Asthma is a chronic condition with unknown pathogenesis, and recent evidence suggests that enhanced airway epithelial chloride (Cl\(^-\)) secretion plays a role in the disease. However, the molecular mechanism underlying Cl\(^-\) secretion and its relevance in asthma pathophysiology remain unknown. To determine the role of the solute carrier family 26, member 9 (SLC26A9) Cl\(^-\) channel in asthma, we induced Th2-mediated inflammation via IL-13 treatment in wild-type and Slc26a9-deficient mice and compared the effects on airway ion transport, morphology, and mucus content. We found that IL-13 treatment increased Cl\(^-\) secretion in the airways of wild-type but not Slc26a9-deficient mice. While IL-13–induced mucus overproduction was similar in both strains, treated Slc26a9-deficient mice exhibited airway mucus obstruction, which did not occur in wild-type controls. In a study involving healthy children and asthmatics, a polymorphism in the 3' UTR of SLC26A9 that reduced protein expression in vitro was associated with asthma. Our data demonstrate that the SLC26A9 Cl\(^-\) channel is activated in airway inflammation and suggest that SLC26A9-mediated Cl\(^-\) secretion is essential for preventing airway obstruction in allergic airway disease. These results indicate that SLC26A9 may serve as a therapeutic target for airway diseases associated with mucus plugging.
SLC26A9-mediated chloride secretion prevents mucus obstruction in airway inflammation

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Introduction

The clinical syndrome of asthma has evolved as one of the most common chronic diseases in children and adults, but its pathogenesis remains incompletely understood. Th2-mediated inflammation triggered by inhaled allergens plays an important role in mucus hypersecretion, airway remodeling, and airway hyperresponsiveness contributing to airflow limitation in asthma, and the airway epithelium is a central effector tissue in this process (1, 2). Recent studies demonstrated that Th2-dependent airway inflammation produces a pro-secretory airway epithelial ion transport phenotype characterized by upregulation of Cl– secretion and inhibition of Na+ absorption in mouse airways, and have identified active modulation of airway ion transport as an additional epithelial response in the complex in vivo pathogenesis of asthma (3). Proper hydration of airway surfaces by coordinate regulation of absorption and secretion of ions and fluid by the airway epithelium plays a critical role in maintaining normal mucociliary clearance (MCC) (4, 5). Because mucin macromolecules are secreted in a condensed dehydrated form into the airway surface liquid (ASL), where they are hydrated to form mucin gels by binding of free water (6), we hypothesized that upregulation of fluid secretion may be required for maintenance of ASL homeostasis and prevention of mucus obstruction in the presence of mucin hypersecretion (3). However, expression of Cfr and Tmem16a, constituting the main molecular determinants of cAMP-regulated and Ca2+-activated Cl– secretion in airway epithelia under physiological conditions (7), was not altered in mouse models of allergic asthma (3), and the molecular identity and pathophysiological relevance of the Cl– conductance underlying increased epithelial Cl– secretion in allergic airway disease remain unknown.

SLC26A9 (solute carrier family 26, member 9) is a member of the SLC26 family of anion transporters predominantly expressed in epithelia of the lung and upper GI tract (8, 9). Recent studies showed that the SLC26A9 protein functions as a Cl– channel with minimal HCO3– conductance that is regulated by CFTR and WNK kinases in heterologous cells (10–13). Further, SLC26A9 contributes to constitutive and cAMP-dependent Cl– secretion in human bronchial epithelial (HBE) cells, where it has been suggested to interact functionally with CFTR in vitro (11, 14). Based on its functional properties in transduced cells and expression pattern in human and mouse airways (8, 15), we hypothesized that SLC26A9 may function as an alternative Cl– channel that may contribute to ASL homeostasis in health and allergic airway disease. To test this hypothesis, we compared transepithelial ion transport in freshly excised bronchial tissues, lung morphology, and airway mucus content in wild-type versus Slc26a9-deficient (Slc26a9–/–) mice under physiological conditions and after intratracheal instillation of IL-13 to model Th2-mediated airway inflammation. Further, in a large human study population of 658 healthy children and 661 children with asthma, we tested whether polymorphisms in

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the SLC26A9 gene are associated with asthma and determined the functional relevance of a select SNP (rs2282430) in the 3′ UTR of SLC26A9 in luciferase gene reporter assays. Our studies demonstrate that SLC26A9-mediated Cl⁻ secretion is activated and prevents airway mucus obstruction in allergic airway disease. These results suggest SLC26A9 as a potential disease modifier and novel therapeutic target in asthma and possibly other diseases caused by deficient ASL hydration, such as CF.

