

Uteroglobin Suppresses SCCA Gene Expression Associated with Allergic Asthma*

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Uteroglobin (UG), the founding member of the *Secretoglobin* superfamily, is a potent anti-inflammatory protein constitutively expressed at a high level in the airway epithelia of all mammals. We previously reported that the lungs of UG-knock-out (UG-KO) mice express elevated levels of Th2 cytokines (e.g. interleukin (IL)-4 and IL-13), which are augmented by allergen sensitization and challenge leading to exaggerated airway inflammation. Notably, these responses are suppressed by recombinant UG treatment (Mandal, A. K., Zhang, Z., Ray, R., Choi, M. S., Chowdhury, B., Pattabiraman, N., and Mukherjee, A. B. (2004) *J. Exp. Med.* 199, 1317–1330). Recent reports indicate that human orthologs of murine squamous cell carcinoma antigen-2 (*SCCA-2/serpinb3a*), a serine protease-inhibitor, are overexpressed in the airways of asthmatic patients. We report here that compared with wild type littermates, UG-KO mouse lungs express markedly elevated levels of *SCCA-2* mRNA and protein, which are augmented by allergen-challenge. Most importantly, these effects are abrogated by recombinant UG treatment. We further demonstrate that treatment of cultured human bronchial epithelial cells with IL-4 or IL-13 stimulates phosphorylation of STAT-1 and STAT-6 leading to *SCCA-1* (*SERPINB3*) and *SCCA-2* (*SERPINB4*) gene expression. We propose that: (i) IL-4- and IL-13-stimulated *SCCA* gene expression is mediated via STAT-1 and STAT-6 activation, and (ii) by suppressing the production, and most likely by interfering with the signaling of these cytokines, UG inhibits *SCCA* gene expression associated with airway inflammation in asthma.

Uteroglobin (UG)¹ is a steroid-inducible, homodimeric, multifunctional secreted protein with potent anti-inflammatory

and immunomodulatory properties (reviewed in Ref. 1). The founding member of the *secretoglobin* superfamily of proteins (2), UG, is constitutively expressed at a high level in the pulmonary mucosal epithelial cells of virtually all mammals including mice. We previously reported that compared with wild type (WT) littermates, the lungs of the UG-knock-out (UG-KO) mice express markedly higher levels of Th2 cytokines and manifest exaggerated airway inflammatory response to allergens marked by elevated levels of eosinophil infiltration (3, 4). All of these are characteristically found in human airway inflammatory diseases such as bronchial asthma in which sensitivity to allergens play a critical pathogenic role.

Recently, it has been reported that the expression of squamous cell carcinoma antigens (*SCCA*), cysteine, and chymotrypsin proteinase inhibitors of the ovalbumin/serpin family (reviewed in Ref. 5; Ref. 6) is markedly elevated in the airway epithelia of patients with bronchial asthma (7). Consistent with these findings, it has also been reported that *SCCA* protein targets a potent allergen and an extrinsic cysteine proteinase in house-dust mites, which is thought to play a pathogenic role in allergic asthma (8), although the physiological role(s) of *SCCA* is yet to be defined.

In humans, *SCCA-1* and *SCCA-2* genes encode cysteine and chymotrypsin proteinase inhibitors, respectively. These genes have also been designated *SERPINB3* and *SERPINB4*, respectively, according to the new nomenclature guidelines (reviewed in Ref. 6). The *SCCA* locus in the mouse contains 4 genes of which *Serpinb3a*, also known as *SQN-5* and *SCCA-2*, is clearly related to both *SCCA-1* and *SCCA-2* in humans. Mouse *serpinb3a* is both a cysteine- and trypsin-like serine proteinase inhibitor (9), and it is expressed in tissues in which human *SCCA-1* and *SCCA-2* are also expressed. Thus, *Serpinb3a*, which is referred to as *SCCA-2* in this paper, is the mouse ortholog of human *SCCA-1* and *SCCA-2* (6).

