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Title: Therapeutic Dosing with Anti-Interleukin-13 Monoclonal Antibody Inhibits Asthma Progression in Mice

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Running title: Anti-IL-13 Inhibits Asthma Progression

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ABSTRACT

In vivo models have demonstrated that interleukin-13 (IL-13) plays an important role in asthma. However, few studies have evaluated the effect of inhibition of IL-13 on established and persistent disease. In the present study we have investigated the effect of a therapeutic dosing regimen with an anti-IL-13 monoclonal antibody (mAb) in a chronic mouse model of persistent asthma. Balb/c mice were sensitized to allergen (ovalbumin, OVA, on days 1 and 8) and challenged with OVA weekly from day 22. Anti-IL-13 mAb or vehicle dosing was initiated following two OVA challenges when disease was established. At this time, mice exhibited airway hyperresponsiveness (AHR), increased mucus production, inflammation and initiation of subepithelial fibrosis compared to saline-challenged mice. Mice received four additional OVA challenges. Treatment with anti-IL-13 mAb inhibited AHR and prevented the further development of subepithelial fibrosis and progression of inflammation. Furthermore, mAb treatment reversed the mucus hyperplasia to basal levels. These effects were associated with an inhibition of cytokines, chemokines and matrix metalloproteinase-9. These data demonstrate that neutralization of IL-13 can inhibit the progression of established disease in the presence of repeated allergen exposures.

INTRODUCTION

Asthma is characterized by the presence of reversible bronchoconstriction, increased sensitivity to specific and non-specific bronchospasmic agents and excessive mucus production. These clinical features are accompanied by an underlying pathology of inflammation and airway remodeling. The pathological changes are thought to contribute to the clinical symptoms of the disease (Fireman, 2003). Current therapies for asthma provide symptomatic control but do not halt the underlying disease highlighting the unmet medical need. Patients are continually exposed to allergens, or other bronchospasmic agents, which may contribute to the maintenance or progression of persistent disease. Evidence to date suggests that interleukin-13 (IL-13) has an important role in asthma; it is associated with human disease and preclinical models have demonstrated that it induces many of the features associated with human asthma.

Elevated levels of IL-13 mRNA and protein (e.g.Huang et al., 1995) have been described in human disease pathogenesis and polymorphisms in the IL-13 gene have been associated with asthma (Wills-Karp, 2000). Multiple studies have demonstrated that administration of recombinant murine (rm) IL-13 to the lungs of mice induces airway mucus hyperplasia, eosinophilia and airway hyperresponsiveness (AHR) (e.g.Grunig et al., 1998; Wills-Karp et al., 1998; Singer et al., 2002; Kibe et al., 2003; Vargaftig and Singer, 2003b; Vargaftig and Singer, 2003a). These effects of IL-13 are reproduced in transgenic mouse systems where IL-13 overexpression is induced in a constitutive or inducible manner (Zhu et al., 1999; Zhu et al., 2001; Lanone et al., 2002). Chronic transgenic overexpression of IL-13 also induces subepithelial fibrosis and emphysema.

Mice deficient in the IL-13 (and IL-4) signaling molecule signal transducer and activator of transcription 6 (STAT6) fail to develop allergen-induced AHR and mucus hyperplasia (Kuperman et al., 2002). Finally, utilization of an IL-13-specific neutralization strategy with soluble IL-13 receptor fusion protein (sIL-13Ra2Fc) has demonstrated the pivotal role of this cytokine in experimental allergen (ovalbumin; OVA)-induced airway disease (Grunig et al., 1998; Wills-Karp et al., 1998; Taube et al., 2002).

Clinically, therapeutic interventions occur in the presence of established disease. To date, experimental therapeutic-intervention studies have not investigated the effects of IL-13 neutralization in the presence of chronic, persistent allergen challenges. In the present study, we used a rat monoclonal antibody (mAb) to neutralize the actions of mouse IL-13 specifically and have initiated treatment with the mAb once disease is established. Further, we have continued mAb treatment in the presence of further allergen challenges. Our data demonstrate that anti-IL-13 mAb treatment inhibits AHR, chronic inflammation, subepithelial fibrosis and reverses mucus hyperplasia. Associated with these effects, neutralization of IL-13 inhibited the production of multiple cytokines, chemokines and matrixmetalloproteinase 9 (MMP-9). These data support the hypothesis that IL-13 is an important upstream mediator involved in asthma pathogenesis and disease progression.

METHODS

Mice

BALB/C female mice (6-8 weeks old, weighing 20-25 g) were from Charles Rivers Laboratories (Raleigh NC). All mice were maintained under specific pathogen free conditions and maintained on an OVA free diet with free access to food and water at Centocor. 20-week old Sprague-Dawley rats were used for the generation of anti-IL-13 mAb. Sprague Dawley rats were housed and cared for at Covance Research Products Inc. (Denver, PA). All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of Centocor or Covance as appropriate.

Generation of rat anti-mouse IL-13 mAb (CNTO 134)

Rat-anti-mouse IL-13 mAb (CNTO 134; IgG2a isotype) was generated at Centocor with 50 μg recombinant murine (rm) IL-13 (R&D Systems, Minneapolis, MN) using conventional immunization protocols and hybridoma technology. Solid phase enzyme immunoassay was used to screen for antibodies specific for rm IL-13. Briefly, plates were coated overnight with rm IL-13 in phosphate-buffered buffer (PBS) and wells were blocked with 1%(w/v) bovine serum albumin (BSA) for 1h at room temperature. Undiluted hybridoma supernatants were added to the rm IL-13 wells and washed. The wells were probed with horseradish peroxidase-labeled goat anti-rat IgG. Plates were washed then incubated with citrate-phosphate substrate solution (0.1M citric acid and 0.2M sodium phosphate, 0.01%H_20_2, and 1mg/ml o-phenylenediamine dihydrochloride).

Substrate development was stopped by addition of 4N sulfuric acid and the optical density determined at 490nm.

