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Innate activation of human primary epithelial cells broadens the host response to *Mycobacterium tuberculosis* in the airways

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Abstract

Early events in the human airways determining whether exposure to *Mycobacterium tuberculosis* (Mtb) results in acquisition of infection are poorly understood. Epithelial cells are the dominant cell type in the lungs, but little is known about their role in tuberculosis. We hypothesised that human primary airway epithelial cells are part of the first line of defense against Mtb-infection and contribute to the protective host response in the human respiratory tract. We modelled these early airway-interactions with human primary bronchial epithelial cells (PBECs) and alveolar macrophages. By combining *in vitro* infection and transwell co-culture models with a global transcriptomic approach, we identified PBECs to be inert to direct Mtb-infection, yet to be potent responders within an Mtb-activated immune network, mediated by IL1β and type I interferon (IFN). Activation of PBECs by Mtb-infected alveolar macrophages and monocytes increased expression of known and novel antimycobacterial peptides, defensins and S100-family members and epithelial-myeloid interactions further shaped the immunological environment during Mtb-infection by promoting neutrophil influx. This is the first in depth analysis of the primary epithelial response to infection and offers new insights into their emerging role in tuberculosis through complementing and amplifying responses to Mtb.

Author summary

*Mycobacterium tuberculosis* (Mtb) is the causative agent of tuberculosis, which remains a major public health burden today. In the majority of cases, infection is acquired by inhalation of aerosolised bacteria. Mtb is thought to target alveolar macrophages in the lower airways to establish infection. However, the cells predominantly lining the respiratory...
tract are epithelial cells and thus are likely crucial during the early host-pathogen interactions. We recovered primary human bronchial epithelial cells from healthy volunteers to assess their global transcriptomic response to direct Mtb-exposure and exposure to Mtb-infected myeloid cells. Our analysis revealed that, while being inert to direct Mtb-infection, epithelial cells were highly responsive to soluble mediators released by infected macrophages. The epithelial response induced by this cellular cross-talk, promoted neutrophil influx in vitro as well as the increase of antimycobacterial host responses. Our data provide novel and unexpected insights into the role of the primary human airway epithelium and define a non-redundant role for epithelial cells in shaping the local immunological environment at the site of initial Mtb infection.

Introduction

The first interactions between Mycobacterium tuberculosis (Mtbd) and its human host occur in the lungs after inhalation of aerosolised bacteria. Approximately fifty percent of individuals exposed to Mtb remain uninfected [1] and host-determinants have been associated with this resistance to or clearance of infection in humans [2]. The absence of a detectable peripheral adaptive immune response in resistant individuals suggests that it is mediated by the local innate immune system and that the respiratory mucosa plays a role in determining the outcome of exposure [3]. It is assumed that the primary target of Mtb, an intracellular pathogen, is the alveolar macrophage in the lower airways. However, the majority of cells in the airway lining are epithelial which constitute a surface of approximately 70 m² [4] and are thus likely to be the first point of contact for Mtb in the human host.

Epithelial cells are known to significantly contribute to the immune responses in the lungs and can sense intra- and extracellular pathogens, such as viruses and bacteria, via a wide range of pattern recognition receptors (PRR) [5]. In contrast to this, their responses in tuberculosis (TB) are poorly defined and little investigated, which presents a surprising knowledge gap.

Recognition by, or infection of, epithelial cells in the human airways may shape early host responses to the bacilli and act as an adjunct to the immune response against infections. Work on human airway epithelial-like cell lines and murine airway epithelial cells has identified potential mechanisms of recognition and response to Mtb-infection, including chemokine release and antimicrobial action [6–8]. However, the response of primary human airway epithelial cells to Mtb infection has barely been investigated and has so far mainly concentrated on isolated pathways rather than the global epithelial response to Mtb-infection [9–11]. The need to investigate the interactions of Mtb with primary human airway epithelial cells is further underscored by detection of Mtb in these cells during infections in vivo. Previous studies reported that in animal models of tuberculosis infection the bronchial and alveolar epithelium is infected after instillation with Mtb [12,13] and Mtb DNA was detected in human epithelial cells of histologically normal lung tissue in healthy latently infected individuals [14].

In this study, we hypothesised that human primary airway epithelial cells contribute to the protective early host response to Mtb-infection in the human respiratory tract. To test this, we interrogated the direct response of epithelial cells to Mtb as well as indirect effects mediated via an immune-network comprising infected myeloid cells, by measuring the global epithelial transcriptome. Through in vitro infection and transwell co-culture models, we identified primary bronchial epithelial cells to be inert to direct Mtb-infection, yet to be potent responders within an Mtb-activated immune network, mediated by IL1β and type I IFN, with the capacity to shape the local antimicrobial and immunological environment.
Results

Bronchial epithelial cells dominate the airway lining and are resistant to Mtb challenge \textit{in vitro}.

In healthy human airways, epithelial cells were the major cell type lining the respiratory tract and harboured less than 10% of leukocyte subsets (Fig 1A), of which macrophages, lymphocytes and neutrophils contributed on average 86.4%, 12.5% and 1.1%, respectively. The abundance of epithelial cells increases the likelihood that Mtb interacts directly with the epithelial lining after inhalation of aerosols. To assess whether human airway epithelial cells are permissive to intracellular Mtb-infection, primary bronchial epithelial cells (PBECs) recovered from bronchial brushes were expanded \textit{in vitro} and infected with Mtb. The total cell-associated mycobacterial burden, after amikacin treatment, was over two logs less in PBECs than the Mtb-burden in THP-1 macrophages after 24h (Fig 1B). The decreased association of Mtb with PBECs was confirmed by microscopy in the presence of an increased multiplicity of infection (MOI) (S1 Fig). The minimal level of epithelial infection was reflected by a lack of...
transcriptomic changes in PBECs after exposure to different MOIs of Mtb for 24h (Fig 1C). Microarray analysis revealed no significant differences in gene expression after statistical adjustment for multiple comparisons. Since PBECs were poor direct responders to Mtb exposure and infection, yet macrophages were readily infected, we reasoned that the role of PBECs during the early host response to Mtb might be as part of the Mtb-activated immune network with myeloid cells as the primary replicative niche for Mtb.

