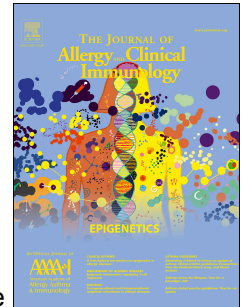


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IL-13 and IL-4, but not IL-5 nor IL-17A, induce hyperresponsiveness in isolated human small airways

Martijn L. Manson, PhD, Jesper S  fholm, PhD, Anna James, PhD, Anna-Karin Johnsson, PhD, Per Bergman, MD, PhD, Mamdoh Al-Ameri, MD, PhD, Ann-Charlotte Orre, MD, PhD, Carina K  rrman M  rdh, PhD, Sven-Erik Dahl  n, MD, PhD, Mikael Adner, PhD



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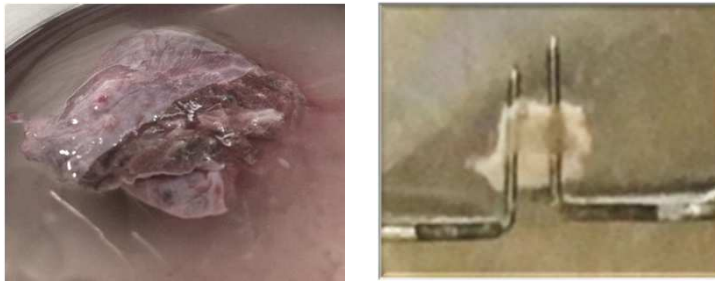
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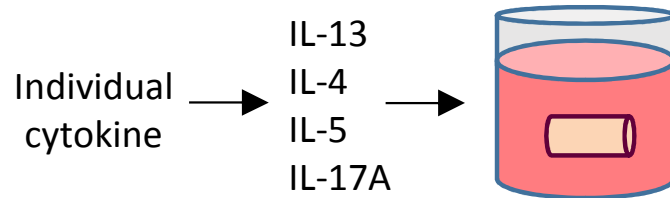


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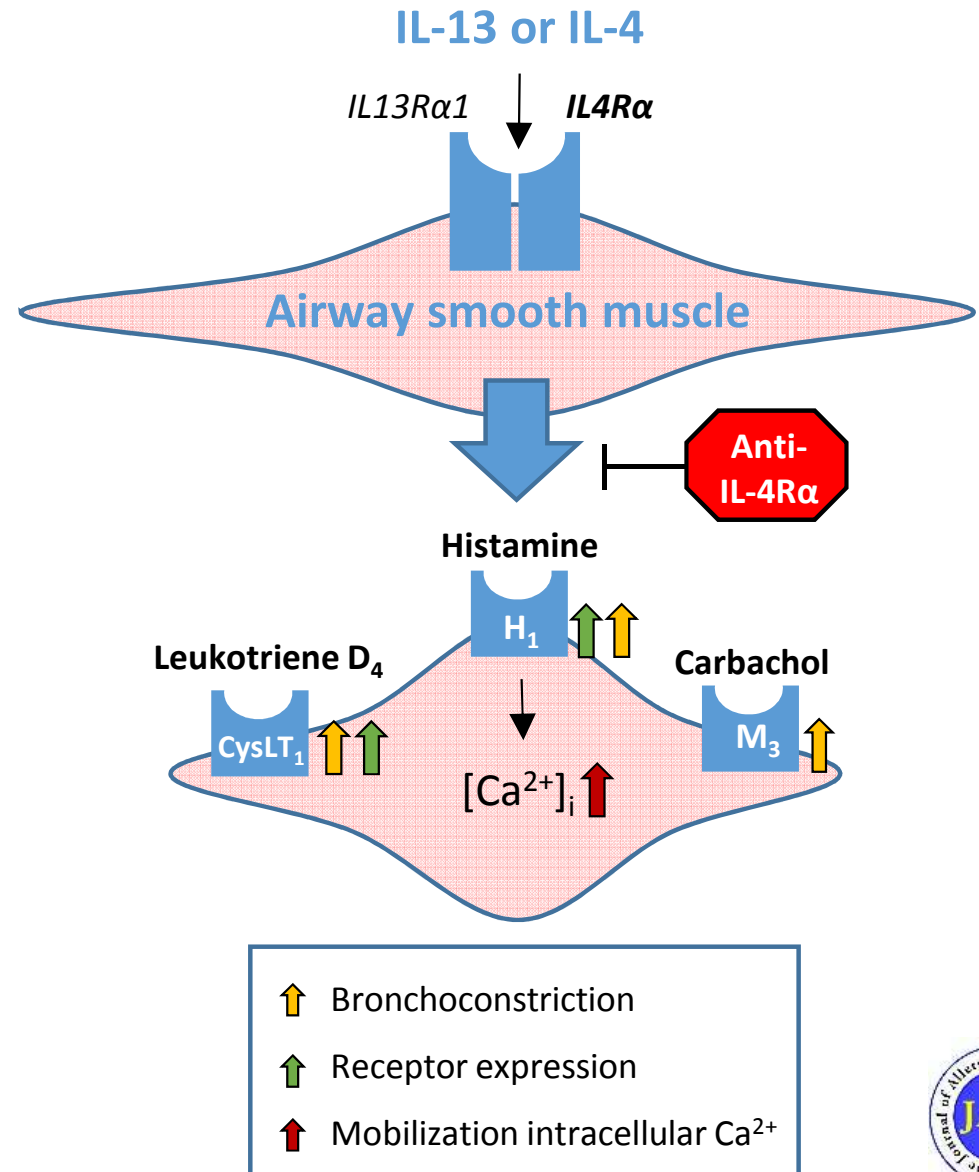
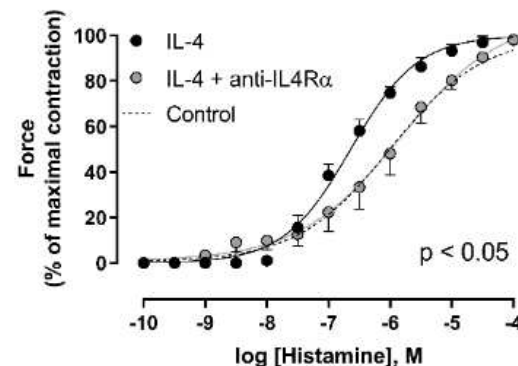
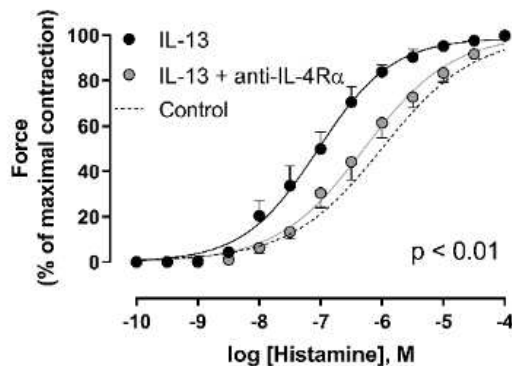
Isolated human small bronchi



Organ culture



Airway smooth muscle contraction



IL-13 and IL-4, but not IL-5 nor IL-17A, induce hyperresponsiveness in isolated human small airways

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	Body of MS	3569
	Intro	404
	Methods	785
	Results	818
	Discussion	1562

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Disclosure of potential conflicts of interests

MLM and CKM were employed at Astra Zeneca during parts of this study. No further conflicts of interest were declared for this study.

