

IL-4 induces IL-13-independent allergic airway inflammation

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Background: The related T_H2 cytokines IL-4 and IL-13 are produced during allergic responses, signal through receptors that contain IL-4 receptor (IL-4R) α , and promote allergic inflammation by activating signal transducer and activator of transcription 6. IL-4 promotes T_H2 response induction, and IL-13 is necessary and sufficient to induce airways hyperresponsiveness (AHR) and goblet cell hyperplasia in some mouse models of asthma. The nonredundant role of IL-13 could reflect unique IL-13 activation of a signaling pathway, inhibitory effects induced by IL-4 but not IL-13, or greater production-potency of IL-13 than IL-4.

Objectives: We sought to distinguish among these possibilities by determining whether IL-4 inhalation can induce acute allergic airways disease in the absence of IL-13.

Methods: Mice were inoculated intratracheally with IL-13 or a long-acting formulation of IL-4. Responses of IL-13-deficient and IL-13-sufficient mice were compared, as were responses in mice treated with a potent IL-13 antagonist, anti-IL-4R α antibody, or control reagents.

Results: IL-4 inhalation stimulated bronchoalveolar lavage fluid eosinophilia, AHR, and goblet cell hyperplasia. These responses were similar in IL-13-deficient and IL-13-sufficient mice and were not inhibited by an IL-13 antagonist but were blocked by anti-IL-4R α antibody.

Conclusion: IL-4 can induce IL-13-independent AHR and goblet cell hyperplasia. Thus the greater role for IL-13 than IL-4 in the induction of these acute allergy-related changes reflects increased production, potency, or both of IL-13 relative to IL-4 rather than a unique IL-13-signaling pathway or a suppressive effect of IL-4.

Clinical implications: Dual IL-4/IL-13 inhibition might be more effective than selective IL-13 inhibition at suppressing allergic

inflammation in some circumstances. (*J Allergy Clin Immunol* 2006;118:410-9.)

Key words: Airways hyperresponsiveness, eosinophil, goblet cell, IL-4, IL-4 receptor, IL-13, mouse, mucus

In addition to regulating lymphopoiesis, lymphocyte survival, and lymphocyte activation, cytokines regulate patterns of lymphocyte differentiation and the proinflammatory effector mechanisms that are induced by an immune response.¹ This is particularly true for allergic inflammation, which is characterized by increased lymphocyte production of T_H2 cytokines (IL-4, IL-5, IL-9, and IL-13) and which can be induced by T_H2 cytokine treatment or overexpression.¹ Two related T_H2 cytokines, IL-4 and IL-13, play the dominant role in the induction and effector phases of allergic inflammation.²⁻¹² Both of these cytokines bind to and activate receptors that include the IL-4 receptor (IL-4R) α polypeptide,¹³ which forms the type 1 IL-4R heterodimer with the cytokine receptor common γ chain¹⁴ and the type 2 IL-4R heterodimer with the IL-13-binding polypeptide, IL-13 receptor (IL-13R) α 1.¹⁵⁻¹⁷ The type 1 IL-4R is only activated by IL-4, whereas the type 2 IL-4R is activated by both IL-4 and IL-13.^{2,18} The 2 IL-4Rs transduce similar signals, including activation of the transcription factor signal transducer and activator of transcription 6 (STAT6) and activation of adaptor proteins, such as insulin receptor substrate 2, that promote activation of phosphatidylinositol-3 kinase.¹⁹⁻²¹ Consequently, it would be reasonable to expect IL-4 to induce all effects that are induced by IL-13, as well as some unique effects.

The unique effects of IL-4 are well established. Because T cells express the type 1, but not the type 2, IL-4R, IL-4, but not IL-13, can directly promote a T_H2 response.²²⁻²⁵ Differences in type 1 and type 2 IL-4R expression might also be responsible for the unique effects of IL-4 on mast cells, dendritic cells, and natural killer cells.^{8,22,26,27}

The putative ability of IL-4 to replicate all effects induced by IL-13 is considerably more controversial. Although IL-4 is often required to induce a T_H2 response, many studies suggest that IL-13 contributes more than IL-4 to the induction of critical inflammatory effector mechanisms, including those that promote host defense against worm infections,^{2,19,28} induce airways hyperresponsiveness (AHR) and goblet cell hyperplasia in mouse models of asthma,^{3,4,7,9,11,27,28-31} and suppress the control of viral infections and the rejection of malignant tumors.^{32,33}

The bases for what seem to be IL-13-specific or IL-13-dominant effects are uncertain. Possibilities include more