PBECs express antimicrobial peptides and immune mediators in response to Mtb-induced myeloid activation

After infection with Mtb, macrophages, the largest leukocyte subset in the healthy airways [15,16], can mount a pro-inflammatory response. To investigate if PBECs, whilst inert to direct interaction with Mtb bacilli, are affected by these events, we established a contact-independent co-culture system to measure the global epithelial response to myeloid-derived Mtb-induced mediators (Fig 2A). THP-1 monocytic cells were used to mimic the myeloid compartment in the Airways and were infected with Mtb or remained uninfected. Microarrays were performed on PBECs derived from eight healthy donors exposed to these two conditions to model cross-talk in the healthy and infected lungs (Fig 2B). Four-hundred-twenty-eight probesets representing 375 genes were differentially expressed in PBECs exposed to Mtb-infected THP-1 cells compared to uninfected THP-1 cells (q-value <5%). Seventy of these were at least 1.5 fold differentially expressed (Fig 2B, see S1 Table for full list). The identified expression signature included cytokines and antimicrobial peptides and 6 of these genes were selected for independent validation by RT-PCR (Fig 3): DEFB4 and S100A7A, which encode known antimicrobial peptides β-defensin 2 (hBD2) and koebnerisin [17,18]; IL36G which is an emerging IL1-family member and may be beneficial during tuberculosis infection [19] the chemokines IL8 and C-X-C motif ligand (CXCL) 10; and two known type I IFN inducible targets interferon-induced protein with tetratricopeptide repeats (IFIT) 1 and interferon-induced protein (IFI) 44. The RT-PCR validation experiment confirmed the gene expression patterns identified by microarray and showed that the signature was strongly dependent on exposure to infected THP-1 cells and was not inducible by direct infection of PBECs with Mtb, which confirmed the epithelial inertia to Mtb. While myeloid infection with virulent Mtb had profound effects on epithelial gene expression, non-pathogenic M. bovis BCG infection of THP-1 cells at a MOI of 10 only increased epithelial CXCL10 expression to 9 or 19% of the gene expression induced by Mtb in two independent experiments, respectively.

THP-1 cells are a valuable model for in vitro studies of myeloid responses to Mtb, and we confirmed that our model reflected responses in the lungs through the use of primary alveolar macrophages (AMΦ). For this, PBECs were exposed to infected primary AMΦs from independent donors in co-culture (n = 2, S2 Fig), which confirmed increased expression of DEFB4, S100A7A, CXCL10 and IFIT1. Transwell co-culture with primary macrophages could only be performed twice and cell-free supernatant of infected and uninfected AMΦs was used to confirm expression patterns in depth (Fig 4). Soluble mediators secreted by AMΦs induced similar transcriptomic responses as observed when THP-1 cells were used. Thus, the epithelial response to Mtb-induced inflammation we observed in our model mimicked paracrine AMΦ-epithelial signalling that may occur early after exposure of the human Airways to Mtb.

PBEC responses to Mtb-induced myeloid inflammation are driven discretely by IL1β and type I IFN

To identify the Mtb-induced inflammatory signals which drove gene expression in PBECs, we performed transcription factor binding site (TFBS) enrichment analysis with oPossum which
Fig 2. Whole transcriptome analysis of PBECs exposed to Mtb-infected myeloid cells. (A) To interrogate the effect of myeloid-epithelial cross-talk during Mtb infection, a transwell co-culture system was established. Separated by a 0.4 μm pore-sized transwell membrane to allow exchange of soluble mediator only, PBECs were seeded in the bottom well of a tissue culture plate and THP-1 cells were added into the transwell insert. Cells were infected as indicated. (B) In the transwell co-culture model, PBECs from eight donors were exposed to uninfected (THP, T) or Mtb-infected THP-1 (THP + Mtb, TM) cells for 24h.
Affymetrix HTA2.0 microarrays were performed on PBECs. Hierarchical clustering of all significantly differentially expressed genes at a fold change of > 1.5 and a q-value < 5% was performed using average linkage and Euclidean distance. Expression range from low (green) to high (red).

![Image](https://doi.org/10.1371/journal.ppat.1006577.g002)

Fig 3. PBEC gene expression in response to infection-driven inflammation is dependent on direct interaction between myeloid cells and Mtb. In the transwell model, PBECs were exposed to THP-1 cells or Mtb H37Rv bacilli (MOI5) for 24h as indicated. Gene expression in PBECs was measured by RT-PCR and is shown as fold change over unstimulated PBECs (n = 5). Friedman test with Dunn’s post-test was used to compare expression with unstimulated controls (fold change = 1, not displayed). Boxplots show median and range.

* p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

![Image](https://doi.org/10.1371/journal.ppat.1006577.g003)
revealed that the epithelial expression signature was associated with nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), including RelA and the p105 subunit (NFKB1), and interferon regulatory factor (IRF)-family members 1 and 2 (Fig 5A). Exposure of PBECs to myeloid Mtb-infection furthermore induced pathways involved in immune response signalling, including interferon-driven pathways (Fig 5B). Similarly, gene ontology was enriched for terms associated with cytokine and IFN signalling (Fig 5C).