In vitro bioactivity of CNTO 134

Neutralization activity of CNTO 134 was measured by determining inhibition of rm IL-13-mediated B9 myeloma cell (a murine B cell hybridoma cell line, ATCC, Rockville, MD) proliferation. B9 cells were maintained in IMDM (containing 5% fetal bovine serum (FBS), 1% l-glutamine, 0.1 mM MEM nonessential amino acids, sodium pyruvate, 50 μM 2-mercaptoethanal and 5 ng/ml rm IL-13 (R&D Systems). $1X10^5$ B9 cells/ml (50 μl/well) cells were seeded in 96-wells culture plates and incubated with a final concentration of 5 ng/ml of mouse IL-13 together with different concentrations of CNTO 134 for 3 days at 37^0C, 5%CO_2. The IL-13-dependent cell proliferation was measured using a luminescent ATP detection assay kit (Packard Bioscience, Meriden, CT). Inhibition of IL-13 resulted in lowered proliferation of B9 cells that could be measured based on the amount of ATP per well. The concentration of CNTO 134 that inhibited B9 cell proliferation by 50% (IC50) was 17 ng/ml (data not shown).

In vivo OVA model protocol

Mice were immunized intraperitoneally (I.P.) with 10 μg OVA (Sigma, St. Louis, MO) in 100 μl of PBS mixed with the same volume of Inject Alum (Pierce, Rockford, IL) on day 1, and boosted in the same way on day 8. On day 22, 29, 36, 43, 50 and 52, mice received an intranasal (I.N.) challenge with 50 μl PBS or 100 μg OVA (2 mg/ml) under ketamine/xylazine anesthesia (90 and10 mg/kg respectively; I.P.). Rat anti-mouse IL-13

mAb (CNTO 134; 500 μg/mouse) or vehicle (PBS; 200 μl/mouse) treatment was initiated on day 36 and agents were administered intravenously (I.V.) one hour prior to each I.N. challenge. Our previous in-house studies have established the appropriateness of using the vehicle (PBS) as the control for CNTO 134 since no biological differences are observed in the OVA model between mice treated with vehicle and those treated with control IgG (data not shown). A subset of mice was sacrificed on day 36; these mice did not receive day 36 I.N. challenge or I.V. treatment (Figure 1).

Airway hyperresponsiveness (AHR)

On day 36 and 53 AHR was measured in mice using whole body plethysmography (Buxco, Sharon, CT). AHR was measured following aerosolization of PBS followed by increasing concentrations of methacholine (10-40 mg/ml, Sigma) for two minutes into the chamber. AHR was expressed as the average enhanced pause (Penh) (Hamelmann et al., 1997) that was measured over a five minute period following aerosol exposure to PBS or methacholine. All mice were exposed to PBS and subsequently exposed to each methacholine dose. There was approximately an interval of sixty minutes between each aerosol exposure and within this period of time the Penh values had returned to baseline.

Bronchoalveolar lavage (BAL)

After AHR measurements, mice were euthanized (CO2 asphyxiation), the trachea cannulated and BAL was performed by slowly injecting 1 ml of PBS (once) into the trachea and the lavage was retrieved. Supernatants were collected after centrifugation

(10 minutes; 1500 rpm) for further analyses. BAL cells were resuspended in 1 ml PBS (containing 2% FCS) for total and differential cell counts.

Histology and morphometric analyses

Following BAL, the right lung was clamped off and removed and, the left lung was fixed with 10% buffered formalin under constant pressure of 15 cm water. After fixation, lungs were dehydrated and embedded in paraffin by routine methods. Lungs were oriented in the blocks so that para-hilar sagital sections were obtained. Five micron serial sections were stained with hematoxylin and eosin (H&E), periodic acid Schiff (counterstained with hematoxylin) (PAS) or picric-Sirius Red (SR). H&E stained sections were used for general evaluation of histopathologic change and PAS for morphometric analysis of mucus inclusions and inflammatory infiltrates. SR, viewed and photographed under crossed polarized light, was used for morphometric analysis of mature collagen fibers (Dolhnikoff et al., 1999). For semi-quantitiative analysis of mucus inclusions, cellularity and mature collagen, sections were analyzed morphometically using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). PAS stained sections were thresh-holded so as to measure only the area of mucus. H&E sections were thresh-holded so as to measure all nuclei. This can be taken as a measure of cellularity. To calculate the % lung occupied by mucus or nuclei (equation 1), the stained area for each constituent was divided by the total area of lung (including blood vessels and air space) included in the 4x field:

$$\% \text{ Lung} = \frac{\text{Stained Area}}{} \times 100$$

Total Area

Equation 1.

To measure mature collagen, Sirius red stained sections were photographed with cross-polarized light, the images converted from RGB to hue-saturation-intensity (HIS). These images were thresh-holded so as to measure only mature, i.e., birefringent, collagen and the integrated optical density (IOD) for collagen measured for each 4x field. A random, low magnification 4x objective field, of the para-hilar region, including the main stem bronchus and its primary and secondary branches, was photographed for each lung using a Nikon E800 equipped with plan apochromatic lenses and a Nikon DXM1200 digital camera. Images were stored as red green blue (RGB).tiff files for morphometric analysis.

Chemokine, cytokine and MMP-9 detection by ELISA

The right lungs from some mice were homogenized in PBS and supernatants assayed chemokines, cytokines (R&D Systems, Minneapolis, MN) and total MMP-9 (Amersham Biosciences, Piscataway, NJ).

Statistical analysis

Data are summarized using mean ± standard error of the mean (S.E.M.). Statistical differences between groups were tested based on analysis of variance (ANOVA) with two-tailed tests. For day 36 AHR, data analyses for significant differences within the entire data set were performed on means and S.E.M. across both days 36 and 53. Comparisons between the groups of animals (both PBS groups *versus* both OVA groups; Group 1 and 3 *versus* Group 2 and 4) were made at all three methacholine concentrations

(10-40 mg/ml) after fitting a mixed effect linear model with Day as a random effect, baseline and PBS as covariates. Statistical testing was performed based on two-tailed tests, and significance was claimed if the *p*-value is less than 0.05. Day 53 AHR data was analyzed by ANOVA. Statistical significance was claimed with *p*-values less than 0.05.

