

Now offering human  
immune cells.

Same day collection, purification,  
and cryopreservation.

charles river



## Thiocyanate Transport in Resting and IL-4-Stimulated Human Bronchial Epithelial Cells: Role of Pendrin and Anion Channels

This information is current as of October 2, 2022.

Nicoletta Pedemonte, Emanuela Caci, Elvira Sondo, Antonella Caputo, Kerry Rhoden, Ulrich Pfeffer, Michele Di Candia, Roberto Bandettini, Roberto Ravazzolo, Olga Zegarra-Moran and Luis J. V. Galietta

*J Immunol* 2007; 178:5144-5153; ;  
doi: 10.4049/jimmunol.178.8.5144  
<http://www.jimmunol.org/content/178/8/5144>

**References** This article cites **53 articles**, 17 of which you can access for free at:  
<http://www.jimmunol.org/content/178/8/5144.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days\*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*\*average*

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:  
<http://jimmunol.org/subscription>

**Permissions** Submit copyright permission requests at:  
<http://www.aai.org/About/Publications/JI/copyright.html>

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:  
<http://jimmunol.org/alerts>

*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
1451 Rockville Pike, Suite 650, Rockville, MD 20852  
Copyright © 2007 by The American Association of  
Immunologists All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Thiocyanate Transport in Resting and IL-4-Stimulated Human Bronchial Epithelial Cells: Role of Pendrin and Anion Channels<sup>1</sup>

Nicoletta Pedemonte,<sup>\*†</sup> Emanuela Caci,<sup>\*</sup> Elvira Sondo,<sup>\*</sup> Antonella Caputo,<sup>\*</sup> Kerry Rhoden,<sup>‡</sup> Ulrich Pfeiffer,<sup>§</sup> Michele Di Candia,<sup>†</sup> Roberto Bandettini,<sup>¶</sup> Roberto Ravazzolo,<sup>\*</sup> Olga Zegarra-Moran,<sup>\*</sup> and Luis J. V. Galietta<sup>2\*†</sup>

SCN<sup>-</sup> (thiocyanate) is an important physiological anion involved in innate defense of mucosal surfaces. SCN<sup>-</sup> is oxidized by H<sub>2</sub>O<sub>2</sub>, a reaction catalyzed by lactoperoxidase, to produce OSCN<sup>-</sup> (hypothiocyanite), a molecule with antimicrobial activity. Given the importance of the availability of SCN<sup>-</sup> in the airway surface fluid, we studied transepithelial SCN<sup>-</sup> transport in the human bronchial epithelium. We found evidence for at least three mechanisms for basolateral to apical SCN<sup>-</sup> flux. cAMP and Ca<sup>2+</sup> regulatory pathways controlled SCN<sup>-</sup> transport through cystic fibrosis transmembrane conductance regulator and Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels, respectively, the latter mechanism being significantly increased by treatment with IL-4. Stimulation with IL-4 also induced the strong up-regulation of an electroneutral SCN<sup>-</sup>/Cl<sup>-</sup> exchange. Global gene expression analysis with microarrays and functional studies indicated pendrin (SLC26A4) as the protein responsible for this SCN<sup>-</sup> transport. Measurements of H<sub>2</sub>O<sub>2</sub> production at the apical surface of bronchial cells indicated that the extent of SCN<sup>-</sup> transport is important to modulate the conversion of this oxidant molecule by the lactoperoxidase system. Our studies indicate that the human bronchial epithelium expresses various SCN<sup>-</sup> transport mechanisms under resting and stimulated conditions. Defects in SCN<sup>-</sup> transport in the airways may be responsible for susceptibility to infections and/or decreased ability to scavenge oxidants. *The Journal of Immunology*, 2007, 178: 5144–5153.

Transport of anions like Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> is important in the airway epithelium to control electrolyte and fluid secretion and to regulate pH (1–3). In particular, Cl<sup>-</sup> transport is recognized as one of the important factors in the regulation of airway surface hydration and therefore in mucociliary clearance (4). Transepithelial anion transport is mediated by a series of channels and transporters which operate in coordination with membrane proteins devoted to cation transport. In cystic fibrosis (CF),<sup>3</sup> the function of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl<sup>-</sup> channel is severely reduced thus causing a deficit in anion permeability (5). In the airways, this defect, together with the dysregulation of the Na<sup>+</sup> channel ENaC (6), causes an impairment of mucociliary clearance and of other antimicrobial mechanisms favoring the colonization of the lungs by antibiotic-resistant

bacteria (4, 5). Anion transport in epithelial cells may be mediated by other membrane proteins in addition to CFTR (7). The role and molecular identity of such proteins is only partially clear.

Anion transport is regulated by acute and chronic stimuli. Acute activation of Cl<sup>-</sup> transport is triggered by signals that elevate intracellular cAMP or Ca<sup>2+</sup> (2). The increase of cAMP activates CFTR through phosphorylation of its R domain (8). In contrast, Ca<sup>2+</sup> elevation causes the activation of Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels, a different class of membrane proteins whose identity is controversial (7). Chronic regulation seems also important as demonstrated by experiments on cultured cells where treatment with IL-4/IL-13 causes an up-regulation of Cl<sup>-</sup> transport, particularly of the Ca<sup>2+</sup>-dependent component (9, 10).

It has been recently postulated that SCN<sup>-</sup> (thiocyanate) transport may also play a very important role in the pathophysiology of the airway epithelium (11). SCN<sup>-</sup> is a pseudohalide normally present in the blood at 30–100 μM and secreted in saliva and milk at concentrations up to 0.5–1.6 mM (12–14). In such fluids, SCN<sup>-</sup> has an antimicrobial role (15–17). Indeed, it is oxidized by H<sub>2</sub>O<sub>2</sub>, a reaction catalyzed by lactoperoxidase, to produce OSCN<sup>-</sup> (hypothiocyanite), a molecule with bactericidal or bacteriostatic activity (18). Recent studies have shown that: 1) lactoperoxidase is secreted on the airway surface (19, 20); 2) the airway epithelium has the ability to generate H<sub>2</sub>O<sub>2</sub> at the apical membrane through dual oxidases 1 and 2 (DUOX1 and DUOX2) (21–23); 3) polarized preparations of airway epithelium transport SCN<sup>-</sup> from the basolateral to the apical side in a way that involves CFTR (11); CF cells show a defective bacterial killing due to lack of SCN<sup>-</sup> transport (24). Such observations suggest that the lactoperoxidase/H<sub>2</sub>O<sub>2</sub>/SCN<sup>-</sup> antimicrobial system is present also in the airways where it could play an important role in innate defense function. They also imply that CFTR dysfunction in CF could favor bacterial colonization

\*Laboratory of Molecular Genetics, Istituto Giannina Gaslini, Genova, Italy; †Advanced Biotechnology Center, Genova, Italy; ‡Unita Operativa Medical Genetics, Policlinico Orsola-Malpighi, Bologna, Italy; §Functional Genomics, National Cancer Research Institute, Genova, Italy; and ¶Laboratorio Centrale di Analisi, Istituto Giannina Gaslini, Genova, Italy

Received for publication September 12, 2006. Accepted for publication February 1, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by grants from Telethon-Italy (GGP05103), the Cystic Fibrosis Foundation Therapeutics, Comitato Interministeriale Programmazione Economica Regione Liguria (Biofarma 2), and the National Institutes of Health (P30 DK072517).

<sup>2</sup> Address correspondence and reprint requests to Dr. Luis J. V. Galietta, Laboratorio di Genetica Molecolare, L.go Gerolamo Gaslini 5, 16147 Genova, Italy. E-mail address: galietta@unige.it

<sup>3</sup> Abbreviations used in this paper: CF, cystic fibrosis; FRT, Fischer rat thyroid; siRNA, short-interfering RNA; YFP, yellow fluorescent protein; CFTR, cystic fibrosis transmembrane conductance regulator; PDS, pendrin.

by causing a deficit in  $SCN^-$  transport and that other anion channels and transporters may compensate this deficit by providing an alternate route for  $SCN^-$ .

In our study, we investigated the mechanisms responsible for  $SCN^-$  transport in human bronchial epithelial cells under resting and cytokine-stimulated conditions to assess the involvement of CFTR and that of other transport mechanisms. We have found that bronchial epithelial cells possess at least three mechanisms for  $SCN^-$  transport. Particularly, interesting is the finding that IL-4 causes a dramatic up-regulation of an electroneutral transport for  $Cl^-$  and  $SCN^-$ . Global analysis of gene expression by microarrays and functional studies indicate that this  $SCN^-$  transport is mediated by pendrin (25), an anion transporter whose expression/function in the respiratory system has not been previously reported. Our findings indicate a novel function for pendrin and anion channels in the innate defense of mucosal surfaces.

## Materials and Methods

### Culture of Fischer rat thyroid (FRT) and bronchial epithelial cells

FRT cells were cultured in Coon's-modified F12 culture medium supplemented with 10% FCS. Human bronchial epithelial cells were collected and cultured as previously described (26). Briefly, dissected bronchi were placed overnight at 4°C in a Hanks' solution containing protease XIV. The bronchi were then gently removed from the protease solution, and epithelial cells were collected by flushing energetically the bronchial lumen with Hanks' solution. Detached epithelial cell layers were pelleted by centrifugation and resuspended in a small volume of saline solution containing trypsin. After 5–10 min at 37°C, single cells were dissociated by repeated pipetting. After this step, trypsin was neutralized with a culture medium containing serum. Cells were then pelleted by centrifugation and plated in culture flasks in a serum-free culture medium consisting in a 1:1 mixture of LHC9 and RPMI 1640 (26). After two to five passages, cells were frozen in several aliquots. When needed, a single aliquot of bronchial cells was thawed and cultured for 1–2 more passages in the serum-free medium. At the end, the cells were plated at high density (500,000 cells/cm<sup>2</sup>) on Snapwell (12-mm diameter) or Transwell (24.5-mm diameter) porous supports (code 3801 and 3450, respectively; Corning Costar). After 24 h, the serum-free medium was replaced with an enriched mixture containing DMEM/F12 (1:1) plus 2% FCS and various hormones and supplements (26). Generation of differentiated epithelia was checked by measuring daily transepithelial electrical resistance and potential difference with an epithelial volt ohmmeter (World Precision Instruments). Experiments were usually done at 10–14 days after plating, when epithelial resistance and potential difference were 1.5–2.5 k $\Omega$  · cm<sup>2</sup> and –40 to –50 mV, respectively.

### Gene expression analysis by microarrays

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) followed by purification with RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed using T7-(dT)<sub>24</sub> oligonucleotide primers and the Custom SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen Life Technologies). Double-stranded cDNAs were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), ethanol precipitated, and used to prepare cRNAs using the Bioarray High Yield RNA Transcription kit (Affymetrix) according to the manufacturer's instructions. cRNAs were purified using the RNeasy Mini kit (Qiagen), controlled by agarose gel electrophoresis and RNA 6000 Pico Assay (Agilent Technologies) and subjected to fragmentation for 35 min at 94°C in fragmentation buffer (40 mM Tris-acetate (pH 8.1), 100 mM CH<sub>3</sub>COOH, 30 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>).

