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

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GRAPHICAL ABSTRACT

IL-13 and IL-4, but not IL-5 nor IL-17A, induce hyperresponsiveness in isolated human small airways
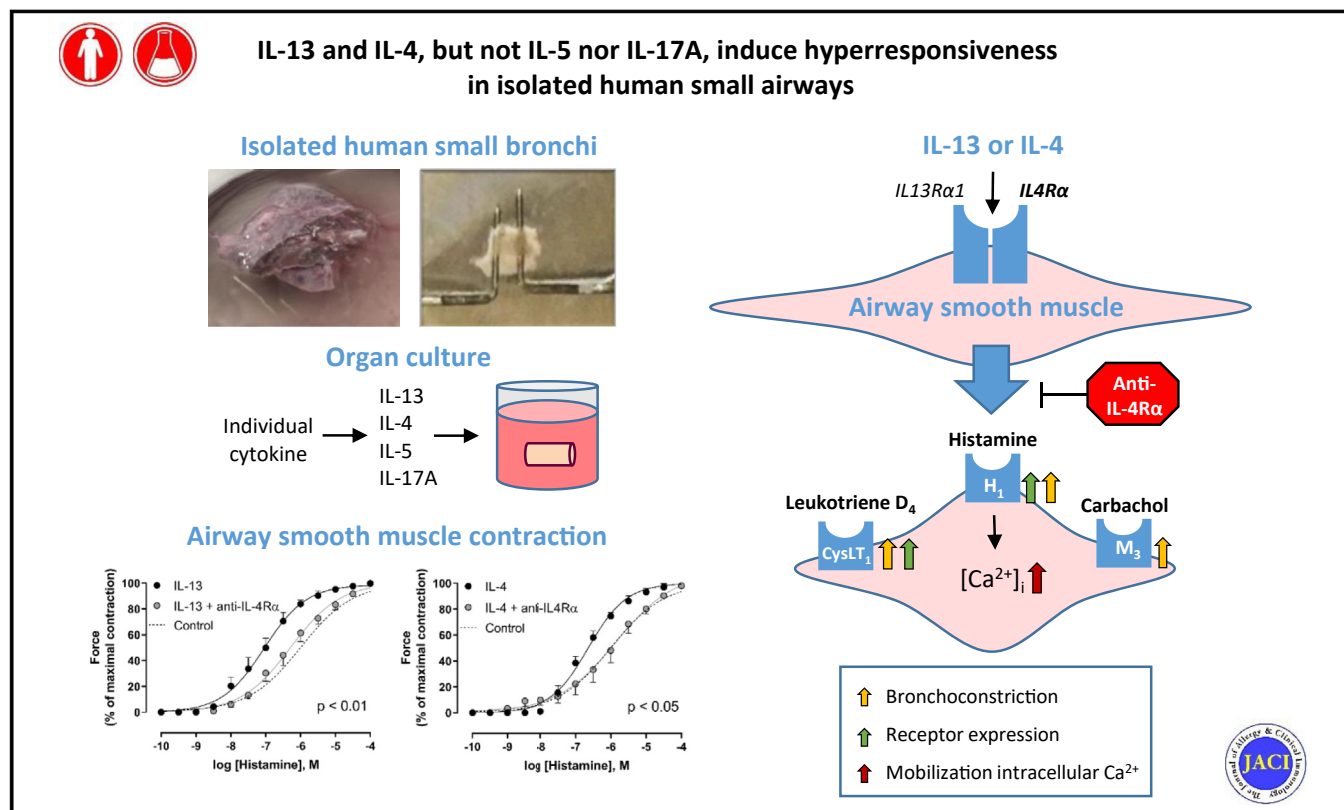
Background: Specific inflammatory pathways are indicated to contribute to severe asthma, but their individual involvement in the development of airway hyperresponsiveness remains unexplored.
Objective: This experimental study in human small bronchi aimed to provide insight into which of the type 2 and type 17 cytokines cause hyperresponsiveness of airway smooth muscle.

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

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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 human airway smooth muscle cells, IL-13 and IL-4, but not IL-5 and IL-17A, enhanced the histamine-induced Ca^{2+} mobilization that was accompanied with increased mRNA expression of histamine H_1 and cysteinyl leukotriene $CysLT_1$ receptors. RNA sequencing of isolated bronchi confirmed the IL-13–mediated upregulation 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+} mobilization, 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 human airway smooth muscle cells.
Conclusions: The glucocorticoid-insensitive hyperresponsiveness in isolated human airways induced by IL-13 and IL-4 provides further evidence that the IL-4Rα pathway should be targeted as a new strategy for the treatment of airway hyperresponsiveness in asthma. (J Allergy Clin Immunol 2020;145:808-17.)

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 stimuli.[1] Although glucocorticoids, the cornerstone therapy in asthma, reduce inflammation and cause some reduction of AHR, individuals with asthma remain hyperresponsive compared with healthy subjects.[2,3] As a result of our increased understanding of the heterogeneity in asthma and its molecular phenotypes,[4] novel therapies are being developed that specifically block the actions of particular cytokines considered to be central to the pathogenesis of asthma.[5-7] Clinical studies have shown that targeting IL-5 and the common receptor for IL-4 and IL-13, IL-4Rα, reduces asthma exacerbations in subsets of patients with asthma 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 aimed 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 mice[9-11] and rabbits[12] have shown that IL-13 can enhance contractions of rodent airways. Similar effects have been observed for IL-5,[13] IL-17A,[14] and IL-4[15] in animal models. Importantly however, very few studies have translated these animal data into human airways.[16-19] The few reports using human tissues create a scattered picture because of the different experimental models used (isolated bronchi vs precision cut lung slices) or varying protocols (eg, duration of exposure). The 4 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 in which isolated human airways are subjected to controlled exposures of inflammatory mediators.[20] Additional studies were performed using human airway smooth muscle cells (HASMCs) to further define the mechanisms implicated by the observations in isolated human bronchi.

METHODS
Human tissue preparation

With permission of the Regional Ethical Review Board in Stockholm (reference 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 women and 9 men; median age, 69 years, range, 44-82; Table I). From each lung, 1 to 3 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 intraindividual variability regarding the responsiveness of human airway preparations.[21]

Organ culture

Human bronchi (0.5-2 mm) were dissected and segments were cultured in Dulbecco modified Eagle medium supplemented with 1% penicillin/streptomycin (Life Technologies, Carlsbad, Calif) and placed in a humidified incubator at 37°C at 95% O_2 and 5% CO_2 for 2 days in the presence of 100 ng/mL IL-13, IL-4, IL-5, or IL-17A and transferred to fresh medium and treatments every day.[20] For reculture experiments, first baseline contractility was evaluated (*day 0*), and subsequently the bronchial rings were placed back into culture for 2 days in the presence of 100 ng/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 1.5 mN, during a 90-minute equilibration period, and viability was tested by administrations of 60 mM KCl.[22] Contractile responses to histamine (0.1 nM to 100 μM), leukotriene D_4 (LTD_4) (0.01 nM to 100 nM), and carbachol (10 nM to 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 segment.[23]

Culture of HASMCs

Human primary bronchial smooth muscle cells (passage 4-6; Promocell [Heidelberg, Germany]/Lonza [Basel, Switzerland]) were grown in Dulbecco modified Eagle medium supplemented with 10% FBS and penicillin/streptomycin. Confluent cells were serum-deprived (0.3% FBS) for 1 day before 1-day stimulations with 100 ng/mL IL-4, IL-5, IL-13, or IL-17A.