RESULTS

Neutralization of IL-13 reverses established AHR

It was important to establish the presence of airway disease at the time CNTO 134 treatment was initiated. Disease was induced in mice, following OVA sensitization, with two allergen challenges a week apart (outlined in Figure 1). One week after the second allergen challenge (day 36) a small subset of mice was analyzed for the presence of allergic disease. AHR to methacholine challenge was observed in mice challenged with OVA compared to PBS-challenged mice (Table 1). Anti-IL-13 treatment was initiated on day 36 and mice continued to receive multiple allergen challenges. Treatment with CNTO 134 inhibited AHR on day 53 (Figure 2).

Neutralization of IL-13 inhibits the progressive increase in BAL inflammation and cellularity in lung tissue

Two OVA challenges also induced an increase in the numbers of eosinophils, mononuclear cells and neutrophils in the BAL on day 36 (Figure 3 a, b and c respectively). The BAL cell numbers revealed that the airway inflammation had progressed in day 53 OVA-challenged mice compared to day 36 mice. The increased airway inflammation was supported by the increased cellularity observed by morphometric analysis of the lung histology (Figure 4 a and c, Figure 5 a). Histopathologic examination of the lung sections of day 36 OVA-challenged mice revealed increased cellularity characterized by perivascular inflammatory cell cuffing, airway smooth muscle cell hypertrophy and, goblet cell hypertrophy and hyperplasia compared to PBS-challenged mice. All these changes progressed between day 36 and 53

and the inflammatory infiltrates made the overwhelming contribution to the increase in cellularity. The perivascular cuffing extended from the primary branches of the pulmonary artery to the level of arterioles and was composed of a mixed inflammatory infiltrate in which eosinphils were prominent. In the more severely affected mice, inflammatory cells infiltrates extended to involve the main stem bronchus and primary, secondary and respiratory bronchioles. The infiltrates occasionally formed follicular structures. Airway smooth muscle cell hypertrophy was confined mainly to the bronchi and primary bronchioles. The epithelium lining the main stem bronchus and primary and secondary bronchioles was markedly hypertrophied and hyperplastic. The epithelium of the respiratory bronchioles was also hypertrophied, but much less so.

Treatment with CNTO 134 significantly inhibited the progressive increase in BAL eosinophilia (Figure 3 a). Increased numbers of BAL mononuclear cells were observed on day 53 compared to day 36 OVA-challenged mice and, treatment of mice with CNTO 134 resulted in significantly fewer mononuclear cells in the BAL (Figure 3 b). Allergen challenge stimulated a BAL neutrophilia that was not significantly modulated by CNTO 134 treatment (Figure 3 c). The inhibition of inflammation in the airways with CNTO 134 treatment was supported by the morphometric analyses of the lung histology. A significant increase in the cellularity was observed in the lung tissue in OVA-challenged mice on day 53 compared to day 36 mice (Figure 4 c and e; Figure 5 a). CNTO 134 treatment prevented this progressive increase in lung cellularity (Figure 4 e and g; Figure 5 a). Histologically, a mixed inflammatory cell infiltrate, rich in eosinophils, was still present but was more sparse and less well organized than observed in the PBS-treated

mice. Similarly, there was still evidence of airway smooth muscle cell hypertrophy. As was seen in the PBS-treated OVA-challenged mice, the inflammatory infiltrates accounted for the majority of lung cellularity.

Neutralization of IL-13 reverses excessive mucus production

Lung histology revealed a significant increase in mucus staining in allergen-challenged mice on day 36 (Figure 4 a and c, 5 b). Mucus was another pathological feature that progressed between day 36 and 53 (Figure 4 c and e, Figure 5 b). The fraction of the epithelium lining the main stem bronchus that was formed by goblet cells in some cases approached 100% and was also increased in the primary and secondary bronchioles. Goblet cells were observed only very rarely in the respiratory bronchioles. Neutralization of IL-13 with CNTO 134 significantly inhibited not only the progressive increase in mucus from day 36 to day 53 (Figure 4 c, e and g; Figure 5 b) but, reversed the mucus levels to near-background levels when compared to OVA-challenged day 36 mice (Figure 4 a, c and g; Figure 5b). Histologically, the fraction of airway epithelium occupied by goblet cells was less than what was observed on Day 36 in the OVA-sensitized and challenged mice and, in some mice almost normal.

Neutralization of IL-13 inhibits the development of fibrosis

Subepithelial fibrosis was assessed by staining the lung sections with Sirius red and measuring birefringence, a measurement of the amount of mature collagen. Although significant subepithelial fibrosis was not apparent on day 36 (Figure 4 b and d, Figure 5 c), subepithelial fibrosis had developed significantly in the airway interstitium by day 53

in the lungs of mice challenged with OVA (Figure 4 f; Figure 5 c). Treatment with CNTO 134 prevented the development of fibrosis (Figure 4 h; Figure 5 c).

IL-13 regulates MMP-9 activity and production of pro-inflammatory mediators

Airway remodeling is associated with MMP activity. To address whether IL-13 was involved in MMP activation, total MMP-9 protein levels was measured in the lung tissue of mice (Figure 6). Significant increases in MMP-9 were observed in OVA-challenged mice on day 53. Neutralization of IL-13 resulted in a reduction in MMP-9. A number of cytokines and chemokines were also modulated with anti-IL-13 treatment. Allergen challenges induced significant increases in the levels of interleukin (IL-)-4, -5, -13, tumor necrosis factor α (TNFα), KC and eotaxin by day 36 and these levels remained elevated on day 53 together with significant increases in JE levels (Table 2). Eotaxin levels increased further on day 53 compared to levels present on day 36. This may reflect the progressive increase in eosinophils in the BAL between the two time points. Neutralization of IL-13 inhibited the levels of all cytokines and chemokines. The effect of CNTO 134 on IL-13 protein levels was not assessed since CNTO 134 interfered with the ELISA assay.

DISCUSSION

The current study has demonstrated that an IL-13-specific neutralization approach with a mAb may be an effective therapy for the treatment of asthma. To our knowledge, this is the first demonstration that therapeutic dosing with an anti-IL-13 mAb in established, persistent, progressive disease in mice modulated lung function as well as the underlying

pathologic features associated with the disease. Further, our data show that anti-IL-13 treatment halts disease progression and even reverses certain features associated with experimental asthma.

