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Blocking K_V1.3 Channels Inhibits Th2 Lymphocyte Function and Treats a Rat Model of Asthma*

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Background: CCR7$^-$ effector memory T lymphocytes are major players in lung inflammation that characterizes allergic asthma.
Results: Blocking K_V1.3 channels reduced the severity of an ovalbumin-induced model of asthma in rats.
Conclusion: K_V1.3 channels are attractive targets for immunomodulation and the treatment of allergic asthma.
Significance: Selective K_V1.3 channel blockers may prove beneficial in the treatment of asthma.

Allergic asthma is a chronic inflammatory disease of the airways. Of the different lower airway-infiltrating immune cells that participate in asthma, T lymphocytes that produce Th2 cytokines play important roles in pathogenesis. These T cells are mainly fully differentiated CCR7$^-$ effector memory T (T_{EM}) cells. Targeting T_{EM} cells without affecting CCR7$^+$ naïve and central memory (T_{CM}) cells has the potential of treating T_{EM}-mediated diseases, such as asthma, without inducing generalized immunosuppression. The voltage-gated K_V1.3 potassium channel is a target for preferential inhibition of T_{EM} cells. Here, we investigated the effects of ShK-186, a selective K_V1.3 channel blocker, for the treatment of asthma. A significant proportion of T lymphocytes in the lower airways of subjects with asthma expressed high levels of K_V1.3 channels. ShK-186 inhibited the allergen-induced activation of peripheral blood T cells from those subjects. Immunization of F344 rats against ovalbumin followed by intranasal challenges with ovalbumin induced airway hyper-reactivity, which was reduced by the administration of ShK-186. ShK-186 also reduced total immune infiltrates in the bronchoalveolar lavage and number of infiltrating lymphocytes, eosinophils, and neutrophils assessed by differential counts. Rats with the ovalbumin-induced model of asthma had elevated levels of the Th2 cytokines IL-4, IL-5, and IL-13 measured by ELISA in their bronchoalveolar lavage fluids. ShK-186 administration reduced levels of IL-4 and IL-5 and induced an increase in the production of IL-10. Finally, ShK-186 inhibited the proliferation of lung-infiltrating ovalbumin-specific T cells. Our results suggest that K_V1.3 channels represent effective targets for the treatment of allergic asthma.

Allergic asthma is a chronic inflammatory disease of the airways. Despite the effectiveness of bronchodilators and anti-inflammatory medications, the prevalence of asthma is on the rise, especially in developed countries where it remains a major health issue (1–4). The inflammatory infiltrates in the airways that are characteristic of asthma can affect the structural cells of the airways, leading to airway hyper-responsiveness (5). Of the different immune cells in those infiltrates, T lymphocytes that produce Th17 and Th2 cytokines, such as IL-4, IL-5, and IL-13, play important roles in the pathogenesis of asthma in a large proportion of patients (5–7). These T lymphocytes are mainly fully differentiated CCR7$^-$ effector memory T (T_{EM})[4] cells (8). Targeting T_{EM} lymphocytes without affecting CCR7$^+$ naïve and central memory (T_{CM}) lymphocytes has the potential to effectively treat T_{EM}-mediated diseases, such as asthma without inducing generalized immunosuppression.

Studies in the last few decades have identified the voltage-gated K_V1.3 potassium channel as a target for preferential inhibition of T_{EM} cells both *in vitro* and *in vivo*. Of the 78 potassium channels in the human genome, only two are expressed by T lymphocytes; these are homotetramers of K_V1.3 (*KCNA3*) and of the calcium-activated K_{Ca}3.1 (IKCa1, *KCNN4*) channel (9, 10). These channels provide the potassium efflux necessary to counterbalance the calcium influx crucial for T lymphocyte activation (9, 10). At rest, T lymphocytes express low levels of both K_V1.3 and K_{Ca}3.1. Upon activation with an antigen or a mitogen, T_{EM} cells up-regulate K_V1.3 (11–15). In contrast, CCR7$^+$ naïve and central memory (T_{CM}) lymphocytes up-regulate K_{Ca}3.1 after activation (13, 16). This differential expression of potassium channels results in a different sensitivity of naïve/T_{CM} and T_{EM} lymphocytes to selective blockers of K_V1.3

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[4] The abbreviations used are: T_{EM}, effector-memory T (lymphocyte); T_{CM}, central-memory T (lymphocyte); BALF, bronchoalveolar lavage fluids; K_V, voltage-gated potassium channel; R_{Rs}, respiratory system resistance.

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TABLE 1

Characteristics of the subjects who donated induced sputum and peripheral blood for this study

FEV1, forced expiratory volume in one second; BID, twice daily; PRN, as needed; OD, once daily.

Subject	Gender	Age	Race	Base-line FEV1 in liters and % predicted	Medications at time of sample collection
Control	F	33	Asian	2.53 (85%)	None
Asthmatic	F	29	Caucasian	2.73 (81%)	Albuterol inhaler (PRN), Advair 250/50 (BID)
Control	F	22	Caucasian	3.38 (107%)	None
Asthmatic	M	31	African-American	2.99 (88%)	Albuterol inhaler (PRN), Symbicort 160/4.5 (BID), Protopic (topical drug- BID for alopecia)
Asthmatic	F	44	African-American	2.40 (94%)	Albuterol inhaler (PRN), Advair 500/50 (BID), Nexium 40 mg OD
Control	F	54	Caucasian	2.83 (97%)	Cozaar for high blood pressure
Control	M	21	Mexican-American	3.84 (84%)	None
Asthmatic	F	32	Mexican-American	2.02 (68%)	Albuterol inhaler (PRN), Advair 250/50 (BID)
Control	F	34	Mexican-American	2.48 (84%)	Zyrtec (PRN for seasonal allergies)
Asthmatic	M	47	African-American	2.23 (64%)	Albuterol inhaler (PRN), Advair 500/50 (BID)
Asthmatic	M	30	Caucasian	3.46 (85%)	Albuterol inhaler (PRN)
Asthmatic	M	31	African-American	3.95 (95%)	Albuterol inhaler (PRN), Claritine tb (PRN for seasonal allergies)
Asthmatic	F	51	African-American	1.97 (74%)	Albuterol inhaler (PRN), Allegra tb (PRN for seasonal allergies)
Asthmatic	M	42	Caucasian	2.84 (78%)	Albuterol inhaler (PRN), Symbicort 160/4.5 (BID)
Control	M	24	Caucasian	4.10 (89%)	None
Asthmatic	F	31	Asian	1.72 (60%)	Asmanex 220 mcg (OD), Albuterol inhaler (PRN)
Control	F	36	Asian	2.24 (83%)	None
Asthmatic	F	52	African-American	1.94 (89%)	Albuterol inhaler (PRN), Advair 100/50 (BID), Lisinopril 10 mg for high blood pressure
Asthmatic	F	48	African-American	1.93 (81%)	Albuterol inhaler (PRN), Advair 250/50 (BID), Claritine tb (PRN for seasonal allergies)
Control	F	25	Caucasian	4.11 (116%)	None
Control	F	39	African-American	2.57 (94%)	Lisinopril/hydrochlorothiazide for high blood pressure

