Int J Clin Exp Pathol 2017;10(4):4377-4388
www.ijcep.com /ISSN:1936-2625/IJCEP0047845

Original Article

Decreased expression of SERCA2 in airway smooth muscle cells leads to increased IL-8 release in asthma

Zhenxi Yu, Jian Shen, Chunming Jiang, Xiaoli Li, Zhanli Liu, Xianmei Huang

Department of Pediatrics, Hangzhou First People's Hospital, Nanjing Medical University, Hangzhou, Zhejiang Province, China

Received January 1, 2017; Accepted January 27, 2017; Epub April 1, 2017; Published April 15, 2017

Abstract: Airway smooth muscle cells (ASMCs) secretory and hyperproliferative phenotype in asthma were modulated by the level of free Ca^{2+} in the cytosol ($[Ca^{2+}]_i$). Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), an important controller of calcium homeostasis is deficient in asthmatic ASMCs. This study aimed to determine whether SERCA2 deficiency in ASMCs is associated with its excessive IL-8 production. SERCA2 expression, $[Ca^{2+}]_i$ and IL-8 production in primary ASMCs isolated from rats with ovalbumin induced asthma was determined using qRT-PCR, Western blotting, fura PE-3 and ELISA assay respectively. SERCA2 knockdown were performed using siRNA to detect its influence on IL-8 release, NF-κB activation, proliferation and migration of normal ASMCs. The NF-κB activation was inhibited using PDTC to evaluate its necessity to IL-8 release. IL-8 was depleted using anti-IL-8 antibody to determine whether IL-8 was responsible for enhanced proliferation and migration of ASMCs transfected with SERCA2 siRNA. SERCA2 expression was reduced in asthmatic ASMCs, leading to decreased SR Ca^{2+} storage and delayed return to base line of $[Ca^{2+}]_i$ after enhanced by bradykinin. SERCA2 knockdown promoted IL-8 mRNA expression and release in normal ASMCs, which was NF-κB dependent. In addition, the proliferation and migration of normal ASMCs were also facilitated by SERCA2 knockdown, and IL-8 depletion significantly attenuated ASMCs migration, whereas showed little influence on proliferation. In conclusion, decreased SERCA2 expression in ASMCs during asthma resulted in excessive IL-8 secretion, which in turn accelerates ASMCs migration.

Keywords: Asthma, airway smooth muscle cells, sarco/endoplasmic reticulum Ca^{2+}-ATPase, IL-8

Introduction

Asthma is one of the most common chronic diseases worldwide [1], which is characterized by chronic airway inflammation and variable remodeling. Phenotypic modulation of airway smooth muscle (ASM) is an important feature of airway remodeling in asthma that is characterized by hypertrophy and abnormal release of inflammatory cytokines. Furthermore, airway smooth muscle cells (ASMCs) isolated from asthmatic patient showed increased proliferative and migratory responses [2-4]. ASMCs is not only the target cells of a variety of proinflammatory cytokines such as interleukin (IL)-1, IL-4, IL-13, transforming growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF-α), but also the source cells of various cytokines and chemokines such as IL-8, eotaxin, CXCL-10 and some components of extracellular matrix, which have been implied in airway inflammation in asthma [5].

IL-8 is a C-X-C chemokine that potently chemoattracts and activates neutrophils, eosinophils and other inflammatory cells, and plays an important role in the pathogenesis of asthma. It was found IL-8 was 19 times higher in tracheal aspirates from asthmatic patients than normal subjects [6]. Moreover, Digiovine et al. proved that ASMCs is the most important source of IL-8 in patients with obliterative bronchiolitis (OB) after lung transplantation [7]. However, the regulation mechanism for IL-8 secretion is not fully understood. Calcium mobilization can regulate a wide range of essential functions of ASMCs, including constriction, migration and proliferation [8, 9]. Increased Ca^{2+} influx after the activation of Ca^{2+} -permeable ion channels and the Na^+-Ca^{2+} -exchanger (NEX, $3Na^+$: $1Ca^{2+}$), and release of stored Ca^{2+} from the sarcoplasmic reticulum (SR) following the in turn activation of inositol 1,4,5-triphosphate (IP_3) and ryanodine receptor (RyR) lead to the elevation of the concentration of free Ca^{2+} in the cytosol

($[Ca^{2+}]_i$), which can be normalized by rapid reuptake of Ca^{2+} into SR stores by Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump [10]. The mammalian SERCA family has 3 tissue-specific members, SERCA1, SERCA2 and SERCA3, each encoded by a separate gene (*ATP2A1*, *ATP2A2* and *ATP2A3*), in which SERCA2 is highly expressed in smooth muscle [11, 12]. It was found that SERCA2 expression in ASMCs in patients with mild and moderate asthma was reduced, and SERCA2 knockdown using siRNA promoted normal ASMCs spreading, proliferation and eotaxin-1 (CCL-11) release [8].

Thus, we hypothesized that increased IL-8 production by ASMCs might arise from reduced SERCA2 expression. To test this, the primary ASMCs were isolated from normal and OVA induced asthmatic rats, and the expression of SERCA2, $[Ca^{2+}]_i$ and IL-8 release were measured. SERCA2 knockdown was performed using siRNA to evaluate the effect of SERCA2 deficiency on IL-8 release, proliferation and migration. In addition, NF-κB activation was determined using western blotting to explore the mechanism responsible for the increased IL-8 production in ASMCs transfected with SERCA2 siRNA. IL-8 depletion using anti-IL-8 antibody was carried out to detect the role of IL-8 in enhanced proliferation and migration of asthmatic ASMCs.

Materials and methods

Rat model of asthma

An asthma model was established in rats as described previously with slight modification [13]. All animal use procedures were approved by the committee of Hangzhou first people's hospital. Thirty male SD rats (SPF grade, 120-160 g, 6-8 weeks old) supplied by SLRC Laboratory Animal (Shanghai, China) were randomly divided into three groups of ten: normal, asthma1 and asthma2 group. The rats in asthma1 and asthma2 group were sensitized on days 1 and 8 with intraperitoneal (*i.p.*) injections of 1 mg ovalbumin (OVA, Sigma; Shanghai, China) adsorbed to 100 mg aluminum hydroxide, and challenged with 1% and 2% OVA saline solution respectively aerosolized using an air compressed atomizer for 30 min per day on days 17-21. The rats in normal group were treated synchronously with physiological saline. Animals were sacrificed on days 23.

