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Bronchial Epithelial Cells Induce Alternatively Activated Dendritic Cells Dependent on Glucocorticoid Receptor Signaling

Michael Weitnauer,* Lotte Schmidt,*,† Nathalie Ng Kuet Leong,* Stephanie Muenchau,* Felix Lasitschka,† Volker Eckstein,‡ Sabine Hübner,§ Jan Tuckermann,§ and Alexander H. Dalpke*

Airway epithelial cells mount a tolerogenic microenvironment that reduces the proinflammatory potential of respiratory dendritic cells (DCs). We recently demonstrated that tracheal epithelial cells continuously secrete soluble mediators that affect the reactivity of local innate immune cells. Using transcriptional profiling, we now observed that conditioning of DCs by tracheal epithelial cells regulated 98 genes under homeostatic conditions. Among the most upregulated genes were Ms4a8a and Ym1, marker genes of alternatively activated myeloid cells. Ex vivo analysis of respiratory DCs from nonchallenged mice confirmed a phenotype of alternative activation. Bioinformatic analysis showed an overrepresentation of hormone-nuclear receptors within the regulated genes, among which was the glucocorticoid receptor. In line with a role for glucocorticoids, pharmacological blockade as well as genetic manipulation of the glucocorticoid receptor within DCs inhibited Ms4a8a and Ym1 expression as well as MHC class II and CD86 regulation upon epithelial cell conditioning. Within epithelial cell–conditioned medium, low amounts of glucocorticoids were present. Further analysis showed that airway epithelial cells did not produce glucocorticoids de novo, yet were able to reactivate inactive dehydrocorticosterone enzymatically. The results show that airway epithelial cells regulate local immune responses, and this modulation involves local production of glucocorticoids and induction of an alternative activation phenotype in DCs. The Journal of Immunology, 2014, 193: 1475–1484.

Human lungs are ventilated by several thousand liters of air every day, and the lung frequently contacts airborne microbes or microbial substances with proinflammatory potential. To avoid detrimental inflammatory processes under homeostatic conditions, a specific respiratory microenvironment exists that controls and limits inflammatory reactivity (1–5). Thus, stromal nonprofessional immune cells regulate organ-specific immunity (6). Disturbances of this state of physiological hyporesponsiveness might give rise to several pathologies including allergies, asthma, or cystic fibrosis, finally leading to pulmonary remodeling processes and loss of modulatory function (7, 8).

At mucosal surfaces, epithelial cells form a tight physical barrier that serves as a first line of defense (9). However, during recent year, a more sophisticated role of epithelial cells in organ-specific immunity has been elucidated (4, 10). Accordingly, epithelial cells are able to produce immune-modulatory substances; examples are retinoic acid or thymic stromal lymphopoietin (TSLP), which directly act on local professional immune cells. Whereas epithelial cells were often considered to suppress reactivity of dendritic cells (DCs) or T lymphocytes, it is now clear that such a view underestimates the immunological function of mucosal epithelial cells. Instead, it seems that mucosal epithelial cells in general and airway epithelial cells specifically shape and modulate the responses of local immune cells, thus setting up an organ-specific microenvironment (6). However, knowledge about the direct crosstalk between respiratory epithelial cells and DCs is still limited.

Recently, we and others (2, 11, 12) demonstrated that respiratory epithelial cells are able to suppress proinflammatory cytokine secretion of LPS-stimulated DCs through the constitutive production of PGE2, TSLP, macrophage-derived chemokine, or thymus- and activation-regulated chemokine. Other soluble, epithelial cell–derived factors with immune-modulatory potential have been described (1, 13, 14). However, most of the studies analyzed the response of epithelial cell–conditioned immune cells after challenge with stimulatory substances. We speculated that epithelial cell–mediated conditioning of DCs might change the phenotype of the latter cells even under homeostatic conditions without further challenge. Therefore, the aim of this study was to evaluate the crosstalk between tracheal epithelial cells and DCs to identify new epithelial cell–derived immune-modulating factors that contribute to the definition of a specific airway microenvironment.

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). FCS was from Life Technologies/Life

Abbreviations used in this article: BMDC, bone marrow–derived DC; COX, cyclooxygenase; Ct, cycle threshold; ECCC, epithelial cell–conditioned medium; GR, glucocorticoid receptor; MHC II, MHC class II; NR, nuclear hormone receptor; qPCR, quantitative PCR; TrEC, murine tracheal epithelial cell; TSLP, thymic stromal lymphopoietin.

