Mycobacteria Manipulate G-Protein-Coupled Receptors to Increase Mucosal Rac1 Expression in the Lungs

Nader Alaridah^a Nataliya Lutay^a Erik Tenland^a Anna Rönnholm^a
Oskar Hallgren^b Manoj Puthia^a Gunilla Westergren-Thorsson^c
Gabriela Godaly^a

^aDivision of Laboratory Medicine, Department of MIG, ^bDivision of Clinical Sciences, Department of Respiratory Medicine and Allergology, and ^cDivision of Vascular- and Respiratory Research Unit of Lung Biology, Department of Experimental Medical Science, Lund University, Lund, Sweden

Key Words

G-protein-coupled receptors · Bacille Calmette-Guérin · Pulmonary tuberculosis

Abstract

Mycobacterium bovis bacille Calmette-Guérin (BCG) is currently the only approved vaccine against tuberculosis (TB). BCG mimics *M. tuberculosis* (Mtb) in its persistence in the body and is used as a benchmark to compare new vaccine candidates. BCG was originally designed for mucosal vaccination, but comprehensive knowledge about its interaction with epithelium is currently lacking. We used primary airway epithelial cells (AECs) and a murine model to investigate the initial events of mucosal BCG interactions. Furthermore, we analysed the impact of the G-protein-coupled receptors (GPCRs), CXCR1 and CXCR2, in this process, as these receptors were previously shown to be important during TB infection. BCG infection of AECs induced GPCR-dependent Rac1 up-regulation, resulting in actin redistribution. The altered distribution of the actin cytoskeleton involved the MAPK signalling pathway. Blocking of the CXCR1 or CXCR2 prior to

infection decreased Rac1 expression, and increased epithelial transcriptional activity and epithelial cytokine production. BCG infection did not result in epithelial cell death as measured by p53 phosphorylation and annexin. This study demonstrated that BCG infection of AECs manipulated the GPCRs to suppress epithelial signalling pathways. Future vaccine strategies could thus be improved by targeting GPCRs.

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Introduction

It has been proposed that mucosal delivery of the bacille Calmette-Guérin (BCG) vaccine provides superior protection against tuberculosis (TB), a disease that kills 1.9 million people each year [1, 2]. Traditionally, the adaptive immune responses, particularly central memory CD4+ and CD8+ cells, are considered to be important for long-term immunity [3–6]. However, emerging evidence indicates that the cells of the innate immune system are equipped with “epigenetic memory” where genes encoding specific host defence molecules increase the response

upon re-stimulation [4–7]. Recent studies further demonstrated that airway epithelial cells (AECs) harbour *Mycobacterium tuberculosis* (Mtb) and are critical during the progression to active disease [8, 9]. These cells could thus be important for both host defence and vaccine development, but a more comprehensive knowledge of direct interaction between AECs and mycobacteria is currently lacking [10].

BCG mimics Mtb in its ability to persist in the body, and is similar to the tubercle bacillus in its physiological, molecular, and metabolic responses [11–14]. This invasive bacterium actively induces its own uptake by macropinocytosis to enter normally non-phagocytic cells such as AECs [15]. Until now, Toll-like receptor (TLR)2, TLR4 and C-type lectins have been implicated in mycobacteria-induced epithelial signalling and uptake [16, 17]. It has also been observed that mycobacteria manipulate epithelial production of the cytokine CXCL8 through the inhibitory G-protein-coupled receptor (GPCR) kinase 2 [18]. To trigger GPCR signal transduction in AECs, intracellular bacteria, such as *Shigella*, are known to engage GTPases in actin polymerization [19–21]. These low-molecular-weight proteins belong to the Ras GTPase superfamily and include Rab and Rho/Rac, with the ability to act as molecular switches by coupling extracellular signals to different cellular responses, cytoskeletal integrity, intracellular vesicular transport, and trafficking of proteins [22]. Inhibition of Rac1 was recently shown to repeal tumour protein p53 suppression of STAT and NF- κ B, and Rho is essential in the establishment and maintenance of tight junctions [23]. Previous studies indicate that signalling through TLRs is important for the phagocytosis of bacteria, as TLR-mediated MyD88-dependent activation of p38 is required for phagosome maturation [24, 25]. Intracellular pathogens such as *Listeria monocytogenes* manipulate TLRs through the MAPK pathway to avoid phagosome maturation and autophagy [26, 27]. The p53 pathway acts in synergy with the p38 MAPK pathway to mediate cell cycle arrest, cellular senescence and apoptosis [28].