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Abbreviations used

AHR:	Airways hyperresponsiveness
APF:	<i>Ascaris</i> species pseudocoelomic fluid
BAL:	Bronchoalveolar lavage
IL-4C:	Anti-IL-4 mAb that is saturated with IL-4
IL-4R:	IL-4 receptor
IL-13R:	IL-13 receptor
OVA:	Ovalbumin
PAS:	Periodic acid-Schiff
Penh:	Enhanced pause
STAT6:	Signal transducer and activator of transcription 6

potent activation of the type 2 IL-4R by IL-13 than IL-4, greater production of IL-13 than IL-4, and suppression of type 2 IL-4R-mediated effects by IL-4 activation of the type 1 IL-4R.³⁴ In addition, recent evidence that IL-13 can uniquely signal through an additional cell membrane polypeptide, IL-13R α 2,³⁵ which had been thought to act solely as a decoy receptor,^{2,36-38} raises the possibility that IL-13-unique or IL-13-dominant effects might involve signaling through this receptor. Taken together, these considerations led us to evaluate whether IL-4, in the absence of IL-13, can induce the 2 asthma-related pulmonary phenomena, AHR and goblet cell hyperplasia, that are IL-13 dominant in mouse models of asthma. Our results demonstrate that both phenomena are induced by IL-4 when IL-13 is absent or suppressed and consequently suggest that IL-13-dominant induction of these effects reflects either greater production, persistence, or potency of IL-13 than IL-4.

METHODS

Mice

BALB/c female mice and athymic nude male mice were purchased from NCI (Frederick, Md). IL-13-deficient mice³⁹ bred for more than 10 generations onto a BALB/c background were obtained from Andrew McKenzie (Cambridge, United Kingdom). All procedures were performed in accordance with the ethical guidelines in the "Guide for the care and use of laboratory animals" of the Institutional Animal Care and Use Committee and approved by the Veterinary Services Departments of the Cincinnati Children's Hospital Medical Center Research Foundation and the Cincinnati Veterans Administration Medical Center.

Immunologic reagents

Murine soluble IL-13R α 2-Fc fusion protein¹⁹ and a control fusion protein were generously provided by Debra Donaldson and Marion Kasaian at Wyeth Research (Cambridge, Mass). Recombinant mouse IL-4 was purchased from PeproTech (Rocky Hill, NJ). Rat IgG2b anti-mouse IL-4 mAb (BVD4-1D11)⁴⁰ was obtained from the American Type Culture Collection (Rockville, Md) with the permission of DNAX Corporation (Palo Alto, Calif) and the help of Robert Coffman. Rat IgG2a anti-mouse IL-4R α mAb (M1)⁴¹ was obtained from Immunex Corporation (now Amgen, Seattle, Wash). Hybridomas that produce monoclonal rat IgG2a and IgG2b control mAbs (GL117 and J1.2, respectively) were obtained from DNAX. Monoclonal antibodies were produced in Pristane-primed (Acros Organic, Morris Plains, NJ) athymic nude mice as ascites and purified

by means of ammonium sulfate precipitation and diethyl-aminoethyl-cellulose column chromatography.

IL-4/anti-IL-4 complexes

To extend the *in vivo* half-life of IL-4, mice were inoculated in some experiments with complexes produced by mixing recombinant mouse IL-4 with a neutralizing anti-IL-4 mAb (BVD4-1D11) at a 2:1 molar (1:5 weight) ratio, which saturates the mAb with IL-4. We have previously demonstrated that these complexes have an *in vivo* half-life of approximately 1 day and slowly dissociate, releasing biologically active IL-4.⁴² Because these complexes contain a single IgG molecule, they neither fix complement nor react more avidly than uncomplexed serum IgG with Fc γ receptors. Because IL-4 cannot bind to IL-4Rs when it is complexed with BVD4-1D11, there is no possibility for these complexes to cross-link IL-4Rs to Fc γ Rs.

Intratracheal inoculation

Mice were anesthetized by means of intraperitoneal injection of ketamine and xylazine and allowed to hang vertically with their mouths open, supported by a taut string placed under their canine teeth. Their tongues were gently withdrawn with a blunt forceps to keep them from swallowing, and 40 μ L of vehicle (1% autologous serum in PBS) with or without anti-IL-4 mAb that is saturated with IL-4 (IL-4C) was pipetted onto the base of the tongue. When the mice had aspirated the pipetted solution, they were placed on their sides in a box flushed with 100% oxygen until they recovered from the anesthesia.

Inoculation protocol

Five to 7 mice per group were used for each experiment. Mice were inoculated intratracheally with saline or IL-4C daily for 7 days. In some experiments mice were also injected intraperitoneally every other day with 100 μ g of IL-13 antagonist (soluble IL-13R α 2-Fc fusion protein) or a control fusion protein or 3 mg of anti-IL-4R α mAb (M1) or an isotype-matched control mAb (GL117), starting simultaneously with the initiation of intratracheal inoculations.

Bronchoalveolar lavage

Mice were killed, and polyethylene catheters (OD, 0.97 mm; Becton Dickinson, Sparks, Md) were inserted into their tracheas. Lungs underwent lavage 3 times with 1 mL of Ca²⁺-free and Mg²⁺-free HBSS (Gibco-BRL Rockville, Md) that contained 0.02% EDTA and ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid. The bronchoalveolar lavage (BAL) fluid was centrifuged at 1000 rpm for 5 minutes at 4°C. Cell pellets were resuspended in 1.5 mL of cold HBSS with 10% newborn bovine serum and 0.2% NaN₃ and counted with a Coulter counter (Coulter Electronics, Hialeah, Fla). Two hundred thousand cells were centrifuged at 700 rpm for 5 minutes onto glass microscopic slides with a cytocentrifuge (Shandon Scientific, Pittsburgh, Pa). Slides were stained with Giemsa stain (Sigma, St Louis, Mo). At least 200 cells per slide were examined microscopically and characterized as neutrophils, lymphocytes, macrophages, or eosinophils.

