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Interleukin-13 inhibits proliferation and enhances contractility of human airway smooth muscle cells without change in contractile phenotype

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Risse P-A, Jo T, Suarez F, Hirota N, Tolloczko B, Ferraro P, Grutter P, Martin JG. 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* 300: L958–L966, 2011. First published April 1, 2011; doi:10.1152/ajplung.00247.2010.—IL-13 is an important mediator of allergen-induced airway hyperresponsiveness. This Th2 cytokine, produced by activated T cells, mast cells, and basophils, has been described to mediate a part of its effects independently of inflammation through a direct modulation of the airway smooth muscle (ASM). Previous studies demonstrated that IL-13 induces hyperresponsiveness in vivo and enhances calcium signaling in response to contractile agonists in vitro. We hypothesized that IL-13 drives human ASM cells (ASMC) to a procontractile phenotype. We evaluated ASM phenotype through the ability of the cell to proliferate, to contract, and to express contractile protein in response to IL-13. We found that IL-13 inhibits human ASMC proliferation (expression of Ki67 and bromodeoxyuridine incorporation) in response to serum, increasing the number of cells in G0/G1 phase and decreasing the number of cells in G2/M phases of the cell cycle. IL-13-induced inhibition of proliferation was not dependent on signal transducer and activator of transcription-6 but was IL-13Rα2 receptor dependent and associated with a decrease of Kruppel-like factor 5 expression. In parallel, IL-13 increased calcium signaling and the stiffening of human ASMC in response to 1 μM histamine, whereas the stiffening response to 30 mM KCl was unchanged. However, Western blot analysis showed unchanged levels of calponin, smooth muscle α-actin, vinculin, and myosin. We conclude that IL-13 inhibits proliferation via the IL-13Rα2 receptor and induces hypercontractility of human ASMC without change of the phenotypic markers of contractility.

Ki67; Kruppel-like factor 5; IL-13Rα2; atomic force microscopy; cell stiffness; calcium signaling

INTERLEUKIN-13 is a Th2 cytokine that has been implicated in the pathophysiology of asthma. It is actively secreted during the late asthmatic response in mild asthmatic subjects (16) and has been implicated in the development of airway hyperresponsiveness (AHR) in models of allergic asthma. IL-13-gene-deleted mice fail to develop allergen-induced AHR (32), and the inhibition of IL-13 through the administration of the soluble IL-13 receptor (IL-13Rα2) reverses ovalbumin-induced AHR in mice (33) and guinea pigs (23). Repeated administration of IL-13 to naïve mice and the overexpression of IL-13 by murine airway epithelium cause AHR (33, 38). Although IL-13 promotes inflammation, AHR is rapidly induced by the administration of IL-13 to experimental animals and precedes inflammation (23, 31).

It is therefore plausible that a significant part of the effects of IL-13 may be mediated through mechanisms unrelated to inflammation. IL-13, for example, is more expressed in the smooth muscle bundle of biopsies from severe asthmatic subjects compared with mild and moderate asthmatics (27), where it might potentially exert significant biological effects. Indeed, IL-13 receptors are expressed on human airway smooth muscle cells (hASMC), and IL-13 affects both contractile and secretory properties of these cells. IL-13 has been shown to augment contractile responses to acetylcholine (ACh) and to impair relaxation to isoproterenol in rabbit tracheal smooth muscle (9) as well as isoproterenol-induced decrease in cell stiffness in cultured hASMC (18). Enhancement by IL-13 of Ca^{2+} responses of cultured hASMC evoked by bradykinin, histamine, and ACh has been reported (30) and attributed to the increased expression of CD38 protein (3) and activation of MAP kinases (24). IL-13 stimulates the secretion of eotaxin (14, 26) and IL-5 from ASMC (7).

The paradigm of ASM phenotype plasticity describes the ability of ASM to switch between a contractile and a proliferative/synthetic phenotype (12). Phenotype change results in the ability of the ASM cells to acquire or to lose specific molecular markers. Increase in proliferation resulting in a lower ability to contract is associated with a lower abundance of contraction-associated proteins such as SM α-actin, SM myosin heavy chain (MHC), calponin, caldesmon, SM22, desmin, and smoothelin (12). The proliferation of ASM in the course of remodeling in asthma may also be associated with changes in cellular phenotype, with loss of contractile proteins, as suggested by experimental models of asthma (22) (17). However, several studies of human asthma tissues have shown that there is either no change in contractile gene expression (36) or there may be increased expression in asthmatic airway tissues (20), suggesting the possibility that certain factors may promote the restoration of muscle contractile phenotype despite remodeling. Transforming growth factor-β (TGF-β) is one such cytokine that promotes the synthesis of contractile protein in cultured SM cells (35). We hypothesized that IL-13 contributes to AHR by modulating the phenotype of SM to a more contractile state. To test this hypothesis, we have used cultured hASMC and examined the effects of IL-13 on their contractile responses to histamine, their proliferative response to a mitogenic stimulus, and their expression of specific contractile markers.

MATERIALS AND METHODS

Cell culture. Primary hASMC were isolated from tracheobronchial tissue procured from donor subjects of the lung transplantation pro-

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gram of the Université de Montréal. The study was approved by a human ethics committee at each of the participating institutions. hASMC were isolated and cultured as previously described (8). Briefly, tissue digestion was performed by gently rocking the tissues in HBSS (in mM: 5 KCl, 0.3 KH_2PO_4, 138 NaCl, 4 $NaHCO_3$, and 5.6 Na_2HPO_4) containing collagenase type IV (0.4 mg/ml), elastase (0.38 mg/ml), and soybean trypsin inhibitor (1 mg/ml) at 37°C for 90 min. The dissociated cells were collected by filtering through 125-μm Nytex mesh followed by centrifugation. The pellet was reconstituted in growth medium consisting of DMEM-Ham's F12 (1:1) medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), and gentamycin (50 μg/ml). Cells were plated in 25-cm^2 flasks, and medium was changed daily for 3 days and subsequently on alternate days. Confluent cells were detached with a 0.025% trypsin solution containing 0.02% EDTA. Cells were used between *passages 2* and *5*. They were identified as SM cells by positive immunohistochemical staining for SM α-actin and positive identification of SM MHC and calponin by Western blot analysis.

Cell-cycle analysis. hASMC were grown in DMEM/F12 medium supplemented with 10% FBS and antibiotics. Sixty percent confluent cells were synchronized at G_0 phase by serum starvation for 48 h and incubated in fresh medium containing 10% FBS to allow the progression through the cell cycle. Cells were treated with IL-13 (10 ng/ml) or its vehicle and then collected at 6, 12, and 24 h after FBS addition and fixed in cold 70% ethanol. Cells were treated with RNAse and stained with propidium iodide (556463; BD Pharmingen, San Jose, CA) for cell-cycle analysis. The samples were analyzed by flow cytometry (FACS Calibur, BD Pharmingen) and commercial software (Cellquest, BD Pharmingen).

