

RESEARCH ARTICLE

KCa3.1 differentially regulates trachea and bronchi epithelial gene expression in a chronic-asthma mouse model

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Abstract

Ion channels are potentially exploitable as pharmacological targets to treat asthma. This study evaluated the role of KCa3.1 channels, encoded by *Kcnn4*, in regulating the gene expression of mouse airway epithelium and the development of asthma traits. We used the ovalbumin (OVA) challenge as an asthma model in wild-type and *Kcnn4*^{−/−} mice, performed histological analysis, and measured serum IgE to evaluate asthma traits. We analyzed gene expression of isolated epithelial cells of trachea or bronchi using mRNA sequencing and gene ontology and performed Ussing chamber experiments in mouse trachea to evaluate anion secretion. Gene expression of epithelial cells from mouse airways differed between trachea and bronchi, indicating regional differences in the inflammatory and transepithelial transport properties of proximal and distal airways. We found that *Kcnn4* silencing reduced mast cell numbers, mucus, and collagen in the airways, and reduced the amount of epithelial anion secretion in the OVA-challenged animals. In addition, gene expression was differentially modified in the trachea and bronchi, with *Kcnn4* genetic silencing significantly altering the expression of genes involved in the TNF pathway, supporting the potential of KCa3.1 as a therapeutic target for asthma.

asthma; KCa3.1; KCNN4

INTRODUCTION

Asthma is a highly prevalent disease affecting over 350 million individuals worldwide. This disease is accompanied by high mortality and produces a substantial economic burden on health systems (1). As the understanding of the disease has advanced, new targets for treating asthma have been identified, and encouraged the design of antibodies to tackle inflammatory T_H2 cytokines mainly, and whose role in asthma is unequivocal. However, these new and expensive therapies do not have beneficial effects on all patients and severe side effects have also been documented (2–4). To face this scenario, a new group of targets with potential benefits correspond to ion channels expressed in different cells that participate in inflammatory airway disease, for example, members of the transient receptor potential (TRP) family, potassium channels activated by voltage like Kv1.3 or calcium like KCa3.1, chloride channels such as CFTR and SLC29A9 among others (5–8).

The KCa3.1 intermediate conductance calcium-activated potassium channel is an ubiquitous protein found in many different cell types including epithelial, immune

cells, neurons, fibroblast, and smooth muscle (9–12). Its potential role as a therapeutic target in asthma was previously documented in animal models using channel blockers, short hairpin RNA, and in the *Kcnn4*^{−/−} mice. These studies showed that KCa3.1 channels are necessary for cytokine release, mast cell activation, smooth muscle proliferation, and changes in fibroblast activity (12–17). KCa3.1 function is increased in human bronchial epithelial cells (HBECs) isolated from patients with asthma and a role in airway remodeling was proposed (9). Nevertheless, little more is known about the specific function of the channel in epithelial cells during asthma.

Here, we propose that *Kcnn4* knockout in a mouse model of asthma modulates airway epithelial-gene expression and attenuates disease severity. To test our hypothesis, we evaluated changes in gene expression of airway epithelial cells and the effects of inactivation of the *Kcnn4* gene, coding for the KCa3.1 channel, in mice treated with ovalbumin (OVA) to induce chronic asthma (18). First, we replicated airway remodeling, including increased collagen, mucus, and epithelial thickness in the mouse model, and observed that these characteristics were significantly

reduced in *Kcnn4*^{-/-} mouse airways. We focused on the effect of *Kcnn4* silencing by evaluating changes in gene expression exclusively in epithelial cells isolated from the mouse bronchi or trachea, observing that KCa3.1 blockade attenuates the expression of genes involved in asthma pathogenesis, supporting its potential as a therapeutic target for asthma.

METHODS

Reagents

All reagents were obtained from Sigma Aldrich unless stated otherwise.

Animals

Mice were housed at the Centro de Estudios Científicos (CECs) animal facility. The *Kcnn4*-null animal generation and genotyping have been previously described (19). Male and female mice were bred on the C57BL/6J background and adult mice aged 2–5 mo were used. All experimental procedures were approved by the CECs Institutional Animal Care and Use Committee (No. 2015-02). The study was carried out in compliance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

Ovalbumin Challenge and IL-4 Instillation

Adult mice (male or female) received three intraperitoneal injections of 100 μ L ovalbumin (OVA) 0.5 mg/mL on days 0, 3, and 7. This was then followed by intranasal instillation of 30 μ L OVA 0.6 mg/mL once a week for an additional 9 wk. For intranasal instillation, animals were anesthetized before treatment (ketamine 1 μ g/xylazine 0.1 μ g/weight g) and left to recover consciousness before being returned to the animal facility. Twenty-four hours after the last treatment, animals received a fatal dose of anesthetic (ketamine 5 μ g/xylazine 0.5 μ g/weight g) and the required samples were taken. Intranasal instillation of 60 ng of IL-4 (Peprotech) resuspended in 30 μ L of PBS was administered to anesthetized animals (males: 20–22 g weight), 48 h before electrophysiological measurements.

Histology

Lungs were cleaned and fixed overnight in 4% formalin. Slides were stained using periodic acid Schiff (PAS). Semiquantitative scoring of PAS staining was assigned by three independent observers who were blinded to the identity of the samples. PAS staining intensity was scored as 0 (0–15%), 1 (16%–25%), 2 (26%–50%), 3 (51%–75%), or 4 (76%–100%). Masson's trichrome staining was performed to observe collagen. Images in the RGB format were analyzed as described previously (20). The same images were also used to quantify epithelial thickness tracing lines on the epithelial layer, as described previously (21). All image analysis was performed using ImageJ (v. 1.48v; ImageJ, RRID:SCR_003070). Mast cells were counted in Toluidine blue-stained slides and degranulated cells were identified as those with extracellular granules in close proximity. Cells per optical field ($\times 400$) were counted by three independent observers in a blinded fashion from tissue slides containing trachea and main bronchi. After 20–30 optical

fields were analyzed, the mean was calculated for each animal (22).

