

Targeted Inhibition of KCa3.1 Channel Attenuates Airway Inflammation and Remodeling in Allergic Asthma

Zhi-Hua Yu¹, Jian-Rong Xu¹, Yan-Xia Wang¹, Guang-Ni Xu¹, Zu-Peng Xu¹, Kai Yang¹, Da-Zheng Wu², Yong-Yao Cui^{1*}, and Hong-Zhuan Chen^{1*}

¹Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai, China; and ²Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China

KCa3.1 has been suggested to be involved in regulating cell activation, proliferation, and migration in multiple cell types, including airway inflammatory and structural cells. However, the contributions of KCa3.1 to airway inflammation and remodeling and subsequent airway hyperresponsiveness (AHR) in allergic asthma remain to be explored. The main purpose of this study was to elucidate the roles of KCa3.1 and the potential therapeutic value of KCa3.1 blockers in chronic allergic asthma. Using real-time PCR, Western blotting, or immunohistochemical analyses, we explored the precise role of KCa3.1 in the bronchi of allergic mice and asthmatic human bronchial smooth muscle cells (BSMCs). We found that KCa3.1 mRNA and protein expression were elevated in the bronchi of allergic mice, and double labeling revealed that up-regulation occurred primarily in airway smooth muscle cells. Triaryl methane (TRAM)-34, a KCa3.1 blocker, dose-dependently inhibited the generation and maintenance of the ovalbumin-induced airway inflammation associated with increased Th2-type cytokines and decreased Th1-type cytokine, as well as subepithelial extracellular matrix deposition, goblet-cell hyperplasia, and AHR in a murine model of asthma. Moreover, the pharmacological blockade and gene silencing of KCa3.1, which was evidently elevated after mitogen stimulation, suppressed asthmatic human BSMC proliferation and migration, and arrested the cell cycle at the G0/G1 phase. In addition, the KCa3.1 activator 1-ethylbenzimidazolinone-induced membrane hyperpolarization and intracellular calcium increase in asthmatic human BSMCs were attenuated by TRAM-34. We demonstrate for the first time an important role for KCa3.1 in the pathogenesis of airway inflammation and remodeling in allergic asthma, and we suggest that KCa3.1 blockers may represent a promising therapeutic strategy for asthma.

Keywords: KCa3.1; airway inflammation; remodeling; asthma; bronchial smooth muscle cells

Asthma is characterized by chronic inflammation of the airways, with increased lung infiltration by inflammatory cells, mainly eosinophils, lymphocytes, macrophages, and neutrophils (1–3). The inflammatory mediators secreted by these cells contribute to changes in airway structure and persistent airway hyperresponsiveness (AHR). Among the histopathological characteristics of

CLINICAL RELEVANCE

KCa3.1 mRNA and protein were up-regulated in the bronchi of ovalbumin (OVA)-challenged mice, and double labeling revealed that up-regulation occurred primarily in airway smooth muscle cells. We show that blocking KCa3.1 inhibited OVA-induced airway inflammation significantly, concomitant with Th2-type cytokine production, subepithelial extracellular matrix deposition, mucus-secreting goblet-cell up-regulation, and airway hyperresponsiveness. A triaryl-methane (TRAM-34) or KCa3.1 gene silencing suppressed the asthmatic human bronchial smooth muscle cell proliferation and migration associated with cell-cycle arrest at the G0/G1 phase, through influences on membrane potential and Ca²⁺ signaling. These findings provide support for the therapeutic potential of KCa3.1-specific blockers in human allergic asthma.

airway remodeling, increased extracellular matrix (ECM) deposition, mucous gland hyperplasia with associated mucus hypersecretion, and airway smooth muscle cell (SMC) proliferation may result in the decline in lung function characteristic of chronic asthma (4–6).

Corticosteroids are currently the cornerstone of asthma management. They are efficacious in inhibiting proinflammatory cell recruitment (7). However, airway remodeling and AHR are less sensitive to steroids, especially in severe persistent asthma, whereas the use of monoclonal antibodies, such as anti-TNF- α , has proven largely ineffective (8–10). Thus a clinical need continues for new asthma drugs with different mechanisms of action that address the other hallmark symptoms of asthma.

The intermediate-conductance, calcium-activated potassium channel, composed of four KCa3.1 subunits, is expressed in T lymphocytes, macrophages (11), mast cells (12), airway epithelium (13), fibroblasts (14), and both normal and asthmatic human airway SMCs (15), where it can communicate directly between Ca²⁺ signaling pathways and changes in the membrane potential required for various cellular processes, such as cell proliferation, migration, and activation (16). Small molecules and peptide toxins have been explored as KCa3.1 blockers, such as triaryl methanes (TRAM-34) and charybdotoxin. They inhibit airway SMC proliferation (15), fibrocyte migration (17), macrophage function (11), T-cell activation (18, 19), and epithelial cell proliferation (20). We thus hypothesized that KCa3.1 might serve as a novel therapeutic target shared by these cell types implicated in asthma.

Platelet-derived growth factor (PDGF) is a potent mitogen for mesenchymal cells, including fibroblasts and smooth muscle cells (21). Interestingly, mitogens (e.g., PDGF, basic fibroblast growth factor [bFGF], and vascular endothelial growth factor) have been found to up-regulate KCa3.1 in several cell types (22, 23). Conversely, in experimental angiogenesis, postinterventional arterial

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*Joint senior authors.

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Correspondence and requests for reprints should be addressed to Yong-Yao Cui, Ph.D., Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, China. E-mail: yongyaocui@yahoo.com.cn

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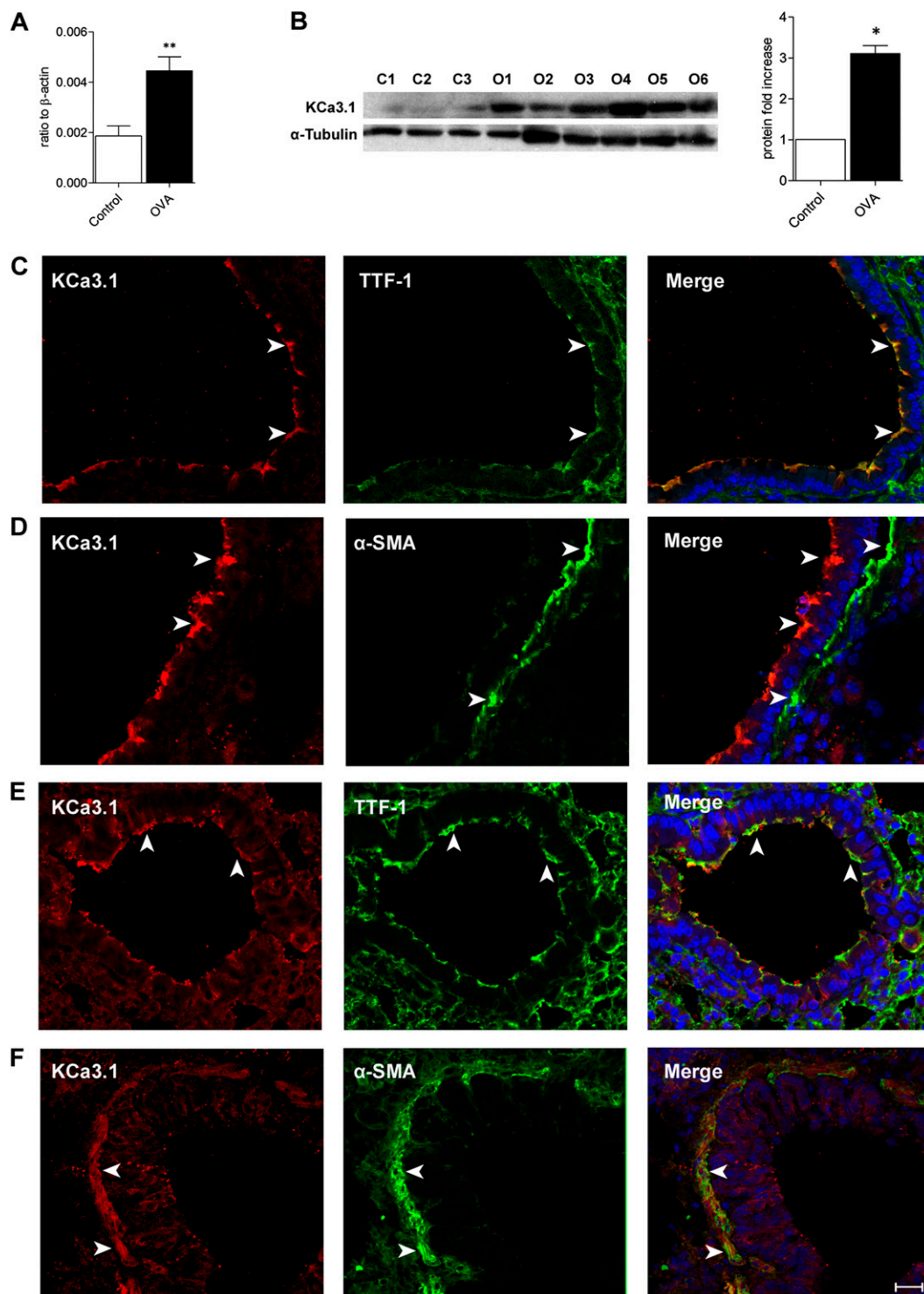