Measurement of cell proliferation. hASMC were grown in six-well plates with DMEM/F12 medium supplemented with 10% FBS and antibiotics. The proliferative responses of hASMC in association with the cell-cycle analysis were determined by flow cytometric quantification of Ki67 (FITC mouse anti-human Ki67 set, no. 556026, BD Pharmingen) as recommended by the manufacturer. The same protocol was used to assess the response of the SM cells to IL-13 on proliferation induced by PDGF-BB (15 ng/ml) and TGF-β (3 ng/ml) and on cells pretreated with indomethacin (10 μM), a nonspecific cyclooxygenase inhibitor, and LY294002 (10 μM), a selective phosphatidylinositol 3-kinase (PI3K) inhibitor. All other assays of proliferation were performed using bromodeoxyuridine (BrdU) uptake (FITC BrdU Flow Kit, BD Pharmingen). Cells were rendered quiescent by culturing for 48 h in 0.5% FBS. The effects of IL-13 on basal and serum-stimulated proliferation were first assessed. Medium was changed for 0.5% or 10% FBS-containing medium with IL-13 (10 ng/ml) or its vehicle (0.0001% BSA). BrdU was added 6 h later, and the cells were harvested after a further 18 h. Cells were stained for BrdU and analyzed by flow cytometry as recommended by the manufacturer. With a similar protocol, we studied the concentration dependence of the effects of IL-13 (0.3 to 10.0 ng/ml) on FBS-induced hASMC proliferation and the effects of 100 μM of leflunomide (Tocris, Ellisville, MO), a signal transducer and activator of transcription (STAT)-6 inhibitor (24), and 10 μM of Secin-H3 (Tocris), an insulin receptor substrate (IRS) phosphorylation inhibitor (11). The vehicle for the inhibitors never exceeded 0.1% DMSO. Each experiment was performed in duplicate and repeated three to five times.

Transfection with siRNA. All siRNA sequences were designed by and purchased from Dharmacon (smartpool L-004598-00 for IL-13Ra2 and D-00180-10-05 for the nontargeting control; Dharmacon, Lafayette, CO). Subconfluent hASMC were transfected with 20 nM siRNA (final concentration) in DMEM/F12 containing 0.1% FBS without antibiotics for 6 h. The siRNA was transfected into cells using 1 μl of siRNA/2.5 μl of lipofectamine 2000 (Invitrogen, Carlsbad, CA). Lipofectamine 2000 and siRNA were first resuspended in Optimem medium (Invitrogen) and then mixed together for 20 min

before the transfection. After 6 h, the medium was replaced with fresh medium containing antibiotics and incubated for a further 48 h. Cells were then used for proliferation assay as previously described.

Measurement of resting cell mechanics and responsiveness to stimulation by atomic force microscopy. hASMC were seeded at 3 × 10^3 cells on 35-mm glass-bottom dishes (MatTek, Ashland, MA) and starved for 48 h in 0.1% FBS. IL-13 (10 ng/ml) or its vehicle was added in the medium 24 h before stiffness measurements. Measurements were performed with a Bioscope atomic force microscope equipped with a G-type scanner, Nanoscope IIIa control electronics, and software version 4.43r8 (Digital Instruments; Veeco Metrology Group, Santa Barbara, CA) mounted over an inverted optical microscope (Axiovert S100TV; Zeiss, Thornwood, NY). Silicon nitride triangular microlevers (TM Microscopes; Veeco Metrology Group) with a nominal spring constant of $k = 0.01$ N/m were used to collect force-distance curves. A latex bead of 7.5 μm diameter (Bangs Laboratories, Fishers, IN) was fixed to the tip of each probe with ultraviolet light-curable adhesive (Elecro-lite, Danbury, CT) to avoid disruption of the cell membrane (29). The deflection sensitivity of the optical lever was calibrated before and after each experiment by measuring the slope of the contact region of a force-distance curve acquired on a clean glass cover slip in HBSS. Stiffness was measured in baseline conditions and during exposure of cells to histamine (1 μM) and KCl (30 mM). Each experiment was performed on six to seven cells.

Reverse transcriptase-PCR and real-time quantitative PCR. hASMC were cultured until confluence and starved for 48 h in 0.5% FBS. Medium was changed, and cells were incubated with IL-13 (10 ng/ml) or its vehicle for 24 h. RNA was extracted using a commercial kit (RNeasy mini kit; Qiagen, Valencia, CA), and integrity was verified with the Experion electrophoresis station (Bio-Rad, Hercules, CA). Reverse transcription was done using 500 ng of RNA for each sample. qPCR was performed with 1 μl of cDNA per reaction with 5 μl of 2× QuantiTect SYBR Green PCR (Qiagen), 0.3 μl of forward and reverse primers (0.3 μM), and 3.4 μl of RNAse-free water for a total volume of 10 μl per well (see Table 1 for primer sequences and PCR product length). The cDNA was amplified in the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA). A negative control (RNAse-free water) was added to the qPCR run. Relative mRNA expression was calculated using the ΔΔCt method. Individual data were normalized for their corresponding housekeeping gene (ribosomal S9). PCR product lengths were verified on an agarose gel. Results were expressed as a fold change in expression over the control.

Assessment of SM proteins by Western blotting. SM α-actin, SM MHC, calponin, and vinculin expression was measured by Western blotting as previously described. hASMC were incubated with IL-13 or its vehicle for 24 h and then washed with ice-cold PBS and lysed in ice-cold extraction buffer containing 62.5 mM Tris·HCl (pH 6.8), 2% SDS, 10% glycerol, and 50 mM DTT. Additional experiments were conducted to examine the protein expression of Kruppel-like factor-5 (KLF5) and cyclin D1. In this case, hASMC were cultured as for the proliferation assay. After 48 h of starvation, subconfluent cells were cultured for 24 h with IL-13 or its vehicle in the presence of 10% FBS. Electrophoresis was carried out using 8% or 12% polyacrylamide gels, and proteins were transferred to nitrocellulose or polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature with 3% powdered milk in Tris·HCl buffer containing Tween 20 (TBST) or 1% BSA with EDTA and NaCl in TBST. Primary antibodies were diluted in TBST and added as follows: anti-SM α-actin (mouse monoclonal IgG2, dilution 1:2,000; Sigma A4700); anti-β-actin (mouse monoclonal, dilution 1:10,000, Sigma); anti-SM myosin (rabbit polyclonal IgG, dilution 1:1,000; BT-562; BTI, Stoughton, MA), anti-calponin (mouse monoclonal IgG1, dilution 1:1,000, Sigma C2687), anti-GAPDH (mouse monoclonal IgG1, dilution 1:4,000, MAB 374; Millipore, Billerica, MA), anti-vinculin (mouse monoclonal IgG1, dilution 1:2,000, Sigma V9131), anti-KLF5

