Allergic airway inflammation induces a pro-secretory epithelial ion transport phenotype in mice

P. Anagnostopoulou, L. Dai, J. Schatterny, S. Hirtz, J. Duerr and M.A. Mall

ABSTRACT: The airway epithelium is a central effector tissue in allergic inflammation and T-helper cell (Th) type 2-driven epithelial responses, such as mucus hypersecretion contribute to airflow obstruction in allergic airway disease. Previous *in vitro* studies demonstrated that Th2 cytokines also act as potent modulators of epithelial ion transport and fluid secretion, but the *in vivo* effect of allergic inflammation on airway ion transport remains unknown.

We, therefore, induced allergic inflammation by intratracheal instillation of *Aspergillus fumigatus* extract or interleukin-13 in mice and determined effects on ion transport in native tracheal and bronchial tissues.

We demonstrate that allergic inflammation enhanced basal Cl^- secretion in both airway regions and inhibited epithelial Na^+ channel (ENaC)-mediated Na^+ absorption and increased Ca^{2+}-dependent Cl^- secretion in bronchi. Allergen-induced alterations in bronchial ion transport were associated with reduced transcript levels of α-, β- and γENaC, and were largely abrogated in signal transducer and activator of transcription (Stat)6$^{-/-}$ mice.

Our studies demonstrate that Th2-dependent airway inflammation produced a pro-secretory ion transport phenotype *in vivo*, which was largely Stat6-dependent. These results suggest that Th2-mediated fluid secretion may improve airway surface hydration and clearance of mucus that is hypersecreted in allergic airway diseases such as asthma, and identify epithelial Stat6 signalling as a potential therapeutic target to promote mucus hydration and airway clearance.

KEYWORDS: Allergic airway inflammation, epithelial sodium channel, interleukin-13, ion transport, mucociliary clearance, signal transducer and activator of transcription 6

Allergic airway diseases, such as asthma, belong to the most common chronic diseases in children and adults, but their pathogenesis remains incompletely understood [1]. The airway epithelium provides important protective functions and constitutes a central effector tissue in allergic airway disease. In healthy airways, the superficial respiratory epithelium acts as a barrier and provides coordinate regulation of mucus secretion, ciliary beating and airway surface liquid (ASL) volume, functions that are essential for effective mucociliary clearance (MCC) and protection of the lung from inhaled allergens, pathogens and other noxious agents [2–4]. A large body of evidence suggests that a T-helper cell (Th) type 1/2 imbalance with a Th2-dominant response to inhaled allergens plays a critical role in the pathogenesis of allergic airway inflammation and identified the Th2 cytokine interleukin (IL)-13 as a key effector molecule in this process [1, 5, 6]. Further studies demonstrated that IL-13, through binding to a common IL-4/IL-13 receptor complex and activation of signal transducer and activator of transcription (Stat)6 signalling, has direct effects on airway epithelial cells, including mucus hypersecretion, goblet cell metaplasia and airway hyperresponsiveness [7], key features contributing to airflow obstruction in the clinical syndrome of asthma.

In addition, more recent *in vitro* studies showed that IL-13 and the related Th2 cytokine IL-4 act as potent modulators of ion transport in cultured human bronchial epithelial cells, identifying another direct effect of these Th2 cytokines on the airway epithelium [8, 9]. By active regulation of transepithelial ion transport, the airway epithelium maintains the thin film of liquid that covers airway surfaces and provides a low-viscosity layer essential for proper ciliary beating and MCC [3]. In this process, the amiloride-sensitive epithelial Na^+ channel (ENaC) is limiting for the absorption of Na^+ and osmotically
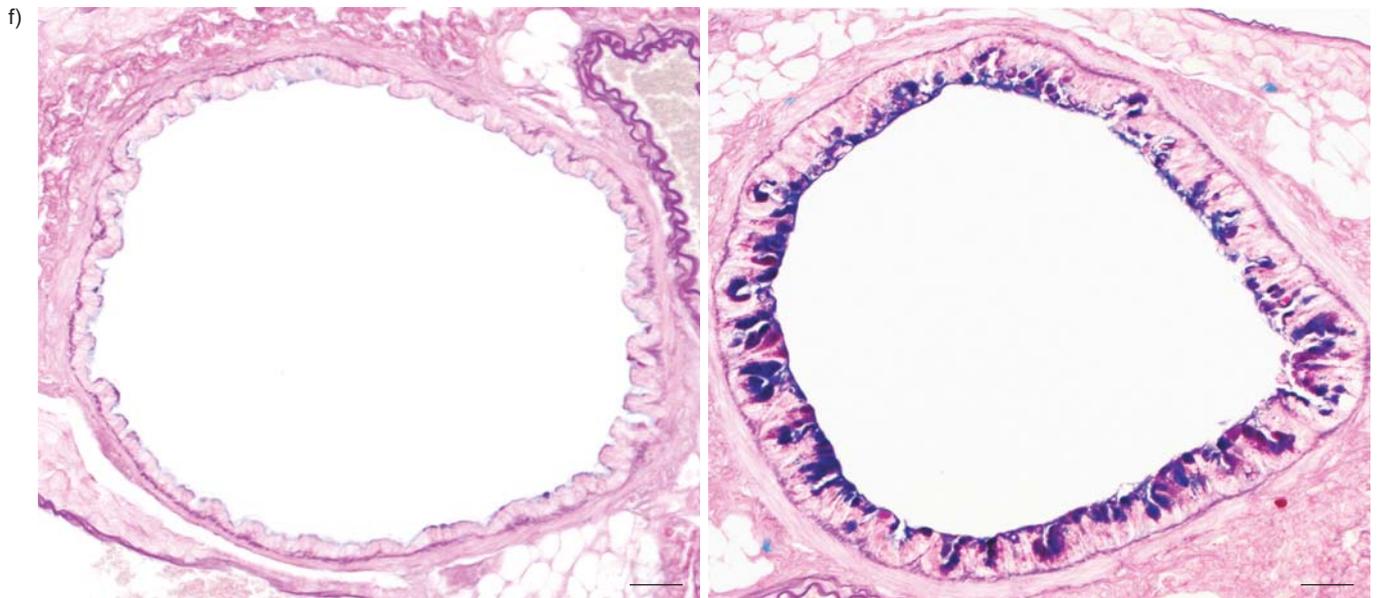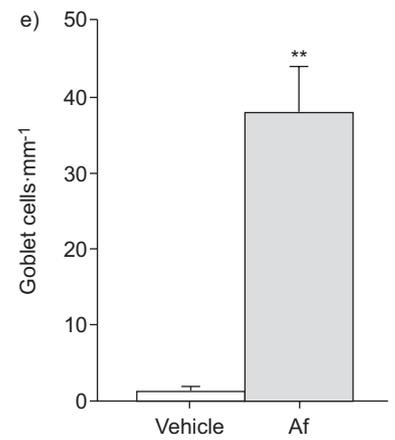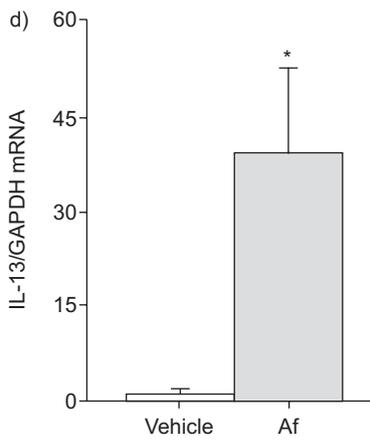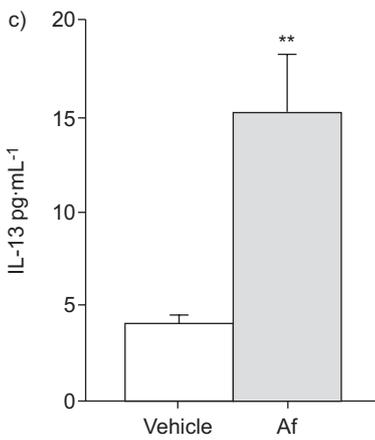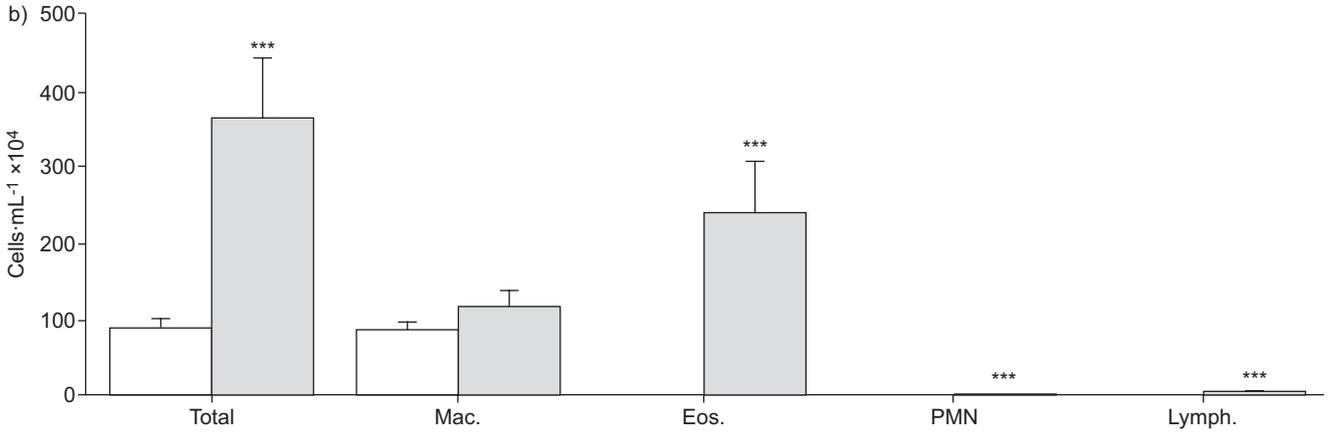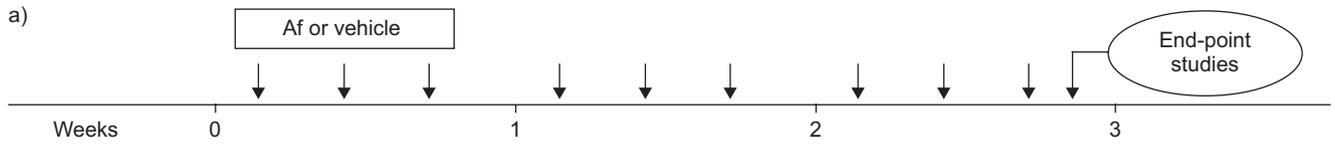
FIGURE 1.   Allergic airway inflammation induced by sensitisation with *Aspergillus fumigatus* extract (Af) in mice. a) Protocol for intratracheal sensitisation with Af, b) effect on total and differential cell counts, c) interleukin (IL)-13 concentration in bronchoalveolar lavage, d) IL-13 transcript levels in lungs, e) airway goblet cell counts and f) airway histology in mice (representative airway sections stained with alcian blue–periodic acid Schiff from Af-sensitised and vehicle-treated mice; scale bars=100 μm) sensitised with Af or vehicle alone for a period of 3 weeks. Data are presented mean±SEM (n=5–8 mice per group). □: vehicle; ■: Af. Mac.: macrophages; Eos.: eosinophils; Lymph.: lymphocytes; GAPDH: glyceraldehyde 3-phosphate dehydrogenase. *: p<0.05 compared with vehicle-treated mice; **: p<0.01 compared with vehicle-treated mice; ***: p<0.001 compared with vehicle-treated mice.

