

An IL-4/IL-13 Dual Antagonist Reduces Lung Inflammation, Airway Hyperresponsiveness, and IgE Production in Mice

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IL-4 and IL-13 comprise promising targets for therapeutic interventions in asthma and other Th2-associated diseases, but agents targeting either IL-4 or IL-13 alone have shown limited efficacy in human clinical studies. Because these cytokines may involve redundant function, dual targeting holds promise for achieving greater efficacy. We describe a bifunctional therapeutic targeting IL-4 and IL-13, developed by a combination of specific binding domains. IL-4-targeted and IL-13-targeted single chain variable fragments were joined in an optimal configuration, using appropriate linker regions on a novel protein scaffold. The bifunctional IL-4/IL-13 antagonist displayed high affinity for both cytokines. It was a potent and efficient neutralizer of both murine IL-4 and murine IL-13 bioactivity in cytokine-responsive Ba/F3 cells, and exhibited a half-life of approximately 4.7 days in mice. In a murine model of ovalbumin-induced ear swelling, the bifunctional molecule blocked both the IL-4/IL-13-dependent early-phase response and the IL-4-dependent late-phase response. In the ovalbumin-induced lung inflammation model, the bifunctional IL-4/IL-13 antagonist reduced the IL-4-dependent rise in serum IgE titers, and reduced IL-13-dependent airway hyperresponsiveness, lung inflammation, mucin gene expression, and serum chitinase responses. Taken together, these findings demonstrate the effective dual blockade of IL-4 and IL-13 with a single agent, which resulted in the modulation of a more extensive range of endpoints than could be achieved by targeting either cytokine alone.

Keywords: cytokine; bifunctional; Th2; asthma

A bifunctional modality that targets both murine IL-4 and IL-13 was generated to achieve the dual inhibition of these related cytokines with a single agent. IL-4 and IL-13 are promising targets for intervention in the treatment of asthma and other Th2-associated disorders. IL-4 acts as a Th2 differentiation agent, whereas IL-13 drives downstream effector responses, and both play a role in asthma. IL-4 and IL-13 interact with cells through a common receptor, consisting of IL-4R α and IL-13R α 1 (Type II receptor), expressed on monocytes, fibroblasts, epithelial cells, smooth muscle cells, and other nonlymphoid cell types (1). IL-4

CLINICAL RELEVANCE

Dual cytokine inhibition is an attractive therapeutic modality for diseases in which multiple agents are known to play a role. This report describes a novel dual antagonist directed against murine IL-4 and IL-13, which effectively targets both cytokines *in vivo* and inhibits a broader range of responses than could be achieved by targeting either cytokine alone. These findings could help advance the design and validation of dual cytokine targeting agents for therapeutic purposes.

can also activate cells through the IL-4R α / γ common complex (Type I receptor), expressed in T cells, B cells, and monocytes (2). The activation of either receptor results in the phosphorylation, dimerization, and nuclear translocation of signal transducer and activator of transcription-6 (STAT6), resulting in an activation of genes that mediate a range of activities associated with inflammation, fibrosis, and mucus production (2).

Multiple lines of preclinical evidence support a role for both IL-4 and IL-13 in the development and maintenance of allergic lung inflammation (3, 4). Despite their similarities and shared use of the Type II receptor, distinct activities of IL-4 and IL-13 have been noted in mouse models, attributable to use of the IL-4-specific Type I receptor, to differences in amounts or localizations of each cytokine produced, or to the specific modulation of IL-13 with the decoy receptor, IL-13R α 2. IL-4 acting through the Type I receptor enhances Th2 differentiation (5–7), whereas IL-4 or IL-13 acting through the Type II receptor could drive airway hyperresponsiveness (AHR) and mucus secretion (5, 6, 8). One or both receptors may contribute to lung inflammation and IgE production, depending on the model (5, 6, 8). Although targeting IL-13 appears to have greater potency than targeting IL-4, the deficiency or neutralization of both cytokines in combination achieves the highest degree of efficacy in mouse models of allergic lung inflammation (4, 5, 9–12).

A single therapeutic agent that can target both cytokines would be expected to provide robust efficacy in the treatment of asthma and other Th2-driven diseases. An antibody to IL-4R α was developed for this purpose (AMG-317; Amgen, Thousand Oaks, CA), but displayed relatively poor pharmacokinetic (PK) parameters and low efficacy in a human asthma trial (13). An IL-4 mutein (Pitrakinra; Aerovance, Berkeley, CA) binds to the IL-4R α chain of both Type I and Type II receptors, and blocks signaling mediated by either IL-4 or IL-13. It showed activity in allergen challenge studies (14), but displayed relatively unfavorable PK properties, with a short half-life (15). Inhibitors of STAT6 signaling, which should block responses to either IL-4 or IL-13, have also been described (16–20), but to date none have been tested in human clinical studies. In the

(Received in original form December 6, 2012 and in final form January 29, 2013)

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 49, Iss. 1, pp 37–46, Jul 2013

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Originally Published in Press as DOI: 10.1165/rcmb.2012-0500OC on February 28, 2013

Internet address: www.atsjournals.org

present study, a biotherapeutic agent targeting both murine IL-4 and IL-13 was generated by combining well-characterized binding domains in an optimal configuration, using appropriate linker regions. Proof of concept for dual IL-4 and IL-13 cytokine targeting was examined in mouse models of ovalbumin (OVA)-induced ear swelling and lung inflammation. Results confirmed that the bifunctional IL-4/IL-13 antagonist blocked IgE responses primarily driven by IL-4, and blocked lung inflammation, AHR, and mucin gene expression responses primarily driven by IL-13. Thus, the dual antagonist inhibited more correlates of disease than could be affected by targeting either cytokine alone.

MATERIALS AND METHODS

Generation of Murine IL-4/IL-13 Bifunctional Antagonist

The mouse IL-13R α 2-Fc (fragment crystallizable) $\times$ mu11B11 bifunctional antagonist was generated on a SCORPION (multispecific protein therapeutic) platform (Emergent BioSolutions, Rockville, MD). It consists of mouse sIL-13R α 2-Fc and mouse C57BL/6 IgG2c Fc domain lacking the C-terminal lysine (21), a linker sequence, anti-mouse IL-4 mu11B11 V_L (light chain variable region), a minimal 16 amino acid glycine-serine linker, and anti-mouse IL-4 mu11B11 V_H (heavy chain variable region). DNA fragments encoding these components were generated by PCR amplification, using Vent polymerase (New England Biolabs, Ipswich, MA) and appropriate oligonucleotide primers (Integrated DNA Technologies, Coralville, IA). Chinese hamster ovary PA-Dukx 153.8 cells were stably transfected using TransIT (Mirus Bio LLC)/Opti-MEM (Gibco; Invitrogen).

Concentrated, conditioned medium was loaded onto Protein A Sepharose Fast Flow (Invitrogen Life Technologies, Grand Island, NY) and washed with PBS (pH 7.2), and bound protein was eluted with 20 mM citric acid and 150 mM NaCl (pH 2.5). Material was immediately neutralized with 5% 2 M Tris (pH 8.0), and then loaded onto a Superdex 200 column (GE Healthcare Biosciences, Pittsburgh, PA) to remove aggregates. The final pool was analyzed by optical density at 280 nm, an endotoxin assay, protein A ELISA, analytical size exclusion, and SDS isoelectric focusing-PAGE. The material (molecular weight, 180 kD) was formulated in Ca²⁺, Mg²⁺-free PBS, pH 7.2.

Airway Hyperresponsiveness

Female C57BL/6 mice were immunized intraperitoneally with 10 μ g OVA emulsified in 4.5 mg alum in 200 μ l water on Days 0 and 14, and challenged via the airways with aerosolized OVA (1%) on Days 26, 27, and 28. Animals were treated intraperitoneally with 200 μ g anti-IL-4 mu11B11, sIL-13R α 2-Fc, or the bifunctional modality, on Days 23, 26, and 28. On Day 30, animals were assessed for changes in lung function, as described elsewhere (22). Aerosolized methacholine (0, 8, 16, 32, 64, and 128 mg/ml) was administered in a dose-escalating manner via tracheal cannula. Immediately after each aerosolized dose, 15 airway perturbations were performed at 8-second intervals. Data were fit by Flexiware software, version 7 (Scireq Inc., Montreal, PQ, Canada), to calculate airway resistance.