Table 1. *List of target genes with primers and PCR product length*

Target Gene	Forward Primer	Reverse Primer	PCR Product Length
S9	TGCTGACGCTTGATGAGAAG	CGCAGAGAGAAGTCGATGTG	307 bp
KLF5	ACCTCCATCCTATGCTGCTAC	TTTGTGCAACCAGGGTAATC	174 bp
Desmin	CAGCCAACAAGAACAACGAC	CAAATCGGTCCTCCAATTCC	153 bp
Smoothelin	ATGCTGCTGGACTGGTGTCG	TTCTGGCGTCGGTTCTGAGG	167 bp
SM α-actin	AAGAGTTACGAGTTGCCTGATG	TGATGCTGTTGTAGGTGGTTT	136 bp
Calponin	AGGTGAACGTGGGAGTGAAG	GGCTGGCAAACTTGTTGGTG	117 bp
MHC	GCAAGAAGAGGCACGAGATG	ACGGCCAGGTACTGAATGAC	160 bp
CDKN1B	TGACTTGCATGAAGAGAAGCA	GCTGTCTCTGAAAGGGACATTAC	81 bp
Vinculin	GCGATACCACAACTCCCATC	GCTGCCCTCTCATCAAATACC	110 bp
MLCK	GATTTTGTGCTGCAGTGCTC	AGCGAGCGTACTGGATGG	88 bp
SMA	CACAAGTATCACGGGAGAGC	TTGTCGTTCTTCACTGTTTTGG	93 bp
TGF-β1	GCAGCACGTGGAGCTGTA	CAGCCGGTTGCTGAGGTA	64 bp
IL-13Ra2	CCTTTGCCGCCAGTCTATCTTA	TCAAAACACCTTGCTGGAATAGG	108 bp

Cysteinyl leukotriene receptor 1 (CysLTR1) real-time PCR was performed using the RT^2 qPCR Primer Assay for Human CYSLTR1: PPH02507A (SABiosciences, Frederick, MD). KLF5: Krüppel-like factor-5; SM, smooth muscle; MHC: smooth muscle myosin heavy chain; CDKN1B: cyclin-dependent kinase inhibitor 1B; MLCK: myosin light chain kinase; SMA, smooth muscle actin; TGF-β1: transforming-growth factor-β1; IL-13Ra2: IL-13 receptor-α2.

(rabbit polyclonal, dilution 1:500, ab24331; Abcam, Cambridge, MA), and anti-cyclin D1 (rabbit polyclonal, dilution 1:200; cyclin D1 Ab-3; Neomarkers, Freemont, CA). Fluorescent secondary antibodies (goat anti-rabbit IgG Dylight-800 and goat anti-mouse IgG Dylight-680, dilution for both 1:15,000; PI35571 and PI35518, respectively; Pierce-Thermo-Fisher, Rockford, IL) were diluted in TBST. Western blots were quantified with the Odyssey imaging system (LI-COR, Lincoln, NE).

Single cell intracellular calcium measurement. Cytosolic calcium measurements were performed as previously described (30a). hASMC were seeded at 1×10^4 cells on sterile glass cover slips, disposed in a six-well plate, and grown in 10% FBS-containing medium. Subconfluent cells were starved in 0.1% FBS-containing medium for 24 h. IL-13 or its vehicle was added for an additional 24 h of incubation. To measure the calcium signaling, cells were incubated in HBSS containing in mM: 137 NaCl, 4.2 NaHCO$_3$, 10 glucose, 3 Na$_2$HPO$_4$, 5.4 KCl, 0.4 KH$_2$PO$_4$, 1.3 CaCl$_2$, 0.5 MgCl$_2$, 0.8 MgSO$_4$, and 5 HEPES, at 37°C with 5 μM of fura-2 AM for 30 min. Cells were then washed three times with HBSS solution and placed in a Leiden chamber (Medical Systems, Greenville, NY) on the stage of an inverted microscope equipped for cell imaging with a ×40 oil-immersion objective (Olympus, Tokyo, Japan). Cells were alternatively excited at 340 and 380 nm, and the intensity of the emitted fluorescence signal at 510 nm was recorded with a camera (CoolSnapPro; Media Cybernetics, Bethesda, MD) and measured with image-master software (Photon Technology International, Birmingham, NJ). Histamine (1 μM) was added 20 s after beginning of measurement, and the fluorescence ratio (340/380) was measured in individual cells ($n = $ 10–15 per slide). Calcium concentrations were calculated according to Grynkiewicz's formula (10) using a Kd of Ca^{2+} to Fura-2 of 224 nM. R$_{max}$ was determined in cells exposed to ionomycin (10 μM) in the presence of 1.3 mM CaCl$_2$ and R$_{min}$ in Ca^{2+}-free buffer to which EGTA (10 mM) and ionomycin (10 μM) had been added.

Statistical analysis. The data are expressed throughout as means ± one SE. Statistical significance was tested using paired *t*-tests for comparison of paired means and by ANOVA and Bonferroni posttest for comparisons among several means. $P < 0.05$ was considered to be significant.

RESULTS

The effects of IL-13 on hASMC proliferation. After 48 h of starvation, hASMC were cultured for 6, 12, and 24 h in 10% FBS. Cells were stained with propidium iodide to study the cell-cycle progression. After a period of starvation, 90.9 ± 0.6% of hASMC were in G0/G1 phase, 1.7 ± 0.2% in S phase, and 5.0 ± 0.8% in G2/M (Fig. 1*A*). The proportions of cells in the different phases of the cell cycle were unchanged at 6 and 12 h (data not shown). After 24 h of incubation with 10% FBS, the percentage of cells in G0/G1, S, and G2/M phases changed compared with control to 64 ± 2.9%, 13.2 ± 1.8%, and 16.5 ± 1.4%, respectively (Fig. 1*D*). IL-13 (10 ng/ml) coincubation did not alter the percentage of cells in S phase (11.8 ± 2.1%) but significantly increased the percentage of cells in G0/G1 (74.0 ± 3.8%) and reduced the percentage of cells in G2/M (8.7 ± 1.1%) ($P < 0.05$, $n = 3$). To quantify the apparent inhibitory effects of IL-13 on hASMC proliferation, Ki67 immunoreactivity was assessed by flow cytometry. After 24 h of incubation in 10% FBS-containing medium, 33.5 ± 6.0% of the cells expressed Ki67 (Fig. 2*A*). Coincubation with IL-13 (10 ng/ml) decreased Ki67-positive cells to 23.3 ± 4.8% ($P < $ 0.05, $n = 3$), corresponding to a 32% decrease of the mitogenic effects of serum. However, IL-13 did not affect the proliferation induced by 15 ng/ml of PDGF-BB (Fig. 2*B*) and potentiated the antiproliferative effect of TGF-β (Fig. 2*C*). The effect of IL-13 on hASMC proliferation was also studied using BrdU incorporation in cells cultured with low (0.5%) or high (10%) concentrations of FBS. IL-13 limited serum-induced hASMC proliferation in a concentration-dependent manner, reaching a plateau at 3 ng/ml (Fig. 2*D*) ($P < 0.05$, $n = 5$). IL-13 (10 ng/ml) led to a reduction of 31% of the mitogenic effects of 10% FBS, whereas in 0.5% FBS IL-13 reduced hASMC proliferation by 20% compared with the control ($P < 0.05$, $n = 5$) (Fig. 2*E*).