Cellular and humoral lung inflammation is a characteristic feature of asthma and in vivo models of asthma mimic this aspect of disease. Our current study demonstrated a progressive increase in lung tissue cellularity and airway inflammation (eosinophils and mononuclear cells) between days 36 and 53 that was inhibited by neutralization of IL-13. A trend towards inhibition of BAL neutrophilia was also observed. The effect of IL-13 inhibition on allergic inflammation is controversial (Grunig et al., 1998; Wills-Karp et al., 1998; Taube et al., 2002) and studies (including the current study) suggest that neutralization of IL-13 may impact the inflammatory response in more chronic settings (Taube et al., 2002). Data also suggest that IL-13 may play a protective role in acute inflammatory settings. IL-13 has been shown to be anti-inflammatory in a guinea pig model of acute allergic inflammation (Watson et al., 1999). Further, neutralization of IL-13 resulted in an exacerbation of inflammation in an experimental model of acute lung injury (Lentsch et al., 1999).The allergen challenge protocol used in the present study differs significantly from previous studies: at the time mAb treatment was initiated persistent disease and pathology was established and mice continued to receive multiple allergen challenges to establish a chronic disease state. The discrepancy in data regarding an effect on cell inflammation may be due to differences in the mouse strain used, the time point at which cell numbers were evaluated together with the chronicity of the model. Since neutralization of IL-13 reduced the levels of the eosinophil-survival

cytokine IL-5 and, IL-13 itself has been shown to be a survival factor for eosinophils (Horie et al., 1997), it is possible that increased apoptosis and clearance in the absence of IL-13 contributes to the diminished cell numbers. Interestingly, although multiple cytokines (including IL-13) and chemokines were elevated in the airways of OVA-challenged mice from day 36, only the level of eotaxin increased further at day 53 suggesting its association with the progressive increase in inflammation. IL-13 has been shown to induce eotaxin expression in epithelial cells (Li et al., 1999). Anti-IL-13 mAb treatment reduced, but did not completely reverse, the levels of all the cytokines and chemokines measured. Exogenous administration (Grunig et al., 1998; Wills-Karp et al., 1998; Singer et al., 2002; Kibe et al., 2003; Vargaftig and Singer, 2003b; Vargaftig and Singer, 2003a) or transgenic pulmonary over-expression of IL-13 itself (Zhu et al., 1999; Zhu et al., 2001; Lanone et al., 2002) induces the infiltration of neutrophils, eosinophils and mononuclear cells into the lungs of mice together with stimulating the expression of many chemokines including eotaxin, JE (a mouse homologue of human monocyte chemoattractant protein 1, MCP-1) and KC. Synergistic actions of cytokines such as TNFα with IL-13 (Li et al., 1999; Moore et al., 2002; Kibe et al., 2003), together with positive feedback loops (Vargaftig and Singer, 2003b), may explain the incomplete inhibition of the lung inflammation by anti-IL-13 treatment. Interestingly, pulmonary-specific overxpression of IL-13 does not induce IL-4 or IL-5 expression in the lungs (Zhu et al., 1999). Further, lymph node cells from OVA-sensitized and challenged IL-13 gene-deleted mice release levels of IL-4 and IL-5 that are similar to that released by wild type challenged mice (Walter et al., 2001). These data indicate that the inhibition of IL-4 and IL-5 observed in the present study and others (Taube et al., 2002) is secondary to IL-13

neutralization. Indeed, it has been shown that eotaxin stimulates eosinophils to secrete IL-4 (Bandeira-Melo et al., 2002).

Multiple studies, including the present study, demonstrate a dissociation between the cellular (particularly eosinophils) inflammatory component following OVA challenge and AHR (e.g.Walter et al., 2001; Taube et al., 2002). Similar to OVA-induced AHR, exogenous administration of IL-13 to mice induces AHR that is dissociated from the inflammation induced by IL-13 (Wills-Karp et al., 1998; Walter et al., 2001; Singer et al., 2002; Venkayya et al., 2002). In agreement with previous studies, we have shown that inhibition of IL-13 can almost completely inhibit AHR with a modest effect on inflammation. Recent data in a chronic OVA-challenge model has demonstrated that AHR and airway remodeling persists for up to 4 weeks following the last OVA challenge in the absence of cellular and humoral inflammation (Leigh et al., 2004). In contrast to the present study, the persistent AHR and remodeling was not modulated by acute inhibition of IL-13 with a soluble IL-13 receptor fusion protein. The Leigh et al model also illustrates clearly that AHR, in mice, may occur in the absence of inflammation and is associated with airway remodeling. It will be interesting to establish whether chronic neutralization of IL-13 is required to modulate the remodeling and persistent AHR observed in the model. The mechanisms underlying IL-13-induced AHR are not fully understood. In vitro, IL-13 is able to enhance nerve-stimulated murine jejunal smooth muscle contraction that is STAT6-dependent (Zhao et al., 2003) and IL-13 also increases carbachol-induced murine and human smooth muscle contraction (Tliba et al., 2003). IL-13 mediates its actions through a heterodimer receptor consisting of the IL-13 receptor α

1 (IL-13Rα1) and the IL-4 receptor α (IL-4Rα) chains. Human smooth muscle cells express the IL-4Rα and IL-13Rα1 receptors (Laporte et al., 2001) and smooth muscle mast cells express IL-13 (Brightling et al., 2003). These in vitro data suggest that IL-13 may have a direct effect on smooth muscle function. However, in vivo evidence suggests an indirect effect of IL-13 on smooth muscle constriction. Reconstitution of STAT6 specifically in lung epithelial cells of STAT6-deficient mice restored IL-13-induced AHR (Kuperman et al., 2002) indicating that IL-13 is able to act on epithelial cells to induce AHR. Further, it has been shown that IL-13-induced AHR is partly mediated by leukotrienes (Vargaftig and Singer, 2003a).

