

IL-13 signaling through IL-13 receptor α 2 mediates airway epithelial wound repair

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ABSTRACT: Asthma is an airway inflammatory disease characterized by epithelial barrier dysfunction and airway remodeling. Interleukin-13 (IL-13) is a pleiotropic cytokine shown to contribute to features of airway remodeling. We have previously demonstrated that IL-13 is an important mediator of normal airway epithelial repair and health. The role of IL-13 signaling *via* its receptor subunits (IL-13R α 1/IL-4R α and IL-13R α 2) in airway epithelial repair and restoration of intact barrier function is not well understood and was investigated in this study using *in vitro* models. The blocking of IL-13 signaling *via* IL-13R α 2 significantly reduced airway epithelial repair by 24 h post-mechanical wounding in 1HAEo[−] cells. Expression and release of repair-mediating growth factor, heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), and subsequent activation of EGF receptor (EGFR) were also significantly reduced in response to wounding when IL-13R α 2 was blocked. Our data support that IL-13 signals *via* IL-13R α 2 to mediate normal airway epithelial repair *via* HB-EGF-dependent activation of EGFR. In human donor lung tissues, we observed that airway epithelium of asthmatics expressed significantly decreased levels of IL-13R α 2 and increased levels of IL-13R α 1 compared with nonasthmatics. Dysregulated expression of IL-13 receptor subunits in the airways of asthmatics may thus contribute to the epithelial barrier dysfunction observed in asthma.—Yang, S. J., Allahverdian, S., Saunders, A. D. R., Liu, E., Dorscheid, D. R. IL-13 signaling through IL-13 receptor α 2 mediates airway epithelial wound repair. *FASEB J.* 33, 000–000 (2019). www.fasebj.org

KEY WORDS: asthma • type 2 inflammation • barrier function • EGFR • HB-EGF

The airway epithelium modulates responses to constant environmental challenges and maintains tissue homeostasis as a first line of defense. Secreted mucus, antimicrobial molecules, and immune modulators expressed by airway epithelial cells (AECs) form the immune barrier responding to environmental insults (1). Junctional proteins of AECs maintain a selectively permeable physical barrier protecting underlying tissue (2). Appropriate activation of cellular repair processes in response to environmental insults and injuries is critical in restoring and maintaining epithelial barrier function. Repair involves

initial inflammation, cell spreading, migration, proliferation, and differentiation, followed by resolution of inflammation. Defects in any of these complex processes can lead to epithelial barrier dysfunction and persistent damage (3). Our objective is to better understand the regulation of normal airway epithelial repair and barrier function to identify defects that contribute to inflammatory disease states, such as asthma.

The airway epithelium of asthmatics exhibits features of barrier dysfunction. Patchy disruption of tight junctions has been observed in bronchial biopsies from asthmatic subjects (4). Differentiated air-liquid interface (ALI) cultures of AECs from asthmatic donors express significantly lower levels of E-cadherin compared with nonasthmatics (5). The presence of Creola bodies, large clusters of shed AECs in induced sputum samples of asthmatics (6), and excessive epithelial shedding observed in asthmatic bronchial biopsies (7) suggests persistent damage and aberrant repair responses in the airway epithelium. As a result of cycles of damage and inappropriate repair, the airway epithelium of asthmatics is chronically activated and releases dysregulated levels of various growth factors and cytokines that are involved in normal repair (8). Particularly, injury-induced, epithelial-derived alarmins, such as IL-33, IL-25, and thymic stromal lymphopoietin, activate type 2 innate lymphoid cells to promote T helper 2

ABBREVIATIONS: AEC, airway epithelial cell; ALI, air-liquid interface; EGFR, epidermal growth factor receptor; GOI, gene of interest; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HPRT, hypoxanthine-guanine phosphoribosyltransferase; HRP, horseradish peroxidase; IL-4R α , IL-4 receptor α subunit; IL-13R α 1, IL-13 receptor α 1; IL-13R α 2, IL-13 receptor α 2; MUC5AC, mucin 5AC; p-EGFR, phosphorylated epidermal growth factor receptor; p-STAT6, phosphorylated signal transducer and activator of transcription 6; qPCR, quantitative PCR; rhIL-13, recombinant human IL-13; sIL-13R α 2, soluble IL-13 receptor α 2; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline-Tween-20

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high inflammation within the airway (9). In murine models of asthma, type 2 innate lymphoid cells have been shown to produce potent levels of the T helper 2 cytokines IL-13 and IL-5 in response to allergen challenges (10).

Our laboratory has previously shown that normal AECs produce and secrete IL-13 in response to mechanical injury (11). We demonstrated that IL-13 promotes normal airway epithelial repair, and this effect is mediated *via* the autocrine/paracrine release of heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) and activation of EGF receptor (EGFR). IL-13 is a pleiotropic cytokine produced by AECs (11) and various immune cell types (9, 12–14). The role for IL-13 on specific airway epithelial biology has not been explored. IL-13 was identified to be genetically associated with asthma (15) and observed to be elevated in the airways of asthmatics (16, 17), suggesting abnormalities in IL-13 production and signaling. Studies in transgenic murine models of asthma have demonstrated that IL-13 is a central mediator of airway remodeling features, including mucus hypersecretion, goblet cell metaplasia, and eosinophilia (18, 19).

The effects of IL-13 are mediated through 2 receptors: the complex of IL-13 receptor $\alpha 1$ (IL-13R $\alpha 1$)/IL-4 receptor α subunit (IL-4R α) and IL-13 receptor $\alpha 2$ (IL-13R $\alpha 2$) (20). IL-13R $\alpha 1$ is expressed by numerous cell types, including B lymphocytes (21), monocytes (22), and AECs (23). IL-13 signaling *via* IL-13R $\alpha 1$ /IL-4R α activates the Janus kinase-signal transducer and activator of transcription (STAT) pathway, specifically STAT6. This has been shown to be the principal pathway that mediates mucus production, goblet cell metaplasia, and other airway remodeling modifications (24). In contrast, IL-13R $\alpha 2$ alone binds IL-13 with high affinity and is thought to be a decoy receptor that regulates IL-13 availability to dampen activation of STAT6 (25).

Several studies, however, have provided evidence that IL-13R $\alpha 2$ can function as a signaling receptor. IL-13R $\alpha 2$ was demonstrated to signal *via* activated protein-1 to induce TGF- $\beta 1$ production in human macrophages and murine models of lung fibrosis and colitis (26, 27). IL-13R $\alpha 2$ is expressed by various cell types, including macrophages, lung fibroblasts, and AECs (28). Both humans and mice express IL-13R $\alpha 2$ on the cell surface as a transmembrane protein and in intracellular pools (29). Soluble IL-13R $\alpha 2$ (sIL-13R $\alpha 2$) is produced from an alternatively spliced transcript in mice, whereas in humans, it is generated *via* matrix metalloproteinase-mediated cleavage of membranous IL-13R $\alpha 2$ (30). It is unclear if humans express sIL-13R $\alpha 2$ *in vivo*, as it was undetectable in bronchoalveolar lavage fluids from healthy and asthmatic subjects who underwent a segmental allergen challenge in addition to plasma samples (31). Further characterization of the relative cellular distribution of IL-13R $\alpha 2$ in human systems will provide insight into its role in regulating IL-13 signaling as it relates to airway epithelial biology.

In our current *in vitro* investigation, we tested the hypothesis that IL-13 signals through IL-13R $\alpha 2$ to mediate normal airway epithelial repair and to re-establish an intact epithelial layer and resulting barrier function. Appropriate repair processes in the airway epithelium are critical in restoring epithelial barrier function and

regulating timely resolution of inflammatory responses elicited by injuries (32). We used mechanical wounding and IL-13 exposure as injury signals to characterize normal airway epithelial repair processes and not as models to simulate the dysregulated, elevated levels of IL-13 in the asthmatic airway. We identified a novel role for IL-13R $\alpha 2$ in mediating normal epithelial wound repair through regulation of downstream production and release of HB-EGF and activation of EGFR. We also characterized differential expression of IL-13 receptor subunits in the asthmatic airway epithelium that may contribute to the dysregulated repair and barrier dysfunction observed in asthma.