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Preparation of primary airway epithelial cells
Isolation and culture of murine tracheal epithelial cells (mTECs) were performed as previously described (2). Briefly, 8–10-week-old female C57BL/6 mice (Charles River Laboratories, Salzfeld, Germany) were sacrificed by CO2 asphyxia, and tracheae were dissected and digested with Pronase E and DNAseI overnight at 4°C. Cell suspensions were allowed to adhere for 2 to 3 h in a Petri dish at 37°C in culture medium (DMEM/ Ham’s F12 1:1 medium mix, completed with 1% penicillin/streptomycin, 5% heat-inactivated FCS, and 120 IU/l human insulin). Nonadherent cells were removed and then grown for 4–7 d until confluence was reached in a Transwell system on collagen-precoated membranes (Corning). Cells were further differentiated for 4 wk by cultivation in air–liquid interface cultures in Ultrasor Medium (DMEM/Ham’s F12 1:1 medium mix, completed with 1% penicillin/streptomycin and 2% UltrasorG serum supplement). For generation of epithelial cell–conditioned medium (ECCM), medium was changed to culture medium and collected after 48 h.

Generation and stimulation of bone marrow–derived DCs
DCs were differentiated from bone marrow from 4–10-week-old female C57BL/6 mice as previously described (2). Culture supernatant of a GM-CSF–transfected cell line was used as a source of GM-CSF. GM-CSF mice were described previously (15). At day 8, 2 × 10^5 bone marrow–derived DCs (BMDCs) were seeded into 48-well plates and incubated with 50% (v/v) ECCM. ECCM derived of cells pretreated with cycloxygenase (COX) inhibitor Diclofenac (25 μM) was used equally. BMDCs were stimulated with LPS (100 ng/ml) overnight, and the supernatants were analyzed by ELISA. Where indicated, DCs were treated with Dexamethasone (10 μM) or RU486 (1 μM).

Gene expression profiling
For microarray analysis, DCs were generated as described above. After differentiation, cells from two C57BL/6 mice were pooled, and 8 × 10^6 cells were seeded into 24-well plates and used in four replicates. Cells were conditioned with 50% (v/v) ECCM (from one batch) overnight, and the next day, cells were stimulated for 4 h with LPS (100 ng/ml) or left unstimulated. Afterwards, cells were lysed, and RNA was isolated using the High Pure RNA Isolation Kit (Roche). RNAs (at least 50 ng/μl) were then sent to the Genome Core Facility of the German Cancer Research Center Heidelberg, where Illumina Mouse Sentrix-8x2 BeadChip assays were performed. RNA quality was checked by RNA gel analysis using the total RNA Nano chip assay on Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with RNA index values >8.5 were selected for expression profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). A total of 250 ng total RNA was used for cDNA synthesis, followed by an amplification/labeling step (in vitro transcription) to synthesize biotin-labeled cRNA according to the MessageAmp II aRNA Amplification kit (Ambion, Austin, TX). Biotin-16-UTP was purchased from Roche Applied Science (Penzberg, Germany). The cRNA was column purified using the TotalPrep RNA Amplification Kit and eluted in 60 μl water. Quality of cRNA was controlled using the RNA Nano Chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified spectrophotometrically. Microarray scanning was done using a Beadstation array scanner, with settings adjusted to a scaling factor of 1 and PMT settings at 430. Data extraction was done for all beads individually, and outliers were removed when fold deviation was >2.5. All remaining data points were used for the calculation of the mean average signal for a given probe, and SDs were calculated for each probe. Raw data were statistically analyzed using Chipster software (www.chipster.csc.fi) for array analysis, creation of a Venn diagram, and pathway analysis. Data were normalized using quantile normalization within the program, multiple test correction (Benjamini-Hochberg) was done, and only probe sets that had a p value ≤0.05 and a fold change ≥2 or ≤0.5 were further investigated. The microarray data from this publication have been submitted to the ArrayExpress database (www.ebi.ac.uk/arrayexpress) and assigned the identifier E-MTAB-2517.