Determination of airway responsiveness to methacholine by means of barometric plethysmography

Mice were placed, unrestrained, in cylindrical plexiglass plethysmograph chambers that were connected to a Buxco nebulized control 10 aerosol delivery system and a Buxco Max II apparatus for analyzing barometric plethysmography (Buxco Electronics, Sharon, Conn). Baseline measurements of enhanced pause (Penh)⁴³ were made over a 5-minute period. Mice were then challenged for 3 minutes by means of inhalation of aerosolized β -methacholine in PBS produced with a DeVilbiss Model 5500D-030 nebulizer (DeVilbiss

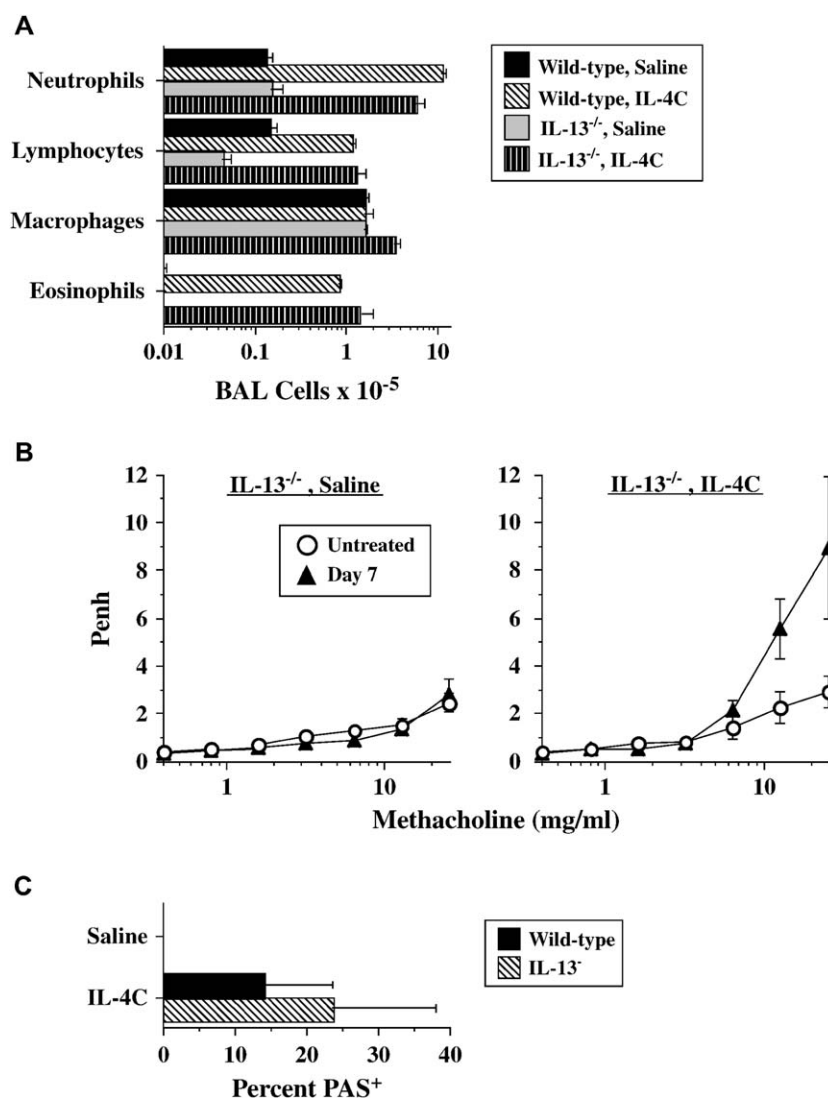


FIG 1. IL-4C induces allergic airway inflammation in IL-13-deficient mice. Wild-type and IL-13-deficient mice were tested for airway responsiveness and then inoculated intratracheally daily for 7 days with IL-4C (2 μ g of IL-4 + 10 μ g of anti-IL-4 mAb per dose). Mice were retested for airway responsiveness on day 8, after which BAL was performed and PAS-stained lung sections were prepared. **A**, Means and SEs of BAL cells. Eosinophils are significantly increased in IL-4C-treated versus saline-treated wild-type and IL-13-deficient mice but similar in IL-4C-treated wild-type and IL-13-deficient mice. **B**, Airway responses (means and SEs) to methacholine. IL-4C caused a significant increase in methacholine responsiveness in IL-13-deficient mice. **C**, Percentages of bronchiolar epithelial cells that were PAS positive are shown for saline- and IL-4C-treated wild-type and IL-13-deficient mice. Percentages of PAS-positive cells are significantly increased in IL-4C-treated versus saline-treated wild-type and IL-13-deficient mice but do not differ significantly between IL-4C-treated groups.

