

The effects of transient receptor potential channel (TRPC) on airway smooth muscle cell isolated from asthma model mice

Xiaoyu Zhang | Zhixin Zhao | Lijun Ma | Yali Guo | Xiaosu Li |
Limin Zhao | Cuijie Tian | Xueyi Tang | Dongjun Cheng | Zhuochang Chen |
Luoxian Zhang 

Department of Respiratory Medicine,
People's Hospital Affiliated to Zhengzhou
University, Zhengzhou, China

Correspondence

Luoxian Zhang, PhD and Zhuochang Chen,
PhD, Department of Respiratory Medicine,
People's Hospital Affiliated to Zhengzhou
University, No. 7 Weiwu Road, Zhengzhou
450003, China.

Email: xianxian_zlx@163.com (L.Z);
phd_chen@sohu.com (Z.C).

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Abstract

This study aimed to validate whether transient receptor potential channel1 (TRPC1) and TRPC3 participate in the regulation the proliferation of airway smooth muscle cells (ASMCs) through modulating calcium ion (Ca^{2+}) influx in vitro. Chronic model of murine asthma was induced and ASMCs isolated from asthmatic mice were used in this whole study. TRPC1 and TRPC3 were upregulated in asthmatic mouse ASMCs and selected for further investigation. Ca^{2+} concentration and the cell viability of asthmatic mouse ASMCs were significantly higher than that from non- asthma mice, however, TRPC channels blocker SKF96365 alleviated these effects. Furthermore, TRPC1 or TRPC3 overexpression markedly increased Ca^{2+} concentration and significantly induced the viability of ASMCs; whereas TRPC1 or TRPC3 knockdown exerted the completely conversed effects. Moreover, knockdown of TRPC1 and TRPC3 also exerted different effects on the protein expression of growth-related proteins p-p38, p-JNK, cleaved caspase-3 and Bcl-2, as well as on cell cycle. Finally, we found Ca^{2+} chelator EGTA or BAPTA-AM significantly diminished the effects of si-TRPC1 and si-TRPC3 on the cell viability, cell cycle, and the protein expression of p-p38, p-JNK, cleaved caspase-3, and Bcl-2 in asthmatic mouse ASMCs. Our findings demonstrated that the effects of TRPC1 and TRPC3 on the cell viability and cell cycle of ASMCs were, at least partially, through regulating Ca^{2+} influx.

KEYWORDS

airway smooth muscle cell, asthma, transient receptor potential canonical (TRPC)

1 | INTRODUCTION

Bronchial asthma is one of the world's most common chronic diseases dangerous to human health. It is characterized by

variable respiratory symptoms and variable airflow limitation.¹ Airway remodeling, also known as airway reconstruction, is one of the key features of asthma. One of the main characteristics of airway remodeling is an increase in the amount of airway smooth muscle (ASM) within the airway wall, which is likely to be the result of increased muscle proliferation.² Therefore, studies concerning the prevention

Xiaoyu Zhang and Zhixin Zhao are co-first authors.

of airway remodeling and the mechanism of airway smooth muscle cells (ASMCs) proliferation is very urgent and important in the prevention and treatment of asthma.

The calcium ion (Ca^{2+}) is a ubiquitous second messenger controlling a broad range of cellular functions including growth and differentiation.³ Ca^{2+} influx in pulmonary ASMCs is mainly regulated by voltage-dependent Ca^{2+} channels and voltage-independent non-selective cation channels, such as store-operated Ca^{2+} channels (SOCC), receptor-operated Ca^{2+} channels (ROCC).⁴ A previous study indicated that the alteration of intracellular Ca^{2+} concentration in ASMCs plays an important role in ASMCs metabolism and cell proliferation.⁵ Another study also suggested that increased Ca^{2+} influx is an important stimulus for pulmonary ASMCs proliferation.⁶ Besides, the transient receptor potential channel (TRPC) genes encode Ca^{2+} channels that are responsible for Ca^{2+} entry.⁷ Thus, it raises the possibility that TRPC genes may strongly associated with the progression of asthma. However, research about it is limited.

TRPC is a superfamily of cation-selective ion channels and makes up a family of non-selective Ca^{2+} -permeable cation channels widely expressed in vertebrate tissues. Members of the mammalian TRPC family can be divided into TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5 subfamilies based on the sequence homology and functional similarities.⁸ Based on the aforementioned studies, we speculated that TRPCs may participate in the regulation of ASMCs proliferation through modulating Ca^{2+} influx.

2 | MATERIALS AND METHODS

2.1 | Chronic model of murine asthma

Wistar mice (female ($n = 12$), male ($n = 12$), weighing 200–220 g) were purchased from Slaccas Shanghai Laboratory Animal Co., Ltd. (Shanghai, China). These mice were separated into two groups, normal (non-asthma) and asthma groups. On day 1, 7, and 15, chronic asthma model mice were sensitized by intraperitoneal injection of 10% ovalbumin (OVA, 1 mL) including 100 mg OVA and 100 mg aluminum hydroxide powder as immunologic adjuvants. The non-asthma mice received the same volume of saline water intraperitoneally. Twenty one days later, mice were placed in a glass barrel for 30 min every day and challenged with inhalation of aerosolized OVA solution (2% in saline) for excitation for 7 days. Mice in the normal group were challenged with an aerosol of saline without OVA. These mice were sacrificed within 18–24 h after the final challenge in each group. All animal procedures were approved by the Institutional Life Science Ethics Committee of ZhengZhou University.

2.2 | Cell culture of ASMCs

ASMCs were prepared by the explant method in Wistar mice as described previously.⁹ Briefly, mice were deeply anesthetized with urethane (5 mL/kg) intraperitoneally and euthanized by exsanguination. Large bronchi was collected and washed three times. Pure airway smooth muscle bundles were cut (1 mm³ fragments) and then seeded in 2–3 mL DMEM (Gibco, Grand Island, NY) containing 20% (v/v) fetal bovine serum (FBS, Gibco). The flasks were placed in a 5% CO_2 humidified incubator at 37°C. Then the cells were digested with 0.25% trypsin (containing 0.02% EDTA, Gibco) and subcultured in DMEM with 10% FBS. Cells between passages three and six were used for this study. The identification of ASMCs was confirmed by the presence of positive immunofluorescent staining for α -smooth muscle actin (α -SMA). The purity of isolated ASMCs reached up to 99%.

