

RESEARCH ARTICLE

Store-operated calcium entry-associated regulatory factor regulates airway inflammation and airway remodeling in asthma mice models

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Abstract

Store-operated calcium entry (SOCE) is involved in the pathogenesis of airway inflammation and remodeling in asthma. Store-operated calcium entry-associated regulatory factor (SARAF) can downregulate SOCE. We sought to investigate the role of SARAF in the regulation of airway inflammation and remodeling in asthma mice models, as well as in the functional regulation of human airway smooth muscle cells (hASMCs). Balb/c mice were sensitized and challenged with ovalbumin to establish the asthma mice models. Mice were transfected with lentivirus, which expressed the SARAF gene + GFP (green fluorescence protein) or the negative control gene + GFP. Airway resistance was measured with the animal pulmonary function system. Airway inflammation and remodeling were evaluated via histological staining. In vitro cultured hASMCs were transfected with scrambled small interfering RNA (siRNA) or SARAF-specific siRNA, respectively. The proliferation, migration rate, hypertrophy, and SOCE activity of hASMCs were examined with Cell Counting Kit-8, wound healing test, bright field imaging, and Ca^{2+} fluorescence imaging, respectively. SARAF expression was measured by quantitative real-time PCR. Asthma mice models showed decreased SARAF mRNA expression in the lungs. SARAF overexpression attenuated airway inflammation, resistance, and also remodeling. Downregulation of SARAF expression with siRNA promoted the proliferation, migration, hypertrophy, and SOCE activity in hASMCs. SARAF plays a protective role against airway inflammation and remodeling in asthma mice models by blunting SOCE; SARAF may also be a functional regulating factor of hASMCs.

asthma; airway smooth muscle cells; airway inflammation; airway remodeling; store-operated calcium entry-associated regulatory factor

INTRODUCTION

Asthma is a chronic inflammatory disease characterized by airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling (1, 2). Severe persistent asthma will lead to irreversible airflow limitation, persistent respiratory symptoms, and airway remodeling. Reducing airway inflammation and airway remodeling has become the mainstay of asthma treatment.

Ca^{2+} is a critical second messenger participating in various physiological and pathological processes of airway inflammation and remodeling in asthma (3–5). Store-operated calcium entry (SOCE) is the major extracellular Ca^{2+} influx pathway for many immune cells and structural cells involved in asthma (4, 6–12), which is crucial to the maintenance of intracellular calcium homeostasis and Ca^{2+} store refilling (13). Stromal interaction molecule 1 (STIM1) acts as an endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) Ca^{2+} sensor (12, 14); upon calcium store depletion, STIM1 interacts with the Ca^{2+} release-activated Ca^{2+}

channel protein 1 (Orai1), the pore-forming subunit of store-operated calcium (SOC) channels, and then activates SOCE (15, 16).

Upregulated expression of STIM1 and Orai1 were observed in the lungs of asthma mice when compared with control mice; in addition, SOCE blockers SKF-96365, BTP-2, and RP3128 attenuated airway inflammation and airway hyperactivity in asthma mice models (17–19). We previously demonstrated that SOCE also participated in the regulation of airway smooth muscle cells (ASMCs) functions, such as proliferation and migration (3, 20). Moreover, SOCE is shown to be involved in the differentiation of fibrocytes, which are important precursors of fibroblasts and sources of collagen deposition (21). All these data suggest a critical role of SOCE in the regulation of airway inflammation and remodeling.

Store-operated calcium entry-associated regulatory factor (SARAF) has been identified as a regulating molecule of SOCE (22, 23). SARAF participates in the interaction between STIM1 and Orai1, promotes slow calcium-dependent inactivation (SCDI), and thus negatively regulates SOCE to prevent

Ca^{2+} overrefilling (23, 24). Overexpression of SARAF attenuated vascular neointimal hyperplasia via inhibition of vascular smooth muscle cell proliferation and migration (21). A recent study demonstrated that the baseline androgen receptor (AR) was greater in human ASMCs (hASMCs) from male asthma patients and increased with inflammation/asthma. The blunting effects of AR on ASM $[\text{Ca}^{2+}]_i$ involve reduced Ca^{2+} influx via L-type Ca^{2+} channels and SOCE, the latter by downregulating STIM1 and Orai1 and increasing TMEM66 (also called SARAF) (25). In addition, in a previous study SARAF was shown to be protective against severe pancreatitis in mice (13), also suggesting a protecting role of SARAF in the inflammation. In consideration of the important role of SOCE in the regulation of airway inflammation and remodeling, we hypothesized that SARAF may be able to attenuate asthma airway inflammation and remodeling by negatively regulating SOCE. In the current study, we investigated the effects of SARAF overexpression on airway inflammation, resistance, and remodeling in acute and chronic asthma mice models. Since ASMCs are major contributors to airway remodeling in asthma, and SOCE is the main Ca^{2+} entry pathway for ASMCs, we also investigated the impacts of SARAF downregulation on the function of hASMCs.

MATERIALS AND METHODS

Animals

Female and male Balb/c mice (6–8 wk) were obtained from the Experiment Animal Center of Wuhan University (Wuhan, China). Mice were housed under a 12-h light/dark cycle at 25°C. All animal experiments were approved by the Animal Care Committee of Zhongnan Hospital of Wuhan University (Approval No.: 2017039).

Establishment of Acute and Chronic Asthma Mice Models

The experimental protocols for the establishment of ovalbumin (OVA)-induced acute and chronic asthma mice models were performed, as described previously (26). Sixty mice were divided into 12 groups ($n = 5$ per group), as shown in Table 1. Mice were sensitized by intraperitoneal injection of 20- μg OVA (Grade V; Sigma, St. Louis, MO) emulsified in 2 mg aluminum hydroxide (Thermo Fisher Scientific Inc., IL)

in 200 μL of phosphate buffer saline (PBS) on *day 0* and *day 14* and then challenged with 100 μg OVA in 50 μL of PBS through intranasal delivery on *day 25*, *day 26*, and *day 27* in acute female and male asthma model, whereas the control mice were sensitized and challenged only with PBS. In the chronic asthma model group, mice were sensitized with 20 μg OVA on *day 0*, *day 7*, and *day 14*. From *day 21*, mice were challenged with 5% aerosolized OVA three times a week for consecutive 6 wk. The control mice were sensitized and challenged with PBS. The experimental protocol for lentiviral vector transfection was performed, as described previously (27). Briefly, the mice were intranasally delivered with 50- μL lentivirus at a concentration of 1×10^8 TU/mL of Lenti-expressing SARAF gene + GFP (green fluorescence protein) (Lenti-SARAF) or lentiviral vector expressing the control gene (Lenti-Ncontrol) (Shanghai Genechem Co., LTD, Shanghai, China), respectively, on *day 23* in acute asthma model and 2 days before each nebulization in the chronic asthma model.

