Interleukin-13 Neutralization by Two Distinct Receptor Blocking Mechanisms Reduces Immunoglobulin E Responses and Lung Inflammation in Cynomolgus Monkeys

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ABSTRACT

Interleukin (IL)-13 is a key cytokine driving allergic and asthmatic responses and contributes to airway inflammation in cynomolgus monkeys after segmental challenge with Ascaris suum antigen. IL-13 bioactivity is mediated through a receptor complex consisting of IL-13Rα1 and IL-4Rα and can be inhibited in vitro by targeting IL-13 interaction with either chain. However, in cytokine systems, in vitro neutralization activity may not always predict inhibitory function in vivo. To address the efficacy of two different IL-13 neutralization mechanisms in a primate model of atopic disease, two humanized monoclonal antibodies to IL-13 were generated, with highly homologous properties, differing in epitope recognition. Ab01 blocks IL-13 interaction with IL-4Rα, and Ab02 blocks IL-13 interaction with IL-13Rα1. In a cynomolgus monkey model of IgE responses to A. suum antigen, both Ab01 and Ab02 effectively reduced serum titers of Ascaris-specific IgE and diminished ex vivo Ascaris-triggered basophil histamine release, assayed 8 weeks after a single administration of antibody. The two antibodies also produced comparable reductions in pulmonary inflammation after lung segmental challenge with Ascaris antigen. Increased serum levels of IL-13, lacking demonstrable biological activity, were seen postchallenge in animals given either anti-IL-13 antibody but not in control animals given human IgG of irrelevant specificity. These findings demonstrate a potent effect of IL-13 neutralization on IgE-mediated atopic responses in a primate system and show that IL-13 can be efficiently neutralized by targeting either the IL-4Rα-binding epitope or the IL-13Rα1-binding epitope.

As an inducer of lung inflammation, IgE production, airway hyper-reactivity, and lung pathology, interleukin (IL)-13 represents a key target for the treatment of asthma (Wills-Karp, 2001; Yasunaga et al., 2003), although it may have some other cell types. This complex recruits IL-4Rα to form the high-affinity receptor that is competent for signal transduction through STAT6 (Andrews et al., 2002). Responses are influenced by IL-13Rα2, which binds IL-13 with high affinity and sequesters it from the IL-13Rα1 complex (Yoshikawa et al., 2003; Andrews et al., 2006). IL-13Rα2 typically inhibits IL-13 function (Feng et al., 1998; Bernard et al., 2001; Yasunaga et al., 2003), although it may have some respiratory disease triggered by segmental lung challenge with the antigen, Ascaris suum (Bree et al., 2007).

IL-13 bioactivity is mediated through a receptor complex consisting of IL-13Rα1 and IL-4Rα chains. Initially, IL-13 undergoes a relatively low-affinity interaction with IL-13Rα1 on the surface of B cells, monocytes, epithelial cells, and other cell types. This complex recruits IL-4Rα to form the high-affinity receptor that is competent for signal transduction through STAT6 (Andrews et al., 2002). Responses are influenced by IL-13Rα2, which binds IL-13 with high affinity and sequesters it from the IL-13Rα1/IL-4Rα complex (Yoshikawa et al., 2003; Andrews et al., 2006). IL-13Rα2 typically inhibits IL-13 function (Feng et al., 1998; Bernard et al., 2001; Yasunaga et al., 2003), although it may have some...
signaling capacity under certain activation conditions (Fichtner-Feigl et al., 2006).

Because the IL-13 receptor is a heterodimer, IL-13 neutralization strategies could be directed at blockade of IL-13 interaction with either the IL-13Rα1 or IL-4Rα chains. To address whether the mechanism of IL-13 neutralization would affect in vivo efficacy, we generated two humanized IgG1 monoclonal antibodies, Ab01 and Ab02, which had comparable in vitro IL-13 neutralization activity and indistinguishable biochemical properties. Ab01 blocked IL-13 interaction with the IL-4Rα chain of the receptor but permitted its interaction with IL-13Rα1 and IL-13Rα2. Ab02 blocked IL-13 interaction with both the activating receptor, IL-13Rα1, and the “decoy” receptor, IL-13Rα2.

In vivo neutralization of cytokine-receptor interactions has proved to be difficult to predict and model in vitro. In some cases, antibodies with strong inhibitory activity in vitro have been observed to have agonistic activity, potentiating cytokine responses in vivo (May et al., 1993; Zabeau et al., 2001; Boyman et al., 2006). This phenomenon may be related to such factors as the epitope seen by the antibody, complex interactions among cytokine, antibody, and multiple receptor chains, and the potential creation of antibody-bound depots of cytokine. Direct comparison of Ab01 and Ab02 in the cynomolgus monkey model allowed us to specifically address the consequences of distinct epitope recognition on serum IgE titers, ex vivo basophil histamine release, and airway inflammation. The results reveal a potent effect of IL-13 neutralization on IgE-mediated allergic responses.