Labeled cRNA was used for hybridization of GeneChip Human Genome U133 plus 2 arrays (Affymetrix). The experiment consisted of four biological replicates (different donors) for control and IL-4-treated cells. Hybridization and scanning was conducted on the Affymetrix platform. Data were normalized following the guanine cytosine robust multiarray average procedure (27) of Bioconductor 1.8 (28) (www.bioconductor.org). Normalization was set to "invariant.set." Statistically significant expression changes were determined using permutation tests, significance analysis of microarrays (29) (www-stat.stanford.edu/~tibs/SAM/) considering treated and untreated cells from the same donor as a pair. Genes regulated at least 2-fold in comparison to untreated controls were considered. The  $\delta$  value was set to return a median false significant number <1. Annotations were

obtained through the DAVID database (<http://david.niaid.nih.gov/david/b/index.htm>) (30).

### Gene expression analysis by RT-PCR

For real-time quantitative PCR, 1  $\mu$ g of total RNA was retrotranscribed with both random hexamer and oligo(dT) primers using the Advantage RT-for-PCR kit (BD Clontech). Quantitation of transcripts for desired genes was conducted using inventoried Assays-on-Demand provided by Applied Biosystems (specifically: Hs001166504\_m1 for pendrin, and Hs00187842\_m1 for  $\beta_2$ -microglobulin as endogenous control). PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Reaction conditions were: 2 min at 50°C for UNG activation and 10 min hot start at 95°C, followed by 40 cycles, each consisting in denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Changes in transcript levels were quantified by using the comparative cycle threshold method (Sequence Detection System Chemistry Guide; Applied Biosystems). Each sample was run in triplicate. Data were analyzed by using the Sequence Detector Systems version 2.0 software (Applied Biosystems).

### Cloning of human pendrin

Total RNA extracted from bronchial epithelial cells was retrotranscribed to cDNA as described above. Amplification of full-length pendrin coding sequence was obtained in a reaction containing: 7  $\mu$ l of bronchial cell cDNA, 2 mM Mg<sup>2+</sup>, 20  $\mu$ M dNTPs, 1  $\mu$ M forward and reverse primers, 0.25  $\mu$ l of AmpliTaq Gold (PerkinElmer), in a final volume of 25  $\mu$ l. Forward primer sequence was: 5'-CCACTGCCCTTCTGAGAGC-3'. Reverse primer sequence was: 5'-CCTAGAAGCAGTCTTAGTGC-3'. The PCR consisted in a first step at 95°C for 12 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 2 min, and a final step of 72°C for 7 min. The amplification product was cloned in the pcDNA3.1 TOPO vector (Invitrogen Life Technologies) following the manufacturer's instructions. The resulting plasmids extracted from bacterial colonies were analyzed with *Sac*II digestion to identify the constructs with the correct orientation of the insert. Full sequencing of the insert was then conducted to confirm identity of the cloned product with human pendrin (SLC26A4).

### Transfection in FRT cells and analysis of anion transport by fluorescence

The plasmid vector carrying pendrin-coding sequence and neomycin-resistance gene was transfected in FRT cells already expressing the yellow fluorescent protein (YFP)-H148Q/I152L by means of the Lipofectamine 2000 reagent (Invitrogen Life Technologies). Cells were then treated with 0.75 mg/ml G418 and 0.5 mg/ml hygromycin B to select clones with stable coexpression of pendrin and the YFP. Determination of pendrin activity was conducted by plating the cells in 96-well microplates (50,000 cells/well). After 24 h, the cells were washed three times with 200  $\mu$ l of PBS (in mM: 137 NaCl, 2.7 KCl, 8.1 Na<sub>2</sub>HPO<sub>4</sub>, 1.5 KH<sub>2</sub>PO<sub>4</sub>, 1 CaCl<sub>2</sub>, 0.5 MgCl<sub>2</sub>). After washing, cells were left for 15–30 min at 37°C in 60  $\mu$ l of PBS per well. The microplate was then transferred to a BMG Fluostar Galaxy plate reader equipped with excitation and emission optical filters for YFP (excitation: HQ500/20X, 500  $\pm$  10 nm; emission: HQ535/30 M, 535  $\pm$  15 nm; Chroma) and injection pumps. The assay consisted, for each well, in the continuous reading of fluorescence for 14 s. At 2 s from start, the plate reader injected 165  $\mu$ l of a modified PBS in which  $Cl^-$  was replaced by  $I^-$ ,  $SCN^-$ ,  $NO_3^-$ , or  $Br^-$ . The decrease of cell fluorescence caused by addition of quenching anions was analyzed to calculate anion transport. Briefly, the fluorescence decay phase was fitted with an exponential function to derive the maximal slope. Maximal slopes were converted to rates of variation of intracellular anion concentration (in millimoles per second) using the equation:  $d[X^-]/dt = K_X[d(F/F_0)/dt]$ , where  $[X^-]$  is the anion concentration,  $K_X$  is the affinity constant of YFP for a given anion, and  $F/F_0$  is the ratio of the cell fluorescence at a given time vs initial fluorescence. Activity in cells transfected with pendrin was compared with that in null cells or in cells expressing CFTR. Before the assay, CFTR was preactivated with 20  $\mu$ M forskolin.

### Transepithelial $SCN^-$ and $Cl^-$ transport

FRT cells (with and without expression of pendrin or CFTR) or bronchial epithelial cells were plated on Transwell permeable supports (500,000 cells/cm<sup>2</sup>) and cultured with the appropriate medium (1.5 ml on the apical side, 2.5 ml on the basolateral side). After 10–12 days, the Transwell inserts with cells were washed on both sides with PBS and then transferred to a 6-well plate seating on the top of metal block heated at 37°C. Three Transwell inserts were processed at each time. Each well (basolateral side) contained 2 ml of PBS plus 10 mM glucose and 0.4  $\mu$ Ci of <sup>36</sup>Cl<sup>-</sup> or

$^{14}\text{CN}^-$  (total concentration of  $\text{SCN}^-$ : 95  $\mu\text{M}$ ). The top of the Transwell (apical side) instead received 1 ml of PBS. For bronchial cells, the apical solution also contained 10  $\mu\text{M}$  amiloride to block the epithelial sodium channel. After incubation of cells for 15 min, the apical fluid was removed and replaced with 1 ml of the same solution preheated at 37°C. This process was repeated every 2 min. The apical fluid collected in the first three time points was discarded. Subsequently, the fluid collected every 2 min was placed in scintillation vials for radioactivity determination.

#### Short-circuit current recordings

Bronchial epithelial cells or FRT cells plated on Snapwell supports were mounted in a self-contained Ussing chamber system (vertical diffusion chamber; Corning Costar). FRT cells were studied with a transepithelial  $\text{Cl}^-$  gradient. Accordingly, the basolateral solution contained (in millimoles): 130 NaCl, 2.7 KCl, 1.5  $\text{KH}_2\text{PO}_4$ , 1  $\text{CaCl}_2$ , 0.5  $\text{MgCl}_2$ , 10 sodium-HEPES (pH 7.3) and 10 glucose. For the apical side, this solution was instead modified by replacing half of NaCl with sodium gluconate and increasing  $\text{CaCl}_2$  to 2 mM to compensate for calcium buffering caused by gluconate. The basolateral membrane was permeabilized with 250  $\mu\text{g}/\text{ml}$  amphotericin B. For, human airway epithelial cells, both apical and basolateral chambers contained (in millimoles): 126 NaCl, 0.38  $\text{KH}_2\text{PO}_4$ , 2.1  $\text{K}_2\text{HPO}_4$ , 1  $\text{MgSO}_4$ , 1  $\text{CaCl}_2$ , 24  $\text{NaHCO}_3$ , and 10 glucose (basolateral membrane not permeabilized). During experiments, solutions in both chambers were continuously bubbled with air (FRT cells) or with 5%  $\text{CO}_2$  in air (bronchial cells). The hemichambers were connected to DVC-1000 voltage clamps (World Precision Instruments) via Ag/AgCl electrodes and 1 M KCl agar bridges. Transepithelial currents were digitized using PowerLab 4/25 and 2/25 data acquisition systems and stored on Macintosh computers. All measurements were done at 37°C.

#### Immunodetection of pendrin protein

Bronchial epithelial cells or FRT cells plated on Snapwell supports were fixed in paraformaldehyde (4%) and permeabilized with Triton X-100 (0.2%). Fixed epithelia were then incubated with anti-pendrin Abs (31) at a concentration of 0.25  $\mu\text{g}/\text{ml}$  for 2 h at 37°C. After washings with PBS, cells were incubated with a Cy3-labeled anti-rabbit secondary Ab (Molecular Probes). At the end, the porous membrane with the stained epithelium was detached from the plastic support and mounted on a slide using the VectaShield mounting medium with 4',6'-diamidino-2-phenylindole (Vector Laboratories). Photographs were acquired with a fluorescence microscope equipped with a digital camera and the IPLab software.

#### Transfection with short-interfering RNA (siRNA)

Previous unpublished studies from our laboratory have shown that best conditions for siRNA transfer to bronchial epithelial cells require transfection at the time of cell plating on permeable supports. Accordingly, bronchial cells were coplated on the apical side of Transwell inserts in 2 ml of LHC9/RPMI 1640 medium without antibiotics together with 500  $\mu\text{l}$  of OPTI-MEM medium (Invitrogen Life Technologies) containing preformed complexes of siRNA (20–100 nM final concentration) and 20  $\mu\text{l}$  of Lipofectamine 2000 (Invitrogen Life Technologies). Stealth RNA interference against human pendrin (code HSS107795) and corresponding negative control (code 12935-200) were from Invitrogen Life Technologies. The anti-pendrin siRNA was chosen as the one inducing the most effective silencing in FRT cells expressing human pendrin. The negative control was a nontargeting siRNA having a guanine cytosine content matching that of the antipendrin duplex. During transfection, the basolateral side contained 2.5 ml of LHC9/RPMI 1640 medium without antibiotics. siRNA complexes were removed after 24 h. Transepithelial  $\text{SCN}^-$  transport was conducted after 10–12 days.

#### Measurement of $\text{H}_2\text{O}_2$

Generation of  $\text{H}_2\text{O}_2$  was measured with the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes/Invitrogen Life Technologies). Bronchial epithelial cells were plated and cultured on Snapwell permeable supports as for short-circuit current recordings. After 10–14 days, the Snapwell supports containing the cells were washed with PBS and positioned in a 6-well plate, four supports each time. The well (basolateral side) contained 2 ml of PBS plus 10 mM glucose. The top of the Snapwell (apical side) instead received 250  $\mu\text{l}$  of PBS without glucose. After 20 min of equilibration at 37°C, 200  $\mu\text{l}$  of the apical solution were replaced with an equal volume of PBS containing Amplex Red reagent and HRP, to a final concentration of 25  $\mu\text{M}$  and 0.1 U/ml. After addition of reagents, measurement of  $\text{H}_2\text{O}_2$  started immediately by reading fluorescence with a BMG Fluostar Galaxy plate reader. The reader was programmed to read fluorescence from each one of the four Snapwell supports every 6 s for 10

min. Excitation and emission wavelengths were 544 and 590 nm, respectively. After this step, UTP (final concentration 100  $\mu\text{M}$ ) was added to the apical solution and fluorescence reading was continued for further 10 min.