coupled fluid [10], whereas Cl$^-$ secretion is mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) [11] and a Ca^{2+}-activated Cl$^-$ channel (CaCC), which was recently identified as transmembrane protein (TMEM)16a [12, 13]. Both IL-4 and IL-13 inhibited ENaC-mediated Na$^+$ absorption, and increased CFTR- and CaCC-mediated Cl$^-$ secretion, thereby producing a pro-secretory ion transport phenotype in cultured human bronchial epithelial cells [8, 9]. These studies suggested that selected Th2 cytokines may be involved in the regulation of ASL volume to improve mucus hydration and promote MCC in allergic airway disease. However, the role of Th2-driven inflammation in regulation of airway ion transport in the complex *in vivo* pathogenesis of allergic airway inflammation remains unknown.

In the present study, we induced allergic inflammation in mice by repeated mucosal exposure with a natural aeroallergen, *Aspergillus fumigatus* extract (Af) [14], and studied the effects on ion transport properties and transcript expression of ENaC subunits (α, β and γ), CFTR and the CaCC candidate TMEM16a in freshly excised airway tissues. We compared bioelectric properties in native tracheal and bronchial epithelia from Af-sensitised and vehicle-treated mice to elucidate effects of allergic inflammation in different airway regions along the tracheo-bronchial tree. Furthermore, we compared the effect of Af-induced inflammation on bioelectric properties in wild-type (WT) and Stat6$^{-/-}$ mice, and studied direct effects of IL-13, in an attempt to obtain mechanistic insights into the link between Th2-driven inflammation and regulation of airway ion transport. These studies show for the first time that allergen-induced airway inflammation induces a pro-secretory ion transport phenotype *in vivo*, yielding novel insights into the interplay between Th2-driven inflammation and epithelial function in the complex pathogenesis of allergic airway disease, which may lead to new therapeutic strategies for asthma and other airway diseases associated with impaired mucus clearance.

MATERIALS AND METHODS
Experimental animals
All animal studies were approved by the Regierungspräsidium, Karlsruhe, Germany. Female BALB/c WT mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Stat6$^{-/-}$ mice in the BALB/c background [15] were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Experimental animals were bred by intercrossing of Stat6$^{+/-}$ mice; offspring were genotyped by PCR of genomic DNA, as previously described [15], and 6–8-week-old Stat6$^{-/-}$ mice and WT litter mates were used for all experiments. Mice were housed in a specific pathogen-free animal facility and had free access to food and water.

Allergen exposure
Allergic airway inflammation was induced by repeated airway sensitisation with Af (Hollister-Stier Laboratories, Spokane,

WA, USA) according to previously established protocols [14]. In brief, mice were anaesthetised by inhalation of isoflurane (3% in oxygen) and 20 μL of either Af (2 mg·mL^{-1}) dissolved in 0.9% NaCl or 0.9 % NaCl alone, were instilled intratracheally three times per week, for a period of 3 weeks. All end-point studies were performed 24 h after the last allergen exposure (fig. 1a).

IL-13 instillation
IL-13 was administered by repeated intratracheal instillation as previously described [16]. In brief, 5 μg recombinant murine IL-13 (Peprotech, Hamburg, Germany) dissolved in 50 μL phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (low-endotoxin; Sigma, Steinheim, Germany) or an equal volume of vehicle alone, were administered by intratracheal instillation once daily for three consecutive days. End-point studies were performed 24 h after the last IL-13 instillation.

Bronchoalveolar lavage cell counts and IL-13 measurements
Mice were deeply anesthetised *via* intraperitoneal injection of a combination of ketamine and xylazine (120 and 16 mg·kg^{-1} respectively) and killed by exsanguination, by cutting the renal arteries. A median sternotomy was performed, the trachea was cannulated, the left mainstem bronchus was ligated and the right lung was lavaged with PBS as previously described [17]. Bronchoalveolar lavage (BAL) samples were centrifuged and the cell-free supernatant was stored at -80°C. Total cell counts were determined using a haemocytometer and differential cell counts were determined in cytospin preparations stained with May–Grünwald–Giemsa [17]. IL-13 concentration in BAL supernatant was measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions [17].

Histology and airway morphometry
Left lungs were removed through the median sternotomy, immersion-fixed in 10% neutral-buffered formalin and embedded in paraffin. Lungs were sectioned transversally at the level of the proximal intrapulmonary main axial airway. 5-μm sections were cut and stained with alcian blue–periodic acid Schiff (AB-PAS) as previously described [17]. Images were captured with an Olympus IX-71 microscope interfaced with a SIS Colorview I Camera Set (Olympus, Hamburg, Germany) using the 10× objective. Goblet cells were identified by the presence of intracellular AB-PAS-positive material and numeric cell densities were determined per mm of the basement membrane using an interactive image measurement tool (celÎF image analysis software; Olympus, Hamburg, Germany), as previously described [17].

Electrogenic ion transport measurements
Anaesthetised mice were killed by exsanguination and airway tissues were dissected using a stereomicroscope as previously

described [18]. In brief, the trachea and mainstem bronchi were exposed by a median incision of the anterior part of the neck and a sternotomy. Airway tissues were freed from surrounding muscle, vessels and connective tissue by blunt dissection *in situ*. Next, tracheae or mainstem bronchi were excised, the anterior part with the cartilage rings was cut open longitudinally, the pars membranacea was exposed and tissues were immediately mounted into perfused micro-Ussing chambers with circular open areas of 0.8 mm^2 for tracheal tissues and 0.5 mm^2 for bronchial tissues [19]. The luminal and basolateral bath was perfused continuously at a rate of 10 mL·min^{-1}, with a solution of the following composition: 145 mM NaCl, 0.4 mM KH_2PO_4, 1.6 mM K_2HPO_4, 5 mM D-glucose, 1 mM $MgCl_2$, 1.3 mM calcium gluconate. The pH was adjusted to 7.4. Experiments were performed at 37°C under open-circuit conditions as previously described [19]. In brief, values for the transepithelial voltage (V_{te}) were referenced to the serosal side. The transepithelial resistance (R_{te}) was determined by applying intermittent (1 s) current pulses ($\Delta I = 0.5$ μA) and the equivalent short-circuit current (I_{sc}) was calculated according to Ohm's law from V_{te} and R_{te} ($I_{sc} = V_{te}/R_{te}$). After an equilibration period of 40 min in the Ussing chamber, basal I_{sc} was determined and amiloride (100 μM, luminal) was added to inhibit electrogenic ENaC-mediated Na$^+$ absorption. Then, 3-isobutyl-1-methylxanthine (IBMX; 100 μM, luminal) and forskolin (1 μM, luminal), and uridine 5'-triphosphate (UTP) (100 μM, luminal) were added sequentially to induce cyclic adenosine 5'-monophosphate (cAMP)-mediated and Ca^{2+}-activated Cl$^-$ secretion, respectively [10]. Additionally, we performed experiments in which bumetanide (100 μM, basolateral), an inhibitor of the basolateral Na$^+$–K$^+$–2Cl$^-$ cotransporter, was added under basal conditions or in the presence of amiloride to block transepithelial Cl$^-$ secretion [20]. Amiloride, IBMX, forskolin and bumetanide were obtained from Sigma (Steinheim, Germany) and UTP was obtained from GE Healthcare (Hatfield, UK). All chemicals were of the highest grade of purity available.