ABSTRACT

Background: Specific inflammatory pathways are indicated to contribute to severe asthma but their individual involvement in the development of airway hyperresponsiveness remains unexplored.

Objective: This experimental study in human small bronchi aimed to provide insight into which of the Type-2 and Type-17 cytokines cause hyperresponsiveness of airway smooth muscle.

Methods: Explanted small bronchi isolated from human lung tissue and human airway smooth muscle cells (HASM) were treated for 2 and 1 day(s) respectively with 100ng/mL of IL-4, IL-5, IL-13 or IL-17A, and contractile responses, Ca^{2+} -mobilization, and receptor expression were assessed.

Results: Treatment with IL-13 increased the potency of histamine, carbachol and leukotriene D_4 as contractile agonists. IL-4, but not IL-5 or IL-17A, also increased the potency of histamine. In HASM, IL-13 and IL-4, but not IL-5 and IL-17A, enhanced the histamine-induced Ca^{2+} -mobilisation that was accompanied with increased mRNA expression of histamine H_1 and CysLT_1 receptors. RNA sequencing of isolated bronchi confirmed the IL-13-mediated up-regulation of H_1 and CysLT_1 receptors, without showing an alteration of muscarinic M_3 receptors. Dexamethasone had no effects on IL-13-induced hyperresponsiveness in human bronchi, the increased Ca^{2+} -mobilisation or the enhanced receptor expression. In contrast, antagonism of the common receptor for IL-13 and IL-4 by the biologic dupilumab, prevented the effects of both IL-13 and IL-4 in human bronchi and HASM.

Conclusion: The glucocorticoid-insensitive hyperresponsiveness in isolated human airways induced by IL-13 and IL-4 provides further evidence that the IL-4R α pathway should be targeted as a new strategy for treatment of airway hyperresponsiveness in asthma.

Word count: (247)

Key messages

- ✓ IL-4 and IL-13 induces hyperresponsiveness of the airway smooth muscle in isolated human small airways.
- ✓ This glucocorticoid-insensitive hyperresponsiveness could be prevented by the IL-4R α antagonist dupilumab.
- ✓ This study warrants follow-up bronchoprovocation studies in asthmatic patients where the effect of combined IL-4/IL-13 antagonism on AHR is examined.

Capsule summary: IL-4 and IL-13 induced profound hyperresponsiveness of airway smooth muscle in isolated human small airways through activation of IL-4R α , suggesting that blockade of this pathway may reduce airway hyperresponsiveness caused by direct spasmogens. (33 words)

Key words: airway smooth muscle; airway hyperresponsiveness; bronchoconstriction explanted human tissue model; calcium signaling; bronchoconstrictor agents, IL-4R α ; dupilumab; glucocorticoid; STAT-6

Abbreviations:

AHR	Airway hyperresponsiveness
CHMR3	Cholinergic receptor muscarinic 3
CYSLTR1	Cysteinyl leukotriene receptor 1
DMEM	Dulbecco's modified eagle's medium
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
HASMC	Human airway smooth muscle cells
HRH1	Histamine receptor H1
IL-13	Interleukin-13
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
LTD ₄	Leukotriene D ₄
PCLS	Precision cut lung slices
RT-PCR	Reverse transcriptase polymerase chain reaction
STAT-6	Signal transducer and activator of transcription 6
TP	Thromboxane A ₂ receptor

INTRODUCTION

Airway hyperresponsiveness (AHR) is a hallmark of asthma that is defined as the increased sensitivity and enhanced narrowing of the airways in response to a broad range of physical or chemical stimuli¹. Although glucocorticoids, the cornerstone therapy in asthma, reduce inflammation and cause some reduction of AHR, asthmatic individuals remain hyper-responsive compared to healthy subjects^{2,3}. As a result of our increased understanding of the heterogeneity in asthma and its molecular phenotypes⁴, novel therapies are being developed that specifically block the actions of particular cytokines considered to be central to the pathogenesis of asthma⁵⁻⁷. Clinical studies have shown that targeting IL-5 and the common receptor for IL-4 and IL-13, IL-4R α , reduces asthma exacerbations in subsets of asthmatic patients displaying the most prominent signs of type-2 inflammation^{5,6}. However, it is not known whether these new biologicals also improve AHR.

This experimental study aims to provide a rationale for which biologics may be considered to target AHR in future clinical investigations. The study was conducted using freshly isolated human airways and builds on the hypothesis that inflammatory mediators may directly alter the responsiveness of airway smooth muscle^{1,8}. The type-2 cytokine IL-13 has mostly been linked to this form of hyperresponsiveness, because studies in mice⁹⁻¹¹ and rabbits¹² have shown that IL-13 can enhance contractions of rodent airways. Similar effects have been observed for IL-5¹³, IL-17A¹⁴ and IL-4¹⁵ in animal models. Importantly however, very few studies have translated these animal data into human airways¹⁶⁻¹⁹. The few reports using human tissues create a scattered picture because of the different experimental models used (isolated bronchi vs. precision cut lung slices [PCLS]) or varying protocols (e.g. duration of exposure). The four key cytokines addressed in the current investigation, IL-4, IL-5, IL-13 and IL-17A, have not previously been compared in the same study in human airways.

The aim of this study was therefore to investigate the effects of the presumed inducer of hyperresponsiveness, IL-13, on smooth muscle function in human small airways and to compare this with the effects of IL-4, IL-5 and IL-17A under the same conditions. The contractile responses of isolated human bronchi were thus examined using an established organ culture method where isolated human airways are subjected to controlled exposures of inflammatory mediators²⁰. Additional studies were performed using HASM cells to further define the mechanisms implicated by the observations in isolated human bronchi.