The innate host defence of the conducting airway depends on the multiple barriers created by layers of mucus and the tight adhesions between epithelial cells. In the human lung, AECs are able to harbour Mtb and are critical during progression to active disease [29]. Recently, mucosal vaccination with an attenuated Mtb strain induced a strong innate immune response, followed by a robust central memory answer [30]. AECs facilitate a protective environment for Mtb replication where it could gain enhanced virulence by modifying envelope structure

and gene expression [31]. AECs also interact with other cells of the innate immune system, such as granulocytes, monocytes, macrophages, and innate lymphoid cells, to mount an effective defence against the invading pathogen as well as to activate the following specific immunity. AECs are now recognized as active participants of the immune response against Mtb [32]. In this study, we analysed mycobacteria-induced epithelial signalling pathways and the contribution of GPCRs to further elucidate these responses. We could conclude that BCG induced Rac1 up-regulation, resulting in long-term actin cytoskeleton distribution. Inhibition of GPCRs decreased BCG-induced Rac1 expression but increased AEC transcriptional activity and epithelial cytokine production.

Material and Methods

Ethical Statement

The Swedish Research Ethical Committee in Lund (FEK 413/2008) approved the isolation of the primary AECs. These were acquired from lung explants of healthy donors with irreversible brain damage and no history of lung disease; the lungs were intended for transplantation, but could instead be used in this study as no matched recipients were available at that moment. Written consent was obtained from the donors' closest relatives. The murine study was approved by the Animal Experiment Ethics Committee at the Lund District Court in Sweden (M7–15).

Bacterial Strain and Growth Condition

Mycobacterium bovis BCG Montreal strain containing the pSMT1 shuttle plasmid was prepared as previously described [33]. Briefly, the mycobacteria were grown in Middlebrook 7H9 broth, supplemented with 10% ADC enrichment (Becton Dickinson, Oxford, UK) and hygromycin (50 mg/L; Roche, Lewes, UK), the culture was washed twice with sterile PBS, re-suspended in broth, and then dispensed into vials. Glycerol was added to a final concentration of 25% and the vials were frozen at -80°C . Prior to each experiment, a vial was defrosted, added to 9 mL of 7H9/ADC/hygromycin medium, and incubated with shaking for 72 h at 37°C . Mycobacteria were then centrifuged for 10 min at 3,000 g, washed twice with PBS, and re-suspended in 10 mL of PBS.

Cell Culture

Bronchial tissue was dissected from lungs and kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin, penicillin, streptomycin, Fungizone, and 10% FCS (all from Gibco, Paisley, UK) until further isolation. After removing intraluminal mucus and surrounding tissue, bronchi were digested in 0.1% protease (Sigma, St. Louis, MO, USA) prepared in a minimum essential medium Eagle, Spinner modification (Sigma-Aldrich) supplemented with gentamicin, penicillin, streptomycin, and Fungizone for 24 h. The cells were recovered by repeated intraluminal rinsing with DMEM supplemented as above. Cells were filtered through a 100- μm strainer (Falcon, Becton Dickinson) and seeded in cell-culture flasks coated with 1% collagen-1 (PureCol, Inamed Biomaterial, Freemont, CA, USA) in bronchial epithelial

cell growth medium (Clonetics). The following day, cells were thoroughly washed with a medium change every other day. Experiments were performed in passage 3 or 4.