Isolation and culture of primary rat airway smooth muscle cells

Tracheas were removed and placed into sterile, ice-cold PBS solution. Epithelium and any unwanted fibrous tissue were removed under a microscope, and the remained tissue was digested in a digestion buffer containing 2 mg/mL bovine serum albumin (BSA), 2 mg/mL collagenase I and 20 U/mL elastase IV at 37°C for 1 h [14]. The resulting cell suspension was centrifuged to remove debris and resuspended in Dulbecco's Modified Eagle's Medium (D-MEM) supplied with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 LG/ml), amphotericin B (1.25 LG/ml) and 10% fetal bovine serum (FBS, Invitrogen, Shanghai, China), and cultured in a humidified environment with 5% CO_2 at 37°C. Cells were sub-cultured by continuous passage. Primary cell cultures used in this experiment showed > 95% of cells staining for α-smooth muscle actin.

SERCA2 gene silencing using siRNA

SERCA2 gene silencing was performed as described previously [15]. Rat ASMCs cultured on fibronectin-coated 6-well plates (1×10^6 cells per well) were transfected with either 50 nM of predesigned rat 'SMARTPOOL' SERCA2 siRNAs (Dharmacon, Shanghai, China) or a scrambled RNA oligo sequence using Dharma FECT2 siRNA transfection reagent (Dharmacon) in accordance with the manufacturer's instructions. Sham transfection was used as a negative control. At 48 or 72 h post-transfection, the cells were harvested and reseeded in D-MEM for further experiments.

Real-time reverse transcription PCR

Total RNA was prepared from whole cell lysates of approximately 1×10^6 cells using RNeasy mini kits (Qiagen, Inc.). RNA quality and integrity were determined by spectrophotometry and agarose gel electrophoresis. Then 1 μg of total RNA was reverse transcribed using the GoScript™ Reverse Transcription System (Promega), and 2 ng of obtained cDNA was quantified by SYBR® Premix ExTaq™ (TaKaRa) using the following conditions: 95°C for 15 min and 45 cycles of 94°C for 15 s, 56°C for 20 s, and 72°C for 20 s. The reaction system was constructed as the manufacturer's proposal and β-actin was used as a reference for quantification, and the relative gene expression level

was calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were conducted in triplicates. The primer sequences used were as follows: SERCA2, F 5'-CCCTGTACAGTTTGCT TA-3' and R 5'-GCTGTGAGGAACTGAACC-3'; IP3R1, F 5'-AGCCATGTTAGAGGCTCACA CGTT-3' and R 5'-CCTGGGAGATGACACTGACTGGT-3'; IL-8, F 5'-GAGCAACCCATACCC ATCGA-3' and R 5'-TGGTCCCACCATATCT TCTTAATCT-3'; β-actin, F 5'-TGGCCTCACTGT CCACCTTCCA-3' and R 5'-CGCAGCTCAGTAACAGTCCGCC-3'.

Western blotting

The cells were harvested and lysed in RIPA buffer containing protease and phosphatase inhibitors (Thermo Scientific, USA), and nuclear extracts were collected by using a nuclear extraction kit (Millipore, Billerica, USA). The total protein concentration was determined with a BCA protein assay kit. Afterwards, equivalent amounts of protein lysates were electrophoresed and transferred to a PVDF membrane. After being blocked with Tris-buffer containing 5% skim milk, the membrane was probed with rabbit antibodies against SERCA2 (ab1370-20, Abcam), IP3R1 (NBP1-95155, Novus), IκBα (9242, Cell Signaling Technology), NF-κB p65 (4764, Cell Signaling Technology), β-actin (49-70, Cell Signaling Technology) at 1:1,000 dilution or GAPDH (ab181603, Abcam) at 1:10,000 dilution followed by HRP-conjugated secondary antibody. The blots were visualized using ECL kits (Amersham) and the optical density of these protein bands was quantified using the ImageJ software.

Calcium measurements

The measurements of $[Ca^{2+}]_i$ were carried out as previously described by Katharina Mahn et al. [10].

IL-8 assay

The ASMCs isolated from normal or asthmatic rats and the cells transfected with SERCA2 siRNA or scrambled siRNA were cultured in the presence or absence of 0.1 ng/ml of recombinant rat IL-17 (R&D Systems) in a humidified environment with 5% CO_2 at 37°C [16]. After 24 h, the apical and basolateral media was collected, centrifuged and supernatant was analyzed for IL-8 release by an ELISA kit (Biosource SA).

Cellular proliferation assay

After transfection of SERCA2 siRNA or scrambled siRNA into the ASMCs isolated from normal or asthmatic rats, the cells (1×10^4 cells/well) were seeded into 96-well plates (3 parallel wells in each group) and cultured in the complete medium (5% CO_2, 37°C, 95% humidity) for 24, 48 and 72 h. After incubation, 10 μl of CCK-8 solution was added to each well. After incubation for 1 h at 37°C, the absorbance of each well at 450 nm was determined using a microplate reader.

Cell migration assay

After transfection of SERCA2 siRNA or scrambled siRNA into the ASMCs isolated from normal or asthmatic rats, the cells were allowed to grow to confluence and maintained in growth factor free medium for 24 h prior to experiments. Then the cell were harvested, resuspended in serum free medium and added to the upper well of a Transwell (Corning Costar) at 2.0×10^5 cells/well. The chemoattractants (600 μl) were added to the lower wells. After 5 h, cells that migrated to the lower face of the membrane were fixed, stained and counted in 6-12 random fields in each well.

Statistical analysis

Results are expressed as mean ± SD. The significance of difference between the experimental groups and controls was assessed by Student's t-test. A difference was deemed significant if $P < 0.05$ or 0.01.

Results

Reduced SERCA2 expression results in altered calcium homeostasis in ASMCs from asthmatic rats

Here we found SERCA2 transcription and translation were both reduced in ASMCs obtained from asthmatic rats relative to that in normal rats (**Figure 1A-C**), and that reduction of SERCA2 expression might be related to disease severity. However, no significant difference was observed in IP3R1 expression between normal and asthmatic ASMCs (**Figure 1A-C**), indicating the change in SERCA2 was not due to a reduction in total SR [10]. To evaluate the effect of SERCA2 deficiency on the Ca^{2+} storage of SR, the cells were stimulated with 1 μM of bradyki-