2.3 | Identification of the ASMCs

Cell morphology, including dimension and arrangement, was observed under an optical microscope (IX71; Olympus, Tokyo, Japan) following culture for 7 days. Immunocytochemical identification by α -SMA was performed as previously described.¹⁰ Briefly, cells were seeded in 24-well culture plates preliminarily coated with poly-L-Lysine in a humidified incubator at 37°C and 5% CO_2 for 24 h. The plates were rinsed using 0.01 mol/L of phosphate-buffered saline and fixed in 4% phosphate-buffered paraformaldehyde for 30 min at room temperature. Following that, they were blocked with 5% bovine serum albumin (Ameresco, Solon, OH) and incubated with α -SMA (1:200, Abcam, Chicago, IL) at 4°C overnight. Twelve hour later, the plates were treated with the DAB staining kit according to the manufacturer's instructions (Muto Pure Chemicals, Tokyo, Japan).

2.4 | Determination of the intracellular Ca^{2+} concentration

ASMCs ($1 \times 10^6/\text{mL}$) were seeded on coverslips and cultured for 48 h. The control group was added the appropriate volume of D-Hank's solution. Before detecting, the cells were incubated in serum-free culture containing 10 mg/L Fluo3-AM (Dojindo, Kyushu, Japan) and 0.1% F-127 (Dojindo) at 37°C for 30 min. For detection, Ca^{2+} -free extracellular fluid containing 0.1 mmol/L EGTA was added. The slides were fixed to the bottom of the recording dish and then placed in a laser scanning confocal microscope (Leica, Solms, Germany), with an excitation wavelength of 488 nm and a detection wavelength of 515 nm. The Ca^{2+} fluorescence intensity of smooth muscle cells was observed under different conditions at 200 $\times$ magnification. Intracellular Ca^{2+} concentration was reflected as Ca^{2+} fluorescence intensity.

2.5 | Construction of TRPC1 or TRPC3 overexpression and TRPC1/3 knockdown

For overexpression of TRPC1 or TRPC3, a cDNA encoding full-length TRPC1 or TRPC3 was amplified by PCR. The PCR-generated full-length TRPC1 or TRPC3 was digested and ligated together, and then cloned to create the expression construct. The prepared plasmid DNA was fully sequenced. TRPC1 or TRPC3 was then transfected into ASMCs with liposome-packaged method.¹¹

For knockdown of TRPC1 or TRPC3, TRPC1 siRNA (si-TRPC1), TRPC3 siRNA (si-TRPC3), and scramble siRNA were purchased from Invitrogen (Carlsbad, CA). siRNAs (100 nm) were transfected into the ASMCs at 60% confluence, using Lipofectamine™ 2000 reagent (Invitrogen) for 48 h according to the manufacturer's instructions. The knockdown efficiency of TRPC1 and TRPC3 was validated by qRT-PCR (Supplementary Figure S1 and S2). si-TRPC1-1 and si-TRPC3-1 with the highest knockdown efficiency was selected for subsequent studies. The sequences of siRNAs were as follows: si-TRPC1-1, forward, AUAUUAGAAGUCCGAAAGCCAAGU, and reverse, ACUUGGCUUCGGACUUCUAAUUAU; si-TRPC3-1, forward, 5'-CCACCCAGUUCACAUGGACAGAAU-3' and reverse, 5'-AUUUCUGUCCAUGUGAACUGGGUGG-3'; the scramble siRNA, forward, 5'-CCAUGACCACUGGUAGACAACCAAU-3' and reverse, 5'-AUUGGUUGUCUACCAGUGGUCAUGG-3'.

2.6 | Cell proliferation assay

Proliferation of ASMCs was measured by counting cell number. Cells (1×10^4 /mL) were seeded in 6-well plates and grew in DMEM containing 10% FBS for 24 h. After 2–3 days, MTT solution (5 mg/mL, Gibco) was added to each well for incubation for 4 h. After removing the entire medium from the wells, 150 μ L of dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) was placed on the oscillator for 10 min. Then the absorbance at 570 nm wavelength with averages from triplicate wells by an enzyme-linked immunosorbent assay reader (Bio-Tek ELX800, Bio-Tek Instruments, Winooski, Vermont) was recorded and normalized to control well.

2.7 | Flow cytometry analysis of cell cycle

The distributions of cells in each of the phases of the cell cycle were determined by flow cytometry. Briefly, cells were trypsinized and suspended in PBS (pH = 7.2). After centrifugation at 1200 r for 5 min, the pellets were suspended in 70% ethanol and fixed overnight at 4°C. The cells were washed by PBS and the pellets were stained with propidium iodide (PI) solution (100 mg/mL PI in

sample buffer containing RNase A). After incubation at 4°C for 30 min, PI-stained nuclei were analyzed by flow cytometry (FACS calibur; BD Biosciences, Heidelberg, Germany). The cell cycle analysis was determined with the computer program MODFIT LT V3.3.11 (Verity Software House, Topsham, ME).

2.8 | Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from proximal tibias using the TRIzol method (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA). cDNA was prepared by reverse transcription of single-stranded RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc), according to the manufacturer's instructions. qRT-PCR was carried out using the SYBR® Premix Ex Taq™ kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's instructions. The reaction mix (20 μ L) consisted of 30-fold diluted 1st-strand cDNA (2 μ L), 2 $\times$ SYBR® Premix Ex Taq™ (10 μ L), 10 μ M forward and reverse primer (0.4 μ L), 50 $\times$ ROX Reference Dye (0.4 μ L), and DEPC-treated water (6.8 μ L). The primer pairs used in these reactions were as follows: TRPC1, forward, 5'-GGTACATCTTCTCCTTAGCGC-3' and reverse, 5'-TGGTCTGTGCTCTGCATCTTC-3'; TRPC3, forward, 5'-TGAGGTGAACGAAGGTGAACTG-3' and reverse, 5'-CCTGTCCCCCAAGGAACTCT-3'; TRPC4, forward, 5'-GCTTCGAAGTTCTGGGATTGC-3' and reverse, 5'-CAC-CACCACCTTCTCCGACTT-3'; TRPC5, forward, 5'-CTATGAGACCAGAGCTATTGATG-3' and reverse, 5'-CTACCAGGGAGATGACGTTGTATG-3'; TRPC6, forward, 5'-ATGCGGATGTGGAGTGGAAG-3' and reverse, 5'-AGCAGGGACTTTGGACTTGG-3'; TRPC7, forward, 5'-GAAGTACGACCACAAGTTCATCG-3' and reverse, 5'-TCAGGTGGTCTTTGTTCAAAT-3'; GAPDH, forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. Reactions were performed in an ABI7300 Real-Time quantitative instrument (Applied Biosystems; Thermo Fisher Scientific, Inc). The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. The expression level of the internal control GAPDH was used as a housekeeping gene, and the comparative $2^{-\Delta\Delta C_q}$ method was used to quantify gene expression levels. The products were then analyzed by electrophoresis on a 1% agarose gel.