TABLE I. Patient characteristics of the 33 patients studied in this study

Characteristic	Value
Sex: female, %	73
Age (y)	69 (44-82)
Body mass index (kg/m^2)	25.5 (18.4-38.9)
C-reactive protein (mg/L)	2 (1-71)
Hemoglobin (g/L)	133 (109-177)
Leukocyte particle concentration ($\times 10^9$/L)	6.9 (4.9-17.4)
Current smoker	12
Ex-smoker	17
Chronic obstructive pulmonary disorder	5
Asthma	2
Allergy	7

Ex-smoker is defined as a person who has not smoked for the last 12 mo. The numbers represent absolute values or median and range.

Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} fluxes were measured in HASMCs according to previously described protocols.[24] In brief, detached cells were incubated in PBS containing 3 μM Fluo-4, 0.02% pluronic F-127, and 2.5 μM probenecid, or Fluo-4 Direct Calcium Assay Kit (ThermoFisher, Waltham, Mass) according to the manufacturer's recommendations. Ca^{2+} fluxes were analyzed by flow cytometry (LSR Fortessa; BD Biosciences, Franklin Lakes, NJ) or using a SpectraMax iD3 (Molecular Devices, San Jose, Calif).

Western blotting

Standard methodology was used to detect phosphoSTAT-6 (phosphoY641; Abcam, Cambridge, United Kingdom) in relation to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (14C10, Cell Signaling Technology, Danvers, Mass).

Polymerase chain reaction

RT-PCR was conducted using Taqman Gene Expression Master Mix and primers of Applied Biosystems (Waltham, Mass). Changes in gene expression were determined using the $^{\Delta\Delta}$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 analyzed using Qubit and Agilent Tapestation. Indexed cDNA libraries were normalized 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 (Santa Clara, Calif).

Drugs and materials

Recombinant human IL-13, IL-4, IL-5, and IL-17A were obtained from R&D Systems (Minneapolis, Minn). Carbachol, dexamethasone, and histamine dihydrochloride were purchased from Sigma-Aldrich (St Louis, Mo). AS1517499 was purchased from Axon Medchem LLC (Groningen, The Netherlands). LTD$_4$ was obtained from Cayman Chemical (Ann Arbor, Mich). Dupilumab was obtained from Apoteket Produktion & Laboratorier AB (Stockholm, Sweden). Interventions with dexamethasone, AS1517499, and dupilumab were administrated 60 minutes before the initial addition of cytokines.

Calculations and statistics

All data are presented as means ± SEM. Information on sample size (n) and the number of individual patients (N) studied for each individual experiment is provided in the figure legends. For the statistical analysis of responses in human bronchi, paired segments of bronchi were used, with 1 segment from an individual bronchi always used as the control (nontreated).

The concentration-response curve values for pEC$_{50}$ were calculated using nonlinear regression analysis. Fold-changes were calculated from these acquired pEC$_{50}$ values using the following formula:

$$fold\ change = \frac{10^{-pEC50(control)}}{10^{-pEC50(cytokine)}}$$

Paired *t* tests were used for comparisons between 2 groups and 1- or 2-way ANOVA with the Bonferroni posttest for comparisons between multiple groups. Graph Pad Prism 5.01 (San Diego, Calif) 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* values were adjusted for multiple testing using the Benjamini and Hochberg method.

Additional information on the applied methodologies can be found in this article's Online Repository at www.jacionline.org.

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 with segments cultured with vehicle (pEC$_{50}$: 6.8 ± 0.1 vs 6.4 ± 0.1; *P* < .01). There was however no change in the near-maximal contractions (113% ± 9% vs 114% ± 11%) induced in the same segments before the 2 days of exposure (*day 0*) (Fig 1, *A*). Likewise, the amplitude of the maximal contractions induced by histamine (Fig 1, *B*), or 60 mM KCl, a non–receptor-mediated contractile agent (Fig 1, *C*), was the same on *day 0* compared with that on *day 2*, for both vehicle (*P* = .99 and .27) and IL-13–treated segments (*P* = .99 and .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 vs 6.5 ± 0.1; *P* < .001), a 3.2-fold increase in the potency of LTD$_4$ (pEC$_{50}$: 9.0 ± 0.1 vs 8.5 ± 0.1; *P* < .01), and a 2.5-fold increase in the potency of carbachol (pEC$_{50}$: 6.3 ± 0.1 vs 5.9 ± 0.1; *P* < .01) (Fig 1, *D-F*). IL-13 also enhanced the maximal contractile response toward LTD$_4$ (*E*$_{max}$: 92.4% ± 3.8% vs 76.2% ± 5.2%; *P* < .05; Fig 1, *E*), whereas the maximal contractile responses toward carbachol were unchanged (Fig 1, *F*).

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 data set for the response to histamine (Fig 1, *D*), which also enabled us to evaluate the effect of IL-13 (pEC$_{50}$: 6.9 ± 0.1) and vehicle (pEC$_{50}$ 6.4 ± 0.1) between patients (see Fig E1 in this article's Online Repository at www.jacionline.org).

Effect of IL-13 on intracellular Ca^{2+} mobilization

To investigate the possible mechanisms involved, the effect of IL-13 on histamine-induced intracellular Ca^{2+} mobilization was examined in cultured HASMCs. Treatment with IL-13 for 24 hours enhanced the concentration-dependent mobilization of intracellular Ca^{2+} by histamine (Fig 2, *A-D*), increasing both amplitude (Fig 2, *C*) and potency compared with vehicle (Fig 2, *D*; pEC$_{50}$: 5.7 ± 0.1 vs 5.4 ± 0.1; *P* < .01).

Effects of IL-4, IL-5, and IL-17A on airway contraction and intracellular Ca^{2+} mobilization

