Latifa Chachi, Aarti Shikotra, S. Mark Duffy, Omar Tliba, Christopher Brightling, Peter Bradding and Yassine Amrani

*J Immunol* 2013; 191:2624-2636; Prepublished online 31 July 2013; doi: 10.4049/jimmunol.1300104

http://www.jimmunol.org/content/191/5/2624
Functional $K_{Ca}$3.1 Channels Regulate Steroid Insensitivity in Bronchial Smooth Muscle Cells

Latifa Chachi,* Aarti Shikotra,* S. Mark Duffy,* Omar Tliba, † Christopher Brightling,* Peter Bradding,§* and Yassine Amrani*§†

Identifying the factors responsible for relative glucocorticosteroid (GC) resistance present in patients with severe asthma and finding tools to reverse it are of paramount importance. In asthma we see in vivo evidence of GC-resistant pathways in airway smooth muscle (ASM) bundles that can be modeled in vitro by exposing cultured ASM cells to TNF-α/IFN-γ. This action drives GC insensitivity via protein phosphatase 5-dependent impairment of GC receptor phosphorylation. In this study, we investigated whether $K_{Ca3.1}$ ion channels modulate the activity of GC-resistant pathways using our ASM model of GC insensitivity. Immunohistochemical staining of endobronchial biopsies revealed that $K_{Ca3.1}$ channels are localized to the plasma membrane and nucleus of ASM in both healthy controls and asthmatic patients, irrespective of disease severity. Western blot assays and immunofluorescence staining confirmed the nuclear localization of $K_{Ca3.1}$ channels in ASM cells. The functional importance of $K_{Ca3.1}$ channels in the regulation of GC-resistant chemokines induced by TNF-α/IFN-γ was assessed using complementary inhibitory strategies, including $K_{Ca3.1}$ blockers (TRAM-34 and ICA-17043) or $K_{Ca3.1}$-specific small hairpin RNA delivered by adenoviruses. $K_{Ca3.1}$ channel blockade led to a significant reduction of fluticasone-resistant CX3CL1, CCL5, and CCL11 gene and protein expression. $K_{Ca3.1}$ channel blockade also restored fluticasone-induced GC receptor-α phosphorylation at Ser211 and transactivation properties via the suppression of cytokine-induced protein phosphatase 5 expression. The effect of $K_{Ca3.1}$ blockade was evident in ASM cells from both healthy controls and asthmatic subjects. In summary, $K_{Ca3.1}$ channels contribute to the regulation of GC-resistant inflammatory pathways in ASM cells: blocking $K_{Ca3.1}$ channels may enhance corticosteroid activity in severe asthma. The Journal of Immunology, 2013, 191: 2624–2636.

Although many patients with asthma are well controlled with inhaled glucocorticoid (GC) therapy, ~5–10% of patients have difficult-to-control or severe disease that is poorly responsive to this treatment. These patients account for a disproportionate share of asthma-related health care costs and morbidity (1). This situation represents a significant unmet clinical need, and novel therapies are urgently required (1).

Despite significant progress in the field, the precise molecular mechanisms mediating GC insensitivity in severe asthma are poorly understood. Several potential mechanisms have been postulated, identified mostly from studies in immune cells, including alveolar macrophages and circulating PBMCs obtained from patients with GC insensitivity (2–6). In addition to infiltrating inflammatory cells, the airway smooth muscle (ASM) is another key player in severe asthma pathophysiology, demonstrating heightened sensitivity to both direct and indirect contractile stimuli leading to exaggerated airway narrowing and airflow obstruction (7). The therapeutic benefit in those with severe asthma that is provided by bronchial thermoplasty, a therapy that attenuates bronchoconstriction via reduction of ASM mass, has strongly supported the idea that ASM plays a central part in the pathogenesis of the severe disease (8–11). In addition to its central role in bronchoconstriction, the role of ASM in the pathogenesis of severe asthma could derive also from its immunomodulatory function, as it secretes a variety of proinflammatory mediators (12). This characteristic is evident both in vitro using cultured ASM cells and in vivo in the ASM bundles of asthmatic patients (12).

Whether the pathogenesis of severe asthma is driven by the steroid-resistant production of proteins from ASM cells represents an interesting hypothesis. Indeed, previous reports convincingly showed that despite high doses of inhaled or oral GC taken by patients, there is ongoing expression of different “proasthmatic” proteins in asthmatic ASM bundles, including the following chemokines: chemokine (C-X-C motif) ligand (CXCL) 1 (13), CCL11 (14), CCL15 (15), and CCL19 (16), and a disintegrin and metalloprotease domain (ADAM) 33 and ADAM8 (17, 18). These studies provide indisputable in vivo evidence for the existence of steroid-resistant pathways in ASM that are potentially driving inflammatory processes and ASM contractile dysfunction in asthmatic airways. A better understanding of the underlying mechanisms driving these steroid-resistant pathways in ASM could therefore lead to novel therapeutic approaches.
The intermediate-conductance Ca2+-activated K+ (KCa3.1) channel KCa3.1 channel (also known as IK1, SK4, or KCNN4) is closely associated with the progression of number of human diseases, and is expressed by human ASM cells (19, 20). In human ASM cells, a KCa3.1 blocker attenuated mitogen-induced ASM cell proliferation (20). Inhibitors of KCa3.1 channels, such as TRAM-34, are effective in treating various inflammatory diseases in preclinical models, including atherosclerosis (21), neurodegenerative disorders (22), autoimmune encephalomyelitis (23), and coronary vasculoproliferative diseases (24). With respect to asthma, we recently provided the first evidence, to our knowledge, that TRAM-34 prevents the development of eosinophilic inflammation, airway hyperresponsiveness, and airway remodeling in a murine model of allergic asthma (25). The underlying mechanisms by which KCa3.1 channels contribute to the pathogenesis of allergic asthma are yet to be defined, but we have shown that KCa3.1 channels regulate mast cell degranulation and migration (26–29), as well as fibrocyte migration (30). Others have implicated KCa3.1 channel (also known as IK1, SK4, or KCNN4) is closely associated with the pathogenesis of allergic asthma. 34 prevents the development of eosinophilic inflammation, airway hyperresponsiveness, and airway remodeling in a murine model of allergic asthma (25). The underlying mechanisms by which KCa3.1 channels contribute to the pathogenesis of allergic asthma are yet to be defined, but we have shown that KCa3.1 channels regulate mast cell degranulation and migration (26–29), as well as fibrocyte migration (30). Others have implicated KCa3.1 channels in the migration of lung dendritic cells to CCL19 and CCL21 (31). These observations demonstrate that activation of KCa3.1 channels on structural (smooth muscle/fibroblasts) airway cells may represent an important pathway driving key features of allergic asthma.

In the current study, we uncovered the surprising finding that KCa3.1 channels are not only essential in driving the production of GC-resistant chemokines by ASM cells but also contribute to the reduced ASM sensitivity to GC therapy via the upregulation of serine/threonine phosphatase protein phosphatase 5 (PP5). To our knowledge, this is the first report to demonstrate a functional interaction between KCa3.1 channels and the impairment of GC function. This study uncovers a novel molecular mechanism contributing to the development of GC insensitivity, a defining feature of severe asthma.