#### Intracellular-free $\text{Ca}^{2+}$ detection

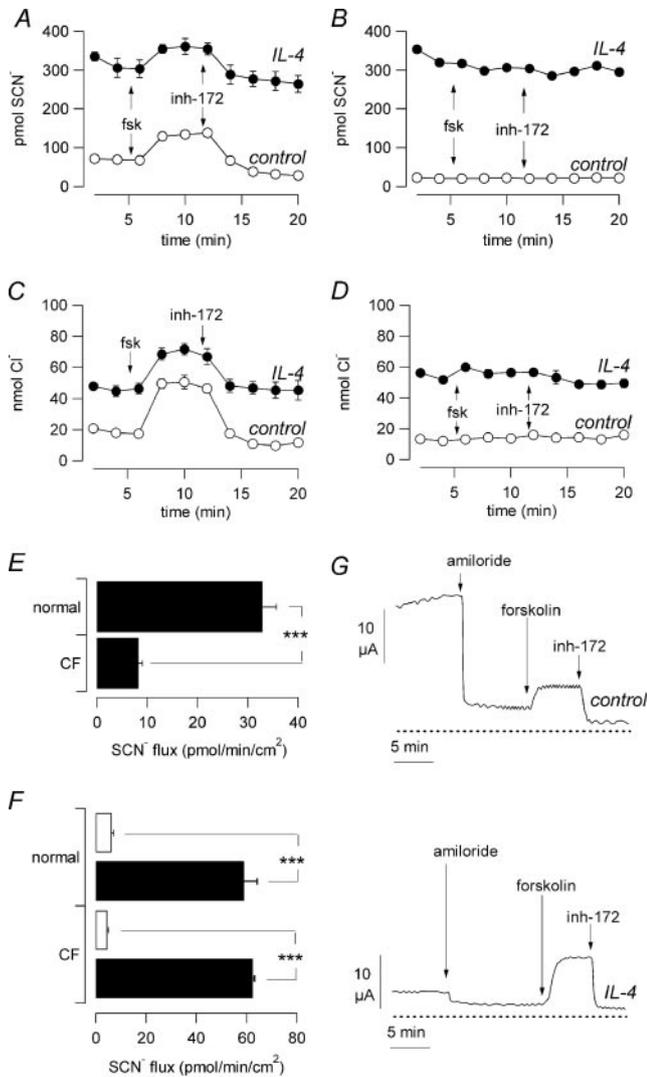
Bronchial epithelial cells, cultured on Snapwell supports, were placed in a six-well plate after washing apical and basolateral sides with PBS. The well (basolateral side) contained 2 ml of PBS plus glucose 10 mM. The apical side of the Snapwell support contained instead 200  $\mu\text{l}$  of PBS. Cells were incubated for 1 h at 37°C with 4  $\mu\text{M}$  Fluo-4/AM (Molecular Probes) and 2 mM probenecid. After Fluo-4 loading, cells were washed leaving 200  $\mu\text{l}$  and 2 ml of PBS plus probenecid in the apical and basolateral sides, respectively. The multiwell plate containing the Snapwell supports was then transferred to the microplate reader for fluorescence measurement. The assay was run for one Snapwell at a time and consisted in continuous fluorescence reading for 35 s. Excitation and emission wavelengths were 485 and 520 nm, respectively. At 8 s, the plate reader was programmed to inject 330  $\mu\text{l}$  of PBS plus UTP (100  $\mu\text{M}$  final concentration) on the apical side.

## Results

We measured  $\text{SCN}^-$  transport from the basolateral to the apical side of bronchial epithelial cells cultured as a polarized monolayer on a porous membrane. In normal cells, stimulation with forskolin, a cAMP-elevating agent, caused a 2-fold increase in transepithelial  $\text{SCN}^-$  transport (Fig. 1A). Forskolin response may be accounted for by CFTR, which is a cAMP-activated anion channel. This conclusion is supported by the strong reduction in  $\text{SCN}^-$  flux caused by addition of CFTR<sub>inh</sub>-172 (Fig. 1A). This is a compound, identified by high-throughput screening, which works as a selective inhibitor of CFTR channel activity (32). Interestingly, the level of  $\text{SCN}^-$  transport reached in the presence of the blocker was lower than that measured in resting conditions, before addition of forskolin. This may be explained by the fact that a fraction of CFTR is active in nonstimulated epithelia, as we have already reported in a previous study (33). The involvement of CFTR was further confirmed by carrying out similar experiments in CF epithelia (Fig. 1B). In CF bronchial cells,  $\text{SCN}^-$  transport was not modified by forskolin stimulation or by CFTR<sub>inh</sub>-172. Furthermore, the level of  $\text{SCN}^-$  flux in CF cells was low and equivalent to that of normal cells following block with CFTR<sub>inh</sub>-172 (compare Fig. 1, A and B). Summarizing, in the presence of forskolin, CF cells showed a 4-fold reduced  $\text{SCN}^-$  transport compared with non-CF cells ( $8.2 \pm 0.8$  vs  $32.9 \pm 2.7$  pM/min/cm<sup>2</sup>; Fig. 1E), a deficit that can be ascribed to CFTR loss of function.

A very interesting result was obtained when cells were pre-treated with IL-4 (10 ng/ml) for 24 h. The basal level of  $\text{SCN}^-$  transport was strongly increased in both normal and CF cells (Fig. 1, A and B). This new component of transepithelial  $\text{SCN}^-$  flux was apparently additive to CFTR because a response to forskolin and a proportional block by CFTR<sub>inh</sub>-172 was still detectable in normal cells treated with IL-4 (Fig. 1A). After removal of the CFTR-dependent transport with CFTR<sub>inh</sub>-172, the effect of IL-4 treatment consisted in a 10-fold increase in  $\text{SCN}^-$  flux, approximately, in both normal and CF cells (Fig. 1F). No effect was found when IL-4 was given acutely (data not shown).

We determined transepithelial  $\text{Cl}^-$  transport in the same conditions and with the same procedure used for  $\text{SCN}^-$ . We found a behavior qualitatively similar to that of  $\text{SCN}^-$  (Fig. 1, C and D). A CFTR-dependent component of  $\text{Cl}^-$  transport (i.e., activation by forskolin and block by CFTR<sub>inh</sub>-172) was detected in normal but not in CF cells. Furthermore, a significant increase in basal  $\text{Cl}^-$  flux was obtained by incubating the cells with IL-4. Although similar, the data obtained with the two anions differed from the quantitative point of view. The stimulation with IL-4 elicited a larger effect on  $\text{SCN}^-$  compared with  $\text{Cl}^-$  (~10- vs 4-fold, respectively). It is also interesting to note that if we normalize for the different



**FIGURE 1.** Anion transport in resting and IL-4-stimulated bronchial epithelial cells. Transepithelial transport of  $\text{SCN}^-$  (A and B) and  $\text{Cl}^-$  (C and D) in bronchial epithelial cells. Highly differentiated epithelia were generated from normal (A and C) or CF bronchi (B and D) on permeable supports. Cells were kept under resting conditions or treated for 24 h with 10 ng/ml IL-4. After equilibrating the cells with  $\text{S}^{14}\text{CN}^-$  or  $\text{S}^{36}\text{Cl}^-$  in the basolateral medium, the radioactive tracer transported to the apical side was collected and measured. Apical amiloride (10  $\mu\text{M}$ ) was maintained throughout the experiments. As indicated by arrows, cells during the experiment were first stimulated with forskolin (20  $\mu\text{M}$ ) and then treated with the selective CFTR blocker inh-172 (20  $\mu\text{M}$ ). E, CFTR-dependent transepithelial  $\text{SCN}^-$  flux. Basolateral to apical  $\text{SCN}^-$  transport was measured in normal and CF epithelia during maximal stimulation with forskolin (6 min after forskolin addition). F, CFTR-independent  $\text{SCN}^-$  flux. Transepithelial  $\text{SCN}^-$  transport was measured in normal and CF epithelia in the presence of inh-172 (20  $\mu\text{M}$ ). Cells were in resting conditions ( $\square$ ) or treated with IL-4 ( $\blacksquare$ ). G, representative traces from Ussing chamber experiments on resting (top) and IL-4 treated (bottom) bronchial epithelial cells. Except for Ussing chamber experiments, all data are presented as mean  $\pm$  SEM of four to six experiments. \*\*\*, A statistically significant difference ( $p < 0.001$ ).

concentrations used in the experiments to mimic physiological conditions (95  $\mu\text{M}$   $\text{SCN}^-$  vs 137 mM  $\text{Cl}^-$  in the basolateral solution), it appears that permeability for  $\text{SCN}^-$ , in particular after IL-4 treatment, is even higher than that for  $\text{Cl}^-$ .

The strong up-regulation of basal anion transport by IL-4, detected by radioisotopic technique, was particularly interesting because

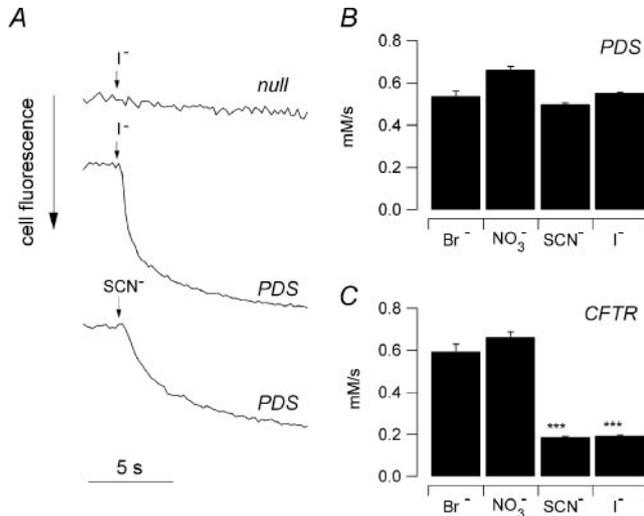
Table I. Expression of SLC26 transporters and other genes<sup>a</sup>

Gene	Microarray			RT-PCR
	Control	IL-4	IL-4/control	IL-4/control
SLC26A1	4.9	5	Absent	
SLC26A2	106.9	244.5	2.29	3.07
SLC26A3	5.6	5.6	Absent	Absent
SLC26A4	139.1	3150.5	22.65	21.72
SLC26A6	35.2	58.2	1.65	1.06
SLC26A7	6.9	7	Absent	Absent
SLC26A8	3.8	3.8	Absent	Absent
SLC26A9	6.6	6.3	Absent	Absent
SLC26A10	4.4	4.5	Absent	
SLC26A11	293.3	458.3	1.56	
SLC4A1	6.3	6.4	Absent	
SLC4A2	196.7	168	0.85	
SLC4A3	5.2	5.2	Absent	
SLC5A5	70.4	98.2	1.39	0.97
DUOX1	94.7	188.7	1.99	1.7
DUOX2	895.9	2592.6	2.89	2.4
LPO	4.2	4.3	Absent	

<sup>a</sup>Expression of SLC26 transporters and other genes in resting and IL-4-treated bronchial epithelial cells by microarray and real-time RT-PCR.

cause we had not detected in previous studies a similar effect during short-circuit current recordings (9), as also shown in Fig. 1G. In agreement with our previous findings, we found that treatment with IL-4 caused a dramatic reduction of  $\text{Na}^+$  absorption as evident from the reduced effect of the ENaC blocker, amiloride. Upon block of ENaC with amiloride, and of CFTR with CFTR<sub>inh</sub>-172, we observed that the residual current was very small and not different between control and IL-4-treated cells (Fig. 1G). This contrasts with the high  $\text{Cl}^-$  and  $\text{SCN}^-$  fluxes evoked by IL-4 treatment. Therefore, we hypothesized that the strong effect elicited by IL-4 on basal  $\text{SCN}^-$  and  $\text{Cl}^-$  transport is mediated by stimulation of a nonelectrogenic anion transporter, possibly by up-regulation of transcription of the corresponding gene.