MATERIAL AND METHODS

Human tissue preparation

With permission of the Regional Ethical Review Board in Stockholm (Ref no 2010/181-31/2), macroscopically healthy human lung tissue was collected after consent from patients undergoing lobectomy for neoplasms (94%) or other reasons (hamartoma) (n=33, 24 females and 9 males; median age 69 years, range: 44-82; **Table 1**). From each lung, one to three bronchi were dissected out, cut into segments and used for paired analyses. In several of the experiments, different bronchi from the same patient were used to control for the intra-individual variability regarding the responsiveness of human airway preparations²¹.

Organ culture

Human bronchi (0.5-2mm) were dissected and segments were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% penicillin/streptomycin (LifeTechnologies) and placed in a humidified incubator at 37°C at 95% O₂ and 5% CO₂ for two days in the presence of 100ng/mL IL-13, IL-4, IL-5 or IL-17A and transferred to fresh medium and treatments every day²⁰. For re-culture experiments, first baseline contractility was evaluated (*Day 0*), and subsequently the bronchial rings were placed back into culture for two days in the presence of 100ng/mL IL-13 or vehicle (*Day 2*). Chosen concentrations were based on previous in-vitro investigations of these cytokines^{9, 15, 18}.

In vitro pharmacology

Human bronchial segments were mounted in myographs for isometric tension measurements. The bronchi were stretched to 1.5mN, during a 90-minute equilibration period and viability was tested by administrations of 60mM KCl²². Contractile responses to histamine (0.1 nM – 100 µM), leukotriene D₄/LTD₄ (0.01 nM – 10 µM) and carbachol (10 nM – 100 µM) were studied by cumulative administrations of the agonists. The next concentration of agonist was administrated once the plateau of the contraction was reached, or in case no contractile response was initiated, following 10 minutes after the administration of the previous agonist. To obtain maximal contractile responses, each segment was exposed to 100 µM histamine, which was used as the reference for the contractile response of that particular bronchial segment²³.

Culture of human airway smooth muscle cells

Human primary bronchial smooth muscle cells (passage 4-6; Promocell/Lonza) were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) and

penicillin/streptomycin. Confluent cells were serum-deprived (0.3% FBS) for one day prior to one day stimulations with 100ng/mL IL-4, IL-5, IL-13 or IL-17A.

Intracellular Ca^{2+} -measurements

Intracellular Ca^{2+} -fluxes were measured in HASMC according to previously described protocols²⁴. In brief, detached cells were incubated in PBS containing 3 μ M Fluo-4, 0.02% pluronic F-127 and 2.5 μ M probenecid or Fluo-4 Direct Calcium Assay Kit (ThermoFisher) according to the manufacturers recommendations. Ca^{2+} -fluxes were analyzed by flow cytometry (LSR Fortessa®, BD Biosciences) or using a SpectraMax iD3 (MolecularDevices).

Western-blotting

Standard methodology was used to detect phosphoSTAT-6 (phosphoY641, Abcam) in relation to GAPDH (14C10, CellSignalingTechnology).

Polymerase-chain reaction

RT-PCR was conducted using and Taqman® Gene Expression Master Mix and primers (AppliedBiosystems). Changes in gene expression were determined using $\Delta\Delta$ Ct method after normalization for endogenous controls β -actin and GAPDH.

RNA sequencing

Paired bronchial segments were cultured and exposed for 24 hours to IL-13 (100 ng/mL) or vehicle. Total RNA was extracted using Trizol and further purified using the RNAeasy protocol. The yield and quality were analysed using Qubit and Agilent Tapestation. Indexed cDNA libraries were normalised and sequenced on the Illumina HiSeq 2000. Raw intensity values were background corrected, log2 transformed and then quantile normalized by the Expression Console software from Affymetrix.

Drugs and materials

Recombinant human IL-13, IL-4, IL-5 and IL-17A were obtained from R&D systems. Carbachol, dexamethasone and histamine dihydrochloride were purchased from Sigma-Aldrich. AS1517499 was purchased from Axon Medchem LLC. LTD₄ was obtained from Cayman Chemical. Dupilumab was obtained from Apoteket Produktion & Laboratorier AB, Stockholm, Sweden. Interventions with dexamethasone, AS1517499 and dupilumab were administrated 60 min prior to the initial addition of cytokines.

Calculations and statistics

All data are presented as means $\pm$ S.E.M. Information on sample size (n) and the number of individual patients (N) studied for each individual experiment are provided in the figure

legends. For the statistical analysis of responses in human bronchi, paired segments of bronchi were used, with one segment from an individual bronchi always used as the control (non-treated).

The concentration-response curve values for pEC_{50} , were calculated using non-linear regression analysis. Fold-changes were calculated from these acquired pEC_{50} values using the following formula: $\frac{10^{-pEC_{50} (veh)}}{10^{-pEC_{50} (IL13)}}$.

Paired t-tests were used for comparisons between two groups and One-or Two-Way ANOVA with the Bonferroni post-test for comparisons between multiple groups. GraphPadPrism 5.01 was used for statistical analyses. For the RNA sequencing, sample group comparisons were performed using the R package DESeq2 with Wald test, where the p-value were adjusted for multiple testing using the Benjamini & Hochberg method.

Additional information on the applied methodologies can be found in the supplement.

RESULTS

The effect of IL-13 on contraction of human small bronchi

In the first set of experiments, culture with IL-13 (*Day 2*) caused a 2.4-fold increase in the potency of histamine compared to segments cultured with vehicle (pEC_{50} : 6.8 ± 0.1 versus 6.4 ± 0.1 ; $p < 0.01$). There was however no change in the near maximal contractions ($113 \pm 9\%$ versus $114 \pm 11\%$) induced in the same segments prior to the two days of exposure (*Day 0*) (**Figure 1A**). Likewise, the amplitude of the maximal contractions induced by histamine (**Figure 1B**), or 60mM KCl, a non-receptor-mediated contractile agent (**Figure 1C**) were the same on *Day 0* compared to *Day 2*, for both vehicle ($p = 0.99$ and 0.27) and IL-13-treated segments ($p = 0.99$ and 0.47).

In the next set of experiments, using paired segments from same bronchial branch, IL-13 caused a 3.2-fold increase in the contractile potency of histamine (pEC_{50} : 7.0 ± 0.1 versus 6.5 ± 0.1 ; $p < 0.001$), a 3.2-fold increase in the potency of LTD₄ (pEC_{50} : 9.0 ± 0.1 versus 8.5 ± 0.1 ; $p < 0.01$) and a 2.5-fold increase in the potency of carbachol (pEC_{50} : 6.3 ± 0.1 versus 5.9 ± 0.1 ; $p < 0.01$) (**Figure 1D-F**). IL-13 also enhanced the maximal contractile response towards LTD₄ (E_{max} : $92.4 \pm 3.8\%$ versus $76.2 \pm 5.2\%$; $p < 0.05$; **Figure 1E**), whereas the maximal contractile responses towards carbachol was unchanged (**Figure 1F**).

