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Epithelial G protein-coupled receptor kinases regulate the initial inflammatory response during mycobacterial infection

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ABSTRACT

The interaction between mycobacteria and epithelium is unexplored, but may determine the outcome of the infection. We have analyzed the role of two G protein-coupled receptors, CXCR1 and CXCR2 that are important regulators of many pulmonary diseases. We found that mycobacteria significantly increased the expression of both CXCR1 and CXCR2 on alveolar epithelial cells and both receptors were found to be important for neutrophil diapedesis across primary endothelial cells towards infected mucosa. Mycobacteria, lipoarabinomannan or 19-kDa glycolipoprotein up-regulated the inhibitory G protein-coupled receptor kinase (GRK)2, while GRK3 was less affected. Mycobacteria-induced GRK2 up-regulation decreased chemokine transcription and secretion thereby affecting the neutrophil recruitment to infected mucosa. These events were completely abolished by blocking these receptors prior to infection as the blocking increased epithelial immune responses. We have identified novel interactions occurring in the initial phase of mycobacterial infections by which mycobacterial manipulate epithelial inflammatory responses.

Introduction

The balance between bacterial proliferation mechanisms and the host immune response to infection may dictate the outcome and progression of mycobacterial disease. Inhaled bacteria interact with epithelial cells, the major constituent of the alveolar lining, but not much is known about the initial contacts. Immunocompetent humans exposed to *Mycobacterium tuberculosis* (*M. tuberculosis*) mount an early innate and late adaptive response that ultimately destroys the bacteria and usually prevents the development of clinical disease (Schluger and Rom 1998; van Crevel et al. 2002). If not cleared, the infection can either progress to an active disease, but is more often contained by the host adaptive immune response (van Crevel et al. 2002). Epithelial invasion of *M. tuberculosis* activates the cells to produce cytokines in order to attract neutrophils to the site of infection (Lin et al. 1998; Wickremasinghe et al. 1999; Mendez-Samperio et al. 2007). The first cell-type to arrive to infected tissue is the neutrophil and these cells are found in great amount in bronchoalveolar lavage fluid of patients with active pulmonary tuberculosis (Werneck-Barroso et al. 2000; Ribeiro-Rodrigues et al. 2002; Andersson et al. 2012). The chemokine CXCL8 is a strong neutrophil chemoattractant, but is also chemotactic for monocytes and lymphocytes (Zhang et al. 1995; Gerszten et al. 1999). CXCL8 enhances killing of mycobacteria in macrophages and neutrophils (O'Kane et al. 2007; Nibbering et al. 1993) and augmented CXCL8 levels are found in plasma and in bronchoalveolar lavage fluids from patients infected with tuberculosis (Zhang et al. 1995; Meddows-Taylor et al. 1999; Pokkali and Das 2009).

G protein-coupled receptors (GPCRs) are important regulators of pulmonary diseases through their binding to a variety of mediators, such as histamine, leukotrienes, prostanoids and chemokines (Barnes et al. 1998). CXCL8 binds to two seven trans-membrane GPCRs, CXCR1 and CXCR2, which are expressed on a variety of cell types and tissues (Baggiolini 2001; Godaly et al. 2000; Schraufstatter et al. 2001). Of these two, the

Abbreviations: GPCR, G protein-coupled receptors; GRK, G protein-coupled receptor kinase; LAM, lipoarabinomannan; PI-3K, phosphoinositide 3-kinases; ERK, extracellular signal-regulated kinase; Akt, serine/threonine protein kinase; COPD, chronic obstructive pulmonary disease; ARDS, acute respiratory distress syndrome; TLR, Toll like receptor; RKIP, Raf kinase inhibitor protein; MEK, mitogen-activated protein kinase kinase; PBE, primary bronchial epithelial; HUVEC, Primary Human Umbilical Vein Endothelial Cells; BCG, *Mycobacterium bovis* bacilli Calmette-Guerin.

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CXCR2 has been identified in the pathology of a wide diversity of pulmonary diseases such as chronic obstructive pulmonary disease (COPD), asthma, acute respiratory distress syndrome (ARDS) and pulmonary fibrosis (Chapman et al. 2009). GPCR function and signalling is regulated by G protein-coupled receptor kinases (GRKs) that are induced upon GPCR activation (Lefkowitz 1998; Kohout and Lefkowitz 2003). GRK binding of GPCR phosphorylates the serine-threonine residues, leading to receptor desensitization and internalization (Lefkowitz 1998). Additionally, GRKs modulate signalling in a phosphorylation-independent manner by interacting with proteins involved in signalling, including the phosphoinositide 3-kinases (PI-3Ks), extracellular signal-regulated kinase (ERK) and serine/threonine protein kinase Akt (Naga Prasad et al. 2002; Day et al. 2003; Premont et al. 1998; Sallese et al. 2000; Jimenez-Sainz et al. 2006; Liu et al. 2005). GRK2 is the best characterized of the GRKs and has been shown to phosphorylate and desensitize the agonist-bound form of many GPCRs (Aragay et al. 1998).