Similar to IL-13, IL-4 caused a 5.1-fold increase in the potency of the histamine-induced contraction (pEC$_{50}$: 6.8 ±
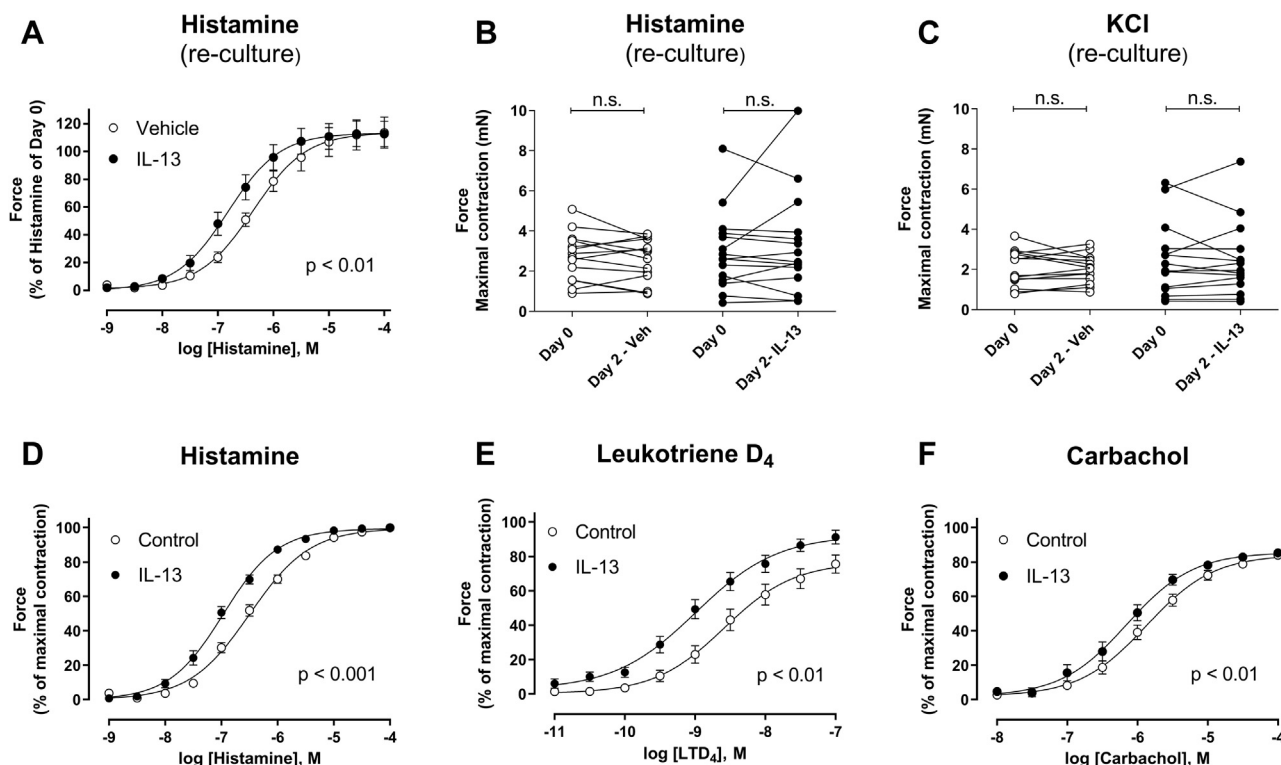
FIG 1. Effects of IL-13 on the potency and amplitude of contractile responses in human small bronchi. Contractile responses in response to histamine and potassium chloride (KCl) were assessed in each individual human bronchial ring preceding any treatment (*day 0*). The same bronchial rings were then placed back into culture and treated for 2 days with 0.1% BSA (○) or 100 ng/mL IL-13 (●), after which the contractile responses to histamine and KCl were reexamined (*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 before (*day 0*) and after treatment (*day 2*) with vehicle (veh) or IL-13. Data are presented as mean ± SEM (N: 7; n: 14-15). Statistical analysis was performed by paired *t* tests comparing the pEC_{50} values (Fig 1, *A*) and absolute contractions (Fig 1, *B* and *C*) on *day 2* with the outcomes of the same bronchial preparation on *day 0*. For subsequent experiments, human bronchial rings were no longer recultured, but instead directly exposed to vehicle or 100 ng/mL IL-13. Cumulative concentration-response curves to (**D**) histamine, (**E**) LTD_4, and (**F**) carbachol after 2 days of exposure of vehicle or IL-13 are presented. Because 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 pEC_{50} values and absolute contraction of IL-13–treated bronchial rings with their matched untreated controls from the same isolated bronchi. *n.s.*, Nonsignificant.

0.1 vs 6.1 ± 0.1; $P < .05$; Fig 3, *A*) and an increased histamine-induced intracellular Ca^{2+} mobilization (Fig 3, *D*). In contrast, culture with IL-5 or IL-17A caused no change in the potency of histamine (pEC_{50}: 6.3 ± 0.2 and 6.4 ± 0.2, respectively) compared with vehicle (6.2 ± 0.1) (Fig 3, *B* and *C*), and no alteration of the histamine-induced intracellular Ca^{2+} mobilization (Fig 3, *E* 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 histamine receptor H1 (*HRH1*) and cysteinyl leukotriene receptor 1 (*CYSLTR1*) were investigated in HASMCs. IL-13 caused a concentration-dependent increase in mRNA for both *HRH1* (Fig 4, *A*) and *CYSLTR1* (Fig 4, *B*). 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 H_1 (*HRH1*), $CysLT_1$ (*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 II). 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 presumed decoy receptor *IL13A2*,[25] which has also been implicated in airway fibrosis,[26] whereas neither the expression of *CHRM3* nor that of 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
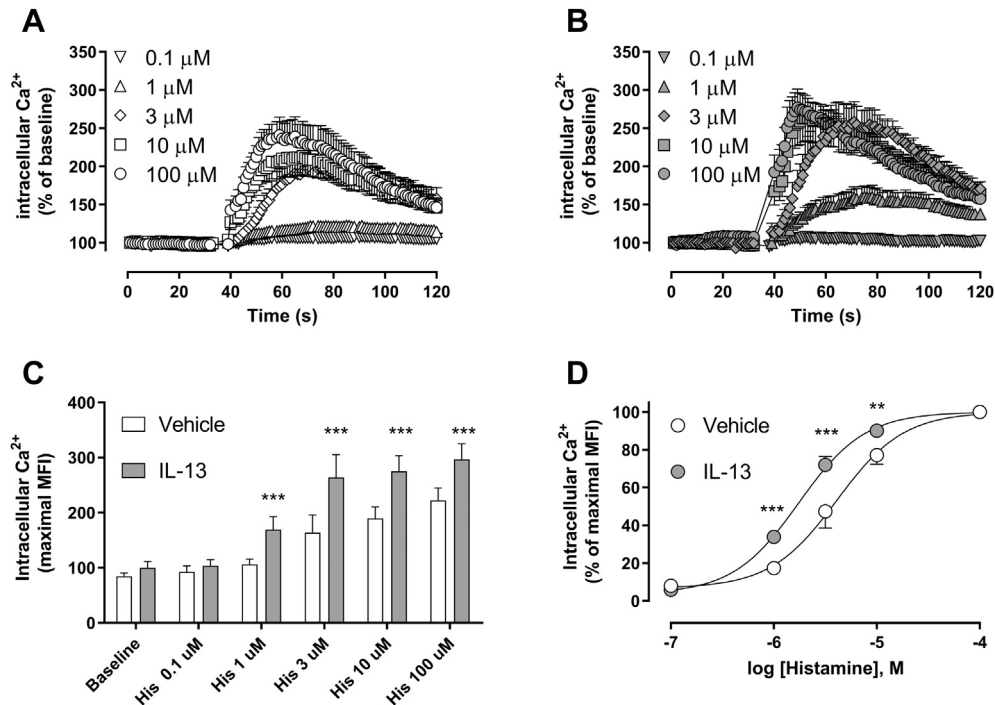
FIG 2. Effect of IL-13 on intracellular Ca^{2+} mobilization in cultured HASMCs. Compiled traces of the relative histamine-induced increases in intracellular Ca^{2+} in cells treated for 24 hours with (**A**) 0.1% BSA or (**B**) 100 ng/mL IL-13. **C,** Intracellular Ca^{2+} mobilization induced by histamine measured as mean fluorescence intensity (MFI). **D,** 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 the mean $\pm$ SEM (N: 3; n: 4-8); significance is presented by *$P < .05$, **$P < .01$, and ***$P < .001$.