Figure 1. Up-regulation of KCa3.1 in the bronchi of ovalbumin (OVA)-challenged mice. (A) Quantitative real-time PCR analysis of KCa3.1 gene expression. Values are shown for steady-state transcripts relative to β -actin in the same preparation. Results are expressed as means $\pm$ SEMs (control bronchi, $n = 4$ animals; OVA-challenged bronchi, $n = 4$ animals). ** $P < 0.01$, versus control bronchi. (B) Western blot analysis of KCa3.1 protein expression in control bronchi and OVA-sensitized and OVA-challenged bronchi. Plasma membrane fractions were probed by immunoblotting with anti-KCa3.1 antibody. The quantification of KCa3.1 protein concentrations is presented (control bronchi, $n = 3$ animals; OVA-challenged bronchi, $n = 6$ animals). * $P < 0.05$, versus control bronchi. (C–F) Representative images show the immunoreaction for KCa3.1 in the bronchus of a control mouse (C and D) and of an OVA-induced and OVA-challenged mouse (E and F). Arrowheads show a positive immunoreaction for KCa3.1, thyroid transcription factor 1 (TTF-1), or α -smooth muscle actin (α -SMA), and the scale bar indicates 25 μ m. (original magnification, $\times 40$).

restenosis (24, 25), and atherosclerosis (18), both pharmacological inhibition and KCa3.1 knockout suppress mitogen-driven cell proliferation, thus ameliorating the progression of these diseases.

The main objective of this study comprised assessing the contribution of KCa3.1 channels to the pathogenesis of chronic allergic asthma. We demonstrate elevated expression levels of KCa3.1 in the airways of mice with asthma, especially in airway SMCs. The pharmacological blockade of KCa3.1 resulted in the suppression of airway inflammation, remodeling, and AHR *in vivo*. The blockade of KCa3.1 suppressed asthmatic human bronchial smooth muscle cell (BSMC) proliferation and migration, and arrested the cell

cycle at the G0/G1 phase *in vitro*. These results suggest that blocking the KCa3.1 channel could offer a potential therapeutic approach to treating chronic asthma.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Mice were aged 5 to 6 weeks and were housed in environmentally controlled, specific pathogen-free conditions for 1 week before the study. All experimental procedures

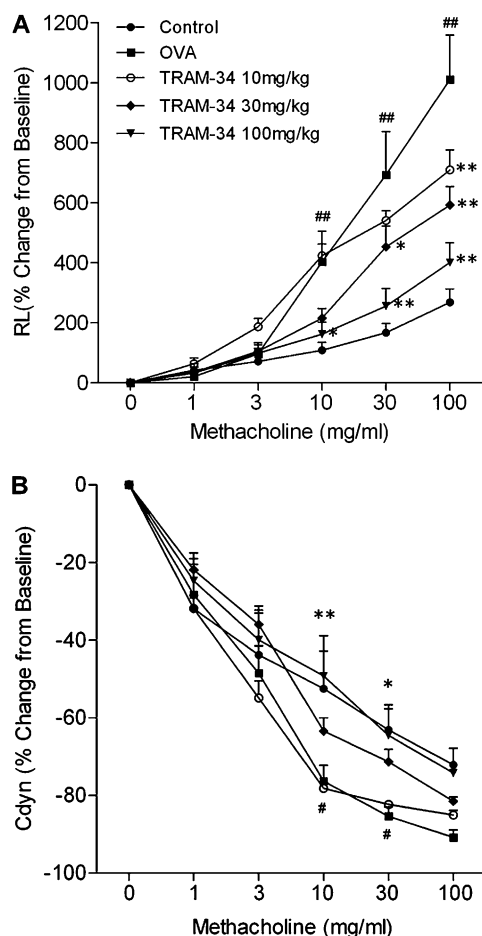


Figure 2. Blockade of KCa3.1 attenuates airway hyperresponsiveness. Airway hyperresponsiveness is expressed as percent change from the baseline level of (A) lung resistance (RL) and (B) dynamic compliance (Cdyn) in control, OVA-sensitized and OVA-challenged mice, and OVA + triaryl methane (TRAM-34; 10, 30, and 100 mg/kg) therapeutic mice. RL and Cdyn values were obtained in response to increasing concentrations of inhaled methacholine. Data represent means $\pm$ SEMs ($n = 6$ in each group). * $P < 0.05$ and ** $P < 0.01$, versus control mice. * $P < 0.05$ and ** $P < 0.01$, versus OVA mice.

were reviewed and approved in accordance with the guidelines for the care and use of laboratory animals at Shanghai Jiao Tong University.

Allergen-Induced Airway Inflammation and Remodeling

Mice were sensitized on Days 1 and 14 by an intraperitoneal injection of 20 μ g ovalbumin (OVA, Grade V; Sigma-Aldrich, St. Louis, MO), which was emulsified in 2.25 mg of aluminum hydroxide in a total volume of 200 μ l PBS. Twenty-one days after the first intraperitoneal injection, the mice were challenged with aerosolized OVA (5%), three times per week for 8 weeks. These OVA-sensitized and OVA-challenged mice were used as a model of *in vivo* chronic asthma. Control mice sensitized with the same volume of PBS and aluminum hydroxide were challenged with PBS. TRAM-34 (10, 30, and 100 mg/kg, intraperitoneal) was administered once daily for the last 8 weeks (30 minutes before the OVA challenges). One day after the final OVA challenge, AHR was assessed (for further details, please see the online supplement), and samples were obtained for further analysis.

Lung Tissue Histopathology

Paraffin-embedded sections (5 μ m) were stained with hematoxylin and eosin, Masson trichrome, and periodic acid–Schiff (PAS). These stains were used to evaluate histological inflammation, matrix deposition, and

mucus production, respectively. Quantitative analyses were performed in a blinded manner, as described previously (26, 27) (for further details, please see the online supplement).