MATERIALS AND METHODS

Cell culture

1HAEO[−] cells are previously characterized simian virus 40-transformed normal human AECs (33) that were cultured in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% CO₂. Ethics approval for the use of human tissue was granted by the University of British Columbia and Providence Health Care Ethics Review Board (Research Ethics Board H00-50100). Primary AECs were isolated by protease digestion (34) from lungs of de-identified, nonasthmatic and asthmatic donors that were deemed unsuitable for transplantation and donated to research (International Institute for the Advancement of Medicine, Edison, NJ, USA). Isolated primary AECs were cultured as submerged undifferentiated monolayers in bronchial epithelial growth medium (CC-3170; Lonza, Mississauga, ON, Canada) at 37°C in 5% CO₂. Differentiated ALI cultures of primary AECs were grown for 21 d in PneumaCult-ALI medium (05001; Stemcell Technologies, Vancouver, BC, Canada).

Immunohistochemistry

IL-13R $\alpha 1$ and IL-13R $\alpha 2$ levels in formalin-fixed, paraffin-embedded tissue sections of de-identified, nonasthmatic and asthmatic donor lungs (Research Ethics Board H00-50100) were detected *via* immunohistochemistry. Tissue sections (4 μ m thick) were de-waxed, rehydrated, and subjected to heat-induced antigen retrieval in citrate buffer (pH 6.0; Thermo Fisher Scientific). Tissue sections were blocked in either universal protein block (Dako, Burlington, ON, Canada) or 10% normal rabbit serum for 30 min, followed by avidin biotin block (Vector Laboratories, Burlington, ON, Canada) for 30 min at room temperature. Tissue sections were incubated with goat anti-human IL-13R $\alpha 1$ antibody, goat anti-human IL-13R $\alpha 2$ antibody, or normal goat IgG as a negative control (AF152, 2 μ g/ml; AF146, 8 μ g/ml; AB-108-C, 8 μ g/ml, respectively; R&D Systems, Minneapolis, MN, USA) in Tris-buffered saline (TBS) overnight at 4°C. Tissues were washed in TBS and incubated with biotin-conjugated rabbit anti-goat IgG (E0466; Dako) for 1 h at room temperature. An avidin-biotin complex-alkaline phosphatase amplification step was next applied (Vector Laboratories) for 30 min, followed by Warp Red Chromogen (Biocare Medical, Concord, CA, USA) as substrate for 7 min. Cell nuclei were counterstained with hematoxylin. Brightfield images (Nikon Eclipse E600 Microscope; Nikon, Tokyo, Japan) of sections from large airways were digitally captured (SpotFlex Camera; Diagnostic Instruments, Sterling Heights, MI, USA), with the observer blinded to donor disease status. A minimum of 3 airways from each section were assessed and averaged for IL-13R $\alpha 1$ and IL-13R $\alpha 2$ positivity *via* color

segmentation using ImagePro Plus (Media Cybernetics, Silver Spring, MD, USA).

Preparation of total cell lysates and Western blot

Confluent monolayer cultures of 1HAEo⁻ cells were subjected to linear cross-hatched mechanical wounding using a rubber styllet as previously established in Allahverdian *et al.* (11). Eighty to 90% confluent 1HAEo⁻ cells were treated with recombinant human IL-13 (213-IL; rhIL-13, 10 ng/ml; R&D Systems). Cultures were treated with either goat anti-human IL-13Rα1 or goat anti-human IL-13Rα2 neutralizing antibodies (AF152; 20 μg/ml, or AF146, 2 μg/ml; R&D Systems) immediately after mechanical injury or 1 h before rhIL-13 (10 ng/ml) treatment. Total cell lysates were collected at indicated times and analyzed *via* Western blot. Fifteen micrograms of reduced protein extracts was electrophoresed in polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were blocked for 30 min at room temperature in TBS-Tween-20 (TBS-T) containing either 5% skim milk or 5% bovine serum albumin for phosphorylated proteins of interest. Membranes were incubated with the following primary antibodies overnight at 4°C: rabbit anti-human IL-13Rα1 antibody (ab79277; 1 μg/ml; Abcam, Cambridge, MA, USA), goat anti-human IL-13Rα2 antibody (0.2 μg/ml; R&D Systems), rabbit anti-phosphorylated EGFR (p-EGFR) antibody (44784G; 1 μg/ml; Thermo Fisher Scientific), sheep anti-EGFR antibody (20-ES04; 1 μg/ml; Fitzgerald, North Acton, MA, USA), goat anti-HB-EGF antibody (sc-1413; 1 μg/ml; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-phosphorylated STAT6 (p-STAT6) antibody (9361; 0.1 μg/ml; Cell Signaling Technology, Danvers, MA, USA), and mouse anti-STAT6 antibody (611,290; 0.25 μg/ml; BD BioSciences, Mississauga, ON, Canada). Membranes were washed 3 times with TBS-T and incubated with appropriately matched secondary antibody for 1 h at room temperature. The following secondary antibodies were used: goat anti-rabbit IgG-horseradish peroxidase (12-348; HRP; 0.5 μg/ml; Millipore, Temecula, CA, USA), bovine anti-sheep IgG-HRP, bovine anti-goat IgG-HRP (sc-2701 and sc-2350; 0.2 μg/ml; Santa Cruz Biotechnology), and goat anti-mouse IgG-HRP (554002; BD BioSciences). Following TBS-T washes, samples were detected with ECL substrate (Supersignal West Femto; Thermo Fisher Scientific), and membranes were imaged on the ChemiGenius Bio-Imaging System (Syngene, Frederick, MD, USA).

RNA isolation and real-time quantitative PCR

RNA was isolated using TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's protocol. RNA concentration was measured using the NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific). RNA quality was assessed based on a 260:280 ratio with the inclusion criteria of values between 1.8 and 2.0. Two-step real-time quantitative PCR (qPCR) was performed to assess mRNA expression of IL-13Rα1, IL-13Rα2, and mucin 5AC (MUC5AC). RNA (1000 ng) was converted into cDNA using qScript cDNA SuperMix (95047-100; Quanta Biosciences, Gaithersburg, MD, USA), according to the manufacturer's protocols in a T100 Thermocycler (Bio-Rad, Hercules, CA, USA). qScript reverse transcriptase within the qScript cDNA SuperMix reverse transcribed RNA into cDNA with a mixture of oligo(dT), random primers, and dNTPs in a total reaction volume of 20 μl. Reaction conditions for cDNA synthesis consisted of 1 cycle at 22°C for 5 min, 1 cycle at 42°C for 30 min, and 1 cycle at 85°C for 5 min. mRNA levels were quantified using commercially available SYBR Green Assays (Qiagen, Mississauga, ON, Canada) on the ViiA7 Real-Time PCR System (Thermo Fisher Scientific). Each reaction had a total volume of 20 μl, consisting of 1000 ng cDNA, 0.5 μM forward and

reverse primers, 10 μl SYBR Green Master Mix, and DNase- and RNase-free water. The following primers were used: IL-13Rα1 (forward) 5'-TCTCACCCAGGAAGGTGATC-3', (reverse) 5'-ATTCCCTCCAGGGAGCCAAG-3'; IL-13Rα2 (forward) 5'-AAACAACAAATGAAACCCGACAA-3', (reverse): 5'-GTCTTCACCTTCCCAGCATTG-3'; MUC5AC (forward) 5'-AGCGTGGAGAATGAGAAGTATG-3', (reverse) 5'-CATGCAGTTCGAGAGTAGGTT-3'; hypoxanthine-guanine phosphoribosyltransferase (HPRT; forward) 5'-TGACACTGGCAAAACAATGCA-3', (reverse) 5'-GGTCCTTTTACCAGCAAGCT-3'. Triplicates of each sample were loaded into a 384-well PCR plate. The reaction conditions for IL-13Rα1 and IL-13Rα2 real-time qPCR consisted of 1 cycle at 95°C for 15 min, 45 cycles of denaturing at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The reaction conditions for MUC5AC qPCR consisted of 1 cycle at 95°C for 15 min, 45 cycles of denaturing at 94°C for 15 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. Following amplification, the resulting products were subjected to melt curve analysis, and the presence of single-sized products was confirmed. Data were analyzed using the ViiA7 Real-Time PCR System Software. Results were expressed using the comparative quantification method (35), where threshold cycle (C_t) values of the gene of interest (GOI) were normalized to C_t values of the housekeeping gene HPRT, and expression fold changes relative to untreated control samples were then calculated as the following: $\Delta\Delta C_t = [(C_{tGOI} - C_{tHPRT})_{TREATED} - (C_{tGOI} - C_{tHPRT})_{CONTROL}]$. Expression fold change was defined as $2^{-\Delta\Delta C_t}$.