It is well known that macrophages secrete several inflammatory mediators in response to Mtb-infection, including IL1β and IFNβ [20–22]. We found that, IL1β levels in culture supernatants were over threefold enhanced when infected THP-1 cells were co-cultured with PBECs (Fig 6A). A similar increase was not observed to the same extent when THP-1 cells were infected with BCG in the presence of PBECs, which resulted in a release of only 28.9–81.2 pg/ml IL1β during co-culture in three independent experiments. IFN signalling was indicated by the pathway enrichment analysis and we confirmed IFNβ expression and release from myeloid cells to identify whether type I IFNs may have been involved in epithelial activation. We detected increases in gene expression and protein secretion during Mtb-infection in AMΦ and THP-1 cells (Fig 6B and 6C). IFNβ induction co-incided with phosphorylation of STAT1, a downstream mediator of type I IFN-signalling, in PBECs exposed to Mtb-infected THP-1 cells (Fig 6D). IFNB was not induced in PBECs during co-culture (S3A Fig) and neither IFNγ nor IFNλ could be detected by ELISA in culture supernatants of THP-1 cells 24h after Mtb-infection (S3B Fig).
Stimulation with recombinant cytokines confirmed that IL1β and IFNβ were sufficient to induce *DEFB4* and *CXCL10* expression respectively, but not vice versa (S3C Fig). To identify whether IL1β or type I IFN could in fact mediate the epithelial signature in response to myeloid Mtb-infection, we abrogated each pathway in co-culture. Neutralising IL1β prevented upregulation of AMP genes *DEFB4* and *S100A7A* (Fig 7A) as well as pro-inflammatory cytokines IL8 and IL36G (S3D and S3E Fig). Neutralisation of TNF in Mtb-infected co-cultures or infected THP-1 macrophage monoculture, enhanced the IL1β mediated effects, likely reflecting autocrine activation of THP-1 cells (S4A–S4C Fig). In contrast, genes associated with interferon-signalling, *CXCL10* and *IFIT1*, were unaffected by IL1β neutralisation (Fig 7A) and dependent on IFNAR2 activation (Fig 7B), which mediates type I IFN signalling. Conversely, abrogation of IFNAR2 signalling did not affect the expression of the tested IL1β-dependent genes. Neutralisation of IFNγ had no effect on *CXCL10* expression (S4D Fig).

**PBECs can shape the antimycobacterial and cellular host response to infection during Mtb-driven inflammation**

*DEFB4* and *S100A7A*, which were strongly expressed in PBECs in response to Mtb-driven inflammation, were not expressed by Mtb-infected THP-1 cells. Mtb-infection also failed to
induce S100A7A expression in AMFs, while DEFB4 induction ranged from 0.84–14.08 fold in three healthy donors. Since the release of AMPs may critically determine the outcome of exposure to Mtb, we tested whether the infection-induced defensins and S100A-family members control free growing Mtb. Human β-defensin 2 (hBD2), which is encoded by DEFB4, has been previously described to diminish Mtb growth in a resazurin assay [17] and was a potent inhibitor of growth of Mtb H37Rv (Fig 8A) and clinical strains in our hands (S5A Fig).

Because, no bioactive form of S100A7A was available, we used the close homologue S100A7 [23], known as psoriasin, which was also induced in an IL1β-dependent manner in co-culture.
Fig 7. Mtb-driven inflammation activates PBECs in an IL1β and type I IFN signalling dependent manner. (A) PBECs were co-cultured with infected or uninfected THP-1 cells in the presence of αL1β or IgG₁ (isotype control) as indicated. After 24h, gene expression was measured by RT-PCR. Expression is shown as fold change over unstimulated (n = 6). Boxplots show median and range. (B) PBECs were exposed to Mtb-infected THP-1 cells (MOI5) in the presence of increasing concentrations of αIFNAR2 (5, 10 and 20 μg/ml) or αIFNAR2.
and reflected the induction pattern observed for S100A7A (Fig 3). Psoriasin decreased the median Mtb burden in liquid culture significantly by 62% (Fig 8B).

In addition to local responses to Mtb, influx of immune cells to the site of infection can shape the human immune response in the lungs. As part of the myeloid-driven epithelial expression signature identified here, chemokines including CXCL10 and IL8 were induced through pro-inflammatory and IFN-driven pathways. Infected myeloid cells secrete chemokines which attract leukocytes [24,25] and in addition to this, constitutive or induced release of mediators by epithelial cells may contribute or amplify timely immune cell recruitment to the site of infection. To address this, we investigated whether the observed gene expression patterns in PBECs were associated with changes in the chemotactic environment during co-culture compared to infected THP-1 monoculture. THP-1 cells were infected with Mtb in the presence or absence of PBECs and cell free culture supernatants were harvested after 48h. The secreted protein content (secretome) was measured through LC-MS. We detected 654 proteins at significantly different levels in co-culture compared to infected THP-1 monocultures (S2 Table). This included increased levels of IL1β in THP-1 PBEC co-culture, which confirmed our previous ELISA data (Fig 6A). To identify which of these proteins were differentially expressed at the gene level in PBECs during co-culture, the gene expression signature was converted into UniProt IDs, which mapped to 341 proteins of which 18 were detected in the transcriptomic and secretome analyses (Fig 9A). Three entities, the leukocyte chemoattractants IL8, CXCL1 and CXCL10, associated with the GO term ‘chemotaxis’ (GO:0006935, AmiGO 2, Gene Ontology Consortium). Myeloid-epithelial cross-talk significantly induced their expression in PBECs and the subsequent release into the culture supernatant, thus directly contributing to the chemotactic environment during Mtb-infection (Fig 9B). In fact, in a transwell chemotaxis assay, confirmed, that the epithelial-driven differences in the mediator environment contributed to cellular influx towards the site of infection. The largest population in peripheral blood are polymorphonuclear (PMN) cells (S6 Fig), of which neutrophils are rapid responders during bacterial infections [26]. PMN migration towards cell free cell-culture supernatants, which was generated in the same ways as the supernatants for secretome analysis, was measured. Supernatants from infected epithelial-myeloid co-cultures attracted on average approximately 9-fold more PMNs than supernatants from infected THP-1 monocultures (Fig 9C–9F), suggesting that epithelial cells contribute crucially to the leukocyte recruitment to the site of infection.

**Discussion**

The first step in the natural history of tuberculosis infection, the host-pathogen interaction in the human airways after Mtb inhalation, is likely shaped by the two dominant cell types in the lungs: epithelial cells and alveolar macrophages. While macrophages are well known to respond and interact with Mtb, airway epithelial cells are understudied. We present the first global transcriptomic profiling of primary human airway epithelial cells in response to Mtb and Mtb-driven inflammation and found that epithelial cells contribute distinctly to the host response against Mtb through antimicrobial effectors as well as by amplifying the chemotactic environment initiated by infected macrophages to shape the local immunological environment.
To interrogate the epithelium in an in vitro setting which resembles early immune interactions in the human respiratory tract more physiologically, we established a co-culture model to allow contact-independent communication between PBECs and infected myeloid cells in real time. We used THP-1 cells to model myeloid responses in the co-culture system developed here and confirmed that the epithelial expression patterns are the same in response to Mtb-infected alveolar macrophages or THP-1 cells.