Epithelial mRNA Isolation and cDNA Preparation

For mRNA isolation, mice were given a fatal dose of anesthesia. Once vital signs were absent, the mice were placed on a sterile petri dish that was then placed on top of ice. The trachea and bronchi were exposed and opened through the ventral side of the tissue to gain access to the epithelial cells. Using a previously sterilized micro-orthodontic brush, the trachea and bronchi were scraped to remove epithelial cells, which were then placed in TRIzol reagent (Life Technologies) to obtain RNA. RNA was obtained using the miRNeasy kit (Qiagen) followed by cDNA production using the QuantiTect Reverse Transcript kit (Qiagen).

RNA Sequencing and Analysis

cDNA samples from trachea and bronchi epithelial tissue were sent to Qiagen for QIAseq targeted RNA extended panel (No. 333015), plus a custom panel (ERMM-10016-005Z-40) including selected gene of interest: *Agr2*, *Tslp*, *Sftpa1*, *Sftpd*, and *Mmp6* normally upregulated in asthma or asthma related; *Muc5ac*, *Foxa3*, *Spdef* and *Muc5b* for goblet cells; *Tuba1a* and *Foxj1* for ciliated cells; *Scgb1a1* for Club cells, and *Rplp0*, *Trp63* and *Krt5* for basal cells. Also ion channels and subunits *Cftr*, *Tmem16a*, *Scnn1a*, *Scnn1b*, *Scnn1g*, *Lrrc8a*, *Lrrc8b*, *Lrrc8c*, *Lrrc8d*, *Lrrc8e*, *Trpc1*, *Trpm6*, *Trpm7*, *Kcnn4*, *Kcnj10*, *Kcnj13*, and *Kcnj16*; and exchangers and cotransporters *Slc4a1*, *Slc4a2*, *Slc4a3*, *Slc4a4*, and *Slc26a9* of interest. And *Slc11a1* as a marker for immune cells. In search of differentially expressed genes, the data were analyzed using DESeq2 (DESeq, RRID:SCR_000154), and limma (LIMMA, RRID:SCR_010943) for gene ontology enrichment analysis, both from the EnrichmentBrowser pipeline (Bioconductor, RRID:SCR_006442; www.bioconductor.org). The analysis of the KEGG (KEGG, RRID:SCR_012773) pathway was performed using the ORA (over representation analysis) method. A *P* value <0.05 (Wald test), *q* value <0.1 (Benjamini and Hochberg procedure for False Discovery Rate or “*P* adjusted”) and log fold change (FC) <−1.5 or FC >1.5 were considered as cut-off criteria for genes of interest. In comparisons of bronchi versus trachea, a positive FC value (bronchi/trachea) indicated higher expression in bronchi, whereas a negative FC value indicated higher expression in trachea.

Mouse Tissue Immunohistochemistry

Briefly, tissue was fixed in paraformaldehyde (PAF) 4% and 4- μ m paraffin sections were incubated with the polyclonal antibody that recognized both high- and low-molecular weight kininogens (1:100; Boehringer, Germany) as we used previously (23). To detect bound antibodies, we used the L.S. U. Vectastain Kit (Vector laboratories). The positive reaction was developed with Vector SG (Vector Labs). At the end of the labeling reaction, sections were washed in 50 mM Tris-HCl pH 7.8. The counterstaining was performed with Nuclear Fast Red (Vector Labs).

Ussing Chamber Experiments

Freshly excised mouse tracheas were mounted in P2306B tissue sliders (0.057 cm²) placed in modified

Ussing chambers (Physiologic Instruments Inc., San Diego, CA) bathed with bicarbonate buffered solution (pH 7.4), of the following composition (in mM): 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, and 10 D-glucose, gassed with 5% CO₂-95% O₂ and kept at 37°C throughout the experiment. Measurement of the potential difference between the mucosal and the serosal compartment (V_{te}) allowed the calculation of tissue resistance (R_{te}) and equivalent short-circuit current (I_{sc}). Tissues with R_{te} values above 50 $\Omega\cdot\text{cm}^2$ were considered for analysis (24). The experiments were performed in the presence of 10 μM amiloride in the apical solution to block sodium absorptive currents. The 1 μM forskolin (FSK) + 100 μM 3-isobutyl-1-methylxanthine (IBMX) cocktail was used to stimulate cAMP-dependent anion secretion, followed by 100 μM uridine-5'-triphosphate (UTP) to evoke Ca²⁺-activated anion secretion. The I_{sc} values were calculated as the area under the curve (AUC) to include peak and plateau components of the responses. The first 2 min after addition of forskolin-IBMX for cAMP-induced or the first 5 min after UTP addition for Ca²⁺-activated currents, were analyzed using the Acquire & Analyze 2.3v software (Physiological Instruments).

Statistical Analysis

Comparisons have been made either with ANOVA on ranks for multiple groups or Mann-Whitney for paired groups. Comparisons for relative gene expression were performed with ANOVA on ranks and fold change as described in previous sections of the METHODS. Tests used are indicated in each figure legend. Calculations were made using the Sigmaplot 12 program (SigmaPlot, RRID: SCR_003210).

RESULTS

Genetic Silencing of *Kcnn4* Prevents Asthmatic Features in OVA-Challenged Mice

First, we established the OVA-challenge model and tested the effect of *Kcnn4* silencing in common signs of allergic inflammation in animals. We quantified serum IgE to monitor the successful induction of asthma in the mice. As shown in Fig. 1A, serum IgE was significantly increased in animals challenged with OVA. Meanwhile, inactivation of the *Kcnn4* gene did not alter serum IgE in OVA-challenged mice (Fig. 1A).

The OVA challenge increased mast cell numbers in wild-type mice but remained unaltered in the absence of KCa3.1 channels (Fig. 1B). Nevertheless, the increase in degranulated mast cells induced by OVA was significantly reduced in the *Kcnn4*^{-/-} tissues (Fig. 1, B and F). Histological analysis of tissue samples of OVA-challenged animals showed that *Kcnn4* silencing prevented an increase in epithelial thickness (Fig. 1C) and collagen accumulation on the subepithelial layer (Fig. 1, D and F), corroborating previous observations (18). Quantification of PAS-stained tissues demonstrated that *Kcnn4* silencing prevented mucus accumulation in airways after the OVA challenge (Fig. 1, E and F).