Airway remodeling is a characteristic underlying feature of asthma and is thought to contribute to the symptoms associated with asthma. The features of airway remodeling assessed in the current study were goblet cell hyperplasia (measured indirectly by staining for mucus positive cells) and subepithelial fibrosis. Neutralization of IL-13 reversed the increase in mucus-producing cells and inhibited the development of subepithelial fibrosis. IL-13-induced goblet cell hyperplasia is not due to cell proliferation in vivo (Vargaftig and Singer, 2003a) but may be due to differentiation of epithelial cells into secretory cells (Zuhdi Alimam et al., 2000; Laoukili et al., 2001). Previous studies have demonstrated that IL-13-induced goblet cell hyperplasia in vivo is partially dependent on inflammation (Shim et al., 2001; Singer et al., 2002). Further, IL-13-induced mucus protein expression may be mediated through the epidermal growth factor receptor (Shim et al., 2001). IL-13 has been shown to stimulate fibroblast proliferation in vitro (Ingram et al., 2003; Jakubzick et al., 2003; Saito et al., 2003) and in

vivo (Vargaftig and Singer, 2003b). IL-13 may activate fibroblast directly through its receptor which is present on fibroblasts (Doucet et al., 1998; Jakubzick et al., 2003) or indirectly via the stimulation of secondary mediators such as leukotrienes, transforming growth factor-β and platelet derived growth factor (Richter et al., 2001; Chibana et al., 2003; Ingram et al., 2003). IL-13 also stimulates collagen deposition from fibroblasts in vitro and in vivo (Doucet et al., 1998; Zhu et al., 1999) which may by mediated through activation of TGFβ by MMP-9 (Lee et al., 2001). Interestingly, in our study, we observed a progressive increase in MMP-9 levels in the lung tissue between day 36 and 53, which was associated with the development of subepithelial fibrosis, and was inhibited by anti-IL-13 mAb. Increased levels of MMP-9 have been shown to be elevated in the airways of asthmatics and immunolocalization studies have demonstrated that MMP-9 expression in the subepithelial basement membrane is associated with fibrosis (e.g.Wenzel et al., 2003).

In summary, by using a therapeutic treatment regimen with an anti-IL-13 mAb in a mouse model of persistent disease, we have demonstrated clearly an important role of IL-13 in allergic asthma. Neutralization of IL-13 inhibited and even reversed multiple features of pathophysiology that are inadequately controlled in the clinic at present. We believe that IL-13 neutralization has the potential to be an important therapeutic for the treatment of persistent asthma.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Schematic diagram illustrating the protocol for OVA sensitization, challenges and mAb treatment. All mice were sensitized to OVA. Mice were challenged weekly with OVA or PBS on days 22, 29, 36, 43, 50 and 52. CNTO 134 or vehicle treatment (weekly) was initiated on day 36. On day 36, a subset of mice were sacrificed and did not receive OVA or PBS challenge. The remaining mice were sacrificed on day 53.

Figure 2. Anti-IL-13 inhibits methacholine-induced AHR. All mice were sensitized to OVA. On day 53, AHR was tested 24 h following the last PBS (open bars; control mice; n=10) or OVA challenge where mice were either treated with i.v. vehicle (filled bars; n=13) or CNTO 134 (gray filled bars; n=11). AHR was measured using whole body plethysmography. Each point represents the mean ± S.E.M. *, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$. Data are representative of two separate studies.

Figure 3. Anti-IL-13 inhibits progression of BAL inflammation. All mice were sensitized to OVA. On day 36 BAL was collected from mice one week after the second PBS (clear column; day 36 control mice; n=4) or OVA (filled column; n=6) challenge. On day 53 BAL was collected 24 h following the last PBS (clear column; day 53 control mice; n=10) or OVA challenge where mice were either treated with i.v. vehicle (filled column; n=12) or CNTO 134 (gray column; n=11). Differential cell counts were performed to determine the total number of (a) eosinophils, (b) mononuclear cells and (c) neutrophils. Each point represents the mean ± S.E.M. *, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$. Data are representative of two separate studies.

Figure 4. Anti-IL-13 affects disease pathology. All mice were sensitized to OVA. On day 36 and 53 lungs were collected for histology as described in Methods. Lung sections were stained with PAS (to show mucus and inflammation) or Sirius red (to show fibrosis). On day 36, lungs were collected from mice one week after the second PBS (a, b) or OVA (c, d) challenge. On day 53, lungs were collected 24 h following the last OVA challenge where mice were either treated with i.v. vehicle (OVA/vehicle; e, f) or CNTO 134 (OVA/CNTO 134; g, h). The pictures are representative of 4-13 mice and illustrate the lung pathology. PAS stained pictures are at x100 magnification; Sirius red stained pictures are at x 40 magnification. Arrows indicate relevant pathology.

Figure 5. Anti-IL-13 inhibits progression of pathology. All mice were sensitized to OVA. On day 36 and 53 morphometric analyses were performed on low magnification photographs (x 4 objective lens) of lung histology sections as described in Methods; (a) H&E-stained slides were used to quantitate cellularity, (b) PAS-stained slides to quantitate mucus and (c) SR-stained slides were used to quantitate fibrosis. Morphometric analyses data quantitating cellularity, mucus and fibrosis are expressed as (a) % area nuclei, (b) % area mucus and (c) IOD (integrated optical density) collagen respectively. On day 36, lungs were collected from mice one week after the second PBS (clear column; day 36 control mice; n=4) or OVA (filled column; n=5) challenge. On day 53, lungs were collected 24 h following the last PBS (clear column; day 53 control mice; n=10) or OVA challenge where mice were either treated with i.v. vehicle (filled column; n=12) or CNTO 134 (gray column; n=11). Each point represents the mean ± S.E.M. *, p<

0.05; **, p<0.01; ***, p<0.001; ###, p<0.001. ns = not significant. Data are representative of two separate studies.

Figure 6. Anti-13 inhibits total MMP-9 protein levels in the airways. All mice were sensitized to OVA. On day 36 BAL was collected in mice one week after the second PBS (clear column; day 36 control mice; n=4) or OVA (filled column; n=6) challenge. On day 53 BAL was collected 24 h following the last PBS (clear column; day 53 control mice; n=5) or OVA challenge where mice were either treated with i.v. vehicle (filled column; n=6) or CNTO 134 (gray column; n=6). MMP-9 levels were measured in tissue homogenate supernatants as described in Methods. Each point represents the mean ± S.E.M. *, p< 0.05; **, p<0.01.

Table 1. Statistical analyses of AHR on days 35 and 53.