The contractile response to histamine was used as a reference for the maximal contraction of the human bronchi and therefore routinely examined in all studied preparations. We therefore have the largest dataset for the response to histamine (**Figure 1D**), which also enabled us to evaluate the effect of IL-13 (pEC₅₀: 6.9±0.1) and vehicle (pEC₅₀ 6.4±0.1) between patients (**Supplementary Figure 1**).

Effect of IL-13 on intracellular Ca²⁺-mobilization

To investigate possible mechanisms involved, the effect of IL-13 on histamine-induced intracellular Ca²⁺-mobilization was examined in cultured HASMC. Treatment with IL-13 for 24 hours enhanced the concentration-dependent mobilization of intracellular Ca²⁺ by histamine (**Figure 2A-D**), increasing both amplitude (**Figure 2C**) and potency compared to vehicle (**Figure 2D**; pEC₅₀: 5.7±0.1 vs 5.4±0.1; *p*<0.01).

Effects of IL-4, IL-5 and IL-17A on airway contraction and intracellular Ca²⁺-mobilization

Similar to IL-13, IL-4 caused a 5.1-fold increase of the potency of the histamine-induced contraction (pEC₅₀: 6.8±0.1 versus 6.1±0.1; *p*<0.05; **Figure 3A**) and an increased histamine-induced intracellular Ca²⁺-mobilization (**Figure 3D**). In contrast, culture with IL-5 or IL-17A caused no change in the potency of histamine (pEC₅₀: 6.3±0.2 and 6.4±0.2, respectively) compared to vehicle (6.2±0.1) (**Figure 3B and C**), and no alteration of the histamine-induced intracellular Ca²⁺-mobilization (**Figure 3E and F**).

Cytokine effects on gene expression

The effects of all tested cytokines (IL-13, IL-4, IL-5 and IL-17A) on the mRNA expression of *HRH1* and *CYSLTR1*, were investigated in HASMC. IL-13 caused a concentration-dependent increase in mRNA for both *HRH1* (**Figure 4A**) and *CYSLTR1* (**Figure 4B**). IL-4 induced a similar increase in *HRH1* and *CYSLTR1*, whereas IL-5 and IL-17 had no effect on receptor expression.

Focusing on the receptors of interest in this study, RNA-sequencing analysis of human small bronchi showed expression of histamine H₁ (*HRH1*), CysLT₁ (*CYSLTR1*) and muscarinic M3 (*CHRM3*) receptors and cytokine receptors for IL-4 (*IL4R/IL2RG* or *IL4R/IL13RA1*), IL-13 (*IL4R/IL13RA1* or *IL13RA2*), IL-5 (*IL5RA/CSF2RB*) and IL-17A (*IL17RA/IL17RC*) (**Table 1**). At baseline, highest expression was observed for the *IL4R/IL13RA1* heterodimer and lowest expression for the G-protein coupled receptors *CYSLTR1* and *CHRM3*. After culture with IL-13, there was a significant increase in *HRH1* and *CYSLTR1* together with the

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presumed decoy receptor *IL13A2*²⁵, which has also been implicated in airway fibrosis²⁶,
whereas neither the expression of *CHMR3* nor the other cytokine receptors was affected.

The effect of dexamethasone on IL-13- induced effects

In the presence of the glucocorticoid dexamethasone and IL-13, the potency of histamine was 2.6 to 3.3-fold greater than in preparations treated with dexamethasone alone, or vehicle-treated bronchi (**Figure 5A**). Similarly in HASMC the IL-13-induced increase in the potency of histamine-induced intracellular Ca^{2+} -mobilisation was not inhibited by dexamethasone (**Figure 5B**). The increase in *HRH1* mRNA expression caused by IL-13 (2.3-fold change versus vehicle) was also replicated in this set of experiments (**Figure 5C**). IL-13 increased the expression of *HRH1* to a similar degree following treatment with dexamethasone (2.0-fold versus dexamethasone), although the glucocorticoid reduced the baseline expression of *HRH1*.

Effect of STAT-6 inhibition on IL-13-induced effects

Signal transducer and activator of transcription 6 (STAT6) is a down-stream signalling pathway of IL-13²⁷. IL-13 induced a profound phosphorylation of STAT-6 in HASMC (**Figure 5D**) which AS1517499 (100nM), a selective STAT-6 inhibitor²⁸, inhibited whereas dexamethasone had no effect (**Figure 5D-E**). However, pre-treatment with AS1517499 (100nM), did not affect the IL-13-induced hyperresponsiveness of the bronchi (**Figure 5F**).

Effect of IL4Rα antibody treatment on IL-13- and IL-4-induced effects in human bronchi and HASMC

Pre-treatment with the IL4Rα antibody dupilumab (1μM) before IL-13 (**Figure 6A**) and IL-4 (**Figure 6B**) administration attenuated the increase in potency for histamine (pEC_{50} : 7.1 ± 0.1 versus 6.3 ± 0.1 and 6.7 ± 0.1 versus 6.1 ± 0.2 , respectively; $p < 0.05$). Similarly, dupilimab blocked the increase in the maximal effect of histamine-induced intracellular Ca^{2+} -mobilization caused by IL-13 (**Figure 6C**) and IL-4 (**Figure 6D**) in HASMC.

DISCUSSION

This investigation was conducted in the small airway segment that is increasingly being recognized as a critical determinant of the severity of AHR^{29, 30}. It was established that IL-13 unambiguously increased the potency of agonists belonging to fundamental endogenous contractile pathways. Moreover, it was shown for the first time that IL-4 shares this effect of IL-13 in the human bronchi. The enhancing effects of IL-13 and IL-4 on histamine responses were replicated in HASMC. In contrast, IL-5 and IL-17A did not enhance responses to histamine in either model. Using unbiased RNA-sequencing methodology and RT-PCR expression analysis, it was also discovered that both IL-4 and IL-13 induced an up-regulation of the receptors for histamine and LTD₄. Furthermore, both dexamethasone and STAT6 inhibition failed to alter the IL-13-induced responses in bronchi and HASMC. In both models, pre-treatment with the IL-4R α inhibitor dupilumab prominently abolished the responses to IL-4 and IL-13, confirming that the effects observed were mediated via activation of their common receptor.

