Check for updates

Received: 20 January 2023 | Revised: 27 March 2023 | Accepted: 19 April 2023

DOI: 10.1111/all.15765

WILEY

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Glucocorticoids are differentially synthesized along the murine and human respiratory tree

Verena M. Merk[1] | Pietro Renzulli[2] | Bart Vrugt[3] | Achim Fleischmann[3] | Thomas Brunner[1]

[1]Biochemical Pharmacology, Department of Biology, University of Konstanz, Konstanz, Germany

[2]Department of Surgery, Cantonal Hospital Münsterlingen, Münsterlingen, Switzerland

[3]Institute of Pathology, Cantonal Hospital Münsterlingen, Münsterlingen, Switzerland

Correspondence
Thomas Brunner, Biochemical Pharmacology, Department of Biology, University of Konstanz, Konstanz, Germany.
Email: thomas.brunner@uni-konstanz.de

Abstract

Background: Synthetic glucocorticoids (GC) are effective in the treatment of inflammatory diseases of the lung. However, long-term use leads to severe side effects. Endogenous GC can be synthesized locally, either de novo from cholesterol in a 11β-hydroxylase (*Cyp11b1*)-dependent manner, or by reactivation from 11-dehydrocorticosterone/cortisone by 11β-hydroxysteroid dehydrogenase 1 (*Hsd11b1*). We aimed to define the molecular pathways of endogenous GC synthesis along the respiratory tree to provide a basis for understanding how local GC synthesis contributes to tissue homeostasis.

Methods: Expression of steroidogenic enzymes in murine lung epithelium was analyzed by macroscopic and laser capture microdissection, followed by RT-qPCR. Flow cytometry analysis was performed to identify the cellular source of steroidogenic enzymes. Additionally, the induction of steroidogenic enzyme expression in the lung was analyzed after lipopolysaccharide (LPS) injection. mRNA and protein expression of steroidogenic enzymes was confirmed in human lung tissue by RT-qPCR and immunohistochemistry. Furthermore, GC synthesis was examined in ex vivo cultures of fresh tissue from mice and human lobectomy patients.

Results: We observed that the murine and human lung tissue differentially expresses synthesis pathway-determining enzymes along the respiratory tree. We detected *Hsd11b1* expression in bronchial, alveolar, club and basal epithelial cells, whereas *Cyp11b1* expression was detectable only in tracheal epithelial cells of mice. Accordingly, de novo synthesis of bioactive GC occurred in the large conducting airways, whereas reactivation occurred everywhere along the respiratory tree. Strikingly, *Cyp11b1* but not *Hsd11b1* expression was enhanced in the trachea upon LPS injection in mice.

Conclusion: We report here the differential synthesis of bioactive GC along the murine and human respiratory tree. Thus, extra-adrenal de novo GC synthesis and reactivation may differentially contribute to the regulation of immunological and inflammatory processes in the lung.

KEYWORDS

Cyp11b1, extra-adrenal, glucocorticoids, Hsd11b1, lung

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2023 The Authors. *Allergy* published by European Academy of Allergy and Clinical Immunology and John Wiley & Sons Ltd.

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI — WILEY

GRAPHICAL ABSTRACT

The lung epithelium expresses steroidogenic enzymes and synthesizes GCs de novo from cholesterol as well as via reactivation from 11-dehydrocorticosterone/cortisone. GC reactivation via 11β-hydroxysteroid dehydrogenase 1 occurs mostly in the alveoli, whereas de novo GC synthesis via 11β-hydroxylase predominantly in the large conducting airways. The concept of differential GC synthesis could be confirmed in murine as well as human lung tissue.

Abbreviations: *Cyp11b1*, 11β-hydroxylase; GC, glucocorticoids; *Hsd11b1*, 11β-hydroxysteroid dehydrogenase; 11-Doc, 11-deoxycorticosterone; 11-Dhc, 11-dehydrocorticosterone

1 | INTRODUCTION

Various inflammatory diseases of the lung, including chronic obstructive pulmonary disease (COPD) and asthma, are successfully treated with synthetic glucocorticoids (GC). GC are lipophilic hormones that exert their strong anti-inflammatory action by binding to the glucocorticoid receptor α (GRα), which leads to transcriptional activation or repression of genes containing a glucocorticoid response element (GRE), or via protein–protein interaction with other transcription factors.[1,2] Despite various side effects upon long-term use, synthetic GC are widely applied due to their excellent efficacy.[3] The role of endogenous GC in the regulation of inflammatory lung diseases, however, is still unknown. It is generally accepted that the adrenal glands are the main source of endogenous GC, but studies on extra-adrenal GC synthesis at epithelial barriers (i.e., intestine, skin, and lung) have highlighted important functions of locally produced GC in regulating immunological processes under homeostatic and inflammatory conditions.[4–12] Bioactive GC are synthesized either de novo from cholesterol or by reactivation from inactive GC (cortisone, resp. 11-dehydrocorticosterone).[13–15] Endogenous GC synthesis involves a number of steroidogenic enzymes, comprising cytochrome P450 enzymes and hydroxysteroid dehydrogenases, with cortisol (main GC in humans) or corticosterone (main GC in mice) as the active hormones. *Cyp11b1* is a gene encoding a crucial GC-activating enzyme of the de novo synthesis pathway (11β-hydroxylase), whereas *Hsd11b1* encodes the enzyme that catalyzes the reactivation reaction (11β-hydroxysteroid dehydrogenase 1, 11β-HSD1). It is well established that for example, skin and intestine are both capable of locally synthesizing GC de novo as well as via corticosterone reactivation.[6,8,16,17] Moreover, de novo GC synthesis appears to be particularly important for the control of intestinal and skin homeostasis and inflammation.[6–8,11,16,18] Our previous study on local GC synthesis in the lung described that all enzymes necessary for de novo synthesis and GC reactivation are expressed in the lung.[5] Experiments in adrenalectomized (ADX) mice further revealed that lung GC synthesis in response to strong systemic inflammation is dependent on adrenal-derived precursors. Immune cell activation promoted lung GC synthesis in a 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1; gene: *Hsd11b1*)-dependent manner, but simultaneously also led to a drastic upregulation of P450(scc) (gene: *Cyp11a1*), the enzyme that catalyzes the initial cholesterol conversion in the de novo synthesis pathway.[5] Therefore, we hypothesized that de novo GC synthesis may also be relevant in the regulation of lung homeostasis and inflammation. Thus, the present study aimed to investigate firstly, whether different GC synthesis pathways exist in the lung, and secondly, whether there are spatial differences regarding GC synthesis along the respiratory tree. Considering the lung in its different functional units and the route of pathogen, resp. allergen exposure, differential GC synthesis via distinct pathways may be biologically relevant. The composition of the lung epithelium changes along the respiratory tree in order to cope with different physiological functions and requirements.[19] The pseudostratified epithelium of the large conducting airways contains basal cells with multipotent stem cell function.[20] They give rise to several specialized epithelial cell types, for example, club cells, which have an immunomodulatory, but also a stem cell function to regenerate the epithelium.[21] The alveolar epithelium contains type I alveolar cells, which form the large surface area of the lungs and allow gas exchange. They develop from alveolar type II cells, which produce the surfactant necessary to maintain alveolar structure and function.[22] This diversity of epithelial cells suggests that there may be specific cell types capable of synthesizing GC in different lung segments. This study aimed to analyze the different GC synthesis pathways to demonstrate the complexity and spatial differences of GC synthesis along the respiratory tree in mice and human. Therefore, we

determined steroidogenic enzyme expression, tissue-specific substrate conversion and bioactivity of synthesized GC in the murine and human lung. In addition, we determined the cellular sources of steroidogenic enzymes in murine lungs.

2 | MATERIALS AND METHODS

2.1 | Mice

Wild-type C57BL/6 were housed in individually ventilated cages at the animal facility of the University of Konstanz. Mice (male and female) were used in experiments between 8 and 10 weeks of age. Experiments were conducted in accordance with the animal experimentation regulations of Germany and were approved by the Review Board of the Regional Council Freiburg i.B.

2.2 | Human samples

Investigations on human patient samples have been conducted in accordance with the ethics committees of the Canton Thurgau and Eastern Switzerland, and the Declaration of Helsinki and informed consent was obtained from all subjects. Formalin-fixed and paraffin-embedded (FFPE) lung tissue sections were collected at the Institute of Pathology, and fresh tissue samples from lobectomy patients were collected by the Department of Surgery, Cantonal Hospital Münsterlingen, Switzerland.