Materials and Methods

Test and Control Articles. Antibody to human IL-13 was generated in BALB/c mice and humanized at Wyeth Research (Cambridge, MA) to generate Ab01 (IgG1, κ). A second antibody was raised in BALB/c mice against a series of overlapping peptides corresponding to the amino acid sequence of cynomolgus monkey IL-13 (Bree et al., 2007) conjugated to KLH and humanized at Wyeth Research to resemble the amino acid sequence of cynomolgus monkey IL-13 (Bree et al., 2007) conjugated to KLH and humanized at Wyeth Research to generate Ab02 (IgG1, κ). Both antibodies were formulated in 10 mM L-histidine, pH 6, containing 5% (w/v) sucrose. Carimune NH im-

Human IL-13 Preparation. Umbilical cord blood mononuclear cells were cultured in RPMI medium containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine in a 37°C incubator at 5% CO2. The cells were skewed to Th2 by 3-day incubation with 1 μg/ml phytohemagglutinin (Sigma-Aldrich, St. Louis, MO), 50 ng/ml recombinant human IL-4 (R&D Systems, Minneapolis, MN), and 0.5 μg/ml anti-human IL-12 (Wyeth Research), then washed and cultured for an additional week with 50 ng/ml recombinant human IL-2 (R&D Systems), added every 2 to 3 days. Cells were activated for 3 days with 100 ng/ml PMA (Sigma-Aldrich) and 2 μg/ml ionomycin (Sigma-Aldrich). Supernatant was collected, dialyzed to remove PMA and ionomycin, and depleted of GM-CSF and IL-4 by incubation with biotinylated antibodies (R&D Systems), followed by Dynabeads M-280 streptavidin (Dynal/Invitrogen, Carlsbad, CA).

ELISA to Detect Ab01 or Ab02 in NHP Serum. Ab01 or Ab02 in serum was detected by competition with biotinylated versions of each antibody for binding to cynomolgus monkey IL-13-FLAG immobilized on ELISA plates coated with anti-FLAG M2 (Sigma-Aldrich), as previously described (Bree et al., 2007). Sensitivity of the assay was approximately 30 ng/ml.

ELISA to Detect IL-13 in the Presence of Ab01 or Ab02. To detect IL-13 in the presence of Ab01, ELISA plates (Nunc Maxi-Sorp; Thermo-Fisher Scientific, Rochester, NY) were coated with mouse anti-IL-13 antibody, J2 (Wyeth Research), washed with PBS containing 0.05% Tween 20 (PBS-Tween), then blocked with 0.5% gelatin in PBS. NHP IL-13 standard (Wyeth Research) or dilutions of NHP serum were added in PBS-Tween containing 2% FCS. After 4 h, plates were washed, and biotinylated mouse anti-IL-13 antibody, Ab64 (Wyeth Research), was added for 1 to 2 h, followed by horseradish peroxidase-streptavidin (Southern Biotechnology Associates, Birmingham, AL). Color was developed using Sure Blue peroxidase substrate (KPL, Gaithersburg, MD). Absorbance was read at 450 nm (SpectraMax; Molecular Devices, Sunnyvale, CA). Serum IL-13 was quantitated by reference to an NHP IL-13 standard curve. Detection of Ab02 in the presence of IL-13 was performed in a similar manner, except that plates were coated with mouse anti-IL-13 antibody, mAb13 (Wyeth Research).

ELISA for Ascaris-Specific IgE. Total serum IgE was quantitated by ELISA. Plates were coated with goat anti-human IgE (KPL) or A. suum extract (Greer Diagnostics), blocked with PBS containing 0.5% gelatin and 1 mM polyvinylpyrrolidone (Sigma-Aldrich), then incubated with NHP serum diluted in PBS-Tween overnight on a shaker at 4°C. The plates were washed, then incubated with biotinylated goat anti-human IgE (BD Biosciences, San Jose, CA) for 2 h. Binding was detected with peroxidase-linked streptavidin (Southern Biotechnology Associates) and Sure Blue substrate (KPL).

Flow Cytometry. HT-29 (American Type Culture Collection, Manassas, VA) or A375 (American Type Culture Collection) cells were washed into ice-cold PBS containing 1% bovine serum albumin and 0.001% sodium azide. Cells were incubated with 20 ng/ml human IL-13 for 20 min, 4°C, washed, and incubated for an additional 20 min at 4°C with biotinylated Ab01 or Ab02. The cells were stained with phosphodiethylthanolamine-labeled streptavidin, fixed in PBS containing 1% paraformaldehyde, and analyzed on a FACScan (BD Biosciences).

