

TRPC1 intensifies house dust mite–induced airway remodeling by facilitating epithelial-to-mesenchymal transition and STAT3/NF-κB signaling

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ABSTRACT: Airway remodeling with progressive epithelial alterations in the respiratory tract is a severe consequence of asthma. Although dysfunctional signaling transduction is attributed to airway inflammation, the exact mechanism of airway remodeling remains largely unknown. TRPC1, a member of the transient receptor potential canonical Ca²⁺ channel family, possesses versatile functions but its role in airway remodeling remains undefined. Here, we show that ablation of TRPC1 in mice alleviates airway remodeling following house dust mite (HDM) challenge with decreases in mucus production, cytokine secretion, and collagen deposition. HDM challenge induces Ca²⁺ influx via the TRPC1 channel, resulting in increased levels of signal transducer and activator of transcription 3 (STAT3) and proinflammatory cytokines. In contrast, STAT3 expression was significantly decreased in TRPC1^{−/−} mouse lungs compared with wild-type controls after HDM challenge. Mechanistically, STAT3 promotes epithelial-to-mesenchymal transition and increases mucin 5AC expression. Collectively, these findings identify TRPC1 as a modulator of HDM-induced airway remodeling *via* STAT3-mediated increase in mucus production, which provide new insight in our understanding of the molecular basis of airway remodeling, and identify novel therapeutic targets for intervention of severe chronic asthma.—Pu, Q., Zhao, Y., Sun, Y., Huang, T., Lin, P., Zhou, C., Qin, S., Singh, B. B., Wu, M. TRPC1 intensifies house dust mite–induced airway remodeling by facilitating epithelial-to-mesenchymal transition and STAT3/NF-κB signaling. *FASEB J.* 33, 000–000 (2019). www.fasebj.org

KEY WORDS: asthma • ion channel • cell signaling • transcription factors

Asthma is a common chronic airway disease with significant mortality and morbidity worldwide (1). Asthma manifests with reversible airway obstruction, recurring airway inflammation, and vascular and airway remodeling (2). Airway remodeling, first described by Hubert in 1922 (3), is characterized by the structural changes occurring in both small and large airways associated with miscellaneous diseases. These structural changes include

enlarged goblet cells and submucosal glands (4), the thickened basement membrane (5), increased smooth muscle mass (6), proliferated airway vascularity (7), impaired epithelial integrity (8), augmented subepithelial fibrosis (9), and decreased cartilage integrity (10). In addition, interactions between lung structural cells and immune cells or their products are attributed to the initiation and development of airway remodeling (11). Furthermore, epithelial-to-mesenchymal transition (EMT) has recently been implicated in pathophysiological alterations during airway remodeling *via* loss of epithelial cell-cell contact and overgrowth of mesenchymal cells (12). The molecular mechanism of EMT is complex and is not completely understood (13). Improved understanding of the critical components of airway EMT in asthma will indicate novel therapeutic targets to prevent and control airway remodeling.

The house dust mite (HDM) is an important source of allergens that can induce asthmatic diseases (14). HDM sensitivity varies, affecting anywhere from 65 to 130 million people worldwide and constituting 50% of all asthmatic disease cases (15); however, the mechanism underlying HDM-related respiratory allergies is not fully

ABBREVIATIONS: EMT, epithelial-to-mesenchymal transition; HDM, house dust mite; muc, mucin; PAS, periodic acid–Schiff; siRNA, small interfering RNA; siTRPC1, silenced transient receptor potential canonical 1; SOCC, store-operated Ca²⁺ channel; SOCE, store-operated Ca²⁺ entry; Tg, thapsigargin; STAT3, signal transducer and activator of transcription 3; TRPC1, transient receptor potential canonical 1; WT, wild type

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elucidated. Improved knowledge about HDMs, including their microhabitats and action mechanisms in causing allergic diseases (16), can facilitate development of novel and efficient approaches to controlling HDM allergy.

The transient receptor potential canonical 1 (TRPC1), a well-characterized, nonselective cation channel required for Ca^{2+} homeostasis, is associated with cell migration, cell proliferation, metabolism and fluid secretion in various organ systems (17, 18). Our previous study reported that TRPC1 is also involved in inflammatory response to bacterial infection through the TLR4/TRPC1/NF- κ B signaling circuit (19). In addition, TRPC1 appears to contribute to the regulation of EMT in cancer (20) and the expression of TRPC1 channel is associated with progression of chronic airway inflammation in asthmatic guinea pigs (21). However, the function of TRPC1 in airway remodeling induced by the physiologic allergen HDM has not been investigated.

To determine the role of TRPC1 in HDM-induced asthmatic allergy, we utilized TRPC1 knockout mice to investigate its impact on a chronic allergic model. We found that TRPC1 may be a new factor of airway remodeling *via* activation of signal transducer and activator of transcription 3 (STAT3) pathway. The latter is implicated in a variety of autoimmune diseases in response to stimulation of cytokines and growth factors (22). In TRPC1^{-/-} mice, HDM challenging mitigated the airway remodeling phenotype with significant reduction of mucus secretion, α -smooth muscle actin expression, collagen deposition, and thickening of the peribronchial smooth muscle layer. Mechanistically, TRPC1 deficiency also led to decreased STAT3, which promoted the EMT process and mucus expression in HDM challenge model.

MATERIALS AND METHODS

Reagents

Control small interfering RNA (siRNA) and TRPC1 siRNA were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). ELISA kits for IL-6, IL-1 β , TNF- α , IL-4, and IL-10 were purchased from R&D Systems (Minneapolis, MN, USA). STAT3 inhibitor WP1066 (573097) and SFK-96365 were purchased from MilliporeSigma (Burlington, MA, USA). All chemicals used were of analytical grade.

Primary cells and cell lines

MLE-12 lung type II epithelial cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained according to the manufacturer's instructions. Alveolar macrophages were isolated by bronchoalveolar lavage from mice and cultured in Roswell Park Memorial Institute 1640 Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin) incubated in a 5% CO_2 environment at 37°C (23, 24).

Mice

B6129SF2/J [wild-type (WT)] or TRPC1 (knockout) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA)

and maintained under pathogen-free conditions. Experiments were initiated when mice were 6–8 wk of age. Genotyping was performed by PCR and Western blot. All animal studies were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee.

Calcium measurements

Cells were incubated with 2 μM fura-2 (Molecular Probes, Eugene, OR, USA) for 45 min, washed twice with Ca^{2+} free SES (Standard External Solution, include: 10 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1 mM MgCl_2 , 10 mM glucose, pH 7.4) buffer. For fluorescence measurements, the fluorescence intensity of Fura-2-loaded control cells was monitored with a CCD camera-based imaging system (Compix, Lake Oswego, OR, USA) mounted on an Olympus XL70 inverted microscope equipped with an Olympus $\times 40$ (1.3 NA) objective. The images of multiple cells collected at each excitation wavelength were processed using Simple Phase-Contrast X-ray Imaging (PCI) Software (Compix, Inc., Cranberry Township, PA, USA) to provide ratios of Fura-2 fluorescence from excitation at 340 nm to that from excitation at 380 nm (F340/F380). Fluorescence traces shown represent $[\text{Ca}^{2+}]$ averaged values from ≥ 30 –40 cells and are representative of results obtained in ≥ 3 –4 individual experiments (19).

Electrophysiology

For patch clamp experiments, coverslips with cells were transferred to the recording chamber and perfused with an external Ringer's solution containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 10 mM HEPES, and 10 mM glucose at a pH of 7.4 (NaOH). All electrophysiological experiments were performed according to previous protocol (24). Briefly, whole-cell currents were recorded using an Axopatch 200B (Molecular Devices, San Jose, CA, USA). The patch pipette had resistances between 3 and 5 M Ω after filling with the standard intracellular solution containing 150 mM cesium methanesulfonate, 8 mM NaCl, 10 mM HEPES, and 10 mM EGTA at a pH of 7.2 (CsOH). Basal leaks were subtracted from the final currents and average currents are shown. The maximum peak currents were calculated at a holding potential of -80 mV. The I–V curves were made using a ramp protocol ranging from -100 to $+100$ mV and 100 ms duration was delivered at 2-s intervals, whereby current density was evaluated at various membrane potentials and plotted. All experiments were carried out in triplicate at room temperature (25).

Immunization and HDM challenge

WT and TRPC1^{-/-} mice were sensitized by intraperitoneal injection with 1 mg aluminum hydroxide and 20 μg HDM on d 0 and 14. Mice were challenged with 10 μg HDM in 30 μl PBS buffer 3 times/wk from wk 3 to 8. WT and TRPC1^{-/-} mice in the control group were also challenged with 30 μl PBS. Mice were euthanized by intraperitoneal injection of a ketamine mixture (40 mg/kg ketamine-HCl and 5 mg/kg xylazine-HCl) 24 h after the final intranasal challenge. Lung tissues were collected in liquid nitrogen or fixed in 10% neutral-buffered formalin and embedded in paraffin. All animal experiments were performed in accordance with the guidelines of the University of North Dakota Institutional Animal Care and Use Committee (26).

ELISA for detecting cytokines

Cytokines were measured by ELISA kits (eBioscience, San Diego, CA, USA). Samples of lung homogenates collected at the

indicated times after HDM challenge. The cytokine concentrations were determined according to the corresponding standards (27).

