Modelling the lung in vitro

Riet, S. van

Chapter 5

Modulation of airway epithelial innate immunity and wound repair by M(GM-CSF) and M(M-CSF) macrophages

Sander van Riet[1], Annemarie van Schadewijk[1], Steve De Vos[2], Nick Vandeghinste[2], Robbert J. Rottier[3], Jan Stolk[1], Pieter S. Hiemstra[1], P. Padmini S. J. Khedoe[1]

[1]Dept. of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands.

[2]Galapagos NV, Mechelen, Belgium

[3]Department of Pediatric Surgery, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

Journal of Innate Immunity 2020;12(5):410-421.

Abstract

Airway epithelial cells and macrophages participate in inflammatory responses to external noxious stimuli, which can cause epithelial injury. Upon injury, epithelial cells and macrophages act in concert to ensure rapid restoration of epithelial integrity. The nature of the interactions between these cell types during epithelial repair are incompletely understood. Here we use an *in vitro* human co-culture model of primary bronchial epithelial cells cultured at the air-liquid interface (ALI-PBEC) and polarized primary monocyte-derived macrophages. Using this co-culture, we studied the contribution of macrophages to epithelial innate immunity, wound healing capacity and epithelial exposure to whole cigarette smoke (WCS). Co-culture of ALI-PBEC with LPS-activated M(GM-CSF) macrophages increased expression of *DEFB4A*, *CXCL8* and *IL6* at 24 hours in the ALI-PBEC, whereas LPS-activated M(M-CSF) macrophages only increased epithelial *IL6* expression. Furthermore, wound repair was accelerated by co-culture with both activated M(GM-CSF) and M(M-CSF) macrophages, also following WCS exposure. Co-culture of ALI-PBEC and M(GM-CSF) macrophages resulted in increased *CAMP* expression in M(GM-CSF) macrophages, which was absent in M(M-CSF) macrophages. *CAMP* encodes LL-37, an antimicrobial peptide with immune modulating and repair enhancing activities. In conclusion, dynamic crosstalk between ALI-PBEC and macrophages enhances epithelial innate immunity and wound repair, even upon concomitant cigarette smoke exposure.

Introduction

Airway epithelial cells play a central role in the first line of defense against inhaled particles, gasses and pathogens.The epithelial lining acts as a physical barrier and epithelial cells produce protective mediators (e.g. cytokines, chemokines, antimicrobial peptides) to prevent intrusion of harmful substances and pathogens into the lungs. Epithelial cells also mediate mucociliary clearance to remove mucus-trapped-particles and pathogens from the airways (Hiemstra, McCray, and Bals 2015; Whitsett and Alenghat 2015). Injury to the epithelial layer, due to e.g. bacterial and/or viral infection or inhalation of toxicants (including cigarette smoke), may cause disruption of epithelial barrier integrity and impair epithelial repair (Hiemstra, McCray, and Bals 2015; Hiemstra et al. 2016). The epithelial repair process is tightly controlled to ensure rapid closure of the wound and restoration of lung tissue homeostasis. However, chronic insults to the epithelial layer contribute to dysfunction of airway epithelial cells and development and progression of lung diseases, such as chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF) and asthma. Epithelial integrity, barrier function and host defense responses are impaired in various lung diseases (Amatngalim and Hiemstra 2018; Hiemstra, McCray, and Bals 2015), predisposing these patients to repeated infections and exacerbations. Inflammatory cells such as macrophages contribute to the epithelial wound repair process by releasing a range of mediators and by providing protection against infections following disruption of the epithelial barrier integrity (Gardner, Borthwick, and Fisher 2010; Alber et al. 2012).

Macrophages constitute a heterogeneous population of cells resulting form their high level of plasticity, and the various subsets contribute to the epithelial repair response and host defense (Gordon and Martinez-Pomares 2017; Snyder et al. 2016). The phenotype of macrophages is tightly controlled by their microenvironment, that provides signals for activation and differentiation. Insight into these mechanisms has resulted in a classification of macrophages based on their activation state and properties. Macrophages can thus be broadly subdivided in pro-inflammatory macrophages (known as classically activated macrophages, also known as M1 macrophages) and anti-inflammatory macrophages (known as alternatively activated macrophages, also known as M2 macrophages) (Arora et al. 2018; Murray 2017). Pro-inflammatory macrophages produce proinflammatory cytokines and their phenotype is driven by pro-inflammatory stimuli, including TNF-α, IFN-γ and lipopolysaccharide (LPS), whereas anti-inflammatory macrophages are

more diverse and can be divided into several subsets, which are involved in defence against parasitic infections (M2a), immunoregulation (M2b) and tissue remodeling and matrix deposition (M2c) (Byrne et al. 2015). In the lungs, macrophages are widely present in the airway lumen (airway macrophages), the alveolar lumen (alveolar macrophages), and in the lung parenchyma and airway wall (interstitial macrophages), whereas monocytes can be recruited upon inflammation (Hu and Christman 2019).

The function and phenotype of these cells depends on the local cytokine milieu (Byrne et al. 2015; Puttur, Gregory, and Lloyd 2019). Following injury to the lung epithelial lining, both resident macrophages and those derived from recruited monocytes contribute to the inflammatory and remodeling phase of epithelial repair, although the precise interaction with airway epithelial cells is insufficiently studied.

Despite the knowledge gained from various *in vivo* models on epithelial repair, the use of laboratory animals becomes more controversial and importantly the translation of results from such animal models to human disease is not always straightforward. However, whereas *in vitro* models with (primary) airway epithelial cells have provided much knowledge on the mechanism of epithelial wound repair (Amatngalim et al. 2016; Gardner, Borthwick, and Fisher 2010), these models do not accurately represent the complex cellular network of airway epithelial cells and inflammatory cells, including macrophages, that are essential during epithelial wound repair. Although various models are available to investigate the interaction between airway epithelial cells and immune and inflammatory cells, many of these studies have been performed using cell lines for either macrophages, airway epithelial cells or both, and are therefore not representative. Furthermore, many of these models lack lung specificity. In the present study, we therefore combined primary airway epithelial cells with primary monocyte-derived macrophages. To study the complex cellular crosstalk and interaction between airway epithelial cells and macrophages in more detail, we developed a co-culture model of human primary bronchial epithelial cells grown at the air-liquid interface (ALI-PBEC) and human peripheral blood $CD14^+$ monocyte-derived macrophages, that were polarized to either a pro-inflammatory M(GM-CSF) or anti-inflammatory M(M-CSF) macrophagephenotype (Van't Wout et al. 2015). Using this model of primary cells, we studied the interaction between M(GM-CSF) or M(M-CSF)macrophages and ALI-PBEC and its effect on epithelial innate immunity and repair.

Materials and methods

Culture of primary bronchial epithelial cells (PBEC)

Primary bronchial epithelial cells (PBEC) were obtained from tumor-free lung tissue of patients undergoing lobectomy for lung cancer at the Leiden University Medical Center (Leiden, The Netherlands). The use of this lung tissue for research following surgery within the framework of patient care was in line with the "Human Tissue and Medical Research: Code of conduct for responsible use" (2011) (www.federa.org), that describes the no-objection system for coded anonymous further use of such tissue. All PBEC donors used for these experiments were considered not to have chronic airflow limitation (i.e. not having chronic obstructive pulmonary disease [COPD]), based on a predicted forced expiratory volume in 1 sec (FEV1) >85% and all had an age of >55 at time of surgery. The cells were isolated, cultured and differentiated at the air-liquid interface (ALI) for 14 days (SFig. 1A), to develop a well-differentiated epithelial layer, in transwell-inserts in 12-well plates ALI as previously described (Amatngalim et al. 2018). During PBEC differentiation, the cells were cultured at ALI with Bronchial Epithelial Cell Medium-basal (BEpiCM-b ScienCell, Carlsbad, CA, USA) diluted 1:1 with DMEM from Stemcell Technologies (Vancouver, Canada) with bronchial epithelial cell growth supplements from ScienCell, further supplemented with the 50 nM EC-23 (synthetic retinoic acid analogue, Tocris, Bio-Techne Ltd. Abingdon, U.K.).Well-differentiated ALI-PBEC were used for further co-culture experiments. Approximately $1x10^6$ ALI-PBEC were present on these inserts at the time of the experiment.