Materials and Methods

Human subjects

Asthmatic subjects, patients with chronic obstructive pulmonary disease (COPD), and healthy volunteers were recruited. Asthmatic and COPD subjects had a consistent history and objective evidence of asthma or COPD, as described previously (32). Asthma severity was defined by British Thoracic Society guidelines on the Management of Asthma treatment steps: mild = step 1, β2-agonist only; moderate = steps 2 and 3, inhaled corticosteroid ≤ 800 μg beclomethasone equivalent per day ± long-acting β2-agonist; severe = steps 4 and 5 (33). Five of 7 patients at step 4/5 undergoing immunohistochemical staining met the American Thoracic Society criteria for refractory asthma (1). All COPD patients used for the study were classified as GOLD (Global Initiative for Chronic Obstructive Lung Disease) I and II. The studies were approved by the Leicestershire, Northamptonshire, and Rutland Research Ethics Committee (references: 4977, 04/Q2502/74 and 08/H0406/189). Written informed consent was gained from all participants prior to their involvement. Tables I and II show the demographics of the patients used in the immunohistochemistry studies and molecular/cellular studies, respectively.

Fiberoptic bronchoscopy

Subjects underwent fiberoptic bronchoscopy, as described previously (32) and according to British Thoracic Society guidelines (34). Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae, fixed in acetone, and embedded in glycol methacrylate (35). Additional biopsy specimens were dissected for the isolation and culture of ASM cells (36).

KCa3.1 staining in endobronchial biopsies

Sequential 2-μm sections were cut from glycol methacrylate–embedded bronchial biopsies and immunostained as described previously (35), using a rabbit polyclonal anti-human KCa3.1 Ab (M20, 2.7 μg/ml; gift from Dr. Chen, GlaxoSmithKline, Stevenage, U.K.; Ref. 37) and isotype control rabbit IgG (2.7 μg/ml; R&D Systems, Minneapolis, MN). Cells staining positively for KCa3.1 in the ASM compartment were quantified using a semiquantitative intensity score: 0 = no staining, 0.5 = sparse and patchy, 1.0 = diffuse but weak, 2.0 = diffuse. The percentage of nuclei that were stained positive within the ASM cells was also calculated. All sections were counted by a blinded observer.

Culture of ASM cells

Primary human ASM cells were obtained from healthy subjects and isolated from endobronchial biopsy specimens from COPD and asthmatic patients, as previously described (36). Briefly, pure ASM bundles in airways were dissected free of surrounding tissue. The small muscle bundles were cultured in DMEM supplemented with 10% FBS, 4 mM l-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 μg/ml amphotericin. ASM characteristics were determined by immunofluorescence and light microscopy, with α-smooth muscle actin–FITC direct conjugate and myosin indirectly conjugated with FITC (Sigma-Aldrich, Dorset, U.K.). Cells were used from passages 2–6.

Treatment of ASM cells

Cells were pretreated with the specific KCa3.1 channel blockers TRAM-34 (200 nM; a gift from Heike Wulff, University of California–Davis, Davis, CA) (38) and ICA-17043 (60 nM; a gift from Leagen, Durham, NC) (39), alone or in combination with fluticasone (100 nM), for 2 h and then stimulated with TNF-α (10 ng/ml)/INF-γ (500 U/ml) for an additional 24 h. Cell viability was assessed in cells from two asthmatic individuals (each performed in triplicate), using the MTT assay, as described in our previous report (40).

ELISA

ELISAs for assessing chemokine levels were performed on 100-μl cell supernatants using the R&D Systems DuoSet kits according to the manufacturer’s instructions (41). DMSO controls were performed, with the final concentration of DMSO 0.1% in all control and drug wells.

Quantitative PCR

Total RNA was extracted using the RNeasy Plus Mini Kit (#74134; QIAGEN). The cDNAs were synthesized using a First Strand cDNA Synthesis Kit (Fermentas Life Sciences). Real-time quantitative PCR was performed using Brilliant SYBR Green QPCR Master Mix (Agilent), using a Stratagene Mx3000P (Santa Clara, CA). Each sample from different patients was assayed in duplicate. For relative quantification, the Ct method, ratio = 2 − [(Ct (gene of interest) − Ct (β actin as internal control)](2−ΔΔCt), was used as described in Ref. 42 to determine the fold change caused by the different treatment conditions (fluticasone alone, KCa3.1 blockers alone, and both drugs in combination). The primers were as follows: β-actin forward: 5′-CCCAAGGCAACCCGGAGAAG-3′ and reverse: 5′-GTCCGGCGACGGTAGCTG-3′; CCL21 forward: 5′-GCTGAAGACCCATCCC-3′ and reverse: 5′-GGAGCTTGGAAGTTGAACA-3′; CCL5 forward: 5′-TCTGCGCTTCTGTCATCTG-3′ and reverse: 5′-GGGCCAAGTGCTGACAGA-3′; CCL11 forward: 5′-AATTGTCGCCAAGCTCTGTG-3′ and reverse: 5′-TCCCAGGCTGCACGGACGAT-3′; CXCL11 forward: 5′-ACTGTTGCGAGGGTATGTCG-3′ and reverse: 5′-ACCTTTTGGATTCCAGGAGC-3′.

Immunofluorescence

ASM cells grown on the chamber slides were fixed with 10% neutral buffered formalin for 15 min at room temperature, washed three times with PBS, permeabilized using 90% ice-cold methanol for 20 min, and washed again three times with PBS. The cells were then incubated with 3% BSA/PBS for 1 h before anti-KCa3.1 (2.5 μg/ml; Bioybt, Cambridge, U.K.), anti-HDAC1 (1 μg/ml; Cell Signaling Technology, Danvers, MA), or appropriate isotype-matched control Abs (all prepared in 1% BSA/PBS) were added overnight at 4°C. After three washes, Alexa Fluor 488 goat anti-rabbit IgG Ab (Invitrogen, Paisley, U.K.) was added at 1:300 dilution for 40 min, then washed with PBS and mounted using VectorMount (Vector Laboratories, Peterborough, U.K.). Images of the cells were viewed on a confocal microscope (Leica TCS SP5 confocal) and captured using Leica LAS AF software (Leica, Milton Keynes, U.K.).
Cruz Biotechnology, Santa Cruz, CA). Immunoblots on nuclear fractions used samples prepared using a reagent kit (NE-PER™ Thermo Fisher Scientific). A total of 30 μg nuclear extracts was analyzed by 10% SDS-PAGE and assayed by immunoblot using anti-KCn3.1 Ab from Sigma-Aldrich (1:500) and anti–β-actin (1:200 dilution). The anti-KCn3.1 Ab from Biornby (1:500 dilution) was used for assessing KCa3.1 expression in total cell lysates (60 μg) in the silencing studies. Anti-mouse IgG-HRP or anti-rabbit IgG-HRP (1:5000 dilution Santa Cruz Biotechnology) was used as a secondary Ab. The bands were visualized by the ECL system (Amersham Biosciences) and autoradiographed.