Airway Hyperresponsiveness Measurement

Twenty-four hours after the last challenge with OVA, mice were anesthetized with 1% pentobarbital, and airway resistance index (RI) was measured, as previously described (28). Mice were intubated with a 19-gauge tracheostomy tube, mechanically ventilated with a tidal volume of 0.2 mL and a rate of 130 beats/min. RI of mice was calculated at baseline (PBS aerosolization) and after provoked with accumulated doses of aerosolized acetyl- β -methacholine (3.125, 6.25, 12.5, 25, and 50 mg/mL; Sigma-Aldrich) with FinePointe RC system (Buxco Electronics, Troy, NY) in a 3-min period following each challenge.

Bronchoalveolar Lavage Fluid Collection

The lungs of mice were lavaged with 0.5 mL of PBS containing 0.6 mM EDTA via a 1-mL syringe for three times, as previously described (29). The collected bronchoalveolar lavage fluid (BALF) was centrifuged, and the supernatants were stored at -80°C for immunoglobulin E (IgE) analysis. Cell smears were made from BALF by cytospin (TXD3 cytocentrifuge Xiangyi, Changsha, China) and stained with Wright-Giemsa (Goodbio technology, Wuhan, China). After staining, differential cell count was performed to measure the numbers of neutrophils, eosinophils, and lymphocytes under a light

Table 1. Mice groups

Group Name	OVA Sensitized and Challenged	Transfected Virus	Model
A-PBS female group	No	No	Acute
A-OVA female group	Yes	No	Acute
A-NC female group	Yes	Negative control lentivirus	Acute
A-SARAF female group	Yes	SARAF lentivirus	Acute
A-PBS male group	No	No	Acute
A-OVA male group	Yes	No	Acute
A-NC male group	Yes	Negative control lentivirus	Acute
A-SARAF male group	Yes	SARAF lentivirus	Acute
C-PBS female group	No	No	Chronic
C-OVA female group	Yes	No	Chronic
C-NC female group	Yes	Negative control lentivirus	Chronic
C-SARAF female group	Yes	SARAF lentivirus	Chronic

A, acute asthma; C, chronic; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, NC, negative control; OVA, ovalbumin; SARAF, store-operated calcium entry-associated regulatory factor.

microscope; 400 cells were counted per slide. Total IgE level in BALF was tested using an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, CA) according to the manufacturer's protocol, with a detection limit of 4 ng/mL.

Lung Histology

The left lungs were removed from mice and fixed in 4% paraformaldehyde buffer for at least 24 h then were dehydrated and embedded in paraffin before slicing (~4–5 μ m per slice). The lung tissue sections were used for hematoxylin-eosin (H&E) staining, Periodic Acid-Schiff (PAS) staining, Masson's trichrome staining, and α -smooth muscle actin (α -SMA) immunostaining to evaluate the inflammatory cell infiltration of the airway, hyperplasia of goblet cells, collagen deposition, and hypertrophy of ASMCs. Five sections were assessed per lung. Airway inflammation scores were classified into 0–3 grades according to the degree of inflammatory cell infiltration around the airways: 0, no inflammatory cell infiltration; 1, few inflammatory cells were found, but no layer was formed; 2, a ring of one to five layers inflammatory cells were found; 3, a ring of more than five layers inflammatory cells were found. Image-Pro Plus 6.0 (IPP 6.0) software was used to determine the positive staining areas, including PAS⁺ area, Masson⁺ area, α -SMA⁺ area, and airway basement membrane perimeter (Pbm), and the ratios of APAS⁺/Pbm, AMasson⁺/Pbm, and A α -SMA⁺/Pbm were calculated to indicate the degree of goblet cell hyperplasia, collagen deposition, and airway smooth muscle hyperplasia, respectively.

Cell Culture

Pulmonary lobar and segmental bronchi were collected from five patients with lung cancer who underwent pneumonectomy or lobectomy in the Department of Thoracic Surgery, Zhongnan Hospital of Wuhan University. The study was approved by the Medical Ethics Committee of Zhongnan Hospital of Wuhan University (approval No.: 2019044). The lobar or segmental bronchi from tumor-free areas were isolated and put into 100-mm petri dishes. The bronchial adventitia, intima, and cartilage were removed carefully in ice-cold PBS under a dissecting microscope, leaving only the smooth muscle layer to adhere to the flask wall. The isolated bronchial smooth muscle was cut into 1 $\times$ 1 mm blocks and then digested in 2-mL PBS containing 0.5% trypsin (Gibco) for 5 min at 37°C. The suspension was centrifuged and washed with Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA). The ASM blocks were seeded in a 25 cm² culture flask. The flask was inverted and added 3–4 mL of DMEM/F-12 supplemented with 10% FBS and 100 U/mL penicillin/streptomycin and incubated at 37°C in 5% CO₂ for 3–4 h to promote the adhesion of ASM to the wall. The flask was then turned back and replenished with culture medium to 5 mL. The medium was replaced with fresh medium every 2 days, as suggested (30). Those hASMCs grown out of the tissue blocks were passaged and cultured in DMEM/F-12. The confluent ASMCs were typically spindle shaped and presented with a hill-and-valley pattern. The purity of cultured hASMCs was >95% when determined with α -SMA immunostaining. All experiments were performed at >80% cell confluence in hASMCs till five passage.

Small Interfering RNA Knockdown of SARAF

Small interfering RNA (siRNA) was purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The target sequence of siRNA was genOFFTM st-h-saraf: GCGGATTC-CTGTAACATGA. hASMCs were transfected with siRNA against SARAF or scrambled RNA, as negative control (NC) diluted in riboFECT CP reagent (RiboBio, Guangzhou, China). The transfection complex (final siRNA concentration: 30 nmol/L) was prepared and added into serum-free DMEM with a final volume of 500 μ L. In the blank group, cells were treated with the same amount of PBS. The transfection complex was removed after incubated with cells for 6 h, and the cells were cultured in 10% FBS-containing DMEM/F12 for additional 24–72 h (20). The silencing efficiency of siRNA was examined by quantitative real-time PCR (qRT-PCR).

Quantitative Real-Time PCR

qRT-PCR was performed to examine the mRNA expression of SARAF in siRNA-treated hASMCs and in lung tissues. Total RNA was extracted from the lung tissues or hASMCs via TRIzol Reagent (Invitrogen, CA). cDNA was reversely transcribed using the ReverTra Ace qPCR RT kit (Toyobo, Tokyo, Japan). qRT-PCR was performed according to the manual of SYBR Premix Ex Taq (TAKARA, Otsu, Japan) provided by the manufacturer. Relative mRNA expression was normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated as fold change compared with the PBS group. The primer sequences of target genes are listed in Table 2.