Health Care, Inc, Somerset, Pa), starting at a methacholine concentration of 0.8 mg/mL. Penh measurements were made for 5 minutes, starting 2 minutes after completion of exposure to the aerosolized methacholine, and average Penh values for the 5-minute period were calculated. The procedure was then serially repeated with methacholine at concentrations of 1.6, 3.2, 6.4, 12.8, and 25.6 mg/mL.

Lung histology

After BAL, lungs were inflated by injecting 1 mL of zinc formalin fixative through the catheter used to perform BAL. Lungs were then

removed and fixed in the same solution. Lung sections were stained with periodic acid-Schiff (PAS) and examined microscopically.

Evaluation of goblet cell hyperplasia

Lung sections stained with PAS were evaluated blindly. Three bronchioles containing 90 to 130 epithelial cells were identified on each section. PAS-positive (goblet) cells and total epithelial cell nuclei per bronchiole section were counted, and the ratio was converted into a percentage.

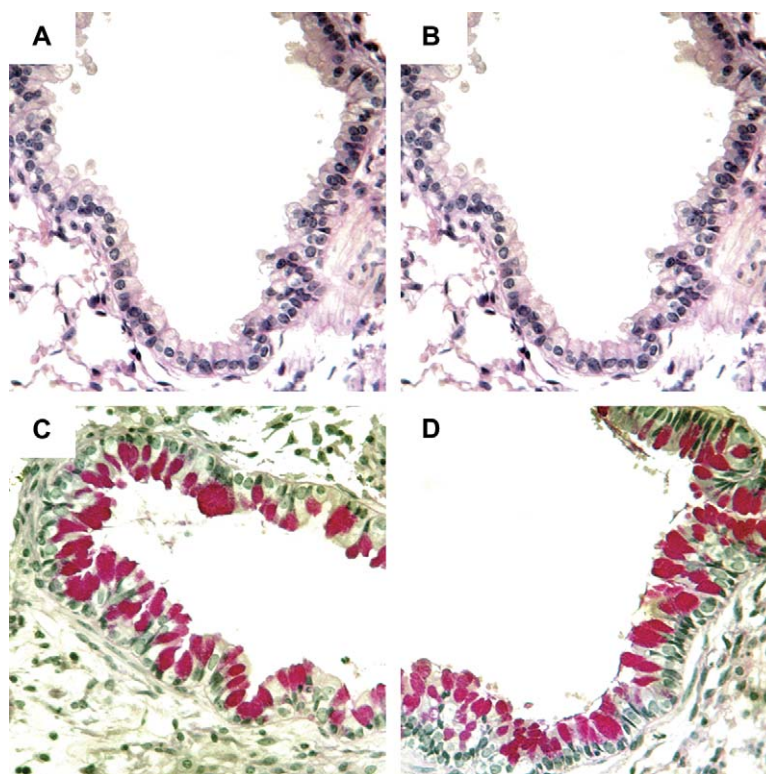


FIG 2. Inhalation of IL-4C stimulates goblet cell hyperplasia in IL-13-deficient mice. PAS staining of lung sections from saline-inoculated wild-type mice (**A**), saline-inoculated IL-13-deficient mice (**B**), IL-4C-inoculated wild-type mice (**C**), and IL-4C-inoculated IL-13-deficient mice (**D**) are shown.

Statistics

Data were analyzed with a Student *t* test. *P* values of less than .05 were considered significant. Figures show means $\pm$ SEs.

RESULTS

Inhalation of IL-4 stimulates allergic inflammation in IL-13-deficient mice

Because IL-4 can promote IL-13 production,^{20,22,44-46} it is possible that the IL-4 effects seen in normal mice are secondary to IL-4-induced IL-13 production. To eliminate this possibility, experiments were performed in which wild-type and IL-13-deficient mice on the same BALB/c background were inoculated daily for 7 days intratracheally with a long-acting form of IL-4, IL-4C, that contained 2 μ g of IL-4 and 10 μ g of a neutralizing anti-IL-4 mAb, and effects on pulmonary inflammatory infiltrates, AHR, and goblet cell hyperplasia were determined. As expected, IL-4 inhalation substantially increased BAL fluid eosinophilia in IL-13-deficient mice (Fig 1, A). IL-4 also caused an increase in AHR, as measured by means of barometric plethysmography, in IL-13-deficient mice (Fig 1, B) that was actually larger than that seen in wild-type mice (not shown). Most importantly, IL-4 inhalation substantially increased airway goblet cell hyperplasia (Fig 1, C, and Fig 2) at least as much in IL-13-deficient mice as in wild-type mice.

As noted in the Methods section, the effects of IL-4C should be qualitatively similar to those of free IL-4 and have been in all previous experiments. However, to demonstrate that the effects of IL-4C can be induced by free IL-4, wild-type and IL-13-deficient mice were inoculated daily intratracheally with 10 μ g of uncomplexed IL-4 for 7 days and studied 1 day after the last dose. The 5-fold increase in IL-4 dose compared with that in our previous experiment compensated for the lower potency of free IL-4 compared with IL-4C (F. Finkelman, unpublished data). Effects induced by inhalation of IL-4 on BAL cells (Fig 3, A), AHR (Fig 3, B), and goblet cells (Fig 3, C) were similar to those observed in mice inoculated with IL-4C (Fig 1).