Isolation of monocytes and differentiation towards M(GM-CSF)and M(M-CSF) macrophagephenotype

CD14 positive monocytes were isolated from fresh buffycoats (Sanquin Blood Bank, Leiden, the Netherlands) obtained from healthy controls as described previously (Van't Wout et al. 2015). We seeded $0.5x10^6$ monocytes per well of a 12-well plate with either 5 ng/ml GM-CSF (R&D Systems, Minneapolis, MN) or 50 ng/ml M-CSF (Myltenyi Biotec, Auburn, CA) to induce polarization to M(GM-CSF) or M(M-CSF) macrophages respectively. Following 7 days of culture in RPMI 1640 medium (Invitrogen, Breda Life Technologies, The Netherlands) containing 10% FCS (Invitrogen), 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Bio Whittaker, Walkersville, MD, USA), M(GM-CSF) or M(M-CSF) macrophages were stimulated with

100 ng/ml lipopolysaccharide (LPS from Pseudomonas aeruginosa, Sigma-Aldrich, St. Louis, MO) during co-culture experiments (experimental outline shown in SFig. 1A). After 7 days, before start of co-culture with ALI-PBEC, similar numbers of M(GM-CSF) and M(CSF) were counted (~3.2x10^6 cells/well). M(GM-CSF) or M(M-CSF) macrophages were characterized by high expression of *CHI3L1* and IL-12/p40 release (M(GM-CSF)) or *CD163* expression and IL-10 release (M(M-CSF)), respectively (SFig. 2).

Co-culture of M(GM-CSF)or M(M-CSF) macrophages and ALI-PBEC

ALI-PBEC were cultured as described above. Twentyfour hours before co-culture, the medium of ALI-PBEC was switched to epithelial culture medium (Amatngalim et al. 2018) without growth factors, hydrocortisone and EC23 (starvation medium, 24h starvation). Co-culturing was performed by transfer of the transwell-inserts seeded with ALI-PBEC to another 12-wells plate that contained the polarized macrophages. All co-culture experiment were performed in starvation medium with or without LPS in the basal compartment, for activation of macrophages. ALI-PBEC were i) co-cultured with (LPS-activated) M(GM-CSF) or M(M-CSF) macrophages for 24 h (SFig. 1A), ii) mechanically wounded and then co-cultured with LPS-activated M(GM-CSF) or M(M-CSF) macrophages until wound closure, or iii) mechanically wounded, exposed to whole cigarette smoke (WCS) and subsequently co-cultured with LPS-activated M(GM-CSF) or M(M-CSF) macrophages until wound closure. Epithelial wounding was performed as described previously (Hiemstra et al. 2016). A list of compounds that were used for mechanistic experiments is shown in Table 1. These compounds were added during co-culture of ALI-PBEC and M(GM-CSF)/ M(M-CSF) macrophages.

Compound	Concentration	Supplier
LL-37	2.5 μg/ml	(37)
TGF-β1	5 ng/ml	R&D system
anti-LL-37 (Clone III D7 (09/02/00 ST)	1:100	(37)
GM6001	25 μM	Merck
SB-431542	5 μM	Sigma

Table 1. Compounds

Whole cigarette smoke exposure

Whole cigarette smoke (WCS) exposure was performed as described previously (Amatngalim et al. 2018). In brief, well-differentiated ALI-PBEC cultures were placed in a modified hypoxic chamber for WCS or air (control) exposure. In these chambers, the cultures were exposed to either 4-5 min of cigarette smoke from 1 3R4F research cigarette (University of Kentucky, Lexington, KY) or room air as control. Following 4-5 min of WCS exposure, the chambers were ventilated for 10 min to remove smoke from the chambers. The transwell inserts were subsequently removed from the plate, and transferred to the 12-well plates containing the macrophages for co-culture and placed back at 37°C.

Quantitative RT-PCR

RNA was isolated from ALI-PBEC (from transwell-insert) and macrophages (from 12-wells plate at bottom), separately, according to manufacturer's instruction using Maxwell RNA extraction kits (Promega, Madison, WI, USA). Quantitative RT-PCR was performed as described previously (Amatngalim et al. 2018) using primer pairs listed in Table 2. qPCR reactions were performed in triplicate, corrected for the geometric mean of expression of 2 reference genes (*ATP5B* and *RPL13A*), selected using the NormFinder algorithm software (Andersen, Jensen, and Orntoft 2004). Expression values were determined by the relative gene expression of a standard curve as determined by CFX manager software, and expressed as fold increase (Bio-Rad).

Gene	Forward primer (5' to 3')	Reverse primer (3' to 5')
ATP5B	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT
RPL13A	AAGGTGGTGGTCGTACGCTGTG	CGGGAAGGGTTGGTGTTCATCC
CAMP	TCATTGCCCAGGTCCTCAG	TCCCCATACACCGCTTCAC
CXCL8	CTGGACCCCAAGGAAAAC	TGGCAACCCTACAACAGAC
IL6	CAGAGCTGTGCAGATGAGTACA	GATGAGTTGTCATGTCCTGCA
CD163	TTTGTCAACTTGAGTCCCTTCAC	TCCCGCTACACTTGTTTTCAC
CHI3L1	CTGTGGGGATAGTGAGGCAT	CTTGCCAAAATGGTGTCCTT
HGF	TCCAGAGGTACGCTACGAAGTCT	CCCATTGCAGGTCATGCAT
MMP9	ACCTCGAACTTTGACAGCGAC	GAGGAATGATCTAAGCCCAGC
PDGFA	CACCACCGCAGCGTCAA	CCTCACCTGGACTTCTTTTAATTTTG
TGFB1	CTAATGGTGGAAACCCACAACG	TATCGCCAGGAATTGTTGCTG
DEFB4A	ATCAGCCATGAGGGTCTTG	GCAGCATTTTGTTCCAGG
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG

Table 2. Primers used for RT-PCR

ELISA

Levels of the interleukins (IL), IL-8 (R&D), IL-10 and IL-12/p70 (BD Bioscience), and human β-defensin 2 (hBD-2) (Antigenix America) were determined in supernatant, basal medium (IL-10, IL-12/p40, IL-8 and hBD-2) or in apical wash (IL-8, hBD-2) according to the manufacturer's instructions.

Apical wash

To assess levels of mediators secreted to the apical side by well-differentiated ALI-PBEC, apical washes were collected as described (Amatngalim et al. 2017). Briefly, apical washes were performed by applying 100 µL PBS for 10 min. After 10 min the fluid was collected, and stored at -80°C pending analysis by ELISA as described above.

Statistical analyses

Statistical significance of differences was assessed using one-way ANOVA or repeated measures analysis, followed by post-hoc analysis using Fisher's LSD multiple comparison test using Graphpad Prism 7. Differences at $p<0.05$ were regarded as statistically significant.

Results

LPS-activated M(GM-CSF) and M(M-CSF) macrophages alter epithelial innate immunity

As host defense is one of the key functions of airway epithelial cells, we first aimed to establish if there is an effect of macrophages on epithelial host defense in our co-culture model. To this end we used well-differentiated primary bronchial epithelial cells cultured at the air-liquid interface (ALI-PBEC) in the presence or absence of lipopolysaccharide (LPS)-stimulated M(GM-CSF) or M(M-CSF) macrophages (SFig. 1A). Following 24 hours of co-culture we measured the epithelial expression of host defense mediators (*IL6, CXCL8,* and *DEFB4A*). In absence of LPS, we found no effect of M(GM-CSF) or M(M-CSF) macrophages on the epithelial mRNA expression of IL6, *CXCL8* or *DEFB4A*. In presence of LPS, expression of *IL6* mRNA was increased in ALI-PBEC upon co-culture with both macrophage subtypes (Fig. 1A). Expression of *CXCL8* was increased in ALI-PBEC upon co-culture with LPS-activated M(GM-CSF) but not M(M-CSF) macrophages. Furthermore, epithelial expression of *DEFB4A*, the gene encoding hBD-2, was increased

upon co-culture with LPS-activated macrophages, and this effect was significantly higher in co-culture with M(GM-CSF) compared to M(GM-CSF) macrophages (Fig. 1A). Expression of other host defense proteins in ALI-PBEC (CAMP, RNASE7) was not altered (data not shown). We further investigated this effect on ALI-PBEC innate immune responses at the protein level (Fig. 1B). Confirming our findings on gene expression level in ALI-PBEC,