Cell Proliferation Assay

The proliferation rates of hASMCs were determined using a Cell Counting Kit-8 reagent (CCK-8; Dojindo, Shanghai, China), as described previously (26). The cells in each group were inoculated in 96-well plates with a density of 5×10^3 cells/well without FBS for a quiescent state, after 24 h, cells were cultured in 10% FBS-containing DMEM/F-12 for 24 h, 4–6 duplicate wells per group. CCK-8 reagents (10 μ L) were then added into each well and incubated for 2–4 h. The absorbance ratio at 450 nm wavelength was examined with a microplate reader (Thermo Fisher Scientific). The corresponding optical density (OD) value represents cell proliferation.

Cell Migration Test

The migration rates of hASMCs were determined using wound healing test. Cells were seeded in 6-well plates and then transfected with siRNA against SARAF or scrambled RNA (1×10^6 cells per well). Upon 100% cell confluence, five parallel lines were scratched on the bottom of each well using a sterile glass pipette tip. After washing with PBS, cells were then cultured for 24 h in serum-free DMEM/F-12. Photos of these lines were taken initially and 24 h later. IPP 6.0 software was used to measure the width of each line at six to eight different locations and the average widths were calculated. The reduction in averaged widths (percentages of recovery of the wound) represents cell migration rate.

Table 2. Primer sequences and product sizes

Clone Name	Primer Sequence	Product Size, bp
PCR of mouse SARAF	Sense	5'-GCTGTTGCTCCTGCTGCTCACTC-3'
	Antisense	5'-GTGCAAACCTTGGCCATTC-3'
PCR of mouse GAPDH	Sense	5'-AAGAAGGTGGTGAAGCAGGCATC-3'
	Antisense	5'-CGGCATCGAAGGTGAAGAGTG-3'
PCR of human SARAF	Sense	5'-CTGGCCATGGTGCAACTTCT-3'
	Antisense	5'-GGTGTTCGCGCTCTATGCT-3'
PCR of human GAPDH	Sense	5'-ATCGTGAAGGACTCATGACCACA-3'
	Antisense	5'-AGAGGCAGGGATGATGTTCTGGA-3'

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SARAF, store-operated calcium entry-associated regulatory factor.

Cell Hypertrophy Assay

The sizes of hASMCs were determined using bright-field imaging. Briefly, hASMCs grown in six-well plates were transfected with siRNA against SARAF or scrambled RNA and cultured in DMEM/F12 with 10% FBS for 24–72 h. The transfection reagents were washed out and the cell sizes were measured via cross-sectional area calculation using Image J. For each group, sizes of 50 individual hASMCs were measured and averaged.

Measurement of Intracellular Ca^{2+}

Intracellular Ca^{2+} fluorescence imaging was used to determine SOCE of hASMCs, the detailed method had been described previously (26). Briefly, 5×10^3 cells/mL ASMCs were seeded on a glass coverslip with a 1 cm diameter for 48 h. Fluo-3/AM (5 μM) (Dojindo, Japan) in a Ca^{2+} -free medium was added to the cells and incubated for 45 min at room temperature. After washing with Ca^{2+} -free physiological salt solution (PSS) (PSS without CaCl_2 and 0.2 mM EGTA), coverslips were placed with the side of the cells upward onto the bottom of a petri dish containing 1 mL Ca^{2+} -free PSS. The dish was then placed under an inverted microscope and observed with a $\times 20$ objective lens. Intracellular Ca^{2+} fluorescence was activated by an Ar-Kr laser (excitation wavelength 488 nm and emission wavelength 530 nm) and recorded with a confocal system (Leica TCS SP2 AOBS MP, Wetzlar, Germany). The cell edge was marked under phase-contrast microscope to calculate the ROI (region of interest) of the cells. On every coverslip, 10–15 cells were recorded at the same time. The laser intensity and the photomultiplier tube gain remained unchanged during the recording. The pinhole value was 1 airy. F_0 and $F_{\max}$ represent the baseline level and the maximum change of Ca^{2+} fluorescence, respectively. F represents the real-time Ca^{2+} fluorescence intensity, which was calculated with the equation of $F = (F_{\max} - F_0)/F_0$.

hASMCs Stimulated with Sera from Patients with Asthma and Healthy Controls

hASMCs (5×10^5) were inoculated into each well of six-well plates, at 80% confluence, cells were treated with 10% sera from patients with allergic asthma or healthy controls for 48 h. Sera collected were all heat inactivated and sterilized. Then the hASMCs were collected for qRT-PCR. Patients with allergic asthma were recruited from those who met the diagnostic criteria of the Global Initiative for

Asthma (GINA) for allergic asthma with a total IgE > 200 KU/L, no acute infection within 1 wk and not treated with oral corticosteroids within 4 wk. Healthy controls were recruited from students in the same university and without any history of allergy. Venous blood (5 mL) was collected from each person. Informed consent was obtained from all blood donors and approved by the Medical Ethics Committee of Zhongnan Hospital of Wuhan University (Approval No.: 2019044).

Statistical Analysis

Data are shown as means $\pm$ SD. Independent sample t test and one-way ANOVA were used for statistical analysis with SPSS 24.0 software (IBM, Chicago). P value less than 0.05 was considered statistically significant.

RESULTS

SARAF Expression in the Lungs of Asthma Mice Models

The protocols for the construction of acute and chronic asthma mice models and timelines for transfection are shown in Fig. 1, A and B. GFP fluorescence could be observed in lung tissues of lentivirus-transfected mice (Fig. 1C). The qRT-PCR assay showed decreased mRNA expression of SARAF in lungs from OVA-sensitized and OVA-challenged acute and chronic asthma mice models when compared with control mice (Fig. 1, D and E). Transfection of Lenti-SARAF increased SARAF mRNA expression in lungs of both acute and chronic mice groups when compared with asthma mice transfected with Lenti-Ncontrol or those without transfection (Fig. 1, D and E).

SARAF Overexpression Attenuated Airway Resistance in Both Acute and Chronic Asthma Mice Models

The airway RIs of the mice models were evaluated at baseline and after provocation with methacholine. In comparison to control mice, the RIs of acute and chronic asthma mice were significantly increased at concentrations of methacholine ≥ 12.5 mg/mL. At the concentration of 25 mg/mL and 50 mg/mL, methacholine-induced RIs were decreased in both acute and chronic asthma mice overexpressed with SARAF (Fig. 1, F and G). Transfection with Lenti-Ncontrol had no significant effect on RIs in both acute and chronic asthma mice (Fig. 1, F and G). These results suggest that up-regulation of SARAF expression could attenuate airway resistance in asthma mice.

