

## Efficacy of IL-13 Neutralization in a Sheep Model of Experimental Asthma

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IL-13 contributes to airway hyperresponsiveness, mucus secretion, inflammation, and fibrosis, suggesting that it plays a central role in asthma pathogenesis. Neutralization of IL-13 with sIL-13R $\alpha$ 2-Fc (sIL-13R) reduces allergen-induced airway responses in rodent models of respiratory disease, but its efficacy in a large animal model has not been previously reported. In this study, we determined whether two different strategies for IL-13 neutralization modified experimental asthma in sheep. Sheep with natural airway hypersensitivity to *Ascaris suum* antigen were treated intravenously either with sIL-13R, a strong antagonist of sheep IL-13 bioactivity *in vitro*, or with IMA-638 (IgG1,  $\kappa$ ), a humanized antibody to human IL-13. Higher doses of IMA-638 were used because, although it is a potent antagonist of human IL-13, this antibody has 20 to 30 times lower binding and neutralization activity against sheep IL-13. Control animals received human IgG of irrelevant specificity. Sheep were treated 24 h before inhalation challenge with nebulized *A. suum*. The effects on antigen-induced early and late bronchial responses, and antigen-induced hyperresponsiveness, were assessed. Both sIL-13R and IMA-638 provided dose-dependent inhibition of the antigen-induced late responses and airway hyperresponsiveness. The highest dose of IMA-638 also reduced the early phase response. These findings suggest that IL-13 contributes to allergen-induced airway responses in this sheep model of asthma, and that neutralization of IL-13 is an effective strategy for blocking these *A. suum*-induced effects.

**Keywords:** sheep; *Ascaris suum*; airway hyperresponsiveness; late phase

IL-13 is a major contributor to generation of asthma pathology (1–3). *In vitro*, IL-13 induces many cellular responses relevant to asthma, including B cell IgE production (4), epithelial cell maturation and mucus production (5), generation of extracellular matrix proteins (6), and enhanced contractility of airway smooth muscle cells (7). In animal models, administration of exogenous IL-13 triggers asthmatic signs, including airway hyperresponsiveness (AHR), lung inflammation, and mucus secretion (8, 9). IL-13-transgenic mice display high levels of mucus production and pulmonary fibrosis, even in the absence of antigen challenge (10). After antigen sensitization, IL-13-transgenic mice generate high levels of IgE, histamine, and mucus, and are prone to anaphylaxis (11). Conversely, neutralization of IL-13 with sIL-13R $\alpha$ 2-Fc (sIL-13R) or with anti-IL-13 antibodies effectively reduces asthmatic changes in several animal models

### CLINICAL RELEVANCE

The current study provides the first evidence that IL-13 blockade can be efficacious in a large animal model of respiratory disease. This is a critical milestone supporting the development of IL-13-neutralizing agents for treating human asthma.

els of respiratory disease (9, 12–15). Consistent with these inhibitor studies are findings in mice deficient in IL-13. These animals fail to develop antigen-induced eosinophilia, mucus cell hyperplasia, or fibrosis in response to allergen challenge (16), although some AHR could still be seen in chronically challenged animals (17). Collectively, these studies suggest that IL-13 contributes significantly to asthma pathology (5, 8, 16).

While receptor-Fc fusion molecules such as sIL-13R are effective IL-13 antagonists and have been used to confirm the role of IL-13 in rodent models of asthma (1, 8, 9), anti-cytokine antibodies can also be effective neutralizing agents (13, 15). Toward that goal an antibody to IL-13 was developed to combine the efficacy of IL-13 neutralization with the stability advantages of a monoclonal antibody. MA613.2 (IgG1,  $\kappa$ ) was isolated from mice immunized with human IL-13, and humanized to generate IMA-638 (IgG1,  $\kappa$ ).

Although sIL-13R has shown activity in small animal models of asthma, its effect against allergen-induced changes in airway function has not previously been tested in a large animal model. IMA-638 lacks neutralization activity against murine IL-13. Sheep that have natural airway hypersensitivity to *Ascaris suum* antigen represent a large animal model that has been used extensively to study the pathophysiology of asthma (18). A primary strength of the model is the ability to repeatedly and accurately assess changes in measures of pulmonary function after provocation with antigen. These functional changes (i.e., early and late bronchial responses and AHR), are well characterized and mechanistically similar to the respective pathophysiologic changes seen in human subjects in clinical studies (18). Therefore, in this study, efficacy of IMA-638 to modify antigen-induced responses in the sheep model was compared with that of sIL-13R. To support the *in vivo* findings, sheep IL-13 was cloned and sequenced, and used to assess binding of sIL-13R and IMA-638 to both sheep and human IL-13. In addition, *in vitro* studies were performed to show that both inhibitors neutralized sheep IL-13 bioactivity.

The results show that both sIL-13R and IMA-638 provide dose-dependent inhibition of antigen-induced late responses and AHR. IMA-638 was ~10-fold less active than sIL-13R *in vivo*, as was predicted by its partial cross-reactivity for sheep IL-13 in the *in vitro* assays. Collectively, these findings suggest that neutralization of IL-13 is an effective strategy for blocking

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antigen-induced late responses and AHR in the sheep model of asthma and so support and extend the possibility that neutralization of IL-13 can be used to treat asthma in humans.

## MATERIALS AND METHODS

### Test and Control Articles

Humanized antibody to human IL-13, IMA-638, was generated at Wyeth Research (Cambridge, MA). IMA-638 (IgG1,  $\kappa$ ) was formulated in 10 mM L-histidine, pH 6, containing 5% (wt/vol) sucrose. Human sIL-13R $\alpha$ 2 fused with human IgG1-Fc (sIL-13R) was generated at Wyeth Research. Carimune NH immune globulin intravenous (human IVIG) was purchased from ZLB Bioplasma Inc. (Berne, Switzerland), depleted of non-Ig components by passage over a protein A column, and formulated in 10 mM L-histidine, pH 6, containing 5% (wt/vol) sucrose. *A. suum* extract (Greer Diagnostics, Lenoir, NC) was diluted with PBS to a concentration of 82,000 protein nitrogen units/ml and delivered to sheep as an aerosol (20 breaths/min). Carbamylcholine (carbachol; Sigma Chemical Co., St. Louis, MO) was dissolved in PBS at concentrations of 0.25, 0.50, 1.0, 2.0, and 4.0% wt/vol and delivered as an aerosol.

