Bronchial Epithelial Cell-Derived Prostaglandin E₂ Dampens the Reactivity of Dendritic Cells

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Airway epithelial cells regulate immune reactivity of local dendritic cells (DCs), thus contributing to microenvironment homeostasis. In this study, we set out to identify factors that mediate this regulatory interaction. We show that tracheal epithelial cells secrete soluble factors that downregulate TNF-α and IL-12p40 secretion by bone marrow-derived DCs but upregulate IL-10 and arginase-1. Size exclusion chromatography identified small secreted molecules having high modulatory activity on DCs. We observed that airway tracheal epithelial cells constitutively release the lipid mediator PGE₂. Blocking the synthesis of PGs within airway epithelial cells relieved DCs from inhibition. Cyclooxygenase-2 was found to be expressed in primary tracheal epithelial cell cultures in vitro and in vivo as shown by microdissection of epithelial cells followed by real-time PCR. Paralleling these findings we observed that DCs treated with an antagonist for E-prostanoid 4 receptor as well as DCs lacking E-prostanoid 4 receptor showed reduced inhibition by airway epithelial cells with respect to secretion of proinflammatory cytokines measured by ELISA. Furthermore, PGE₂ mimicked the effects of epithelial cells on DCs. The results indicate that airway epithelial cell-derived PGE₂ contributes to the modulation of DCs under homeostatic conditions. The Journal of Immunology, 2011, 186: 2095–2105.

Because airways are frequently exposed to a variety of inhaled Ags and microbes, local immune reactivity has to be tightly regulated. Recently, the concept emerged that epithelial cells exert simple immune functions (1, 2). Airway epithelial cells express different pattern recognition receptors (3) and respond to microbes by induction of immunological mediators. It has been proposed that epithelial cells create an anti-inflammatory microenvironment that modulates the phenotype of local APCs (4). Similar observations within the intestine support the concept that the local microenvironment of individual organs induces organ-specific immune response, with the degree of microbial contact being an important restriction variable (5).

Respiratory tract dendritic cells (DCs) represent only a small cell population, and different DC subtypes have been identified that can be separated based on their location as well as their function (6). APCs can be activated under different conditions, and at least for macrophages, two seemingly opposing activation modes have been identified. IFN-γ induces classically activated macrophages that are characterized by a vigorous inflammatory response; in contrast, IL-10 and PGE₂ induce alternatively activated macrophages (7). The latter cells express several marker genes, such as chitinase-like lectin Ym1, resistin-like secreted protein Fizz-1, and arginase-1, as well as the anti-inflammatory cytokine IL-10 (8). A similar phenotype exists in tolerogenic DCs, which are immature DCs that are modulated by suppressive factors (9).

Prostanoids are soluble lipid mediators that are produced in two enzymatic steps from C20-unsaturated fatty acids. Cyclooxygenase (Cox)-1 and Cox-2 form the unstable intermediates PGG₂ and PGH₂. PGH₂ is then converted by specific enzymes to the different PGs and thromboxane A₂ (10). PGE₂ has a variety of biological effects, with DCs exposed to PGE₂ having reduced secretion capacity of proinflammatory cytokines (11, 12). Prostanoids are potent immune modulators and their production is tightly controlled to avoid damage of neighboring cells (10). E-prostanoid (EP) receptors mediate the biological effects of PGE₂, and knockout studies have revealed that PGE₂ exerts not only proinflammatory, but also anti-inflammatory actions depending on the specific context (13–15). EP1 and EP3 receptors signal through Ca²⁺ levels, whereas EP2 and EP4 receptors elevate the level of cAMP in cells (10, 13).

In this study, we investigated how airway epithelial cells induce an inhibitory DC phenotype. Specifically, we set out to identify and characterize soluble factors that are secreted by epithelial cells and that downregulate the secretion of proinflammatory cytokines by DCs. We were able to identify PGE₂ as one of those factors and characterized its effects in the interplay of airway epithelial cells with DCs.

Materials and Methods

Reagents and Abs

RPMI 1640 and a 1:1 mix of DMEM/Ham’s F12 medium were obtained from Biochrom (Berlin, Germany). FBS was from BioWest (Nuaille, France). PBS, penicillin, and streptomycin were obtained from PAA (Coelbe, Germany). LPS from Salmonella minnesota was provided by U. Seydel.
(Research Center Borstel, Borstel, Germany). DNase I was from Roche (Mannheim, Germany) and protein A/G Plus-agarose beads were from Santa Cruz Biotechnology (Heidelberg, Germany). Murine Cox-1 and Cox-2 polyclonal Abs and EP antagonists GW 627368X and AH8909 were purchased from Cayman Chemicals (Ann Arbor, MI). The polyclonal Cox-2 Ab used for immunoenzyme staining was from Abcam. IL-10 was from ImmunoTools (Friesoythe, Germany), and the IL-10 blocking Ab as well as CD3 and CD28 Abs were from eBioscience (Frankfurt, Germany).