IL-13 antagonist fails to inhibit IL-4-induced inflammation in wild-type mice

Transgenic mice in which one signaling pathway is genetically deleted might have compensatory enhancement of a partially redundant pathway.⁴⁷ Consequently, the IL-4 induction of IL-13-independent goblet cell hyperplasia observed in IL-13-deficient mice might not be characteristic of wild-type, IL-13-sufficient mice. To evaluate this possibility, we stimulated wild-type BALB/c mice with intratracheal IL-4C and concurrently treated them with a soluble IL-13 antagonist (soluble IL-13R α 2-Fc) at a dose that suppresses IL-13 induction of

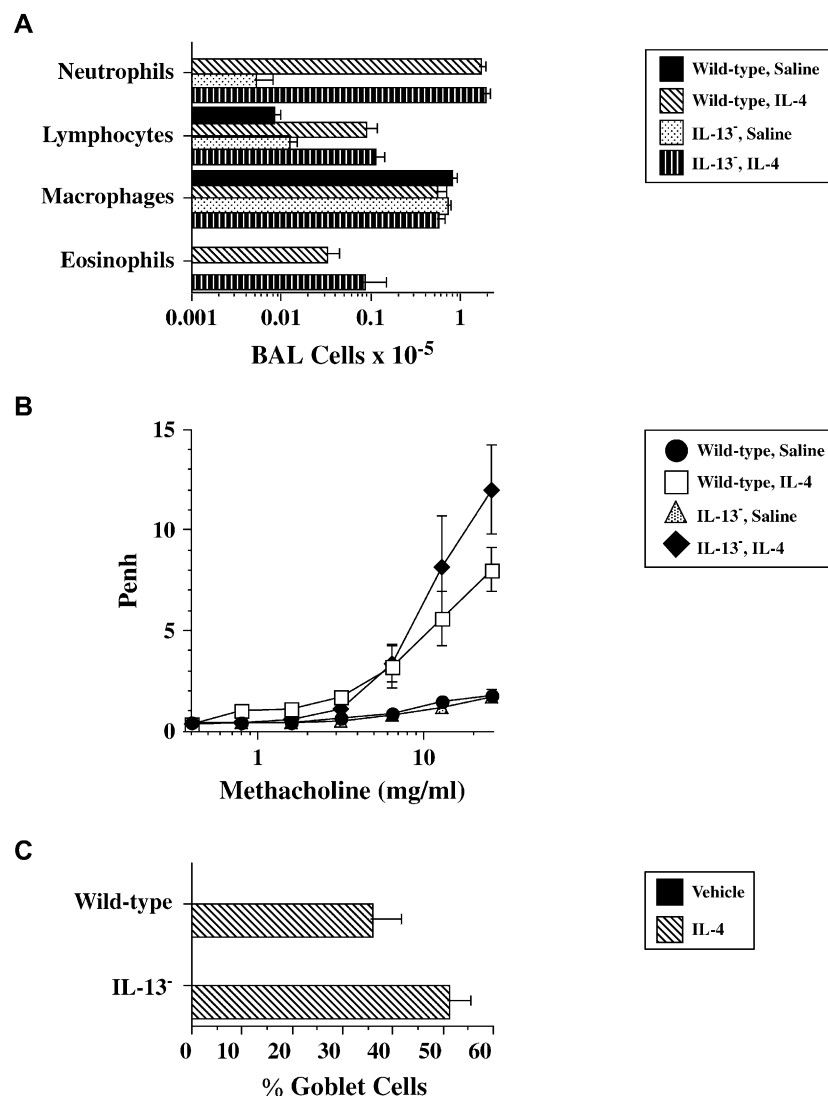


FIG 3. Inhalation of free IL-4 induces allergic airway inflammation in IL-13-deficient mice. The experiment shown in Figs 1 and 2 was repeated, with the exception that IL-4C was replaced by free IL-4 (10 μ g per dose). Results show BAL cellularity (A), airway responsiveness (B), and percentage of PAS-positive bronchiolar epithelial cells (C).

AHR and goblet cell hyperplasia or with a control reagent. IL-13 antagonist therapy had no effect on IL-4 induction of BAL eosinophilia (Fig 4, A), AHR (Fig 4, B), or goblet cell hyperplasia (Fig 4, C, and 5).

Anti-IL-4R α mAb blocks IL-4-induced inflammation in wild-type mice

The failure of IL-13 deficiency or an IL-13 antagonist to block IL-4C or IL-4 induction of AHR or goblet cell hyperplasia might reflect a contaminant or unexpected activity in our IL-4C preparations that induced these phenomena independently of IL-4. To eliminate this possibility, we evaluated the ability of an mAb (M1) that blocks IL-4 signaling through IL-4Rs to inhibit IL-4C induction of AHR, BAL eosinophilia, and goblet cell

hyperplasia. All of these phenomena were blocked by M1 but not by equal doses of an isotype-matched control mAb, although nonatopic inflammation induced by IL-4C (lymphocytosis and neutrophilia) was not reversed (Fig 6). This might reflect incomplete neutralization of IL-4 effects by M1.