Basophil Histamine Release. Peripheral blood was drawn into sodium heparinized tubes. An equal volume of PACM buffer (25 mM PIPES, pH 7.2, 110 mM NaCl, 5 mM CaCl2, 2.5 mM MgCl2) with 0.005% human serum albumin (Sigma-Aldrich) was added to fresh whole blood, within 12 h of draw. Diluted whole blood was challenged

Quantitation of BAL Inflammation and Cytokine. The cell fraction of BAL fluid was analyzed for total and differential leukocyte count. The fluid fraction was concentrated approximately 16-fold using Centriprep-YM3 concentrators (Millipore Corporation, Billerica, MA). Eotaxin was quantitated by ELISA specific for the human protein (Biosource International, Camarillo, CA). The limit of assay sensitivity was 7.5 pg/ml.
with *Ascaris* antigen (Greer Diagnostics) in PACM containing 0.005% human serum albumin, for 30 min at 37°C. Histamine in supernatants was quantitated by ELISA (Beckman Coulter, Fullerton, CA). Background histamine release was determined by challenging cells with PACM in the absence of antigen. Total histamine release was measured after lysis of the cells with 0.1% Triton X-100. Histamine nanomolar values were converted to percentage maximal release according to the equation: (sample – background)/(total – background) × 100.

**Cell-Based Assays for IL-13.** The monocyte CD23 expression assay was performed using human peripheral blood mononuclear cells, as described previously (Kasaian et al., 2007). The STAT6 phosphorylation assay was performed using TF-1 cells (American Type Culture Collection), as described previously (Kasaian et al., 2007). The STAT6 assay was performed using human peripheral blood mononuclear cells, as described previously (Kasaian et al., 2007). The STAT6 phosphorylation assay was performed using HT-29 cells (American Type Culture Collection), as described previously (Kasaian et al., 2007). The STAT6 assay was performed using human peripheral blood mononuclear cells, as described previously (Kasaian et al., 2007). The STAT6 phosphorylation assay was performed using HT-29 cells (American Type Culture Collection), as described previously (Kasaian et al., 2007). The STAT6 assay was performed using human peripheral blood mononuclear cells, as described previously (Kasaian et al., 2007). The STAT6 phosphorylation assay was performed using HT-29 cells (American Type Culture Collection), as described previously (Kasaian et al., 2007). The STAT6 assay was performed using human peripheral blood mononuclear cells, as described previously (Kasaian et al., 2007). The STAT6 phosphorylation assay was performed using HT-29 cells (American Type Culture Collection), as described previously (Kasaian et al., 2007).

**Surface Plasmon Resonance Analysis.** A Biacore 3000 instrument (Biacore, Piscataway, NJ) was used with BioEvaluation software version 3.1. Immobilization buffers, coupling reagents, and sensor chips were obtained from Biacore. The surface of a Research grade CM5 sensor chip (Biacore) was activated for amine coupling with a mixture of N-ethyl-N-(2-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide. hIL13Rα1 monomer (R&D Systems), IL-4Rα, or Ab01 was injected at 10 μg/ml in 10 mM sodium acetate, pH 4.5. The surface was deactivated with 1 M ethanolamine-HCl. Control surfaces were generated with N-ethyl-N-(2-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide followed by 1 M ethanolamine-HCl. All measurements were performed at 22°C with analytes diluted in PBS containing 0.01% bovine serum albumin, 3.4 mM EDTA, and 0.005% Tween 20. Surfaces were regenerated with a 30% solution of 1.83 M MgCl₂, 0.46 M KSCN, 0.92 M urea, and 1.83 M guanidine-HCl followed by two consecutive 15-s buffer injections. The experimental data were corrected for instrumental and bulk artifacts by double referencing using blank sensor chip surface and buffer injections as controls.

**Results**

**Generation of Ab01 and Ab02.** Ab01 is the humanized form of a mouse anti-human IL-13, and Ab02 is the humanized form of a mouse antibody raised against KLH-conjugated peptides corresponding to the sequence of cynomolgus monkey IL-13 (Bree et al., 2007). These antibodies were selected for potency in IL-13 neutralization bioassays and humanized by a CDR grafting approach, using the same human VH and VL framework region genes. The antibodies differ only in their CDR sequences, sharing 74% identity in the VH region, and 81% identity in the VL region. Both are human IgG1, κ.

