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Nebulized anti-IL-13 monoclonal antibody Fab' fragment reduces allergen-induced asthma

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Authors' contributions

JH and MG carried out experiments, participated in the experimental design and in the interpretation of data and write the manuscript. KT and RP provided the monoclonal antibody (mAb) Fab' fragment (CA154_582) and revised the manuscript critically. LM, GP and NR did take part to bench work. JMF initiated the project and revised the manuscript. AN did take part to study design and revised the manuscript critically. DC initiated and supervised the

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whole project, was responsible to find grants, participated in the design of the study, reviewed results and reviewed and finalized the manuscript.

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Abstract

Rationale: Interleukin-13 (IL-13) is a prototypic T_h2 cytokine and a central mediator of the complex cascade of events leading to asthmatic phenotype. Indeed, IL-13 plays key roles in IgE synthesis, bronchial hyperresponsiveness, mucus hypersecretion, subepithelial fibrosis and eosinophil infiltration.

Objectives: We assessed the potential efficacy of inhaled anti-IL-13 monoclonal antibody Fab' fragment on allergen-induced airway inflammation, hyperresponsiveness and remodeling in an experimental model of allergic asthma. Anti-IL-13 Fab' was administered to mice as a liquid aerosol generated by *inExpose*® inhalation system in a tower allowing a nose-only exposure.

Methods: BALB/c mice were treated by PBS, anti-IL-13 Fab' or A33 Fab' fragment and subjected to ovalbumin (OVA) exposure for 1 and 5 weeks (short term (ST) and long term (LT) protocols).

Measurements and Main Results: Our data demonstrate a significant anti-asthma effect following nebulization of anti-IL-13 Fab' in a model of asthma driven by allergen exposure as compared to saline and non-immune Fab fragments. In short and long terms protocols, administration of the anti-IL-13 Fab' by inhalation significantly decreased bronchial responsiveness to methacholine, BALF eosinophilia, inflammatory cell infiltration in lung tissue, and many features of airway remodeling. Levels of pro-inflammatory mediators and matrix metalloprotease levels were significantly lower in lung parenchyma of mice treated with anti-IL-13 Fab'.

Conclusions: These data demonstrate that an inhaled anti-IL-13 Fab' significantly reduces airway inflammation, hyperresponsiveness and remodeling. Specific neutralization of IL-13

in the lungs using an inhaled anti-IL-13 Fab' could represent a novel and effective therapy for the treatment of asthma.

Keywords: Inflammation, inhalation, mouse model, pulmonary disease, therapy

Introduction

Asthma is an inflammatory disease of the airways of increasing prevalence characterized by typical symptoms and disordered airway physiology on a background of airway inflammation and remodeling (1). There are crucial unmet clinical needs in asthma care since up to 10% of asthmatic patients are refractory to current therapy. The inflammatory process underlying asthma is coordinated by a complex cytokine network (2). Designing drugs able to interfere with this network represents a new potential strategy for asthma treatment (3).

IL-13 is produced by T cells subtypes including $CD4^+$ T_h cells, T_h1 cells, T_c2 cells, and invariant NKT cells, but also mast cells, basophils and eosinophils (4). Recent results indicate that alternatively activated macrophages can also become a major source of IL-13 production (5). During helminthic infection, nuocytes, recently described as new innate helper cells, represent a predominant source of IL-13 (6).

IL-13 binds to a heterodimeric receptor complex constituted by the IL-4 receptor α-chain (IL-4Rα) and a specific IL-13–binding chain (IL-13Rα1), mediating the biological effects via the activation of the signal transducer and activator of transcription factor 6, STAT6 (7). IL-13 also binds to a second receptor (IL-13Rα2) playing a crucial role in pathways leading to IL-13 clearance. Indeed, it has recently been reported that cell surface IL-13Rα2 acts as a scavenger for IL-13 therefore playing a crucial role in IL-13 clearance pathway (8). IL-13 also promotes airway hyperresponsiveness (AHR) as demonstrated in mice (9, 10) and drives many of the structural changes reported in chronic asthma, including goblet cell hyperplasia, airway smooth muscle proliferation, and subepithelial fibrosis (11, 12). There is therefore much evidences to attribute a central role in asthma pathology to IL-13 rendering IL-13 to be considered as a suitable therapeutic target for the treatment of asthma (13, 14). Hence IL-13-specific blocking agents appear to be more promising than previous therapies designed to

target other cytokines (e.g. IL-4 and IL-5), as they exhibit more sustained anti-inflammatory effects in vivo (15). Applying an asthma model to IL-13 knock-out mice led to the conclusion that deletion of IL-13 prevents the development of airway hyperresponsiveness after allergen challenge, despite a sustained eosinophilic response (16). Moreover, multiple studies using monoclonal antibodies neutralizing IL-13 given systemically significantly in mouse models of asthma suppressed airway hyperresponsiveness, eosinophilic infiltration, pro-inflammatory cytokine/chemokine production, serum IgE and airway remodeling (17, 18). Very recently, the efficacy of a new systemically administered monoclonal antibody to IL-13, lebrikizumab, to improve lung function has been demonstrated in humans (19). In the recent study of Corren et al., lebrikizumab or placebo was given to humans subcutaneously once a month for a total of 6 months. Targeted pulmonary drug administration requires direct delivery of drug formulations into the lower pulmonary tract and alveoli of the lung in the form of inhaled particles or droplets, providing a distinct advantage over other methods for the treatment of respiratory diseases. Indeed, inhaled drug can be delivered directly to the site of inflammation, thus reducing the need for systemic exposure and the probability of adverse events. In this study, we have assessed the potential efficacy of a murinised anti-IL-13 monoclonal antibody fragment, CA154_582, administered for the first time by inhalation, in two mouse models of allergic asthma in order to study inflammation and airway hyperresponsiveness as well as bronchial remodeling.

Methods

Antibody generation

The purified recombinant Fab' fragment of a high affinity murinised anti-mouse IL-13 mAb (CA154_582) was provided by UCB (Slough, UK). Generation and characterization of the parent mAb has previously been reported by Berry at al. (20). CA154_582 binds IL-13 such that the interaction between IL-13 and IL-13Rα1 and the interaction between IL-13 and IL-13Rα2 is prevented. A control non-immune Fab antibody was included in our study to demonstrate the specific effect of the monoclonal antibody Fab' fragment (CA154_582). We used as control an A33 Fab' fragment purified by UCB (UCB Celltech, Slough, UK).

Animals and study design

All protocols were conducted in accordance with the guidelines of the University of Liege Ethical Committee. We designed two distinct murine models of allergic airways inflammation: the acute (short term) and chronic (long term) OVA sensitization and exposure models, which mimic bronchial allergic inflammation, hyperresponsiveness and airway remodeling, respectively. Male BALB/c mice of 6 to 8 weeks of age were sensitized to OVA (10 μg) (Sigma Aldrich, Germany) by intraperitoneal injection with $Al(OH)_3$ (Perbio, Belgium). Sensitized mice were randomly assigned to a group receiving PBS (placebo group), a group receiving anti-IL-13 Fab' or a group receiving A33 Fab' fragment. Anti-IL-13 Fab', A33 Fab' fragment and placebo were nebulized by the *inExpose*® system from days 18 to 25 or 21 to 52 as described in figure 1. A group of mice receiving PBS, anti-mouse IL-13 Fab' fragment or A33 Fab' fragment by intravenous injection at 10mg/kg was also used. Each mouse was then subjected to the inhalation of 10% OVA for 5 consecutive days (d21-d25) in

the short-term exposure protocol or for 3 periods of 5 days on alternate weeks from day 24 in the long term exposure protocol (figure 1).

Administration of PBS, anti-mouse IL-13 Fab fragment or control A33 Fab' fragment by the inExpose® system

The *inExpose*® (SCIREQ Scientific Respiratory Equipment Inc, Canada) system is a nose-only inhalation exposure system that significantly reduces the quantity of substances to be used for animal exposure and prevents any oral absorption from mice licking their fur. Additional information was described on Online Data Supplement.

Determination of airway reactivity

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (10 mg/ml, Merial, Belgium) and xylazine (1 mg/ml, VMD, Belgium). Mice were intubated and ventilated with a *flexiVent* small animal ventilator® (SCIREQ, Canada) as previously described (22). The mean airway resistance after methacholine exposure was the primary parameter measured during the challenge.

Bronchoalveolar lavage (BAL)

A bronchoalveolar lavage was performed as previously described (23). The differential cell counts were performed using morphologic criteria on cytocentrifuged preparations (Cytospin) after staining with Diff-Quick (Dade, Belgium). A skilled observer blinded to the experimental treatment performed differential cell counts.

Statistical analysis

All results were expressed as mean ± S.E.M. and the comparison between the groups was performed using non-parametric Kruskal–Wallis test (GRAPHPAD INSTAT).

