Dendritic cell functional properties in a three-dimensional tissue model of human lung mucosa

Anh Thu Nguyen Hoang,1 Puran Chen,1 Julius Juarez,1 Patty Sachamitr,2 Bo Billing,3 Lidija Bosnjak,1 Barbro Dahlén,3 Mark Coles,2 and Mattias Svensson1

1Center for Infectious Medicine at the Department of Medicine, Karolinska Institutet, and 2Division of Respiratory Medicine and Allergy at Karolinska University Hospital, Huddinge, and Center for Allergy Research, Karolinska Institutet, Stockholm, Sweden; 3Centre for Immunology and Infection, at Hull York Medical School and Department of Biology, University of York, York, United Kingdom

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Human tissue models can also capture important aspects of tissue-associated responses to infections because many important human pathogens [e.g., *Mycobacterium tuberculosis* (*M. tuberculosis*), group A streptococcus, and *Staphylococcus aureus*) induce species specific responses (2, 25).

Several approaches have been used to study immune responses of human lung mucosa to external stimuli in vitro, including monolayers of epithelial cells isolated from lung tissue bronchoscopies (37). Epithelial cells isolated from bronchoscopy are terminally differentiated, short-lived cells that generally have lower metabolic capacity than actively growing cells in vivo or in vitro (14). Although, primary bronchial epithelial cells can develop tight junctions and adherence junctions as well as deposit extracellular matrix proteins found in functional mucosal barriers, they may have a variable constitutive and inducible expression of proteins, depending on the donor as previously reported (42). These monolayer systems are additionally limited by the lack of polarized cell phenotype and the lack of a large number of cell-cell contacts, which will affect their function and response to external stimuli (31). Instead, the complexity of two-cell layer-based systems are advantageous as demonstrated when studying, for example, early events in *M. tuberculosis* infection (2).

More recently, protocols describing the setup of bronchial 3D mucosal tissue models that better recapitulate human lung mucosa have been developed (6). This includes models with a physiologically relevant fibroblast matrix layer and epithelial differentiation into ciliated, mucus-secreting cells (6, 9). Under such conditions, fibroblasts proliferate slowly, and their extracellular matrix is likely to provide better conditions for growth and differentiation of lung epithelial cells in vitro compared with artificial gels or membranes, possibly via the release of growth and survival factors (5, 15). The fibroblast component is, not only critical in promoting survival, remodeling, and deposition of matrix components, but also ensures the resemblance to the human lung mucosa and submucosa (4, 5).

Fibroblasts actively interact with the adjacent epithelial layer and have a key role in inflammation and repair of lung tissue (36). What has only recently been recognized are the cellular interactions between epithelial cells, fibroblasts, and immune cells, such as dendritic cells (DC), in many aspects of tissue homeostasis and inflammation. Thus, in vitro 3D models combining DC with tissue-specific cells provides a useful tool to study mechanisms involved in local inflammatory processes previously not achievable, and will have a great potential in replacing, in some cases, experiments performed on animals.
DC belong to a heterogeneous population of widely distributed immune cells that play a central role in initiation and regulation of immune responses (1). Immune responses to pathogens are initiated and orchestrated by DC located in peripheral tissues, including the skin and mucosa. DC orchestrate immune responses locally by producing cytokines and chemokines that are important in the activation and recruitment of other inflammatory cells (22). Within lung tissue, DC mainly associate with the epithelial layer, and the interactions with the epithelium likely influence the phenotype and function of DC, including their ability to orchestrate immune responses locally (12, 18). There are also data supporting that epithelial cell dysfunction leads to overzealous immune activation (17). However, dissecting how tissue-specific nonimmune cells and DC cooperate in coordinating tissue homeostasis and inflammation in human tissue has multiple practical problems. Although 3D lung tissue models that resemble “real” tissue have been engineered to include a fibroblast extracellular matrix and differentiating epithelial cells (Fig. 1A), few, however, are utilizing the combination of fibroblasts, epithelial cells, and immune cells, such as DC that can be imaged in 4D (x, y, z, time, Fig. 1B).

In this study, therefore, we developed a standardized human 3D organotypic lung model using the “normal” bronchial epithelial cell line 16HBE14o− (16HBE) immortalized with the SV40 large T antigen, the MRC-5 fibroblasts derived from fetal lung tissue, and human monocyte-derived DC. The model recapitulates key anatomical and functional features of lung mucosal tissue, including deposition of extracellular matrix proteins and the formation of tight junction and adherence junction proteins. Analyses of fixed-tissue models also revealed that DC distribute in close association with the epithelial layer, and this was confirmed also in live tissue models using multiphoton microscopy analysis. In addition, we found that the lung tissue microenvironment affects DC function and that the chemokines CCL17, CCL18, and CCL22 are differentially regulated in DC within the 3D model. Thus this system provides a new platform to study human DC functional properties associated to lung mucosal tissue, previously not achievable.

**MATERIALS AND METHODS**

**Cell lines.** MRC-5 is a human lung fibroblast cell line (American Type Culture Collection, Manassas, VA) derived from normal lung tissue of a 14-wk-old male fetus. MRC-5 were maintained in complete DMEM (Invitrogen, Carlsbad, CA) with 1 mM sodium pyruvate, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 0.1 mM nonessential amino acids, and 10% heat-inactivated FBS (all from Invitrogen). The MRC-5 fibroblasts were thawed and expanded on tissue-culture flasks in complete DMEM until 70–80% confluent (~7–9 days) before use in the model. The cells were used at passages 24–26 in the model.