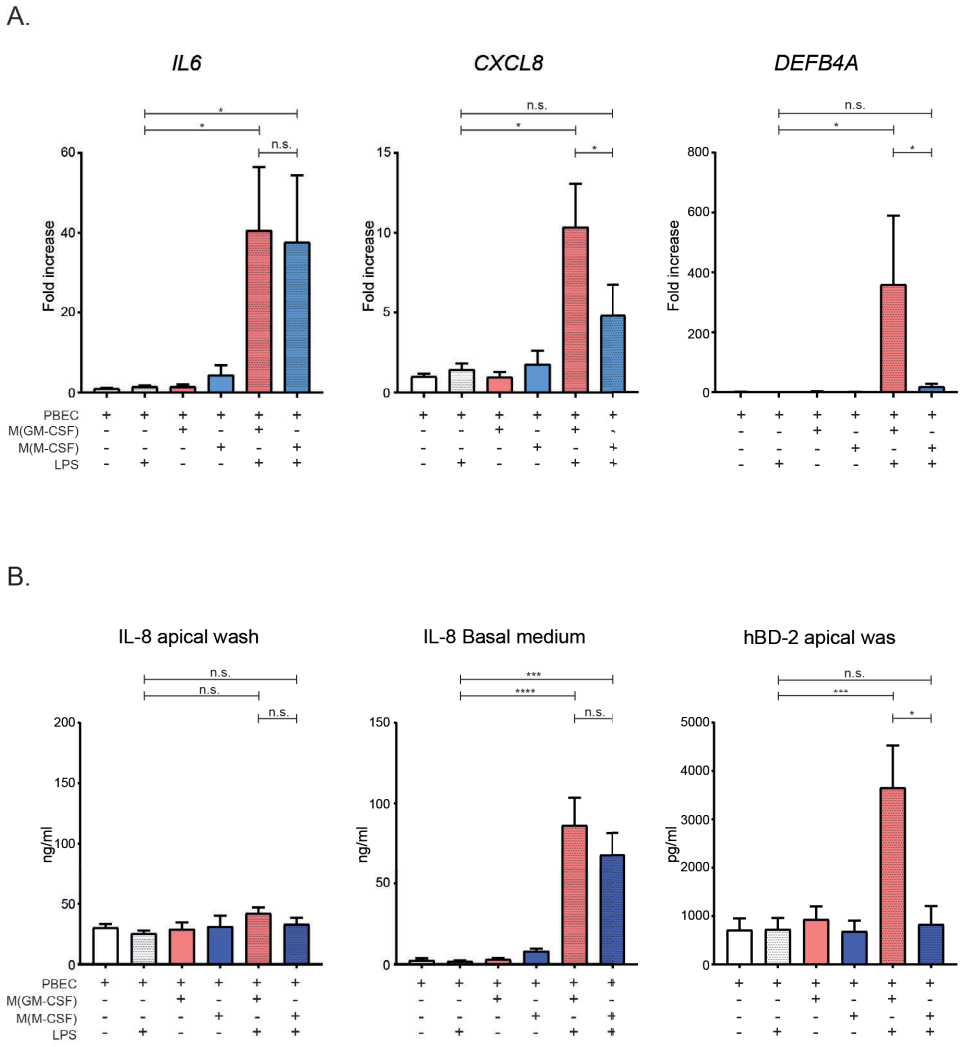


Figure 1. Activated M(GM-CSF) and M(M-CSF) macrophages modulate epithelial innate immunity. Well-differentiated ALI-PBEC were co-cultured with M(GM-CSF) or M(M-CSF) macrophages in the presence and absence of LPS and mRNA and protein levels of cytokines and antimicrobial peptides were measured after 24 hours in ALI-PBEC. (A) mRNA expression levels of *IL6*, *CXCL8* and *DEFB4A* were measured in ALI-PBEC upon co-culture with (activated) M(GM-CSF) (red bars) and M(M-CSF) (blue bars) macrophages (n= 7 independent ALI-PBEC donors). (B) IL-8 and hDB-2 protein levels were measured at 24 hours in basal medium and apical washes (n= 7 independent ALI-PBEC donors). Data are shown as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

IL-8 was found to be increased in the basal medium of co-cultures with LPS-activated macrophages but not in the unstimulated controls. No increased IL-8 levels were observed in the apical washes. hBD-2 was not detected in the basal medium (data not shown) but was secreted on the apical side of the ALI-PBEC co-cultured with activated M(GM-CSF), but not M(M-CSF) macrophages. Since airway epithelial cells do not respond to LPS (Fig. 1A+B) (Jia et al. 2004), these findings show that ALI-PBEC initiate host defense responses in co-culture with LPS-activated M(GM-CSF) or M(M-CSF) macrophages.

Both activated M(GM-CSF) and M(M-CSF) macrophages enhance epithelial wound repair

After demonstrating that LPS-activated macrophages can modulate epithelial host defense responses, we next continued by investigating whether LPS-activated M(GM-CSF) or M(M-CSF) macrophages can alter epithelial wound repair. To this end, circular wounds were mechanically created in the epithelial layer (Hiemstra et al. 2016), and subsequently co-cultured with activated macrophages (SFig. 1B, Fig. 2A). LPS alone in the absence of macrophages did not affect epithelial wound closure. However, co-culture with both M(GM-CSF) and M(M-CSF) macrophages significantly increased epithelial wound closure compared to epithelial mono-cultures. Complete wound closure was reached at 30 and 50 hours post mechanical wounding in the presence of activated M(GM-CSF) or M(M-CSF) macrophages respectively, whereas mono-cultures reached only 75% at this time-point, indicating that epithelial wound closure was enhanced consistently in co-cultures with both LPS-activated M(GM-CSF) and M(M-CSF) macrophages.

Since we previously reported that exposure to whole cigarette smoke (WCS) decreases epithelial wound closure (Luppi et al. 2005; Hiemstra et al. 2016), we investigated whether LPS-activated macrophages also enhanced epithelial wound closure in WCS exposed ALI-PBEC cultures. To this end, ALI-PBEC were exposed to WCS following wounding and subsequently co-cultured with either M(GM-CSF) or M(M-CSF) macrophages (Fig. 2B). In line with our previous findings (Amatngalim et al. 2016), WCS exposure delayed wound closure especially at early time points (t=8 hours; p=0.065), irrespective of co- or mono-culture of ALI-PBEC. Co-culture with both M(GM-CSF) and M(M-CSF) macrophages also significantly increased epithelial wound closure in WCS-exposed cultures. These data suggest that LPS-activated

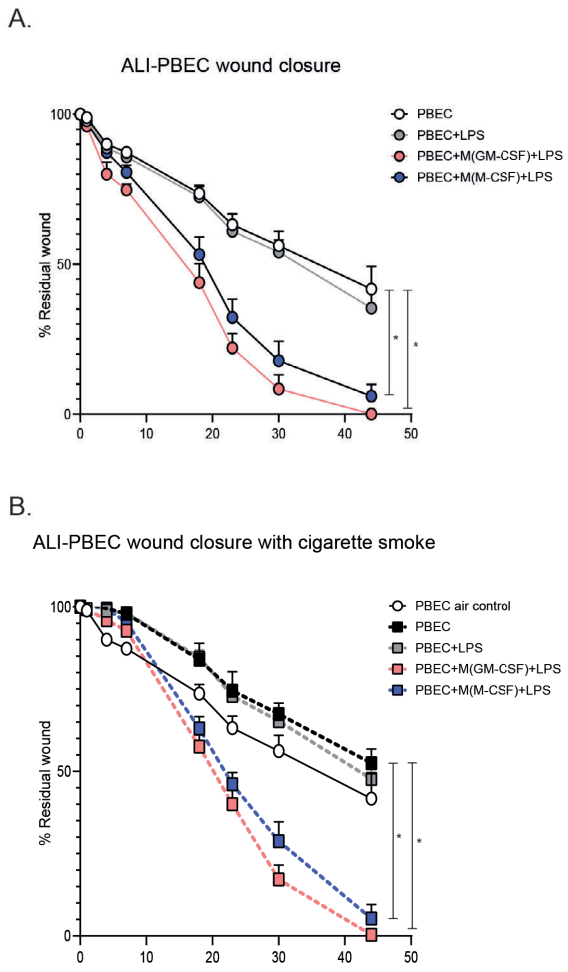


Figure 2. Activated M(GM-CSF) and M(M-CSF) macrophages enhance epithelial wound repair. ALI-PBEC were mechanically injured and subsequently co-cultured with either activated M(GM-CSF) or M(M-CSF) macrophages. Wound closure was monitored in time using phase-contrast light microscopy. Wound closure is shown as percentage residual wound area. (A) Upon mechanical wounding, ALI-PBEC were cultured alone (black line) or co-cultured with activated M(GM-CSF) (red line) or M(M-CSF) (blue line) macrophages. (n=4 independent ALI-PBEC donors). (B) ALI-PBEC were exposed to whole cigarette smoke (WCS) and subsequently co-cultured with activated M(GM-CSF) (red line) or M(M-CSF) (blue line) macrophages (n=7 independent ALI-PBEC donors). Data are shown as mean ± SEM. * $p < 0.05$

macrophages increase epithelial wound repair, also following exposure to cigarette smoke.