Ear Swelling

Female BALB/c mice ($n = 10$ /group) were sensitized with OVA/alum intraperitoneally. On Day 13, mice received 20 μ g of the test agent intradermally in both ears. On Day 14, mice were challenged intradermally in the left ear with 10 μ g of OVA or vehicle in a total volume of 20 μ l endotoxin-free sterile normal saline (0.9%). Ear swelling was measured using an engineer's micrometer (Mitutoyo Corp., Aurora, IL), and expressed as the change in ear thickness (Δ) from baseline preinjection values.

Statistical Analysis

All data shown are representative of two to four independent experiments. Differences between treatment groups were determined by a Student unpaired t test. Variations of lung mechanics were analyzed with two-way ANOVA, followed by a Bonferroni *post hoc* test. Analysis was performed using GraphPad Prism for Windows (version 5.02; Graphpad Software, San Diego, CA). Results were considered significant at $P < 0.05$.

Online Supplement

The online supplement describes our methods for the generation of murine 11B11, surface plasmon resonance, PK study design, the quantitation of IL-4 and IL-13 antagonist, IgE, and chitinase activity in serum, STAT6 phosphorylation, the analysis of bronchoalveolar lavage fluid (BALF) inflammation, and gene expression analysis.

RESULTS

Generation of Murine IL-4/IL-13 Bifunctional Antagonist

A bifunctional molecule with specificity for murine IL-4 and IL-13 was constructed with an N-terminal IL-13-binding domain derived from the murine IL-13R α 2 extracellular domain sequence, linked to the C57BL/6 IgG2c Fc. This was connected by a linker sequence to a C-terminal IL-4-binding domain, comprised of the single chain variable fragment (scFv) generated by engrafting complementarity-determining region sequences of the rat anti-mouse IL-4 monoclonal antibody (mAb) 11B11 (23) onto the murine IgG1 framework. A schematic of the bifunctional molecule is shown in Figure 1. The construct was designed to bind two molecules of IL-4 and two molecules of IL-13 per molecule of bifunctional antagonist.

Murine IL-4/IL-13 Binding Kinetics

The IL-4 and IL-13 binding kinetics of the bifunctional molecule were examined by surface plasmon resonance. For IL-13, the K_D and binding capacity were highly comparable to those of sIL-13R α 2-Fc, with 2 moles of IL-13 bound per mole of

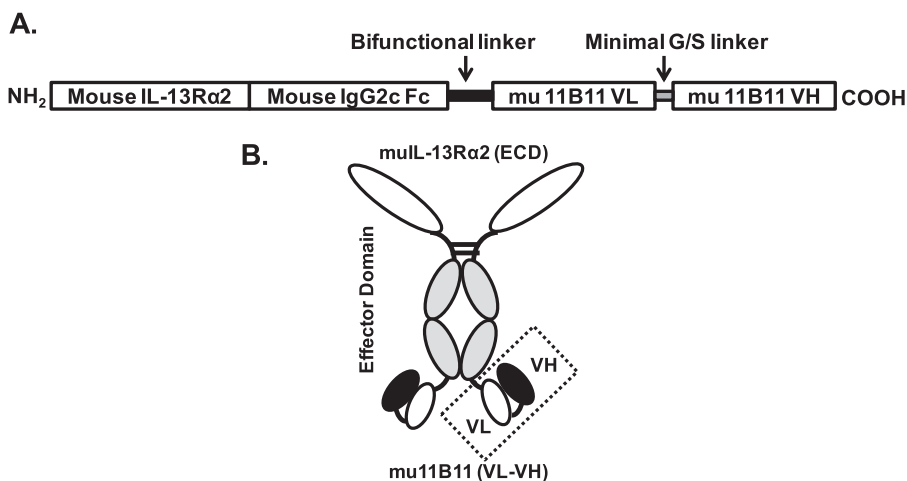
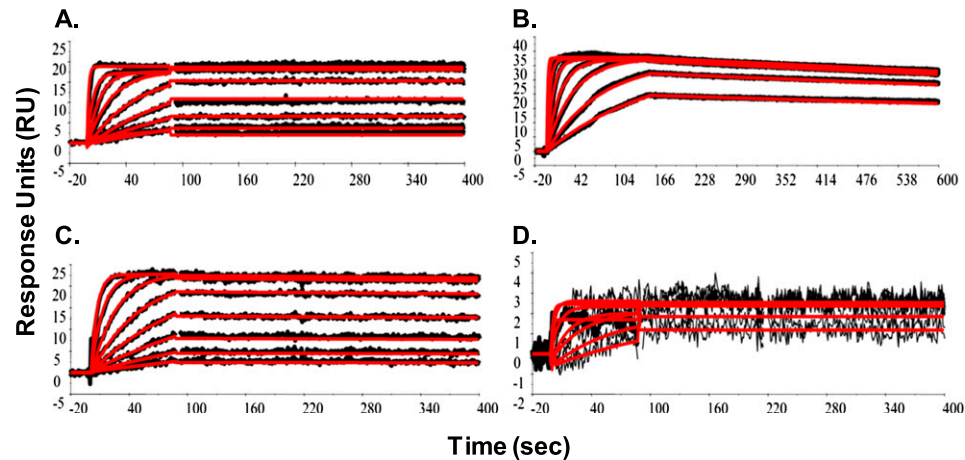


Figure 1. Schematic of murine bifunctional IL-4/IL-13 antagonist. (A) Configuration of IL-4/IL-13 bifunctional antagonist, from the N-terminal mouse IL-13R α 2 extracellular domain (ECD) to the C-terminal single chain variable fragment of anti-mouse IL-4 monoclonal antibody (mAb) mu11B11. (B) Schematic indicates overall structure of the IL-4/IL-13 dual antagonist, with two binding sites for each cytokine. COOH, C-terminus; Fc, fragment crystallizable; G/S, glycine/serine; VH, heavy chain variable region; VL, light chain variable region.

bifunctional entity (Figures 2A, 2C, and 2E). For IL-4, the K_D was similar to that of anti-mouse IL-4 mAb mu11B11, but the binding capacity suggested that approximately 50% of the theoretical maximum number of IL-4 binding sites was occupied (Figure 2E), indicating either that one half of the bifunctional molecules bound two molecules of IL-4 whereas the other half did not bind IL-4, or that each bifunctional molecule bound only one molecule of IL-4. Of note, the bifunctional molecule could be bound to both cytokines simultaneously, such that when immobilized through IL-13, it retained the ability to bind to IL-4 (Figure 2F). The order of cytokine addition did not affect the dual binding activity (data not shown).

Cytokine Binding and Neutralization Activity of Bifunctional IL-4/IL-13 Antagonist

The bifunctional molecule competed for the binding of murine IL-4 to anti-IL-4 mAb 11B11 (Figure 3A), and competed for the binding of murine IL-13 to anti-IL-13 mAb MJ2-7 (Figure 3B). It showed full IL-13 binding activity compared with sIL-13R α 2-Fc on a molar basis (Figure 3B), but a 2–3-fold reduced ability to compete for IL-4 binding to anti-IL-4 mAb 11B11 (Figure 3A), which is consistent with data from our surface plasmon resonance analysis. An assay to detect the bifunctional antagonist by the capture of immobilized IL-13 and the detection of biotinylated IL-4 (Figure 3C) confirmed that both cytokines could be bound together. Because the



E.	murine IL-13				murine IL-4			
	k_a 1 x E+06 (1/Ms)	k_d 1 x E-05 (1/s)	K_d (pM)	% Theoretical Rmax (2:1)	k_a 1 x E+06 (1/Ms)	k_d 1 x E-04 (1/s)	K_d (pM)	% Theoretical Rmax (2:1)
Bifunctional	3 ± 1	9 ± 1	31 ± 1	74 ± 3	5 ± 2	4 ± 2	75 ± 1	51 ± 4
sIL-13Rα2-Fc	3.0 ± 0.6	1.7 ± 0.4	5.9 ± 0.1	95 ± 3				
Anti IL-4 mu11B11					5 ± 2	2.8 ± 0.3	66 ± 2	92 ± 4