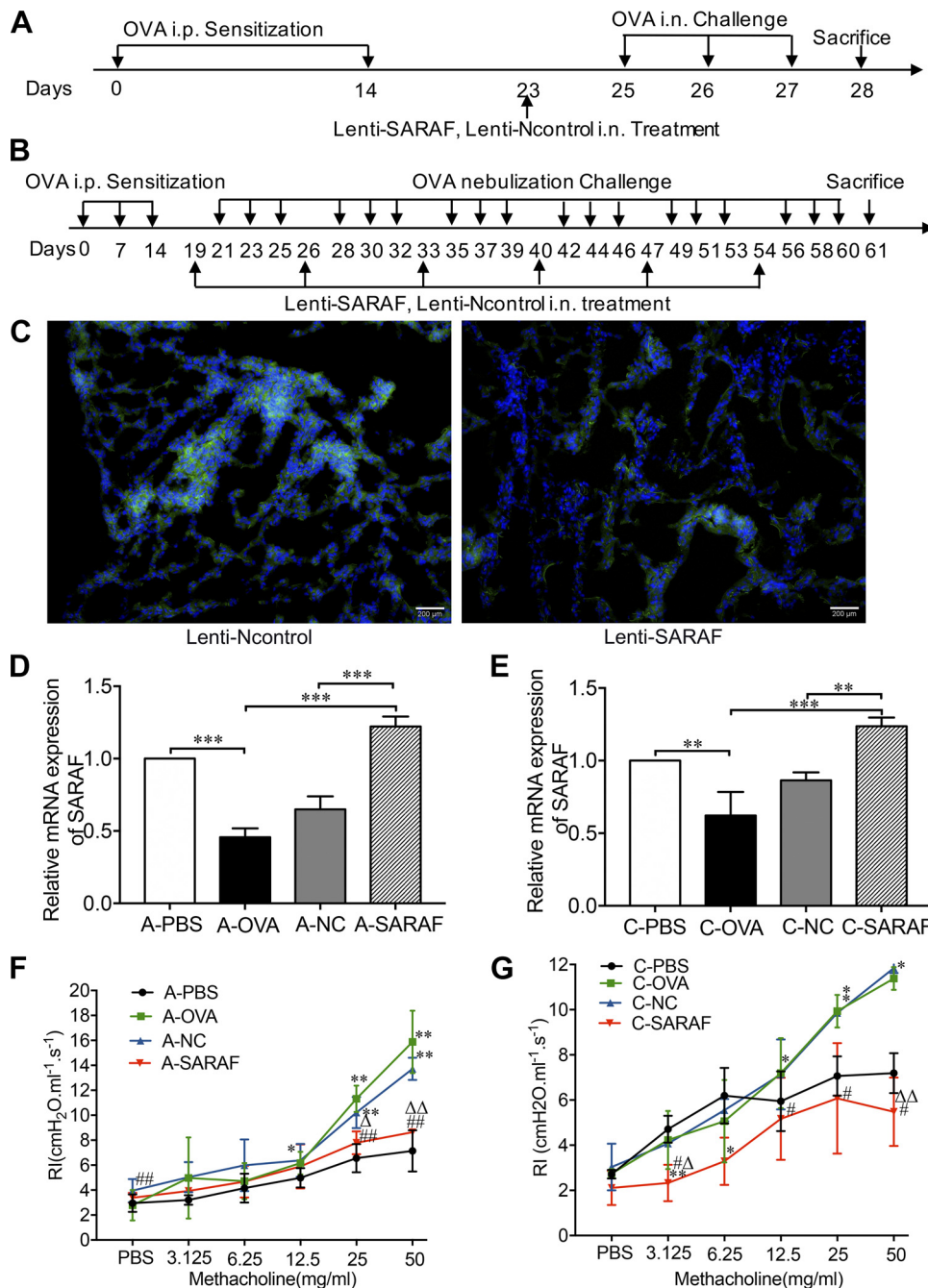


Figure 1. Store-operated calcium entry-associated regulatory factor (SARAF) mRNA expression in lungs and effects of overexpression of SARAF on airway resistance in acute and chronic asthma mice models. Experimental protocols of ovalbumin (OVA)-induced acute (A) and chronic (B) asthma mice models. C: representative fluorescence imaging showed SARAF expression in the lungs from mice transfected with lentivirus loaded with SARAF (Lenti-SARAF) or negative control (Lenti-Ncontrol) gene, respectively ($\times 200$). SARAF mRNA expression examined by qRT-PCR in the lungs from control or acute asthma mice models (D) and chronic asthma mice models (E) with or without lentivirus transfection ($n = 3$ mice in each group). ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA. F and G: methacholine-induced airway resistance in the respective mice groups ($n = 5$ mice in each group). * $P < 0.05$, ** $P < 0.01$ compared with PBS group. ## $P < 0.01$ compared with OVA group. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ compared with NC group, one-way ANOVA. A-NC, acute asthma mice model transfected with lentivirus loaded with negative control gene; A-OVA, acute asthma mice model; A-PBS, control for acute asthma models; i.n., intranasally; i.p., intraperitoneal; A-SARAF, acute asthma mice model transfected with lentivirus loaded with SARAF gene; C-NC, chronic asthma mice model transfected with lentivirus loaded with negative control gene; C-OVA, chronic asthma mice model; C-PBS, control from chronic asthma mice models; C-SARAF, chronic asthma mice model transfected with lentivirus loaded with SARAF gene; RI, resistance index.

SARAF Overexpression Alleviated Airway Inflammation in Asthma Mice Models

The potential regulating role of SARAF in airway inflammation in asthma mice models was explored. The numbers of total cells, macrophages, neutrophils, lymphocytes, and eosinophils in BALF of both acute and chronic asthma mice were all higher than those of control mice. Eosinophils were hardly observed in the BALF of control mice. In both acute and chronic asthma mice models overexpressed with SARAF, total cell numbers, numbers of macrophages, and eosinophils in BALF were lower than in control and Lenti-Ncontrol mice (Fig. 2, A and B). However, lymphocyte

numbers in BALF were decreased only in chronic but not in acute asthma mice model overexpressed with SARAF compared with control and Lenti-Ncontrol mice (Fig. 2, A and B). H&E staining did not demonstrate obvious inflammatory cell infiltration and airway epithelial goblet cell metaplasia in the lungs of control mice, whereas a large number of inflammatory cells and goblet cells were observed in the lungs of both acute and chronic asthma mice groups, and most of the infiltrating inflammatory cells were more than five layers thick (Fig. 2C). Inflammation scores demonstrated similar results (Fig. 2, D–F). Thus, SARAF overexpression attenuated both airway inflammatory cell infiltration and inflammatory scores. Transfection with Lenti-Ncontrol in