The effects of IL-13 on the expression of regulators of cell proliferation. Cyclin-dependent kinase inhibitor 1B (CDKN1B) and KLF5 are transcription factors involved in cell-cycle progression (6). Twenty-four hours of incubation with IL-13 significantly reduced KLF5 mRNA expression ($n = 4$, $P < $ 0.05) without affecting CDKN1B mRNA expression (Fig. 3*A*). Protein expression of KLF5 was also significantly decreased by IL-13 after 24 h of culture in presence of 10% FBS (Fig. 3*B*). In parallel, we observed a decreased expression of cyclin D1 expression (Fig. 3*C*).

Involvement of STAT6, IRS pathway, and autocrine mediator production in IL-13-induced inhibition of proliferation. We examined the role of STAT6 and IRS in IL-13-induced inhibition of proliferation using the inhibitors leflunomide (100 μM), a STAT6 inhibitor, Secin-H3 (10 μM), an IRS phosphor-

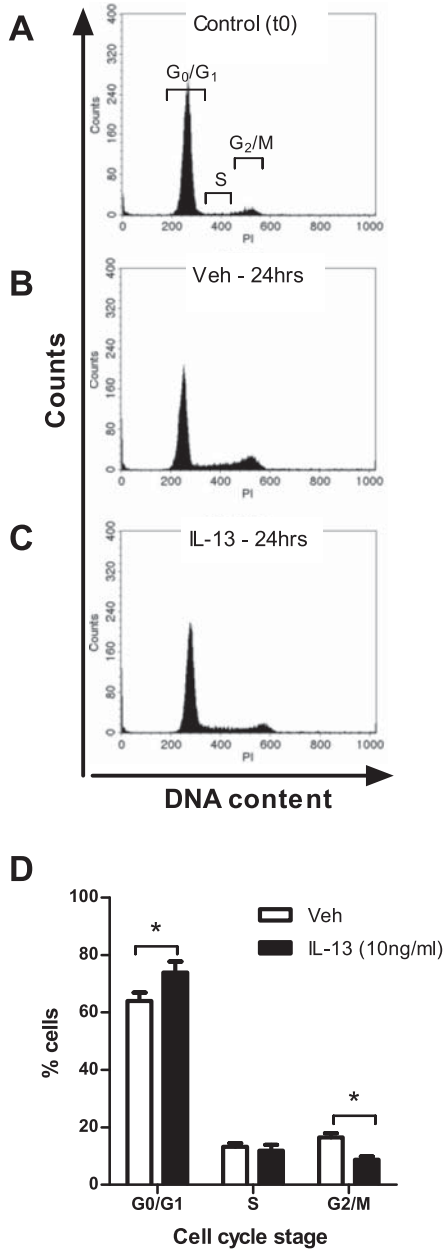Fig. 1. IL-13 (10 ng/ml) represses cell-cycle progression in airway smooth muscle cells (ASMC). A: representative example of flow cytometric measurement of DNA content in ASMC stained with propidium iodide (PI) after 2 days of starvation in 0.1% FBS. B and C: representative example of DNA content in cells incubated for 24 h with 10% FBS in presence of 10 ng/ml of IL-13 (*bottom*) or its vehicle (*top*). D: after 24 h of culture in 10% FBS, IL-13-treated cells exhibited a significantly higher population in quiescent phase (G0/G1) and a lower population in mitosis phase (G2/M) compared with vehicle (Veh) ($n = 3$, *$P < 0.05$) Means + 1 SE are shown.

ylation inhibitor, and LY294002, a PI3K inhibitor. The proliferation of hASMC was assessed by BrdU incorporation or Ki67 expression after 24 h. Leflunomide and Secin-H3 did not affect the reduction of hASMC proliferation by IL-13 (Fig. 4A). LY294002, per se, inhibits the serum-induced proliferation, but IL-13 still further reduced the proliferation (4.2 ± 1.9 vs. $13.4 \pm 3.9\%$ of Ki67-positive cells). Additionally, indomethacin, a nonselective cyclooxygenase inhibitor, did not alter the effect of IL-13 on serum-induced proliferation (Fig. 4B), and IL-13 did not increase the mRNA expression of TGF-β1 (Fig. 4C).

Involvement of IL-13Rα2 receptor in IL-13-induced inhibition of proliferation. Forty-eight hours after transfection, the mRNA expression of IL-13Rα2 was decreased by 56% compared with the scrambled siRNA ($n = 3$, $P < 0.05$, Fig. 4D). The inhibitory effect of IL-13 on serum-induced proliferation was maintained in control cells at 35% (16.03 ± 0.3 vs. $25.7 \pm 1.1\%$ of Ki67-positive cells; $n = 3$, $P < 0.05$), consistent with previous results. However, in cells transfected with siRNA for IL-13Rα2, the inhibitory effect of IL-13 was only 15.2% ($21.8 \pm 1.13\%$ of Ki67-positive cells), significantly less than the cells treated with scrambled siRNA ($P < 0.05$), a reduction of the inhibitory effect of IL-13 by 56% comparable to the magnitude of the decrease of mRNA expression ($n = 3$, Fig. 4E).

IL-13 affects hASMC stiffening in response to histamine. We assessed the contractile responses of hASMC in culture using atomic force microscopy (AFM). Subconfluent cells were starved 1 day in 0.1% FBS before IL-13 (10 ng/ml) or its vehicle addition for 24 h of incubation. Stiffness of cells was measured with AFM. IL-13 did not induce change in the resting stiffness of hASMC (365.4 ± 45.7 Pa in control group vs. 375.7 ± 43.5 Pa in IL-13 treated group; $n = 13$–15; Fig. 5A). Histamine (1 μM) induced an increase of stiffness (Fig. 5B) that was enhanced in IL-13-treated cells ($+288.7 \pm 54.7$ vs. $+540.7 \pm 54.7$ Pa; $n = 7$, $P < 0.05$; Fig. 5C). However, KCl (30 mM)-induced stiffening of hASMC was not significantly modified by IL-13 ($+396.3 \pm 61.5$ Pa; $n = 6$ vs. $+480.9 \pm 71.2$ Pa; $n = 8$; Fig. 5D).