In the first set of experiments, the potency of histamine-induced contractions was increased in segments cultured with IL-13 without alteration of the amplitude of the contraction. The unaltered maximal responsiveness was documented using a re-culture protocol that mitigates possible limitations caused by variability in size and smooth muscle structure between different isolated airway segments studied in parallel. The amplitude of 60 mM KCl, which elicits its contractions through membrane depolarisation³¹, was not altered after culture with IL-13. As the concentration of KCl used in this study was submaximal^{32, 33}, this observation indicates that IL-13 does not potentiate this receptor-independent activation. The findings therefore support that the increased sensitivity to histamine after exposure to IL-13 occurs via receptor-operated signalling, upstream of the contractile machinery. The potential impact of these changes in smooth muscle hyperresponsiveness are more profound in the *in vivo* situation as air flow is determined by airway resistance according to Poiseuille's law which is inversely related to the 4th power of the airway radius³⁴.

In the second experimental design, using paired segments that enable a higher throughput, the effect of IL-13 on histamine-responsiveness was replicated. Using this procedure, we discovered that IL-4 also increased responsiveness to histamine in explanted human bronchi, which has not been shown before. Furthermore, it was demonstrated that IL-13 also increased the responses to another mast cell mediator, LTD₄, as well as the cholinergic agonist carbachol. These findings are in line with previous observations that IL-13 increased

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responses to histamine and the TP agonist U46619 in human bronchi¹⁷, and to carbachol in human PCLS¹⁶. These studies primarily observed an increase in the amplitude of the contractions. In our setting, only the contractile response to LTD₄ was increased in amplitude, most likely because LTD₄ did not cause maximal airway contractions in the absence of cytokine treatment. Taken together, the findings document that both IL-13 and IL-4 can induce smooth muscle hyperresponsiveness for the major contractile pathways in asthma.

By measuring the effect of histamine-induced Ca²⁺-influx, which precedes airway contraction, this study confirmed previous findings in HASMC showing that IL-13 increases Ca²⁺-mobilisation^{9, 35, 36}. Moreover, IL-13 caused an increase in the potency of histamine-mediated effects, strengthening the link to the effects observed in the bronchi. Previous studies in HASMC, observed that IL-13 increased the effects of other agonists activating G-protein coupled receptors, such as bradykinin and thrombin³⁵, but not KCl³⁷. The absence of effect on sub-maximal KCl contraction was also shown for the airway segments used in the present study. Thus, the IL-13-induced increase in Ca²⁺-influx indicates that the alteration in contraction is specific for the G-protein-coupled receptor pathway and regulated at the level of Ca²⁺-mobilization or above.

Both IL-13 and IL-4 increased in agreement with previous studies the expression of *HRH1* and *CYSLTR1* in HASMC^{36, 38}. Our RNA-sequencing confirmed and extended the importance of this phenomenon, by showing for the first time that IL-13 also increases the expression of these receptors in intact human airways. An increase in receptor density can cause a combined increase in the amplitude and the potency of the agonist, or only an increase in potency if the agonist already at the basal state elicits a maximal tissue effect³⁹. These processes are exactly what was observed for the effects of IL-13 on the contractions to LTD₄ and histamine respectively in human bronchi, indicating that one component of the cytokine-induced increase in airway contractility is potentially due to the up-regulation of contractile receptors.

Earlier studies in HASM cells have however shown that concurrent inhibition of ERK and JNK could prevent the IL-13 driven enhancement of histamine-induced Ca²⁺ mobilization without affecting the up-regulation of HRH1 mRNA, raising the possibility of alternative mechanisms other than receptor regulation³⁶. This outcome does not however exclude a role for receptor regulation, as the effects of these interventions were solely examined on the maximal mobilisation of intracellular Ca²⁺ and not on the potency for which an evaluation of

the full concentration-response would be needed. Nonetheless, the lack of effect of IL-13 on the expression of the muscarinic M3 receptor (*CHRM3*) in our study also suggests that the enhanced response to activation of muscarinic M₃ receptors by IL-13 should be attributed to mechanisms other than receptor upregulation, which may involve different kinase(s) important in the homeostasis of intracellular Ca²⁺^{9, 11, 35, 36}. Taken together, this indicates that at least two distinct pathways can explain the increased sensitivity induced by IL-13 and IL-4 in the bronchi. Future studies need to further address the signaling downstream of the IL4R α /IL-13R α 1.

Of particular relevance to the clinical perspective, we found that pre-treatment with dexamethasone failed to inhibit the IL-13-induced increase in sensitivity of the bronchi and Ca²⁺ mobilization in HASMC. The IL-13-induced increase in *HRH1* expression was also not affected by dexamethasone, further suggesting that the IL-13-induced hyperresponsiveness of airway smooth muscle is insensitive to the actions of glucocorticoids. The positive effects of glucocorticoids probably relate to their capacity to inhibit the production of inflammatory mediators⁴⁰, whereas as this study shows, once these cytokines are generated, their actions cannot be overcome by glucocorticoids.

STAT-6 signaling has been targeted for the treatment of asthma⁴¹. We found that the STAT-6 inhibitor AS1517499 markedly decreased IL-13-induced phosphorylation in HASMC, but it did not affect the IL-13-induced contractile hyperresponsiveness to histamine in the bronchi. In line with previous data showing no inhibitory effect of STAT-6 inhibition in HASMC³⁶, it remains for future studies to define the signaling mechanisms activated by IL-13 in human airways.

In this study, treatment with dupilumab, an antibody against IL4R α , markedly blocked the effects induced by both IL-13 and IL-4. This suggests that IL4R α probably mediates the effects of IL-13 and IL-4 through dimerization with IL13R α 1⁴². This hypothesis gains circumstantial support from the high basal expression of both parts of the common receptor (*IL4R* and *IL13RA1*) observed in this study. Indeed, simultaneously inhibiting the actions of both IL-13 and IL-4 with dupilumab has shown significantly lower rates of severe asthma exacerbation, better lung function, asthma control improvements and reduced oral glucocorticoid use in patients with uncontrolled asthma⁵ and in patients with glucocorticoid-dependent severe asthma⁴³. Dupilumab may therefore have effects over and above those of glucocorticoids, which also fits with our data showing that dexamethasone did not have any

effect on IL-13-induced hyperresponsiveness. Our study may be one explanation why the combined blockade of IL-4 and IL-13 activity is more beneficial in asthma patients than antagonism of either alone, which is underpinned by the limited effects of IL-13 monoclonals in previous clinical studies^{44, 45}.