Incubation of AECs with Mycobacteria

AECs were grown in 6-well plates (2.0×10^5 cells/well; Fisher Scientific, Loughborough, UK) for 2 days until confluent (80%). Before the experiment, the medium was changed and the cells were treated with monoclonal mouse anti-human CXCR1 and/or monoclonal mouse anti-human CXCR2 antibodies (10 μ g/mL; R&D Systems) and/or Rac1 inhibitor (50 μ M; Merck Millipore) for 30 min on ice, followed by infection with BCG. The cells were infected with a multiplicity of infection (MOI) of 1:1 at 37°C for up to 3 days. As a control, we used uninfected cells treated with CXCR1, CXCR2 antibodies, or the Rac1 inhibitor.

Western Blot

The primary cells were washed with PBS containing 0.2 mM phenylmethylsulfonyl fluoride, 1 μ g/mL pepstatin A, and 5 μ g/mL leupeptin (Sigma-Aldrich), and then lysed with modified mammalian protein extraction reagent solution (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 50 mM ZnCl₂, 1% NP-40, 0.1% deoxycholate, 0.1% SDS; Pierce) containing phosphatase and complete protease inhibitor cocktail (1:100). The cells were then placed on a shaker for 5 min, collected, and then centrifuged at 10,000 g for 5 min. Protein samples were used immediately for Western blot analysis or stored at -80°C. Protein levels were measured with a NanoDrop™ 8000 spectrophotometer using the Pierce 660 nm assay (Thermo Scientific). Medium alone and only BCG-infected cells were used as controls. Protein samples were mixed with PBS, 4× NuPAGE LDS sample buffer (Life Technologies), and 0.1 M DTT, and incubated at 90°C for 10 min followed by centrifugation at 218 g for 5 min. Equal amounts of protein (20 μ g/well) were loaded on a NuPAGE 4–12% Bis-Tris Gel (Life Technologies) and separated by SDS-PAGE. A molecular weight marker (Novex® Sharp Prestained; Life Technologies) was loaded onto each gel for protein-band identification. After separation, the proteins were transferred to a PVDF membrane (GE Healthcare, Little Chalfont, UK). The membrane was then blocked with either 5% dry milk (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 5% BSA (Santa Cruz Biotechnology) for 1 h on a shaker at room temperature. Membranes were then incubated on a shaker overnight at 4°C with mouse monoclonal antibodies against human Rac1 (1:500; 23A8 Millipore), Rho A (1:500; 26c4; Santa Cruz Biotechnology), Rab4 (1:500; 46-K; Santa Cruz Biotechnology) and goat polyclonal against human actin (1:500; I-19; Santa Cruz Biotechnology). Bound primary antibodies were detected with goat-anti-mouse IgG horseradish peroxidase (HRP) or rabbit-anti-goat IgG HRP (1:5,000; Santa Cruz Biotechnology) using ECL (GE Healthcare) and GelDoc equipment (Bio-Rad Laboratories). The house-keeping protein GAPDH was used to confirm equal loading on the wells. Blot intensity was quantified using ImageJ v1.49, and normalized against GAPDH. If required, membranes were stripped with Restore Western Blot stripping buffer (Pierce, Rockford, IL, USA), blocked, and re-probed with new antibodies.

Phospho-Kinase Array

A proteome human phospho-kinase array kit (Proteome Profiler Array, R&D Systems), a membrane-based sandwich immunoassay, was used to measure protein phosphorylation. The assay was

performed according to the manufacturer's instructions. Briefly, total cell extracts were prepared from stimulated near-confluent cultures of AECs grown in 6-well plates. Untreated cells were used as controls. The cell extracts containing 500 μ g of total protein were incubated with the human phospho-kinase array. The proteins present in a lysate sample were captured by discrete antibodies printed in duplicate across the nitrocellulose membranes. The array was washed 3 times with 1× wash buffer for 10 min on a rocking platform shaker to remove unbound proteins. Washing was followed by incubation with a cocktail of biotinylated detection antibodies (monoclonal anti-human of phosphorylated PYK2 (Y402), MEK1/2 (S218/S222, S222/S226), JNK pan (T183/Y185, T221/Y223), p38, p53 (S15), p53 (S46), p53 (S392), STAT1 (Y701), STAT5b (Y699), STAT6 (Y641), and a subsequent addition of streptavidin-HRP conjugate. The signals were detected with the ECL Plus Western blotting detection system (GE Healthcare). Developed signals were analysed using ImageJ v1.49 analysis software.