Effects of IL-13 on histamine-induced calcium release. We tested the effect of IL-13 (10 ng/ml) on Ca^{2+} signaling in response to 1 μM histamine. IL-13 augmented significantly the increase of cytoplasmic Ca^{2+} concentrations induced by histamine ($[Ca^{2+}]_{peak} = 1,452.0 \pm 175.5$ vs. 786.2 ± 86.13 nM; $n = 5$, $P < 0.05$) without affecting the resting Ca^{2+} level ($[Ca^{2+}]_{baseline} = 132.6 \pm 12.7$ vs. 134.1 ± 16 nM, $n = 5$) (Fig. 6).

Effects of IL-13 on the expression of contractile proteins. To assess whether a broad change in phenotype of hASMC was induced by IL-13, we examined the expression of a number of contractile proteins by real time q-PCR (Fig. 7A). MHC, SM actin, and desmin mRNA expressions were not changed after 24 h of incubation with IL-13. However, mRNA expression of SM α-actin was not significantly increased, whereas calponin, vinculin, and CysLT1 receptor expression were significantly increased ($n = 4$; $P < 0.05$). Western blot for MHC confirmed PCR results but also showed no change in SM α-actin expression, vinculin, and calponin at the protein level ($n = 3$, Fig. 7B). To verify the effect of serum on SM contractile protein expression, we also studied the effect of IL-13 in serum-free conditions. After 24 h of incubation in the presence of IL-13, the level of contractile proteins was also unchanged (Supplemental Fig. S1; supplemental material for this article is available online at the *American Journal of Physiology Lung Cellular and Molecular Physiology* website).

DISCUSSION

The purpose of this study was to examine the effect of IL-13 on the phenotype of hASMC. We observed that IL-13 inhibited the proliferation of ASMC that was stimulated to proliferate with FBS. At the same time IL-13 increased contractility and

Fig. 2. IL-13 limited human ASMC (hASMC) proliferation. *A*: IL-13 (10 ng/ml) significantly reduced the number of Ki67-positive cells after 24 h of incubation with 10% FBS (*n* = 3, *P* < 0.05). *B*: PDGF-BB (15 ng/ml) induced proliferation of hASMC that was not inhibited by IL-13. *C*: transforming growth factor (TGF)-β1 (3 ng/ml) reduced the proliferation of hASMC. IL-13 has an additional effect on this inhibition. *D*: IL-13 reduced bromodeoxyuridine (BrdU) incorporation in ASMC cultured with 10% FBS for 24 h in a concentration-dependent manner (*n* = 5, *P* < 0.05). *E*: IL-13 significantly reduced incorporation of BrdU in ASMC cultured in 0.5% FBS for 24 h (*n* = 5, *P* < 0.05). The mean + 1 SE is shown.
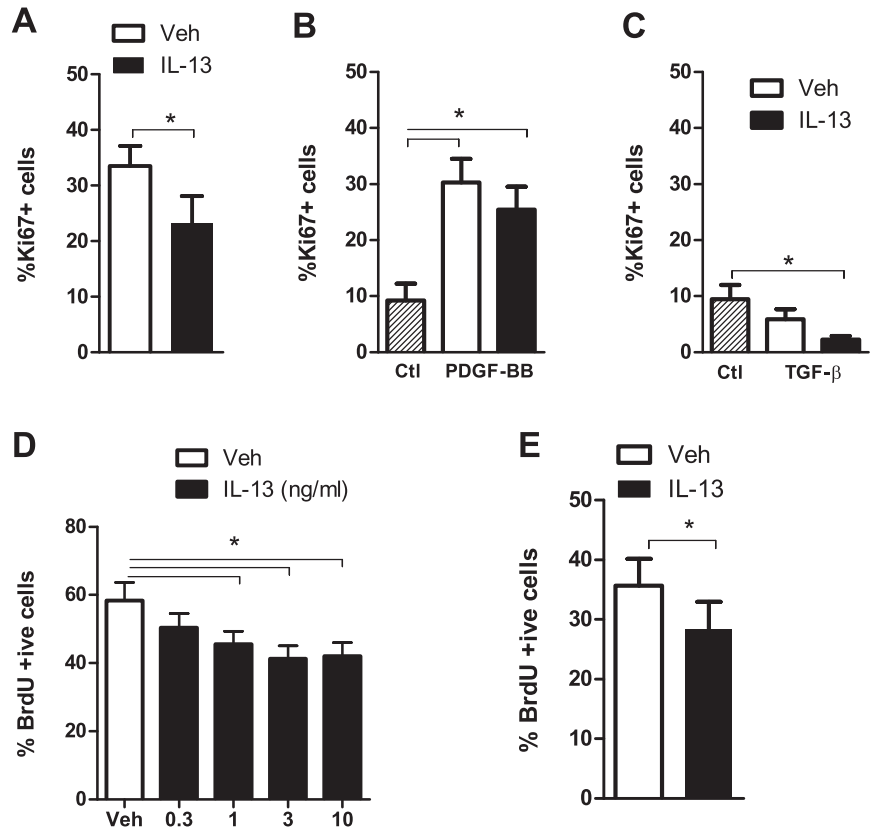
calcium signaling in response to histamine. However, despite the inhibition of proliferation, there was no substantive effect on the contractile phenotype as reflected in contractile protein expression. Despite the generally postulated idea of reciprocal relationships between contractile and proliferative phenotypes, we demonstrate that IL-13 may modify hASM contractile signaling responses and proliferation without causing a significant change in contractile protein expression.

Using cell-cycle analysis we demonstrated that IL-13 regulated the cell cycle of hASMC by limiting the passage of the cells from G0/G1 to S and from S to G2/M, increasing the number of cells in the quiescent phase, and decreasing the number of cells in the passage to mitosis. In addition, we used two markers expressed during the S phase of the cycle, Ki67 and BrdU incorporation, to confirm the inhibition of proliferation in response to stimulation by FBS. However, it appears that IL-13 has an effect only on the particular mitogenic factors present in the serum because the inhibitory effect observed with the serum was not reproduced with PDGF-BB. On the basis of previously published work (37), we chose to examine