and K_{Ca}3.1 channels. Selective blockers of K_V1.3, such as the peptide ShK-186, inhibit the production of Th1 and Th17 cytokines and the proliferation and migration of T_{EM} cells, whereas selective blockade of K_{Ca}3.1 channels preferentially targets naïve/T_{CM} cells (11–13, 17). Consequently, ShK-186 prevents and treats disease expression in rat models of delayed type hypersensitivity, multiple sclerosis, and rheumatoid arthritis mediated by Th1/Th17 lymphocytes without preventing the clearance of acute bacterial and viral infections and without inducing toxicity in rodents or non-human primates (11, 12, 18, 19). ShK-186 has successfully completed Phase 1a first-in-man safety clinical trials (20).

Here we show that T lymphocytes from the airways of patients with allergic asthma express large numbers of K_V1.3. ShK-186 inhibited the allergen-induced proliferation and production of IL-4 by peripheral blood T cells from patients with asthma. A similarly high expression of K_V1.3 by T lymphocytes was detected in the lungs of rats using a model of asthma. Administration of ShK-186 at the time of antigen challenge reduced immune cell infiltration in the BALF and also reduced production of IL-4, IL-5, and antigen-induced T cell proliferation, resulting in a reduction in airway reactivity. Our results suggest that K_V1.3 represents effective targets for the treatment of allergic asthma.

EXPERIMENTAL PROCEDURES

Human Samples—We obtained sputum and peripheral blood samples from subjects with mild to moderate asthma and from healthy volunteers using methods previously described (21). Subjects were non-smokers and had a base-line forced expiratory volume in 1 s (FEV1) of ≥60% predicted at the time of collecting the induced-sputum. Subjects were on no systemic corticosteroids or other immunosuppressive medications, but some were taking short- or long-acting β2 agonists and/or inhaled corticosteroids. Subject demographics are shown in Table 1. The human protocol was reviewed and approved by the Baylor College of Medicine Institutional Review Board.

Patch Clamp Electrophysiology—Mononuclear cells isolated from peripheral blood or induced sputum of subjects with asthma or healthy volunteers and from BALF or spleens of rats with a model of asthma were stained with AlexaFluor 488-conjugated anti-human or anti-rat CD3 antibodies (eBioscience and Biolegend, San Diego, CA). $CD3^+$ cells were immediately patch-clamped in the whole-cell configuration to determine numbers of functional K_V1.3 channels at the plasma membrane, as described (12, 13, 22, 23).

Flow Cytometry—Cells isolated from the induced sputum of subjects with asthma and healthy volunteers or from single-cell suspensions prepared from the lungs and spleens of rats with ovalbumin-induced asthma were washed with FACS-PBS (calcium-free PBS + 2% goat serum + 2% BSA). They were stained with anti-human or anti-rat CD3 antibodies conjugated to allophycocyanin (BD Biosciences) and either 20 nM ShK-F6CA (24) or anti-human CCR7 and anti-human CD45RA antibodies conjugated to FITC and V450, or anti-rat CD45RC antibodies conjugated to phycoerythrin (all from BD Biosciences) in FACS-PBS for 20 min on ice in the dark. After 3 washes with FACS-PBS, cells were fixed for 20 min at room temperature with PBS + 1% paraformaldehyde, and data were acquired on a BD CantoII or a BD LSRII flow cytometer (BD Biosciences) using FACSDiva and analyzed using FlowJo (Treestar, Ashland, OR).

[^{3}H]Thymidine Incorporation and Detection of Intracellular Cytokines—Proliferation assays were performed as previously described (11–13, 23, 25, 26). Intracellular levels of IL-4, IL-5, and IL-13 were detected by flow cytometry after a 6-h allergen-

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induced activation in the presence of 100 nM ShK-186 or vehicle and of the protein transport inhibitor brefeldin A.

Animals and Induction of Airway Inflammation—All procedures were conducted after approval by the Institutional Animal Care and Use Committee at Baylor College of Medicine. Baylor College of Medicine follows the requirements of the Guide for the Care and Use of Laboratory Animals (National Research Council, eighth edition). Female F344 rats (7–9 weeks old) were purchased from Harlan-Sprague-Dawley (Indianapolis, IN) and housed in autoclaved setups with food and water *ad libitum*. Airway inflammation to endotoxin-free ovalbumin (Biovendor R&D, Asheville, NC) was induced as previously described (27). Briefly, F344 rats received a subcutaneous immunization with 500 μl/rat of an ovalbumin-alum solution prepared by dissolving 5 ml of 500 μg/ml endotoxin-free Ova in emulsion with 5 ml of 0.5 g/5 ml of aluminum potassium sulfate dodecahydrate adjuvant (Sigma) in sterile, pyrogen-free 0.9% NaCl. Animals received a subcutaneous booster injection of the same emulsion 7 days later. Fourteen days after the last immunization, rats were challenged with an intratracheal bolus (500 μl/rat) of 500 μg/ml endotoxin-free ovalbumin in PBS. Challenges were given every day for three consecutive days.

Administration of ShK-186—ShK-186 (synthetic analog of ShK; accession number P29187) is a well characterized, potent, and selective peptide blocker of K_V1.3 channels (14, 15). It was manufactured using solid-phase synthesis as described and dissolved in P6N buffer (10 mM $NaHPO_4$, 0.8% NaCl, 0.05% Tween 20, pH 6.0) for verification of selectivity and potency by patch clamp electrophysiology (11, 12, 17, 19). Rats received a daily subcutaneous injection of P6N buffer (vehicle) or ShK-186 (0.1 mg/kg) in the scruff of the neck at time of challenge with ovalbumin.

Airway Hyper-responsiveness Measurement—Measurements of airway hyper-responsiveness were conducted 24 h after the last intra-tracheal challenge with ovalbumin as previously described, with some modifications (28, 29). Briefly, rats were anesthetized with etomidate and intubated for continuous ventilation inside a whole body plethysmograph using 100% oxygen to maintain physiologic pH and arterial PCO_2. A stable base line was established for respiratory system resistance as determined by continuously quantitating $\Delta Pt/V$ (where ΔPt = change in tracheal pressure, and V = change in air flow) at 70% tidal volume. Acetylcholine chloride was administered via tail vein injection over 1 s in increasing doses. The provocative concentration of acetylcholine that caused a 200% increase in resistance was calculated and plotted.

