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Therapeutic Effect of IL-13 Immunoneutralization During Chronic Experimental Fungal Asthma

Kate Blease, Claudia Jakubzick, John Westwick, Nicholas Lukacs, Steven L. Kunkel, and Cory M. Hogaboam

IL-13 and IL-4 are key contributors to the asthmatic phenotype. The temporal role of these cytokines in airway function, inflammation, and remodeling were assessed in a chronic murine model of Aspergillus fumigatus-induced allergic asthma. IL-13 and IL-4 protein levels were significantly elevated by 30 days after conidia challenge in A. fumigatus-sensitized mice. Furthermore, IL-13Rα1 mRNA expression was significantly elevated 7 days after conidia challenge and remained elevated until day 21. In contrast, IL-13Rα2 mRNA expression, although constitutively expressed in naive lung, was absent in the lungs of A. fumigatus-sensitized mice both before and after conidia challenge. Membrane-bound IL-4R mRNA expression was significantly elevated 7 days after conidia challenge; however, soluble IL-4R mRNA expression was increased 30 days after conidia challenge. Immunoneutralization of IL-13 between days 14 and 30 or days 30 and 38 after fungal sensitization and challenge significantly attenuated airway hyperresponsiveness, collagen deposition, and goblet cell hyperplasia at day 38 after conidia challenge; however, the effects of IL-4 immunoneutralization during the same time periods were not as marked. IFN-γ and IL-12 release after Aspergillus Ag restimulation was elevated from spleen cells isolated from mice treated with IL-4 anti-serum compared with IL-13 anti-serum or normal rabbit serum-treated mice. This study demonstrates a pronounced therapeutic effect of IL-13-immunoneutralization at extended time points following the induction of chronic asthma. Most importantly, these therapeutic effects were not reversed following cessation of treatment, and IL-13 anti-serum treatment did not alter the systemic immune response to Ag restimulation, unlike IL-4 immunoneutralization. Therefore, IL-13 provides an attractive therapeutic target in allergic asthma.

Materials and Methods

Sensitization of mice to A. fumigatus

Sensitization of mice CBA/J mice to a commercially available preparation of soluble A. fumigatus Ags (Greer Laboratories, Lenoir, NC) was performed as previously described in detail (12).

Immunoneutralization of IL-13 or IL-4

Immunoneutralization of IL-13 or IL-4 was conducted by i.p. injection of 0.5 ml of anti-murine IL-13 or IL-4 anti-serum (titer of 10^6/ml; Ref. 13) at 2-day intervals according to three dosing protocols (see Fig. 1). The volume of antiserum was considered to be sufficient to neutralize systemic endogenous IL-13 or IL-4 in that the biological half-life of the Ab was ~48 h (13). Anti-murine IL-13 or IL-4 polyclonal Abs were raised by immunizing New Zealand White rabbits with recombinant murine IL-13 or IL-4 (R&D Systems, Minneapolis, MN). Polyclonal Abs were tiered by direct ELISA, and these Abs recognized murine IL-13 or IL-4 at a dilution of 1 × 10^{-6}. The Abs did not cross-react with other assayed murine recombinant cytokines and chemokines. As a control, preimmune normal rabbit serum (NRS) was used. The endotoxin content in both anti-IL-13 and IL-4 anti-serum and NRS was below detection level (<0.05 endotoxin U/ml Pyrogen; BioWhittaker, Walkersville, MD).
Measurement of bronchial hyperresponsiveness

Thirty-eight days after intratracheal A. fumigatus conidia challenge, bronchial hyperresponsiveness to methacholine (10 μg i.v.) in A. fumigatus-sensitized mice was assessed in a Buxco (Troy, NY) plethysmograph (14). At the conclusion of the assessment of airway responsiveness, blood was collected for serum isolation, bronchoalveolar lavage was performed, and whole lungs were finally dissected from each mouse and snap-frozen in liquid N2 or prepared for histological analysis.