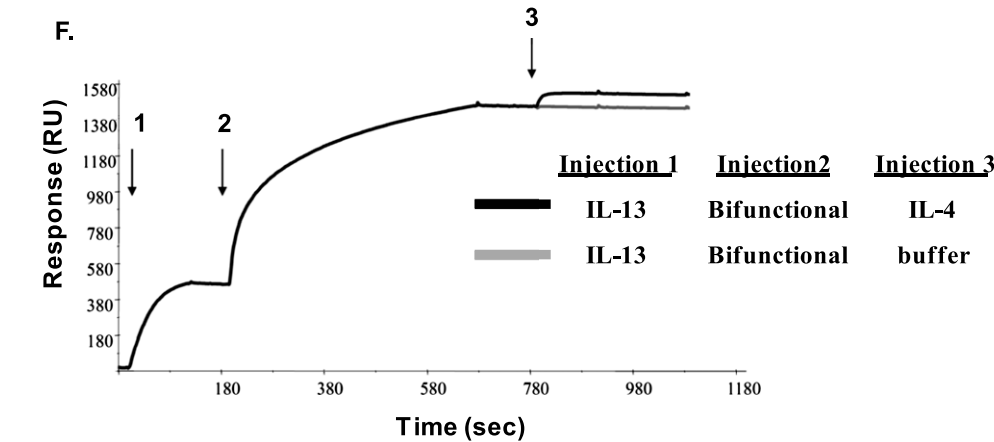


Figure 2. Surface plasmon resonance analysis of IL-13 and IL-4 binding to the dual antagonist. (A) sIL-13R α 2 fragment crystallizable (Fc), (B) anti-IL-4 mAb mu11B11, or (C and D) bifunctional IL-4/IL-13 antagonist was immobilized on a biosensor chip, and solutions of IL-13 or IL-4 were flowed over the chip, as indicated, to evaluate the association rate. At 90 or 150 seconds, the cytokine solution was exchanged for buffer, and dissociation was monitored for the remainder of the time course (up to 1 hour). All data were fit to a 1:1 Langmuir binding model in BiaEvaluation software, version 4.1.1 (GE Healthcare) (fit lines shown in gray). For each cytokine concentration (0.195–100 nM IL-4 or IL-13), raw data are shown in red, and the fit line in black. (E) Kinetic constants. Assuming two binding sites for each cytokine per molecule of bifunctional antagonist, the percent theoretical binding capacity (Rmax) indicates that approximately 74% of the IL-13 binding sites, but only approximately 53% of the theoretical IL-4 binding sites, are accessible. For IL-4 and the bifunctional molecule, the percent theoretical Rmax was calculated based on the binding of IL-4 to the bifunctional protein. (F) The IL-4/IL-13 bifunctional binds both cytokines. At time point 1, 100 nM of IL-13 were injected over an anti-IL-13 antibody immobilized on a biosensor chip. At time point 2, the bifunctional IL-4/IL-13 antagonist was injected and captured on IL-13 through the IL-13-binding domain. At time point 3, either PBS-NET buffer

(gray line) or murine IL-4 (black line) was injected over the dual antagonist, and additional binding was monitored. Data shown are representative of two independent surfaces of anti-human IL-13. No binding of the dual antagonist or murine IL-4 alone to the anti-human IL-13 antibody surface was observed (data not shown). Results are representative of two independent experiments. E+06, E–04, and E–05 indicate numbers to the power of +6, –4, or –5.

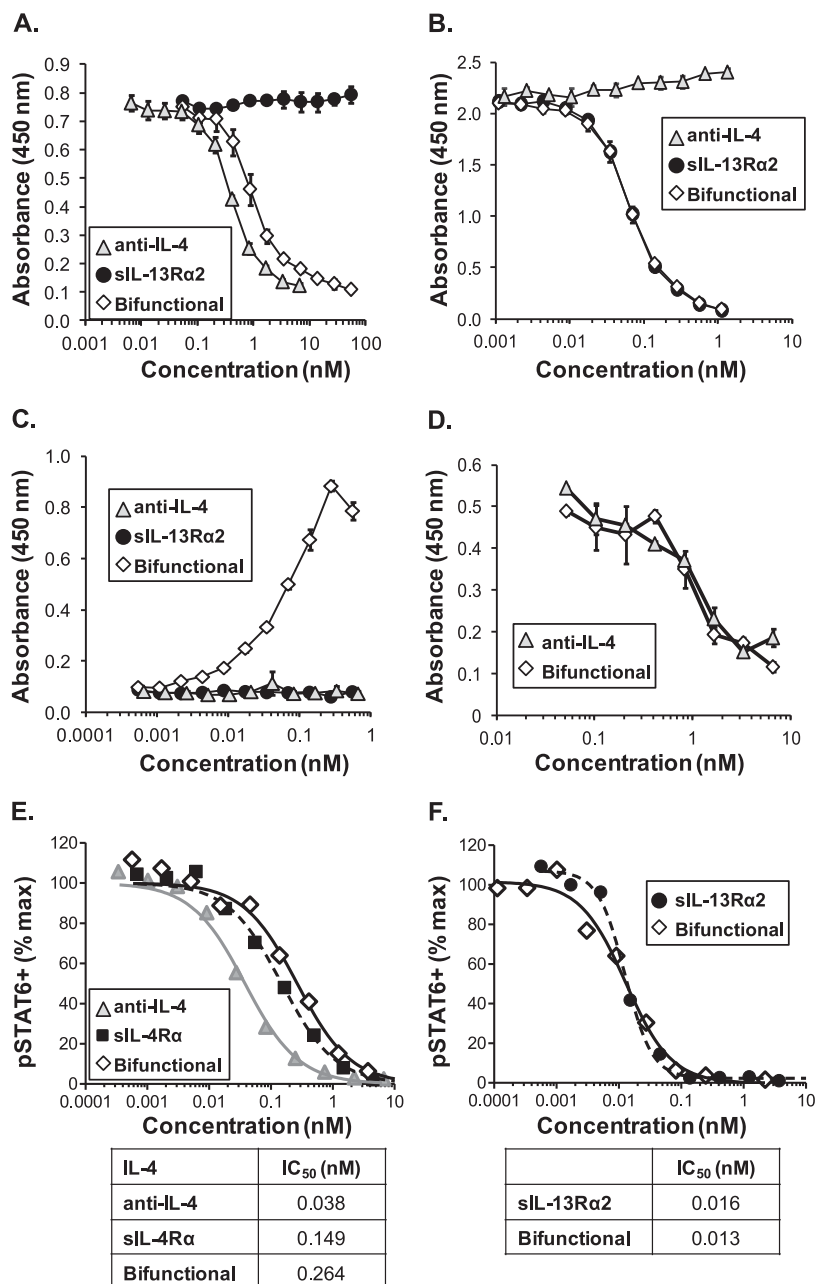


Figure 3. Assays to detect (A) IL-4 binding activity, assayed as competition for IL-4 binding to biotinylated anti-IL-4 mAb 11B11; (B) IL-13 binding activity, assayed as competition for IL-13FLAG binding to anti-IL-13 antibody; and (C) IL-4/IL-13 bifunctional activity, assayed through the capture of IL-13FLAG and the detection of bound, biotinylated IL-4. Anti-IL-4 mAb mu11B11, IL-13 blocker murine sIL-13Rα2 Fc, or IL-4/IL-13 bifunctional was tested. (D) Competition for biotinylated IL-4 binding to murine sIL-4Rα Fc. The neutralization of (E) IL-4 bioactivity and (F) IL-13 bioactivity by IL-4/IL-13 bifunctional antagonist was examined. Ba/F3 cells expressing mouse IL-4 and IL-13 receptors were incubated for 30 minutes at 37°C with a suboptimal concentration of IL-4 or IL-13, along with the indicated concentrations of bifunctional IL-4/IL-13 antagonist, anti-IL-4 (mu11B11), murine sIL-4Rα Fc, or murine sIL-13Rα2 Fc. After incubation, cells were fixed, permeabilized, and stained with Alexa-Fluor 488-labeled antibody to phosphorylated signal (ps) transducer and activator of transcription-6 (STAT6). The 50% inhibitory concentration (IC₅₀) was determined as the concentration of each agent that produced a 50% inhibition of the cytokine response.

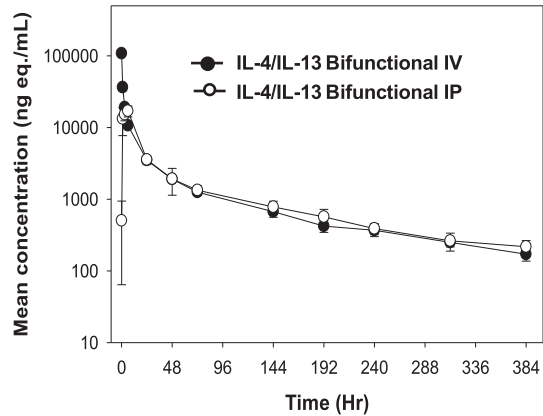
bifunctional molecule demonstrated reduced binding to IL-4, its potency in blocking IL-4 interactions with IL-4Rα was assessed. Murine IL-4 binding to soluble murine IL-4Rα-Fc was competed with comparable potency by mu11B11 and by the IL-4/IL-13 bifunctional antagonist (Figure 3D).