Airway inflammation and airway remodeling evaluation

Samples were centrifuged (500 g for 5 min at 4°C) and resuspended with PBS. Cells in bronchoalveolar lavage fluid were enumerated with a hemocytometer. Slides were air dried and stained by Hema 3 Stat Pack (Thermo Fisher Scientific). Differential cell counts were administered in duplicate 150 cells from each specimen. The lung tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. To evaluate the tissue histologic alterations, specimens were stained with standard hematoxylin and eosin methods. Periodic acid–Schiff (PAS) reagent was used to stain lung sections for detecting airway mucus production. Subepithelial was assessment with fibrosis Masson's trichrome staining. The tissues were assessed for general morphology and cellular infiltration. The index was calculated by multiplying by extent (with a maximum possible score of 9) and degree of cellular infiltration was scored by previously described methods (28). Masson's trichrome staining was used to detect peribronchial collagen deposition. A score ranging from 0 to 3 was applied to each observed bronchus, with ~10 areas being scored in total (29).

Western blot analysis

The samples obtained from lung homogenates and cells were lysed in T-Per Tissue Protein Extraction Reagent Buffer, separated by electrophoresis with SDS-PAGE gels and transferred to polyvinylidene fluoride transfer membranes (GE Healthcare Biosciences, Piscataway, NJ, USA). Proteins testing were performed by using primary antibodies at a concentration of 1/1000 (Santa Cruz Biotechnology) and were incubated overnight. Corresponding secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) were used for detecting the primary antibodies and detected using ECL reagents (Santa Cruz Biotechnology). Protein levels were quantified by 3 independent immunoblotting membranes (30).

Transfection of small interfering RNA or full length TRPC1

MLE-12 cells were transfected with 50 nM TRPC1 or control siRNAs or full length TRPC1 plasmid using Lipofectamine 2000 (Thermo Fisher Scientific), following manufacturer's instruction (31).

Confocal microscopy and immunofluorescent staining

Tissue sections and MLE-12 cells were incubated with matched primary antibodies respectively (Santa Cruz Biotechnology). FITC-conjugated or tetramethylrhodamine isothiocyanate-conjugated secondary antibodies were used to recognize primary antibodies. Tissue sections were viewed with a Zeiss Meta 510 confocal microscope (Carl Zeiss, Oberkochen, Germany) (26).

Quantitative RT-PCR

Total RNA of MLE-12 cells were extracted using Trizol (Thermo Fisher Scientific) according to the manufacturer's instruction. The quantitative RT-PCR used iTag Universal SYBR Green Supermix

(Bio-Rad, Hercules, CA, USA) and gene-specific primers in a CFX Connect System (Bio-Rad). (p34:5'-AACCACCTTTCCACGG-AGACT-3' (forward), 5'-ACAGGTTCCCAGACTTCCACT-3' (reverse), Mus5ac: 5'-GGACCAAGTGGTTTGACACTGAC-3' (forward), 5'-CCTCATAGTTGAGGCACATCCCAG-3' (reverse). GAPDH: 5'-TCAACGGCACAGTCAAGG-3' (forward) 5'-ACC-AGTGGATGCAGGGA-3' (reverse). The mRNAs were expressed as the fold difference to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and all these genes showed fold change compared with the indicated reference group in each individual experiment (32).

Lipid peroxidation assay

Lipid peroxidation process was measured in a colorimetric assay (Calbiochem) by directly testing its end product malondialdehyde in homogenized lung according to the manufacturer's instructions. Duplicates were done for each sample and control (33).

Myeloperoxidase assay

Samples were homogenized in 50 mM hexadecyltrimethylammonium bromide (50 mM KH₂PO₄, pH 6.0, 0.5 mM EDTA) (33). After centrifuged the samples at 12,000 rpm at 4°C, supernatants were decanted and 100 ml of reaction buffer (0.167 mg/ml O-dianisidine, 50 mM KH₂PO₄, pH 6.0, 0.0005% mM H₂O₂) were added to per 100 ml of sample. Read absorbance at 460 nm at 2-min intervals. Duplicates were done for each sample and control (34).

Flow cytometry

Cells were washed 3 times with PBS and digested with trypsin for 5 min. The cells were centrifuged at 800 rpm for 3 min to remove the trypsin and washed with PBS. The cells were then fixed with 70% ethanol and washed again with PBS. The cells were incubated with 40 µg/ml propidium iodide for 30 min and were tested cell cycle by flow cytometry (35).

Cell survival

Cells were transfected with TRPC1 siRNA or plasmid for 24 h and were then challenged with 20 µg/ml HDM for 12 h. Cell survival was tested *via* 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide treatment and values of OD₅₇₀ were used to represent relative cell survival compared with the control group (36).

Colony-forming units

MLE-12 cells were incubated in 6-well plates (250 cells/well) to observe the clone formation at d 7; most cell clones had >50 cells. Then cells were washed 3 times with PBS, fixed with methanol for 15 min, and stained with crystal violet for 15 min. After removing the dye, we dissolved the crystal violet with 100% ethanol. An absorbance of 590 was recorded (37). The experiment was performed in triplicate.

Statistical analysis

All experiments were repeated ≥3 times. Statistical analysis was performed by 1-way ANOVA with Tukey *post hoc* tests. The

results were expressed as the means $\pm$ SD and the significance level between 2 groups was defined as $P < 0.05$ (26).

RESULTS

TRPC1^{-/-} mice exhibited decreased inflammatory responses following repetitive HDM challenge

To investigate the role of TRPC1 in chronic airway inflammation, we performed initial sensitizations by intraperitoneal injection of HDM followed by repetitive intranasal instillation to induce chronic airway inflammation in age-matched WT and TRPC1^{-/-} control mice (Fig. 1A). After HDM challenges, TRPC1^{-/-} mice exhibited reduction in both total inflammatory cells and eosinophils in the lungs (Fig. 1B, C), along with a decreased inflammation index (Fig. 1D) when compared with WT mice. In addition, myeloperoxidase activity was found to be significantly decreased in the lungs of TRPC1^{-/-} mice following HDM challenges (Fig. 1E). Consistent with this observation, lipid peroxidation was reduced in TRPC1^{-/-} mice (Fig. 1F). Histologic alterations were also decreased in the lungs and airways in HDM-challenged TRPC1^{-/-} mice compared with sham-challenged controls (Fig. 1G). Moreover, compared with WT mice, inflammatory cell infiltration was also markedly decreased in the airway, around blood vessels, and along alveoli of TRPC1^{-/-} mice

(Fig. 1G). Finally, in comparison with WT mice, TRPC1^{-/-} mice showed significant airway smooth muscle thickening and angiogenesis. These findings indicate that TRPC1 may play an important role in HDM-induced airway chronic remodeling and chronic inflammation with largely reduced pathophysiological parameters including polymorphonuclear cell penetration and tissue alterations in TRPC1^{-/-} mice *vs.* WT mice.

TRPC1^{-/-} mice showed a decline in mucus secretion and peribronchial collagen deposition following repetitive HDM challenge

Airway remodeling and chronic inflammation are characterized by mucus metaplasia, wall thickening, sub-epithelial fibrosis, myocyte hyperplasia, myofibroblast hyperplasia, and hypertrophy (38). To examine how TRPC1 impacts HDM-induced airway remodeling and inflammation, we detected the levels of mucus in airway tissue and found that chronic HDM exposure caused higher mucus secretion (PAS staining in lung tissue) in WT mice than TRPC1^{-/-} mice (Fig. 2A). Peribronchial collagen deposition, another important marker of airway remodeling, was examined by Masson's trichrome staining. The results showed that chronic exposure to HDM increased peribronchial collagen deposition in WT mice compared with the TRPC1^{-/-} mice (Fig. 2A), providing