ELISA analysis

Murine IL-4, IL-13, IL-12, IFN-γ, and TNF-β protein levels were determined in 50 μl of whole lung homogenates, using a standardized sandwich ELISA technique previously described in detail (15).

Whole lung histological analysis

Whole lungs from A. fumigatus-sensitized mice before and after A. fumigatus conidia challenge were fully inflated by intratracheal perfusion with 4% paraformaldehyde. Lungs were then dissected and placed in fresh paraformaldehyde for 24 h. Routine histological techniques were used to paraffin-embed this tissue, and 5-μm sections of whole lung were stained with Masson trichrome (for determination of collagen deposition) or periodic acid-Schiff (PAS; for identification of goblet cell hyperplasia and mucus production).

Measurement of collagen

Collagen content in whole lung homogenates from A. fumigatus-sensitized mice treated with NRS, polyclonal anti-IL-13, or anti-IL-4 Abs was measured according to instructions using a collagen assay purchased from Bio-color (Westbury, NY).

Preparation of cDNA and RT-PCR amplification

Total RNA samples were prepared from whole lungs removed from mice at 0, 3, 7, 21, and 30 days after conidia challenge using the one-step Trizol isolation procedure (Life Technologies). RNA from specific samples were reverse transcribed into cDNA using a BRL reverse transcription kit and oligo(dT)12-18 primer. The amplification solution contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 mM MgCl2. Specific oligonucleotide primers were added (200 ng/sample) to the buffer, along with a 5-μl reverse transcribed cDNA sample. The following oligonucleotide primers were used. IL-4R primer sequences: sense 5'-AGTGAGTGGAGTCCTAGCATC-3' and antisense, 5'-GCTCGGCCGTGGTGGTC-3'. IL-13Rα1 primer sequences: sense 5'-GAATTTGAGCGTCTCTGTC-3' and antisense, 5'-GCTCGGCCGTGGTGGTC-3'. GAA-3' and 308-bp product. For soluble and membrane IL-4R primer sequences: sense 5'-ATGGCTTTTGTGAGATCAGATGCT-3' and antisense, 5'-CAGGTTGTCCTACATTCTCATAAT-3'. The cDNA was amplified using the following cycling parameters. The mixture was first incubated for 4 min at 94°C and cycled 38 times at 94°C (denatured), annealing at 55°C or 66°C (IL-4R or IL-13R, respectively) for 45 s, and elongated at 72°C for 45 s. After amplification, the mixture was first incubated for 4 min at 94°C and cycled 38 times at 94°C (denatured), annealing at 55°C or 66°C (IL-4R or IL-13R, respectively) for 45 s, and elongated at 72°C for 45 s. After amplification, the samples were separated on a 2% agarose gel containing 0.3 μg/ml ethidium bromide, and bands were visualized and photographed using a transilluminator.

Ag restimulation of isolated spleen cells

Whole spleens were isolated from mice following protocol 2 at day 38. RBC were lysed following mechanical separation, and spleen cells were plated onto six-well plates at 1 × 107 cells/well. Supernatants were collected, and IFN-γ or IL-12 levels were measured by ELISA after incubation of the spleen cells with medium or A. fumigatus-soluble Ag (50 μg) for 24 h.

Statistical analysis

All results are expressed as mean ± SEM. ANOVA and Dunnett’s test for multiple comparisons were used to determine statistical significance in both groups at various times after conidia challenge; p < 0.05 was considered statistically significant.