Bronchoalveolar Lavage, Lung, and Spleen Collection—BALF and lungs were collected after measurement of airway responsiveness for differential counts, functional assays on T lymphocytes, and detection of K_V1.3 channels on lung-infiltrating T lymphocytes and of cytokines as described (27, 28).

Cytokine ELISAs—Kits to measure cytokine levels in the BALF from rats were purchased from R&D Systems (Minneapolis, MN) for IL-10, from Qiagen (Valencia, CA) for IL-13, from Ray Biotech (Norcross, GA) for IL-4, and from Signosis (Sunnyvale, CA) for IL-5 and were used following manufacturers' instructions.

Statistical Analysis—Data are expressed as the mean ± S.E. Statistical analysis was performed using the non-parametric Mann-Whitney *U* test for all tests but the dose-response to acetylcholine, for which we used a two-way analysis of variance (GraphPad Prism, La Jolla, CA). p values of less than 0.05 were considered significant in all statistical analyses.

RESULTS

K_V1.3 Channels Are Expressed at High Levels by CCR7$^-$CD45RA$^-$ T_{EM} Lymphocytes in the Induced Sputum of Patients with Asthma—We used the whole-cell technique of patch clamp electrophysiology to detect functional K_V1.3 channels at the plasma membrane of CD3$^+$ cells in the induced sputum of subjects with asthma and control subjects. The biophysical properties of K_V1.3 channels in all samples analyzed by whole-cell patch clamp were similar to those previously described for native K_V1.3 channels in human and rat T lymphocytes and for cloned K_V1.3 channels (13, 23, 30). The channels displayed a slow inactivation, typical of K_V1.3, when pulsed every 30 s at 40 mV (Fig. 1*A*); they had a half-activation voltage of ≈−32 mV (Fig. 1, *D* and *E*). Pulsing every 1 s induced a progressive reduction in current amplitude, known as the "use dependence" characteristic for K_V1.3 (data not shown). We measured the total K^+ conductance through K_V1.3, and knowing that the single-channel conductance for this channel is 12 picosiemens (30), we calculated the number of functional K_V1.3 channels at the plasma membrane of each T lymphocyte. T cells from the induced sputum of subjects with asthma displayed higher numbers of K_V1.3 channels (channel numbers ranged from 615 to 1559 per cell) and a higher density of K_V1.3 channels than T cells in the induced sputum of healthy controls (channel numbers ranged from 492 to 809 per cells; $p < 0.05$) or T cells in the peripheral blood of either subjects with asthma or controls (channel numbers ranged from 411 to 636 and from 427 to 627, respectively; $p < 0.01$ for both) (Fig. 1, *A–C*).

Flow cytometry using ShK-F6CA, a fluorophore-conjugated K_V1.3-binding peptide (24), detected staining on CD3$^+$ cells only in the induced sputum from subjects with asthma (Fig. 1, *F* and *G*). The other samples displayed minimal staining ($p < 0.01$ when comparing mean fluorescence intensity of T cells from asthma-induced sputum with T cells from the other three samples), suggesting a number of channels below the 600-channel detection threshold described for ShK-F6CA (11, 24). Using flow cytometry, we also found that 33.3 ± 3.3% of T lymphocytes in the induced sputum of subjects with asthma are CD45RA$^-$CCR7$^-$ T_{EM} cells, a number significantly higher than found in the induced sputum of control subjects (14.9 ± 1.1%; $p < 0.05$) or in the peripheral blood of subjects with asthma (9.9 ± 3%; $p < 0.01$) or of healthy subjects (6.4 ± 0.5%; $p < 0.01$) (Fig. 1, *H* and *I*).

ShK-186 Inhibits the Activation of Allergen-specific Th2 Lymphocytes Isolated from the Blood of Patients with Asthma—ShK-186 is known to inhibit the proliferation and secretion of cytokines (IL-2, IFNγ, IL-17A) by human Th1 and Th17 T_{EM} lymphocytes (14, 15). To determine its effects on the function of Th2 lymphocytes, we stimulated peripheral blood T lymphocytes from human subjects with asthma with a panel of allergens or the mitogen phytohemagglutinin and determined the

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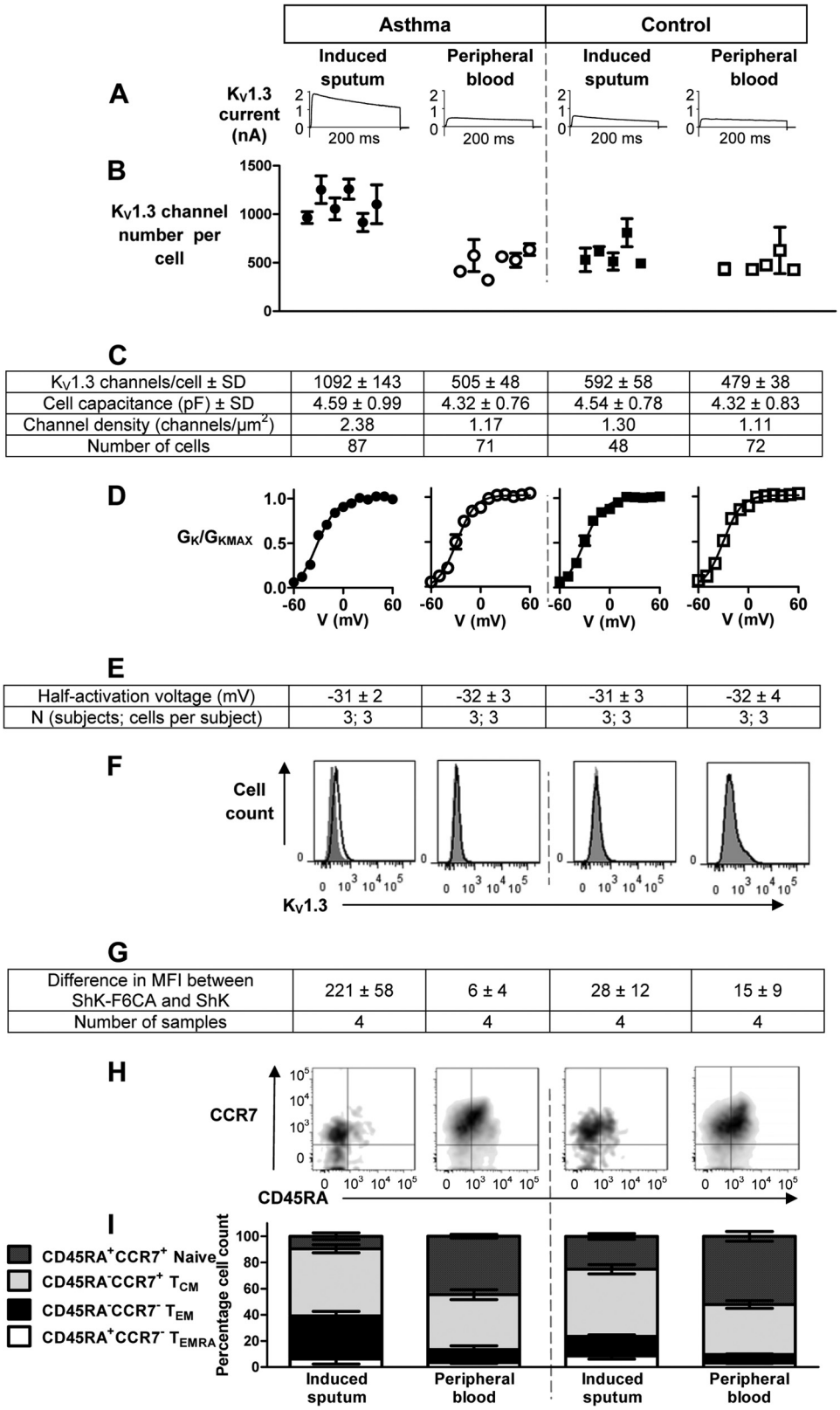