			Treatment				
Time	**Group**	**Summary**	**Baseline**	**PBS**	**10mg**	**20mg**	**40mg**
Day 36	PBS (1)	n	4	4	4	4	4
		Mean	0.6157	1.0248	0.7860	1.2973	2.3197
		SD	0.167	0.327	0.218	0.310	1.678
	OVA (2)	n	6	6	6	6	6
		Mean	0.7067	0.8014	1.7382	3.4697	3.9899
		SD	0.305	0.257	0.888	2.219	1.944
Day 53	***PBS (3)***	***n***	***10***	***10***	***10***	***10***	***10***
		Mean	0.5508	0.7566	1.3229	1.7527	2.3679
		SD	0.156	0.251	0.313	0.606	0.844
	OVA (4)	n	14	14	14	14	13
		Mean	0.8060	0.8608	2.2176	3.6011	4.3504
		SD	0.191	0.218	0.776	1.268	1.557
		p-value	n/a	n/a	0.0189	0.0102	0.0296

Table 1. All mice were sensitized to OVA. On day 36, AHR was tested in mice one week after the second PBS or OVA challenge. On day 53, AHR was tested 24 h following the last PBS or OVA challenge. Data analyses of AHR for significant differences within the

entire data set were performed on means and S.E.M. across both days 36 and 53. Comparisons between the groups of animals (both PBS groups *versus* both OVA groups; Group 1 and 3 *versus* Group 2 and 4) were made at all three methacholine concentrations (10-40 mg/ml). A two-tailed *p*-value based on a Mixed Effect Linear Model with Day as a random effect, baseline and PBS as covariates for comparing Groups 1 and 3 *versus* Group 2 and 4.

Table 2. Anti-IL-13 treatment inhibits cytokine and chemokine levels in the lungs.

	Cytokine/chemokine levels (pg/ml)						
Group	IL-13	IL-4	IL-5	TNFα	eotaxin	JE	KC
Day 36							
PBS	178±18	103±8	121±11	68±6	29±4	89±10	58.2 ± 4
OVA	472±20[a]***	272±9[a]***	349±11 [a]***	218±7 [a]***	213±18[a]*	323±27	146 ± 3 [a]***
Day 53							
PBS	162±9	88±5	97±6	59±3	28±3	104±5	48 ± 2
OVA/vehicle	387±30 [b]***	235±13 [b]***	280±17 [b]***	174±12 [b]***	858±44 [b,d]***	413±72 [b]***	167 ± 6 [b]***
OVA/CNTO 134	-	144±8 [c]***	176±10 [c]***	102±88 [c]***	150±15 [c]***	201±14 [c]***	100 ± 4 [c]***

Table 2. All mice were sensitized to OVA. On day 36 and 53 lungs were homogenized and supernatants were analyzed for levels of cytokines (IL-13, IL-4, IL-5, TNFα) and chemokines (eotaxin, KC and JE) as described in Methods. On day 36, lungs were collected from mice one week after the second PBS (n=4) or OVA challenge (n=6). On day 53, lungs were collected 24 h following the last PBS (n=10) or OVA challenge where mice were either treated with i.v. vehicle (OVA/vehicle; n=13) or CNTO 134 (OVA/CNTO 134; n=13). Each point represents the mean ± S.E.M. *, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$. *a* compared to day 36 PBS-challenged mice; *b* compared to day 53 PBS-challenged mice; *c* compared to day 53 vehicle-treated mice; *d* compared to day 36 OVA-challenged mice.

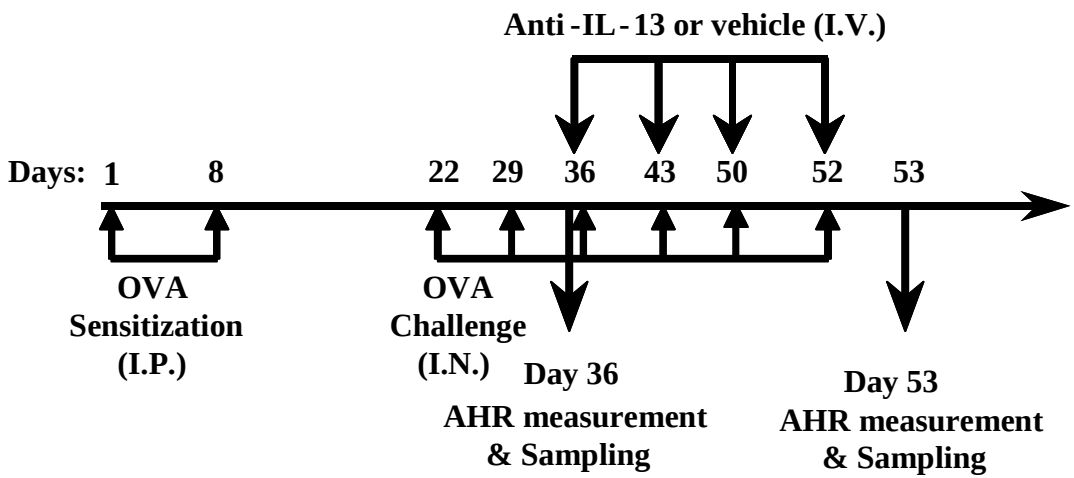
Anti-IL-13 or vehicle (I.V.)
Days: 1 8 22 29 36 43 50 52 53
OVA Sensitization (I.P.)
OVA Challenge (I.N.)
Day 36
AHR measurement & Sampling
Day 53
AHR measurement & Sampling