Macrophage-derived mediators enhance epithelial wound repair

To determine which macrophage-derived mediators contributed to the observed enhanced epithelial wound repair, we measured macrophage expression of various growth factors (*TGFB1*, *HGF*, *IL10* and *PDGFA*), the metalloproteinase *MMP9* and the antimicrobial peptide *CAMP*, all of which have been implicated in epithelial wound repair (Gardner, Borthwick, and Fisher 2010) (Fig. 3A+B). Activated M(M-CSF) macrophages showed higher expression of *IL10*, and to a lesser extent *HGF* compared to activated M(GM-CSF)macrophages. Macrophage expression of *TGFB1*, *MMP9* and PDGFA was not altered (Fig. 3A+B). Interestingly, LPS-activated M(GM-CSF)

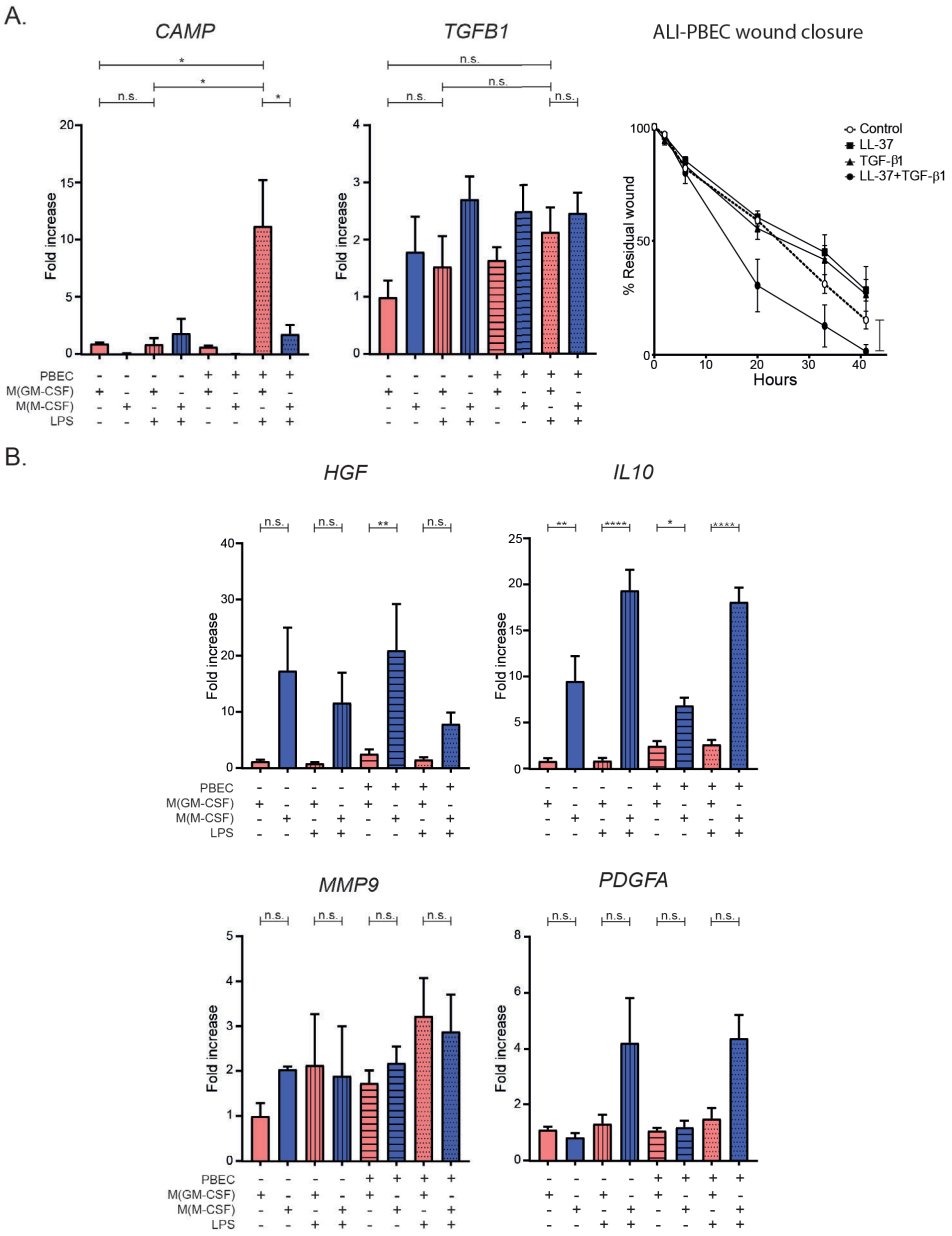


Figure 3. Macrophages-derived mediators enhance epithelial wound repair. mRNA expression levels of various genes in M(GM-CSF) and M(M-CSF) macrophages were measured upon co-culture with ALI-PBEC and epithelial wounding. (A) Expression levels of *CAMP* and *TGFB1* were measured in (LPS-activated) M(GM-CSF) (red bars) or M(M-CSF) (blue bars) macrophages in mono-cultures on upon co-culture with ALI-PBEC. (n=3 independent buffy donors). The role of these mediators in epithelial wound closure was assessed by addition of LL-37 and/or TGF-β1 in wounded ALI-PBEC (n=3 independent ALI-PBEC donors). (B) mRNA expression levels of *HGF*, *IL10*, *MMP9* and *PDFGA* in M(GM-CSF) (red bars) and M(M-CSF) (blue bars) macrophages was measured by q-PCR (n=3 independent buffy donors). Data are shown as mean ± SEM. * $p < 0.05$, ** $p < 0.01$

macrophages showed increased *CAMP* (encodes LL-37, an antimicrobial peptide with immune modulating and wound repair enhancing activities) expression only upon co-culture with ALI-PBEC (Fig. 3A). As *CAMP* expression in M(GM-CSF) macrophages was increased in presence of LPS and concommitant co-culture with ALI-PBEC, we investigated whether this increased *CAMP* expression may contribute to the observed enhanced wound closure in ALI-PBEC. We therefore added LL-37 and/or TGF-β1 (which is known to contribute to epithelial repair) in wounded ALI-PBEC (Fig. 3A). Neither LL-37 nor TGF-β1 alone affected wound closure, whereas their combination enhanced wound closure, with complete wound closure at 41 hours, as opposed to wound closure at t=30h in presence of activated M(GM-CSF) macrophages (Fig. 2A). The prolonged time till wound closure upon stimulation with LL-37/TGF-β1 compared to M(GM-CSF) macrophage induced wound closure, suggests that additional factors in concert with LL-37 and TGF-β1 contribute to the observed M(GM-CSF) macrophage-enhanced epithelial wound repair.

Therefore, we investigated whether inhibition of the TGF-β pathway (SB-431542), matrix metalloproteinases (GM6001) or LL-37 (neutralizing antibody) altered macrophage-induced enhanced epithelial wound repair (SFig. 3). Macrophage-induced epithelial wound closure was delayed by both SB-431542 and GM6001. However, since wound closure of ALI-PBEC monocultures was delayed as well in presence of these compounds, we concluded that the observed delayed wound repair was independent of the presence of macrophages (SFig. 3). The contribution of these pathways to macrophage-induced epithelial wound closure, therefore remained inconclusive. We studied the contribution of LL-37 to M(GM-CSF) enhanced epithelial wound repair using a selective LL-37 neutralizing antibody, however, this did not affect epithelial wound repair (SFig. 3). Collectively, these findings suggest that there is crosstalk between ALI-PBEC and macrophages and that this contributes to epithelial wound repair. We could demonstrate involvement of macrophage-derived factors in enhanced epithelial wound closure, including LL-37 (and TGF-β1), but also found that cross-talk is not restricted to these factors alone. Additionally, M(M-CSF) macrophage-derived IL-10, HGF and MMP9 are likely candidates as driving factors of M(M-CSF) enhanced epithelial wound repair, which we did not further investigate in view of our observation that both types of macrophages enhanced wound repair.

Two-way crosstalk between ALI-PBEC and M(GM-CSF)macrophages

We established that activated M(GM-CSF) macrophages increase *CAMP* expression only in the presence of ALI-PBEC (Fig. 3A). We hypothesized that the increased *CAMP* in M(GM-CSF) macrophages resulted from crosstalk between the M(GM-CSF) macrophages and epithelial cells. We tested this hypothesis by assessing gene expression in macrophages in experiments using conditioned media from ALI-PBEC cultures and epithelial/macrophage co-cultures (Fig. 4A). Conditioned medium (CM) was collected from : i. ALI-PBEC only (ALI-PBEC-CM); ii. co-cultures of ALI-PBEC and macrophages (Co-culture-CM); and iii. conditioned medium that was obtained first from activated macrophages (24h), and then added to ALI-PBEC mono-cultures (24h) (Double-CM) (Fig. 4A). M(M-CSF) macrophages did not alter *CAMP* expression upon stimulation with ALI-PBEC-CM, co-culture-CM or Double-CM (Fig 4B, blue bars). In contrast, *CAMP* expression was increased in M(GM-CSF) macrophages upon addition of co-culture-CM or Double-CM (Fig 4B, red bars). ALI-PBEC-CM was not able to increase *CAMP* expression in M(GM-CSF) macrophages, suggesting that *CAMP* in M(GM-CSF) macrophages is induced only as a result of a two-way crosstalk between the M(GM-CSF) macrophages and ALI-PBEC.

These data suggest that soluble mediators released by M(GM-CSF) macrophages, trigger the release of soluble mediators from ALI-PBEC that enhance CAMP expression in M(GM-CSF) macrophages.

Discussion

In this study, we investigated the crosstalk between primary bronchial epithelial cells cultured at the air-liquid interface (ALI-PBEC) and polarized macrophages. We found that co-culture of well-differentiated ALI-PBEC and activated macrophages displayed interactive crosstalk, and influenced epithelial innate immune responses and wound repair.