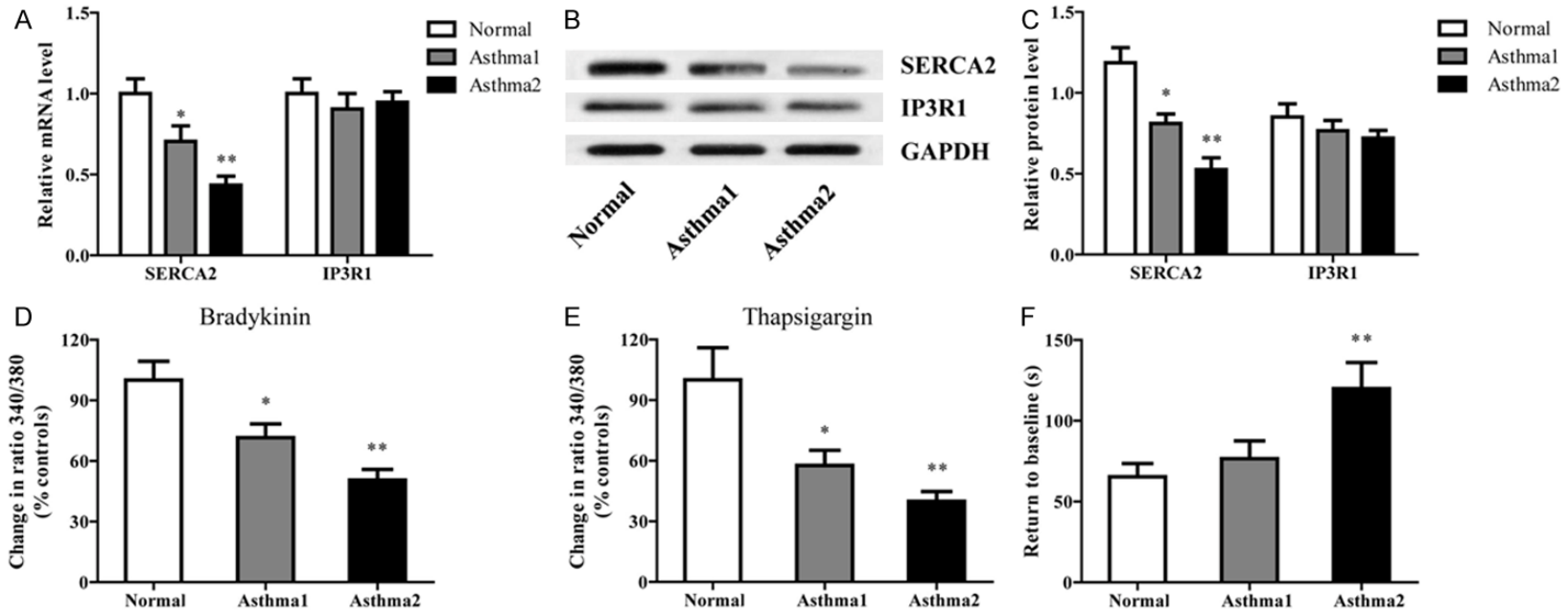

Figure 1. SERCA2 and IP3R1 expression levels and calcium store release in primary airway smooth muscle cells (ASMCs) isolated from rats with or without OVA induced asthma. The rats in asthma1 and asthma2 group were sensitized and challenged with 1% and 2% OVA saline solution respectively, whereas the rats in normal group were treated synchronously with physiological saline. Animals were sacrificed on days 23, and the ASMCs were isolated, purified and identified. The expression of SERCA2 and IP3R1 in normal and asthmatic ASMCs were evaluated using qRT-PCR (A) and western blotting (B, C) assay. Transient peak Ca^{2+} responses was measured by fura PE-3 in normal and asthmatic ASMCs after stimulation with 1 μM of bradykinin (D) or 0.1 μM of thapsigargin (E). (F) The time interval taken for the peak response to return to baseline following stimulation with 1 μM of bradykinin was also recorded. $*P < 0.05$ versus Normal, $**P < 0.01$ versus Normal.

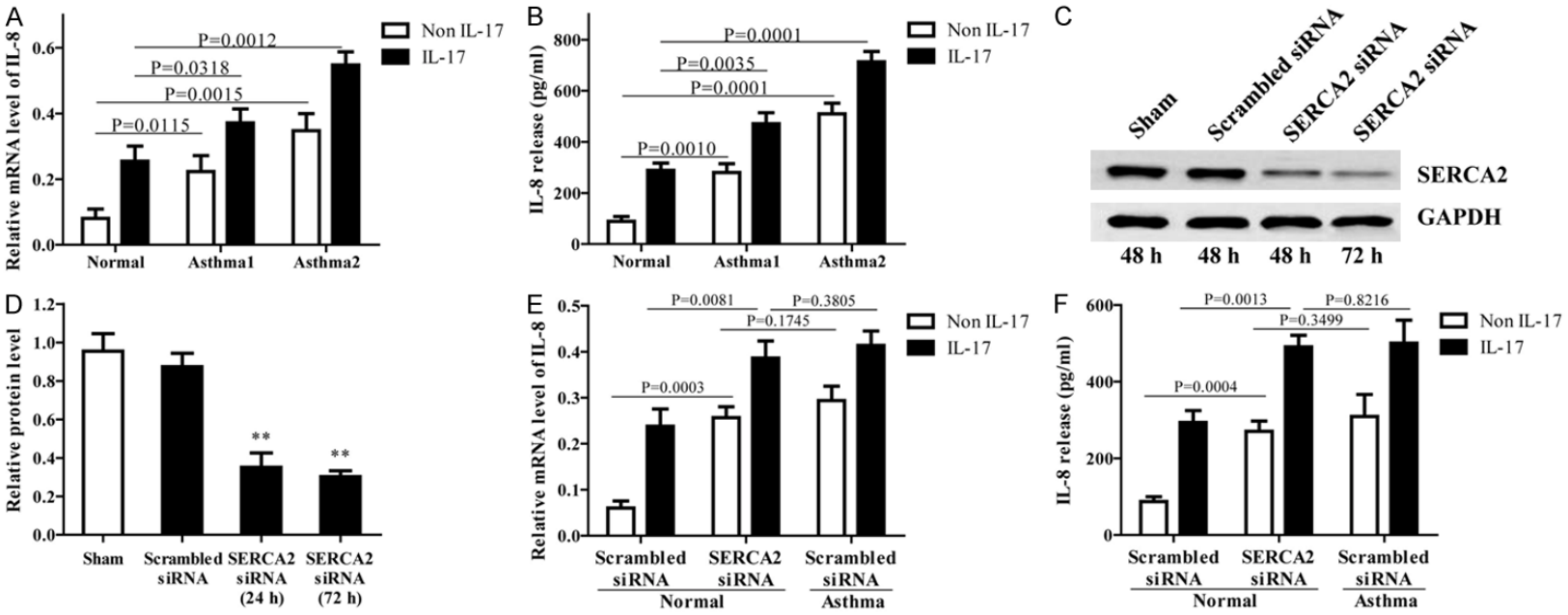

Figure 2. IL-8 mRNA expression and release in normal, asthmatic ASMCs, and normal ASMCs transfected with SERCA2 siRNA. The ASMCs isolated from normal or asthmatic rats were cultured in the presence or absence of 0.1 ng/ml of recombinant rat IL-17 for 24 h, and then the cells and the media were collected and analyzed for IL-8 mRNA expression and release using qRT-PCR and ELISA assay (A, B). Rat ASMCs were either sham transfected, transfected with scrambled non-targeting siRNA or SERCA2 siRNA. At 48 or 72 h post-transfection, the cells were harvested and lysed for the determination of SERCA2 protein expression using western blotting (C) and the relative expression were quantified by densitometry (D). After confirmation of successful transfection the cells were stimulated with or with not IL-17 for 24 h, and IL-8 expression and release were measured as mentioned above (E, F). $*P < 0.05$ versus Sham, $**P < 0.01$ versus Sham.