The cell line 16HBE (a gift from Dr. Dieter Gruenert, Mt. Zion Cancer Center, University of California, San Francisco, CA) is an immortalized human bronchial epithelial cell line developed by transformation of human surface epithelial cell from 1-yr-old male with SV40 large T-antigen. 16HBE were cultured in fibronectin/collagen-coated flasks (7) and maintained in complete MEM (Sigma-Aldrich, St. Louis, MO) supplemented with Earle’s salts, 0.292 g/l l-glutamine, 0.03 mg/l sodium pyruvate, 2 mM l-glutamine, and 10% heat-inactivated FBS (all from Invitrogen).
dmEM and incubated for 30 min at 37°C. A suspension of MRC-5 bovine type I collagen (1.1 mg/ml; Organogenesis, Canton, MA) in DMEM mixture was added onto the precoated collagen layer and allowed to dry at 37°C. The cells were used at passage 74. 16HBE were then seeded of the epithelial cells. Epithelial cells were seeded by adding 5 x 10^5 cells from day 6 were conditioned by replacing the original medium with 500 µl of culture supernatants from the lung tissue model composed of only fibroblasts and epithelial cells, or with supernatants from either fibroblasts or air-exposed epithelial cells, or with supernatants from both fibroblast and epithelial cells. The DC were conditioned for 24 h and then extracted for RNA, and supernatants were collected for protein measurement using ELISA. Ethical permission for the use of human tissue and cells for RNA, and supernatants were collected for protein measurement using ELISA. Ethical permission for the use of human tissue and cells was obtained from the regional ethics committee at Karolinska Institutet, Stockholm, Sweden, and where needed informed consent has been obtained.

Organotypic model preparation. The models were generated on 3.0-µm Transwells inserts (Becton Dickinson, Franklin Lakes, NJ) in six-well plates based on a protocol for human oral mucosal models (9). The procedure of establishing model tissue takes ~4-5 wk (Fig. 1C) and includes cell expansion, culturing of the cells in the model, and air exposure (AE) of the tissue model. To set up the model, the inner chamber of the six-well inserts were coated with a solution of bovine type I collagen (1.1 mg/ml; Organogenesis, Canton, MA) in DMEM and incubated for 30 min at 37°C. A suspension of MRC-5 fibroblasts at 2.3 x 10^5 cells/ml diluted in 1.1 mg/ml of collagen DMEM mixture was added onto the precoated collagen layer and incubated for 2 h in 5% CO_2 at 37°C. Following polymerization, 2 ml of complete DMEM was added to the outer chamber, and the cultures were incubated for an additional 24 h. The following day, culture media was removed from the outer and the inner chamber. Then 2 ml of fresh complete DMEM was added to both chambers. To allow remodeling of the stroma/matrix layer, cultures were incubated for 7 days with the medium being replaced every second day.

After 7 days, the culture media was removed from the outer and inner chambers followed by the addition of 1.5 ml of fresh complete DMEM to the outer chamber. Then 2 x 10^5 monocyte-derived DC in 50 µl of complete DMEM were added to the fibroblast collagen matrix and incubated for 2 h in 5% CO_2 at 37°C. After incubation 1.5 ml of culture media was gently added into the insert, and the culture was incubated for an additional 24 h in 5% CO_2 at 37°C before seeding of the epithelial cells. Epithelial cells were seeded by adding 5 x 10^5 epithelial cells in 50 µl complete DMEM to each insert. Submerged cultures were incubated for an additional 3 days in 5% CO_2 at 37°C to allow the epithelial cells to form a confluent mono-layer on the collagen gel. After 3 days, the tissue models were air exposed by removing the medium from the insert and reducing the volume to 1.5 ml in the outer chamber. Air-exposed tissue models were incubated for up to 10 days in 5% CO_2 at 37°C, and the culture media in the outer chamber was changed every second day. At days 5 to 10, tissue models were used for immunohistological immunofluorescence and live-cell imaging analyses, as well as DC functional analyses.

Histological and immunohistological analyses. Lung tissue model for cryosectioning was prepared by embedding the tissue in optimal cutting temperature compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) and then slowly frozen in −20°C for 24 h and transferred to −80°C until required. Eight-micron cryosections were cut onto diagnostic microscope glass slides (Thermo Scientific, Waltham, MA) using a MICROM cryostat HM 560 MV (Carl Zeiss, Jena, Germany) and fixed in 2% freshly prepared formaldehyde in PBS for 15 min at room temperature or in ice-cold acetone for 10 min at −20°C. For hematoxylin and eosin staining, the sections were stained for 15 s in Mayes Hematoxylin and counterstained for 2 min in eosin.

For immunohistochemistry, 8-µm frozen sections were blocked with 10% FBS in balanced salt solution (BSS) with 0.1% saponin for 30 min at room temperature, followed by additional blocking with 2% H_2O_2 in BSS-saponin and an avidin biotin blocking reagent (Vector Laboratories, Burlingame, CA). Primary monoclonal rat anti-human leukocyte antigen (HLA)-DR (1.25 µg/ml, clone YE2/36-HLK; AbD Serotec, Düsseldorf, Germany) antibody was diluted in BSS solution containing saponin and incubated overnight at room temperature as previously described (27). After incubation, tissue sections were washed and blocked with 1% normal rabbit serum in BSS-saponin before addition of biotinylated rabbit anti-rat IgG (4.2 µg/ml; Dako, Glostrup, Denmark) diluted in 1% normal rabbit serum in BSS-saponin. After wash avidin-peroxidase solution was added (Vectorstain Elite; Vector Laboratories), and the color reaction developed by the addition of 3,3-diaminobenzidine (Vector Laboratories) followed by counterstaining with hematoxylin. Tissue sections were visualized using a Leica DMR microscope (Wetzlar, Germany). Imaging and quantification of positive immunostaining were performed using acquired computerized image analysis by transferring digital images of the stained tissue samples taken by a DMR-X microscope (Leica) to a computerized Quantimet 550W image analyzer (Leica) (3). Single-positive stained cells were quantified in 25 high-power fields, and protein expression was determined as the percent positive area of the total relevant cell area using a Qwin 550 software program (Leica Imaging Systems). The total cell area was defined as the nucleated and cytoplasmic area within the tissue. Tissue sections stained with secondary antibodies only were used as negative controls.