Figure 1

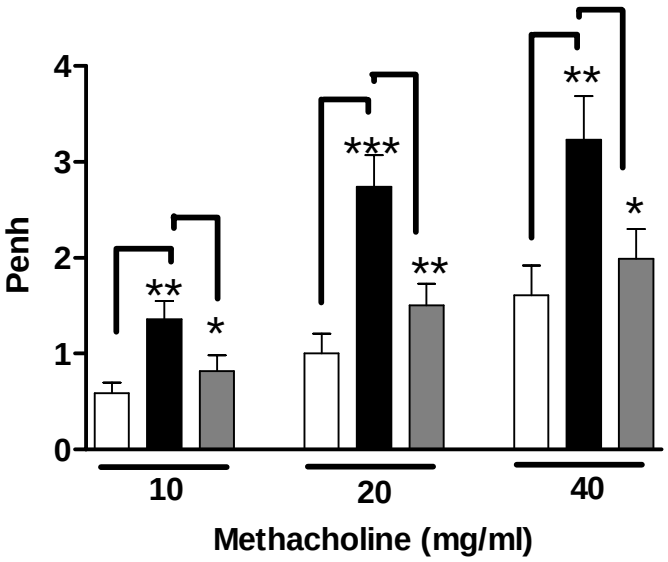

Figure 2

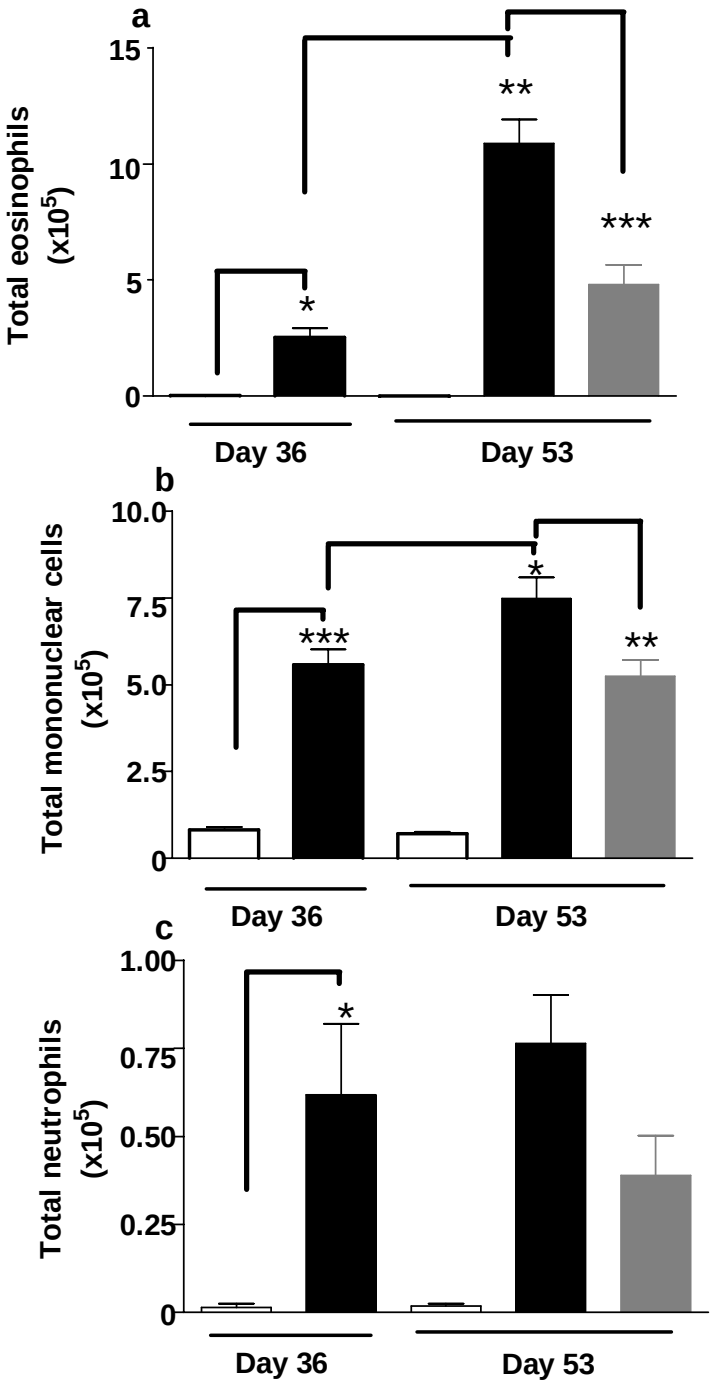
a
Total eosinophils (x10^5)
15
10
5
0
*
**
***
Day 36
Day 53
b
Total mononuclear cells (x10^5)
10.0
7.5
5.0
2.5
0
***
*
**
Day 36
Day 53
c
Total neutrophils (x10^5)
1.00
0.75
0.50
0.25
0
*
Day 36
Day 53

Figure 3

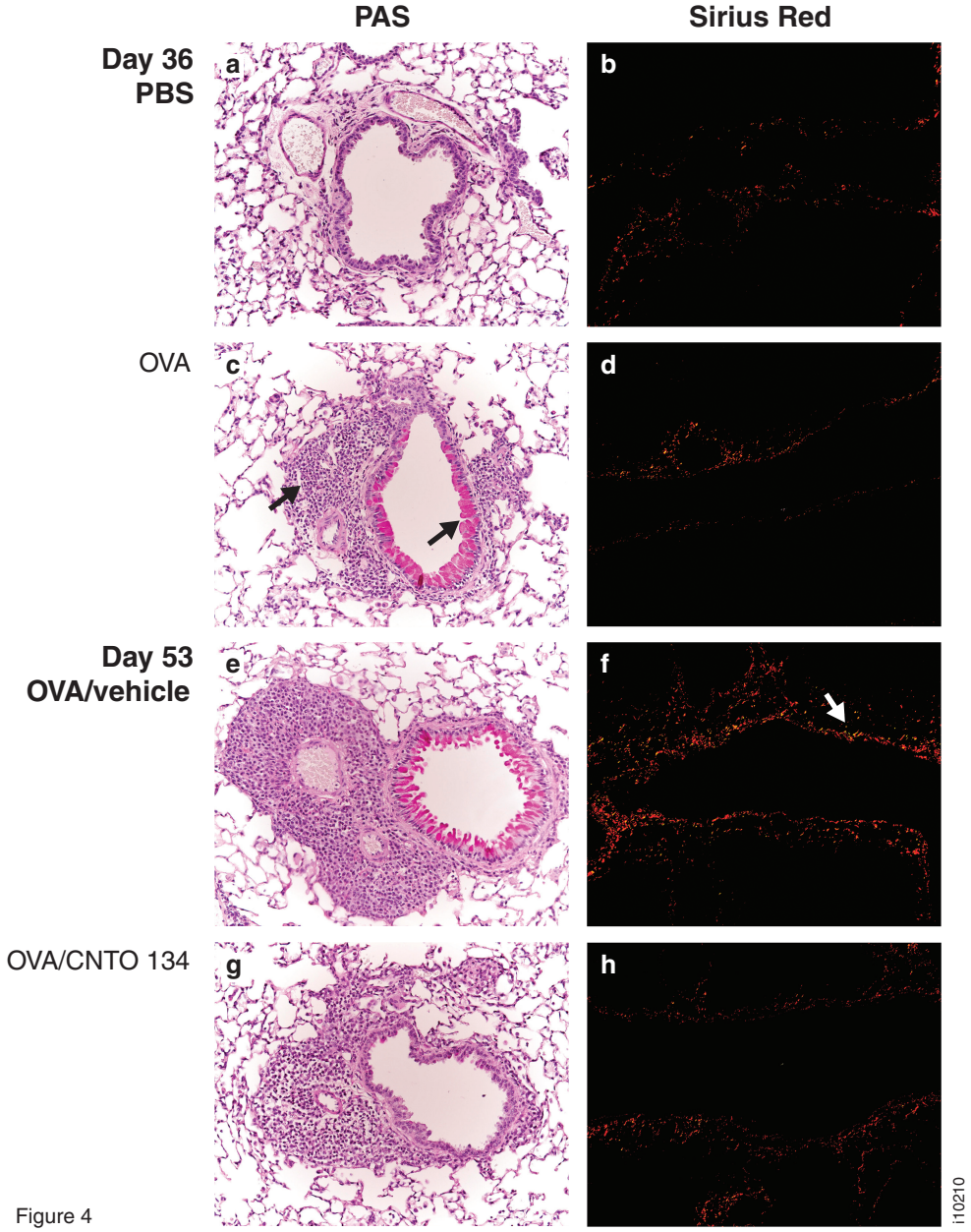
PAS
Sirius Red
Day 36
PBS
a
b
OVA
c
d
Day 53
OVA/vehicle
e
f
OVA/CNTO 134
g
h
i10210