Cytokine neutralization activity was tested in the STAT6 phosphorylation bioassay, using Ba/F3 cells responsive to murine IL-4 and IL-13. The bifunctional molecule inhibited the bioactivity of both cytokines. Its activity against IL-4 was approximately 3–5-fold lower than that of mu11B11 (Figure 3E), consistent with the reduced binding capacity for IL-4. The bifunctional modality demonstrated complete neutralization activity against IL-13, compared with the sIL-13Rα2-Fc standard (Figure 3F).

Pharmacokinetics of the Bifunctional Murine IL-4/IL-13 Antagonist

For PK analysis, the bifunctional molecule was iodinated, and delivered at an intraperitoneal or intravenous dose of 8 mg/kg

to female C57BL/6 mice. Data were collected for 16 days (384 h) (Figure 4). After a single intravenous dose, the total body clearance of the [¹²⁵I] murine IL-4/IL-13 bifunctional antagonist was 13.7 ml/h/kg, which appeared to be faster than that of a typical murine IgG in mice (< 2 ml/h/kg), and the elimination half-life (t_{1/2}) of approximately 4.7 days (112 h; Figure 4) was reduced compared to that of murine IgG (24, 25). The volume of distribution at steady state (V_{dss}) was approximately 1,102 ml/kg, and appeared to be larger than the total body water and larger than a typical V_{dss} estimate for murine IgG in mice (typically less than 500 ml/kg). After a single intraperitoneal dose, the bioavailability was approximately 99%, the time to peak concentration (T_{max}) was 6 hours, and t_{1/2} was 4.8 days, in line with the t_{1/2} estimate in the intravenous dose group. We observed no evidence of an immune response directed against the IL-4/IL-13 bifunctional antagonist in these mouse studies. PK profiles from single-dose studies were used to predict trough test article concentration for the pharmacology studies to be described.



Route	C _{5min} or C _{max} (μg.eq/mL)	t _{max} (hr)	AUC _{0-∞} (hr*μg.eq/mL)	AUC _{0-∞} /Dose (hr*μg.eq/mL)/(mg/kg)	CL _p (mL/hr/kg)	V _{dss} (mL/kg)	t _{1/2} (hr)
IV	108	NA	586	73.2	13.7	1102	112
IP	17.0	6	582	72.8	NA	NA	116

Activity of the Bifunctional IL-4/IL-13 Antagonist in an OVA-Induced Ear Swelling Model

Mice were sensitized on Day 0 with OVA/alum, and challenged intradermally in the ear with OVA on Day 14. Early phase (1–2 h; Figures 5A and 5B) and late phase (25 h; Figures 5A and 5C) phase ear-swelling responses were measured. In this model, IL-4 blockade with anti-IL-4 mAb mu11B11 eliminated late-phase ear swelling, but did not reduce the early-phase response. In contrast, IL-13 blockade with sIL-13Rα2-Fc exerted a partial but not statistically significant effect on the early-phase response, but did not affect the late phase. The IL-4/IL-13 bifunctional molecule significantly reduced both the early-phase and

late-phase responses (Figures 5B and 5C). Because both cytokines contribute to early-phase ear swelling, the activity of the bifunctional molecule in the early phase could not be attributed to a blockade of IL-4 or IL-13. In contrast, the reduction in late-phase response confirms that the bifunctional antagonist blocked IL-4 activity *in vivo*.

Activity of the Bifunctional IL-4/IL-13 Antagonist in an OVA-Induced Lung Inflammation Model

After appropriate allergen sensitization and airways allergen challenge, total BALF inflammation (Figure 6A) and BALF eosinophilia (Figure 6B) were significantly reduced by IL-13 blockade, but not by IL-4 blockade, as measured on Day 30.

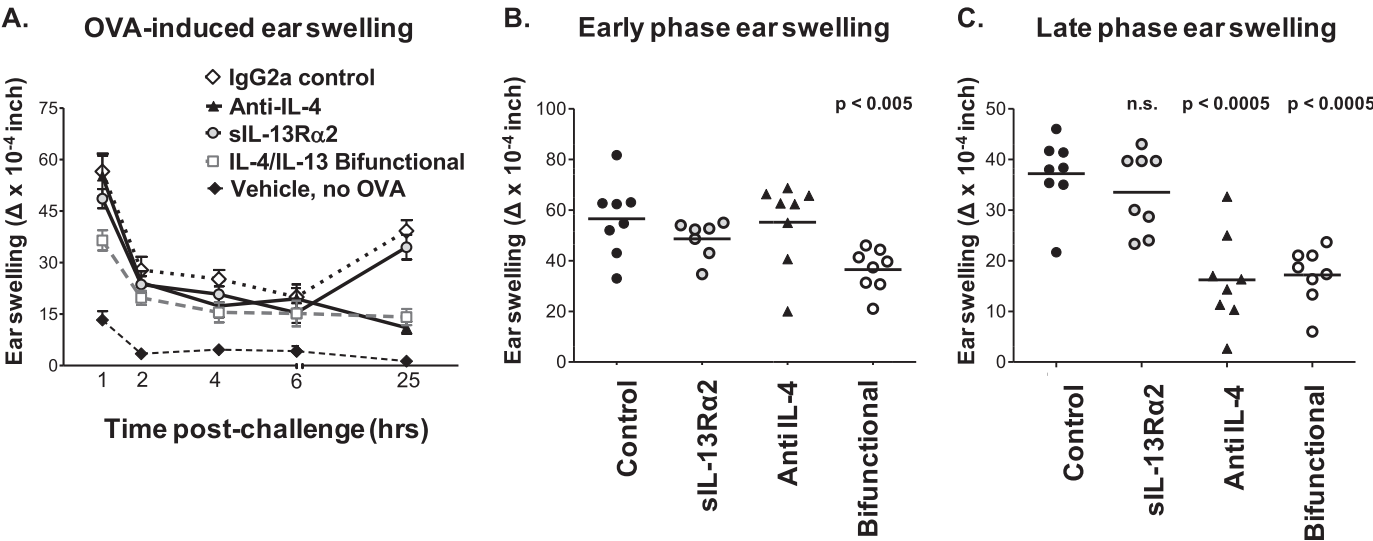


Figure 5. Bifunctional IL-4/IL-13 antagonist exerts IL-4 neutralization activity in an ear-swelling model. Mice were immunized intraperitoneally with OVA/alum on Day 0. On Day 13, the animals were treated with murine 11B11, sIL-13Rα2 Fc, IgG2a control, or the IL-4/IL-13 bifunctional intradermally in both ears. On Day 14, they were challenged intradermally with vehicle (right ear) or ovalbumin (OVA; left ear). The swelling response was measured at various time points after the challenge. (A) Early-phase and late-phase responses. (B) Late-phase responses were measured at 25 hours for individual animals. P values were determined by a t test for groups treated with sIL-13Rα2 Fc, anti-IL-4 mu11B11, or bifunctional IL-4/IL-13 antagonist, compared with those treated with IgG control. Results with the IL-13 blocker, IL-4 blocker, or bifunctional antagonist are representative of two to four independent experiments. Δ, change in ear thickness.