K_V1.3 channels/cell ± SD	1092 ± 143	505 ± 48	592 ± 58	479 ± 38
Cell capacitance (pF) ± SD	4.59 ± 0.99	4.32 ± 0.76	4.54 ± 0.78	4.32 ± 0.83
Channel density (channels/μm^2)	2.38	1.17	1.30	1.11
Number of cells	87	71	48	72

Half-activation voltage (mV)	-31 ± 2	-32 ± 3	-31 ± 3	-32 ± 4
N (subjects; cells per subject)	3; 3	3; 3	3; 3	3; 3

Difference in MFI between ShK-F6CA and ShK	221 ± 58	6 ± 4	28 ± 12	15 ± 9
Number of samples	4	4	4	4

FIGURE 1. **K_V1.3 channel expression in T lymphocytes isolated from the induced sputum and the peripheral blood of patients with asthma and healthy volunteers.** *A*, representative whole-cell K_V1.3 currents in T lymphocytes. *B*, K_V1.3 channel number measured by whole-cell patch clamp. Each symbol represents the mean ± S.E. of 8–15 cells from a different donor. *C*, table detailing the values obtained in *panel B*. *pF*, picofarads. *D*, normalized peak K^+ conductance-voltage relations fitted to a Boltzmann function to determine the half-activation voltage of the K_V current. *E*, half-activation voltages determined in *D*. *F*, representative K_V1.3 channel expression detected by staining of $CD3^+$ cells with ShK-F6CA (*black line*) in cells from a donor in each group shown in *A*. Cells incubated with unlabeled ShK are shown in *gray*. *G*, difference in mean fluorescence intensity (*MFI*) for each type of samples shown in *panel F*. *H*, representative flow cytometric profile of $CD3^+$ cells stained for CCR7 and CD45RA. *I*, quantification of the different subsets of T cells based on expression levels of CCR7 and CD45RA in the subjects analyzed. Data are shown as the mean ± S.E. ($n = 5$ subjects with asthma and 5 control subjects).

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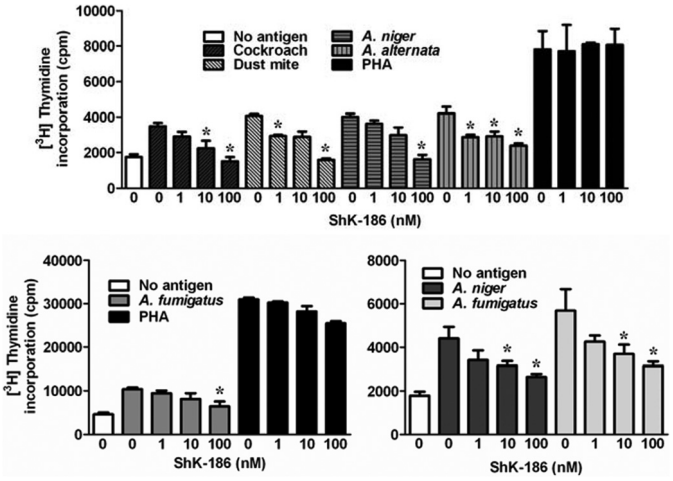

FIGURE 2. **ShK-186 inhibits the allergen-induced proliferation of T lymphocytes isolated from the peripheral blood of patients with asthma.** Mean ± S.E. of data obtained in triplicate with cells from three different patients with asthma (one per plot) are shown. *, $p < 0.05$ (Mann-Whitney *U* test).

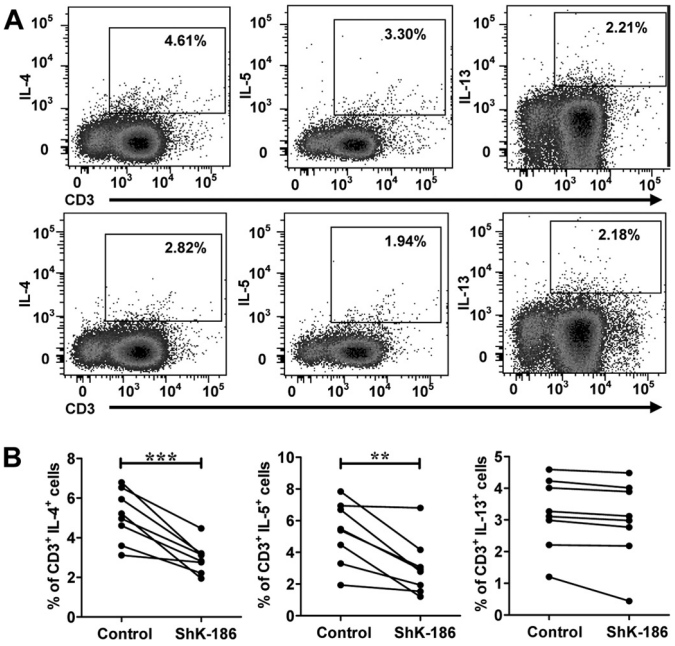

FIGURE 3. **ShK-186 inhibits the allergen-induced production of IL-4 by T lymphocytes isolated from the peripheral blood of patients with asthma.** *A*, representative flow cytometric profiles of IL-4 (*left*), IL-5 (*middle*), and IL-13 (*right*) production in $CD3^+$ cells without (*top*) and with (*bottom*) 100 nM ShK-186. *B*, percentages of $CD3^+IL\text{-}4^+$ (*left*), $CD3^+IL\text{-}5^+$ (*middle*), and $CD3^+IL\text{-}13^+$ cells; each symbol represents a different donor. **, $p < 0.01$; ***, $p < 0.001$ (Mann-Whitney *U* test).

effect of ShK-186 on their proliferation and production of Th2 cytokines. ShK-186 caused a dose-dependent inhibition of allergen-induced T cell proliferation regardless of the allergen used (Fig. 2). In contrast, the phytohemagglutinin-induced proliferation of T lymphocytes was not significantly affected by the $K_V1.3$ blocker.