Flow cytometry

Cell-surface expression of membranous IL-13Rα2 was assessed *via* flow cytometry in monolayers of 1HAEo⁻ cells postmechanical wounding and rhIL-13 (10 ng/ml) treatment. Cells were detached at designated time points using 5 mM EDTA at 37°C in 5% CO₂ for 10 min. Cells were collected and washed with PBS and centrifuged at 250 g for 7 min. Cell pellets were resuspended in PBS and stained with Live/Dead Fixable Violet Viability Dye (L34955; Thermo Fisher Scientific) for 30 min on ice in dark. Cells were centrifuged at 350 g at 4°C for 5 min and then washed with PBS. Following centrifugation, cells were resuspended and blocked with 5% bovine serum albumin in PBS on ice for 15 min in dark. Each sample was then incubated with 10 μl of either phycoerythrin-conjugated mouse anti-human IL-13Rα2 antibody (852.122.010; Cell Sciences, Canton, MA, USA) or isotype mouse IgG-phycoerythrin (IC002P; R&D Systems) in the dark for 30 min on ice. Cells were washed with PBS and centrifuged as previously detailed. Cell pellets were resuspended and fixed in 1% paraformaldehyde in PBS. Cells were enumerated with a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA). Data were analyzed using Kaluza v.1.2 (Beckman Coulter).

ELISA

Released HB-EGF levels were measured *via* ELISA (DY259; R&D Systems), according to the manufacturer's protocol in cell-free supernatants frozen at -80°C before analysis.

Small interfering RNA transfection

Synthetic small interfering RNA (siRNA) targeting human IL-13Rα2 (1299001, identification: HSS105391; Thermo Fisher Scientific) was used to knockdown production of IL-13Rα2 protein in 1HAEo⁻ cells. Stealth RNAi Negative Control (45-2002; Thermo Fisher Scientific) served as scrambled siRNA negative control. 1HAEo⁻ cells were transfected with 100 nM siRNA using

the Lipofectamine RNAiMax reagent (13778-075; Thermo Fisher Scientific), where cells were simultaneously seeded with an siRNA-containing, lipid-mediated delivery system. 1HAEo⁻ cells were seeded at a density of 50,000–60,000 cells/ml in 12- or 6-well dishes with antibiotic-free DMEM containing 10% fetal bovine serum. IL-13Rα2-specific and scrambled siRNA were prepared separately from the Lipofectamine RNAiMax reagent in serum and antibiotic-free DMEM and incubated at room temperature for 5 min. siRNA and the Lipofectamine RNAiMax reagent were then combined and incubated for 15 min at room temperature before addition. Cells were then incubated at 37°C and 5% CO₂ for 48 h before protein extraction or any treatment. Western blotting was performed and determined a knockdown efficiency of 77% in IL-13Rα2-specific siRNA-transfected cultures.

Wound repair assay

With the use of our previously established wound kinetics assay (11), we examined epithelial repair in 1HAEo⁻ cells treated with either IL-13Rα1 or IL-13Rα2 neutralizing antibodies or transfected with IL-13Rα2-specific or scrambled siRNA. All cultures were subjected to circular wounding (<2.5 mm²) using a rubber styllet and photographed at 0 and 24 h postwounding using a Nikon Eclipse TE200 inverted scope equipped with a Nikon Coolpix E995. Wound areas were determined using ImagePro Plus (Media Cybernetics), and percentage of repair over 24 h was calculated using corresponding wound areas at 0 and 24 h postinjury. Wound repair kinetics of small (<3 mm²) and large (>3 mm²) circular wounds were also assessed in monolayer cultures of primary AECs from nonasthmatic and asthmatic donor lungs over 24 h.

Statistical analysis

Data were analyzed with the GraphPad Prism 5.0 software package (GraphPad Software, La Jolla, CA, USA). Values are presented as means ± SEM. Means were compared with 2-tailed unpaired Student's *t* and Mann-Whitney *U* tests for non-parametric data sets or for multiple comparisons, 1-way ANOVA and 2-way ANOVA with appropriate *post hoc* tests. Significance is noted at *P* < 0.05.

RESULTS

Epithelium of asthmatic airways expresses higher levels of IL-13Rα1 and lower levels of IL-13Rα2 compared with nonasthmatic airways

Expression of IL-13Rα1 and IL-13Rα2 in nonasthmatic and asthmatic lung tissue was examined *via* immunohistochemistry (Fig. 1A). Mean area of airway epithelium positive for IL-13Rα1 expression/basement membrane length in the asthmatic group was significantly elevated compared with nonasthmatics (Fig. 1B), whereas IL-13Rα2 expression was significantly lower in the asthmatic group compared with nonasthmatics (Fig. 1C). Baseline expression of IL-13Rα2 in both differentiated ALI and undifferentiated monolayer cultures of primary AECs from nonasthmatic and asthmatic donor lungs detected *via* Western blotting reflected levels observed in lung tissue, where the asthmatic group expressed significantly lower levels of IL-13Rα2 compared with the nonasthmatic group

(Fig. 1D, E). To determine the effect of differential IL-13Rα2 expression in the asthmatic airway epithelium on wound repair, we examined repair kinetics in monolayer cultures of nonasthmatic and asthmatic primary AECs. In large wounds (>3 mm²), the percentage of epithelial repair at 24 h postinjury was significantly reduced in primary asthmatic AECs compared with nonasthmatics (Fig. 1F).

Expression of IL-13Rα2 is upregulated in AECs in response to mechanical wounding and IL-13 exposure

We previously demonstrated that AECs produce IL-13 in response to mechanical wounding to mediate normal airway epithelial repair (11). As asthmatic AECs exhibit reduced capacity to repair and differential IL-13 receptor subunit expression levels, we aim to characterize the regulation of these receptors in the context of normal airway epithelial repair and restoration of barrier function. Better understanding of epithelial repair processes in normal systems will provide insight into identifying defects in the disease process of asthma. We investigated the role of IL-13Rα1 and IL-13Rα2 in repair and examined their expression in response to previously established injury signals, such as exogenous IL-13 exposure and mechanical wounding in monolayer cultures of 1HAEo⁻ cells, a normal AEC cell line. mRNA levels of IL-13Rα2 were significantly increased at 4 and 6 h post-mechanical wounding relative to unwounded cultures at 0 h (Fig. 2A). There was no significant change in IL-13Rα1 mRNA expression in response to mechanical wounding (Fig. 2B). Exposure to exogenous IL-13 significantly increased expression of IL-13Rα2 mRNA at 4 h post-treatment (Fig. 2C). At 0.5 and 6 h post-IL-13 exposure, IL-13Rα1 mRNA levels were significantly decreased (Fig. 2D). Expression of IL-13Rα2 protein in 1HAEo⁻ cells was significantly increased by 24 h after mechanical wounding and IL-13 exposure compared with untreated monolayers at 0 h (Fig. 3A, C), whereas expression of IL-13Rα1 protein was not significantly different (Fig. 3B, D). Expression of membranous IL-13Rα2 on the cell surface of 1HAEo⁻ cells was confirmed *via* flow cytometry (Fig. 3E, F). In contrast, 1HAEo⁻ cells do not release sIL-13Rα2 as there were no detectable levels of sIL-13Rα2 in the cell-free supernatants collected at either baseline or post-mechanical injury (data not shown). These data demonstrate that IL-13Rα2 expression is dynamic and suggest that IL-13Rα2 may serve as more than just a decoy receptor that sequesters excess IL-13.