The cytokines IL-5 and IL-17A were included because they are also implicated in the pathogenesis of asthma⁴, but their effects on human airways have not been extensively studied. In contrast to previous reports^{13, 14, 18}, neither of these cytokines enhanced the contractile responses in the small airways, nor did they increase Ca^{2+} -mobilization or receptor expression. The differing results obtained in previous studies for the effects of IL-5¹³ and IL-17A on human airways^{14, 18} may relate to our use of small bronchi ranging from the 8th-13th generation, compared to 1st-5th generation in previous studies. For IL-17A, the lack of effect on smooth muscle may be one reason why the anti-IL-17 receptor monoclonal antibody brodalumab failed to show efficacy in asthmatic patients⁷. Given the success of anti-IL-5 therapies^{6, 46}, the current findings support that their effects on exacerbations and other asthma outcomes are a consequence of their established effects on eosinophils rather than direct effects on airway smooth muscle. This would also be in line with the limited effects of anti-IL-5 therapies on lung function^{6, 43}.

Taken together, this is the first study where the influence of four established pro-inflammatory cytokines, all of which are currently targeted by new biologic treatments, have been compared under identical and standardized conditions in human small airways. In particular, the demonstration that activation of the IL-4/IL-13 pathway can promote profound hyperresponsiveness of the airway smooth muscle indicates that these two cytokines can directly contribute to the development of AHR at the level of the smooth muscle. These findings warrant follow-up bronchoprovocation studies where the effect of combined IL-4/IL-13 antagonism on AHR is examined in asthmatic patients. Brittle asthma with pronounced AHR is one example of a phenotype that is difficult to treat, and might show particular benefit from such interventions.

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AUTHOR CONTRIBUTIONS

M.L.M, A.J., S-E.D and M.A. planned the conception and design of research; P.B, M.A-A and A-C.O provided human lung material. M.L.M, A.J., A-K.J. and J.S. performed experiments; M.L.M, J.S., A.J., A-K.J., S-E.D., and M.A. analyzed the data; M.L.M, J.S., A.J., A-K.J., C.K.M, S-E.D and M.A. interpreted the results of experiments; M.L:M, J.S., A.J., A-K.J., and M.A. prepared figures; M.L.M, A.J., S-E.D., and M.A. drafted the manuscript; all authors read and revised the manuscript.

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Table 1: Patient characteristics of the 33 patients studied in this study.

		Unit
Sex	73	% females
Age	69 (44-82)	years
Body mass index	25.5 (18.4-38.9)	Kg
C-reactive protein	2 (1-71)	mg/L
Haemoglobin	133 (109-177)	g/L
Leukocyte Particle Conc.	6.9 (4.9-17.4)	$\times 10^9/\text{L}$
Current smoker	12	N
Ex-smoker	17	N
COPD	5	N
Asthma	2	N
Allergy	7	N

Ex-smoker is defined as a person that has not smoked for the last 12 months. The numbers represent absolute values or median and range. N: Number of patients.

Table 2. RNA sequencing analysis of human small bronchi. Analysis of RNA expression from segments that were cultured one day in absence and presence with IL-13 focusing on the receptors of interest in this study.

Receptors	baseMean	FoldChange	<i>p</i> -value	<i>p</i> -adj
<i>HRH1</i>	548±73	1.73	6.09E-07	0.000152
<i>CYSLTR1</i>	75±10	2.67	2.13E-09	9.66E-07
<i>CHRM3</i>	33±3	0.98	0.911604	0.999951
<i>IL4R</i>	1989±43	1.11	0.263766	0.999951
<i>IL2RG</i>	983±24	0.88	0.339479	0.999951
<i>IL13RA1</i>	2000±109	0.83	0.10588	0.999951
<i>IL13RA2</i>	704±87	2.38	9.62E-09	3.45E-06
<i>IL5RA</i>	347±72	1.11	0.604956	0.999951
<i>CSF2RB</i>	566±17	1.10	0.535423	0.999951
<i>IL17RA</i>	675±28	1.05	0.545536	0.999951
<i>IL17RC</i>	205±26	0.94	0.721519	0.999951

The data are expressed as baseMean (the mean of optical intensity values corrected for background), FoldChange (real number), *p*-value and *p*-adj (adjusted *p*-value for multiple comparisons). Data are obtained from 4 donors. *HRH1*: histamine H₁ receptor; *CYSLTR1*: CysLT₁ receptor; *CHRM3*: muscarinic M3 receptors; *IL4R*: IL-4 receptor α; *IL2RG*: IL-2 receptor γ (forms a heterodimer with *IL4R*, with IL-4 as agonist); *IL13RA1*: IL-13 receptor α1 (forms a heterodimer with *IL4R*, with IL-4 and IL13 as agonists); *IL13A2*: IL-13 receptor α2 (soluble IL-13 receptor α2); *IL5RA*: IL-5 receptor α; *CSF2RB*: colony stimulating factor 2 receptor β (forms heterodimer with *IL5RA*, with IL-5 as agonist); *IL17RA*: IL-17 receptor A; *IL17RC*: IL-17 receptor C (*IL17RA* and *IL17RC* form a heterodimer with IL-17A as agonist). *N* = 4; *n* = 4.

FIGURE LEGENDS

Figure 1. Effects of IL-13 on the potency and amplitude of contractile responses in human small bronchi.

Contractile responses in response to histamine and potassium chloride (KCl) were assessed in each individual human bronchial ring preceding any treatment (*Day 0*). The same bronchial rings were then placed back into culture and treated for two days with 0.1% BSA (○) or 100 ng/ml IL-13 (●), following which the contractile responses to histamine and KCl were re-examined (*Day 2*). **A**) The histamine-induced contraction on *Day 2*, expressed in relation to the maximal contraction measured on *Day 0*. Maximal contractions (mN) in response to **B**) histamine and **C**) KCl of the same individual bronchial rings prior (*Day 0*) and after treatment (*Day 2*) with vehicle or IL-13. Data are presented as mean±SEM (N:7; n:14-15). Statistical analysis was performed by paired t-tests comparing the pEC₅₀ values (**A**) and absolute contractions (**B-C**) on *Day 2* with the outcomes of the same bronchial preparation on *Day 0*.

For subsequent experiments, human bronchial rings were no longer re-cultured, but instead directly exposed to vehicle or 100 ng/ml IL-13. Cumulative concentration response curves to **D**) histamine, **E**) leukotriene D₄, and **F**) carbachol following 2-days of exposure of vehicle or IL-13 are presented. As histamine caused the strongest maximal effect, this response was used as a reference for the force generated. Data are presented as mean±SEM (N:4-9; n:8-32). Statistical analysis was performed by paired t-tests comparing the pEC₅₀ values and absolute contraction of IL-13 treated bronchial rings with their matched untreated controls from the same isolated bronchi.