DISCUSSION

Our observations demonstrate that IL-4 can induce all of the acute changes in airway inflammation that have been associated with IL-13, including BAL fluid eosinophilia, AHR, and goblet cell hyperplasia, in the absence of IL-13 signaling. IL-4 induced each of these changes in

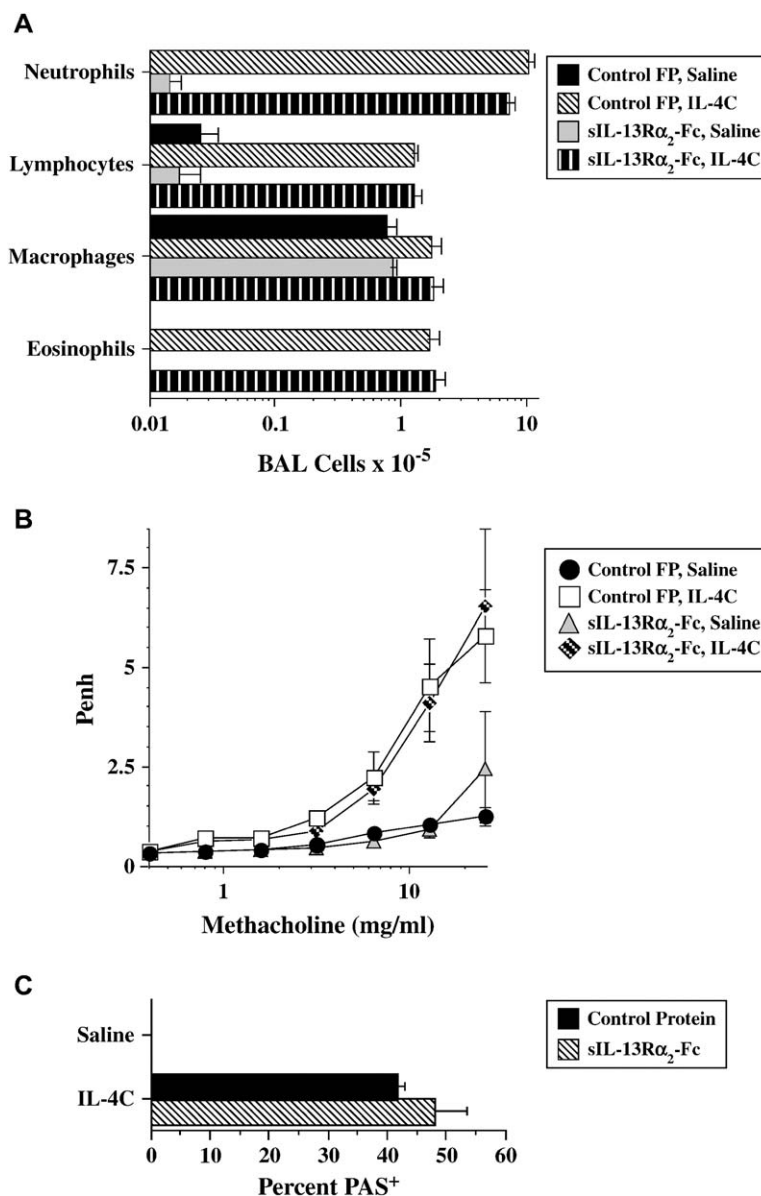


FIG 4. IL-4C induces allergic airway inflammation in IL-13 antagonist-treated mice. BALB/c mice were injected with soluble IL-13R α_2 -Fc or control fusion protein and inoculated with saline or IL-4C, as in Fig 1. BAL eosinophil numbers (**A**), methacholine responsiveness (**B**), and percentages of PAS-positive bronchiolar epithelial cells (**C**) are all significantly increased in both groups of IL-4C-treated mice but not significantly affected by soluble IL-13R α_2 -Fc.

both IL-13-deficient mice and IL-13-sufficient mice that were treated with a potent IL-13 antagonist. IL-4 induction of BAL eosinophilia in the absence of IL-13 is expected; intratracheal IL-13 is less effective than intratracheal IL-4 at inducing this phenomenon, and IL-13 antagonists are relatively ineffective at suppressing BAL eosinophilia in allergen-immunized mice.^{4,48} In contrast, IL-13 antagonists, but not IL-4 antagonists, have generally been effective at suppressing established AHR and goblet cell hyperplasia in mouse models of asthma.^{3,4,7,9,29,48,49} Consequently, it has been hypothesized that the role of

IL-4 in the induction of AHR, goblet cell hyperplasia, or both is limited to stimulating T cells to differentiate into IL-13-secreting cells.^{30,50}