Mycobacterium bovis bacilli Calmette-Guerin (BCG) mimics *M. tuberculosis* in its ability to persist in the body and is similar to the tubercle bacillus in the physiological, molecular, and metabolic responses (Fritz et al. 2002; Hutter and Dick 2000; Lim et al. 1999; Murugasu-Oei and Dick 2000). The mycobacterial adhesion heparin-binding haemagglutinin (Menozzi et al. 2006) is believed to be involved in invasion of human alveolar epithelial cells (Pethe et al. 2001). Furthermore, as the genome of *M. tuberculosis*, BCG is equipped with several genes coding for invasin/adhesin-like proteins (Cole et al. 1998; Garnier et al. 2003; Ahmad et al. 2005; Gao et al. 2006; Brosch et al. 2007; Florio et al. 2009). Several groups have previously shown that mycobacteria invades, survives, manipulates and replicates within human alveolar epithelial cells (Lin et al. 1998; Mendez-Samperio et al. 2007; Bermudez and Goodman 1996; Bermudez et al. 2002; Mehta et al. 1996; Garcia-Perez et al. 2008; Mendez-Samperio et al. 2008a; Mendez-Samperio et al. 2009; Mendez-Samperio et al. 2008b), but not much is known about the initial epithelial contacts with mycobacteria. Until now, Toll like receptor (TLR) 2, TLR4 and C-type lectins have been implicated in mycobacteria-induced epithelial signalling and uptake (Mendez-Samperio et al. 2008a,b; Torrelles et al. 2008). In the present study we characterized epithelial GPCRs CXCR1 and CXCR2 expression and signalling in the active phase of mycobacterial infection. Taken together, our data indicate that mycobacteria manipulate alveolar epithelial inflammatory responses by GRK2 dependent GPCRs desensitization.

Materials and methods

Bacterial strains and growth conditions

Mycobacterium bovis bacilli Calmette-Guerin (BCG) Montreal strain containing the pSMT1 shuttle plasmid was prepared as previously described (Snewin et al. 1999). The reporter-plasmid pSMT1 contains the *Vibrio harveyi luxA* and *luxB* genes to provide a rapid and simple alternative to the counting of CFU as a means of monitoring mycobacterial viability (Snewin et al. 1999). Briefly, the mycobacteria were grown in Middlebrook 7H9 culture medium, supplemented with 10% ADC (Becton Dickinson, Oxford, UK) and hygromycin (50 mg/ml; Roche, Lewes, UK), the culture was 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 3000 × *g*, washed two times with sterile PBS, and re-suspended in sterile PBS.

Cell culture

A549 alveolar epithelial cells (ATCC no. HTB-44) were cultured in Roswell's Park Memorial Institute medium (RPMI) 1640 (Invitrogen, Stockholm, Sweden) supplemented with 0.05 mg/ml gentamicin (PAA Laboratories, Austria) and 5% Foetal Bovine Serum (FBS; PAA Laboratories, Austria), with change of medium every third day. The cells were detached by Versene-treatment (140 mM NaCl, 2.4 mM KCl, 8 mM Na_2HPO_4, 1.6 mM KH_2PO_4, 0.5 mM EDTA, and pH 7.2).

Primary bronchial epithelial (PBE) cells were used as control to the A549 epithelial cell line throughout the study. Bronchial material for primary cell cultures was obtained from lung explant from healthy donors with no history of lung disease. Bronchial tissue was dissected from lungs and kept in DMEM supplemented with gentamicin, PEST, Fungizone and 10% FBS (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) prepared in S-MEM supplemented with gentamicin, PEST and Fungizone for 24 h. Bronchial epithelial cells (HBEC) were recovered by repeated intraluminar rinsing with DMEM supplemented with gentamicin, PEST, Fungizone and 10% FBS. 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) in BEGM cell culture medium (Clonetics). The following day cells were thoroughly washed with a medium change every other day. Experiments were performed in passage 3. This study was approved by the Swedish Research Ethical Committee in Lund (FEK 91/2006).

Primary Human Umbilical Vein Endothelial Cells (HUVEC) (Clonetics, Lonza, Germany) were obtained from Lonza and cultured in endothelial cell growth medium EGM-2 (Clonetics, Lonza, Germany) supplemented with the EGM-2 bulletkit (hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% heparin, 2% FBS, 0.1% hEGFMl and 0.1% GA-1000) (Clonetics, Lonza, Germany) (37 °C, 5% CO_2). The cells were used at passages 2–5, according to manufacturers' instructions.

Human venous blood neutrophils were isolated from healthy volunteers by the Polymorphprep density gradient (Axis-Shield, Oslo, Norway) according to manufacturers instructions as described previously (Godaly et al. 1997).

Incubation of cells with mycobacteria

For the infection experiments, epithelial A549 or PBE cells were grown in 6-well plates (2.0×10^5 cells/well; Fisher Scientific, UK), infected with BCG (one bacterium per cell; 1 MOI) or phenol purified LPS (1 ng/ml; Sigma–Aldrich), lipoarabinomannan (LAM, 1 μg/ml, Sigma–Aldrich) or 19-kDa glycolipoprotein (1 μg/ml, Sigma–Aldrich) at 37 °C for up to three days. BCG infected cells and medium alone were used as controls. Samples were taken before infection and after 1, 2, 3, 5, 6, 24, 48 and 72 h for cytokine measurements.

For the blocking experiments, epithelial cells were treated with monoclonal mouse anti-human CXCR1 and/or monoclonal mouse anti-human CXCR2 antibodies (R&D Systems) 10 μg/ml for 30 min at 37 °C before the addition of BCG. The cells were then incubated with BCG at 37 °C for three days.

To investigate whether mycobacteria enters and survives within alveolar epithelial cells, alveolar epithelial cells were infected as described above and stained with 0.01% acridine orange in PBS for 45 s (Miliotis 1991). After washing, A549 cells were incubated with 0.01% crystal violet in PBS for 45 s to quench extracellular fluorescence, washed and intracellular bacteria was visualized using a Nikon Microphot (LRI Instrument AB), FX epifluorescence microscope equipped with a Olympus UC30 digital colour camera. Cell

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