Alcian blue staining. Eight-µm-thick frozen tissue sections were hydrated in distilled water and stained in Alcian blue solution (pH 2.5, Sigma Aldrich) for 30 min at room temperature. The sections were washed for 2 min in tap water and then rinsed in distilled water.

After that, sections were counterstained in nuclear Fast Red solution (Sigma) for 2 min and then washed in tap water for 1 min. The sections were dehydrated through 95% alcohol and twice in absolute alcohol for 3 min each. The sections were cleared in xylene and mounted in DPX mountant (VWR International, Radnor, PA). Mucins and mucous substances were thus stained in blue, nuclei in pink to red, and cytoplasm in pale pink.

Immunofluorescence analysis. Immunofluorescent labeling of 8-µm frozen sections was performed with the following primary antibodies: mouse anti-type IV collagen (13.5 µg/ml, clone COL-94; Abcam, Cambridge, UK), rabbit anti-tropoelastin (1:200, polyclonal; Elastin Products, Owensville, MO), mouse anti-laminin-5 (α3) (2.5 µg/ml; clone P3H9–2; R&D Systems, Minneapolis, MN), mouse anti-vimentin (1:100, clone VM3B4; Novocastra Laboratories, Newcastle, UK), rabbit anti-claudin I (1 µg/ml, polyclonal; Abcam), mouse anti-occludin (2.5 µg/ml, clone OC-3F10; Invitrogen), mouse anti-E-cadherin (2 µg/ml, clone HEC-D1; Invitrogen), rat anti-HLA-DR (2.5 µg/ml, clone YE2/36-HLK; AbD Serotec), mouse anti-DC-specific ICAM-grabbing nonintegrin (SIGN) (0.25 µg/ml, clone 120507; R&D Systems), and mouse anti-CD11c (1.25 µg/ml, clone B-ly-6; BD Pharmingen, San Diego, CA). Sections were washed in PBS containing 0.2% saponin and blocked with 1.5% normal goat serum (NGS) in 0.2% saponin in PBS for 45 min at room temperature. Sections were incubated with primary antibodies (see above) diluted in PBS containing 1.5% NGS and 0.2% saponin for 45 min at room temperature and then washed three times in PBS with 0.2% saponin. Specific staining was detected by the following secondary antibodies: Alexa 488-conjugated goat anti-rat IgG, Alexa 488-conjugated goat anti-mouse IgG, Alexa 555-conjugated goat anti-mouse IgG2b, Alexa 568-conjugated goat anti-mouse IgG1, and Alexa 647-conjugated goat anti-mouse IgG3.
488-conjugated goat anti-mouse IgG1, Alexa 594-conjugated goat anti-rabbit IgG (all from Molecular Probes, Invitrogen). The sections were incubated with secondary antibodies diluted to 3.3 μg/ml for Alexa 488 and 4 μg/ml for Alexa 555 and 594 in PBS containing 1.5% NGS and 0.2% saponin for 30 min at room temperature. Sections were mounted in SlowFade Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen) and visualized using a Leica TCS SP2 confocal microscope (Leica).

Quantification of DC numbers over time was performed in live tissue models by imaging the models at days 1, 3, 5, and 8 using a confocal microscope. To enable DC visualization in live tissue, DC were labeled with the far-red cell tracker, dimethyl dodecylamine oxide-succinimidyl (Molecular Probes, Invitrogen), before implantation into the models. Day 0 monocyte-derived DC were washed twice with PBS, resuspended at 10^6 cells/ml of PBS, and labeled with 5 μM of the cell tracker at room temperature for 15 min. Cells were washed twice with PBS after being labeled and resuspended to 2 × 10^5 cells/50 μl of complete DMEM. The labeled DC were seeded in 50 μl directly on top of the fibroblast collagen layer as described above. After 24 h, epithelial cells were added to the models followed by the additional 3D models with DC air-exposed and cultured for DC quantification at days 3, 5, and 8. Tissue models were visualized using a Nikon A1 confocal microscope (Nikon Instruments, Amstelveen, The Netherlands), and the obtained z-stack images were used for reconstruction of 3D projections. To quantify DC in 3D microscopic projections, fluorescently labeled cells within a defined field of each 3D projection were counted. A minimum of 250 cells was counted in each field, and, to exclude enumeration of dead cells, propidium iodide was added to the models before imaging. Then the number of cells per defined field was multiplied with a factor reflecting the total 3D tissue model area.