Real-time RT-PCR
Right lungs and tracheae were immediately stored in RNAlater® (Applied Biosystems, Darmstadt, Germany). RNA was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany). For transcript expression studies in tracheal tissues, tracheae from three mice of the same genotype and treatment group were pooled. RNA purity and quantity were determined using a NanoDrop ND100 spectrophotometer (PeqLab, Erlangen, Germany) and integrity was verified by agarose gel electrophoresis. cDNA was obtained by reverse transcription of 1 μg of total RNA (Superscript III RT; Invitrogen, Karlsruhe, Germany). Quantitative real-time RT-PCR for αENaC, βENaC, γENaC, CFTR, TMEM16a, IL-13 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed on an Applied Biosystems 7500 Real Time PCR System using TaqMan universal PCR master mix and inventoried TaqMan gene expression assays according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). Relative fold changes in target gene expression were determined from the efficiency of the PCR reaction and the crossing point deviation between Af-sensitised and vehicle-treated mice, and normalised to the expression of the reference gene GAPDH, as previously described [17].

Statistics
All experiments were performed by an investigator blinded to the treatment group and the genotype of the mice. Data were analysed with SigmaStat version 3.1 (Systat Software, Erkrath, Germany) and are presented as mean±SEM. We performed statistical analyses using unpaired t-test, Mann–Whitney Rank Sum test, one-way ANOVA and Kruskal–Wallis ANOVA on ranks as appropriate, and $p < 0.05$ was the cut-off to indicate statistical significance.

RESULTS

Induction of allergic airway inflammation by intratracheal sensitisation with Af in mice
Previous studies have demonstrated that IL-4 and IL-13 act as potent modulators of epithelial ion transport *in vitro* [8, 9]; however, the role of this regulatory function in the complex *in vivo* pathogenesis of allergic airway inflammation has not been studied. To elucidate the effect of allergic inflammation on airway ion transport *in vivo*, we sensitised mice with a natural aeroallergen, *A. fumigatus*, by repeated intratracheal instillation for a period of 3 weeks (fig. 1a). Similarly to previous studies [14], this sensitisation protocol produced a robust allergic inflammation characterised by airway eosinophilia (fig. 1b), increased levels of the Th2 cytokine IL-13 in BAL fluid (fig. 1c), increased IL-13 transcript levels in lung tissues (fig. 1d), airway goblet cell metaplasia and mucus hypersecretion (figs 1e and f) in Af-sensitised compared with vehicle-treated mice.

Effects of allergic inflammation on epithelial ion transport in the trachea
To determine the role of allergen-induced inflammation in regulation of airway ion transport *in vivo*, we first studied its effects on tracheal epithelia, a tissue commonly used for studies of airway ion transport in mice [10, 13, 18]. Freshly excised tracheal tissues were mounted in perfused micro-Ussing chambers and basal bioelectric properties, amiloride-sensitive Na$^+$ absorption, and cAMP-mediated and UTP-induced Cl$^-$ secretion were compared in Af-sensitised and vehicle-treated mice. I_{sc} under basal conditions, as well as amiloride-sensitive I_{sc}, was not different in tracheal tissues from Af-sensitised compared with vehicle-treated mice (figs 2a–d). The amiloride-insensitive residual I_{sc} in the presence of amiloride block was significantly increased by allergic airway inflammation (fig. 2e), whereas the cAMP-induced (IBMX and forskolin) and Ca^{2+}-activated (UTP) Cl$^-$ secretory responses were not different in tracheal tissues from Af-sensitised compared with vehicle-treated mice (figs 2f and g). Taken together, these results demonstrate that allergic inflammation caused an increase in amiloride-insensitive I_{sc} in native tracheal tissues. However, our *in vivo* model of allergic airway inflammation did not produce modulation of ENaC-mediated Na$^+$ absorption or agonist-induced Cl$^-$ secretion in native tracheal tissues.

Next, we determined the effect of Af-induced airway inflammation on mRNA transcript levels of these ion channels in tracheal tissues by quantitative real-time RT-PCR. Consistent with the results obtained from ion transport measurements (fig. 2), expression levels of αENaC, βENaC, γENaC and TMEM16a did not differ in tracheal tissues from Af-sensitised compared with vehicle-treated mice (fig. 3). In agreement with previous functional studies that demonstrated that CFTR is
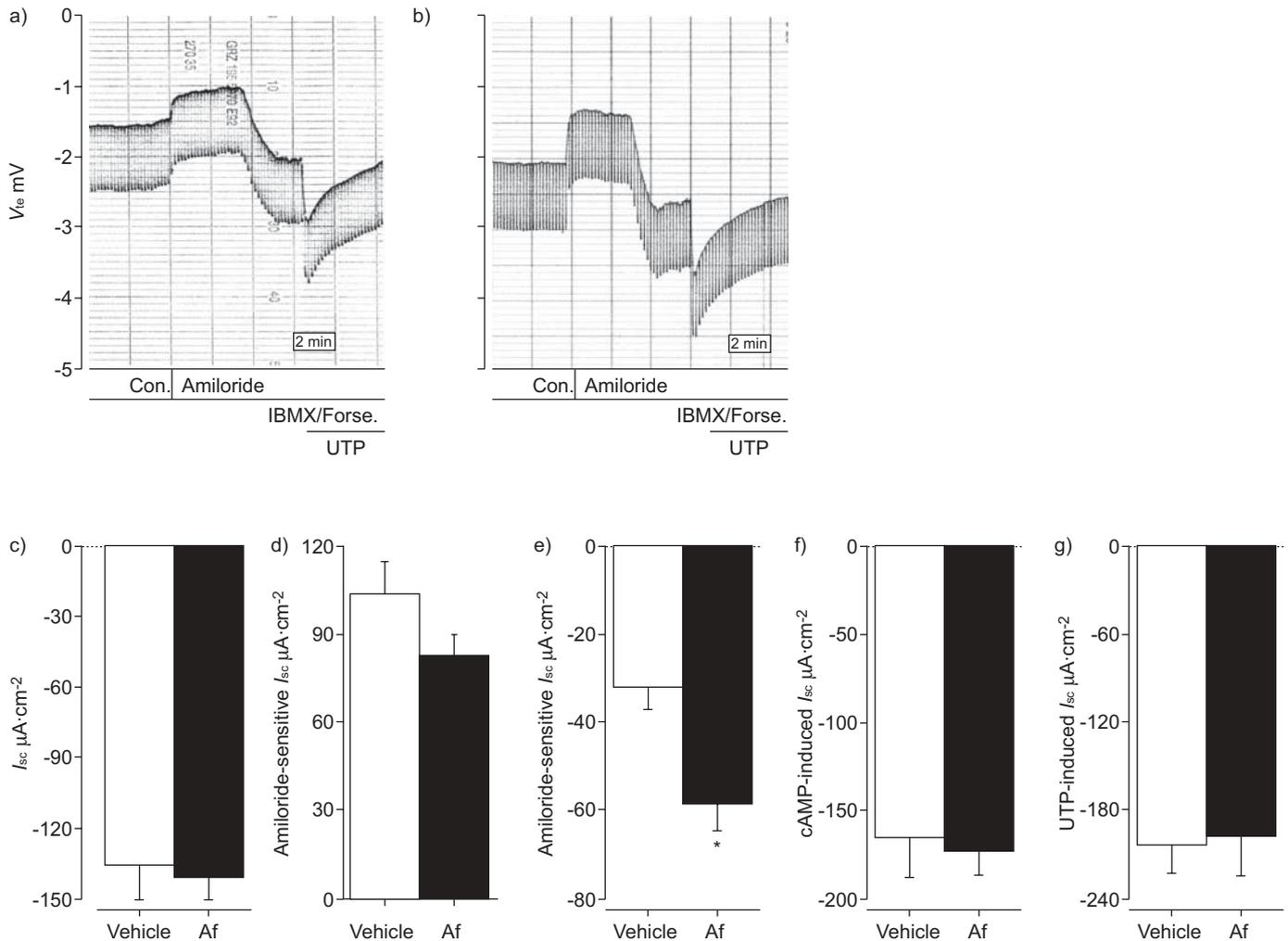
FIGURE 2. Effect of allergic inflammation on ion transport in native tracheal epithelia. a, b) Original recordings of the effects of amiloride, cyclic adenosine 5'-monophosphate (cAMP)-dependent activation and uridine 5'-triphosphate (UTP)-dependent activation on transepithelial voltage (V_{te}) and transepithelial resistance (R_{te}) across freshly excised tracheal tissues from a) vehicle-treated and b) *Aspergillus fumigatus* extract (Af)-sensitised mice. R_{te} was determined from V_{te} deflections obtained by pulsed current injection. Representative results of n=27–31 mice per group. c–g) Summary of c) basal equivalent short-circuit current (I_{sc}), d) amiloride-sensitive I_{sc}, e) amiloride-insensitive I_{sc}, f) cAMP-induced I_{sc} and g) UTP-induced I_{sc} in freshly excised tracheal tissues from Af-sensitised and vehicle-treated mice. Data are presented as mean ± SEM (n=27–31 mice per group). Con.: control; IBMX: 3-isobutyl-1-methylxanthine; Forse.: forskolin. *: $p < 0.001$ compared with vehicle-treated mice.

expressed at very low levels in mouse trachea [18], signals for CFTR transcripts were below the detection limit in tracheal tissues from both groups (data not shown).