We show that epithelial IL-6 expression was increased upon co-culture with activated M(GM-CSF) and M(M-CSF) macrophages compared to epithelial monoculture. IL-6 is a multifunctional cytokine that has also been shown to promote intestinal epithelial proliferation (Kuhn et al. 2014). Co-culture with activated M(GM-CSF) macrophages furthermore increased epithelial expression of *DEFB4A* and *CXCL8*. We also confirmed this at protein level for *DEFB4A*, as hBD-2 levels in apical washes were increased upon co-culture with activated M(GM-CSF) macrophages. The effect on IL-8 secretion was

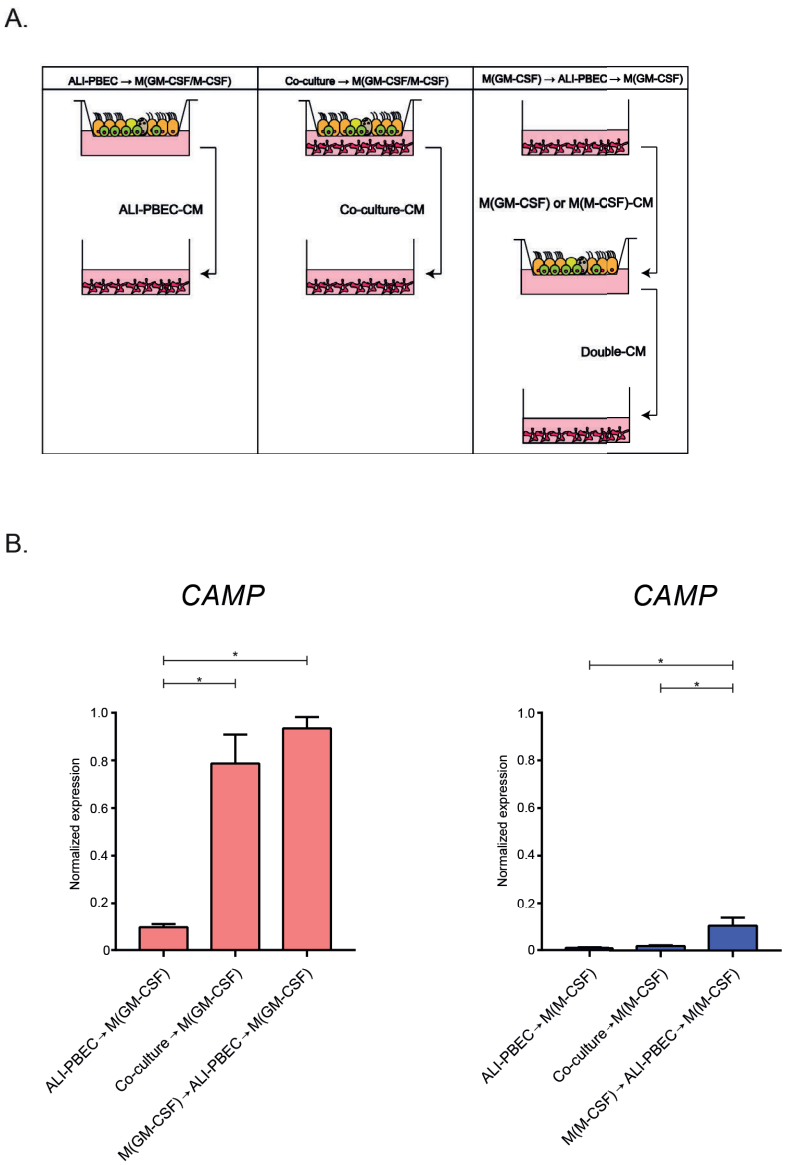


Figure 4. Effect of ALI-PBEC on *CAMP* expression in M(GM-CSF) macrophages. The mechanisms underlying increased *CAMP* expression in M(GM-CSF) macrophages in co-culture with ALI-PBEC were investigated by exposing macrophages to conditioned media (CM) collected from various culture conditions. (A) Overview of the culture condition from which the conditioned medium was collected (n=3 independent buffy donors). (B) *CAMP* expression at 24h was measured by q-PCR in M(GM-CSF) (red bars) or M(M-CSF) macrophages (blue bars) upon stimulation with the various conditioned media. Data are shown as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

less clear, which may in part be explained by the fact that IL-8 is produced by epithelial cells as well as by activated macrophages and can be secreted in the basal compartment. hBD-2 however, is produced mainly by the epithelial cells and secreted apically. The induction of hBD-2 in epithelial cells is in line

with studies which show that pro-inflammatory cytokines induce hBD-2 (Kao et al. 2004; O'Neil et al. 1999) and that microbial products (e.g. LPS) activate macrophages to release inflammatory mediators. Activated pro-inflammatory macrophages also produce pro-inflammatory cytokines including IL-1β, that subsequently enhance epithelial production of antimicrobial peptides and inflammatory cytokines (Bals and Hiemstra 2004). This cascade may act as an amplifying response to microbial products, as ALI-PBEC are less responsive to LPS compared to macrophages (Jia et al. 2004), which we also found in our study. These findings suggest that activated macrophages alter innate immune responses of well-differentiated ALI-PBEC through the release of soluble mediators.

In addition to an altered epithelial innate immune response, we show that co-culture with activated macrophages significantly enhanced epithelial wound repair. LPS addition to ALI-PBEC alone did not alter wound closure compared to unstimulated control. Our observations are in line with an *in vivo* response upon epithelial damage: following epithelial damage, pro-inflammatory macrophages are activated during the inflammatory phase, produce inflammatory cytokines and display antimicrobial activity (Yamada, Fujino, and Ichinose 2016). In the subsequent remodeling phase, anti-inflammatory macrophages will contribute to epithelial proliferation and migration followed by further restoration of the epithelial barrier and resolution of inflammation (Smigiel and Parks 2018).

There was no significant difference between M(GM-CSF) and M(M-CSF) polarized macrophages in their wound healing capacity. However, M(GM-CSF) polarized macrophages consistently induced faster epithelial wound closure compared to M(M-CSF) polarized macrophages, but this did not reach statistical significance at any of the time points investigated. At present, data on the wound healing capacity of pro- versus anti-inflammatory macrophages are conflicting (Wynn and Vannella 2016). Whereas one study also showed that M2 macrophage administration was not beneficial in murine cutaneous wound healing (Jetten et al. 2014), this is in contrast with other *in vitro* studies. These studies show that anti-inflammatory M(IL-10) macrophages increased wound repair of A549 epithelial cells, compared to pro-inflammatory M(IFN-γ) macrophages. This effect may be mediated through IL-10 (26) or hepatocyte growth factor (HGF) (Garnier et al. 2018), which is a prominent growth factor produced by alveolar macrophages (Garnier et al. 2018) and intestinal macrophages (D'Angelo et al. 2013). Also

in our study, HGF was also expressed in M(M-CSF) macrophages and lower in M(GM-CSF) macrophages. Interestingly, expression *IL10* was significantly higher in M(M-CSF) macrophages compared to M(GM-CSF) macrophages, again suggesting that there is cellular crosstalk between ALI-PBEC and activated macrophages, although we did not further examine this.

Interestingly, macrophages in bronchoalveolar lavage display higher levels of $CD163^{+}$ anti-inflammatory macrophages compared to levels in induced sputum (Kunz et al. 2011), suggesting that macrophages in the airways display a pro-inflammatory phenotype, which may aid in the defense against the heterogeneity of inhaled substances/pathogens. In contrast, M2-type activity of macrophages in the alveolar compartment may protect against excessive inflammation and contributes to repair. This is supported by the proposed role of M2 macrophages in alveolar repair in a mouse pneumonectomy model (Lechner et al. 2017). In our model, we used ALI-PBEC as a model using epithelial cells isolated from the large conducting airways, where indeed M1 macrophages may induce rapid wound closure, to prevent intrusion of harmful pathogens or substances. Dependent on the localization and the micro-environment, the phenotype and function of macrophages may be adapted, and thereby influence repair processes (Puttur, Gregory, and Lloyd 2019). In COPD, macrophage polarization has been described to be dysregulated (Hiemstra 2013; Shaykhiev et al. 2009), which suggests that epithelial wound repair may be affected.

In a previous study we showed that cigarette smoke exposure delayed epithelial wound closure especially at early time points (Amatngalim et al. 2016). We confirmed this in the present study, irrespective of co- or mono-culture of ALI-PBEC. Co-culture with both M(GM-CSF) and M(M-CSF) macrophages however, significantly increased epithelial wound closure in WCS-exposed. In our model, only airway epithelial cells were exposed to WCS, whereas macrophages were remained non-exposed. Other studies showed that cigarette smoke exposure also affects macrophage function (Strzelak et al. 2018), which we did not further investigate.