Small hairpin RNA gene knockdown of KCn3.1 channels

KCa3.1 (V1 and V2), control (V5), and GFP–small hairpin RNA (shRNA)–expressing adenoviruses (Ad5C20Δ301) were purchased from BioFocus DPI (Leden, the Netherlands). Preliminary transduction experiments using this adenovirus expressing cDNA for GFP demonstrated high transduction efficiency (~80% of cells). Gene knockdown of KCn3.1 channels with different shRNA adenoviruses was confirmed using 2 × 10⁵ ASM cells transduced using a multiplicity of infection of 30 and subsequent Western blotting. For experiments, 24 h after transduction, the cells were deprived for 3 h and then exposed to fluticasone (100 nM) for 2 h, followed by addition of control media or TNF-α/IFN-γ for an additional 22 h. After 24 h, protein supernatants were collected for ELISA assays (CCL5, CX3CL1, CCL11), and cells were lysed for total cell extract preparation, as described above.

Flow cytometry

ASM cells were fixed and permeabilized in 4% paraformaldehyde/0.1% saponin on 15 min on ice. Cells were then stained with 2 μg/ml rabbit anti-human PP5 Ab (Cell Signaling Technology) or isotype-matched control (rabbit IgG; IMMUNOSTEP, Salamanca Spain) overnight, followed by secondary sheep anti-rabbit IgG-FITC Ab (Cell Signaling Technology) for 2 h. Staining was examined by flow cytometry using the Becton Dickinson FACScan (Oxford, U.K.).

Patch-clamp electrophysiology

The whole-cell variant of the patch-clamp technique was used as previously described (20). Briefly, ASM cells were cultured until confluent and then maintained in insulin–transferrin–sodium selenite media as normal. Cells were incubated overnight with TNF-α and IFN-γ for an additional 22 h. Prior to electrophysiological recording, the cells were trypsinized and resuspended in ASM culture media. Cells were pipetted into a heated (30˚C) recording chamber ready for patching. Individual (nonadhered) cells were voltage clamped using the whole-cell variant of the patch-clamp technique. Briefly, voltage commands were applied to potentials ranging from −100 to +1000mV from a holding potential of −20mV. Membrane currents were recorded and subsequently plotted against command potential. Solutions used were as follows: external (mM)—NaCl (140), KCl (5), MgCl2 (1), and CaCl2 (2) (pH was adjusted to 7.4 using NaOH); internal (mM)—KCl (140), MgCL2 (2), HEPES (10), ATP (2), and GTP (0.1) (pH was adjusted to 7.3 using KOH).

Statistical analysis

For cell culture studies across groups, comparisons were made using one-way ANOVA or repeated-measures ANOVA, as appropriate, with the Bonferroni post hoc test for comparisons between specific groups. Immunohistochemical staining was analyzed across groups using the Kruskal–Wallis test. A p value <0.05 was taken as statistically significant.

Results

Expression of KCn3.1 in ASM bundles

The clinical characteristics of the subjects assessed by immunohistochemistry are described in Table I. There was no immunostaining in isotype control sections (Fig. 1A), but KCn3.1 immunoreactivity was positive in ASM bundles (Fig. 1B–D), irrespective of disease status (Fig. 1E). The overall intensity of staining of KCn3.1 in the ASM was not different between healthy subjects (Fig. 1B) and asthmatic patients with moderate (Fig. 1C) or severe disease (Fig. 1D) (quantification not shown). Of interest, not only was KCn3.1 localized to the plasma membrane and cytoplasm (consistent with channel trafficking), but it was also localized to the nuclear membrane (Fig. 1E). The immunostaining taken at a higher magnification clearly shows a marked expression of KCn3.1 in the nucleus of three different cells in one asthmatic patient (×1000). The proportion of ASM cells expressing the KCn3.1 channel in the nucleus did not differ between normal and asthmatic conditions (Fig. 1F).

Nuclear expression of KCn3.1 in cultured ASM cells

The demographics of patients used for the immunostaining, chemokine expression assays, and immunoblot are shown in Table II. To further investigate whether KCn3.1 channel was also present in the nuclear compartment in cultured ASM cells, we conducted immunofluorescence staining in permeabilized cells derived from

| Table I. Demographics of patients used in the immunohistochemistry studies |
|-----------------|----------------|----------------|-----------------|----------------|
|                   | Healthy Control | Patients with Mild to Moderate Asthma (BTS steps 1–5) | Patients with Severe Asthma (BTS steps 4 and 5) | p Value |
| Number            | 7              | 5                | 7                | —          |
| Age (mean ± SEM)  | 35.71 ± 6.98   | 36 ± 7.86        | 42.29 ± 4.18     | 0.590     |
| Sex (M/F)         | 2/5            | 2/3              | 3/4              | 0.843     |
| Asthma duration (mean ± SEM) | N/A | 17 ± 5.38 | 33.86 ± 5.63 | 0.004 |
| Inhaled corticosteroid dose (beclomethasone equivalents (mg)) | N/A | 512.5 ± 234.9 | 1400 ± 130.9 | 0.006 |
| Number at BTS step 5 | N/A | 0               | 4                | —         |
| Number on long-acting β-agonist | N/A | 2               | 7                | —         |
| Exacerbations in last year (median [range]) | N/A | 0 (0–1) | 1 (0.0–3.0) | 0.238 |
| Mean daytime symptom score (median [range]) | N/A | 0.25 [0.18–0.54] | 0.93 [0.14–1.39] | 0.176 |
| Mean daily night time symptom score (median [range]) | N/A | 0.0 [0.0–0.0] | 0.28 [0.0–0.07] | 0.109 |
| Reliever use/week (median [range]) | N/A | 1.5 [0.0–5.25] | 2.20 [0.0–3.70] | 0.037 |
| Sputum eosinophil count (%) (geometric mean [95% CI]) | 0.34 [0.20–0.56] | 8.86 [2.08–37.75] | 2.07 [0.43–9.98] | 0.006 |
| PEF amplitude % of the mean (mean ± SEM) | N/A | 20.08 ± 6.43 | 34.50 ± 9.45 | 0.272 |
| FEV1 (predicted) | 102.4 ± 3.45 | 91.80 ± 7.37 | 66.57 ± 6.71 | 0.006 |
| PC20 methacholine (mg/ml)/geometric mean [95% CI]) | 84.0 ± 3.90 | 76.40 ± 5.17 | 59.57 ± 6.47 | 0.018 |
| Serum IgE (kU/L) (geometric mean [95% CI]) | 31.59 [12.57–79.4] | 501.5 [43.07–5840] | 203.1 [28.98–1424] | 0.021 |
| Number with positive skin prick test | 5 | 4 | 5 | 0.368 |
| Number with positive skin prick test to Aspergillus fumigatus | 0 | 0 | 0 | 1 |

*p value <0.05 was taken as statistically significant.

†Ratio for budesonide Turbuhaler calculated as 1.5.

