-specific IgE and IgG2a in OVA-sensitized/challenged mice were 2946 ± 519 and 3207 ± 489 EU/ml, respectively \((n = 12)\). Administration of mIL-4Ra during OVA challenge significantly inhibited \((p < 0.05)\) development of the IgE response \((899 ± 196\) EU/ml; \(n = 12)\), while levels of IgG2a were increased \((6174 ± 2158\) EU/ml; \(n = 12)\). In contrast, hIL-4Ra had no significant effect on either OVA-specific IgE or IgG2a.

Effect of mIL-4Ra on lung cell composition

No significant differences in the number of cells expressing CD3, CD4, or CD8 were seen between mice receiving OVA challenge

![Figure 5](http://www.jimmunol.org/) Murine IL-4Ra significantly inhibits tissue MBP+ eosinophil number following OVA sensitization and challenge. Immunohistochemical localization of airway tissue eosinophils using MBP Ab was performed in OVA-challenged mice (a), OVA-sensitized/challenged mice (b), OVA-sensitized/challenged mice given mIL-4Ra (c), and OVA-sensitized/challenged mice given hIL-4Ra (d). Magnification, ×64. Peribronchial eosinophil number (counts per field) were quantified (e) for each group \((n = 8)\) as described in Materials and Methods. The results of each group are expressed as the mean ± SEM. * Significant differences \((p < 0.05)\) from OVA-challenged mice; #, significant differences \((p < 0.05)\) from OVA-sensitized/challenged mice given mIL-4Ra.

![Figure 6](http://www.jimmunol.org/) Murine IL-4Ra inhibits mucus hyperproduction in the airway epithelium of OVA-sensitized/challenged mice. Histochemical staining (PAS) was performed to identify mucus-containing cells in the airway epithelium of OVA-challenged mice (a), OVA-sensitized/challenged mice (b), and OVA-sensitized/challenged mice given mIL-4Ra (c). The number of mucus-positive cells per millimeter basement membrane (d) and the number of mucus-positive cells scored 1 per millimeter basement membrane (e) were quantified for each group \((n = 4)\) as described in Materials and Methods. The results of each group are expressed as the mean ± SEM. * Significant differences \((p < 0.05)\) from OVA-challenged mice; #, significant differences \((p < 0.05)\) from OVA-sensitized/challenged mice given mIL-4Ra.
alone (CD3, 34 ± 5%; CD4, 21 ± 4%; CD8, 7 ± 0.4%) and OVA-sensitized/challenged (CD3, 33 ± 2%; CD4, 20 ± 2%; CD8, 5 ± 0.4%) mice. However, the numbers of cells expressing B220 (B cells; OVA challenge alone, 23 ± 1%; OVA-sensitized/challenged, 31 ± 2%) and CD23 (OVA challenge alone, 14 ± 1%; OVA-sensitized/challenged, 27 ± 3%) as well as double-positive cells (OVA challenge alone, 11 ± 1%; OVA-sensitized/challenged, 24 ± 3%) were significantly increased. The number of cells expressing CD23 (20 ± 1%) was significantly reduced (p < 0.05) in OVA-sensitized/challenged mice pretreated with mIL-4Ra. The numbers of cells expressing B220 (21 ± 2%) or CD23/B220 double-positive cells (16 ± 1%) were reduced, although not significantly. As in the lung, only increases in the number of cells expressing CD23 and B220 were seen in the spleen and PBLN after OVA sensitization and challenge. However, in contrast to lung digest cells, mIL-4Ra had little effect on their expression (data not shown).

Discussion

In the present study, we demonstrate that a truncated murine IL-4 mutant (C118 deletion) protein (mIL-4Ra) that was effective in preventing both IL-4- and IL-13-induced signal transduction and IgE production also inhibited the development of allergic airway eosinophilia and AHR in OVA-sensitized mice when given during challenge. Overlap of the functions of IL-4 and IL-13 results from the IL-4R α-chain forming an important functional signaling component of both cytokine receptor complexes (15, 31). Here, IL-4 and IL-13 induced STAT6 phosphorylation in murine peritoneal macrophages and murine splenocytes as well as isolated T cells and B cells, confirming previous observations (40–46). Importantly, not only IL-4-induced, but also IL-13-induced, phosphorylation of STAT6 was inhibited following preincubation of the cells with the C118 deletion mIL-4Ra. Previously, the murine IL-4 double-mutant protein, Q116D/Y119D, was also shown to inhibit IL-4-induced STAT6 phosphorylation in BA/F3 cells (36). The critical role of IL-4 and the STAT6 pathway in IgE isotype switching in B cells is well established (17, 46, 47). As we show, IL-13 can activate STAT6 and can induce IgE production in mice (4, 18, 19, 48, 49). Increased levels of IgE were measured following culture of murine splenocytes with anti-CD40 and IL-4 and to a lesser extent with anti-CD40 and IL-13. Both IL-4- and IL-13-induced IgE production were reduced in a concentration-dependent manner following coculture with mIL-4Ra, consistent with the inhibitory action on STAT6 phosphorylation in splenocytes. This effect of a murine IL-4 mutant on IL-4- and IL-13-induced IgE production has not previously been demonstrated in vitro, although the human IL-4 single-mutant protein, Y124D, was shown to inhibit IL-4- and IL-13-induced IgG4 and IgE production from cultured human B cells (50).