Allergic inflammation induces a pro-secretory ion transport phenotype in bronchi

In an attempt to explain the differences in ion transport modulation by Th2 cytokines in our studies in native tracheal tissues (figs 2 and 3) and previous *in vitro* studies in cultured human bronchial epithelial cells [8, 9], we hypothesised that these differences may be caused by regional differences in ion transport properties and/or regulation along the tracheobronchial tree. To test this hypothesis, we extended our studies in the Af model of allergic airway inflammation to freshly excised bronchial tissues.

First, we compared the bioelectric properties in freshly excised bronchial and tracheal tissues from vehicle-treated

WT mice to determine regional differences in the absence of allergic inflammation. As shown in table 1, several distinct ion transport properties were detected in bronchial *versus* tracheal epithelia. Under baseline conditions, R_{te} was not different in the two airway regions, whereas V_{te} and I_{sc} were lower in bronchial compared to tracheal tissues (table 1). Overall, the absolute values for amiloride-sensitive I_{sc} ($p < 0.001$), residual amiloride-insensitive I_{sc} ($p < 0.001$), cAMP-induced I_{sc} ($p < 0.01$) and UTP-induced I_{sc} ($p < 0.001$) were significantly lower in bronchial compared to tracheal tissues (table 1). However, when the different I_{sc} responses were expressed as a fraction of the basal I_{sc} in each tissue, it became apparent that the fractional amiloride-sensitive I_{sc} ($p < 0.01$) as well as the fold-increase in I_{sc} upon cAMP-dependent activation ($p < 0.001$) were significantly higher, whereas the fractional amiloride-insensitive I_{sc} ($p < 0.01$), as well as the fold-increase in I_{sc} in response to UTP-induced activation ($p < 0.05$), were significantly lower in bronchial
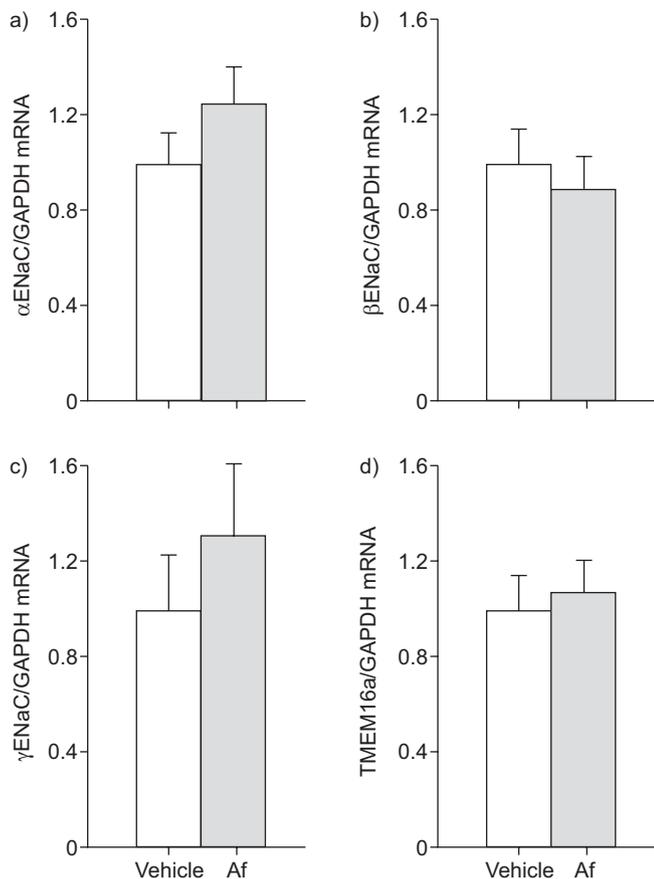
FIGURE 3. Effect of allergic inflammation on expression of epithelial Na$^+$ channel (ENaC) subunits and transmembrane protein (TMEM)16a in native tracheal epithelia. a–d) Transcript levels of a) αENaC, b) βENaC, c) γENaC and d) TMEM16a in freshly excised tracheal tissues from *Aspergillus fumigatus* extract (Af)-sensitised and vehicle-treated mice. Data are expressed as fold changes over vehicle-treated mice. Data are presented as mean±SEM. n=5 mice per group.

versus tracheal tissues. These results indicate that, in comparison to trachea, 1) bronchial tissues are more absorptive under normal conditions, 2) the relative capacity to induce Cl⁻ secretion is greater in bronchi and 3) Cl⁻ secretory responses

are, to a greater extent, elicited by cAMP agonists and, to a lesser extent, by Ca^{2+}-dependent activation in native bronchial *versus* tracheal epithelia.

Next, we determined the effect of allergic inflammation on ion transport in bronchi by comparing bioelectric properties of freshly excised bronchial tissues from Af-sensitised and vehicle-treated mice. Similar to trachea, the basal I_{SC} was not altered by allergen-induced inflammation (fig. 4e). In contrast to tracheal tissues, amiloride-sensitive I_{SC} was significantly inhibited ($p < 0.05$) in freshly excised bronchi from Af-sensitised compared with vehicle-treated mice (fig. 4f). As observed in tracheal tissues, the amiloride-insensitive residual I_{SC} was significantly increased ($p < 0.01$) in bronchi from Af-sensitised mice (fig. 4g). The cAMP-mediated Cl⁻ secretion in bronchial tissues was attenuated by allergic inflammation (fig. 4h), whereas the UTP-induced Cl⁻ secretory response was significantly ($p < 0.05$) increased in native bronchial tissues from Af-sensitised mice compared to vehicle-treated controls (fig. 4i). Taken together, these results demonstrate that inhibition of ENaC-mediated Na$^+$ absorption and up-regulation of Ca^{2+}-activated Cl⁻ secretion by allergic inflammation produced a pro-secretory ion transport phenotype in bronchi (fig. 4).

Th2-mediated modulation of ENaC-mediated Na$^+$ absorption and Ca^{2+}-activated Cl⁻ secretion in bronchi is Stat6-dependent

Previous studies demonstrated that Stat6 plays a central role in the regulation of Th2-mediated allergic airway inflammation in asthma, including airway eosinophilia, mucus hypersecretion and airway hyperresponsiveness [7, 15], but the role of Stat6 signalling in regulation of epithelial ion transport has not been studied. In an attempt to elucidate the mechanisms underlying regulation of airway ion transport by allergic inflammation, we compared responses to Af sensitisation in bronchial tissues from WT and Stat6$^{-/-}$ mice. As shown in figure 4, Stat6 knockout had no effect on basal I_{SC} in the absence or presence of Af-induced inflammation (fig. 4e). However, the inhibition of amiloride-sensitive Na$^+$ absorption that was caused by allergic inflammation in bronchi from WT mice was completely abrogated in Stat6$^{-/-}$ mice (fig. 4f). While the Af-induced upregulation of amiloride-insensitive I_{SC}

TABLE 1	Bioelectric properties of freshly excised native tracheal and bronchial tissues									
	Mice n	Baseline			Amiloride		Post-amiloride		IBMX/Forskolin		UTP	
		V_{te} mV	I_{SC} µA·cm^{-2}	R_{te} Ω·cm^2	ΔI_{SC} µA·cm^{-2}	% basal I_{SC}	I_{SC} µA·cm^{-2}	% basal I_{SC}	ΔI_{SC} µA·cm^{-2}	% basal I_{SC}	ΔI_{SC} µA·cm^{-2}	% basal I_{SC}
Trachea	27	-2.1±0.3	-135.4±15.4	18.6±2.9	103.6±11.2	77.4±1.8	-31.8±5.1	22.6±1.8	-165.2±22.7	124.7±8.6	-204.0±19.5	163.5±13.4
Bronchus	20	-1.1±0.2**	-36.9±4.4***	24.6±3.2	32.7±3.8***	91.8±4.6**	-4.5±1.7***	8.2±4.4**	-85.9±7.6**	267.1±36.0***	-45.8±7.1***	118.1±14.2*