2.9 | Western blot analysis

Proteins were lysed in radioimmunoprecipitation buffer (RIPA) containing protease inhibitors for at 4°C for 30 min. Cell lysates were prepared with a RIPA lysis buffer kit (Santa Cruz Biotechnology, Inc., Santa Cruz,

CA), and the protein concentrations were quantified using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins (30 µg) were separated on 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham; GE Healthcare, Chicago, IL). The membranes were blocked in 5% non-fat milk (Merck, Darmstadt, Germany) overnight at 4°C. Transferred membranes were then stained with the following primary antibodies: anti-TRPC1 (1:100, Alomone Labs Ltd, Jerusalem, Israel), anti-TRPC3 (1:500, Alomone Labs Ltd), anti-TRPC4 (1:100, Alomone Labs Ltd), anti-TRPC5 (1:100, Alomone Labs Ltd), anti-TRPC6 (1:500, Alomone Labs Ltd), anti-TRPC7 (1:100, Alomone Labs Ltd), anti-p38 (1:500, Santa Cruz Biotechnology, Inc.), anti-p-p38 (1:500, Santa Cruz Biotechnology, Inc.), anti-JNK (1:200, Santa Cruz Biotechnology, Inc.), anti-p-JNK (1:200, Santa Cruz Biotechnology, Inc.), anti-cleaved caspase-3 (1:500, Santa Cruz Biotechnology, Inc.), anti-caspase-3 (1:1000, Cell Signaling Technology, Inc. (Danvers, MA), anti-Bcl-2 (1:200, Abcam) and anti-GAPDH (1:20 000, Santa Cruz Biotechnology, Inc.) overnight at 4°C. Subsequently, protein bands were detected by incubation with a horseradish peroxidase-conjugated secondary antibody (1:1000, Golden Bridge Biotechnology Co., Ltd, Beijing, China) at room temperature for 1 h. Signals were detected using an enhanced chemiluminescence kit (ECL; Wuhan Booute Biotechnology Co., Ltd, China) and exposed to Kodak X-OMAT film (Kodak, Rochester, NY). Each experiment was performed at least three times.

2.10 | Statistical analysis

All data were expressed as mean ± standard deviation (SD). Each experiment was performed at least three times. Statistical analyses were performed using SPSS statistical software package standard version 16.0 (SPSS, Inc., Chicago, IL). Statistical differences between two independent groups were determined using Student's *t* test. Statistical differences among multiple independent groups were determined using a one-way analysis of variance (ANOVA). Values of *P* less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | TRPC1, TRPC3, and TRPC6 are upregulated in asthmatic mouse ASMCs

To explore the role of TRPCs in asthma, we detected the expression of TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7 in ASMCs from non-asthma and asthma-model mice. As shown in Figure 1A, the mRNA expression levels of TRPC1, TRPC3, and TRPC6 were significantly upregulated in ASMCs from asthma-model mice compared

with that from non-asthma mice. However, the mRNA expression levels of TRPC4 and TRPC5 showed no significant difference between ASMCs from asthma-model and that from non-asthma mice. Furthermore, no expression level of TRPC7 mRNA was detected in ASMCs from both asthma-model and non-asthma mice (data not shown). Similar to the mRNA expression pattern in ASMCs, a significant increase in protein expression of TRPC1, TRPC3, and TRPC6 was also observed in ASMCs from asthma-model mice compared with that from non-asthma mice (Figures 1B and 1C). These results implied that TRPC1, TRPC3, and TRPC6 expression might be associated with the progression of asthma. Since the role of TRPC6 is well-studied in the progression of asthma,¹² TRPC1 and TRPC3 were selected for further investigation.

3.2 | TRPC channels blocker SKF96365 decreases the elevated Ca²⁺ concentration and inhibits the increased cell viability of asthmatic mouse ASMCs

For identification of ASMCs, microscope photograph was used to observe the cultured primary airway epithelial cells; and the typical peak-valley pattern of ASMCs was able to be observed (Figure 2A). Additionally, data from immunocytochemical staining with specific α-smooth muscle actin (α-SMA) antibody also demonstrated that the cultured cells were ASMCs (Figure 2B).

Furthermore, Ca²⁺ concentration in ASMCs from asthma-model mice was significantly higher than that from non-asthma mice (Figure 2C). The viability of ASMCs from asthma-model mice was also significantly higher than that from non-asthma mice (Figure 2D). However, when asthmatic mouse ASMCs were treated with TRPC channels blocker SKF96365, the Ca²⁺ concentration in ASMCs was significantly decreased compared with that without SKF96365 treatment (Figure 2E). Furthermore, the proliferation ability of asthmatic mouse ASMCs was also significantly inhibited by SKF96365 (Figure 2F). These findings indicated that blocking TRPC channels by SKF96365 significantly decreased the elevated Ca²⁺ concentration and inhibited the increased cell viability of ASMCs from asthma-model mice. It may suggest that, to some extent, the involvement of TRPCs in regulating Ca²⁺ concentration and proliferation of ASMCs from asthma-model mice.