2.3 | RT-qPCR

RNA isolation was performed with peqGOLD TriFast (PeqLab, Erlangen, Germany) (murine tissue), ReliaPrep (PROMEGA, Madison, USA) (LCM samples), RNeasy FFPE Kit (Qiagen, Hilden, Germany) (human samples (FFPE)), and RNeasy Mini Kit (Qiagen) (human samples (fresh)), according to the manufacturer's protocols. SYBRGreen-based RT-qPCR was performed with StepOnePlus Real-Time PCR system/QuantStudio™ 3 System (Applied biosystems, Waltham, USA) (Primers: Table S1). Expression was normalized to *β-Actin/β-ACTIN*. Expression ($2^{-\Delta CT}$) was calculated as follow: $\Delta CT = CT$ (gene of interest)−CT (*β-Actin*, *resp.β-ACTIN)*.

2.4 | Murine lung ex vivo culture

Lungs were perfused with phosphate-buffered saline (PBS) and macroscopically dissected to obtain trachea, main stem bronchi, and lung lobes. Ex vivo organ cultures were performed as previously published.[5,8,23,24] Tissues (10 mg) were cut and cultured in Dulbecco's Modified Eagle Medium (DMEM) (containing 50 µg/mL gentamycin, 2,5 mM L-glutamine), in the absence or presence of 200 µg/mL metyrapone (MET), 100 µM 11-dehydrocorticosterone (11-Dhc) (VWR, Radnor, USA), or 11-deoxycorticosterone acetate (11-Doc) (Sigma, St. Louis, USA), and incubated for 16 h (37°C, 5% CO_2).

2.5 | Human lung ex vivo culture

Noncancerous tissues from distant areas were obtained from eight cancer patients (6x lobar bronchus and peripheral alveolar tissue, 2x peripheral alveolar tissue only). Tissue samples were washed in PBS (containing 100 U/mL penicillin, 100 µg/mL streptomycin) and stored in Improved Minimum Essential Medium (IMDM) (containing penicillin and streptomycin) until processing. Tissues (200 mg) were cut into small pieces and cultured in DMEM medium (containing 5% charcoal-stripped Fetal Calf Serum (FCS), 50 µg/mL gentamycin, and 2,5 mM L-glutamine), in the presence or absence of 200 µg/mL metyrapone or 100 µM cortisone for 6 h (37°C, 5% CO_2).

2.6 | Luciferase-based glucocorticoid (GC) bioassay

GC synthesis in ex vivo cultures was determined via a previously published GR-regulated luciferase reporter assay.[5,6,23,25] HEK293T cells were transfected with a GRE-containing luciferase reporter construct (GRE2tk-LUC), a GR-expression plasmid (SVGR1) and a β-galactosidase expression plasmid for normalization. Cells were exposed to ex vivo culture supernatant (SN), and luciferase activity was assessed after overnight incubation. GC concentrations were calculated using a cortisol/corticosterone standard curve. Δ conversion of 11-Dhc, 11-Doc, or cortisone corresponds to the difference between the values of untreated and precursor-treated SN, after subtraction of background signals.

2.7 | Fluorescence activated cell sorting (FACS)

Murine lung and trachea were digested separately, and single cell suspensions were prepared as previously published (Table S2).[20,26,27] After red blood cell lysis, lung cells were negative selected for CD45 and CD16/32 with streptavidin magnetic beads (STEMCELL Technologies, Vancouver, Canada). Cells were stained (Table S3) for 30 min (lung) or 40 min (trachea) on ice, and respective cell populations were sorted on a FACSAria™III multicolor cell sorter (Becton Dickinson (BD), Franklin Lakes, USA) into CnT-17 medium (CELLnTec, Bern, Switzerland).

2.8 | Laser capture microdissection (LCM)

Frozen sections of Tissue-Tek-O.C.T (Sakura Finetek, Umkirch, Germany)-embedded murine lungs were fixed on RNAse-free membrane-covered slides (Zeiss) and stained with 1% cresyl violet

(Sigma-Aldrich). LCM was performed using a PALM MicroBeam System and PALM RoboSoftware (Zeiss, Jena, Germany).

2.9 | Histological analysis

FFPE human lung tissue sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). For immunohistochemistry heat-induced epitope retrieval was performed prior to antibody staining with anti-11β-HSD1 antibody (sodium citrate pH 6,0), anti-CYP11B1 antibody (Tris-EDTA pH 9,0), or isotype control (Table S5), and a biotin-labeled secondary antibody.[28] Vectastain ABC-kit (Vector Laboratories, Burlingham, USA) was used to convert DAB (3,3'-D'diaminobenzidine) (Roche, Basel, Switzerland), and nuclei were counterstained with hematoxylin (Carl Roth, Karlsruhe, Germany).

2.10 | Lipopolysaccharide (LPS) challenge

C57BL/6 mice were intraperitoneally injected with 100 μL of PBS or 100 μg LPS in PBS (Sigma-Aldrich). After 3 h lungs were processed for ex vivo culture or frozen for RNA isolation.

2.11 | ELISA

Tumor necrosis factor (TNF), interleukin-1β (IL1β), and interleukin-6 (IL6) concentrations in murine lung homogenates were quantified by ELISA according to the manufacturer's protocols (Table S4).

2.12 | Statistical analysis

Statistical analyses were performed using GraphPad Prism (v.8.0; La Jolla, CA, USA). Normal distribution tests: D'Agostino-Pearson omnibus, Shapiro Wilk, and Kolmogorov–Smirnov test. Details are indicated in the figure legends.

3 | RESULTS

3.1 | Steroidogenic enzymes are differentially expressed along the murine respiratory tree

We have previously shown that the lung is a potent extra-adrenal source of GC and expresses all steroidogenic enzymes necessary for de novo synthesis as well as reactivation of GC.[5] However, this study did not investigate potential differences of GC synthesis in the different lung segments. Hence, we aimed to investigate the different GC synthesis pathways along the respiratory tree. Firstly, we determined the mRNA expression of several P450 enzymes and hydroxysteroid dehydrogenases involved in the different GC synthesis pathways (Figure 1A) in macroscopically dissected murine lung tissue (Figure 1B, Figure S1A). Strikingly, whereas there were no differences in *Star*, *Cyp11a1*, *Cyp21*, and *Hsd3b3* expression, we found that *Cyp11b1* and *Hsd11b1* are inversely expressed from the trachea to the alveoli, indicating different GC synthesis pathways depending on the lung segments (Figure 1C, Figure S1A–C). 11β-hydroxylase (*Cyp11b1*) and 11β-HSD1 (*Hsd11b1*) use different substrates to catalyze a final step to active GC, and are therefore good markers of the respective pathway, that is, de novo synthesis versus reactivation. We confirmed these data using laser capture microdissection (LCM) to specifically isolate RNA from the epithelial cells in the different lung segments (Figure 1D). The expression of *Cyp11b1* and *Hsd11b1* in isolated tracheal, bronchiolar, and alveolar epithelium was consistent with the result from macroscopically dissected tissue. Thus, steroidogenic enzymes are differentially expressed in different epithelial lung tissues.

3.2 | Murine alveolar, bronchial, and tracheal tissue differentially produce GC

Although gene expression is a good indicator for cellular processes, it only provides limited information about the functionality of the gene product, for example, enzymes. Thus, we next determined whether the differential enzyme expression pattern leads to functional differences in the synthesis of bioactive GC. We examined the production of corticosterone in ex vivo cultures of murine tracheal, bronchial, and alveolar tissue. GC synthesis and bioactivity were confirmed in all parts of the lung investigated in a GR-based luciferase assay (Figure 2A). Importantly, the synthesis of bioactive GC could be blocked by treatment with metyrapone, an inhibitor of 11β-hydroxylase (11β-OH) and 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), thus excluding potential contaminations by serum-derived corticosterone (Figure 2A). This implies that GC are generally synthesized ex vivo along the entire respiratory tree regardless of the respective lung segment, however, does not provide any information about the relevant GC synthesis pathway. In order to distinguish between de novo GC synthesis and reactivation, we developed a functional assay to measure substrate conversion of the respective enzymes. Interestingly, the addition of 11-deoxycorticosterone, the substrate of 11β-OH, to the culture medium enhanced the GC synthesis in tracheal tissue relative to alveolar tissue (Figure 2B). By contrast, addition of 11-dehydrocorticosterone, the substrate of 11β-HSD1, resulted in increased GC synthesis in alveolar tissue compared with tracheal tissue (Figure 2C). The specificity of the conversion assay was confirmed in ex vivo cultures of large intestine and liver (Figure. S2A, B). The large intestine is known to synthesize GC ex vivo and expresses both *Cyp11b1* and *Hsd11b1*, whereas the liver expresses only *Hsd11b1*.[7,29] As expected, conversion of 11-dehydrocorticosterone was detected in both organs, whereas 11-deoxycorticosterone was converted only in the large intestine. Thus, the pattern of substrate conversion is consistent with the respective enzyme expression along the respiratory epithelium.