Generation of 16HBE cells expressing tdTomato. pCMV expression vector encoding the fluorescent tdTomato protein was a kind gift from R. Tsien (San Diego, CA). Plasmids were purified using HiSpeed MidiPrep kit (Qiagen, Crawley, UK) according to manufacturer's instructions. Cells were cultured in 35-mm culture dishes (MaTek, Ashland, MA), and, at 50–80% confluency, cells were transfected utilizing 4 μg of pCMV-tdTomato plasmid diluted in 100 μl OptiMEM and 14 μl Lipofectamine (Invitrogen) diluted in 100 μl OptiMEM. Both solutions were mixed and incubated at room temperature for 30 min. Media was removed from 35-mm dish and replaced with 600 μl OptiMEM and transfection mixture. After 6 h of incubation at 5% CO2, 37°C, 1.2 ml of complete DMEM with 20% FBS was added to the dish. The media was replaced with complete DMEM the next morning. Transfection efficiencies were analyzed using a CyAn flow cytometer (Beckman Coulter, Brea, CA). Cells were selected in 400 μG418 for 2 wk, and then the highest expression fraction was isolated using high-speed MoFlo cell sorter (Beckman Coulter). Nontransfected 16HBE cells were used to define the background fluorescence and gating strategy. Postsort purity of cells was analyzed using the CyAn ADP flow cytometer.

Live imaging of the tissue model using multiphoton microscopy. The lung tissue model was generated for live imaging using fluorescent tdTomato 16HBE and carboxyfluorescein diacetate succinnimidy l ester (CFSE)-labeled monocyte-derived DC. Day 6 DC were washed twice with PBS, resuspended at 10^6 cells/ml of PBS, and labeled with 5 μM of CFSE (Molecular Probes, Invitrogen) at room temperature for 15 min. Cells were washed twice with PBS after labeling and resuspended to 2 × 10^5 cells/50 μl of complete DMEM. The labeled DC were seeded directly on top of the fibroblast collagen layer as described above. After 7 days of AE, the fluorescent model was removed from the membrane insert and placed onto a 35-mm glass-bottom dish and imaged on a Zeiss LSM 510 NLO Meta Laser Scanning Confocal Microscope (Carl Zeiss) with three detectors for non-descanned detection (NDD) and a Coherent Chameleon Ultra laser (Coherent, Santa Clara, CA) with a blacked out, temperature controller chamber (Solenic Scientific, Segensworth, UK). Imaging was performed at 872–930-nm wavelengths using a C-Apochromat ×40 1.1 NA water dipping objective.

Real-time qRT-PCR analysis. Total RNA was extracted from DC and from the lung tissue model by using a Ribopure kit (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol. To compare chemokine expression between the 3D model containing DC and the control DC, we used 1.5 × 10^5 DC (similar numbers of cells that was implanted in each 3D model) cultured in 1.5 ml of complete DMEM without CSF-2 and IL-4 for 24 h before analysis. Similar conditions for control DC and DC implanted in the 3D model were obtained by lysing control DC in TRIzol together with one 3D model (fibroblasts and epithelial cells only) before RNA extraction. RNA was converted into cDNA by using a CDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. The primers and probes for HLA-DR, DC-SIGN, CD11c, CSF-2, IL-4, IL-1β, TNF, IL-10, IL-13, transforming growth factor (TGF)-β, CX3CL1, CXCL8, CCL17, CCL18, CCL22 and ubiquitin C were purchased as Predeveloped TaqMan Gene Expression Assays (Applied Biosystems). Ubiquitin C served as an endogenous control to normalize the amount of sample cDNA. Amplification and quantification of cDNA was performed as previously described (27). Relative amounts of the two chemokines were calculated using the comparative threshold cycle (CT) method. The threshold correlates to the cycle number where there is sufficient amplified product to give a detectable reading, and, if the threshold is not attained after 35–40 cycles, the mRNA is considered undetectable. The data are presented as relative expression of chemokine mRNA in untreated DC conditioned with supernatant compared with the chemokine mRNA in control DC. The data are also presented as relative expression of mRNA in the lung tissue model containing DC compared with control DC (mixed 1:1 with RNA extracted from a 3D model without DC) and 3D model composed of epithelial cells and fibroblasts only.

Chemokine measurement. Supernatants from control DC and tissue model were collected for protein measurement. A sandwich ELISA construction kit to detect CCL18 (R&D Systems), CCL17, and CCL22 (Antigenix, Huntington, NY) was used according to the manufacturer’s protocols. CCL18 was detected using 1.0 μg/ml capture of antibody and 200 ng/ml of detection antibody. CCL17 was detected using 1.0 μg/ml capture of antibody and 0.2 μg/ml detection antibody. CCL22 was detected using 1.0 μg/ml of capture antibody and 0.25 μg/ml detection antibody. Assays were developed by adding streptavidin-peroxidase and 3,3,5,5-tetramethyl-benzidine as the substrate. Absorbance was read at 450 nm using a Microplate Manager 6 reader (Bio-Rad, Hemel Hempstead, UK). Recombinant CCL18 (1 ng/ml), CCL17 (10 ng/ml) and CCL22 (10 ng/ml) were used as standards, and the concentration of chemokines in the test samples was calculated using the linear part of a “four-parameter fit” standard curve run in parallel with the samples.

Statistical analysis. Data are presented as means ± SD for the indicated number of experiments. All analyses are based on three or more separate experiments performed with monocyte-derived DC from different donors. The data were analyzed with use of the GraphPad Prism v5.5 software (GraphPad, San Diego, CA) using one-way ANOVA with Bonferroni multiple-comparison test, and differences between groups were determined to be statistically significant at P < 0.05.

