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Original Article

Interaction Between Serum/Glucocorticoid-Regulated Kinase 1 and Interleukin-6 in Chronic Rhinosinusitis

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ABSTRACT

Purpose: Serum/glucocorticoid-regulated kinase 1 (SGK1) has recently emerged as a critical regulator of inflammatory diseases. In this study, we examined SGK1 expression and its possible pathogenic roles in chronic rhinosinusitis (CRS).
Methods: Immunohistochemistry, western blotting, Bio-Plex assay, enzyme-linked immunosorbent assays, and quantitative real-time polymerase chain reaction were performed to assess protein and gene expression levels. The mRNA expression levels of SGK1 and interleukin-6 (IL-6) were extracted from a CRS database to perform correlation analysis. Stable cell lines with SGK1 overexpression (16HBE) and knockdown (A549) were constructed to investigate the interaction between SGK1 and IL-6 *in vitro*.
Results: SGK1 exhibited strong cytoplasmic and nuclear staining in the epithelial layers and the lamina propria of nasal polyps (NPs) and in the mucosal tissues of CRS without nasal polyps (CRSsNP). The mRNA and protein expression levels of SGK1 and IL-6 were significantly increased in NPs and CRSsNP tissues, compared to control tissues. SGK1 phosphorylation was significantly greater in NPs than in CRSsNP tissues ($P < 0.01$). The mRNA levels of SGK1 and IL-6 were significantly correlated ($P < 0.001$, $r = 0.649$). Exposure to IL-6 significantly increased SGK1 expression in cultured dispersed NP cells, 16HBE cells, and A549 cells. IL-6 expression was significantly down-regulated in SGK1-overexpressing 16HBE cells ($P < 0.01$) and significantly up-regulated in SGK1-knockdown A549 cells ($P < 0.05$). Administration of GSK650394, a SGK1 inhibitor, significantly increased IL-6 self-induced mRNA expression in cultured dispersed NP cells and 16HBE cells.
Conclusions: The interaction between SGK1 and IL-6 may play an anti-inflammatory role in IL-6-induced inflammation in the pathogenesis of CRS.

Keywords: SGK1; IL-6; chronic rhinosinusitis; nasal polyps; inflammation; rhinitis; airway; nasal cavity

INTRODUCTION

Chronic rhinosinusitis (CRS) is a complicated, chronic inflammation that affects the nasal cavity and sinuses.[1] Because its pathogenesis involves specific molecules or cells, we have gained valuable information on disease progression, recurrence, and the best available

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There are no financial or other issues that might lead to conflict of interest.

treatment.[2] According to the 2012 European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS 2012), CRS is classified into 2 subtypes: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP).[3]

Interleukin -6 (IL-6) is a proinflammatory cytokine that plays critical roles in the pathogenesis of CRS.[2,4] IL-6 induces the differentiation of naive T cells into T helper type (Th) 17 cells and maintains IL-17 production in CRSwNP patients.[5] Moreover, IL-6 exaggerates the epithelial response to external factors and promotes polyp formation,[6] while inhibition of IL-6 trans-signaling is associated with impaired epithelial barrier function that contributes to the pathogenesis of asthma and nasal polyp (NP) formation in CRS.[7]

The expression of serum/glucocorticoid-regulated kinase 1 (SGK1) is up-regulated by serum and glucocorticoids, and was first identified in rat mammary tumor cells.[8] Excessive SGK1 production is involved in the pathophysiology of several disorders, including hypertension, obesity, diabetes, thrombosis, stroke, inflammation, autoimmune disease, fibrosis, and tumor growth.[9] In inflammatory diseases, SGK1 may exert favorable or unfavorable effects. SGK1 promotes Th2 differentiation and represses the production of interferon-γ (IFN-γ). Mice with selective deletion of *SGK1* in T cells are resistant to experimentally induced asthma and produce high levels of IFN-γ in response to viral infection.[10] Estradiol-induced SGK1 activation reduces the secretion of proinflammatory Th1 cytokines and promotes the generation of Th2 cytokines in lipopolysaccharide (LPS)-incubated decidual stromal cells.[11] Augmentation of SGK1 phosphorylation with prolonged exposure to LPS activates nuclear factor (NF)-κB in airway epithelial cells, whereas inhibition of SGK1 abrogates airway inflammation *in vitro* and *in vivo*.[12] SGK1 is also critical for the development and function of proinflammatory Th17 cells in autoimmune-related tissue inflammation, SGK1 deficiency protects against autoimmunity, and enhances self-tolerance by promoting the development of regulatory T cells and disarming Th17 cells.[13] In LPS-stimulated primary human monocytes, the increased phosphorylation of glycogen synthase kinase-3β, Akt, and SGK1 suppresses the release of proinflammatory cytokines and the innate/adaptive bridging cytokines while simultaneously augmenting the production of anti-inflammatory cytokines.[14] Currently, however, it is unknown whether SGK1 is involved in inflammatory diseases of the upper respiratory tract, like CRS. Therefore, in this study we investigated the expression of SGK1 in NPs of CRSwNP patients and in mucosal tissues of CRSsNP patients to explore its roles in the pathogenesis of CRS.

MATERIALS AND METHODS

Subjects

The study complied with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the Eye and ENT Hospital affiliated with Fudan University (Shanghai, China) (IRB2016009). Written informed consent was obtained from each participant. CRS was diagnosed according to the EPOS 2012 criteria.[3] We also enrolled a control group of patients diagnosed with ossifying fibroma, osteoma, hypophysoma, or optic neuropathy without sinusitis. Normal mucosal tissues were obtained from the nasal cavity and taken as controls. Patients with any of the following were excluded: allergic fungal sinusitis, autoimmune diseases, acute sinusitis, aspirin-exacerbated respiratory disease, cystic fibrosis, and primary ciliary dyskinesia. Patients with CRS who received oral/nasal steroids or other medications (*e.g.*, antibiotics or antileukotrienes) within 1 month before

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Table 1. Characteristics of study subjects

Characteristics	CRSwNP	CRSsNP	Control
No. of subjects	66	19	15
Sex (M/F)	47/21	11/8	9/6
Age (yr)	40 (25–57)	38 (26–59)	32 (22–45)
SPT (positive rate; %)	21.2	15.8	-
Asthma history (positive rate; %)	9.1	5.3	-
Methodologies used			
IHC	30	12	8
WB	32	16	8
qRT-PCR	32	16	8
ELISA	42	12	8
In vitro DPCs culture assay	9	-	-

CRSwNP, chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without nasal polyps; SPT, skin prick test; IHC, immunohistochemistry; WB, western blot; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; DPC, dispersed nasal polyp cell.

sample collection were also excluded. A total of 66 patients with CRSwNP, 19 patients with CRSsNP, and 15 control subjects were recruited. The demographic characteristics of patients in these 3 groups are listed in **Table 1**.