Isolation of tissue DCs
Lung was perfused with 10 ml PBS/heparin by cannulating the right ventricular chamber. Lung tissue was dissected and cut into small pieces. To generate single-cell suspensions, tissue was digested with bicine (100 μg/ml), collagenase XI (250 μg/ml), hyaluronidase (1 mg/ml), and DNAse I (200 μg/ml) for 45 min at 37°C in RPMI 1640 (10% FCS). Mononuclear cells were isolated by removing the upper phase after differential centrifugation of single-cell suspensions (15 min, 1300 rpm, 4°C) using Pancoll mouse (PAA Laboratories). For Isolation of CD11c-positive cells, DC11c microbeads were used (Miltenyi Biotec), and isolation was performed according to protocol. FACS staining of the cells was performed with the desired Abs for 30 min at 4°C. Analysis was performed at the FACS Core Facility of Department of Medicine V, University Hospital Heidelberg. Purity of the samples was always >90%.

Quantitative RT-PCR
Total RNA was isolated usingpeqGOLD (Peqlab Biotechnology, Erlangen, Germany) total RNA Kit and reverse transcribed with a cDNA synthesis kit (Verso cDNA kit; Thermo Fisher Scientific) using random hexamers. Two microletters cDNA (diluted 1:5) was used as template in a total reaction volume of 15 μl (quantitative FastSYBRGreen Mastermix; Applied Biosystems, Foster City, CA) and analyzed on an ABI StepOnePlus or 7900HT Fast Real-Time PCR System (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 was used. Cycle threshold (Ct) values were subtracted from the Ct values of housekeeping genes (Actb, 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.

Immunohistochemistry staining
Immunoenzyme staining of 11β-HSD1 was performed on μm paraformaldehyde fixed murine lung sections. After deparaffinization and rehydration of sections, samples were boiled at high pressure in 10 mM citrate (pH 6) using a steamer, peroxidase blocked (LSAB+ Kit; DakoCytomation, Hamburg, Germany) for 15 min. and BSA blocked (4% in PBS, room temperature) for 30 min. Primary Ab anti–11β-HSD1 (1:50) was added overnight at 4°C. Visualization was performed using the LSAB+ Kit according to the manufacturer’s protocol.

Statistics
All experiments were repeated at least three times unless indicated otherwise. Means ± SD are shown. Significant differences were evaluated by the unpaired Student t test with two-tailed distributions or ANOVA with Bonferroni posttest if multiple comparisons were performed using GraphPad Prism (GraphPad). Statistical tests on quantitative PCR (qPCR) data were performed on log-normalized data to produce normal distributed data. The p values <0.05 (*) were considered significant.

Results
Airway epithelial cells induce a phenotype of alternative activation in DCs
Recently, our group showed that airway ECCM induces a tolerogenic phenotype in immune cells, including reduced T cell responsiveness (1, 2) and decreased production of proinflammatory cytokines IL-12p40 and TNF-α after LPS stimulation of BMDCs (Fig. 1A) (2). To further investigate the crosstalk between epithelial cells and DCs, especially under unstimulated, homeostatic conditions, we performed expression profiling comparing unchallenged BMDCs, BMDCs treated with ECCM, and LPS-stimulated BMDCs as well as LPS-stimulated ECCM DCs. (Fig. 1B, 1C). LPS stimulation regulated 1197 gene probes, and of those, 391 (roughly one-third) were not regulated anymore when DCs were preconditioned with ECCM. We thus confirm that epithelial cell conditioning has an enormous impact on the responsiveness of DCs to a subsequent
infection challenge, mimicked by LPS treatment. Of note, and of utmost interest for our actual working hypothesis, the epithelial conditioning process itself already changed the gene expression pattern of DCs. In total, 98 genes were at least 2-fold regulated, of which 57 were induced and 41 were repressed after overnight incubation with ECCM (Table I). Pathway analysis revealed that among the regulated genes, immune response and regulation of NF-κB cascade processes were significantly overrepresented (Fig. 1C). Strikingly, the two most strongly induced genes were Ms4a8a (18.9-fold) and Ym1 (18.5-fold) (Table I), both of which are known to identify alternatively activated M2 macrophages (16, 17). We verified by qPCR that ECCM induced exemplary genes, including Ms4a8a and Ym1 (Fig. 1D). In line with this, Flt3L-derived BMDCs also showed increased levels of Ms4a8a and Ym1 expression after ECCM treatment, indicating that various DCs can express M2 macrophage marker genes (data not shown). Moreover, we also used supernatant of nonepithelial cells (NIH3T3 fibroblasts) and observed no relevant induction of Ms4a8a and Ym1 (Fig. 1E). Thus, tracheal epithelial cell conditioning specifically induced genes as-sociated with an alternatively activated DC phenotype. In the same line, we have previously shown that ECCM-treated DCs produce more IL-10 and present less MHC II and CD86 on their surface after LPS stimulation (1, 2).