bronchi (Fig 5, *A*). Similarly in HASMCs, the IL-13–induced increase in the potency of histamine-induced intracellular Ca^{2+} mobilization was not inhibited by dexamethasone (Fig 5, *B*). The increase in *HRH1* mRNA expression caused by IL-13 (2.3-fold change vs vehicle) was also replicated in this set of experiments (Fig 5, *C*). IL-13 increased the expression of *HRH1* to a similar degree after treatment with dexamethasone (2.0-fold vs dexamethasone), although the glucocorticoid reduced the baseline expression of *HRH1*.

Effect of signal transducer and activator of transcription 6 inhibition on IL-13–induced effects

Signal transducer and activator of transcription 6 (STAT6) is a downstream signaling pathway of IL-13.[27] IL-13 induced a profound phosphorylation of STAT6 in HASMCs (Fig 5, *D*), which AS1517499 (100 nM), a selective STAT6 inhibitor,[28] inhibited, whereas dexamethasone had no effect (Fig 5, *D* and *E*). However, pretreatment with AS1517499 (100 nM) did not affect the IL-13–induced hyperresponsiveness of the bronchi (Fig 5, *F*).

### Effect of IL-4Rα antibody treatment on IL-13– and IL-4–induced effects in human bronchi and HASMCs

Pretreatment with the IL-4Rα antibody dupilumab (1 μM) before IL-13 (Fig 6, *A*) and IL-4 (Fig 6, *B*) administration attenuated the increase in potency for histamine (pEC$_{50}$: 7.1 $\pm$ 0.1 vs 6.3 $\pm$ 0.1 and 6.7 $\pm$ 0.1 vs 6.1 $\pm$ 0.2, respectively; $P < .05$).

Similarly, dupilimab blocked the increase in the maximal effect of histamine-induced intracellular Ca^{2+} mobilization caused by IL-13 (Fig 6, *C*) and IL-4 (Fig 6, *D*) in HASMCs.

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 HASMCs. 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 upregulation of the receptors for histamine and LTD$_4$. Furthermore, both dexamethasone and STAT6 inhibition failed to alter the IL-13–induced responses in bronchi and HASMCs. In both models, pretreatment with the IL-4Rα 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 reculture protocol that mitigates possible limitations caused by
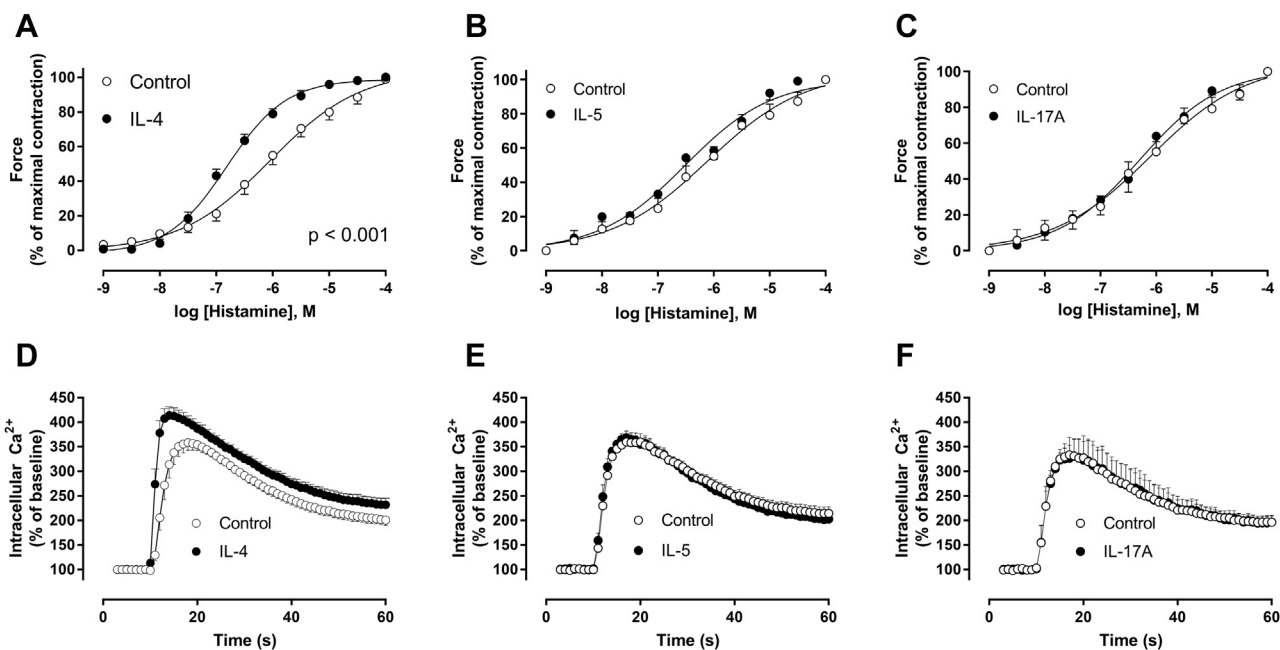
FIG 3. Effects of IL-4, IL-5, and IL-17A on airway contraction of human bronchi and Ca^{2+} mobilization in HASMCs. Cumulative concentration-response curves for histamine in human bronchial rings cultured for 2 days in the presence of (**A**) IL-4, (**B**) IL-5, or (**C**) IL-17A. Data are presented as mean $\pm$ SEM (N: 5; n: 6-11). Statistical analysis was performed by paired t tests comparing the pEC_{50} values of cytokine-treated bronchial rings with their matched untreated controls from the same isolated bronchi. Effects of 24-hour stimulation with (**D**) IL-4, (**E**) IL-5, or (**F**) IL-17A on the mobilization of intracellular Ca^{2+} in HASMCs following 100 μM of histamine. Data are presented as mean $\pm$ SEM (N: 3-5; n: 3-5).
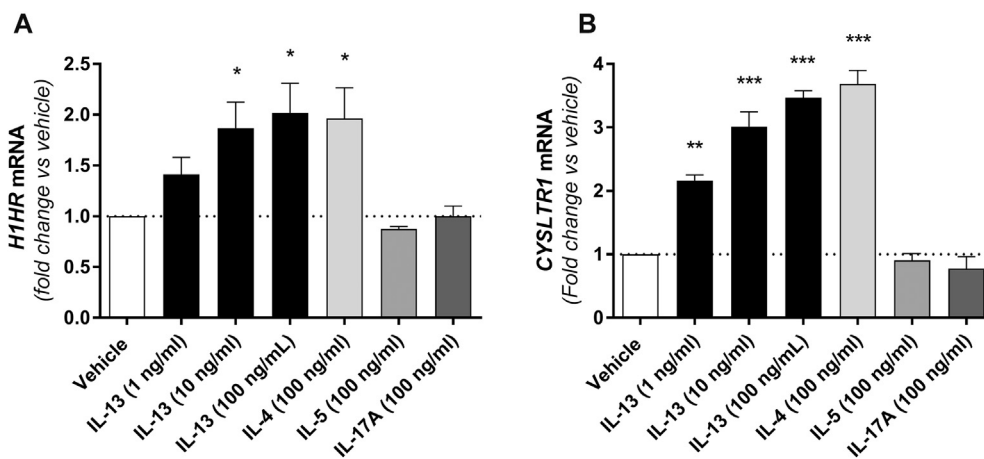