Fig. 3. Effects of IL-13 on the expression of regulators of cell proliferation. *A*: expression of hASMC mRNA transcripts for Kruppel-like factor-5 (KLF5) was significantly reduced in the presence of IL-13 (10 ng/ml), whereas there was no change for cyclin-dependent kinase inhibitor 1B (CDKN1B) (*n* = 4, *P* < 0.05). The mean + 1 SE is shown. *B* and *C*: hASMC cultured for 24 h in 10% FBS exhibited a significant inhibition of KLF5 (*B*) and cyclin D1 (*C*) expression in presence of IL-13 (*n* = 4, *P* < 0.05).
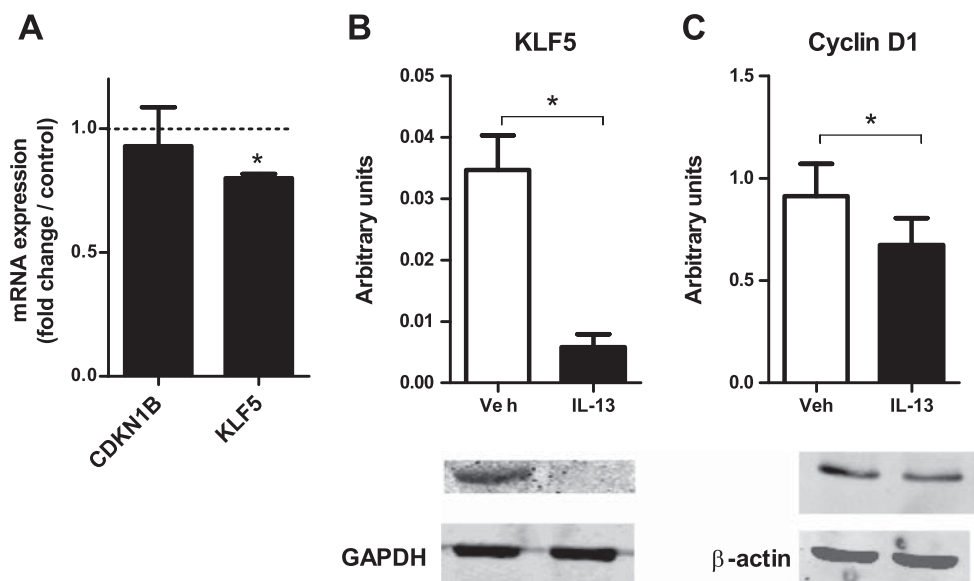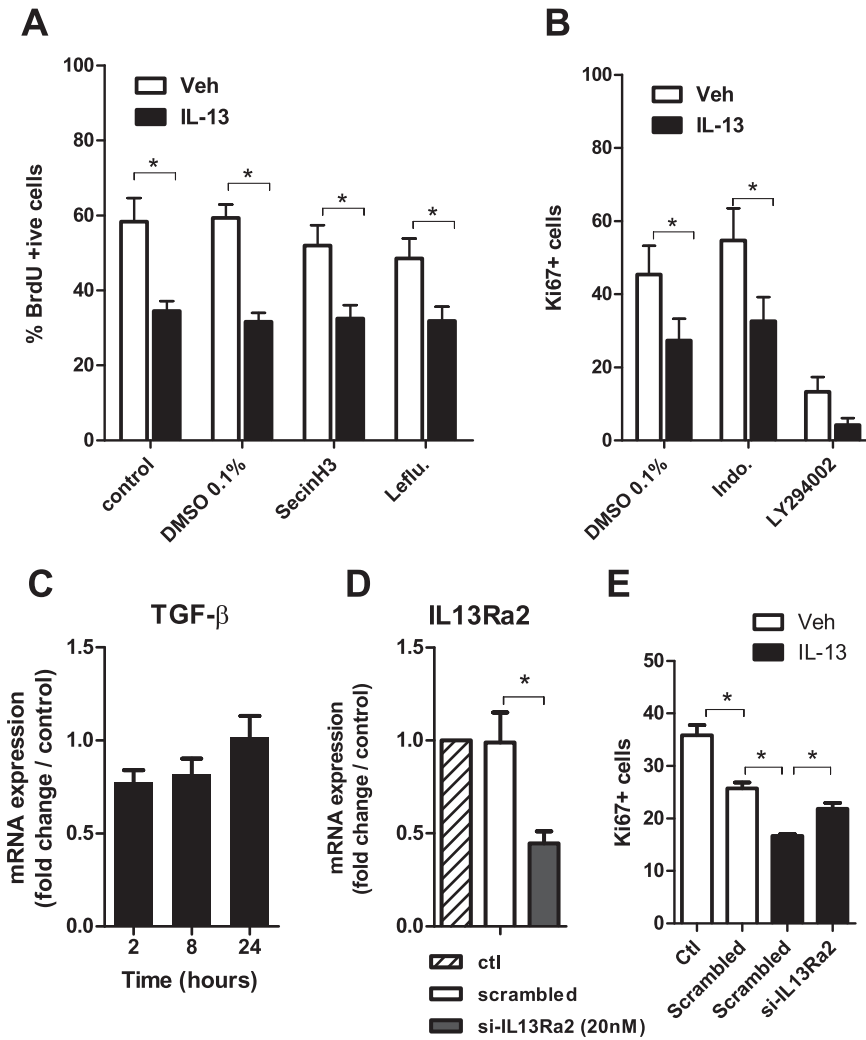
Fig. 4. IL-13-induced reduction of ASMC proliferation was not dependent on signal transducer and activator of transcription (STAT)6 or on insulin receptor substrate (IRS) phosphorylation but dependent on the IL-13Rα2. *A*: addition of a STAT6 phosphorylation inhibitor (100 μM leflunomide, Leflu.) or an IRS phosphorylation inhibitor (10 μM Secin-H3) did not reverse the effects of IL-13 on hASMC proliferation ($n = 3$). *B*: inhibition of phosphatidylinositol 3-kinase by LY294002 (10 μM) inhibited the serum-induced proliferation, but IL-13 retained its inhibitory effect. Addition of indomethacin (10 μM) (Indo.) did not counteract the effect of IL-13 ($n = 3$, *$P < 0.05$). *C*: TGF-β mRNA expression was not increased by IL-13 over a 24-h period ($n = 3$). *D*: 48 h after transfection with siRNA expression of IL-13Rα2 was reduced by 56% ($n = 3$, *$P < 0.05$) *E*: transfection with siRNA for IL-13Rα2 receptor reduced the inhibitory effect of IL-13 on hASMC proliferation ($n = 3$, *$P < 0.05$). The mean + 1 SE is shown.

the effect of IL-13 on ASM treated with TGF-β1 because it had been reported to cause proliferation. However, we observed an inhibitory effect of TGF-β1 treatment, similar to the findings of Cohen and Panettieri (2). IL-13 combined with TGF-β1 showed additive effects of the two cytokines in inhibiting

proliferation. Hawker and colleagues (13) observed similar effects on serum-induced ASM proliferation with IL-4 and showed that inhibition was attributable to a decrease of cyclin D1 expression. In the present study, we found that the reduction of the ASM cell proliferation was associated with a
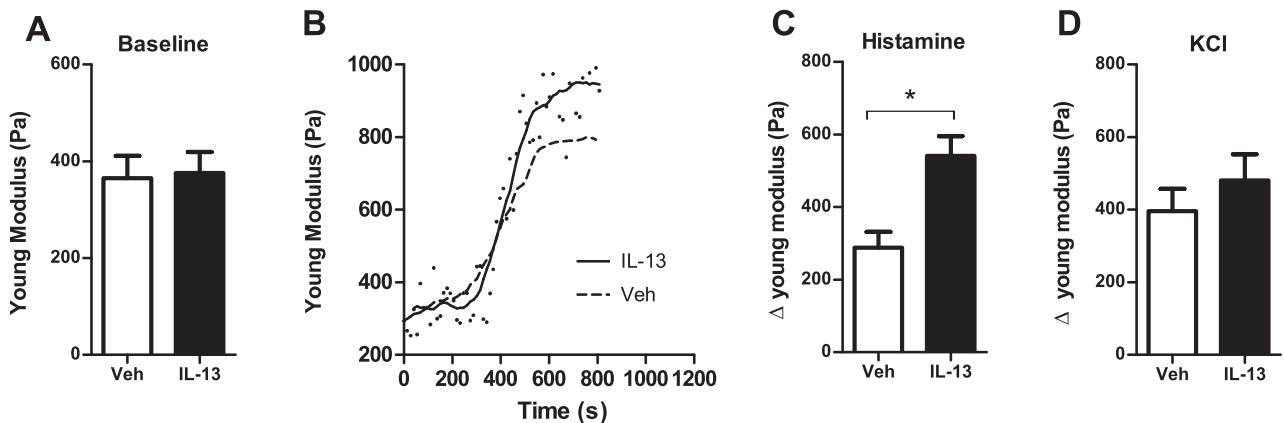