Primary respiratory DCs express markers of alternative activation

To verify that the phenotype of DCs conditioned in vitro by epithelial cells reflects the phenotype of primary respiratory DCs, we ex vivo sorted DCs from murine lungs and analyzed them for expression of genes, previously identified to be regulated by tracheal epithelial cells. We sorted DCs for CD103 and CD11b expression (Fig. 2A) and compared expression of Ms4a8a and Ym1 in these cells to those from splenic DCs (Fig. 2B, 2C). All DCs expressed both genes but CD11b+ pulmonary DCs had a significantly higher expression of Ms4a8a and Ym1. In line with these findings, we could detect higher Ms4a8a protein levels in lung CD11b+ DCs compared with spleen DCs (Fig. 2D). Moreover, alveolar macrophages also expressed high levels of Ms4a8a (data not shown). We therefore confirmed that ex vivo respiratory CD11b+ DCs also resemble alternatively activated DCs. We hypothesize that airway epithelial cells contribute to this specific respiratory phenotype in vivo.

Lipophilic epithelial-derived factors induce alternative activation in DCs

As we recently showed an involvement of epithelial cell derived PGE2 in the reduction of DC responsiveness to LPS stimulation, we speculated that soluble factors might also be responsible for the upregulation of Ms4a8a and Ym1. To further characterize such factors, we extracted lipophilic substances from either ECCM or control medium using ethylacetate. Afterwards, we incubated BMDCs with complete medium or ethylacetate extracts from ECCM and analyzed Ms4a8a and Ym1 expression as well as LPS-induced IL-12p40 secretion of these cells (Fig. 3A, 3B). Confirming our previous results for lipophilic PGE2, we show that the lipophilic fraction mediated the suppressive effect of ECCM on
LPS-induced IL-12 within DCs. Moreover, we observed that the lipophilic extract was also potent enough to induce Ms4a8a and Ym1 expression similar to complete medium. Based on our previous results, we analyzed whether epithelial cell–derived PGs might also explain the induction of Ms4a8a and Ym1 in conditioned DCs. To test if COX-derived PGs played a role, we treated tracheal epithelial cells with the unselective COX1/2 inhibitor diclofenac and tested the supernatant of these cells for the potential to directly induce Ms4a8a or Ym1 and to repress IL-12p40 secretion in BMDCs (Fig. 3C, 3D). Even though diclofenac treatment resulted in the expected rise in IL-12p40 (Fig. 3C) and TNF-α (data not shown), it did not show any significant effect on the regulation of Ms4a8a or Ym1. In line with this, direct addition of PGE2 failed to induce Ms4a8a or Ym1 expression in BMDCs (data not shown). Thus, we conclude that lipophilic factors different from PGs mediate the alternative activation of DCs.

The glucocorticoid receptor is involved in epithelial cell–mediated expression of Ms4a8a and Ym1 in DCs

Nuclear hormone receptors (NRs) are responding to a number of lipophilic ligands. Thus, we speculated that NRs activated by lipophilic substances might be responsible for the observed ECCM-induced gene expression in BMDCs. To investigate which transcription factor and whether NRs in particular might be involved in modulating BMDC gene expression, we analyzed the expression data for overrepresented transcription factor binding sites in ECCM-induced genes using oPOSSUM-3.0 software (Table II) (18). The analysis revealed that among the 10 most overrepresented transcription factor binding sites, 5 belong to the family of hormone–nuclear receptor. Among those was the glucocorticoid receptor (GR; Nr3c1). Indeed, promoter regions of Ms4a8a and Ym1 contain multiple putative GR binding sites (data not shown). Because glucocorticoids are able to induce alternatively activated macrophages and known to be extractable by ethylacetate and considering that the Ms4a8a and Ym1 inducing activity is contained within the lipophilic fraction of ECCM, we now measured corticosterone concentration in ECCM and control medium directly. Using a highly sensitive detection method, we can show

### Table I. List of the 10 most up- or downregulated genes in ECCM-treated BMDCs identified in whole genome expression analysis