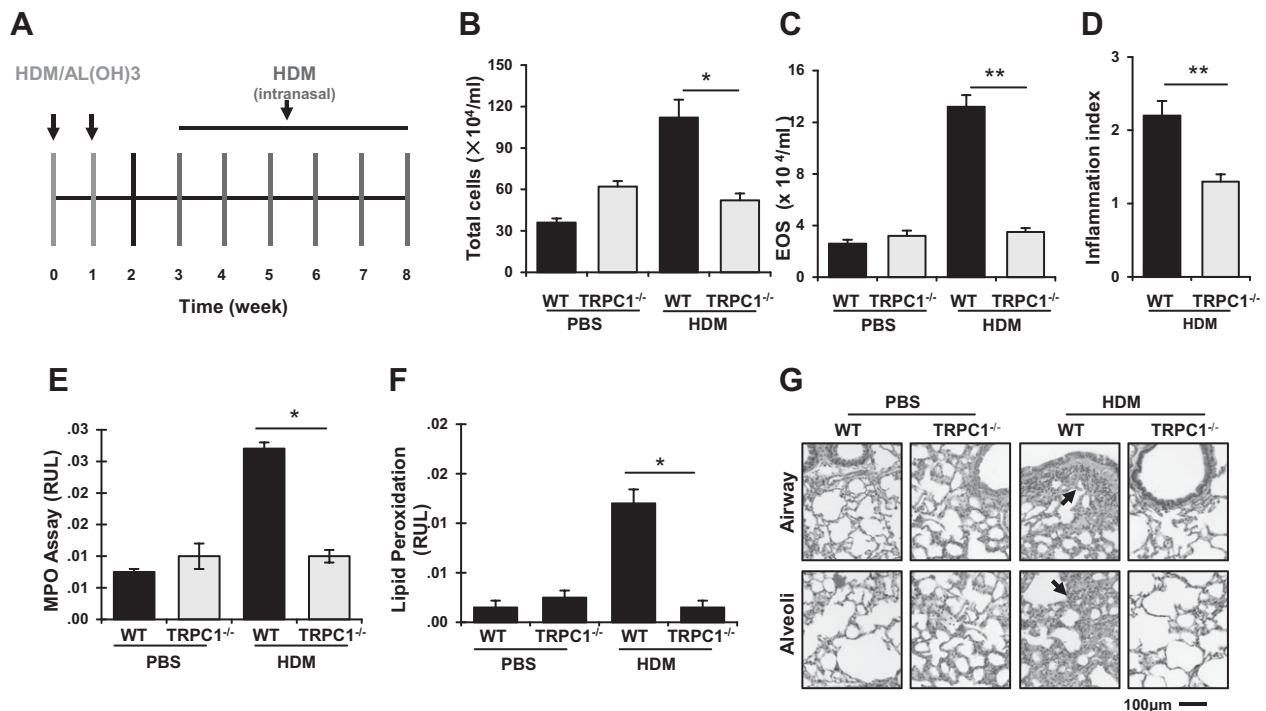


Figure 1. TRPC1^{-/-} mice exhibited reduced inflammation after chronic allergen exposure. A) Sensitization and challenge protocol for TRPC1^{-/-} and WT mice with HDM ($n = 8$ mice/group). The number of total cells (B) and eosinophils (EOS) (C) in the bronchoalveolar lavage of mice exposed to PBS or HDM were determined by differential cell analysis and plotted on a bar graph. D) Inflammatory cell infiltration was examined in the lungs of TRPC1^{-/-} and WT mice that were subject to the allergen challenge (10 random areas). E) Myeloperoxidase activity in lungs following HDM challenge in WT mice and TRPC1^{-/-} mice. F) Lipid peroxidation in TRPC1^{-/-} mouse lung compared with WT mice following HDM challenge. G) Lung tissues from WT mice and TRPC1^{-/-} mice treated with PBS (control) or allergen challenge (HDM) were stained with hematoxylin and eosin (arrows indicating typical inflammatory regions; original magnification, $\times 3200$). Data are displayed as means $\pm$ SEM and are representative of 8 mice evaluated in each group. * $P < 0.05$, ** $P < 0.01$.

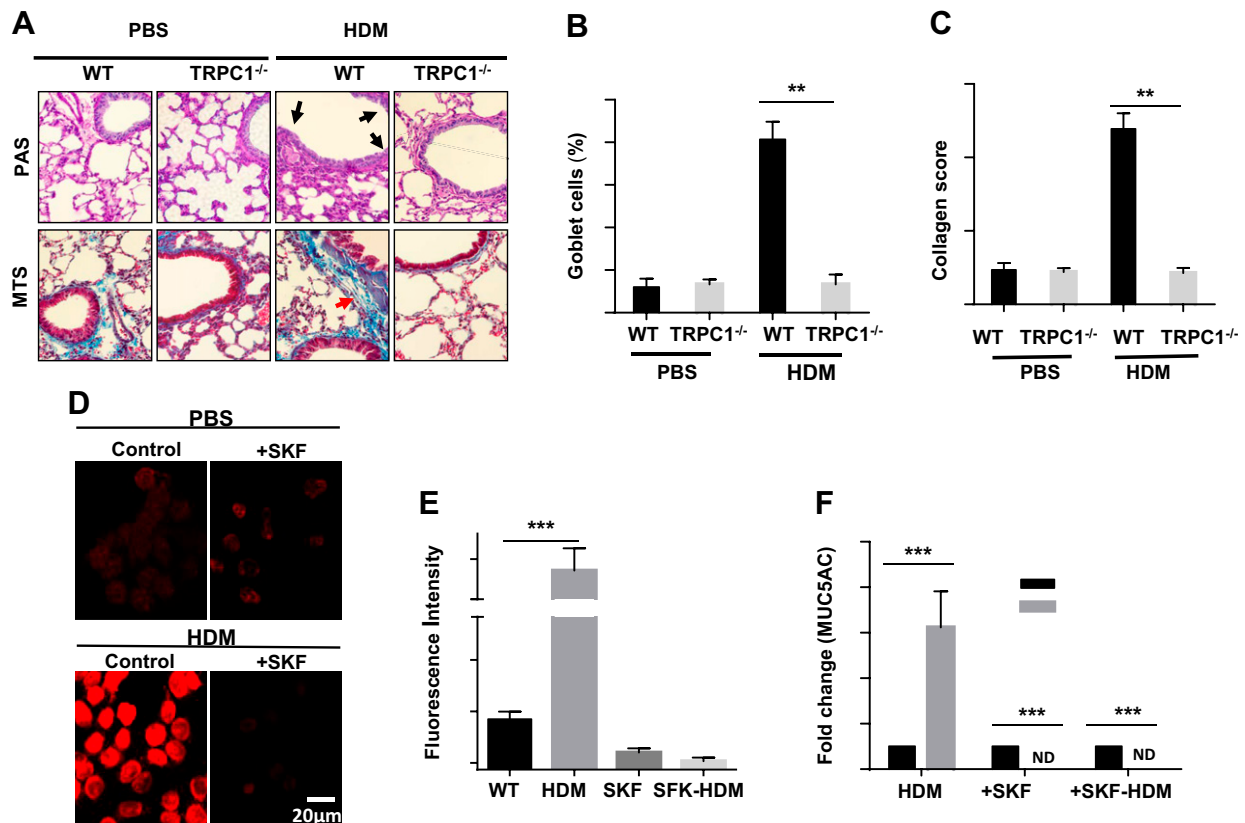


Figure 2. TRPC1^{-/-} mice showed reduced mucus and peribronchial collagen deposit after chronic allergen exposure. *A*) PAS staining of polysaccharides and mucins (from goblet cells) in the lungs of WT and TRPC1^{-/-} mice exposed to HDM or PBS (control) and Masson's trichrome staining for collagen in the lungs of WT and TRPC1^{-/-} mice. Arrows indicate typical inflammatory regions. *B*, *C*) Quantification of goblet cell percentage in 10 random airway epithelial cell fields (50 cells) and quantitation of collagen in the lungs of WT and TRPC1^{-/-} mice is shown. Original magnification, $\times 200$. *D*) Images of muc5AC immunofluorescence in control and SKF-96365-treated MLE-12 cells exposed to HDM and PBS, respectively. *E*) The fluorescence intensity per micrometer of *D*). *F*) Muc5AC mRNA expression was determined in the control or SKF-96365-treated MLE-12 cells exposed to HDM or PBS using quantitative RT-PCR; $n = 3-5$ ** $P < 0.05$, *** $P < 0.001$.

additional evidence that TRPC1 is strongly associated with airway chronic remodeling. In addition, we observed a decrease in collagen score as well as the number of goblet cells in the airway of HDM-challenged TRPC1^{-/-} mice when compared with the WT mice (Fig. 2*B*, *C*). To delve into the mechanisms of TRPC1-associated inflammation, we used a lung epithelial cell line (MLE-12) and evaluated the cell signaling pathways. Our data showed a significant increase in mucin (muc) 5AC immunostaining in the HDM-challenged MLE-12 cells, which was attenuated in cells pretreated with a nonspecific TRPC1 antagonist (-96365) (Fig. 2*D*, *E*). Consistent with these results, we also noted a decrease in muc5AC mRNA levels of MLE-12 cells when treated with SKF-96365 compared with sham control cells (Fig. 2*F*). Together, these data demonstrate that TRPC1 might be involved in airway remodeling and chronic inflammation.

HDM challenge induced Ca²⁺ influx via the TRPC1 channel

In lung epithelial cells, Ca²⁺ signaling is essential for regulating various physiologic functions including inflammation. HDM can induce barrier dysfunction and

induce severe inflammation through Ca²⁺ signaling mechanisms (39). TRPC1 is thought to mediate calcium entry in response to depletion of endoplasmic calcium stores, termed store-operated Ca²⁺ entry (SOCE), or *via* the activation of receptors coupled to the PLC system (40). To evaluate whether HDM challenge induces airway remodeling *via* TRPC1-mediated Ca²⁺ signaling, we examined Ca²⁺ entry and found that HDM treatment significantly increased the calcium influx (SOCE) induced by the addition of thapsigargin (Tg) (Fig. 3*A*, *B*). To evaluate the properties of the Ca²⁺ currents, membrane recordings were performed. Addition of Tg induced a nonselective inward current that reversed between 0 and -5 mV (Fig. 3*C*, *D*). Importantly, the current properties observed in lung epithelial cells were consistent with previous recordings performed in other cell types, which have been shown to be linked to TRPC1 channels (41-43), suggesting that TRPC1 contributes to the endogenous SOCE channel in these cells. Furthermore, the inward currents were significantly facilitated after HDM treatment (-80 mV was -6.40 ± 0.43 pA/pF in the untreated control, whereas -8.61 ± 0.38 pA/pF was observed with HDM treatment) (Fig. 3*C-E*). Although TRPC1 allows plasma membrane Ca²⁺ influx in response to endoplasmic reticulum Ca²⁺ depletion, thus far there are no reports showing that TRPC1