Kcnn4 Silencing Modulates Inflammatory Gene Expression in the Airways of the OVA-Challenged Mice

Analysis of gene expression showed that epithelial cells isolated from trachea displayed differences from those isolated from bronchi. We detected that after the OVA challenge 11 genes in the trachea and 22 genes in bronchi were significantly modified, including seven genes shared between trachea and bronchi (Fig. 2A). Of these common genes *Crp*, *Kng1*, and *Mbl2* increased their expression in the trachea but decreased in bronchi after the OVA challenge (Fig. 2A). These observations prompted us to examine differential gene expression in trachea and bronchi epithelia that identified 13 genes preferentially expressed in the bronchi and two in trachea of control animals (Table 1). Table 1 shows that after wild-type mice were challenged with OVA, the pattern of differential gene expression was altered. Except for gene expression of the epithelial Na⁺ channel (ENaC) subunits *Scnn1b* and *Scnn1g*, and lysozyme *Lyz1*, which remained higher in bronchi and trachea, respectively, 21 different genes were upregulated in bronchi and the secreted phosphoprotein 1 *Spp1* gene in trachea after OVA challenge. Silencing of *Kcnn4* greatly reduced expression gradients, leaving three genes more represented in the trachea and none in bronchi (Table 1). As presented in Table 1 and Supplemental Fig. S1A (all Supplemental Figures are available at <https://doi.org/10.6084/m9.figshare.19890424.v1>), the kininogen 1 gene (*Kng1*) was preferentially expressed in bronchi of control wild-type mice. We tested if differences in *Kng1* gene expression were reflected at the protein level. As observed in Supplemental Fig. S1B, KNG1 was more abundant in bronchi epithelial cells than in the trachea of wild-type PBS animals. However, when challenged with OVA there was an increase of KNG1 in the trachea, whereas *Kcnn4* silencing maintained increased KNG1 staining in both trachea and bronchi.

Analysis of the KEGG pathways indicated that the OVA challenge affected genes related to the TNF pathway in airway epithelial cells isolated from bronchi. We found that 12 out of 27 of these genes were significantly changed after the OVA challenge (Fig. 2B). Silencing of *Kcnn4* prevented changes in gene expression induced by OVA including those of the TNF pathway (Fig. 2, A and B).

Differential Expression of Cell-Specific and Asthma-Related Genes in Trachea and Bronchi after Challenge with OVA

We include sets of genes differently expressed in epithelial cell types of the airways and others associated with the development of asthma. As observed in Supplemental Fig. S2A, OVA challenge increased in the expression of *Foxj1* and *Tuba1a* in ciliated cells and *Krt5* in basal cells from trachea of wild-type mice. Although the asthma-related genes *Mmp9* and *Arg2* increased their expression, *Clca1* was decreased in the trachea of wild-type mice treated with OVA (Supplemental Fig. S2A). By contrast, in epithelial cells isolated from the bronchi of wild-type mice treated with OVA, only the expression of *Mmp9* and *Clca1* changed (Supplemental Fig. S2A). Even though *Kcnn4* silencing prevented OVA-induced changes in gene expression, it also induced increased expression of *Tuba1a*, *Muc5b*, and *Scgb1a1* under OVA treatment in the bronchi. Nevertheless, we do not observe significant changes in the