Detection of Apoptosis and Necrosis

After incubation of AECs with BCG, the cells were detached by EDTA for about 5 min at room temperature, followed by the addition of a trypsin inhibitor, washed twice in PBS for 5 min (1,000 rpm), and stained with annexin V-Alexa Fluor® 488 (1:100; Life Technologies Europe BV, Stockholm, Sweden) for 15 min in the dark on ice to detect early apoptosis and 7-aminoactinomycin D (1:100; BD Via-Probe, BD Pharmingen Biosciences, San Diego, CA, USA) for 15 min in the dark on ice to test for late apoptosis and necrosis. Mean fluorescence intensity was analysed by flow cytometry (Accuri, Becton Dickinson).

Murine BCG Infection

Male BALB/c mice, aged 8–10 weeks, were obtained from Jackson Laboratories (Bar Harbor, ME, USA). They were maintained in the animal facilities at the Department of Microbiology, Immunology, and Glycobiology, Lund University, Lund, Sweden. They were anaesthetized by isoflurane inhalation for 10–20 s. The mice were divided into 2 groups: BCG infected ($n = 5$) and uninfected ($n = 3$). For infections, BCG at 5×10^4 CFU in 10 μ L PBS was given intranasally, and 10 μ L PBS was given to the control mice. After 5 weeks, the mice were sacrificed by intraperitoneal administration of pentobarbitone (60 mg/mL, 0.05–0.1 mL/mice). The largest lobe was saved in paraformaldehyde (4%), and used for immunohistochemistry staining. Remaining lobes were homogenised and plated on 7H11 agar plates and incubated at 37°C for 4 weeks for CFU measurements.

Immunofluorescence Microscopy

The expression of actin and Rac in the sections of lung tissue from BCG-infected BALB/c mice was detected by immunofluorescence staining. The fixed tissue samples were dehydrated by overnight incubation in ethanol, followed by xylene, and placed in Histowax (Histolab Products, VästraFrölunda, Sweden), according to the manufacturer's recommendations. The samples were embedded in paraffin; sections (4–5 μ m) were cut and placed on glass slides. Deparaffinization, rehydration, and antigen retrieval of the specimens were done, followed by blocking in 10% FCS with 1% BSA in TBS for 2 h at room temperature, followed by incubation overnight, shaking in a cold room with PBS with 1% BSA; the goat polyclonal anti-actin (sc-1616) (Santa Cruz Biotechnology) or

Fig. 1. Mycobacteria increase Rac1 expression. The impact of BCG infection, CXCR1 and CXCR2 on modulated epithelial Rac1 expression was studied in a murine model and by Western blot. **a, b** Mycobacterial infection significantly altered Rac1 expression in vivo as detected by immunofluorescence microscopy compared

to uninfected mice. **c, d** BCG infection increased epithelial Rac1 expression, but CXCR1/2 blocking prior to infection decreased Rac1. Data are presented as representative images or as mean $\pm$ SEM of 3 separate experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

mouse monoclonal antibody against Rac1 clone 23A8 (Millipore) antibodies were used. As a control, we used an isotype antibody by replacing the primary antibody with antibody diluent (blocking buffer). The specimens were washed twice with PBS plus 0.0025% Triton X-100 for 5 min, and then incubated with fluorophore-conjugated rabbit anti-goat or goat anti-mouse secondary antibody (1:2,000; Invitrogen) in PBS with 1% BSA for 2 h in the dark. Slides were then examined by fluorescence microscopy (AX60, Olympus Optical).