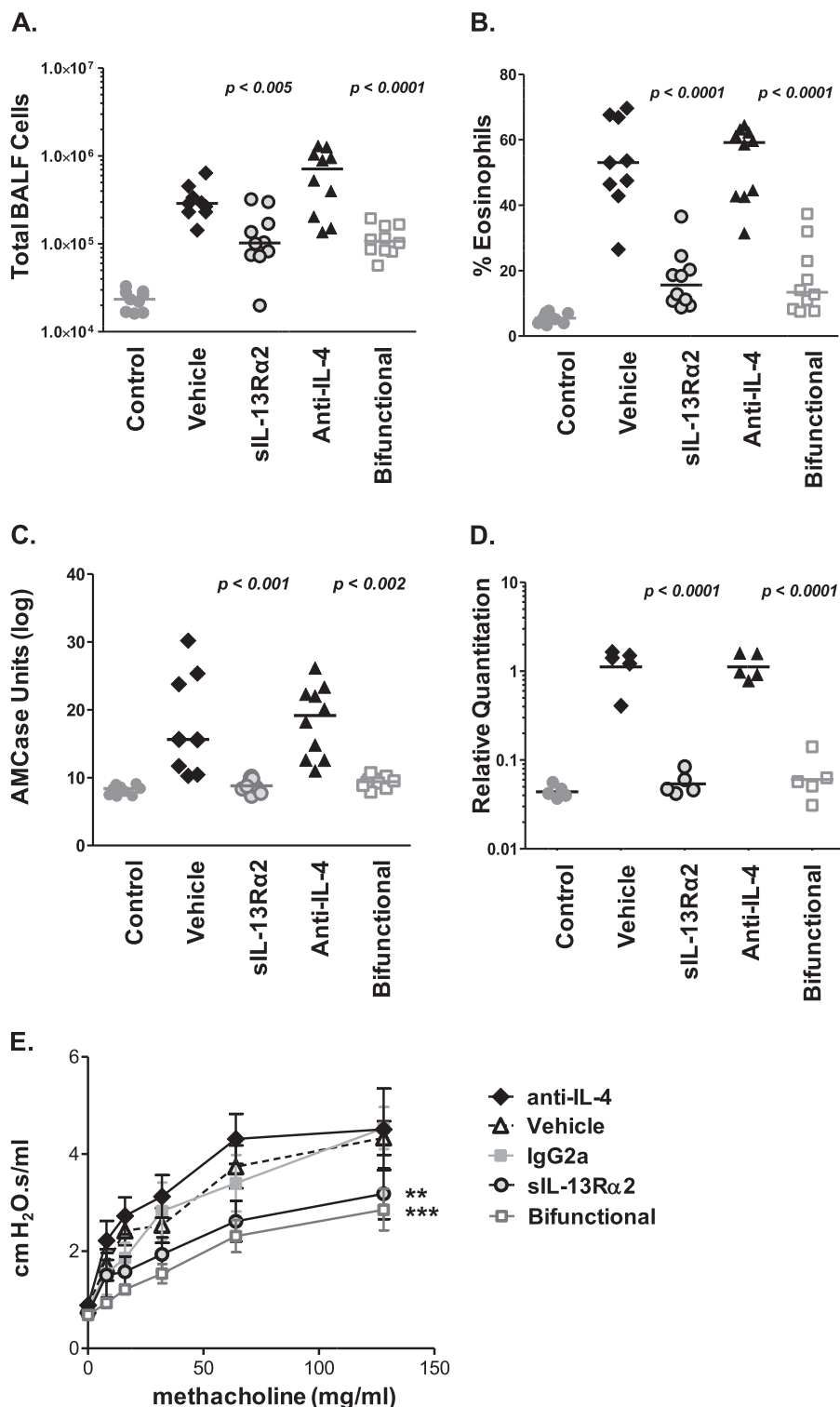


Figure 6. Bifunctional IL-4/IL-13 antagonist exerts IL-13 neutralization activity in OVA-induced lung inflammation and airway hyper-responsiveness. Female C57BL/6 mice were immunized intraperitoneally with OVA/alum on Days 0 and 14, received an inhalation challenge with OVA aerosol on Days 26 and 27. Mice were treated with vehicle, IL-4/IL-13 bifunctional, sIL-13Rα2 Fc, or anti-IL-4 mAb mu11B11 at challenge (Days 23, 26, and 28). Total cellular infiltrate (A) and percent eosinophils (B) were determined in BALF harvested on Day 30. (C) Acidic mammalian chitinase (AMCase) activity was quantitated in serum at end bleeds. (D) Muc5ac gene expression in lung tissue, expressed relative to glyceraldehyde 3-phosphate dehydrogenase. Results are representative of three independent experiments. (E) On Day 30, mice received increasing doses of aerosolized methacholine. Airway resistance was measured using the Scireq Flexivent system (Scireq Inc., Montreal, PQ, Canada) ($n = 10$ per group). Statistical significance was analyzed by two-way ANOVA in comparison with IgG2a control (** $P < 0.02$ and *** $P < 0.0001$). Results are representative of two independent experiments.

The bifunctional modality was also efficacious in this model, consistent with IL-13 neutralization activity (Figures 6A and 6B).

Additional biomarkers of the response to OVA immunization and challenge were assayed. Activity of the IL-13-regulated chitinase, acidic mammalian chitinase (AMCase) (26, 27), was reduced by sIL-13Rα2-Fc or by the bifunctional IL-4/IL-13 antagonist, but not by anti-IL-4 treatment at time of challenge (Figure 6C). The lung-tissue expression of several IL-13-regulated genes was also analyzed. Expression of the lung epithelial gene Muc5ac, known to be regulated primarily by IL-13 (28–30), was reduced by treatment with sIL-

13Rα2-Fc or the bifunctional IL-4/IL-13 antagonist, but not by the anti-IL-4 mAb (Figure 6D). For all of these endpoints, the magnitude of treatment effect was directly proportional to the concentration of IL-4/IL-13 bifunctional antagonist that was recovered in the end bleeds of the mice (Figure E1 in the online supplement).

Activity of the Bifunctional IL-4/IL-13 Antagonist on OVA-Induced Lung Resistance

To assess lung function, OVA-immunized mice were challenged on Day 30 with increasing doses of inhaled methacholine. Both

sIL-13R α 2-Fc and the bifunctional IL-4/IL-13 antagonist significantly reduced airway resistance (Figure 6E) to a level comparable to that seen with animals that did not receive the OVA challenge (data not shown). In contrast, IL-4 neutralization with anti-IL-4 exerted no effect (Figure 6E). Both sIL-13R α 2-Fc and the IL-4/IL-13 bifunctional antagonist produced statistically significant reductions in airway resistance, whether compared with vehicle or control IgG2a (Figure 6E). Taken together, the activity of the bifunctional IL-4/IL-13 antagonist on the OVA-induced, IL-13-dependent inflammatory endpoints, including lung eosinophilia, serum chitinase, lung Muc5ac expression, and airway resistance, confirmed its IL-13 neutralizing activity *in vivo*.

Activity of Bifunctional IL-4/IL-13 Antagonist in OVA-Induced IgE Production

OVA-immunized mice were treated with vehicle, bifunctional IL-4/IL-13 antagonist, sIL-13R α 2-Fc, or anti-IL-4 mAb mu11B11 every 2 days, starting on Day -1 (before OVA immunization) and continuing until Day 24. IgE titers were assayed in end bleeds, along with concentrations of the test article. IL-13 neutralization did not affect serum IgE concentrations in this model, whereas IL-4 blockade effectively reduced IgE titers to undetectable concentrations in most animals (Figure 7A). The bifunctional antagonist also significantly reduced IgE titers, but was less effective than anti-IL-4 mu11B11. We compared IL-4 blocking activity in sera at sacrifice in mice treated with anti-IL-4 or with the bifunctional IL-4/IL-13 antagonist, using the IL-4-binding ELISA described in Figure 3A. Much higher IL-4-binding activity was recovered in the sera of animals dosed with anti-IL-4, compared with those dosed with the bifunctional modality in this repetitive dosing protocol, resulting in an approximately 100-fold difference at termination (Figure 7B). Measured trough serum concentrations of the bifunctional IL-4/IL-13 antagonist were in good agreement with projected values, based on PK data from single-dose experiments (Figure 4). This difference in trough serum concentrations most likely accounts for the lower potency of the bifunctional IL-4/IL-13 antagonist in reducing serum IgE titers, compared with anti-IL-4 treatment. Nevertheless, the reduced serum IgE titers after treatment with the bifunctional antagonist confirmed that this molecule effectively neutralized IL-4 *in vivo*.