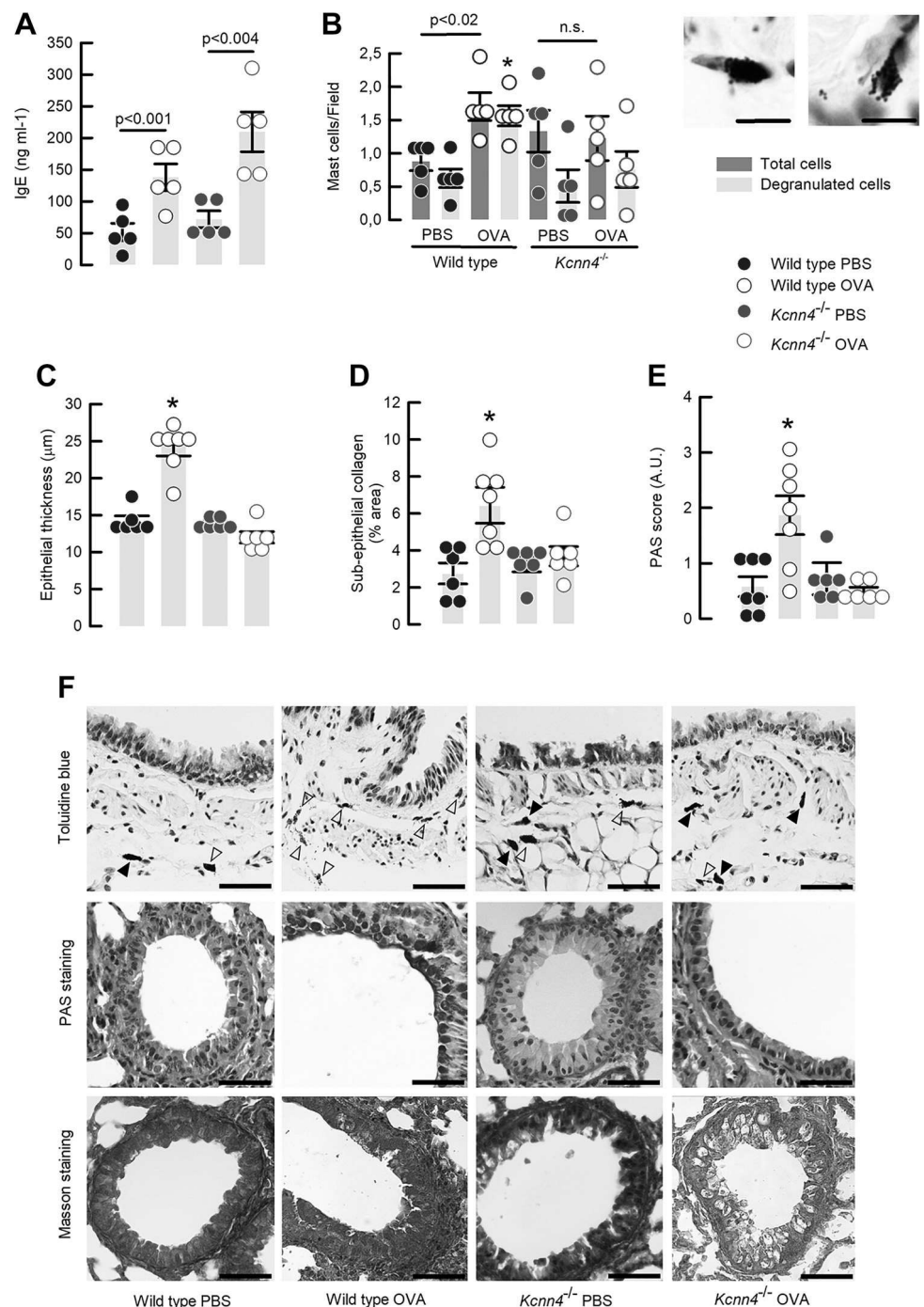


Figure 1. Silencing of *Kcnn4* expression protects airways in ovalbumin (OVA)-challenged mice. **A:** serum IgE concentration in wild-type and *Kcnn4*^{-/-} animals subjected to a long-term OVA challenge; *n* = 5 each group; *P* values were calculated using the Mann–Whitney test. **B:** summary of total and degranulated mast cells in wild-type and *Kcnn4*^{-/-} lung tissue of mice treated with PBS or OVA; **P* < 0.05 one-way ANOVA on ranks, *n* = 5 different animals for each group. **Picture insets** show examples of a nondegranulated (*left* image) and a degranulated (*right* image) mast cell. Scale bar = 10 μ m. Measurements of epithelial thickness (**C**), collagen accumulation (**D**), and evaluation of periodic acid Schiff (PAS)-positive staining (**E**) in airway samples. **P* < 0.05 using one-way ANOVA on ranks; *n* = 6 or 7 different animals for each group. **F:** representative images of Toluidine blue staining (*top*) indicating nondegranulated (filled black triangles) and degranulated (open triangles) mast cells; PAS staining (*middle*), and Masson staining (*bottom*). Scale bar = 50 μ m.

bronchi/trachea ratio of expression under any experimental conditions tested (Supplemental Fig. S2B).

Regional Differences in Gene Expression for Ion Channels and Transporters in Mouse Airway Epithelial Cells Are Affected by the Asthmatic Challenge and *Kcnn4* Silencing

Major components of electrogenic anion transport in the mouse airways correspond to calcium-activated anion secretion and sodium absorption (25). Accordingly, both *Ano1* (coding for TMEM16A) and *Scnn1a* (α -ENaC) transcripts, are the most abundantly expressed

ion channels found in our study (Fig. 3A). The β - and γ -ENaC subunits (*Scnn1b* and *Scnn1g*) are more abundant in the bronchus along with *Cftr* (Fig. 3B). As observed in Fig. 3A, the OVA challenge in wild-type animals significantly increased the expression of *Cftr*, *Lrrc8b*, *Lrrc8c*, *Slc26a9*, *Slc4a4*, and *Trpm6* in the trachea and decreased *Scnn1g* in bronchi, which was all prevented by *Kcnn4* silencing (Fig. 3A). *Kcnn4* silencing also induced an increase in the expression of *Slc4a1* and *Kcnj13* transcripts in bronchi. After the OVA challenge, both *Scnn1b* and *Scnn1g* are the only genes preferentially expressed in the bronchi (Fig. 3B).