RESULTS
Characterization of the lung tissue model. To reveal pathways by which human DC and tissue-specific nonimmune cells cooperate to orchestrate lung tissue physiology and immune responses at the mucosal surface, we sought to establish a 3D organotypic lung model combining human DC with two distinct cellular compartments: a fibroblast/matrix layer and an epithelial cell layer. The lung tissue model was cultured on a
permeable membrane for a total of 11–14 days, including submersion in medium for 4 days and AE for 7–10 days. After AE histological analysis of the tissue model revealed that the epithelial cells had formed a stratified squamous structure on top of the fibroblast matrix consistent with reports in similar systems containing lung epithelial cells (Fig. 1D). Also, a structure equivalent to a basement membrane was observed at the boundary between epithelial cells and the underlying fibroblast matrix layer (Fig. 1D, inset). These data demonstrate that our 3D organotypic lung model is fully developed and has a structure that resembles normal lung mucosa tissue (Fig. 1E).

**Extracellular matrix proteins are deposited in the lung tissue model.** Extracellular matrix proteins, such as collagens, elastin, and laminins are essential for tissue to function properly. To confirm that the tissue-specific cells in our model produce and deposit important tissue components, immunofluorescent analysis of frozen tissue sections was performed. Sections were labeled with antibodies against type IV collagen and tropoelastin, known to be important for formation of basement membranes. This revealed that both epithelial cells and fibroblasts in the tissue model produce type IV collagen and tropoelastin and that a continuous basement membrane is formed at the boundary between epithelial cells and the underlying fibroblast matrix layer (Fig. 2, A and B). Tissue sections were also analyzed for the presence of laminin-5, an extracellular matrix protein important in the initiation and maintenance of epithelial cell anchorage to the underlying connective tissue layer (35). Laminin-5 was found associated to epithelial layer as well as the basement membrane at the boundary between fibroblasts and epithelial cells (Fig. 2D). Immunofluorescence analysis also confirmed that yet another protein, vimentin, that is important for tissue fibroblast function was produced by the tissue model (39). Vimentin staining was only found in the fibroblast collagen matrix layer and not in the epithelial layer (Fig. 2C). Together, this indicates that molecules forming essential structures for lung tissue function are produced and deposited in the model.

The lung tissue model form tight and adherence junctions and a mucus layer. One key function of mucosal barriers resides in epithelial cell impermeability that is mediated by the formation of tight and adherence junctions (19). To verify that the epithelial cell layer of the lung tissue model has the capacity to form tight junctions and adhesion junctions, we analyzed the tissue sections for the presence of claudin I and occludin, two proteins important for formation of mucosal junction structures (19). As shown in Fig. 3A, epithelial cells in the lung tissue model express claudin I. In addition, occludin that interacts directly with claudins and actin (19) was detected in the lung tissue model (Fig. 3B). In mucosal tissue, production of E-cadherin by epithelial cells is important for formation and preservation of stratified epithelial barriers (19). Therefore, the model was analyzed for the presence of E-cadherin, and this revealed that E-cadherin is preferentially expressed by the epithelial cells and not by the fibroblasts in the tissue model (Fig. 3A and data not shown).

The production of mucus by the epithelium plays an important role in the protection of epithelial barriers against infectious agents and toxins that enter the respiratory system. To investigate whether our epithelial cells secret mucus, we stained the model with Alcian blue, a reagent that stains acid mucosubstances and acetic mucins. This showed mucus production already after 5 days of AE (Fig. 3C). Mucus production was also detected at 10 days of AE (Fig. 3D). These data indicate that the tissue model has a functional mucosal barrier and produce the tight junction proteins, claudin I and occludin, the cell-cell adhesion molecule E-cadherin, and mucus. To conclude, we have created a human 3D

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Fig. 2. The lung tissue model produces structural proteins important for lung tissue function. Immunofluorescence analyses of structural proteins essential for lung tissue function were carried out on cryosections (8 μm) of frozen lung tissue models. A and B: images show distribution of collagen IV (green) and tropoelastin (red) in the lung tissue model. Higher magnification (original magnification ×630) shows localization of collagen IV and tropoelastin fibrous structures (arrowed) at the boundary between the stroma matrix and epithelial cell layer (B). C and D: images show distribution of vimentin (C) and laminin-5 (D) in the lung tissue model (original magnification ×200). 4,6-Diamidino-2-phenylindole (DAPI) was used to reveal cell nuclei. All results are representatives of at least 3 independent experiments. In each experiment, 6 tissue models were analyzed (original magnification ×200).
lung tissue model that has basic structural and typical features of a normal lung mucosa.

The lung tissue model supports DC survival independent of exogenous growth factors. Next, we sought to confirm that DC in our lung tissue model survived equally well in both the absence and presence of CSF-2 and IL-4. Monocyte-derived DC were added to the fibroblast/matrix layer before seeding epithelial cells, and subsequently the models were cultured either with or without addition of exogenous CSF-2 and IL-4 for 11 days. To visualize DC, lung tissue models were sectioned and analyzed for the presence of HLA-DR\(^+\) cells using immunohistochemical analysis. The HLA-DR staining not only revealed that DC have survived in the tissue model but also that the DC are closely distributed to the epithelial layer consistent with reports of DC location in the lung tissue (24). Acquired computerized image analysis of HLA-DR immunostaining (Fig. 4, A–C) revealed that DC in the tissue model survived equally well in the absence and the presence of CSF-2 and IL-4 (Fig. 4D). These findings demonstrate that the lung tissue model supports the survival of DC independent of exogenous growth factors, CSF-2 and IL-4, for at least 11 days. To investigate whether there is endogenous expression of CSF-2 and IL-4 in the 3D lung model, we performed real-time RT-PCR analysis of lung tissue model without DC, DC cultured in medium only, and the lung tissue model with DC (Fig. 4E). CSF-2 mRNA was abundantly expressed, in all samples compared, and CSF-2 expression was lower in DC (average Ct value = 27.1 ± 0.8) and the 3D model with DC (average Ct value = 28.9 ± 1.4) compared with the 3D model only (average Ct value = 26.7 ± 0.5). In contrast, IL-4 mRNA was undetectable in all samples compared. Recent studies have established CX3CL1/fractalkine, the ligand for CX3CR1, as an important chemokine for DC interaction with the epithelial layer in lung tissue. The CX3CL1 mRNA was abundantly expressed in the lung tissue model without DC (average Ct value = 31.7 ± 1.2) and in DC only (average Ct value = 31.4 ± 0.5), and, as shown in Fig. 4E, CX3CL1 expression was higher in the 3D model with DC (average Ct value = 30.2 ± 0.5) compared with the 3D model without DC. In addition, we analyzed the expression of IL-10, IL-13, IL-1β, TNF, TGF-β, and CXCL8. Similarly to IL-4, IL-10 and IL-13 were undetectable in all samples compared, and, although IL-1β (Ct values in the range of 24.0–27.9), TNF (Ct-values in the range of 26.9–29.8), TGF-β (Ct values in the range of 27.2–29.9), and CXCL8 (Ct-values in the range of 19.6–23.4) were abundantly expressed in all samples compared, no significant differences were observed (data not shown).