Immunohistochemical Analyses

The evaluation of KCa3.1 channel distribution was achieved by immunohistochemistry in murine airways (for further details, please see the online supplement).

Western Blot Analysis

Protein lysates from murine airways and asthmatic human BSMCs were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted with anti-KCa3.1 and anti-KCa1.1 (Alomone Labs, Ltd., Jerusalem, Israel; for further details, please see the online supplement).

Real-Time PCR

Quantitative analyses of the mRNA concentrations of target genes, both in murine airways and in human BSMCs, were examined by quantitative real-time PCR (for further details, please see the online supplement).

Human BSMC Culture

Asthmatic human BSMCs (Lonza, Walkersville, MD) were purchased and cultured according to the manufacturer's instructions (90% of the cells were positively stained for anti- α -smooth muscle actin [α -SMA], and 90% were positively stained for smooth muscle heavy chain myosin). All experiments were performed between Passages 4 and 8. After a quiescent state of the cells was achieved by a 48-hour incubation in serum-free medium (Lonza), cells were stimulated with 20 ng/ml PDGF in each assay. Asthmatic human BSMC proliferation and migration, and small interfering (si)RNA transfection cell cycle assays, are described in the online supplement.

Statistical Analysis

All data are presented as means $\pm$ SEMs. For multiple comparisons, one-way ANOVA was used, followed by the Dunnett test for selected pairs if appropriate. For individual comparisons, the statistical analysis was performed using the Student *t* test. Data analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). The threshold for statistical significance was set at $P < 0.05$.

RESULTS

KCa3.1 Expression Is Increased in Asthmatic Airway Smooth Muscle

We examined the expression of KCa3.1 mRNA in control and OVA-sensitized and OVA-challenged mice bronchi, using real-time PCR analyses. As shown in Figure 1A, KCa3.1 mRNA expression in OVA-sensitized and OVA-challenged mice was approximately 2.4-fold higher than in control mice. Western blot analyses of the bronchi also indicated elevated KCa3.1 expression (i.e., a 3.1-fold increase) in OVA-sensitized and OVA-challenged mice, compared with control mice (Figure 1B).

Two-color immunofluorohistochemistry was performed in the bronchi of control and OVA-sensitized and OVA-challenged mice, using cell-specific antibodies to determine which cell types contributed to the increased KCa3.1 expression in asthma. In the bronchi of control mice, a KCa3.1 immunoreaction was found mainly in the epithelial cell layer (Figure 1C). A merged image shows the coimmunostaining of KCa3.1 with the epithelial marker thyroid transcription factor-1 (TTF-1) (Figure 1C), whereas no colocalization with the smooth muscle marker α -SMA, and thus no immunoreaction for KCa3.1 in the smooth muscle layer, were evident (Figure 1D). In the bronchi of OVA-sensitized and OVA-challenged mice, a positive immunoreaction for KCa3.1 was detected in both the epithelial and smooth muscle layers, which was positive for

TTF-1 and α -SMA, respectively (Figures 1E and 1F). Overall, these results demonstrate that the up-regulation of KCa3.1 occurred primarily in bronchial smooth muscle.

Blockade of KCa3.1 Attenuates AHR

We tested whether a pharmacological KCa3.1 blockade exerted beneficial effects on lung function in OVA-sensitized and OVA-challenged mice. Here, the KCa3.1 blocker TRAM-34 was used. TRAM-34 was administered once daily (10, 30, and 100 mg/kg, intraperitoneally) for 8 weeks. Mice sensitized and challenged with OVA exhibited a robust decline in lung function, as shown by developing AHR, significant increases in lung resistance (RL) ($P < 0.01$), and decreases in dynamic compliance (C_{dyn}) ($P < 0.01$) versus the results in control mice (Figures 2A and 2B). In contrast, mice in the TRAM-34 (10, 30, and 100 mg/kg) therapeutic group managed to retain significantly lower RL than the OVA group ($P < 0.01$; Figure 2A). When C_{dyn} was monitored, only the TRAM-34 (100 mg/kg) group showed a significant increase in methacholine-induced C_{dyn}, and the level of C_{dyn} was significantly higher than in the OVA group ($P < 0.01$; Figure 2B).

Blockade of KCa3.1 Reduced Established Airway Inflammation

Inflammatory cell recruitment to the airways is thought to constitute a major factor in the development of allergen-induced pathophysiology. Therapeutic treatment with TRAM-34 (30 and 100 mg/kg) significantly reduced total cell numbers in bronchoalveolar

lavage (BAL) fluid ($P < 0.01$; Figure 3A). Differential counts of cells recovered from BAL fluid revealed that TRAM-34 (30 and 100 mg/kg) treatment significantly reduced the numbers of eosinophils, neutrophils, and lymphocytes, in comparison with OVA-sensitized and OVA-challenged mice (Figure 3B).

Changes in inflammatory infiltrates were examined in hematoxylin and eosin-stained lung sections (Figure 3C). The degree of inflammation was assessed in a blinded manner, and was assigned a semiquantitative score (Figure 3D). OVA sensitization and challenge resulted in inflammatory cell influx into the lamina propria, perivascular, and peribronchiolar regions of the lung. After treatment with TRAM-34 in OVA-sensitized and OVA-challenged mice, the inflammatory influx into the lung tissue was reflected by significantly reduced gross lung lesion scores (Figures 3C and 3D), consistent with the analysis of BAL fluid.

To investigate whether the presence of the KCa3.1 channel resulted in dominant T cell-derived cytokines after OVA challenge, cytokine concentrations in supernatants were measured in BAL fluid. As we expected, OVA-sensitized and OVA-challenged mice demonstrated increased concentrations of Th2-type cytokines (specifically, IL-4, IL-5, and IL-13). In fact, these cytokine concentrations appeared to be lower in control mice. In contrast, the Th1-type cytokine IFN- γ showed a decrease, compared with control mice. Interestingly, the administration of TRAM-34 (100 mg/kg) significantly decreased concentrations of Th2-type cytokines and increased concentrations of Th1-type cytokines, in comparison with OVA-sensitized and OVA-challenged mice (Figure 3E). Thus, these results may partly reflect the possibility of promising clinical