PGE2 was from Sigma-Aldrich (Taunton, Germany), 2000 (the Cox inhibitors NS-398 and SC-560 were obtained from Merck (Mannheim, Germany). CD4 T cell isolation kit II was from MACS Miltenyi Biotec (Bergisch-Gladbach, Germany).

Preparation of primary airway epithelial cells

The isolation and culture of tracheal epithelial cells were performed with small adaptations as previously described (4). Four- to 10-wk-old female C57BL/6 mice (Charles River Laboratories) were sacrificed by CO2 treatment, and tracheae were prepared and digested with pronase E and DNase I overnight. Cell suspensions were allowed to adhere for 2–3 h in a petri dish at 37 °C. Nonadherent cells were grown for 4–7 d until confluent. This was reached in a Transwell system on collagen-coated membranes (Costar). They were further cultured as air–liquid interface cultures.

Generation and stimulation of bone marrow-derived DCs

DCs were differentiated from bone marrow from 4- to 10-wk-old female BALB/c or C57BL/6 mice as previously described (16). Cultured cells of a GM-CSF–transfected cell line was used as a source of GM-CSF. EP knockout cells were obtained from frozen bone marrow from Maria Belvisi (London). DCs from mice devoid of one of the following genes—Ptger1 (EP1), Ptger2 (EP2), Ptger3 (EP3), or Ptger (DP) —had been backcrossed at least eight times onto the C57BL/6 background. Ptger4−/− (EP4) mice do not survive on the C57BL/6 background due to patent ductus arteriosus, and so they were backcrossed on a mixed background of 129Ola × C57BL/6 mice. Mice were provided by Dr. Shuh Narumiya, Kyoto University, and breeding colonies were maintained at Imperial College, London.

At day 8, 2 × 105 bone marrow–derived DCs (BMDCs) were seeded into 96-well plates and incubated with 50% (v/v) epithelial cell-conditioned medium (ECM). ECM derived of cells pretreated with Cox inhibitors NS-398 (1 μM), SC-560 (1 μM), or with EP antagonists (EP4, GW627368X or EP2, AH8909; 10 μM) was used equally. BMDCs were stimulated with LPS (100 ng/ml) overnight, and the supernatants were analyzed by ELISA (OptEIA ELISA; BD Biosciences). Where indicated, cells were treated with IL-10 (40 ng/ml) or an IL-10–blocking Ab (10 μg/ml).

Measurement of PGE metabolites (PGE2)

PGE2 metabolites were measured in 48-h supernatants of airway epithelial cells with a commercially available PGE metabolite (PGE2) ELISA from Cayman Chemicals. This competitive assay converts the intermediates 13, 14-dihydro-15-keto PGA2 and 13,14-dihydro-15-keto PGE2 into stable derivatives.

Gas chromatography-mass spectrometry/mass spectrometry identification and quantification of PGE2 in cultured epithelial cells

PGE2 from cultured epithelial cells was analyzed by gas chromatography-mass spectrometry/mass spectrometry (GC-MS/MS) as described elsewhere (17, 18). The internal standard [3,3′,4,4′-tetrakis(4-H)]-PGE2 (d4-PGE2; 98 atom% 2H) was added to 0.9-ml aliquots of samples, resulting in a final concentration of 500 pg/ml. Samples were applied directly to 4 ml PGE2-immunoaffinity columns. d4-PGE2 and PGE2-immunoaffinity columns were obtained from Cayman Chemicals. Columns were washed with 2 ml column buffer (0.1 M potassium phosphate buffer [pH 7.4], 3.7 mM NaCl, 0.5 M NaCl), followed by 2 ml distilled water. Compounds were eluted by allowing 2 ml elution solution, consisting of absolute ethanol/diluted water (95:5, v/v) to pass. Solvents were removed under a stream of nitrogen, and the pentafluorobenzyl ester methoxime trimethylsilyl ether derivatives were prepared. GC-MS/MS analyses were performed on a ThermoQuest TSQ 7000 triple-stage quadrupole mass spectrometer interfaced to a ThermoQuest gas chromatograph (Thermodex Trace 7) from ThermoQuest (Geel, Belgium). Fused silica capillary columns (Optima 35MS; 30 × 30.25 mm interior diameter, 0.25 mm film thickness) from Macherey–Nagel (Düren, Germany) were used. The product ions at m/z 268 for PGE2, and m/z 272 for d4-PGE2, which were generated by collision-induced dissociation of the parent ions [M-pentafluorobenzyl]− at m/z 524 and m/z 528, respectively, were monitored in the selected-reaction monitoring mode. The dwell time was 400 ms for each ion. Peak areas were used for calculation of concentrations.