nin (BK) to induce Ca^{2+} release from the SR and the cytosolic calcium was measured by fura PE-3. Compared with normal ASMCs, transient peak Ca^{2+} responses to BK were significantly decreased in asthmatic ASMCs (**Figure 1D**), which indicated that SR stores are partially depleted in ASMCs from asthmatic rats [10]. To test this hypothesis, SERCA were inhibited using 0.1 μM of thapsigargin (TSG) to empty SR, and the resulting change in $[Ca^{2+}]_i$ was used as an estimate of the Ca^{2+} content of the SR. The following increase in $[Ca^{2+}]_i$ was also markedly higher in ASMCs from normal rats (**Figure 1E**). Furthermore, asthmatic ASMCs took longer time to restore BK-elevated $[Ca^{2+}]_i$ to baseline than normal ASMCs (**Figure 1F**), which revealed SR dysfunctions in asthmatic ASMCs.

SERCA2 knockdown enhances IL-8 mRNA expression and release in ASMCs

Interleukin (IL)-8 is a kind of important inflammatory mediators derived from ASMCs, which may evoke the migration of neutrophils, monocytes and eosinophils to the sites of inflammation [17], and plays an important role in the pathogenesis of asthma. Here we found both basal mRNA expression and IL-17 induced expression of IL-8 were significantly higher in asthmatic ASMCs when compared with normal ASMCs (**Figure 2A**). IL-8 release was correspondingly increased in asthmatic ASMCs (**Figure 2B**). Whether IL-8 release in asthmatic ASMCs is modulated by SERCA2 deficiency is unclear. To address this, Inhibition of SERCA2 expression was performed using siRNA approach. After confirmation of SERCA2 knockdown using western blotting (**Figure 2C**, **2D**), the mRNA expression and release of IL-8 by normal ASMCs transfected with scrambled siRNA or SERCA2 siRNA, and asthmatic ASMCs transfected with scrambled siRNA with or without IL-17 stimulation were detected. It was found that SERCA2 silence markedly facilitated IL-8 mRNA expression and release in normal ASMCs, and no significant difference was observed when compared with asthmatic ASMCs transfected with scrambled siRNA (**Figure 2E**, **2F**), presenting a vital role of SERCA2 in regulating IL-8 production by ASMCs.

SERCA2 knockdown enhances NF-κB activation that mediates IL-17 induced IL-8 release

Accumulating studies have related intracellular calcium mobilization to the activation of NF-κB and inflammatory signaling responses [18, 19]. To examine whether NF-κB activation is involved in SERCA2 dysfunction induced IL-8 release in asthmatic ASMCs, we first examined NF-κB activation in normal and asthmatic ASMCs using western blotting. It was found both IκBα phosphorylation and NF-κB p65 translocation were significantly increased in asthmatic ASMCs relative to normal ASMCs (**Figure 3A**, **3B**). Then we evaluate the effect of SERCA2 on the activation of NF-κB in normal ASMCs. We found both SERCA2 knockdown using siRNA and inhibition using TSG promote NF-κB p65 translocation (**Figure 3C**, **3D**), suggesting that decreased SERCA2 expression may be responsible for the activation of NF-κB in ASMCs during asthma. To examine whether NF-κB activation is necessary for the IL-17 induced IL-8 production in ASMCs, an NF-κB inhibitor, PDTC, was used. It was found IL-17 induced IL-8 mRNA expression and release was remarkably attenuated by 10 μM of PDTC in normal ASMCs transfected with either scrambled siRNA or SERCA2 siRNA, and asthmatic ASMCs transfected with scrambled siRNA (**Figure 3E**). Overall, in asthmatic ASMCs, reduced SERCA2 expression results in enhanced NF-κB activation, which mediates IL-8 synthesis and release.

Asthmatic ASMCs released IL-8 promotes their migration in vitro

As the promoting effect of SERCA2 dysfunction on IL-8 release in asthmatic ASMCs has been confirmed, we wondered whether SERCA2 dysfunction was related to the phenotypic modulation of ASMCs in asthma, including enhanced proliferation and migration. To address this, CCK-8 and transwell assays were carried out. It was found that both proliferation and migration were increased in asthmatic ASMCs when compared with normal ASMCs, and SERCA2 knockdown markedly facilitate these activities of normal ASMCs (**Figure 4**), which presented that SERCA2 deficiency not only promotes IL-8 production in ASMCs but also enhances their proliferation and migration, but the corresponding mechanism is not clear. As previously mentioned, ASMCs express functional IL-8 receptors, CXCR1 and CXCR2 [17], we speculated that IL-8 derived from ASMCs promoted their proliferation and migration. To test this, the asthmatic ASMCs transfected with scrambled siRNA were pretreated with 1 ng/ml anti-IL-8 antibody before using in proliferation and