FIG 4. Effects of asthma-related interleukins on gene expression of *HRH1* and *CYSLTR1* in HASMCs. HASMCs were incubated for 24 hours with IL-13, IL-4, IL-5, and IL-17A and mRNA expression was examined for (**A**) *HRH1* and (**B**) *CYSLTR1*. Data are presented as mean $\pm$ SEM (N: 3; n: 3). Significance is shown as *$P < .05$, **$P < .01$, and ***$P < .001$ vs vehicle control.

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 depolarization,[31] was not altered after culture with IL-13. Because 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 signaling,

upstream of the contractile machinery. The potential impact of these changes in smooth muscle hyperresponsiveness is more profound in the *in vivo* situation because air flow is determined by airway resistance according to Poiseuille's law, which is inversely related to the fourth power of the airway radius.[34]

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
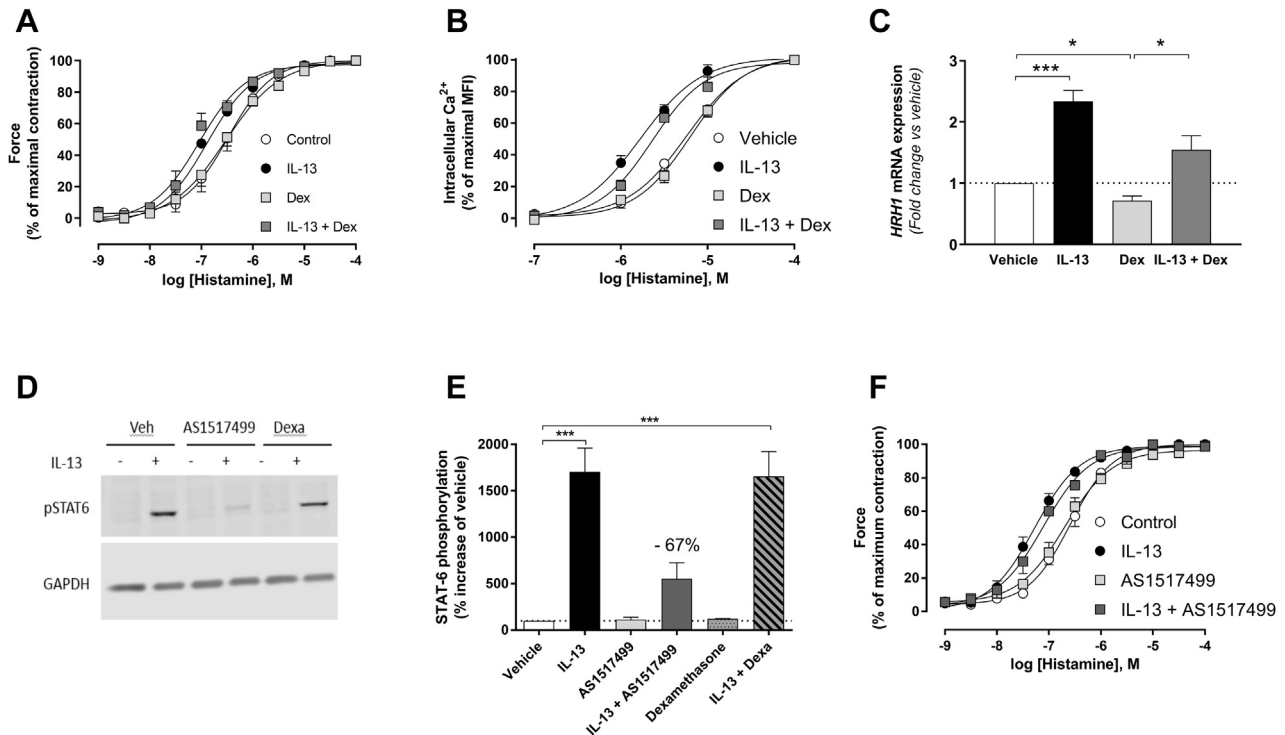
FIG 5. The effect of dexamethasone and STAT6 inhibition on IL-13–induced hyperreactivity. **A,** Cumulative contractile responses to histamine in human bronchi cultured for 2 days in the presence of IL-13 and/or 100 nM dexamethasone (Dex). **B,** Mobilization of intracellular Ca^{2+} induced by histamine (0.1-100 μM). **C,** Expression of *HRH1* in HASMCs 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 ± SEM, from investigations in human bronchi (N: 4; n: 6) and HASMCs (N: 3; n: 4-9). **D,** Representative western blot of STAT6 phosphorylation in HASMCs exposed for 1 hour to IL-13 (100 ng/mL), vehicle (veh) control, the STAT6 inhibitor AS1517499 (100 nM), or dexamethasone (100 nM). **E,** Densitometric analysis of STAT6 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). pEC$_{50}$ values for the different treatments in human bronchi were statistically compared with each other by a 1-way ANOVA with a Bonferroni posttest. AS1517499 and dexamethasone were administered 1 hour before treatment with IL-13. *MFI,* Mean fluorescence intensity.

in explanted human bronchi, which has not been shown before. Furthermore, it was demonstrated that IL-13 increased the responses to another mast cell mediator, LTD$_4$, as well as the cholinergic agonist carbachol. These findings are in line with previous observations that IL-13 increased responses to histamine and the thromboxane A2 receptor agonist U46619 in human bronchi,[17] and to carbachol in human precision cut lung slices.[16] These studies primarily observed an increase in the amplitude of the contractions. In our setting, only the contractile response to LTD$_4$ was increased in amplitude, most likely because LTD$_4$ 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 Ca^{2+} influx, which precedes airway contraction, this study confirmed previous findings in HASMCs, showing that IL-13 increases Ca^{2+} mobilization.[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 HASMCs observed that IL-13 increased the effects of other agonists activating G protein–coupled receptors, such as bradykinin and thrombin,[35] but not KCl.[37] The absence of effect on submaximal

KCl contraction was also shown for the airway segments used in the present study. Thus, the IL-13–induced increase in Ca^{2+} influx indicates that the alteration in contraction is specific for the G protein–coupled receptor pathway and regulated at the level of Ca^{2+} mobilization or above.