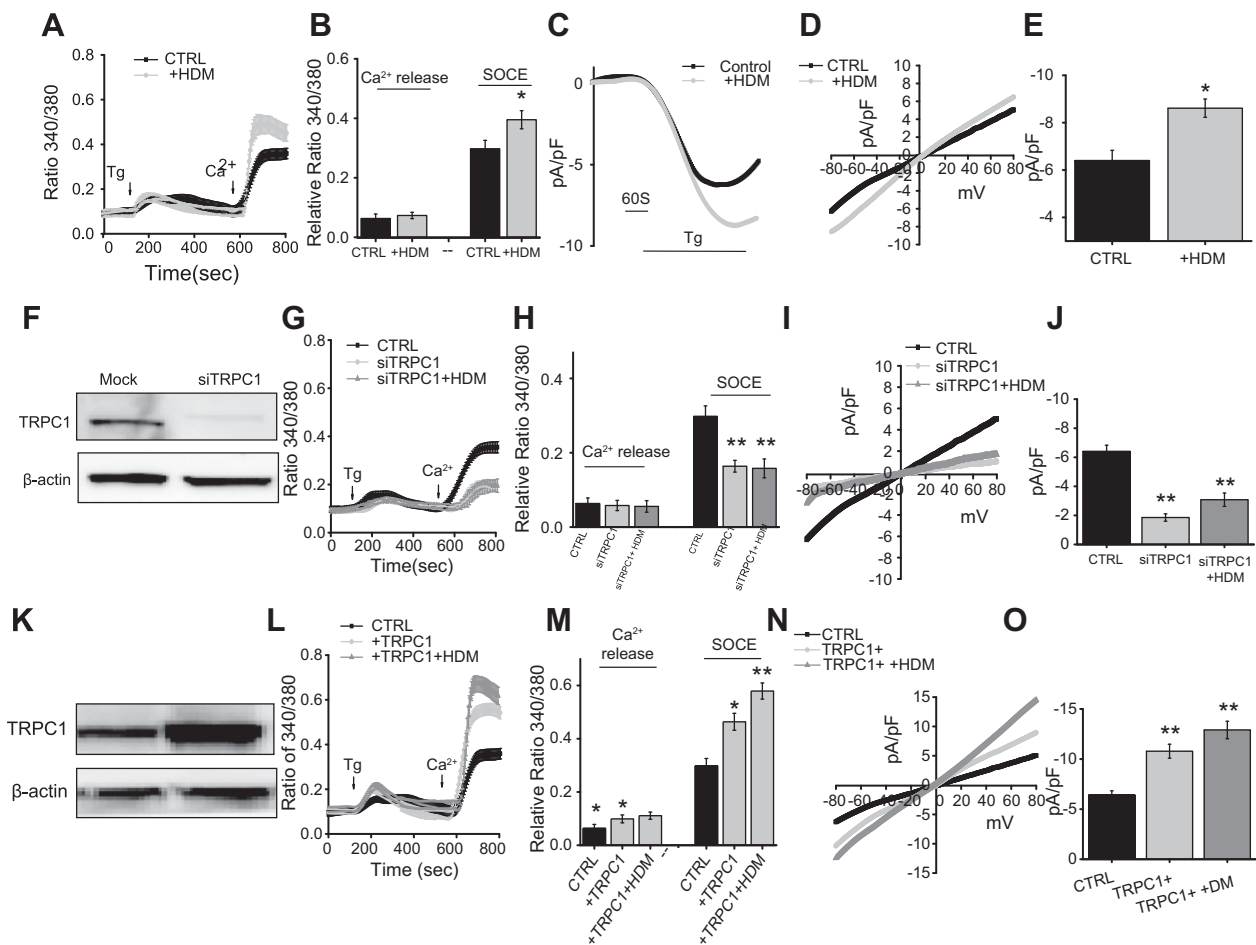


Figure 3. HDM treatment facilitated TRPC1 channel function in lung epithelial cells. **A)** Ca^{2+} imaging was performed in the presence or absence of HDM in MLE-12 cells. Analog plots of the fluorescence ratio (340/380) from an average of 40–60 cells are shown. **B)** Quantification (mean $\pm$ sd) of fluorescence ratio (340/380). **C)** Whole-cell patch recording showed that bath application of 1 μM Tg-induced a nonselective inward current in MLE-12 cells. Average I - V curves at -80 mV under these conditions are shown in **D** and current density from 8 to 10 cells are quantified in **E**. **F)** MLE-12 cell lysates in control (mock-treated) and TRPC1 siRNA-treated were resolved and analyzed by Western blot. Antibodies used are labeled; β -actin was used as a loading control. **G)** Ca^{2+} imaging was performed in MLE-12 cells in control and various conditions as labeled in the figure. Analog plots of the fluorescence ratio (340/380) from an average of 40–60 cells are shown. **H)** Quantification (mean $\pm$ sd) of fluorescence ratio (340/380) for both calcium release and calcium entry is shown as a bar graph. **I, J)** Whole-cell patch recording showed average I - V curves and current intensity ($n = 8$ –10 cells) at -80 mV under these conditions. **K)** MLE-12 cell lysates showing TRPC1 and β -actin expression in control (mock-treated) and TRPC1-overexpressing cells. **L)** Analog plots of the fluorescence ratio (340/380) from an average of 45–70 cells in each condition. **M)** Quantification (mean $\pm$ sd) of fluorescence ratio (340/380). **N, O)** Average I - V curves from whole-cell patch recording and current intensity at -80 mV under conditions as stated. $*P < 0.005$, $**P < 0.001$.

mediates SOCE in MLE-12 cells. To fill in this knowledge gap, we silenced TRPC1 (siTRPC1) using an siRNA approach. As shown in Fig. 3F, expression of TRPC1 was significantly decreased in lung epithelial cells that overexpress siTRPC1, but actin (control) was not. Importantly, Tg-induced Ca^{2+} influx was also inhibited in cells expressing siTRPC1 (Fig. 3G, H). Furthermore, there was no significant difference in Ca^{2+} influx between siTRPC1 cells and siTRPC1 treated with HDM, again suggesting that the effects observed here are caused by TRPC1. Consistent with these results, Tg-induced Ca^{2+} currents were significantly inhibited in lung epithelial cells that expressed siTRPC1 (Fig. 3I, J). Similar results were also observed with HDM treatment and no significant difference was noticed in siTRPC1 group

and siTRPC1 treated with HDM. To further establish that the effect of HDM on cells was dependent on TRPC1 expression, we transfected lung epithelial cells with a plasmid expressing full-length TRPC1 and evaluated the effect of HDM on lung epithelial cells. Western blot analysis showed increased TRPC1 expression in lung epithelial cells after transfection (Fig. 3K). Overexpression of TRPC1 increased Tg-induced Ca^{2+} influx, which was significantly increased by HDM treatment (Fig. 3L, M). In addition, the Ca^{2+} current induced by Tg was markedly increased by HDM treatment in cells overexpressing TRPC1 (Fig. 3N, O). Overall, these results demonstrate that TRPC1 is essential for SOCE-mediated Ca^{2+} entry and that ablation of TRPC1 affects endoplasmic reticulum and cytosolic Ca^{2+} homeostasis.

TRPC1 deficiency down-regulated STAT3 and NF- κ B in TRPC1^{-/-} mice and in lung epithelial cells

Our previous studies have shown that silencing TRPC1 suppresses the JNK/NF- κ B pathway during Gram-negative bacterial infection (19). However, TRPC1 function and its downstream signaling pathways have not been studied in HDM-induced allergic models. To determine the downstream signals, we evaluated presumptively relevant kinase proteins and cytokines in WT and TRPC1^{-/-} mice and in control MLE-12 cells or cells overexpressing full-length TRPC1 (ovTRPC1) or in siTRPC1 condition. Western blot analysis demonstrated a decrease in the phosphorylation of STAT3 and NF- κ B p65 in TRPC1 siRNA-transfected MLE-12 cells (Fig. 4A). In contrast, STAT3 and NF- κ B phosphorylation was increased in TRPC1 overexpressed MLE-12 cells compared with control MLE-12 cells (Fig. 4A). Importantly, STAT3 inhibition further decreased phosphorylation of STAT3 but not of NF- κ B (Fig. 4B), and stimulation with IL-6 was able to increase STAT3 phosphorylation (Fig. 4B). Consistently, loss of TRPC1 was also associated with decreased secretion of IL-6, TNF- α , IL-4, IL-10, and IL-1 β (Fig. 4C–G). Collectively, these findings illustrate that TRPC1 deficiency exhibits impaired proinflammatory

responses following HDM challenge through the NF- κ B and STAT3 pathway.

TRPC1 and STAT3 impacted EMT following HDM challenge

EMT is a significant contributor to airway wall thickening in the pathologic course of severe asthma (44), and HDM exposure promoted EMT in human bronchial epithelium (45). To elucidate the relationship between EMT and airway remodeling, we examined whether HDM exposure promoted EMT in MLE-12 cells. NF- κ B is associated with EMT and plays a crucial role in inflammatory airway diseases (46–48). Whereas autocrine STAT3 was associated with EMT in lung adenocarcinoma (49), the role of STAT3 in EMT in airway remodeling has not been established. We found that either HDM challenge or TRPC1 siRNA-transfected MLE-12 cells decreased the level of E-cadherin, a marker for EMT (Fig. 5A). Importantly, following HDM challenge, TRPC1 was colocalized with E-cadherin, and collagen type I (Fig. 5A, B). Moreover, STAT3 inhibitor abolished the effect of HDM challenge on E-cadherin expression in MLE-12 cells (Fig. 5C). These results confirmed the critical role of STAT3 in inducing EMT and inflammatory response. To explore the upstream regulator of STAT3, we examined the