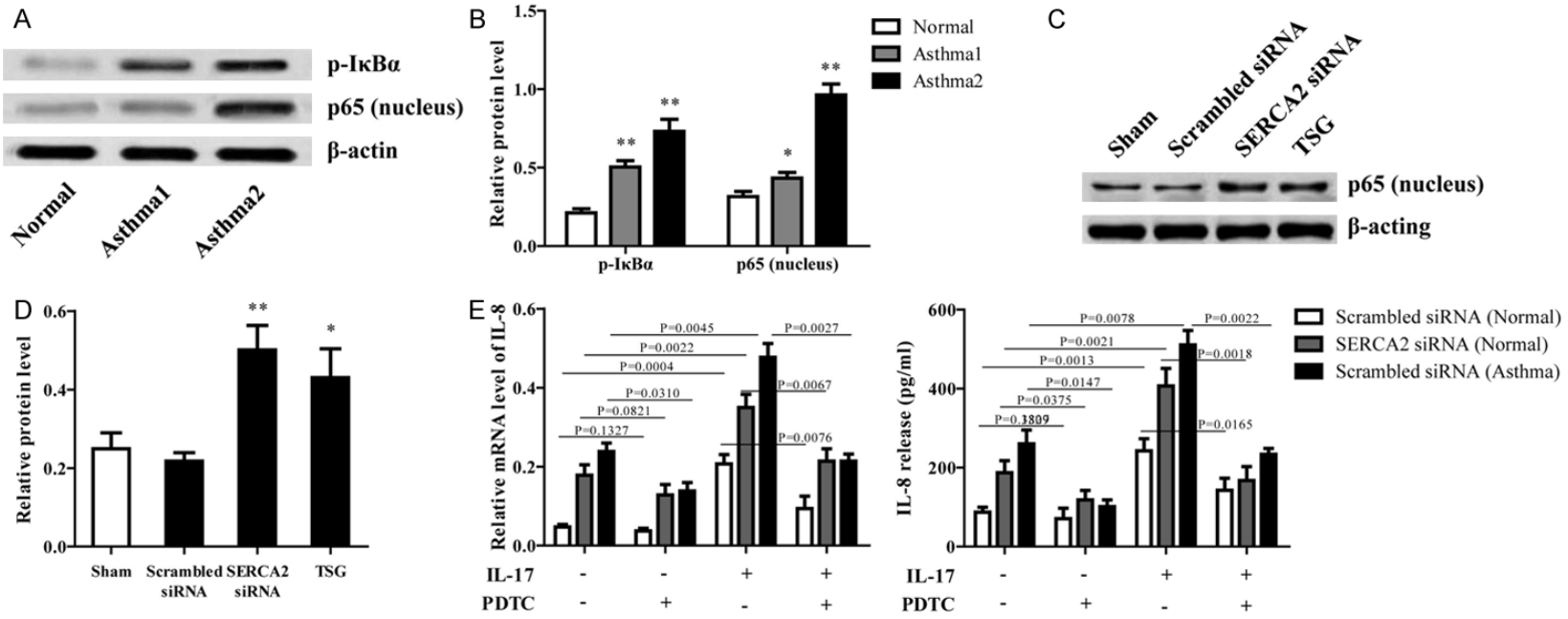

Figure 3. NF-κB activation in normal, asthmatic ASMCs and SERCA2 knockdown normal ASMCs, and IL-17 induced IL-8 mRNA expression and release in the absence or presence of PDTC. The phosphorylation of IκBα and NF-κB p65 translocation in primary airway smooth muscle cells (ASMCs) isolated from rats in normal, asthma1 and asthma2 groups were determined using western blotting assay (A), and the relative protein levels were quantified by densitometry (B). The SERCA2 in normal ASMCs were knock using siRNA or inhibited using 0.1 μM of thapsigargin (TSG) as mentioned above, and the NF-κB p65 translocation were determined using western blotting (C, D). (E) The IL-17 induced IL-8 mRNA expression and release in normal ASMCs transfected with either scrambled siRNA or SERCA2 siRNA, and asthmatic ASMCs transfected with scrambled siRNA were measured using qRT-PCR and ELISA assays. **P* < 0.05 versus Sham, ***P* < 0.01 versus Sham.

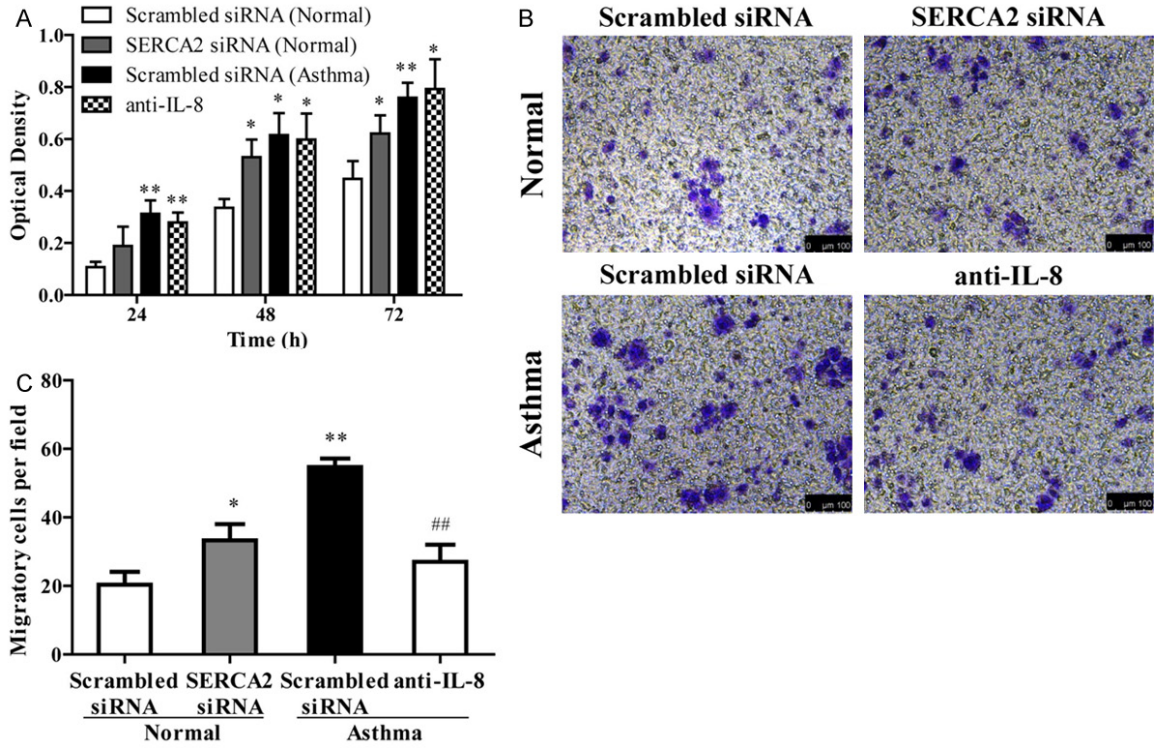

Figure 4. Proliferation and migration of normal ASMCs transfected with either scrambled siRNA or SERCA2 siRNA and asthmatic ASMCs transfected with either scrambled siRNA, and the effect of IL-8 depletion on these activities of asthmatic ASMCs. (A) After transfection of SERCA2 siRNA or scrambled siRNA into the ASMCs isolated from normal or asthmatic rats, the cells were cultured in the complete medium for 24, 48 and 72 h, and the proliferation was determined using CCK-8 assay. (B) After transfection, the cells were allowed to grow to confluence and maintained in growth factor free medium for 24 h prior to experiments. Then the cell were harvested, resuspended in serum free medium and added to the upper well of a Transwell at 2.0×10^5 cells/well. After 5 h, cells that migrated to the lower face of the membrane were fixed, stained and counted in 6-12 random fields in each well (C). For IL-8 depletion, the cells were pretreated with 1 ng/ml anti-IL-8 antibody before using in proliferation and migration assays. $^*P < 0.05$ versus scrambled siRNA (Normal), $^{**}P < 0.01$ versus scrambled siRNA (Normal). $^{\#}P < 0.05$ versus scrambled siRNA (Asthma), $^{\#\#}P < 0.01$ versus scrambled siRNA (Asthma).