**IL-13 Binding Characterization of Ab01.** The binding interactions of Ab01 with IL-13 were evaluated by surface plasmon resonance. To establish a model system to characterize the dynamics of IL-13-receptor interactions, IL-13 was injected over a human IL-13Rα1 sensor chip surface, followed immediately by injection of either buffer or soluble IL-4Rα. In the absence of IL-4Rα (buffer), IL-13 rapidly dissociated from IL-13Rα1 (Fig. 1A). When sIL-4Rα was injected immediately after IL-13, binding to the IL-13/IL-13Rα1 complex was observed, indicative of formation of the IL-13Rα1/IL-13/IL-4Rα ternary complex (Fig. 1A). The addition of sIL-4Rα stabilized the interaction of IL-13 with IL-13Rα1, resulting in a slower dissociation rate.

To probe the effect of Ab01 on IL-13 binding to receptor components, IL-13 was injected over an IL-13Rα1 surface, followed immediately by buffer or Ab01. In the presence of buffer, IL-13 dissociated rapidly from IL-13Rα1. In contrast,

![Image](https://via.placeholder.com/150)

**Fig. 1.** Characterization of Ab01 effects on IL-13 receptor binding by surface plasmon resonance. A, sIL-13Rα1 monomer was immobilized on a CM5 sensor chip (980 RU). IL-13 was injected, followed by either sIL-4Rα or buffer. B, IL-13 was injected on an IL-13Rα1-coated sensor chip surface (8915 RU), followed by either Ab01 or buffer. C, IL-13 was injected on an Ab01-coated sensor chip surface (1033 RU), followed by IL-13Rα1 alone, a mixture of IL-13Rα1 and IL-4Rα, or buffer. D, mixture of IL-13 and IL-13Rα1 was injected onto an IL-4Rα-coated sensor chip surface (10945 RU), in the absence or presence of Ab01. The inflection point in all panels indicates replacement of IL-13 receptor components or IL-13 binding antibody with buffer.
Ab01 bound to the IL-13/IL-13Rα1 heterodimer, forming a ternary complex (Fig. 1B). There was no binding of Ab01 to the chip in the absence of IL-13 (data not shown). The complex among Ab01, IL-13, and IL-13Rα1 dissociated rapidly upon substitution of Ab01 solution with buffer (Fig. 1B). These data indicated that Ab01 recognized an epitope of human IL-13 distinct from that seen by IL-13Rα1.

To determine whether Ab01 prevents IL-4Rα binding to the IL-13/IL-13Rα1 complex, IL-13 was first bound to an Ab01-coated surface (Fig. 1C). Upon buffer injection, very slow dissociation was observed, consistent with the strong kinetics of this interaction (Andrews et al., 2002). When IL-13Rα1 was injected immediately after IL-13, binding to the IL-13/Ab01 complex was observed (Fig. 1C), indicating that Ab01 binding to IL-13 did not obscure the IL-13Rα1 binding site. The same profile was seen when IL-13Rα1 was added in combination with IL-4Rα immediately after IL-13 (Fig. 1C). Because additional signal would have been observed if IL-4Rα had bound to the IL-13/IL-13Rα1 complex, this result indicated that there was no formation of a ternary complex in the presence of Ab01.

Finally, we evaluated whether Ab01 blocked IL-13 interaction with the IL-4Rα chain. A preformed mixture of IL-13 and IL-13Rα1 was injected onto an IL-4Rα sensor chip surface, and binding was observed (Fig. 1D). Previous observations indicated that IL-4Rα could not capture free IL-13 in this format but that IL-13Rα1 was required (data not shown). However, when a mixture of IL-13, IL-13Rα1, and Ab01 was added, binding of IL-13 and IL-13Rα1 to IL-4Rα was prevented (Fig. 1D). This observation supports the hypothesis that Ab01 prevents IL-13 interaction with the IL-4Rα chain, which would block formation of the active IL-13 signaling complex.

**IL-13 Binding Characterization of Ab02.** To target the IL-13Rα1 binding epitope of IL-13, Ab02 was generated in mice immunized with a series of overlapping peptides corresponding to the IL-13 sequence. To ensure that this antibody would have full recognition of NHP IL-13, the amino acid sequence of cynomolgus monkey IL-13 (M. fascicularis; accession no. DQ679797) (Bree et al., 2007) was used. Ab02 was selected for its activity in blocking IL-13 binding to IL-13Rα1 (Fig. 2A). Because the IL-13 binding sites for IL-13Rα1 and IL-13Rα2 overlap (Madhankumar et al., 2002), Ab02 also interferes with IL-13 binding to IL-13Rα2 (Fig. 2B). Peptide epitope analysis confirmed that Ab02 recognized the C-terminal region of IL-13 (data not shown), which contains the major sites for interaction with IL-13Rα1 and IL-13Rα2 (Madhankumar et al., 2002; LaPorte et al., 2008).