Through biological over-representation analysis of the epithelial transcriptome, we have uncovered the paracrine activation of epithelial cells by type I IFNs and IL1β. The interplay of type I IFN and IL1 has recently been elegantly delineated within infected macrophages and has been proposed as a point of therapeutic intervention during active tuberculosis [27]. While the cross-regulation of IL1 and type I IFN signalling in the Mtb-infected macrophage is an important well-documented rapidly-evolving area, our findings revealed for the first time that these pathways also act on the epithelial lining. In contrast to the induction patterns of IL1 and type I IFN within macrophages [20] and their respective cross-regulation, the paracrine epithelial activation induced by these two mediators occurred independently of each other with
Fig 9. PBECs upregulate chemokines and enhance neutrophil recruitment to the site of Mtb-infection. (A) The Venn diagramme shows the overlap between significantly induced genes in PBECs exposed to Mtb-infected THP-1 cells at 24h (Expression) and significantly increased secreted proteins during infected PBEC-THP-1 co-culture over infected THP-1 monoculture at 48h (Secretome). Three of the overlapping proteins were associated with chemotaxis (GO:0006935) and their fold changes in their respective comparisons are shown in (B). PBLs were added into a transwell insert on top of a culture well containing conditioned medium from Mtb-infected THP-1 cells ± PBECs or medium control (BEBM). Supernatants were generated from five different PBEC donors (SN1-5). After 3 h cells from the insert and the bottom well were collected and enumerated by flow cytometry. (C) Shows representative plots of the gating strategy to define...
abrogation of either signalling pathway leaving gene expression of the opposite mediator untouched.

The activation of epithelial cells through Mtb-driven myeloid-derived type I IFNs has not previously been reported. Despite the association of type I IFNs with neutrophil-driven disease severity of tuberculosis in humans [28] and mice [29], the effect of type I IFNs on the outcome of Mtb-infection early after exposure is yet to be determined. Early myeloid-driven activation of IFN-signalling may be beneficial for the host as evidenced by the finding that IFN I and IFNγ are both required for optimal immune cell recruitment during the initial pre-adaptive phase of Mtb-infection in a mouse model [30]. Our findings further support this concept and showed that type I IFN signalling is required to induce epithelial transcription of the chemotactic factor CXCL10 during Mtb-driven inflammation in humans. This activation, together with IL1β-mediated chemokine expression, amplifies cellular influx initiated by infected myeloid cells.

We observed that PBECs significantly enhance the neutrophil influx induced by infected myeloid cells and suggest that they may support rapid recruitment to the initial site of infection in vivo. Neutrophils are important early antimicrobial effectors with poor specificity. While they can cause substantial tissue damage and inflammation, their antimicrobial properties are crucial to control and prevent recurrent infections in humans [31]. Tissue neutrophilia is associated with pathogenesis during active TB; however, influx of neutrophils early after infection has been suggested to be beneficial for host control of the pathogen. Absence of neutrophils during early mycobacterial infection results in increased bacterial burden in mice and zebrafish [32,33] and growth control of mycobacteria by whole blood in vitro is strongly dependent on neutrophils [34] which may be an important function at the site of infection.

Besides the release of chemokines, myeloid-derived IL1β mediated the antimicrobial responses elicited in PBECs, including induction of DEFB4 expression, in accordance with previously reported findings [9]. We further confirmed that DEFB4 was a potent direct antimycobacterial effector. While β-defensins are known to kill mycobacteria [17], IL1β-induced S100-family members have not previously been considered to be active against Mtb. Whilst both S100A7A (encoding koebnerisin) and S100A7 (encoding psoriasin) are antibacterial effectors in the skin, neither were known to have antimycobacterial activity prior to our current discovery that psoriasin limited Mtb growth in liquid culture.

IL1β which mediated AMP-expression, is increased in the airways during active TB [35]; and may already be elevated early after Mtb-infection of alveolar macrophages before the onset of detectable lung pathology. This would then result in the activation of epithelial antimicrobial responses at the earliest stages of the natural history of tuberculosis in the human airways. Interestingly, IL1β-dependent epithelial AMP-expression was further enhanced by TNF in PBECs during co-culture with infected myeloid cells. While augmentation of TNF signalling by IL1β has been described in macrophages before [36], we believe that this is the first time that IL1β activation was shown to be enhanced by TNF in Mtb-infection. Epithelial antimicrobial peptides during infection may contribute to host defense against free extracellular mycobacteria thus preventing or diminishing cell to cell spread in early infection. They may also be a promising therapeutic target, since their activity is not affected by multidrug resistance of Mtb. Additionally, the identification of epithelial-specific AMP-expression with anti-
mycobacterial activity may complement myeloid responses to Mtb in the airways, since monocytes and macrophages do not express high levels of the AMPs identified here [37,38].

Prior studies of macrophage-driven activation of epithelial cells focussed solely on specific pathways [9,11], involving matrix metalloproteinases (MMPs) or DEFB4. Our data provide the first global perspective on the epithelial response to Mtb infection, and reveal important new targets in the epithelial response to Mtb-infection while simultaneously confirming the previously published findings. In transwell co-culture, a large proportion of the expression signature (112 genes) was associated with the innate immune response, including type I IFN and cytokine-mediated signalling. IL36G, which is an emerging IL1-family member, was dependent on myeloid-derived IL1β. It has recently been described to have a host-protective role during Mtb-infection [39] and its epithelial release may support macrophage function further. As part of the type I interferon-inducible mediators, the known antiviral factor ISG15 was upregulated and its secretion during mycobacterial infection is involved in the appropriate induction of IFNγ in humans [40]. These are only two of several genes uncovered by the coculture model we have established, which provide interesting targets for future investigations, but where further analysis into their function was beyond the scope of this study.