migration assays. It was found that IL-8 depletion exhibited little influence on proliferation rate of asthmatic ASMCs, but observably suppressed the enhanced migration (**Figure 4**), which suggests that ASMCs released IL-8 promotes their migration during asthma, but may have no influence on their proliferation.

Discussion

Reversible airway obstruction is an important clinical symptom of bronchial asthma (asthma), and its main mechanism is suggested to be dysfunction of airway smooth muscle (ASM), which is characterized by increased contractile response to agonists, hyperplasia, hypertrophy and abnormal secretion of inflammatory cytokines. Extensive research has implied that these abnormalities may be related to the imbalance of cytosolic calcium homeostasis [5].

The regulation of cytosolic calcium homeostasis is a complex process involved multiple receptors and ion channels located in the sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER), and cytoplasmic calcium buffer systems. The binding of agonists such as acetylcholine and bradykinin (BK) to corresponding G protein coupled receptors results in the activation of phospholipase C, which promotes phosphatidylinositol biphosphate to decompose into inositol trisphosphate (IP3) and diacylglycerol (DAG). The combination of IP3 with IP3 receptors (IP3R) in the SR leads to the release of SR calcium, which consequently active sarcoplasmic reticulum ryanodine receptors (RyRs) in the SR and causes further release

of SR calcium. SR calcium release induced $[Ca^{2+}]_i$ rise is not static, but a cyclical rise and fall, that is, calcium oscillation. Calcium release also activates multiple calcium influx pathways. Calcium influx can maintain the amplitude and duration of calcium oscillations. The rise in $[Ca^{2+}]_i$ can be normalized by extracellular removal of cytosolic Ca^{2+} by the Na^+-Ca^{2+}-exchanger (NCX, $3Na^+$: $1Ca^{2+}$) and plasma membrane Ca^{2+} ATPase (PMCA), and by its rapid sequestration into SR stores by the sarco/endoplasmic reticulum Ca^{2+} (SERCA) pump [20].

Accumulating studies have presented that dysregulation of Ca^{2+} homeostasis caused at least partly by a downregulation in expression and activity of SERCA pump, which is central to the abnormal asthmatic phenotype of ASM, including exaggerated contractile response [21, 22], hyperplasia, migration, and excessive release of chemokines and cytokines [8]. Katharina Mahn et al. have reported previously that SERCA2 protein expression in both native and cultured ASM from endobronchial biopsies of patients with mild and moderate/severe asthma is reduced when compared with healthy subjects and the extent of this effect correlated with disease severity [8]. Although the mechanisms account for this effect have not been fully elucidated, Sathish et al. found that SERCA expression and function of healthy ASM were suppressed by proinflammatory cytokines, IL-13 and TNF-α exposure, which related airway inflammation to reduced ASM SERCA2 expression in asthma. In accordance with previous reports, we also found that expression and activity of SERCA2 were both decreased in ASMCs isolated asthmatic rats with OVA induced asthma. Asthmatic ASMCs exhibited the typical asthmatic phenotype, including enhanced proliferation and migration, and increased IL-8 release. More important, SERCA2 knockdown using siRNA in ASMCs derived from normal rats recapitulated the asthmatic phenotype. The results indicated that reduced SERCA2 plays an important, if not critical role in the imbalance of ASM Ca^{2+} homeostasis in asthma and the consequent phenotypic modulation.

IL-8, the CX-C chemokine, is an important neutrophil chemoattractant and may play a critical role in acute severe asthma. Claudia et al. found that the number of neutrophils and IL-8 level in tracheal aspirates collected within 12 h of intubation from patients intubated emergently for acute severe asthma were both significantly increased, whereas the numbers of eosinophils were 8-fold less than neutrophils and therefore, they demonstrated neutrophils are the dominant inflammatory leukocyte characterizing airway inflammation in acute severe asthma that requires mechanical ventilation and IL-8 is an important mediator of this neutrophilia [6]. An analysis of a panel of 48 cytokines in bronchoalveolar lavage (BAL) fluids found that neutrophils and IL-8 are the only inflammatory components in BAL fluids that distinguish controlled asthma from uncontrolled asthma [23]. *In vitro* studies have identified various potential sources of IL-8 in the airway, including airway epithelial cells [24], macrophages [25], mast cells [26] and fibroblasts [27]. ASMCs is not only the target cells of multiple proinflammatory cytokines, but also a rich source of inflammatory mediators, such as IL-8, eotaxin, CXCL-10, and may play a critical role in perpetuating the inflammatory process in the airway [28]. Digiovine et al. proved that ASMCs is the most important source of IL-8 in patients with obliterative bronchiolitis (OB) after lung transplantation [7]. Fong et al. found transforming growth factor (TGF)-β1 stimulation could significantly promote cultured human ASMCs IL-8 release in a concentration and time-dependent manner [29]. However, the underlying mechanism responsible for the increased IL-8 generation in ASMCs is not clear yet. Pang et al. demonstrated that bradykinin (BK) treatment enhanced IL-8 release of confluent serum deprived human ASMCs in a concentration- and time-dependent fashion (maximum 50-fold increase over basal). Given the reality that BK is an inhibitor of SERCA2, we speculated that increased IL-8 production might rise from the reduced SERCA2 expression and activity in asthmatic ASMCs. We found that SERCA2 knockdown in normal ASMCs using siRNA remarkably facilitated IL-17 induced IL-8 expression and release, which mimicked the IL-8 generation by asthmatic ASMCs.