Results

*Allergen-induced airway hyperresponsiveness is attenuated by anti-mouse IL-13 **Fab'** treatment*

In order to determine whether anti-mouse IL-13 Fab' treatment was able to decrease airway hyperresponsiveness when administered either by inhalation or IV, airway resistance was measured by *flexiVent*® in OVA-sensitized and -challenged mice treated by nebulization with *inExpose*® system (figure 2A) or intravenous injection (figure 2B) of anti-mouse IL-13 Fab',PBS (placebo group) or A33 Fab' fragment in the short-term protocol as described in material and methods section. When compared to placebo-treated mice, the decrease of airway resistance upon anti-mouse IL-13 Fab' treatment was significant from the lowest dose of MCh used (3g/l) to the highest dose (12g/l) in both IV-treated and nebulization-treated groups (*:$p<0.05$). Administration of A33 Fab' fragment (black discontinuous line) by the *inExpose*® system (INH) (figure 2A) or by intravenous injection (IV) (figure 2B) did not significantly modulate the bronchial reactivity to increasing doses of methacholine when compared to mice exposed to PBS (placebo, black continuous line).

Study of bronchoalveolar lavage fluid (BALF) and lung tissue inflammation

In order to determine whether anti-mouse IL-13 Fab' administration by inhalation has an influence on inflammatory cell recruitment, cells in BALF were counted (table 2 and table 3). When compared to placebo-treated mice, eosinophil percentages were significantly decreased in the groups of mice receiving the anti-mouse IL-13 Fab' by inhalation through *inExpose*® system (INH) in both long-term and short-term protocols (*: $p<0.01$). By contrast, when anti-mouse IL-13 Fab' was administered by intravenous injection (IV), the decrease in eosinophil percentage was observed only in the short-term exposure protocol (*: $p<0.01$). The percentages of neutrophils were significantly decreased after anti-mouse IL-13 Fab' by

inhalation in the long term exposure protocol (†: $p<0.05$) when the percentage of macrophages was decreased after Fab' administration (*: $p<0.01$). Administration of A33 Fab' fragment by inhalation using the *inExpose*® system (INH) or by intravenous injection (IV) did not induce modulations of the cellular composition of the bronchoalveolar lavage fluid when compared to the placebo group. Histological analysis was performed on lung from mice exposed to placebo, A33 Fab' fragment or anti-IL-13 administered by INH or IV (figure 3A-R). Peribronchial tissue infiltration by inflammatory cells was analyzed in lung tissue sections stained with haematoxylin-eosin (figure 3A-F). The peribronchial inflammation score (see material and methods section) was significantly decreased after administration of the anti-mouse IL-13 Fab' by both inhalation and intravenous injection in short and long-term protocols (figure 3S-T). As lung eosinophilic inflammation is a hallmark of asthma, we examined peribronchial eosinophil numbers in lung tissue sections stained with Congo Red (figure 3G-L). The number of eosinophils was significantly decreased after Fab' administration, by inhalation or IV, in the two protocols used (figure U-V). Number of mast cells per field, specifically detected by Toluidine Blue staining (figure 3M-R), was also significantly decreased after exposure to anti-mouse IL-13 Fab' by either route of administration in the short term protocol (figure 3W-X). In addition, inhalation of anti-IL-13 Fab' reduced the number of mast cells following long-term allergen exposure. The administration of A33 Fab' Fragment during a short period by INH or IV did not significantly modulate the peribronchial inflammation score, the number of eosinophils and mast cells in lung tissue when compared to placebo exposed mice (figure 3S, V and W).

*Effects of anti-mouse IL-13 **Fab'** administration on allergen-induced airway remodeling*

Chronic allergen exposure in mice promotes the development of a bronchial remodeling. Ovalbumin challenge for 5 weeks in placebo-treated mice induced a significant increase in the number of mucus producing cells detected by Alcian Blue staining (figure 4A-D). Anti-mouse

IL-13 Fab' administered to mice by inhalation or IV induced a significant decrease in the percentage of mucus producing cells in the epithelium (figure 4E). The peribronchial area occupied by collagen (figure 4F-I) was significantly lower in anti-mouse IL-13 Fab' treated mice (figure 4J). Smooth muscle cell layers were measured by immunohistochemistry performed with an antibody raised against α-smooth muscle actin (figure 4K-N). Anti-mouse IL-13 Fab' administrated by the *inExpose®* system induced a significant decrease of the peribronchial smooth muscle cell layer. However, IV administration of anti-mouse IL-13 Fab' failed to do so (figure 4O).

Measurement of serum IgE levels

Measurement of total IgE levels was performed by ELISA in order to verify that mice were adequately immunized and to evaluate any potential influence of the anti-mouse IL-13 Fab' administrated with *inExpose®* system on the IgE production process. Our results show that Fab' administration did not modulate total IgE levels either in the short-term exposure protocol or in the long-term exposure protocol when compared to control mice (Figure E1).

*Effects of anti-mouse IL-13 **Fab'** administration on cytokine and chemokine levels*

Levels of IL-13 in protein extracts were determined in crushed lung tissue. As expected, IL-13 levels were decreased upon treatment with anti-IL-13 Fab' fragment in both protocols when compared to OVA-challenged mice exposed to placebo (figure 5A). IL-4 levels were decreased in anti-mouse IL-13 Fab'-treated mice when compared to control mice but this decrease failed to reach statistical significance in the long-term exposure protocol (figure 5B). IL-5 levels were significantly decreased in both short-term and long-term exposure protocols (figure 5C) since IL-17 levels were not significantly modulated following the administration of the anti-mouse IL-13 Fab' (figure 5D). Anti-mouse IL-13 Fab' treatment also inhibited the

expression of CCL11, a chemokine involved in recruiting eosinophils in both short-term and long-term exposure protocols (figure 5E).

*Anti-mouse IL-13 **Fab'** effects on matrix metalloproteases*

As MMP-2 and MMP-9 have been described to play a crucial role in asthma, levels of expression for these proteases as well as their natural inhibitor, TIMP-1 were measured by RT-PCR analysis. A zymography study was performed to detect MMP-9 and MMP-2 proteins in pro and activated form in crushed lung protein extracts (figure 6A). Quantitative analysis reveals that anti-mouse IL-13 Fab' given by inhalation using the *inExpose*® system (INH) significantly decreased levels of MMP-2 and MMP-9 in the short-term and in the long-term exposure protocols (figure 6B).

*Effect of anti-mouse IL-13 **Fab'** on transcription factor STAT6 expression*

As the transcription factor STAT6 is activated downstream of IL-13 receptor activation and as the phosphorylation will more accurately illustrate activation of STAT6, we measured both the levels of total STAT6 and phospho-STAT6 by western blot. When treated with anti-mouse IL-13 Fab' trough the *inExpose*® system (INH), allergen-exposed mice displayed a marked decrease both total STAT6 and phospho-STAT6 expression in lung parenchyma as compared to placebo-treated controls (figure 7AB and C).

Discussion

In the current study, we demonstrate that IL-13-specific neutralization using an inhaled high affinity monoclonal antibody (mAb) Fab' fragment may be an effective approach for the treatment of asthma. To the best of our knowledge, this is the first demonstration of the efficacy of an anti-IL-13 antibody fragment administered by nebulization in animals. Our results show that anti-IL-13 Fab' administration by inhalation effectively prevents the development of airway hyperresponsiveness, inflammation and bronchial remodeling associated with experimental asthma and caused by acute or repeated allergen exposure. Indeed, the effectiveness of anti-IL-13 has been assessed in two distinct protocols with different allergen exposure durations therefore mimicking different clinically relevant features of asthma pathology. In this study, we observed significantly decreased bronchial hyperresponsiveness after treatment with inhaled anti-IL-13. The presence of inflammatory cells, and especially eosinophils, was decreased both in BALF and in lung tissue and different features of airway remodeling were also significantly reduced after anti-IL-13 Fab' administration by inhalation. Modulation of key mediators such as IL-13, IL-5, IL-4, CCL11, matrix metalloproteinases (MMP-2 and MMP-9), STAT6 and phospho-STAT6 levels were also decreased after anti-IL-13 Fab' exposure. In our preliminary experiments, we showed that the anti-IL-13 monoclonal antibody Fab' fragment, when administrated by inhalation, did not induce toxicity in mice not exposed to allergens as assessed by clinical assessment and lung histology (data not shown). We also demonstrate in the present study that topical application of this antibody fragment in inflamed lungs is safe and does not induce any toxicity in a murine model of asthma. In order to demonstrate the specific effect of the monoclonal anti-IL-13 antibody Fab' fragment, we used an A33 Fab' fragment provided by UCB. A33 Fab' fragment was administered both by inhalation and intravenous injection following the short-term exposure protocol described in the manuscript. Results of the

measurement of bronchial hyperresponsiveness, cellular composition of the BALF, peribronchial inflammation score and number of eosinophils and mast cells in lung tissue were presented in this manuscript.