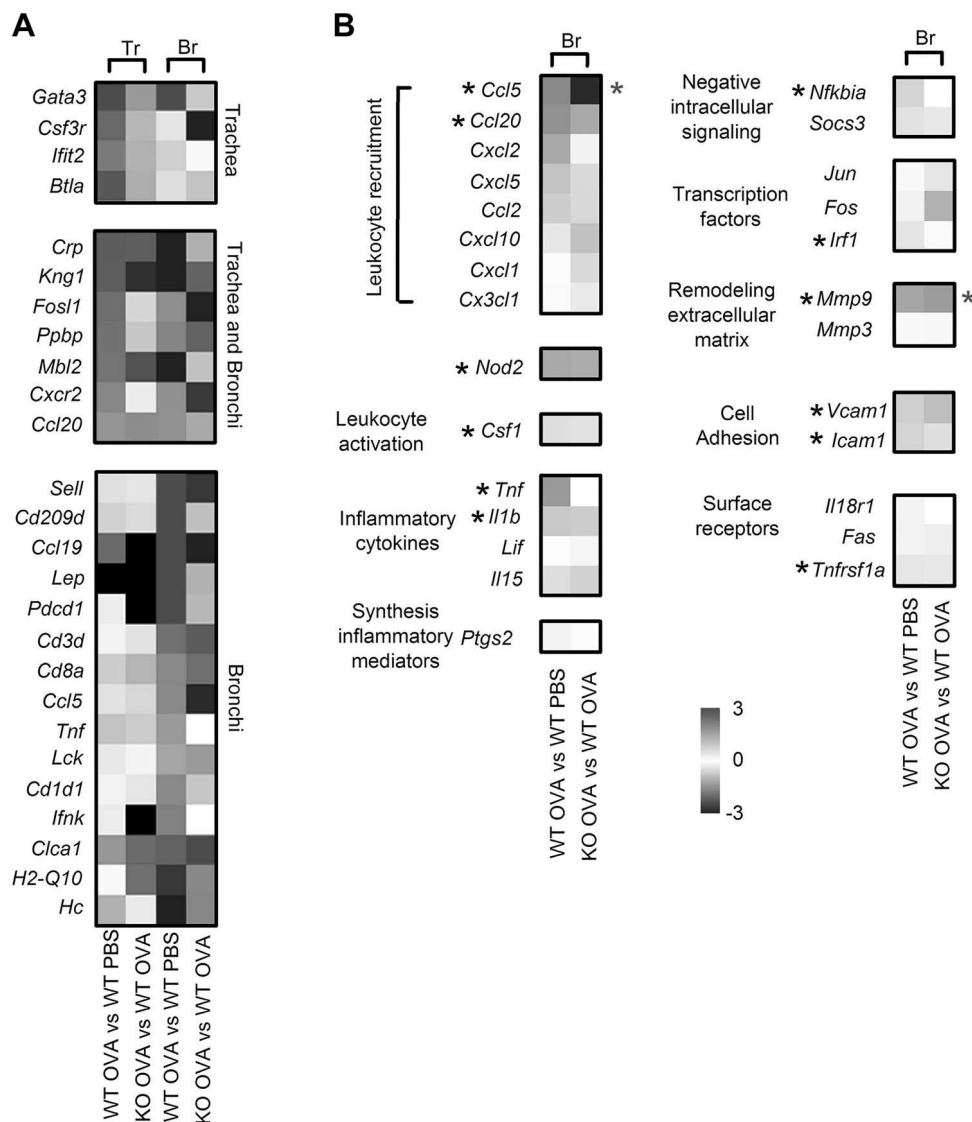


Figure 2. Gene expression is regulated differently by ovalbumin (OVA) and KCNN4 in epithelial cells of trachea and bronchi. **A:** heatmap corresponding to fold changes (FC) in gene expression after OVA (wild-type OVA/wild-type PBS) or the effect of *Kcnn4* silencing (*Kcnn4*^{-/-} OVA/wild-type OVA), for trachea (Tr), or bronchi (Br); *n* = 4 different animals per group. Genes were selected when they fulfilled $-1.5 < \text{fold change (FC)} < 1.5$, $P < 0.05$ and $q < 0.1$ criteria. **B:** heatmaps of FC for genes corresponding to the TNF-pathway in bronchi; the * (black) genes significantly modified after KEGG analysis and the * (red) genes significantly modified after *Kcnn4* silencing ($P < 0.05$ and $q < 0.1$; *n* = 4 different animals per group).

OVA Treatment Reduces Ca²⁺-Activated Anion Secretion in Mouse Trachea

We tested if the OVA challenge induced changes in anion secretion. We observed that cAMP-induced currents remained unchanged (-37 ± 8 vs. -27 ± 2 $\mu\text{A}\cdot\text{cm}^{-2}$ for wild-type PBS and wild-type OVA, respectively) (Fig. 4, A and B). However, there was a significant decrease in Ca²⁺-activated anion secretion in response to UTP in the mouse trachea (-140 ± 8 vs. -76 ± 15 $\mu\text{A}\cdot\text{cm}^{-2}$ for wild-type PBS and wild-type OVA, respectively) (Fig. 4, A and C). A key cytokine of T_H2 response corresponds to IL-4, which is known to increase anion secretion in HBECS (26). Instillation of IL-4 in wild-type animals produced a significant increase in the Ca²⁺-activated secretion evoked by UTP (-233 ± 8 $\mu\text{A}\cdot\text{cm}^{-2}$) but cAMP-induced anion secretion remained unaffected (-42 ± 6 $\mu\text{A}\cdot\text{cm}^{-2}$), confirming that anion secretion is highly modulated by inflammatory signaling (Fig. 4, A–C). The *Kcnn4*^{-/-} mouse treated with PBS showed a reduction of Ca²⁺-dependent anion secretion as we have shown previously (27). Additionally, the analysis of trans-epithelial resistance, voltage, and basal short-circuit

current determined that there were no differences among groups (Supplemental Fig. S3, A–C).

DISCUSSION

The KCa3.1 channel has been the subject of several studies, including in the area of inflammatory airway disease and previous work proposed that inhibition of the channel is a potential therapy for asthma (6). We observed that animals lacking KCa3.1 presented a significant reduction in asthma features such as mast cell degranulation, epithelial hyperplasia, collagen, and mucus accumulation, which correlates with the observations of previous studies (12, 14, 16, 17, 28). Even though these studies did not evaluate a detailed role for epithelial cells in the development of asthma, it has been suggested that KCa3.1 activity in HBECS might favor the development of the disease (9).

We observed that the OVA challenge induced different gene expression profiles. We identified genes that were modified exclusively in the trachea or bronchi or both epithelial cell types. Moreover, some genes behaved differently

Table 1. Significant up- and downregulated genes

Gene	Fold Change (Bronchi/Trachea)	P Value	Q Value
Wild-type PBS			
<i>Knq1</i>	5.83	2.5e-10	1.85e-8
<i>Crp</i>	5.32	1.1e-6	3.71e-5
<i>Hc</i>	4.88	1.27e-6	3.95e-5
<i>Mbl2</i>	4.72	3.94e-8	1.59e-6
<i>H2-Q10</i>	3.20	4.58e-6	0.0001
<i>Csf3r</i>	2.68	0.002	0.02
<i>Igf1</i>	2.37	0.0003	0.004
<i>Cd1d1</i>	2.07	0.0009	0.01
<i>Scnn1g</i>	1.88	9.5e-15	1.4e-12
<i>Ciita</i>	1.84	0.008	0.07
<i>Cftr</i>	1.78	0.0005	0.006
<i>Scnn1b</i>	1.67	3.26e-8	1.45e-6
<i>Bst2</i>	1.62	1.62e-5	0.0004
<i>Cxcl13</i>	-1.52	6.7e-11	6.02e-9
<i>Liz1</i>	-2.04	5.3e-29	2.3e-26
Wild-type OVA			
<i>Ccl19</i>	4.60	0.0003	0.005
<i>Ccr6</i>	4.37	0.0009	0.009
<i>Osm</i>	3.63	0.01	0.06
<i>Prf1</i>	3.62	0.01	0.06
<i>Ccl5</i>	3.6	1.48e-7	5.59e-6
<i>Adipoq</i>	2.87	3.5e-34	1.6e-31
<i>Irf4</i>	2.83	0.005	0.04
<i>Btla</i>	2.53	0.008	0.05
<i>Ccr7</i>	2.49	0.0001	0.002
<i>Ccr5</i>	2.44	0.02	0.09
<i>Cd3d</i>	2.11	0.003	0.03
<i>Fcer2a</i>	2.06	0.0008	0.009
<i>Il27ra</i>	1.98	0.01	0.06
<i>Cd2</i>	1.95	0.002	0.02
<i>Sell</i>	1.91	0.004	0.03
<i>Scnn1b</i>	1.89	5.32e-9	2.68e-7
<i>Cd27</i>	1.86	0.01	0.06
<i>Pbbp</i>	1.81	0.0008	0.009
<i>Cd3g</i>	1.73	0.002	0.02
<i>Cd8a</i>	1.72	0.005	0.04
<i>Scnn1g</i>	1.68	2.3e-10	1.34e-8
<i>Ms4a1</i>	1.51	0.02	0.06
<i>Il2rb</i>	1.51	0.02	0.084
<i>Lyz1</i>	-1.67	1.2e-7	4.93e-6
<i>Spp1</i>	-1.84	2.0e-10	1.33e-8
<i>Kcnn4</i> ^{-/-} OVA			
<i>Fos1</i>	-2.35	0.002	0.06
<i>Cxcl2</i>	-2.42	0.003	0.065
<i>Ccl4</i>	-3.26	0.0022	0.066