Sample collection

All samples were collected from patients who underwent endoscopic sinus surgery at the Eye and ENT Hospital. NP tissues from patients with CRSwNP, and mucosal tissues from CRSsNP patients and control subjects were collected during surgery. Each specimen was separated into 3 parts. The first part was fixed overnight in 4% paraformaldehyde and then embedded in paraffin for immunohistochemistry (IHC). The second one was stored at −80°C for western blotting. The third part was immersed in RNAlater (Tiangen, Beijing, China) for 24 hours at 4°C and then stored at −80°C for quantitative reverse transcription polymerase chain reaction (qRT-PCR). All subjects underwent an allergen skin prick test to assess their atopic status. Asthma was diagnosed by a pneumologist. Dispersed nasal polyp cells (DPCs) were collected for *in vitro* assays.

Cell culture and stimulation

DPCs were randomly collected from patients with CRSwNP by enzymatic digestion to establish *in vitro* cultures as previously described.[15] Briefly, fresh polyp tissues were sequentially washed with PBS, iodophor disinfectant, and gentamicin 3 times each, cut into pieces of approximately 1 mm^3, digested in incomplete RPMI-1640 medium (Gibco, ThermoFisher Scientific, Waltham, MS, USA) containing 1 mg/mL collagenase I (Worthington, Lakewood, NJ, USA) and 200 μg/mL DNase I (Sigma-Aldrich, Missouri, MO, USA), and then placed in a 37°C/5% CO_2 incubator for 1 hour. After repeated vortexing, the cell suspensions were filtered through 100-μm cell strainers to remove undigested tissue and centrifuged twice at 300 × *g* for 5 minutes at 4°C, and the sediments were retained. The cells within the sediment were then transferred into fresh RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco) and cultured on a 6-well plate in a humidified incubator (5% CO_2) at 37°C for 8 hours. The cells were incubated with recombinant human IL-6 protein (R&D Systems, Minneapolis, MN, USA) for 8 hours, and then harvested for qRT-PCR.

Human bronchial epithelial cells (16HBE) and human pulmonary epithelial cells (A549 cells) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS at 37°C under 5% CO_2. At the indicated time in each experiment, the cells and supernatants

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were harvested. The cells were stored at −80°C for qRT-PCR and western blotting. The supernatants were centrifuged at 2,000 rpm for 5 minutes at 4°C and stored at −80°C to measure the secreted IL-6 protein levels.

To measure self-induced IL-6 secretion, the supernatant was removed after exposure to IL-6 for 8 hours. The plate was washed with PBS twice and an equivalent volume of supernatant was added to each well. The supernatants were harvested 16 hours later. The SGK1 inhibitor (GSK650394, 50 μM; TOCRIS, Minneapolis, MN, USA) was diluted in the medium and added to the corresponding well 2 hours before adding IL-6, while the same volume of medium was added to other wells simultaneously.

Establishment of cell lines with SGK1 overexpression or SGK1 knockdown

To clone full-length 16HBE SGK1 DNA coding regions, we used 16HBE cDNA as the template and the following primers for *SGK1*: 5′-ATGACGGTGAAAACTGAGGCTG-3′ and 3′-ACGGCTCTGACTGACAACTGG-5′. For SGK1 overexpression, the full-length 16HBE *SGK1* DNA was cloned into pLVX-IRES-blasticidin (Clontech). For SGK1 knockdown, the annealed sense and antisense short hairpin RNA oligonucleotides were cloned into the *EcoR*I and *Age*I site of pLKO.1 (Addgene), as previously described,[16] with target sequence 3′-GTCTTGCAATGACTCGTATTC-5′. The construct or empty vector was transfected into 16HBE and A549 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Blasticidin (10 μg/mL; Gibco) and puromycin (1 μg/mL; Gibco) were added to select colonies of cells with stable SGK1 overexpression or SGK1 knockdown. The stable cells were tested by qRT-PCR and western blotting.

IHC

Paraffin sections (4-μm thick) were deparaffinized in xylene and rehydrated in a decreasing alcohol gradient. Endogenous peroxidase was blocked by incubating the sections in 1.5% H_2O_2 for 10 minutes. After blocking nonspecific binding, the slides were incubated overnight at 4°C with primary antibodies for SGK1 (1:800; Abcam, Cambridge, MA, USA) and phosphorylated SGK1 (p-SGK1) (1:400; Abcam). The slides were subsequently incubated for 30 minutes at room temperature with a secondary antibody, and then incubated with streptavidin-conjugated peroxidase (Zhongshanjinqiao, Beijing, China) for 30 minutes at room temperature, washed with phosphate-buffered saline (PBS), and counterstained with hematoxylin. After washing 3 times in PBS, the primary antibody staining was visualized by monitoring the development in diaminobenzidine substrate medium. Sections were examined under an Olympus microscope (Olympus Optical, Hamburg, Germany).
The numbers of positive cells in the lamina propria (LP) in 3 high-power fields (HPFs; magnification × 400) were counted by 2 independent observers in a blinded manner.
Given the diffuse staining pattern, the positive staining in the epithelial layer in 3 areas was analyzed using Image Pro-Plus version 6.0 software (Media Cybernetics, Inc., Silver Spring, MD, USA), and the results are presented as the mean optical density per unit area.[17,18]