Quantitative RT-PCR

Total RNA was isolated using a High Pure RNA isolation kit (Roche) and was reverse transcribed with a cDNA synthesis kit (Verso cDNA kit; Thermo Fisher Scientific). Two microliters of cDNA (diluted 1:4) was used as template in a total reaction volume of 20 μl (quantitative PCR mix; Abgene) and analyzed on ABI Prism 7700 (Applied Biosystems). Quantifications were made using SYBR Green, including no-template and no-RT controls. Gene expression was measured in duplicates, and automatic detection of baseline and threshold values were subtracted from the Ct values of housekeeping genes (β-actin, GAPDH), resulting in ΔCt for each target gene, which was then used to calculate the relative expression (2−ΔΔCt). All primer sequences are available on request.

Size exclusion chromatography

Forty-eight hour epithelial supernatant was analyzed on an AKTaPrime (Amersham Pharmacia, Uppsala, Sweden) using a 200-kDa Sepharose S-100 column (GE Healthcare) with a pressure of 0.24 MPa and a speed of 1.6 ml/min. To compare and to get a size for the different fractions, we also used the Gel Filtration Calibration Kit LMW (GE Healthcare). The resulting fractions were collected and 50% (v/v) were added to BMDCs, which were then stimulated with LPS.

Western blotting

Protein concentrations of cell lysates were measured by Pierce assay (Thermo Scientific), and equal amounts were fractionated by 12% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. Unspecific binding was blocked with 5% nonfat milk blocked by TBS (pH 7.8) with 5% nonfat milk and 0.05% Tween 20. After blocking, proteins were visualized using specific Abs, HRP-marked secondary Abs, and ECL (Amersham Pharmacia).

Immunoprecipitation of Cox-2

In vitro–cultured epithelial cells were stimulated with 100 ng/ml LPS for 4 h. Cells were harvested, pelleted at 4 °C and lysed in RIPA buffer containing 1 μg/ml proteinase inhibitors (aprotinin, leupeptin, and pepstatin), 1 mM Na3VO4, NaF, and PMSF for 20 min on ice. The lysates were treated with 30 μl protein A/G Plus-agarose beads and 5 μl CoX-2 Ab and incubated at 4 °C in an overhead shaker for 4 h. Beads were washed three times (13,000 rpm, 4°C, 1 min) with RIPA buffer containing 2% SDS. Samples were incubated at 95°C for 5 min, centrifuged again, and the supernatant was taken off and used for Western blot analysis.

Immunoenzyme staining

Immunoenzyme staining of Cox-2 was performed on 4-μm cryostat sections of fresh-frozen tissues, which were postfixed in 2% paraformaldehyde and further processed by the paraformaldehyde–saponin protein precipitation in combination with the standard alkaline phosphatase anti-alkaline phosphatase technique (Dako), as previously published (19). As primary Ab, anti-mouse Cox-2 (1:100) (Abcam) was added overnight at room temperature. Normal rabbit Ig (Dianova) served as negative control. A mouse anti-rabbit mAb (1:50) (Dako), was used as secondary reagent (30 min at room temperature). Naphthol AS/BI phosphate (Sigma-Aldrich) with new fuchsin (Merck, Darmstadt, Germany) served as substrate.

Immunofluorescence

Trachea of female C57BL/6 mice were isolated and immediately fixed in formalin. Paraffin sections (5 μm) were stained after Ag retrieval in steamer with citrate buffer (pH 6.0) for 20 min, cooled down to room temperature, and then incubated with anti-cytokeratin K8 (1:50) mouse Ab (Progen Biotechnik, Heidelberg, Germany). K8 was detected with TSA kit number 23 (Alexa Fluor 546 tyramide; Invitrogen/Molecular Probes, Eugene, OR). This was followed by incubation with Alexa Fluor 488 anti-rabbit (1:200) (Invitrogen). Nuclei were depicted with DRAQ5. Sections were mounted with fluoromount-G (SouthernBiotech).
Laser microdissection and pressure catapulting

Fresh-frozen tracheal samples were cut into 18-μm-thick sections using a cryostat (Leica CM1850; Leica Microsystems, Wetzlar, Germany) and processed as follows. First, the sections were mounted on membrane slides (polyethylene naphthalate membrane, 1 mm glass; Carl Zeiss MicroImaging, Bernried, Germany). For further preservation, samples were fixed in ethanol and stained in cresyl violet acetate (1% [w/v]) in American Chemical Society-grade ethanol; Sigma-Aldrich, Munich, Germany) for 15 s. Subsequently, the slides were washed in ethanol and incubated for 5 min in xylene. After air-drying the slides were mounted on the stage of an inverse microscope, which is a component of a MicroBeam LMPC system (Carl Zeiss MicroImaging). We employed the RoboLPC method to microdissect and capture the tracheal epithelium (~7 mm² epithelium, ∼70,000–200,000 cells). For sample collection, we applied 0.5 ml AdhesiveCaps (opaque) (Carl Zeiss MicroImaging).