Values are gene expression differences in bronchi compared with trachea in wild-type PBS, wild-type OVA, and *Kcnn4*^{-/-} OVA groups (*n* = 4 animals each group), expressed as fold change, with positive numbers indicating higher expression in bronchi and negative values indicating higher expression in trachea; *P* and *Q* values are also listed. OVA, ovalbumin.

between the trachea and bronchi, indicating that epithelial cells are specialized depending on their location. This differential response to inflammatory signaling was previously observed in the mouse when cells isolated from distal airways (bronchi) displayed a stronger response to IL-13 stimulation than cells from the proximal airway (trachea) (29). Similarly, our gene enrichment analysis determined that the TNF pathway was significantly upregulated after the OVA challenge in epithelial cells of the bronchi but not in the trachea. Silencing of *Kcnn4*^{-/-} inhibited OVA-induced changes in trachea and bronchi gene expression, including those of the TNF pathway. It was previously shown that KCa3.1 inhibition negatively affected TNF- α production in lymphocytes

(10), reduced mRNA and protein levels of TNF- α in the spinal cord of a mouse model for autoimmune encephalomyelitis (30), and reduced circulating TNF- α in mice affected by cystic fibrosis (CF) (22), suggesting an important role for KCa3.1 in the regulation of the TNF pathway. Sensitization of *Tnf*^{-/-} mice with OVA produced partial reduction of inflammation and cytokine release in airways suggesting a downstream location in T_H2 signaling that dominates the asthma inflammatory milieu (31). The dynamics of cytokine release in acute allergic inflammation demonstrated that IL-4 and IL-13 rapidly increased their levels in the bronchoalveolar fluid, whereas TNF- α levels remained undisturbed for up to 48 h (32). Even though the role of epithelial TNF- α in asthma has not been extensively studied, epithelial TNF- α signaling is necessary to produce hyperresponsiveness and increased contractility in asthmatic airways, suggesting that epithelial TNF plays an active role in the disease (33). Anti-TNF- α therapies have been tested, but have not successfully managed all asthma phenotypes. Moreover, severe side effects have been reported in humans, and therapies tackling upstream targets like anti-IgE or anti-T_H2 cytokines not are favorable in all asthma phenotypes (4, 34–36). Therefore, new therapeutic strategies are necessary.

The remodeling of the epithelial layer in asthma is characterized by increased mucus-containing goblet cells, a hallmark of T_H2 inflammation and airflow obstruction observed in this disease (37). Nevertheless, other cell types in the epithelium might also play a role in the pathology (38). We used selected markers of cell populations to monitor changes in epithelial airway cell types between trachea and bronchi during the asthmatic trial. We observed that the OVA challenge did not alter the expression of genes in goblet and club cells (both of the mucoid/secretory lineage) in either trachea or bronchi. Nevertheless, expression of the *Foxj1* and *Tuba1a* genes, which are preferentially found in ciliated cells was significantly increased in the trachea and might reflect the functional adaptation of the proximal airway epithelium to increase mucus transport by mucociliary clearance mechanism. Gene expression of *Krt5*, a marker for basal cells, was increased after the OVA challenge in the trachea only. In agreement with the reported location of basal cells in mouse and human proximal airways, this supports the view that repair of the airway epithelium arises from basal cells in the trachea (38). These data indicate that pathophysiological changes in epithelial cell composition results from the OVA challenge. Moreover, gene expression of asthma-related genes *Mmp6* and *Arg2* were also increased after the OVA challenge in accordance with previous observations that link their activity with asthma exacerbations in humans (39, 40). Silencing of *Kcnn4* prevented the increase of both genes. Surprisingly, the *Muc5ac* gene was barely increased after the OVA challenge, even though the histological analysis determined increased mucus and goblet cells in the airways. Such a discrepancy might be explained by the fact that other groups had used mouse lung extracts instead of isolated epithelial cells to study gene expression (41, 42). Nevertheless, a recent and detailed study using single-cell mRNA sequencing in patients with asthma reported an increased number of goblet cells expressing *MUC5AC* along with newly identified goblet cell genes *NOS2*, *CST1*, and *CEACAM5* that were not included in our study (43).