Western blotting

The tissues or cells were dissociated on ice and homogenized in radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), 10 μL/mL sodium fluoride (NaF; Sangon Biotech, Shanghai, China), 2 μL/mL protease inhibitor cocktail (Merck-Millipore, Bedford, MA, USA), and 1 μL/mL sodium orthovanadate (Na_2VO_3; Sangon Biotech). The supernatant protein concentration was determined using the BCA method. Samples containing 30 μg of protein were separated

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by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred onto a nitrocellulose filter membrane. The membrane was blocked with 5% fat-free milk for 1 hour at room temperature and incubated with rabbit anti-human SGK1 (1:1,000; Cell Signaling Technology, Danvers, MA, USA), and rabbit anti-human p-SGK1 (1:1,000; Cell Signaling Technology) overnight at 4°C. The membrane was then washed and incubated with a corresponding secondary antibody, and processed using an electrochemiluminescence reaction kit (Merck-Millipore), followed by exposure to medical film. The density of the target protein band relative to that of β-actin (1:10,000; Beyotime) was quantified using Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA).

qRT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) and evaluated with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription was performed to synthesize cDNA for quantitative PCR from 1 μg of total RNA using an oligo (dT)15 primer and PrimeScript RT Reagent Kit (Takara, Shiga, Japan). The mRNA expression levels were determined using an ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR® Premix Ex Taq™ (Takara). The qRT-PCR amplification protocol consisted of 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Melting curve analysis was performed to control for amplification specificity. The mean cycle threshold (Ct) values were normalized to those of β-actin in tissue samples or to glyceraldehyde 3-phosphate dehydrogenase for cell samples, and the relative mRNA levels of the target genes were analyzed using the $2^{-\Delta\Delta Ct}$ method. Experiments were performed in triplicate for each data point. The primer sequences are listed in **Table 2**.

Enzyme-linked immunosorbent assay (ELISA)

The IL-6 protein levels in tissue extracts were determined using a Bio-plex Pro™ Human Th17 Cytokine Panel (Bio-Rad Laboratories) according to the manufacturer's instructions. Briefly, tissues stored at −80°C were dissociated on ice, weighed, and placed in PBS (100 mg tissue/200 μL PBS) containing 10 μL/mL PMSF, 10 μL/mL NaF, 2 μL/mL cocktail, and 1 μL/mL Na_2VO_3, and homogenized on ice for 1 minute. The suspension was then centrifuged at 13,000 rpm for 20 minutes at 4°C. The total protein concentration in the supernatant was determined using the BCA method. Each sample was diluted to 5,000 μg/mL with sample diluent and then adjusted to 0.5% with bovine serum albumin. The cytokine concentrations were measured on a Bio-Plex® 200 (Bio-Rad Laboratories), according to the manufacturer's instructions. The lower and upper limits of quantitation for IL-6 were 1.65 and 27,078 pg/mL, respectively. For convenience, all values outside this range were defined as zero. To reduce errors, the measured levels were normalized to the total protein levels.

Table 2. Primers used for qRT-PCR analysis

Gene name	Sequence (5′-3′)
SGK1	Forward: 5′ TCATGCCAACATCCTGACCAA 3′
	Reverse: 5′ TGAATAAAGTCGTTCAGACCCATCC 3′
IL-6	Forward: 5′ GATGAGTACAAAAGTCCTGATCCA 3′
	Reverse: 5′ CTGCAGCCACTGGTTCTGT 3′
β-actin	Forward: 5′ CCTGGCACCCAGCACAAT 3′
	Reverse: 5′ GGGCCGGACTCGTCATAC 3′
GAPDH	Forward: 5′ GAAGGTGAAGGTCGGAGTC 3′
	Reverse: 5′ GAAGATGGTGATGGGATTTC 3′

qRT-PCR, quantitative reverse transcription polymerase chain reaction; SGK1, serum/glucocorticoid-regulated kinase 1; IL-6, interleukin-6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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The cell culture supernatants were dissociated on ice, centrifuged at 1,500 rpm for 2 minutes at 4°C, and diluted as necessary prior to assays. The IL-6 protein levels in cell supernatants were measured in duplicate using a human IL-6 ELISA kit (Neobioscience, Shenzhen, China) according to the manufacturer's instructions. The optical density of each sample was measured using a multi-detection microplate reader (Synery Mx; Biotek, Winooski, VT, USA).

Correlation analysis

We conducted searches of the GSE36830 microarray dataset on the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/).[19] The dataset comprised microarray data of 24 human samples, including NPs and uncinate processes from 6 CRSwNP patients, the uncinate processes from 6 CRSsNP patients, and uncinate processes from 6 control subjects. Gene expression profiles were analyzed using GPL570 Affymetrix Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA, USA). We extracted the mRNA expression values for SGK1 and IL-6 to perform correlation analysis.

Statistical analysis

Data analysis was performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). For analyses of tissue samples, statistical comparisons between 2 groups were made using the nonparametric Mann-Whitney *U* test, and correlation analysis was performed with Pearson's correlation test. For results of *in vitro* assays, 2-tailed Student's *t*-test was used for comparisons between 2 groups. Data are expressed as means ± standard deviation. Values of $P < 0.05$ were considered statistically significant. All imaging experiments were repeated at least 3 times, with similar results.