For in vitro actin expression, AECs were seeded on glass slides (12 mm $\varnothing$), and allowed to attach for 1 day at 37°C in a 5% CO₂ atmosphere. Rac1 inhibitor was added for 30 min on ice before BCG infection. Bacteria were added to the AECs at MOI 1:1, and incubated for 72 h, followed by fixation in 4% paraformaldehyde. The glass slides were washed twice in PBS with 5% FCS, and blocked and mounted overnight with CytoPainter Phalloidin-iFluor 488 Reagent (Abcam) and anti-Mtb antibody (ab905; 1:200; Abcam).

ELISA

IL-6 (D6050) and IL-10 (D1000B) secretion by the infected cells was quantified in supernatants by human quantikine ELISA kits (R&D Systems) according to the manufacturer's instructions. NF- κ B (EK1111) and AP-1 (c-Jun, EK1041) were quantified with nuclear extraction kits containing ELISA kit according to manufacturer's instructions (Affymetrix Panomics, UK).

Statistical Analysis

Prism 6f for Mac OS X was used for statistical analysis. The statistical difference between two groups was investigated by means of the nonparametric Mann-Whitney U test. Multiple comparisons were done by one-way analysis of variance followed by the Kruskal-Wallis test with the Bonferroni correction and the Dunnett post hoc test. Significance was accepted at $p < 0.05$, $p < 0.01$, or $p < 0.001$.

Results

Mycobacteria Utilize CXCR1 and CXCR2 to Induce Epithelial Rac1 Activation

Rac1, a member of the Ras superfamily of small GTPases, regulates the basal level of actin assembly and the reorganization of the actin cytoskeleton in response to GPCR stimulation [34]. BCG-induced mucosal Rac1 expression was analysed in vivo by immunohistochemistry staining of mycobacteria-infected mouse lung sections (Fig. 1a). BCG infection induced sustained mucosal Rac1 expression 5 weeks after infection ($p = 0.0001$; Fig. 1a). We confirmed that BCG infection of primary AECs increased Rac1, compared to uninfected cells ($p = 0.015$; Fig. 1c, d). Blocking of CXCR1 or CXCR2 decreased Rac1 production down to basal levels ($p = 0.092$, $p = 0.001$, and $p = 0.001$ for CXCR1, CXCR2, and CXCR1/2, respectively; Fig. 1c, d).

Mycobacteria Up-Regulate Actin Distribution

During inflammation, actin cytoskeletal changes regulate junctional integrity leading to disturbed barrier function [35]. Actin expression of BCG-infected AEC was studied by Western blot and immunofluorescence (Fig. 2a, b). BCG infection induced increased actin expression compared to uninfected cells ($p = 0.002$; Fig. 2a). Blockage of the CXCR2 decreased BCG-induced actin production ($p = 0.07$, $p = 0.001$, and $p = 0.006$ for CXCR1, CXCR2, and Rac1 inhibition respectively; Fig. 2a). With immunofluorescence, we observed that BCG infection induced epithelial actin redistribution (Fig. 2b; online suppl. Fig. 2; for all online supplementary material, see www.karger.com/doi/10.1159/000453454). To confirm that BCG infection regulates actin expression and distri-

bution in vivo, lung tissues from BCG-infected mice were stained for actin cytoskeletal changes (Fig. 2c, d). Compared to in the uninfected animals, BCG infection increased sustained actin up-regulation for 5 weeks after infection (Fig. 2d).

Mycobacteria Activate the Epithelial MAPK Pathway

Previous studies indicated that mycobacteria regulate the epithelial inflammatory response through GPCR kinases [18]. GPCR-mediated signalling is further known to activate the pathways of major kinases, including MAPK and JNK, new targets in drug discovery [36]. To investigate mycobacterial epithelial kinase activation, we used the human phospho-kinase array on primary AECs (Fig. 3). Mycobacterial infection led to increased epithelial phosphorylation of PYK2 ($p = 0.0045$; $p = 0.0003$), p38 kinase ($p = 0.0001$; $p = 0.0005$) and MEK1/2 (MAP2K1/2; $p = 0.0003$; $p = 0.0015$) compared to medium control at both 6 and 72 h after infection (Fig. 3). In contrast, JNK had decreased after 6 h, and increased after 72 h, compared to the control (Fig. 3). Actin distribution requires p38 and MEK1/2, and both of these molecules were activated by BCG infection of AECs (Fig. 3).