Figure 2. Effect of IL-13 on intracellular Ca²⁺ mobilization in cultured human airway smooth muscle cells.

Compiled traces of the relative histamine-induced increases in intracellular Ca²⁺ in cells treated for 24-hours with **A**) 0.1% BSA or **B**) 100 ng/ml IL-13. **C**) Intracellular Ca²⁺ mobilization, induced by histamine measured as mean fluorescence intensity (MFI). **D**) Changes in intracellular Ca²⁺ expressed as percentages of each treatment's own maximal change in MFI (indexed at 100%). All data are presented as the mean±SEM (N:3; n:4-8), significance is presented by * p < 0.05, ** p < 0.01 and *** p < 0.001.

Figure 3. Effects of IL-4, IL-5 and IL-17A on airway contraction of human bronchi and Ca^{2+} mobilization in HASMC.

Cumulative concentration response curves for histamine in human bronchial rings cultured for 2 days in the presence of **A)** IL-4, **B)** IL-5 or **C)** IL-17A. Data are presented as mean $\pm$ SEM (N:5; n:6-11). Statistical analysis was performed by paired t-tests comparing the pEC₅₀ values of cytokine-treated bronchial rings with their matched untreated controls from the same isolated bronchi.

Effects of 24-hours stimulation with **D)** IL-4 **E)** IL-5 or **F)** IL-17A on the mobilization of intracellular Ca^{2+} in HASMC following 100 μM of histamine. Data are presented as mean $\pm$ SEM (N:3-5; n:3-5).

Figure 4. Effects of asthma related interleukins on gene expression of *HRH1* and *CYSLTR1* in HASMC.

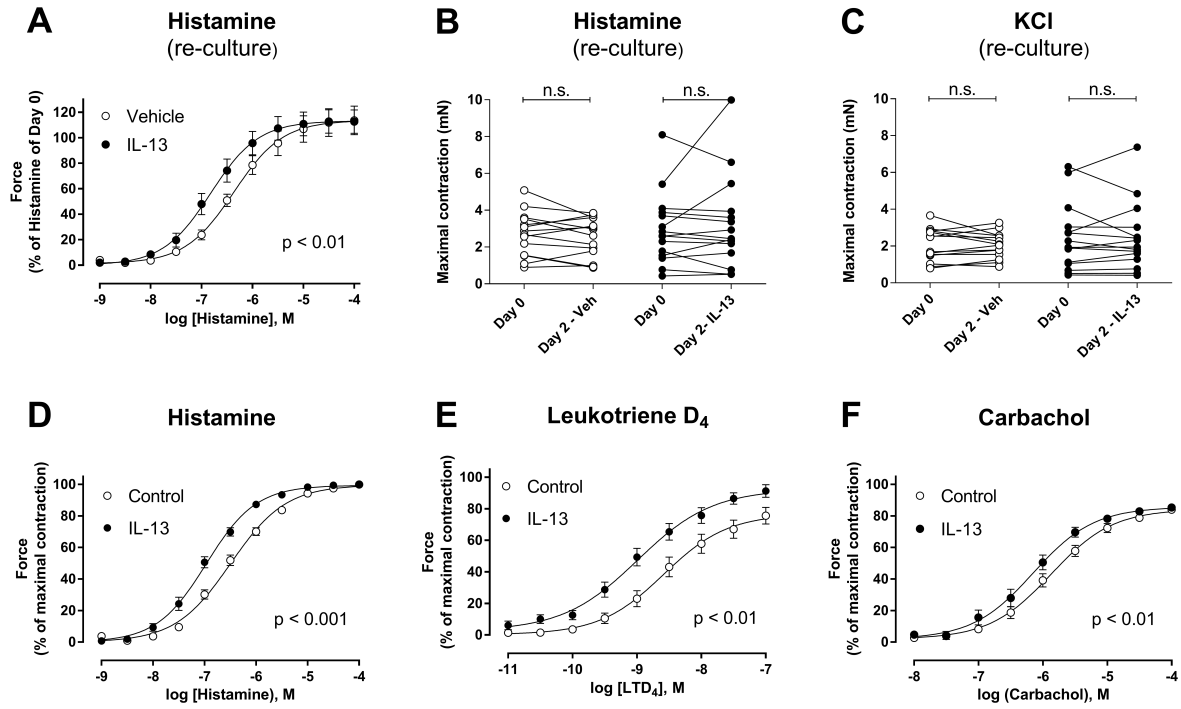
HASMC were incubated for 24-hours with IL-13, IL-4, IL-5 and IL-17A and mRNA expression was examined for **A)** the histamine 1 receptor (*HRH1*), **B)** the cysteinyl leukotriene receptor 1 (*CYSLTR1*). Data are presented as mean $\pm$ SEM (N:3; n:3). Significance is shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus vehicle control.

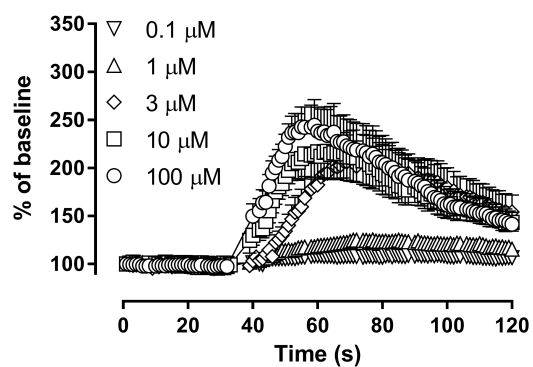
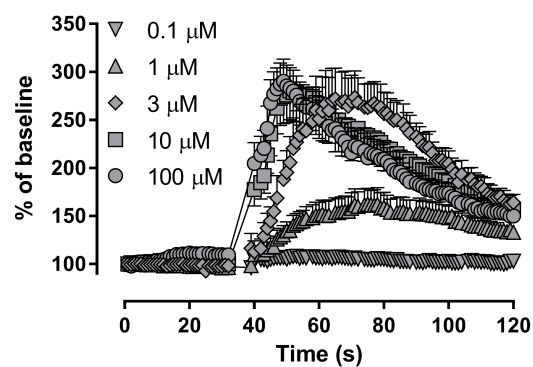
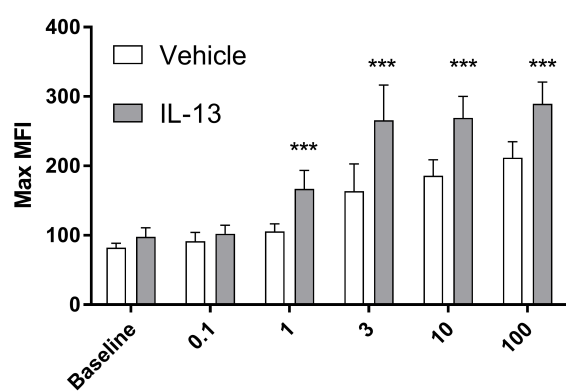
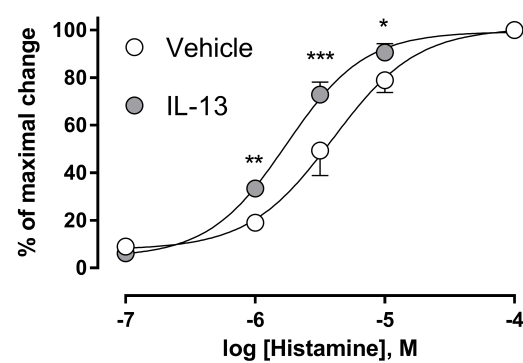
Figure 5: The effect of dexamethasone and STAT-6 inhibition on IL-13 induced hyperreactivity.