Results and Discussion

IL-4 and IL-13 expression in whole lung

Transgenic overexpression of IL-13 or IL-4 in the murine lung has been shown to generate a complex phenotype that recapitulates many of the features of clinical asthma (3, 17). These observations have led to several investigations into the role of IL-13 and IL-4 in acute allergic airway disease in mice. In this study we have examined the role of IL-13 and IL-4 in a murine model of A. fumigatus-induced chronic fungal asthma (7–9) that persists for several weeks following fungal sensitization and conidia challenge. IL-4 and IL-13 levels in whole lung were increased following intratracheal conidia challenge in A. fumigatus-sensitized mice (Fig. 1). A significant (p < 0.05) increase in the levels of both IL-4 and IL-13 were measured at day 30 after conidia challenge compared with the levels in lungs before conidia; however, levels of IL-13 were over 10-fold greater than IL-4. To identify the temporal role of IL-13 and IL-4 in this model, cytokine immunoneutralization was conducted at delayed time points after the establishment of allergic airway inflammation and airway remodeling. IL-13 or IL-4 was neutralized according to each protocol shown in Fig. 1. IL-4 or IL-13 anti-sera or NRS was administered every 2 days between days 0 and 14 (protocol 1), days 14 and 30 (protocol 2), or days 30 and 38 (protocol 3) after the conidia challenge. Airway function, inflammation, and remodeling were assessed at 38 days after A. fumigatus live conidia challenge following cessation of immunoneutralization therapy to determine the therapeutic benefit after attenuation of IL-13 or IL-4.

Time course of IL-13 and IL-4 receptor expression

IL-4 and IL-13 signal through multimeric receptor complexes. The IL-4Rα subunit plays a role in both IL-13 and IL-4 signaling, whereas the role of the IL-13Rα subunits is less clear. Two IL-13Rα subunits have been identified, IL-13Rα1 and IL-13Rα2 (18). IL-13Rα1 binds weakly to IL-13, but a heterodimer consisting of IL-4Rα and IL-13Rα1 acts as a functional receptor for both IL-4 and IL-13. In the present model IL-13Rα1 mRNA was constitutively expressed in whole lung before conidia challenge, but was increased after conidia challenge (Fig. 2). Seven
days after conidia challenge a significant \((p < 0.001)\) increase in IL-13\(\alpha_1\) mRNA expression was measured (Fig. 2), which remained elevated at day 21. By 30 days after conidia challenge IL-13\(\alpha_1\) mRNA levels had returned to similar levels measured at day 0 (Fig. 2). In contrast, the IL-13\(\alpha_2\) subunit, although constitutively expressed in naive whole lung (Fig. 2A, Con), was not detected at any time point after conidia challenge (Fig. 2A). IL-13\(\alpha_2\) alone binds to its ligand with higher affinity than the IL-13\(\alpha_1\) subunit; however, its functional role, decoy or active, remains unknown (19). In the context of its function as a decoy receptor, the absence of IL-13\(\alpha_2\) expression in \(A.\) fumigatus-sensitized lung after conidia challenge may leave the actions of IL-13 unchecked, resulting in exacerbated fibrosis and goblet cell hyperplasia.

The IL-4R subunit is an essential component for both IL-4 and IL-13 signaling. This receptor subunit exists in two forms and is produced naturally both as a membrane-associated and as a truncated, soluble form (mIL-4R and sIL-4R, respectively) (20). The two IL-4R forms are encoded by distinct mRNA species that arise from alternative splicing of a primary transcript of the IL-4R gene (20). mIL-4R expression significantly \((p < 0.001)\) increased 7 days after conidia challenge in \(A.\) fumigatus-sensitized mice (Fig. 3). In contrast, sIL-4R mRNA expression was not significantly \((p < 0.001)\) elevated until 30 days after conidia challenge (Fig. 3). These results are similar to those found following infection of mice with \(Leishmania major\) (20), whereby sIL-4R expression was increased. Whereas the function of the mIL-4R in ligand binding and subsequent signaling appears obvious, the role of the sIL-4R is less clear. Because the sIL-4R binds IL-4 with high affinity, it can act as a natural antagonist of IL-4 activity, competing with the mIL-4R on target cells for the binding of IL-4.