Both IL-13 and IL-4 increased in agreement with previous studies the expression of *HRH1* and *CYSLTR1* in HASMCs.[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 potency of the agonist, or only an increase in potency if the agonist already at the basal state elicits a maximal tissue effect.[39] These processes are exactly what was observed for the effects of IL-13 on the contractions to LTD$_4$ and histamine, respectively, in human bronchi, indicating that one component of the cytokine-induced increase in airway contractility is potentially due to the upregulation of contractile receptors.

Earlier studies in HASMCs have however shown that concurrent inhibition of extracellular signal–regulated kinases and c-Jun N-terminal kinase could prevent the IL-13–driven enhancement of histamine-induced Ca^{2+} mobilization without
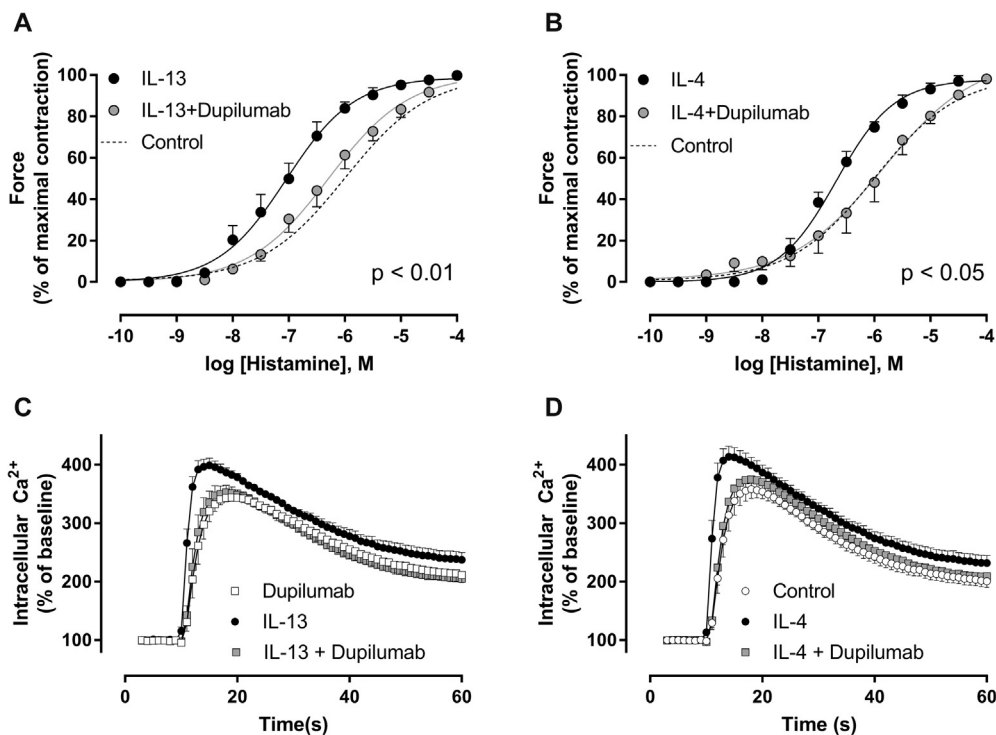
FIG 6. The effect of dupilumab on IL-13– and IL-4–driven hyperreactivity in human bronchi and HASMCs. Cumulative concentration-response curves to histamine were created in human bronchial rings treated for 2 days with (**A**) IL-13 or (**B**) IL-4 in the presence or absence of 1 μM dupilumab. Paired t tests were used to statistically compare the pEC$_{50}$ 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 Ca^{2+} by 100 μM histamine in HASMCs 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 HASMCs (N: 1; n: 3).

TABLE II. RNA-sequencing analysis of human small bronchi*

Receptor	BaseMean	Fold change	P value	P adjusted
HRH1	548 $\pm$ 73	1.73	6.09×10^{-07}	.000152
CYSLTR1	75 $\pm$ 10	2.67	2.13×10^{-09}	9.66×10^{-07}
CHRM3	33 $\pm$ 3	0.98	.911604	.999951
IL4R	1989 $\pm$ 43	1.11	.263766	.999951
IL2RG	983 $\pm$ 24	0.88	.339479	.999951
IL13RA1	2000 $\pm$ 109	0.83	.10588	.999951
IL13RA2	704 $\pm$ 87	2.38	9.62×10^{-09}	3.45×10^{-06}
IL5RA	347 $\pm$ 72	1.11	.604956	.999951
CSF2RB	566 $\pm$ 17	1.10	.535423	.999951
IL17RA	675 $\pm$ 28	1.05	.545536	.999951
IL17RC	205 $\pm$ 26	0.94	.721519	.999951

CHRM3, Cholinergic receptor muscarinic 3; *CSF2RB*, colony-stimulating factor 2 receptor β (forms heterodimer with *IL5RA*, with IL-5 as agonist); *IL4R*, IL-4 receptor; *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 IL-13 as agonists); *IL13A2*, IL-13 receptor α2 (soluble IL-13 receptor α2); *IL5RA*, IL-5 receptor subunit α; *IL17RA*, IL-17 receptor A; *IL17RC*, IL-17 receptor C (*IL17RA* and *IL17RC* form a heterodimer with IL-17A as agonist).
The data are expressed as baseMean (the mean of optical intensity values corrected for background), fold change (real number), *P* value, and *P* adjusted (adjusted *P* value for multiple comparisons). Data are obtained from 4 donors. *N* = 4; *n* = 4.
*Analysis of RNA expression from segments that were cultured 1 d in absence and presence with IL-13 focusing on the receptors of interest in this study.

affecting the upregulation of HRH1 mRNA, raising the possibility of alternative mechanisms other than receptor regulation.[36] This outcome does not however exclude a role for receptor regulation, because the effects of these interventions were solely examined on the maximal mobilization of intracellular Ca^{2+} 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 *CHRM3* in our study also suggests that the enhanced response to activation of muscarinic M$_3$ 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 Ca^{2+}.[9,11,35,36] Taken together, this indicates that at least 2 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 IL-4Rα/IL-13Rα1.

Of particular relevance to the clinical perspective, we found that pretreatment with dexamethasone failed to inhibit the IL-13–induced increase in sensitivity of the bronchi and Ca^{2+} mobilization in HASMCs. 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 mediators,[40] whereas as this study shows, once these cytokines are generated, their actions cannot be overcome by glucocorticoids.

STAT6 signaling has been targeted for the treatment of asthma.[41] We found that the STAT6 inhibitor AS1517499

markedly decreased IL-13–induced phosphorylation in HASMCs, 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 STAT6 inhibition in HASMCs,[36] 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 IL-4Rα, markedly blocked the effects induced by both IL-13 and IL-4. This suggests that IL-4Rα probably mediates the effects of IL-13 and IL-4 through dimerization with IL13Rα1.[42] 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 asthma[5] and in patients with glucocorticoid-dependent severe asthma.[43] 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 patients with asthma 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 asthma,[4] 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-5[13] and IL-17A on human airways[14,18] may relate to our use of small bronchi ranging from the 8th to the 13th generation, compared with 1st to 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 mAb brodalumab failed to show efficacy in patients with asthma.[7] 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 in which the influence of 4 established proinflammatory 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 2 cytokines can directly contribute to the development of AHR at the level of the smooth muscle. These findings warrant follow-up bronchoprovocation studies in which the effect of combined IL-4/IL-13 antagonism on AHR is examined in patients with asthma. Brittle asthma with pronounced AHR is one example of a phenotype that is difficult to treat, and might show particular benefit from such interventions.