The blocking of IL-13 signaling through IL-13Rα2 inhibits normal airway epithelial repair

The direct role of IL-13 receptor subtypes in IL-13-mediated epithelial repair was examined using mechanically wounded monolayer cultures of 1HAEo⁻ cells treated with IL-13Rα1 and IL-13Rα2 neutralizing antibodies. The blocking of IL-13 binding to IL-13Rα2 *via* antibody neutralization significantly reduced epithelial repair in a dose-dependent manner compared with untreated controls over 24 h (Fig. 4A), suggesting that IL-13 signals through

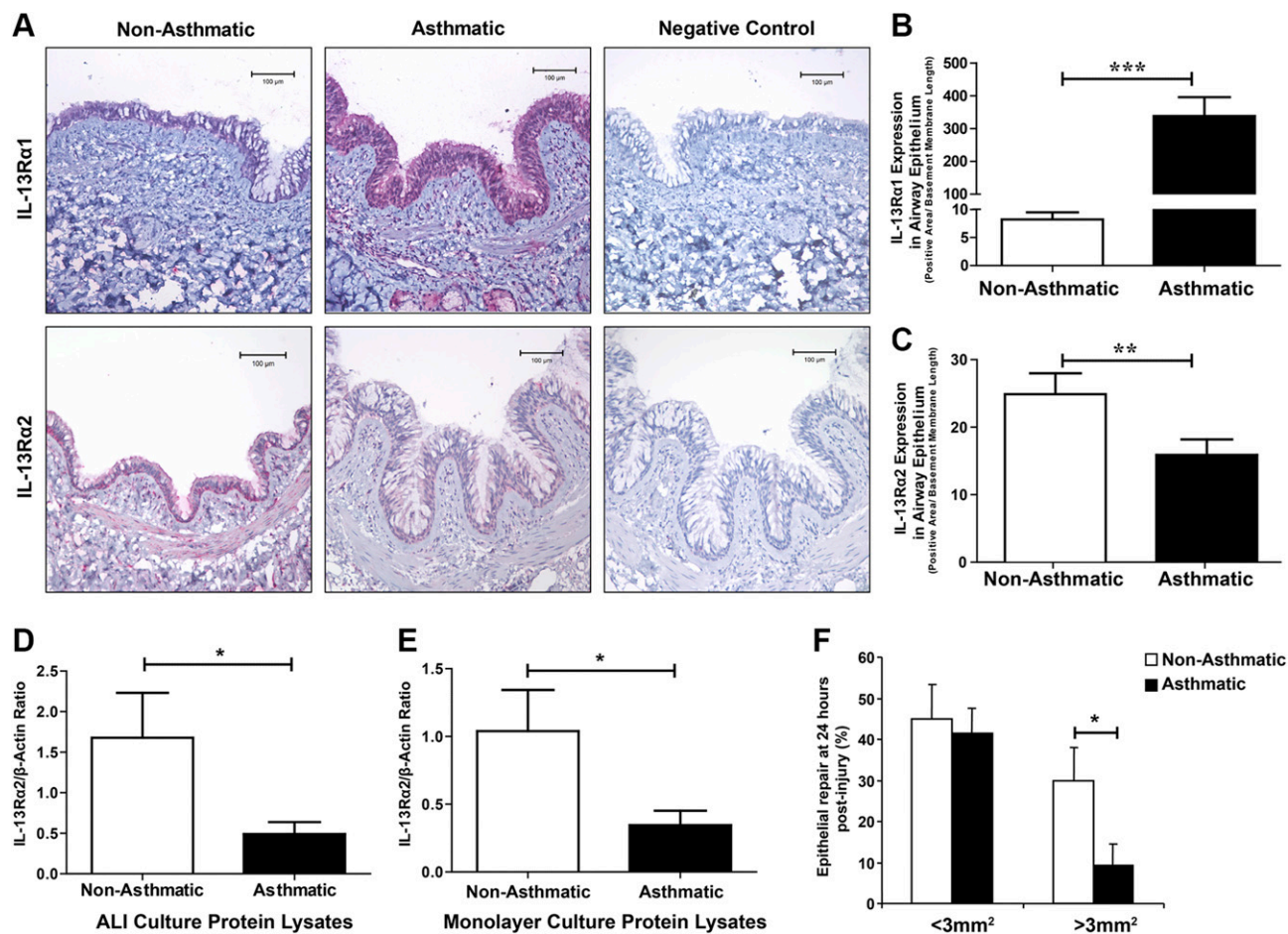


Figure 1. Airway epithelium of asthmatic donors express higher levels of IL-13Rα1 and lower levels of IL-13Rα2 compared with nonasthmatic donors. *A*) Expression of IL-13Rα1 and IL-13Rα2 in the airways of nonasthmatic and asthmatic donor lungs was detected *via* immunohistochemistry, where pink was indicative of positivity. Scale bars, 100 μm. *B*, *C*) Epithelial area positive for IL-13Rα1 (*B*) and IL-13Rα2 (*C*) was quantified *via* color segmentation in ImagePro Plus (Media Cybernetics). Data are means ± SEM of multiple airways from 5 to 10 donors in each group. ****P* < 0.001, ***P* < 0.01, Mann-Whitney *U* test. *D*, *E*) IL-13Rα2 protein expression is significantly lower in both differentiated ALI and monolayer cultures of asthmatic primary AECs compared with nonasthmatics. **P* < 0.05, *n* = 6, Mann-Whitney *U* test. *F*) The percentage of epithelial repair at 24 h postinjury in monolayer cultures was significantly reduced in primary asthmatic AECs compared with nonasthmatics in large circular wounds that were >3 mm² in size. **P* < 0.05, *n* = 4, 2-way ANOVA with Bonferroni multiple comparison test.

IL-13Rα2 to mediate repair. The percentage of repair observed in cultures treated with IL-13Rα1 neutralizing antibody was similar to control. Representative images in Fig. 4*B* demonstrate that repair is inhibited in IL-13Rα2 neutralizing antibody-treated cultures, whereas the control and IL-13Rα1 neutralizing antibody-treated cultures repair by 24 h postwounding.

The blocking of IL-13 binding to IL-13Rα1 reduces STAT6 activation and MUC5AC expression

The effectiveness of IL-13Rα1 antibody neutralization on the blocking of IL-13 binding and signaling in 1HAEo⁻ cells was assessed to confirm that IL-13Rα1 does not play a direct role in mediating wound repair. IL-13 signaling *via* IL-13Rα1/IL-4Rα induces p-STAT6 and activates subsequent transcription of airway remodeling-related genes, such as MUC5AC. Exposure to exogenous IL-13 in the

absence of antibody pretreatment induced p-STAT6 in 1HAEo⁻ cells (Fig. 4*C*). Conversely, pretreatment with IL-13Rα1 neutralizing antibodies significantly attenuated STAT6 activation, 2 h post-IL-13 exposure, compared with time-matched controls, demonstrating that IL-13 binding to IL-13Rα1 was effectively blocked. Expression of MUC5AC mRNA was decreased in IL-13Rα1 neutralizing antibody-treated 1HAEo⁻ monolayer cultures at 4 and 6 h post-IL-13 exposure compared with time-matched control cultures (Fig. 4*D*).

The blocking of IL-13 binding to IL-13Rα2 reduces HB-EGF production and release in response to mechanical injury

We previously demonstrated that IL-13 induces production and secretion of HB-EGF and subsequent activation of EGFR and wound repair-related pathways in response to mechanical injury in normal AECs (11). As the