### Animal Manipulations

The Mount Sinai Medical Center (Miami, Beach FL) Animal Research Committee that is responsible for assuring the humane care and use of laboratory animals approved the procedures used in this study. Two or three female Florida Native sheep (29–40 kg) were used per group. The sheep had airway hypersensitivity to *A. suum* antigen and documented antigen-induced early and late bronchial responses and AHR after allergen provocation (18–22). For the described studies, the sheep were conscious and were restrained in a modified shipping cart in the prone position with their heads immobilized. Nasal passages were anesthetized with topical 2% lidocaine, and a balloon catheter was advanced through one nostril into the lower esophagus. The animals were intubated with a cuffed endotracheal tube through the other nostril.

### Airway Mechanics

Breath-by-breath determination of mean pulmonary airflow resistance ( $R_L$ ) was measured with the esophageal balloon technique as previously described (18–22). The mean of at least five breaths, free of swallowing artifact, was used to obtain  $R_L$  in cm H<sub>2</sub>O/Ls.

### Concentration Response Curves to Carbachol Aerosol

Airway responsiveness was determined from cumulative concentration response curves to inhaled carbachol using a medical nebulizer-dosimeter system extensively described for use in the sheep (18–22). The cumulative carbachol concentration (in breath units [BU]) that increased  $R_L$  by 400% over the post-PBS value ( $PC_{400}$ ) was calculated by interpolation from the concentration response curve. One BU was defined as one breath of a 1% wt/vol carbachol aerosol solution.

### Protocols

IMA-638 was administered intravenously at doses of 20, 5, and 2 mg/kg, 24 h before antigen challenge. Three sheep were treated with each dose of antibody. Groups of two sheep each were administered sIL-13R intravenously at doses of 2, 1, and 0.5 mg/kg. As a control for both the IMA-638 and sIL-13R treatments, additional animals were administered IVIG at 20 mg/kg (two sheep) and at 5 mg/kg (five sheep). Allergen-induced responses (early and late bronchial responses and airway responsiveness) after the various treatments were compared with each animal's responses after allergen challenge when the sheep were given saline. To assess the pharmacokinetics of sIL-13R and IMA-638 in these studies, serum samples were collected before dosing (pre), and at 30 min, 24 h, 26 h, 30 h, and 48 h after dosing, and stored at  $-80^\circ\text{C}$  for all dose groups. To determine the effects of these agents on antigen-induced responses, we measured baseline airway responsiveness ( $PC_{400}$ ) 1–3 d before dosing with IMA-638, sIL-13R, or IVIG. Antigen challenge was then performed 24 h after treatment. On the challenge day, values of mean pulmonary flow ( $R_L$ ) were determined at baseline (pre-*Ascaris*) and then immediately after *Ascaris* challenge and 1, 2, 3, 4, 5, 6, 6.5, 7, 7.5, and 8 h after challenge. A post-challenge

$PC_{400}$  was determined 24 h after inhalation challenge with *Ascaris* antigen to assess the development of AHR. *A. suum* antigen aerosol was generated using the medical nebulizer dosimeter system extensively described for use in the sheep (18–22).

### Data Presentation and Statistical Analysis

To assess the effects of the different treatments on the antigen-induced responses, the pre-challenge  $R_L$  value was subtracted from each post-challenge  $R_L$  reading and the resulting value was expressed as percent increase over the pre-challenge value (% resistance). This was calculated for each individual sheep in each treatment group and was compared with the antigen response of the same sheep when given saline only. To assess the effects of the treatments on airway responsiveness, the  $PC_{400}$  value obtained post-*Ascaris* challenge for each sheep was expressed as a percentage of the value obtained at baseline (pre-*Ascaris* challenge). The pre-*Ascaris* challenge response was normalized to a  $PC_{400}$  value of 100, and the study response was expressed as a percentage of that level.

A paired *t* test was used to compare the early and late bronchoconstrictor responses after treatment with sIL-13R or IMA-638, with the responses in the absence of drug. Comparisons were made for the peak early phase response, average early phase response (time 0–4 h), and average late phase response (5–8 h). To assess the response to carbachol pre-*Ascaris*  $PC_{400}$  values were normalized to 100% and the post-*Ascaris* response was determined in the presence or absence of drug. These effects were also analyzed using a paired *t* test. Significance was accepted as a *P* value of  $< 0.05$  using a two-tailed analysis. Data in the text and figures are presented as mean  $\pm$  SEM.

### Serum Antibody

For each animal, serum levels of IMA-638 or of sIL-13R were determined by enzyme-linked immunosorbent assay (ELISA) for total human IgG. ELISA plates (MaxiSorp; Nunc, Rochester, NY) were coated with 1:1,500 dilution of goat anti-human Ig(M+G+A)Fc (ICN-Cappel, Costa Mesa, CA), blocked for 1 h at room temperature with 0.5% gelatin in PBS, and washed in PBS containing 0.05% Tween-20 (PBS-Tween). IMA-638 standard or dilutions of sheep serum ranging from 1:500–1:50,000 were added and incubated for 2 h at room temperature. Plates were washed with PBS-Tween, and biotinylated mouse anti-human IgG (Southern Biotechnology Associates, Birmingham, AL) was added for 2 h at room temperature. Binding was detected with peroxidase-linked streptavidin (Southern Biotechnology Associates) and Sure Blue substrate (KPL, Gaithersburg, MD). Absorbance at 450 nm was read in a plate reader (Molecular Devices Corp., Sunnyvale, CA). The assay was sensitive to 0.5 ng/ml human IgG.