FIGURE 1 Steroidogenic enzymes are differentially expressed along the respiratory tree. (A) Scheme of the glucocorticoid (GC) synthesis pathway in humans and rodents. (B) Scheme for sample collection for macroscopic dissection. (C) Expression of *Star*, *Cyp11a1*, *Cyp11b1*, and *Hsd11b1* in macroscopically dissected murine trachea, bronchi, and alveoli was assessed by RT-qPCR. Expression levels were normalized to *β-Actin*. Bars indicate mean values + SD of $n = 6$ mice. (D) Representative images of selected epithelial structures of trachea, bronchioles, and alveoli for laser capture microdissection (LCM). Left panel: circles frame tracheal epithelium. Right panel: dashed lines frame alveolar epithelia, continuous lines frame epithelium of bronchioles. (E) Expression levels of *Cyp11b1* and *Hsd11b1* of samples collected via LCM were assessed by RT-qPCR. Expression levels are normalized to *β-Actin*. Bars show mean values + SD of $n = 5–6$ mice. Statistical analyses were performed using the RM one-way ANOVA and Sidak's multiple comparisons. * $p < 0.05$; ns, not significant. ND, not detectable.

Taken together, these results indicate that GC are indeed differentially synthesized along the respiratory tree, which was not only confirmed by mRNA expression but also by substrate conversion and synthesis of bioactive corticosterone in ex vivo tissue cultures.

3.3 | Murine stem cell-like progenitor cells are a likely source of de novo synthesized GC

It is well established that the source of extra-adrenal GC in the skin and the intestine are stem cell-like progenitor cells.[6,30] The cellular

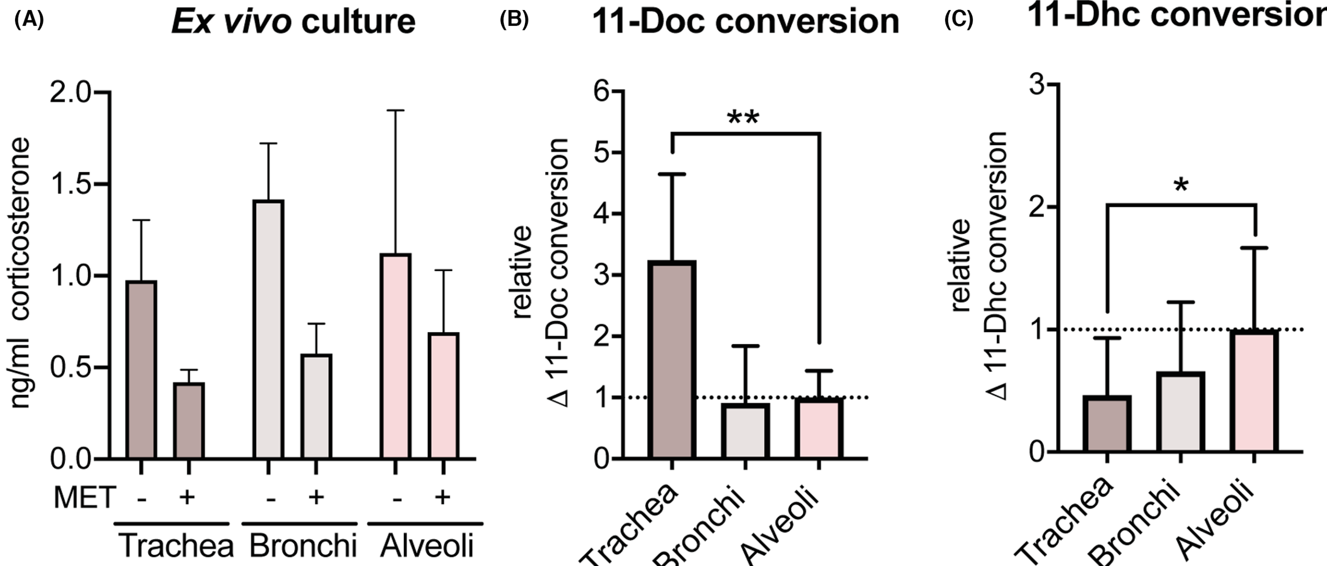

FIGURE 2 Glucocorticoids are synthesized in situ in murine trachea, bronchi, and alveoli. (A–C) Macroscopically dissected tissue samples of murine trachea, bronchi, and alveoli (10 mg/sample) were cultured ex vivo for 16 h. GC concentrations in supernatants were assessed in a GC receptor (GR)-based luciferase assay. (A) Supernatants of control or metyrapone (MET)-treated ex vivo cultures were analyzed ($n=6$ mice). GC concentration was assessed using a corticosterone standard curve. (B), (C) Ex vivo cultures were treated with either 100 μM 11-dehydrocorticosterone (11-Dhc) or 11-deoxycorticosterone acetate (11-Doc). Conversion to corticosterone was calculated as the difference between samples cultured with and without substrate (Δ conversion). Data were normalized to in situ GC synthesis in alveolar tissue. Bars show mean values ± SD of samples from $n=7$ mice. Statistical analysis was performed by using RM one-way ANOVA and Sidak's multiple comparisons * $p<0.05$; ** $p<0.01$.

source of lung-derived GC, however, is unknown. Because the cell type composition of the conducting and alveolar epithelium differs, we hypothesized that also the cellular source of de novo synthesized and reactivated GC may be different. Therefore, we aimed to identify the specific cell types of the lung epithelium that express *Cyp11b1* or *Hsd11b1*. Hence, we performed fluorescence activated cell sorting (FACS) followed by RT-qPCR analysis. The different methods used to isolate the respective epithelial cells required separate sorting of tracheal epithelial cells and cells derived from the lung lobe epithelium (Figure 3A). The epithelial cells of the lung lobes were defined as $CD45^{neg}$, $CD16/32^{neg}$, and $Ep\text{-}CAM^{pos}$. Bronchial epithelial cells, mainly consisting of ciliated cells, were identified by $CD24^{high}$ (Figure 3B).[31] Alveolar epithelial cells were sorted based on the expression of podoplanin (AEC type I) or MHCII (AEC type II) (Figure 3B).[32] Epithelial cells from the tracheal epithelium were defined as $CD45^{neg}$ and $Ep\text{-}CAM^{pos}$. Basal cells were identified by their expression of CD49f and podoplanin (Figure 3C).[33] Club cells were sorted based on their expression of $Ep\text{-}CAM^{high}$ and $CD49f^{int}$ (Figure 3C). The identities of the sorted cell populations were confirmed by RT-qPCR-based detection of typical cell type markers (Figure 3D).[34,35] Whereas the expression of *Hsd11b1* was detectable in all sorted cell populations, *Cyp11b1* expression was only detectable in basal and club cell populations isolated from the trachea (Figure 3E). Both cell types, basal and club cells, are known to have stem cell function,[20,21] representing an interesting similarity to other extra-adrenal organs with barrier function (i.e., skin and intestine) and further highlights that the cells of the large conducting airways have the potential to synthesize GC de novo.