In the present study, we sought to determine whether: (a) the level of murine *SCCA-2* (*Serpinb3a*) gene expression is elevated in the lungs of UG-KO mice compared with that of WT littermates, (b) OVA sensitization and challenge of UG-KO mice influence the level of *SCCA-2* gene expression, and (c) rUG treatment has any effect on *SCCA-2* gene expression in the lungs of UG-KO mice. Our results show that compared with WT mice, the lungs of UG-KO mice express markedly elevated levels of *SCCA-2* mRNA and protein, which are further augmented by OVA sensitization and challenge. Most importantly, treatment of the OVA-sensitized UG-KO mice with rUG prior to OVA challenge dramatically suppresses the expression of *SCCA-2*. Using cultured human bronchial epithelial cells, we further demonstrate that both IL-4 and IL-13 stimulate the expression of *SCCA-1* and *SCCA-2* genes, which are human orthologs of mouse *SCCA-2* (10–12) and that IL-4- and IL-13-induced *SCCA* expression requires STAT-1 and/or STAT-6 phosphorylation. Since UG inhibits IL-4 and IL-13 production (4) and these cytokines stimulate *SCCA* gene expression, we propose that by down-regulating the expression and signaling of these Th2 cytokines UG inhibits *SCCA* gene expression associated with allergic asthma.

EXPERIMENTAL PROCEDURES

Materials—Chicken OVA (grade V), protease inhibitor mixture, phosphatase inhibitor mixture, and leflunomide were purchased from Sigma. Human bronchial epithelial cells (HBEC), the small airway

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¹ The abbreviations used are: UG, uteroglobin; UG-KO, uteroglobin-knock-out; WT, wild type; OVA, ovalbumin; IL, interleukin; HRP, horseradish peroxidase; *SCCA*, squamous cell carcinoma antigen; HBEC, human bronchial epithelial cells; RT, reverse transcription; STAT, signal transducers and activators of transcription.

epithelial growth medium bullet kit, and SABM basal medium were purchased from Bio-Whittaker, Inc. (Walkersville, MD). Human IL-4 and IL-13 were purchased from Peprotech (Rocky Hill, NJ). Antibodies against STAT-1, phospho-Tyr-STAT-1, STAT-6, phospho-Tyr-STAT-6, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibody for human SCCA-1 and SCCA-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pre-cast SDS-polyacrylamide (4–15%) gels were obtained from Bio-Rad.

Mice—UG-KO mice were generated as described previously (13). Both UG-KO and WT mice were maintained under germ-free conditions, and all experiments were performed according to an institutionally approved animal care and use protocol. The methodology for inducing airway inflammation by OVA is previously reported (3, 4).

Human Bronchial Cell Culture and Treatment with IL-4 and IL-13—Normal HBEC were cultured in small airway epithelial growth medium according to the manufacturer's protocol. Nearly 60–70% confluent cells were washed twice with SABM basal medium and then cultured in the basal medium in presence or absence of human IL-4 (10 ng/ml) and IL-13 (50 ng/ml) for 24 h as described previously (14). To determine the signaling pathway(s) of IL-4- and IL-13-induced SCCA expression the cells were treated with leflunomide (100 μ M–250 μ M) in the presence and absence of each of these cytokines.

RNA Isolation and SCCA-2 mRNA Expression by Quantitative Real-time PCR—Total RNA was isolated using TRIzol (Invitrogen) following the manufacturer's protocol. Total RNA (~600 ng) was reverse-transcribed using Taq-Man RT reagent (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems) as described previously (4). The primers for SCCA-2 (sense, 5'-ATG ATT GTC CTG TTG CCA GT-3'; antisense, 5' TTC TGC TCT TGT CCA CTC CA-3') and 18S (sense, 5'-GAC TCA ACA CGG GAA ACC TC-3'; antisense, 5'-CTC CAC CAA CTA AGA ACG GC-3') were used for PCR. The data from each PCR run were analyzed using ABI Prism Software version 1.01 (Applied Biosystems). The final data were normalized to 18 S rRNA and presented as fold increase compared with the expression level in WT mice. Quantitation was performed using three independent total RNA samples for each treatment group.