M(GM-CSF) macrophages may enhance wound repair in part by the selective increased expression of *CAMP* upon co-culture with ALI-PBEC. We have previously shown that LL-37 drives macrophage polarization towards a pro-inflammatory macrophage phenotype (van der Does et al. 2010). Part of the observed effect of M(GM-CSF) macrophages on epithelial innate immunity and wound repair may have been caused by an increased susceptibility of

epithelial cells to LPS resulting from exposure to macrophage-derived LL-37 (Shaykhiev et al. 2005). Apart from its prominent role in host defense, the antimicrobial peptide LL-37 has been shown to be involved in wound repair both *in vivo* and in *in vitro* skin models (Ramos et al. 2011; Carretero et al. 2008). LL-37 is able to activate airway epithelial cells through EGFR transactivation (Tjabringa et al. 2003), which may contribute to wound repair. In our model, exogenously added LL-37 enhanced wound repair only upon concomitant addition of TGF-β1. A possible explanation for the synergistic effect of LL-37 or TGF-β1 on wound repair could be an interaction between the putative induction of an epithelial migratory (by TGF-β1) and proliferatory phenotype (by LL-37). We also determined the contribution of other mediators that may enhance wound repair, but since inhibition of the TGF-β1 pathway or MMPs also markedly reduced epithelial wound closure in the ALI-PBEC mono-cultures, we could not determine the role of these pathways in M(GM-CSF) or M(M-CSF) macrophage-enhanced epithelial wound repair.

We did not observe alterations in macrophage polarization during co-culture (data not shown). These findings suggest that in our experimental set-up ALI-PBEC do not produce strong polarizing factors or the time in co-culture is insufficient to influence macrophage polarization. In this study we focused on only 2 subsets of macrophages (Van't Wout et al. 2015), however, for future studies our co-culture setup allows incorporation of various other macrophage subsets to study epithelial-macrophage interaction (Gindele et al. 2017; Boyette et al. 2017; Murray and Wynn 2011). Whereas other studies of airway epithelial cell co-cultures either focus solely on the host defense aspect of macrophages or use cell lines (Bodet, Chandad, and Grenier 2006; Blom et al. 2016; Bauer et al. 2015; Reuschl et al. 2017), we used both primary monocyte-derived macrophages and well-differentiated primary airway epithelial cells, which better reflects *in vivo* responses. Furthermore, other cell-types, such as structural cells may be incorporated as well, to better mimic the *in vivo* cellular niche. Previous studies have shown that macrophage-derived mediators have an effect on dermal fibroblasts (Ploeger et al. 2013), and this may also occur in the lung, assisting in modulation of epithelial cell function.

Although a limitation of our model is that the cells are cultured in separate compartments, and therefore cell-cell interactions are excluded (Leoni et al. 2015), we were able to reveal crosstalk between macrophages and epithelial

cells and show that they interact in part through secreted mediators. In recent years it has been found that cellular crosstalk may occur through extracellular vesicles, and this has also been described for the interaction between epithelial cells and macrophages (Lee et al. 2018). Another limitation is that we did not extend the co-culture beyond 72 hours, to avoid potential problems with different media requirements for primary bronchial epithelial cells and macrophages. Future studies are necessary to further optimize this model with prolonged co-culture time, without affecting ALI-PBEC integrity and/or M(GM-CSF) or M(M-CSF) macrophage polarization. Finally, we used CD14-monocyte-derived M(GM-CSF) and M(M-CSF) macrophages in this study, whereas these may not fully reflect the repertoire of airway, alveolar and interstitial macrophages in the lung (Byrne et al. 2015; Puttur, Gregory, and Lloyd 2019). The current culture set-up with 2-weeks differentiated ALI-PBEC limited the use of freshly isolated lung-derived macrophages, however, we did mimic the interaction between airway epithelial cells and recruited monocyte-derived macrophages. Furthermore, whereas macrophage phenotype *in vivo* displays plasticity, we simplified our model by using polarized macrophages, and thereby could investigate macrophage subtype specific responses on airway epithelial innate immunity and repair.

In summary, using primary cells, we show that ALI-PBEC and activated macrophage co-culture alters epithelial innate immune responses, enhances epithelial wound repair and induces interactive crosstalk between epithelial cells and macrophages, thereby better representing the *in vivo* situation compared to mono-cultures of airway epithelial cells.

Acknowledgements

We thank Marloes Hofstee and Bram van der Linden (Dept. of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands) for the technical support during this study, and Dr. Anne van der Does (Dept of Pulmonology, Leiden University Medical Center, Leiden the Netherlands) for critical reviewing the manuscript.

Author contributions

SR designed, performed and analyzed the experiments, interpreted the data, prepared the figures and drafted the manuscript; AS provided technical support and helped with the data acquisition; SV and NV provided input during experimental design and manuscript preparation; JS, RR, PH and PK designed the study, and supervised experiments and manuscript writing. All authors have read and approved the final version of this manuscript.

Statement of Ethics

The use of lung tissue for research following surgery within the framework of patient care was in line with the "Human Tissue and Medical Research: Code of conduct for responsible use" (2011) (www.federa.org), that describes the no-objection system for coded anonymous further use of such tissue.

Disclosure statements

Dr. De Vos and Dr. Vandeghinste are employees of Galapagos NV. Prof. Hiemstra reports grants from Galapagos NV, grants from Lung Foundation Netherlands, during the conduct of the study; grants from Boehringer Ingelheim, outside the submitted work.

Funding

This study was supported by grants from the Lung Foundation Netherlands (grant 6.1.14.010 and grant 6.1.14.009) and Galapagos.

References

Alber, A., S. E. Howie, W. A. Wallace, and N. Hirani. 2012. 'The role of macrophages in healing the wounded lung', Int J Exp Pathol, 93: 243-51.

Amatngalim, G. D., W. Broekman, N. M. Daniel, L. E. van der Vlugt, A. van Schadewijk, C. Taube, and P. S. Hiemstra. 2016. 'Cigarette Smoke Modulates Repair and Innate Immunity following Injury to Airway Epithelial Cells', PLoS One, 11: e0166255.

Amatngalim, G. D., and P. S. Hiemstra. 2018. 'Airway Epithelial Cell Function and Respiratory Host Defense in Chronic Obstructive Pulmonary Disease', Chin Med J (Engl), 131: 1099-107.

Amatngalim, G. D., J. A. Schrumpf, F. Dishchekenian, T. C. J. Mertens, D. K. Ninaber, A. C. van der Linden, C. Pilette, C. Taube, P. S. Hiemstra, and A. M. van der Does. 2018. 'Aberrant epithelial differentiation by cigarette smoke dysregulates respiratory host defence', Eur Respir J, 51.

Amatngalim, G. D., J. A. Schrumpf, A. Henic, E. Dronkers, R. M. Verhoosel, S. R. Ordonez, H. P. Haagsman, M. E. Fuentes, S. Sridhar, J. Aarbiou, R. A. J. Janssen, A. N. Lekkerkerker, and P. S. Hiemstra. 2017. 'Antibacterial Defense of Human Airway Epithelial Cells from Chronic Obstructive Pulmonary Disease Patients Induced by Acute Exposure to Nontypeable Haemophilus influenzae: Modulation by Cigarette Smoke', J Innate Immun, 9: 359-74.

Andersen, C. L., J. L. Jensen, and T. F. Orntoft. 2004. 'Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets', Cancer Res, 64: 5245-50.

Arora, S., K. Dev, B. Agarwal, P. Das, and M. A. Syed. 2018. 'Macrophages: Their role, activation and polarization in pulmonary diseases', Immunobiology, 223: 383-96.

Bals, R., and P. S. Hiemstra. 2004. 'Innate immunity in the lung: how epithelial cells fight against respiratory pathogens', Eur Respir J, 23: 327-33.

Bauer, R. N., L. Muller, L. E. Brighton, K. E. Duncan, and I. Jaspers. 2015. 'Interaction with epithelial cells modifies airway macrophage response to ozone', Am J Respir Cell Mol Biol, 52: 285-94.

Blom, R. A., S. T. Erni, K. Krempaska, O. Schaerer, R. M. van Dijk, M. Amacker, C. Moser, S. R. Hall, C. von Garnier, and F. Blank. 2016. 'A Triple Co-Culture Model of the Human Respiratory Tract to Study Immune-Modulatory Effects of Liposomes and Virosomes', PLoS One, 11: e0163539.

Bodet, C., F. Chandad, and D. Grenier. 2006. 'Inflammatory responses of a macrophage/epithelial cell co-culture model to mono and mixed infections with Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia', Microbes Infect, 8: 27-35.

Boyette, L. B., C. Macedo, K. Hadi, B. D. Elinoff, J. T. Walters, B. Ramaswami, G. Chalasani, J. M. Taboas, F. G. Lakkis, and D. M. Metes. 2017. 'Phenotype, function, and differentiation potential of human monocyte subsets', PLoS One, 12: e0176460.