Data are presented as mean±SEM, unless otherwise stated. Summary of basal bioelectric properties (transepithelial voltage (V_{te}), transepithelial resistance (R_{te}) and short-circuit current (I_{SC})) and effects of amiloride, cyclic adenosine 5′-monophosphate-dependent activation and uridine 5′-triphosphate (UTP)-induced activation on transepithelial I_{SC} in freshly excised tracheal and bronchial tissues from vehicle-treated wild-type mice. Responses are expressed as absolute change in I_{SC} (ΔI_{SC}) and as percentage of basal I_{SC} (% I_{SC}). IBMX: 3-isobutyl-1-methylxanthine. *: $p < 0.05$ compared with tracheal tissues; **: $p < 0.01$ compared with tracheal tissues; ***: $p < 0.001$ compared with tracheal tissues.
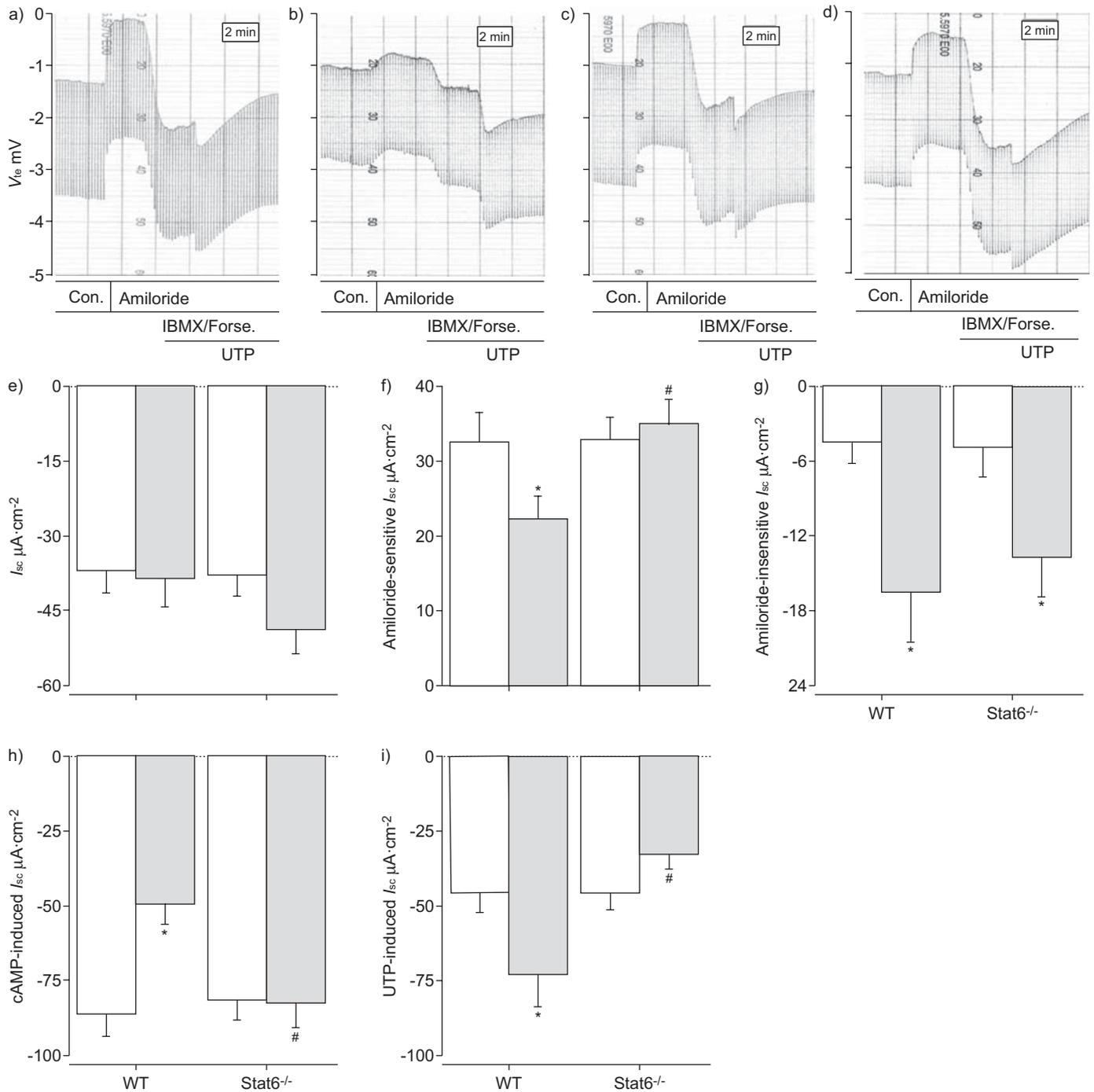
FIGURE 4. Allergic inflammation modulates ion transport in native bronchial epithelia in a signal transducer and activator of transcription (Stat)6-dependent manner. a–d) Original recordings of the effects of amiloride, cyclic adenosine 5'-monophosphate (cAMP)-dependent activation and uridine 5'-triphosphate (UTP)-dependent activation on transepithelial voltage (V_{te}) and transepithelial resistance across freshly excised bronchial tissues from a, c) vehicle-treated and b, d) *Aspergillus fumigatus* (Af) sensitised wild-type (WT) mice (a, b) and Stat6$^{-/-}$ littermates (c, d). Summary of e) basal equivalent short-circuit current (I_{sc}), f) amiloride-sensitive I_{sc}, g) amiloride-insensitive I_{sc}, h) cAMP-induced I_{sc} and i) UTP-induced I_{sc} in freshly excised bronchial tissues from Af-sensitised (■) and vehicle-treated (□) WT mice and Stat6$^{-/-}$ littermates. Data are presented as mean ± SEM (n=18–25 mice per group). Con.: control; IBMX: 3-isobutyl-1-methylxanthine; Forse.: forskotin. *: p<0.05 compared with vehicle-treated mice of the same genotype; #: p<0.005 compared with Af-sensitised WT mice.

observed in WT mice was not affected in Stat6$^{-/-}$ littermates (fig. 4g), both the reduction in the cAMP response, as well as the upregulation of UTP-induced Cl$^-$ secretion observed in bronchial tissues from Af-sensitised WT mice was completely abolished in Stat6$^{-/-}$ mice (figs 4h and i). These results demonstrate that Stat6 signalling is directly involved in regulation of epithelial ion transport in allergic airway disease.

### Stat6 reduces expression of α, β and γENaC in allergic airway inflammation

Because many effects of Stat6 are mediated through regulation of target gene expression [7, 15, 21], we next sought to determine if Stat6-dependent regulation of ion transport by allergic inflammation was associated with changes in transcript levels of ENaC, CFTR and the CaCC candidate TMEM16a in lungs from Af-sensitised mice. These studies were performed in lung tissues, because the yield of mRNA from bronchial tissues was too low for reliable quantification of these genes by real-time RT-PCR. Consistent with the results from ion transport measurements in bronchial tissues (fig. 4f), allergic inflammation caused a significant reduction in expression levels of αENaC ($p < 0.01$), βENaC ($p < 0.01$) and γENaC ($p < 0.05$) subunits in lungs from WT mice (fig. 5a–c). This effect was entirely abolished in Af-sensitised Stat6$^{-/-}$ mice (fig. 5a–c). CFTR and TMEM16a expression remained unchanged in lungs from Af-sensitised, compared with vehicle-treated, WT mice and transcript levels were not altered in Stat6$^{-/-}$ mice in the absence or presence of allergic inflammation (figs 5d and e). These results indicate that Stat6-dependent regulation of ENaC function in allergic airway inflammation was mediated by transcriptional regulation of ENaC genes, whereas Stat6-dependent regulation of cAMP-mediated and UTP-induced Cl$^-$ secretion was independent of transcriptional regulation of CFTR or TMEM16a.