In contrast to their multifaceted responses to myeloid Mtb-infection, PBECs were surprisingly inert to direct stimulation with Mtb. This was not due to a general non-responsiveness to PRR-stimulation, as PBECs mount a substantial IL8 response towards synthetic TLR-agonists and in response to Streptococcus pneumoniae (Reuschl et al, manuscript in preparation). The global assessment of the transcriptomic response to direct stimulation with live Mtb revealed that no genes were significantly upregulated after 24h and this was complemented by poor invasion and adherence of Mtb to epithelial cells. This finding is consistent with reports of poor invasion of Mtb into primary tracheal epithelial cells in comparison to dendritic cells [41]. Interestingly, two days after intra tracheal Mtb-infection of mice, which results in locally very high density of Mtb bacilli, the majority of bacilli are found in macrophages and only approximately 10% of intracellular Mtb can be detected in epithelial cells [13]. Epithelial inertia to Mtb and the lack of uptake in humans may thus ensure the uptake of Mtb by local professional phagocytes, the preferred target host cell of Mtb after inhalation in the human lungs. Our co-culture model did not include endothelial cells and it remains to be seen whether interaction with an endothelial layer would augment the epithelial response to Mtb. Our findings contrast with previous studies, which described epithelial susceptibility to infection and upregulation of chemokines in A549 cells within 24h [10,42–44]. This most likely reflects differences between primary cells and cell lines, as A549 cells are adenocarcinoma-derived and may not truly mimic the responses of healthy human respiratory epithelium [45]. Through the recovery of PBECs from several donors, we have overcome the limitations of using a single cell line and reflected true biological variation in humans.

In vivo, the airway epithelium is at an air-liquid-interface, polarised and differentiated. However, in our model, the epithelial cells were undifferentiated and in submerged culture which allowed for comparison with the existing literature. Mediator release of undifferentiated epithelium to viral infection or air pollution particles surpasses the release by differentiated epithelium [46,47] suggesting that the inert phenotype of PBECs in response to Mtb is not an underestimate of their in vivo response and is moreover consistent with the lack of epithelial infection observed in mice in vivo [13].

We expanded and studied human primary cells in vitro, as opposed to using respiratory epithelial cell lines or mouse models. A limitation of our study is the focus on primary bronchial epithelial cells which, unlike alveolar epithelial cells, can be obtained by bronchoscopy. The recovery of pure alveolar epithelial cells requires samples from resections of dissected lungs or post-mortem cadaveric explants which may not reflect the responsiveness of healthy
epithelium, and their phenotype is difficult to maintain \textit{in vitro} \cite{48}. Primary alveolar epithelial cells should be used to address whether our findings extend to human alveolar epithelial cells. This would be of interest as differences in the responsiveness to pro-inflammatory mediators between upper and lower airway epithelial cells have been previously described \cite{49,50}. Myeloid-epithelial cross-talk was independent of cell-cell contact, providing evidence that the bronchial epithelium is able to respond to Mtb-infection upon activation by secreted mediators which diffuse from the site of infection throughout the airspaces. In the future, it would be important to assess the mechanisms identified in our study in PBECs derived from patients with active pulmonary tuberculosis, where epithelial responsiveness may be altered. Most of the epithelial cells within the human airways will not be in direct contact with the small number of infected alveolar macrophages. The majority of the initial epithelial response would therefore be expected to be driven by soluble mediators. Our experimental rationale therefore focussed on contact-independent interactions between infected myeloid and primary epithelial cells. Based on our findings, future work should also include direct interactions between the myeloid and epithelial compartment during infection to assess how cell-cell contact modulates the response to Mtb.

Our findings shed light on the contribution of the respiratory epithelial lining to the response against Mtb as well as the complexity of the immune response in the airways early after infection. It is known from challenge studies with rhinovirus in humans and \textit{ex vivo} infection of bronchial tissue that only a limited number of epithelial cells are actively infected \cite{51,52}, yet potent immune activation occurs throughout the epithelial lining. Given that infection with Mtb requires only very few aerosolised mycobacteria \cite{53,54}, amplification of the macrophage-response to infection by uninfected surrounding epithelium may very well be decisive for the outcome of infection. Appropriate animal models of Mtbinfection should be employed to assess the reported epithelial responses \textit{in vivo} during early infection.

In summary, we show that PBECs are inert to direct early Mtb-infection, but potent responders to infected alveolar macrophages. PBECs contributed to host defense as part of an Mtb-activated immune network through pathways, which are likely protective early after infection, by creating an extracellular antimicrobial milieu and promoting early neutrophil influx. Through these cell-specific differential responses to myeloid-derived cytokines, airway epithelial cells likely fulfil a non-redundant role in the human pulmonary host response to Mtb as vital players with the potential to shape the human innate immune response to infection and influence the outcome of exposure to Mtb.

\textbf{Methods}

\textbf{Recruitment and ethics statement}

PBECs and AMFs were collected through bronchial brushings and bronchoalveolar lavage from the airways of healthy volunteers during bronchoscopic procedure at St. Mary’s Hospital, London, United Kingdom. All volunteers were adults and recruited through advertisements or from Contact and TB clinics at Imperial College NHS Trust (St. Mary’s Hospital), London North West Healthcare NHS Trust (Ealing Hospital, Northwick Park Hospital) and Barts Health NHS Trust (Newham Chest Clinic). All samples were collected in accordance with the Human Tissue Act 2004 and written informed consent was obtained from all participants (National Research Ethics Service approval reference 07/H0712/85+5).

\textbf{Cell culture}

Bronchial epithelium was recovered from the right lower lobe of healthy volunteers through cytological brushes (Olympus Keymed, Southend-on-Sea, UK). PBECs were cultured from up
to three brushes per volunteer and expanded in supplemented bronchial epithelial growth medium (BEGM) (Lonza, Walkersville, USA) as previously described [55]. To prevent the outgrowth of recovered contaminating bacteria or fungi, the growth medium was supplemented with gentamycin and amphotericin-B as per the manufacturer’s instruction. All cultures showed the characteristic cobblestone appearance of bronchial epithelial cells. PBECs were used in experiments after two passages. Cells were seeded at 8x10^4/ml in collagen/fibrinogen-coated tissue culture plates and grown to confluence. BEGM was then changed to bronchial epithelial basal medium (BEBM) over night before experimentation. To identify cell subsets in the epithelial lining, *ex vivo* differential cell counts of one separate brush was obtained. Sample sizes given in figure legends refer to biological replicates from independent donors.