Comparison of Effective Dose Ranges for the IL-4/IL-13 Bifunctional Antagonist and Single Inhibitors

To compare the relative *in vivo* efficacy of the bifunctional IL-4/IL-13 antagonist with single IL-4 and IL-13 inhibitors, a dose range of each agent was administered to mice. Because lung eosinophilia was inhibited by sIL-13R α 2-Fc or bifunctional antagonist administered at the time of challenge, but was not inhibited by anti-IL-4 under the same conditions (Figure 6B), this endpoint was used to evaluate the effects of IL-13 antagonism. The dose dependence of the IL-4/IL-13 bifunctional antagonist in reducing eosinophilia was comparable to that of sIL-13R α 2-Fc (Figure E2A). In the same model, the administration of anti-IL-4 or IL-4/IL-13 dual antagonist at the time of sensitization rather than challenge blocked the expression of thymus and activation regulated chemokine (TARC) in the lung (Figure E2B). IL-13 blockade at sensitization did not reduce TARC expression, although sIL-13R α 2-Fc administered at challenge was effective (not shown). Compared with anti-IL-4 mAb mu11B11, the dual antagonist was somewhat less effective at blocking TARC expression (Figure E2B), consistent with its relatively reduced activity against IL-4 (Figures 2 and 3). These results show that the magnitude of *in vivo* inhibitory activity of the bifunctional antagonist against responses dependent on IL-4 or IL-13 did not exceed that of the single antagonists.

DISCUSSION

A bispecific immunotherapeutic agent targeting murine IL-4 and IL-13 was generated on a novel protein therapeutic scaffold. The molecule consists of N-terminal IL-13-binding domains derived from the murine sIL-13R α 2 extracellular region, linked to murine IgG2c Fc, followed at the C-terminus by scFv domains targeting IL-4, derived from the anti-mouse IL-4 mAb, 11B11. The dual antagonist potently binds to and neutralizes both cytokines, and was used to establish preclinical proof of concept for the efficacy of the bifunctional strategy in allergic inflammation. In different models of immune activation in response to immunization and challenge with OVA, IL-4 blockade reduced late-phase ear swelling and IgE production, whereas IL-13 blockade reduced airway hyperresponsiveness, lung eosinophilia, serum chitinase activity, and mucin gene expression. The bifunctional molecule blocked all of these responses, indicating that a bifunctional therapeutic agent that recognizes mouse IL-4 and IL-13 could effectively target both cytokines *in vivo*, and could exert

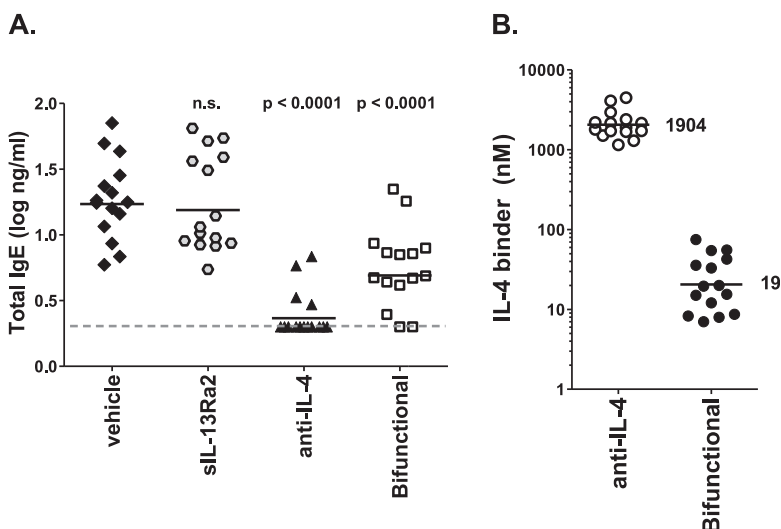


Figure 7. Repeated dosing with bifunctional IL-4/IL-13 antagonist reduces titers of OVA-specific IgE. Female C57BL/6 mice were immunized and challenged with OVA/alum, and received repeated doses of IL-4 binder, IL-13 binder, or IL-4/IL-13 bifunctional every 2 days, starting on Day -1 (before OVA immunization), and continuing until termination on Day 24. (A) IgE concentrations were assayed in sera collected at sacrifice, using a specific ELISA. (B) Serum concentrations of IL-4 binder were assayed in end bleeds, using a specific ELISA. Results are representative of two independent experiments. n.s., no significance. Sera of mice treated with anti-IL-4 and bifunctional antagonist contained median concentrations of 1,904 nM and 19 nM IL-4 binder, respectively.

a broader range of activity than a therapeutic targeting either cytokine alone.

IL-4 and IL-13 are well-established drivers of airway responses in mouse models of respiratory disease. A deficiency in IL-4 or IL-13, or the blockade of either cytokine with specific antagonists, confirmed that both IL-4 and IL-13 contribute to the development of atopy and other correlates of respiratory disease (4, 5, 12, 31). In mice, IL-4 appears to play a primary role in IgE generation, whereas IL-13 is the dominant driver of inflammation, mucus production, and fibrosis (31). A lack of response to both IL-4 and IL-13, through the combined absence of both cytokines (9), a deficiency in the shared receptor element IL-4R α (6, 32), or a lack of the shared signaling molecule, STAT6 (32, 33), confers strong protection in murine models of respiratory disease.

Genetically deficient animals underscore the potential consequences of the complete developmental absence of these cytokines, but do not fully mimic the therapeutic potential of targeting IL-4 and IL-13 pharmacologically. Human clinical studies with therapeutic antibodies to IL-13 have shown signs of efficacy in treating asthma (34, 35), but appear to exert suboptimal potency (36, 37). IL-4-targeted agents, including anti-IL-4 antibody and sIL-4R, have so far failed to meet endpoints in asthma trials (38). Given the overlapping functions of these cytokines, mediated in part through a shared receptor, the partial activity of single antagonists may not be unexpected. Dual targeting strategies hold promise for greater efficacy (39), but present design and engineering challenges. Several strategies for the bifunctional targeting of IL-4 and IL-13 have been proposed or are being developed, including anti-IL-4R (13), small interfering RNA to IL-4R α (40), IL-4 mutein (41), and bifunctional inhibitors on novel protein scaffolds (39, 42, 43), and some have shown promising signs of activity in human clinical studies (13, 14).

The bifunctional IL-4/IL-13 antagonist is comprised of separate IL-4 and IL-13 binding domains, and is able to bind and neutralize both cytokines. Agents targeting two soluble cytokines could be attractive candidates for development as therapeutic agents, but because of potential differences in the localization and quantity of each target, their utility must be validated *in vivo*. Several novel configurations have been constructed for the dual targeting of soluble agents, based on antibody scaffolds. Structural analysis confirmed that a bispecific chimeric antibody targeting IL-13 and extracellular matrix metalloproteinase inducer (EMMPRIN) maintained the antigen-binding interfaces of the parent monoclonal antibodies, but biological engagement of the targets was not demonstrated (44). A bispecific antibody targeting IL-17A and IL-23p19 was developed by combining scFv targeting each cytokine. This agent was found to neutralize both targets in cell-based assays, and bound both targets simultaneously as determined by surface plasmon resonance, but *in vivo* activity could not be validated in a disease model because the agent did not react with rodent cytokines (45). A dual variable domain immunoglobulin (DVD-Ig) targeting IL-12 and IL-18, engineered by fusing the V_L and V_H domains of individual antibodies in tandem using a short linker sequence, bound and neutralized both cytokines, and effectively reduced IFN- γ production in a humanized mouse model (46). A DVD-Ig targeting mouse IL-1 α and IL-1 β , produced to allow validation in rodent models, showed potency in collagen-induced arthritis comparable to that seen with a combination of antibodies to the individual cytokines (46).

In the present study, the bifunctional IL-4/IL-13 antagonist was targeted to mouse cytokines to allow for the assessment of *in vivo* potency and comparisons with its well-characterized constituents targeting the single cytokines. The late-phase response to intradermal challenge with OVA was dependent on IL-4 but not IL-13, consistent with the previously described IL-4

dependence of late-phase reactions (47–49). Anti-IL-4 also reduced serum IgE titers after OVA immunization, whereas sIL-13R α 2-Fc did not. Although serum IgE titers are reduced in IL-13-deficient mice (9, 10, 50, 51) and elevated in IL-13 transgenic mice (52–54), neither anti-IL-13 (55) nor sIL-13R α 2-Fc (56, 57) affected IgE responses in short-term OVA immunization and challenge models. In contrast, IL-4 antibody has been shown to reduce IgE responses to OVA (58) and other antigens (23, 59–61). The activity of the IL-4/IL-13 dual antagonist in reducing serum IgE confirmed its IL-4-blocking activity *in vivo*. Immunization and airway challenges with OVA also elicit a panel of IL-13-dependent effector responses, including BALF eosinophilia (62), AHR (56, 62), increased chitinase activity (22, 63), and the induction of mucus and Mu5ac expression in the airways (56, 62, 64). The bifunctional molecule blocked all of these responses, confirming its IL-13 neutralization activity *in vivo*.