Mycobacterial Induction of p53 Does Not Affect Epithelial Survival

Intracellular bacteria trigger actin polymerization in AECs by engaging GTPases such as Rac and Rab [20, 37, 38]. Inhibition of Rac1 was recently shown to abolish tumour protein p53 suppression of the transcription factors STAT and NF- κ B [23]. We investigated the mycobacterial influence on epithelial p53 with a phosphorylation assay (Fig. 4). BCG infection of AECs had increased the phosphorylation of p53 at the regulatory S319 and apoptotic S46 domains, at 6 and 72 h post infection, but not at the S15 domain, which activates transcription and cell survival. Rac1 inhibition prior to infection decreased p53 (S392 and S46), possibly suggesting that Rac1 is involved in transcription and cell survival (online suppl. Fig. 1).

To analyse if mycobacteria induced cellular death, we measured epithelial apoptosis and necrosis (data not shown). We found that mycobacteria did not affect epithelial survival.

BCG Manipulate Epithelial STAT Phosphorylation

Compared to our observation using BCG, pathogenic mycobacteria are known to suppress the MAPK and JAK/

Fig. 2. Mycobacteria up-regulate actin distribution. **a, b** BCG-induced increased actin expression was detected in BCG-infected AECs. CXCR2 blocking or the addition of Rac1 inhibitor prior to infection decreased the actin expression. **c** In vivo, the infection increased actin distribution in the BCG-infected mice compared

to the uninfected controls, visualised by immunofluorescence microscopy. **d** The images were further analysed by ImageJ software. Data are presented as representative images or as mean $\pm$ SEM of 3 separate experiments. ** $p < 0.01$; *** $p < 0.001$.

STAT signalling pathways that are crucial for many innate and adaptive immune responses [36]. Patients with disseminated BCG infection have been found to have dominant-negative mutations in *STAT1* that affect IFN γ signalling [39]. BCG infection in primary AECs reduced

the phosphorylation of STAT1 (Y701), STAT5b (Y699), and STAT6 (Y641) 6 h after infection, and increased phosphorylation on STAT1 (Y701) and STAT5b (Y699) at 72 h after infection, but the STAT6 (Y641) levels were reduced (Fig. 4).

Fig. 3. Mycobacteria activate the epithelial MAPK pathway. We used BCG to investigate epithelial kinase modulation using the human phospho-kinase array. Mycobacterial infection led to increased epithelial phosphorylation of PYK2, p38 kinase, and MEK1/2 (MAPK1/2) compared to medium control 6 and 72 h after infection. JNK had decreased after 6 h but increased after 72 h compared to the control. Data are presented as mean $\pm$ SEM of 3 experiments. *** $p < 0.001$.

Fig. 4. Mycobacteria modulate epithelial p53 and STAT pathways. Mycobacterial influence on epithelial p53 and STAT was investigated with the phosphorylation assay. BCG infection of primary AECs increased the phosphorylation of p53 at the regulatory S319 and apoptotic S46 domains, 6 and 72 h after infection. In contrast, the phosphorylation at S15, which activates transcription and cell survival, was not induced by the infection. STAT1 (Y701), STAT5b (Y699), and STAT6 (Y641) were suppressed 6 h after infection, while BCG induced phosphorylation of STAT1 (Y701) and STAT5b (Y699) 72 h after infection. STAT6 (Y641) levels remained reduced during the study. Data are presented as mean $\pm$ SEM of 3 separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. Mycobacteria regulate NF-κB and c-Jun through CXCR1 and CXCR2. We determined the impact of CXCR1 and CXCR2 blockade on mycobacteria-induced NF-κB and c-Jun regulation. BCG infection did not affect epithelial NF-κB and AP-1 activation. However, the blockade of CXCR1 or CXCR2 prior to infection increased NF-κB and c-Jun protein levels compared to uninfected cells. Data are presented as mean ± SEM of 3 separate experiments. * $p < 0.05$; *** $p < 0.001$.