SCCA-2 Antibody Generation—Rabbits were immunized using recombinant glutathione *S*-transferase-SerpinB3a fusion protein, which was produced and purified as described previously (12). Serum was diluted as described for Western applications.

Western Blot Analysis of Mouse SCCA-2 Protein Expression—To detect the SCCA-2 protein, lungs were homogenized in extracting buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40) containing protease and phosphatase inhibitor mixtures (Sigma-Aldrich). Total protein (20 μ g) from each sample was resolved by electrophoresis using 4–15% SDS-polyacrylamide gels under denaturing and reducing conditions and electrotransferred onto the nitrocellulose membranes (Bio-Rad). Immunoblot analysis was performed using rabbit polyclonal SCCA-2 antibody at 1:2000 dilution. HRP-conjugated donkey anti-rabbit IgG (1:10,000) served as the secondary antibody. Chemiluminescent detection was performed by using an ECL system (Amersham Biosciences) according to the manufacturer's protocol. Total protein loading standard β -actin was detected using an actin (Ab-1) kit (Oncogene Research Products, Boston, MA) according to the manufacturer's protocol.

Immunodetection of Human SCCAs and STATs in HBEC—Total protein (20 μ g) from cell lysates was resolved and electrotransferred on the nitrocellulose membrane as described above. Human SCCA-1 and SCCA-2 proteins were detected by using mouse monoclonal anti-human SCCA-1 and SCCA-2 antibodies (1:400), and HRP-conjugated rabbit anti-mouse IgG served as the secondary antibody (1:2000). The levels of non-phospho- and phospho-STAT-1 and STAT-6 were detected by following the manufacturer's protocol.

Tissue Fixation and Immunofluorescence—The lung tissues were fixed in 3.7% paraformaldehyde, and the sections were stained with rabbit SCCA-2 antibody (1:500) followed by counter staining with fluorescein isothiocyanate-conjugated anti-rabbit (1:1000) secondary antibody. The stained slides were examined by using a Zeiss Axiotroph Microscope, and digital photomicrographs were recorded.

RESULTS AND DISCUSSION

In this study, we demonstrate that SCCA-2 gene expression in UG-KO mouse lungs is markedly higher than in those of WT littermates. In addition, we show that allergen (OVA) sensitization and challenge further augments these levels and, most