RNA isolation of microdissected samples

Total RNA was isolated from each sample using the RNaseasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions by applying 300 μl RLT lysis buffer supplemented with 1% 2-ME (Sigma-Aldrich, Steinheim, Germany). For precipitation, 20 μg glycogen (from mussels; Sigma-Aldrich) per sample was used. The quality of the RNA was assessed on RNA 6000 Nano microfluidics chips (Agilent Technologies, Waldbronn, Germany). For precipitation, 20 μg glycogen (from mussels; Sigma-Aldrich) per sample was used. The quality of the RNA was assessed on RNA 6000 Nano microfluidics chips (Agilent Technologies, Waldbronn, Germany). For precipitation, 20 μg glycogen (from mussels; Sigma-Aldrich) per sample was used. The quality of the RNA was assessed on RNA 6000 Nano microfluidics chips (Agilent Technologies, Waldbronn, Germany).

In vitro proliferation assay

BMDCs (4 × 10⁴) from C57BL/6 mice were incubated with or without ECM 50% (v/v) and stimulated with LPS (100 ng/ml) or left unstimulated overnight. Subsequently, cells were washed three times and pulsed with 100 μg/well EndoGrade OVA (Hyglos) for 3 h and washed three times again. CD4⁺ T cells were isolated via MACS beads from OT-II mice, and 10⁵ T cells were cocultured for 72 h with the pretreated DCs. After 3 d, proliferation of OT-II cells was measured by [³H]thymidine incorporation.

Statistics

All experiments were repeated at least twice unless indicated otherwise. Means + SD are shown. Significant differences were evaluated by the unpaired Student t test with two-tailed distributions; p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***). Results shown are means ± SD from three to five experiments.
type for 48 h after removal of the epithelial supernatant (still up to 40% inhibition of IL-12p40). Using DCs from C57BL/6 mice instead of BALB/c mice we observed the same suppressive activity of the ECM on TNF and IL-12p40 secretion (Fig. 1B). Moreover, we observed that ECM-conditioned cells showed enhanced IL-10 secretion in LPS-stimulated BALB/c and to a lesser extent C57BL/6 DCs (Fig. 1A, 1B).

Furthermore, transcription of some genes, including arginase-1 and the lectin Ym1 (Fig. 1C, 1D), was specifically induced by the treatment with ECM alone. The latter genes have been identified as markers for alternative activation of macrophages (8). The results of the phenotypic analysis of preconditioning of DCs with epithelial cell-derived soluble mediators indicate that DCs are not suppressed globally but instead adopt a phenotype that is characterized by increased anti-inflammatory properties. An important function of DCs is activation of T cells. Whereas LPS-matured, OVA-loaded DCs showed high induction of OT-II T cell proliferation (Fig. 1E), this capacity was reduced for ECM DCs. Because ECM-treated DCs showed increased IL-10 secretion, we included a role of DC-derived autocrine IL-10 by adding a blocking IL-10 Ab in BALB/c DCs (Fig. 1F). Exogenous added IL-10 was entirely blocked in its inhibitory actions on IL-12p40 secretion. However, ECM conditioning was still effective in suppressing IL-12p40, showing that IL-10 does not play a major role in this context. Similar results were observed with C57BL/6 DCs (data not shown).

The inhibitory capacity of epithelial supernatant is mediated by multiple soluble factors

Because DCs could be modulated by epithelial cell-conditioned medium, we concluded that soluble factors contribute to the modulation of DC reactivity. Because epithelial cells did not produce IL-10 (data not shown), and because we already excluded IL-10 as a mediator (Fig. 1F), we next wanted to investigate the influence of other soluble factors. We first analyzed the supernatant via size exclusion chromatography, thus separating molecules in molecular mass range of 1–200 kDa. Fractions were tested for modulation of IL-12p40 secretion (and TNF-α, data not shown) from LPS-stimulated BMDCs. We identified at least four different fractions that contained inhibitory activity (Fig. 2A). Of note, small molecules <6.5 kDa showed considerable if not highest inhibitory activity. In fact, targeted separation of molecules that were >5000 Da showed reduced inhibition of DC reactivity as compared with the nonseparated ECM (Fig. 2B). Proteinase K digestion did not significantly reduce the modulation by ECM (data not shown), which prompted us to conclude that nonproteinaceous small compounds are constitutively released by airway epithelial cells and modulate DCs.