To possibly identify this transporter, we performed a global analysis of gene expression by means of Affymetrix U133 Plus 2.0 microarrays. This last version of microarrays contains 54,000 probe sets corresponding to >47,000 human transcripts. The mRNA was extracted from untreated and IL-4-treated cells of cultured polarized epithelia of four different individuals. We looked for possible up-regulation of anion channels and transporters. We found no up-regulation of  $\text{Cl}^-$  channels of the CLC family like CLC2 (data not shown). The same negative findings were found for putative  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels of the CICA or bestrophin family. Actually, the genes belonging to the latter family appeared as poorly expressed in our cells (data not shown). Interestingly, the only anion transporter that appeared strongly up-regulated by IL-4 was SLC26A4, also known as pendrin (25) (see Table I). The up-regulation consisted in a 23-fold increase of SLC26A4 signal in cytokine-treated cells. This finding was surprising because pendrin expression in airway epithelial cells has not been previously reported. Pendrin is a transporter which mediates anion exchange at the apical membrane of cells in the kidney, inner ear, and thyroid follicle (34–37). When mutated, the pendrin gene is responsible for Pendred syndrome or nonsyndromic deafness (38, 39). Besides pendrin, no other known anion transporter was strongly up-regulated by IL-4. Many other members of the SLC26 family, like SLC26A1, SLC26A6, SLC26A7, SLC26A8, SLC26A9, and SLC26A10, were expressed at a low level in our cells and not affected by IL-4. SLC26A2, a sulfate transporter, was the only member of the same family with a significant expression under basal levels and a modest induction by

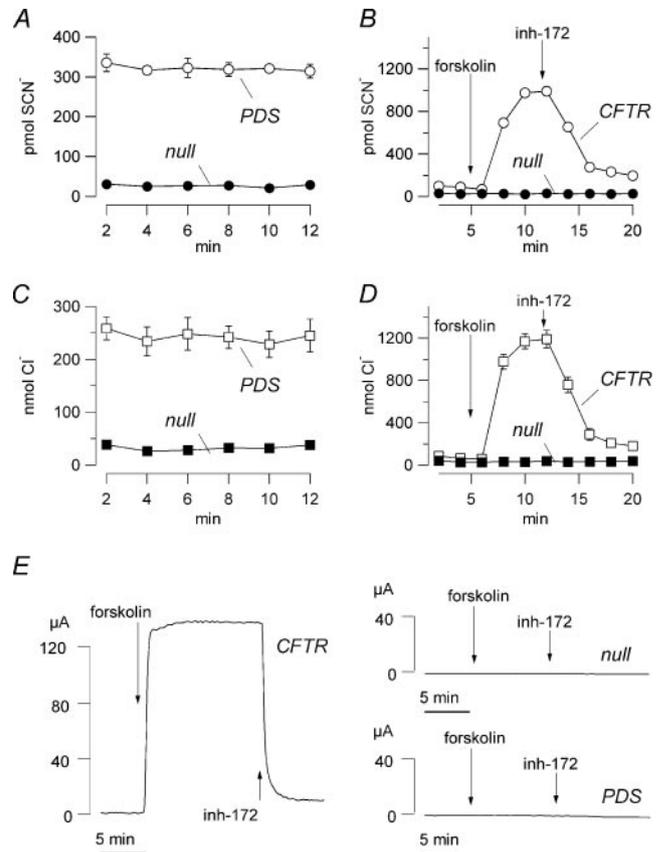


**FIGURE 2.** Anion permeability in pendrin (PDS) and CFTR-expressing cells. **A**, Representative traces showing quenching of YFP fluorescence in null and PDS-transfected FRT cells by addition of anions. Cells were plated in 96-well microplates and assayed with a microplate reader as described in *Materials and Methods*. **B** and **C**, Rates of anion transport (mean  $\pm$  SEM) in PDS- and CFTR-expressing cells. Data are from 8–22 experiments per condition. \*\*\*, A significantly lower transport of  $\text{SCN}^-$  or  $\text{I}^-$  vs  $\text{Br}^-$  and  $\text{NO}_3^-$ .

IL-4. Similarly, other anion transporters belonging to the SLC4 family (anion exchangers) were poorly expressed and/or not affected by cytokine treatment. To confirm the findings obtained with the microarrays, we determined the changes in expression of specific genes by real-time RT-PCR. We found that pendrin mRNA was indeed strongly up-regulated after treatment with IL-4 ( $24.7 \pm 4.9$ -fold,  $n = 6$ ). Other transporters of the same family, including SLC26A3, A6, A7, A8, and A9 were instead poorly expressed and not up-regulated. We also measured by the same method the expression of SLC5A5, the  $\text{Na}^+/\text{I}^-$  symporter, which is also able to mediate  $\text{SCN}^-$  uptake (11). This gene was not up-regulated by IL-4 in agreement with data deriving from microarrays (Table I).

To demonstrate the expression of full-length pendrin mRNA in bronchial cells, we conducted an RT-PCR using two primers allowing amplification of the whole coding sequence. The reaction generated a fragment of  $\approx 2500$  bp, compatible with the expected pendrin amplification product (data not shown). This fragment was cloned in a plasmid and the resulting clones were fully sequenced. Determination of nucleotide sequence revealed that the clones contained the full coding sequence of pendrin, as reported in GenBank (accession number: NM\_000441).

To determine the functional properties of cloned pendrin we decided to take advantage of the halide and pseudohalide sensitivity of YFPs (40, 41). Accordingly, we transfected the expression vector carrying the pendrin-coding sequence in FRT cells that already expressed the YFP-H148Q/I152L (41). FRT cells are thyroid cells that have lost expression of endogenous pendrin. The resulting cell line coexpressing the two desired proteins was used in fluorescence assays using a microplate reader equipped with syringe pumps for liquid injection during the assay. Injection in the well of a modified PBS containing  $\text{I}^-$  instead of  $\text{Cl}^-$  generated a decrease in fluorescence that was much faster in pendrin-expressing cells compared with null cells (Fig. 2A). This is in agreement with the reported ability of pendrin to mediate exchange of  $\text{Cl}^-$  with  $\text{I}^-$  (25, 39). In the same way, a fast fluorescence quenching was also obtained by injecting a  $\text{SCN}^-$ - or a  $\text{NO}_3^-$ -rich solution



**FIGURE 3.** Transepithelial anion transport in transfected FRT cells. (**A** and **B**) transepithelial  $\text{SCN}^-$  transport and (**C** and **D**) transepithelial  $\text{Cl}^-$  transport in null, PDS-transfected, and CFTR-transfected FRT cells (mean  $\pm$  SEM;  $n = 3$ –4 per condition). Arrows indicate addition of forskolin ( $20 \mu\text{M}$ ) and inh-172 ( $20 \mu\text{M}$ ). **E**, Representative traces (from triplicate experiments) of transepithelial  $\text{Cl}^-$  currents in null and in PDS- or CFTR-transfected FRT cells.

thus indicating that pendrin is also able to transport these anions (Fig. 2A). We calculated anion fluxes by taking into account the different affinity of the YFP for the different anions (41). After this correction, the rates of transport for the different anions in pendrin-expressing cells were comparable (Fig. 2B). We performed the same type of experiments also in FRT cells expressing CFTR. In this case, we found that  $\text{I}^-$  and  $\text{SCN}^-$  transport rates were lower than those measured for  $\text{NO}_3^-$  and  $\text{Br}^-$  (Fig. 2C). This is in agreement with the CFTR pore being partially blocked by such anions (42).

The cells expressing pendrin were also plated at high density on Transwell porous supports to perform studies of transepithelial ion transport. When plated on porous supports, FRT cells form tight monolayers with high electrical resistance. We measured the rate of transepithelial  $\text{SCN}^-$  and  $\text{Cl}^-$  transport by using radioactive isotopes, as done for bronchial cells. Pendrin-expressing cells showed a transepithelial anion transport that was significantly higher than that of null cells (Fig. 3, A and C). In contrast, CFTR-expressing cells showed small anion fluxes under resting conditions but a rapid and marked increase of transport was obtained by stimulation with forskolin (Fig. 3, B and D). As expected for CFTR-mediated transport, this forskolin-activated component was fully blocked by CFTR<sub>inh</sub>-172. To investigate the mechanism of anion transport, we measured short-circuit current on monolayers of FRT cells expressing pendrin or CFTR. Cells expressing CFTR generated a large transepithelial  $\text{Cl}^-$  current ( $>100 \mu\text{A}/\text{cm}^2$ ) following stimulation with forskolin (Fig. 3E). This current was

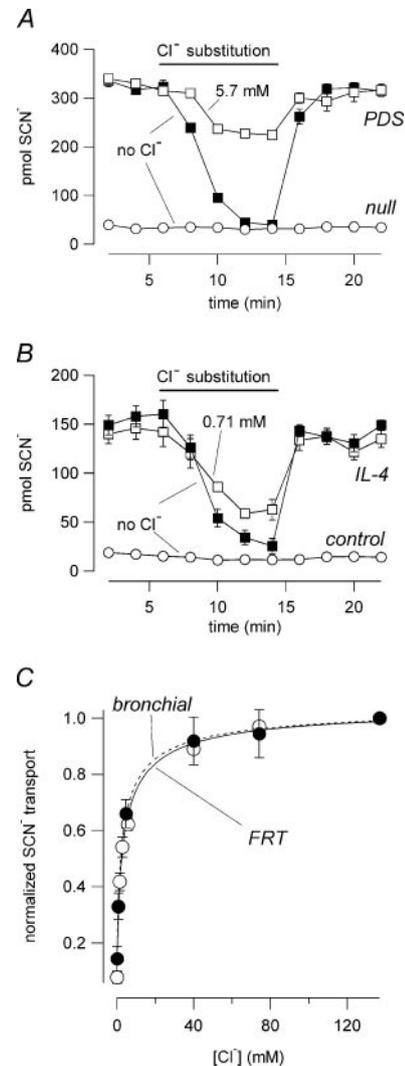
sensitive to CFTR<sub>inh</sub>-172. On the contrary, cells expressing pendrin did not show a significant Cl<sup>-</sup> current compared with null cells (Fig. 3E). The finding that CFTR- and pendrin (PDS) expressing cells show significant rates of anion transport using radioactive tracers, but only CFTR cells display measurable Cl<sup>-</sup> currents, supports the present knowledge that pendrin mediates electroneutral anion transport (43).