<table>
<thead>
<tr>
<th>Fold Regulated</th>
<th>Gene Symbol</th>
<th>Name</th>
</tr>
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<tbody>
<tr>
<td>18.914</td>
<td>Ms4a8a</td>
<td>Membrane-spanning 4-domain subfamily A member 8A</td>
</tr>
<tr>
<td>18.531</td>
<td>Chi3l3</td>
<td>Chitinase-3-like protein 3/Ym-1</td>
</tr>
<tr>
<td>9.649</td>
<td>F13a1</td>
<td>Coagulation factor XII A chain</td>
</tr>
<tr>
<td>7.94</td>
<td>Fkbp5</td>
<td>Peptidyl-prolyl cis-trans isomerase FKBP5</td>
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<tr>
<td>6.158</td>
<td>Tsc2d3</td>
<td>TSC22 domain family protein 3</td>
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<tr>
<td>6.018</td>
<td>Klh6</td>
<td>Kelch-like protein 6</td>
</tr>
<tr>
<td>5.946</td>
<td>Amica1</td>
<td>Junctional adhesion molecule-like</td>
</tr>
<tr>
<td>5.724</td>
<td>Sult1a1</td>
<td>Sulfotransferase 1A1</td>
</tr>
<tr>
<td>5.186</td>
<td>Lcn2</td>
<td>Neutrophil gelatinase-associated lipocalin</td>
</tr>
<tr>
<td>4.795</td>
<td>Ear11</td>
<td>Eosinophil-associated RNase A family member 11</td>
</tr>
<tr>
<td>0.422</td>
<td>Pdlim4</td>
<td>PDZ and LIM domain protein 4</td>
</tr>
<tr>
<td>0.41</td>
<td>Srxn1</td>
<td>Sulfiredoxin-1</td>
</tr>
<tr>
<td>0.404</td>
<td>Galnt9</td>
<td>UDP-N-acetylα-N-galactosamine: polypeptide N-acetylgalactosaminyltransferase 9</td>
</tr>
<tr>
<td>0.389</td>
<td>Oasl2</td>
<td>Protein kinase C β type</td>
</tr>
<tr>
<td>0.387</td>
<td>Prkcb1</td>
<td>Protein kinase C β type</td>
</tr>
<tr>
<td>0.373</td>
<td>Clecfs12</td>
<td>C-type lectin domain family 7 member A</td>
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<tr>
<td>0.337</td>
<td>Slamf8</td>
<td>SLAM family member 8</td>
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<tr>
<td>0.325</td>
<td>Ifit3</td>
<td>IFN-induced protein with tetratricopeptide repeats 3</td>
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<tr>
<td>0.315</td>
<td>Cxcl14</td>
<td>Cxcl14</td>
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<tr>
<td>0.231</td>
<td>Egr2</td>
<td>Early growth response protein 2</td>
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</table>

Only probe sets that had a p value ≤0.05 and a fold change ≥2 or ≤0.5 were further investigated.

### FIGURE 2. Markers of alternative activation are expressed in vivo by CD11b+ pulmonary DCs. (A) FACS strategy for the isolation of pulmonary DCs and macrophages (Mph). Expression levels of Ym1 (B) or Ms4a8a (C) were determined by qPCR in FACs-isolated pulmonary (CD11c+CD11b+ CD103− [CD11b] and CD11c+CD11b+ CD103+ [CD103]) or FACs-isolated splenic DCs (CD11c+). Data are shown as relative expression (RE) to Actb. (D) Ms4a8a protein levels were analyzed by flow cytometry in CD11c+ CD11b+ pulmonary and CD11c+ splenic DCs. *p < 0.05. MFI, mean fluorescence intensity.
that in medium that was incubated for 48 h in the presence of mTrECs, 40–50 pg/ml corticosterone was detectable (Fig. 4A). In contrast, no relevant corticosterone levels were detectable in mTrECs, 40–50 pg/ml corticosterone was detectable (Fig. 4A). In

FIGURE 3. PGs do not modulate expression of Ms4a8a and Ym1. (A and B) Extracts of lipophilic substances (lipo) of ECCM or control medium (Ctrl) were prepared using ethylacetate. After evaporation of the solvent, remaining lipophilic compounds were resuspended in culture medium. (A) BMDCs were incubated for 16 h either with complete (compl) medium or the lipophilic extracts (lipo) derived from Ctrl or ECCM. Expression levels of Ym1 and Ms4a8a were measured after RNA extraction by qPCR. Data are shown as relative expression (rE) to Actb. (B) Levels of secreted IL-12p40 were determined in the cell-culture supernatant by ELISA. (C and D) mTrECs were treated with ethanol or diclofenac (Diclo) for 48 h. Afterwards, BMDCs were incubated with ECCM derived from these cell cultures for 16 h (50% v/v) in the presence or absence of LPS (100 ng/ml). (C) Secretion of IL-12p40 and TNF-α were analyzed in cell-culture supernatant by ELISA. (D) Expression levels of Ms4a8a or Ym1 were determined by qPCR. Data are shown as fold induction normalized to BMDCs treated with Ctrl in the presence of ethanol. *p < 0.05.