FIGURE 2. Epithelial cell supernatant contains different soluble factors with DC inhibitory capacity. A, FPLC analysis via Sephacryl S-100 column from 48 h supernatant derived from primary tracheal epithelia cells. BMDCs (2 × 10⁵) were incubated with 50% (v/v) supernatant of the different fractions and stimulated with LPS (100 ng/ml) overnight. IL-12p40 production was measured by ELISA. The gray shadowed region indicates the mean + SD of LPS control stimulation without ECM. B, Forty-eight hour supernatant was concentrated with a 5000 Da cut-off column. Concentrated fractions were incubated with BMDCs as indicated above and stimulated with LPS (100 ng/ml) overnight. Supernatants were analyzed by IL-12p40 ELISA. C and D, Forty-eight hour supernatants were qualitatively analyzed via GC-MS/MS chromatography against an internal standard (d₄-PGE₂) on a Thermo TSQ 7000 triple-stage quadrupole mass spectrometer interfaced with a ThermoQuest gas chromatograph model Trace 2000. Results of A show representative data for three independent experiments, and results in B show representative data for two independent experiments.
PGs are bioactive lipid mediators capable of inhibiting the secretion of proinflammatory cytokines from BMDCs (11, 12). We therefore decided to directly test ECM for the presence of PGs by GC-MS/MS. After specific extraction by immunoaffinity chromatography, presence of PGE2 was identified in epithelial supernatant. Because of the keto group of PGE2, methoximation yields two methoxyamine isomers that have distinct chromatographic and MS/MS properties. The GC-MS/MS spectra of both peaks (Fig. 2C, 2D) obtained from the PGE2 in the supernatant were identical with those obtained from synthetic PGE2 (17). Thus, PGE2 was identified in epithelial cell-conditioned medium from nonstimulated ex vivo cultured tracheal epithelial cells.

**Resting primary tracheal epithelial cells produce PGE2 in vitro**

Previous publications showed that intestinal epithelial cells (20) and human airway epithelial cells are capable of producing PGE2 in vitro and in vivo (21, 22). Having observed that murine primary epithelial cells cultured in air–liquid interface cultures are capable of producing PGE2 in vitro (Fig. 2C, 2D), we quantified the amounts of PGE2 that are released by those cells (Fig. 3A, 3B). Using GC-MS/MS chromatography and analyzing 48 h epithelial supernatants, airway epithelial cells secreted between 950 and 1600 pg/ml bioactive PGE2 in our experimental setting. In contrast, no significant PGE2 was detected in normal culture medium. Further experiments revealed that there was a time-dependent accumulation of PGE2 in the supernatant with higher production after 48 h (1500 pg/ml) compared with 16 h supernatant (500 pg/ml) (Fig. 3C). This parallels the observation that ECM collected after 48 h has superior inhibitory capacity compared with earlier or later time points (data not shown). Next, we compared dose-dependency of ECM conditioning (Fig. 3D) to direct administration of PGE2 (Fig. 3E) on LPS activation of DCs. ECM con-

**FIGURE 3.** Tracheal epithelial cells produce PGE2 in vitro. A, Forty-eight hour supernatant from different epithelial cell preparations was tested for PGE2 production via GC-MS/MS after immunoaffinity chromatography using an internal standard (d4-PGE2). B, One representative result of A is shown as a chromatogram. C, PGE2 concentrations of supernatants from different ages were analyzed as in A. D, BMDCs (2 × 10^5) were incubated with different amounts of 48 h supernatant of epithelial cells (70–12.5% [v/v]) or (E) PGE2 (0.05–500 ng/ml) and stimulated overnight with LPS (100 ng/ml). Supernatants were analyzed for secretion of IL-12p40 ELISA. F, BMDCs (8 × 10^5) were seeded in a 24-well plate and incubated with ECM (50% [v/v]) or PGE2 (50 ng/ml) overnight and stimulated with LPS (100 ng/ml) or left unstimulated for 4 h. Cells were lysed and analyzed for gene expression of arginase-1 by quantitative RT-PCR. rE, relative expression. G, Four different preparations of ECM were measured by PGEM ELISA. BMDCs (2 × 10^5) were incubated with 50% (v/v) of these supernatants and stimulated with LPS (100 ng/ml) overnight. IL-12p40 production was measured and normalized against the LPS control (100%). pEp, primary epithelial cells. Results in A, C, F, and G show data representative for two independent experiments, and results in D and E show data representative for four independent experiments.
ditioning with 12.5–70% (v/v) dose-dependently inhibited IL-12p40 secretion (and TNF-α, data not shown) by DCs. Similarly, PGE₂ inhibited the activation of DCs as shown by diminished TNF-α and IL-12p40 secretion in concentrations between 0.05 and 500 ng/ml. Thus, the inhibitory capacity of ECM when calculated in comparison with the administration of pure PGE₂ was slightly more effective, confirming our observation that additional factors are operative. To further examine the role of PGE₂ in the interplay of epithelial cells with DCs, we analyzed genes that had been shown to be specifically regulated in DCs when conditioned with ECM. We observed that similar to the activity of ECM, PGE₂ also upregulated expression of arginase-1 (Fig. 3F). Furthermore, we examined different epithelial cell supernatants that varied in content of PGE₂ and analyzed their inhibitory capacity on DC-mediated IL-12p40 secretion. We observed a strict correlation between inhibition of IL-12p40 secretion and concentration of stable PGEMs within ECM. These results confirm our notion that PGE₂ or its metabolites contribute to the modulation of DC activation by airway epithelial cells.