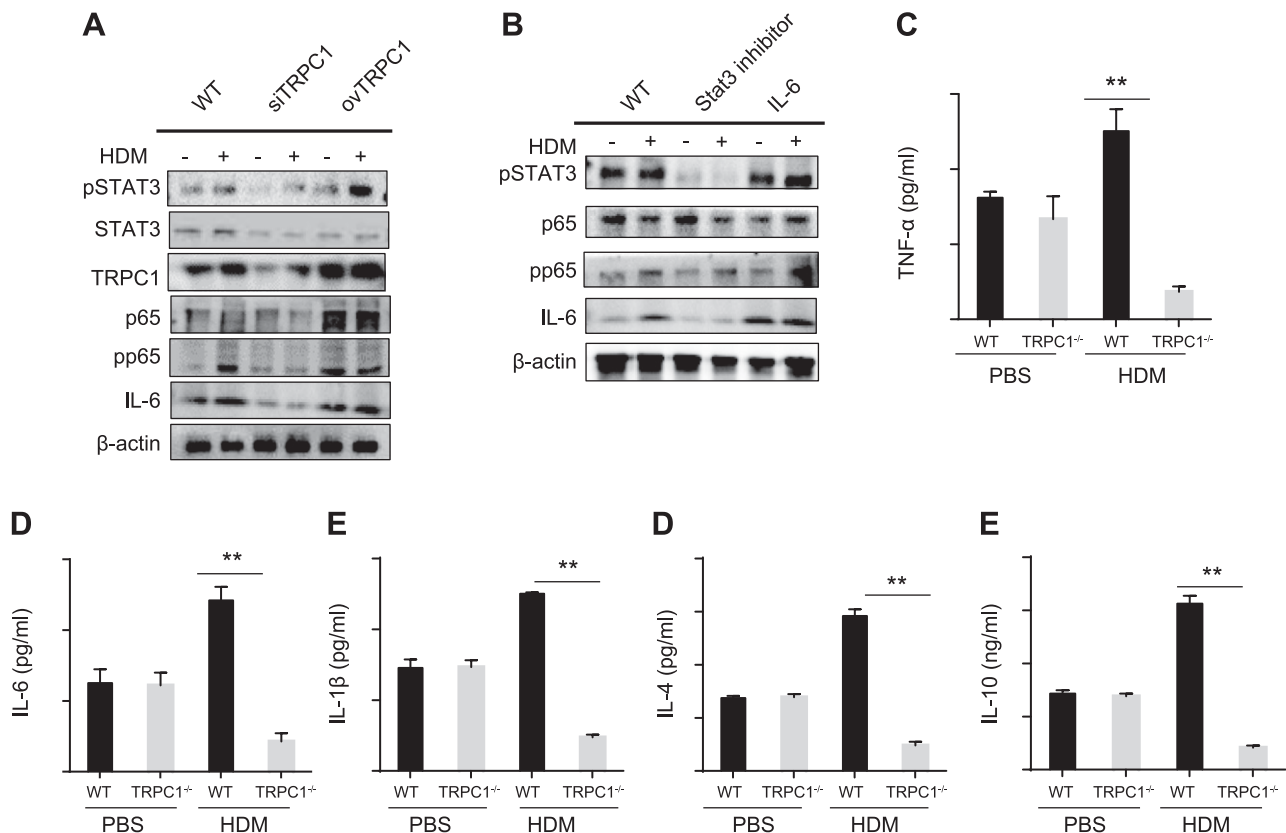


Figure 4. TRPC1 silencing suppressed the STAT3/NF- κ B pathway following HDM challenge. A) Western blot analysis of total and phosphorylation of STAT3, NF- κ B, and STAT3 in control (WT) MLE-12 cells, siTRPC1 cells, or overexpressing full length TRPC1 (ovTRPC1) that were exposed to HDM or PBS. B) Western blot analysis of NF- κ B and STAT3 in MLE12 cells (WT), cells treated with STAT3 inhibitor III (WP1066), and IL-6-treated cells that were exposed to HDM or PBS. C–G) Cytokines as labeled in the lung tissues were measured using ELISA (from 4 independent experiments performed in duplicate). ** $P < 0.01$.

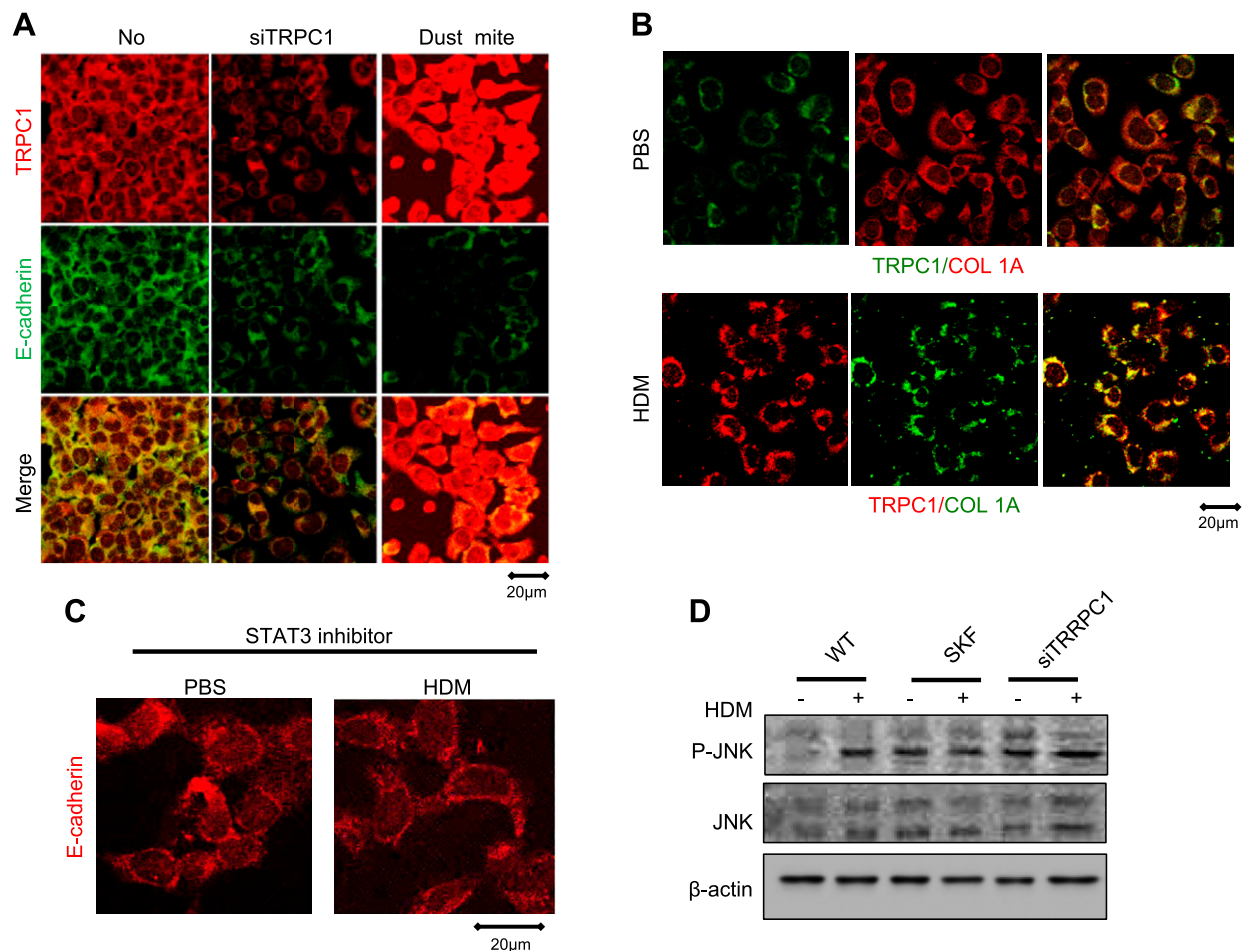


Figure 5. TRPC1 and STAT3 were critical for EMT in epithelial cells. *A*) Immunofluorescence of E-cadherin and TRPC1 in the WT (mock-treated; No) TRPC1 siRNA-transfected cells exposed to HDM and WT cells exposed to HDM. *B*) Immunofluorescence of collagen type 1 (COL 1A) and TRPC1 in the MLE-12 cells exposed to PBS or HDM. *C*) MLE-12 cells were treated with STAT3 inhibitor III (WP1066) and then treated with 20 mg/ml HDM or PBS. Fluorescence intensity was detected by confocal imaging. *D*) Western blot analysis of total and phosphorylation of JNK proteins in MLE-12 cells after exposed to HDM (+) or PBS (–) in control (WT) or SKF (+ SKF 96365), or siTRPC1 cells.

phosphorylation of JNK, which was found to be increased after HDM challenge (Fig. 5D) and could be blocked by the addition of TRPC1 blocker (SKF 96365) or in siTRPC1 cells. These data imply that JNK was also involved in HDM-induced airway remodeling.

TRPC1 impacted cell cycle during HDM-induced EMT in airway epithelial cells

To further dissect the role of TRPC1 in regulating EMT and cell homeostasis during HDM challenge, we explored epithelial cell survival and colony formation ability. Our results showed that decreasing TRPC1 expression and HDM challenge had no impact on cell survival (Fig. 6A). In contrast, blocking TRPC1 function (adding SKF) significantly inhibited their colony-forming ability (Fig. 6B). Interestingly, TRPC1 overexpression influenced both cell survival and the colony formation (Fig. 6A, B). To further probe how TRPC1 alters cell growth during HDM challenge, we evaluated whether TRPC1 can alter cell cycle, and found that there was a significant difference in the S-phase of cell cycle progression upon TRPC1 blocking (Fig. 6C). Importantly, p34, which is

activated at the start of the S phase, was also decreased under these conditions (Fig. 6D, E). These results argue that the cell cycle was arrested at the S phase when TRPC1 was inhibited and the G₂ and M phases were accelerated when TRPC1 was overexpressed in the presence of HDM. Taken together, these data indicate that TRPC1 may impact the cell cycle and EMT following HDM challenge through activation of the STAT3/NF- κ B pathway (Fig. 6F).