Due to the vital role of SERCA2 in the generation of Ca^{2+} oscillations [30, 31], the decreased expression and/or function of SERCA2 would lead to altered dynamics of Ca^{2+} oscillations [32]. Ricardo E. Dolmetsch et al. demonstrated that rapid oscillations stimulate all three proinflammatory transcription factors NF-AT, Oct/OAP and NF-κB, whereas infrequent oscillations

activate only NF-κB [33]. Zhu et al. found $[Ca^{2+}]_i$ oscillation regulates NF-κB transcriptional activity, phosphorylation of IκBα and Ca^{2+}-dependent gene expression all in a way actually dependent on cumulated $[Ca^{2+}]_i$ spike duration whether or not frequency varies. There is growing evidence that Ca^{2+} signaling is important in the regulation of NF-κB activation in other cell types. It was found that phospholipase C/Ca^{2+} inhibitors could block recombinant human LIGHT, a ligand of herpes virus entry mediator, mediated IκBα degradation, generation of reactive oxygen species and TNF-α production in human monocytes [34]. Dolmetsch et al. found NF-κB are selectively activated by a large transient $[Ca^{2+}]_i$ rise in B lymphocytes, whereas NFAT is activated by a low, sustained Ca^{2+} plateau [35]. In addition, $[Ca^{2+}]_i$ homeostasis is also involved in NF-κB in ASMCs. Amrani et al. demonstrated that thapsigargin could reduce TNF-α induced expression of intercellular adhesion molecule 1 (ICAM-1) by suppressing the activation of NF-κB [36]. Moreover, nickel ions (Ni^{2+}), a broad Ca^{2+} channel blocker to reduce Ca^{2+} influx, abrogated CD40-induced NF-κB activation in human ASMCs [37]. Here, we observed that SERCA2 knockdown using siRNA or inhibition by thapsigargin induced NF-κB activation, and that was integral for the expression and release of IL-8 in ASMCs. Thus, we speculated that SERCA2 deficiency in asthmatic ASMCs contribute to altered Ca^{2+} oscillations and subsequent NF-κB activity, which eventually results in increased IL-8 generation.

Given the fact that human ASMCs constitutively express functional IL-8 receptors (CXCR1 and CXCR2) linked to cell contraction and migration [38], we hypothesized that IL-8 might act directly on ASMCs and contribute to the enhanced airway remodeling in asthma. The effect of IL-8 on ASMCs proliferation, migration and contraction has been extensively studied. Govindaraju et al. found stimulation with IL-8 induced an evaluation of $[Ca^{2+}]_i$ and migration in human ASMCs, and a decrease in the length of cells [38]. Tang et al. reported IL-8 derived from bronchial epithelium caused bronchial smooth muscle proliferation and migration [39]. In accordance with previous reports, our work showed that IL-8 depletion using anti-IL-8 antibody exhibited little influence on proliferation of asthmatic ASMCs, but observably suppressed the enhanced migration, indicating that IL-8 released by ASMCs could in turn promote their migration during asthma, but may have little influence on their proliferation.

In conclusion, the expression and function of SERCA2 are decreased in asthmatic ASMCs, which lead to altered Ca^{2+} homeostasis and subsequent NF-κB activation. Increased NF-κB activation results in the upregulation of IL-8 expression and release, which in turn promote ASMCs migration. SERCA2 deficiency also accounts for the increased proliferation of ASMCs, but the corresponding mechanism remains unknown. SERCA2 may server as a new target in asthma treatment.

Acknowledgements

The authors wish to thank the Medical and Health Technology Projects of Hangzhou (Grant No.: 2015A0), Northern Regional Special Disease Center of Zhejiang Province Fund and Hangzhou Key Disciplines Fund.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xianmei Huang, Department of Pediatrics, Hangzhou First People's Hospital, Nanjing Medical University, 261 Huansha Road, Hangzhou 310006, Zhejiang Province, China. Tel: +86-571-56005600; Fax: +86-571-87914773; E-mail: hxianmei630715@163.com

References

[1] Bousquet J, Khaltaev N, Bousquet J and Khaltaev N. Global surveillance, prevention and control of chronic respiratory diseases: a comprehensive approach. Journal of Pharmacy Technology Jpt Official Publication of the Association of Pharmacy Technicians 2008; 24: 122-122.

[2] Berair R, Saunders R and Brightling CE. Origins of increased airway smooth muscle mass in asthma. BMC Med 2013; 11: 145.

[3] Chang Y, Alalwan L, Risse PA, Roussel L, Rousseau S, Halayko AJ, Martin JG, Hamid Q and Eidelman DH. TH17 cytokines induce human airway smooth muscle cell migration. J Allergy Clin Immunol 2011; 127: 1046-53, e1-2.

[4] Bara I, Jm OADL, Marthan R and Berger P. Pathophysiology of bronchial smooth muscle remodelling in asthma. Eur Respir J 2010; 36: 1174-1184.

[5] Perez-Zoghbi JF, Karner C, Ito S, Shepherd M, Alrashdan Y and Sanderson MJ. Ion channel regulation of intracellular calcium and airway