BTS, British Thoracic Society; CI, confidence interval; FEV1, force expiratory volume in 1 s; FVC, force vital capacity; PEF, peak expiratory flow.
in vivo in three different muscle areas. The nuclei staining for KCa3.1 in normal subjects (47 kDa) contained discreet bands recognized by an anti-KCa3.1 Ab of 47 kDa acting beta agonist (3). Interestingly, the nuclear fraction of KCa3.1 in a healthy control (48 kDa) provided additional evidence for the nuclear expression of KCa3.1. We performed Western blots directly on isolated nuclear extracts to assess KCa3.1 membrane expression in human colon carcinoma 116 cells (45). We also detected KCa3.1 is 48 kDa. The larger band is likely to represent alternative splicing variants (20, 37, 46) (Fig. 2C). The predicted size of KCa3.1 is 48 kDa, as described previously in the cytoplasmic extracts of several cell types (20, 37, 46) (Fig. 2C). The predicted size of KCa3.1 is 48 kDa. The larger band is likely to represent alternative splicing variants (46). The quantitative analysis of KCa3.1 expression in the nuclear fractions by immunoblot assay revealed no significant changes between healthy and asthmatic subjects (Fig. 2C, panel bottom). This finding confirms the nuclear expression of intracellular KCa3.1 in human ASM cells.

Using patch-clamp electrophysiology, we also assessed whether channel activity was affected in cells treated with TNF-α/IFN-γ. As shown in Fig. 2A, KCa3.1 channel was identified within the nucleus in ASM cells. Staining for HDAC1 (Fig. 2B), a protein exclusively found in the nucleus (44), was used as a positive control for the immunostaining procedure. The intracellular expression of KCa3.1 in ASM cells is highly novel, although one report identified KCa3.1 channels in mitochondrial membranes in human colon carcinoma 116 cells (45). We also performed Western blots directly on isolated nuclear extracts to provide additional evidence for the nuclear expression of KCa3.1 channel in human ASM cells. Interestingly, the nuclear fraction contained discreet bands recognized by an anti-KCa3.1 Ab of 47 and 53 kDa, as described previously in the cytoplasmic extracts of several cell types (20, 37, 46) (Fig. 2C). The predicted size of KCa3.1 is 48 kDa. The larger band is likely to represent alternative splicing variants, although several smaller splice variants are also described (46). The quantitative analysis of KCa3.1 expression in the nuclear fractions by immunoblot assay revealed no significant changes between healthy and asthmatic subjects (Fig. 2C, bottom panel). This finding confirms the nuclear expression of intracellular KCa3.1 in human ASM cells.

In vivo KCa3.1 expression in human ASM bundles. Representative immunostaining in bronchial biopsies for isotype control Ab (×200) (A), KCa3.1 in a healthy control (×400) (B), or in an asthmatic individual treated with inhaled corticosteroid (×400) (C) or with inhaled corticosteroid + long-acting beta agonist (×400) (D). (E) Representative immunostaining at higher magnification (×1000) showing the nuclear expression of KCa3.1 channel in vivo in three different muscle areas. The upper left image is the tissue incubated with the isotype-matched control Ab. (F) The percentage of ASM cell nuclei staining for KCa3.1 in normal subjects (n = 7) and in patients with moderate (n = 5) and severe asthma (n = 6). Two sections at least 10 μm apart were analyzed for each subject, and the mean of the two sections taken as the value for each subject. A minimum of 15 cells per section were counted (range, 15–100 cells; median, 49.5 cells). Data are expressed as means ± SEM. Ep, Epithelium.

FIGURE 1. In vivo KCa3.1 expression in human ASM bundles. Representative immunostaining in bronchial biopsies for isotype control Ab (×200) (A), KCa3.1 in a healthy control (×400) (B), or in an asthmatic individual treated with inhaled corticosteroid (×400) (C) or with inhaled corticosteroid + long-acting beta agonist (×400) (D). (E) Representative immunostaining at higher magnification (×1000) showing the nuclear expression of KCa3.1 channel in vivo in three different muscle areas. The upper left image is the tissue incubated with the isotype-matched control Ab. (F) The percentage of ASM cell nuclei staining for KCa3.1 in normal subjects (n = 7) and in patients with moderate (n = 5) and severe asthma (n = 6). Two sections at least 10 μm apart were analyzed for each subject, and the mean of the two sections taken as the value for each subject. A minimum of 15 cells per section were counted (range, 15–100 cells; median, 49.5 cells). Data are expressed as means ± SEM. Ep, Epithelium.

Production of steroid-resistant chemokines induced by TNF-α/IFN-γ in ASM cells from healthy controls and patients with asthma and COPD

We first determined whether production of four different chemokines—CCL5, CCL11, CX3CL1, and chemokine (C-X-C motif) ligand 10 (CXCL10)—in response to TNF-α/IFN-γ was affected by disease status (Fig. 3). We found that levels of CCL5 (Fig. 3A), CCL11 (Fig. 3B), and CXCL10 (Fig. 3C) produced in response to TNF-α/IFN-γ at 24 h were similar in the different groups studied. Thus net increases (induced minus basal levels) of CCL5 were 72666 pg/ml in healthy, asthmatic, and COPD subjects, respectively (Fig. 3A). Net increases of CCL11 were 417.2 pg/ml in healthy, asthma and COPD subjects, respectively (Fig. 3B). Net increases of CXCL10 were 7269 pg/ml in healthy, asthmatic, and COPD subjects, respectively (Fig. 3C). Of interest, we found that ASM cells from COPD patients produced significantly less CX3CL1.

Table II. Demographics of patients used for the chemokine expression assays/immunoblot and immunostaining studies

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Asthma</th>
<th>COPD</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>12</td>
<td>10</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>5/7</td>
<td>5/5</td>
<td>6/0</td>
<td></td>
</tr>
<tr>
<td>Age (mean ± SEM)</td>
<td>42.25 ± 4.15</td>
<td>49.4 ± 2.98</td>
<td>66 ± 4.31</td>
<td>0.0046</td>
</tr>
<tr>
<td>FEV1 (± SEM)</td>
<td>3.7 ± 0.27</td>
<td>2.48 ± 0.22</td>
<td>1.9 ± 0.31</td>
<td>0.0004</td>
</tr>
<tr>
<td>FEV1% predicted (± SEM)</td>
<td>88.75 ± 4.08</td>
<td>70.92 ± 9.97</td>
<td>61.5 ± 7.04</td>
<td>0.0116</td>
</tr>
<tr>
<td>FEV1/FVC (%) (± SEM)</td>
<td>88.7 ± 8.3</td>
<td>64.4 ± 3.32</td>
<td>53.82 ± 5.16</td>
<td>0.0044</td>
</tr>
</tbody>
</table>

*Statistical analysis across subject groups. Bold indicates significance.
in comparison with healthy controls (774 ± 142 versus 4763 ± 1400 pg/ml, p < 0.05), whereas no difference was noticed when compared with levels produced by asthmatic individuals (2927 ± 501 pg/ml) (Fig. 3D).