Fig. 5. IL-13 increased histamine-induced stiffening of hASMC. *A*: resting stiffness was not affected after 24 h of treatment in presence of 10 ng/ml of IL-13 ($n = 13–15$). *B*: representative example of hASMC stiffening induced by 1 μM histamine. *C*: histamine (1 μM) induced hASMC stiffening that was more pronounced in cells treated with IL-13 (10 ng/ml) during 24 h ($n = 7$). *$P < 0.05$ D*: KCl (30 mM) induced cell stiffening that was not modified by 24 h of IL-13 (10 ng/ml) treatment ($n = 6–8$). The mean + 1 SE is shown.

Fig. 6. Calcium signaling increased in response to 1 μM histamine. *A*: representative example of intracellular calcium concentration increase in response to 1 μM histamine. *B*: resting calcium level was not modified by 24-h treatment with IL-13 (10 ng/ml) ($n = 5$). *C*: calcium influx in response to 1 μM histamine was significantly increased after 24 h of incubation in presence of 10 ng/ml of IL-13 ($n = 5$, *$P < 0.05$). The mean $+$ 1 SE is shown.
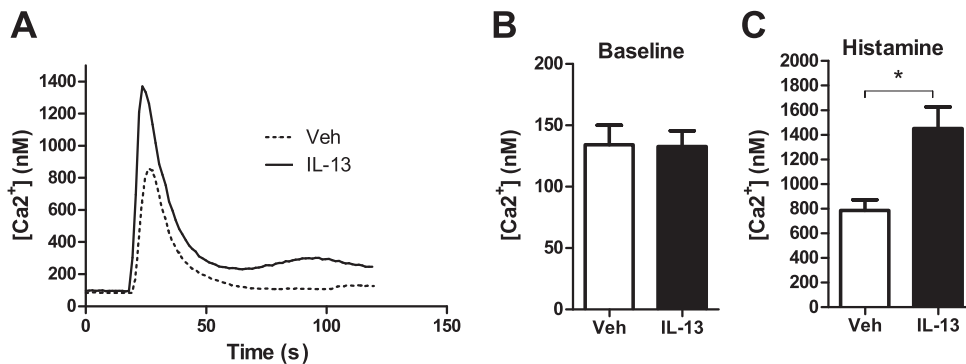
decrease of KLF5 mRNA and protein expression. KLF5 is a transcription factor regulated by the MAP kinases (4) via H-ras activation that promotes cell proliferation directly by increasing cyclin D expression. We observed a decreased expression of cyclin D1. KLF5 also acts indirectly by competing with KLF4 through binding to the KLF4 promoter site. This latter factor inhibits cyclin D that is responsible for the entry into the S phase but also cyclin B that is responsible for the passage from the S phase to the G2/M phase, as we observed with the cell-cycle analysis. A reduction in KLF5 expression, such as we observed, would therefore be expected to result in less proliferation. CDKN1B maintains the cell cycle in the G1 phase by preventing the activation of cyclin D. However,

CDKN1B expression was unchanged, suggesting that KLF5 is the main pathway by which IL-13 limits hASMC proliferation.

To explore the intracellular signaling pathway involved in IL-13-induced reduction of hASMC proliferation, we used leflunomide, Secin-H3 and LY294002, three pharmacological inhibitors of the two major downstream regulators associated with the transactivation of the IL-13Rα1/IL-4R complex, STAT6, and IRS-1 (25), respectively. IL-13-reduced hASMC proliferation was not affected by these three inhibitors. We also investigated the potential role of autocrine mediators involved in the reduction of the proliferation such as cyclooxygenase products or TGF-β. The use of indomethacin, a nonselective COX inhibitor did not counteract the antiproliferative effect of