Previous authors have presented therapeutic inhibition of T_h2 cytokines, including anti-IL-13 antibody administered by intraperitoneal injections (25) or by subcutaneous administration in humans (26). Although anti-IL-13 suppressed eosinophil recruitment and partially reduced airway remodeling, this treatment displayed limited ability to inhibit airway hyperreactivity in mice (25). More recently, Tomlinson et al. reported that neutralization of IL-13 in mice, following the subcutaneous administration an anti-IL-13 mAb, prevents airway pathology caused by chronic exposure to house dust mite (27). The safety of intravenous administration of a human anti-IL-13 monoclonal antibody was also studied in a model of allergic asthma in macaques and demonstrates that the anti-IL-13 mAb was well tolerated in both normal and allergic macaques and decreased significantly BAL and serum eotaxin concentrations (28). Recently, it was described that a human anti-IL-13 mAb given by intravenous injection or by sub-cutaneous administration displays an acceptable safety profile and can be administered safely in patients with asthma (29, 30). Furthermore, lebrikizumab, an anti-IL-13 mAb administered subcutaneously significantly improved lung function in patients with moderate-severe asthma who had elevated serum periostin at baseline (26). Relate to Kasaian, study and highlight inhalation of an anti-IL-13 entity with a short plasma half-life prevents lung pathology without blocking Il-13 systemically, thus would not impede endogenous mechanisms of IL-13 clearance (8). Whether in humans or mouse, majority of monoclonal antibody actually develop and use for the treatment of asthma was administered either by intravenous injection or by subcutaneous injection. To the best of our knowledge, this study is the first demonstration of the efficacy of a high affinity monoclonal antibody (mAb) fragment

administered by inhalation in animals. This topic way of administration was never used in humans for the administration of antibody in the treatment of asthma.

We report that key T_h2 cytokines (IL-4, IL-5, IL-13) as well as eosinophil chemoattractants (CCL11) are significantly decreased upon anti-IL-13 treatment. These results indicate that targeting IL-13 specifically in the lung tissue profoundly modifies the inflammatory reaction and decreases not only IL-13 levels but also other inflammatory triggers that normally participate in the cascade of events leading to an established asthma phenotype suggesting that IL-13 biological effects are upstream of the other T_h2 cytokines in vivo. IL-17 is also a pivotal cytokine and acts on a variety of cells, which respond by overexpressing proinflammatory cytokines, chemokines, and metalloproteases (4). Previous authors also reported that depleting IL-13 leads to an increase in IL-17 levels (31). However, in our experimental setting, we were not able to show any significant difference between the groups regarding IL-17 levels.

In our proof of concept study in animals, the IV administration route was also investigated for the Fab' fragment. In the long term protocol, anti-IL-13 mAb Fab' administered by inhalation through the *inExpose*® system induced a significant decrease of the peribronchial smooth muscle cell layer and of the mast cells number in lung tissue, but the IV injection of Fab' failed to do so. This difference might be explained by some discrepancies regarding the half-life of the antibody fragment depending on the method of administration suggesting a longer half-life in the bronchial wall after inhalation as compared to IV injection. In previous studies, it was indeed measured that the anti-IL-13 Fab' fragment displays a half-life of 4 hours in the systemic circulation. We hypothesize that such a half-life should be more important in the lungs where clearance mechanisms are less efficient than the vascular reticulo-endothelial system. Future studies will measure the levels and pharmacokinetic aspects of Fab' present in the airway tissue.

In our experimental study, treatment of mice with anti-mouse IL-13 Fab' led to markedly decreased STAT6 and phospho-STAT6 levels as compared to placebo. As STAT6 is a key component of cell activation following stimulation of the IL-4/IL-13 receptor, biological compounds targeting these molecules also appear as potentially interesting therapies and indeed some compounds targeting this pathway currently under development (32). Interestingly, we also demonstrate in our both experimental protocols that infiltration of mast cells in lung tissue was decreased following anti-IL-13 Fab' exposure. This could indicate that mast cells, which are key players of allergic reaction, are sensitive to activation by IL-13 (33) and are therefore a target for anti-IL-13 therapeutics.

In conclusion, our data in a murine model of allergic lung inflammation and dysfunction indicate that an inhaled anti-IL-13 Fab' fragment may be a useful therapeutic for the treatment of asthma. The molecular mechanisms leading to decreased inflammation and airway hyperresponsiveness after IL-13 inhibition are complex, and further studies are needed to decipher the changes induced in the asthma-related cascade of interactions between inflammatory and structural cells and their control by cytokine networks.

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Figure legends

Figure 1: Experimental design of sensitization (by intraperitoneal injection) and allergen (OVA) exposure (by inhalation) in the short-term (26 days) and the long-term (53 days) exposure protocols.

Figure 2: Effects of anti-mouse IL-13 Fab' and A33 Fab' fragment on airway resistance following administration of increasing doses of methacholine (MCh) by inhalation as assessed by *flexiVent*® system. Results are represented for OVA-sensitized and -challenged mice treated with PBS (placebo, black continuous line), A33 Fab' fragment (black discontinuous line) or anti-mouse IL-13 Fab' (grey discontinuous line) administrated by inhalation (INH) (A) or intravenous injection (B). *: $p<0.05$ when compared to placebo group receiving PBS or A33 Fab' fragment.

Figure 3: Lung sections stained with haematoxylin-eosin (magnification 100X) (A-F), Congo Red (magnification 100X) (G-L) and toluidine blue (magnification 400X) (M-R). Quantification of staining and immunohistochemistry were presented in panel S-X. Peribronchial inflammation score following administration of PBS, A33 Fab' fragment or anti-mouse IL-13 Fab' trough *inExpose*® system (INH) or intravenous injection (IV) were presented for short-term (S) and long-term (T) exposure protocols. Quantification of the number of eosinophils normalized to the perimeter of the bronchi after administration of PBS (placebo), A33 Fab' fragment or anti-mouse IL-13 Fab' by inhalation (INH) or intravenous injection (IV) were measured in short-term (U) and long-term protocols (V). Number of mast cells per field of lung tissue section in mice receiving placebo or anti-mouse IL-13 Fab' by inhalation (INH) or intravenous injection (IV) were quantified in short-term (W) and long-term (X) protocols.

Figure 4: Study of the bronchial remodeling in the long-term exposure protocol. Representative pictures of Alcian Blue staining (magnification 400X) (A-D), Masson's Trichrome staining (magnification 100X) (F-I) and α-smooth muscle actin immunohistochemistry (magnification 100X) (K-N) after administration of PBS (placebo) or anti-mouse IL-13 Fab' by inhalation (INH) using the *inExpose®* system or intravenous injection (IV). Quantification of mucous producing cells in the airway epithelium (E), the area occupied by collagen normalized to the area of bronchi in the airways (J) and of the thickness of smooth muscle layer normalized to bronchi circumference (O) were performed in allergen-exposed mice following treatment with placebo or anti-mouse IL-13 Fab' given by inhalation with *inExpose®* system (INH) or intravenous injection (IV).

Figure 5: (A-E) Levels of IL-13, IL-4, IL-5, IL-17 and CCL11 (expressed in pg/mg total protein) measured in lung protein extracts obtained from mice expose to placebo or anti-mouse IL-13 Fab' by inhalation with *inExpose®* system (INH) in short-term and long-term exposure protocols.

Figure 6: Representative blot of MMP-2 and MMP-9 protein activity in experimental groups from short-term and long-term exposure protocols by zymography analysis. Different dilutions (1/2 to 1/16) of culture medium conditioned by HT1080 cells were used as internal standard. (A). Levels of total MMP-2 and MMP-9 were quantified in protein extracts from lung of mice expose to placebo or anti-mouse IL-13 Fab' by inhalation with *inExpose®* system (INH) in short-term and in long-term protocols (B).

Figure 7: Representative results of Western blots performed with an anti-STAT6 and an anti-phospho-STAT6 antibody in group of mice receiving anti-mouse IL-13 Fab' or placebo by inhalation (INH) (A). Results were expressed as a ratio between density of STAT6 bands (94kDa), phospho-STAT6 bands (94kDa) and density of β-actin bands (47kDa) used as

internal control. Levels of STAT6 and phosphor-STAT6 were significantly decreased in anti-mouse IL-13 Fab' treated mice with *inExpose*® system when compared with control mice receiving placebo ($p<0.05$) (B).

Table 1: Route of administration and dose of PBS, A33 Fab' fragment or anti-IL-13 Fab' administered per mouse in the six experimental groups. The total deposited amount of inhaled anti-mouse IL-13 Fab' and A33 Fab'fragment with *InExpose*® system were estimated to 0.0617mg per mouse as estimated by a formula described in Supplement data section.