*KCa3.1* channel inhibition differentially suppresses the production of TNF-α/IFN-γ–induced steroid-resistant chemokines

We next investigated the role of *K*<sub>Ca</sub>3.1 inhibitors in our ASM cell model of GC resistance (TNF-α/IFN-γ–treated cells) by assessing the expression of four different chemokines in ASM cells from healthy (*n* = 4), asthmatic (*n* = 6), and COPD subjects (*n* = 4). As reported for tracheal ASM cells (12), induction of CX3CL1, CCL5, CCL11, and CXCL10 by TNF-α/IFN-γ was completely resistant to fluticasone treatment in ASM cells derived from the bronchial tree (Figs. 4–7). Chemokine production was not affected in cells treated with 0.1% DMSO, the final concentration of the inhibitor solvent.

The specific *K*<sub>Ca</sub>3.1 blockers TRAM-34 (200 nM) (38) and ICA-17043 (60 nM) (39) alone did not affect TNF-α/IFN-γ–induced production of CCL5 (Fig. 4) or CCL11 (Fig. 5), with the exception of cells derived from asthmatic patients (Fig. 5B). The inhibitory effect was further enhanced by > 90% in the presence of fluticasone (Fig. 5B). TNF-α/IFN-γ–induced production of CCL5 in all tested individuals (Fig. 4) or CCL11 in healthy and COPD subjects (Fig. 5A–C) was inhibited only when fluticasone was combined with either *K*<sub>Ca</sub>3.1 channel blocker. Fluticasone and ICA-17043 in combination reduced TNF-α/IFN-γ–induced CCL5 levels to 57 ± 6 ± 12%, 61 ± 14.5%, and 52 ± 14% in ASM cells from healthy, asthmatic, and COPD subjects, respectively (Fig. 4). Fluticasone and TRAM-34 reduced TNF-α/IFN-γ–induced CCL5 levels to 57 ± 11%, 51 ± 21%, and 54 ± 9.6% in cells from

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Nuclear expression of *K*<sub>Ca</sub>3.1 channel in cultured human ASM cells. Permeabilized ASM cells were stained for *K*<sub>Ca</sub>3.1 (original magnification ×200) (**A**), the nuclear marker HDAC1 (original magnification ×200) (**B**), or were incubated with the corresponding isotype-matched Abs used as negative controls. DAPI counterstaining was performed on the same cell population to demonstrate the nuclear location of *K*<sub>Ca</sub>3.1. Images are representative of experiments performed on cells from three different healthy subjects. (**C**) Top, *K*<sub>Ca</sub>3.1 expression in the nucleus was also confirmed by assaying ASM nuclear fractions for *K*<sub>Ca</sub>3.1 by immunoblot analysis. Bottom, Scanning densitometric measurement of *K*<sub>Ca</sub>3.1 expression normalized over the corresponding β-actin showed no significant difference in *K*<sub>Ca</sub>3.1 expression in ASM nuclear fractions between nonasthmatic and asthmatic subjects. Results are representative of blots performed in six subjects (three healthy and three asthmatic subjects). (**D**) Current voltage curves showing the lack of detectable *K*<sub>Ca</sub>3.1 currents in ASM cells at baseline and after cytokine treatment. Data are expressed as means ± SEM of measurement performed in *n* = 16 cells per condition taken from a healthy subject.
Inhibition by KCa3.1 blockade was not changed by the presence of TRAM-34 alone (200 nM) (Fig. 6A–C). The degree of CX3CL1 inhibited by TNF-α/IFN-γ was reduced to 31.4% in healthy, COPD, and asthmatic subjects, respectively (Fig. 4). Proportion of CCL11 by TNF-α/IFN-γ was reduced to 36 ± 14%, 12 ± 14%, and 28 ± 12% of control by the fluticasone and ICA-17043 combination in cells from healthy, asthmatic, and COPD subjects, respectively. TNF-α/IFN-γ-induced CCL11 production was reduced to 38.5 ± 14.2%, 9 ± 9%, and 31 ± 15% by the fluticasone and TRAM-34 combination in cells from healthy, asthmatic, and COPD subjects, respectively (Fig. 5).

Of note, induction of CX3CL1 by TNF-α/IFN-γ in cells from healthy, COPD, and asthmatic subjects was reduced to 31.4 ± 12.1%, 8.8 ± 3.4%, and 33.2 ± 8.9% by ICA-17043 alone (60 nM) and to 28.9 ± 10.8%, 21.1 ± 10.9%, and 26.4 ± 14.7% by TRAM-34 alone (200 nM) (Fig. 6A–C). The degree of CX3CL1 inhibition by KCa3.1 blockade was not changed by the presence of fluticasone (Fig. 6A–C, last two columns).

In contrast, production of CXCL10 by TNF-α/IFN-γ was not affected by either KCa3.1 inhibitors used alone or in combination with fluticasone (Fig. 7), ruling out a toxic or nonspecific effect of KCa3.1 inhibitors. Measuring cell viability using the MTT assay did not show any signs of cytotoxicity in cells treated with the above concentrations of KCa3.1 inhibitors. No differences were observed between relative MTT-reducing activities in control cells (with 0.1% DMSO) and in cells treated with either ICA-17043 or TRAM-34: 0.344 ± 0.019, 0.317 ± 0.022, and 0.346 ± 0.063, respectively.

**shRNA silencing of KCa3.1 channels**

To confirm the previous findings with pharmacological blockers of KCa3.1, we also used knockdown of KCa3.1 channels, using shRNA delivered by adenoviruses. An shRNA control adenovirus (V5) did not affect KCa3.1 channel protein expression (Fig. 8A), but this was completely decreased by KCa3.1 shRNAs V1 and V2 (Fig. 8A, top panel). None of the shRNAs affected β-actin expression (Fig. 8A). TNF-α/IFN-γ treatment alone or in the presence of fluticasone had no effect on KCa3.1 expression or on the efficacy of shRNA adenoviruses to silence the channel (Fig. 8A, bottom panel). The following functional assays were then performed using shRNA-KCa3.1 adenoviruses V1 and V2, and shRNA control adenovirus (V5) as a control.

**KCa3.1 downregulation in ASM cells attenuates TNF-α/IFN-γ-induced steroid-resistant chemokine expression**

As in untransduced ASM cells, expression of CX3CL1 (Fig. 8B) and CCL5 (Fig. 8C) in V5 control transduced cells was increased by TNF-α/IFN-γ and unaffected by fluticasone. In contrast, CCL5 production was inhibited by ~30% by KCa3.1 shRNAs V1 and V2, with further inhibition of secretion with the addition of fluticasone to almost 50% (Fig. 8C). In agreement with the data obtained with the soluble inhibitors, CX3CL1 expression was inhibited by KCa3.1 shRNAs V1 and V2 irrespective of fluticasone treatment (Fig. 8B). The percent inhibition of CX3CL1 induced by shRNA V1 in the absence or presence of fluticasone when normalized to CX3CL1 production in the presence of the control shRNA was 68% or 49%, respectively. The percent inhibition of CX3CL1 induced by shRNA V2 was 45% or 30% in the absence or presence of fluticasone, respectively. These data confirm that KCa3.1 channels are essential in the regulation of TNF-α/IFN-γ-inducible GC-resistant chemokine in ASM cells.