IL-13 mimics effects of allergen-induced inflammation on airway ion transport

To determine more directly the role of Th2 cytokines in modulation of airway ion transport, we treated WT mice by intratracheal instillation of IL-13 and studied the effects on bioelectric properties in freshly excised airway tissues. In bronchi, similar to Af-induced allergic inflammation, IL-13 had no effect on I_{SC} under basal conditions; however, the amiloride-sensitive I_{SC} was significantly reduced ($p < 0.001$) and the residual I_{SC} in the presence of amiloride was significantly increased ($p < 0.01$) in IL-13 treated compared with vehicle-treated mice (fig. 6a–c). Furthermore, effects of IL-13 on the cAMP-induced I_{SC} and UTP-dependent I_{SC} (figs 6d and e) were also similar to Af-sensitisation (fig. 4). In the trachea, IL-13 produced a significant increase in amiloride-insensitive I_{SC} ($p < 0.05$), as observed in the Af model (data not shown). These results suggest that the Th2 cytokine IL-13 plays an important role in modulation of epithelial ion transport in the complex *in vivo* pathogenesis of allergic airway inflammation in mice.
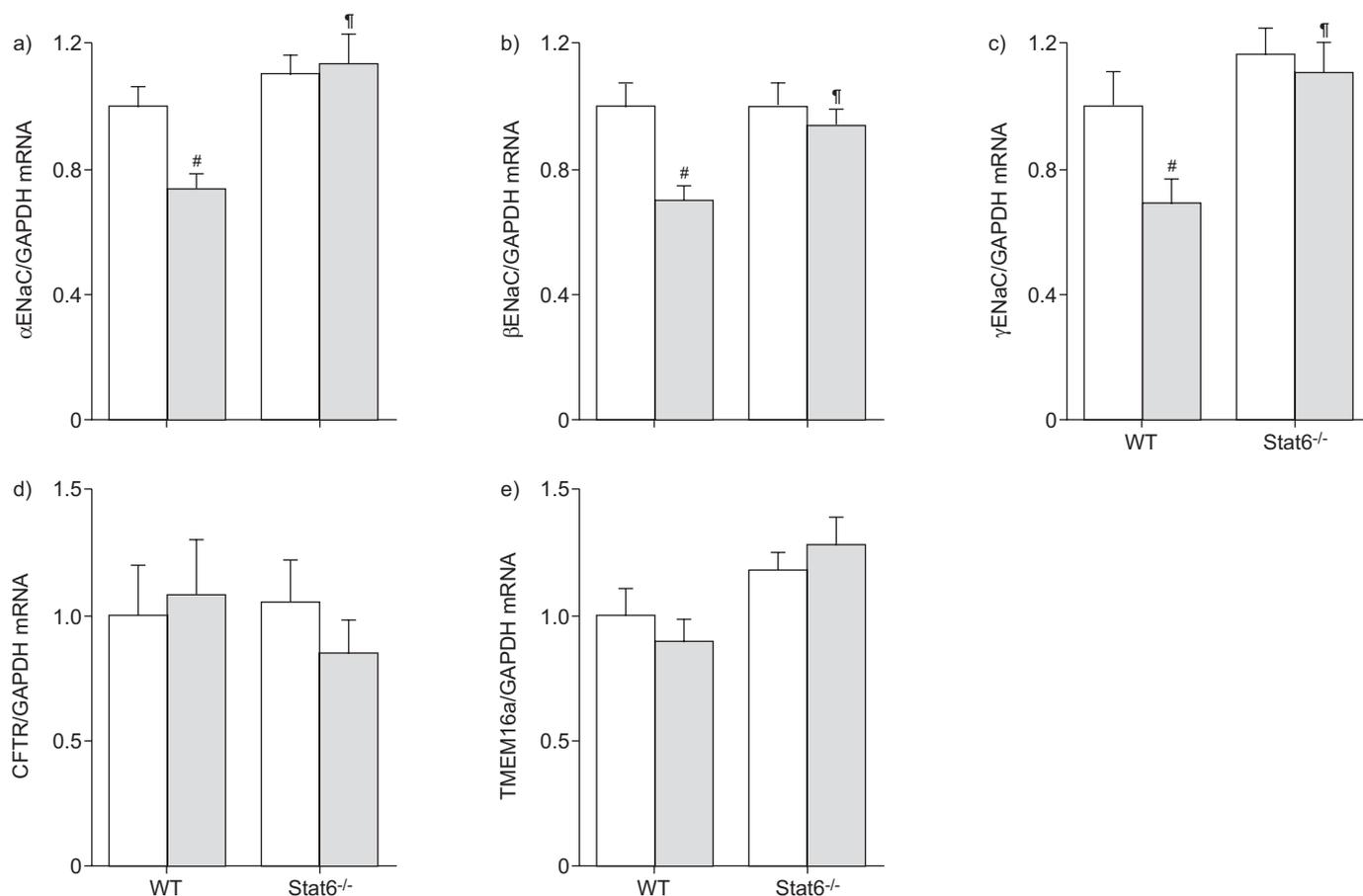
FIGURE 5. Allergic inflammation modulates pulmonary expression of epithelial Na$^+$ channel (ENaC) subunits, but not cystic fibrosis transmembrane conductance regulator (CFTR) and transmembrane protein (TMEM)16a, in a signal transducer and activator of transcription (Stat)6-dependent manner. Transcript levels of a) αENaC, b) βENaC, c) γENaC, d) CFTR and e) TMEM16a in lungs from *Aspergillus fumigatus* extract (Af)-sensitised (■) and vehicle-treated (□) wild-type (WT) mice and Stat6$^{-/-}$ littermates. Data are presented as mean±SEM of fold changes from vehicle-treated WT mice (n=5–6 mice per group). $^{\#}$: $p < 0.05$ compared with vehicle-treated WT mice; ¶: $p < 0.05$ compared with Af-sensitised WT mice.
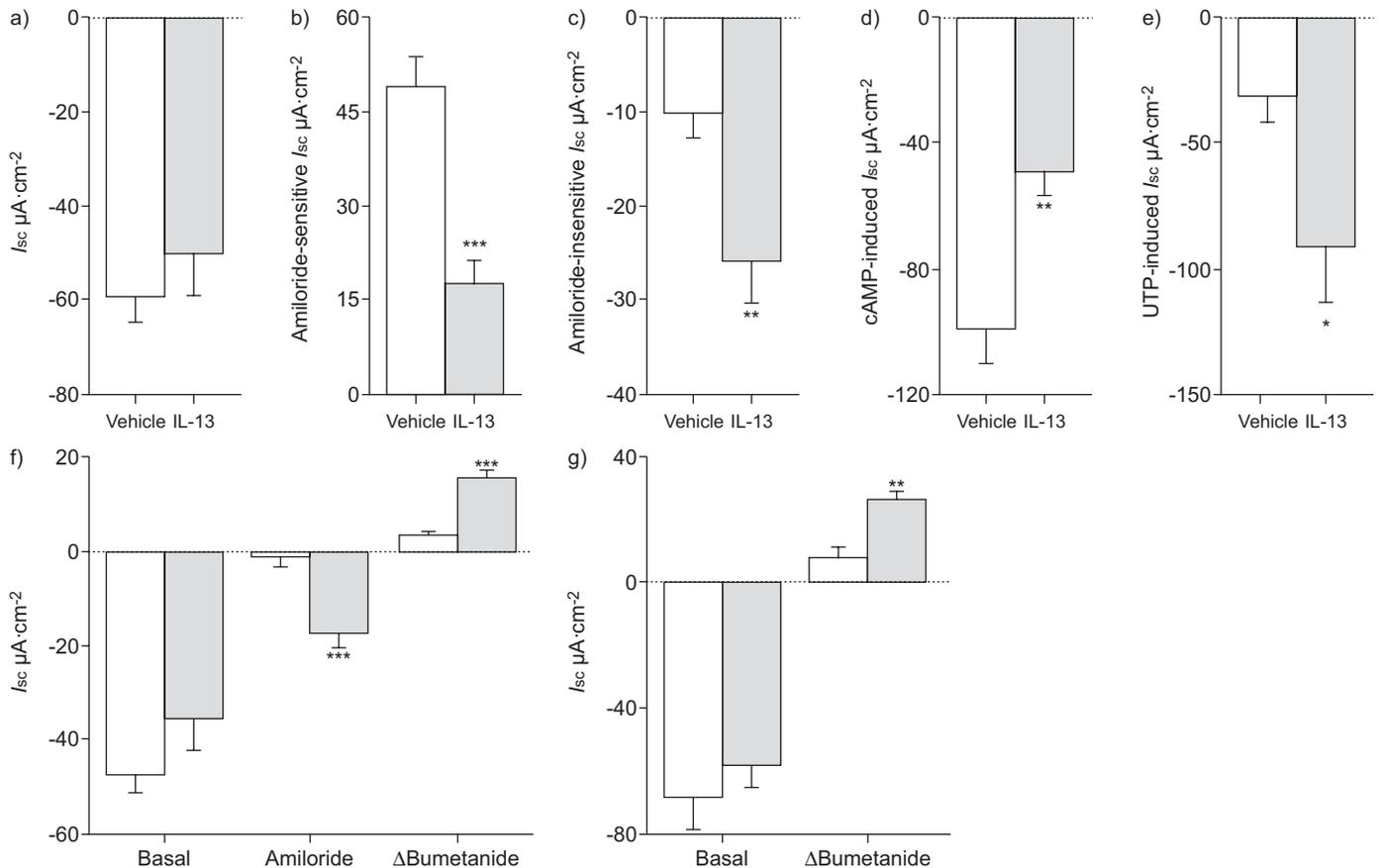
FIGURE 6. Interleukin (IL)-13 mimics effects of allergen-induced inflammation on airway ion transport. Adult wild-type mice were treated with intratracheal instillation of IL-13 (■) or vehicle alone (□) and effects on ion transport were determined in freshly excised bronchial epithelia. Summary of a) basal equivalent short-circuit current (I_{SC}), b) amiloride-sensitive I_{SC}, c) amiloride-insensitive I_{SC}, d) cyclic adenosine 5'-monophospate (cAMP)-induced I_{SC} and e) uridine 5'-triphosphate (UTP)-induced I_{SC} in freshly excised bronchi from IL-13- and vehicle-treated mice. f, g) Effects of T-helper 2-mediated inflammation on transepithelial Cl^- secretion were determined by adding bumetanide under basal conditions and in the presence of amiloride. f) Amiloride-insensitive I_{SC} and bumetanide-sensitive I_{SC} (ΔBumetanide) in the presence of amiloride in bronchi from IL-13-treated *versus* vehicle-treated mice. g) Bumetanide-sensitive I_{SC} in the absence of amiloride in bronchi from IL-13-treated *versus* vehicle-treated mice. Data are presented as mean ± SEM (n=5–8 mice per group). *: $p < 0.05$ compared with vehicle-treated mice; **: $p < 0.01$ compared with vehicle-treated mice; ***: $p < 0.001$ compared with vehicle-treated mice.