Three previous observations argue against this possibility. First, CC-10-IL-4 transgenic mice, which selectively oversecrete IL-4 in the lungs, have prominent goblet cell hyperplasia, although AHR might not develop.⁵¹ Second, inhalation of IL-4 has been reported to rapidly induce AHR.⁵² Third, IL-13 induction of AHR and goblet cell hyperplasia is STAT6 dependent.⁵³ All receptors known to activate STAT6 include IL-4R α as a signaling

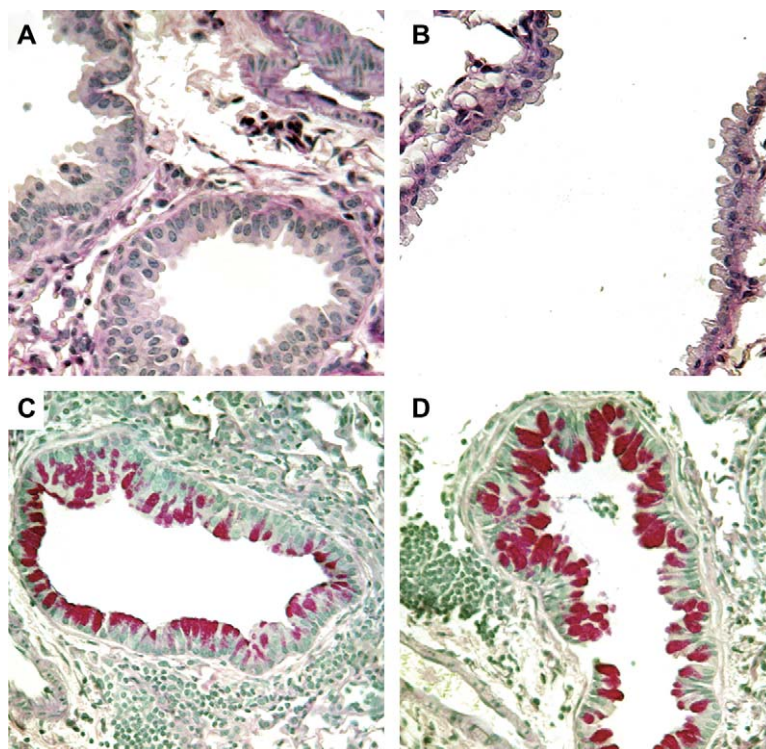


FIG 5. IL-13 antagonist does not prevent IL-4 induction of goblet cell hyperplasia. Lung sections made from the mice used for Fig 3 were PAS stained. Results from mice inoculated with saline-control fusion protein (A), saline/soluble IL-13R α 2-Fc (B), IL-4C/control fusion protein (C), and IL-4C/soluble IL-13R α 2-Fc (D) are shown.

chain, and the only known IL-4R α -containing receptor that is activated by IL-13 (the type 2 IL-4R) is also activated by IL-4.⁵⁴ None of these observations, however, definitively demonstrates that IL-4 can directly induce AHR and goblet cell hyperplasia. Because IL-4 can stimulate naive CD4⁺ T cells to differentiate into T_H2 cells that secrete IL-13 and might also promote IL-13 secretion by basophils and natural killer T cells, IL-4 might indirectly promote AHR and goblet cell hyperplasia by stimulating IL-13 production. This possibility has precedents: both IL-9 and IL-25 induce allergic airway inflammation by stimulating production of other T_H2 cytokines, particularly IL-13.^{55,56} Although experiments with IL-13-treated STAT6-deficient and IL-4R α -deficient mice suggest a critical role for IL-4R α /STAT6-dependent signaling and thus type 2 IL-4R signaling in the induction of AHR and goblet cell hyperplasia,^{12,53,57} they do not eliminate the possibility that induction of these phenomena requires combined signaling through both the type 2 IL-4R and a second receptor that is uniquely triggered by IL-13, such as IL-13R α 2. For these reasons, our current observations provide the first definitive evidence that IL-4 induction of AHR and goblet cell hyperplasia can be totally IL-13 independent.

Why then are established AHR and goblet cell hyperplasia suppressed by IL-13 antagonists in mouse models of asthma without the need to also suppress IL-4? The

possibilities most consistent with our data are that IL-13 can be made in larger quantity than IL-4, that IL-13 might have a longer *in vivo* half-life than IL-4, and that IL-13 might be a more potent ligand than IL-4 for the type 2 IL-4R. Although it is difficult to compare *in vivo* production of 2 cytokines, *ex vivo* restimulation studies in which a potent allergen, *Ascaris* species pseudocoelomic fluid (APF), is used to restimulate bronchial lymph node cells from mice previously immunized intratracheally with the same allergen frequently show production of approximately 10-fold more IL-13 than IL-4 (C. Perkins and F. Finkelman, manuscript in preparation). Studies of IL-4 and IL-13 affinity for the type 2 IL-4R suggest that the 2 ligands bind this receptor with similar affinities¹³; however, some *in vitro* studies suggest that IL-13 is a more potent stimulator of endothelial and smooth muscle cells than IL-4.^{24,58} Thus greater potency for IL-13 remains possible, although a mechanism for increased potency is lacking. *In vivo* half-lives of IL-4 and IL-13 are difficult to compare for 2 reasons: (1) half-life studies have been performed with recombinant molecules, which might not have the same half-lives as naturally produced cytokines, and (2) the *in vivo* half-lives of both molecules are modified by the presence of soluble receptors^{36,59} that vary in concentration (M. Khodoun and F. Finkelman, manuscript in preparation). Thus it is not currently possible to tell whether greater production, *in vivo* half-life, potency, or