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**Fig. 2.** Ab01 and Ab02 recognize distinct epitopes of IL-13. Ab02, but not a control antibody of irrelevant specificity, competes for IL-13 binding to ELISA plates coated with the following: A, IL-13Rα1; or B, IL-13Rα2. Data shown are mean ± S.D. for two to three independent experiments. C, surface plasmon resonance analysis confirms that Ab02 and Ab01 recognize distinct epitopes of IL-13. sIL-13Rα2-Fc was immobilized on the chip. NHP IL-13 was added, followed by Ab01, which recognized an epitope distinct from the IL-13Rα2 binding site, or Ab02, which did not. D, binding of biotinylated Ab01 or biotinylated Ab02 to IL-13Rα1-expressing A375 cells or to IL-13Rα1-expressing HT-29 cells preloaded with IL-13, detected with phosphatidylethanolamine-streptavidin. Each finding is representative of at least three separate experiments.
Ab01 and Ab02 Recognize Distinct Epitopes of IL-13. Surface plasmon resonance analysis was performed to confirm that Ab01 and Ab02 recognized distinct epitopes of IL-13. IL-13 bound rapidly to IL-13Rα2 immobilized onto a Biacore chip (Fig. 2C). In a second injection, either Ab01 or Ab02 was added. Ab01 was able to bind, confirming that it recognizes an epitope of IL-13 distinct from that seen by IL-13Rα2. In contrast, Ab02 did not bind, indicating that its epitope was blocked when IL-13 was bound to IL-13Rα2.

These experiments confirmed that the epitope of IL-13 recognized by Ab01 was distinct from that required for binding to IL-13Rα1 and IL-13Rα2. Because of this, we explored the possibility that Ab01 could bind to cells expressing either IL-13Rα1 or IL-13Rα2. The A375 human melanoma cell line expresses high levels of IL-13Rα2 but no detectable IL-13Rα1, whereas the HT-29 human epithelial cell line expresses IL-13Rα1, but no detectable IL-13Rα2 (data not shown). Flow cytometry indicated that biotinylated Ab01 could interact with IL-13 bound to the surface of A375 cells (Fig. 2D). Under the same experimental conditions, biotinylated Ab02 showed no detectable binding to A375 cells, confirming its distinct epitope specificity. No binding of Ab01 to HT-29 cells was seen, either in the presence (Fig. 2D) or absence (data not shown) of IL-13, which may have been due to the higher affinity of IL-13 binding to Ab01 than to IL-13Rα1. Both Ab01 and Ab02 were constructed with mutations in the human IgG1 Fc sequence, which reduced their potential effector activity. Neither Ab01 nor Ab02 had detectable activity in ADCC or C1q binding assays (data not shown).

Comparison of IL-13 Binding and Neutralization Activities of Ab01 and Ab02. The IL-13 neutralization properties of Ab01 and Ab02 were compared in several different bioassay formats. Ab01 and Ab02 had comparable neutralization activity for STAT6 phosphorylation in HT29 cells, induced by human IL-13 (Fig. 3A) or NHP IL-13 (Fig. 3B) in a 30-min assay. In the 72-h TF1 proliferation bioassay, Ab01 had an IC50 approximately 2-fold lower than that of Ab02 (Fig. 3C). In a third assay format, FcRII (CD23) was induced on the surface of freshly isolated human peripheral blood monocytes after 24-h treatment with IL-13. Ab01 had an IC50 approximately 3-fold lower than that of Ab02 for neutralization of NHP IL-13 in this assay (Fig. 3D). Neither antibody inhibited the bioactivity of IL-4 (Fig. 3D). The kinetic parameters of Ab01 and Ab02 binding to NHP IL-13 were characterized by surface plasmon resonance analysis. Ab02 displayed relatively more rapid association and dissociation kinetics than Ab01, and the two antibodies had a comparable overall affinity for NHP IL-13 (Table 1).

Both Ab01 and Ab02 Reduce Ascaris-Induced Lung Inflammation. The in vivo efficacy of Ab01 and Ab02 was evaluated in a cynomolgus monkey model of lung inflammation induced by segmental airway challenge with A. suum antigen. In this model, a single airway challenge with Ascaris antigen, administered by bronchoscope, results in a reproducible pulmonary inflammation 24 h later, with increased total cell counts in the BAL fluid (Bree et al., 2007). Although it induces strong inflammation, the segmental challenge protocol precludes accurate evaluation of bronchoconstriction. Ab01 and Ab02 were tested in two separate studies and were

![Fig. 3](https://jpet.aspetjournals.org/article-pdf/10.1124/jpet.106.106206/10.1124/jpet.106.106206-fig3.pdf)
administered at 10 mg/kg by slow i.v. infusion. Control animals were administered human IgG of irrelevant specificity (IVIG; 10 mg/kg) or an equivalent volume of saline. Twenty-four hours later, baseline BAL was collected from the left lung, and *Ascaris* antigen was instilled into the right lung by bronchoscope. Twenty-four hours post-*Ascaris* challenge, BAL was collected from the right lung. All study agents, including Ab01 and Ab02, were well tolerated. Each antibody had a circulating half-life of approximately 14 days in NHP (Vugmeyster et al., 2008).