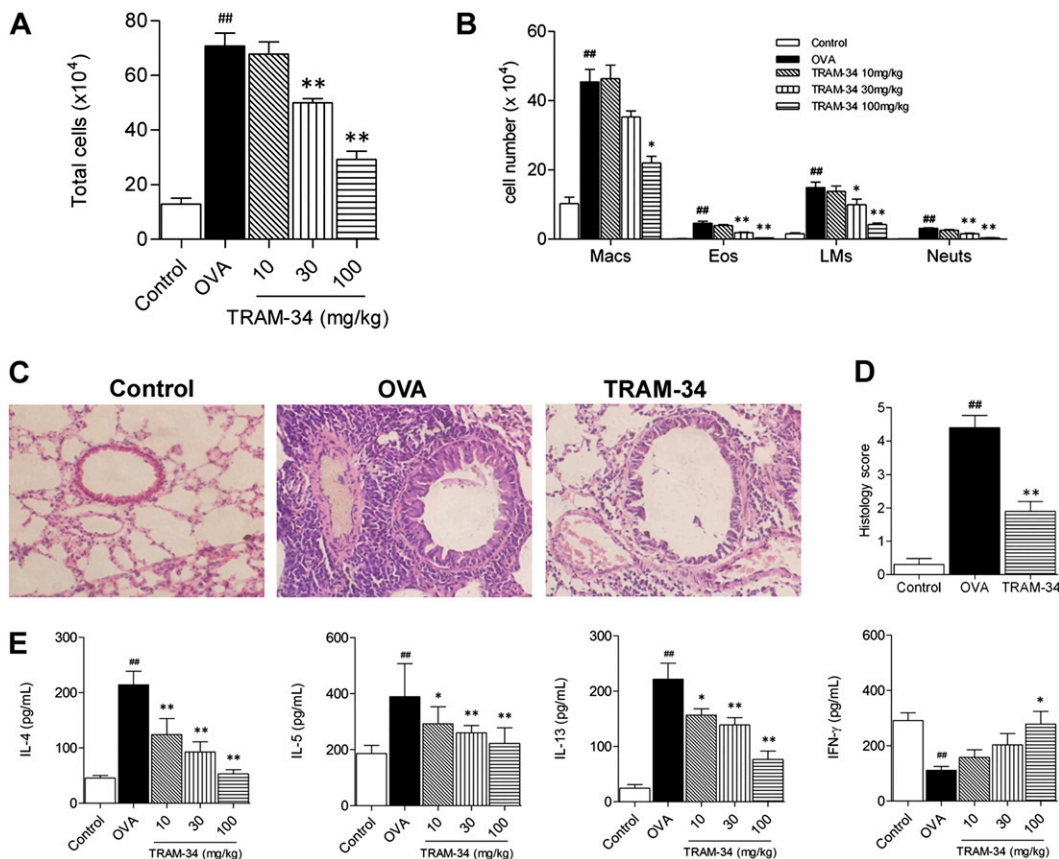


Figure 3. Blockade of KCa3.1 reduced established airway inflammation. (A) Inflammatory cell counts in bronchoalveolar lavage (BAL) fluid were obtained from sensitized and challenged mice 24 hours after their final OVA challenge. (B) Differential cell counts were performed on a minimum of 400 cells to identify macrophages (Macs), eosinophils (Eos), lymphocytes (LMs), and neutrophils (Neuts). Values are expressed as means $\pm$ SEMs ($n = 6$). (C) Representative photomicrographs of hematoxylin and eosin-stained lung sections from control, OVA-sensitized and OVA-challenged, and OVA + TRAM-34 (100 mg/kg) mice (original magnification, $\times 30$). (D) Semiquantitative analyses of inflammatory cell infiltration in lung sections. Data are expressed as means $\pm$ SEMs ($n = 5$). (E) Effects of KCa3.1 blockade on BAL fluid cytokine concentrations in a murine model of asthma. Mice were challenged by OVA for 8 weeks (three times a week) for asthma induction. Three ex-

perimental groups were treated with different concentrations of TRAM-34 (10, 30, and 100 mg/kg) for the last 8 weeks. BAL fluid was collected 24 hours after the final OVA challenge, and concentrations of IL-4, IL-5, IL-13, and IFN- γ were analyzed using ELISA. Values shown are the means $\pm$ SEMs ($n = 6$). ^{##} $P < 0.01$, versus control mice. ^{*} $P < 0.05$ and ^{**} $P < 0.01$, versus OVA mice.

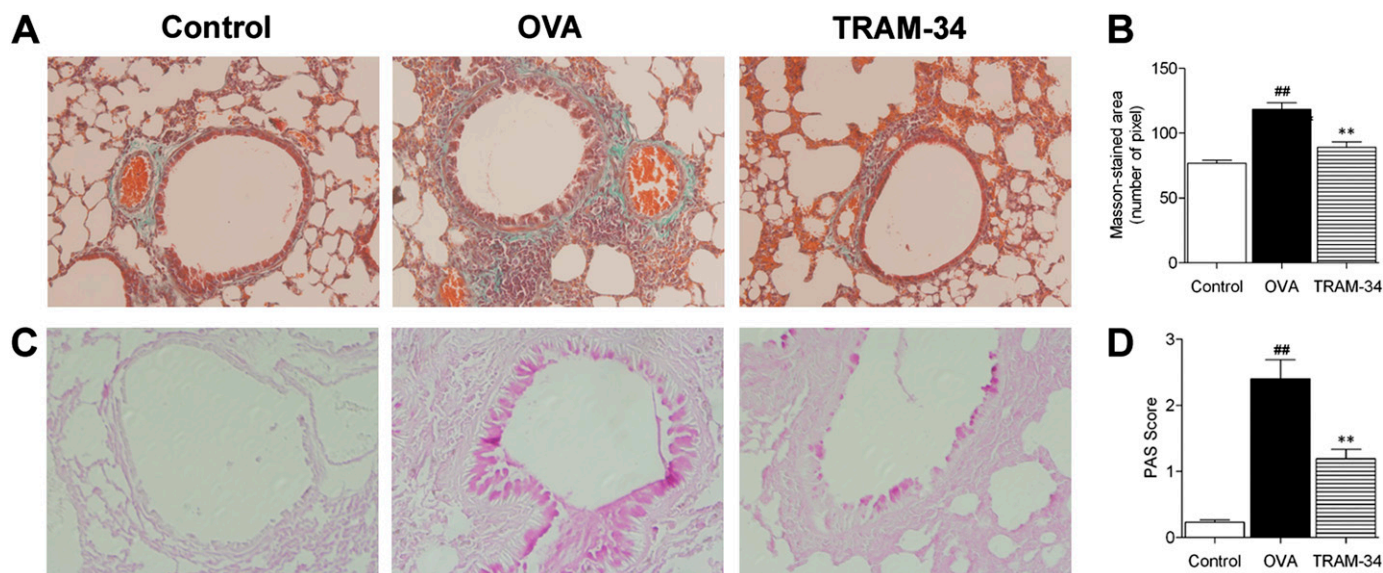


Figure 4. Blockade of KCa3.1 prevented established airway remodeling. (A) Representative photomicrographs were obtained of stained paraffin lung sections with Masson trichrome from control, OVA-sensitized and OVA-challenged, and OVA + TRAM-34 (100 mg/kg) groups (original magnification, $\times 30$). (B) Semiquantitative analyses were performed on Masson trichrome-stained lung sections from mice treated with TRAM-34. Random measurements from the basement membrane into the submucosa (10 measurements of 20 μ m in length) were taken, and the mean density was calculated from four bronchi per mouse ($n = 5$ mice per group). (C) Representative photomicrographs were obtained of stained paraffin lung sections with periodic acid–Schiff (PAS) from control, OVA-sensitized and OVA-challenged, and OVA + TRAM-34 (100 mg/kg) groups (original magnification, $\times 30$). (D) PAS-stained sections were assessed by a blinded observer and scored. Data are presented as means $\pm$ SEMs ($n = 5$). ^{##} $P < 0.01$, versus control mice. ^{**} $P < 0.01$, versus OVA mice.

effects for the KCa3.1 channel in Th2-dominated allergic asthma.

Blockade of KCa3.1 Prevented Airway Remodeling

The prolonged allergen challenge of sensitized mice results in increased ECM deposition in the subepithelial regions, as visualized by Masson trichrome staining, in comparison with control mice. These data support the observations of McMillan and Lloyd with a similar murine model using OVA (5). The administration of TRAM-34 exerted a substantial effect on reducing ECM deposition in bronchiolar subepithelial regions (Figure 4A). To quantify these differences in matrix deposition, we performed image analysis to determine the relative density of matrix in 20- μ m lengths of the basement membrane in lung sections from each group of mice. Figure 4B shows that treatment with TRAM-34 (100 mg/kg) significantly decreased the density of the subepithelial region of mice during prolonged allergen challenge, in comparison with mice from the OVA group.