RESULTS

SGK1 expression is increased in CRS

To evaluate the expression level of SGK1 in NPs and CRSsNP tissues, we first measured its protein expression in the nasal mucosa. As indicated in **Fig. 1A**, SGK1 and p-SGK1 exhibited strong cytoplasmic and nuclear staining in the epithelium and the lamina propria (LP). IHC revealed that the SGK1 and p-SGK1 expression levels in the epithelium were significantly increased in NPs and CRSsNP tissues, compared to the control tissues ($P < 0.05$; **Fig. 1A, B, and C**), but no difference was found between the 2 CRS groups (**Fig. 1A**). The relative number of SGK1-positive cells per HPF in the LP was increased in both CRSwNP and CRSsNP groups, compared to the control group ($P < 0.05$; **Fig. 1D**), but no difference was observed between the 2 CRS groups. The relative number of p-SGK1-positive cells per HPF in the LP was significantly higher in NPs than in CRSsNP tissues ($P < 0.01$; **Fig. 1E**); these cell counts in the 2 CRS groups were also significantly greater than those in the control subjects ($P < 0.001$; **Fig. 1E**). To confirm whether there were similar changes in SGK1 and p-SGK1 protein expression levels, we also performed western blotting of nasal tissues. The results showed that the protein expression levels of SGK1 ($P < 0.05$) and p-SGK1 ($P < 0.01$) in nasal tissues were more significantly increased in both CRSwNP and CRSsNP patients than in the control subjects, and the p-SGK1 protein expression level was significantly increased in the NPs, compared to those in CRSsNP ($P < 0.01$; **Fig.1F-H**). Consistent with these findings at the protein level, qRT-PCR revealed that the SGK1 mRNA levels were significantly higher in NPs and CRSsNP tissues than in control tissues ($P < 0.05$; **Fig. 1I**), but was similar between the 2 CRS groups. The significant increases in protein and mRNA expression levels of SGK1 in NPs and CRSsNP suggest that SGK1 may play a pathogenic role in CRS.

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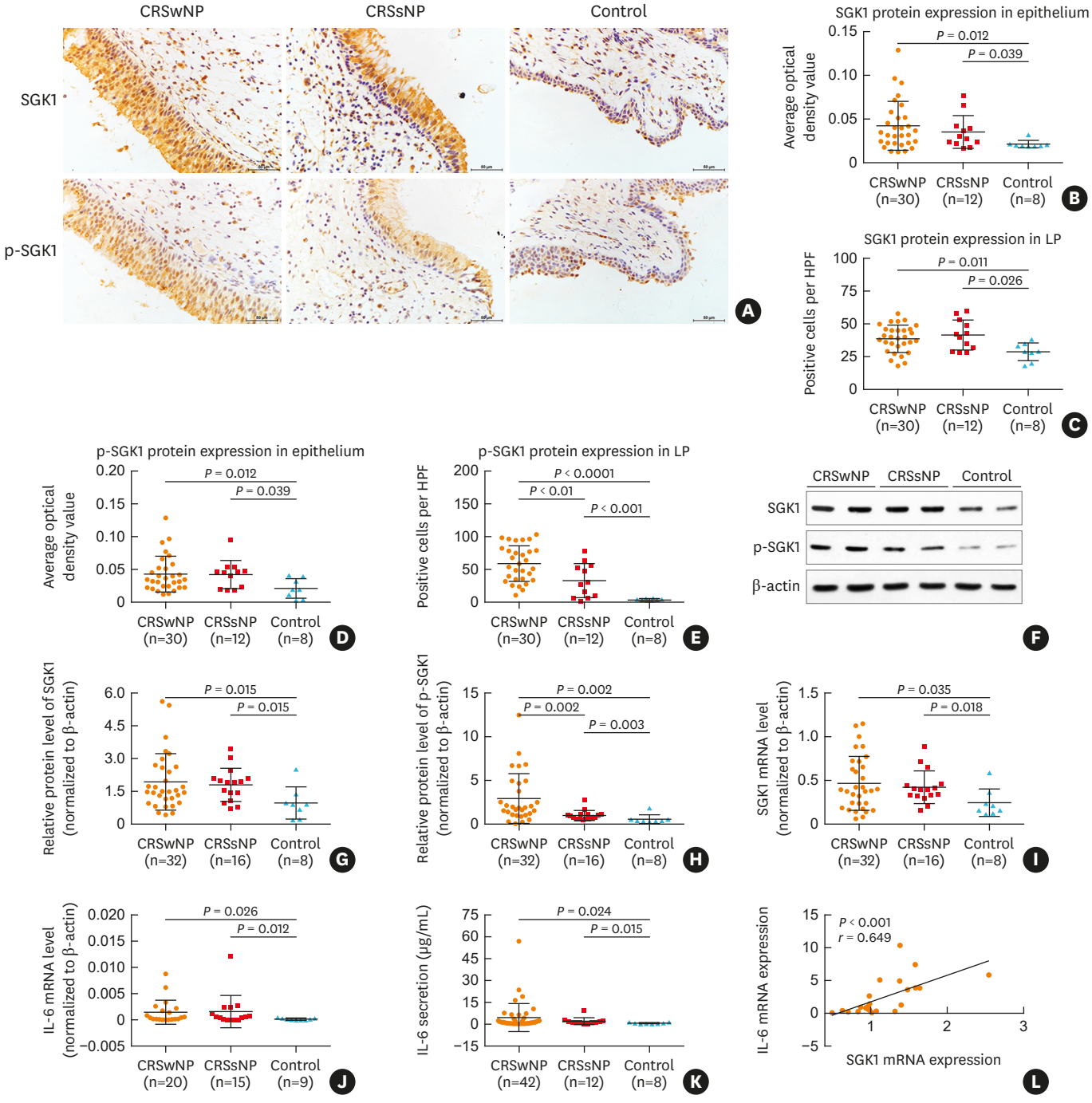

Fig. 1. The SGK1, p-SGK1, and IL-6 expression levels are increased in the NPs and CRSsNP. (A) Representative immunohistochemical staining of SGK1 and p-SGK1 in nasal tissues (magnification × 400). (B, C) Mean optical densities of epithelial SGK1 (B) and p-SGK1 (C) expression in 3 regions. (D, E) Number of SGK1-positive cells (D) and p-SGK1-positive cells (E) within the LP in 3 HPFs (magnification × 400). (I-K) The mRNA expression levels of SGK1 (I) and IL-6 (J), and IL-6 protein secretion (K) were significantly increased in the NPs and in CRSsNP tissues, compared to those in control subjects. (F-H) Representative western blots (F) and densitometric analysis (G, H) of SGK1 and p-SGK1, relative to β-actin. (L) The relative SGK1 and IL-6 mRNA levels were positively correlated based on data from the CRS database. (K) IL-6 protein expression levels in nasal tissues.

SGK1, serum/glucocorticoid-regulated kinase 1; p-SGK1, phosphorylated serum/glucocorticoid-regulated kinase 1; IL-6, interleukin-6; NP, nasal polyp; CRS, chronic rhinosinusitis; CRSwNP, chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without nasal polyps; LP, lamina propria; HPF, high-power field.