### TF-1 Proliferation Assay

TF-1 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI containing 10% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin, 2 mM L-glutamine, and 5 ng/ml recombinant human GM-CSF (R&D Systems, Inc., Minneapolis, MN). Before assay, cells were starved of GM-CSF overnight, plated in flat-bottom microtiter plates (Costar/Corning, Acton, MA), and challenged with human IL-13 concentrations ranging from 100–0.01 ng/ml. After 72 h in a  $37^\circ\text{C}$  incubator with 5%  $\text{CO}_2$ , the cells were pulsed with 1  $\mu\text{Ci}$  / well 3H-thymidine (Perkin Elmer/New England Nuclear, Boston, MA), incubated an additional 4.5 h, then harvested onto filter mats using a TomTek harvester (EG&E Wallace, Gaithersburg, MD). 3H-thymidine incorporation was assessed by liquid scintillation counting. The standard deviation of replicate assays was typically  $< 10\%$ .

### Monocyte CD23 Expression Assay

Mononuclear cells were isolated from human peripheral blood by layering over Histopaque 1077 (Sigma). Cells were washed into RPMI containing 10% heat-inactivated FCS, 50 U/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin, 2 mM L-glutamine, and plated in a 48-well tissue culture plate (Costar/Corning) in the presence of 100–0.01 ng/ml human IL-13. For assays testing the ability of antibody to inhibit the IL-13 response, 1 ng/ml human IL-13 was added along with dilutions of antibody ranging from 500–0.4 ng/ml. Cells were incubated overnight at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator, harvested using nonenzymatic Cell Dissociation Solution (Sigma), then washed into ice-cold PBS containing 1% bovine serum

albumin (BSA). Cells were incubated with PE-labeled antibody to human CD23 (BD Biosciences, San Jose, CA), and Cy-Chrome-labeled antibody to human CD11b (BD Biosciences). By flow cytometry (FACS-Scan; BD Biosciences), monocytes were gated based on high forward and side light scatter, and expression of CD11b. The percentage of CD23+ monocytes was quantitated with CellQuest software (BD Biosciences). The standard deviation of replicate assays was typically < 15%.

### STAT6 Phosphorylation Assay

HT-29 human colonic epithelial cells (ATCC) were grown as an adherent monolayer in McCoy's 5A medium containing 10% fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, and 0.15% sodium bicarbonate. For assay, the cells were dislodged from the flask using trypsin, washed into fresh medium, and challenged with 100–0.01 ng/ml human IL-13. For assays testing the ability of antibody to inhibit the IL-13 response, 1 ng/ml human IL-13 was added along with dilutions of antibody ranging from 500–0.4 ng/ml. Cells were incubated in a 37°C water bath for 30–60 min, then washed into ice-cold PBS containing 1% BSA. Cells were fixed in 1% paraformaldehyde in PBS, then washed into PBS containing 1% BSA, incubated overnight at –20°C in absolute methanol, then stained with AlexaFluor 488-labeled antibody to STAT6 (BD Biosciences). Fluorescence was analyzed with a FACScan and CellQuest software (BD Biosciences). The standard deviation of replicate assays was typically < 15%.

### Surface Plasmon Resonance Analysis

To prepare the biosensor surface, either humanized antibody IMA-638 or sIL-13R was immobilized onto a research-grade carboxymethyl dextran chip (CM5) using standard amine coupling. The surface was activated with N-ethyl-N'-(3-dimethyl aminopropyl)-carbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS), and mAb13.2 or IMA-638 injected at a concentration of 1 µg/ml in sodium acetate buffer (pH 5.5). Remaining activated groups were blocked with 1.0 M ethanolamine (pH 8.0). Data were collected using a Biacore 2000 (Biacore International, Uppsala, Sweden). As a control, the first flow cell was used as reference surface to correct for bulk refractive index, matrix effects, and nonspecific binding. The second, third, and fourth flow cells were coated with the capturing molecule.

Solutions of human or sheep IL-13 were flowed over the chip at 600, 200, 66.6, 22.2, 7.4; 2.5, 0.8 and 0 nM in triplicates, at 100 µl/min, and the amount of bound material as a function of time was recorded as sensorgrams. The dissociation phase was monitored in 10 mM HEPES, pH 7.4 containing 150 mM NaCl, 3 mM EDTA and 0.005% (vol/vol) Surfactant P20 (HBS/EP buffer) for 60 min at the same flow rate. This was followed by two 5-µl injections of glycine pH 1.5 to regenerate a fully active capturing surface. All kinetic experiments were done at 22.5°C in HBS/EP buffer. Blank and buffer effects were subtracted for each sensorgram using double referencing.

### Cloning and Sequencing of Sheep IL-13

IL-13 was cloned from sheep peripheral blood lymphocytes using a cDNA library screening approach. Sheep PBLs were stimulated for 4 d with *Ascaris* antigen, centrifuged over Ficol1 Hypaque to remove dead cells, and replated in 10% conditioned media. On Day 7, the cells were replated again. On Day 9, cells were stimulated with 100 ng/ml PMA (Sigma) and 2 µg/ml ionomycin (Sigma). Two days later, RNA was harvested and used to generate cDNA. PCR primers corresponding to a semi-conserved internal region of IL-13 from various species, including bovine (accession # NM\_174089) and human (accession # U31120), amplified a band of the correct size from the activated sheep lymphocyte cDNA. The PCR product was cloned into the pCR4 vector using the TA cloning kit (Invitrogen Corp., Carlsbad, CA). The cloned 200-bp PCR fragment was sequenced and showed five nucleotide differences from bovine IL-13 (NM\_174089). This fragment was labeled with <sup>32</sup>P and used as a probe for screening an *Ascaris*/PMA-ionomycin-stimulated sheep PBL cDNA library (Open Biosystems, Huntsville, AL). The library (1.97 × 10<sup>6</sup> colony-forming units [cfu]/ml) was plated on 20 plates at 5,000 cfu per plate, and probed with the internal probe. Only one clone was identified out of 100,000 cfu plated. This 1.3-kb transcript was sequenced and found to contain a small 5-prime UTR and a long 3-prime UTR.