Mucus-secreting goblet cells were quantified in PAS-stained sections. These sections were derived from mice treated with TRAM-34 (100 mg/kg; Figures 4C and 4D). TRAM-34 significantly reduced the number of mucus-secreting goblet cells within the respiratory epithelium, in comparison with those of mice in the OVA group.

Involvement of KCa3.1 in Asthmatic Human BSMC Proliferation and Migration

To study the role of KCa3.1 in the activation, proliferation, and migration of airway SMCs, we stimulated asthmatic human BSMCs with PDGF. PDGF induced in a time-dependent increase in KCa3.1 mRNA expression, peaking at 4 hours (Figure 5A). A corresponding increase in KCa3.1 protein expression was evident (Figure 5B). In contrast, our results

also demonstrated that KCa3.1 protein expression levels decreased after stimulation with PDGF (Figure 5C), indicating that KCa3.1 expression in asthmatic human BSMCs is a feature of airway SMC remodeling, paralleling the phenotypic conversion of airway SMCs from a normal contractile state

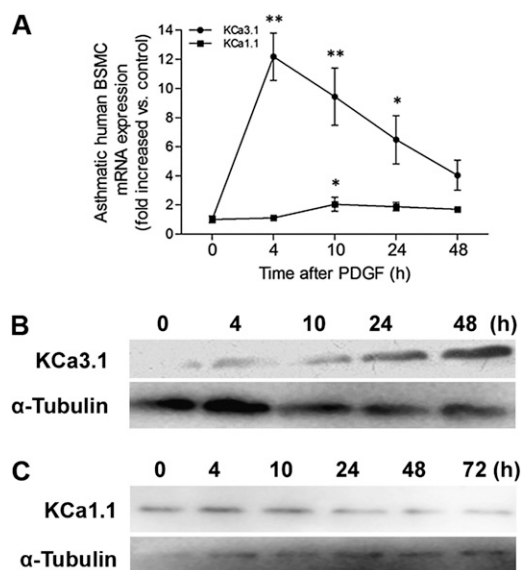


Figure 5. Up-regulation of KCa3.1 in asthmatic human bronchial smooth muscle cells (BSMCs). (A) Stimulation with 20 ng/ml platelet-derived growth factor (PDGF) increased KCa3.1 mRNA in asthmatic human BSMCs ($n = 5-9$). ^{*} $P < 0.05$, ^{**} $P < 0.01$ versus control. (B) Total KCa3.1 protein expression (40 μ g whole-cell lysates) was increased in asthmatic human BSMCs in a time-dependent fashion during PDGF stimulation. (C) Total KCa1.1 protein expression (30 μ g whole-cell lysates) was decreased in asthmatic human BSMCs in a time-dependent fashion during PDGF stimulation.

to a proliferative, dedifferentiated state during the prolonged allergen challenge of sensitized mice.

We then applied a pharmacological blockade and gene silencing with siRNA to assess the functional consequences of KCa3.1 in asthmatic human BSMCs. At concentrations that block the KCa3.1 channel, TRAM-34 and charybdotoxin suppressed the PDGF-induced proliferation (Cell Counting Kit-8 assay; Figure 6A), *de novo* DNA synthesis (bromodeoxyuridine incorporation; Figure 6B), and migration (Transwell migration assay; Figure 6C) of asthmatic human BSMCs in a dose-dependent fashion. Similar results were obtained with gene silencing. KCa3.1-specific siRNA inhibited the PDGF-stimulated increases in KCa3.1 protein concentrations and suppressed proliferation (Figure 6D), *de novo* DNA synthesis (Figure 6E), and migration (Figure 6F) in asthmatic human BSMCs, whereas control siRNA exerted no effect.

We also examined the effects of TRAM-34 on cell-cycle progression in asthmatic human BSMCs, to determine more fully the mechanisms by which inhibiting KCa3.1 suppressed the proliferation of asthmatic human BSMCs. In the presence of different concentrations of TRAM-34, the percentage of asthmatic human BSMCs during the G0/G1 phase was significantly enhanced, whereas the percentage of cells in the S phase was markedly reduced in a dose-dependent fashion (Figures 6G and 6H). Moreover, in comparison with the control profile, the cell-cycle profile of asthmatic human BSMCs that were transfected with siRNA against KCa3.1 was also characterized by an increase in the percentage of cells in the G0/G1 phase, and a reduction of the fraction of cells in the S phase (Figure 6I). Overall, these

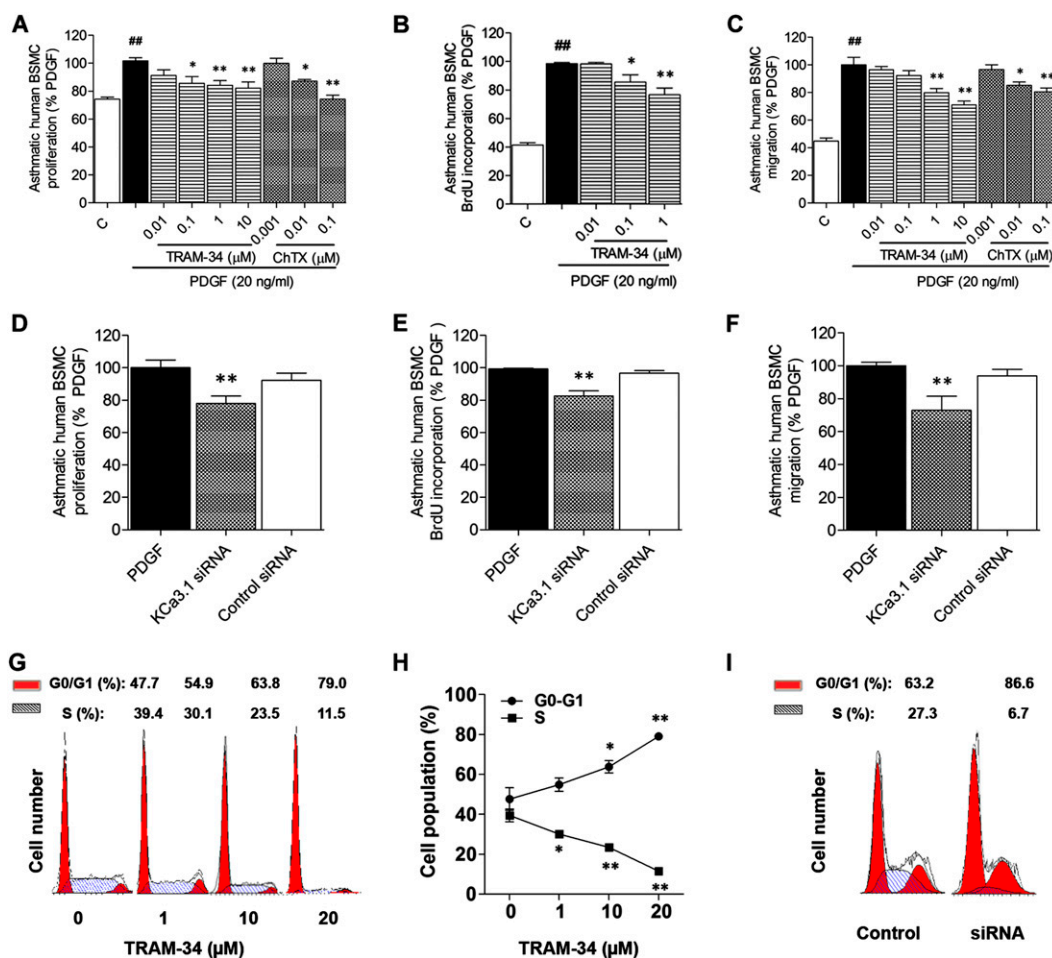
results suggest that the up-regulation of KCa3.1 channels is involved in asthmatic human BSMC migration, proliferation, and cell-cycle progression.