Both Ab01 and Ab02 effectively reduced BAL inflammation in this model (Fig. 4A). Total BAL inflammation in groups treated with Ab01 and Ab02 differed significantly postchallenge from that of control animals that were given saline or IVIG (*p*/H11005 < 0.02 by ANOVA). There was also a trend toward reduced eosinophil counts in the BAL fluid (Fig. 4B), but the difference did not achieve statistical significance. The saline- and IVIG-treated control groups did not differ from each other in any parameter (data not shown).

Both Ab01 and Ab02 Reduced *Ascaris*-Specific IgE Titers and *Ascaris*-Specific Basophil Histamine Release in Cynomolgus Monkeys. IL-13 is a key driver of IgE production in vivo. Sera were collected from cynomolgus monkeys 8 weeks post-treatment and *Ascaris* challenge, and serum dilutions were assayed for *Ascaris*-specific and total IgE. Although no significant changes in total IgE were seen after antibody treatment (data not shown), both Ab01 and Ab02 reduced titers of *Ascaris*-specific IgE by 8 weeks postchallenge (Fig. 5, A and B).

To evaluate basophil histamine release as an indicator of allergen sensitivity in the cynomolgus monkeys, peripheral

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### Table 1

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<th>Kd</th>
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*Surface plasmon resonance analysis of human or cynomolgus monkey IL-13 binding to immobilized Ab01 or Ab02 was performed. Kinetic constants were derived from Langmuir model analysis.

\( R_{\text{max}} \), maximal signal.

\( R^2 \), measure of curve fit accuracy.

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Fig. 4. Anti-IL-13 treatment reduces BAL inflammation and cytokine levels. Cynomolgus monkeys were administered 10 mg/kg Ab01 or Ab02 by i.v. infusion. Control animals were given human Ig of irrelevant specificity (IVIG, 10 mg/kg) or saline. Twenty-four hours later, the animals were administered a lung segmental challenge with *A. suum* antigen. Total cell number (A) and eosinophil number (B) in BAL fluid, pre- or 24 h post-*Ascaris* challenge, shown as median ± S.E.M. Total inflammation differed significantly among Ab01-, Ab02-, and control-treated animals postchallenge (*p* = 0.02 by ANOVA) but not prechallenge. Differences in eosinophil number were not statistically significant. Eosinophil levels in concentrated BAL fluid, harvested 24 h post-*Ascaris* challenge. The reductions with anti-IL-13 treatment were not statistically significant.
blood was challenged ex vivo with *Ascaris* antigen, and histamine release into the supernatant was quantitated by ELISA. At 8 weeks postchallenge, animals given saline or IVIG exhibited a boost in the histamine release response. Those animals treated with Ab01 or Ab02 failed to show this increase in histamine release and in some cases displayed reduced basophil sensitivity to *Ascaris* antigen (Fig. 6A).

Compared with controls given saline or IVIG, animals treated with either Ab01 or Ab02 had significantly lower basophil histamine release responses at 8 weeks postchallenge (Fig. 6B).

Both Ab01 and Ab02 Trap IL-13 in Sera of Cynomolgus Monkeys. Administration of anti-cytokine antibody has the potential to trap the cytokine in serum, resulting in detectable titers of circulating cytokine bound to the antibody (Suffredini et al., 1995; Charles et al., 1999; Margolin et al., 2001; Ito et al., 2004). To examine this phenomenon in cynomolgus monkeys treated with Ab01 or Ab02, assays were developed to detect NHP IL-13 in the presence or absence of each antibody. These assays showed that, after antibody administration and *Ascaris* challenge, titers of IL-13 rose in the circulation of animals treated with Ab01 or Ab02. The IL-13 was detectable by 24 h postchallenge in each animal, peaked between 24 h and 2 weeks postchallenge, then declined over time (Fig. 7, A and B). The level of IL-13 trapped in the serum was directly related to the degree of *Ascaris*-induced lung inflammation (Fig. 7C). Bioassay confirmed that this serum IL-13 had no biological activity (Fig. 7D). Furthermore, sera of treated animals had the capacity to neutralize at least 10 ng/ml of additional, exogenously added IL-13 (Fig. 7D). Thus, both Ab01 and Ab02 seemed to trap biologically inactive IL-13 in the sera of treated animals.