Multiple, independent RT-PCRs revealed a clear consensus nucleotide sequence (Figure 1A) and amino acid sequence (Figure 1B). The cloned sheep IL-13 gene was transfected into Chinese hamster ovary (CHO) cells. The cells produced protein, as determined by Western blot analysis, and by intracellular staining with hIL-13Rα2Fc (data not shown). Supernatant was harvested and sheep IL-13 quantitated by

**A** *ggtaccggtccggaattccgggaATCTTAGGCCAGCCTATGCGTCTGCTTCTCAGTCTCTCTGTTGTG*  
*TCTAGGCTCTTAGGCGCTCTCTTCTGACCGCGTCTGTTCTTCTACCTGCTTTGGTGGCCCTCGCCCTCCC*  
**AAACCTCTGTCCTTCTCTTCTATCCCTCAAGGAGCTCATTTGAAGAGCTGGTAAATATCACCAAGCAACA**  
**GAAAGTCCGCTGTCATGTCAGCAGTGGTGGGACCTCAACCTGACGAGCAGCAGTATGCTATGTCAGC**  
**CCTGGACTCCCTGATCAGCATCTCCAACCTGCAGTGTATCCACGAGCAAGAGGATGCTGAGTCGGCT**  
**CTGTCTCTCAAGCCCTCAGCTAAGCATGTTTCAGTGAAGTACGTCGAGACACCAAAATTAAGATGGC**  
**CCAGTCTCTAAAAGACCTGCTCAGACACTCAAGGATCATTTTTCGCAATGGAAGTTACAACTGACAT**  
 GAAAGCTAGCATTAATATCTGAGAGGACAGCCTGACCACTAAGTTGACAGTTATTTTCTTTCTCA  
 GATGTCCAAATTTCTTGGGAGACTAAAGAGGGTTTAGGAGGGGGGTAAGATGCTCTTATGCTTTAGA  
 TCTGAGCTGTGATGCTGTGAGGCTAGCCACTTACGCTGCCCTGTGCTTGATATCCGGGACTC  
 AGCCCGCAGGCTCCTCTCATCTCAGGACTCCGAGCTCGCTGAGCAGGGTGACCTTGCCCACTAGACC  
 TCCCTCTCTCAGAACCAACTGTATGTTGATTAGACACTGTTGAGGAGGACCACTGCTTTGACAGAG  
 GGCACTGAGGACAGGAGGAGCAGGCGAGGCACATCTCTTCTGGCTTATTATTATGATGTTATTAA  
 ACAAGTCTTTTCTGCTGCTAGGGTTAGAGGGGACTGTTACTGCCAGACTGTAGGACAGCTGGGTGG  
 AGACTGTGGGGCTCAGCAGCTAAAGCAGTGGGCACTAGGAATCCCTGGCAGTAAGTACTGTATGCAAACT  
 TCTGCTCACTCAGTGGGTCTGGGGCAAGGCAAGGACAGGAGAGGGCCAGAGTACGACTCTTG  
 TCTGACAGCAGCAGTGGGCTCAGCCCAAGTAATGTTATTTTCTAGTATTTAAAGTTAAGTATT  
 AAAATATGTTCAAAAGGTTAATAATATATAGCTGCTAGAACACTGATATGGTATGTTGGGACGTA  
 GGGGGGGGGTCACTGAATAAAGTGTTCATCGATTTTGTCAAGCTAAAGCTAAAAGTAAAGATGGTAC  
 AGAGAAAAAAGAGGCGCGCTGTAGATATCCCTGAGGGGCCAAGCTT

**Figure 1.** (A) Nucleotide sequence of sheep IL-13 (accession # DQ679798). Sheep IL-13 was cloned from a cDNA library generated from splenocytes stimulated *in vitro* with *A. suum* antigen, to which the sheep was sensitized. The open reading frame is boxed and shown in bold. ATG start and TGA stop codons are highlighted. Lowercase italics represent vector sequence. Uppercase (not bold) represents 5-prime and 3-prime UTR. (B) The amino acid sequences of human (accession # U31120) and sheep IL-13 are 70% identical. Sheep residues that differ from the human sequence are highlighted. The arrow indicates the predicted N-terminus of the mature protein. Numbering corresponds to the mature protein.

**B**

|       |          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     |
|-------|----------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-----|
| human | MAILLTVI | AL | TL | GL | GF | SP | GP | VP | ST | AL | RE | LI | EL | LV | NI | QN | QK | 25  |
| sheep | MAILLTVI | VL | TL | GL | GG | AS | PP | VP | SS | SS | LI | EL | EL | LV | NI | QN | QK |     |
| human | PL       | NC | GS | MS | W  | VS | I  | N  | L  | T  | A  | G  | M  | Y  | C  | A  | E  | 69  |
| sheep | PL       | NC | GS | MS | W  | VS | I  | N  | L  | T  | S  | M  | Y  | C  | A  | E  | SL |     |
| human | CP       | HK | VS | A  | G  | Q  | F  | SS | L  | H  | R  | D  | T  | K  | I  | E  | V  | 112 |
| sheep | CP       | HK | S  | A  | K  | H  | V  | S  | S  | E  | Y  | R  | D  | T  | K  | I  | E  |     |

determination of the concentration producing half-maximal bioactivity in the TF-1 bioassay.