Byrne, A. J., S. A. Mathie, L. G. Gregory, and C. M. Lloyd. 2015. 'Pulmonary macrophages: key players in the innate defence of the airways', Thorax, 70: 1189-96.

Carretero, M., M. J. Escamez, M. Garcia, B. Duarte, A. Holguin, L. Retamosa, J. L. Jorcano, M. D. Rio, and F. Larcher. 2008. '*In vitro* and *in vivo* wound healing-promoting activities of human cathelicidin LL-37', J Invest Dermatol, 128: 223-36.

D'Angelo, F., E. Bernasconi, M. Schafer, M. Moyat, P. Michetti, M. H. Maillard, and D. Velin. 2013. 'Macrophages promote epithelial repair through hepatocyte growth factor secretion', Clin Exp Immunol, 174: 60-72.

Gardner, A., L. A. Borthwick, and A. J. Fisher. 2010. 'Lung epithelial wound healing in health and disease', Expert Rev Respir Med, 4: 647-60.

Garnier, M., A. Gibelin, A. A. Mailleux, V. Lecon, M. Hurtado-Nedelec, J. Laschet, G. Trebbia, M. Neuville, S. Tanaka, B. Crestani, M. Dehoux, and C. Quesnel. 2018. 'Macrophage Polarization Favors Epithelial Repair During Acute Respiratory Distress Syndrome', Crit Care Med, 46: e692-e701.

Gindele, J. A., S. Mang, N. Pairet, I. Christ, F. Gantner, J. Schymeinsky, and D. J. Lamb. 2017. 'Opposing effects of *in vitro* differentiated macrophages sub-type on epithelial wound healing', PLoS One, 12: e0184386.

Gordon, S., and L. Martinez Pomares. 2017. 'Physiological roles of macrophages', Pflugers Arch, 469: 365-74.

Hiemstra, P. S. 2013. 'Altered macrophage function in chronic obstructive pulmonary disease', Ann Am Thorac Soc, 10 Suppl: S180-5.

Hiemstra, P. S., G. D. Amatngalim, A. M. van der Does, and C. Taube. 2016. 'Antimicrobial

Peptides and Innate Lung Defenses: Role in Infectious and Noninfectious Lung Diseases and Therapeutic Applications', Chest, 149: 545-51.

Hiemstra, P. S., P. B. McCray, Jr., and R. Bals. 2015. 'The innate immune function of airway epithelial cells in inflammatory lung disease', Eur Respir J, 45: 1150-62.

Hu, G., and J. W. Christman. 2019. 'Editorial: Alveolar Macrophages in Lung Inflammation and Resolution', Front Immunol, 10: 2275.

Jetten, N., N. Roumans, M. J. Gijbels, A. Romano, M. J. Post, M. P. de Winther, R. R. van der Hulst, and S. Xanthoulea. 2014. 'Wound administration of M2-polarized macrophages does not improve murine cutaneous healing responses', PLoS One, 9: e102994.

Jia, H. P., J. N. Kline, A. Penisten, M. A. Apicella, T. L. Gioannini, J. Weiss, and P. B. McCray, Jr. 2004. 'Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2', Am J Physiol Lung Cell Mol Physiol, 287: L428-37.

Kao, C. Y., Y. Chen, P. Thai, S. Wachi, F. Huang, C. Kim, R. W. Harper, and R. Wu. 2004. 'IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways', J Immunol, 173: 3482-91.

Kuhn, K. A., N. A. Manieri, T. C. Liu, and T. S. Stappenbeck. 2014. 'IL-6 stimulates intestinal epithelial proliferation and repair after injury', PLoS One, 9: e114195.

Kunz, L. I., T. S. Lapperre, J. B. Snoeck-Stroband, S. E. Budulac, W. Timens, S. van Wijngaarden, J. A. Schrumpf, K. F. Rabe, D. S. Postma, P. J. Sterk, P. S. Hiemstra, and Group Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease Study. 2011. 'Smoking status and anti-inflammatory macrophages in bronchoalveolar lavage and induced sputum in COPD', Respir Res, 12: 34.

Lechner, A. J., I. H. Driver, J. Lee, C. M. Conroy, A. Nagle, R. M. Locksley, and J. R. Rock. 2017. 'Recruited Monocytes and Type 2 Immunity Promote Lung Regeneration following Pneumonectomy', Cell Stem Cell, 21: 120-34 e7.

Lee, H., E. Abston, D. Zhang, A. Rai, and Y. Jin. 2018. 'Extracellular Vesicle: An Emerging Mediator of Intercellular Crosstalk in Lung Inflammation and Injury', Front Immunol, 9: 924.

Leoni, G., P. A. Neumann, R. Sumagin, T. L. Denning, and A. Nusrat. 2015. 'Wound repair: role of immune-epithelial interactions', Mucosal Immunol, 8: 959-68.

Luppi, F., J. Aarbiou, S. van Wetering, I. Rahman, W. I. de Boer, K. F. Rabe, and P. S. Hiemstra. 2005. 'Effects of cigarette smoke condensate on proliferation and wound closure of bronchial epithelial cells *in vitro*: role of glutathione', Respir Res, 6: 140.

Murray, P. J. 2017. 'Macrophage Polarization', Annu Rev Physiol, 79: 541-66.

Murray, P. J., and T. A. Wynn. 2011. 'Protective and pathogenic functions of macrophage subsets', Nat Rev Immunol, 11: 723-37.

O'Neil, D. A., E. M. Porter, D. Elewaut, G. M. Anderson, L. Eckmann, T. Ganz, and M. F. Kagnoff. 1999. 'Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium', J Immunol, 163: 6718-24.

Ploeger, D. T., N. A. Hosper, M. Schipper, J. A. Koerts, S. de Rond, and R. A. Bank. 2013. 'Cell plasticity in wound healing: paracrine factors of M1/M2 polarized macrophages influence the phenotypical state of dermal fibroblasts', Cell Commun Signal, 11: 29.

Puttur, F., L. G. Gregory, and C. M. Lloyd. 2019. 'Airway macrophages as the guardians of tissue repair in the lung', Immunol Cell Biol, 97: 246-57.

Ramos, R., J. P. Silva, A. C. Rodrigues, R. Costa, L. Guardao, F. Schmitt, R. Soares, M. Vilanova, L. Domingues, and M. Gama. 2011. 'Wound healing activity of the human antimicrobial peptide LL37', Peptides, 32: 1469-76.

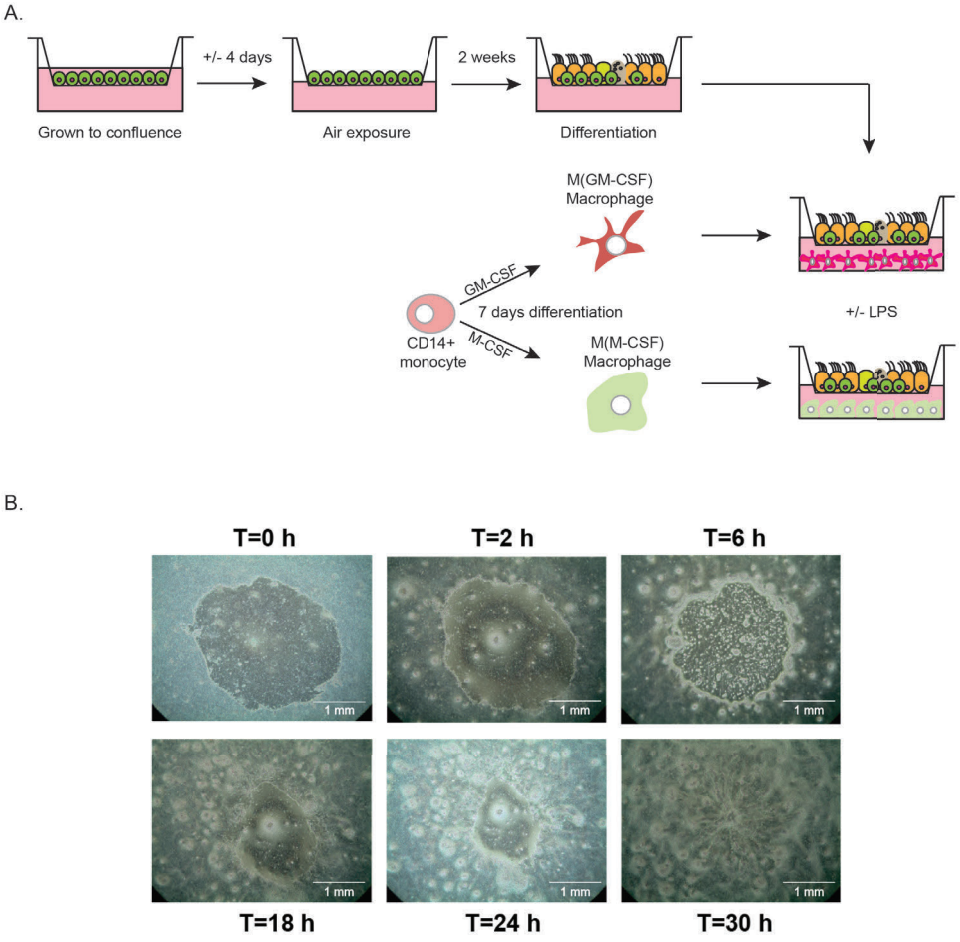
Reuschl, A. K., M. R. Edwards, R. Parker, D. W. Connell, L. Hoang, A. Halliday, H. Jarvis, N. Siddiqui, C. Wright, S. Bremang, S. M. Newton, P. Beverley, R. J. Shattock, O. M. Kon, and A. Lalvani. 2017. 'Innate activation of human primary epithelial cells broadens the host response to Mycobacterium tuberculosis in the airways', PLoS Pathog, 13: e1006577.