Following OVA sensitization and challenge, mice developed increased airway responsiveness to aerosolized MCh. This development of AHR was associated with significant airway eosinophilia as well as increased OVA-IgE in the serum. Treatment of sensitized mice with mIL-4Ra, but not hIL-4Ra, during OVA challenge significantly inhibited AHR, the associated airway eosinophilia, and OVA-IgE production. These are the first results demonstrating the effect of a mIL-4Ra on Ag-induced airway inflammation and AHR in previously sensitized mice and are consistent with the inhibitory activity of an Ab to the IL-4R in SRBC-sensitized/challenged mice (51).

The role of IL-4 in the development of allergic airway eosinophilia and AHR has been evaluated in a number of studies. Using IL-4 Ab administered during the sensitization phase in mice or mice deficient in IL-4, the increase in serum IgE and the development of airway eosinophilia and/or AHR seen following sensitization and allergen provocation were reduced or abolished (25, 27, 52). In contrast, administration of Ab to IL-4 or soluble IL-4R, during the allergen challenge phase in sensitized animals generally had less effect on airway eosinophilia and no effect on AHR (25, 26, 28, 52). Together, these observations suggested that while IL-4 may be important during the initial sensitization or allergen-priming event, alternate mechanisms play a role in the subsequent response to allergen challenge.

Administration of a soluble hIL-13R fusion protein (sIL-13Rα2-Fc) during challenge of sensitized mice, which specifically binds and neutralizes mIL-13, was shown to inhibit AHR in one study and both AHR and airway eosinophilia in a second report (29, 30). Moreover, administration of IL-13 or overexpression of IL-13 in the airways induced airway eosinophilia and AHR (24, 29, 30). Thus, the effectiveness of mIL-4Ra in inhibiting allergen-induced airway eosinophilia and AHR when administered to previously sensitized mice during the allergen challenge phase may not solely be due to its ability to block IL-4-induced effects, but may reflect the capacity to inhibit IL-13-induced responses as well. Importantly, neither IL-13 nor IL-4 triggered airway eosinophilia and AHR in IL-4R α-chain-deficient mice (30). This is consistent with the present study, demonstrating the effectiveness of a mIL-4Ra that binds to the IL-4R α-chain in preventing both IL-4- and IL-13-induced responses.

FIGURE 7. Murine IL-4Ra reduced cytokine levels in BALF of OVA-sensitized/challenged mice. The levels of BALF IL-4 (a), IL-13 (b), IL-5 (c), and IFN-γ (d) were measured in OVA-challenged mice, OVA-sensitized/challenged mice, and OVA-sensitized/challenged mice given mIL-4Ra.
Mucus (goblet) cell hyperplasia and mucus hyperproduction are common features in the asthmatic airway and have been demonstrated in murine models of allergic AHR as well as in the present study (53–55). Treatment of mice with mIL-4Ra inhibited mucus cell hyperplasia and mucus hyperproduction, indicating the critical role of IL-4 and/or IL-13 in regulating these responses. Overexpression or administration of IL-4 or IL-13 in mice has been shown to induce MUC5AC gene expression and/or mucus hypersecretion (23, 24, 29, 30). This is consistent with the lack of mucus production seen in the airways of OVA-sensitized/challenged STAT6-deficient mice (55). Although both IL-4 and/or IL-13 can induce mucus production, it is less apparent if either is critically required. OVA challenge of naïve mice receiving transfer of OVA-specific, IL-4-deficient Th2 cells resulted in marked mucus hyperproduction in the airways, suggesting that IL-4 was not necessary (56). In contrast, administration of a soluble IL-4R (which blocks only IL-4-mediated responses) before challenge of OVA-sensitized mice inhibited mucus hyperproduction (28). Although both IL-4 and IL-13 seem to be able to regulate mucus hyperproduction, the contribution of mucus to the development of AHR is less clear. Previously, we demonstrated the reconstitution of airway eosinophilia and AHR in STAT6-deficient mice given IL-5 during challenge, but in the absence of mucus production (55). In addition, mucus production appears to be a later event, following the peak of AHR (our unpublished observations).