## RESULTS

### Binding of IMA-638 and sIL-13R to Sheep IL-13

Sheep IL-13 (accession # DQ679798) was cloned from an *Ascaris*-stimulated PBL library, using primers based on the bovine (accession # NM\_174089) and human (accession # U31120) IL-13 sequences. The nucleotide sequence is shown in Figure 1A, and amino acid sequence in Figure 1B. There were 17 amino acid differences between sheep and bovine IL-13 (87% amino acid identity) (not shown), and 39 amino acid differences between sheep and human IL-13 (70% identity) (Figure 1B). Both sIL-13R and anti-human IL-13 antibody, IMA-638, bind to human IL-13 with high affinity, as confirmed by surface plasmon resonance analysis (Figures 2A and 2C; Table 1). Sheep IL-13 showed similar high affinity for sIL-13R (Figure 2D), but had almost 100-fold lower affinity for IMA-638 (Figure 2B), driven primarily by rapid dissociation kinetics (Table 1).

### Neutralization of Sheep IL-13 Bioactivity by IMA-638 and sIL-13R

Sheep IL-13 was found to have bioactivity on human cells. Three bioassays were used to evaluate the ability of sIL-13R or IMA-638 to neutralize sheep IL-13. In the monocyte CD23 expression assay, IL-13 induces expression of the low-affinity IgE receptor, CD23, on primary human monocytes (23). The TF-1 proliferation assay, in which IL-13 drives expansion of a human erythroleukemia cell line, is a standard assay for evaluation of cytokine responses (24). The STAT6 phosphorylation assay measures an early signaling response of the HT-29 human epithelial cell line to IL-13 (25). In each case, the bioactivity of IMA-638 was compared with that of sIL-13R. Results showed that 50% inhibition of sheep IL-13 bioactivity in each assay required 20–30 times higher concentration of IMA-638 as compared with sIL-13R (Figures 3A–3C). Thus, sIL-13R has comparable potency for neutralization of human and sheep IL-13 (Figure 4A), whereas IMA-638 has higher neutralization activity against the human cytokine (Figure 4B).

TABLE 1. KINETIC PARAMETERS FOR IMA-638 OR sIL-13R BINDING TO HUMAN OR SHEEP IL-13

| Ligand Analyte   | IMA-638 Human IL-13    | IMA-638 Sheep IL-13   | sIL-13R Human IL-13    | sIL-13R Sheep IL-13    |
|------------------|------------------------|-----------------------|------------------------|------------------------|
| $k_a$ (1/Ms)     | $4.84 \times 10^3$     | $1.25 \times 10^4$    | $4.64 \times 10^4$     | $6.46 \times 10^4$     |
| $k_d$ (1/s)      | $5.22 \times 10^{-3}$  | $3.36 \times 10^{-3}$ | $2.55 \times 10^{-4}$  | $1.63 \times 10^{-4}$  |
| $K_d$ (1/M)      | $9.27 \times 10^2$     | $3.70 \times 10^4$    | $1.82 \times 10^{-10}$ | $3.95 \times 10^{-10}$ |
| $K_D$ (M)        | $1.08 \times 10^{-10}$ | $2.79 \times 10^{-9}$ | $5.49 \times 10^{-11}$ | $2.53 \times 10^{-11}$ |
| $R_{max}$ (RU)   | 45.7                   | 61.4                  | 13                     | 20.9                   |
| Chi <sup>2</sup> | 0.407                  | 0.668                 | 0.555                  | 1.29                   |

Langmuir model analysis from surface plasmon resonance experiments.

### Effects of IMA-638 on Early and Late Phase Bronchoconstriction Responses, and AHR to Carbachol

Sheep treated intravenously with IMA-638 at 20, 5, or 2 mg/kg demonstrated dose-dependent inhibition of the *Ascaris*-induced responses seen in the same animal in the absence of drug. IMA-638 at 20 mg/kg produced a partial but significant reduction in the peak early bronchoconstriction response ( $P < 0.05$ , with reductions of 46, 35, and 45%, respectively, for the three sheep tested), and completely eliminated the late phase response ( $P < 0.05$ , with reductions of 80, 87, and 80%, respectively, for the average late phase response in individual sheep) (Figure 5A). The 5 mg/kg dose of IMA-638 did not affect the early response to *Ascaris*, but partially reduced the late response (reductions of 46, 46, and 51% in the three sheep;  $P < 0.05$ ; Figure 5B). The 2 mg/kg dose of IMA-638 (Figure 5C) was ineffective. As a control for IMA-638, human IgG of irrelevant specificity (IVIG) was tested in the same model. IVIG had no effect on either the early or late phase response, at either the 20 mg/kg (Figure 5D) or 5 mg/kg (Figure 5E) doses.

The 20 mg/kg dose of IMA-638 completely prevented the AHR to carbachol after *Ascaris* challenge ( $P < 0.05$ ) (Figure 6A). The 5 mg/kg dose of IMA-638 produced complete inhibition of AHR in one sheep, and a partial reduction in the other two sheep ( $P < 0.05$ ; Figure 6A). The 2 mg/kg dose of IMA-638 produced a small reduction in AHR to carbachol (Figure 6A), which was seen in each of the three sheep tested at this dose (data not shown). In contrast, IVIG at the 20 mg/kg and 5 mg/kg

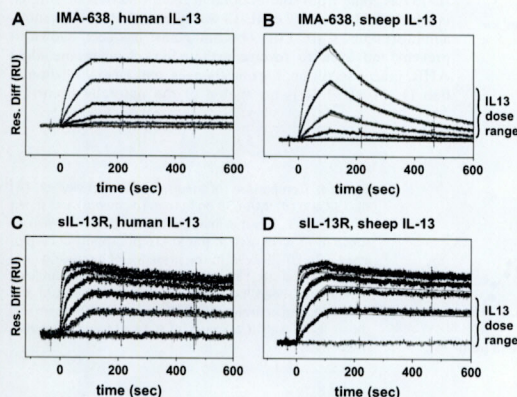


Figure 2. Surface plasmon resonance (Biacore) analysis of human or sheep IL-13 binding to immobilized sIL-13R or IMA-638. (A) Human IL-13 binding to IMA-638; (B) sheep IL-13 binding to IMA-638; (C) human IL-13 binding to sIL-13R; and (D) sheep IL-13 binding to sIL-13R.