ShK-186 significantly reduced IL-4 and IL-5 production by allergen-stimulated peripheral T cells from subjects with asthma ($p < 0.001$ and $p < 0.01$, respectively; Fig. 3). The production of IL-13 was not affected by ShK-186 ($p > 0.05$) Thus, consistent with its ability to inhibit T_{EM} cell function, ShK-186 selectively inhibited antigen-specific proliferation and cytokine secretion from human T_{EM} cells.

Infiltrating T Cells in the Lungs of Rats with an Ovalbumin-induced Model of Asthma Are CD45RC⁻ Memory T Cells and Express High Levels of $K_V1.3$ Channels—Whole-cell patch clamp demonstrated that lung-infiltrating T lymphocytes in rats with a model of asthma express a large number of $K_V1.3$ channels (1156 ± 297 channels/cell), whereas splenic T lymphocytes from the same rats express only 153 ± 108 $K_V1.3$ channels/cell (Fig. 4, *A–C*). We next used flow cytometry to detect $K_V1.3$ channels on T lymphocytes in the lungs and spleens of rats with a model of asthma induced against ovalbumin. Splenic T cells expressed fewer than 600 $K_V1.3$ channels per cell, as demonstrated by a lack of staining with ShK-F6CA (Fig. 4, *D* and *E*). In contrast, expression of $K_V1.3$ was detectable by T lymphocytes in the lungs. Finally, flow cytometry also demonstrated that approximately half of the lung-infiltrating T lymphocytes in those rats are $CD45RC^-$, a phenotype of memory T cells (11, 31), whereas fewer than 15% of splenic T cells exhibited this phenotype (Fig. 4, *F* and *G*).

ShK-186 Reduces Immune Infiltrates in the BALF of Rats with Ovalbumin-induced Asthma and Inhibits the ex Vivo Proliferation of Ovalbumin-specific T Lymphocytes Isolated from the BALF—BALF was collected from the rats immediately after measurement of airway reactivity, 24 h after the last intra-tracheal challenge with ovalbumin. The number of total cells and of eosinophils, neutrophils, and lymphocytes in BALF was significantly increased in rats immunized and challenged with ovalbumin when compared with healthy rats (Fig. 5, *A* and *B*). Systemic administration of ShK-186 reduced the number of total BALF cells by 63% (Fig. 5*A*). ShK-186 treatment also reduced the number of BALF eosinophils by 78%, of neutrophils by 76%, and of lymphocytes by 55% (Fig. 5*B*).

To determine the effects of the *in vivo* administration of ShK-186 on the proliferation of lung-infiltrating ovalbumin-specific T lymphocytes, we measured the base-line and ovalbumin-induced proliferation of BALF cells (Fig. 5*C*). The base-line total cell proliferation was higher in the ShK-186-treated rats than in the vehicle-treated rats ($p < 0.05$). The *ex vivo* stimulation with ovalbumin induced a 54% increase in the proliferation of cells isolated from the BALF of the vehicle-treated rats ($p < 0.05$ when compared with baseline proliferation). In contrast, ovalbumin did not increase the proliferation of the cells from the BALF of ShK-186 treated rats ($p = 0.68$). These results suggest that the *in vivo* administration of ShK-186 inhibits the proliferation of lung-infiltrating ovalbumin-specific T cells.

ShK-186 Inhibits the Production of IL-4 and IL-5 and Induces the Production of IL-10 in the BALF of Rats in a Model of Asthma—Because ShK-186 reduced airway inflammation and a Th2 response is an important feature of the ovalbumin model of asthma in F344 rats, we determined the effects of ShK-186 on Th2 cytokine levels in BALF (32). Immunization and challenge with ovalbumin significantly increased the amounts of IL-4, IL-5, and IL-13 in the BALF of rats (Fig. 6, *A*, *B*, and *C*). Treatment with ShK-186 significantly reduced the levels of IL-4 and IL-5 ($p < 0.01$ and $p < 0.05$, respectively) but only marginally those of IL-13 ($p > 0.05$).

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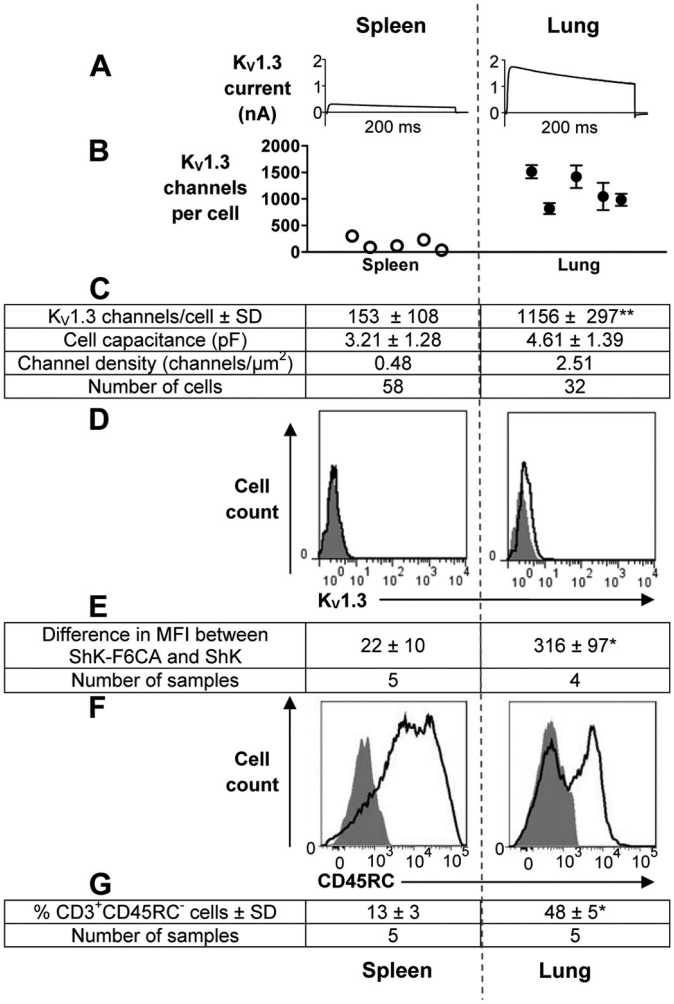