Because we established a link between corticosterone production and expression of Ms4a8a and Ym1, we were trying to identify the source of glucocorticoids in ECCM. Two possibilities of how glucocorticoids are synthesized have been shown (20); one is the de novo synthesis using cholesterol as a precursor in which the final step is catalyzed by P450c11, and the other is via reactivation of the oxidized inactive corticosterone derivate 11-dehydrocorticosterone by 11β-HSD1. To identify which pathway plays a role in the crosstalk between pulmonary epithelial cells and DCs, we analyzed the expression levels of P450c11, which is encoded by Cyp11b1, and 11β-HSD1/2, which is encoded by Hsd11b1/2, in various tissues or mTrECs (Fig. 5A, 5B). RNA expression of Cyp11b1 was absent in most tissues analyzed despite the adrenal gland and also absent in mTrECs, whereas Hsd11b1 was expressed in all tissues analyzed. 11β-HSD1 expression in airway epithelial cells could also be confirmed by immunohistochemistry (Fig. 5C). These results do not support a role of de novo synthesis of glucocorticoids by mTrECs. In line with this, ECCM from mTrECs treated with metyrapone, a specific inhibitor for P450c11, did not have any significant effects on the expression of Ms4a8a or Ym1 (Fig. 5E), nor did it affect corticosterone levels in ECCM (Fig. 5D). Similar effects where observed using fluvastatin, an inhibitor of the HMG-CoA reductase that inhibits biosynthesis of cholesterol and thereof derived corticosterone (data not shown).

Therefore, we speculated that reactivation of inactive dehydrocorticosterone by 11β-HSD1 might be the way by which airway epithelial cells produce bioactive corticosterone. Indeed, treatment of mTrECs with a specific inhibitor of 11β-HSD1 lead to a 50% reduction of corticosterone in ECCM (Fig. 5F). A potential source of inactive dehydrocorticosterone under physiological conditions is serum. In our cell-culture system, we were using FCS. To deplete cell-culture medium from FCS-derived dehy-

Table II. List of 10 transcription factors with overrepresentation in ECCM-treated genes

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein Name</th>
<th>JASPAR Identification Number</th>
<th>z-Score</th>
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<tbody>
<tr>
<td>Ar</td>
<td>Androgen receptor</td>
<td>MA0007.1</td>
<td>26.607</td>
</tr>
<tr>
<td>PPARG::RXRA</td>
<td>PPARγ</td>
<td>MA0065.2</td>
<td>16.207</td>
</tr>
<tr>
<td>ESR2</td>
<td>Estrogen receptor</td>
<td>MA0258.1</td>
<td>13.698</td>
</tr>
<tr>
<td>NR2F1</td>
<td>COUP transcription factor 1</td>
<td>MA0017.1</td>
<td>11.605</td>
</tr>
<tr>
<td>Klf4</td>
<td>Kruppel-like factor 4</td>
<td>MA0039.2</td>
<td>11.347</td>
</tr>
<tr>
<td>MZF1_5-13</td>
<td>MZF1</td>
<td>MA0057.1</td>
<td>10.934</td>
</tr>
<tr>
<td>NR3C1</td>
<td>GR</td>
<td>MA0113.1</td>
<td>9.407</td>
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<tr>
<td>MZF1_1-4</td>
<td>MZF1</td>
<td>MA0056.1</td>
<td>8.937</td>
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<tr>
<td>ZNF354C</td>
<td>Zinc finger protein 354C</td>
<td>MA0130.1</td>
<td>8.647</td>
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<td>Spz1</td>
<td>Spermatogenic leucine zipper protein 1</td>
<td>MA0113.1</td>
<td>8.626</td>
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</table>