Results and Discussion
SLC26A9 does not contribute to airway Cl⁻ secretion and is not essential for lung health under physiological conditions. We first compared lung morphology and ion transport properties in native bronchial tissues from naive wild-type and Slc26a9⁻/⁻ mice. Consistent with previous immunolocalization studies (8, 15), Slc26a9 mRNA expression was detected by RT-PCR in bronchi from naive wild-type mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI60429DS1). Morphological evaluation demonstrated that the conducting airways and alveolar airspaces were structurally normal and that lungs of Slc26a9⁻/⁻ mice did not show evidence of spontaneous lung disease (Figure 1, A and B).

To determine whether SLC26A9 contributed to constitutive Cl⁻ secretion in native airway epithelia, as previously described for cultured HBE cells (11), we first compared bioelectric properties of freshly excised bronchial tissues from wild-type and Slc26a9⁻/⁻ mice under HCO₃⁻-free conditions. These analyses demonstrated that transepithelial voltage (Vₑ), transepithelial resistance (Rₑ), and short circuit current (Iₑ) were not different in wild-type versus Slc26a9⁻/⁻ mice (Figure 1, C–E). Further, amiloride-sensitive Na⁺ absorption mediated by epithelial Na⁺ channels (ENaCs) and amiloride-insensitive Iₑ reflecting constitutive Cl⁻ secretion in the presence of amiloride block (3) were normal in bronchial epithelia from Slc26a9⁻/⁻ mice (Figure 1, F and G). The amiloride-insensitive Iₑ was not inhibited by CFTR inh-172, but was largely abolished by bumetanide in both wild-type and Slc26a9⁻/⁻ mice (Figure 1G).

Because previous studies showed that SLC26A9 stimulated cAMP/PKA-regulated CFTR Cl⁻ conductance in HBE cell lines (14), we next compared the magnitude of cAMP-induced Cl⁻ secretion and effects of CFTR inh-172 in airseways from wild-type and Slc26a9⁻/⁻ mice. In wild-type bronchi, cAMP-mediated stimulation induced a sustained Cl⁻ secretory response that was significantly inhibited by CFTR inh-172 (Figure 1H). In contrast to previous results in cultured HBE cells transduced with SLC26A9 (14), our studies in native bronchial epithelia detected no differences in either cAMP-induced Iₑ or inhibition by CFTR inh-172 in tissues from Slc26a9⁻/⁻ compared with wild-type mice (Figure 1H).
insensitive $I_{sc}$ was completely inhibited by bumetanide in tissues from both wild-type and $Slc26a9^{-/-}$ mice (Figure 1H). To determine, whether SLC26A9-mediated Cl– transport and functional interaction with CFTR required the presence of HCO$_3$– (10, 11, 14), we compared bioelectric properties in wild-type and $Slc26a9^{-/-}$ bronchi in the absence and presence of HCO$_3$–. HCO$_3$– had no effect on basal $I_{sc}$, constitutive Cl– secretion (amiloride-insensitive $I_{sc}$), and, as in HCO$_3$–-free conditions, constitutive and cAMP-dependent Cl– secretion were not different in wild-type compared with $Slc26a9^{-/-}$ tissues (Supplemental Figure 2). This lack of functional interaction between SLC26A9 and CFTR in native mouse airway epithelia may reflect species-dependent differences or differences in regulation of SLC26A9 and CFTR function in native airway epithelia versus transduced cell lines (14). Finally, we tested a potential contribution of SLC26A9 to Ca$^{2+}$-activated Cl– conductance (CaCC) by comparing UTP-induced Cl– secretory responses in bronchi from wild-type and $Slc26a9^{-/-}$ mice. As expected, Ca$^{2+}$-dependent activation induced a transient Cl– secretory response that was not different in wild-type versus $Slc26a9^{-/-}$ bronchi (Figure 1I). Taken together, these results argue against a role of SLC26A9 Cl– channels in constitutive or agonist-induced (cAMP-mediated and Ca$^{2+}$-activated) Cl– secretion in native airway tissues and indicate that SLC26A9 function is not essential for lung health under physiological conditions in vivo.

SLC26A9 constitutes an epithelial Cl– conductance induced in Th2-mediated airway inflammation. Our previous studies demonstrated that Th2-mediated airway inflammation induces a substantial (~2- to 3-fold) increase in constitutive and Ca$^{2+}$-activated Cl– secretion without changing expression levels of CFTR or TMEM16A in mouse airways (3). To test the hypothesis that SLC26A9 Cl– channels may be activated and contribute to enhanced Cl– secretion in allergic airway disease, we induced inflammation by intratra-