Mycobacteria Regulate NF-κB and c-Jun through CXCR1 and CXCR2

The data on NF-κB activation by pathogenic mycobacteria is still conflicting. BCG was previously shown to bypass NF-κB activation, but activate the signalling through

Fig. 6. Controlled epithelial cytokine secretion. Mycobacterial control of transcriptional factors was analysed as epithelial cytokine secretion. BCG infection of AECs induced significant IL-6 and IL-10 secretion. Blockage of CXCR1/2 prior to infection increased IL-6 secretion even further. IL-10 levels were decreased significantly by CXCR2 blockage, but not by CXCR1 antibodies. Data are presented as mean ± SEM of 3 separate experiments. *** $p < 0.001$.

ERK1/2 and cFos instead [40]. We could confirm that BCG did not increase the activation of NF-κB or AP-1 (c-Jun). Interestingly, blocking the epithelial GPCRs CXCR1 and CXCR2 prior to mycobacterial infection increased the NF-κB and c-Jun levels compared to uninfected cells (Fig. 5).

Mycobacteria Control Epithelial Cytokine Secretion

Mycobacteria infection has been suggested to evoke the pro-inflammatory immune response in order to evade host immune responses. IL-6 bridges innate and adaptive host immune responses, while the anti-inflammatory IL-10 suppresses inflammation and postpones the generation of adaptive immunity. Mycobacterial engagement of CXCR1 and CXCR2 to control cellular transcriptional factors was analysed as epithelial cytokine secretion. Infection of AECs induced significant IL-6 and IL-10 secretion (Fig. 6). Blockage of CXCR1 or CXCR2 prior to infection increased IL-6 secretion even further ($p = 0.0001$; $p = 0.0004$). However, IL-10 levels were significantly decreased by CXCR2 blockage compared to BCG-infected cells ($p = 0.0001$), while CXCR1 antibodies did not affect BCG-stimulated epithelial IL-10 production (Fig. 6). The treatment of uninfected cells with CXCR1 or CXCR2 antibodies did not affect IL-10 and IL-6 secretion (data not shown).

Discussion

Reprogramming of AECs could be beneficial for the development of vaccines and treatment strategies [4–6]. Evidence is now accumulating that innate immunity can “remember” previous exposure to a microorganism and could contribute to host defence against infection and vaccine-induced immunity. We found that BCG infection of mucosal AECs induced epithelial signalling pathways leading to actin remodelling and selective cytokine secretion. Actin cytoskeletal changes regulate junctional integrity that leads to disturbed barrier function during inflammation [35]. Mtb invasion of AECs is both receptor- and actin-mediated [41]. BCG is also known to invade AECs [18]; in this study, we show that the infection resulted in Rac1 up-regulation followed by altered epithelial actin distribution. Actin distribution by BCG infection requires p38 that is activated by phosphorylation at the T180/Y182 sites. Recently, macropinocytosis, in contrast to phagocytosis, was shown to lead to rapid elimination of mycobacteria, suggesting that the cytoskeletal mobilization could be involved in the initiation of the adaptive immune response [42]. A previous study showed that Mtb infection increased the oxidative stress in AECs through the p38-ERK-NF- κ B axis and led to the cytotoxicity of human lung epithelial cells [43, 44]. Mtb could thus facilitate its own dissemination by compromising the epithelial lining. Contrary to this finding, our results demonstrated that BCG stimulated the MAPK

pathway by signalling through the PYK2-p38-MAPK2-JNK axis, pointing to a sustained epithelial barrier after BCG infection.