**FIGURE 2.** RT-PCR analysis of IL-13\(\alpha_1\) mRNA expression compared with \(\beta\)-actin mRNA expression in whole lung homogenates 0, 7, 21, and 30 days after conidia challenge in \(A.\) fumigatus-sensitized mice. Blots for \(\beta\)-actin or IL-13\(\alpha_1\) (A) of a representative experiment and a bar graph (B) of the means \(\pm\) SEM of IL-13\(\alpha_1/\beta\)-actin ratios for four experiments are shown. Whole lung from naive animals is shown as a positive control (Con).

**FIGURE 3.** RT-PCR analysis of membrane and soluble IL-4R mRNA expression compared with \(\beta\)-actin mRNA expression in whole lung homogenates 0, 7, 21, and 30 days after conidia challenge in \(A.\) fumigatus-sensitized mice. Total RNA was extracted from freshly isolated whole lung homogenates and used to amplify sIL-4R (241 bp) and mIL-4R (127 bp) as a control. Blots for \(\beta\)-actin or IL-14R (A) of a representative experiment and a bar graph (B) of the means \(\pm\) SEM of IL-4R/\(\beta\)-actin ratios for four experiments are shown. Whole lung from naive animals is shown as a positive control (Con).

**FIGURE 4.** Following neutralization of IL-4 or IL-13 between days 0 and 14 (A), days 14 and 30 (B), or days 30 and 38 (C), AHR in \(A.\) fumigatus-sensitized mice at 38 days after \(A.\) fumigatus conidia challenge were assessed. Peak increases in airway resistance or hyperresponsiveness \((U = \text{cm H}_2\text{O/ml/s})\) were determined after the i.v. injection of methacholine and compared with basal levels \((2.1 \pm 0.3 \text{ cm H}_2\text{O/ml/s})\). Values are expressed as mean \(\pm\) SEM; \(n = 4–5\) group/time point. \(*, p < 0.05\) denotes significant inhibition of AHR compared with NRS-treated controls.
Greater attenuation of airway hyperreactivity by IL-13 compared with IL-4 neutralization

AHR is a common feature of human asthma and is defined as an exaggerated bronchoconstrictor response to various provocative agents. Both IL-4 and IL-13 have been established as primary effector molecules in experimental AHR (4, 5); however, IL-13 appears to play a more important role, as revealed by receptor neutralization and recombinant cytokine administration. During chronic fungal asthma, AHR following methacholine challenge increases between 3 and 7 days after conidia challenge (7–9) and remains significantly elevated at 38 days after challenge (basal = 2.1 ± 0.3 to 33.6 ± 4.8 cm H2O/ml/s following methacholine; Fig. 4). Although anti-IL-4 reduced airway hyperreactivity by ~2-fold in both protocol 2 and 3, this effect did not reach statistical significance (Fig. 4, A–C). Similarly, immunoneutralization of IL-13 between days 0 and 14 (protocol 1) did not significantly reduce AHR at day 38 (Fig. 2A). However, following immunoneutralization of IL-13 between days 14 and 30 (protocol 2; Fig. 4B) or days 30 and 38 (protocol 3; Fig. 4C), a statistically significant (p < 0.05) inhibition of AHR to a methacholine injection was measured compared with controls that correlated with IL-13Rα1 mRNA expression during these time points of immunoneutralization. This inhibitory effect was noted despite the fact that anti-IL-13 therapy was stopped 8 days before assessment of AHR in protocol 2. Although our results are consistent with previous studies, this study is the first, to date, that demonstrates that attenuation of IL-13, 2 wk after the induction of airway inflammation, affords a therapeutic effect. Most notably these therapeutic effects persisted despite the cessation of immunoneutralization therapy.