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Figure 2
SLC26A9 determines constitutive Cl– secretion induced in Th2-mediated airway inflammation. (A) Original recordings of effects of amiloride, cAMP-mediated (IBMX/forskolin [Fors]) activation, and UTP on $V_{m}$ and $R_{ml}$ across freshly excised bronchial tissues from vehicle- and IL-13–treated wild-type and $Slc26a9^{-/-}$ mice. Ctrl, control. (B–F) Summary of basal $I_{sc}$ (B), amiloride-sensitive $I_{sc}$ (C), amiloride-insensitive $I_{sc}$ (D), cAMP-induced $I_{sc}$ (E), and UTP-induced $I_{sc}$ (F) in bronchi from vehicle-treated and IL-13–treated wild-type and $Slc26a9^{-/-}$ mice. (G) Summary of bumetanide-sensitive $I_{sc}$ in amiloride-pretreated tissues from vehicle- and IL-13–treated wild-type and $Slc26a9^{-/-}$ mice (G). $n = 8–11$ mice per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle-treated mice of the same genotype. †$P < 0.05$, ‡$P < 0.01$ compared with IL-13–treated wild-type mice.
cheal application of the key Th2 signaling molecule IL-13 (1) and compared effects on airway ion transport in wild-type and Slc26a9−/− mice (Figure 2). Treatment with IL-13 had no effect on R_{te} but reduced ENaC-mediated Na+ absorption and produced a significant increase in amiloride-insensitive I_{sc} as well as Ca2+-activated Cl− secretion, as expected (ref. 3 and Figure 2, A–F). In bronchi from Slc26a9−/− mice, IL-13 treatment resulted in significantly reduced basal I_{sc} compared with tissues from vehicle-treated Slc26a9−/− mice and wild-type mice of both treatment groups (Figure 2, A and B). Inhibition of basal I_{sc} was not due to differences in ENaC-mediated Na+ absorption, because IL-13 treatment reduced amiloride-sensitive ENaC currents to similar levels in bronchi from Slc26a9−/− and wild-type mice (Figure 2C). However, in contrast to wild-type tissues, bronchi from IL-13-treated Slc26a9−/− mice failed to exhibit increased amiloride-insensitive I_{sc} (Figure 2D). As in bronchi from naïve mice (Figure 1G), the amiloride-insensitive I_{sc} was completely inhibited by bumetanide in tissues from vehicle- and IL-13–treated wild-type and Slc26a9−/− mice (data not shown). Of note, the bumetanide-sensitive I_{sc} was significantly increased by IL-13 in wild-type tissues, but remained unchanged in tissues from Slc26a9−/− mice (Figure 2G). Effects of IL-13 on ion transport in wild-type and Slc26a9−/− bronchi were not different when tissues were studied in the absence or presence of HCO3− (Supplemental Figure 2). A similar bioelectric pattern with abrogation of IL-13–induced constitutive Cl− conductance was also observed in native tracheal tissue from Slc26a9−/− mice (data not shown), demonstrating that Th2-mediated inflammation induced SLC26A9-dependent Cl− secretion in different regions of the conducting airways. In contrast to effects on constitutive Cl− secretion, IL-13–induced upregulation of CaCC activity did not differ in Slc26a9−/− compared with wild-type airways (Figure 2F).

Taken together, these results demonstrate that SLC26A9 constitutes the Cl− channel responsible for increased constitutive Cl− secretion in Th2-mediated inflammation, and link SLC26A9 function to allergic airway disease. On the other hand, our data indicate that SLC26A9 is not implicated in Th2-mediated upregulation of Ca2+-activated Cl− secretion (Figure 2G). Of note, we previously showed that upregulation of Ca2+-activated Cl− secretion in allergic airway disease is mediated by STAT6 signaling, whereas the increase in constitutive Cl− secretion is STAT6 independent (3), indicating that SLC26A9 and CaCC are regulated by distinct mechanisms. In our present study, expression analyses by semi-quantitative real-time RT-PCR demonstrated that increased SLC26A9 Cl− channel function did not correlate with transcript levels in native airway tissues from IL-13–treated wild-type mice (Supplemental Figure 1), suggesting that SLC26A9 Cl− conductance was regulated on the post-transcriptional level. In this context, it is noteworthy that the activity of SLC26A9, similar to several other ion channels and transporters implicated in electrolyte homeostasis in Cl−-handling epithelia, is inhibited by WNK4 and other WNK kinases via inhibition of SLC26A9 protein expression at the cell surface (13). Given that WNK4 is expressed in the lung (16), we speculate that WNKs may inhibit SLC26A9 in airway epithelia under physiological conditions and act as a molecular switch to activate SLC26A9-mediated Cl− secretion in response to stimuli released during allergic airway inflammation.
SLC26A9-mediated Cl− secretion prevents mucus obstruction in Th2-mediated airway inflammation. To determine the functional consequences of SLC26A9-mediated Cl− secretion on mucus homeostasis in allergic airway disease, we next compared the airway mucus phenotype in naive and IL-13–treated wild-type and Slc26a9−/− mice (Figure 3). Bronchi from IL-13–treated wild-type mice exhibited goblet cell metaplasia with increased epithelial mucin production and epithelial hypertrophy, as expected (Figure 3, A–C, and Supplemental Figure 3), indicating that SLC26A9 was not involved in epithelial remodeling and mucin overproduction caused by Th2-mediated inflammation. Consistent with previous studies in mouse models of asthma (4), accumulation of mucus in the airway lumen was rarely observed (Figure 3D), and morphometric analyses identified only small amounts of intraluminal mucus in airways from IL-13–treated wild-type mice (Figure 3E). In contrast, airway mucus obstruction was observed in the majority of IL-13–treated Slc26a9−/− mice (Figure 3F). Further, airway morphometry demonstrated that airway mucus content was significantly increased in IL-13–treated Slc26a9−/− compared with wild-type mice (Figure 3E). When viewed in combination with the substantial airway mucus obstruction, which remains difficult to treat, responds poorly to current anti-asthmatic drugs such as bronchodilators and antiinflammatory agents, and continues to contribute to asthma-related deaths (18). In this context, it is noteworthy that upregulation of SLC26A9 as a potential disease modifier and novel therapeutic target, especially in asthma phenotypes associated with hypersecretion, may also serve as a novel therapeutic target to counteract impaired epithelial Cl− secretion in CF.