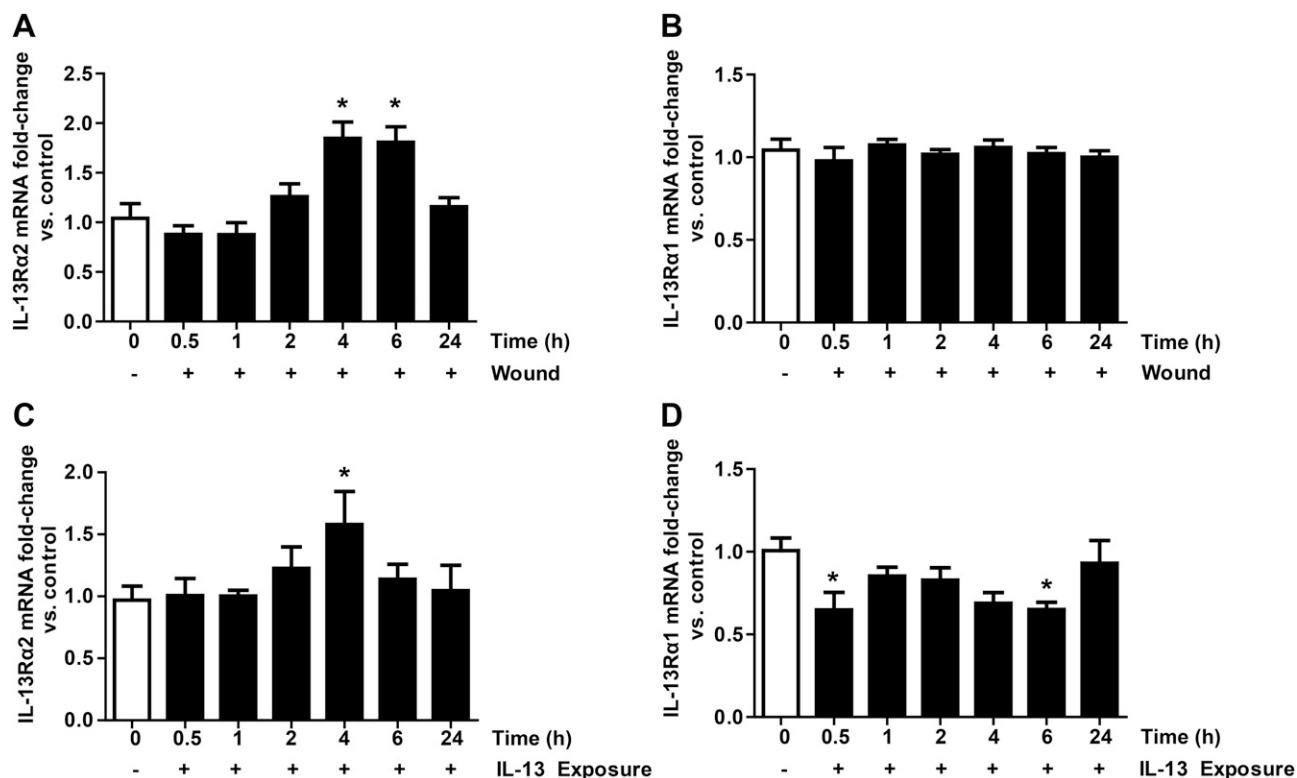


Figure 2. Expression of IL-13Rα2 mRNA is up-regulated in response to mechanical injury in 1HAEo⁻ cells. mRNA expression of IL-13Rα1 and IL-13Rα2 in 1HAEo⁻ cells was examined *via* qPCR and quantified as fold change relative to untreated controls at 0 h. A, B) Mechanical wounding induced significant increases in the expression of IL-13Rα2 mRNA at 4 and 6 h postinjury (A) and did not alter mRNA expression of IL-13Rα1 (B). C, D) IL-13 exposure (10 ng/ml) significantly increased IL-13Rα2 mRNA expression at 4 h post-treatment (C) and significantly decreased IL-13Rα1 mRNA expression (D). **P* < 0.05 vs. 0 h control, *n* = 3, 1-way ANOVA with Bonferroni multiple comparison test.

blocking of IL-13 binding to IL-13Rα2 inhibited wound repair, we next investigated expression and release of HB-EGF, as mediated through this pathway in 1HAEo⁻ cells. Release of HB-EGF from IL-13Rα2 neutralizing antibody-treated cells, detected in cell-free supernatants *via* ELISA, was significantly reduced compared with the media control group at 6 and 24 h postinjury (Fig. 5A). Total expression of HB-EGF protein in response to mechanical wounding was also reduced by IL-13Rα2 antibody neutralization (Fig. 5B). In our mechanical wounding model, we observed that increased HB-EGF release at 6 and 24 h postinjury led to EGFR phosphorylation in media control cultures (Fig. 5C). In contrast, EGFR phosphorylation postinjury was reduced in IL-13Rα2 neutralizing antibody-treated cells compared with time-matched media control groups.

Gene silencing of IL-13Rα2 attenuated wound-induced HB-EGF release and repair in AECs

We further confirmed the direct role of IL-13Rα2 in mediating HB-EGF expression and release by knocking down expression of IL-13Rα2 using specific siRNA in 1HAEo⁻ cells. HB-EGF release in response to mechanical wounding was up-regulated in media control and scrambled siRNA-transfected and vehicle control groups. Release of HB-EGF from IL-13Rα2 knocked-down cells was significantly reduced compared with all 3 control groups at 24 h

post-mechanical wounding (Fig. 6A). Total expression of HB-EGF was also significantly reduced in IL-13Rα2 knocked-down cells at 24 h postwounding compared with all control groups (Fig. 6B). Consequently, p-EGFR was also down-regulated in IL-13Rα2 siRNA-transfected cells compared with all control groups at 24 h postinjury (Fig. 6C). Gene silencing of IL-13Rα2 resulted in significantly reduced repair by 24 h postinjury compared with all 3 control groups (Fig. 6D).

DISCUSSION

Asthma is an airways inflammatory disease with features of persistent epithelial damage (7) and abnormal epithelial barrier function (4). Studies elucidating the regulation of normal epithelial barrier function and repair are necessary to gain insight into defects in asthma that are not well understood. Our laboratory has previously shown that IL-13 is important in normal airway health (36) and epithelial repair (11). IL-13 signaling is regulated through IL-13Rα1/IL-4Rα and IL-13Rα2. IL-13-IL-13Rα1/IL-4Rα signaling *via* STAT6 has been well characterized, and IL-13Rα2 has been highlighted as a decoy receptor (25, 37). The signaling role of IL-13Rα2, however, remains to be further explored.

In an *in vitro* model of monolayer cultures of 1HAEo⁻ cells, we demonstrated that normal airway epithelial repair was inhibited in a dose-dependent manner when