Only probe sets that had a p value ≤0.05 and a fold change ≥2 or ≤0.5 were further investigated.
The role of GR in epithelial cell–mediated gene expression in DCs. Treatment of BMDCs with the synthetic glucocorticoid dexamethasone resulted in an induction of Ms4a8a and Ym1 expression to a similar extent as observed with ECCM stimulation (Fig. 6A). Moreover, ECCM treatment had no additional effect on dexamethasone treatment. Next, we used RU486 to block GR activity in epithelial cell–conditioned DCs. Pharmacological inhibition with RU486 inhibited ECCM-mediated expression of Ms4a8a and Ym1 (Fig. 6B). To confirm the findings, we next generated BMDC from CD11cCre+GRfl/fl mice which have a specific knockout of the GR in CD11c-positive DCs (15). BMDCs were treated with ECCM as before and then analyzed for expression of Ms4a8a and Ym1 (Fig. 7A). Loss of GR in BMDC resulted in a marked and significant reduction of Ms4a8a (81% reduction) and Ym1 expression (64% reduction) upon ECCM treatment. In contrast and as a control for specificity, the potential of ECCM to inhibit IL-12p40 secretion from LPS-stimulated BMDC (known to be dependent on PGE2) was only affected to a minor extent (Fig. 7B). In line with this, the PGE2–independent inhibition of MHC II and CD86 surface presentation after LPS stimulation was also released in GR-deficient BMDCs (Fig. 7C). To further verify our results, we directly analyzed ex vivo isolated and sorted DCs. We isolated CD11c-positive cells from lung and spleen of either GRfl/fl mice or CD11c-Cre+GRfl/fl mice and analyzed the levels of Ms4a8a and Ym1 expression (Fig. 7D). DCs isolated from the lung of CD11c-Cre+GRfl/fl mice showed a significant reduction in the expression levels of Ms4a8a and Ym1 compared with cells derived from GRfl/fl mice. We could not observe any significant differences in expression of the two genes between splenic DCs isolated from GR knockout and control mice consistent with the previous observation of specific upregulation of Ms4a8a and Ym1 in respiratory DCs. These findings would argue for more active pulmonary DCs in CD11c-Cre+GRfl/fl mice. To substantiate this idea, we performed differential cell counting of bronchoalveolar lavage fluid cells in GRfl/fl mice or CD11c-Cre+GRfl/fl mice. We did not observe any difference in total cell counts (data not shown), but we detected significantly more lymphocytes in BALF derived from CD11c-Cre+GRfl/fl mice (Fig. 7E). This indicates that already under homeostatic conditions, pulmonary DCs are inhibited by glucocorticoids.

**Discussion**

In the past, mucosal epithelial cells were mostly considered to contribute to immune defense by forming a tight physical barrier toward the outside of the body. However, a more complex role of these cells in immunity became evident in recent years based on the observation that receptors of innate immune cells, including TLRs and RIG-I–like receptors (22–25), are expressed in airway epithelial cells, thus ascribing a potential immune function to the epithelial barrier itself. Moreover, it has been described repeatedly that epithelial cells actively shape the organ-specific immune response. This can be achieved by secretion of several immune modulators, among which are, e.g., TSLP (12) or retinoic acid (13), as well as by direct cell contacts with local professional immune cells (1). Such an immune modulatory function seems to be of special importance in the conducting airways because these are frequently in contact with inhaled Ags and harmless as well as harmful microbes or microbial substances (26). It has been suggested that mucosal epithelial cells in general induce a specific microenvironment that adjusts the level of reactivity of classical immune cells (2, 11, 12). To avoid repeated inflammation and associated tissue destruction in the fragile lung, the pulmonary immune system has to be kept under tight control because an uncontrolled and hyperreactive immune response is associated with several pulmonary diseases like asthma or cystic fibrosis, resulting in pulmonary remodeling and loss of function (7, 8, 27).
Recently, we demonstrated that pulmonary epithelial cells secrete PGE2 constitutively, thereby suppressing proinflammatory cytokine secretion of DCs upon TLR triggering (2). We now add the observation that even under homeostatic, unstressed conditions, DCs that were conditioned by soluble epithelial cell–derived factors change their phenotype. Transcriptional profiling demonstrated that the expression of a number of genes was affected by epithelial conditioning of DCs, and a significant number of these genes have a well-described function in the immune response. This finding indicates that epithelial cells are actively able to modulate the immune response mediated by pulmonary DCs prior to an inflammatory insult. Moreover, the two most strongly induced genes, \textit{Ms4a8a} and \textit{Ym1}, are marker genes of murine alternatively activated macrophages (16, 17). In addition, other markers of M2 macrophages, e.g., \textit{Stab1} or \textit{Arg1} were also observed to be upregulated by ECCM in BMDCs, even though these genes were not as much regulated as \textit{Ym1} or \textit{Ms4a8a} and were not among the 10 most regulated genes (data not shown) (2). Alternatively activated macrophages have been well described in the literature (17, 21) and have been correlated with an immunosuppressive function or a Th2-type immune response. Both functions fit well in the proposed hyporesponsive environment observed in the pulmonary tissue.