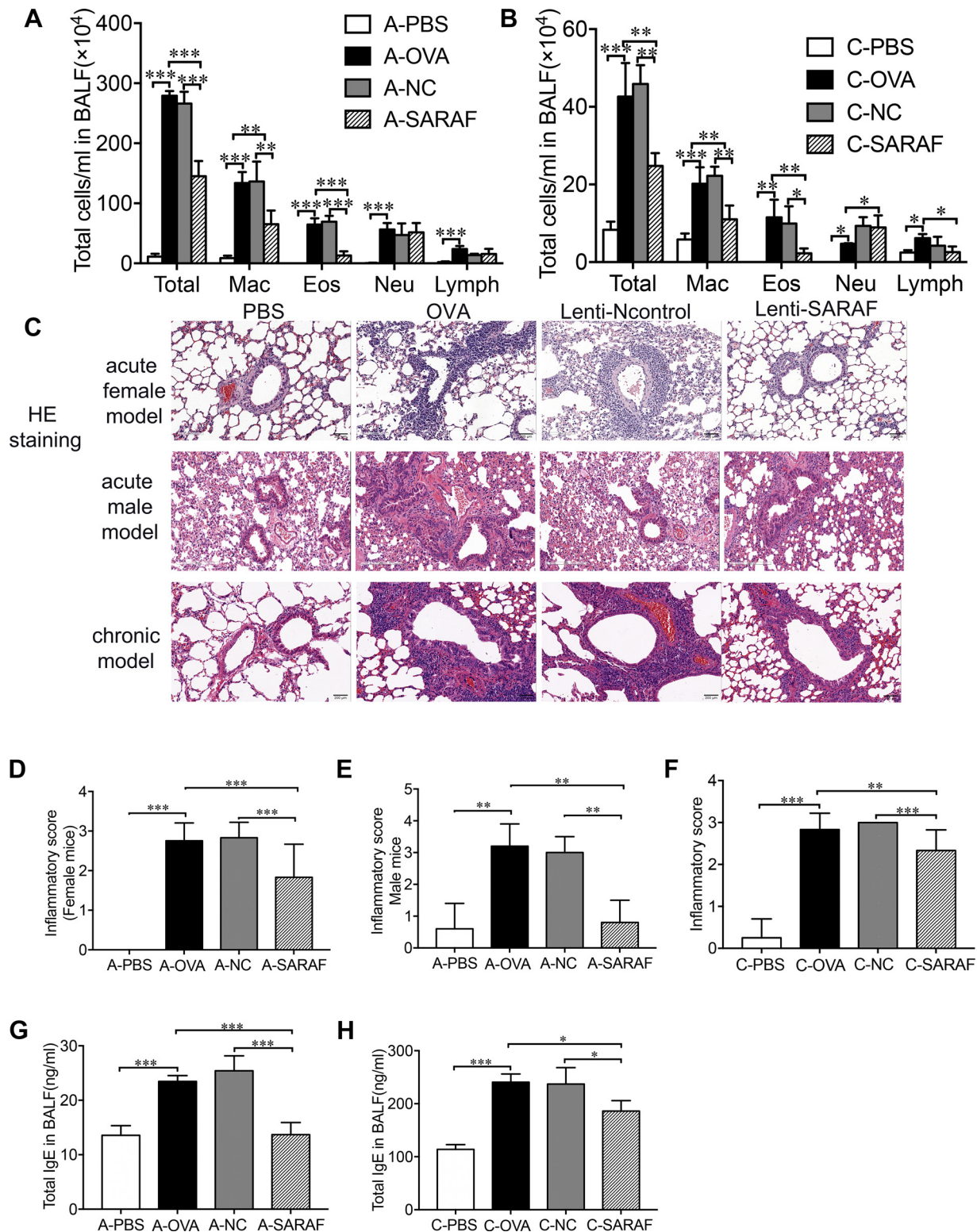


Figure 2. Airway inflammation in acute and chronic asthma mice models. **A** and **B**: absolute numbers of total inflammatory cells, macrophages, eosinophils, neutrophils, and lymphocytes in bronchoalveolar lavage fluid (BALF) from the respective mice groups ($n=3$ mice for PBS group and $n=5$ mice for asthma groups). **C**: representative hematoxylin-eosin (HE) staining of lung tissue sections from control and asthma mice models with or without lentivirus transinfection ($\times 200$). Airway inflammation scores in control mice (A-PBS or C-PBS) and acute female and male (**D** and **E**) or chronic (**F**) asthma mice models with or without lentivirus transfection ($n=12$ counts per field-of-view). Total IgE levels in bronchoalveolar lavage fluid (BALF) from control mice (PBS) and acute (**G**) or chronic (**H**) asthma mice models with or without lentivirus transfection ($n=3$ mice in each group). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, one-way ANOVA. A-NC, acute asthma mice model transfected with lentivirus loaded with negative control gene; A-OVA, acute asthma mice model; A-PBS, control for acute asthma models; A-SARAF, acute asthma mice model transfected with lentivirus loaded with SARAF gene; C-NC, chronic asthma mice model transfected with lentivirus loaded with negative control gene; C-OVA, chronic asthma mice model; C-PBS, control for chronic asthma mice models; C-SARAF, chronic asthma mice model transfected with lentivirus loaded with SARAF gene.

both acute (female and male) and chronic asthma mice groups (A-NC and C-NC) had no significant effect on airway inflammation (Fig. 2, C–F). IgE levels in BALF were significantly higher in both acute and chronic asthma groups of mice when compared with control mice. IgE levels in BALF from both acute and chronic asthma mice models overexpressed with SARAF were lower than asthma mice without transfection or transfected with Lenti-Ncontrol; there were no significant differences between IgE levels in BALF from asthma mice without transfection or transfected with Lenti-Ncontrol (Fig. 2, G and F). These data showed that SARAF overexpression attenuated airway inflammation in both acute and chronic asthma mice models, suggesting a possible protective role of SARAF against airway inflammation of asthma.

SARAF Overexpression Attenuated Airway Remodeling in Chronic Asthma Mice Model

Histological examination showed significant goblet cell hyperplasia, subepithelial collagen deposition, and ASM hyperplasia in airways of chronic asthma mice when compared with control mice (Fig. 3A). Quantitative analysis using A_{PAS}^{+}/Pbm , A_{Masson}^{+}/Pbm , and $A_{\alpha-SMA}^{+}/Pbm$ confirmed the abovementioned three parameters of airway remodeling in the chronic asthma mice model when compared with control mice (Fig. 3, B–D). Noticeably, in the chronic asthma mice model, overexpression of SARAF attenuated airway remodeling, as indicated by the decrease of the abovementioned three parameters (Fig. 3, A–D). However, transfection with Lenti-Ncontrol had no significant effect on airway remodeling in chronic asthma mice (Fig. 3, A–D). These data demonstrated that SARAF overexpression could inhibit airway remodeling in the chronic asthma mice model.