To correlate the pendrin-dependent SCN<sup>-</sup> transport in FRT cells with the IL-4-stimulated SCN<sup>-</sup> transport in bronchial cells, we performed ion replacement experiments. Such experiments were based on the hypothesis that SCN<sup>-</sup> is exchanged with apical Cl<sup>-</sup>. Accordingly, the apical Cl<sup>-</sup> was lowered to various concentrations by replacement with an equimolar amount of gluconate while measuring the basolateral to apical SCN<sup>-</sup> flux. In both FRT and bronchial epithelial cells, there was a strong decrease in SCN<sup>-</sup> transport when apical Cl<sup>-</sup> was lowered below 5 mM (see Fig. 4, A and B). The plot of SCN<sup>-</sup> transport vs the apical Cl<sup>-</sup> concentration, fitted with the Michaelis-Menten equation, gave an apparent  $K_m$  of 3.4 and 2.6 mM for pendrin-expressing FRT cells and for IL-4-stimulated bronchial epithelia, respectively (Fig. 4C). Conversely, nonstimulated bronchial epithelia did not show a significant reduction of SCN<sup>-</sup> transport after removal of apical Cl<sup>-</sup> (Fig. 4B). These data indicated that IL-4 induced the appearance in bronchial cells of a SCN<sup>-</sup> transport with properties similar to cloned pendrin.

We also attempted at obtaining pharmacological evidence of pendrin expression in bronchial epithelia. To our knowledge, there are no potent and selective inhibitors of pendrin. For example, it has been reported that DIDS causes only a weak inhibition of anion transport (25). To find pendrin inhibitors, we screened the Spectrum library (44), which contains 2000 compounds, using the functional assay based on the halide-sensitive YFP. No compounds were able to decrease the pendrin-mediated anion transport. We tested also various known inhibitors of different anion transporters and channels like DIDS, glibenclamide, NPPB, CFTR<sub>inh</sub>-172, and GlyH-101, without finding a significant block. The inability of DIDS to block pendrin has been also reported by others (41). The only active compound was niflumic acid (100  $\mu$ M), which elicited a partial inhibition (30–40%) in both FRT and bronchial cells (data not shown). We also tested the possibility of pendrin function regulation using activators/inhibitors of regulatory pathways. Short-term incubation (30–60 min) of FRT cells expressing pendrin with wortmannin (1  $\mu$ M), staurosporin (1  $\mu$ M), U73122 (2  $\mu$ M), thapsigargin (1  $\mu$ M), forskolin (10  $\mu$ M), or phorbol ester (1  $\mu$ M) did not alter anion transport (data not shown).

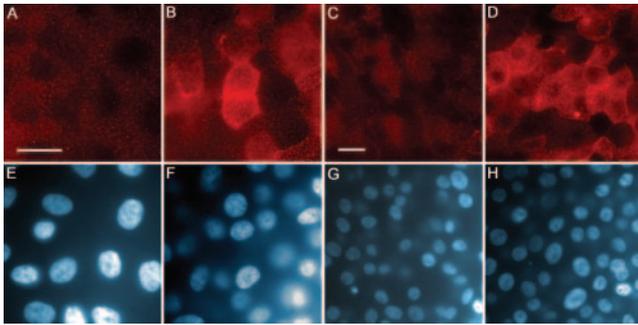
We looked for pendrin protein expression by immunofluorescence using Abs directed against pendrin N and C termini (Fig. 5). Immunostaining was clearly detected with either Ab when bronchial epithelial cells were treated with IL-4. A similar signal was also detected in FRT cells expressing human pendrin whereas untransfected cells were indistinguishable from cells incubated with second Ab alone (data not shown).

To get further evidence of pendrin involvement in cytokine-induced SCN<sup>-</sup> transport, we followed two approaches. In the first approach, we treated bronchial epithelial with IL-1 $\beta$ . In fact, preliminary results with microarrays have shown that this cytokine also induces pendrin expression (our unpublished results). We have conducted real-time PCR to evaluate relative pendrin expression in treated and untreated cells. We found that treatment for 24 h with 2.5 ng/ml IL-1 $\beta$  caused a 23-fold induction of pendrin mRNA. Consequently, we performed transport studies in IL-1 $\beta$ -treated cells. We found that treatment with this cytokine caused a significant increase in SCN<sup>-</sup> transport which was affected by removal of apical Cl<sup>-</sup> as in IL-4-treated cells (Fig. 6A). In the second



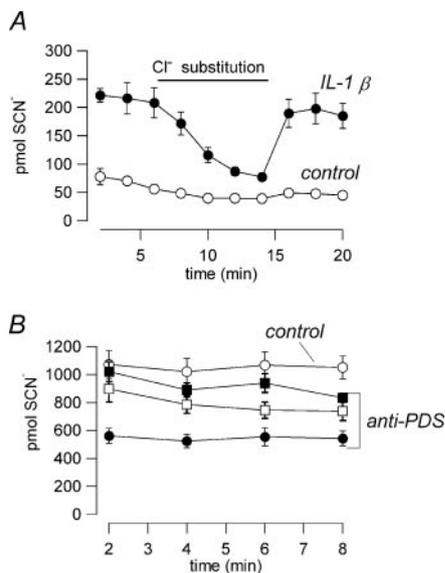
**FIGURE 4.** Effect of apical Cl<sup>-</sup> concentration on transepithelial SCN<sup>-</sup> transport in FRT and bronchial epithelial cells. *A* and *B*, Time course of basolateral to apical SCN<sup>-</sup> transport in FRT (*A*) and bronchial cells (*B*) upon lowering apical Cl<sup>-</sup> to indicated values. Data were from PDS-expressing FRT cells (*A*) and from IL-4-treated bronchial cells (*B*). The graphics show also data obtained from null FRT cells and from untreated bronchial cells (empty circles in both cases; replacement with 0 Cl<sup>-</sup>). All experiments on bronchial cells were conducted in the continuous presence of inh-172 (20  $\mu$ M) to block CFTR. Each point is the mean  $\pm$  SEM of three to six experiments. *C*, Dependence of transepithelial SCN<sup>-</sup> transport on apical Cl<sup>-</sup> concentration in FRT cells expressing PDS and in bronchial cells treated with IL-4. Data are fitted with a Michaelis-Menten relationship. Fits gave  $K_m$  values of 3.4 and 2.6 mM for FRT and bronchial cells, respectively.

approach, we directly attempted to silence pendrin mRNA with siRNA. siRNA against pendrin (20–100 nM) was transfected in bronchial epithelial cells at the time of cell plating. After generation of the polarized epithelium, cells were treated with IL-4. We found that treatment with anti-pendrin siRNA generated a dose-dependent decrease of SCN<sup>-</sup> transport with respect to cells transfected with a nontargeting siRNA (Fig. 6B). Maximum inhibition of SCN<sup>-</sup> transport (50–60%) was obtained with 100 nM siRNA. By real-time RT-PCR, we found that pendrin mRNA was 80% decreased in cells treated with specific pendrin siRNA (100 nM) compared with negative control at the same concentration (data not shown). We found no effect of transfection reagent alone on either anion transport or pendrin mRNA.

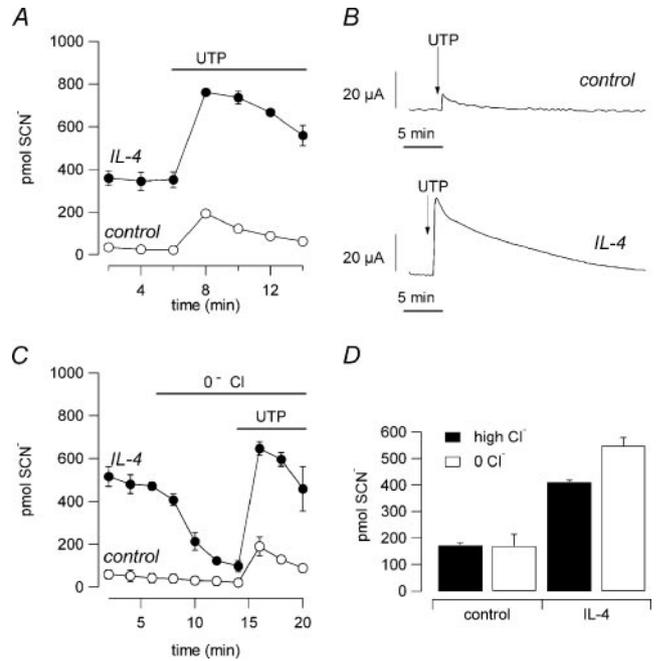


**FIGURE 5.** Immunodetection of pendrin protein in human bronchial epithelial cells. Immunostaining was performed using primary Abs recognizing human pendrin N-terminal (A and B) or C-terminal (C and D) epitopes, and a Cy3-labeled anti-rabbit secondary Ab. Cells were in resting conditions (A and C) or stimulated for 24 h with IL-4 (B and D). Exposure time for acquisition of images was kept at 50 ms for all pictures. Magnification was  $\times 60$  (A and B) or  $\times 100$  (C and D). Scale bar is 20  $\mu\text{m}$ . Images from the same fields showing labeling with 4',6'-diamidino-2-phenylindole are shown (E–H).

To obtain a more complete view of the mechanisms of  $\text{SCN}^-$  transport in bronchial epithelial cells, we also evaluated the contribution of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels. Such channels can be activated with UTP, a purinergic agonist that causes intracellular increase of free  $\text{Ca}^{2+}$  (46). The result is a transient stimulation of  $\text{Cl}^-$  secretion. We have previously shown that IL-4 causes an up-regulation of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  secretion (9). We hypothesized that  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels may also allow permeation of  $\text{SCN}^-$  and, consequently, that treatment with IL-4 may induce an



**FIGURE 6.** Induction of  $\text{SCN}^-$  transport by IL-1 $\beta$  and silencing with anti-pendrin siRNA. **A**, Time course of transepithelial  $\text{SCN}^-$  transport in control and IL-1 $\beta$ -treated bronchial epithelial cells. IL-1 $\beta$  concentration was 2.5 ng/ml. The graphic shows the effect of removal of apical  $\text{Cl}^-$ . Each point is the mean  $\pm$  SEM of three experiments. **B**, Transepithelial  $\text{SCN}^-$  transport in bronchial cells treated with IL-4 (10 ng/ml). Cells were transfected with siRNA against pendrin or with nontargeting siRNA. Concentration of anti-pendrin siRNA was 20 nM (■), 50 nM (□), or 100 nM (●). For simplicity, only data obtained with 100 nM nontargeting siRNA are shown (○). Each point is the mean  $\pm$  SEM of four experiments. A significant reduction of  $\text{SCN}^-$  transport ( $<0.01$ ) was obtained at all time points using 100 nM siRNA, and for the last two time points for 50 nM siRNA.