Although, our results show that DC survive equally well in the absence and presence of exogenous growth factors, this raises the question of DC longevity in the tissue model. To assess DC survival and to quantify DC numbers over time, we utilized live confocal microscopy to image lung tissue models that were set up with fluorescently labeled DC. Confocal microscopy images of the developing 3D models were acquired at days 1, 3, 5, and 8 (Fig. 5, A and B). From the microscopic projections, it is evident that DC in the tissue model acquire a more elongated phenotype and that the layer where DC are localized expands over time (Fig. 5A). Furthermore, DC numbers were enumerated in microscopic projections (Fig. 5B), and this revealed that the number of DC in models was significantly lower at day 8 compared with day 1 (Fig. 5C). Thus this approach avoiding tissue digestion can be used as a noninvasive method to observe and quantify the longevity of DC in the 3D lung tissue model.

Next, we conducted experiments analyzing the expression of HLA-DR, DC-SIGN, and CD11c, surface molecules typically expressed by hematopoietic cells but not by nonimmune cells. In the 3D model without DC, HLA-DR, DC-SIGN, and CD11c mRNA were undetectable, whereas the mRNA expression was abundantly expressed in DC only (average Ct value = 28.7 ± 1.4, 30.2 ± 32.4 ± 0.9, respectively) and in the 3D model with DC (average Ct value = 29.5 ± 1.7, 29.1 ± 2.5, 33.7 ± 1.1, respectively). As shown in Fig. 5E, the expression of HLA-DR, DC-SIGN, and CD11c was not significantly different comparing the expression between DC and the 3D model with DC, thus confirming the presence of DC in the lung tissue model at day 8 of culture and that DC surface molecule expression is not significantly affected by the introduction into the lung tissue model.

DC are widely distributed in the epithelial layer of the lung tissue model. To further characterize and investigate the distribution of DC in the lung tissue model, we performed immunofluorescence staining of tissue sections using antibodies against HLA-DR, DC-SIGN, and CD11c. This revealed that all...
three markers can be used in immunofluorescence analysis to identify DC in the lung tissue model and confirmed that DC are closely distributed to the epithelial layer within the lung tissue model (Fig. 6, A–C). As shown in Fig. 6A, HLA-DR DC could be detected within the epithelial layer. The immunofluorescence analysis also revealed that HLA-DR DC-SIGN DC localized to the boundary between the epithelial and the fibroblast/matrix layers (Fig. 6B). Interestingly, CD11c DC-SIGN DC localized at the apical side of the epithelial layer (Fig. 6C). To confirm the expression CX3CR1, the receptor for CCL1, on DC in the lung tissue model, tissue sections were stained with antibodies against CX3CR1. As shown in Fig. 6D, CX3CR1-positive cells could be detected in the lung tissue model. This, together with previous reports establishing CCL1 as important for the capacity of lung DC to interact with the epithelial layer, may indicate that CCL1 and its ligand contribute to the epithelial organization of DC observed in the lung tissue model. Together, these data indicate that DC can be easily identified in the tissue model and that DC are strategically located underneath, within, and at the apical side of the epithelium coherent with previous reports (24).

Although the data above indicate that DC are widely distributed in the epithelial layer of the model, it is possible that the sectioning procedure introduces damage to the tissue and possible artifacts regarding DC distribution. Therefore, we established a modified model that could be imaged in real time using multiphoton microscopy. To visualize the different interacting cell types, epithelial cells expressing tdTomato and CFSE-labeled DC were utilized. Second, the model containing fibroblasts, fluorescent epithelial cells, and DC was monitored using multiphoton microscopy analysis. As shown in Fig. 6E, live imaging analysis of the lung tissue model confirmed that DC are localized at the apical side of the epithelial cell layer of the lung tissue model. Taken together, these data demonstrate that the tissue model comprises a fibroblast collagen matrix rich in extracellular matrix proteins and a tightly joined stratified squamous epithelium that sits on top of a continuous basement membrane as well as DC that are closely associated to the epithelial layer.