The mechanism of action through which IL-13 modulates AHR is still unclear; however, direct instillation of IL-13 but not IL-4 into the airways of naive mice has been shown to induce AHR (4, 5, 21). IL-13-induced AHR in naive mice correlates with eosinophil accumulation in the airways in a process dependent on signaling through the IL-4Rα subunit (4); however, the role of eosinophils in IL-13-induced AHR, to date, remains controversial (22, 23). Indeed, Wills-Karp et al. (5) suggested that IL-13-induced AHR could be disassociated from eosinophil inflammation. These findings are consistent with observations in our chronic fungal asthma model because eosinophil infiltration into the airways peaks at day 7 following conidia challenge, and eosinophil numbers largely disappear by day 14, after the conidia are cleared from the lungs. Thus, in this study the attenuation of eosinophil activity probably does not account for the therapeutic effects of IL-13 immunoneutralization during chronic fungal asthma.

Collagen deposition and subepithelial fibrosis was inhibited using a therapeutic IL-13 neutralization protocol

Collagen deposition and subepithelial fibrosis are characteristic pathological features of chronic human asthma (24); however, the role that IL-13 and IL-4 play in the peribronchial fibrosis associated with chronic asthma has been difficult to investigate due to the paucity of appropriate in vivo models of allergic airway disease. Masson trichrome staining (Fig. 5) revealed marked collagen deposition and subepithelial fibrosis around the airways of A. fumigatus-sensitized mice at 38 days after conidia challenge. Compared with the appropriate serum controls, collagen staining was markedly decreased following the immunoneutralization of IL-13 between days 14 and 30 (protocol 2) and days 30 and 38 (protocol 3; Fig. 5, B and E). In contrast, immunoneutralization of IL-4 did not appear to reduce collagen staining (Fig. 5, C and F). Further confirmation of these histological findings was observed using a collagen assay. After cessation of IL-13 immunoneutralization, the total soluble collagen content in the lungs of mice at day 38 was significantly (p < 0.05) lower than controls when mice were treated according to protocol 2 with anti-IL-13 (Table I); however, immunoneutralization of IL-4 did not have a similar effect. No significant effect on total soluble collagen lung levels was observed in mice treated according to protocol 3 (anti-IL-13). However, it was noted that significantly (p < 0.001) attenuated levels of the profibrotic cytokine, TGF-β, were measured in whole lung homogenates from anti-IL-13-treated mice (protocol 3; 0.337 ± 0.01 to 0.24 ± 0.01 ng/mg protein). Noticeable differences in pulmonary

![FIGURE 5](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)  Representative photomicrographs of Masson trichrome-stained whole lung sections from A. fumigatus-sensitized mice at day 38 after a live A. fumigatus conidia challenge. Mice were treated between days 14 and 30 with NRS (A), anti-IL-13 (B), or anti-IL-4 (C) or between days 30 and 38 with NRS (D), anti-IL-13 (E), or anti-IL-4 (F). Collagen deposition around the airways appears as light blue material. Original magnification was ×200 for each photomicrograph.
fibrosis have been previously shown between IL-13- and IL-4-transgenic mice. IL-4-transgenic mice do not develop airway fibrosis; however, IL-13-transgenic mice manifest significant degrees of subepithelial and adventitial fibrosis (3, 25, 26). These results have been clarified in IL-4R knockout and STAT6-deficient mice, an intracellular signaling pathway that is used by IL-13 (27, 28) whereby hepatic collagen deposition was reduced in Schistosoma mansoni-infected animals, whereas collagen responses were normal in IL-4-deficient mice (29).

Goblet cell hyperplasia was inhibited by therapeutic immunoneutralization of IL-13 but not IL-4