**KCa3.1 channel inhibition suppresses TNF-α/IFN-γ–induced steroid-resistant chemokine mRNA expression**

Quantitative PCR analyses were performed to determine whether KCa3.1 channel blockers modulate TNF-α/IFN-γ–induced chemokine expression at the transcriptional level. In cells exposed to TNF-α/IFN-γ for 6 h, CCL5, CX3CL1, and CCL11 mRNA expression was increased significantly by 35.2- ± 19-fold, 96- ± 41-fold, and 1.8- ± 0.43-fold, respectively (p < 0.05). Fluticasone did not alter the expression of CCL5 or CCL11 mRNA, but nonsignificant trends were noted for reduced expression of CCL5 and CCL11 mRNA with KCa3.1 blockade (Fig. 9). Combining fluticasone with TRAM-34 or ICA-17043 led to a drastic inhibition of both CCL5 and CCL11 expression (p < 0.05, n = 4 combined from 2 asthmatic patients, 1 control, and 1 COPD patient)(Fig. 9A–C).

---

**FIGURE 3.** Chemokine production induced by TNF-α/IFN-γ in ASM cells from healthy, asthmatic, and COPD subjects. Cells were stimulated with TNF-α/IFN-γ for 24 h. CCL5, CX3CL1, CCL11, and CXCL10 levels in the supernatants were assessed by ELISA assays, as described in Materials and Methods. Data are expressed as means ± SEM of the net TNF-α/IFN-γ–induced increase of CCL5 (A), CCL11 (B), CXCL10 (C), and CX3CL1 (D) in n = 5 controls, n = 7 asthmatic patients, and n = 5 COPD patients, all performed in triplicate. All chemokines were significantly increased compared with those in unstimulated control. **⁎⁎⁎ p < 0.01 compared with healthy cells.**
Fluticasone alone had no significant effect on TNF-α/IFN-γ–induced CX3CL1 expression in response to TNF-α/IFN-γ, but this expression was almost completely reduced in cells treated with either K_Ca3.1 blocker alone, irrespective of fluticasone treatment (Fig. 9B). These data demonstrate that K_Ca3.1 channel inhibition regulates the expression of GC-resistant chemokines in ASM cells at the transcriptional level.

Fluticasone-induced GRα phosphorylation at Ser211 and GILZ expression are impaired in steroid-resistant states but restored in the presence of K_Ca3.1 channel inhibitors

Cytokine-induced steroid resistance in human ASM cells involves the inhibition of GRα transcriptional activity via multiple mechanisms (12). In this study, we confirmed that fluticasone-induced GRα phosphorylation on Ser211, which is essential for its transcriptional activity, was almost completely inhibited in the presence of TNF-α/IFN-γ (Fig. 10A, 10B). This reduced GC-induced GR phosphorylation induced by TNF-α/IFN-γ was associated with an impaired expression of the well-defined GRE-dependent gene called GILZ (Fig. 10C). Although these data were generated in cells from healthy subjects, similar findings were also observed in cells derived from n = 2 persons with nonsevere asthma (data not shown). More importantly, in the presence of the K_Ca3.1 channel blockers ICA-17043 or TRAM-34, the inhibitory effect of TNF-α/IFN-γ on both fluticasone-induced GRα phosphorylation and GILZ expression was completely prevented (n = 3, p < 0.01) (Fig. 10A–C). This finding demonstrates that K_Ca3.1 channel inhibition restores a key signaling component required for normal GRα transcriptional function in this ASM model of steroid resistance.

K_Ca3.1 channel inhibition suppresses cytokine-induced PP5 expression

We have recently uncovered that TNF-α/IFN-γ–induced upregulation of PP5 is the key factor by which this cytokine combination
impairs GC-induced GRα phosphorylation at Ser211 (43, 47). Flow cytometry assays confirmed that PP5 was indeed upregulated following treatment with TNF-α/IFN-γ in nine subjects (four asthmatic subjects and five healthy subjects), but interestingly, this response was significantly inhibited by both KCa3.1 blockers (Fig. 11). This finding suggests that although KCa3.1 channel activity directly regulates the expression of CX3CL1 (Fig. 6), it is also involved in the induction of PP5, which in turn promotes GC resistance by dephosphorylating GRα at Ser211 (Fig. 12).

Discussion

KCa3.1 channels may represent major players in the pathogenesis of several lung diseases (19). The present article now reveals that KCa3.1 channels may figure importantly in the regulation of GC-insensitive pathways. We found that TNF-α/IFN-γ–induced expression of GC-resistant CX3CL1 was inhibited by KCa3.1 channel blockade or knockdown at both the mRNA and the protein levels, irrespective of GC presence. In contrast, the inhibition of GC-resistant CCL5 was visible only in cells cotreated with both fluticasone and KCa3.1 channel inhibition. The failure of fluticasone to induce GRα phosphorylation at Ser211 or to promote the expression of the GC-inducible gene GILZ in the GC-resistant state was fully prevented by the presence of KCa3.1 inhibitors as a consequence of reduced PP5 expression. To our knowledge, this is the first report in any given cell type or tissue that describes a functional interaction between KCa3.1 channels and GC signaling.

The mechanisms underlying GC resistance in asthma are not clearly defined (48). However, combined in vitro and in vivo studies have led to the important conclusion that GC-resistant pathways are operative in ASM cells within asthmatic airways (49). Indeed, many studies performed on bronchial biopsies from asthmatic individuals showed marked expression of inflammatory proteins such as CX3CL1 (13), CCL11 (14), CCL15 (15), CCL19...
ADAM33, and ADAM8 (17, 18) within ASM bundles, despite treatment of patients with either high-dose inhaled or oral GC. We have previously modeled this GC-resistant state in vitro by exposing cultured human tracheal ASM cells to a combination of TNF-α/IFN-γ, which results in the production of an array of inflammatory proteins, including CD38, CCL5, CX3CL1, and IFN regulatory factor 1, that are completely resistant to GC treatment (12, 43, 47, 50, 51). In this study, we found that in addition to CCL5 and CX3CL1, production of other chemokines, CCL11 and CXCL10, is also insensitive to fluticasone treatment; more importantly, this GC-resistant state also occurs in primary bronchial ASM cells in health, asthma, and COPD (as opposed to tracheal ASM cells in our previous studies). Generally, TNF-α/IFN-γ–dependent steroid-resistant chemokine production was similar irrespective of disease status (healthy status, COPD, asthma), although production of CX3CL1 was dramatically reduced by >80% in COPD patients when compared with levels produced by cells from healthy subjects. The reasons for this are unknown, and additional studies are clearly required to confirm this observation.