Activation of KCa3.1 Induces Membrane Hyperpolarization and Intracellular Ca^{2+} Increase

Membrane potential and intracellular Ca^{2+} are generally accepted as playing an important role in almost all cellular processes, including the regulation of cell migration and proliferation. As shown in Figures 7A and 7B, TRAM-34 (100 nM and 1 μM) abolished KCa3.1 pharmacological activator 1-ethylbenzimidazolone (EBIO)-induced (200 μM) membrane hyperpolarization, as indicated by a decrease in bis-(1,3-dibutylbarbituric acid) trimethine oxonol fluorescence intensity at 530 nm, a consequence caused by potassium efflux upon the activation of KCa3.1 in asthmatic human BSMCs. In addition, we measured the intracellular free Ca^{2+} kinetics when 1-EBIO was present. We found that 1-EBIO induced an instantaneous increase in intracellular free Ca^{2+} that was completely reversed by TRAM-34 (1 μM) (Figures 7C and 7D). These results provide a mechanism for the KCa3.1 channel in the proliferation and migration of asthmatic human BSMCs by regulating the membrane potential and Ca^{2+} signaling.

DISCUSSION

The major finding in this study was of augmented KCa3.1 mRNA and protein expression in the bronchi of allergic-challenged mice. Treatment with TRAM-34 resulted in a striking reduction



for 48 hours. Data represent the means $\pm$ SEMs of three independent experiments. (I) The down-regulation of KCa3.1 arrested asthmatic human BSMCs at the G0/G1 phase. * $P < 0.05$ and ** $P < 0.01$, versus 10% FBS alone. C, control; ChTX, charybdotoxin.

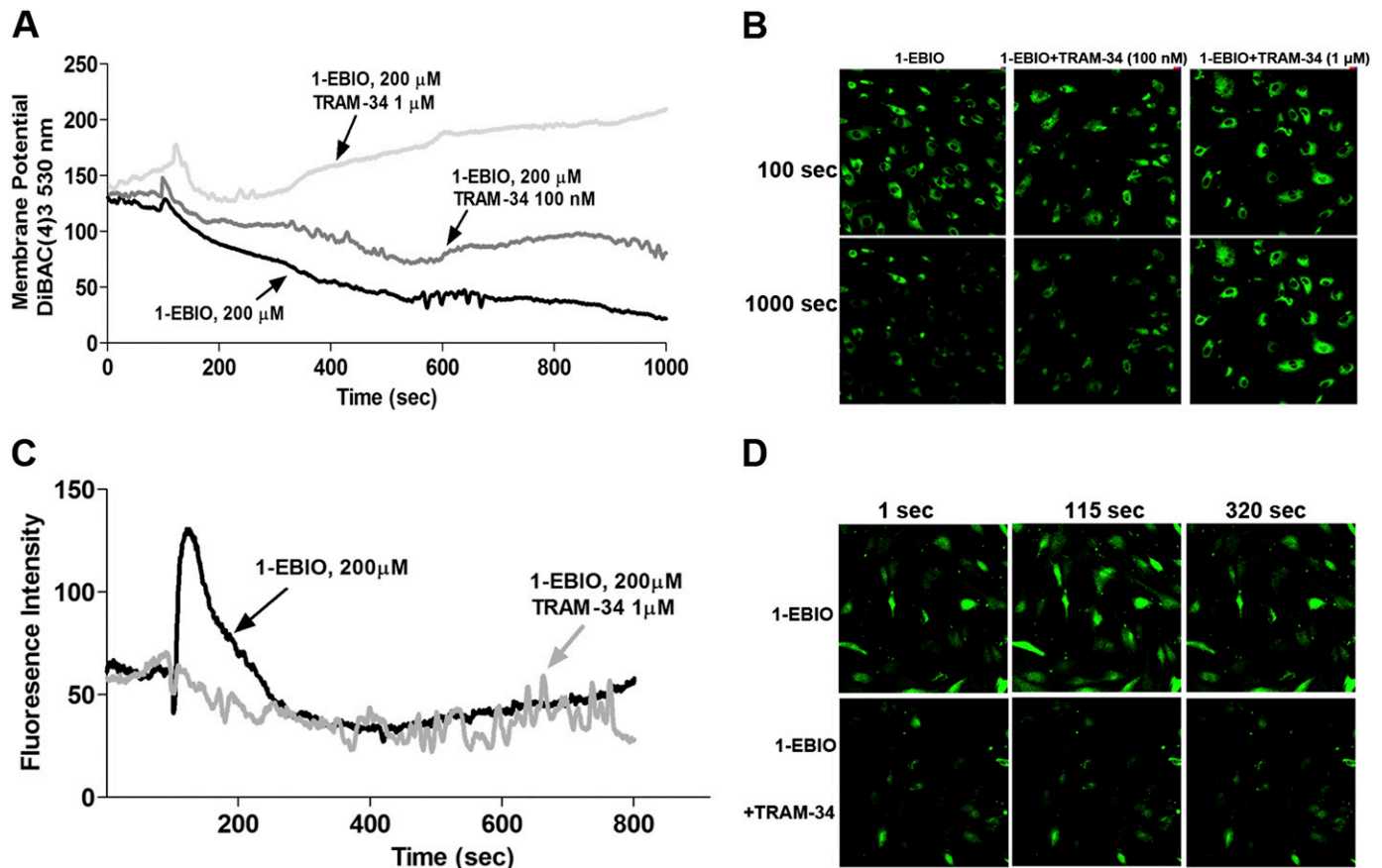


Figure 7. The blockade of KCa3.1 inhibited 1-ethylbenzimidazolone (EBIO)-induced membrane hyperpolarization and intracellular calcium increase in asthmatic human BSMCs. Asthmatic human BSMCs were loaded with membrane potential dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (A and B) or Fluo-4 AM (C and D) at 37°C for 45 minutes. (A and B) The KCa3.1 activator, 1-EBIO (200 μ M), was added to asthmatic human BSMCs at 100 seconds. TRAM-34 (100 nM and 1 μ M) was added to 1-EBIO-treated asthmatic human BSMCs at 300 seconds. (C and D) To study the impact of KCa3.1 activity on intracellular calcium, 1-EBIO (200 μ M) was used to activate the channel in calcium imaging studies of asthmatic human BSMCs. In the presence of TRAM-34 (1 μ M), 1-EBIO failed to induce a calcium increase in asthmatic human BSMCs ($n = 10$). Signal acquisition lasted for 800–1,000 seconds.

of AHR, airway inflammation, and airway remodeling in a murine model of asthma. Pharmacological blockers and the siRNA gene silencing of KCa3.1, which was evidently elevated after mitogen stimulation, attenuated the proliferation and migration processes of asthmatic human BSMCs through regulating their membrane potential and Ca^{2+} signaling. These findings highlight a novel role for the KCa3.1 channel in allergic airway disease, and suggest that KCa3.1 blockers may represent a promising therapeutic strategy for human allergic asthma.