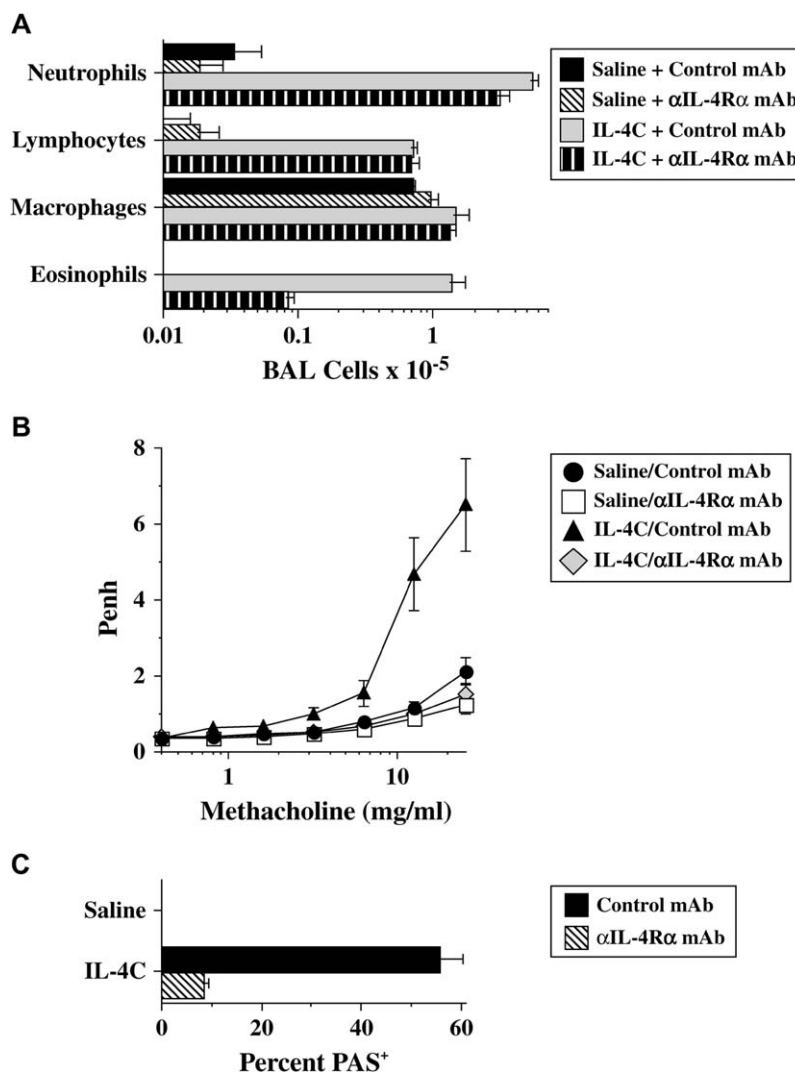


FIG 6. Anti-IL-4R α mAb blocks IL-4 induction of AHR, BAL eosinophilia, and goblet cell hyperplasia. BALB/c mice were inoculated daily intratracheally with saline or IL-4C, as described for Fig 1, and injected intraperitoneally with 3 mg of anti-IL-4R α mAb or an isotype-matched control mAb. Mice were evaluated for BAL cells (A), AHR (B), and goblet cell hyperplasia (C).

some combination of these is primarily responsible for making IL-13 more important than IL-4 as a stimulus of AHR and goblet cell hyperplasia in mouse models of asthma. It should also be considered that most studies with mouse models of asthma have used the same antigen, chicken ovalbumin (OVA), to stimulate allergic airway inflammation.^{3,4} OVA is a less potent inducer of a T_H2 response than dust mite allergen or APF and might stimulate less IL-4 production. In this regard it is noteworthy that inhibition of both IL-4 and IL-13 is required to significantly suppress goblet cell hyperplasia in mice immunized with APF instead of OVA, although neutralization of IL-13 completely suppresses AHR in mice treated with either antigen (C. Perkins and F. Finkelman, manuscript in preparation).

Observations made in animal models of human diseases often translate to the human disorder, but this is not invariable. General principles (eg, the importance of T_H2 cytokines in allergic airways inflammation) are more like to translate than specific details (eg, the greater role of IL-13 than IL-4 in induction of AHR and goblet cell hyperplasia). Although only human studies can definitively reveal the relative roles of IL-4 and IL-13 in human asthma, our observations that IL-4 can stimulate goblet cell hyperplasia and AHR in mouse lung and that IL-4 contributes to goblet cell hyperplasia in at least one mouse model of asthma raise the possibility that suppression of both IL-4 and IL-13 might be necessary to optimally suppress goblet cell hyperplasia and excessive mucus production in some patients with asthma.

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