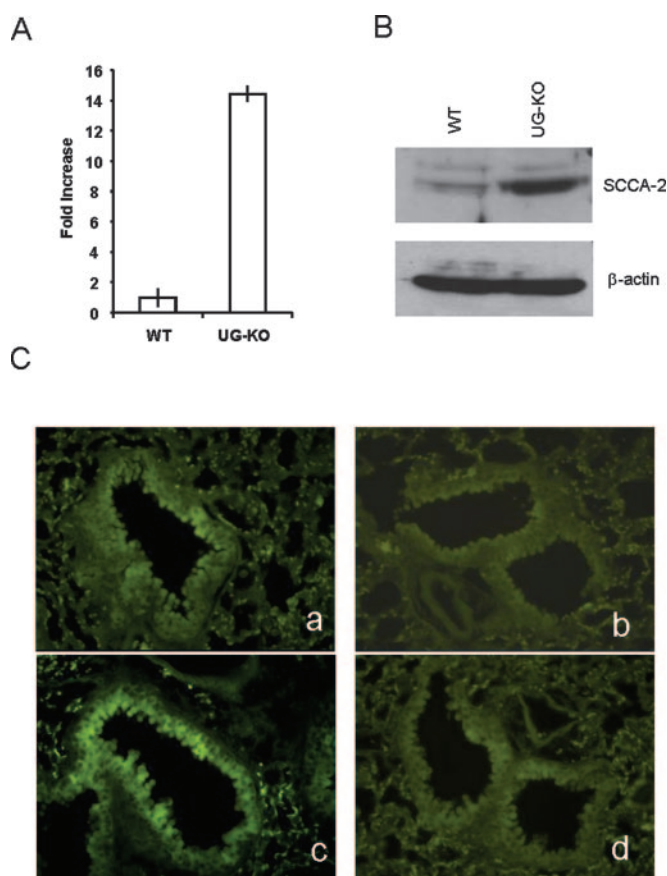
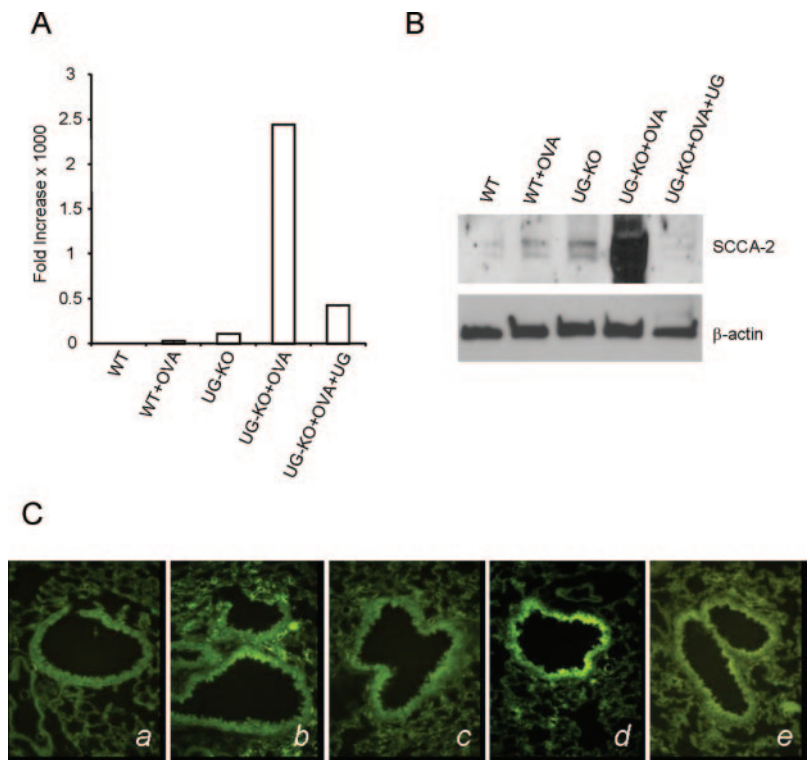


FIG. 1. SCCA-2 gene is overexpressed in the lungs of UG-KO Mice. A, expression of SCCA-2 mRNA as determined by quantitative real-time RT-PCR using total RNA from the lungs of WT and UG-KO mice. Results are expressed as the mean of at least three experiments $\pm$ S.D. B, SCCA-2 protein was detected by Western blot analysis using total protein extract from the lungs of WT and UG-KO mice. C, immunolocalization of SCCA-2 in the lungs of WT (panel a) and UG-KO (panel c) mice using rabbit anti-SCCA-2 antibody, and preimmune sera were used as controls: WT (panel b) and UG-KO mice (panel d).

importantly, that rUG treatment suppresses SCCA-2 expression. Furthermore, using HBEC we demonstrate that IL-4 and IL-13 stimulate the expression of both SCCA-1 and SCCA-2 genes (orthologs of murine SCCA-2), and these cytokines mediate SCCA gene expression via STAT-1 and/or STAT-6 activation.