3.4 | Murine tracheal *Cyp11b1* expression is induced upon immune cell activation

Although immune regulation by GC under steady-state conditions is important to maintain immune homeostasis, the anti-inflammatory action of GC is also known to efficiently control acute inflammation. Intraperitoneal injection (i.p) of lipopolysaccharide (LPS) is an established model to induce systemic inflammation and associated extra-adrenal GC synthesis in steroidogenic tissues.[5-7] Specifically, it is known that LPS injection results in enhanced GC synthesis in the intestine and the skin, which is associated with induced gene expression of *Cyp11b1*.[6,7] To address de novo GC synthesis in the lung also under inflammatory conditions, we investigated whether *Cyp11b1* expression in the trachea can also be induced by LPS injection and associated immunological stress (Figure 4A). LPS i.p. injection resulted in a strong inflammatory response in the lung, as indicated by significantly increased TNF, IL1β, and IL6 levels in lung homogenates (Figure 4B). Additionally, ex vivo GC synthesis was increased in alveolar and tracheal tissue. Furthermore, we observed an up to 14-fold induction of tracheal *Cyp11b1* expression in mice injected with LPS compared to PBS controls (Figure 4C, D). This increase in *Cyp11b1* expression was

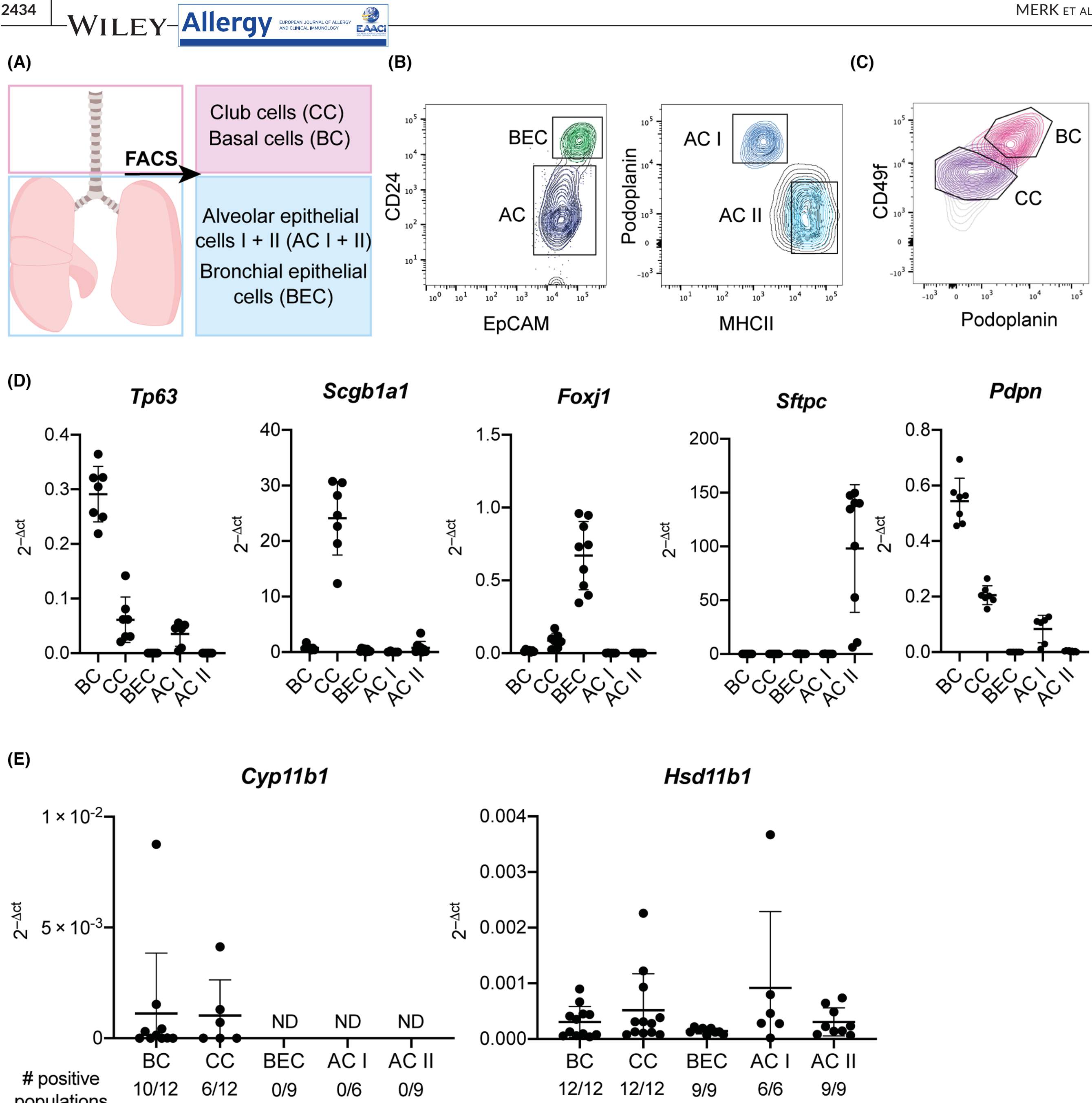

FIGURE 3 Identification of lung epithelial cell types with *Cyp11b1* and *Hsd11b1* expression. (A) Scheme of murine lung epithelial cell isolation and sorting strategy. Club cells (CC) and basal cells (BC) were isolated and sorted only from tracheal tissue. Bronchial epithelial cells (BEC) and alveolar epithelial cells type I and II (AC I+II) were isolated and sorted from the lung lobes. (B) The panels display the gating strategy for BC and CC sorting based on CD45-, Ep-CAM+ and the different expression patterns of podoplanin and CD49f. (C) The panels display the gating strategy for BEC, AEC I, and AEC II sorting based on CD45-, CD16/32-, Ep-CAM+ and the different expression patterns of CD24, podoplanin and MHC II. (D) The cellular identity of sorted populations was verified by typical epithelial cell marker expression via RT-qPCR. The expression of *Tp63*, *Scgb1a1*, *Foxj1*, *Pdpn*, and *Sftpc* was normalized to *β-Actin*. Lines display mean ± SD of $n = 6–9$ mice. (E) Expression of *Cyp11b1* and *Hsd11b1* in the sorted cell population was assessed by RT-qPCR and normalized to *β-Actin*. Violin plots show values of populations with detectable expression. Numbers of positive populations are indicated below the graph. ND, not detectable.

not observed in alveolar tissue of the same mice (Figure S3A). Also, the expression of *Hsd11b1* remained unchanged in tracheal and alveolar tissue (Figure 4B, Figure S3A). We additionally analyzed *Cyp11b1* and *Hsd11b1* expression in trachea and lung of mice with airway hypersensitivity caused by house dust mite (HDM) extract, but did not observe significant differences (Figure S3B, C). This supports the hypothesis that, depending on the trigger and type of immune response, de novo synthesis in the tracheal epithelium responds to acute immunological stress.

In summary, the murine lung epithelium not only differentially synthesizes GC under steady-state conditions but is also capable to respond to immunological stress via the induction of *Cyp11b1*