**Epithelial cell-mediated inhibition of DCs depends on Cox activity**

To further investigate and confirm the role of epithelial cell-derived PGE₂, we blocked the synthesis of PGs by inhibition of the two isoforms of Cox, Cox-1 and Cox-2, with specific inhibitors. Pretreatment of epithelial cells with SC-560 or NS-398, specific inhibitors for Cox-1 and Cox-2, respectively, resulted in a marked reduction of PGE₂ in the epithelial supernatant (Fig. 4A) as determined by GC-MS/MS (Fig. 4B). Applying both inhibitors resulted in a near complete inhibition of PGE₂ secretion. We then incubated DCs with supernatants of epithelial cells that had been blocked by SC-560 or NS-398 and stimulated the DCs with LPS. Whereas untreated ECM inhibited IL-12p40 secretion to ~22% of LPS control, ECM derived from NS-398–treated cells showed a marked reduction in inhibitory capacity (90%) (Fig. 4C). In contrast to Cox-2 inhibition, inhibition of Cox-1 by SC-560 was less effective with respect to de-repression of DC activity (55%). Applying higher concentrations of the Cox-1 inhibitor did not result in increased relief of repression (data not shown). Treatment of the epithelial cells with both inhibitors did not result in a more pronounced de-repression of DC activation (data not shown). Moreover, none of the inhibitors affected reactivity of DCs alone (Fig. 4C and data not shown), allowing us to conclude that the observed effects were due to the action of Cox inhibitors on the epithelial cells. Again, we also examined markers that were specifically induced in DCs upon ECM conditioning. Confirming the above findings, ECM treatment increased LPS-mediated IL-10 secretion in DCs, and this increase was inhibited or reduced when using ECM derived from NS-398– or SC-560–pretreated epithelial cells (Fig. 4D). The data allow for the conclusion that functional Cox isoforms (resulting in production of PGE₂) within epithelial cells are necessary to induce the modulating effects of ECM on LPS-activated DCs.

**FIGURE 4.** Blocking cyclooxygenases diminishes the inhibitory capacity of epithelial cell supernatant. A and B, Epithelial cells were treated with either 1 μM NS398 (Cox-2 inhibitor) or 1 μM SC560 (Cox-1 inhibitor) overnight. Supernatants were harvested and concentrations of PGE₂ were measured via GC-MS/MS chromatography using an internal standard (d₄-PGE₂). pEp, primary epithelial cells. C and D, BMDCs (2 × 10⁵) were incubated with 50% (v/v) supernatants after epithelial cell pretreatment as in A and stimulated overnight with LPS (100 ng/ml). BMDCs were analyzed for secretion of IL-12p40 (C) and IL-10 (D) by ELISA. Results in A and B show data representative for two independent experiments, and results in C and D show data representative for three independent experiments.
Cox isoforms are constitutively expressed in resting tracheal epithelial cells

In most tissues Cox-1 is the constitutive form of the cyclooxygenases expressed under homeostatic conditions, and Cox-2 is only induced during infections or after stimulation of the cells (23). However, our data obtained so far, as well as single reports in the literature (24, 25), suggested that Cox-2 might also be expressed constitutively within airway epithelial cells. Therefore, we investigated constitutive expression of Cox-2 within the used primary tracheal epithelial cells in more detail. We first analyzed the gene expression levels of Cox-1 and Cox-2 in primary cultures of epithelial cells by quantitative RT-PCR. We observed expression of Cox-1 as well as Cox-2 mRNA in different epithelial cell preparations (Fig. 5A). Expression levels of Cox-2 were even slightly higher as compared with Cox-1. None of these epithelial cell cultures showed contamination with myeloid cells as determined by absence of CD11b expression (data not shown). Furthermore, we detected expression of Cox-1 and Cox-2 protein by immunoprecipitation in untreated cultures of tracheal epithelial cells (Fig. 5B, 5C). Stimulation of epithelial cells with LPS, although being effective with respect to increased IL-8 secretion (data not shown), did not affect protein expression of Cox-2.

We next wanted to verify that constitutive in vitro expression of Cox-2 is not a consequence of culture conditions but in fact reflects the physiological in vivo situation. We first tried to confirm Cox-2 expression by immunofluorescence in murine tracheae of C57BL/6 mice. Although we observed strong expression of Cox-1 in epithelial cells (marked by K8 cytokeratin, Fig. 5D), as well as in macrophages, we hardly detected any constitutive expression of Cox-2 in epithelial cells (data not shown). However, using a different Ab for Cox-2 and applying immunoenzyme staining we detected constitutive Cox-2 protein expression in tracheal epithelial cells (Fig. 5E, control with IgG in Fig. 5F). Tissue of a colon carcinoma stained with the Cox-2 Ab served as positive control (data not shown). To further confirm our findings we decided to use an additional approach for detecting Cox-2 expression in untreated epithelial cells. We prepared tracheal samples from untreated mice and microdissected epithelial tissue using laser capture microscopy. Frozen tracheae were cut into pieces and epithelial cells were stained, successfully cut out with the RoboLPC method (Fig. 5G), and total RNA was isolated. RNA integrity was sufficient (RNA integrity number 5.7) to analyze the samples by quantitative RT-PCR. By measuring gene expression levels of the different isoforms of Cox, we detected high expression levels of Cox-1 in resting epithelial cells comparable to those from in vitro-cultivated epithelial cells (Fig. 5H). Purity of the samples was proven by lack of CD11b expression and by detection of cystic fibrosis transmembrane conductance regulator. Moreover, we also detected high gene expression of the microsomal PGE synthase 1, which is coexpressed with Cox-2 (27). With respect to expression of Cox-2, we confirm that this isoform was expressed in microdissected, pure epithelial samples from murine tracheae in similar amounts as observed for in vitro-cultivated cells. Overall, the data strongly indicate that murine tracheal epithelial cells constitutively express Cox-1 and Cox-2 as well as the further enzyme machinery to produce PGE2 without any specific stimulation.