SARAF Expression Was Different between hASMCs Stimulated with Sera from Patients with Asthma and from Healthy Controls

The phenotype of ASM was characterized based on morphology and α -SMA staining. Figure 4A showed typical morphology of in vitro cultured hASMCs with and without α -SMA staining. We first examined the different effects of sera from patients with asthma and healthy controls on the expression of SARAF in in vitro cultured hASMCs. After incubated with the sera for 48 h, the mRNA expression of SARAF in hASMCs treated with sera from patients with asthma was significantly lower than that in hASMCs treated with sera from healthy controls (Fig. 4B).

Knockdown of SARAF Expedited Human ASMCs Proliferation, Migration, Hypertrophy, and SOCE

Since ASMCs are the key cell types involved in airway hyperactivity and remodeling, the effects of SARAF on functional regulation of hASMCs were then determined by knockdown of SARAF. qRT-PCR showed that SARAF-specific siRNA significantly reduced the mRNA level of SARAF when compared with scrambled siRNA transfected cells (NC) or control (PBS) hASMCs (Fig. 4C). SARAF knockdown with specific siRNA dramatically increased hASMCs proliferation rates when compared with scrambled siRNA-transfected hASMCs or control (PBS) hASMCs (Fig. 4D). Wound healing

tests showed that SARAF-specific siRNA expedited the migration rates of hASMCs when compared with scrambled siRNA-transfected (NC) or control (PBS) hASMCs (Fig. 4E). SARAF knockdown also aggravated the hypertrophy of hASMCs, in that the cell sizes were higher in SARAF siRNA-transfected hASMCs than in scrambled siRNA-transfected (NC) or control (PBS) hASMCs (Fig. 4, F and G). In addition, Ca^{2+} fluorescence measurement showed that thapsigargin-induced SOCE was higher in SARAF-specific siRNA-treated hASMCs than in scrambled siRNA-treated hASMCs (Fig. 3, H and I). SKF-96365, a relatively specific SOC channel blocker, could partly inhibit SOCE in NC cells (Fig. 3, H and I). These experiments confirmed the critical role of SARAF in preventing hASMCs from expedited proliferation, migration, hypertrophy, and increased SOCE.

DISCUSSION

This study showed a decreased expression of SARAF in the lungs from OVA-induced asthma mice models. Overexpression of SARAF alleviated airway inflammation, resistance, and remodeling, whereas knockdown of SARAF expedited proliferation, migration, hypertrophy, and SOCE of hASMCs.

The role of SOCE in airway inflammation and remodeling has been demonstrated previously in animal models of asthma (17). A recent study showed that a 70% ethanolic extract of *Flos Magnoliae* (FMEtOH) suppressed allergic inflammation by inhibiting SOCE (31). SOCE has been demonstrated to participate in the functional regulation of diverse immune cells that may contribute to the development of allergic airway inflammation (4, 8–11, 15, 28, 31). SOCE is critical for the differentiation of Th17 cells (32), regulatory T cells (Tregs) (33), type I and type II helper T (Th1 and Th2) cells (34), T follicular helper (Tfh), and T follicular regulatory (Tfr) cells (35). In addition, SOCE regulates dendritic cell maturation and antigen presentation (36). SOCE is also essential to the proper function of B lymphocytes (37) and mast cells (38). Since SARAF downregulates SOCE activity by promoting SCDI, all the abovementioned cell types may be involved in the effects of SARAF overexpression on airway inflammation in asthma mice models and this should be confirmed in future research. Moreover, SOCE could be directly activated by aeroallergens such as house dust mites and *Alternaria alternata* in airway epithelial cells and participated in the pathogenesis of type 2 airway inflammation (39). There is evidence showing that SOCE is also involved in the functional regulation of different types of cells that may contribute to airway remodeling (6, 12, 40, 41). We previously demonstrated that Th2 cytokine interleukin-4 could promote circulating fibrocytes differentiation and airway remodeling via regulation of SOCE (42). Ca^{2+} signaling through SOCE is pivotal to the proliferation of ASMCs (40, 41) and the proliferation and migration of lung fibroblasts (6). In consistent with these results, we found that SARAF, the newly identified regulating molecule of SOCE, was also involved in airway remodeling in the asthma mice model.

We have previously demonstrated the indispensable role of SOCE in the proliferation, hyperplasia, and hypertrophy of ASMCs (19, 41, 43). ASMCs are the key cell types involved in the airway remodeling of asthma (7, 10), and SOCE is the