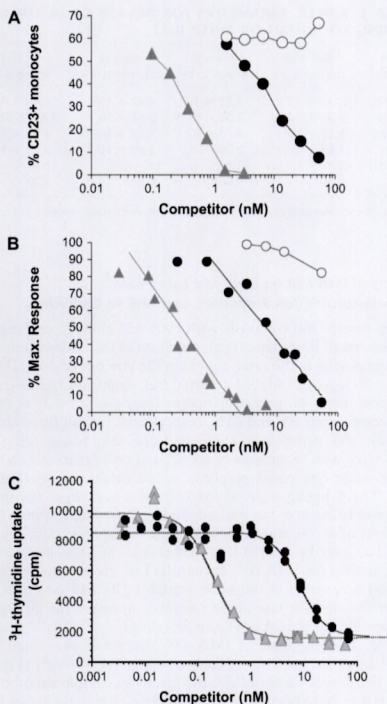


**Figure 3.** Neutralization of sheep IL-13 bioactivity with sIL-13R (triangles), IMA-638 (solid circles), or control human IgG (IVIG; open circles). Bioactivity of CHO-expressed sheep IL-13 was tested as: (A) induction of CD23 expression on primary human monocytes.  $IC_{50}$  values for sIL-13R and IMA-638 were 0.43 nM and 13 nM, respectively; (B) induction of STAT-6 phosphorylation in HT-29 human epithelial cells.  $IC_{50}$  values for sIL-13R and IMA-638 were 0.28 nM and 7 nM, respectively; (C) proliferation of human TF-1 erythroleukemia cells.  $IC_{50}$  values for sIL-13R and IMA-638 were 0.15 nM and 8 nM, respectively.

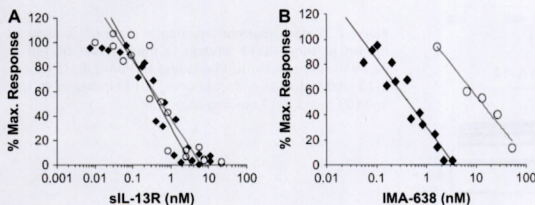


**Figure 4.** Comparison of neutralization activity of (A) sIL-13R and (B) IMA-638 on human (diamonds) and sheep (circles) IL-13. Sub-maximal concentrations of human or sheep IL-13 were used in the STAT6 phosphorylation bioassay with HT-29 cells, in the presence of increasing concentrations of sIL-13R or IMA-638. sIL-13R neutralized human and sheep IL-13 with  $IC_{50}$  values of 0.46 nM and 0.56 nM, respectively. IMA-638 neutralized human and sheep IL-13 with  $IC_{50}$  values of 0.44 nM and 12.6 nM, respectively.

doses had no effect on antigen-induced AHR to carbachol (Figure 6B).

#### sIL-13R Reduces Early and Late Phase Bronchoconstriction, and AHR

Figures 7A–7C illustrate the effects of intravenous sIL-13R (2, 1, or 0.5 mg/kg, respectively) when compared with the control challenge responses for each animal in the absence of drug. sIL-13R did not affect the early phase response at any dose tested (Figures 7A–7C). The 2 mg/kg dose of sIL-13R produced statistically significant inhibition of the late phase response ( $P < 0.05$  for the average late response; Figure 7A). The reduction in the late phase response was 66% and 58% for the two sheep treated at this dose. The 1 mg/kg dose of sIL-13R also inhibited the late phase response, by 48% and 38% in the two sheep ( $P < 0.05$ ) (Figure 7B). The 0.5 mg/kg dose of sIL-13R had no effect (Figure 7C).

The 2 mg/kg dose of sIL-13R completely prevented the post-antigen-induced AHR to carbachol (Figure 8;  $P < 0.05$ ), whereas the 1 mg/kg dose gave variable results, resulting in a nonsignificant effect (Figure 8). The 0.5 mg/kg dose had no effect (Figure 8). Because sIL-13R is a fusion of the extracellular portion of human IL-13Rα2 with the Fc portion of human IgG1, human IgG of irrelevant specificity (IVIG) served as the control for this reagent, as well as the control for IMA-638. As shown above, treatment of sheep with IVIG, even at doses of 5 or 20 mg/kg, did not affect early or late phase bronchoconstriction (Figures 5D and 5E) or carbachol-induced AHR (Figure 6B) after *Ascaris* challenge. Therefore, we were confident that the reduced late phase response seen at 2 and 1 mg/kg sIL-13R, and the reduced AHR seen at 2 mg/kg sIL-13R, were effects of the drug.

#### Pharmacokinetics of sIL-13R and IMA-638 in Sheep

sIL-13R is a fusion of sIL-13Rα2 with human IgG1 Fc, and IMA-638 is a humanized IgG1, κ. To quantitate the sIL-13R and antibody after infusion, sera of sheep were tested for levels of total human IgG by ELISA. The half-lives of sIL-13R and IMA-638 in the sheep were estimated as ~36 h and 14 d, respectively (data not shown).