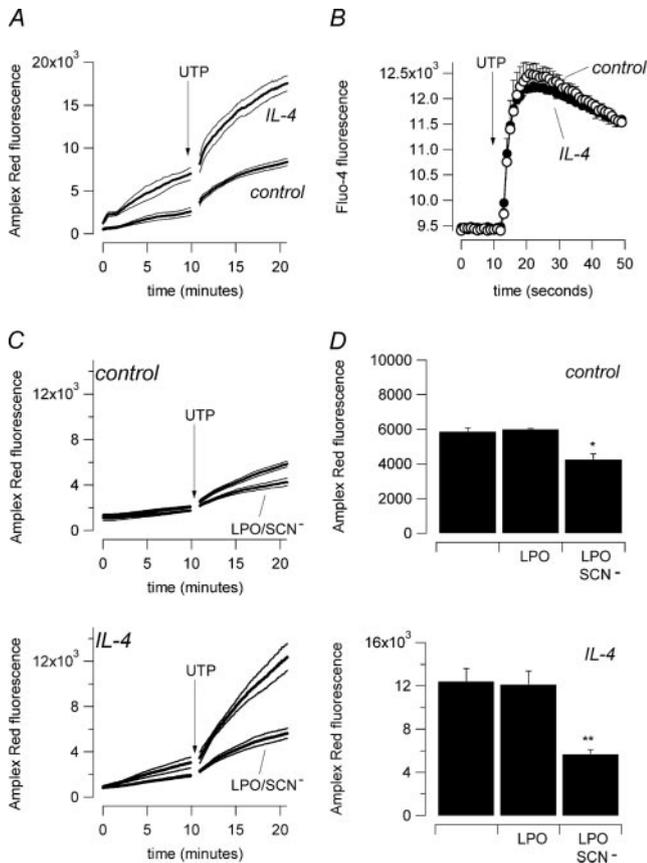


**FIGURE 7.** Stimulation of  $\text{SCN}^-$  transport in bronchial cells by apical UTP. **A**, Time course of transepithelial  $\text{SCN}^-$  transport elicited by apical UTP (100  $\mu\text{M}$ ) in resting and IL-4-treated epithelial cells. All experiments were performed in the presence of inh-172 (20  $\mu\text{M}$ ) to block CFTR. Each point is the mean  $\pm$  SEM of four to five experiments. **B**, Representative traces showing activation of  $\text{Cl}^-$  secretion (short-circuit current) by UTP in resting and IL-4-treated epithelial cells. **C**, Effect of apical  $\text{Cl}^-$  removal on basal and UTP-stimulated  $\text{SCN}^-$  transport. Cells were treated with and without IL-4 ( $n = 4$  per condition). **D**, Peak of UTP-activated  $\text{SCN}^-$  transport in the various conditions (control vs IL-4 treated cells, presence vs absence of apical  $\text{Cl}^-$ ).

enhancement of  $\text{Ca}^{2+}$ -dependent  $\text{SCN}^-$  transport. In agreement with this hypothesis, we found that stimulation of bronchial cells with apical UTP triggered a flux of  $\text{SCN}^-$ , which was markedly potentiated in cells treated with IL-4 (Fig. 7A). Similar results were obtained in CF cells (data not shown). An increase in UTP-dependent  $\text{Cl}^-$  secretion was confirmed in IL-4-treated cells (Fig. 7B) in agreement with previous studies (9).

We wanted to assess whether the basal and  $\text{Ca}^{2+}$ -stimulated  $\text{SCN}^-$  transport in IL-4-treated bronchial cells are distinct. Therefore, apical UTP was added in the absence of  $\text{Cl}^-$ . Under  $\text{Cl}^-$ -free conditions, the basal (electroneutral)  $\text{SCN}^-$  transport was strongly inhibited. On the contrary, the peak of  $\text{SCN}^-$  induced by UTP was similar to that measured in the presence of  $\text{Cl}^-$  (Fig. 7, C and D).

The physiological meaning of  $\text{SCN}^-$  transport across airway epithelium is probably to provide one of the substrates for lactoperoxidase, the other substrate being  $\text{H}_2\text{O}_2$ . We asked whether up-regulation of  $\text{SCN}^-$  transport in IL-4-treated cells is paralleled by a change in the ability to generate  $\text{H}_2\text{O}_2$ . We measured the production of  $\text{H}_2\text{O}_2$  on the apical membrane of bronchial epithelial cells using the Amplex Red fluorescent reagent. Under resting conditions, we detected a significant generation of  $\text{H}_2\text{O}_2$ , whose rate was markedly accelerated by addition of UTP (Fig. 8A). This stimulation is considered to be the consequence of intracellular  $\text{Ca}^{2+}$  increase and activation of dual oxidases (DUOX1 and DUOX2), the membrane enzymes responsible for extracellular  $\text{H}_2\text{O}_2$  production (21, 47). Interestingly, cells pretreated with IL-4 showed an up-regulation of  $\text{H}_2\text{O}_2$  release, particularly of the UTP-stimulated component.  $\text{H}_2\text{O}_2$  production was strongly inhibited in the presence of 20  $\mu\text{M}$  diphenyleneiodium, an inhibitor of dual oxidases (data not

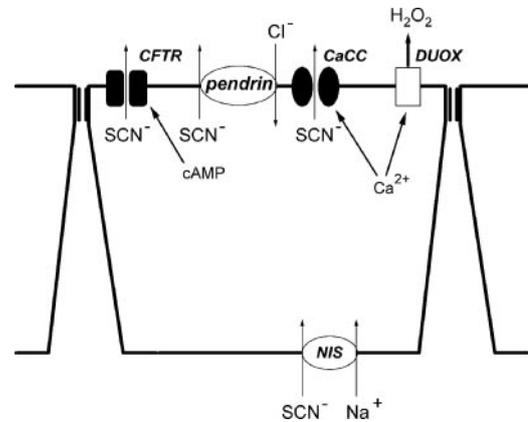


**FIGURE 8.** Apical  $\text{H}_2\text{O}_2$  generation by bronchial epithelial cells. *A*, Time course of  $\text{H}_2\text{O}_2$  generation by resting and IL-4-treated bronchial cells under basal conditions and after stimulation with apical UTP (100  $\mu\text{M}$ ). Continuous lines report the mean value (thick line)  $\pm$  SEM (thin lines) of Amplex Red fluorescence ( $n = 4/\text{condition}$ ). *B*, Time course of Fluo-4 fluorescence (cytosolic  $\text{Ca}^{2+}$ ) in resting and IL-4-treated bronchial cells upon stimulation with apical UTP ( $n = 3/\text{condition}$ ). *C*, Effect of apical lactoperoxidase/basolateral  $\text{SCN}^-$  on  $\text{H}_2\text{O}_2$  generation in resting (*top*) and IL-4-treated (*bottom*) cells (mean  $\pm$  SEM;  $n = 4/\text{condition}$ ). Data with LPO alone overlapped those obtained in its absence and are therefore not shown. *D*, Total  $\text{H}_2\text{O}_2$  production generated in all conditions. The data report the Amplex Red fluorescence at the end of experiments as those shown in *C*. The presence of LPO plus  $\text{SCN}^-$  significantly decreased the amount of  $\text{H}_2\text{O}_2$  detected by Amplex Red reagent (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

shown). Examination of microarray data revealed that both DUOX1 and DUOX2 transcripts are increased (2- to 3-fold) by IL-4 treatment (Table I). These data were confirmed by real-time RT-PCR.

We tested the hypothesis that up-regulation of  $\text{H}_2\text{O}_2$  production by IL-4 is not only due to DUOX overexpression but also to a potentiation of the intracellular  $\text{Ca}^{2+}$  signal. Actually, such a mechanism could also account for the increased response to UTP of  $\text{Ca}^{2+}$ -dependent anion channels in IL-4-treated cells. Accordingly, we measured intracellular  $\text{Ca}^{2+}$  using the Fluo-4 fluorescent probe. Nevertheless, the pattern and size of the  $\text{Ca}^{2+}$  increase triggered by extracellular UTP was essentially identical in control and IL-4-treated epithelia (Fig. 8*B*).

We asked whether the rate of  $\text{SCN}^-$  transepithelial transport was adequate to sustain  $\text{H}_2\text{O}_2$  conversion by lactoperoxidase. Accordingly, we mimicked the physiological situation by measuring  $\text{H}_2\text{O}_2$  in the presence of apical lactoperoxidase and basolateral  $\text{SCN}^-$ . We found that the amount of detectable  $\text{H}_2\text{O}_2$  on the apical side was strongly decreased when both  $\text{SCN}^-$  and lactoperoxidase were present during the experiment, but unchanged with lactoperoxidase alone (Fig. 8, *C* and *D*).



**FIGURE 9.**  $\text{SCN}^-$  transport in the bronchial epithelium. The cartoon shows the pathways for  $\text{SCN}^-$  transport as indicated by our results. CFTR is responsible for cAMP-regulated  $\text{SCN}^-$  transport.  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (CaCCs) are instead involved in the  $\text{SCN}^-$  transport triggered by purinergic agonists and, possibly, other  $\text{Ca}^{2+}$ -dependent stimuli. Finally, an electroneutral anion transporter (possibly pendrin) mediates exchange of  $\text{Cl}^-$  with  $\text{SCN}^-$ . Both CaCC and the anion exchanger are up-regulated by IL-4. The basolateral membrane contains NIS (the sodium-dependent  $\text{I}^-$  symporter, also known as SLC5A5) which mediates  $\text{Na}^+$ -dependent  $\text{SCN}^-$  accumulation (11).  $\text{SCN}^-$  transport occurs in parallel with  $\text{H}_2\text{O}_2$  generation at the apical membrane by  $\text{Ca}^{2+}$ -dependent dual oxidases (DUOX1 and 2).  $\text{SCN}^-$  and  $\text{H}_2\text{O}_2$  are substrates of lactoperoxidase to generate  $\text{OSCN}^-$ , an antimicrobial molecule.

## Discussion

$\text{SCN}^-$  is emerging as an important molecule for innate defense mechanisms of mucosal surfaces. Recent studies have shown that the lactoperoxidase/ $\text{H}_2\text{O}_2$ / $\text{SCN}^-$  system (12–15) has a critical role also in the airways (18). In this context, the activity of anion channels and transporters is probably crucial in determining the availability of apical  $\text{SCN}^-$  for the reaction catalyzed by lactoperoxidase. Indeed, CFTR seems to be involved in cAMP-dependent  $\text{SCN}^-$  transport and deficit of CFTR channel activity in CF cells causes an impairment of bactericidal activity (11, 24). Our study further supports the importance of CFTR in cAMP-stimulated  $\text{SCN}^-$  transport in the airway epithelium. We have found that this mechanism is absent in CF bronchial epithelial cells and fully blocked in normal cells by a highly selective CFTR inhibitor. Comparison of normal and CF cells revealed a 4-fold difference in total  $\text{SCN}^-$  transport under cAMP-stimulated conditions. This difference indicates that defective  $\text{SCN}^-$  transport is an important feature of CF cells that may limit the efficacy of the lactoperoxidase-based antimicrobial system.