Alveolar macrophages (AMΦ) were recovered from bronchoalveolar lavage and isolated through overnight adherence to tissue culture plastic. AMΦ were cultured in RPMI with 10% human serum and 50μg/ml gentamycin. For infection experiments, medium was changed to medium with 5% human serum or BEBM. AMΦ were used at 10^6/ml for experiments.

Peripheral blood leukocytes (PBL) were isolated from heparinised whole blood. Red blood cells were removed trough addition of 10 fold excess RBC lysis buffer (Biolegend, San Diego, USA) for 10 min.

THP-1 cells were obtained from American Type Culture Collection (ATCC) and grown RPMI with 10% FBS, 50000 U penicillin, 50 mg streptomycin (Sigma-Aldrich, Poole, UK) and 0.05 mM β-mercaptoethanol. For macrophage experiments, THP-1 cells were differentiated with 50nM phorbol 12-myristate 13-acetate (PMA) for 24h, seeded in tissue culture plates and rested overnight in complete medium without PMA. THP-1 cells were used at 10^6/ml for experiments. Before infection experiments, culture medium was replaced with infection medium containing 5% human serum or BEBM.

**Mycobacterial culture**

*Mtb H37Rv*, *M. bovis* BCG (SSI) or the clinical isolates *Mtb CH* [56] and *Mtb NPH4216* [57] were cultured in 7H9 Middlebrook medium supplemented with 10% OADC, 0.5% glycerol, 0.05% Tween-80 and 10 μg/ml amphotericin. Cultures were harvested in mid-log phase and frozen down in 15% glycerol. To standardise infection doses, cells were infected from frozen stocks and multiplicity of infections determined based on the mycobacterial counts of the stocks. For determination of colony forming units (CFU), mycobacteria were grown on 7H10 agar plates supplemented with 10% OADC, 0.5% glycerol and 0.05% Tween-80 and cultured for 3–4 weeks at 37°C. Antimycobacterial effects of selected peptides were performed in 96 well plates. hBD2 (PeproTech, London, UK), S100A7/Psoriasin (a kind gift from Prof. Joachim Grötzing [23]) and controls were diluted in fresh 7H9 and *Mtb* bacilli harvested in mid-log phase was added. Plates were incubated shaking for up to 7 days at 36°C. Culture growth was monitored by measuring optical density at 595nm. OD-readings from 7H9-only controls were subtracted from all culture conditions before the data was analysed. On day 7, cultures were plated on 7H10 for CFU enumeration.

**Mtb-infection experiments**

For infections, *Mtb* bacilli were diluted in cell culture medium, washed by centrifugation and taken up in cell culture medium for infection experiments. *Mtb* was sonicated for 40 seconds in an ultrasonic waterbath (Grant) to disperse clumps before inoculation of cell culture. For *Mtb*-uptake and adherence to THP-1 MΦ or PBECs. *Mtb* bacilli were added onto cells for 24h. Infection was determined by cell lysis with 0.1% Triton-X/PBS-Tween80. Lysates were serially diluted in PBS-Tween80 and plated on 7H10 agar. Association of *Mtb* with PBECs was
visualised for representative experiments by Kinyoun stain with a Tb-color kit (Merck, Darmstadt, Germany) and images were acquired with an Axio Scope. A1 microscope (Zeiss, Rugby, UK). For some experiments, 200 μg/ml amikacin (Sigma) was added for 2h before cell lysis. To measure mediator release or gene expression, myeloid cells or PBECs were infected with Mtb at the indicated MOI and supernatants or RNA harvested at the indicated time.

For transwell experiments, PBECs were seeded into tissue culture plates then 0.4 μm cell culture inserts (Millipore) were placed on top with THP-1 cells added to them. Mtb was added to either compartment as desired (MOI5 over THP-1). For blocking and neutralisation of cytokine signalling, 20 μg/ml of antibodies (αIFNα/β R2 (MMHAR-2, R&D Systems), αIFNγ (K3.53, R&D Systems), αIL1β (2805, R&D Systems), αTNF (1825, R&D Systems), mIgG1 (11711, R&D), mIgG2A (20102, R&D Systems) were added to the bottom well 45 min before transwell inserts and THP-1 cells were added. Culture supernatants were harvested from the tissue culture wells after inserts were removed.

**Supernatant transfer experiments**

PBECs were exposed to soluble mediators released from uninfected or infected AMΦ. Culture supernatants from AMΦ or cell-free incubation controls ± Mtb bacilli were harvested after 24h and 0.22 μm sterile filtered. Supernatants were diluted 1:10 in fresh BEBM, added to PBECs and total RNA was harvested after 24h.

**Microarrays and RT-PCR**

RNA was extracted with the TRIzol Plus RNA Purification Kit (Ambion) and treated with DNase I (Thermo, Epsom, UK) to remove residual genomic DNA. For RT-PCR, RNA quality and quantity was measured by Nanodrop. RNA was converted to cDNA using Maxima reverse transcriptase and random hexamers. Solaris Gene Expression and Taqman Expression assays were used to determine gene expression levels by RT-PCR. RT-PCR reactions were performed in duplicate with 12.5 ng of cDNA. Expression data were analysed with Biogazelle qbase+ and normalised to ACTB and PGK according to [58]. Solaris Gene Expression assays: ACTB (AX-003451), CXCL10 (AX-007871), DEFB4A (AX-012997), IFIT1 (AX-019616), IFNB1 (AX-019656), IL36G (AX-007959), IL8 (AX-004756), PGK (AX-006767), S100A7A (AX-027145). Taqman Expression assays: S100A7 (Hs01923188_u1).

For microarrays, RNA quality was assessed by Bioanalyzer (Agilent, Stockport, UK) (RIN > 9 for all samples) and RNA was quantified by Qubit (Thermo). 50-100ng total RNA was prepared for GeneChip Human Transcriptome Array 2.0 with the GeneChip WT PLUS Reagent Kit (Affymetrix, High Wycombe, UK). Samples were hybridized and scanned at the MRC Genomics Laboratory (Hammersmith Campus, Imperial College London).