#### DISCUSSION

IL-13 is a key cytokine driving pathogenic changes associated with asthma. Major validation supporting the importance of IL-13 has come from studies done in rodent models of asthma, in which the bioactivity of IL-13 was neutralized by exogenous administration of sIL-13R. This antagonist has been shown to prevent and even to reverse asthma-like changes, including AHR, mucus secretion, fibrotic changes, and airway inflammation (1, 3). sIL-13R is an analog of the naturally occurring

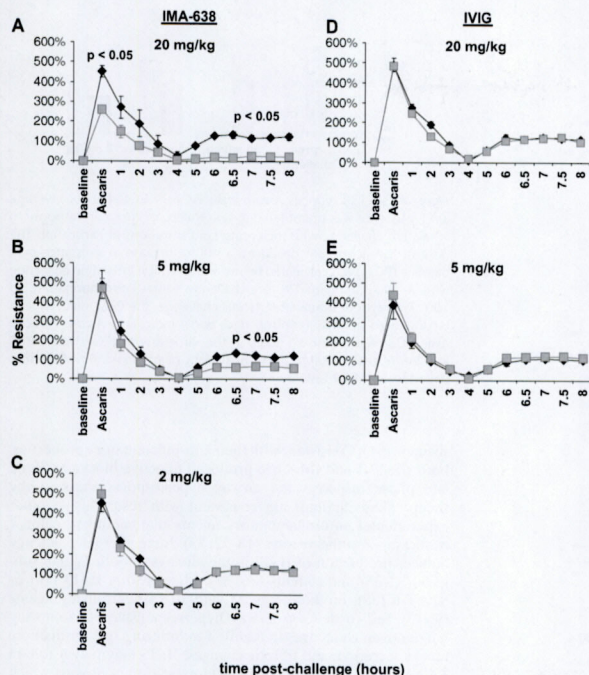


**Figure 5.** IMA-638 produced a dose-dependent reduction in the late phase response to *Ascaris*. Average lung resistance in three sheep per group treated with (A) 20 mg/kg, (B) 5 mg/kg, or (C) 2 mg/kg IMA-638; or (D) 20 mg/kg or (E) 5 mg/kg IVIG. For each cohort, resistance was measured at baseline, at the time of inhalation challenge with *A. suum* antigen, and at intervals up to 8 h after challenge. In a control trial, *Ascaris* was administered to untreated sheep (diamonds). In a second trial, *Ascaris* was administered to the same animals 24 h after intravenous infusion of IMA-638 (squares). The early phase response is seen from time 0 to 4 h after challenge, and the late phase is seen starting at 5 h after challenge. The peak early phase response was significantly reduced by treatment with 20 mg/kg IMA-638. The late phase response was significantly reduced by treatment with 20 mg/kg or 5 mg/kg IMA-638. Significance was determined at  $P < 0.05$  in a paired two-tailed *t* test. Data shown are mean  $\pm$  SEM for each group.

IL-13 antagonist, IL-13 $\alpha$ 2, which exists in cell-surface and soluble forms (26, 27). Because it lacks apparent signaling capacity, IL-13 $\alpha$ 2 is thought to act as a decoy receptor (28, 29), although evidence has recently been presented implicating a role for cell-bound IL-13 $\alpha$ 2 in the profibrotic activity of IL-13 (30).

Anti-human IL-13 antibody IMA-638 is a high-potency antagonist of the human cytokine, combining the IL-13-neutralizing properties of sIL-13R with the pharmacologic properties of an

antibody. Before evaluating sIL-13R and IMA-638 in the sheep model, it was important to verify that these antagonists would interact with sheep IL-13. Thus, sheep IL-13 was cloned from sheep splenocytes and expressed recombinantly in CHO cells. Sheep IL-13 was found to share 70% amino acid identity with the human cytokine, and showed complete cross-reactivity with human sIL-13R. In contrast, IMA-638 had 20–30 times lower reactivity with sheep IL-13 as compared with human IL-13, demonstrating less efficient neutralization of sheep IL-13 in three different bioassay formats. Biacore analysis confirmed this result and indicated that IMA-638 bound rapidly to sheep IL-13, but

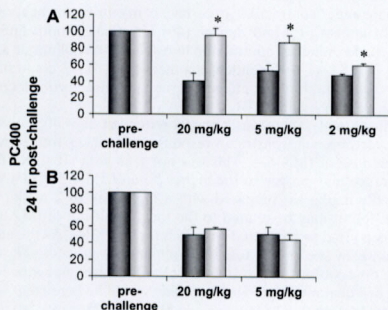


**Figure 6.** IMA-638 produced a dose-dependent reduction in antigen-induced AHR. Average lung resistance was quantitated in three sheep per group in response to inhalation challenge with increasing concentrations of carbachol. The dose of carbachol that produced a 400% increase in resistance over baseline ( $PC_{400}$ ) was calculated before and 24 h after inhalation challenge with *A. suum* antigen. The pre-challenge values were normalized to 100. Twenty-four hours after *Ascaris* challenge, the  $PC_{400}$  was reduced in untreated animals (control; dark grey bars), indicating *Ascaris*-induced AHR.  $PC_{400}$  was measured again in the same animal after intravenous infusion (light grey bars) of the indicated doses of (A) IMA-638, or (B) IVIG. \* $P < 0.05$  in a paired two-tailed *t* test.

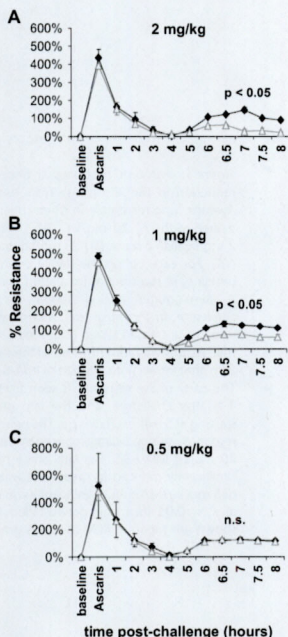


**Figure 7.** sIL-13R reduces *Ascaris*-induced late phase bronchoconstriction. The average lung resistance in two sheep per group treated with (A) 2 mg/kg, (B) 1 mg/kg, or (C) 0.5 mg/kg sIL-13R is shown. For each animal, resistance was measured at baseline, at the time of inhalation challenge with *A. suum* antigen, and at intervals of up to 8 h after challenge. In a control trial, *Ascaris* was administered to untreated sheep (diamonds). In a second trial, *Ascaris* was administered to the same animals 24 h after intravenous infusion of sIL-13R (triangles). The early phase response is seen from time 0 to 4 h after challenge, and the late phase is seen starting at 5 h after challenge. The late phase response was significantly reduced by treatment with 2 mg/kg sIL-13R, and by 1 mg/kg sIL-13R. Significance was determined by a  $P < 0.05$  in a paired two-tailed  $t$  test. Data shown are mean  $\pm$  SEM for each group.

then dissociated rapidly, accounting for the low affinity of the interaction.