Goblet cell hyperplasia is a characteristic of the remodeled airway during allergic disease. Mucus hyperproduction from these cells appears to play a key role in exacerbation of AHR and may contribute significantly to morbidity and mortality associated with asthma (30). Furthermore, IL-13-transgenic mice exhibit goblet cell hypertrophy and mucus hypersecretion (3, 31). The presence of airway goblet cells was assessed by PAS staining at day 38 after conidia challenge, following treatment with NRS (A), anti-IL-13 (B), or anti-IL-4 (C) between days 14 and 30 (protocol 2) or NRS (D), anti-IL-13 (E), or anti-IL-4 (F) between days 30 and 38 (protocol 3; Fig. 6). Goblet cell hyperplasia in large and small airways was detected in control A. fumigatus-sensitized mice at 38 days after the conidia challenge (Fig. 6, A and D). However, immunoneutralization of IL-13 between days 14 and 30 (protocol 2; Fig. 6B) or days 30 and 38 (protocol 3; Fig. 6E) completely ablated goblet cell hyperplasia. Although goblet cells were less prominent following IL-4 immunoneutralization between days 14 and 30 (Fig. 6C) and days 30 and 38 (Fig. 6F), this effect was not as marked as that following IL-13 immunoneutralization. These results are in concordance with results obtained from IL-13-transgenic studies (3), whereby they demonstrate a critical role for IL-13 in mucus cell metaplasia; however, the contribution of IL-4 to goblet cell hyperplasia and mucus production is less well defined. Similarly to IL-13-transgenic mice, IL-4 overexpressing mice have also shown increases in PAS-positive material in lavage fluid, suggesting that IL-4, in addition to IL-13, stimulates mucus production within the airways (31). However, because IL-4 has been shown to induce the production of IL-13 from mast cells (32), the effect of IL-4 on mucus production may be indirect via the release of IL-13. Therefore, IL-13 immunoneutralization appears to exhibit a more profound therapeutic effect on the attenuation of goblet cell hyperplasia than IL-4 that is maintained up to 7 days following cessation of anti-IL-13 treatment.

**IL-4- but not IL-13-neutralization alters the cytokine release from restimulated spleen cells**

In addition to their effects on allergic airway inflammation and collagen deposition, IL-4 and IL-13 have important systemic immunomodulatory effects. Spleen cells isolated from mice treated with anti-IL-4 Abs responded to restimulation with soluble A. fumigatus with a robust Th1 response, involving IL-12 and IFN-γ secretion (Fig. 7), similar to previous studies in the absence of IL-4 (29, 33). This demonstrates that IL-4 neutralization appears to have important systemic immunomodulatory effects that IL-13 does not possess. In this respect, IL-13 appears to be a more attractive therapeutic target than IL-4 because it does not alter the systemic immune response.

In conclusion, this study demonstrates a marked therapeutic effect of IL-13 immunoneutralization on the chronic functional and structural changes that take place in the airways during chronic allergic disease.

<table>
<thead>
<tr>
<th>Days</th>
<th>NRS</th>
<th>Anti-IL-13 (µg/ml/lung)</th>
<th>Anti-IL-4 (µg/ml/lung)</th>
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<td>0–14</td>
<td>74.4 ± 4.8</td>
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<td>14–30</td>
<td>88.2 ± 7.7</td>
<td>60.9 ± 1.6*</td>
<td>62.8 ± 8.2</td>
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<tr>
<td>30–38</td>
<td>75.2 ± 5.9</td>
<td>87.7 ± 2.2</td>
<td>84.2 ± 5.4</td>
</tr>
</tbody>
</table>

* Total collagen levels in whole lung (µg/ml/lung) at day 38 after live A. fumigatus conidia challenge were assessed following neutralization of IL-4 or IL-13 between days 0 and 14, days 14 and 30, or days 30 and 38. Values are expressed as mean ± SEM; n = 4–5/group/time point.

* p < 0.05 denotes significant inhibition of collagen levels compared to NRS-treated controls.

**FIGURE 6.** Representative photomicrographs of PAS-stained whole lung sections from A. fumigatus-sensitized mice at day 38 after a live A. fumigatus conidia challenge. Mice were treated between days 14 and 30 with NRS (A), anti-IL-13 (B), or anti-IL-4 (C) or between days 30 and 38 with NRS (D), anti-IL-13 (E), or anti-IL-4 (F). Goblet cells stained dark magenta were readily apparent in whole lung sections from NRS controls but not anti-IL-13-treated mice. Original magnification was ×200 for each photomicrograph.
cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by