3.3 | The effects of TRPC1 and TRPC3 on Ca²⁺ concentration and cell viability of asthmatic mouse ASMCs

To further determine the role of TRPC1 in regulating Ca²⁺ concentration and viability of ASMCs, we overexpressed and knocked down TRPC1 in asthmatic mouse ASMCs,

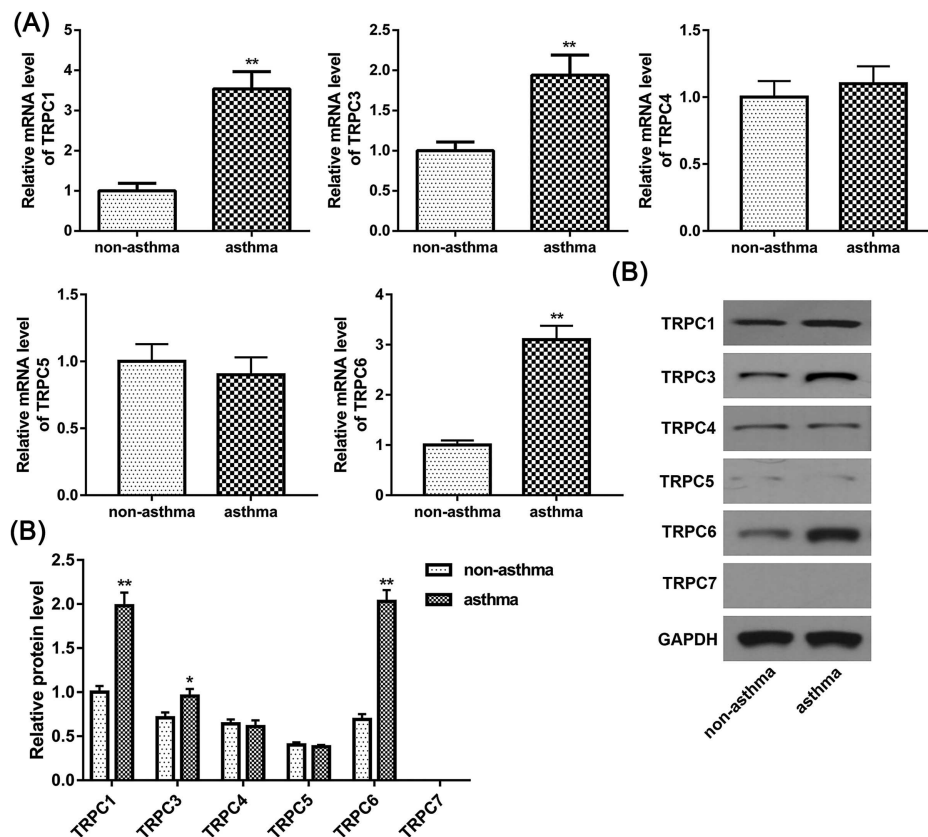


FIGURE 1 TRPC1, TRPC3, and TRPC6 are upregulated in asthmatic mouse ASMCs. A, The mRNA expression levels of TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 in ASMCs from asthma-model mice and non-asthma mice were detected by qRT-PCR. B, Western blot images of TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7 in ASMCs from asthma-model mice and non-asthma mice. C, Quantitative analysis of the relative protein expression of TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7. GAPDH served as the loading control. * $P < 0.05$ and ** $P < 0.01$ versus the non-asthma group

respectively. The overexpression and knockdown efficiency were validated by Western blot (Figure 3A). As shown in Figure 3B, TRPC1 overexpression markedly increased Ca^{2+} concentration as compared to the control cells; whereas TRPC1 knockdown significantly decreased Ca^{2+} concentration compared with the control cells. Furthermore, TRPC1 overexpression markedly induced the viability of ASMCs compared with the control cells; whereas TRPC1 knockdown significantly inhibited the viability of ASMCs compared with the control cells (Figure 3C).

TRPC3 was also overexpressed and knocked down in ASMCs from asthma-model mice, respectively, which was validated by Western blot (Figure 4A). Data revealed that the effects of TRPC3 on Ca^{2+} concentration and viability of ASMCs were similar to TRPC1. TRPC3 overexpression also significantly increased Ca^{2+} concentration and induced the viability of ASMCs compared with the control cells; whereas TRPC3 knockdown markedly decreased Ca^{2+} concentration and inhibited the viability of ASMCs compared with the control cells (Figures 4B and 4C).

3.4 | The effects of TRPC1 and TRPC3 on protein expression of p38, JNK, cleaved caspase-3, and Bcl-2 in asthmatic mouse ASMCs

It is well-accepted that the mitogen-activated protein kinases (MAPKs), which including c-Jun amino-terminal kinase (JNK), extracellular-regulating kinase (ERK), and p38 MAPK, were strongly associated with growth of ASMCs.^{13,14} Therefore, we investigated whether or not the effects of TRPC1 and TRPC3 knockdown on the cell viability of asthmatic mouse ASMCs were related to p38 and JNK pathways. Here, we found that the protein expression of p-p38 and p-JNK was significantly increased in asthmatic mouse ASMCs transfected si-TRPC1 or si-TRPC3 compared with that in the scramble siRNA-transfected control cells. Furthermore, the protein expression of cleaved caspase-3 was significantly increased and anti-apoptotic Bcl-2 was decreased in asthmatic mouse ASMCs transfected si-TRPC1 or si-TRPC3 compared with that in the scramble siRNA-