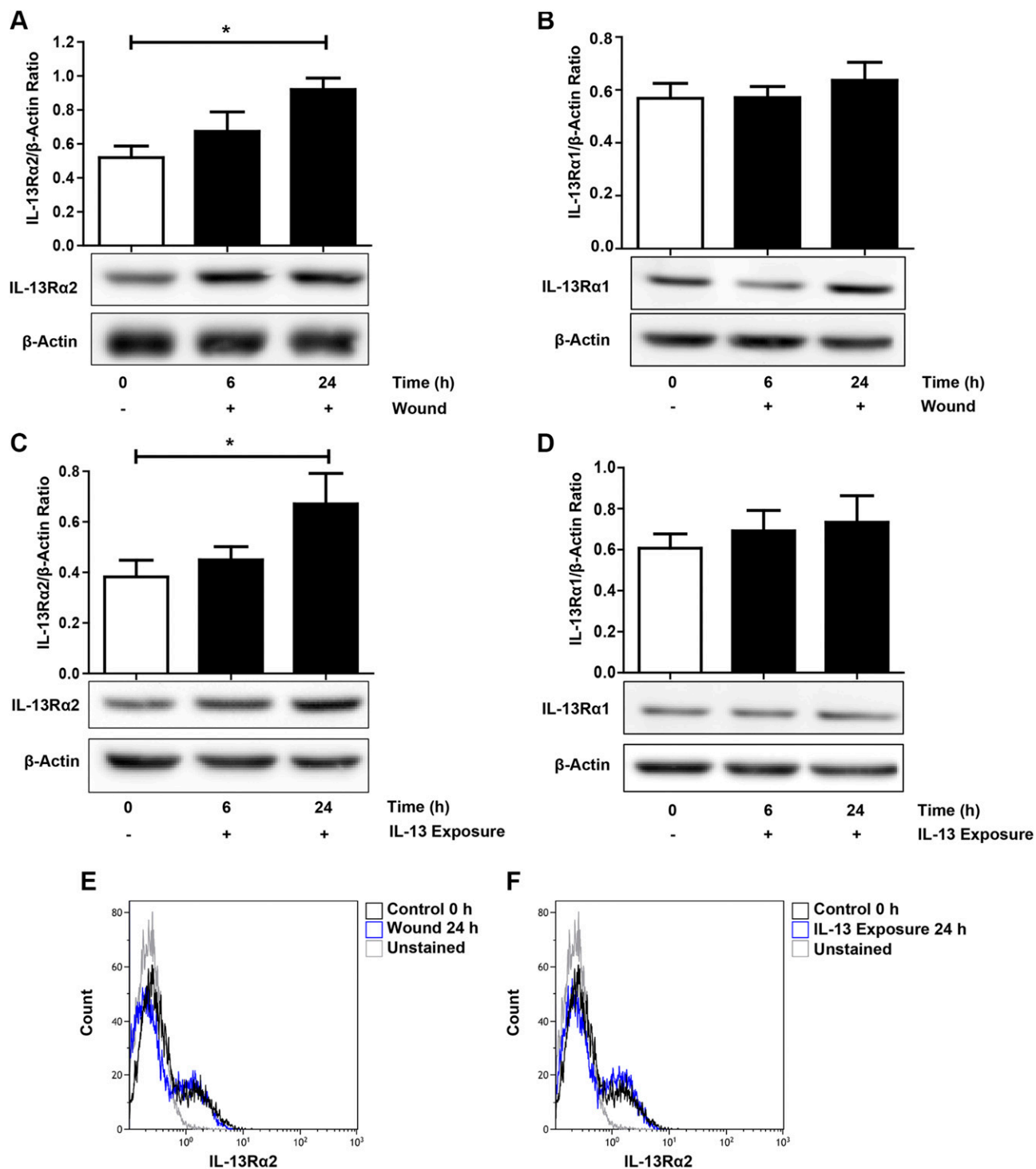


Figure 3. Expression of IL-13Rα2 protein is up-regulated in response to injury signals in 1HAEO⁻ cells. *A, C*) IL-13Rα2 protein expression in 1HAEO⁻ cells was significantly increased at 24 h postinjury and post-IL-13 exposure compared with untreated cultures at 0 h. *B, D*) There was no difference in IL-13Rα1 protein expression. **P* < 0.05 vs. 0 h control, *n* = 5–7, 1-way ANOVA with Bonferroni multiple comparison test. *E, F*) Representative histograms demonstrate that AECs (1HAEO⁻ cells) express membranous IL-13Rα2 on the cell surface, as detected by flow cytometry.

IL-13 binding to IL-13Rα2 was blocked with antibody neutralization. Conversely, the blocking of IL-13 binding to IL-13Rα1 did not inhibit repair. Downstream expression and release of HB-EGF were decreased in response to mechanical wounding when IL-13Rα2 was blocked and led to decreased EGFR activation. These data provide

further mechanistic evidence of our previous study, where wound-induced IL-13 was shown to mediate normal airway epithelial repair *via* HB-EGF-dependent EGFR activation (11). Here, we demonstrate that HB-EGF is a downstream product of IL-13 signaling *via* IL-13Rα2. Experiments conducted using IL-13Rα2-specific siRNA

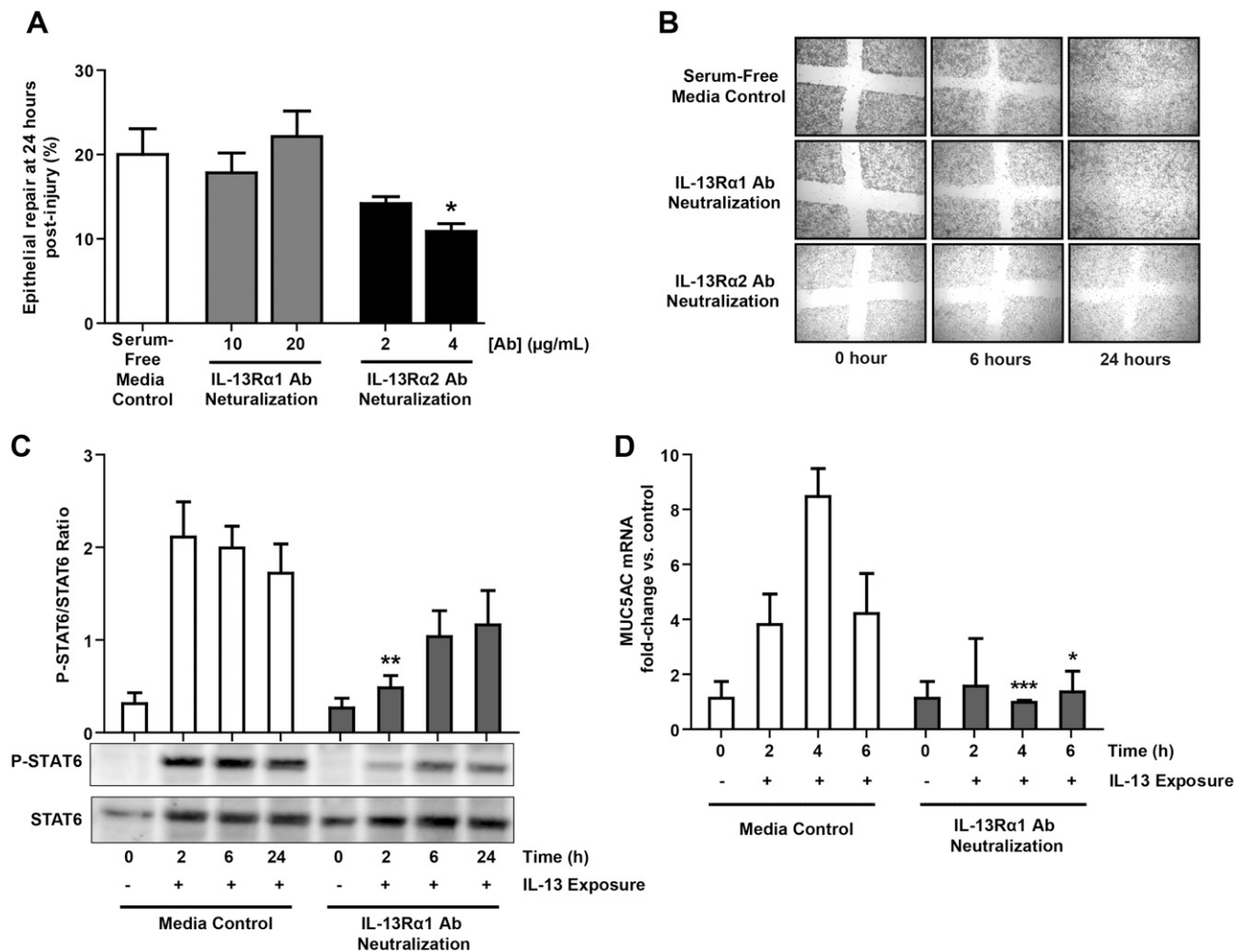


Figure 4. The blocking of IL-13Rα2 *via* antibody neutralization reduces epithelial repair. Mechanically wounded monolayers of 1HAEo⁻ cells were treated with increasing concentrations of IL-13Rα1 and IL-13Rα2 neutralizing antibodies immediately after injury. 1HAEo⁻ cells in the control group were wounded and maintained in serum-free medium. *A*) Corresponding wound areas at time 0 and 24 h were used to determine the percentages of repair over 24 h. The percentage of repair at 24 h postinjury was significantly reduced in the IL-13Rα2 neutralizing antibody (4 μg/ml) treatment group compared with the serum-free media control group. **P* < 0.05 *vs.* serum-free media control, *n* = 4, 1-way ANOVA with Bonferroni multiple comparison test. *B*) Images represent mechanically wounded monolayer cultures of 1HAEo⁻ cells treated with IL-13Rα1 or IL-13Rα2 neutralizing antibody. Cultures were imaged at 0, 6, and 24 h postinjury to assess repair. *C*) Pretreatment with IL-13Rα1 neutralizing antibody before IL-13 exposure reduced STAT6 activation compared with the time-matched control at 2 h post-treatment in 1HAEo⁻ cells. ***P* < 0.01, *n* = 3, 2-way ANOVA with Bonferroni posttest. *D*) 1HAEo⁻ cells that were pretreated with IL-13Rα1 neutralizing antibody have reduced MUC5AC transcript expression, 4 and 6 h post-IL-13 exposure, as measured by qPCR. ****P* < 0.001, **P* < 0.05 *vs.* control, *n* = 3, 2-way ANOVA with Bonferroni posttest.

provided similar confirmatory results. Our data demonstrate that IL-13Rα2 functions as a signaling receptor mediating airway epithelial repair *via* HB-EGF synthesis and release and subsequent EGFR activation.

IL-13Rα2 has been long thought to act only as a decoy receptor; however, our current study and others have shown that IL-13Rα2 can also function as a signaling receptor. In mouse models of colitis and bleomycin-induced lung injury, IL-13 was shown to signal *via* IL-13Rα2 to activate transcription factor activated protein-1 to induce production of TGF-β1 (26, 27). IL-13 signaling through IL-13Rα2 has also been demonstrated to activate MAPK pathways in primary intestinal epithelial cells from normal subjects and Crohn's disease patients (38) and in ovarian cancer cell lines (39). Another study found that IL-13 mediates the proliferation of human pulmonary artery

smooth muscle cells through IL-13Rα2-dependent induction of arginase 2 (40). There is mounting evidence from our study and others supporting the hypothesis that IL-13Rα2 is a functional signaling receptor that mediates IL-13 responses.