DISCUSSION

The etiology and progression mechanisms of asthma are complex and are not fully understood. HDM-induced airway inflammation influences both innate and adaptive immunity, making it very difficult to totally reveal the mechanisms, despite intense research efforts. Previous studies have identified that a rise in cytosolic Ca²⁺ concentration serve as a shared signal transduction element that causes bronchial wall thickening and bronchial constriction in asthma (50). TRPC1, mediate Ca²⁺ entry in response to depletion of endoplasmic Ca²⁺ stores (40), thus has the potential function in asthma. In addition to

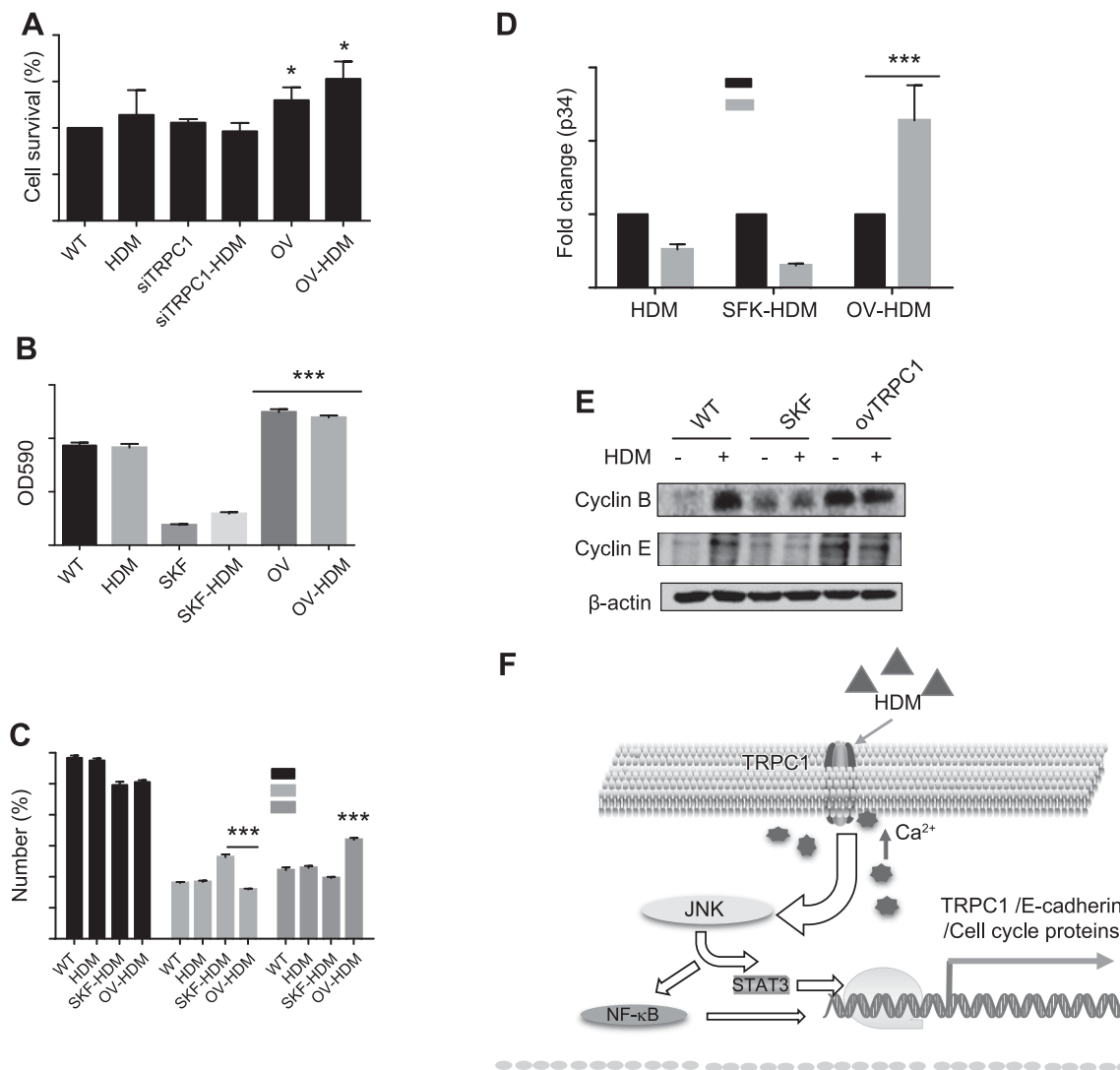


Figure 6. TRPC1 and HDM altered the cell cycle of airway epithelial cells. *A, B*) Cell survival and colony-forming units were determined in inhibitor-treated (SKF) or TRPC1-overexpressing MLE-12 cells. *C*) Flow cytometry analysis of cell cycle progression of MLE-12 cells using propidium iodide stain. *D, E*) Quantitative RT-PCR and Western blot analysis of cell cycle-associated genes at the mRNA and protein levels. *F*) Schematic of the underlying pathway revealed in the study. * $P < 0.05$, *** $P < 0.001$.

bronchial wall thickening and bronchial constriction, airway remodeling is also an important marker of asthma (51), but whether this is associated with Ca^{2+} or TRPC1 has not been demonstrated. In this study, we showed that HDM challenge facilitated the activation of Ca^{2+} influx *via* the TRPC1 channel, exhibiting an apparent phenotype related to TRPC1. Furthermore, TRPC1^{-/-} mice show decreased inflammation and mucus secretion following repetitive HDM challenge. Although mucus produced by goblet cells serves to protect epithelial cells in the respiratory tract (52) by catching small particles such as dust, allergens, and infectious agents, in asthma conditions they are oversecreted and cause harm to patients by plugging their airways.

TRPC1 plays an important role in Ca^{2+} entry, which belongs to the group of store-operated Ca^{2+} channels (SOCCs) and is the main Ca^{2+} influx pathway for non-excitable cells. For non- Ca^{2+} -selective SOCCs, TRPC1

together with the molecules STIM1 (stromal interaction molecule 1) and Orai1 (ORAI calcium release-activated calcium modulator 1) form a macromolecular complex that constitutes the basic structure of the SOCCs. SOCCs could control the muscle contractility and cellular inflammatory factors (chemokines) and regulates cell growth and airway pathology (53). Importantly, when TRPC1 was knocked out, the airway phenotype, including mucus secretion, was altered. The decrease in mucus indicated that the airway remodeling was mitigated after the loss of TRPC1 expression, and blocking TRPC1 function also decreased muc5AC expression. This is consistent with down-regulated inflammation pathways, especially the STAT3/NF- κ B signaling pathway, along with decreased proinflammatory cytokines including IL-4, IL-1 β , and TNF- α (Fig. 4). STAT3 is important for the differentiation of immune cells, such as Th17 helper cells, that are involved in a variety of autoimmune

diseases (22). During infection, mice lacking STAT3 showed more severe inflammatory response (54). Over the years, the role of STAT3 in airway inflammation is becoming quite conspicuous since Simeone *et al.* (39) reported that STAT3 was necessary for allergic inflammation in HDM-treated mice. Similarly, subsequent studies also showed that phospho-STAT3 was up-regulated in HDM-induced airway inflammation (55–57). These lines of evidence provide strong support for the regulation of STAT3 *via* TRPC1 activation in murine asthma models. In addition, the Janus kinase 2 (JAK2)/STAT3 signaling pathway also function in cell growth by directly or indirectly regulating cell cycle.

Importantly, TRPC1 silencing led to the down-regulation of E-cadherin, which promotes EMT. It has been confirmed that airway remodeling is involved differentiation of airway epithelial cells into myofibroblasts through EMT to intensify the level of subepithelial fibrosis (58). Our results indicate that TRPC1 was involved in EMT, thus revealing a novel mechanism by which TRPC1 facilitates HDM-induced airway remodeling and inflammation through elevation of the STAT3/NF- κ B pathway and through regulation of the cell cycle. Although TRPC1 was reported to arrest cell cycle in both cancer cells and bone marrow stromal cells (59–61), this is seldom reported in HDM-induced asthma. Our observations provide a new perspective on how the cell cycle is altered during HDM challenge. Overall, our data demonstrate a typical phenotype of HDM challenge in TRPC1^{-/-} mice and suggest an important role for TRPC1 in airway remodeling. TRPC1 elevates the STAT3 pathway, leading to the dysregulated proinflammatory response during HDM challenge and EMT. Either inhibiting or regulating TRPC1 expression influences the dysregulated proinflammatory response by different but overlapping pathways. HDM increases the activity of TRPC1, providing new insight into the role of TRPC1 in the airway remodeling following HDM challenge and indicating novel drug targets for treating clinical asthma.

Although only a small proportion of patients experience severe asthma, it is the principal element of asthma-related morbidity (62). Targeted treatments for its phenotypes indicate major advancement in the therapy of severe asthma. Monoclonal antibody treatment with the eosinophilic asthma phenotype seems to benefit patients (62), and new therapeutic procedures such as bronchial thermoplasty, which reduces airway smooth muscle mass in patients with severe uncontrolled asthma, have been developed (62). A targeted approach will provide a major advancement in better understanding of the mechanism of asthma and help develop more effective treatment strategies. As the study progressed, many functions of TRPC1, including intrauterine immunoregulation, were discovered, such as stem cell proliferation (63), neuron distribution (64), and lipid raft integrity (65). Thus, the potential of TRPC1 as an asthma therapy target is increasing. However, the multiple roles of TRPC1 and calcium signaling raised additional questions: What is the identity of the SOCE channel in different cell types (lung cells, smooth muscle cells, immune cells, *etc.*) that are influenced during asthma? Does TRPC1 form multimeric channels with other TRPCs and

Orais? How does TRPC1 interact with other molecules that influence calcium entry (including STIM1, STIM2, SARAF, SIGMA, and others)? Does TRPC1 function change in response to different pathogens or allergens? Does it have a universal effect or is it specific to HDM treatment? Hence, further studies on TRPC1 and its related signaling mechanisms are necessary to identify novel therapeutic targets for treating asthma and chronic obstructive pulmonary disease. FJ

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AUTHOR CONTRIBUTIONS

Q. Pu, Y. Zhao, and M. Wu designed research; Q. Pu, T. Huang, P. Lin, and C. Zhou contributed to analyze data; Q. Pu, B. B. Singh, and M. Wu wrote the paper; and Q. Pu, Y. Zhao, Y. Sun, and S. Qin performed research.