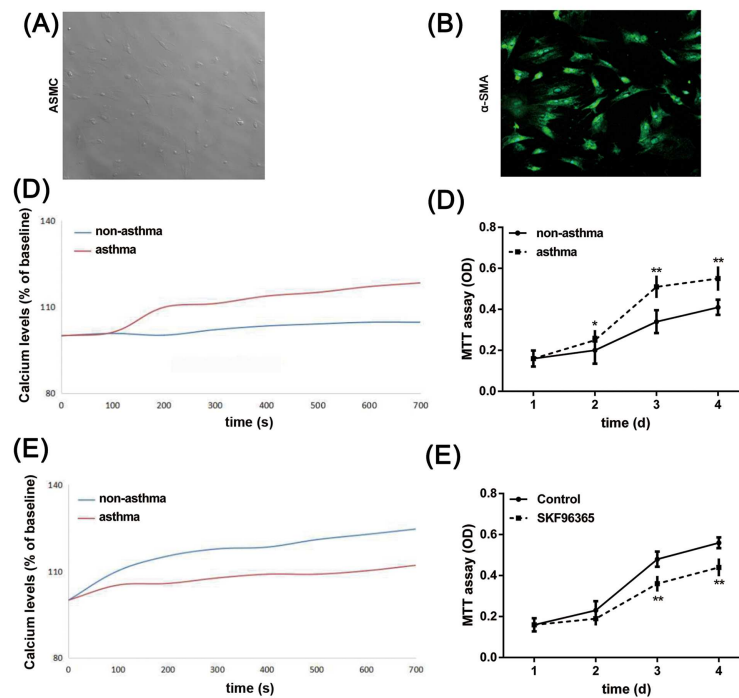


FIGURE 2 TRPC channels blocker SKF96365 decreases the elevated Ca^{2+} concentration and inhibits the increased cell viability of asthmatic mouse ASMCs. A, The typical peak-valley pattern of ASMCs was observed under microscope photograph ($\times 100$). B, The immunocytochemical staining with specific α -SMA antibody ($\times 400$). α -SMA, α -smooth muscle actin. C, Ca^{2+} concentration in ASMCs from asthma-model and non-asthma mice was examined by detecting fluorescence intensity. D, The viability of ASMCs from asthma-model and non-asthma mice was examined by MTT assay. E, After treatment with TRPC channels blocker SKF96365 or not, Ca^{2+} concentration in ASMCs isolated from asthma-model mice was examined by detecting fluorescence intensity. ASMCs without treatment with SKF96365 served as the control group. F, After treatment with TRPC channels blocker SKF96365 or not, the cell viability in ASMCs isolated from asthma-model mice was examined by MTT assay. ASMCs without treatment with SKF96365 served as the control group. $*P < 0.05$ and $**P < 0.01$ versus the non-asthma group (C and D). $**P < 0.01$ versus the control group (E and F)

transfected control cells (Figure 5A). These results indicated the regulative role of TRPC1 and TRPC3 on p-p38, p-JNK, cleaved caspase-3, and Bcl-2 expression.

3.5 | PD169316 and SP600125 significantly abrogates the effects of si-TRPC1 and si-TRPC3 on cell viability and cell cycle of asthmatic mouse ASMCs

To test whether or not si-TRPC1- or si-TRPC3-mediated suppression of cell viability of ASMCs was through the activation of p38-MAPK and JNK, we treated scramble-siRNA-, si-TRPC1-, or si-TRPC3-transfected ASMCs with p38-MAPK inhibitor PD169316 or JNK inhibitor SP600125. As shown in Figure 5B, the cell viability of asthmatic mouse ASMCs transfected with si-TRPC1 or si-TRPC3 was significantly decreased compared with that of scramble siRNA-transfected cells. However, treatment with PD169316 or SP600125 significantly increased cell viability of si-TRPC1- or si-TRPC3-transfected ASMCs compared with cells without treatment with PD169316 or SP600125.

Next, we explored the effects of TRPC1 and TRPC3 on the cell cycle of ASMCs. As shown in Figures 5C and 5D, compared with the scramble controls, the cell population in G1 phase of si-TRPC1 or si-TRPC3-transfected ASMCs was significantly decreased, whereas the cell population in S and G2 phases in si-TRPC1 or si-TRPC3 group was significantly increased. Furthermore, treatment with PD169316 or SP600125 significantly reversed the effects on cell fraction exerted by knockdown of TRPC1 or TRPC3. Taken together, these results indicated that p38-MAPK inhibitor PD169316 and JNK inhibitor SP600125 blocked the effects of TRPC1 and TRPC3 knockdown on the cell viability and cell cycle of asthmatic mouse ASMCs.

3.6 | EGTA or BAPTA-AM significantly diminishes the effects of si-TRPC1 and si-TRPC3 on cell viability and cell cycle of asthmatic mouse ASMCs

To test whether or not the effects of TRPC1 or TRPC3 on cell viability and cell cycle of asthmatic mouse ASMCs was

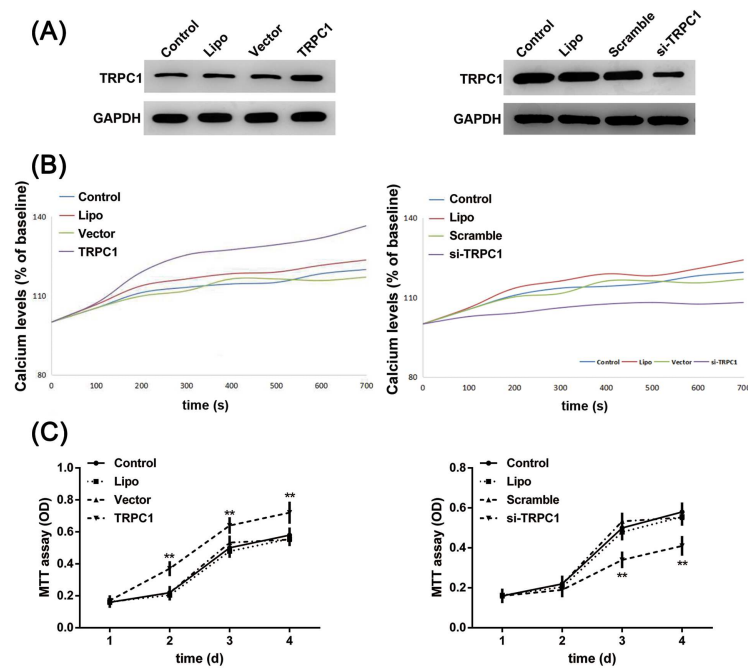


FIGURE 3 The effects of TRPC1 on Ca^{2+} concentration and cell viability of asthmatic mouse ASMCs. A, TRPC1 was overexpressed and knocked down in ASMCs from asthma-model mice, and TRPC1 overexpression and knockdown efficiency were validated by Western blot. GAPDH served as the loading control. B, The effects of overexpression and knockdown of TRPC1 on Ca^{2+} concentration in ASMCs were examined by detecting fluorescence intensity. C, The effects of overexpression and knockdown of TRPC1 on the proliferation of ASMCs were examined by MTT assay. $**P < 0.01$ versus the control group