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IL-6 expression is increased and positively correlated with SGK1 expression in CRS

IL-6 plays critical pathogenic roles in CRS and has regulatory effects. In the present study, the genetic analysis showed that the IL-6 mRNA levels were significantly increased in the NPs and CRSsNP tissues, compared to control tissues, but was not significantly different between the CRSwNP and CRSsNP samples ($P < 0.05$; **Fig. 1J**). The ELISA of IL-6 yielded similar results ($P < 0.05$; **Fig. 1K**). These findings indicate that IL-6 may be upregulated at the protein and mRNA levels, like SGK1, in CRS. Furthermore, the correlation analysis using the GSE36830 database revealed a significant positive correlation between the IL-6 and SGK1 mRNA expression levels ($P < 0.001$, $r = 0.649$; **Fig. 1L**). Overall, these results suggest an interaction between SGK1 and IL-6 in CRS.

IL-6 induces SGK1 expression *in vitro*

To investigate whether IL-6 regulates SGK1 expression, we stimulated DPCs with IL-6 (10 ng/mL) for different time (0, 2, 4, 6, or 8 hours) and collected the cells for qRT-PCR. Interestingly, we found that SGK1 mRNA expression was up-regulated in a time-dependent manner, with a significant increase at 8 hours ($P < 0.01$; **Fig. 2A**). When cells were exposed to 10 or 20 ng/mL IL-6 for 8 hours, we found no difference in the SGK1 mRNA level between these groups (**Fig. 2B**). Unfortunately, we could not measure the change in SGK1 protein expression in response to IL-6 exposure using DPCs *in vitro* because of the low protein concentration in the supernatants. To confirm these findings, we also stimulated 16HBE and A549 cells with IL-6 for different times (10 ng/mL for 0, 4, 8,16, 20, or 24 hours) or with 2 concentrations (10 or 20 ng/mL). In both cell lines, the SGK1 mRNA and protein expression levels were significantly elevated after stimulation for 4 hours (**Fig. 2C-F**). The expression levels increased in a time-dependent manner up until 8 hours, reaching a plateau thereafter. There were no differences in SGK1 expression levels between 16HBE and A549 cells at the tested concentrations (**Fig. 2G-J**). These data indicate that IL-6 may induce SGK1 expression *in vitro*.

SGK1 overexpression and knockdown regulate the expression of IL-6

We next investigated whether SGK1 affects IL-6 expression. To this end, we established cells with stable SGK1 overexpression (16HBE cells) or SGK1 knockdown (A549 cells) as confirmed by the relevant changes in mRNA and protein expressions (**Fig. 3A-D**). Surprisingly, SGK1 overexpression significantly reduced IL-6 expression in 16HBE cells in terms of its mRNA level ($P < 0.05$) and protein secretion ($P < 0.001$; **Fig. 3E and F**). By contrast, IL-6 expression was significantly increased by SGK1 knockdown in A549 cells in terms of its mRNA level ($P < 0.05$) and protein secretion ($P < 0.01$; **Fig. 3G and H**). Additionally, IL-6 self-induced mRNA and protein secretion were significantly decreased in 16HBE cells with SGK1 overexpression (both $P < 0.01$; **Fig. 3I and J**), although no clear change was observed in A549 cells with SGK1 knockdown (**Supplementary Fig. S1**). Overall, these results suggest that SGK1 may negatively regulate IL-6 expression *in vitro* (**Fig. 3K**).

Inhibition of SGK1 enhances IL-6 self-induced mRNA expression *in vitro*

Next, we examined the effect of a SGK1 inhibitor on IL-6 self-induced expression. DPCs, 16HBE cells, and A549 cells were simulated with IL-6 (10 ng/mL) for 8 hours. Pretreatment with the SGK1 inhibitor, GSK650394, significantly increased IL-6 self-induced mRNA expression in DPCs ($P < 0.05$; **Fig. 4A**). Similar results were found in cultured 16HBE cells ($P < 0.01$; **Fig. 4B**), but not in A549 cells (**Supplementary Fig. S2A**). Thus, these results confirm that SGK1 has negative effects on IL-6 expression in DPCs and 16HBE cells. IL-6

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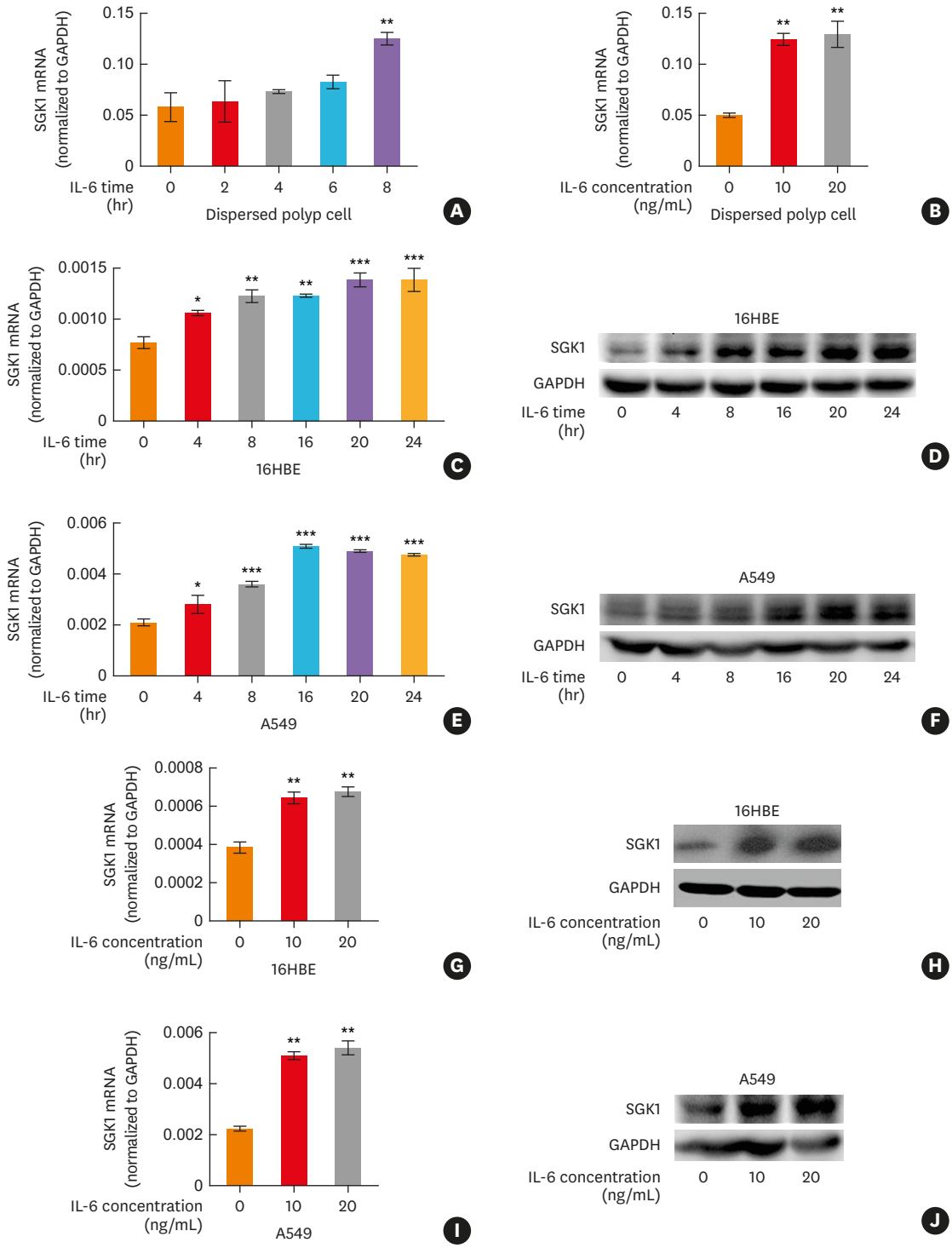