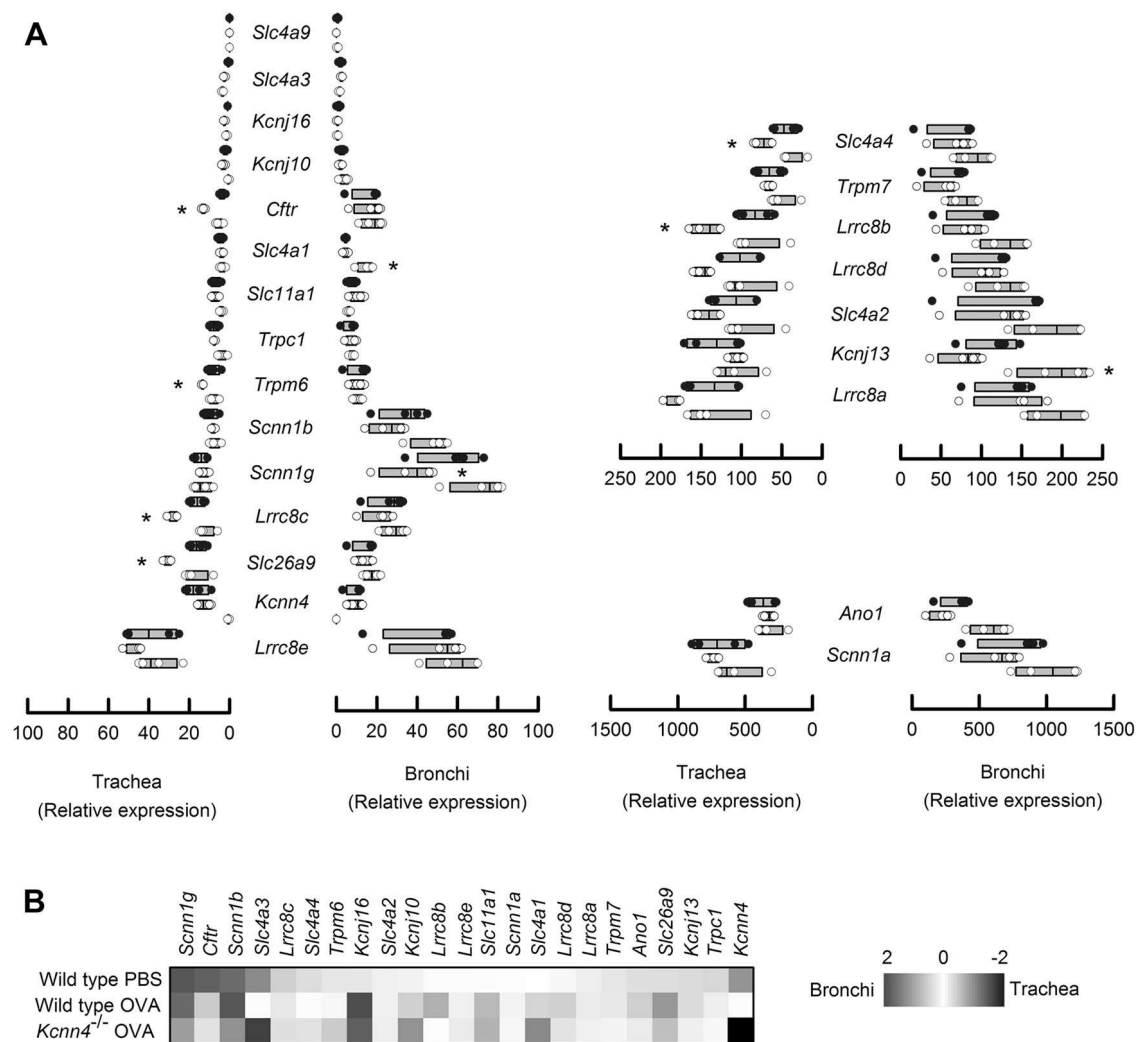


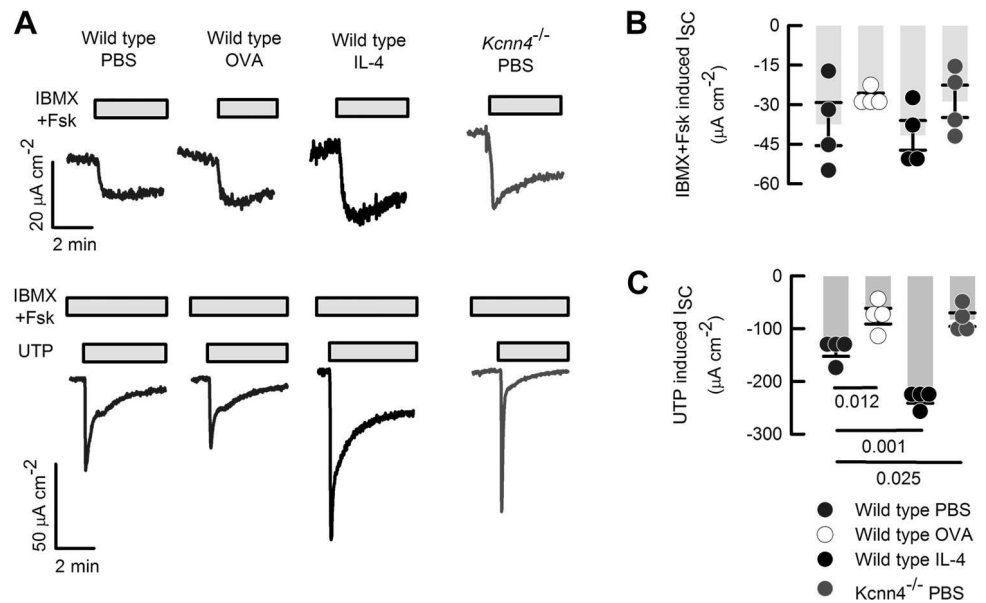
Figure 3. Ovalbumin (OVA) challenge differentially affects the expression of ion channels and transporters in tracheal and bronchial epithelial cells. **A:** relative gene expression in epithelial cells of the trachea or bronchi, blue filled, blue open, and red open circles indicate wild type-PBS, wild type-OVA, or *Kcnn4*^{-/-} OVA, respectively; **P* < 0.05 determined by ANOVA on ranks, *n* = 4 different animals for each group. **B:** heatmap summary of fold changes (bronchi/trachea) showing changes in gene expression after OVA challenge or *Kcnn4* silencing. The black box indicates nondetected.

Ion channels and transporters are important in maintaining fluid balance and mucus homeostasis in the lumen of the airways. The best example is observed in cystic fibrosis, where deficiencies in ion transport due to CFTR-inactivating mutations produce severe malfunctions in airway clearance (44). We observed that *Ano1* (coding for the calcium-activated TMEM16A channels) and *Scnn1a* (coding for α -ENaC) are the most abundant transcripts expressed in both the trachea and bronchi. The β - and γ -ENaC subunits *Scnn1b* and *Scnn1g* are more abundant in the bronchus and are accompanied by *Cftr*. These observations correlate with previous electrophysiological studies showing that calcium-activated anion secretion (occurring through TMEM16A) is higher in the trachea, whereas amiloride-sensitive sodium absorption through ENaC and cAMP-activated anion secretion by CFTR are higher in the bronchi of wild-type mice (25). The expression of genes coding for ion channels and transporters was affected by the asthmatic trait. We observed an increased expression of *Trpm6* and the anionic channels, subunits, and transporters *Slc26a9*, *Lrrc8b*, *Lrrc8d*, *Cftr*, and *Slc4a4* in

the trachea. Increased expression of anionic channels and transporters might relate to the need for bicarbonate secretion for proper mucin maturation and deployment in the airway lumen, and for chloride secretion to increase air surface liquid (ASL) fluidity (45). The latter can be boosted by CFTR-dependent chloride secretion as observed in human cells (46). Nevertheless, the participation of CFTR in ASL homeostasis in mouse airways is disputed by the absence of lung disease in CF mouse models lacking active CFTR channels and is explained by the presence of alternative chloride channels or the low expression of ATP12 H⁺-pump (47, 48). LRRC8 family heteromers can extrude chloride in different cell types (49), and are functional in human bronchial cells of normal donors and patients with CF (50). But whether LRRC8 channels participate in ASL homeostasis is not known.