smooth muscle function. Pulm Pharmacol Ther 2009; 22: 388-397.
[6] Ordoñez CL, Shaughnessy TE, Matthay MA and Fahy JV. Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma. Am J Respir Crit Care Med 2000; 161: 1185-1190.
[7] DiGiovine B, Lynch JP 3rd, Martinez FJ, Flint A, Whyte RI, Iannettoni MD, Arenberg DA, Burdick MD, Glass MC, Wilke CA, Morris SB, Kunkel SL, Strieter RM. Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. J Immunol 1996; 157: 4194-4202.
[8] Mahn K, Hirst SJ, Ying S, Holt MR, Lavender P, Ojo OO, Siew L, Simcock DE, McVicker CG, Kanabar V, Snetkov VA, O'Connor BJ, Karner C, Cousins DJ, Macedo P, Chung KF, Corrigan CJ, Ward JP, Lee TH. Diminished sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) expression contributes to airway remodelling in bronchial asthma. Proc Natl Acad Sci U S A 2009; 106: 10775-10780.
[9] Sathish V, Thompson MA, Bailey JP, Pabelick CM, Prakash YS and Sieck GC. Effect of proinflammatory cytokines on regulation of sarcoplasmic reticulum Ca2+ reuptake in human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 2009; 297: 26-34.
[10] Mahn K, Hirst SJ, Ying S, Holt MR, Lavender P, Ojo OO, Siew L, Simcock DE, McVicker CG, Kanabar V, Snetkov VA, O'Connor BJ, Karner C, Cousins DJ, Macedo P, Chung KF, Corrigan CJ, Ward JP, Lee TH. Diminished sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) expression contributes to airway remodelling in bronchial asthma. Proc Natl Acad Sci U S A 2009; 106: 10775-10780.
[11] Grover AK and Khan I. Calcium pump isoforms: diversity, selectivity and plasticity. Review article. Cell Calcium 1992; 13: 9-17.
[12] Brini M and Carafoli E. Calcium pumps in health and disease. Physiol Rev 2009; 89: 1341-1378.
[13] Yang M, Zhao X, Liu Y, Tian Y, Ran X and Jiang Y. A role for WNT1-inducible signaling protein-1 in airway remodeling in a rat asthma model. Int Immunopharmacol 2013; 17: 350-357.
[14] Dai YR, Wu HY, Wu LQ, Xu H, Yin J, Yan SS and Zeng WX. Roxithromycin reduces the viability of cultured airway smooth muscle cells from a rat model of asthma. Eur Rev Med Pharmacol Sci 2014; 18: 3564-3572.
[15] Ahmad S, Nichols DP, Strand M, Rancourt RC, Randell SH, White CW and Ahmad A. SERCA2 regulates non-CF and CF airway epithelial cell response to ozone. PLoS One 2011; 6: e27451.
[16] Vanaudenaerde BM, Wuyts WA, Dupont LJ, Van Raemdonck DE, Demedts MM and Verleden GM. Interleukin-17 stimulates release of interleukin-8 by human airway smooth muscle cells in vitro: a potential role for interleukin-17 and airway smooth muscle cells in bronchiolitis obliterans syndrome. J Heart Lung Transplant 2003; 22: 1280-1283.
[17] Govindaraju V, Michoud MC, Alchalabi M, Ferraro P, Powell WS and Martin JG. Interleukin-8: novel roles in human airway smooth muscle cell contraction and migration. Am J Physiol Cell Physiol 2006; 291: C957-965.
[18] Fu Z, Bettega K, Carroll S, Buchholz KR, Machen TE. Role of Ca2+ in responses of airway epithelia to pseudomonas aeruginosa, flagellin, ATP, and thapsigargin. Am J Physiol Lung Cell Mol Physiol 2007; 292: 353-364.
[19] Hybiske K, Fu Z, Schwarzer C, Tseng J, Do J, Huang N and Machen TE. Effects of cystic fibrosis transmembrane conductance regulator and DeltaF508CFTR on inflammatory response, ER stress, and Ca2+ of airway epithelia. Am J Physiol Lung Cell Mol Physiol 2007; 293: L1250-1260.
[20] Pozzan T, Rizzuto R, Volpe P and Meldolesi J. Molecular and cellular physiology of intracellular calcium stores. Physiol Rev 1994; 74: 595-636.
[21] Triggle DJ. Calcium, the control of smooth muscle function and bronchial hyperreactivity. Allergy 1983; 38: 1-9.
[22] Perez JF and Sanderson MJ. The frequency of calcium oscillations induced by 5-HT, ACH, and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. J Gen Physiol 2005; 125: 535-553.
[23] Hosoki K, Ying S, Corrigan C, Qi H, Kurosky A, Jennings K, Sun Q, Boldogh I and Sur S. Analysis of a panel of 48 cytokines in BAL fluids specifically identifies IL-8 levels as the only cytokine that distinguishes controlled asthma from uncontrolled asthma, and correlates inversely with FEV1. PLoS One 2015; 10: e0126035.
[24] Marini M, Vittori E, Hollemborg J and Mattoli S. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. J Allergy Clin Immunol 1992; 89: 1001-1009.
[25] Folkard SG, Westwick J and Millar AB. Production of interleukin-8, RANTES and MCP-1 in intrinsic and extrinsic asthmatics. Eur Respir J 1997; 10: 2097-2104.
[26] Möller A, Lippert U, Lessmann D, Kolde G, Hamann K, Welker P, Schadendorf D, Rosenbach T, Luger T and Czarnetzki BM. Human mast cells produce IL-8. J Immunol 1993; 151: 3261-3266.
[27] Rolfe MW, Kunkel SL, Standiford TJ, Chensue SW, Allen RM, Evanoff HL, Phan SH and Striet-

er RM. Pulmonary fibroblast expression of interleukin-8: a model for alveolar macrophage-derived cytokine networking. Am J Respir Cell Mol Biol 1991; 5: 493-501.
[28] Johnson SR and Knox AJ. Synthetic functions of airway smooth muscle in asthma. Trends Pharmacol Sci 1997; 18: 288-292.
[29] Fong CY, Pang L, Holland E and Knox AJ. TGF-β1 stimulates IL-8 release, COX-2 expression, and PGE2release in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2000; 279: L201-7.
[30] Berridge MJ. Smooth muscle cell calcium activation mechanisms. J Physiol 2008; 586: 5047-5061.
[31] Parameswaran K, Janssen LJ and O'Byrne PM. Airway hyperresponsiveness and calcium handling by smooth muscle: a "deeper look". Chest 2002; 121: 621-624.
[32] Mahn K, Ojo OO, Chadwick G, Aaronson PI, Ward JP and Lee TH. Ca(2+) homeostasis and structural and functional remodelling of airway smooth muscle in asthma. Thorax 2010; 65: 547-552.
[33] Dolmetsch RE, Xu K and Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. Nature 1998; 392: 933-936.
[34] Heo SK, Yoon MA, Lee SC, Ju SA, Choi JH, Suh PG, Kwon BS and Kim BS. HVEM signaling in monocytes is mediated by intracellular calcium mobilization. J Immunol 2007; 179: 6305-6310.
[35] Dolmetsch RE, Lewis RS, Goodnow CC and Healy JI. Differential of transcription factor induced by Ca2+ response amplitude and duration. Nature 1997; 386: 855-858.
[36] Amrani Y, Lazaar AL, Hoffman R, Amin K, Ousmer S, Panettieri RA Jr. Activation of p55 tumor necrosis factor-alpha receptor-1 coupled to tumor necrosis factor receptor-associated factor 2 stimulates intercellular adhesion molecule-1 expression by modulating a thapsigargin-sensitive pathway in human tracheal smooth muscle ce. Mol Pharmacol 2000; 58: 237-245.
[37] Lazaar AL, Amrani Y, Hsu J, Panettieri RA, Fanslow WC, Albelda SM and Puré E. CD40-mediated signal transduction in human airway smooth muscle. J Immunol 1998; 161: 3120-3127.
[38] Govindaraju V, Michoud MC, Al-Chalabi M, Ferraro P, Powell WS and Martin JG. Interleukin-8: novel roles in human airway smooth muscle cell contraction and migration. Am J Physiol Cell Physiol 2006; 291: C957-965.
[39] Tang H, Sun Y, Shi Z, Huang H, Fang Z, Chen J, Xiu Q and Li B. YKL-40 induces IL-8 expression from bronchial epithelium via MAPK (JNK and ERK) and NF-κB pathways, causing bronchial smooth muscle proliferation and migration. J Immunol 2013; 190: 438-446.