The prolonged allergen challenge of sensitized mice was reported to result in a Th2 response, the accumulation of inflammatory eosinophils and leukocytes in the lung parenchyma and airways, peribronchial collagen deposition, epithelial goblet-cell hyperplasia, and AHR in response to the nonspecific inhaled bronchoconstrictor methacholine, similar to what has been observed by others in persistent asthma (28). In fact, concentrations of Th2 cell-derived cytokines, such as IL-4, IL-5, and IL-13, are elevated in BAL fluid after airway allergen challenge in patients with asthma (29, 30). The observed reduction in pulmonary inflammation may result (as observed in BAL fluid after TRAM-34 treatment) from the concomitant reduction in lung-tissue concentrations of the Th2-type cytokines IL-4, IL-5, and IL-13, and an increase in the Th1 cytokine IFN- γ in BAL fluid after treatment with TRAM-34 (31, 32). Thus, these results may partly reflect the possibility of promising clinical effects of the KCa3.1 channel in Th2-dominated allergic asthma. AHR

has been linked to IL-4 (33, 34), whereas IL-5 directly modulates allergic airway disease by regulating eosinophilic inflammation (35). IL-13, on the other hand, does not affect eosinophils or IgE production, but affects airway epithelial and smooth muscle cells, where it mediates AHR, mucus hypersecretion, and subepithelial fibrosis (36). Furthermore, IL-13 induces the production of matrix metalloproteinases, underlining its importance for airway remodeling in typical asthma. We thus predicted that TRAM-34 inhibition, leading to reduced IL-13 as well as IL-4 and IL-5, might exert beneficial effects on the expression of genes associated with lung remodeling.

Airway inflammation is a characteristic feature of allergic airway disease, and inflammatory cells that infiltrate the airways are thought to contribute to remodeling by increasing the proliferation and migration of airway SMCs. In normal murine airways, the KCa3.1 channels are normally localized in epithelial cells, whereas the smooth muscle layer contains contractile airway SMCs that express KCa3.1 channels (37). In contrast, asthmatic murine airways demonstrated increased KCa3.1 expression in the smooth muscle layer. Because PDGF-induced asthmatic human BSMC activation is accompanied by an increase in KCa3.1 expression and a decrease in KCa1.1 expression, the enhanced concentrations of KCa3.1 in asthmatic airway SMCs may, in part, be a consequence of the up-regulation of this channel during the activation process. However, the putative *in vivo* role of KCa3.1 in the pathogenesis of asthma had not been previously investigated. We thus

treated allergen-challenged wild-type mice with the small molecule KCa3.1 blocker, TRAM-34, and showed that a pharmacological inhibition of KCa3.1 was effective in reducing airway inflammation, AHR, and airway remodeling. Airway remodeling is increasingly recognized as a consequence of chronic asthma.

Our present findings were consistent with those of small clinical trials performed by Wulff and Castle with TRAM-34 analog ICA-17043 in allergen-induced asthma, but not in exercise-induced asthma (38). However, these two small clinical trials may have been too short (2 or 4 wk) to evaluate the effects of KCa3.1 blockers on airway remodeling. Our present findings, obtained in a murine model of chronic allergen exposure for a period of 11 weeks, exhibit a benefit of TRAM-34 in features of chronic allergic asthma, such as the persistent AHR and airway remodeling concomitant with the up-regulation of the KCa3.1 channel in the airways. A recent study also demonstrated that treatment with TRAM-34 abolished bronchial smooth muscle remodeling and subbasement collagen deposition, and attenuated airway eosinophilia in a murine model of asthma (39). Targeting the KCa3.1 channel may therefore prove beneficial in allergic airway remodeling.

KCa3.1 is a regulator of cell proliferation in undifferentiated smooth muscles and lymphocytes, and it provides a new therapeutic target in restenosis and autoimmune diseases (40). Indeed, PDGF, a human BSMC mitogen that is expressed in asthmatic airways, induced a dramatic increase of KCa3.1 expression in airway SMC. Other growth factors that are released in asthmatic lesions, such as epidermal growth factor, transforming growth factor- β , and bFGF, exert a similar effect on KCa3.1 expression. The major signal transduction pathway of this mitogen-induced KCa3.1 up-regulation appeared to be the Ras/Raf/MAP kinase or ERK kinase/extracellular signal-regulated kinases signaling cascade, consistent with observations in other cell types (22, 41). In contrast, expression levels of the related KCa channel KCa1.1 decline in response to PDGF, underscoring the specificity of KCa3.1 up-regulation and suggesting an important role for KCa3.1 in the promotion of airway SMC proliferation.

In support of these observations, we show that pharmacological inhibition and KCa3.1 gene silencing are capable of suppressing mitogen-driven proliferation and the migration of human BSMCs. The importance of KCa3.1 in airway SMC mitogenesis may be explained by its ability to enhance the driving force for Ca^{2+} influx, via membrane hyperpolarization, and thus to sustain the high intracellular Ca^{2+} concentration needed for gene transcription, as reported in vascular SMC, T lymphocytes, and cancer cells (25, 42, 43). Recent studies have shown that KCa3.1 and the small-conductance Ca^{2+} -activated K^{+} channel KCa2.2 participate in modulating several types of cell proliferation, and that their pharmacological blockade and down-regulation inhibit cell proliferation via G0/G1 arrest (44).

Our data suggest roles for KCa3.1 channels in human BSMC proliferation and migration. Cell membrane hyperpolarization promotes Ca^{2+} influx during the G1 phase of the cell cycle, allowing passage through the G1-S checkpoint (45). Because KCa3.1 channels cause K^{+} efflux, and thus membrane hyperpolarization, the inhibition of KCa3.1 channels leads to membrane depolarization and cell-cycle arrest during the early G1 phase. Here, we demonstrate that KCa3.1 inhibition, with TRAM-34, suppressed, in a dose-dependent manner, asthma human BSMC PDGF-induced proliferation, *de novo* DNA synthesis, and migration. The induction of KCa3.1 may comprise an important step in the initiation of asthma human BSMC proliferation in response to mitogenic factors.

KCa3.1 is expressed in many of the cell types implicated in asthma (airway SMCs, T cells, macrophages, and mast cells), and is a major modulator of the calcium influx that is necessary for substantially maintaining cellular activation processes, such as

proliferation, migration, and cytokine production. Thus, KCa3.1 blockers are likely to induce antiasthmatic effects, thereby treating both inflammation and remodeling issues. Treatment with KCa3.1 blockers alone, or in combination with corticosteroids, may represent new therapeutic approaches to the management of chronic asthma.

KCa3.1 is an attractive pharmacological target because its blockade does not exert major adverse effects on normal physiology. Two generated KCa3.1^{-/-} mice did not show any gross abnormalities in their major organs, and exhibited rather mild phenotypes (22, 46). Moreover, in a postangioplasty restenosis model, a KCa3.1 blocker prevented the up-regulation of both KCa3.1 and activator protein 1 in coronary arteries (47). In addition, the daily administration of TRAM-34 at a relatively high concentration (120 mg/kg) did not induce any changes in blood chemistry or hematology. The results of ICA-17043 safety studies further support these findings (48). Thus we offer the intriguing suggestion that KCa3.1 may emerge as a promising target for modulating the progression of complex proliferative disorders, such as chronic asthma, via its effects in airway remodeling.

In conclusion, KCa3.1 channel expression is increased in the airways of allergen-induced asthmatic mice (especially in airway SMCs). KCa3.1 blockers markedly reduce the development of airway inflammation, remodeling, and AHR. Increased KCa3.1 activity plays a crucial role in regulating the proliferation, migration, and activation of airway SMCs that contribute to asthma. The targeted disruption of KCa3.1 may provide a therapeutic rationale for combating obstructive airway diseases such as asthma.