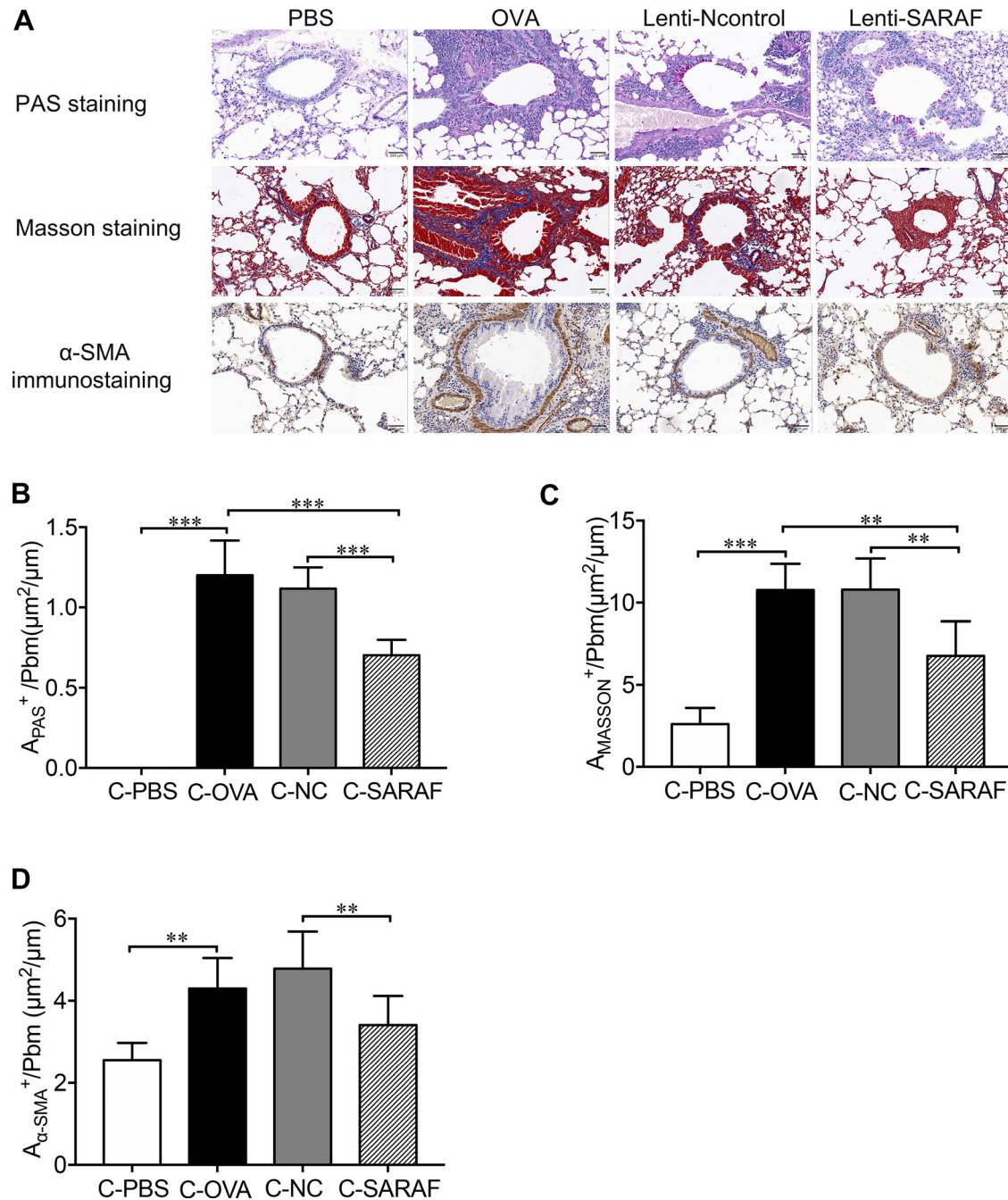


Figure 3. Airway remodeling in chronic asthma mice model. A: representative Periodic Acid-Schiff (PAS) staining, Masson staining, and α -SMA immunostaining of lung tissue sections from PBS control mice or ovalbumin (OVA)-induced chronic asthma mice model transfected with lentivirus loaded with negative control gene (Lenti-Ncontrol) or store-operated calcium entry-associated regulatory factor (SARAF) gene (Lenti-SARAF) or without transfection (OVA), samples were collected 24 h after the last challenge ($\times 200$). A_{PAS^+}/Pbm (B); A_{MASSON^+}/Pbm (C); $A_{\alpha-SMA^+}/Pbm$ (D) were measured from different group of mice shown in A to verify the goblet cell hyperplasia, subepithelial collagen deposition, and smooth muscle hypertrophy, respectively ($n=5$ counts per field-of-view). ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA.

major extracellular Ca^{2+} influx pathway of ASMCs (9, 44). SOCE blocker Ni^{2+} could markedly attenuate bronchial SMCs proliferation (45). Volatile anesthetics prevented sarcoplasmic reticulum refilling by inhibiting SOCE and enhancing cyclic nucleotide, which blunted Ca^{2+} influx in ASM and resulted in substantial airway relaxation (46). Our previous study identified a promoting effect of IL-13 on Ca^{2+} release and SOCE in ASMCs, which may partially contribute

to the pathogenesis of airway remodeling (43). In addition, IL-13 enhanced the frequency of leukotriene D4-induced Ca^{2+} oscillations in hASMCs, possibly through its effect on SOCE (47). Both protein kinase C (PKC) δ -selective inhibitor, rottlerin, and PKC downregulation attenuated SOCE dramatically, confirming a regulating role of PKC on SOCE in ASMCs (40). Moreover, SOCE was upregulated during ASMC proliferation accompanied by increased STIM1 and Orai1