A wide variety of cellular processes are controlled by a complicated regulatory network consisting of positive and negative regulators. Additionally, post-translational modifications often affect points of regulation in a protein, which allows switching activities [45]. S15/S20 phosphorylation at p53 promotes the recruitment of transcriptional co-activators [46], while the phosphorylation of S46 is critical for p53-mediated induction of pro-apoptotic genes [47, 48]. Phosphorylation of C-terminal S392 in response to ultraviolet light activates specific DNA binding through the stabilization of the p53 tetramer [49]. S392 phosphorylation was recently shown to promote cell survival by stabilizing p53 and enhancing its transcriptional activity [50]. Interestingly, inhibition of Rac1 resulted in decreased p53 S392 and S46 phosphorylation, possibly affecting transcription and cell survival in BCG-infected AECs. Our results indicate that BCG infection of AECs stimulate cell survival, which was also confirmed by the negative result of the cell death assay.

Bacterial adherence to cells is also known to trigger signal transduction events involving the G-proteins in actin polymerization, with the subsequent uptake of the bacteria [21, 37, 51]. GPCRs, such as CXCR1 and CXCR2, are important regulators in pulmonary diseases [52]. Previous research revealed that mycobacteria decrease epithelial cytokine production by manipulating these receptors [18], and active TB patients were found to have increased CXCR1 expression [53]. CXCR2 is important in the pathology of a wide diversity of chronic pulmonary diseases, and the modulation of CXCR2 function is considered as a possible therapeutic strategy [54]. Interestingly, blocking of these GPCRs prior to mycobacterial infection repealed the mycobacteria-induced actin redistribution and suppression of epithelial NF- κ B and c-Jun, further supporting the theory that mycobacteria utilize GPCRs to manipulate cellular signalling [23]. GPCRs are also required for the activation of STAT, and *STAT1* mutations were recently identified in patients with disseminated BCG infection [55, 56]. The effect of STAT is to increase the transcriptional activity of quiescent genes and the transcription of less active genes [56]. BCG induced the phosphorylation of the transcription supporting STAT1 and STAT5b, while the STAT6 levels were reduced. Blocking CXCR1/R2 increased epithelial NF- κ B and c-Jun activation, and the secretion of IL-6

was increased. IL-6 is a multi-potent cytokine that acts in both pro- and anti-inflammatory ways. Exerting its pro-inflammatory qualities, IL-6 suppresses the development of regulatory T cells and favours the differentiation of effector T helper 17 cells during inflammation or infection [57]. Locally produced IL-6 could thus increase CD4+ T cell memory formation. Blocking CXCR2, but not CXCR1, decreased mycobacteria-induced IL-10 secretion. This cytokine modulates the anti-inflammatory mechanisms by targeting NF- κ B [58, 59], which could have been the mechanism of suppressed transcriptional activity that we observed in our study. Impaired cellular activation and recruitment was previously related to increased IL-10 production and decreased CXCR2 expression during septic conditions [60, 61]. In our study, blocking of epithelial CXCR2 reduced BCG-induced epithelial IL-10 secretion, possibly suggesting that CXCR2 signalling is important for BCG-induced IL-10 production.

It has been proposed that AECs and innate immune cells drive respiratory diseases [62]. AECs express MHC-I/II [63], and the macropinocytosis of mycobacteria by AECs was recently shown to lead to rapid bacterial elimination [42]. The cytoskeletal mobilization due to macropinocytosis could thus be involved in antigen capture and presentation for incoming T cells [42]. More knowl-

edge is needed on how mycobacteria manipulate cellular kinases and phosphatases, as these molecules constitute potential targets for future TB therapeutics [64]. We showed that the early event of mycobacterial AEC infection led to sustained manipulation of epithelial Rac1 and actin remodelling. Furthermore, our finding that mycobacteria utilized CXCR1 and CXCR2 to manipulate the inflammatory response clarifies the role of GPCRs in pulmonary disease, and possibly towards chronic disease [65]. Future vaccine strategies could thus be improved by targeting GPCRs.

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Disclosure Statement

The authors have declared that no conflict of interests exists.

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