In this study, we used *in vitro* models of injury signals of mechanical wounding and IL-13 exposure in monolayer cultures of 1HAEo⁻ cells to investigate the role of IL-13 receptor subtypes in normal airway epithelial repair and restoration of intact barrier function. In particular, exogenous IL-13 exposure was not intended to serve as a signal of the asthmatic cytokine milieu but as an injury signal and mediator of normal repair processes, as we have previously shown that normal AEC release IL-13 in response to mechanical injury (11). Our data demonstrate the dynamic nature of IL-13Rα2 expression, where both

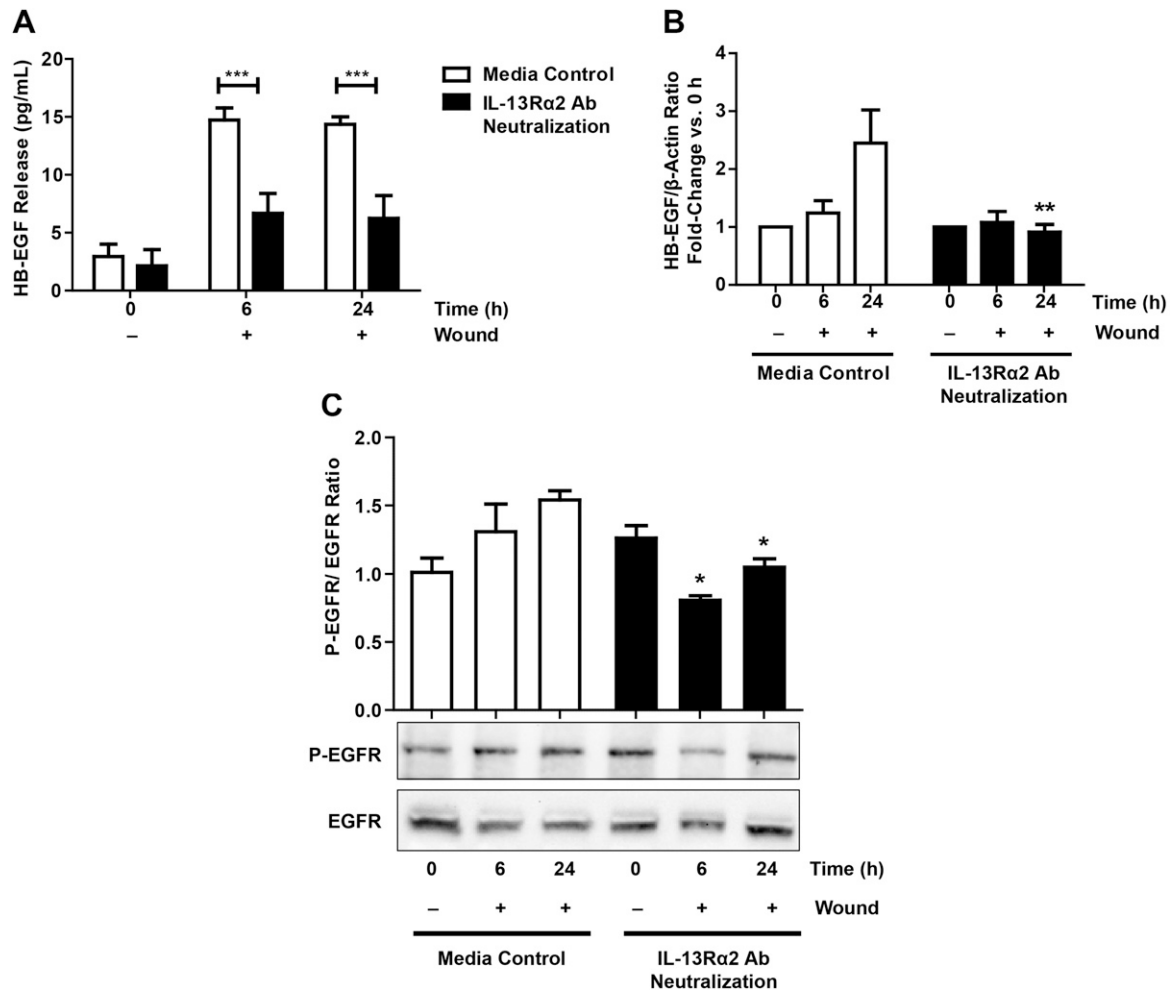


Figure 5. The blocking of IL-13Rα2 reduces HB-EGF release and production and subsequent p-EGFR in response to mechanical injury in 1HAEo⁻ cells. *A, B*) HB-EGF release and total expression in response to mechanical wounding are significantly reduced in 1HAEo⁻ cells treated with anti-IL-13Rα2 neutralizing antibodies compared with time-matched controls. **** $P < 0.001$, $n = 6$ (*A*), ** $P < 0.01$, $n = 5$ (*B*), 2-way ANOVA with Bonferroni posttest. Data in *B* are expressed as fold-change relative to unwounded controls at 0 h. *C*) EGFR activation, as detected *via* p-EGFR, was also reduced in IL-13Rα2 neutralizing antibodies treated with 1HAEo⁻ cells postwounding at 6 and 24 h compared with time-matched media control. * $P < 0.05$, $n = 3$, 2-way ANOVA with Bonferroni posttest.

IL-13Rα2 mRNA and protein levels were up-regulated in response to mechanical wounding and IL-13 exposure. As baseline expression of IL-13Rα2 is lower relative to IL-13Rα1, and IL-13Rα2 is a high-affinity receptor, even modest up-regulation of protein expression in response to mechanical wounding and IL-13 exposure would still have biological effects and yield functional changes. Our findings are consistent with previous studies that demonstrate IL-13-induced up-regulation of IL-13Rα2 mRNA transcript (41, 42). Other studies have also shown that IL-13Rα2 expression is tightly regulated in response to other injury signals, including viral antigens and viral response cytokines. IFN-γ induced cell-surface expression of IL-13Rα2 in nasal epithelial cells and monocytes *via* mobilization of existing intracellular pools (43). In bronchial fibroblasts, IFN-β and polyinosinic:polycytidylic acid exposure induced IL-13Rα2 mRNA production and increased cell-surface expression of IL-13Rα2 through *de novo* protein synthesis (44). Similar to our findings, IL-13Rα1 mRNA and cell-surface protein expression was unchanged in response to IFN-β and polyinosinic:

polycytidylic acid treatment. As IL-13 signals *via* both IL-13Rα1 and IL-13Rα2, the regulation of receptor levels plays a modulatory role for downstream signaling responses.

Appropriate control of inflammatory and repair processes is tightly regulated by the balance of IL-13Rα1 and IL-13Rα2 expression and function in response to injury. We demonstrated that airway epithelial expression of IL-13Rα2 was decreased in asthmatic donor lung tissue compared with nonasthmatics, whereas levels of IL-13Rα1 were found to be increased. In agreement with tissue levels observed, IL-13Rα2 expression in both monolayer and differentiated ALI cultures of primary asthmatic AECs was also decreased compared with nonasthmatics. We showed that monolayer cultures of primary asthmatic AECs have reduced repair in >3 mm²-sized wounds compared with nonasthmatic AECs at 24 h postinjury, suggesting that dysregulated IL-13 receptor expression may contribute to the inappropriate IL-13 response and dysfunctional repair phenotype observed in asthma. In asthmatic airway biopsies, IL-13 receptor subunit levels