Interestingly, our study demonstrates for the first time that the bronchial epithelium expresses also other pathways for  $\text{SCN}^-$  transport in addition to CFTR. These alternative pathways are particularly sensitive to regulation by IL-4. In a previous study, we reported that IL-4 has a pleiotropic effect on transepithelial ion transport in bronchial epithelial cells (9). In particular, we found that anion transport is in general stimulated by the cytokine. We interpreted this effect by postulating that IL-4 stimulates  $\text{Cl}^-$  secretion as a way to improve airway surface hydration and mucociliary clearance. Given the importance of  $\text{SCN}^-$  in host defense, we have now tested the possibility that  $\text{SCN}^-$  transport is also stimulated by IL-4. We have found that cells treated with IL-4 show a marked increase in transepithelial  $\text{SCN}^-$  flux. This type of response is independent of CFTR because it is preserved in CF cells and is not blocked by CFTR<sub>inh</sub>-172. Actually, our data indicate that the  $\text{SCN}^-$  transport induced by IL-4 is mediated by an

electroneutral transporter which exchanges  $\text{SCN}^-$  with  $\text{Cl}^-$ . We hypothesized that the mechanism of IL-4 activity is based on the increased expression of a membrane  $\text{SCN}^-/\text{Cl}^-$  transporter. Using microarray analysis of gene expression, we have found that the only known transporter that appears strongly up-regulated in IL-4-stimulated cells is pendrin (SLC26A4). The cytokine effect consists in a >20-fold elevation of pendrin signal. Expression of cloned pendrin in FRT cells induced the appearance of a  $\text{SCN}^-$  transporter whose properties (e.g., affinity for  $\text{Cl}^-$ ) were similar to those observed in bronchial epithelial cells treated with IL-4. Besides these observations, other three lines of evidences are in support of pendrin involvement in cytokine-stimulated  $\text{SCN}^-$  transport. First, we treated bronchial cells with IL-1 $\beta$ . This treatment also resulted in up-regulation of either  $\text{SCN}^-$  transport and pendrin mRNA. Second, treatment with IL-4 caused the appearance of immunostaining of bronchial cells with a pendrin Ab. Third, treatment with an anti-pendrin siRNA caused a significant reduction in  $\text{SCN}^-$  transport.

We investigated whether  $\text{SCN}^-$  transport is also mediated by  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels. To this aim, we measured the response of bronchial cells to UTP. This nucleotide induces transient activation of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels through P2Y2 purinergic receptors (46). We hypothesized that this activation could result in increased  $\text{SCN}^-$  transport, because  $\text{Cl}^-$  channels generally allow transport of various anions besides  $\text{Cl}^-$  (7). We found that UTP really stimulated transepithelial  $\text{SCN}^-$  transport. The response was transient, with a fast peak followed by a progressive decay that ended in a few minutes, a behavior that reproduced the time course of the  $\text{Cl}^-$  secretion activated by UTP in short-circuit current conditions. Furthermore, the UTP-dependent  $\text{SCN}^-$  transport was up-regulated in IL-4-treated cells, by an extent similar to that of UTP-dependent  $\text{Cl}^-$  secretion, as expected if both processes occur through the same channels. The purinergic-activated transport was not affected by removal of apical  $\text{Cl}^-$ , thus showing that it is different from the electroneutral  $\text{SCN}^-$  transport induced by IL-4. In addition, the up-regulation of  $\text{Ca}^{2+}$ -dependent anion channels by IL-4 does not depend on a modification of the  $\text{Ca}^{2+}$  signal because in control and cytokine-treated cells UTP triggered a similar  $\text{Ca}^{2+}$  increase. Therefore, the mechanism of IL-4 lies in a step downstream of the  $\text{Ca}^{2+}$  increase, possibly involving the channels themselves or channel regulators.

It is interesting to point out that we found also an increase in the ability to produce  $\text{H}_2\text{O}_2$  after treatment with IL-4. This may reflect the up-regulation of DUOX1 and DUOX2 transcripts as evidenced by microarray data. A recent report also indicates that *DUOX* genes transcription is affected by cytokines (48).

In conclusion, we have identified three mechanisms for  $\text{SCN}^-$  transport in bronchial epithelial cells (Fig. 9). One mechanism involves CFTR as indicated by previous findings (11, 24). Lack of this transport could be important in the pathogenesis of CF lung disease. In addition, we have found that an alternative pathway for  $\text{SCN}^-$  transport involves  $\text{Ca}^{2+}$ -dependent anion channels. Such channels are considered as an important pharmacological target to overcome the CFTR defect in CF patients (49). Indeed, a purinergic agonist, deflunisol, is in clinical trials to demonstrate the efficacy in the improvement of lung function in CF patients (50). The hypothesis is that stimulation of  $\text{Cl}^-$  secretion through  $\text{Ca}^{2+}$ -dependent channels would improve mucociliary clearance. Our findings, by showing that UTP also stimulates  $\text{SCN}^-$  transport, could further enhance the relevance of purinergic stimulation as a therapeutic strategy in CF. Indeed, this approach would increase the availability of apical  $\text{SCN}^-$ , which seems to be defective in CF patients due to CFTR loss of function. The finding that the purinergic signal regulates  $\text{SCN}^-$  transport is particularly interesting. Because  $\text{H}_2\text{O}_2$  production is also dependent on purinergic regula-

tion, it is possible to envision a coordinated response where local activation of receptors may enhance antimicrobial activity by providing the two substrates needed by lactoperoxidase.

Interestingly, we found that IL-4 treatment enhances the ability of the bronchial epithelium to transport  $\text{SCN}^-$ . This effect occurs through two apparently independent mechanisms. One is based on the up-regulation of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels. The other one is dependent on the strong up-regulation of pendrin which is represents the third pathway for  $\text{SCN}^-$  transport. It is important to point out that in IL-4-stimulated cells the extent of  $\text{SCN}^-$  transport through pendrin becomes even larger than that occurring through CFTR.

Various intriguing questions arise about the physiological meaning of  $\text{SCN}^-$  transport and the up-regulation by IL-4.  $\text{SCN}^-$  reacts with  $\text{H}_2\text{O}_2$ , a reaction catalyzed by lactoperoxidase, to generate OSCN $^-$ , a bacteriostatic/bactericidal molecule (18). Hence, up-regulation of  $\text{SCN}^-$  transport by IL-4, and possibly by other cytokines like IL-1 $\beta$ , may be considered as a mechanism to potentiate antimicrobial activity. However, it has to be noted that  $\text{H}_2\text{O}_2$  has a bactericidal activity by itself, but also a toxicity to epithelial cells, stronger than OSCN $^-$ . Therefore,  $\text{SCN}^-$  transport may also be important to scavenge excessive  $\text{H}_2\text{O}_2$  generation under particular inflamed conditions. In addition, it has been reported that  $\text{SCN}^-$  is also a substrate for neutrophil and eosinophil peroxidases (myeloperoxidase and eosinophil peroxidase, respectively) (51–53). Actually, availability of  $\text{SCN}^-$  in the airway periciliary fluid may switch the activity of cellular peroxidases toward the production of OSCN $^-$  instead of OCl $^-$  and OBr $^-$ , which are highly cytotoxic oxidants. Accordingly, up-regulation of transepithelial  $\text{SCN}^-$  transport by IL-4 could be a way to scavenge oxidants and to dampen the toxicity arisen from cells like eosinophils and neutrophils that are recruited in the airway lumen during inflammatory conditions.

Future studies will have to determine which of the possible effects of IL-4-stimulated  $\text{SCN}^-$  transport (i.e., antimicrobial vs antioxidant) is most important. In addition, it will be interesting to assess whether and how  $\text{SCN}^-$  transport through non-CFTR channels and transporters affects the lung disease in CF patients. There is a large variability in the severity of lung pathology in CF which is attributed to the role of other proteins in addition to CFTR. Genes involved in the function of the lactoperoxidase/ $\text{SCN}^-/\text{H}_2\text{O}_2$  system like  $\text{SCN}^-$  transporters may be important as modifiers of the CF phenotype in the lung. Regarding pendrin, the presence of cytokines in inflamed airways may lead to expression of this transporter with a possible compensation of the defective  $\text{SCN}^-$  transport in CF airways and partial recovery of antimicrobial activity. However, this hypothesis needs to be verified in vivo because inflamed airways contain a complex mixture of soluble mediators whose net on pendrin expression cannot be anticipated. Another intriguing question regards the absence of a clear respiratory disease in individuals affected by mutations in the pendrin gene (which cause Pendred syndrome or nonsyndromic deafness). It is possible that absence of pendrin function in the lung of such patients is compensated by the expression of other transporters. In addition, it is possible that deficit of pendrin function modulates the severity of acute/chronic pathologies of the lung in a way that has not been detected so far.

## Acknowledgments

We are indebted to Dr. Barbara Czarnocka for the generous gift of pendrin Abs. We also thank Prof. Carlo Tacchetti and Dr. Caterina Valetti for help with immunofluorescence.

## Disclosures

The authors have no financial conflict of interest.