Key messages

- **IL-4 and IL-13 induce hyperresponsiveness of the airway smooth muscle in isolated human small airways.**
- **This glucocorticoid-insensitive hyperrresponsiveness could be prevented by the IL-4Rα antagonist dupilumab.**
- **This study warrants follow-up bronchoprovocation studies in patients with asthma in which the effect of combined IL-4/IL-13 antagonism on AHR is examined.**

REFERENCES

1. Cockcroft DW, Davis BE. Mechanisms of airway hyperresponsiveness. J Allergy Clin Immunol 2006;118:551-9, quiz 60-1.
2. Juniper EF, Kline PA, Vanzieleghem MA, Ramsdale EH, O'Byrne PM, Hargreave FE. Effect of long-term treatment with an inhaled corticosteroid (budesonide) on airway hyperresponsiveness and clinical asthma in nonsteroid-dependent asthmatics. Am Rev Respir Dis 1990;142:832-6.
3. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. Am J Respir Crit Care Med 1999;159:1043-51.
4. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med 2012;18:716-25.
5. Castro M, Corren J, Pavord ID, Maspero J, Wenzel S, Rabe KF, et al. Dupilumab efficacy and safety in moderate-to-severe uncontrolled asthma. N Engl J Med 2018;378:2486-96.
6. Bel EH, Ten Brinke A. New anti-eosinophil drugs for asthma and COPD: targeting the trait! Chest 2017;152:1276-82.
7. Busse WW, Holgate S, Kerwin E, Chon Y, Feng J, Lin J, et al. Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. Am J Respir Crit Care Med 2013;188:1294-302.
8. An SS, Bai TR, Bates JH, Black JL, Brown RH, Brusasco V, et al. Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma. Eur Respir J 2007;29:834-60.
9. Tliba O, Deshpande D, Chen H, Van Besien C, Kannan M, Panettieri RA Jr, et al. IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle. Br J Pharmacol 2003;140:1159-62.
10. Farghaly HS, Blagbrough IS, Medina-Tato DA, Watson ML. Interleukin 13 increases contractility of murine tracheal smooth muscle by a phosphoinositide 3-kinase p110delta-dependent mechanism. Mol Pharmacol 2008;73:1530-7.
11. Chiba Y, Nakazawa S, Todoroki M, Shinozaki K, Sakai H, Misawa M. Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA protein. Am J Respir Cell Mol Biol 2009;40:159-67.
12. Grunstein MM, Hakonarson H, Leiter J, Chen M, Whelan R, Grunstein JS, et al. IL-13-dependent autocrine signaling mediates altered responsiveness of IgE-sensitized airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 2002;282:L520-8.
13. Rizzo CA, Yang R, Greenfeder S, Egan RW, Pauwels RA, Hey JA. The IL-5 receptor on human bronchus selectively primes for hyperresponsiveness. J Allergy Clin Immunol 2002;109:404-9.
14. Kudo M, Melton AC, Chen C, Engler MB, Huang KE, Ren X, et al. IL-17A produced by alphabeta T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. Nat Med 2012; 18:547-54.
15. Bryborn M, Adner M, Cardell LO. Interleukin-4 increases murine airway response to kinins, via up-regulation of bradykinin B1-receptors and altered signalling along mitogen-activated protein kinase pathways. Clin Exp Allergy 2004;34:1291-8.

16. Cooper PR, Lamb R, Day ND, Branigan PJ, Kajekar R, San Mateo L, et al. TLR3 activation stimulates cytokine secretion without altering agonist-induced human small airway contraction or relaxation. Am J Physiol Lung Cell Mol Physiol 2009;297:L530-7.

17. Khaddaj-Mallat R, Sirois C, Sirois M, Rizcallah E, Morin C, Rousseau E. Reversal of IL-13-induced inflammation and Ca(2+) sensitivity by resolvin and MAG-DHA in association with ASA in human bronchi. Prostaglandins Other Lipid Mediat 2015;121:145-54.

18. Willis CR, Siegel L, Leith A, Mohn D, Escobar S, Wannberg S, et al. IL-17RA signaling in airway inflammation and bronchial hyperreactivity in allergic asthma. Am J Respir Cell Mol Biol 2015;53:810-21.

19. Danov O, Jimenez Delgado SM, Obernolte H, Seehase S, Dehmel S, Braubach P, et al. Human lung tissue provides highly relevant data about efficacy of new anti-asthmatic drugs. PLoS One 2018;13:e0207767.

20. Adner M, Rose AC, Zhang Y, Sward K, Benson M, Uddman R, et al. An assay to evaluate the long-term effects of inflammatory mediators on murine airway smooth muscle: evidence that TNFalpha up-regulates 5-HT(2A)-mediated contraction. Br J Pharmacol 2002;137:971-82.

21. De Jongste J, Mons H, Van Strik R, Bonta I, Kerrebijn K. Human small airway smooth muscle responses in vitro: actions and interactions of methacholine, histamine and leukotriene C4. Eur J Pharmacol 1986;125:29-35.

22. Safholm J, Manson ML, Bood J, Al-Ameri M, Orre AC, Raud J, et al. Mannitol triggers mast cell-dependent contractions of human small bronchi and prostacyclin bronchoprotection. J Allergy Clin Immunol 2019;144:984-92.

23. Safholm J, Manson ML, Bood J, Delin I, Orre AC, Bergman P, et al. Prostaglandin E2 inhibits mast cell-dependent bronchoconstriction in human small airways through the E prostanoid subtype 2 receptor. J Allergy Clin Immunol 2015;136:1232-9.e1.

24. Larsson OJ, Manson ML, Starkhammar M, Fuchs B, Adner M, Kumlien Georen S, et al. The TLR7 agonist imiquimod induces bronchodilation via a nonneuronal TLR7-independent mechanism: a possible role for quinoline in airway dilation. Am J Physiol Lung Cell Mol Physiol 2016;310:L1121-9.

25. David M, Ford D, Bertoglio J, Maizel AL, Pierre J. Induction of the IL-13 receptor alpha2-chain by IL-4 and IL-13 in human keratinocytes: involvement of STAT6, ERK and p38 MAPK pathways. Oncogene 2001;20:6660-8.

26. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. Nat Med 2006;12:99-106.

27. Laporte JC, Moore PE, Baraldo S, Jouvin MH, Church TL, Schwartzman IN, et al. Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. Am J Respir Crit Care Med 2001;164:141-8.

28. Nagashima S, Yokota M, Nakai E, Kuromitsu S, Ohga K, Takeuchi M, et al. Synthesis and evaluation of 2-{[2-(4-hydroxyphenyl)-ethyl]amino}pyrimidine-5-carboxamide derivatives as novel STAT6 inhibitors. Bioorg Med Chem 2007;15:1044-55.

29. van der Wiel E, ten Hacken NH, Postma DS, van den Berge M. Small-airways dysfunction associates with respiratory symptoms and clinical features of asthma: a systematic review. J Allergy Clin Immunol 2013;131:646-57.