The capacity of DC to produce chemokines is regulated by the 3D organotypic model. Having established a lung tissue model with human DC, functional assays were performed to confirm that the 3D organotypic lung model contributed to regulate chemokine production related to physiological conditions. We first determined whether the lung tissue model could affect the capacity of DC to produce CCL18, a chemokine that is constitutively expressed in lung at steady state and is elevated in several human disorders, including various malignancies and inflammatory lung, skin, and joint diseases (20, 32). As shown in Fig. 7, expression of CCL18 was detectable in the lung tissue model with DC, whereas CCL18 expression was undetectable in the lung tissue model without DC. Furthermore, comparing CCL18 mRNA accumulation in the DC-

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Fig. 4. The lung tissue model supports survival of DC without the addition of exogenous growth factors, cerebrospinal fluid (CSF-2) and IL-4. To evaluate the capacity of the lung tissue model to support survival of DC, immunohistochemistry analyses detecting human leukocyte antigen (HLA)-DR molecules were performed on 8-μm cryosections from lung tissue models cultured either in the absence or presence of CSF-2 and IL-4. (A–C) Immunohistochemical staining of lung tissue model cryosections for HLA-DR is shown. Tissue sections from models containing DC and cultured in the absence (left) (A) or presence (middle) (B) of exogenous growth factors or tissue sections from models without DC (C), were stained with an anti-HLA-DR antibody (brown) (original magnification ×200). D: quantification of HLA-DR-positive area within the lung tissue model is shown. To quantify the HLA-DR staining in tissue sections, acquired computerized image analyses (ACIA) were used. Bar graphs indicate the amount of HLA-DR-positive staining in tissue sections from models absent of DC or models with DC that were cultured in the absence or presence of exogenous growth factors. To determine whether cytokines and chemokines important for DC survival and function are expressed in the lung tissue model, RNA was extracted and supernatants were collected from 3D models absent of DC, 3D models with DC, or control DC (cultured in medium only). Analyses of mRNA levels were determined in tissue and cells using real-time RT-PCR. E: bar graphs indicate the relative expression of CSF-2, IL-4, and CCL1 mRNA accumulated in control DC or 3D models with or without DC. All results are representatives of at least 3 independent experiments. In each experiment, 6 tissue models were analyzed. Data presented in bar charts are shown as mean values in triplicate from 1 representative experiment (± SD). The statistical significance (1-way ANOVA) of the indicated P values was determined: *P < 0.05, **P < 0.01, ***P < 0.005, ns = nonsignificant.
containing 3D model to DC cultured in medium only revealed enhanced CCL18 expression within the 3D model (Fig. 7A). That CCL18 production by DC is regulated in the 3D model was further supported by the fact that CCL18 protein was readily detected in the culture supernatant of the lung tissue model with DC (Fig. 7B). Next, we analyzed the capacity of DC in the 3D model to produce CCL17 and CCL22, chemokines that are expressed constitutively in thymus but are barely detectable in other peripheral tissues at steady state and that are induced during inflammatory reactions and associated to human skin and pulmonary inflammatory diseases (13, 21). Compared with DC only, the expression of CCL17 and CCL22 was relatively low in lung tissue models regardless of whether the model was set up with or without DC (Fig. 7, C and E). Furthermore, the levels of CCL17 and CCL22 protein were reduced in the culture supernatants of 3D models with DC compared with DC cultured in medium only (Fig. 7, D and F). Together these data suggest that the 3D model has the capacity to differentially regulate the production of chemokines in DC, and this involves enhanced expression of CCL18 and a reduced expression of CCL17 and CCL22.

Soluble components secreted from the lung tissue model are responsible for the induction of CCL18 in DC. Next, we focused our studies to determine whether soluble tissue-de-
derived components were responsible for the selective induction of enhanced CCL18 production in DC. When the impact of soluble tissue model-derived components on CCL18 production by DC was monitored, we found that exposure of DC to medium conditioned with the lung tissue model induced enhanced CCL18 mRNA expression (Fig. 8A) and CCL18 protein secretion by DC (Fig. 8B). Thus the lung tissue model secretes compounds involved in regulating CCL18 production by DC. To determine whether the composition of cells, i.e., lung epithelial cells and fibroblasts, into an AE 3D structure is necessary for the induction of enhanced production of CCL18 in DC, DC were also exposed to medium conditioned with air-exposed epithelial cells or fibroblast as well as epithelial cells and fibroblasts cultured as a mixed (1:1) submerged monolayer. This revealed that only the complete lung tissue model has the capacity to condition medium that can be used to induce enhanced CCL18 expression in DC (Fig. 8C).

**DISCUSSION**

Tissue-specific niches, such as gut and lung mucosa, are increasingly recognized as influencing a broad range of events involved in immune homeostasis and inflammation as well as the host response to infection (18, 30, 33). Although, in vitro 3D organotypic lung models have been established in the past and are commercially available ranging from models of normal airway epithelium to models of lung cancer, models with relevant features and functions of the human airway wall combined with human immune cells are lacking. Here, we have established a 3D organotypic lung tissue model allowing studies of DC regulation by lung tissue specific cells. We first showed that molecules involved in the formation of essential structures for lung tissue function are produced and deposited in the model. We then showed that the lung tissue model secretes compounds involved in regulating CCL18 production by DC. To determine whether the composition of cells, i.e., lung epithelial cells and fibroblasts, into an AE 3D structure is necessary for the induction of enhanced production of CCL18 in DC, DC were also exposed to medium conditioned with air-exposed epithelial cells or fibroblast as well as epithelial cells and fibroblasts cultured as a mixed (1:1) submerged monolayer. This revealed that only the complete lung tissue model has the capacity to condition medium that can be used to induce enhanced CCL18 expression in DC (Fig. 8C).
The finding that our human 3D organotypic lung model has the capacity to support survival and allow functional studies of human DC has important implications for increasing our understanding of local immune regulation in lung tissue. This novel 3D organotypic lung model composed of human lung epithelial cells and fibroblasts in combination with human DC shares similarities with real tissue, including the overall structure and deposition of matrix proteins essential for lung tissue function and forms a functional mucosal barrier including production of proteins necessary for tight junction and adhesion junction formation. In this context, the 3D AE tissue model provides a unique system in which to further elucidate mechanisms of tissue organization and remodeling at steady state and in response to external stimuli, including viral and bacterial infection.