Discussion

IL-13 bioactivity is mediated through a receptor complex consisting of IL-13Rα1 and IL-4Rα chains. We generated Ab01 and Ab02, two humanized monoclonal antibodies (IgG1, κ) that neutralize IL-13 bioactivity. Ab01 permitted IL-13 binding to IL-13Rα1 but prevented recruitment of IL-4Rα, whereas Ab02 directly blocked IL-13 interaction with IL-13Rα1. These antibodies were humanized by CDR grafting onto the same germine V_H and V_L frameworks, had comparable kinetic parameters for IL-13 binding, were potent neutralizers of IL-13 bioactivity in vitro, and had half-lives of approximately 14 days in cynomolgus monkeys. Minor distinctions were apparent because Ab02 had relatively more rapid association and dissociation kinetics for NHP IL-13, and Ab01 had relatively lower IC_50 for IL-13 neutral-
ization in some bioassay formats. To compare the consequences of targeting different IL-13 epitopes, the in vivo efficacy of Ab01 and Ab02 was compared in a cynomolgus monkey model of airway inflammation and IgE responsiveness after lung segmental challenge with *Ascaris* antigen.

In many cytokine antibody systems, in vivo neutralization activity has proved to be more complex than could be modeled in vitro. Several monoclonal antibodies to IL-2 actively induced murine T cell expansion in vivo, despite their inhibitory effect on T cell proliferation in vitro (Boyman et al., 2006). It was proposed that immune complex formation between cytokine and antibody effectively extended the half-life of IL-2 in vivo, accounting for the activity (Boyman et al., 2006). Antibodies to IL-4 (Finkelman et al., 1993), IL-3 (Finkelman et al., 1993), IL-5 (Zabeau et al., 2001), and IL-6 (May et al., 1993) have also been described to prolong half-life of the cytokine in circulation, but this property is difficult to predict and does not seem to be dependent on the strength of interaction with the antigen (Zabeau et al., 2001; Boyman et al., 2006). The complex nature of cytokine receptor systems, in addition to the unique epitopes seen by the antibodies, may contribute to this phenomenon.

With IL-13 signaling mediated by a multicomponent receptor shared between IL-4 and IL-13 and modulated by IL-13Rα2, the consequences of IL-13 inhibition by Ab01 and Ab02 are difficult to model. IL-13 initially forms a relatively low-affinity interaction with IL-13Rα1, leading to recruitment of IL-4Rα to form the high-affinity receptor complex that mediates downstream cellular responses (Andrews et al., 2002). The binding sites on IL-13 for IL-13Rα1 and the antagonist, IL-13Rα2, largely overlap (Madhankumar et al., 2002), allowing IL-13Rα2 to compete for the cytokine. Ab01 allowed IL-13 interaction with IL-13Rα1 and IL-13Rα2, both in solution and on the cell surface, but blocked recruitment of IL-4Rα. To avoid any unintended effector function, both Ab01 and Ab02 were engineered with mutations in the lower hinge region of IgG1 Fc (data not shown).

In contrast to Ab01, Ab02 blocked binding of IL-13 to both IL-13Rα1 and IL-13Rα2. Thus, Ab02 is predicted to have a mechanism of inhibitory activity in common with that of IL-13Rα2 and may compete. The impact of this is unknown. Mice deficient in IL-13Rα2 have exaggerated IL-13 responses, as expected due to absence of the IL-13 antagonist function (Wood et al., 2003; Zheng et al., 2008). The potential that IL-13Rα2 can have a cell signaling function under appropriate circumstances (Fichtner-Feigl et al., 2006) or that it may act as an antagonist of IL-4 in addition to IL-13 (Rahaman et al., 2002; Andrews et al., 2006; Kellner et al., 2003).
suggestion that the role of IL-13Rβ2 is not fully appreciated and raise further questions regarding the in vivo consequences of IL-13 neutralization strategy.

Segmental challenge with Ascaris antigen, delivered through a bronchoscope, induces lung inflammation (Bree et al., 2007). Both Ab01 and Ab02 significantly reduced total BAL cellular infiltrate 24 h postchallenge and produced trends toward reduced eosinophil and neutrophil inflammation. These findings support the utility of targeting IL-13 in reducing allergic lung inflammation. In cynomolgus monkeys, Ascaris elicits a strong IgE response (Patterson and Harris, 1992). In the present study, all animals had pre-existing titers of Ascaris-specific IgE, as a consequence of natural sensitization. Because IL-13 is a critical regulator of IgE production (Bacharier and Geha, 2000; Wills-Karp, 2004), we examined the effects of Ab01 and Ab02 on IgE responses. Treatment with anti-IL-13 antibodies significantly reduced Ascaris-specific IgE titers 8 weeks postchallenge. Basophil histamine release reflects IgE responsiveness of NHP sensitized to Ascaris (Patterson et al., 1983). In accordance with their reduced titers of Ascaris-specific IgE, NHP treated with Ab01 or Ab02 did not undergo the increase in basophil histamine release that accompanied Ascaris challenge in control animals. In human subjects, basophil histamine release has proved to be a useful correlate of allergen sensitivity, showing associations with skin test reactivity, specific IgE, and symptom score (Lichtenstein et al., 1967; Shim et al., 2003). IgE neutralization using a humanized antibody (omalizumab; Xolair) has proved to be an effective therapeutic strategy for asthma (Fahy, 2006), leading to reductions in circulating IgE titer, FcεRI expression, and basophil sensitivity to allergens (MacGlashan et al., 1997). The present findings predict that IL-13 neutralization may lead to similar modulation of IgE-mediated effector activity.