Groups	Route of administration	Compound	Administered dose/mouse
INH placebo	Inhalation (nose-only aerosol exposure with the inExpose® system)	PBS	250 µl
INH A33 Fab' fragment	Inhalation (nose-only aerosol exposure with the *inExpose*® system)	A33 Fab' fragment	250 µl - 5 mg/ml
INH anti-IL-13	Inhalation (nose-only aerosol exposure with the inExpose® system)	Anti-IL-13 Fab'	250 µl - 5 mg/ml
IV placebo	Intravenous injection	PBS	100 µl
IV A33 Fab' fragment	Intravenous injection	A33 Fab' fragment	100 µl – 2.5 mg/ml
IV anti-IL-13	Intravenous injection	Anti-IL-13 Fab'	100 µl - 2.5 mg/ml

Table 2: Percentage of inflammatory cells of the bronchoalveolar lavage fluid (BALF) in the short-term exposure protocol after receiving placebo or anti-IL-13 Fab' either by inhalation (INH) or intravenous injection (IV).

%	Short-term protocol					
	INH			IV		
	Placebo	Fab' A33	Anti-IL-13	Placebo	Fab' A33	Anti-IL-13
Eosinophils	20.41 ± 2.62	23.51 ± 4.2	10.19± 1.58*	18.18 ± 3.21	22.64 ± 4.24	8.12 ± 1.71 *
Neutrophils	0.36 ± 0.17	0.69 ± 0.54	0.47 ± 0.16	0.48 ± 0.23	0.35 ± 0.41	0.45 ± 0.24
Lymphocytes	0.2 ± 0.15	0.11 ± 0.08	0.1 ± 0.06	0.5 ± 0.3	0.48 ± 0.14	0.11 ± 0.05
Epithelial cells	7.41 ± 0.75	7.81 ± 0.5	6.73 ± 0.72	7.29 ± 0.78	8.13 ± 1.26	6.41 ± 0.62
Macrophages	71.51 ± 2.4	67.7 ± 4.26	81.9± 1.19*	73.44 ± 3.24	68.25 ± 4.11	84.6± 2.11*

* : $p<0.01$ when compared to placebo

Table 3: Percentage of inflammatory cells of the bronchoalveolar lavage fluid (BALF) in the long-term exposure protocol after receiving placebo or anti-IL-13 Fab' either by inhalation (INH) or intravenous injection (IV).

%	Long-term protocol			
	INH		IV	
	Placebo	Anti-IL-13	Placebo	Anti-IL-13
Eosinophils	19.22 ± 2.63	4.76 ± 0.39 *	10.46 ± 1.38	9.55 ± 1.43
Neutrophils	3.13 ± 0.7	1.34 ± 0.34 †	0.55 ± 0.14	0.88 ± 0.19
Lymphocytes	0.42 ± 0.08	0.28 ± 0.09	0.35 ± 0.11	0.49 ± 0.13
Epithelial cells	7.27 ± 0.41	7.12 ± 0.53	6.89 ± 0.6	8.32 ± 0.76
Macrophages	69.78 ± 2.49	86.39 ± 0.75 *	81.59 ± 1.4	80.07 ± 1.48

* : $p<0.01$ when compared to placebo ; †: $p<0.05$ when compared to placebo

Figure 1:

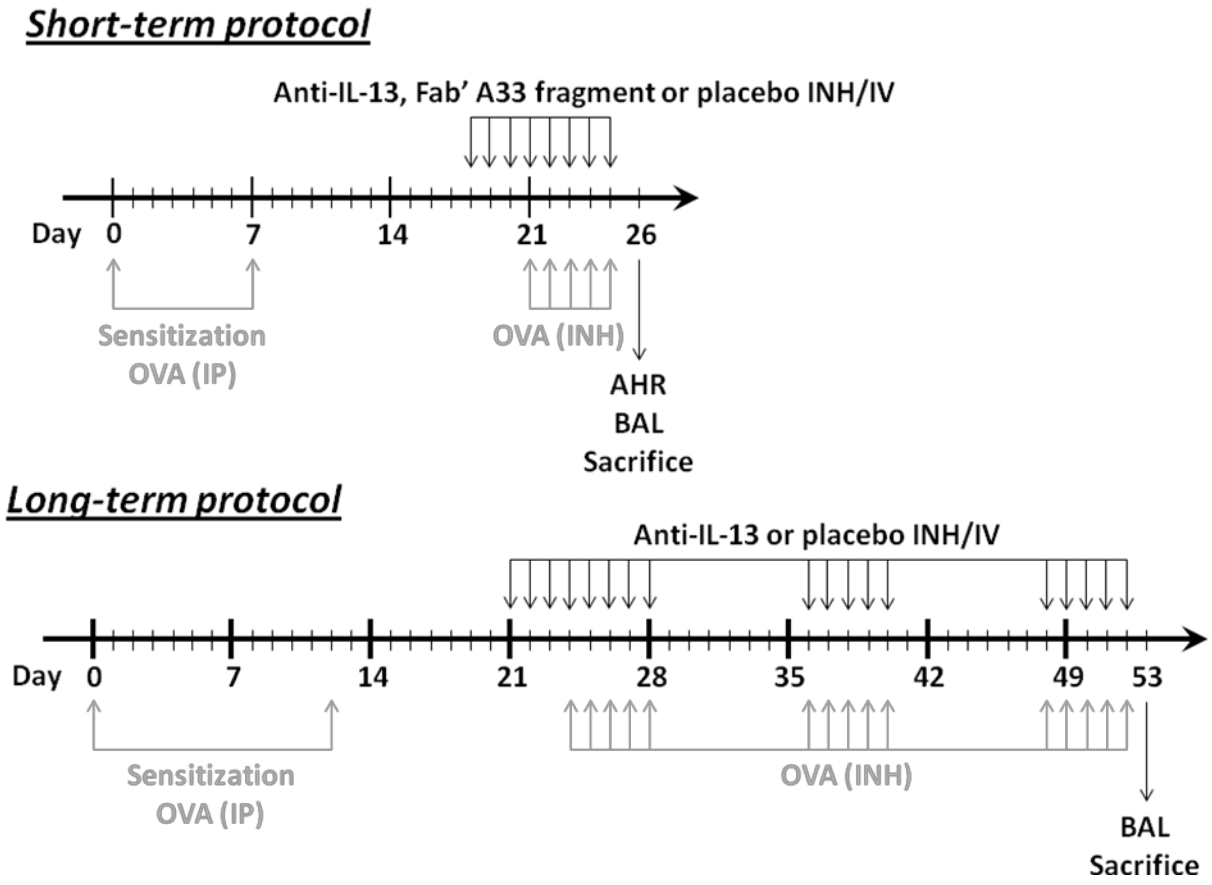
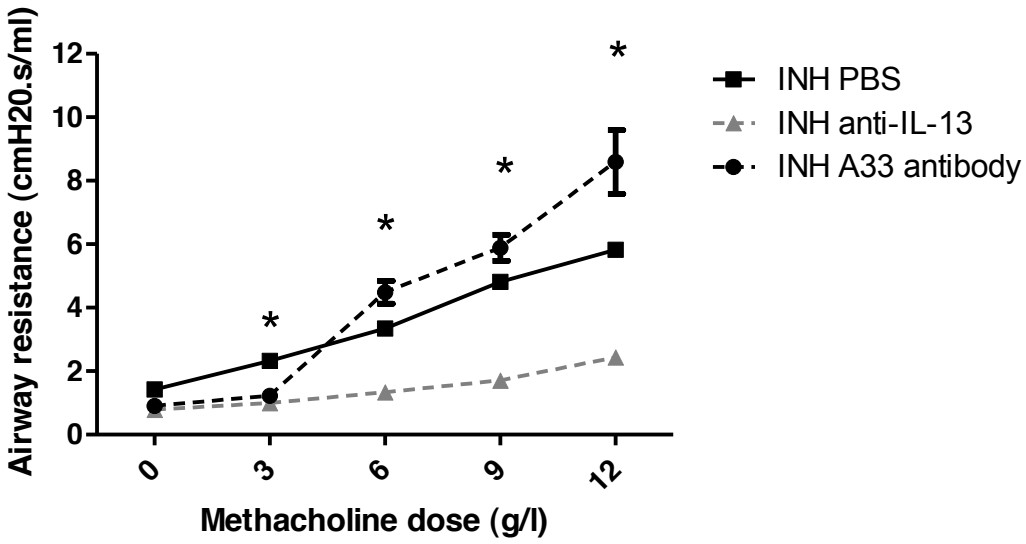

Short-term protocol
Anti-IL-13, Fab' A33 fragment or placebo INH/IV
Day 0
7
14
21
26
Sensitization
OVA (IP)
OVA (INH)
AHR
BAL
Sacrifice
Long-term protocol
Anti-IL-13 or placebo INH/IV
Day 0
7
14
21
28
35
42
49
53
Sensitization
OVA (IP)
OVA (INH)
BAL
Sacrifice

Figure 2:

A


Airway resistance (cmH20.s/ml)
12
10
8
6
4
2
0
0
3
6
9
12
Methacholine dose (g/l)
*
*
*
*
INH PBS
INH anti-IL-13
INH A33 antibody