Immunohistochemistry on bronchial biopsies demonstrated that KCa3.1 channels are expressed in vivo in ASM bundles in asthmatic patients, and interestingly, this included a nuclear distribution. This observation was further confirmed by assessing KCa3.1 expression in cultured ASM cells, using immunoblot analysis directly on nuclear extracts, and immunofluorescence staining showing a nuclear distribution of KCa3.1. KCa3.1 expression in asthmatic ASM bundles was not affected by disease severity and the associated intensity of antiasthma treatment, and no differences in immunostaining intensity were evident between healthy subjects and asthmatic patients. These observations suggest that changes in channel activity rather than protein expression within ASM bundles could explain their contribution to the pathogenesis of asthma. In contrast to the study showing that TGF-β increased both KCa3.1 expression and activity in ASM cells (20), we found that TNF-α/IFN-γ failed to significantly stimulate KCa3.1 protein levels or plasma membrane KCa3.1 channel activity, suggesting that modulation of KCa3.1 expression or function in ASM is highly stimulus dependent (growth factors versus cytokines). The fact that no change in KCa3.1 activity occurs when measured at the cell surface supports the concept that compartmental changes in Ca²⁺ levels rather than cytoplasmic changes could explain the activation of KCa3.1 in GC sensitivity. Our data showing that KCa3.1 inhibition does not affect CXCL10 induction strongly suggest that the inhibitory effect of KCa3.1 blockade is gene specific and not due to generalized nonspecific effects linked to overall changes in Ca²⁺ levels inside the cells. In this study we also confirmed our previous

**FIGURE 8.** shRNA KCa3.1 modulates TNF-α/IFN-γ–induced chemokine expression. (A) Top, Representative immunoblot of KCa3.1 protein expression in cells transduced with KCa3.1 shRNA (V1 and V2) and control (V5) adenoviruses in the presence or absence of cytokines and fluticasone (FP). Bottom, Scanning densitometric measurement of KCa3.1 expression by immunoblot assays normalized over the corresponding β-actin showed complete knockdown of KCa3.1 expression in ASM cells, using shKCa3.1 V1 and V2, when compared with control adenovirus. Note that the TNF-α/IFN-γ combination had no effect on KCa3.1 expression. Results are shown as means ± SEM of blots performed in three healthy donors. (B and C) Effect of the same adenoviruses on TNF-α/IFN-γ–induced expression of CX3CL1 (B) and CCL5 (C) assessed by ELISA. Results are shown as means ± SEM of experiments performed in triplicate in three donors. **p < 0.01 compared with respective control shRNA.
Expression levels of CCL5 were determined by real-time quantitative PCR. Cells pretreated with TRAM-34 (200 nM) or ICA-17043 (60 nM), with or without fluticasone (100 nM, 2 h), were stimulated with TNF-α/IFN-γ for an additional 4 h. Total RNA was extracted, and real-time quantitative PCR was performed, as described in Materials and Methods. Results are expressed as fold induction of chemokine expression by calculating the negative inverse of the 2^(-ΔΔCT) value for each condition. Expression levels of CCL5, CX3CL1, and CCL11 were expressed as % of TNF-α/IFN-γ response. Means ± SEM of experiments performed in duplicate in four different subjects (two asthmatic patients, one control, and one COPD patient). **p < 0.01, ***p < 0.001 compared with TNF-α/IFN-γ/DMSO control.

![Figure 9](http://www.jimmunol.org/)

**FIGURE 9.** KCa3.1 blockers modulate the transcription of GC-resistant chemokines. Cells pretreated with TRAM-34 (200 nM) or ICA-17043 (60 nM), with or without fluticasone (100 nM, 2 h), were stimulated with TNF-α/IFN-γ for an additional 4 h. Total RNA was extracted, and real-time quantitative PCR was performed, as described in Materials and Methods. Results are expressed as fold induction of chemokine expression by calculating the negative inverse of the 2^(-ΔΔCT) value for each condition. Expression levels of CCL5 (A), CX3CL1 (B), and CCL11 (C) were expressed as % of TNF-α/IFN-γ response. Means ± SEM of experiments performed in duplicate in four different subjects (two asthmatic patients, one control, and one COPD patient). **p < 0.01, ***p < 0.001 compared with TNF-α/IFN-γ/DMSO control.

reports (20) showing that KCa3.1 inhibitors had no cytotoxic action on ASM cells. Because KCa3.1 channels were found to be expressed in the nuclear compartment, our study raises the possibility that nuclear modulation of KCa3.1 function by TNF-α/IFN-γ could explain their involvement in modulating GC signaling. Of interest, the expression of KCa3.1 channels has been reported in cytoplasmic organelles such as mitochondria in a human colon tumor cell line (45). Additional studies are clearly needed to define how intracellular KCa3.1 channels regulate cellular function in ASM cells.

We found that combining fluticasone with KCa3.1 blockers or KCa3.1 downregulation was effective in inhibiting GC-resistant CCL5, CCL11, and CX3CL1 in ASM cells. It is interesting that although KCa3.1 blockade had a strong inhibitory effect on cytokine-induced CCL5 expression at the mRNA level, production of CCL5 protein was reduced by only 50%. We believe that in addition to transcriptional mechanisms, CCL5 induction by cytokines is also regulated at the posttranscriptional level. Previous studies performed in A549 cells (52) and in human ASM cells (53) support this hypothesis by showing that although IFN-γ on its own did not stimulate CCL5 expression, it does enhance TNF-α–induced CCL5 expression via posttranscriptional mechanisms. We also have some preliminary evidence, using a different pharmacological inhibitor, demonstrating that the degree of CCL5 inhibition seen at the mRNA level does not correlate with similar changes at the protein level (Y. Amrani, unpublished observations).

The ability to reproduce the same results when channels were downregulated by shRNA shows unequivocally that KCa3.1 regulates, at least in part, TNF-α/IFN-γ–dependent GC-resistant chemokine expression. In our study, we noted some expected discrepancy between the two inhibitory strategies. For example, cytokine-induced CCL5 expression was significantly affected by KCa3.1 shRNA, but not by the pharmacological blockers, and the degree of CX3CL1 inhibition was somewhat greater when combining fluticasone with pharmacological blockers and not with shRNA vectors. In contrast to soluble inhibitors, channel knockdown could indirectly have an impact on other cellular signaling pathways through the loss of interactions between the target protein (in this case, the KCa3.1 channel) and other key binding partners. In the case of KCa3.1, very little is known about the nature of proteins that associate with the channel. The direct binding of 5′-AMP–activated protein kinase (54), mammalian protein histidine phosphatase 1 (55), or nucleoside diphosphate kinase B to KCa3.1 was found to be essential in regulating channel activity (56). Considering the fact that these proteins have multifunctional properties, it is therefore plausible that reducing KCa3.1 levels could lead to downstream effects not evoked by channel blockers alone. The apparent differences observed between the data obtained with the soluble inhibitors and silencing adenoviruses could also be due to the heterogeneity in patients’ responses because different subjects were used with the two inhibitory strategies.