Interestingly, the extent of inhibition for any given endpoint did not exceed that of the single antagonist, even though IL-4 and IL-13 are known to interact at several points. For example, by promoting Th2 differentiation, IL-4 can induce IL-13 production. IL-4 may influence the expression of IL-13R α 1 (65), which could differentially affect the responsiveness to IL-4 and IL-13 by shifting the balance of Type I and Type II receptors (66). Either cytokine may also affect the expression of IL-13R α 2, in association with cofactors such as TNF- α (67), which could in turn regulate responsiveness to IL-13. Within the parameters of the present study, however, cooperative effects such as these were not readily demonstrable. A chronic disease model, induced by house dust mite or fungal allergen, or an examination of longer-term endpoints such as fibrosis, may be required to reveal these effects.

Although a dual antagonist blocking two soluble agents may achieve the same functional effect as a mixture of antibodies, it does present practical advantages. By targeting both IL-4 and IL-13 with a single molecule, a bifunctional antagonist provides the neutralization capacity of two antibodies with half as much protein, offering the potential to deliver a higher effective dose efficiently. It also avoids the need to formulate separate antibody components that may differ in solubility, stability, or half-life. The findings presented here support that a single bifunctional therapeutic agent targeting IL-4 and IL-13 can reduce asthmatic responses driven by either cytokine, resulting in an inhibition of a broader range of responses than could be achieved by the neutralization of either IL-4 or IL-13 alone.

Author disclosures are available with the text of this article at www.atsjournals.org.

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An IL-4/IL-13 Dual Antagonist Reduces Lung Inflammation, Airway Hyperresponsiveness, and
IgE Production in Mice

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ONLINE SUPPLEMENT

MATERIALS AND METHODS

Mice. Female C57BL/6 or BALB/c mice were purchased from Jackson labs (Bar Harbor, ME).

All animal studies were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals and Pfizer Institutional Animal Care and Use Committee approved all aspects of these studies.

Construction of Murine Anti-Mouse IL-4 Antibody. Heavy chain and light chain variable regions were cloned from rat 11B11 hybridoma cells (ATCC; Manassas, VA) using the SMART cDNA synthesis system (Clontech Laboratories Inc, Mountain View, CA) followed by PCR amplification. The V_H and V_L sequences were sub-cloned into the pSMED2-mouse IgG1 and pSMEN3-mouse Kappa expression vectors, respectively, to construct the 11B11-mouse IgG1/kappa version of this antibody (mu11B11 mAb). DNA was transiently transfected into COS-1 (M6) cells, using TransIT (Mirus Bio LLC, Madison, WI)/Opti-MEM system (Gibco; Invitrogen Life Technologies, Carlsbad, CA), and maintained in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 100 IU penicillin, 100 µg/mL streptomycin, and 2 mM glutamine, in a 37°C incubator at 10% CO₂.

Surface Plasmon Resonance. To characterize the binding of IL-13 or IL-4, the bifunctional antagonist was diluted from 5 to 0.1 µg/mL in 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.2, 150 mM NaCl, 2.7 mM KCl, 3.4 mM EDTA and 0.01% Tween 20 (PBS-NET) and injected onto anti-murine IgG immobilized on a carboxymethylcellulose (CM5) sensor chip for 2 min at a flow rate of 5µL/min to achieve a captured level of 50 to 200 Response Units (RU), using a Biacore

T100, 3000 or 2000 instrument (GE Healthcare, Piscataway, NJ). The flow rate was increased to 100 μ L/min and various concentrations of recombinant murine IL-13 or IL-4 (R&D Systems) ranging from 0.195 to 100 nM in PBS-NET were injected for a 2.5 min association and allowed to dissociate for 10 to 60 min. At the end of each cycle, the entire anti-IgG surface was regenerated by a 30 second pulse of 10 mM glycine HCl pH 1.5 followed by two consecutive 15-second pulses of PBS-NET. All injections were performed at 25°C at a collection rate of 10 Hz. Rate constants for IL-13 and IL-4 were determined by fitting the data to a 1:1 model in Biaevaluation software version 4.1 (GE Healthcare) and the equation $K_d = k_{off}/k_{on}$. Theoretical Rmax was calculated as follows: MW of analyte / MW of ligand x captured level of ligand (response units) x apparent stoichiometry.

To confirm the dual binding capability of the bifunctional molecule, an anti-human IL-13 antibody was immobilized by amine coupling onto a CM5 sensor surface to levels of 2200-6700 RU, using a Biacore 3000 instrument. Human IL-13 was diluted in PBS-NET to 100 nM and injected for 2 min at 10 μ L/min, followed by an 8 min injection of 100 nM IL-4/IL-13 dual antagonist. For the third injection, either PBS-NET or 100 nM murine IL-4 was injected for 2 min. At the end of each cycle, surfaces were regenerated by a 30 second pulse of 0.1% Trifluoroacetic acid (TCA). All sensorgrams were double referenced using a reference sensor surface and buffer injections.

PK study design, serum concentration analysis, and PK calculations. Test article was radiolabeled with 125-iodine using IODO BEADS (Pierce, Rockford, IL) and separated from free iodine as described previously (22). A dosing solution was prepared by mixing unlabeled

test article and a trace amount of ^{125}I -labeled test article in PBS, pH 7.2. Specific activity was 14.8 $\mu\text{Ci}/\text{mg}$ and % free iodine was 3%, as determined by TCA precipitation. Radiolabeled test article was administered to female C57BL/6 mice via a single 8 mg/kg IV (tail vein) or IP dose at a volume of 4 mL/kg, based on the body weights prior to dosing. Serum samples were taken over 0.08-384 hr using non-serial sampling design (n=6 per time point), with 1-3 blood draws per mouse (either by retro-orbital bleeding or by cardiac puncture from isoflurane-anesthetized animals). Radioactive equivalent concentrations were calculated from the total and TCA-precipitable radioactivity in serum samples (20-100 μL , in duplicates), as previously described (22). The non compartmental analysis module (Model 201 and Model 200 for analysis of serum data after IV and IP administration, respectively) of WinNonlin ver. 5.1 (Pharsight) was used for PK calculations.

Measurement of IL-4 Binding Activity, IL-13 Binding Activity, or Bifunctional IL-4/IL-13

Antagonist Concentration in Serum. ELISA plates (MaxiSorp; Nunc, Rochester, NY) were coated with anti-mouse IL-4 mAb 11B11 (BD Pharmingen, San Diego, CA) for detection of IL-4 binding activity, or with anti-FLAG M2 (Sigma Aldrich, St. Louis, MO) for detection of IL-13 binding activity or bifunctional antagonist, blocked with 0.5% gelatin in PBS, and washed in PBS containing 0.05% Tween-20 (PBS-Tween). Protein standards or serum samples were mixed with the following reagents. For detection of IL-4 binding activity, murine IL-4 (R&D Systems, Minneapolis, MN) was diluted to 20 ng/mL final concentration in PBS Tween. For detection of IL-13 binding activity, human IL-13FLAG (PGRD), diluted to a 20 ng/mL final concentration, was mixed with biotinylated anti-human IL-13 antibody MJ2-7 (PGRD). Human and murine IL-13 display species cross-reactivity (22, 23), and human IL-13 binds to murine IL-13 α 2-Fc and

the bifunctional antagonist. For detection of IL-4/IL-13 bifunctional antagonist, a mixture of human IL-13 FLAG (PRGD) and murine IL-4 (R&D Systems) was added at 20 ng/mL final concentration each. IL-4 binding activity or bifunctional antagonist was detected with biotinylated goat anti-mouse IL-4 (R&D Systems), HRP-streptavidin (SouthernBiotech, Birmingham, AL) and Sure Blue substrate (KPL Inc, Gaithersburg, MD). The reaction was stopped with 0.01 M sulfuric acid, and absorbance read at 450 nm in a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). The lower limit of quantification (LLOQ) was 5 ng/mL anti-mouse IL-4 antibody, 10 ng/mL mouse IL-13R α 2-Fc, or 2 ng/mL IL-4/IL-13 bifunctional antagonist.

Competition for IL-4 Binding to IL-4R α . ELISA plates were coated with mouse IL-4R α -Fc (PGRD) in PBS, and blocked with 0.5% gelatin in PBS. Recombinant murine IL-4 (R&D Systems) diluted to 40 ng/mL in PBS-Tween was bound at room temperature for 4 hours, in the presence of competitor IL-4 binding agents. Binding was detected using biotinylated goat anti-mouse IL-4 (R&D Systems), followed by HRP-streptavidin and Sure Blue substrate, as described above. LLOQ was 5 ng/mL anti-mouse IL-4 antibody.