30. Telenga ED, van den Berge M, Ten Hacken NH, Riemersma RA, van der Molen T, Postma DS. Small airways in asthma: their independent contribution to the severity of hyperresponsiveness. Eur Respir J 2013;41:752-4.

31. Kohrogi H, Horio S, Ando M, Sugimoto M, Honda I, Araki S. Nifedipine inhibits human bronchial smooth muscle contractions induced by leukotrienes C4 and D4, prostaglandin F2 alpha, and potassium. Am Rev Respir Dis 1985;132:299-304.

32. Black JL, Marthan R, Armour CL, Johnson PR. Sensitization alters contractile responses and calcium influx in human airway smooth muscle. J Allergy Clin Immunol 1989;84:440-7.

33. Advenier C, Naline E, Renier A. Effects of Bay K 8644 on contraction of the human isolated bronchus and guinea-pig isolated trachea. Br J Pharmacol 1986;88:33-9.

34. Burgel PR. The role of small airways in obstructive airway diseases. Eur Respir Rev 2011;20:23-33.

35. Deshpande DA, Dogan S, Walseth TF, Miller SM, Amrani Y, Panettieri RA, et al. Modulation of calcium signaling by interleukin-13 in human airway smooth muscle: role of CD38/cyclic adenosine diphosphate ribose pathway. Am J Respir Cell Mol Biol 2004;31:36-42.

36. Moynihan B, Tolloczko B, Michoud MC, Tamaoka M, Ferraro P, Martin JG. MAP kinases mediate interleukin-13 effects on calcium signaling in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2008;295:L171-7.

37. Risse PA, Jo T, Suarez F, Hirota N, Tolloczko B, Ferraro P, et al. Interleukin-13 inhibits proliferation and enhances contractility of human airway smooth muscle cells without change in contractile phenotype. Am J Physiol Lung Cell Mol Physiol 2011;300:L958-66.

38. Espinosa K, Bosse Y, Stankova J, Rola-Pleszczynski M. CysLT1 receptor upregulation by TGF-beta and IL-13 is associated with bronchial smooth muscle cell proliferation in response to LTD4. J Allergy Clin Immunol 2003;111:1032-40.

39. Black JW, Leff P. Operational models of pharmacological agonism. Proc R Soc Lond B Biol Sci 1983;220:141-62.

40. Barnes PJ. Glucocorticosteroids: current and future directions. Br J Pharmacol 2011;163:29-43.

41. Oh CK, Geba GP, Molfino N. Investigational therapeutics targeting the IL-4/IL-13/STAT-6 pathway for the treatment of asthma. Eur Respir Rev 2010;19:46-54.

42. Eisenmesser EZ, Horita DA, Altieri AS, Byrd RA. Solution structure of interleukin-13 and insights into receptor engagement. J Mol Biol 2001;310:231-41.

43. Rabe KF, Nair P, Brusselle G, Maspero JF, Castro M, Sher L, et al. Efficacy and safety of dupilumab in glucocorticoid-dependent severe asthma. N Engl J Med 2018;378:2475-85.

44. Hanania NA, Korenblat P, Chapman KR, Bateman ED, Kopecky P, Paggiaro P, et al. Efficacy and safety of lebrikizumab in patients with uncontrolled asthma (LAVOLTA I and LAVOLTA II): replicate, phase 3, randomised, double-blind, placebo-controlled trials. Lancet Respir Med 2016;4:781-96.

45. Panettieri RA Jr, Sjobring U, Peterffy A, Wessman P, Bowen K, Piper E, et al. Tralokinumab for severe, uncontrolled asthma (STRATOS 1 and STRATOS 2): two randomised, double-blind, placebo-controlled, phase 3 clinical trials. Lancet Respir Med 2018;6:511-25.

46. Bleecker ER, Wechsler ME, FitzGerald JM, Menzies-Gow A, Wu Y, Hirsch I, et al. Baseline patient factors impact on the clinical efficacy of benralizumab for severe asthma. Eur Respir J 2018;52.

METHODS
Reculture

For reculture experiments, the bronchi were cultured overnight in Dulbecco modified Eagle medium in the absence of any treatment and after that their baseline contractility was evaluated (day 0). The same bronchial segments were placed back into culture, and treated for 48 hours in the presence of IL-13. Responses after this reculture on day 2 were compared with each segment's own individual response on day 1.

Western blotting

Cell lysates were size-fractionated on 4% to 12% gradient NuPage Bis-Tris acrylamide gels (Invitrogen, Carlsbad, Calif) and transferred to nitrocellulose membranes. GAPDH (14C10, Cell Signaling Technology) and phosphoSTAT-6 (phosphoY641, Abcam) were detected using IRDye800CW antirabbit antibody and read for immunocomplexes using the OdysseyCLx imaging system (LI-COR Biosciences, Lincoln, Neb).

A

Histamine
(for individual patients)

B

Histamine - pEC50
(for individual patients)
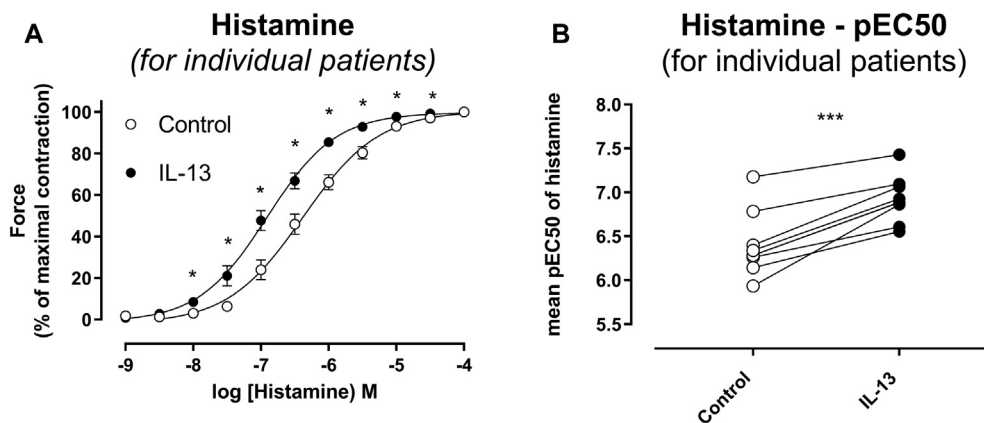
FIG E1. The effect of IL-13 on the potency of histamine in human small bronchi presented for individual patients. To determine the mean effect of 2-day culture with IL-13 for the individual patients, the effects of IL-13 on the contractile response of different bronchial preparations from the same patient were averaged. (**A**) The average cumulative concentration-response curve to histamine for these patients and (**B**) their individual corresponding pEC50 after 2-day culture with IL-13 or vehicle are presented. A mixed-effect model was applied to statistically compare the effect of IL-13 for each individual concentration, whereas a paired *t* test was used for the comparison of the pEC_{50} values. Significance is shown as *$P < .05$ and ***$P < .001$. Data are presented as mean $\pm$ SEM (N: 9).