Th2-mediated airway inflammation promotes Cl^- secretion

To determine the origin of the increased amiloride-insensitive I_{SC} observed in Th2-mediated airway inflammation, airway tissues were perfused with bumetanide to block transepithelial Cl^- secretion [20]. In the presence of amiloride, the bumetanide-sensitive I_{SC} was significantly increased in bronchial (p<0.001; fig. 6f) and tracheal (p<0.05; data not shown) tissues from IL-13 treated mice compared with vehicle-treated mice. Because amiloride hyperpolarises the apical membrane voltage and may thus generate a driving force for Cl^- secretion that is not present in the absence of ENaC blockade, we next determined the effects of bumetanide under basal conditions. In the absence of amiloride, bumetanide had no effect on basal airway ion transport in vehicle-treated mice, but inhibited basal I_{SC} significantly in bronchi (p<0.01; fig. 6g) and tracheae (p<0.05; data not shown) from IL-13 treated mice. Notably, the bumetanide-sensitive I_{SC} was in the same range in the absence and presence of amiloride (fig. 6f and g). These results indicate that Th2-mediated inflammation induced basal Cl^- secretion, contributing to a pro-secretory ion transport phenotype in allergic airway disease.

DISCUSSION

The results of the present study 1) demonstrate, for the first time, that Th2-mediated allergic airway inflammation in mice, induced by mucosal sensitisation with Af as natural aeroaller-gen or by intratracheal instillation of the Th2 cytokine IL-13, produces modulation of airway epithelial ion transport, in addition to eosinophilic inflammation, goblet cell metaplasia and mucus hypersecretion, and 2) identify alterations in airway ion transport as an additional abnormality in the complex *in vivo* pathogenesis of allergic airway disease.

In the trachea, a tissue commonly used for airway ion transport studies in mice, Af-induced airway inflammation produced a substantial increase in residual I_{SC} in the presence of amiloride block, but had no effects on ENaC-mediated Na^+ absorption or cAMP-dependent and UTP-induced Cl^- secretion (fig. 2). In bronchi, the response to allergic inflammation was more complex (fig. 4). Similar to the trachea, amiloride-insensitive I_{SC} was increased in allergen-sensitised mice. In addition, ENaC-mediated Na^+ absorption and cAMP-induced Cl^- secretion were reduced, whereas CaCC-mediated Cl^- secretion was

increased in bronchi from Af-sensitised mice compared to controls. Intratracheal instillation of IL-13 produced similar changes of airway ion transport, demonstrating that the effects of allergic inflammation were Th2-dependent (fig. 6). Addition of bumetanide, a blocker of the Na^+–K^+–$2Cl^-$ cotransporter that inhibits basolateral Cl^- uptake and transepithelial Cl^- transport, to amiloride pretreated tissues demonstrated that the increase in amiloride-insensitive I_{SC} in Th2-mediated inflammation reflected elevated Cl^- secretion (fig. 6f) [20]. Similarly, the bumetanide-sensitive I_{SC} was also increased in the absence of amiloride, indicating that Th2-mediated airway inflammation increased Cl^- secretion under basal conditions (fig. 6g). The modulation of ion transport induced by allergic inflammation in native bronchial tissues largely resembled the effects previously observed in human bronchial epithelial cells after stimulation with selected Th2 cytokines (IL-4 and IL-13) *in vitro* [8, 9]. Taken together, these results demonstrate that airway ion transport is actively regulated during an allergen-driven, Th2-mediated airway immune response *in vivo*. The net effect of the ~2–3-fold increase in basal Cl^- secretion in both tracheal and bronchial tissues, together with the reciprocal regulation of airway Na^+ absorption and CaCC-mediated Cl^- secretion in bronchi (figs 2, 4 and 6) is a pro-secretory ion transport phenotype that promotes fluid secretion and hydration of airway surfaces in allergic airway inflammation.

Proper regulation of ASL volume by coordinate ENaC-mediated Na^+ absorption and Cl^- secretion, mediated by CFTR and CaCC, plays a critical role in maintaining normal MCC, providing an important defense mechanism of the lung [3, 4]. Previous studies in patients with loss of function mutations in ENaC or CFTR [19, 22–24], and studies in mice with airway-specific overexpression of βENaC (βENaC-Tg) [10, 25] demonstrated that alterations in the balance between airway Na^+ absorption and Cl^- secretion have profound consequences on ASL homeostasis and mucociliary function, and showed that a pro-secretory ion transport phenotype accelerates MCC, whereas hyperabsorptive phenotypes cause mucociliary dysfunction and chronic airway disease. In this context, the results from our present studies predict that the induction of a pro-secretory ion transport phenotype by Th2-driven inflammation improves mucus clearance in the presence of mucus overproduction in allergic airway disease. This hypothesis is supported by a comparison of the pulmonary phenotypes of the βENaC-Tg mouse with mouse models of allergic asthma, as well as recent human studies. First, while airway mucus plugging is an invariant feature of the βENaC-Tg mouse [10, 17], mucus plug formation was not observed in lungs from Af-sensitised mice (fig. 1 and data not shown) or in other asthma mouse models [5–7, 15], despite the presence of striking goblet cell metaplasia and mucus hypersecretion. Secondly, a recent human study demonstrated that MCC was significantly increased in patients with mild, stable allergic asthma compared to healthy controls [26]. These data are consistent with the hypothesis that a coordinate increase in fluid and mucin secretion induced by Th2-driven airway inflammation may provide a protective mechanism to promote mucus clearance and prevent airway mucus plugging in allergic airway diseases such as asthma.

Conversely, MCC is impaired in patients with acute asthma [2, 27]. A recent study demonstrated that excess plasma proteins exudated into the airways of patients with acute asthma inhibit protease-dependent degradation of mucins and suggested that a change in viscoelastic properties may impair airway mucus clearance [28]. Since mucin macromolecules are secreted ''dry'' onto airway surfaces, where they are hydrated by binding water from the ASL [29], we speculate that exuberant mucin secretion may exceed Th2-driven ion transport compensation and cause relative ASL depletion, which may provide an additional mechanism for reduced MCC in acute asthma. Furthermore, we speculate that a reduced capacity to raise fluid secretion in allergic inflammation, *e.g.* by genetic and/or functional variants of ENaC, CFTR or other ion channels involved in airway fluid secretion, may be a hitherto unrecognised risk factor in the development of mucociliary dysfunction and severe airway mucus plugging observed in fatal asthma [30].

Our study also provides new mechanistic insights into the modulation of airway ion transport by allergic inflammation. First, by comparing effects of Af-induced airway inflammation on bioelectric properties in bronchi of WT and Stat6$^{-/-}$ mice, we demonstrate that important components of the pro-secretory phenotype induced by allergic inflammation, *i.e.* ENaC-mediated Na^+ absorption and CaCC-mediated Cl^- secretion, depend on Stat6 signalling (fig. 4), which also plays an important role in other components of allergic airway disease, including eosinophilic inflammation, mucus hypersecretion and airway hyperresponsiveness [7, 15, 21]. Because many Stat6-dependent asthma responses are regulated at the level of target gene expression [15, 21] and because previous studies in cultured human bronchial epithelial cells showed that stimulation with the Th2 cytokine IL-4 had effects on expression of ENaC subunits, CFTR and TMEM16a [9, 12], we compared the effects of allergic airway inflammation on transcript expression levels of these ion channels in airways from WT and Stat6$^{-/-}$ mice. These studies demonstrated that inhibition of ENaC-mediated Na^+ absorption in bronchi from Af-sensitised WT mice was associated with a significant reduction in pulmonary transcript levels of all three ENaC subunits (α, β and γ) and that this effect was Stat6-dependent (fig. 5). Recent evidence suggests that post-translational regulation, *e.g.* by proteolytic cleavage of ENaC by extracellular proteases, plays an important role in controlling Na^+ absorption from airway surfaces [31]. While we did not address possible effects of allergic inflammation on posttranslational regulation, our studies indicate that Stat6-dependent transcriptional regulation of ENaC may provide a mechanism that contributes to inhibition of epithelial Na^+ absorption and switching of the epithelium to a pro-secretory ion transport phenotype in Th2-driven airway inflammation.