B

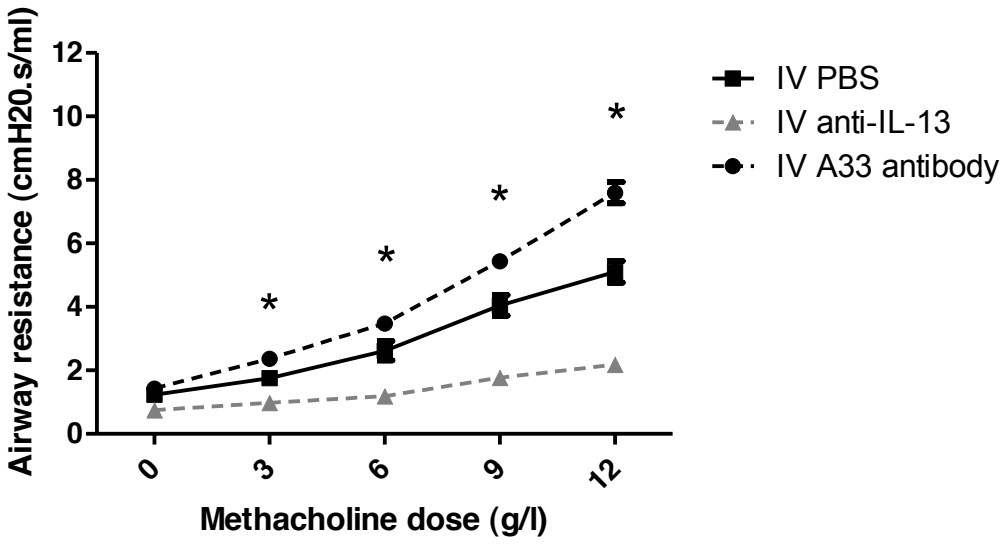
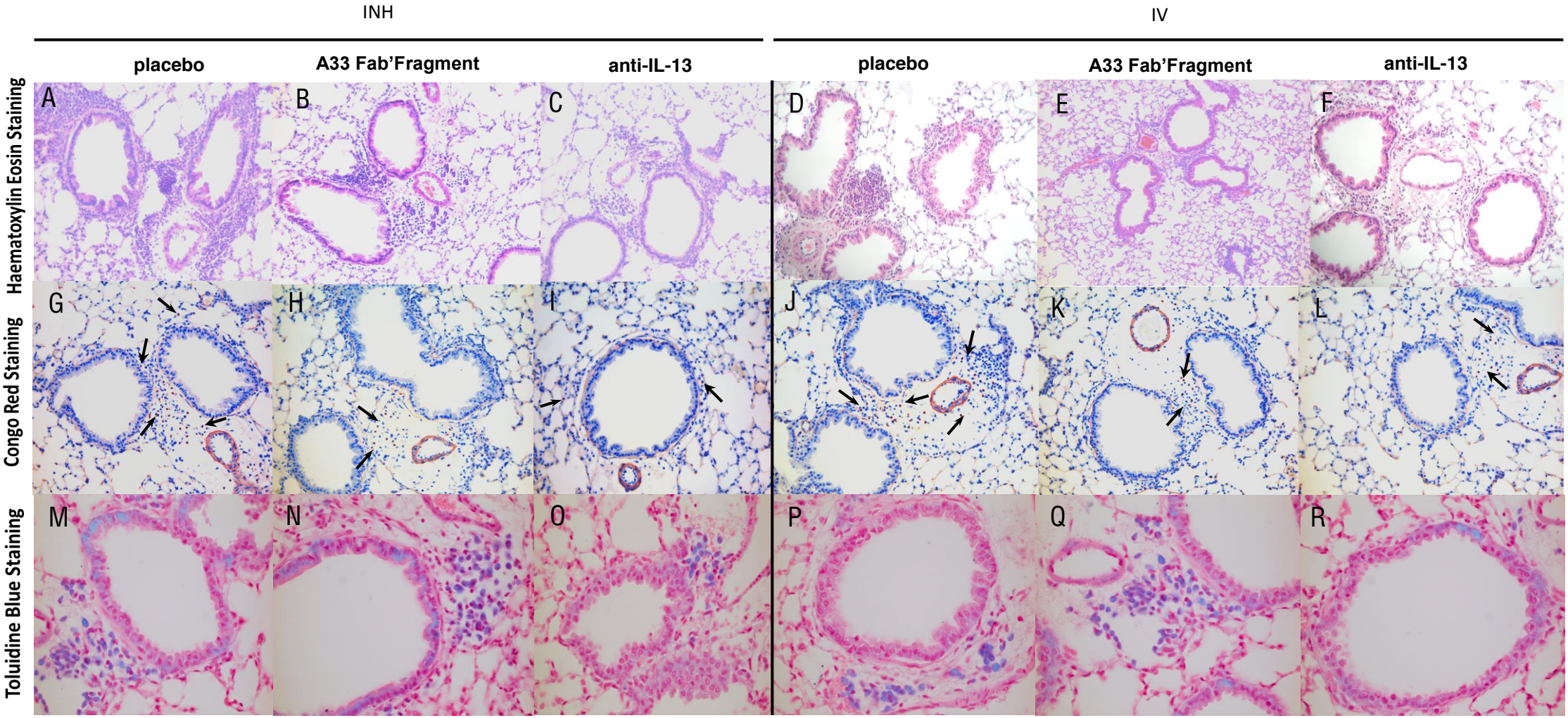

Airway resistance (cmH20.s/ml)
12
10
8
6
4
2
0
0
3
6
9
12
Methacholine dose (g/l)
*
*
*
*
IV PBS
IV anti-IL-13
IV A33 antibody

Figure 3:


INH
IV
placebo
A33 Fab'Fragment
anti-IL-13
placebo
A33 Fab'Fragment
anti-IL-13
Haematoxylin Eosin Staining
Congo Red Staining
Toluidine Blue Staining
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R