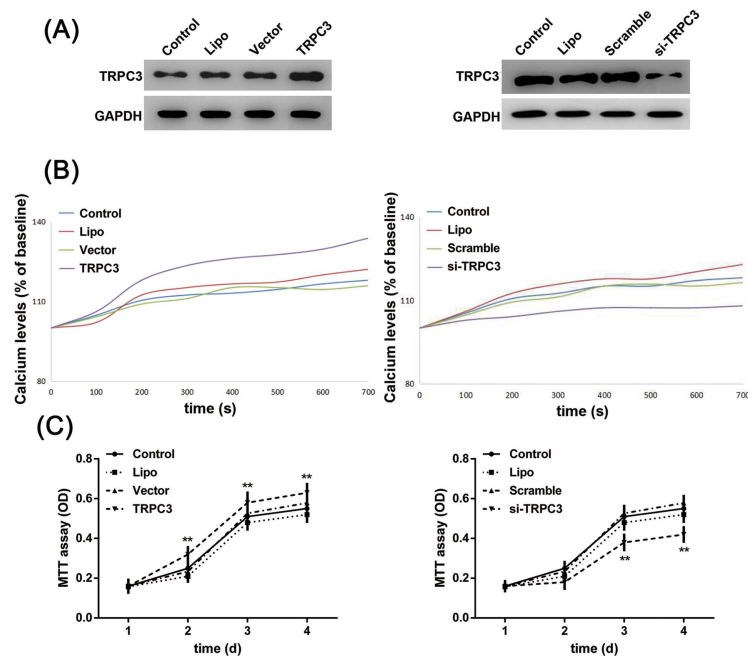


FIGURE 4 The effects of TRPC3 on Ca^{2+} concentration and cell viability of asthmatic mouse ASMCs. A, TRPC3 was overexpressed and knocked down in ASMCs from asthma-model mice, and TRPC3 overexpression and knockdown efficiency were validated by Western blot. GAPDH served as the loading control. B, The effects of overexpression and knockdown of TRPC3 on Ca^{2+} concentration in ASMCs were examined by detecting fluorescence intensity. C, The effects of overexpression and knockdown of TRPC3 on the proliferation of ASMCs were examined by MTT assay. $**P < 0.01$ versus the control group

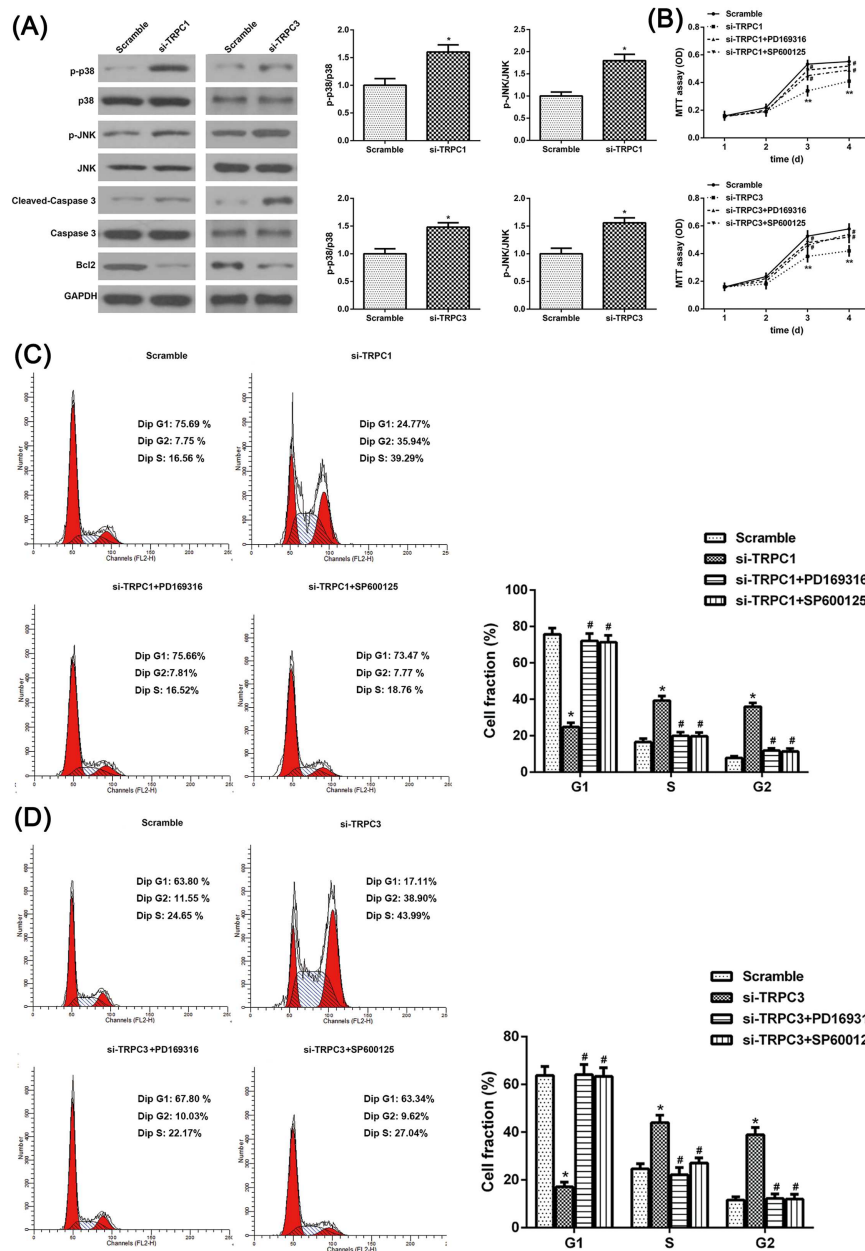


FIGURE 5 PD169316 and SP600125 significantly abrogates the effects of si-TRPC1 and si-TRPC3 on cell viability and cell cycle of asthmatic mouse ASMCs. A, The protein expression of p-p38, p-JNK, cleaved caspase-3, and Bcl-2 in scramble siRNA-, si-TRPC1- or si-TRPC3-transfected ASMCs was examined by Western blot. Quantitative analysis of the relative protein expression of p-p38/p38 and p-JNK/JNK was shown. B, After treatment with p38-MAPK inhibitor PD169316 or JNK inhibitor SP600125 or not, the cell viability of si-TRPC1- or si-TRPC3-transfected asthmatic mouse ASMCs was evaluated by MTT assay. After treatment with p38-MAPK inhibitor PD169316 or JNK inhibitor SP600125 or not, the cell cycle distribution of si-TRPC1- (C) or si-TRPC3- (D) transfected asthmatic mouse ASMCs was evaluated by flow cytometry. * $P < 0.05$, ** $P < 0.01$ versus the scramble group. # $P < 0.05$ versus the si-TRPC1 or si-TRPC3 group

associated with the elevated Ca^{2+} concentration, we treated scramble-siRNA-, si-TRPC1-, or si-TRPC3-transfected ASMCs with an extracellular EGTA (2 mM, ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid) or an intracellular Ca^{2+} chelator BAPTA-AM (10 mM, 1,2-bis-(2-amino-phenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester). As shown in Figures 6A and