The eosinophil is a characteristic feature of allergic asthma and is thought to be a major effector cell in the pathogenesis of clinical and experimental allergic AHR (12, 27, 39, 55, 57). Indeed, mIL-4Ra inhibited both AHR and the associated eosinophilic infiltration in the present study. One mechanism of mIL-4Ra-mediated reduction of the eosinophilic infiltrate may reflect inhibition of VCAM-1 expression on endothelial cells. IL-4 and IL-13 have both been shown to up-regulate VCAM-1, facilitating the preferential recruitment of eosinophils (21, 22). However, recent observations have confirmed that STAT6, important in IL-4 and IL-13 signaling, is not essential for the allergen-induced expression of VCAM-1, and that TNF-α can also increase VCAM-1 expression (58, 59).

The development of airway eosinophilia and AHR was also associated with increased levels of IL-4, IL-5, and IL-13 in the BALF, consistent with the development of a Th2-mediated allergic response. Treatment with mIL-4Ra significantly inhibited these elevations of BALF IL-4, IL-13, and IL-5 levels. This inhibitory effect of mIL-4Ra on BALF IL-5 is notable, as IL-5 appears critical in the development of allergic airway eosinophilia and subsequent AHR in mice (10–12, 27, 39). The crucial role of IL-4 in the priming of T cells for commitment to an IL-4-, IL-13-, and IL-5-producing, Th2 phenotype is well established (13). Whether the inhibitory effect of mIL-4Ra is an effect on commitment, expansion, recruitment, or activation of T cells remains to be determined. Murine IL-4Ra had no significant effect on the numbers of CD4+ and CD8+ T cells in the lung. Furthermore, one would largely expect IL-4-induced T cell commitment to occur during the initial sensitization response, rather than during challenge, the period when mIL-4Ra was administered. Sensitization of IL-4-deficient mice or administration of IL-4-neutralizing Ab during sensitization of mice is known to prevent the commitment of T cells to the Th2 phenotype, but not when IL-4 Ab is given during the challenge phase (13, 25). IL-13 has also been demonstrated to have some regulatory effect on T cells and has now been shown to induce STAT6 phosphorylation in human NK and T cells (43). Moreover, IL-13-deficient mice have an impaired Th2 response following Ag sensitization (16). The effect of mIL-4Ra on Th2 cytokine production may thus reflect an inhibitory activity that is not directly on T cells. Both IL-4 and IL-13 modulate macrophage function and IL-12 release, which can promote a Th1 response (60–62). Other mechanisms may also contribute to the reduced cytokine response and inhibition of allergic eosinophilia and AHR by mIL-4Ra. IL-4 and IL-13 are known to up-regulate CD23 expression and MHC II on the surface of B cells and other cells, including macrophages, enhancing Ag presentation (18, 19, 63, 64). Indeed, the expression of CD23 on B cells in the lung was significantly reduced in mice treated with mIL-4Ra, concordant with the inhibitory effects of the murine IL-4 double mutant, Q116D/Y119D, in vitro (36).

Furthermore, the elevations in serum OVA-specific IgE seen following OVA sensitization and challenge were also reduced in mIL-4Ra-treated mice. The role of IL-4 and or IL-13 in the production of IgE, and the inhibitory effect of mIL-4Ra on this response in vitro have been discussed. The role of allergen-specific IgE in airway eosinophilia and AHR is nevertheless controversial. Studies in IL-4-deficient mice and with IL-4-neutralizing Ab, like the present study, demonstrate absence or reduced levels of serum IgE as well as abrogated airway eosinophilia and AHR (25–27, 65). One mechanism could be the absence of IgE-mediated mast cell activation, which may lead to bronchoconstriction and enhanced airway responsiveness (66). However, mast cell-deficient mice develop normal airway eosinophilia and AHR in response to allergen sensitization and challenge (67). Furthermore, it has been shown that following systemic sensitization and repeated airway challenge, B cell-deficient mice and CD40-deficient mice develop airways eosinophilia and AHR without Ab production (25, 68).

In summary, we demonstrate that a mutation in the C-terminus region (C118 deletion) of IL-4 generates a murine IL-4 mutant protein that inhibits both IL-4- and IL-13-induced signal transduction and IgE production. Moreover, when administered to sensitized mice during allergen (OVA) challenge, the protein inhibited the development of allergic airway eosinophilia, mucus hyperproduction, and AHR. This is of significance given the recent observations reported by Henderson et al. (28). Using soluble IL-4R administered to the airways before allergen challenge, they demonstrated an inhibitory effect on airway eosinophilia and mucus hyperproduction, but without an effect on AHR. While the overlapping and independent roles IL-4 and IL-13 play in the generation of the allergic airway response are still not fully understood, it is apparent that an antagonist of both IL-4 and IL-13, rather than either alone, may have significant benefits for the treatment of allergic asthma.

References