We previously reported that OVA sensitization and challenge in UG-KO mice elicit exaggerated inflammatory response in the airways, which is suppressed by rUG treatment (4). We also reported that lack of UG causes overexpression of Th2 cytokines, including IL-4 and IL-13, which play critical role(s) in airway inflammation in asthma (reviewed in Ref. 15). Recently, it has been reported that overexpression of SCCA-1 and SCCA-2 in the lungs is associated with human bronchial asthma (14), an airway inflammatory disease in which allergens play critical pathogenic roles. To determine whether there is a relationship between UG and SCCA-2 gene expression, we first studied the expression of SCCA-2 mRNA in the lungs of UG-KO mice and in those of their WT littermates by quantitative RT-PCR. We found that the level of SCCA-2 mRNA is markedly elevated in the lungs of UG-KO mice (Fig. 1A). Consistent with these results, the Western blot analysis showed that the SCCA-2 protein level is also markedly elevated in UG-KO mouse lungs (Fig. 1B). Detection of SCCA-2 protein by immunofluorescence shows that while a very low level of SCCA-2 is present in the lungs of WT mice (Fig. 1C, panel a), intense immunofluorescence is present in the bronchial epithe-

FIG. 2. UG suppresses allergen-induced SCCA-2 expression in the lungs. A, expression of SCCA-2 mRNA was determined by quantitative real-time RT-PCR using total RNA from the lungs of WT, OVA-sensitized and -challenged WT (WT+OVA), UG-KO, OVA-sensitized and -challenged UG-KO (UG-KO+OVA), and OVA-sensitized UG-KO mice pretreated with rUG prior to OVA challenge (UG-KO+OVA+rUG). B, detection of SCCA-2 protein by Western blot analysis using the total protein extract from the lungs of WT, WT + OVA, UG-KO, UG-KO + OVA, and UG-KO + OVA + rUG. C, SCCA-2 immunofluorescence in the lungs of WT (panel a), WT + OVA (panel b), UG-KO (panel c), UG-KO + OVA (panel d), and UG-KO + OVA + rUG (panel e). Magnification: $\times 400$.



lial cells of UG-KO mice (Fig. 1C, panel c). Preimmune serum failed to detect any SCCA-2 in WT (Fig. 1C, panel b) and in UG-KO (Fig. 1C, panel d) mice. Taken together, these results suggest that lack of UG causes elevated expression of the SCCA-2 gene in mice.

To determine whether allergen sensitization and challenge in UG-KO mice alters the levels of SCCA-2 gene expression, we determined the SCCA-2 mRNA and protein expression in the lungs of allergen (OVA)-sensitized and -challenged UG-KO mice and compared the results with those of WT mice. We also carried out experiments in which the UG-KO mice were OVA-sensitized and then treated with rUG prior to OVA challenge and analyzed the production of SCCA-2. The results show that the SCCA-2 mRNA level in the lungs of OVA-sensitized and -challenged WT mice is higher than that of the control WT mice, which were not sensitized or challenged with OVA (Fig. 2A). However, in the lungs of OVA-sensitized and -challenged UG-KO mice markedly elevated levels of SCCA-2 mRNA were detected compared with those of the UG-KO littermates that were not sensitized or challenged with OVA. Most importantly, rUG treatment of the OVA-sensitized UG-KO mice prior to OVA challenge markedly inhibited the level of SCCA-2 mRNA (Fig. 2A). Consistent with these results, Western blot analysis showed a high level of SCCA-2 protein expression (Fig. 2B). The results of the Western blot analysis were further confirmed by immunofluorescence using SCCA-2 antibody (Fig. 2C, panels a–e). These results show that compared with the bronchial epithelia of control WT mice (Fig. 2C, panel a), OVA-sensitized and -challenged WT mice (Fig. 2C, panel b) manifested a slightly higher level of SCCA-2 immunofluorescence. However, although the untreated UG-KO mouse lungs (Fig. 2C, panel c) had a higher intensity of SCCA-2 immunofluorescence compared with that of WT control, the bronchial epithelia of OVA-sensitized and -challenged UG-KO mice yielded the highest immunofluorescence (Fig. 2C, panel d). Most importantly, treatment of the OVA-sensitized UG-KO mice with rUG prior to OVA challenge markedly suppressed the level of SCCA-2 immunofluorescence (Fig. 2C, panel e). These results demon-