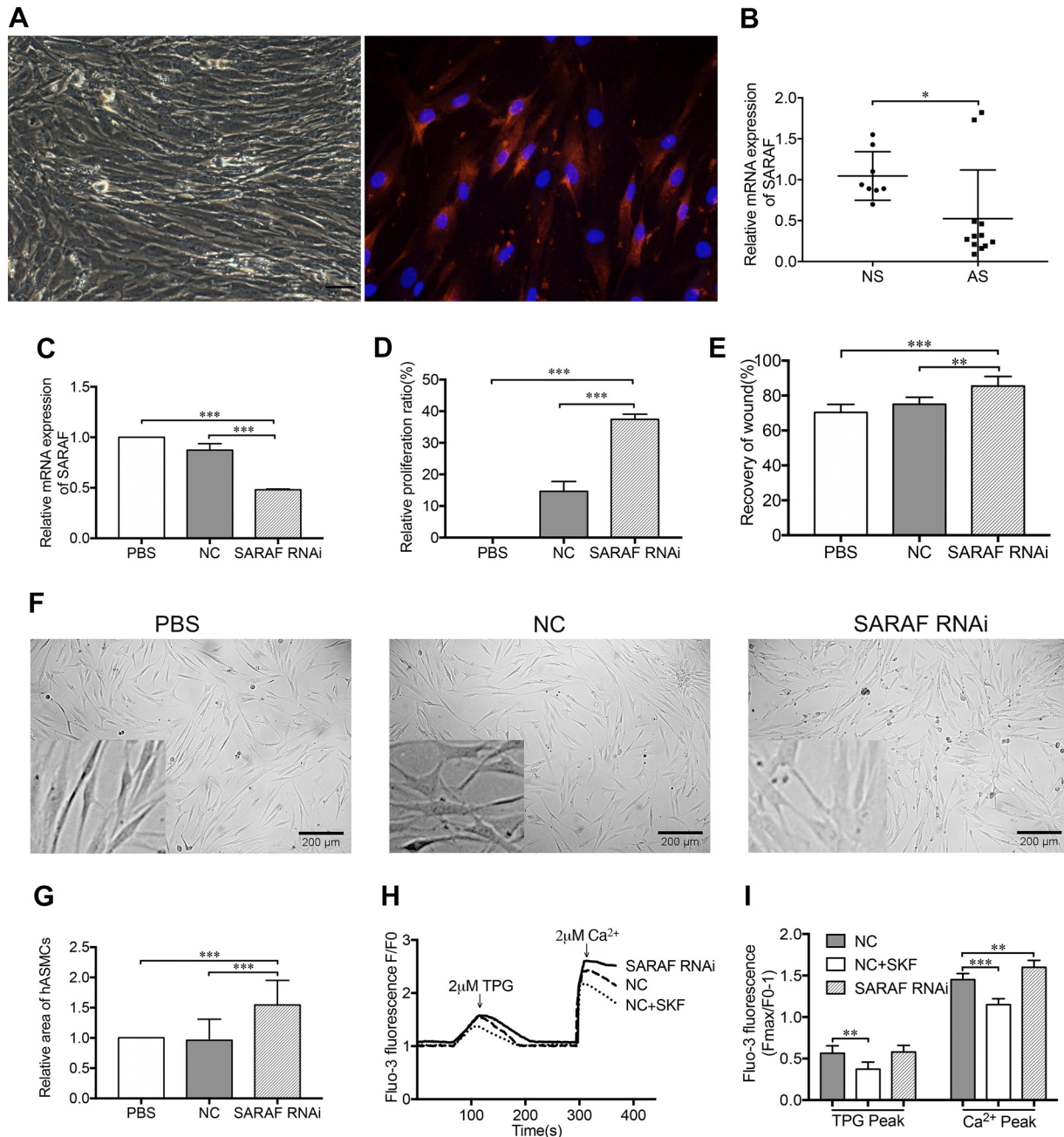


Figure 4. Effects of store-operated calcium entry-associated regulatory factor (SARAF) downregulation with siRNA on proliferation, migration, hypertrophy, and store-operated calcium entry (SOCE) in human airway smooth muscle cells (hASMCs). *A*: typical morphology of cultured hASMCs. *Left*: hASMCs under optical microscope (×200); *right*: hASMCs stained with anti- α -SMA (red) and (×200). *B*: SARAF mRNA expression in hASMCs treated with sera from healthy control (NS, $n=8$ subjects) and from patients with asthma (AS, $n=12$ subjects). *C*: relative expression of SARAF mRNA in cultured hASMCs. *D*: proliferation rates in control hASMCs (PBS), hASMCs transfected with scrambled siRNA (NC) and with SARAF-specific siRNA (SARAF RNAi). Data were of three independent experiments in each group. *E*: cell migration rates in control, NC- and SARAF-RNAi-treated hASMCs groups. Data were of five independent experiments in each group. *F*: representative bright-field imaging of control hASMCs (PBS), hASMCs transfected with siRNA against SARAF or negative control gene to show the differences in cell sizes and indicate hypertrophy of hASMCs. Inserts show the corresponding zoom-in views (×800). *G*: cell sizes in control, NC- and SARAF siRNA-transfected hASMCs. Data were of five independent experiments in each group. *H*: representative time courses of Ca^{2+} fluorescence intensity in NC hASMCs, NC hASMCs in the presence of SKF96365 and SARAF siRNA-treated hASMCs. The first peak indicates Ca^{2+} release induced by thapsigargin (TPG) and the second peak represents SOCE after readdition of 2 mM Ca^{2+} into Ca^{2+} -free physiological salt solution (PSS). SKF, SKF96365. *I*: the averaged peak amplitude of Ca^{2+} release and Ca^{2+} influx via SOCE (F_{max}/F_0-1) in three groups of hASMCs as in *G*. Data were of three independent experiments in each group. * $P < 0.01$; ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA followed by independent sample *t* test.

mRNA expression, whereas the proliferation of ASMCs was partially inhibited by the SOC channel blockers SKF-96365, Ni^{2+} , and BTP-2. Decreased mRNA expression of STIM1 or Orai1 resulted in the attenuation of SOCE and

ASMCs proliferation (19). Furthermore, SOCE also participated in the regulation of hypertrophy and migration of ASMCs by regulating intracellular calcium homeostasis (3). ASMCs hypertrophy contributes to increased

contractility and thickness of the airways, an important characteristic of airway remodeling (48, 49). In the current study, we found that downregulation of SARAF promoted proliferation, migration, hypertrophy, and SOCE of hASMCs. Since SARAF is capable of downregulating SOCE through SCDI, our data provide additional evidence to confirm the role of SOCE in the functional regulation of ASMCs.

Different regulatory molecules of SOCE have been identified recently, including STIM-activating enhancer (50), calcium release-activated channel regulator 2A (51, 52), and SARAF (23). SARAF downregulates SOCE by preventing Ca^{2+} overload through SCDI. It was shown that downregulation of SARAF led to persistent Ca^{2+} influx and thus disturbed Ca^{2+} homeostasis (53). SARAF exhibited threefold higher expression in patients with multiple sclerosis (MS) than in healthy subjects. SARAF had marked immunological activity relevant to MS and augmented the production of proinflammatory cytokines and chemokines (11). Furthermore, overexpression of SARAF in Cajal cells prevented spontaneous intracellular Ca^{2+} oscillations in the small intestine of mice, suggesting a regulating role of SARAF in the slow-wave pattern of the gastrointestinal tract (54). Interestingly, SARAF inhibited the up-regulated expression of STIM1 and Orai1 in mice with cardiac hypertrophy and may play a protective role in cardiac hypertrophy (8). SARAF was also shown to inhibit cardiac growth by activation of mammalian target of rapamycin complex 1 (55). Furthermore, SARAF-knockout mice developed more severe pancreatitis and increased SOCE in pancreatic acinar cells than in control mice, and overexpression of SARAF in acini of pancreas reduced the severity of acute pancreatitis and SOCE (56). This protective role of SARAF in acute pancreatitis is similar to our results in asthma mice models that SARAF had a protective role against airway inflammation and airway remodeling in asthma.

We showed that sera from patients with asthma induced a lower mRNA expression of SARAF in cultured hASMCs when compared with sera from healthy controls, suggesting that some serum factors in patients with asthma, e.g., IgE (57), could directly regulate the expression of SARAF in hASMCs. These serum factors, and also the protein expression of SARAF in serum-treated hASMCs, need to be examined later. This result is also consistent with what we found in mice models, which showed decreased expression of SARAF in the lungs of asthma mice when compared with that in control mice. It is necessary to directly compare the expression levels of SARAF in ASMCs from patients with asthma and healthy controls, which will add more clinical significance to the present study.

In summary, our present study suggests that SARAF may be a potential target for asthma treatment. There are a few limitations of this study. First, the change of SOCE detected with Ca^{2+} fluorescence imaging was not confirmed by the patch-clamp technique. In addition, the detailed factors in the serum from patients with asthma that attributed to the inhibition of SARAF expression in vitro cultured hASMCs need to be further explored. Finally, the functional interaction between the SARAF, STIM1, and Orai1 in ASMCs should be investigated later. To conclude, our results indicate that SARAF plays a protective role in airway inflammation and airway remodeling

and also modulates biological functions of hASMCs by inhibition of SOCE.

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Y.D.G. conceived and designed research; Y.M.W., W.J.X., L.L.X., and B.J.X. performed experiments; Y.M.W., L.L.X., J.J.Z., J.Y.L., and Y.D.G. analyzed data; W.J.X. and J.Y.L. interpreted results of experiments; Y.M.W. prepared figures; Y.M.W. drafted manuscript; M.D. and J.J.Z. edited and revised manuscript; Y.D.G. approved final version of manuscript.

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