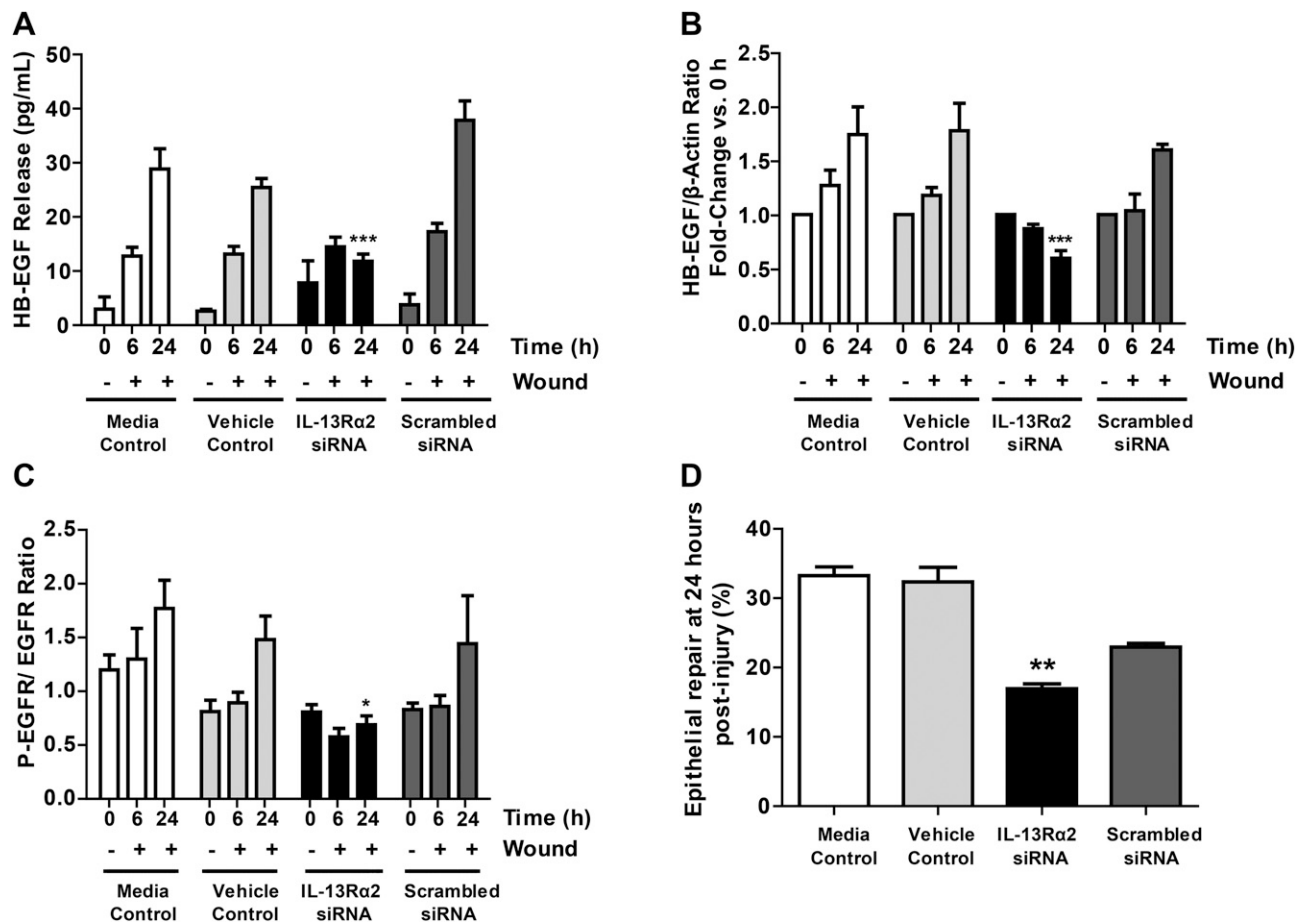


Figure 6. IL-13Rα2 siRNA knockdown inhibits wound closure of 1HAEO⁻ cells at 24 h. Confluent monolayers of IL-13Rα2 siRNA and scrambled siRNA-transfected 1HAEO⁻ cells were subjected to linear cross-hatched (A–C) and circular (D) mechanical wounding. A, B) Release (A) and total expression (B) of HB-EGF in response to wounding were significantly decreased in IL-13Rα2 siRNA-transfected cells compared with time-matched media control, vehicle control, and scrambled siRNA-transfected cells. *** $P < 0.001$, $n = 3$ (A), *** $P < 0.001$, $n = 4$ (B), 2-way ANOVA with Bonferroni posttest. C) p-EGFR was consequently down-regulated in the IL-13Rα2 siRNA-transfected cells in response to wounding compared with time-matched media control, vehicle control, and scrambled siRNA-transfected cells. * $P < 0.05$, $n = 4$, 2-way ANOVA with Bonferroni posttest. D) IL-13Rα2 gene silencing resulted in significantly reduced percentages of wound repair when compared with untreated media control, vehicle only, and scrambled siRNA-treated cells at 24 h postinjury. ** $P < 0.01$, $n = 12$, 2-way ANOVA with Bonferroni posttest.

have previously been shown to be altered and elevated compared with normal subjects when quantified in all cell types (45). Other disease states, such as idiopathic pulmonary fibrosis (41), pulmonary hypertension (46), ulcerative colitis (38), and several types of cancers, including ovarian, colorectal, and pancreatic cancer (38, 47), also exhibit altered IL-13 receptor subunit expression. In colorectal cancer cells, varying levels of IL-13Rα2 cell-surface expression were shown to result in differential responses to IL-13 (38). Our data with others highlight the importance of balanced IL-13 receptor subunit expression in regulating/mediating IL-13 responses.

Many studies published to date, characterizing the regulation of IL-13Rα2, have been performed in murine models. However, there are distinct differences in IL-13Rα2 biology between human and mice that need to be considered. Mice constitutively express both membranous and soluble forms of IL-13Rα2 translated from 2 alternatively spliced transcripts, whereas humans only express 1 form of IL-13Rα2 transcript, and sIL-13Rα2 is generated *via* proteolytic cleavage. Much of the characterization of

human sIL-13Rα2 has been performed on immune cells, where sIL-13Rα2 is generated *via* proteolytic cleavage of membranous IL-13Rα2 (30, 48); however, there may be cell type specific differences in receptor function and regulation. In this study, we demonstrated that AECs do not release sIL-13Rα2 in response to wounding and IL-13 exposure, providing evidence of regulatory differences from immune cells. IL-13 responses may be alternatively regulated in AEC, as our data demonstrated that IL-13 exposure down-regulated IL-13Rα1 mRNA transcript expression. This suggests that IL-13 may act directly on AECs to down-regulate IL-13Rα1 expression and thus, downstream responses *in lieu* of sIL-13Rα2.

The use of monolayer cultures of 1HAEO⁻ cells in our study was an appropriate initial model to characterize specific molecular mechanisms involved in IL-13-mediated airway epithelial wound repair, as IL-13Rα2 expression levels in monolayer cultures of primary AECs were concordant with lung tissue levels. We recognize the limitations in using undifferentiated cell line models of the airway epithelium. We plan to address these limitations in

future studies using polarized, differentiated ALI cultures of primary AECs that are more complex and physiologically relevant. ALI cultures retain concordant IL-13 receptor subunit expression with both monolayer cultures and human lung tissue, as our data have shown, and IL-13R α 2 expression has also been shown to be up-regulated in response to mechanical wounding in ALI cultures (49). IL-13 signaling is also retained in ALI cultures of primary AEC, where IL-13R α 1 neutralization also reduced IL-13-induced STAT6 activation (50). Furthermore, future experiments using coculture models with mesenchymal cells or immune cells will address the complex intercellular interactions required for maintaining airway tissue homeostasis in response to injury signals.

In summary, our current study identified IL-13R α 2 as the signaling receptor that mediates IL-13-induced normal airway epithelial repair and demonstrated the dynamic nature of its expression in response to mechanical injury. Our data show that differential expression of IL-13R α 1 and IL-13R α 2 in the asthmatic airway epithelium may contribute to aberrant repair. Regulation of IL-13 receptor subunits expression is therefore essential in mediating appropriate inflammatory and injury responses and maintaining airway epithelial barrier function. The beneficial role of IL-13 signaling *via* IL-13R α 2 in mediating normal airway epithelial repair and restoring barrier function may be mitigated by anti-IL-13 antibody therapies that block all IL-13 signaling responses. Our data demonstrate the complexity of IL-13 signaling *via* its receptor subunits, which may provide insight into the variable efficacy observed in multiple anti-IL-13 therapy trials for asthma (51, 52). Further studies are required for a complete understanding of the role of IL-13 in asthma. **[F]**

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AUTHOR CONTRIBUTIONS

S. J. Yang contributed intellectual input, study and experimental design, performing experiments and collecting data, data analysis and interpretation, figure assembly, and drafting, editing, and finalizing the manuscript; S. Allahverdian contributed intellectual input, study and experimental design, data analysis and interpretation, and editing and approving the final manuscript; E. Liu and A. D. R. Saunders performed experiments, data analysis, and approved the final manuscript; and D. R. Dorscheid contributed study idea conception, intellectual input, study and experimental design, data interpretation, and editing and approving the final manuscript.

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