Fig. 2. SGK1 expression in cultured dispersed polyp cells, 16HBE cells, and A549 cells in response to IL-6. (A, B) SGK1 mRNA expression was increased in dispersed polyp cells stimulated with IL-6 for different time (A) and at 2 concentrations (B; 10 or 20 ng/mL). (C-J) SGK1 mRNA expression and representative western blotting of 16HBE cells and A549 cells exposed to IL-6 for different time (C-F) and at 2 concentrations (G-J). Results are expressed as the mean of 3 independent experiments. Data are expressed as means ± standard deviation.
SGK1, serum/glucocorticoid-regulated kinase 1; 16HBE, human bronchial cells; A549, human pulmonary epithelial cells; IL-6, interleukin-6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
$^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. the relevant control group.

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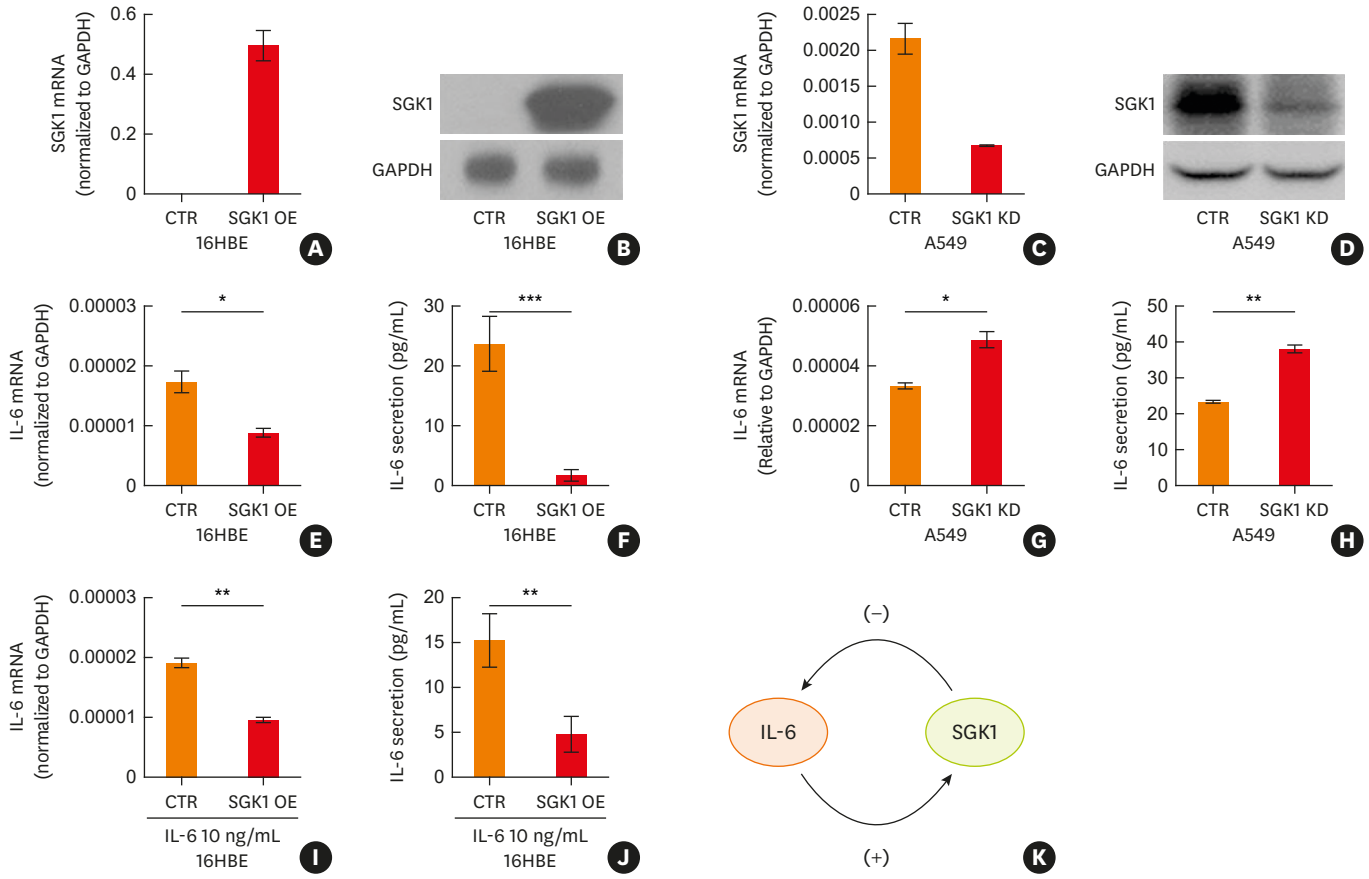