Genetic variants in SLC26A9 are associated with reduced protein expression and increased risk for asthma. To investigate whether SLC26A9 may also play a role in human asthma, we analyzed 661 children with asthma and 658 healthy subjects by chip genotyping and imputation (17) for effects of genetic variants within the SLC26A9 locus on asthma risk (Supplemental Figure 4 and Supplemental Table 1). We identified several SNPs in the 3′ UTR of SLC26A9 (rs12031234, rs2282429, rs2282430) that were associated with asthma in this population (highest odds ratio, 1.48; 95% confidence interval [CI], 1.1–2.0; P = 0.012). In silico analyses predicted that the A allele of rs2282430 (SNP) strengthens binding of has-miR-632 to the SLC26A9 3′ UTR (Figure 4A). In luciferase reporter assays in A549 cells, known to express SLC26A9 endogenously (8), luciferase activity was significantly reduced in cells transfected with the polymorphic compared with the wild-type 3′ UTR (P = 0.02) (Figure 4B). Further, studies in HEK293 cells demonstrated that transfection of precursor has-miR-632 reduced luciferase activity significantly in cells co-transfected with the SNPs compared with the wild-type 3′ UTR (P < 0.05) (Figure 4C). Taken together, these results suggest that the SNP in the SLC26A9 3′ UTR identified by our genetic analyses reduces protein expression, indicate that binding of has-miR-632 is involved in this process, and provide initial evidence that SLC26A9 may be involved in asthma pathogenesis in humans.
brief report

Methods
Mice. Experiments were performed with 8- to 20-week-old Slc26a9−/− mice and wild-type littermates on the 129/SVJ background (9).

IL-13 instillation. IL-13 was instilled intratracheally as previously described (3) and detailed in Supplemental Methods.

Histology and airway morphometry. See Supplemental Methods for details.

Electrogenic ion transport measurements. See Supplemental Methods for details.

Real time RT-PCR. See Supplemental Methods for details.

Genetic association studies. Asthma patients from the Multicenter Asthma Genetics in Childhood (MAGIC) study were compared with randomly selected children from the cross-sectional International Study of Asthma and Allergies in Childhood, phase II (ISAAC II) study (17). A total of 1,319 subjects (661 with asthma) were analyzed in this study as described in Supplemental Methods.

Cloning of SLC26A9 3′ UTR and luciferase gene reporter assays. See Supplemental Methods for details.

Statistics. Data were analyzed with SigmaStat version 3.1 (Systat Software) and are reported as mean ± SEM. We performed statistical analyses using 2-tailed Student’s t test, Mann-Whitney rank-sum test, 1-way ANOVA, and Fisher exact test as appropriate, and P < 0.05 was accepted to indicate statistical significance.

Study approval. All animal studies were approved by the local animal welfare authorities responsible for the Hannover Medical School (Regierungspräsidium Oldenburg, Niedersachsen) and the University of Heidelberg (Regierungspräsidium Karlsruhe, Baden-Württemberg). Protocols of the MAGIC and ISAAC studies (17) were approved by the ethics committees of the participating centers (University Children’s Hospital, Ludwig Maximilian University, Munich; Department of Experimental Pneumology, Ruhr-University Bochum; University Children’s Hospital, University of Cologne; Children’s Department, Marien-Hospital, Wesel; University Children’s Hospital, Albert Ludwig University, Freiburg; Children’s Department, Feldkirch Hospital; University Children’s Hospital Vienna), and written informed consent was obtained from the parents of all children.

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