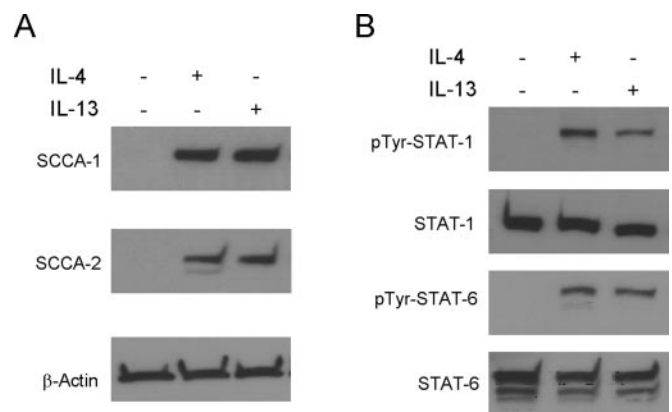
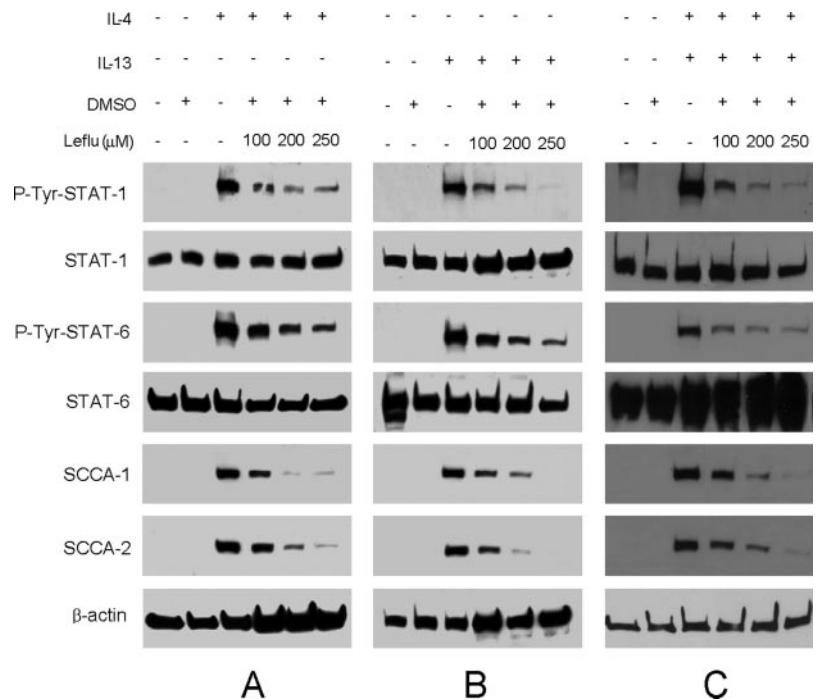


FIG. 3. Th2 cytokine-induced SCCA expression and STAT-phosphorylation in HBEC. A, detection of IL-4- and IL-13-induced SCCA-1 and SCCA-2 gene expression in HBEC by Western blot analysis. B, detection of IL-4- and IL-13-induced phosphorylation on tyrosine residues of STAT-1 and STAT-6 in HBEC by Western blot analysis.

strate that lack of UG in the lungs of UG-KO mice up-regulates SCCA-2 gene expression in the bronchial epithelia and this expression is further augmented by allergen sensitization and challenge. Most importantly, rUG treatment markedly suppresses SCCA-2 gene expression.