Coordinated expression of chemokines by nonimmune and immune cells is essential in mediating adhesion and migration of diverse populations of immune cells from blood to tissue at steady state and during inflammation and infection. The second major outcome from this study is that this organotypic model regulates the capacity of DC to produce one homeostatic chemokine, CCL18, and two inducible chemokines, CCL17 and CCL22. CCL18 is a constitutively expressed chemoattractant produced by immune cells and predominantly associated to lymphnode and lung tissue, and that is detected at high levels in human serum at homeostatic conditions (20). The influence of tissue in the involvement of regulating production of CCL18 in DC was confirmed by demonstrating that DC in the organotypic model make more CCL18 than those cultured under conventional conditions. This finding, therefore, supports the fact that the lung tissue model microenvironment induces changes to DC and selectively modulates the production of chemokines resembling physiological conditions (13, 20, 21). Further support of a functional distinction between DC in the 3D organotypic model compared with DC cultured under conventional conditions comes from our findings that implanting DC in the model reduced the levels of CCL17 and CCL22 detected. CCL17 and CCL22 are not detectable at steady state but are induced during inflammatory reactions and associated to human skin and pulmonary inflammatory diseases (16, 41), thus implicating that specific tissue is able to indirectly exert immune regulatory function via their influence on DC function locally. Furthermore, the results from our study also demonstrate that soluble components secreted by 3D organotypic models are responsible for the enhanced production of CCL18 in DC. Although, differential expression of cytokines and chemokines by DC in response to monolayer of lung epithelial cells has been observed (26, 29), the effect of 3D tissue models on DC function is limited. Therefore, we investigated whether there are differences between the 3D organotypic model, AE epithelial cells, AE fibroblasts, or submerged monolayer culture of epithelial cells and fibroblasts in terms of altering the capacity of DC to produce CCL18. Medium conditioned with the 3D organotypic model was more potent than conditioned medium from the single cell cultures or the monolayer culture.

![Graphs](http://ajplung.physiology.org)
in promoting enhanced CCL18 production, suggesting that the 
3D organotypic model secretes additional factors contributing 
to this process, thus indicating that the complex structure of the 
lung tissue model favors the production of factors involved in 
regulating CCL18 production in DC under homeostatic conditions. 
This finding is quite intriguing and merits further studies.

In addition to 3D organotypic tissue models, various alter-
native cell culture systems are available as tools for studying 
the impact of local tissue on immune cell function. Each 
system has its distinct advantages and disadvantages, and the 
choice of system depends on various factors, including the 
experimental question being addressed and the cost, training, 
and expertise required to successfully establish, validate, and 
apply the model. One common technique involves utilizing 
monolayers that are permitting infection but are usually based 
on a single nonpolarized cell type in the absence of 3D cellular 
interactions. Culture in Transwells allows culturing of mono-
layers on a permeable membrane that, unlike traditional mono-
layers systems, allows the cells to develop an apical-basal polarity. 
These polarized surfaces can be independently exposed to 
different factors, including pathogens and toxins, and thereby 
exposing the cells in a physiologically relevant setting and 
taking into account transepithelial migration (10). One 
disadvantage of the method is that these cultures are often 
based on one type of cell only. Also, such models lack 
extensive 3D cellular interactions that are achieved with 3D 
matrix scaffold cultures, which are crucial for normal tissue 
function as well as infection processes (8).

The engineering of organotypic models involves implanting 
of cells or tissue into a 3D matrix scaffold composed of 
collagen, extracts of extracellular matrix, synthetic or semisyn-
thetic materials, or a combination of these materials. However, 
models with relevant features and functions of the human 
airway wall combined with human immune cells are lacking. 
In this study, therefore, we sought to develop a novel human 
3D lung tissue model that utilizes appropriate human cell 
populations, including bronchial epithelial cells, fibroblasts, 
and human immune cells. In our model epithelial (16HBE) and 
fibroblast (MRC-5) cell lines are used, as they allow for 
reproducibility compared with using potentially impure 
primary cells. The choice of this epithelial line was based on the 
fact that 16HBE cells resemble primary lung epithelial cells 
(38) and thus are a valuable and reproducible model of normal 
lung epithelial tissue (7). Although, quite laborsome, this 
relatively inexpensive approach provides a platform that also 
allows studies in conjunction with manipulation of gene expres-
sion in tissue-specific nonimmune cells. Thus the model is well 
suited for studies on tissue-specific regulation of human DC 
functional properties and migratory behavior in a physiologically 
relevant setting previously not achievable. Furthermore, the model 
allows exposure of cells under physiological conditions where 
important aspects of host-pathogen interactions can be monitored 
in real time. In conclusion, we have established a human 3D 
organotypic lung model in which tissue-specific mechanisms that 
regulate DC function locally during steady state and in response to 
inflammation can be studied. By further understanding the mech-
anisms whereby specific tissue niches control the functional prop-
erties of DC, we might uncover potential targets for manipulating 
the DC function in therapeutic settings to restore tissue homeo-
stasis not only in acute and chronic infectious diseases but also 
other chronic inflammatory diseases.
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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

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