Figure 4

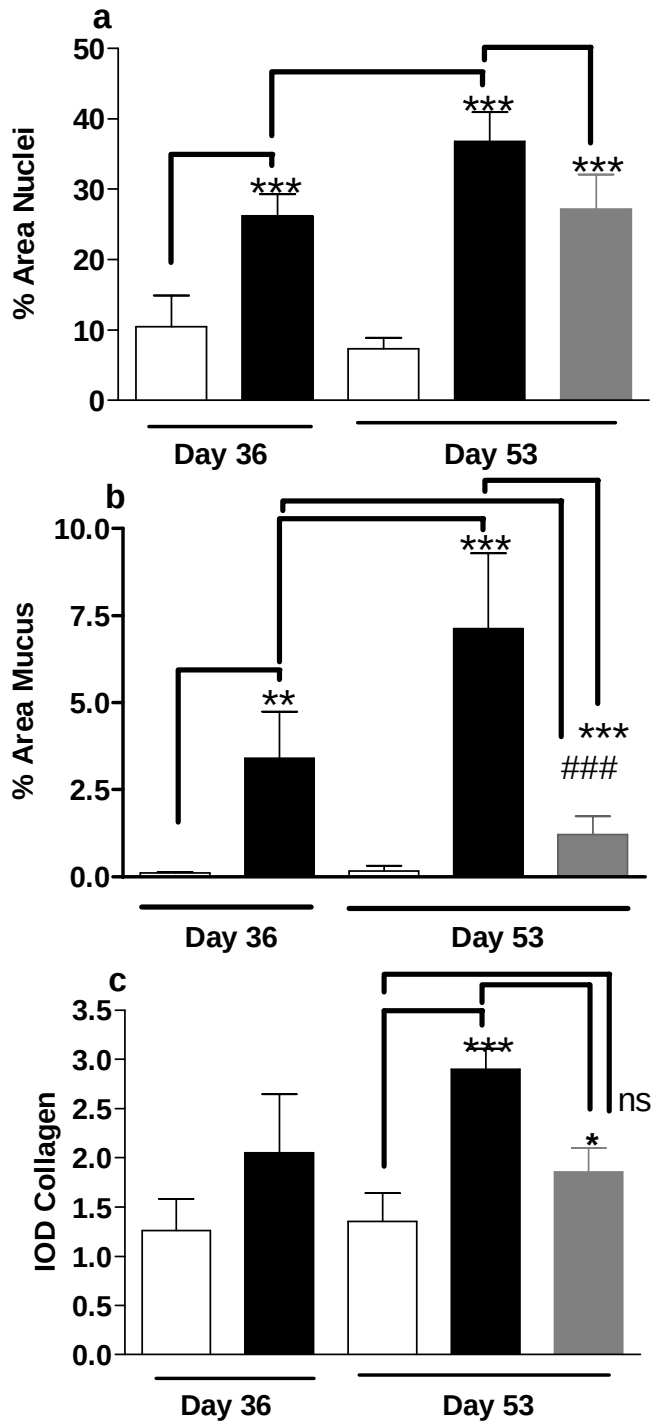
a
% Area Nuclei
50
40
30
20
10
0
***
***
***
Day 36
Day 53
b
% Area Mucus
10.0
7.5
5.0
2.5
0.0
**
***
***
###
Day 36
Day 53
c
IOD Collagen
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
ns
*
Day 36
Day 53

Figure 5

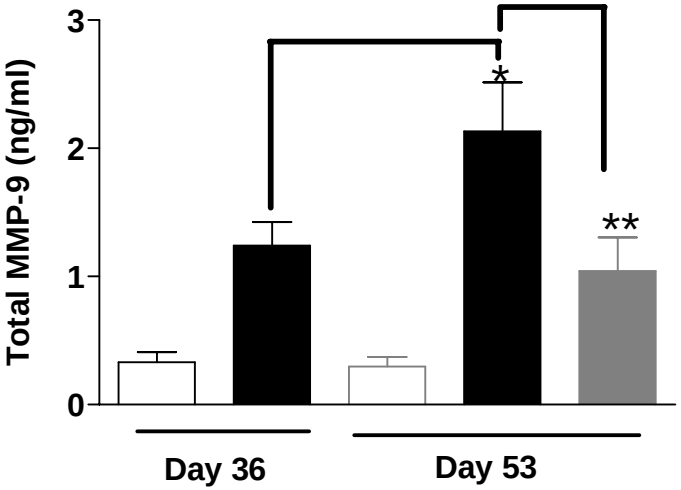

Figure 6