REFERENCES

1. Makino, S., and Sagara, H. (2010) Evolution of asthma concept and effect of current asthma management guidelines. *Allergy Asthma Immunol. Res.* **2**, 172–176
2. Su, X., Ren, Y., Yu, N., Kong, L., and Kang, J. (2016) Thymoquinone inhibits inflammation, neoangiogenesis and vascular remodeling in asthma mice. *Int. Immunopharmacol.* **38**, 70–80
3. Redington, A. E., and Howarth, P. H. (1997) Airway wall remodelling in asthma. *Thorax* **52**, 310–312
4. Aikawa, T., Shimura, S., Sasaki, H., Ebina, M., and Takishima, T. (1992) Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* **101**, 916–921
5. Roche, W. R., Beasley, R., Williams, J. H., and Holgate, S. T. (1989) Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* **1**, 520–524
6. Carroll, N., Lehmann, E., Barret, J., Morton, A., Cooke, C., and James, A. (1996) Variability of airway structure and inflammation in normal subjects and in cases of nonfatal and fatal asthma. *Pathol. Res. Pract.* **192**, 238–248
7. Tanaka, H., Yamada, G., Saikai, T., Hashimoto, M., Tanaka, S., Suzuki, K., Fujii, M., Takahashi, H., and Abe, S. (2003) Increased airway vascularity in newly diagnosed asthma using a high-magnification bronchovideoscope. *Am. J. Respir. Crit. Care Med.* **168**, 1495–1499
8. Bergeron, C., Tulic, M. K., and Hamid, Q. (2010) Airway remodelling in asthma: from benchside to clinical practice. *Can. Respir. J.* **17**, e85–e93
9. Frank, P. I., Frank, T. L., Cropper, J., Hirsch, S., Niven, R. M., Hannaford, P., and McNamee, R. (2001) The use of a screening questionnaire to identify children with likely asthma. *Br. J. Gen. Pract.* **51**, 117–120
10. Yilmaz, O., and Yuksel, H. (2016) Where does current and future pediatric asthma treatment stand? Remodeling and inflammation: bird's eye view. *Pediatr. Pulmonol.* **51**, 1422–1429

11. Gregory, L. G., Mathie, S. A., Walker, S. A., Pegorier, S., Jones, C. P., and Lloyd, C. M. (2010) Overexpression of Smad2 drives house dust mite-mediated airway remodeling and airway hyperresponsiveness via actin and IL-25. *Am. J. Respir. Crit. Care Med.* **182**, 143–154
12. Sohal, S. S., Ward, C., and Walters, E. H. (2014) Importance of epithelial mesenchymal transition (EMT) in COPD and asthma. *Thorax* **69**, 768
13. Zeisberg, M., and Neilson, E. G. (2009) Biomarkers for epithelial–mesenchymal transitions. *J. Clin. Invest.* **119**, 1429–1437
14. Saver, B. G., and Doescher, M. P. (2003) Managing asthma should not focus on dust mite control. *Am. Fam. Physician* **67**, 465–466
15. Calderón, M. A., Linneberg, A., Kleine-Tebbe, J., De Blay, F., Hernandez Fernandez de Rojas, D., Virchow, J. C., and Demoly, P. (2015) Respiratory allergy caused by house dust mites: what do we really know? *J. Allergy Clin. Immunol.* **136**, 38–48
16. Biagtan, M., Viswanathan, R., and Bush, R. K. (2014) Immunotherapy for house dust mite sensitivity: where are the knowledge gaps? *Curr. Allergy Asthma Rep.* **14**, 482
17. Pani, B., Bollimuntha, S., and Singh, B. B. (2012) The TR_(i)P to Ca²⁺ signaling just got STIMY: an update on STIM1 activated TRPC channels. *Front. Biosci.* **17**, 805–823
18. Kuang, C. Y., Yu, Y., Wang, K., Qian, D. H., Den, M. Y., and Huang, L. (2012) Knockdown of transient receptor potential canonical-1 reduces the proliferation and migration of endothelial progenitor cells. *Stem Cells Dev.* **21**, 487–496
19. Zhou, X., Ye, Y., Sun, Y., Li, X., Wang, W., Privratsky, B., Tan, S., Zhou, Z., Huang, C., Wei, Y. Q., Birnbaumer, L., Singh, B. B., and Wu, M. (2015) Transient receptor potential channel 1 deficiency impairs host defense and proinflammatory responses to bacterial infection by regulating protein kinase C α signaling. *Mol. Cell. Biol.* **35**, 2729–2739
20. Stewart, T. A., Azimi, I., Thompson, E. W., Roberts-Thomson, S. J., and Monteith, G. R. (2015) A role for calcium in the regulation of ATP-binding cassette, sub-family C, member 3 (ABCC3) gene expression in a model of epidermal growth factor-mediated breast cancer epithelial–mesenchymal transition. *Biochem. Biophys. Res. Commun.* **458**, 509–514
21. Li, N., He, Y., and Li, M. C. (2015) Role of transient receptor potential canonical 1 in airway remodeling and effect of budesonide on its pulmonary expression in asthmatic guinea pigs [in Chinese]. *Nan Fang Yi Ke Da Xue Xue Bao* **35**, 1374–1379
22. Yang, X. O., Panopoulos, A. D., Nurieva, R., Chang, S. H., Wang, D., Watowich, S. S., and Dong, C. (2007) STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J. Biol. Chem.* **282**, 9358–9363
23. Zhang, D., Wu, M., Nelson, D. E., Pasula, R., and Martin, W. J. II. (2003) Alpha-1-antitrypsin expression in the lung is increased by airway delivery of gene-transfected macrophages. *Gene Ther.* **10**, 2148–2152
24. Kannan, S., and Wu, M. (2006) Respiratory stem cells and progenitors: overview, derivation, differentiation, carcinogenesis, regeneration and therapeutic application. *Curr. Stem Cell Res. Ther.* **1**, 37–46
25. Sun, Y., Selvaraj, S., Varma, A., Derry, S., Sahmoun, A. E., and Singh, B. B. (2013) Increase in serum Ca²⁺/Mg²⁺ ratio promotes proliferation of prostate cancer cells by activating TRPM7 channels. *J. Biol. Chem.* **288**, 255–263
26. Li, G., Fox, J. III, Liu, Z., Liu, J., Gao, G. F., Jin, Y., Gao, H., and Wu, M. (2013) Lyn mitigates mouse airway remodeling by downregulating the TGF- β 3 isoform in house dust mite models. *J. Immunol.* **191**, 5359–5370
27. Li, G. P., Liu, Z. G., Qiu, J., Ran, P. X., and Zhong, N. S. (2005) DNA vaccine encoding Der p 2 allergen generates immunologic protection in recombinant Der p 2 allergen-induced allergic airway inflammation mice model. *Chin. Med. J. (Engl.)* **118**, 534–540
28. Suzaki, Y., Hamada, K., Nomi, T., Ito, T., Sho, M., Kai, Y., Nakajima, Y., and Kimura, H. (2008) A small-molecule compound targeting CCR5 and CXCR3 prevents airway hyperresponsiveness and inflammation. *Eur. Respir. J.* **31**, 783–789
29. Di Valentín, E., Crahay, C., Garbacki, N., Hennuy, B., Guéders, M., Noël, A., Foidart, J. M., Grooten, J., Colige, A., Piette, J., and Cataldo, D. (2009) New asthma biomarkers: lessons from murine models of acute and chronic asthma. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **296**, L185–L197
30. Li, X., He, S., Zhou, X., Ye, Y., Tan, S., Zhang, S., Li, R., Yu, M., Jundt, M. C., Hidebrand, A., Wang, Y., Li, G., Huang, C., and Wu, M. (2016) Lyn delivers bacteria to lysosomes for eradication through TLR2-initiated autophagy related phagocytosis. *PLoS Pathog.* **12**, e1005363
31. Li, R., Fang, L., Tan, S., Yu, M., Li, X., He, S., Wei, Y., Li, G., Jiang, J., and Wu, M. (2016) Type I CRISPR-Cas targets endogenous genes and regulates virulence to evade mammalian host immunity. *Cell Res.* **26**, 1273–1287
32. Yin, X. M., Wu, L. J., Zheng, L., Mao, X. R., Zhang, S. M., and Wang, Q. (2016) Quantification of colony-forming units for *M. tuberculosis* complex using *gyrB*-based real-time PCR assay. *Int. J. Tuberc. Lung Dis.* **20**, 967–972
33. Yuan, K., Huang, C., Fox, J., Gaid, M., Weaver, A., Li, G., Singh, B. B., Gao, H., and Wu, M. (2011) Elevated inflammatory response in caveolin-1-deficient mice with *Pseudomonas aeruginosa* infection is mediated by STAT3 protein and nuclear factor κ B (NF- κ B). *J. Biol. Chem.* **286**, 21814–21825
34. Wu, M., Audet, A., Cusic, J., Seeger, D., Cochran, R., and Ghribi, O. (2009) Broad DNA repair responses in neural injury are associated with activation of the IL-6 pathway in cholesterol-fed rabbits. *J. Neurochem.* **111**, 1011–1021
35. He, S., Li, X., Li, R., Fang, L., Sun, L., Wang, Y., and Wu, M. (2016) Annexin A2 modulates ROS and impacts inflammatory response via IL-17 signaling in polymicrobial sepsis mice. *PLoS Pathog.* **12**, e1005743
36. Guo, Q., Shen, N., Yuan, K., Li, J., Wu, H., Zeng, Y., Fox III, J., Bansal, A. K., Singh, B. B., Gao, H., and Wu, M. (2012) Caveolin-1 plays a critical role in host immunity against *Klebsiella pneumoniae* infection by regulating STAT5 and Akt activity. *Eur. J. Immunol.* **42**, 1500–1511
37. He, Y. H., Wu, M., Kobune, M., Xu, Y., Kelley, M. R., and Martin II, W. J. (2001) Expression of yeast apurinic/aprimidinic endonuclease (APN1) protects lung epithelial cells from bleomycin toxicity. *Am. J. Respir. Cell Mol. Biol.* **25**, 692–698
38. Elias, J. A., Zhu, Z., Chupp, G., and Homer, R. J. (1999) Airway remodeling in asthma. *J. Clin. Invest.* **104**, 1001–1006
39. Post, S., Nawijn, M. C., Jonker, M. R., Kliphuis, N., van den Berge, M., van Oosterhout, A. J., and Heijink, I. H. (2013) House dust mite-induced calcium signaling instigates epithelial barrier dysfunction and CCL20 production. *Allergy* **68**, 1117–1125
40. Skopin, A., Shalygin, A., Vigot, V., Zimina, O., Glushankova, L., Mozhayeva, G. N., and Kaznacheyeva, E. (2013) TRPC1 protein forms only one type of native store-operated channels in HEK293 cells. *Biochimie* **95**, 347–353
41. Liu, X., Groschner, K., and Ambudkar, I. S. (2004) Distinct Ca²⁺-permeable cation currents are activated by internal Ca²⁺-store depletion in RBL-2H3 cells and human salivary gland cells, HSG and HSY. *J. Membr. Biol.* **200**, 93–104
42. Pani, B., Ong, H. L., Liu, X., Rauser, K., Ambudkar, I. S., and Singh, B. B. (2008) Lipid rafts determine clustering of STIM1 in endoplasmic reticulum-plasma membrane junctions and regulation of store-operated Ca²⁺ entry (SOCE). *J. Biol. Chem.* **283**, 17333–17340
43. Selvaraj, S., Sun, Y., Watt, J. A., Wang, S., Lei, S., Birnbaumer, L., and Singh, B. B. (2012) Neurotoxin-induced ER stress in mouse dopaminergic neurons involves downregulation of TRPC1 and inhibition of AKT/mTOR signaling. *J. Clin. Invest.* **122**, 1354–1367
44. Johnson, J. R., Roos, A., Berg, T., Nord, M., and Fuxe, J. (2011) Chronic respiratory aeroallergen exposure in mice induces epithelial–mesenchymal transition in the large airways. *PLoS One* **6**, e16175
45. Heijink, I. H., Postma, D. S., Noordhoek, J. A., Broekema, M., and Kapus, A. (2010) House dust mite-promoted epithelial-to-mesenchymal transition in human bronchial epithelium. *Am. J. Respir. Cell Mol. Biol.* **42**, 69–79
46. Kalita, M., Tian, B., Gao, B., Choudhary, S., Wood, T. G., Carmical, J. R., Boldogh, I., Mitra, S., Minna, J. D., and Brasier, A. R. (2013) Systems approaches to modeling chronic mucosal inflammation. *BioMed Res. Int.* **2013**, 505864
47. Itoigawa, Y., Harada, N., Harada, S., Katsura, Y., Makino, F., Ito, J., Nurwidya, F., Kato, M., Takahashi, F., Atsuta, R., and Takahashi, K. (2015) TWEAK enhances TGF- β -induced epithelial–mesenchymal transition in human bronchial epithelial cells. *Respir. Res.* **16**, 48
48. Hung, J. Y., Chiang, S. R., Tsai, M. J., Tsai, Y. M., Chong, I. W., Shieh, J. M., and Hsu, Y. L. (2015) LIGHT is a crucial mediator of airway remodeling. *J. Cell. Physiol.* **230**, 1042–1053
49. Huang, Q., Han, J., Fan, J., Duan, L., Guo, M., Lv, Z., Hu, G., Chen, L., Wu, F., Tao, X., Xu, J., and Jin, Y. (2016) IL-17 induces EMT via Stat3 in lung adenocarcinoma. *Am. J. Cancer Res.* **6**, 440–451
50. Sweeney, M., McDaniel, S. S., Platoshyn, O., Zhang, S., Yu, Y., Lapp, B. R., Zhao, Y., Thistlethwaite, P. A., and Yuan, J. X. (2002) Role of capacitative Ca²⁺ entry in bronchial contraction and remodeling. *J. Appl. Physiol.* **92**, 1594–1602