FIGURE 4. **K_V1. 3 channel phenotype of T lymphocytes in the lungs and spleens of rats with ovalbumin-induced asthma.** *A*, representative whole-cell K_V1.3 currents in T lymphocytes. *B*, K_V1.3 channel number measured by whole-cell patch clamp. Each symbol represents the mean ± S.E. of a minimum of six cells from a different rat. *C*, table detailing the values obtained in *panel B*. *pF*, picofarads. *D*, representative K_V1.3 channel expression detected by staining of $CD3^+$ cells with ShK-F6CA (*black lines*) in single-cell suspensions. CD3-stained cells incubated with unlabeled ShK are shown in *gray*. *E*, difference in mean fluorescence intensity (*MFI*) for each type of samples shown in *panel D*. *F*, representative flow cytometric histograms of $CD3^+$ cells stained for CD45RC. *G*, quantification of $CD3^+CD45RC^-$ cells. *, $p < 0.05$; **, $p < 0.01$ (Mann-Whitney *U* test).

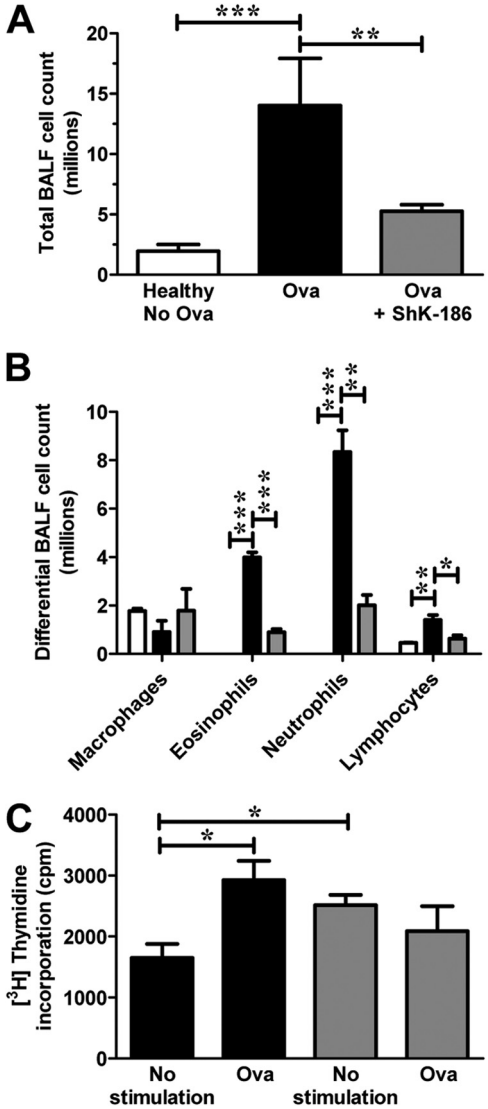

FIGURE 5. **ShK-186 induced a decrease in the number of infiltrating immune cells in the BALF of rats.** *A*, total BALF cell count. $n = 8$ rats per group. *B*, differential cell count. Rats were left untreated (*white*) or were immunized against ovalbumin (*Ova*) and received intra-tracheal ovalbumin followed by treatment with vehicle (*black*) or ShK-186 (*gray*). $n = 8$ rats per group. *C*, [^{3}H]thymidine incorporation over a 72-h period by cells in the BALF of rats immunized and challenged against ovalbumin and treated with vehicle (*black*) and from rats immunized and challenged against ovalbumin and treated with ShK-186 (*gray*). $n = 3$ rats per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Mann-Whitney *U* test).

Patients with asthma have lower levels of IL-10 in their lungs, and an increase in IL-10 production has been proposed for immunotherapy (33). Interestingly, the knockdown of K_V1.3 in mice induces overexpression of IL-10 by lymphocytes (34); we, therefore, tested the effects of ShK-186 in IL-10 levels in the BALF of rats. Rats with ovalbumin-asthma had a similar amount of IL-10 in their BALF as healthy rats (Fig. 6*D*). Treatment with ShK-186 at time of ovalbumin challenge induced a 2-fold increase in the amount of IL-10 in rat BALF.

ShK-186 Reduces Airway Hyper-responsiveness in a Rat Model of Asthma—Immunization of F344 rats against ovalbumin and subsequent intra-tracheal instillations of ovalbumin induced airway hyper-reactivity, measured as a percentage increase of respiratory system resistance (R_{Rs}) after challenges with increasing doses of acetylcholine challenge (Fig. 7). In rats sensitized and challenged with ovalbumin, the dose-response curve of R_{Rs} was shifted to the left when compared with healthy animals. Moreover, the percentage of R_{Rs} induced by 3 μg/g of body weight acetylcholine increased significantly in the ovalbumin-challenged rats compared with control animals ($p < 0.001$). The subcutaneous injection of ShK-186 at the time of intra-tracheal instillation of Ova significantly reduced airway reactivity ($p < 0.01$). At the highest dose of acetylcholine, the percentage of R_{Rs} induced in ShK-186 treated rats was similar to that measured in control rats. These results suggest that ShK-186 reduces ovalbumin-induced airway hyper-responsiveness.