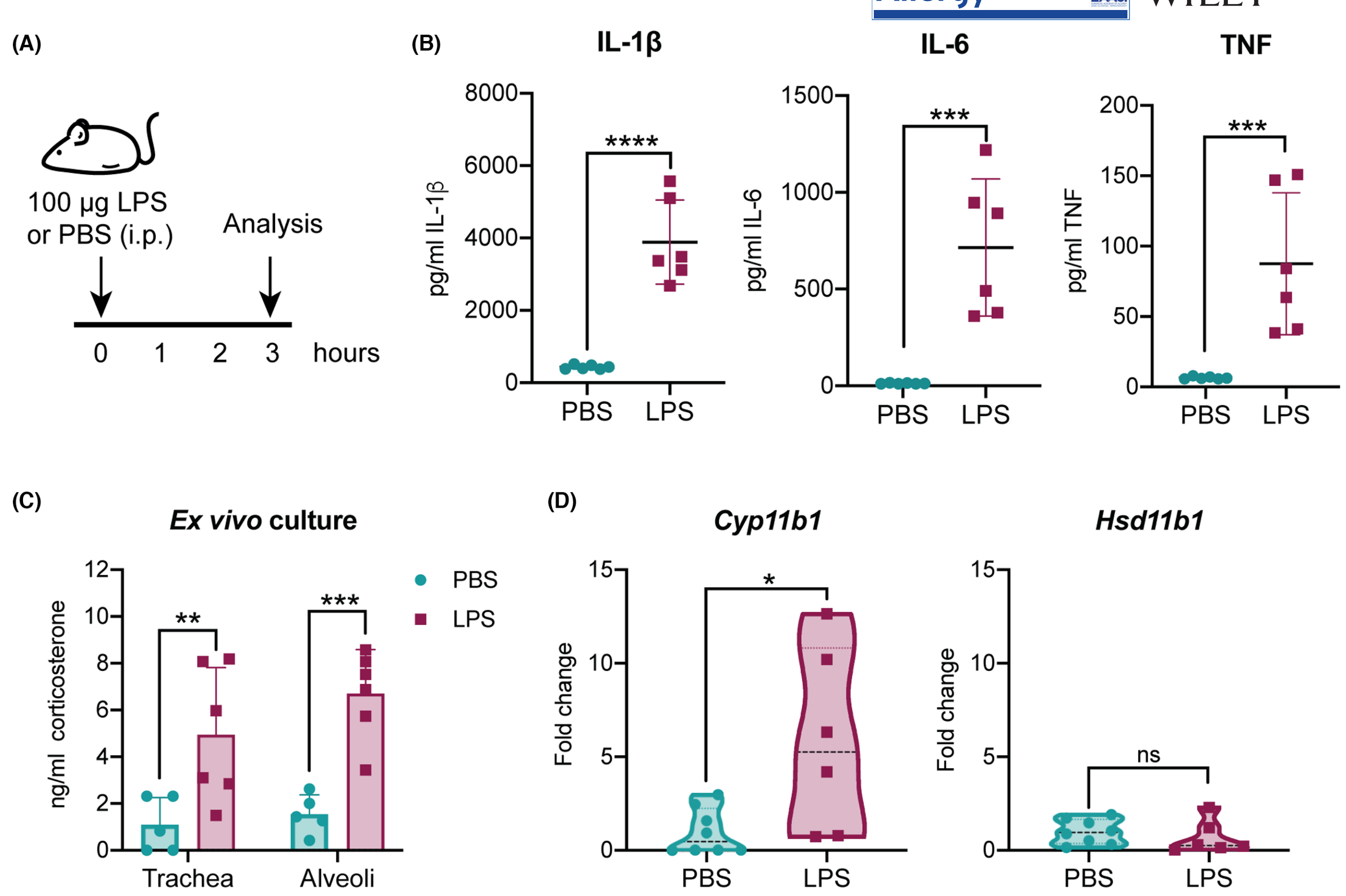

FIGURE 4 *Cyp11b1* expression is induced in murine trachea upon lipopolysaccharide (LPS) injection. (A) A schematic overview of the experimental setup. C57BL/6 mice were injected intraperitoneally (i.p.) with 100 µg LPS or PBS, and analyzed after 3 h. (B) IL1β, TNF, and IL6 in lung homogenates of mice treated with LPS or PBS were assessed by ELISA. Lines show mean values ± SD ($n = 6$). (C) GC concentration in supernatants of lung ex vivo cultures were analyzed in a GC receptor (GR)-based luciferase assay. Bars show mean values ± SD ($n = 3$). (D) RT-qPCR of RNA isolated from tracheas of mice injected for 3 h with 100 µg LPS or PBS. Expression values of *Cyp11b1* and *Hsd11b1* were normalized to *β-Actin*. Violin plots show fold increase of expression levels of $n = 6–8$ mice relative to PBS treated mice. Statistical analysis was performed using (C) Two-way ANOVA Sidak's multiple comparisons, (B), (D) two-tailed unpaired *t* test * $p < 0.05$; *** $p < 0.005$; **** $p < 0.001$, ns, not significant.

expression in the trachea. This indicates an immunoregulatory pathway that is independent of adrenal gland-derived substrate availability.

3.5 | *CYP11B1* is differentially expressed in human lung

Thus far, our data suggest that both GC synthesis pathways, de novo synthesis and reactivation, exist in different lung segments in mice. In order to address the relevance of these findings in human lungs, we investigated the expression and function of steroidogenic enzymes in human lung samples. FFPE human lung sections were visually inspected using H&E staining. Tissue samples containing predominantly large conducting airways and alveolar tissue were collected and their expression of *CYP11B1* and *HSD11B1* was analyzed by RT-qPCR. While *HSD11B1* appeared to be uniformly expressed in the lung regions examined, *CYP11B1* expression was increased in the large conducting airways (Figure 5A). This distinct expression of *CYP11B1* was further confirmed at the protein level using immunohistochemistry. While expression of 11β-HSD1 was detectable throughout the lung epithelium, 11β-OH was absent in alveoli and small conducting airways, but detectable in the large conducting airways (Figure 5B, C; Figure S4). More specifically, 11β-OH expression in the conducting airways appeared to be restricted to the basal cells of the pseudostratified epithelium (Figure 5B, C; Figure S4). These results suggest differential synthesis of local GC also in human lungs.

3.6 | Human alveolar and bronchial tissue produce active glucocorticoids

Since mRNA and protein expression of steroidogenic enzymes alone do not necessarily correlate with functionality, that is, the differential GC synthesis in different sections of the human lung, we next addressed this issue in fresh human lung tissue. We analyzed GC synthesis in human tissue samples from lobectomy patients that

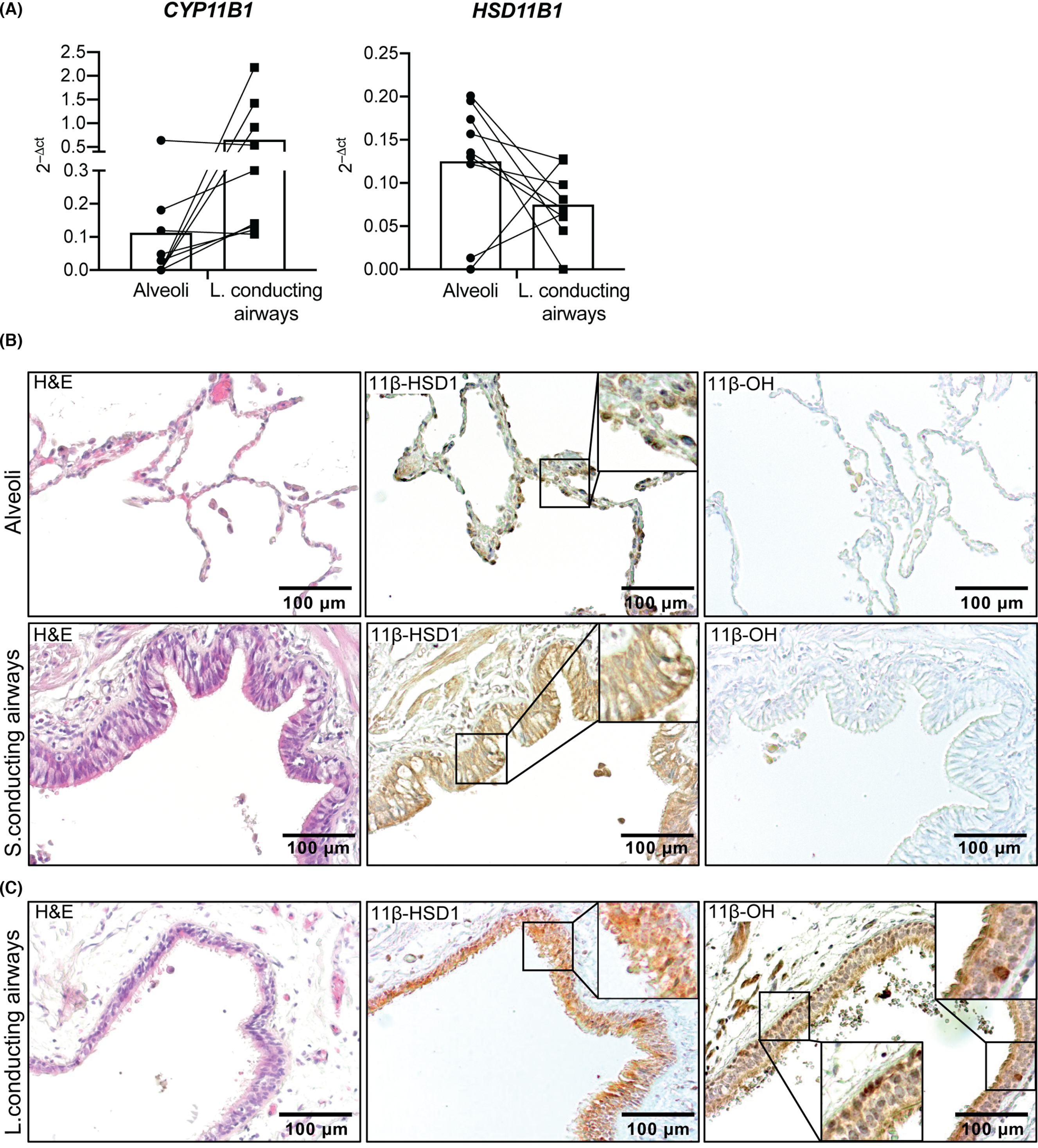

FIGURE 5 *CYP11B1* and *HSD11B1* are expressed along the human respiratory tree. (A) Expression of *CYP11B1* and *HSD11B1* was assessed in tissue sections of alveolar tissue and large conducting airways from human lung sections by RT-qPCR. Bars indicate mean values and lines connect samples from the same patient ($n=4$). (B, C) Representative images of immunohistochemistry staining for 11β-hydroxylase (11-βOH, *CYP11B1*) and 11β-hydroxysteroid dehydrogenase 1 (11-βHSD1, *HSD11B1*) in human lung tissue sections (four patients) of (B) alveoli, small conducting airways and (C) large conducting airways. H&E staining are shown for comparison. Scale bar = 200 μm. Inlays show magnifications. L, large, S, small.