In contrast, expression levels of CFTR and TMEM16a were not changed in allergen-sensitized WT and Stat6$^{-/-}$ mice (fig. 5). Given that TMEM16A was recently identified as a CaCC in human bronchial epithelial cell cultures by global gene expression analysis in a search for genes that are differentially regulated by IL-4 [12] and that the CaCC function of its mouse homologue was confirmed in a recent study in TMEM16a$^{-/-}$ mice [13], it was surprising that the Stat6-dependent up-regulation of UTP-induced Cl^- secretion in allergen-sensitised mice was not associated with changes in TMEM16a expression in our study (fig. 5). These results suggest that a CaCC

genetically different from TMEM16a participates in UTP-mediated Cl⁻ secretion. We speculate that the lack of up-regulation of TMEM16a expression, despite a significant increase in UTP-mediated Cl⁻ secretion induced by allergic airway inflammation in our studies (figs 4 and 5), may reflect species differences between expression patterns of different CaCC candidates in human and mouse airway epithelial cells. Alternatively, regulation of TMEM16a expression may differ under cell culture *versus in vivo* conditions [12, 13]. Furthermore, we demonstrate that the increase in basal Cl⁻ secretion constituting an important component of the pro-secretory ion transport phenotype induced by allergic inflammation was Stat6-independent and not associated with changes in transcript levels of CFTR or TMEM16a (figs 4 and 5). Therefore, our results indicate that future studies are required to define the relative roles of TMEM16a and other Cl⁻ channel candidates in the *in vivo* regulation of the pro-secretory ion transport phenotype in allergic airway disease.

A comparison of ion transport properties of bronchi *versus* tracheae in vehicle-treated WT mice (table 1), as well as the different responses to allergen-induced inflammation (figs 2 and 4), demonstrated profound differences in regulation of airway ion transport in these two regions of the tracheobronchial tree. Interestingly, the concept of regional differences in ion and fluid transport in small *versus* large airways is supported by a previous study that compared primary human bronchial with bronchiolar epithelial cell cultures [32]. The regional differences in native tracheal *versus* bronchial epithelium may be related to: 1) differences in expression levels of ENaC subunits and genes contributing to basal and agonist-induced Cl⁻ secretion; 2) differences in local concentrations of mediators, such as Th2 cytokines (Il-4 and Il-13), extracellular nucleotides and nucleosides (ATP and adenosine), which are released in allergic inflammation and act as potent modulators of airway ion transport [8, 9, 33–35]; or 3) local differences in receptor densities, in particular the common IL-4/IL-13 receptor and/or differences in epithelial Stat6 signalling. We predict that further elucidation of the mechanisms underlying these regional differences in basal and regulated airway ion transport may provide novel insights into local (dys)regulation of MCC in response to noxious stimuli in different airway regions of the lung.

In summary, our study shows that Th2-mediated, allergen-driven airway inflammation triggers a pro-secretory ion transport phenotype *in vivo* and indicates that active modulation of airway ion transport is an important effector function in the complex *in vivo* pathogenesis of allergic airway disease. Furthermore, we demonstrate that important components of this response are mediated by epithelial Stat6 signalling. Our results indicate that coordinate increase in fluid and mucin secretion in Th2-driven airway inflammation may provide a protective mechanism to promote mucus clearance and prevent airway mucus plugging in the presence of mucus overproduction in allergic airway disease, and suggest that dysregulation of this epithelial response may provide a clue to severe airway mucus plugging in fatal asthma.

REFERENCES

1 Elias JA, Lee CG, Zheng T, *et al.* New insights into the pathogenesis of asthma. *J Clin Invest* 2003; 111: 291–297.

2 Wanner A, Salathe M, O'Riordan TG. Mucociliary clearance in the airways. *Am J Respir Crit Care Med* 1996; 154: 1868–1902.

3 Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest* 2002; 109: 571–577.

4 Mall MA. Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models. *J Aerosol Med Pulm Drug Deliv* 2008; 21: 13–24.

5 Wills-Karp M, Luyimbazi J, Xu X, *et al.* Interleukin-13: central mediator of allergic asthma. *Science* 1998; 282: 2258–2261.

6 Zhu Z, Homer RJ, Wang Z, *et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999; 103: 779–788.

7 Kuperman DA, Huang X, Koth LL, *et al.* Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002; 8: 885–889.

8 Danahay H, Atherton H, Jones G, *et al.* Interleukin-13 induces a hypersecretory ion transport phenotype in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002; 282: L226–L236.

9 Galietta LJ, Pagesy P, Folli C, *et al.* IL-4 is a potent modulator of ion transport in the human bronchial epithelium *in vitro*. *J Immunol* 2002; 168: 839–845.

10 Mall M, Grubb BR, Harkema JR, *et al.* Increased airway epithelial Na⁺ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med* 2004; 10: 487–493.

11 Anderson MP, Gregory RJ, Thompson S, *et al.* Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science* 1991; 253: 202–205.

12 Caputo A, Caci E, Ferrera L, *et al.* TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science* 2008; 322: 590–594.

13 Rock JR, O'Neal WK, Gabriel SE, *et al.* Transmembrane protein 16A (TMEM16A) is a Ca²⁺-regulated Cl⁻ secretory channel in mouse airways. *J Biol Chem* 2009; 284: 14875–14880.

14 Mehlhop PD, van de RM, Goldberg AB, *et al.* Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc Natl Acad Sci USA* 1997; 94: 1344–1349.

15 Kuperman D, Schofield B, Wills-Karp M, *et al.* Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J Exp Med* 1998; 187: 939–948.

16 Tachdjian R, Mathias C, Al Khatib S, *et al.* Pathogenicity of a disease-associated human IL-4 receptor allele in experimental asthma. *J Exp Med* 2009; 206: 2191–2204.

17 Zhou Z, Treis D, Schubert SC, *et al.* Preventive but not late amiloride therapy reduces morbidity and mortality of lung disease

in βENaC-overexpressing mice. *Am J Respir Crit Care Med* 2008; 178: 1245–1256.

18 Grubb BR, Paradiso AM, Boucher RC. Anomalies in ion transport in CF mouse tracheal epithelium. *Am J Physiol* 1994; 267: C293–C300.

19 Mall M, Bleich M, Greger R, *et al.* The amiloride inhibitable Na$^+$ conductance is reduced by CFTR in normal but not in cystic fibrosis airways. *J Clin Invest* 1998; 102: 15–21.

20 Mall M, Gonska T, Thomas J, *et al.* Activation of ion secretion *via* proteinase-activated receptor-2 in human colon. *Am J Physiol Gastrointest Liver Physiol* 2002; 282: G200–G210.

21 Zimmermann N, Mishra A, King NE, *et al.* Transcript signatures in experimental asthma: identification of STAT6-dependent and -independent pathways. *J Immunol* 2004; 172: 1815–1824.

22 Kerem E, Bistritzer T, Hanukoglu A, *et al.* Pulmonary epithelial sodium-channel dysfunction and excess airway liquid in pseudo-hypoaldosteronism. *N Engl J Med* 1999; 341: 156–162.

23 Matsui H, Grubb BR, Tarran R, *et al.* Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 1998; 95: 1005–1015.

24 Regnis JA, Robinson M, Bailey DL, *et al.* Mucociliary clearance in patients with cystic fibrosis and in normal subjects. *Am J Respir Crit Care Med* 1994; 150: 66–71.

25 Mall MA, Harkema JR, Trojanek JB, *et al.* Development of chronic bronchitis and emphysema in β-epithelial Na$^+$ channel-over-expressing mice. *Am J Respir Crit Care Med* 2008; 177: 730–742.

26 Lay JC, Alexis NE, Zeman KL, *et al. In vivo* uptake of inhaled particles by airway phagocytes is enhanced in patients with mild asthma compared with normal volunteers. *Thorax* 2009; 64: 313–320.

27 Messina MS, O'Riordan TG, Smaldone GC. Changes in mucociliary clearance during acute exacerbations of asthma. *Am Rev Respir Dis* 1991; 143: 993–997.

28 Innes AL, Carrington SD, Thornton DJ, *et al. Ex vivo* sputum analysis reveals impairment of protease-dependent mucus degradation by plasma proteins in acute asthma. *Am J Respir Crit Care Med* 2009; 180: 203–210.

29 Verdugo P. Mucin exocytosis. *Am Rev Respir Dis* 1991; 144: S33–S37.

30 Dunnill MS. The pathology of asthma, with special reference to changes in the bronchial mucosa. *J Clin Pathol* 1960; 13: 27–33.

31 Garcia-Caballero A, Rasmussen JE, Gaillard E, *et al.* SPLUNC1 regulates airway surface liquid volume by protecting ENaC from proteolytic cleavage. *Proc Natl Acad Sci USA* 2009; 106: 11412–11417.

32 Blouquit S, Regnier A, Dannhoffer L, *et al.* Ion and fluid transport properties of small airways in cystic fibrosis. *Am J Respir Crit Care Med* 2006; 174: 299–305.

33 Mall M, Wissner A, Gonska T, *et al.* Inhibition of amiloride-sensitive epithelial Na$^+$ absorption by extracellular nucleotides in human normal and cystic fibrosis airways. *Am J Respir Cell Mol Biol* 2000; 23: 755–761.

34 Rollins BM, Burn M, Coakley RD, *et al.* A2B adenosine receptors regulate the mucus clearance component of the lung's innate defense system. *Am J Respir Cell Mol Biol* 2008; 39: 190–197.

35 Lazarowski ER, Boucher RC. Purinergic receptors in airway epithelia. *Curr Opin Pharmacol* 2009; 9: 262–267.