Of note, GC-resistant CX3CL1 expression, at both protein and mRNA levels, was inhibited by KCa3.1 inhibitors irrespective of GC treatment, suggesting that KCa3.1 channel activity is a key factor regulating the transcriptional expression of this chemokine in response to TNF-α/IFN-γ. The putative role of KCa3.1 in regulating expression of inflammatory mediators has been mostly described in T lymphocytes, in which KCa3.1 blockade (either via pharmacological inhibitors or the use of T cells deficient in the channel) significantly reduces TCR-induced expression of IL-2, TNF-α, and IFN-γ (57–59). In T cells it is likely that this results from the instrumental role of KCa3.1 in regulating Ca2+ entry through the plasma membrane, a vital signal for optimal T cell activation and cytokine secretion. The fact that TNF-α/IFN-γ–dependent CX3CL1 expression in ASM cells is inhibited by two selective blockers of the KCa3.1 pore demonstrates that the ion conductance of K+ is key in the mediation of this effect, and not a regulatory channel domain. Interestingly, we and others (60, 61) have shown that TNF-α alters Ca2+ handling in ASM cells via the induction of ectoenzyme CD38 or transient receptor potential C3 channels (62). It is therefore plausible that KCa3.1 regulates CX3CL1 via activation of Ca2+-dependent pathways. However, as discussed above, it seems unlikely that this is due to channel activity located in the plasma membrane.

Another major observation in our study is the functional interaction between KCa3.1 channels and GC signaling pathways. Specifically, KCa3.1 inhibitors restored the ability of fluticasone to inhibit the production of CCL5 and CCL11 in TNF-α/IFN-γ–induced GC-resistant conditions, irrespective of the KCa3.1 inhibition strategy used. In addition, this restoration of cell sensitivity to fluticasone by KCa3.1 blockade was concomitantly associated with a reinstatement of the GRα phosphorylation at Ser311 that was impaired in TNF-α/IFN-γ–treated cells. This finding indicates that KCa3.1 activity drives TNF-α/IFN-γ–induced GC insensitivity in
ASM cells, in part by via the modulation of GRα phosphorylation status (Fig. 10A) and GRα transactivation activity (Fig. 10B). Our data support the growing evidence showing the importance of transactivation properties in the anti-inflammatory action of GC (63, 64). Although GRα phosphorylation on three major residues located on its N terminus (Ser 203, Ser 211, and Ser 226) affects key functions of the receptor, including turnover and subcellular trafficking, it is phosphorylation on Ser211 that is essential for optimal GRα transcriptional activity (65). Indeed, in agreement with our previous reports (12, 47, 50, 51), we confirmed that fluticasone-induced GRα-dependent transactivation was significantly impaired in the TNF-α/IFN-γ-induced GC-insensitive state (Fig. 10C). More importantly, KCa3.1 blockade was also able to fully restore fluticasone-induced GRα-dependent transactivation, suggesting a role of KCa3.1 channel in impairing GC function in ASM cells. Our previous report showed that the impaired GRα transactivation was due to the upregulation of the serine/threonine phosphatase PP5, which mediated cytokine-induced GRα dephosphorylation at Ser211 (43). In this article, we confirm not only that PP5 levels are increased by TNF-α/IFN-γ in ASM cells independently of disease status (healthy status and asthma) but also, more importantly, that PP5 induction was dependent on functional KCa3.1 channels.

**FIGURE 10.** Impaired fluticasone-induced GRα phosphorylation and GRE-dependent GILZ expression by cytokines is restored by KCa3.1 inhibitors. Cells pretreated with TRAM-34 (200 nM) or ICA-17043 (60 nM), with or without fluticasone (100 nM, 2 h), were stimulated with TNF-α/IFN-γ for 4 h. (A) Total cell lysates were prepared and assayed for total GR, phosphoserine 211 GR Abs, and β-actin for loading by immunoblot analysis. (B) Means ± SEM of scanning densitometric analyses of blots from n = 3 healthy patients, with each value normalized over the mean density of the corresponding total GR bands. *p < 0.05 compared with fluticasone, *p < 0.05 compared with TNF-α/IFN-γ/fluticasone as indicated. (C) RNA was extracted and purified using the PureLink RNA Mini Kit according to the manufacturer’s instructions. Total mRNA (2 μg) was subjected to real-time RT-PCR with GILZ and β-actin primers, and the relative quantification in each condition was performed using the standard curve method and expressed as fold increased over basal. Each experiment was performed in duplicate and repeated in cells from three different healthy donors. **p < 0.01 compared with TNF-α/IFN-γ/fluticasone, ***p < 0.001 compared with TNF-α/IFN-γ/fluticasone.

**FIGURE 11.** PP5 upregulation by TNF-α/IFN-γ is inhibited by KCa3.1 inhibitors. Cells pretreated with TRAM-34 (200 nM) or ICA-17043 (60 nM) for 2 h were stimulated with TNF-α/IFN-γ for 22 h. PP5 levels assessed by flow cytometry [representative histogram of one cell line shown in (A)] were expressed as the fold increase in mean fluorescence intensity (B) over basal ± SEM of experiments performed in 9 subjects (n = 4 asthmatic patients and n = 5 healthy controls). *p < 0.05, **p < 0.01.
Although a direct link between PP5 and steroid insensitivity has been suggested by Goleva and colleagues (66), who found that PP5 knockdown restored GC responsiveness in estrogen-treated breast cancer cells, our report, to our knowledge, is the first to show that this functional link between proinflammatory cytokines and PP5 occurs via KCa3.1-dependent pathways. Our present study supports the novel model that TNF-α/IFN-γ impairs GC sensitivity in ASM cells by promoting GRs dephosphorylation via KCa3.1-dependent upregulation of PP5 expression.

In summary, we have shown that KCa3.1 channels contribute to TNF-α/IFN-γ-associated GC insensitivity (Fig. 12). Transcription of some proinflammatory genes such as CX3CL1 is driven by KCa3.1-dependent pathways. Our study reveals the potential therapeutic value of targeting the KCa3.1–PP5 axis in the treatment of lung diseases such as severe asthma, in which relative GC resistance is evident. The available-a well-tolerated orally bioavailable KCa3.1 blocker that has been used in phase III trials of sickle cell disease (ICA-17043 [Senicapoc]) (19) means that the potential exists for the rapid translation of these findings to the clinic.

Disclosures

C.B. is on the advisory board for GlaxoSmithKline, AstraZeneca, HoffmanLaRoche, Novartis, Genentech, and MedImmune and has received consultancy fees from Medimmune and Novartis and travel expenses to attend scientific meetings from Boehringer-Ingelheim. P.B. has acted as an advisor to Icagen (2007–2010). The other authors have no financial conflicts of interest.

References

40. Amrani, Y., R. A. Panettieri, Jr., N. Frossard, and C. Bronner. 1996. Activation of
37. Chen, M. X., S. A. Gorman, B. Benson, K. Singh, J. P. Hieble, M. C. Michel,
39. Stocker, J. W., L. Cui, L. Yang, C. Pugliese-Sivo, A. Golovko, M. Petro,
44. Bhavsar, P., T. Ahmad, and I. M. Adcock. 2008. The role of histone deacetylases
23. Reich, E. P., L. Cui, L. Yang, C. Pugliese-Sivo, A. Golovko, M. Petro,
2636 KCa3.1 CHANNEL AND STEROID RESISTANCE
dependent and calcium-activated ion channels in the human mast cell line HMC-