contained lobar bronchus and peripheral alveolar tissue. Firstly, we isolated RNA from the different lung segments and analyzed their *CYP11B1* and *HSD11B1* expression to confirm our results from the FFPE samples. As expected, *CYP11B1* expression was significantly higher in bronchial tissue than in alveolar tissue, whereas no difference in *HSD11B1* expression was detectable (Figure 6A). In

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI — WILEY

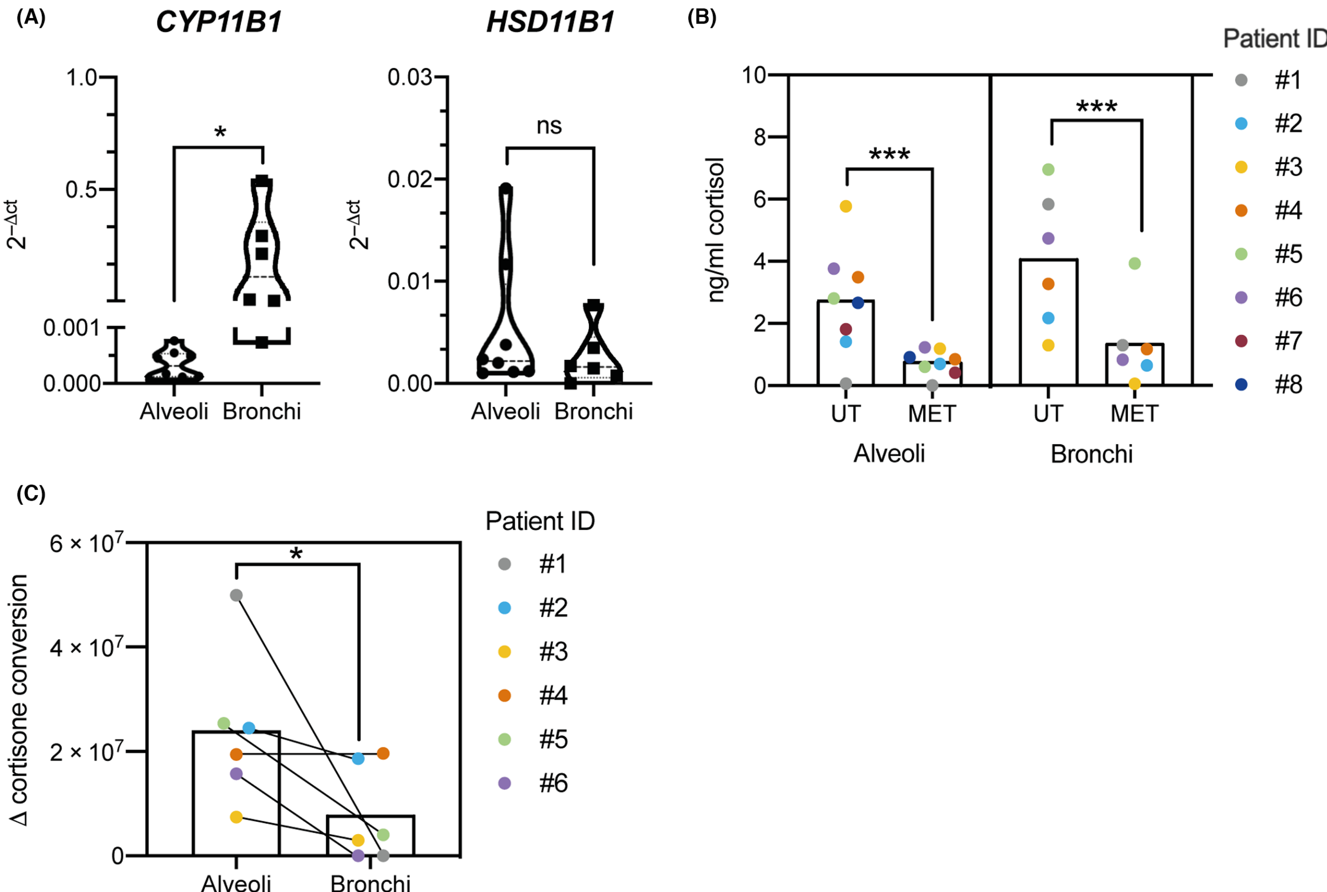

FIGURE 6 Human lungs differentially produce glucocorticoids (GC) along the respiratory tree. (A) Expression of *CYP11B1* and *HSD11B1* in fresh lobar bronchial and alveolar human tissue from lobectomy patients was assessed by RT-qPCR. Violin plots show individual values for alveolar tissue of $n = 8$ patients and lobar bronchial tissue of $n = 6$ patients. (B) GC concentration in supernatant of control (UT) or metyrapone-treated (MET) ex vivo cultures of fresh human bronchial and alveolar tissue (200 mg/sample) was assessed in a GC receptor (GR)-based luciferase assay. GC concentration was calculated using a cortisol standard curve. Bars indicate mean values of $n = 6–8$ patients. The same dot color refers to the same patient. Statistically analyses were performed using the Two-way ANOVA. (C) Ex vivo cultures were treated with cortisone (100 μM). Conversion to cortisol was calculated as the difference between samples cultured with and without substrate (Δ conversion). Bars show mean values and lines connect samples from the same patient ($n = 6$ patients). Statistically analyses were performed using the (A) paired two-tailed *t* test, (B) Two-way ANOVA, (C) Mann–Whitney test. * $p < 0.05$; *** $p < 0.005$, ns: not significant.

order to investigate the actual synthesis of bioactive GC in these different sections, we cultured human alveolar and bronchial tissue ex vivo and quantified the release of GC using the luciferase-based GC assay. Excitingly, we confirmed cortisol synthesis in ex vivo cultured human lung tissues, which was blockable by metyrapone (Figure 6B). Similar to the ex vivo cultured murine lung tissues, inactive cortisone was converted to active cortisol more efficiently in alveolar tissue than in bronchial tissue (Figure 6C). Conversion of 11-deoxycortisol could, however, not be assessed due to high background in the luciferase-based GC bioassay. Nevertheless, these findings strongly suggest differential GC synthesis in human lung tissue, similar to that observed in murine lungs.

In conclusion, the human lung ex vivo experiments confirm our hypothesis that GC are differentially synthesized along the respiratory tree, which could open interesting novel therapeutic approaches for the treatment of inflammatory lung diseases.

4 | DISCUSSION

The immunoregulatory properties and anti-inflammatory actions of extra-adrenal GC have been well investigated in several organs.[14] Exceeding the important regulatory functions in the maintenance of tissue homeostasis, local GC synthesis may also have great potential for the development of new therapies for inflammatory diseases. The lung in particular is affected by various acute and chronic inflammatory diseases, such as asthma or COPD, which represent a major burden for the affected patients and public health. Although the therapeutic use of synthetic GC is very established and effective, long-term use can cause severe side effects, and thus alternatives are required.[3] Stimulation of endogenous GC synthesis in the lung epithelium could represent such an alternative, but the putative complex regulation and function of local GC synthesis requires detailed investigation.