Fig. 3. (A-D) SGK1 mRNA expression and representative western blots in cells with stable SGK1 overexpression (16HBE cells; A, B) or SGK1 knockdown (A549 cells; C, D). (E-H) IL-6 mRNA expression and cytokine secretion (evaluated by enzyme-linked immunosorbent assay) in cells with SGK1 overexpression (E, F) or SGK1 knockdown (G, H). (I, J) Effects of IL-6 stimulation for 8 hours on IL-6 mRNA expression and cytokine expression in 16HBE cells overexpressing SGK1. (K) The interaction between IL-6 and SGK1 forms a closed loop, in which IL-6 upregulates SGK1 and SGK1 downregulates IL-6.
SGK1, serum/glucocorticoid-regulated kinase 1; IL-6, interleukin-6; SGK1 OE, serum/glucocorticoid-regulated kinase 1 overexpressing 16HBE cells; SGK1 KD, serum/glucocorticoid-regulated kinase 1 knockdown A549 cells; CTR, control; +, upregulation; −, downregulation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
$^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. the CTR group.

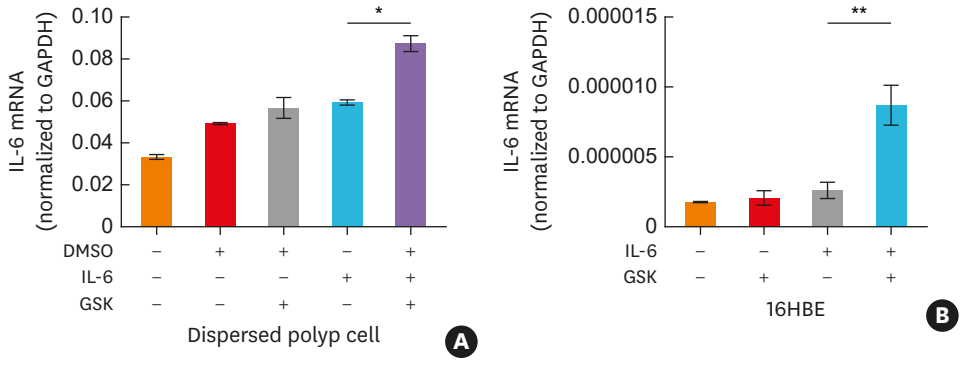

Fig. 4. IL-6 self-induced mRNA expression was significantly enhanced in cultured dispersed polyp cells (A) and 16HBE cells (B) exposed to the SGK1 inhibitor, GSK650394.
DMSO, dimethyl sulfoxide; IL-6, interleukin-6; GSK, GSK650394; SGK1, serum/glucocorticoid-regulated kinase 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
$^{*}P < 0.05$ and $^{**}P < 0.01$.

protein expression was not increased in 16HBE cells pretreated with the SGK1 inhibitor (**Supplementary Fig. S2B**).

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DISCUSSION

Although SGK1 plays critical roles in a variety of inflammatory diseases,[10,13,20,21] there are no reports describing SGK1 expression or its roles in the pathogenesis of CRS. In this study, we first observed strong cytoplasmic and nuclear staining of SGK1 in the epithelial and subepithelial layers of NPs and CRSsNP tissues. The increased mRNA and protein expression levels of SGK1 in the NPs and CRSsNP indicate that SGK1 may be involved in the pathogenesis of CRS. As previously reported, types 1, 2 and 3 inflammatory cytokines, as well as tissue remodeling, participate in the pathogenesis of CRS,[22] while SGK1 promotes the development of Th2 and Th17 inflammation as well as tissue fibrosis, and represses Th1 inflammation.[10,13]

IL-6 is a pleiotropic cytokine involved in innate and adaptive immune responses, including plasma cell differentiation, myeloma cell proliferation and plasmablastic cell survival.[4] Immunohistological staining showed that IL-6 is expressed in the cytoplasm of epithelial cells and submucosal gland cells from CRS patients.[23] A variety of toll-like receptor agonists and proinflammatory cytokines can induce primary human nasal epithelial cells collected from NPs to secret more IL-6 than cells from control subjects.[24] Consistent with prior studies,[5,25-28] our results revealed up-regulation of IL-6 at the mRNA and protein levels in NPs and mucosal tissues from CRS patients, compared to those in control subjects. The increase in IL-6 expression is of particular significance to the development of CRS.

The similar trends toward up-regulated mRNA expression of SGK1 and IL-6 prompted us to investigate their relationship in CRS. Using data from a CRS dataset, we detected a positive correlation between SGK1 and IL-6 mRNA levels. Additionally, it was reported that IL-6 production is associated with up-regulation of SGK1 in adult retinal pigment epithelium-19 cells.[29] Furthermore, in malignant biliary tract epithelia, IL-6 stimulation and p38 mitogen-activated protein kinase activation or overexpression preceded the expression and phosphorylation of SGK.[30] Due to the lack of a stable cell line of nasal epithelial cells and considering the "one airway, one disease" concept,[31] we used 16HBE and A549 cells as tools to explore the interaction between SGK1 and IL-6. The present study revealed that IL-6 increased SGK1 mRNA and protein expression *in vitro*; these findings expand our knowledge for the IL-6 signaling pathway. Although the precise mechanism by which IL-6 affects SGK1 should be studied, we think that SGK1 may play a vital role in the pathogenic effects of IL-6 in CRS.