**Soluble mediators**

Soluble mediators were measured in cell culture supernatants after double-filtration through 0.22 μm centrifuge spin filters. Human IL1β, IFNγ and IFNα were measured by DuoSet ELISAs (R&D Systems). IFNβ was measured by VeriKine Human IFN Beta ELISA kit (pbl interferon source).

**Secretome analysis**

The secretome of Mtb-infected THP-1 cells and Mtb-infected PBEC-THP-1 co-cultures was measured by liquid chromatography—mass spectrometry (LC-MS). Six paired cell culture supernatants were analysed using two independent PBEC cultures. Cell culture supernatants
were harvested at 48h and sterile filtered twice through 0.22 μm centrifuge filters. 500 μl of culture supernatants were buffer exchanged twice with 450 μl of 2 M urea, 100 mM Tris-HCL (pH 8.0) using a 3 kDa nominal molecular weight limit cut-off spin filter (Millipore). A volume of 80 μl of sample was reduced with 5 mM DTT (Sigma) for 30 min at 60°C and alkylated with 10 mM of iodoacetamide (Sigma) in the dark at room temperature for 30 min. 1 μg Trypsin/Lys-C mix (Promega) was added for 18 h at 37°C. Samples were adjusted to 1% (v/v) trifluoroacetic acid (TFA) (Sigma) and peptide digests were purified using the C18 Stop And Go Extraction (STAGE) tips [PMID: 16602707] and resuspended in 2% (v/v) acetonitrile, 0.1% (v/v) formic acid for LC-MS. Samples were analysed using an EASY-nLC 1000 Liquid chromatography system coupled to a Q-Exact mass spectrometer. The separation column and emitter was an EASY-Spray column, 50 cm x 75 μm ID, PepMap C18, 2 μm particles, 100 Å pore size. Buffer A was 2% acetonitrile, 0.1% formic acid and buffer B 100% (v/v) acetonitrile, 0.1% (v/v) formic acid. A gradient from 5% to 40% acetonitrile over 120 min was used to elute peptides for ionization by electrospray ionisation (ESI) and data dependent MS/MS acquisition consisting of 1 full MS1 (R = 70K) scan acquisition from 350-1500m/z, and 10 HCD type MS2 scans (R = 15K). MS/MS charge targets were limited to 1E6 and isolation window set to 2.0 m/z, monoisotopic precursor selection, charge state screening and dynamic exclusion were enabled, charge states of +1, >4 and unassigned charge states were not subjected to MS2 fragmentation. Raw mass spectra were identified and quantified using Maxquant 1.5.15 using a 1% peptide and protein FDR. Searches were conducted against the Uniprot SwissProt database downloaded on 06/06/2014. The database was supplemented with common contaminant proteins introduced during proteomic experiments. Searches were specified as tryptic with 1 missed cleavage, 7 ppm precursor ion mass tolerance, 0.05 Da fragment ion mass tolerance, fixed modifications of carbamidomethylation (C), and variable modification of oxidation (M), acetylation (N-term, Protein). Protein Label free quantitation (LFQ) intensity measured in medium alone was subtracted from all paired samples, and LFQ values were log2 transformed. To identify both qualitative and quantitative effects of stimulation on extracellular all missing values were replaced assuming a normal distribution (width 0.3, and a down shift of 1.8) for only control/unstimulated samples as described in [59].

**Western blot**

For protein lysates PBECs were treated with RIPA buffer (Pierce) supplemented with 2x Halt Protease and Phosphatase Inhibitor Cocktail (Pierce) and 250 U Benzonase (Sigma) for 15 min on ice. Lysates were centrifuged at 14000 rpm for 15 min at 4°C. Total protein concentration was measured by BCA protein assay (Pierce). 12 μg of protein lysates lysates were mixed with Laemmli buffer (BioRad, Hertfordshire, UK) and 25 mM DTT and boiled for 7 min. Proteins separated on 4%-20% Mini PROTEAN gels (BioRad) and transferred onto nitrocellulose membranes by iBlot (Invitrogen). Membranes were blocked in 5% milk/TBST for 1h and probed with primary antibodies (βeta-Actin (D6A8, New England Biolabs), αPhospho-Stat1 (Tyr701) (D4A7, New England Biolabs), αStat1 (#9172, New England Biolabs)) at 1:1000 in 5% BSA/TBST over night at 4°C. HRP-linked IgG (New England Biolabs) (1:2000) in 5% BSA/TBST was added the next day for 1h. Membranes were developed with ECL Western blotting substrate (Pierce) and imaged with FUSION FX7 SPECTRA (Vilber).

**PBL phenotyping by flow cytometry**

PBLs were isolated and resuspended in PBS with 0.5% BSA and 2mM EDTA (FACS buffer). Before staining, 10% human serum in FACS buffer was added to cells for 20 min to block Fc receptors. For surface staining, PBLs were incubated with α CD14-Brillian Violet 421 (M5E2,
Biolegend), α CD15-Brillian Violet 605 (SSEA-1, Biolegend), α CD3-PE-CF594 (UCHT1, BD) and α CD66b-PerCP/Cy5.5 (G10F5, Biolegend). Cells were treated with Cytofix Fixation buffer, resuspended in FACS buffer and left overnight before flow cytometric acquisition using either a BD LSR II or BD Fortessa. Anti-Mouse Ig compensation beads (BD) were used to determine compensation parameters.

PBL transwell migration assay

Cell free culture supernatants were generated through direct co-culture of Mtb-infected THP-1 cells in the presence of absence of PBECs for 48h. BEBM was used as a background control for unspecific migration. Conditioned or control medium was added to the bottom well of a tissue culture plate and 0.5 μm transwell inserts (Corning) placed on top. The plates were placed in a humified 37˚C CO₂ incubator for 30 min to equilibrate. 2.5x10⁵ PBLs were added to the transwell insert and the plate was placed back into the incubator for cell migration. After 3h, 2mM EDTA was added to the bottom compartment to dislodge cells from the transwell membrane. Cells were collected from the insert and bottom compartment, fixed and resuspended in equal volumes of FACS buffer. Cells in each sample were enumerated through acquisition for 120 s using a BD LSR II flow cytometer.