In this study, we investigated the spatial differences of extra-adrenal GC synthesis along the respiratory tree of mice and humans. We observed a differential gene expression pattern of *Cyp11b1* and *Hsd11b1*. Using the LCM technology, we could first confirm the expression of these enzymes in lung tissue, and second identify a spatial gene expression pattern (Figure 1D). The reversed gene expression pattern of *Cyp11b1* and *Hsd11b1* from the trachea to the alveoli suggests that different GC synthesis pathways are involved, depending on the location along the respiratory tree and the exposure of the tissue to inflammatory challenges (Figure 1C, D). Accordingly, this differential enzyme expression could be confirmed by the different efficiencies in substrate conversion. 11-deoxycorticosterone, the substrate of 11β-OH is more efficiently converted to corticosterone in ex vivo cultured tracheal tissue than in alveolar tissue. By contrast, differences in the synthesis of corticosterone from 11-dehydrocorticosterone in a 11β-HSD1-dependent manner are less pronounced (Figure 2B, C). Our own studies on extra-adrenal GC synthesis in other epithelial barriers, that is, the skin and the intestine, already revealed the importance of local GC in regulating the immune system during homeostasis and inflammation.[6,8,9,16] Interestingly, the source of locally de novo synthesized GC in the skin and the intestine are organ-specific stem cell-like progenitor cells.[6,30] Our investigation on the cellular source of de novo synthesized GC in the lung also indicates that stem cell-like basal and club cells in the trachea express *Cyp11b1* and produce GC from cholesterol (Figure 3D). *Hsd11b1*, however, appears to be expressed in all cell types, suggesting that reactivation of inactive GC is important along the entire respiratory tree (Figure 3D). In our previous study we described that 11β-HSD1-dependent reactivation of GC in the lung is dependent on systemic GC synthesis, since adrenalectomy strongly reduced the release of GC from lung tissue.[5] However, it is reasonable to assume that the maintenance of tissue homeostasis could also benefit from local mechanisms responding to immunological stress without involving the entire system, as observed upon release of GC from the adrenal glands. 11β-OH-dependent de novo synthesis could represent such a mechanism. The hypothesis that both GC synthesis pathways likely play important roles in the lung is strengthened by findings from other extra-adrenal organs. Thus, keratinocyte-specific deletion of *Hsd11b1* or *Cyp11b1* showed an exacerbated phenotype in ear swelling and pro-inflammatory cytokine expression in response to hapten-induced allergic dermatitis in mice.[6,17] This indicates an important role of local reactivation of inactive GC as well as de novo synthesis. Remarkably, *Cyp11b1*-deleted mice developed skin pathology even without additional immunological challenge of the skin, suggesting a crucial role of de novo GC synthesis in skin homeostasis.[6] Since the skin, similar to the conducting airway epithelium, is constantly exposed to bacteria and pathogens, immunological processes are continuously triggered and it might be beneficial if local GC synthesis occurs independently of substrate availability from the adrenal glands. That the expression of *Cyp11b1* and consequently de novo GC synthesis in the trachea are indeed induced by immunological stress was confirmed upon immune cell activation by LPS injection (Figure 4C, D). LPS elicits a strong and rapid type 1 inflammatory response and was shown before to trigger GC synthesis in the skin and intestinal tissues,[5–7] and as shown here also in the lung. HDM-induced airway hypersensitivity, which is associated with type 2 immune responses, however, did not induce *Cyp11b1* expression in the trachea, indicating that the type of immune response is critical (Figure S3B, C). This is consistent with the assumption that GC predominantly suppress type 1 and 17, and not type 2 immune responses.[36,37]

The underlying mechanisms that promote and regulate either de novo synthesis or reactivation of inactive GC in the lung epithelium remain, however, to be investigated. While TNF can trigger GC synthesis in the lung, it is not required for pulmonary GC synthesis in response to immune cell activation, while it is critical in the intestine.[5,7] Thus, the relevant immune cell-derived factors, likely cytokines, that regulate local GC synthesis in the lung need to be examined. In this regard, it might be also conceivable that direct activation of lung epithelial cells via pattern recognition receptors (PPR) may promote GC synthesis. Full elucidation of these processes would be important for the development of new treatment strategies.

Although murine and human lungs share similar characteristics, species-specific differences, such as organ size or cell type distribution,[22] must be considered and we thus extended our studies to human samples. The role, regulation, and relevance of local GC synthesis in human lungs are currently poorly defined. Of interest, though, enhanced *CYP11B1* and *HSD11B1* expression, accompanied by increased cortisol levels have been found in patients with chronic rhinosinusitis, suggesting a role of local GC synthesis in the regulation of inflammatory responses in the upper respiratory tract.[38] Similarly, we could now demonstrate *CYP11B1* and *HSD11B1* expression also in human conducting and respiratory airways (Figures 5A, 6A). Additionally, protein expression in patient samples was further confirmed by immunohistochemistry (Figure 5B, C), which also allows to investigate the relative distribution of the steroidogenic enzymes within a tissue. Our analyses show that 11β-HSD1 is expressed fairly uniformly along the respiratory tree with a tendency toward higher levels in the alveoli, but a clear restricted expression pattern is evident for 11β-OH (Figures 5A–C, 6A), which is limited to the conducting airways, especially to the basal cells.

The findings of this study suggest that local GC synthesis in the conducting airways is able to respond to local immune cell activation in order to fine-tune inflammatory processes independent of the adrenal glands. By contrast, inflammation of the alveolar epithelium may have very severe and potentially fatal consequences, as for example seen during SARS-CoV-2 infections,[39] and may thus require an immediate and strong suppression of inflammatory responses in order to prevent tissue damage and associated drop in oxygen exchange. Reactivation of serum-derived precursors by 11β-HSD1 ensures high local concentrations of immunosuppressive GC, thereby preventing tissue damage and ensuring organ functionality.

In conclusion, our present study documents that both GC synthesis pathways, that is, de novo and reactivation, exist in the murine

and human lung in a spatially dependent manner. While the role of the specific synthesis pathways needs to be investigated in detail and in different experimental models of lung inflammation (e.g., allergies, and viral or bacterial infections), it is very likely that locally produced GC contribute to the maintenance of lung immune homeostasis, as has been previously shown for other epithelial tissues.[9] Finally, therapies based on the induction of local GC synthesis may hold great promises in the treatment of lung inflammation and in the replacement of synthetic corticosteroid therapies.

AUTHOR CONTRIBUTIONS

VMM designed the study, performed the experiments, created the figures, and wrote the manuscript. PR provided the fresh tissue samples from human lobectomy patients and revised the manuscript. BV helped with the processing of the human tissue samples. AF selected and provided the paraffin-embedded human lung tissue sections and revised the manuscript. TB designed the study and edited the manuscript.

ACKNOWLEDGEMENT

We thank the Cantonal Hospital Münsterlingen for the cooperation. We further thank the animal facility (TFA) and the flow cytometry core facility FlowKon of the University of Konstanz for technical support. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in relation to this work.

ORCID

Verena M. Merk https://orcid.org/0000-0003-2470-681X
Thomas Brunner https://orcid.org/0000-0002-1594-4712