Author disclosures are available with the text of this article at www.atsjournals.org.

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**Targeted Inhibition of KCa3.1 Channel Attenuates Airway Inflammation and
Remodeling in Allergic Asthma**

Zhi-hua Yu, Jian-rong Xu, Yan-xia Wang, Guang-ni Xu, Zu-peng Xu, Kai Yang, Da-zheng
Wu, Yong-yao Cui and Hong-zhuan Chen

ONLINE DATA SUPPLEMENT

METHODS

Reagents

The primary antibodies used were mouse monoclonal KCa3.1 (Alomone Labs.; MW 46 kDa, 1:25 for immunofluorescence and 1:100 for Western blotting), rabbit polyclonal KCa3.1 (Santa Cruz) 1:100 for two-color immunofluorescence, mouse monoclonal α -tubulin (MW 50 kDa: 1:1000, Santa Cruz), mouse monoclonal TTF-1 (Santa Cruz) 1:25 for two-color immunofluorescence, and mouse monoclonal α -SMA 1:400 (Sigma). Secondary antibodies were a HRP-labeled goat anti-rabbit 1:5,000, or, for Western blotting, a goat anti-mouse 1:5,000 (Santa Cruz). The Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was used. TRAM-34 (Apptec, Wuxi, China) for *in vitro* experiments was dissolved in dimethyl sulfoxide. For *in vivo* studies, TRAM-34 was dissolved in peanut oil. Charybdotoxin (Sigma-Aldrich) was dissolved in cell culture media. Recombinant human PDGF-BB was reconstituted according to the manufacturer's instructions (ProSpec, ProSpec-Tany TechnoGene Ltd., Israel). All other chemicals were of analytical grade.

Bronchoalveolar lavage (BAL) fluid measurement

Immediately after the AHR assessment, lungs were lavaged through the tracheal tube with 1 mL of BAL fluid (1 mM EDTA, 10% FBS, 0.01M PBS). The BAL fluid supernatant was stored at -80°C and used for interleukin (IL)-4, IL-5, IL-13, and interferon- γ (IFN- γ) measurements, by ELISA (Rapidbio Labs., Langka Trade Co. Ltd., Shanghai, China). Total cell numbers were counted with a hemocytometer.

Differential cell counts were performed by counting at least 400 cells, which were stained with Wright's-Giemsa (NJJC, Nanjing, China); these cells were differentiated through standard hematological procedures.

Assessment of Airway Hyperresponsiveness

AHR was assessed in mice 24 h after the final OVA challenge by measuring changes in lung resistance (RL) and dynamic compliance (Cdyn) in response to inhaled methacholine (Sigma-Aldrich; at concentrations of 3-100 mg/mL for 1 min). Data are expressed as percentage of changes from baseline RL and Cdyn values obtained after inhalation of PBS (Buxco, RC system).

Protein expression analysis

KCa3.1 protein expression was analyzed using immunoblotting. Briefly, the proteins were subjected to SDS-polyacrylamide gel electrophoresis (10%) and then transferred to a nitrocellulose membrane. Membranes were blocked and incubated, according to the instructions provided, with the primary antibody. Protein bands were identified through a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents (Pierce, Rockford, IL). Images were captured on a Kodak Image Station; the immunoreactive band density was normalized to the β -actin band's intensity.

Lung tissue histopathology

Peribronchial inflammatory cell counts were performed by a blinded observer, based on a 5-point scoring system: 0, no cells, 1, a few cells, 2, a ring of cells one cell layer deep, 3, a ring of cells two to four cells deep, and 4, a ring of cells of more than four cells deep. PAS-stained goblet cells in the airway epithelium were scored in each bronchus individually (0 = < 5% goblet cells, 1 = 5-25%, 2 = 25-50%, 3 = 50-75%, and 4 > 75%). Scoring of inflammatory cells and mucus production was performed in at least three different fields for each lung section. Mean scores were obtained from five animals. Masson's trichrome staining intensity was quantified using the Image J software (NIH, Bethesda, MD).

Cell proliferation and migration

Cell proliferation (CCK-8 and BrdU incorporation assays) and migration assays were performed as reported previously (E1). Human BSMCs were stimulated with 20 ng/mL PDGF for 6 h in the migration assay and for 48 h in the proliferation assay. All control experiments were performed in the presence of vehicle for the blockers.

Transfection of siRNA

The transfection of siRNA was performed as previously reported (E2). Briefly, 24 h before transfection, human BSMCs were seeded onto culture plates, so that the cells were 70-80% confluent at the time of transfection. KCa3.1-specific siRNA and a negative control were incubated with TurboFect siRNA Transfection Reagent (Fermentas) in DMEM (Gibco). Cells were transfected in the DMEM transfection

mixture for 24 h. After washout, cells were stimulated with PDGF. Optimization of siRNA concentration, transfection time, cell density, and other experimental conditions were determined by quantifying, prior to experiments, KCa3.1 mRNA and protein expression.

Real-time PCR

Quantitative analyses of mRNA levels of target genes, both in mice airways and human BSMCs were examined by quantitative real-time PCR analysis. Briefly, cDNAs were prepared from total RNA using a RevertAid First Strand cDNA Synthesis kit (Fermentas). The Primer sequences were as follows: for human KCa1.1, 5'- AAA ACA ACC AGG CTC TCACCTA -3' (sense) and 5'-ATA AAC ATC CCC ATA ACC AACG-3' (antisense); for human KCa3.1, 5'- GTG CTA CAA GAA GCC TGG ATG-3' (sense) and 5'-AGC GTG TCA ATC TGT TTC TCC-3' (antisense); for human GAPDH, 5'-AGA AGG CTG GGG CTC ATTTG-3' (sense) and 5'- AGG GGC CAT CCA CAG TCT TC-3' (antisense); for mouse KCa3.1, 5'-GGC TGA AAC ACC GGA AGCTC-3' (sense) and 5'-CAG CTC TGT CAG GGC ATCCA-3' (antisense); for mouse β -actin, 5'-CAA CCG TGA AAA GAT GAC-3' (sense) and 5'- CAG GAT CTT CAT GAG GTAGT-3' (antisense). Quantitative real-time PCR was performed using SYBR Green (TaKaRa Biotechnology, Dalian, China) on an ABI 7500 sequencing system (Applied Biosystems). KCa1.1 and KCa3.1 gene expression levels were normalized using GAPDH or β -actin. The final results, expressed as fold differences in target gene expression, relative to both endogenous control gene expression and the calibrator, were determined using the $2^{-\Delta\Delta CT}$ method.

Intracellular free calcium and membrane potential measurements

A confocal laser scanning microscope (Zeiss LSM 510 Meta) was used to evaluate relative changes in intracellular calcium concentration ($[Ca^{2+}]_i$) and cell membrane potential by monitoring Fluo-4 and DiBAC4(3) fluorescence, respectively. Cells were loaded with Fluo-4 AM (Sigma-Aldrich) and DiBAC4(3) for 45 min in an incubator; they were then washed three times with 0.01 M PBS. 1-EBIO (200 μ M) was added to the cells at 100 s. Then, TRAM-34 (100 nM, 1 μ M) was added at 300 s. Fluo-4 fluorescence was measured at 510 nm with excitation at 488 nm and DiBAC4(3) fluorescence was measured at 530 nm with excitation at 488 nm. Confocal images were taken and stored every 1 s for 800-1000 s. Data were obtained by evaluating the fluorescence from selected whole areas of cell bodies, and then by taking the average of ten cells.

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