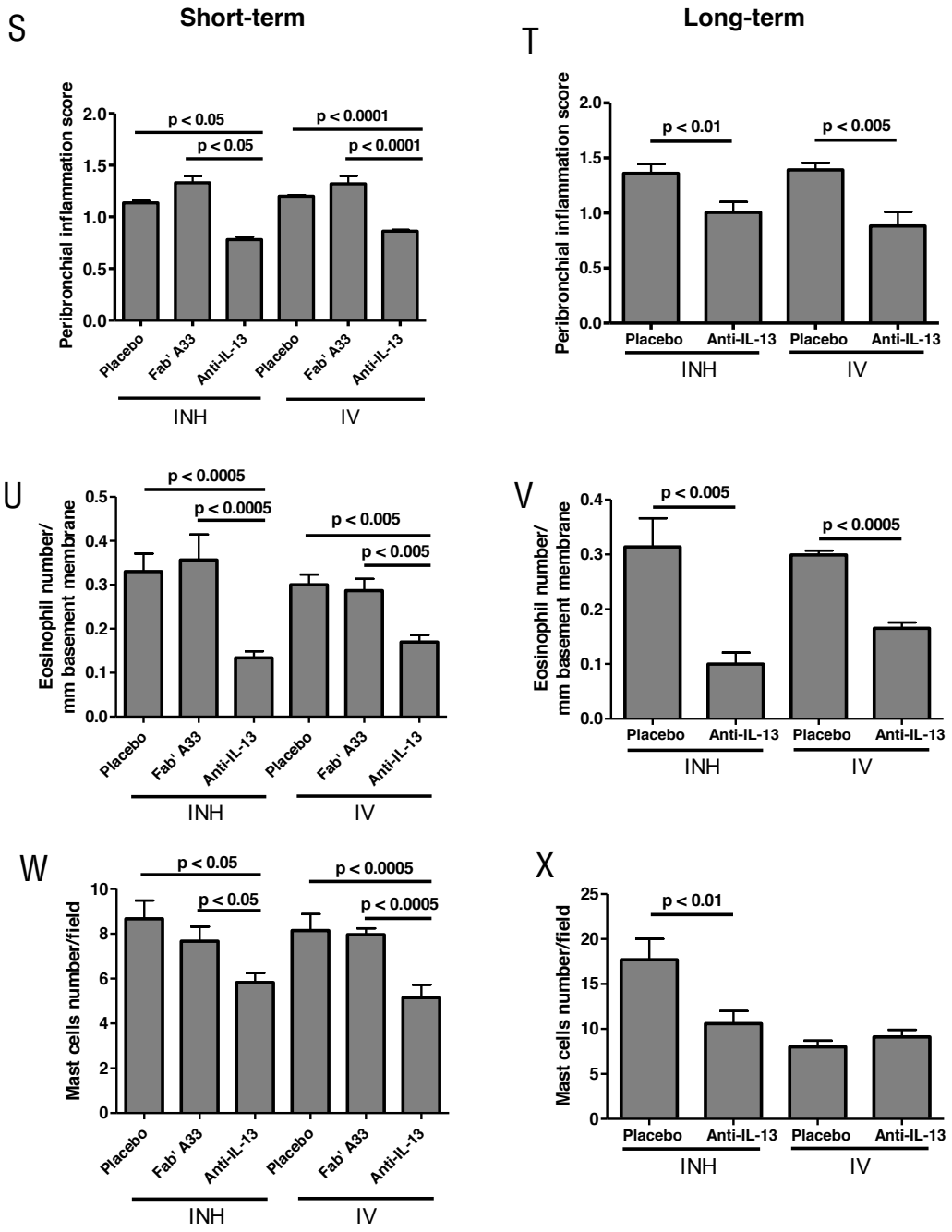

S
Short-term
Peribronchial inflammation score
p < 0.05
p < 0.05
p < 0.0001
p < 0.0001
Placebo
Fab' A33
Anti-IL-13
Placebo
Fab' A33
Anti-IL-13
INH
IV
T
Long-term
Peribronchial inflammation score
p < 0.01
p < 0.005
Placebo
Anti-IL-13
Placebo
Anti-IL-13
INH
IV
U
Eosinophil number/ mm basement membrane
p < 0.0005
p < 0.0005
p < 0.005
p < 0.005
Placebo
Fab' A33
Anti-IL-13
Placebo
Fab' A33
Anti-IL-13
INH
IV
V
Eosinophil number/ mm basement membrane
p < 0.005
p < 0.0005
Placebo
Anti-IL-13
Placebo
Anti-IL-13
INH
IV
W
Mast cells number/field
p < 0.05
p < 0.05
p < 0.0005
p < 0.0005
Placebo
Fab' A33
Anti-IL-13
Placebo
Fab' A33
Anti-IL-13
INH
IV
X
Mast cells number/field
p < 0.01
Placebo
Anti-IL-13
Placebo
Anti-IL-13
INH
IV

Figure 4:

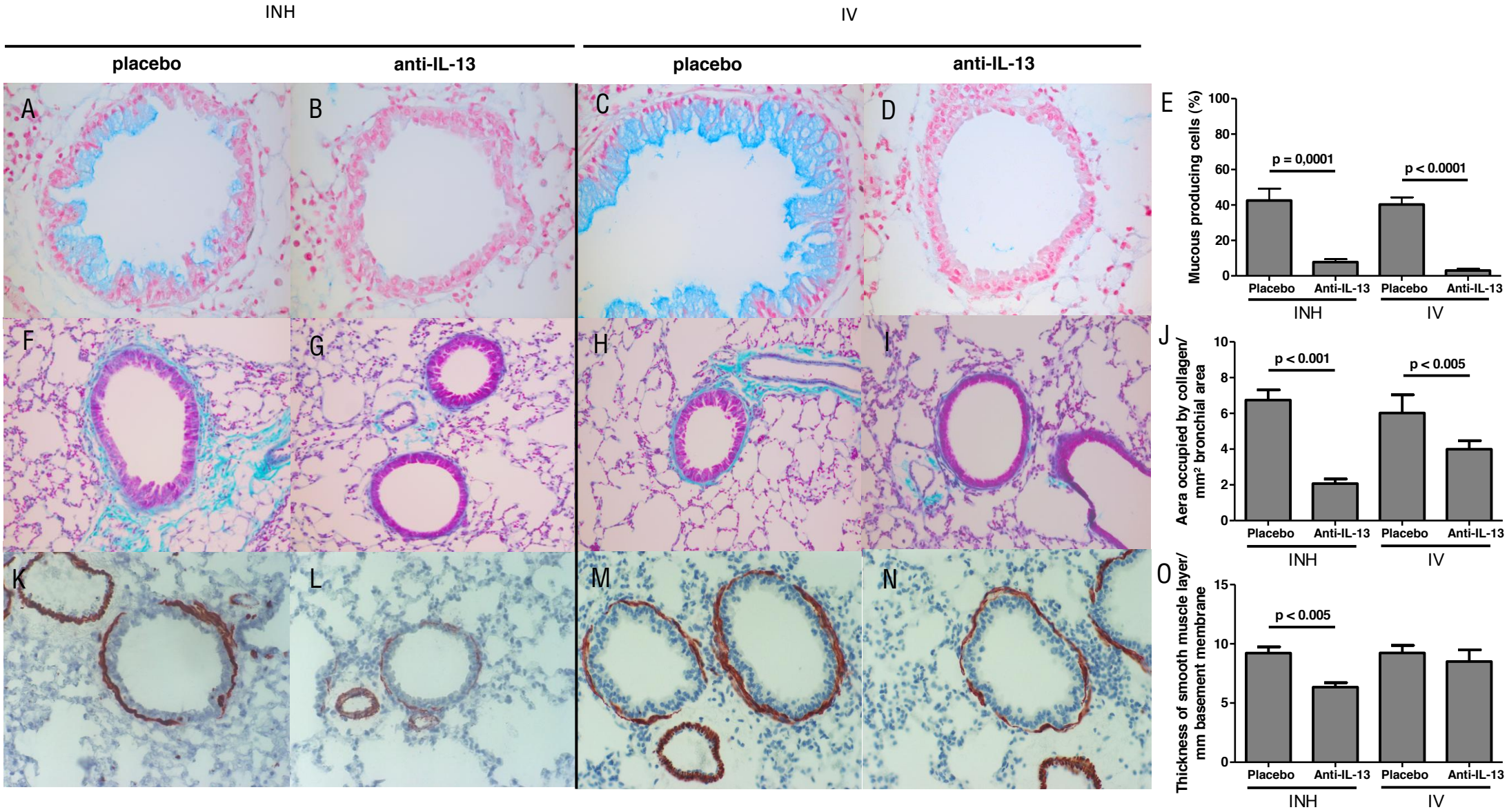

INH
IV
placebo
anti-IL-13
placebo
anti-IL-13
Alcian blue
Masson's Trichrome
α-SMA
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Mucous producing cells (%)
p = 0,0001
p < 0.0001
Placebo
Anti-IL-13
Placebo
Anti-IL-13
INH
IV
Aera occupied by collagen/ mm² bronchial area
p < 0.001
p < 0.005
Thickness of smooth muscle layer/ mm basement membrane
p < 0.005

Figure 5:

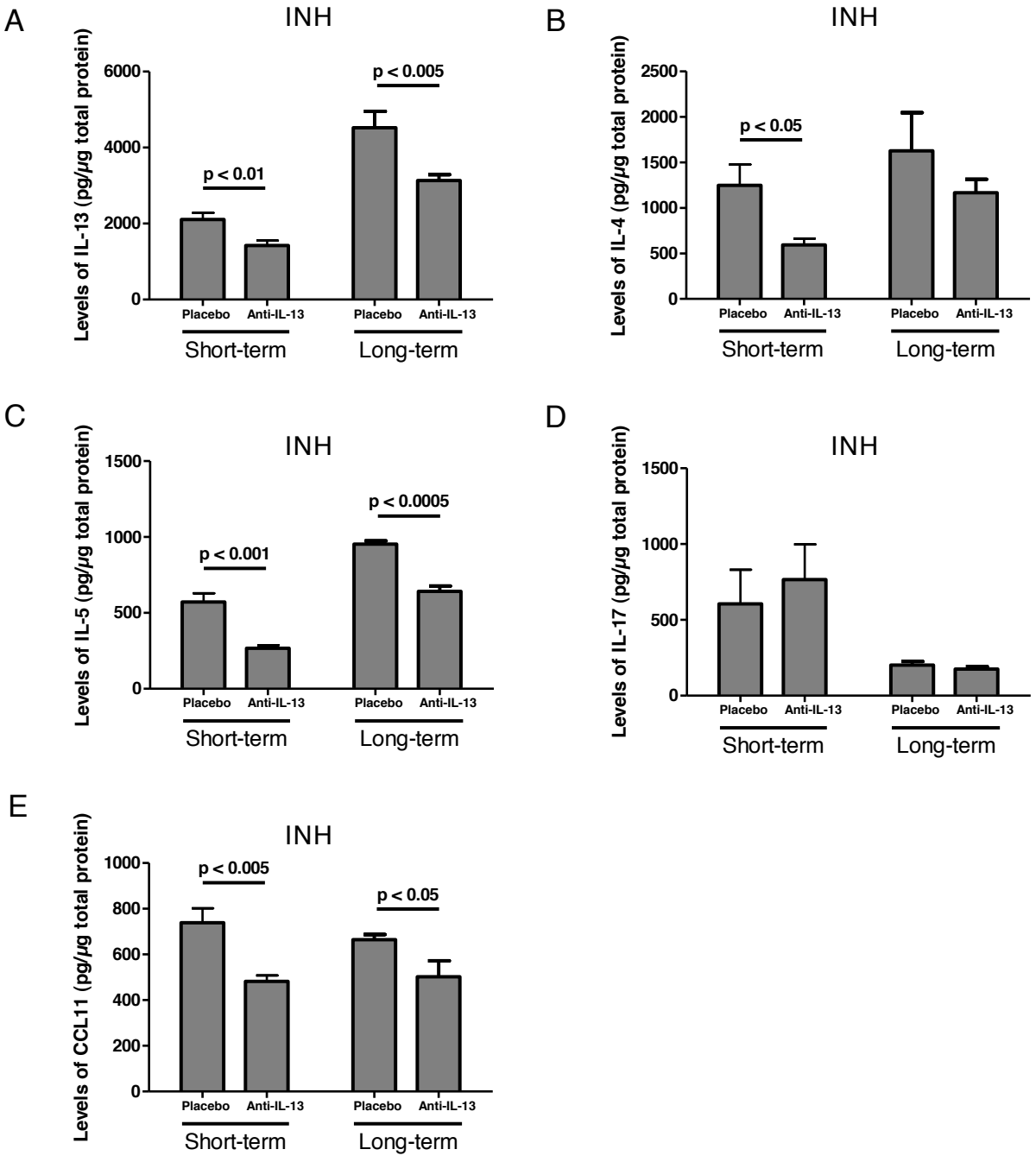

A
INH
Levels of IL-13 (pg/μg total protein)
6000
4000
2000
0
p < 0.01
p < 0.005
Placebo
Anti-IL-13
Placebo
Anti-IL-13
Short-term
Long-term
B
INH
Levels of IL-4 (pg/μg total protein)
2500
2000
1500
1000
500
0
p < 0.05
Placebo
Anti-IL-13
Placebo
Anti-IL-13
Short-term
Long-term
C
INH
Levels of IL-5 (pg/μg total protein)
1500
1000
500
0
p < 0.001
p < 0.0005
Placebo
Anti-IL-13
Placebo
Anti-IL-13
Short-term
Long-term
D
INH
Levels of IL-17 (pg/μg total protein)
1500
1000
500
0
Placebo
Anti-IL-13
Placebo
Anti-IL-13
Short-term
Long-term
E
INH
Levels of CCL11 (pg/μg total protein)
1000
800
600
400
200
0
p < 0.005
p < 0.05
Placebo
Anti-IL-13
Placebo
Anti-IL-13
Short-term
Long-term

Figure 6 :

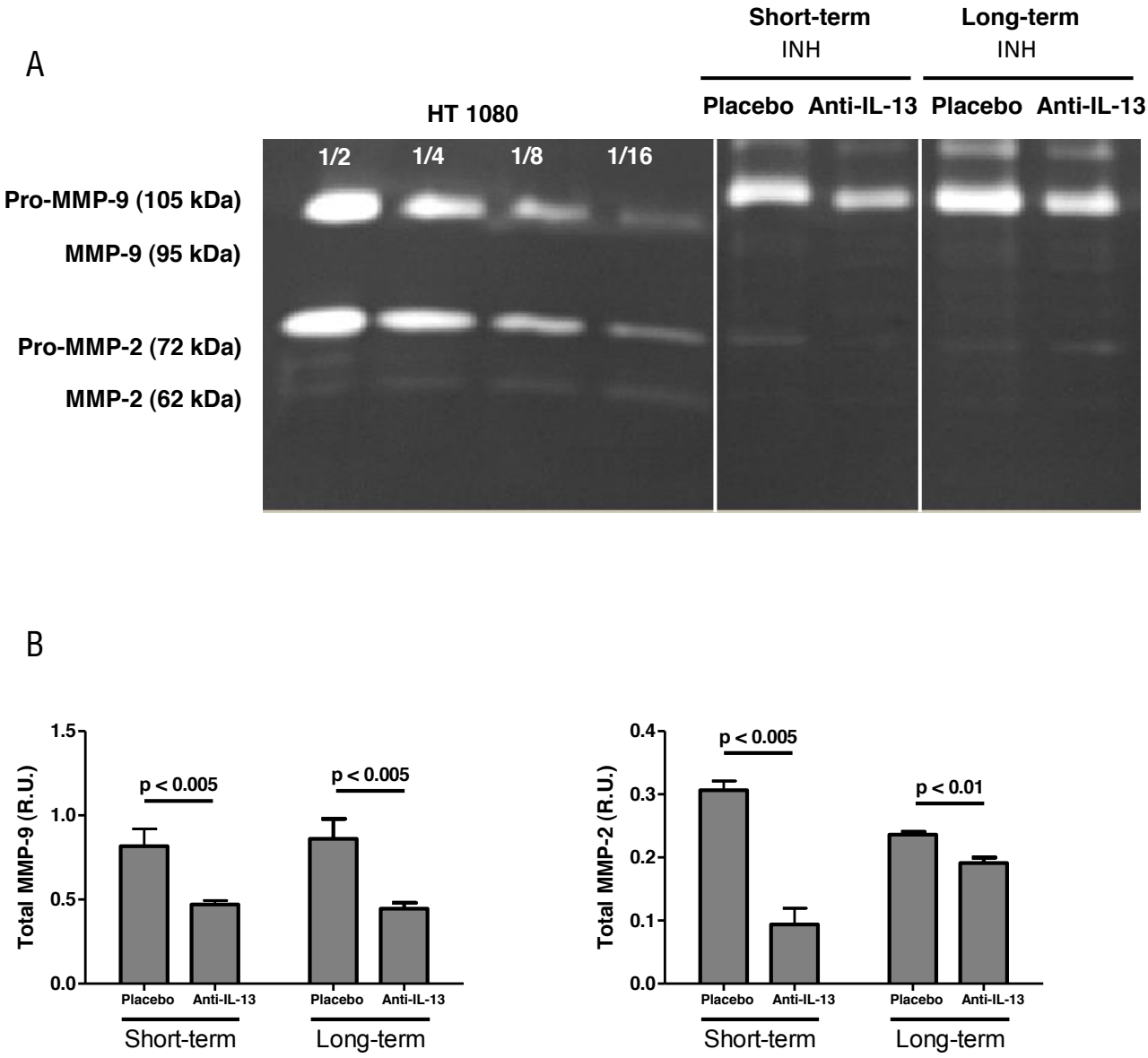
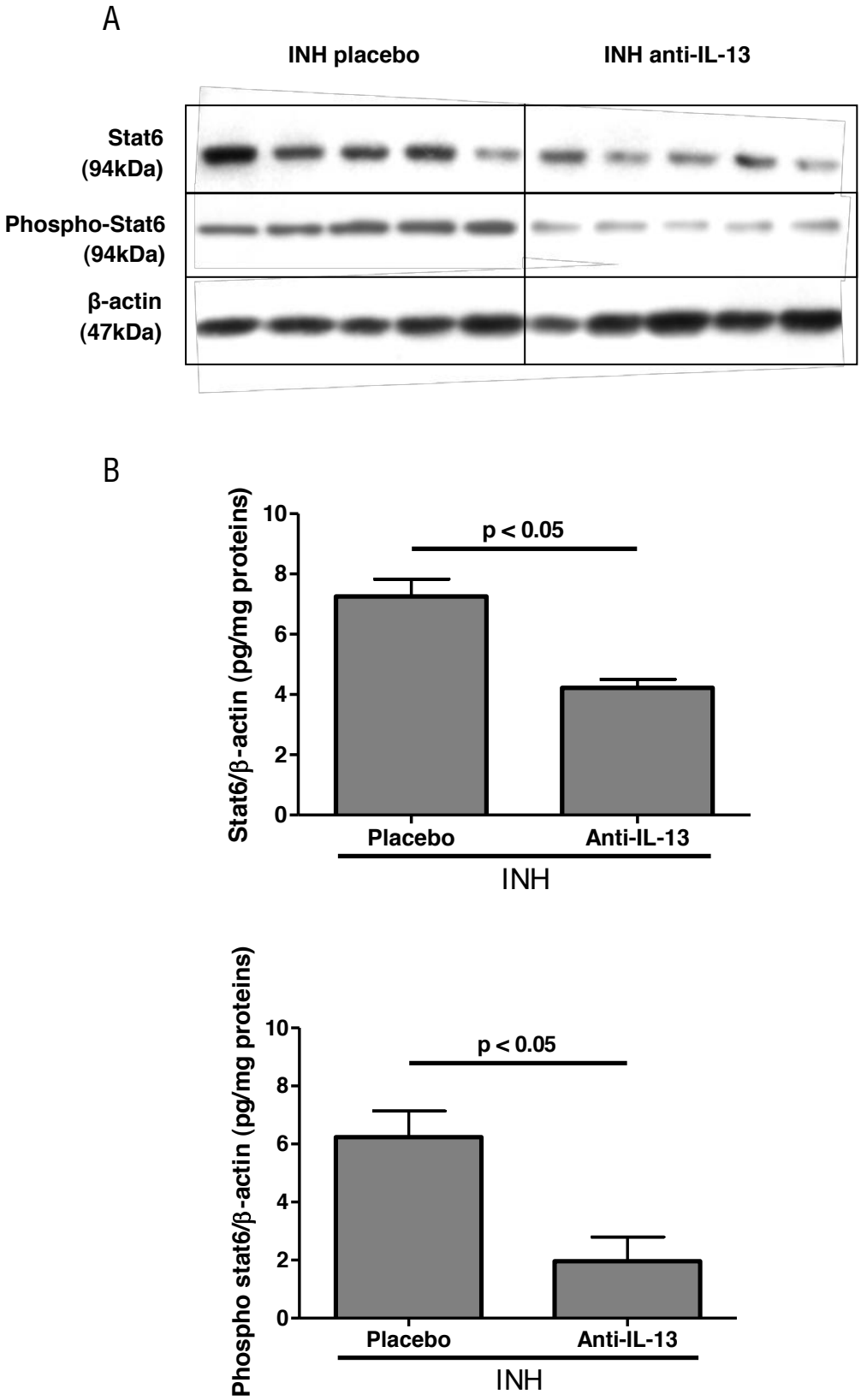