Statistical analysis

Microarray data was normalised by Robust Multi-Array Average (RMA) using Partek Genomic Suite 6. All genes annotated by NCBI Reference Sequence Database (RefSeq), NIH GenBank or Ensembl were taken forward for statistical analysis. One-Way ANOVA on microarray data was performed with PGS. For SAM analysis of expression and secretome data with TIGR MultiExperiment Viewer 256 or 64 permutations were used, respectively. Pathway analysis was performed with InnateDB for over-representation analysis [60]. Transcription factor binding site enrichment on differentially expressed genes was performed using oPossum [61].

Flow cytometry data was analysed using FlowJo v10. Statistical analysis of all other experiments was performed with GraphPad Prism 6 and is indicated where appropriate.

Supporting information

S1 Fig. Association of Mtb with PBECs visualised by Kinyoun stain. PBECs were infected with Mtb H37Rv (MOI50) for 24h. The association of Mtb with PBECs was confirmed microscopically via Kinyoun stain after 24h of infection at MOI 50. Shown are representative images from two donors at a 200x magnification. Arrowheads indicate Mtb.

S2 Fig. PBEC gene expression in response to AMΦ in transwell co-culture. AMΦ from two healthy donors were co-cultured in the transwell model with PBECs and infected with Mtb H37Rv (MOI15) as indicated. Gene expression was measured by RT-PCR.

S3 Fig. Interferon-release and gene expression in response to infection, transwell co-culture and recombinant cytokines.

A) PBECs were stimulated in the transwell co-culture system as indicated and IFNB expression measured after 24h. Fold change was calculated over unstimulated cells. Boxplots show median and range (n = 5). No significant differences were detected.
B) IFNγ and IFNλ release by THP-1 cells after 24h of Mtb-infection (MOI5) was measured by ELISA in two independent experiments. Mean ± SD. n.d., not detected; ϕ, extrapolated values below the detection limit; horizontal lines indicate the detection limits of the assays.

C) PBECs were stimulated with 1 ng/ml IL1β or IFNβ for 24h and gene expression was measured by RT-PCR (n = 3). Mean ± SD are shown.

D) PBECs were co-cultured with Mtb-infected THP-1 cells in the presence of 20 μg/ml αL1β or IgG1 (isotype control) as indicated. After 24h, gene expression was measured by RT-PCR. Expression is shown as fold change over unstimulated (n = 6). Boxplots show median and range.

E) PBECs were co-cultured with Mtb-infected THP-1 cells in the presence of 20 μg/ml αIFNAR2 or IgG2 (isotype control). After 24h, gene expression was measured by RT-PCR and is shown as fold change over unstimulated (n = 3). Median is shown.

Friedman test with Dunn’s post-test was used to compare groups against isotype control. n.s., not significant; *, p<0.05.

(TIF)

S4 Fig. Effects of TNF and IFNψ on PBEC-myeloid co-culture.

(A) PBECs were co-cultured with Mtb-infected (MOI5) THP-1 cells with αTNF or IgG1 for 24h. DEFB4 expression was measured by RT-PCR and is shown as fold change over unstimulated PBECs (n = 3).

(B) IL1β release was measured in the co-culture supernatants of (A) by ELISA.

(C) THP-1 MΦs were infected with Mtb (MOI5) in the presence of αTNF or IgG1 and IL1β release measured after 24h. Cytokine levels are shown as % of IL1 β release during infection in the presence of IgG1 (n = 5).

(D) PBECs were exposed to Mtb-infected THP-1 cells (MOI5) in co-culture in the presence of αIFNγ or IgG2a. After 24h, CXCL10 expression was measured by RT-PCR and is shown as fold change over unstimulated PBECs. Mean ± SD are shown. (A, B and D) Wilcoxon signed rank test was used to compare groups; (C) was compared by repeat-measure ANOVA with Holm-Sidak’s multiple comparisons test. **, p<0.01 or exact p-values are given.

(TIF)

S5 Fig. Antimycobacterial effects of hBD2 and expression of S100A7 in PBECs during transwell co-cultures.

(A) Clinical isolates Mtb NPH4216 and Mtb CH were incubated with 5 μg/ml recombinant hBD2 or vehicle control as described in Fig 8. Colony forming units (CFU) were determined at day 7. Effects of hBD2 was compared with vehicle control by Student t-test. Mean ± SD of triplicate measurements are shown. * p<0.05; ** p<0.01

(B) In the transwell model, PBECs were exposed to THP-1 cells or Mtb H37Rv (MOI5 over THP-1) for 24h as indicated. S100A7 expression in PBECs was measured by RT-PCR and is shown as fold change over unstimulated PBECs (n = 5).

(C) PBECs were co-cultured with infected or uninfected THP-1 cells in the presence of αL1β or IgG1 as indicated. After 24h, S100A7 expression was measured by RT-PCR and is shown as fold change over unstimulated PBECs (n = 5).
Friedman test with Dunn’s post-test was used to compare expression with unstimulated or respective isotype control. Boxplots show median and range. * p<0.05; ** p<0.01.

S6 Fig. Gating strategy for PBL transwell migration experiments. PBLs were isolated from whole blood and stained for CD3, CD14, CD15 and CD66b. Shown are representative plots for the gating strategy from one of three donors. After gating for singlets, forward (FSC) and side (SSC) scatter were used to define PBL subsets. PMN, polymorphonuclear cells.

S1 Table. Differentially expressed genes in PBECs exposed to Mtb-infected THP-1 cells in transwell co-culture. Significantly differentially expressed genes at a q-value < 5% were determined by Significance Analysis of Microarrays.

S2 Table. Secretome of Mtb-infected THP-1 monocultures and co-cultures with PBECs. Significantly differentially secreted proteins identified in cell-free culture supernatants of Mtb-infected THP-1 cells co-cultured with PBECs compared to infected THP-1 monoculture at a q-value < 5% (determined by SAM).

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