Administration of Ab01 or Ab02 to cynomolgus monkeys resulted in their exposure to high-affinity IL-13 binding antibodies, at high concentration, for an extended period of time. To ask whether these antibodies would affect levels of IL-13 cytokine in the circulation, assays were developed to detect IL-13 in the presence of Ab01 or Ab02. In untreated animals, circulating IL-13 titers were undetectable. However, after dosing with either Ab01 or Ab02. IL-13 concentrations of 1 ng/ml or above were found in the circulation. No IL-13 was measurable in any animal that received saline or IVIG. Other cytokine-binding agents have also been shown to effectively increase circulating levels of cytokine complexed with antagonist. Subjects given the anti-tumor necrosis factor (TNFα) antibody, Remicade, showed a dose-dependent increase in circulating TNFα levels (Charles et al., 1999). Likewise, administration of humanized anti-IL-6R mAb to
Crohn’s disease patients resulted in increased secretion concentrations of IL-6R and IL-6 (Ito et al., 2004), injection of murine anti-human IL-6 resulted in a spike in levels of serum IL-6 in a leukemia patient (Klein et al., 1991) and administration of neutralized anti-VEGF mAb to patients with cancer increased serum titers of VEGF (Margolin et al., 2001).

The increases in serum IL-13 titer peaked between 24 h and 2 weeks post-Ascaris challenge in each animal. Because no animal was given antibody without a subsequent Ascaris challenge, we cannot state definitively whether the increased circulating IL-13 titer was due to antibody administration or was a consequence of the challenge. Both scenarios have been described for other cytokine-neutralizing agents. Antibody-complexed with CCL2/MCP-1 was detectable in peripheral blood of RA patients dosed with a monoclonal antibody to the chemokine (Haringman et al., 2006). It was proposed that a depot was created from which bioactive MCP-1 could be released over time, accounting for a lack of clinical efficacy of the antibody (Haringman et al., 2006). Of note in this study, circulating levels of chemokine rose precipitously beginning 30 min after antibody administration (Haringman et al., 2006). This observation contrasts with the present study, in which circulating IL-13 levels did not become detectable until after the animals received the Ascaris challenge. Our findings are similar to those from a study in which humans were dosed with TNFR-Fc (Enbrel). No change in circulating TNFα levels was seen under resting conditions, but after LPS challenge, much higher serum TNFα concentrations were found in patients treated with Enbrel than in those given placebo (Suffredini et al., 1995). This TNFα was biologically inactive, presumably because it was bound to and neutralized by Enbrel (Suffredini et al., 1995). Likewise, in the present study, serum IL-13 concentrations were sufficient to be detectable by bioassay, but no cytokine bioactivity was found. Furthermore, the serum had residual neutralization capacity for additional, exogenously added, IL-13. A direct and statistically significant correlation was found between IL-13 titer in the circulation post-Ascaris challenge and the extent of total cellular inflammation in the BAL fluid. In addition to indicating that endogenous IL-13 is released as a consequence of the Ascaris challenge, this observation implicates a direct role for IL-13 in driving lung inflammation.

Cytokine neutralization is a major focus of therapeutic antibody development. For many cytokines, several strategies exist to achieve neutralization by targeting various aspects of receptor interaction or downstream signaling events. In some cases, differences in epitope specificity have been associated with differences in potency or in the range of activities that are neutralized (Phillips et al., 2000; Zhu et al., 2006). The two anti-IL-13 antibodies that were directly compared in this study had high sequence homology, differing only in the V\textsubscript{H} and V\textsubscript{L} CDR regions. They had comparable in vitro potency, and indistinguishable in vivo efficacy, despite their distinct epitope specificity and mechanisms of IL-13 neutralization. Although these findings may not be generalizable to all in vivo functions of IL-13, the observations that short-term (lung inflammation, cytokine) and long-term (specific IgE titer, basophil histamine release) activities were similarly affected indicates that IL-13 neutralization effectively blocked atopic responses, whether cytokine interaction with IL-4Ra or with IL-13Ro1 was targeted.

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References


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