A
Short-term
INH
Long-term
INH
Placebo
Anti-IL-13
Placebo
Anti-IL-13
HT 1080
1/2
1/4
1/8
1/16
Pro-MMP-9 (105 kDa)
MMP-9 (95 kDa)
Pro-MMP-2 (72 kDa)
MMP-2 (62 kDa)
B
Total MMP-9 (R.U.)
1.5
1.0
0.5
0.0
p < 0.005
p < 0.005
Placebo
Anti-IL-13
Placebo
Anti-IL-13
Short-term
Long-term
Total MMP-2 (R.U.)
0.4
0.3
0.2
0.1
0.0
p < 0.005
p < 0.01
Placebo
Anti-IL-13
Placebo
Anti-IL-13
Short-term
Long-term

Figure 7:


A
INH placebo
INH anti-IL-13
Stat6 (94kDa)
Phospho-Stat6 (94kDa)
β-actin (47kDa)
B
Stat6/β-actin (pg/mg proteins)
p < 0.05
Placebo
Anti-IL-13
INH
Phospho stat6/β-actin (pg/mg proteins)
p < 0.05
Placebo
Anti-IL-13
INH

Nebulized anti-IL-13 monoclonal antibody Fab' fragment reduces allergen-induced asthma

Jonathan HACHA, Kate TOMLINSON, Ludovic MAERTENS, Geneviève PAULISSEN, Natacha ROCKS, Jean-Michel FOIDART, Agnès NOEL, Roger PALFRAMAN, Maud GUEDERS, Didier D CATALDO

ONLINE DATA SUPPLEMENT

Online Data Supplement

Administration of PBS or anti-mouse IL-13 monoclonal antibody by the inExpose® system

The *inExpose*® system consisting of 12 ports located peripherally around a central delivery plenum was utilized for PBS and anti-mouse IL-13 Fab' aerosol exposure. For comparison, anti-mouse IL-13 Fab' was prepared at a final concentration of 5 mg/ml or 2.5 mg/ml in PBS, as described in table 1. Aerosols were generated by nebulization with a Pari LC Star jet nebulizer using dry compressed air at a flow rate of 2 l/min. Male Balb/c mice were restrained in SoftRestraints constructed of flexible coated wire adapted to the animal's body shape and placed in an inhalation tower such that only the nose of each mouse was exposed to the aerosol cloud. The nebulizer was connected to the top part of the inhalation chamber from which the generated aerosol flowed down the central tower to the peripherally restrained 12 mice. The total amount of PBS and Fab' was nebulized to animals during maximum 15 minutes of exposure time. The total amount of anti-mouse IL-13 monoclonal antibody deposited into each mouse was calculated based on the following formula: $D = C \times V \times DI \times T$ where C = concentration of monoclonal antibody in aerosol volume (5mg/ml/364.828 cm^3), V = volume of air inspired by the animal during 1 min (for mice, V=1.0 l min/kg), DI=estimated deposition index (fraction of inhaled dose deposited throughout the respiratory tract for mice DI=0.3), and T = duration of treatment in min (T=15 min) (21). In this study, the estimated total deposited amount of inhaled anti-mouse IL-13 Fab' was 0.0617 mg for each mouse.

Protein extraction

The left lung was excised and frozen immediately at −80 °C and then disrupted in liquid N_2 by using a Mikro-Dismembrator (Braun Biotech International, Germany) to form a

homogenized powder. This crushed lung tissue was incubated overnight at 4 °C in a solution containing 2 M urea, 1 M NaCl and 50 mM Tris (pH 7.5) and subsequently centrifuged 15 min at 16 000 x g for protein extraction.

Pulmonary histology

The right lung was infused with 4% paraformaldehyde, embedded in paraffin and processed for histology. Sections were stained with haematoxylin–eosin to estimate the extent of peribronchial inflammation by scoring sections as previously described and validated (24). Since 5–7 randomly selected tissue sections per mouse were scored, inflammation scores are expressed as a mean value and can be compared between groups. Eosinophilic inflammatory score was determined on paraffin sections stained with Congo Red by manual count in randomly selected bronchi and normalized to the perimeter of corresponding epithelial basement membrane. Goblet cell hyperplasia was observed using Alcian Blue staining in randomly selected bronchi. Masson's Trichrome staining was used to detect peribronchial collagen deposition. We developed software in Java to obtain the area of peribronchial collagen relative to the size of the bronchi (23). α-Smooth Muscle Actin (α-SMA) was detected by immunohistochemistry as previously described (23). Mast cells were specifically detected in paraffin section using Toluidine Blue staining (Sigma Aldrich, Germany). Two different observers blinded to the experimental conditions assessed quantification.

Measurements of IgE and cytokines by ELISA

Blood samples were taken via cardiac puncture for measurement of OVA-specific serum IgE levels by ELISA. Ninety-six-well microtiter plates were coated with 300 µl/well of an OVA solution (5 mg/L) (Albumin from chicken egg white, Grade V, Sigma-Aldrich, Belgium). Sample of serum was added and incubated with a biotinylated polyclonal rabbit anti-mouse IgE (Calbiochem, USA) used at 1/1000. A serum pool from OVA-sensitized animals was

used as internal laboratory standard to design a curve. Modulation of IL-13, IL-4, IL-5, IL-17 and CCL11 levels was confirmed in non-pooled lung protein extract by commercially available Duoset ELISA kits (R&D Systems, UK). Following the manufacturer's instructions, a specific recombinant protein was used at different dilution (from 15.6pg/ml to 8000pg/ml) to construct a standard curve. Plates were coated using the adequate capture antibody. A conjugated detection antibody and a streptavidin-HRP (Horse Radish Peroxydase) were used for the detection of the protein of interest (R&D Systems, UK).

Western blotting

Total protein extracts (20 mg) were separated under reducing conditions on 12% polyacrylamide gels and transferred on PVDF membranes (Perkin Elmer Life Sciences, Boston, CA, USA). Primary antibody rabbit polyclonal anti-STAT6 (1:200) (Abcam, UK) and rabbit polyclonal antibody to phospho Y641 STAT6 (1:500) (Abcam, UK) were applied on membranes overnight at 4°C and then incubated with the same secondary antibody conjugated with HRP (goat anti-rabbit) (1:1000) for 1 h at room temperature. Predicted band sizes were expected at 94kDa. The enhanced chemiluminescence (ECL) detection kit (Perkin Elmer Life Sciences) allowed visualization of immunoreactive proteins. To normalize Western blot data, β-actin was detected in all samples with a rabbit anti-mouse HRP antibody diluted in PBS (1:1000) (Sigma Aldrich, Germany).

Measurement of MMP-2 and -9 levels by zymography

We performed zymography on lung protein extracts as previously described by using dilutions of culture medium conditioned by HT1080 cells as an internal standard (23). Gelatinolytic activity of the murine MMP-2 and -9 was determined by the lysis band in the 72 kDa and the 95 kDa area, respectively.

Figure E1: Levels of total IgE

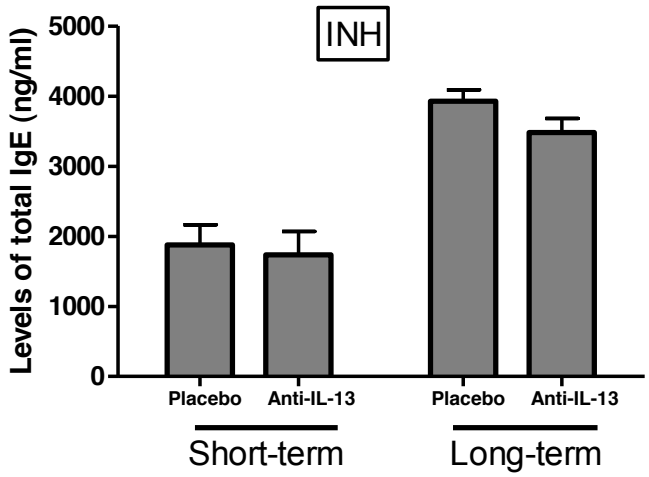


Figure legends

Levels of serum IgE (ng/ml) measured by ELISA following administration of PBS (placebo) or anti-mouse IL-13 Fab' (anti-IL-13) by inhalation (INH) in the short-term and in the long-term protocols.