## References

- Smith, J. J., and M. J. Welsh. 1993. Fluid and electrolyte transport by cultured human airway epithelia. *J. Clin. Invest.* 91: 1590–1597.
- Boucher, R. C. 1994. Human airway ion transport: part two. *Am. J. Respir. Crit. Care Med.* 150: 581–593.
- Coakley, R. D., B. R. Grubb, A. M. Paradiso, J. T. Gatzky, L. G. Johnson, S. M. Kreda, W. K. O'Neal, and R. C. Boucher. 2003. Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. *Proc. Natl. Acad. Sci. USA* 100: 16083–16088.
- Matsui, H., B. R. Grubb, R. Tarran, S. H. Randell, J. T. Gatzky, C. W. Davis, and R. C. Boucher. 1998. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 95: 1005–1015.
- Boucher, R. C. 2004. New concepts of the pathogenesis of cystic fibrosis lung disease. *Eur. Respir. J.* 23: 146–158.
- Boucher, R. C., C. U. Cotton, J. T. Gatzky, M. R. Knowles, and Y. R. Yankaskas. 1988. Evidence for reduced  $\text{Cl}^-$  and increased  $\text{Na}^+$  permeability in cystic fibrosis human primary cell cultures. *J. Physiol.* 405: 77–103.
- Jentsch, T. J., V. Stein, F. Weinreich, and A. A. Zdebik. 2002. Molecular structure and physiological function of chloride channels. *Physiol. Rev.* 82: 503–568.
- Sheppard, D. N., and M. J. Welsh. 1999. Structure and function of the CFTR chloride channel. *Physiol. Rev.* 79: S23–S45.
- Galiotta, L. J. V., P. Pagesy, C. Folli, E. Caci, L. Romio, B. Costes, E. Nicolis, G. Cabrini, M. Goossens, R. Ravazzolo, and O. Zegarra-Moran. 2002. IL-4 is a potent modulator of ion transport in the human bronchial epithelium in vitro. *J. Immunol.* 168: 839–845.
- Danahay, H., H. Atherton, G. Jones, R. J. Bridges, and C. T. Poll. 2002. Interleukin-13 induces a hypersecretory ion transport phenotype in human bronchial epithelial cells. *Am. J. Physiol.* 282: L226–L236.
- Fragoso, M. A., V. Fernandez, R. Forteza, R. H. Randell, M. Salathe, and G. E. Conner. 2004. Transcellular thiocyanate transport by human airway epithelia. *J. Physiol.* 561: 183–194.
- Lundquist, P., B. Kagedal, and L. Nilsson. 1995. An improved method for determination of thiocyanate in plasma and urine. *Eur. J. Clin. Chem. Clin. Biochem.* 33: 343–349.
- Schultz, C. P., M. K. Ahmed, C. Dawes, and H. H. Mantsch. 1996. Thiocyanate levels in human saliva: quantitation by Fourier transform infrared spectroscopy. *Anal. Biochem.* 240: 7–12.
- Tsuge, K., M. Kataoka, and Y. Seto. 2000. Cyanide and thiocyanate levels in blood and saliva of healthy adult volunteers. *J. Health Sci.* 46: 343–350.
- Cowman, R. A., S. S. Baron, S. D. Obenauf, and J. J. Byrnes. 1983. Evidence for thiocyanate-sensitive peroxidase activity in human saliva. *J. Clin. Microbiol.* 18: 1177–1182.
- Gaya, P., M. Medina, and M. Nunez. 1991. Effect of the lactoperoxidase system on *Listeria monocytogenes* behavior in raw milk at refrigeration temperatures. *Appl. Environ. Microbiol.* 57: 3355–3360.
- Ihalin, R., V. Loimaranta, and J. Tenovou. 2006. Origin, structure, and biological activities of peroxidases in human saliva. *Arch. Biochem. Biophys.* 445: 261–268.
- Ratner, A. J., and A. Prince. 2000. Lactoperoxidase: new recognition of an "old" enzyme in airway defenses. *Am. J. Respir. Cell Mol. Biol.* 22: 642–644.
- Gerson, C., J. Sabater, M. Scuri, A. Torbati, R. Coffey, J. W. Abraham, I. Lauredo, R. Forteza, A. Wanner, M. Salathe, et al. 2000. The lactoperoxidase system functions in bacterial clearance of airways. *Am. J. Respir. Cell Mol. Biol.* 22: 665–671.
- Wijkstrom-Frei, C., S. El-Chemaly, R. Ali-Rachedi, C. Gerson, M. A. Cobas, R. Forteza, M. Salathe, and G. E. Conner. 2003. Lactoperoxidase and human airway host defense. *Am. J. Respir. Cell Mol. Biol.* 29: 206–212.
- Forteza, R., M. Salathe, F. Miot, R. Forteza, and G. E. Conner. 2005. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 32: 462–469.
- Ameziane-El-Hassani, R., S. Morand, J. L. Boucher, Y. M. Frapart, D. Apostolou, D. Agnandji, S. Gnidehou, R. Ohayon, M. S. Noel-Hudson, J. Francon, K. Lalaoui, A. Virion, and C. Dupuy. 2005. Dual oxidase-2 has an intrinsic  $\text{Ca}^{2+}$ -dependent  $\text{H}_2\text{O}_2$ -generating activity. *J. Biol. Chem.* 280: 30046–30054.
- Schwarzer, C., T. E. Machen, B. Illek, and H. Fischer. 2004. NADPH oxidase-dependent acid production in airway epithelial cells. *J. Biol. Chem.* 279: 36454–36461.
- Moskwa, P., D. Lorentzen, K. J. D. A. Excoffon, J. Zabner, P. B. McCray, W. M. Nauseef, C. Dupuy, and B. Banfi. 2007. A novel host defense system of airways is defective in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 175: 174–183.
- Scott, D. A., R. Wang, T. M. Kreman, V. C. Sheffield, and L. P. Karniski. 1999. The Pendred syndrome gene encodes a chloride-iodide transport protein. *Nat. Genet.* 21: 440–443.
- Galiotta, L. J. V., L. Musante, L. Romio, U. Caruso, A. Fantasia, A. Gazzolo, L. Romano, O. Sacco, G. A. Rossi, L. Varesio, and O. Zegarra-Moran. 1998. An electrogenic amino acid transporter in the apical membrane of cultured human bronchial epithelial cells. *Am. J. Physiol.* 275: L917–L923.
- Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* 31: e15.
- Zhang, J., V. Carey, and R. Gentleman. 2003. An extensible application for assembling annotation for genomic data. *Bioinformatics* 19: 155–156.
- Tusher, V. G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* 98: 5116–5121.
- Dennis, G. Jr., B. T. Sherman, D. A. Hosack, J. Yang, W. Gao, H. C. Lane, and R. A. Lempicki. 2002. DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.* 4: P3.
- Skubis-Zegadlo, J., A. Nikodemka, E. Przytula, M. Mikula, K. Bardadin, J. Ostrowski, B. E. Wenzel, and B. Czarnocka. 2005. Expression of pendrin in benign and malignant human thyroid tissues. *Br. J. Cancer* 93: 144–151.
- Taddei, A., C. Folli, O. Zegarra-Moran, P. Fanen, A. S. Verkman, and L. J. V. Galiotta. 2004. Altered channel gating mechanism for CFTR inhibition by a high-affinity thiazolidinone blocker. *FEBS Lett.* 558: 52–56.
- Caci, E., C. Folli, O. Zegarra-Moran, T. Ma, M. F. Springsteel, R. E. Sammelson, M. H. Nantz, M. J. Kurth, A. S. Verkman, and L. J. V. Galiotta. 2003. CFTR activation in human bronchial epithelial cells by novel benzoflavone and benzimidazolone compounds. *Am. J. Physiol.* 285: L180–L188.
- Everett, L. A., H. Morsli, D. K. Wu, and E. D. Green. 1999. Expression pattern of the mouse ortholog of the Pendred's syndrome gene (Pds) suggests a key role for pendrin in the inner ear. *Proc. Natl. Acad. Sci. USA* 96: 9727–9732.
- Royaux, I. E., K. Suzuki, A. Mori, R. Katoh, L. A. Everett, L. D. Kohn, and E. D. Green. 2000. Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells. *Endocrinology* 141: 839–845.
- Royaux, I. E., S. M. Wall, L. P. Karniski, L. A. Everett, K. Suzuki, M. A. Knepper, and E. D. Green. 2001. Pendrin, encoded by the Pendred syndrome gene, resides in the apical region of renal intercalated cells and mediates bicarbonate secretion. *Proc. Natl. Acad. Sci. USA* 98: 4221–4226.
- Soleimani, M., T. Greeley, S. Petrovic, Z. Wang, H. Amlal, P. Kopp, and C. E. Burnham. 2001. Pendrin: an apical  $\text{Cl}^-/\text{OH}^-/\text{HCO}_3^-$  exchanger in the kidney cortex. *Am. J. Physiol.* 280: F356–F364.
- Everett, L. A., B. Glaser, J. C. Beck, J. R. Idol, A. Buchs, M. Heyman, F. Adawi, E. Hazani, E. Nassir, A. D. Baxevasis, et al. 1997. Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). *Nat. Genet.* 17: 411–422.
- Scott, D. A., R. Wang, T. M. Kreman, M. Andrews, J. M. McDonald, J. R. Bishop, R. J. Smith, L. P. Karniski, and V. C. Sheffield. 2000. Functional differences of the PDS gene product are associated with phenotypic variation in patients with Pendred syndrome and non-syndromic hearing loss (DFNB4). *Hum. Mol. Genet.* 9: 1709–1715.
- Jayaraman, S., P. Haggie, R. M. Wachtler, S. J. Remington, and A. S. Verkman. 2000. Mechanism and cellular applications of a green fluorescent protein-based halide sensor. *J. Biol. Chem.* 275: 6047–6050.
- Galiotta, L. J. V., P. M. Haggie, and A. S. Verkman. 2001. Green fluorescent protein-based halide indicators with improved chloride and iodide affinities. *FEBS Lett.* 499: 220–224.
- Linsdell, P., J. A. Tabcharani, and J. W. Hanrahan. 1997. Multi-Ion mechanism for ion permeation and block in the cystic fibrosis transmembrane conductance regulator chloride channel. *J. Gen. Physiol.* 110: 365–377.
- Dossena, S., A. Maccagni, V. Vezzoli, C. Bazzini, M. L. Garavaglia, G. Meyer, J. Furst, M. Ritter, L. Fugazzola, L. Persani, et al. 2005. The expression of wild-type pendrin (SLC26A4) in human embryonic kidney (HEK 293 Phoenix) cells leads to the activation of cationic currents. *Eur. J. Endocrinol.* 153: 693–699.
- Pedemonte, N., T. Diena, E. Caci, E. Nieddu, M. Mazzei, R. Ravazzolo, O. Zegarra-Moran, and L. J. V. Galiotta. 2005. Antihypertensive 1,4-dihydropyridines as correctors of the cystic fibrosis transmembrane conductance regulator channel gating defect caused by cystic fibrosis mutations. *Mol. Pharmacol.* 68: 1736–1746.
- Dossena, S., V. Vezzoli, N. Cerutti, C. Bazzini, M. Tosco, C. Sironi, S. Rodighiero, G. Meyer, U. Fascio, J. Furst, et al. 2006. Functional characterization of wild-type and a mutated form of SLC26A4 identified in a patient with Pendred syndrome. *Cell. Physiol. Biochem.* 17: 245–256.
- Mason, S. J., A. M. Paradiso, and R. C. Boucher. 1991. Regulation of transepithelial ion transport and intracellular calcium by extracellular ATP in human normal and cystic fibrosis airway epithelium. *Br. J. Pharmacol.* 103: 1649–1656.
- Geiszt, M., J. Witta, J. Baffi, K. Lekstrom, and T. L. Leto. 2003. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J.* 17: 1502–1504.
- Harper, R. W., C. Xu, J. P. Eiserich, Y. Chen, C. Y. Kao, P. Thai, H. Setiadi, and R. Wu. 2005. Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. *FEBS Lett.* 579: 4911–4917.
- Tarran, R., M. E. Loewen, A. M. Paradiso, J. C. Olsen, M. A. Gray, B. E. Argent, R. C. Boucher, and S. E. Gabriel. 2002. Regulation of murine airway surface liquid volume by CFTR and  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductances. *J. Gen. Physiol.* 120: 407–418.
- Deterding, R., G. Retsch-Bogart, L. Milgram, R. Gibson, C. Daines, P. L. Zeitlin, C. Milla, B. Marshall, L. Lavange, J. Engels, et al. 2005. Safety and tolerability of denufosal tetrasodium inhalation solution, a novel P2Y2 receptor agonist: results of a phase 1/phase 2 multicenter study in mild to moderate cystic fibrosis. *Pediatr. Pulmonol.* 39: 339–348.
- Thomas, E. L., and M. Fishman. 1986. Oxidation of chloride and thiocyanate by isolated leukocytes. *J. Biol. Chem.* 261: 9694–9702.
- Slungaard, A., and J. R. Mahoney. 1991. Thiocyanate is the major substrate for eosinophil peroxidase in physiologic fluids: implications for cytotoxicity. *J. Biol. Chem.* 266: 4903–4910.
- van Dalen, C. J., and A. J. Kettle. 2001. Substrates and products of eosinophil peroxidase. *Biochem. J.* 358: 233–239.