Airway epithelial cells modulate DCs via the PGE2/EP4 axis
PGE2 signals via the E-PG receptors EP1–4. The receptors are widely distributed in different tissues, including hematopoietic...
cells (13). Assuming that epithelial cell-derived PGE₂ drives DCs to adopt a noninflammatory phenotype, we hypothesized that EP receptors should be activated in the ECM DCs. We first verified expression of EP1–4 within DCs by quantitative RT-PCR (Fig. 6A). We observed that all four receptors were expressed, with EP2 and EP4 showing the highest mRNA expression levels, followed by EP3 and EP1. Expression did not change much upon stimulation of the cells with LPS (despite a small decrease for EP2 expression), preconditioning with ECM, or application of PGE₂.

To block the signaling of epithelial cell-derived PGE₂ in DCs, we next used specific antagonists for EP2 and EP4 receptors, which had been linked to PGE₂-mediated modulation of DC function previously (27). Preincubating DCs with the specific antagonists GW 627368X (EP4) or AH 6809 (EP2) and subsequent conditioning with epithelial supernatant diminished the inhibitory capacity of the supernatant on DCs when blocking EP4 but not EP2 (Fig. 6B). EP4 antagonist increased the production of IL-12p40 from 13 ng/ml in ECM-blocked DCs to nearly 50 ng/ml. However, release of inhibition did not result in complete derepression (LPS, 120 ng/ml IL-12p40). The little increase of IL-12p40 secretion in DCs was observed after the treatment with the EP2 antagonist together with ECM was not significant. Importantly, treatment of nonconditioned DCs with either of the antagonists did not affect IL-12p40 secretion by LPS, thus ruling out a role of DC-derived PGs. Combinations of the antagonist were not more effective (data not shown).

To confirm these findings, we made use of DCs from knockout mice for all four individual EP receptors (Fig. 6D). In comparison with wild-type mice, LPS-stimulated DCs from EP1 and EP4 knockout mice secreted slightly more TNF-α; however, those differences were not significant. ECM conditioning resulted in a strong reduction of TNF-α in wild-type mice as observed before. In EP1, EP2, and EP3 knockout DCs, ECM similarly and significantly inhibited TNF-α secretion. This inhibition was reduced only in EP4 knockout mice. However, even EP4 knockout mice still showed residual inhibition, again confirming that additional inhibitory factors are operative. Interestingly, we observed no significant increase of IL-12p40 in the EP4 receptor knockout cells (data not shown). Again, we also analyzed expression of the ECM-regulated marker arginase-1 in the EP knockout DCs. In DCs of wild-type mice, arginase-1 expression was increased by ECM conditioning but not by LPS stimulation. Differences were only observed in EP4 knockout mice: in those DCs, ECM did not upregulate arginase-1 expression anymore (Fig. 6E).

Taken together, these results confirm that tracheal epithelial cell-derived PGE₂ acts on DCs via the EP4 receptor and that PGE₂–EP4 signaling contributes to downregulation of proinflammatory cytokines and upregulation of arginase-1 in DCs.

**Discussion**

Over the years, the prevailing view of the immunological function of airway epithelial cells had been that they build up a tight barrier and physically exclude inhaled microbes. However, it has now been convincingly shown that epithelial cells express pattern rec-
ognition receptors found otherwise in professional innate immune cells. Thus, airway epithelial cells are able to sense infectious danger and actively contribute to the induction of immune responses (1, 2). As the airways encounter dangerous as well as harmless microbes, mucosal immune recognition has to be tightly regulated. Indeed, a number of reports show that intestinal epithelial cells (28, 29) but also bronchial epithelial cells (4, 30) modulate the activation of local professional immune cells. However, the molecular mechanisms that mediate the interplay between epithelial and hematopoietic cells are only beginning to be understood.