51. Yoon, S. Y., Hong, G. H., Kwon, H. S., Park, S., Park, S. Y., Shin, B., Kim, T. B., Moon, H. B., and Cho, Y. S. (2016) S-adenosylmethionine reduces airway inflammation and fibrosis in a murine model of chronic severe asthma via suppression of oxidative stress. *Exp. Mol. Med.* **48**, e236
52. Singh, P. K., Parsek, M. R., Greenberg, E. P., and Welsh, M. J. (2002) A component of innate immunity prevents bacterial biofilm development. *Nature* **417**, 552–555
53. DeHaven, W. L., Jones, B. F., Petranka, J. G., Smyth, J. T., Tomita, T., Bird, G. S., and Putney, J. W., Jr. (2009) TRPC channels function independently of STIM1 and Orai1. *J. Physiol.* **587**, 2275–2298
54. Zhang, C., Li, Y., Wu, Y., Wang, L., Wang, X., and Du, J. (2013) Interleukin-6/signal transducer and activator of transcription 3 (STAT3) pathway is essential for macrophage infiltration and myoblast proliferation during muscle regeneration. *J. Biol. Chem.* **288**, 1489–1499
55. Simeone-Penney, M. C., Severgnini, M., Tu, P., Homer, R. J., Mariani, T. J., Cohn, L., and Simon, A. R. (2007) Airway epithelial STAT3 is required for allergic inflammation in a murine model of asthma. *J. Immunol.* **178**, 6191–6199
56. Ito, T., Hirose, K., Saku, A., Kono, K., Takatori, H., Tamachi, T., Goto, Y., Renauld, J. C., Kiyono, H., and Nakajima, H. (2017) IL-22 induces Reg3 γ and inhibits allergic inflammation in house dust mite-induced asthma models. *J. Exp. Med.* **214**, 3037–3050
57. Gavino, A. C., Nahmod, K., Bharadwaj, U., Makedonas, G., and Twardy, D. J. (2016) STAT3 inhibition prevents lung inflammation, remodeling, and accumulation of Th2 and Th17 cells in a murine asthma model. *Allergy* **71**, 1684–1692
58. Fischer, K. D., Hall, S. C., and Agrawal, D. K. (2016) Vitamin D supplementation reduces induction of epithelial–mesenchymal transition in allergen sensitized and challenged mice. *PLoS One* **11**, e0149180
59. Weber, L. V., Al-Refae, K., Wölk, G., Bonatz, G., Altmüller, J., Becker, C., Gisselmann, G., and Hatt, H. (2016) Expression and functionality of TRPV1 in breast cancer cells. *Breast Cancer (Dove Med. Press)* **8**, 243–252
60. Lepannetier, S., Zanou, N., Yerna, X., Emeriau, N., Dufour, I., Masquelier, J., Muccioli, G., Tajeddine, N., and Gailly, P. (2016) Sphingosine-1-phosphate-activated TRPC1 channel controls chemotaxis of glioblastoma cells. *Cell Calcium* **60**, 373–383
61. Ichikawa, J., and Inoue, R. (2014) TRPC6 regulates cell cycle progression by modulating membrane potential in bone marrow stromal cells. *Br. J. Pharmacol.* **171**, 5280–5294
62. Trivedi, A., Pavord, I. D., and Castro, M. (2016) Bronchial thermoplasty and biological therapy as targeted treatments for severe uncontrolled asthma. *Lancet Respir. Med.* **4**, 585–592
63. Fiorio Pla, A., Maric, D., Brazer, S. C., Giacobini, P., Liu, X., Chang, Y. H., Ambudkar, I. S., and Barker, J. L. (2005) Canonical transient receptor potential 1 plays a role in basic fibroblast growth factor (bFGF)/FGF receptor-1-induced Ca²⁺ entry and embryonic rat neural stem cell proliferation. *J. Neurosci.* **25**, 2687–2701
64. Wu, X., Zagranichnaya, T. K., Gurda, G. T., Eves, E. M., and Villereal, M. L. (2004) A TRPC1/TRPC3-mediated increase in store-operated calcium entry is required for differentiation of H19-7 hippocampal neuronal cells. *J. Biol. Chem.* **279**, 43392–43402
65. Kunzelmann-Marche, C., Freyssinet, J. M., and Martínez, M. C. (2002) Loss of plasma membrane phospholipid asymmetry requires raft integrity. Role of transient receptor potential channels and ERK pathway. *J. Biol. Chem.* **277**, 19876–19881

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