Fig. 7. IL-13 did not change contraction-associated protein expression. *A*: expression of mRNA transcripts varied from 0.8- to 1.2-fold over the control (vehicle). Calponin, vinculin, and CysLTR1 appear significantly upregulated ($n = 4$, *$P < 0.05$). *B*: densitometry and representative example of Western blots of smooth muscle myosin heavy chain (SM MHC), smooth muscle α-actin, calponin, and vinculin. No differences of these contractile protein expressions were observed after 24 h of treatment with IL-13 (10 ng/ml). The mean $+$ 1 SE is shown. MLCK, myosin light chain kinase.
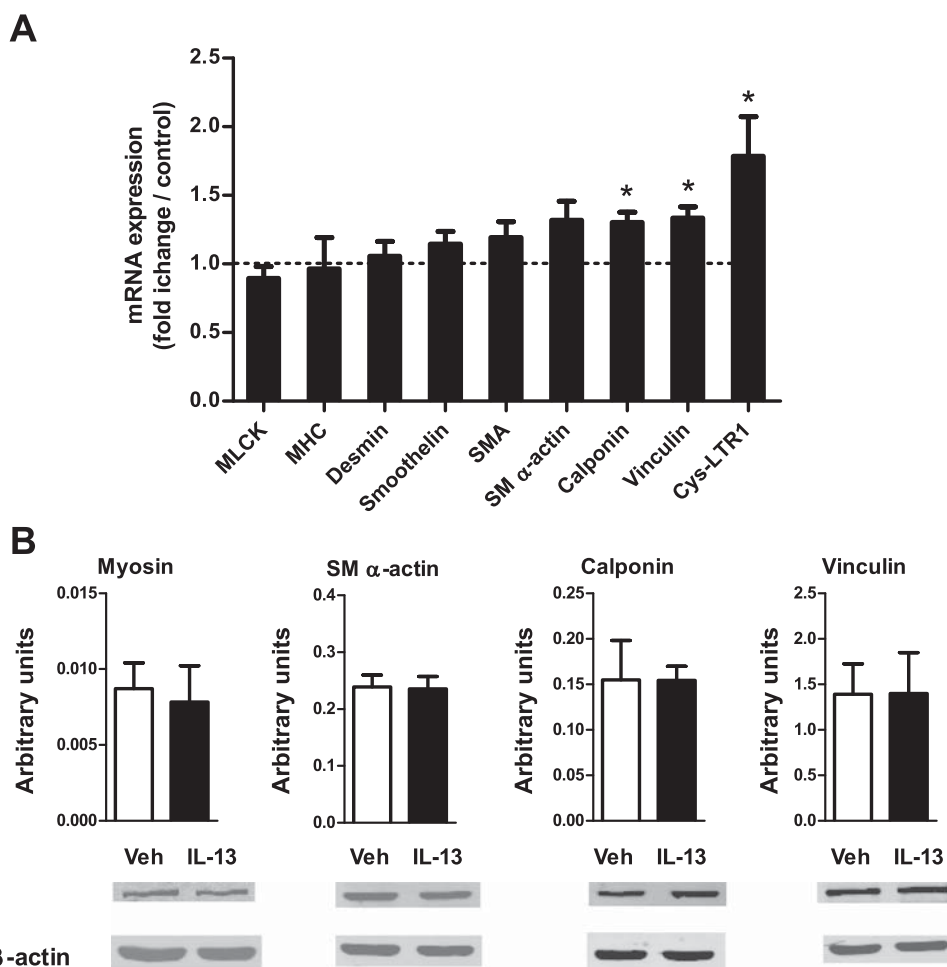
IL-13, and the expression of TGF-β was not upregulated by IL-13. The lack of inhibition of IL-13 effect suggested an alternative pathway that could be mediated by IL-13Rα2 receptor, formerly considered as a decoy. This receptor has been more recently involved in the production of reactive oxygen species induced by IL-13 in human colorectal cell line via ERK activation (21) and in the expression of TGF-β in several murine cell types, including pancreatic cells, macrophages, and colonic mucosal epithelial cells. Additionally IL-13Rα2 and IL-10 coordinately suppress airway inflammation, AHR, and fibrosis in mice (34). IL-13Rα2 has high affinity for IL-13, and consistent with a possible role for this receptor in the observed effects of IL-13 is the finding that low concentrations of IL-13 (3 ng/ml) are sufficient to limit ASMC proliferation, compared with concentrations reportedly required to influence RhoA expression (1). By using siRNA, we reduced both expression of this receptor and the inhibitory effect of IL-13 on serum-induced proliferation by 56%, suggesting a direct consequence of the downregulation of the receptor.

In parallel with the inhibition of hASM proliferation, IL-13 exerted an effect on the hASMC contractility. Several previous studies have demonstrated effects on Ca^{2+} signaling (3, 24, 28). However, there is little information on the effects of IL-13 on the contractile responses of cultured ASMC. AFM was used to study the stiffness of hASMC, and with this methodology we demonstrated that IL-13 augmented not only Ca^{2+} signals but also mechanical responses to histamine. Our results support the work done by Laporte and coworkers (18), where they demonstrated with magnetic twisting cytometry that IL-13 did not influence the resting stiffness of hASMC but induced hyporesponsiveness to the β2-adrenergic agonist isoproterenol. Interestingly, the effect of IL-13 was dependent on ERK but was not reproduced by IL-4, suggesting a mechanism that is STAT6/JAK1 independent. Additionally, we previously demonstrated that IL-13 increased Ca^{2+} signaling in response to histamine via the MAP kinases, JNK and ERK, but this phenomenon was also STAT6 independent (24). Here we confirm that IL-13 potentiated Ca^{2+} signaling in response to 1 μM histamine. In contrast KCl-induced ASMC contraction was not affected by incubation with IL-13. This finding suggests that IL-13 modifies mechanisms involved in agonist-induced intracellular Ca^{2+} release but not in the depolarization of the cell membrane leading to a Ca^{2+} influx. This finding is also consistent with effects that are related to Ca^{2+} sensitization; the small G protein RhoA has been implicated in IL-13-induced hypercontractility via an upregulation of its expression (1). However, upregulation of RhoA reportedly required high concentrations of IL-13 (100 ng/ml) and was not observed at concentrations (10 and 30 ng/ml) closer to those we have employed. Furthermore, the effect of IL-13 was STAT6 dependent. It also appears that KCl-induced contraction may lead to RhoA and Rho-kinase activation (15). These observations suggest that low concentration of IL-13 may lead to a form of Ca^{2+} sensitization that does not involve RhoA and Rho-kinase.

How exactly IL-13 affects Ca^{2+} release is not clear, but recent investigations suggest potential mechanisms. Gao and coworkers (5) demonstrated that 10 ng/ml of IL-13 promoted calcium release and store-operated Ca^{2+} in rat ASMC. However, in their model, IL-13 affected the resting concentrations of Ca^{2+} and enhanced serum-induced proliferation in contrast to our observations. Sathish and colleagues (28) showed that 20 ng/ml of IL-13 decreased the sarcoplasmic reticulum Ca^{2+} ATPase SERCA2 expression in hASMC, contributing to a slower Ca^{2+} reuptake following contractile agonist stimulation. CD38 and cyclic adenosine diphosphate ribose (cADPR) may also be involved in this process. Deshpande and coworkers (3) demonstrated that treatment of ASMC with of IL-13 (50 ng/ml) increased CD38 expression and ADP-ribosyl cyclase activity and that a cADPR antagonist attenuated IL-13-induced augmented intracellular Ca^{2+} responses to agonists in hASMC (3).

We anticipated the possibility that the increase in contractile response of the hASMC to histamine associated with a reduction in proliferation might be associated with the development of a hypercontractile phenotype of cell. To address this issue, we studied the expression of several contraction-associated proteins by real time-qPCR. Among the proteins studied, only calponin and vinculin were significantly upregulated, and these at very modest levels (1.2-fold). To verify whether changes of mRNA expression affected the protein level in ASMC, we performed Western blots for SM myosin, SM α-actin, calponin, and vinculin and found no differences in protein. Thus, although IL-13 increased ASMC contractility, it did so in a limited manner, without affecting contractile protein expression. So far, few studies have addressed whether IL-13 modulates gene expression of contraction-associated proteins in hASMC. Using microarray, Lee and coworkers (19) looked for gene expression after 6 h of incubation in the presence of 100 ng/ml of IL-13. Among these genes, SM MHC expression was increased 3.6-fold. However, we were not able to detect any changes in SM MHC at mRNA or protein level. However, differences in experimental conditions may account for the discrepancy.

In conclusion, IL-13 inhibits hASMC proliferation and induces hASMC hypercontractility in response to histamine. This latter phenomenon seems to be calcium dependent because the expression of contractile proteins is not modified and the contractile response to KCl is unchanged. Additionally, this study demonstrates the importance of alternative pathways to STAT6 in the mechanisms of action of IL-13, a concept for which there is increasing evidence in recent years. There does not appear to be a simple binary regulation of the SM phenotype; hypercontractility of the SM cells is not necessarily associated with an increase of contractile protein expression.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

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