These *in vitro* studies indicated that the sheep model could be used to evaluate and compare the therapeutic efficacy of sIL-13R and IMA-638 (18). Based on the binding data, sIL-13R was given at doses of 2, 1, and 0.5 mg/kg, whereas IMA-638 was tested at higher doses of 20, 5, or 2 mg/kg. Both sIL-13R and IMA-638 had dose-dependent inhibitory effects on the late response and the post-antigen-induced AHR. This is in contrast to the control human Ig (IVIg), which was ineffective, a result that is consistent with previous control studies using intravenously administered antibodies of irrelevant antigen specificity in the model (20, 22).

The development of an antigen-induced late response is thought to be the initial signal of a heightened inflammatory process that leads to a prolonged increase in airway respon-

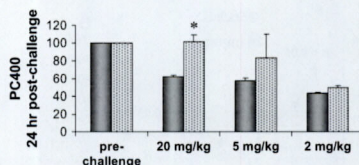


**Figure 8.** sIL-13R reduces *Ascaris*-induced AHR to carbachol. Average lung resistance was quantitated in two sheep per group in response to inhalation challenge with increasing concentrations of carbachol. The dose of carbachol that produced a 400% increase in resistance over baseline ( $PC_{400}$ ) was calculated before and 24 h after inhalation challenge with *A. suum* antigen. The pre-challenge values were normalized to 100. Twenty-four hours after *Ascaris* challenge, the  $PC_{400}$  was reduced in untreated animals (control; dark bars), indicating *Ascaris*-induced AHR.  $PC_{400}$  was measured again in the same animal after intravenous infusion of the indicated doses of sIL-13R (light bars). \* $P < 0.05$  in a paired two-tailed  $t$  test.

siveness (31). Consistent with their anti-inflammatory properties, both sIL-13R and IMA-638 produced effective blockade of the late phase response and airway hyperresponsiveness in the sheep. These findings are consistent with results using other experimental anti-inflammatory agents that modulate cellular trafficking into the airways (18–22, 32). Lung allergen challenge induces production of IL-13 in the airways of both human subjects (33–35) and rodents (36, 37). Mice lacking IL-13 (16) or STAT-6 (38), or those treated with IL-13 neutralizing agents (9, 13), fail to develop airway hyperresponsiveness following lung antigen challenge. In addition to inducing IgE production, mucus secretion, and fibrotic changes, IL-13 may play a role in VCAM/VLA4-mediated cell recruitment (39), a process which contributes to late phase responses in mice, sheep and humans (20, 40, 41).

IL-13 has also been found to directly trigger AHR in mice (8), in a manner independent of its effects on inflammation (42). These events could be related to its regulation of smooth muscle contractility (43). Thus, in addition to its inflammatory actions, IL-13 could play a direct role in mediating late phase bronchoconstriction. IL-13 has been found to trigger contraction of collagen gels embedded with human lung mesenchymal cells (44), and to directly mediate calcium transients in cultured murine (15) and human (7) airway smooth muscle cells. Furthermore, IL-13 has been shown to enhance the contractile response of murine airway smooth muscle to leukotriene D<sub>4</sub> (15) and to mediate hyper-contraction to carbachol in murine tracheal rings (7). In human (45) and murine (46) intestinal smooth muscle cells, IL-13 enhances contraction in response to cholinergic agonists, in a STAT-6-dependent manner. Thus, IL-13 could have both direct and indirect effects on the late phase bronchoconstrictor response and AHR.

Interestingly, there was a partial reduction of  $\sim 40\%$  on the peak early bronchoconstrictor response in the sheep treated with a high dose of IMA-638. This was not seen with sIL-13R. This difference may be due to the higher dose of IMA-638 that was used (20 mg/kg as compared with a high dose of 2 mg/kg for sIL-13R), or may be related to the longer half-life of IMA-638 in sheep (16 d as compared with 36 h for sIL-13R). As the early response in sheep is a mast cell-dependent process (47, 48), these observations suggest that IL-13 antagonism may influence mast cell degranulation. Such an effect would be consistent with reports that mast cells may express IL-13 receptor (49, 50), and

that IL-13 transgenic mice have heightened mast cell responses (11). Thus, an influence of IL-13 antagonism on mast cell activation cannot be ruled out.

In conclusion, this study has shown that both sIL-13R and IMA-638 were effective treatments for respiratory symptoms induced by inhaled allergen exposure in the sheep model. Despite its low level of reactivity with sheep IL-13, IMA-638 effectively reduced late phase bronchoconstriction and AHR, and demonstrated a partial effect on the early response at the highest dose examined. This is the first study to confirm the role of IL-13 to asthmatic responses in the sheep model of experimental asthma. These findings support and extend the possibility that IL-13 neutralization may be a therapeutic strategy for the treatment of human asthma.

**Conflict of Interest Statement:** M.T.K. is an employee of Wyeth. D.D.D. is an employee of Millennium Pharmaceuticals. L.T. is an employee of Wyeth. K.M. is an employee of Wyeth. X.-Y.T. is an employee of Wyeth. A.A.'s institution (Mount Sinai Medical Center, FL) received funds to support some of the studies described herein. B.A.J. is an employee of Wyeth. A.W. is an employee of Wyeth. T.A.C. is an employee of Wyeth. X.X. is a Wyeth employee who owns Wyeth Stock Options via Performance Award Program. A.B.B. is a full-time employee of Wyeth. S.J.G. is an employee of Wyeth. W.M.A.'s institution (Mount Sinai Medical Center, FL) received funds to support some of the studies described herein.

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