Although multiple studies have demonstrated the proinflammatory effects of SGK1 in inflammatory diseases, some findings were contradictory. Maturation of dendritic cells and production of proinflammatory cytokines are suppressed via the phosphoinositide 3-kinase–SGK1–N-myc downstream regulated 1 pathway.[32] The survival time of neutrophils was prolonged by SGK1, with enhanced anti-inflammatory effects for neutralizing invading bacteria by phagocytosis and the release of proteases and reactive oxygen species into the extracellular environment.[33] Additionally, inhibition of SGK1 or suppression of SGK1 by small interfering RNA enhanced the production of proinflammatory cytokines (tumor necrosis factor, IL-12, and IL-6) in toll-like receptor-engaged monocytes, a finding that was confirmed in Cre-loxP-mediated SGK1-deficient cells,[34] indicating that SGK1 may negatively regulate IL-6. In the present study, we confirmed that SGK1 has a negative effect on IL-6 expression in 16HBE cells with stable SGK1 overexpression and in A549 cells with stable SGK1 knockdown. These findings are particularly interesting and suggest the presence of a closed regulatory feedback loop between IL-6 and SGK1, in which the up-regulation of SGK1 by IL-6 serves to down-regulate IL-6 expression (**Fig. 3K**). Thus, there may be a balance between the

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increase in SGK1 expression in response to IL-6 stimulation and the feedback control of IL-6 secretion. This may also explain why SGK1 expression was maintained at the same level in cells exposed to IL-6 for more than 8 hours. These findings also suggest that IL-6 secretion is inhibited if the balance is disturbed by further stimulation that would otherwise increase SGK1 expression, helping control IL-6-induced inflammation. For example, glucocorticoids, such as dexamethasone and budesonide, were reported to induce SGK1 expression and inhibit IL-6 expression.[35-37] However, IL-6 expression was increased by inhibiting SGK1 with GSK650394 in cultured DPCs and 16HBE cells in the present study.

We think a greater amount of IL-6 is secreted in the mucosal tissue of CRS under continuous inflammatory irritations. At the same time, IL-6 induces SGK1 expression and SGK1 down-regulates IL-6 expression. However, the inhibition effects of SGK1 are not sufficient to contradict IL-6 secretion in response to inflammatory irritations. The expressions of IL-6 and SGK1 are increased in the mucosal tissues of CRS. Thus, we suppose that SGK1 could be regarded as a therapeutic target when extra drugs are administered to elevate SGK1 expression in order to control IL-6 secretion and to cure IL-6-induced inflammation in CRS (**Fig. 5**).

We also demonstrated the counterbalancing role of SGK1, in which SGK1 overexpression inhibited IL-6 self-induced mRNA and protein expression. Unexpectedly, we did not detect enhanced IL-6 self-induced mRNA or protein expression in A549 cells with SGK1 knockdown. Pretreatment with the SGK1 inhibitor GSK650394 significantly enhanced IL-6 self-induced mRNA expression in 16HBE cells, but not in A549 cells. This may be explained by the fact that the increase in SGK1 elicited by IL-6 overcame the effects of the inhibitor or the effects of SGK1 knockdown in A549 cells, which is more easily induced by IL-6 in A549 cells. We also failed to observe a significant increase in IL-6 protein expression following inhibition of SGK1 in 16HBE cells, possibly because the cell supernatants were collected 16 hours after stimulation was finished and the cellular self-repair activities counteracted the effects of SGK1 inhibition. In consideration of these findings, we did not examine whether IL-6 protein expression was enhanced by inhibiting SGK1 in DPCs or A549 cells.

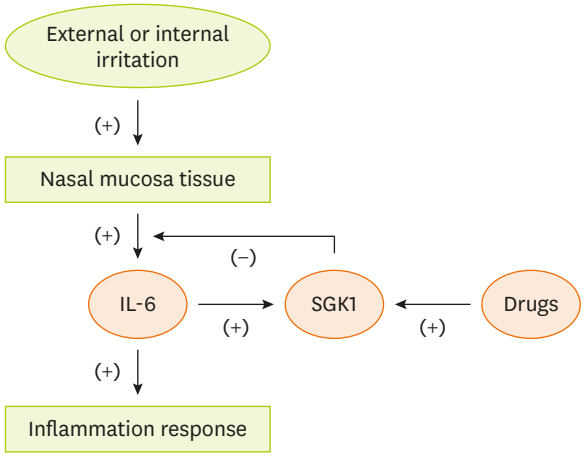

Fig. 5. The interaction between IL-6 and SGK1 in the mucosal tissue of CRS is illustrated schematically. IL-6 is secreted under continuous stimulation of multiple external or internal irritations. IL-6 upregulates SGK1 expression and SGK1 downregulates IL-6 expression. SGK1 could be regarded as a therapeutic target to control IL-6 secretion and to cure IL-6-induced inflammation in CRS, when extra drugs are administered to elevate SGK1 expression. SGK1, serum/glucocorticoid-regulated kinase 1; IL-6, interleukin-6; CRS, chronic rhinosinusitis; +, upregulation; −, downregulation.

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Given the limited number of subjects, we did not prepare dispersed cells from the mucosal tissues collected from CRSsNP patients. Another limitation of our study is that we did not assess SGK1 protein expression in response to IL-6 in DPCs *in vitro* due to the low protein concentrations of the supernatants. Further studies are required to elucidate precise molecular mechanisms involved in the interaction between IL-6 and SGK1 in order to better understand the pathogenic role of SGK1 in CRS. Because there are no known chemicals that stimulate SGK1 expression, we could not investigate whether IL-6 is decreased in response to the stimulation of SGK1 in DPCs *in vitro*. Further studies are needed to investigate the negative effects of SGK1 in the regulation of CRS in animal models.

In conclusion, we showed that the expression levels of SGK1 and IL-6 were significantly increased in CRS. We also provided evidence that SGK1 is elevated in response to IL-6 and that SGK1 down-regulates IL-6 mRNA and protein expression. These findings indicate that SGK1 may exert anti-inflammatory effects in response to IL-6 in the pathogenesis of CRS.

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SUPPLEMENTARY MATERIALS

Supplementary Fig. S1

IL-6 self-induced mRNA (A) and protein (B) expression levels in A549 cells.

Click here to view

Supplementary Fig. S2

The effect of the SGK1 inhibitor, GSK650394 on IL-6 self-induced mRNA expression in A549 cells (A) and 16HBE cells (B).

Click here to view

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