How might lack of UG stimulate SCCA-2 gene expression in the lungs of UG-KO mice? It has been reported that in the bronchial epithelia of patients suffering from allergic asthma the SCCA-2 gene expression is markedly elevated (7, 8, 14). Furthermore, in asthma, a chronic airway inflammatory disease, Th2 cytokines, especially IL-4 and IL-13, play critical pathogenic roles. Interestingly, in patients suffering from asthma lower than normal UG levels have also been reported (17). Moreover, a single nucleotide polymorphism in the UG gene (18) that down-regulates UG expression (21) positively correlated with asthma (18). We previously reported that in OVA-sensitized and -challenged UG-KO mice, IL-4 and IL-13 levels are elevated, which are suppressed by rUG treatment

FIG. 4. Inhibition of STAT phosphorylation suppresses SCCA expression. Human bronchial epithelial cells were grown to 60–70% confluence and treated with either IL-4 (A), IL-13 (B), or a combination of IL-4 and IL-13 (C) for 24 h in serum-free basal medium in absence and presence of varying concentrations of leflunomide. Note that leflunomide inhibits tyrosine phosphorylation of STAT-1 and STAT-6 as well as the expression of SCCA-1 and SCCA-2 protein in a dose-dependent manner. *P-Tyr*, phosphorylated on tyrosine.



(4). Since both IL-4 and IL-13 stimulate *SCCA-2* gene expression (7), it is likely that by inhibiting the levels of IL-4 and IL-13, UG suppresses *SCCA-2* gene expression. However, the mechanism of IL-4- and IL-13-induced *SCCA-2* gene expression, until now, remained unclear.

To determine the mechanism(s) by which IL-4 and IL-13 stimulate *SCCA-2* expression, we treated HBEC with either IL-4 or IL-13 and determined the production of *SCCA-2* by Western blot analysis. We found that both IL-4 and IL-13 treatments stimulate the expression of human *SCCA-1* and *SCCA-2* (Fig. 3A). To determine the mechanism of regulation of *SCCA* gene expression, we first studied the phosphorylation of the transcription factors, STAT-1 and STAT-6, as it has been previously reported that the 5'-regulatory region of the *SCCA* genes contain a STAT-binding site (11, 12). It has also been reported that both STAT-1 and STAT-6 activation require phosphorylation on tyrosine. Accordingly, we studied tyrosine phosphorylation of STAT-1 and STAT-6 by Western blot analysis. Our results show that both IL-4 and IL-13 treatments of the cells stimulate STAT-1 and STAT-6 phosphorylation on tyrosine without altering the STAT-1 and STAT-6-protein levels (Fig. 3B).

To further confirm that STAT-1 and STAT-6 phosphorylation is essential for IL-4- and IL-13-mediated *SCCA* expression, we performed experiments in which human bronchial epithelial cells were stimulated with IL-4, IL-13, or a combination of IL-4 and IL-13 in the presence and absence of an inhibitor of tyrosine phosphorylation, leflunomide. Our results show that leflunomide inhibits tyrosine phosphorylation of STAT-1 and STAT-6 induced by IL-4 (Fig. 4A), IL-13 (Fig. 4B), and IL-4 plus IL-13 (Fig. 4C) in a dose-dependent manner. Most importantly, leflunomide-mediated inhibition of IL-4-, IL-13-, and IL-4 + IL-13-induced tyrosine phosphorylation in STAT-1 and STAT-6 leads to the suppression of *SCCA-1* and *SCCA-2* expression (Fig. 4, A–C). Taken together, our results for the first time demonstrate that IL-4 and IL-13 signaling is mediated via STAT-1 as well as STAT-6 and that tyrosine phosphorylation of these transcription factors is critical for IL-4- and IL-13-induced *SCCA* gene expression.

Although the physiological role(s) of the *SCCA* proteins are not yet clear, its association with asthma, an inflammatory

disease of the airways, and its overexpression in the lungs of mice lacking UG, an anti-inflammatory protein, may suggest that *SCCA* gene product(s) may have pro-inflammatory effects.

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