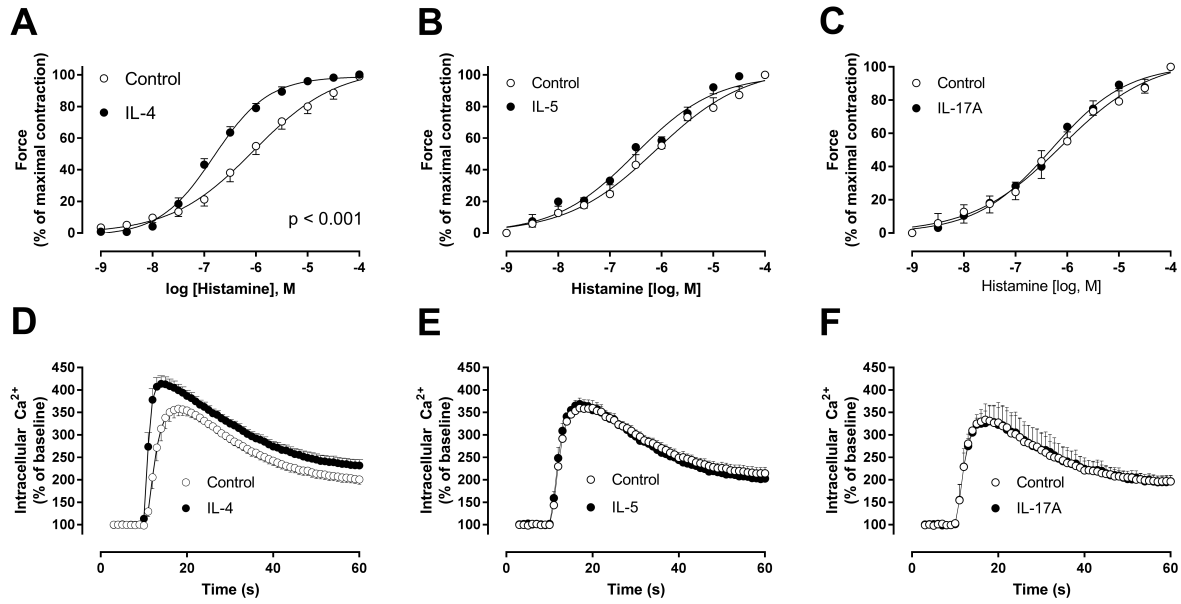
A) Cumulative contractile responses to histamine in human bronchi cultured for 2 days in the presence of IL-13 and/or 100 nM dexamethasone (Dex). **B)** Mobilization of intracellular Ca^{2+} induced by histamine (0.1-100 μM) and **C)** expression of *HRH1* in HASM cells incubated for 24-hours with BSA (0.1%), dexamethasone (100 nM), IL-13 (100 ng/ml) or IL-13 + dexamethasone. Changes in intracellular Ca^{2+} expressed as percentages of each treatment's own maximal change in MFI (indexed at 100%). All data are presented as mean $\pm$ SEM, from investigations in human bronchi (N:4; n:6) and HASM cells (N:3; n:4-9). **D)** Representative western blot of STAT-6 phosphorylation in HASM cells exposed for 1-hour to IL-13 (100 ng/ml), vehicle control, the STAT-6 inhibitor AS1517499 (100 nM) or dexamethasone (100 nM). **E)** Densitometric analysis of STAT-6 phosphorylation, normalized for GAPDH and presented as % increase from vehicle (n:3). **F)** Cumulative contractile responses to histamine in human bronchi cultured for 2-days in the presence of IL-13 and 100 nM AS1517499 (N:3; n:5). pEC₅₀ values for the different treatments in human bronchi were statistically compared to each other by a one-way ANOVA with a Bonferroni post-test. AS1517499 and dexamethasone were administered 1-hour before treatment with IL-13.

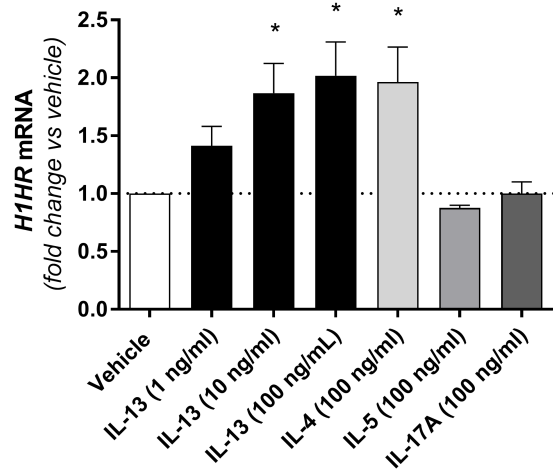
Figure 6. **The effect of dupilumab on IL-13 and IL-4 driven hyperreactivity in human bronchi and HASM cells.**

Cumulative concentration response curves to histamine, were performed in human bronchial rings treated for two days with **A)** IL-13 or **B)** IL-4 in the presence or absence of 1 μ M dupilumab. Paired t-tests were used to statistically compare the pEC₅₀ values of cytokine plus dupilumab treated bronchial rings with their matched IL-14 or IL-13-treated segment isolated from the same bronchi. Maximal induction of intracellular Ca²⁺ by 100 μ M histamine in HASMC stimulated for 24-hours with **C)** IL-13 or **D)** IL-4 in the presence or absence of dupilumab. Data are presented as mean $\pm$ SEM in human bronchi (N:3; n:7) and HASMC (N:1; n:3).



A**B****C****D**



A**B**