6B, EGTA or BAPTA-AM treatment significantly diminished the si-TRPC1- and si-TRPC3-mediated suppression on cell viability of asthmatic mouse ASMCs. Furthermore, EGTA or BAPTA-AM treatment significantly reversed si-TRPC1- and si-TRPC3-mediated upregulation of p-p38, p-JNK, cleaved caspase-3, and downregulation of Bcl-2 in asthmatic mouse ASMCs. Moreover, EGTA or BAPTA-AM

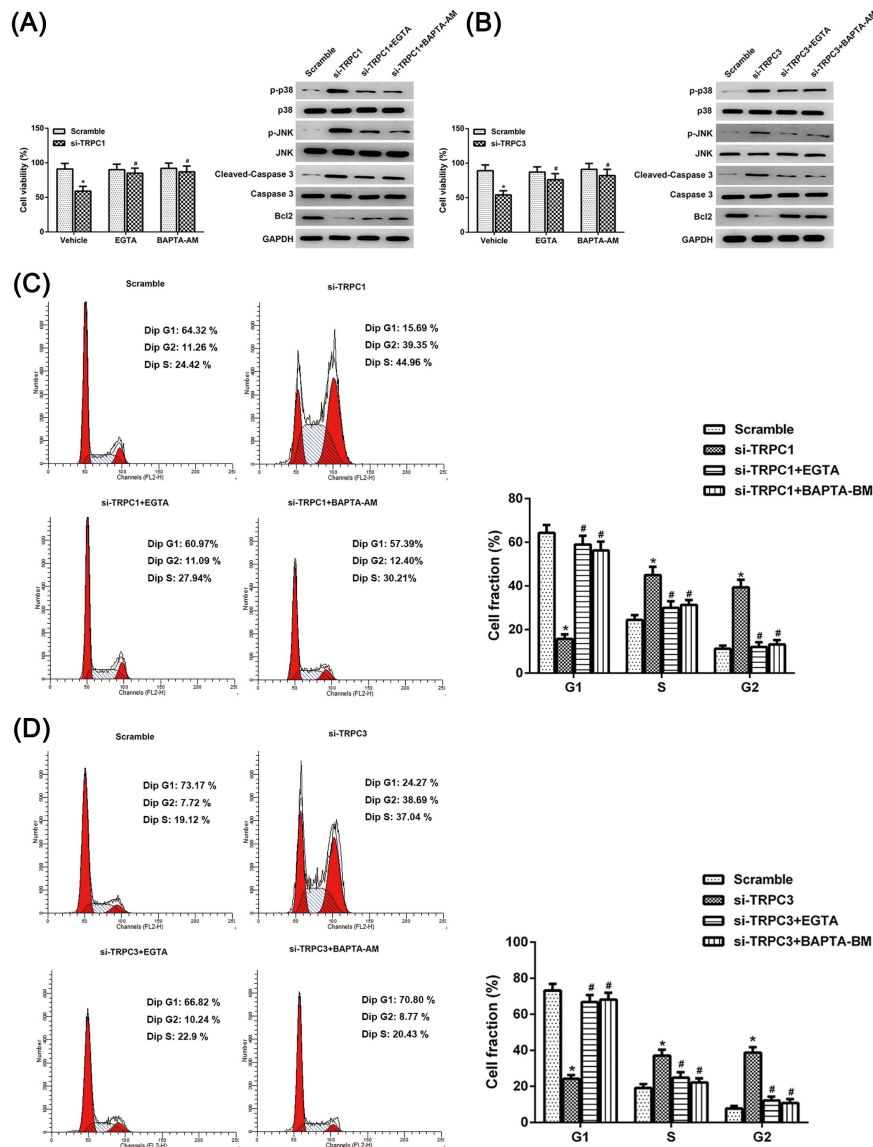


FIGURE 6 EGTA or BAPTA-AM significantly diminishes the effects of si-TRPC1 and si-TRPC3 on cell viability and cell cycle of asthmatic mouse ASMCs. After treatment with Ca^{2+} chelator EGTA or BAPTA-AM or not, the cell viability of si-TRPC1- (A) or si-TRPC3- (B) transfected ASMCs was evaluated by MTT assay, and the protein expression of p-p38, p-JNK, cleaved caspase-3, and Bcl-2 in si-TRPC1- (A) or si-TRPC3- (B) transfected ASMCs was examined by Western blot. C, After treatment with Ca^{2+} chelator EGTA or BAPTA-AM or not, the cell cycle distribution of si-TRPC1- (C) or si-TRPC3- (D) transfected ASMCs was evaluated by flow cytometry. * $P < 0.05$ versus the scramble group. # $P < 0.05$ versus the si-TRPC1 or si-TRPC3 group

significantly diminished the effects of si-TRPC1 and si-TRPC3 on the cell cycle of asthmatic mouse ASMCs (Figures 6C and 6D).

4 | DISCUSSION

TRPCs were expressed in almost every tissue and cell type, where they play unique roles as cellular sensors, and signal integrators of a plethora of Ca^{2+} -mediated cellular functions.^{15,16} Firstly, our study showed that TRPC1, TRPC3, and

TRPC6 were upregulated in ASMCs from asthma model mice, suggesting their potential functional importance in ASMCs. Wang et al¹⁷ reported that hypoxia induced pulmonary ASMC proliferation with increases in basal (Ca^{2+})_i and Ca^{2+} entry via SOC. However, hypoxia-induced pulmonary ASMC proliferation was inhibited by SKF96365 and NFAT blocker (VIVIT and Cyclosporin A). Consistently, we found that Ca^{2+} concentration and the cell viability of asthmatic mouse ASMCs was significantly enhanced compared with that from non-asthma mice. However, TRPC channels blocker SKF96365 alleviated the phenomena. Thus,

our investigations suggest the involvement of TRPCs in regulating Ca^{2+} concentration and proliferation of asthmatic mouse ASMCs.