DISCUSSION

K_V1.3 channels expressed by T lymphocytes have been proposed as targets for the treatment of chronic inflammatory dis-

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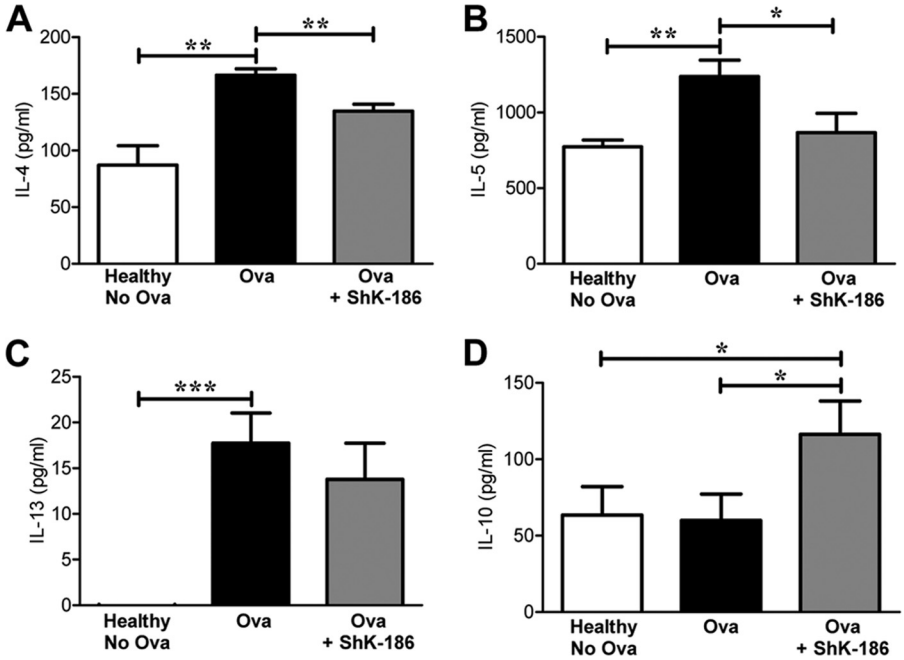

FIGURE 6. **ShK-186 induces a decrease in the BALF concentration of IL-4 and IL-5 and an increase in the concentration of IL-10.** Concentrations of IL-4 (*A*), IL-5 (*B*), IL-13 (*C*), and IL-10 (*D*) were measured in the BALF of rats by ELISA. $n = 6$ rats per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Mann-Whitney *U* test). *Ova*, ovalbumin.

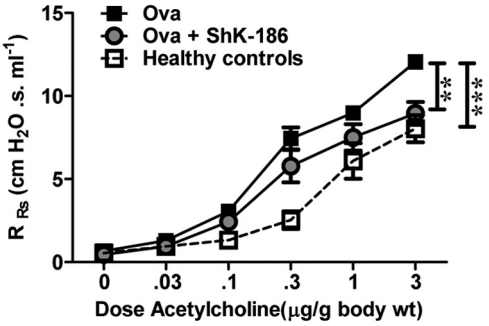

FIGURE 7. **The subcutaneous administration of ShK-186 (0. 1 mg/kg) significantly decreased R_{RS} in response to increasing doses of acetylcholine.** Rats were left untreated (*open square*) or were immunized against ovalbumin (*Ova*) and received intra-tracheal ovalbumin followed by treatment with vehicle (*black square*) or ShK-186 (*gray circle*). $n = 6$ rats per group. **, $p < 0.01$; ***, $p < 0.001$ (two-way analysis of variance).

eases mediated by Th1/Th17 lymphocytes (14, 15). Here, we demonstrate that the $K_V1.3^{high}$ phenotype of T lymphocytes described in those diseases is also a feature of a significant proportion of lower airway- and lung-infiltrating T cells in human and experimental asthma. By using ShK-186, a potent and selective blocker of $K_V1.3$ channels, we show that these channels are attractive targets for the treatment of asthma by inhibiting the allergen-induced proliferation and production of Th2 cytokines by T cells from subjects with asthma. In addition, ShK-186 treatment of rats with ovalbumin-induced asthma induced a decrease in inflammation and airway reactivity.

The majority of T lymphocytes in the induced sputum of subjects with asthma belonged to the $CD45RA^-$ memory phenotype (~85% of $CD3^+$ cells), of which almost half were $CD45RA^-CCR7^-$ T_{EM} cells and expressed high levels of $K_V1.3$ channels at their plasma membranes. These findings are consistent with the infiltrating T lymphocytes being activated $CD45RA^-CCR7^-$ T_{EM} cells, as previously described in asthma (8). The $K_V1.3^{high}$ phenotype of T cells was also previously observed at sites of inflammation in other T cell-mediated chronic inflammatory diseases, such as the synovium and synovial fluid in rheumatoid arthritis, the brain and cerebrospinal fluid in multiple sclerosis, and the skin in psoriasis (12, 35, 36). However, all of these diseases are mediated by Th1/Th17 lymphocytes, whereas Th2 lymphocytes play a role in disease pathogenesis in a large number of patients with asthma. The targeting of $K_V1.3$ channels in T lymphocytes for the treatment of asthma has been hypothesized in recent reviews by us and our collaborators based on the successful inhibition of Th1/Th17 lymphocytes in autoimmune diseases as ShK-186, and other $K_V1.3$ channel blockers have been shown to inhibit the secretion of IL-2, IFNγ, and IL-17A by T_{EM} lymphocytes (14, 15, 20, 37). However, this study is to our knowledge the first to test the efficacy of a $K_V1.3$ channel blocker in inhibiting the function of human Th2 lymphocytes isolated from subjects with asthma and in treating an animal model of asthma.

The isolation of T lymphocytes from the induced sputum of human subjects used a relatively harsh protocol, but the cells remained viable. Each patch clamp assay to detect $K_V1.3$ channels involved pulsing to 40 mV every 30 s for 3–5 min until a stable current was obtained, indicating complete perfusion of the cell with the pipette solution, after which cells underwent a use-dependent pulse protocol (10 pulses for 200 ms, each at a 1-s interval). In addition a few cells from each subject were further used for measuring K_V currents at different voltages (−60 to 60 mV every 30 s) to determine the half-activation voltage of the channel. Such protocols would not yield clean currents if the cells were non-viable. T lymphocyte maintenance in culture for more than a few hours was, however, hindered by the presence of fungi in the samples, a common occur-

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rence in patients with asthma (38–40), and the use of sufficient amounts of amphotericin B in the culture media to prevent fungal growth also affected T cell viability.

Cell capacitance is directly correlated to cell volume and, therefore, gives a good indication of cell size. Previous reports showed that human resting peripheral blood T lymphocytes have a cell capacitance of ~2 picofarads (13, 16, 41). In contrast, our data show a capacitance of ~ 4 picofarads in both peripheral blood and induced sputum. This discrepancy cannot be explained by the method used to collect blood (heparinized tubes) or to isolate the mononuclear cells as we used standard technique. The only differences between the T cells we have analyzed here and those in previous reports are the fact that blood was drawn from subjects after the procedure for inducing and collecting sputum and that the cells were stained with a fluorophore-conjugated anti-CD3 antibody immediately before patch clamp.