Measurement of IgE in Mouse Serum. ELISA plates were coated with rat anti-mouse IgE mAb clone R35-72 (BD Pharmingen) in PBS, and blocked with 0.5% gelatin in PBS. Dilutions of mouse IgE standard (BD Pharmingen) or serum were incubated for 2 to 4 hr at room temperature. Biotinylated rat anti-mouse IgE clone 23G3 (SouthernBiotech) was added in PBS-Tween, with 1 μ g/mL mouse IgG (Sigma Aldrich) as blocker, and incubated for 2 hr at room

temperature. Binding was detected with HRP-streptavidin (SouthernBiotech) and Sure Blue substrate (KPL Inc). LLOQ was 10 ng/mL mouse IgE.

Measurement of Serum Chitinase Activity. Chitinase activity was measured by monitoring cleavage of the β -glycosidic linkage in 4-methylumbelliferyl β -D-N,N'-diacetylchitobioside (chitobiose 4-MU; Sigma Aldrich) to generate the fluorescent product, 4-MU. Serum samples were diluted in McIlvaines buffer (100 mM sodium citrate/citric acid, 200 mM sodium phosphate pH 6.0, 0.005% Brij-35), and transferred to a 96 well flat bottom black plate (Corning Inc, Corning, NY). Substrate was diluted to 30 μ M final concentration in McIlvaines buffer, and mixed. Fluorescence was read in a Safire plate reader (Tecan, Männedorf, Switzerland) with excitation at 320 nm, and emission at 455 nm. Reactions were monitored for 30 min. The slope of the response from each sample well was corrected for dilution factor and reported as chitinase activity units (U/mL).

STAT6 Phosphorylation Assay. The murine Ba/F3 pro-B cell line (ATCC), which expresses IL-4R α and γ common receptor chains, was transfected with murine IL-13R α 1 to generate a line responsive to IL-13 in addition to IL-4. Cells were challenged with 1 ng/mL IL-13 or 0.5 ng/mL IL-4 in the presence or absence of antagonist at 37°C for 30 min, washed into ice-cold PBS containing 1% BSA, then fixed in 1% paraformaldehyde for 15 min at 37°C. To permeabilize the nucleus, cells were incubated overnight at -20°C in absolute methanol, washed into PBS-BSA, then stained with AlexaFluor 488-labeled antibody to STAT6 (pY641; BD Biosciences, San Jose, CA). Fluorescence was analyzed with a FACSCalibur (BD Biosciences), and FlowJo software version 7.2.4 (Tree Star Inc, Ashland, OR).

Bronchoalveolar Lavage Fluid (BALF) Inflammation. Female C57BL/6 mice, immunized and challenged with OVA as described above, were treated IP with 200 µg test article on Days 23, 26, and 28. On Day 30, animals were euthanized, and BALF was harvested by inserting a 23-gauge Luer stub adapter (BD Diagnostic Systems) as a cannula into the trachea. The lungs were lavaged 3 times with 0.7 mL CMF-PBS. Cells were pelleted, resuspended in CMF-PBS and total cell counts obtained using the Cell Dyn 3700 (Abbott Labs, Abbott Park, IL). Cytospins (Thermo Fisher Scientific, Pittsburgh, PA) were stained with Diff-Quick (Dade Being Inc, Newark, DE). The percentage of eosinophils was quantified by manual scoring of 200 cells per slide.

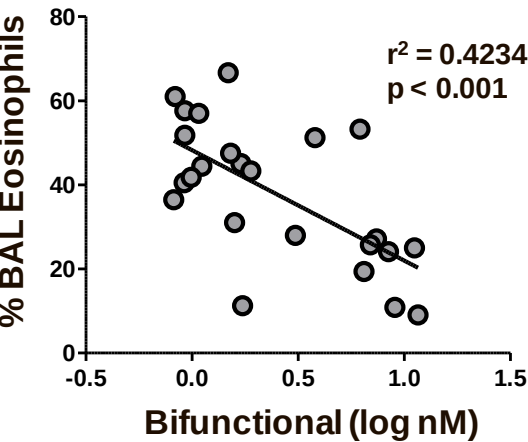
Gene Expression Analysis by Taqman. Lungs were harvested, snap frozen in liquid nitrogen, homogenized in RNeasy lysis buffer (Qiagen, Valenica, CA), passed through a QIAshredder (Qiagen), then stored at -80°C. RNA was extracted using the Qiagen RNeasy Kit with DNase. PCR Master Mix (Quanta Qscript 1Step; Quanta Biosciences, Gaithersburg, MD) was mixed with gene-specific forward and reverse primers and probes (Applied Biosystems, Carlsbad, CA), and run on the ABI 7900 (Applied Biosystems) using the following protocol: 30' 48°C, 10' 95°C, 40 X (15' 95°C, 1' 60°C).

FIGURE LEGENDS

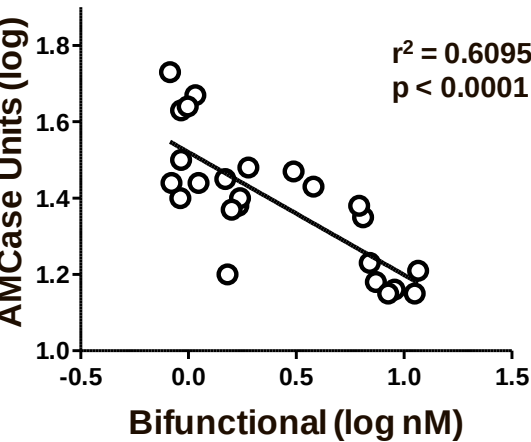
Figure E1. Dose dependence of *in vivo* activity of bifunctional IL-4/IL-13 antagonist. Female C57BL/6 mice were immunized IP with OVA/alum on Days 0 and 14, given inhalation challenge with OVA aerosol on Days 26, 27, 28, and sacrificed on Day 30. Mice were treated with IL-4/IL-13 bifunctional at doses of 8, 2, or 0.5 mg/ml at challenge (Days 23, 26, 28). Concentration of the bifunctional molecule was assayed in sera of terminal bleeds, and plotted in relation to: (A) % BALF eosinophils; (B) Serum AMCase activity; (C) Lung Muc5ac gene expression; (D) Lung AMCase (Chia) gene expression.

Figure E2. Comparison of effective *in vivo* dose range for bifunctional IL-4/IL-13 antagonist and single cytokine inhibitors. Female C57BL/6 mice were immunized IP with OVA/alum on Days 0 and 14, given inhalation challenge with OVA aerosol on Days 26, 27, 28, and sacrificed on Day 30. (A) Mice were treated with bifunctional IL-4/IL-13 antagonist or sIL-13R α 2-Fc at the indicated doses at challenge (Days 23, 26, 28), and % BALF eosinophils determined at sacrifice. (B) Mice were treated with bifunctional IL-4/IL-13 antagonist or anti-IL-4 mu11B11 at the indicated doses at sensitization (Days 0, 7, 14), given inhalation challenge on Days 26, 27, 28, and TARC gene expression analyzed in lungs harvested on Day 30. The molecular weights of mu11B11, sIL-13R α 2-Fc and bifunctional antagonist are 150 kD, 180 kD, and 180 kD, respectively.

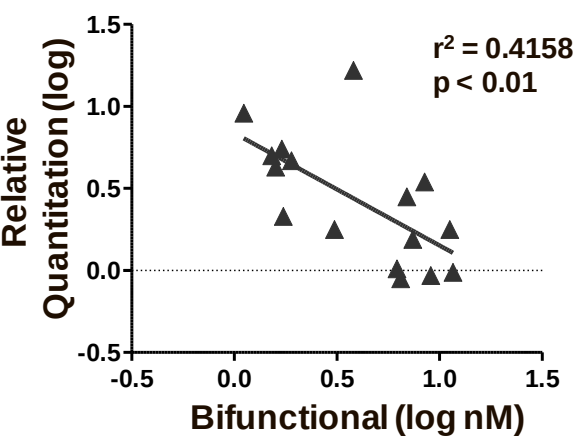
A. % BALF Eosinophils



B. Serum AMCase



C. Lung Muc5ac Expression



D. Lung AMCase Expression