Besides, in our study, overexpression of TRPC1 or TRPC3 markedly increased Ca^{2+} concentration and significantly induced the viability of ASMCs; whereas knockdown of TRPC1 or TRPC3 exhibited the completely conversed effects. Besides, TRPC1 and TRPC3 also exerted effects on growth-related proteins including p-p38, p-JNK, cleaved caspase-3, and Bcl-2 expression, as well as on cell cycle of asthmatic mouse ASMCs. Consistent with this, Wang et al¹⁷ reported that hypoxia induced pulmonary ASMC proliferation with increases in basal (Ca^{2+})_i and Ca^{2+} entry via SOC, which were accompanied by up-regulation of TRPC1 gene and protein expression in pulmonary ASMC. Sweeney et al¹⁸ suggested that the upregulated TRPC1 is, at least in part, responsible for the increased amplitudes of the store depletion-activated currents and capacitative Ca^{2+} entry during bronchial smooth muscle cell proliferation.^{4,19} Revealed that TRPC1 protein level was significantly increased and store-operated Ca^{2+} entry (SOCE) was augmented in pulmonary arterial of monocrotaline-treated rats as compared to the control. After treatment of TRPC inhibitor BTP-2, the proliferation ability of pulmonary ASMC was significantly reduced. Another study indicated that Angiotensin II (Ang II) increased proliferation of cultured human coronary artery smooth muscle cells, and TRPC1 markedly increased in response to Ang II stimulation.²⁰ Besides, TRPC1 small interference RNA (siRNA) decreased SOCE and prevented Ang II-induced hypertrophy.²⁰ In particular, TRPC1 and TRPC3 were reported to form SOCC that are involved in Ca^{2+} homeostasis.^{21,22} Besides, Wang et al²³ suggested that, in human coronary artery smooth muscle cells, silencing TRPC1 or TRPC3 countered the lysophosphatidylcholine-induced increase of Ca^{2+} influx and apoptosis, and the pro-apoptotic proteins (Bax and cleaved caspase-3), and decrease of the anti-apoptotic protein Bcl-2 and the survival kinase p-Akt, which is partially consistent with our results.

With regard to the association between TRPC3 and Ca^{2+} influx, the upregulation of TRPC3 expression and enhanced SOCE had also been shown in pulmonary ASMCs of idiopathic pulmonary arterial hypertension patients.^{6,24} Furthermore, TRPC3 knockdown reduced the Ang II-induced Ca^{2+} entry by ~30%, and TRPC3 overexpression increased this Ca^{2+} entry by ~55%.²⁵ Xiao et al²⁶ showed that the mRNA and protein expression levels of TRPC1 and TRPC3 were expressed in freshly isolated airway smooth muscle tissues. They proposed that TRPC3 blockade inhibited the nonselective cationic currents and caused membrane hyperpolarization in ASMCs. Particularly, Grayson et al²⁷ highlighted the recent advances in understanding the functional diversity of TRPC3, with an emphasis on vascular

health and disease. They concluded that TRPC3 was an attractive target for therapies directed at attenuating tissue and organ damage attributable to Ca^{2+} influx.

Moreover, our results demonstrated that Ca^{2+} chelator EGTA or BAPTA-AM significantly diminished the effects of si-TRPC1 and si-TRPC3 on the cell viability, cell cycle and on the protein expression levels of p-p38, p-JNK, cleaved caspase-3 and Bcl-2 in asthmatic mouse ASMCs, which suggested the effects of TRPC1 and TRPC3 on the ASMCs were, at least through regulating Ca^{2+} influx. The mechanism underlying the effects of TRPC1 and TRPC3 on the regulation of Ca^{2+} influx was unclear in our study, but a previous study suggested the contribution of caveolin-1 and STIM1 in the regulation of TRPC1-SOCE that occurs within the endoplasmic reticulum-plasma membrane junctional region in response endoplasmic reticulum Ca^{2+} store depletion.²⁸ Another study indicated that capacitative Ca^{2+} entry is mediated by TRPC1 channel through activation of STIM1 in mouse pulmonary ASMCs.²⁹ Besides, further study suggested that TRPC1 and Orai1 channels mediate capacitative Ca^{2+} entry through activation of STIM1 in acute hypoxic mouse pulmonary ASMCs.^{30,31} Importantly, STIM1 is reported to interact with TRPCs 1, 4, and 5, but not TRPC3, 6 and 7.³² Ong et al³² indicated that SOCE is activated within the endoplasmic reticulum-plasma membrane junctions in the plasma membrane and involves assembly of the channels, Orai1 and TRPC1 with the regulatory protein STIM1, in response to depletion of Ca^{2+} in the endoplasmic reticulum. It seems that TRPC1 is more important than TRPC3 in regulating Ca^{2+} influx. Since STIM1 does not interact with TRPC3, other mechanisms underlying the effects of TRPC3 on regulating Ca^{2+} influx need to be further elucidated.

Wu et al³³ indicated that co-expression of siRNA specific for TRPC1 and TRPC3 in proliferating rat-derived hippocampal H19-7 cells inhibited approximately the same amount of SOCE as observed with expression of either siRNA alone, suggesting that TRPC1 and TRPC3 work in tandem to mediate SOCE. However, co-immunoprecipitation revealed that TRPC1 and TRPC3 had protein-protein interaction in a recent study.²³ It seems that the interaction between TRPC1 and TRPC3 remain conflict, and further study should be also performed to validate it.

Collectively, our study revealed that TRPC1 and TRPC3 were up-regulated in ASMCs isolated from asthma model mice. TRPC1 and TRPC3 had the ability to regulate cell viability and cell cycle of asthmatic mouse ASMCs in vitro. The effects of TRPC1 and TRPC3 on the ASMCs growth were strongly associated with the growth-related proteins p-p38, p-JNK, cleaved caspase-3, and Bcl-2. Furthermore, our further investigation into the underlying mechanism revealed that the effects of TRPC1 and TRPC3 on the cell viability and cell cycle of asthmatic mouse ASMCs were, at least partially, through regulating Ca^{2+} influx.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ORCID

Luoxian Zhang  <http://orcid.org/0000-0002-0792-4658>

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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