ShK-186 inhibited the allergen-induced proliferation of peripheral blood T lymphocytes from subjects with asthma but did not affect their proliferation induced by phytohemagglutinin. These results are in agreement with previous work showing that ShK-186 and other blockers of $K_V1.3$ preferentially affect $CD45RA^-CCR7^-$ T_{EM} cells (11–13, 18) and that disease-relevant T cells in asthma are $CD45RA^-CCR7^-$ T_{EM} cells (8). Phytohemagglutinin is a mitogen that activates all human T lymphocytes. Because the majority of circulating T cells are $CCR7^+$ naïve and T_{CM} cells, our results are consistent with previous findings showing that naïve and T_{CM} cells escape $K_V1.3$ block by up-regulating $K_{Ca}3.1$ upon activation (11–13, 18). Moreover, the IC_{50} for inhibition of allergen-induced T lymphocyte proliferation was in the low nM range, consistent with published values for ShK-186 (12, 17, 18).

The detection of fewer than 10% T lymphocytes producing Th2 cytokines in the blood of subjects with asthma after allergen-induced *ex vivo* activation is consistent with previously published results using a similar procedure (42–44). ShK-186, used at a concentration of 100 nM, inhibited the allergen-induced production of IL-4 and IL-5 by human T lymphocytes by ~50%. This result is in agreement with previous data showing a similar inhibition of thapsigargin-induced IL-4 production by human T cells (15). The allergen-induced production of IL-13 by human T lymphocytes was not affected by ShK-186 nor were the IL-13 levels in the BALF of rats with the ovalbumin-induced model of asthma. These results are in agreement with previous reports that showed blocking $K_V1.3$ channels at the plasma membrane does not affect all signaling pathways in T lymphocytes (14, 15).

The systemic administration of ShK-186 (0.1 mg/kg) after intra-tracheal challenge with ovalbumin reduced airway reactivity, immune infiltrates, and T lymphocyte activation in a rat model of asthma. This dose, regimen, and delivery method of ShK-186 is consistent with previous studies using the same $K_V1.3$ blocker to prevent or treat rat models of delayed type hypersensitivity, multiple sclerosis, and rheumatoid arthritis (12, 17–19). Pharmacokinetic and pharmacodynamic studies in rats have shown that, after a single subcutaneous injection, ShK-186 reaches a maximum plasma concentration in less than 5 min and has a half-life of less than 10 min (19). However, ShK-186 forms a slow-release depot at the site of injection with a biphasic half-life of 1.5 h initially and a terminal half-life over 48 h (19). This leads to sufficient blood concentrations of free ShK-186 to block $K_V1.3$ for a minimum of 2 days. In addition, the high affinity of ShK-186 for $K_V1.3$ channels (K_d = 70 pM) means that once the blocker is bound to the channel it does not easily detach (14, 15). The *in vivo* half-life of channel-bound ShK-186 is likely longer than 2 days.

The *in vivo* administration of ShK-186 resulted in a higher base-line proliferative rate of BALF cells. Blocking KV1.3 channels has never been associated with the increase of cell proliferation; it is, therefore, unlikely that this increased proliferative rate in the absence of exogenous mitogen or antigen is directly caused by the ShK-186 effect on cells of the BALF, but such an effect cannot be ruled out. A more likely explanation for this difference in proliferative rate resides in the different cellular composition of the sample as the cells from vehicle-treated rats contained more eosinophils and neutrophils and those from ShK-186-treated rats contained more macrophages, and the different cell subsets proliferate at different rates in culture in the absence of exogenous antigens or mitogens. Human and mouse eosinophils and neutrophils express ATP-dependent and K_{Ca} potassium channels, but no currents resembling $K_V1.3$ were observed in those cells (45–48). ShK-186 is 100-fold selective for $K_V1.3$ over the closely related $K_V1.1$ and 700-fold or more selective for $K_V1.3$ over all other ion channels tested (11, 14, 15). Thus, at the concentrations used *in vivo* and *in vitro* during this study, ShK-186 does not affect the function of ion channels expressed by human and mouse eosinophils and neutrophils. If the potassium channel phenotype of human and mouse cells holds true in F344 rats, ShK-186 would have no direct effects on these cell types in the model of asthma used in this study. Macrophages express a number of potassium channels, including $K_V1.3$ (48, 49). In macrophages, however, $K_V1.3$ subunits associate with $K_V1.5$ subunits to form heterotetrameric channels (49). Because ShK-186 has no effect on $K_V1.5$ at the concentrations used here, $K_V1.3/K_V1.5$ heterotetramers are less sensitive to this blocker (14, 15, 49).

The knockdown of $K_V1.3$ in mice biases T lymphocytes toward an immunoregulatory phenotype with increased production of IL-10 (34). Our results demonstrate that blocking $K_V1.3$ channels with ShK-186 induces an increase in IL-10 in the BALF of rats with ovalbumin-induced asthma, suggesting that blocking $K_V1.3$ may have the same effect of T lymphocyte differentiation into a regulatory phenotype as deletion of the channel.

The findings presented here support the efficacy of blocking $K_V1.3$ channels in reducing inflammation and airway reactivity in a rat model of ovalbumin-induced asthma. $K_V1.3$ channels represent an attractive family of potential therapeutics for both asthma and autoimmune diseases because in humans $K_V1.3$ channels selective blockers preferentially target $CCR7^-CD45RA^-$ T_{EM} lymphocytes, leaving $CCR7^+$ naïve and T_{CM} lymphocytes and cells of the innate immune systems free to fight tumors and acute infections. In addition, because of the restricted tissue distribution of homotetramers of the $K_V1.3$ channels to lymphocytes and the olfactory bulb (14, 15, 50) and the requirement of ShK-186 to bind to all 4 subunits of the

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tetramer (51, 52), ShK-186 and similar K_V1.3 blockers lack side effects in other systems. Indeed, blocking the K_V1.3 channel does not prevent the clearance of acute viral or bacterial infections and does not induce overt toxicity in rodents or non-human primates (9, 14, 15). Furthermore, ShK-186 does not prevent all subsets of human natural killer lymphocytes from killing tumor cells, suggesting it will not increase the risk of cancer (53). In contrast, current approved medications that target T lymphocytes to treat these diseases are not selective for $CD45RA^{-}CCR7^{-}$ T_{EM} lymphocytes and are known to induce significant immune side effects, such as an increase in the risk of infections or tumors in addition to non-immune side effects through affecting other organ systems. ShK-186 has already successfully undergone Phase 1a first-in-man safety clinical trials and is currently undergoing Phase 1b clinical trials (20).

Our study was conducted by the systemic administration of ShK-186 but, because ShK-186 and other ShK analogs are stable and resistant to proteases (17), they may be amenable to aerosolization and direct delivery into the lumen of the airways for the treatment of asthma.

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IMMUNOLOGY

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