Analyzing epithelial cell-derived factors that contribute to the generation of a specific immunological microenvironment in the airways was aim of this study. We used an established experimental approach (30) that examined the effects of soluble epithelial cell-derived factors on BMDCs. We can definitively show that epithelial cells, through the constitutive secretion of PGE2, drive DCs to adopt an anti-inflammatory phenotype. DC populations in general show tremendous heterogeneity and exist in many phenotypes that adapt to the local microenvironment (5, 30). Intestinal epithelial cells are known to inhibit DC activation, resulting in low MHC class II and CD86 expression as well as reduced secretion of proinflammatory cytokines (28, 29). In a similar manner, we previously observed that airway epithelial cells regulate DCs to adopt an inhibitory phenotype indicated by diminished IL-12p40 and TNF-α production and downregulation of costimulatory molecules (CD40, CD86) (4). In this study, we show that soluble factors that are secreted constitutively by epithelial cells are sufficient to achieve this specific DC phenotype. Moreover, we report that epithelial cell-conditioned DCs are not globally inhibited but instead modulate their gene expression profile with upregulation of certain genes (arginase-1, IL-10, Ym1). ECM DCs therefore resemble alternatively activated macrophages (8). Alternatively activated DCs produce high amounts of IL-10 and expand regulatory T cells (31, 32).

Our phenotypic characterization of ECM DCs allows for the speculation that airway epithelial cells modulate local DCs toward an alternatively activated, tolerogenic state similar to a report on intestinal cells (9). In support of such a model, preliminary results show that purified primary airway DCs have high expression of Ym1 and arginase-1 and secrete less IL-12 (data not shown). Moreover, ECM DCs were less effective in inducing T cell proliferation. Previously it was shown that ECM drives induction of Ym1 and arginase-1 and secrete less IL-12 (data not shown).

We show that Cox-2–mediated PGE2 derived from airway epithelial cells, but Cox-2 is considered to be inducibly expressed during inflammation and infection (23). By blocking the activity of Cox enzymes in epithelial cells, we observed that mainly the Cox-2 inhibitor affected epithelial modulation of DCs. In line with this, some reports claimed a “quasiconstitutive” expression of Cox-2 in resting lung epithelial cells (42). Our own results support the notion that there is basal expression of Cox-2 under resting conditions in airway epithelial cells. Although inducible Cox-2 is implicated to play a role in cytokine-induced inflammation, other reports indicate that Cox-2 also affects conditions beyond inflammation. Thus, specific inhibition of Cox-2 resulted in increased airway inflammation and airway hyperresponsiveness (43). In the gastrointestinal tract Cox-2 is suggested to play a homeostatic role because Cox-2 inhibition resulted in enhanced colonic mucosal injury (15, 44). Cox-2 has been suggested to be preferred over Cox-1 in PGE2 synthesis under conditions of low arachidonic acid concentrations (45).

We show that Cox-2–mediated PGE2 derived from airway epithelial cells affects DC reactivity. PGE2 signals mainly via four different G protein-coupled EP receptors, that is, EP1–4 (10, 13). BMDCs express all four different receptors, and EP2 and EP4 are important receptors in PGE2-mediated DC signaling (27). Inhibiting EP2 and EP4 receptors on DCs and using EP receptor knockout mice, we identify EP4 to play an exclusive role in epithelial cell-mediated inhibition of DCs. In line with our findings, the PGE2/EP4 axis is important for gut mucosal integrity (15) and homeostatic regulation of vascular remodeling in airway inflammation (46). It was also shown recently that PGE2 inhibits IFN-α secretion and Th1 costimulation in plasmacytoid DCs via EP2 and EP4 engagement (47). However, in a model of murine contact hypersensitivity, PGE2–EP3 signaling exerted an anti-inflammatory signal on keratinocytes, which argues for organ-specific adaptions (48). In T cells PGE2–EP2/EP4 signaling was shown to promote Th1 and TH17 differentiation, and therefore cell-specific adaptations might add further complexity (49). Indeed, previous work of our own showed increased production of IL-17 by T cells directly treated with ECM (4). EP4 signaling is coupled to cAMP production, which has inhibitory effects in DCs.
Also, cAMP is currently seen as a universal regulator for innate immune functions by enhancing the production of IL-10 or the negative regulator suppressor of cytokine signaling 3 (SOCS-3). For the factors that were shown to be inhibited in this study, it has been reported that IL-12p40 contains a PGE2-responsive repressor element (52).

PGE2 is not the only prostanoi found in the microenvironment of the airways. Under inflammatory conditions, leukotriene B4 and under asthmatic conditions PGD2 have been reported (53, 54). It is also known that the crosstalk between PGE2 and leukotriene B4 regulates the phagocytic activity of alveolar macrophages, and leukotriene B4 opposes the immunosuppressive effect of PGE2 (55). Thus, it might well be that during infectious diseases the functions of PGE2 within the airway microenvironment change.

Taken together, we show that airway epithelial cell-derived PGE2 modulates DCs. As a result, PGE2–EP4 receptor signaling generates DCs with reduced proinflammatory properties, thereby limiting DC activation. Local immunity might therefore be shaped and fine-tuned by epithelial cells to serve the organ-specific needs.

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Disclosures
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