REFERENCES

1. McKay LI, Cidlowski JA. Cross-talk between nuclear factor-κB and the steroid hormone receptors: mechanisms of mutual antagonism. *Mol Endocrinol*. 1998;12(1):45-56. doi:10.1210/mend.12.1.0044
2. Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science*. 1990;249(4974):1266-1272. doi:10.1126/science.2119054
3. Oray M, Abu Samra K, Ebrahimiadib N, Meese H, Foster CS. Long-term side effects of glucocorticoids. *Expert Opin Drug Saf*. 2016;15(4):457-465. doi:10.1517/14740338.2016.1140743
4. Cima I, Corazza N, Dick B, et al. Intestinal epithelial cells synthesize glucocorticoids and regulate T cell activation. *J Exp Med*. 2004;200(12):1635-1646. doi:10.1084/jem.20031958
5. Hostettler N, Bianchi P, Gennari-Moser C, et al. Local glucocorticoid production in the mouse lung is induced by immune cell stimulation. *Allergy*. 2012;67(2):227-234. doi:10.1111/j.1398-9995.2011.02749.x
6. Phan TS, Schink L, Mann J, et al. Keratinocytes control skin immune homeostasis through de novo–synthesized glucocorticoids. *Sci Adv*. 2021;7(5):1-20. doi:10.1126/SCIADV.ABE0337
7. Noti M, Corazza N, Tuffin G, Schoonjans K, Brunner T. Lipopolysaccharide induces intestinal glucocorticoid synthesis in a TNFα-dependent manner. *FASEB J*. 2010;24(5):1340-1346. doi:10.1096/fj.09-140913
8. Noti M, Corazza N, Mueller C, Berger B, Brunner T. TNF suppresses acute intestinal inflammation by inducing local glucocorticoid synthesis. *J Exp Med*. 2010;207(5):1057-1066. doi:10.1084/jem.20090849
9. Merk VM, Phan TS, Brunner T. Regulation of tissue immune responses by local glucocorticoids at epithelial barriers and their impact on interorgan crosstalk. *Front Immunol*. 2021;12:1-12. doi:10.3389/fimmu.2021.672808
10. Slominski AT, Zmijewski MA, Skobowiat C, Zbytek B, Slominski RM, Steketee JD. Sensing the environment: regulation of local and global homeostasis by the skin neuroendocrine system. *Adv Anat Embryol Cell Biol*. 2012;212:1-6. doi:10.1007/978-3-642-19683-6_1
11. Slominski A, Zbytek B, Nikolakis G, et al. Steroidogenesis in the skin: implications for local immune functions. *J Steroid Biochem Mol Biol*. 2013;137:107-123. doi:10.1016/j.jsbmb.2013.02.006
12. Slominski AT, Manna PR, Tuckey RC. On the role of skin in the regulation of local and systemic steroidogenic activities. *Steroids*. 2015;103:72-88. doi:10.1016/j.steroids.2015.04.006
13. Phan TS, Merk VM, Brunner T. Extra-adrenal glucocorticoid synthesis at epithelial barriers. *Genes Immun*. 2019;20:627-640. doi:10.1038/s41435-019-0058-z
14. Talabér G, Jondal M, Okret S. Extra-adrenal glucocorticoid synthesis: immune regulation and aspects on local organ homeostasis. *Mol Cell Endocrinol*. 2013;380(1–2):89-98. doi:10.1016/j.mce.2013.05.007
15. Mahata B, Pramanik J, van der Weyden L, et al. Tumors induce de novo steroid biosynthesis in T cells to evade immunity. *Nat Commun*. 2020;11(1):3588. doi:10.1038/s41467-020-17339-6
16. Ahmed A, Schwaderer J, Hantusch A, Kolho KL, Brunner T. Intestinal glucocorticoid synthesis enzymes in pediatric inflammatory bowel disease patients. *Genes Immun*. 2019;20(7):566-576. doi:10.1038/s41435-019-0056-1
17. Terao M, Itoi S, Matsumura S, Yang L, Murota H, Katayama I. Local glucocorticoid activation by 11β-hydroxysteroid dehydrogenase 1 in keratinocytes the role in Hapten-induced dermatitis. *Am J Pathol*. 2016;186(6):1499-1510. doi:10.1016/j.ajpath.2016.01.014
18. Slominski RM, Tuckey RC, Manna PR, et al. Extra-adrenal glucocorticoid biosynthesis: implications for autoimmune and inflammatory disorders. *Genes Immun*. 2020;21(3):150-168. doi:10.1038/s41435-020-0096-6
19. Rackley CR, Stripp BR. Building and maintaining the epithelium of the lung. *J Clin Invest*. 2012;122(8):2724-2730. doi:10.1172/JCI60519
20. Rock JR, Onaitis MW, Rawlins EL, et al. Basal cells as stem cells of the mouse trachea and human airway epithelium. *PNAS*. 2009;106(31):12771-12775. doi:10.1073/pnas.0906850106
21. Rawlins EL, Okubo T, Xue Y, et al. The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, Epithelium. *Cell Stem Cell*. 2009;4(6):525-534. doi:10.1016/j.stem.2009.04.002
22. Hogan B, Tata PR. Cellular organization and biology of the respiratory system. *Nat Cell Biol*. 2019;2. doi:10.1038/s41556-019-0357-7
23. Sidler D, Renzulli P, Schnoz C, et al. Colon cancer cells produce immunoregulatory glucocorticoids. *Oncogene*. 2011;30(21):2411-2419. doi:10.1038/onc.2010.629
24. Ahmed A, Reinhold C, Breunig E, et al. Immune escape of colorectal tumors via local LRH-1/Cyp11b1-mediated synthesis of immunosuppressive glucocorticoids. *Mol Oncol*. 2023. doi:10.1002/1878-0261.13414
25. Merk VM, Grob L, Fleischmann A, Brunner T. Human lung carcinomas synthesize immunoregulatory glucocorticoids. *Genes Immun*. 2023;24:52-56. doi:10.1038/s41435-023-00194-y

26. Hasegawa K, Sato A, Tanimura K, et al. Fraction of MHCII and EpCAM expression characterizes distal lung epithelial cells for alveolar type 2 cell isolation. *Respir Res*. 2017;18(1):1-13. doi:10.1186/s12931-017-0635-5
27. Sinha M, Lowell C. Isolation of highly pure primary mouse alveolar epithelial type II cells by flow cytometric cell sorting. *Bio-Protocol*. 2016;6(22):1-13. doi:10.21769/bioprotoc.2013
28. Jakob S, Corazza N, Diamantis E, Kappeler A, Brunner T. Detection of apoptosis in vivo using antibodies against caspase-induced neo-epitopes. *Methods*. 2008;44(3):255-261. doi:10.1016/j.ymeth.2007.11.004
29. Semjonous NM, Sherlock M, Jeyasuria P, et al. Hexose-6-phosphate dehydrogenase contributes to skeletal muscle homeostasis independent of 11β-hydroxysteroid dehydrogenase type 1. *Endocrinology*. 2011;152(1):93-102. doi:10.1210/en.2010-0957
30. Botrugno OA, Fayard E, Annicotte J-S, et al. Synergy between LRH-1 and β-catenin induces G1 cyclin-mediated cell proliferation. *Mol Cell*. 2004;15(4):499-509. doi:10.1016/J.MOLCEL.2004.07.009
31. Nakano H, Nakano K, Cook DN. Isolation and purification of epithelial and endothelial cells from mouse lung. *Methods Mol Biol*. 2018;1799:59-69. doi:10.1007/978-1-4939-7896-0_6
32. Donati Y, Blaskovic S, Ruchonnet-Métrailler I, Maillard JL, Barazzone-Argiroffo C. Simultaneous isolation of endothelial and alveolar epithelial type I and type II cells during mouse lung development in the absence of a transgenic reporter. *Am J Physiol Lung Cell Mol Physiol*. 2020;318(4):L619-L630. doi:10.1152/AJPLUNG.00227.2019
33. McQualter JL, Yuen K, Williams B, Bertoncello I. Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. *Proc Natl Acad Sci U S A*. 2010;107(4):1414-1419. doi:10.1073/pnas.0909207107
34. Chernaya O, Shinin V, Liu Y, Minshall RD. Behavioral heterogeneity of adult mouse lung epithelial progenitor cells. *Stem Cells Dev*. 2014;23(22):2744-2757. doi:10.1089/scd.2013.0631
35. de Mello F, Costa M, Weiner AI, Vaughan AE. Basal-like progenitor cells: a review of dysplastic alveolar regeneration and remodeling in lung repair. *Stem Cell Reports*. 2020;15(5):1015-1025. doi:10.1016/j.stemcr.2020.09.006
36. Elenkov IJ. Glucocorticoids and the Th1/Th2 balance. *Ann N Y Acad Sci*. 2004;1024:138-146. doi:10.1196/annals.1321.010
37. Cain DW, Cidlowski JA. Immune regulation by glucocorticoids. *Nat Rev Immunol*. 2017;17(4):233-247. doi:10.1038/nri.2017.1
38. Jun YJ, Park SJ, Kim TH, et al. Expression of 11β-hydroxysteroid dehydrogenase 1 and 2 in patients with chronic rhinosinusitis and their possible contribution to local glucocorticoid activation in sinus mucosa. *J Allergy Clin Immunol*. 2014;134(4):926-934.e6. doi:10.1016/j.jaci.2014.03.033
39. Lamers MM, Haagmans BL. SARS-CoV-2 pathogenesis. *Nat Rev Microbiol*. 2022;20(5):270-284. doi:10.1038/s41579-022-00713-0

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Merk VM, Renzulli P, Vrugt B, Fleischmann A, Brunner T. Glucocorticoids are differentially synthesized along the murine and human respiratory tree. *Allergy*. 2023;78:2428-2440. doi:10.1111/all.15765