IL-4 is a key T_H2 cytokine in the pathogenesis of asthma (35). IL-4 induced a strong increase in genes related with goblet cell hyperplasia and increased the expression of *ANO1* that correlated with increased Ca²⁺-induced anion secretion in HBECs (26). Electrophysiological measurements demonstrated

Figure 4. Ovalbumin (OVA) treatment reduces Ca^{2+} -activated anion secretion. A: representative Ussing chamber experiments for cAMP-induced (top) or Ca^{2+} -induced (bottom) anion secretion in mouse tracheas isolated from wild-type PBS, wild-type OVA, wild-type IL-4, or *Kcnn4*^{-/-} PBS animals and in the presence of amiloride (10 μM) ($n = 4$ animals, for each group). Summary for 3-isobutyl-1-methylxanthine (IBMX) + forskolin (FSK)-induced (B) or uridine-5'-triphosphate (UTP)-induced (C) short-circuit currents from mouse tracheas. Calculated P values using ANOVA on ranks are indicated.



that while mice instilled with IL-4 mimicked the increase in Ca^{2+} -activated secretion observed in human cells, the tracheas isolated from OVA-challenged animals bear a reduction of such currents. The observed reduction does not correlate with a change in *Ano1* gene expression. Nevertheless, TMEM16A channel function is enhanced by the action of secreted protein CLCA1, whose transcripts were severely reduced in our study after the OVA challenge and might explain the decrease in UTP-evoked current (51).

In accordance with our data, the absence of TMEM16A critically decreases mucociliary clearance in the mouse trachea and is necessary for mucus release from goblet cells (52–54). Thus, the observed reduction in Ca^{2+} -activated anion secretion observed in the OVA-challenged mouse indicate a role in the deterioration of airway clearance and might explain the observed increase in mucus accumulation. Along the same lines, MUC5AC-overexpressing mice do not produce mucus plugging, inflammation, or goblet cell hyperplasia, indicating that mucus overproduction must be accompanied by reduced fluid secretion to produce obstructive airway disease (55). Nevertheless, the role of TMEM16A in mucus release is still a controversial topic, as new evidence questions such function (56, 57). In addition, the increased *Cftr* expression observed in the trachea did not correlate with increased cAMP-induced anion secretion, reaffirming the idea that Ca^{2+} -activated anion secretion is more relevant for airway function in mice. Finally, our data indicate that *Kcnn4* expression remained unaltered after the OVA challenge, rejecting the idea that reduced Ca^{2+} -activated anion secretion after OVA is caused by decreased *Kcnn4* expression as observed with the *Kcnn4*^{-/-} tracheas.

What epithelial cells express KCa3.1 and can be involved in the mechanism for regulating gene expression in the airways? KCa3.1 has been detected in most airway epithelial cells in rats (58), and electrophysiological studies confirmed their presence in HBECs (9). Single-cell mRNA sequencing techniques do not shed much light on *Kcnn4* expression, and information available from www.lungcellatlas.org indicates that basal cells have the highest expression in airways

(43). KCa3.1 regulation of transcription is an understudied topic but it is known that its inhibition affects intracellular Ca^{2+} signaling by reducing hyperpolarization-dependent Ca^{2+} entry (10, 59). In particular, Ca^{2+} -dependent activation of the nuclear factor of activated T-cells NFATc1 in inflammatory osteoclastogenesis was shown to be dependent on KCa3.1 activity (60). Interestingly, this study also showed that after *Kcnn4* silencing one of the affected genes was *Mmp9*, which we also found downregulated in the bronchi of the *Kcnn4*^{-/-} mouse treated with OVA, suggesting common pathways for the inflammatory response between different cell types where KCa3.1 participates. Nevertheless, the restricted amount of genes included in our study excluded the identification of other possible KCa3.1-regulated targets of chronic airway inflammation in mouse airways.

Even though a detailed description of how KCa3.1 activity regulates gene expression remains to be determined, the inhibition of the channel has been proved to decrease the inflammatory response. This has been the case for animal models of diseases affecting the airways including chronic asthma and allergic rhinitis (12, 14, 15, 17, 28, 61), and a beneficial role for *Kcnn4* silencing was also observed in a chronic obstructive pulmonary disease (COPD) animal model (27). Several cell types that participate in asthma are affected by *Kcnn4* silencing including mast cells, neutrophils, macrophages, and smooth muscle (11, 12, 16, 62), suggesting that *Kcnn4* silencing in these cells might be a therapeutic strategy. Consistent with this idea, our data indicate that KCa3.1 inhibition reduces several asthma-related features including a reduction in TNF- α signaling pathway, paving the way for the testing of new specific inhibitors of KCa3.1 to ameliorate airway damage as observed in asthma.

DATA AVAILABILITY

The RNA-seq data that support this study are available in Figshare at <https://doi.org/10.6084/m9.figshare.20061152>.

SUPPLEMENTAL DATA

Supplemental Figs. S1–S3: <https://doi.org/10.6084/m9.figshare.19890424.v1>.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.A.F. conceived and designed research; A.R.P., A.G., A.M., P.S., I.M., G.V., and C.A.F. performed experiments; A.R.P., F.M., A.G., P.S., G.V., C.D.F., L.J.V.G., J.M.S., and C.A.F. analyzed data; A.R.P., L.J.V.G., J.M.S., and C.A.F. interpreted results of experiments; A.G., P.S., L.J.V.G., J.M.S., and C.A.F. prepared figures; C.A.F. drafted manuscript; A.R.P., C.D.F., L.J.V.G., J.M.S., and C.A.F. edited and revised manuscript; A.R.P., F.M., A.G., A.M., P.S., I.M., G.V., C.D.F., L.J.V.G., J.M.S., and C.A.F. approved final version of manuscript.

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