In the lung, several subpopulation of DCs have been described (28, 29). Under homeostatic conditions, DCs can be subdivided into CD11b+ and CD103 + DCs, which get accompanied by monocyte-derived CD11b+ DC under inflammatory conditions. Surprisingly, only CD11b+ DCs, which are more heterogeneous in...
origin, expressed the markers for alternative activation in vivo, e.g., Ym1 and Ms4a8a, to a higher extent than splenic DCs do. This observation fits to the proposed function of CD11b+ pulmonary DCs to be less migratory (30, 31) and mediate Th2 cell immunity (32–34). The data suggest that ECCM DCs adopt a phenotype that resembles a phenotype of alternative activation in macrophages and therefore represent alternatively activated or tolerogenic DCs. As we also observed downregulated MHC II and CD86 surface presentation of BMDCs was analyzed using flow cytometry either in presence or absence of ECCM (50% v/v, 16 h) after LPS stimulation (100 ng/ml, 16 h). (D) CD11c+ cells were isolated by MACS from lung or spleen tissue of Gr^{fl/fl} and Gr^{fl/fl} CD11c-Cre+ mice, and expression levels of Ms4a8a and Ym1 were determined by qPCR analysis. Data are shown as relative expression (rE) to Actb. (E) Differential cell count of bronchoalveolar lavage fluid of Gr^{fl/fl} and Gr^{fl/fl} CD11c-Cre+ mice. *p < 0.05.

**FIGURE 7.** Genetic deletion of GR in CD11c⁺ cells modulates gene expression of DCs in vitro and in vivo. BMDCs were generated from Gr^{fl/fl} and Gr^{fl/fl} CD11c-Cre+ mice and incubated for 16 h with ECCM in the presence or absence of LPS (100 ng/ml). (A) After RNA extraction, expression of Ms4a8a and Ym1 was analyzed by qPCR. Data are shown as fold induction normalized to BMDCs derived from Gr^{fl/fl} mice. (B) Levels of secreted IL-12p40 in the cell-culture supernatant were analyzed by ELISA. (C) MHC II and CD86 surface presentation of BMDCs was analyzed using flow cytometry either in presence or absence of ECCM (50% v/v, 16 h) after LPS stimulation (100 ng/ml, 16 h). (D) CD11c⁺ cells were isolated by MACS from lung or spleen tissue of Gr^{fl/fl} and Gr^{fl/fl} CD11c-Cre+ mice, and expression levels of Ms4a8a and Ym1 were determined by qPCR analysis. Data are shown as relative expression (rE) to Actb. (E) Differential cell count of bronchoalveolar lavage fluid of Gr^{fl/fl} and Gr^{fl/fl} CD11c-Cre+ mice. *p < 0.05.
various inflammatory diseases including topic and systemic treatment of asthma. Moreover, glucocorticoids do not only have a suppressive function on DCs; instead, they are able to induce a tolerogenic phenotype in these cells, which is comparable to CD4+ T cells in pathogen-specific tolerance. It has been shown that glucocorticoids are able to suppress markers of activation, like MHC II, or costimulatory molecule expression and to induce phagocytic activity as well as IL-10 production (19). All of these processes have been associated with EDCM DCs (1, 2). Indeed, EDCM-derived glucocorticoids seem to participate in the suppression of IL-12p40, because in GR-deficient DCs, the attenuation was present, but not to a certain extent impaired. This indicates that PGE2 in concert with glucocorticoids suppress proinflammatory cytokines in DCs.

Glucocorticoid synthesis occurs in the cortex of the adrenal gland, but extra-adrenal glucocorticoid synthesis has been reported in the murine intestine and pulmonary tissue (45, 46) as well as in skin and thymus (47). In murine intestine, de novo synthesis of glucocorticoids is controlled by TNF under inflammatory conditions (48), whereas increased reactivation of inactive glucocorticoids is controlled by TNF under inflammatory conditions. Mechanistically, epithelial cells reactivate inactive glucocorticoids in the lung has been described in the pulmonary system (48), whereas increased reactivation of inactive glucocorticoids is controlled by TNF under inflammatory conditions (48).

 Activation, like MHC II, or costimulatory molecule expression and proinflammatory cytokines in DCs.

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