Shaykhiev, R., C. Beisswenger, K. Kandler, J. Senske, A. Puchner, T. Damm, J. Behr, and R. Bals. 2005. 'Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure', Am J Physiol Lung Cell Mol Physiol, 289: L842-8.

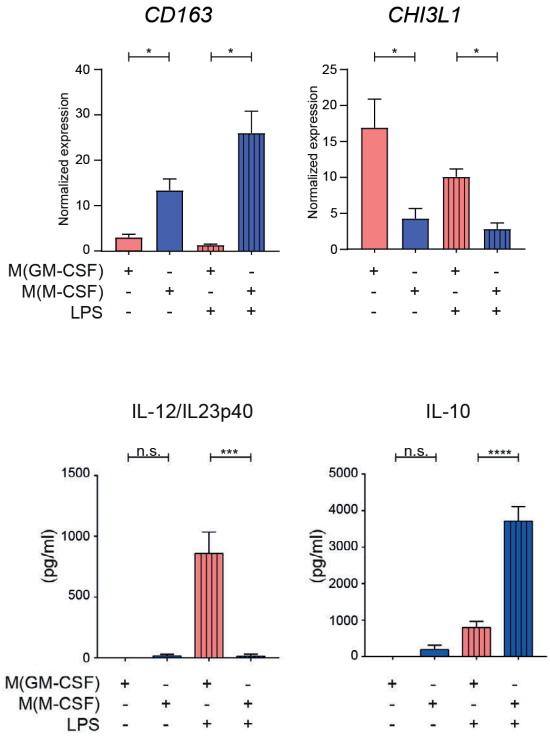
Shaykhiev, R., A. Krause, J. Salit, Y. Strulovici-Barel, B. G. Harvey, T. P. O'Connor, and R. G. Crystal. 2009. 'Smoking-dependent reprogramming of alveolar macrophage polarization: implication for

pathogenesis of chronic obstructive pulmonary disease', J Immunol, 183: 2867-83.
Smigiel, K. S., and W. C. Parks. 2018. 'Macrophages, Wound Healing, and Fibrosis: Recent Insights', Curr Rheumatol Rep, 20: 17.
Snyder, R. J., J. Lantis, R. S. Kirsner, V. Shah, M. Molyneaux, and M. J. Carter. 2016. 'Macrophages: A review of their role in wound healing and their therapeutic use', Wound Repair Regen, 24: 613-29.
Strzelak, A., A. Ratajczak, A. Adamiec, and W. Feleszko. 2018. 'Tobacco Smoke Induces and Alters Immune Responses in the Lung Triggering Inflammation, Allergy, Asthma and Other Lung Diseases: A Mechanistic Review', Int J Environ Res Public Health, 15.
Tjabringa, G. S., J. Aarbiou, D. K. Ninaber, J. W. Drijfhout, O. E. Sorensen, N. Borregaard, K. F. Rabe, and P. S. Hiemstra. 2003. 'The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor', J Immunol, 171: 6690-6.
Van't Wout, E. F., A. van Schadewijk, D. A. Lomas, J. Stolk, S. J. Marciniak, and P. S. Hiemstra. 2015. 'Function of monocytes and monocyte-derived macrophages in alpha1-antitrypsin deficiency', Eur Respir J, 45: 365-76.
van der Does, A. M., H. Beekhuizen, B. Ravensbergen, T. Vos, T. H. Ottenhoff, J. T. van Dissel, J. W. Drijfhout, P. S. Hiemstra, and P. H. Nibbering. 2010. 'LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature', J Immunol, 185: 1442-9.
Whitsett, J. A., and T. Alenghat. 2015. 'Respiratory epithelial cells orchestrate pulmonary innate immunity', Nat Immunol, 16: 27-35.
Wynn, T. A., and K. M. Vannella. 2016. 'Macrophages in Tissue Repair, Regeneration, and Fibrosis', Immunity, 44: 450-62.
Yamada, M., N. Fujino, and M. Ichinose. 2016. 'Inflammatory responses in the initiation of lung repair and regeneration: their role in stimulating lung resident stem cells', Inflamm Regen, 36: 1

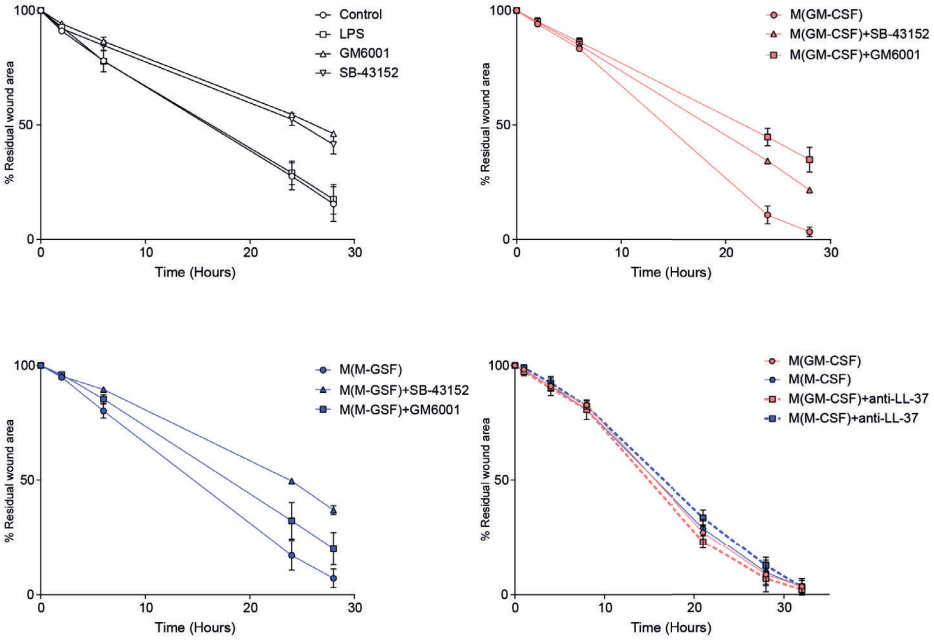
Supplementary figures



Supplementary Figure 1. Schematic overview of experimental setup. (A) Peripheral blood-derived CD14^{+} monocytes were cultured in presence of GM-CSF or M-CSF to induce a M(GM-CSF) or M(GM-CSF) macrophage phenotype, respectively. After 7 days in culture, macrophages were activated with 100 ng/mL LPS after which they were used in co-culture experiments. PBEC were cultured until confluent after which they were cultured at the air-liquid interface (ALI-PBEC) and differentiated for 2 weeks. Upon 2 weeks differentiation, ALI-PBEC and (LPS-activated) macrophages were co-cultured for 24h or until wound closure (B) Epithelial wounding was performed by mechanically scraping well-differentiated ALI-PBEC, using a template to ensure identical wound surface areas (Amatngalim et al. 2016). Wound closure was measured over time; representative light microscopic images were taken at various time points and are shown here.



Supplementary Figure 2. Macrophage polarization. (A) mRNA expression of *CD163* (M(M-CSF) macrophages – blue bars) and *CHI3L1* (M(GM-CSF) macrophages – red bars) in (LPS-activated) M(GM-CSF) and M(M-CSF) macrophages was measured by q-PCR (n=3 independent experiments). (B) Protein levels of IL-10 (M(M-CSF) macrophages – blue bars) and IL-12/IL-23p40 (M(GM-CSF) macrophages – red bars) were measured at 24h after LPS stimulation and upon co-culture (n=3 independent experiments). Data are shown as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



Supplementary Figure 3. Inhibition of key repair pathways and molecules during epithelial wound repair. To determine the contribution of several pathways that play a role in epithelial wound repair to M(GM-CSF) (red line) or M(M-CSF) (blue line)-induced enhanced epithelial wound closure, various inhibitors were added in the epithelial mono (black lines)- and co-cultures upon epithelial wounding. Inhibitors of the TGF-β pathway (SB-434215), and MMP inhibitor (GM6001) and an anti-LL-37 antibody were added upon wounding and the residual wound area was measured over time (n=3 independent ALI-PBEC donors). Data are shown as mean ± SEM.