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CR3-dependent negative regulation of human eosinophils by *Mycobacterium bovis* BCG lipoarabinomannan

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ABSTRACT

Eosinophils have recently been shown to participate in innate immune responses against mycobacteria. We have investigated whether *Mycobacterium bovis* BCG regulate the human eosinophil immune response. A negative correlation between mycobacteria internalization and eosinophil activation was observed. In addition, mannose-capped lipoarabinomannan from *M. bovis* BCG (ManLAM) failed to induce a significant release of eosinophil peroxidase and TNF-α. Noteworthy, ManLAM exhibited a potent inhibitory effect on eosinophil peroxidase release by TLR2-activated eosinophils involving the complement receptor-3 molecule and the phosphatidylinositol-3 kinase pathway. ManLAM, generally present in pathogenic mycobacteria, plays an important role in modulating eosinophil-dependent immune response.

1. Introduction

The mycobacterial cell wall comprises a vast array of proteins, lipids and lipoglycans, including lipoarabinomannan (LAM) and its related biosynthetic precursor lipomannan (LM), known to modulate the host immune system [1,2]. Both *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG express mannose-capped LAM (ManLAM), inhibiting IL-12 production in LPS-stimulated human mononuclear phagocytes and in dendritic cells (DCs) through binding to mannose receptor (MR) [3]. LAM from *M. tuberculosis* also recognizes dendritic cell-specific ICAM 3-grabbing non-integrin (DC-SIGN) molecule [4] and prevents mycobacteria- or LPS-induced DC maturation [5]. Moreover, other immune receptors, such as complement receptors (CR), are also involved in the interactions with mycobacteria [6]. Taken together, these studies illustrate close relationships between innate receptors and cell wall-associated lipoglycans in regulating innate immune responses.

Most studies have focused on macrophages and DCs to study receptor/ligand interactions influencing host resistance or susceptibility to mycobacterial infection. However, little is known regarding the eosinophil surface receptors potentially involved in eosinophil-mycobacteria interactions, despite the fact that eosinophil recruitment within mycobacterial granuloma has been observed in different animal models [7]. We have recently provided evidence for a direct TLR2-dependent interaction of *M. bovis* BCG with human eosinophils [8]. Since eosinophils have the potential to express CR, especially CR3 (CD11b/CD18) [9], this study aims to investigate whether *M. bovis* BCG can interact with eosinophil CR and may influence the subsequent CR-mediated activation process.

2. Materials and methods

2.1. Eosinophil and lipoglycan purification

Eosinophils were isolated from peripheral blood of donors following informed consent. Eosinophils were separated from

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peripheral-blood mononuclear cells (PBMCs) by Percoll centrifugation as previously described [10]. Eosinophil purity checked by cytocentrifugated preparations after RAL555 coloration was found to be >98%.

Lipomannan and lipoarabinomannan from *M. bovis* BCG were purified as previously described [11].

2.2. Mycobacterial cultures

M. bovis BCG Pasteur strain was obtained from Dr. C. Locht (Inserm U1019, Pasteur Institute, Lille, France) and maintained at 37 °C in Sauton's liquid medium.

2.3. Eosinophil degranulation

Eosinophils (2×10^5 cells in 100 μL) were incubated for 2 h in RPMI 1640 without phenol red, with stimuli at 37 °C in 5% CO_2. EPO activity in eosinophil supernatants was measured by oxidation of H_2O_2 by luminol as previously described [10]. Chemiluminescence was measured with a luminometer (Victor™ Wallac).

For inhibition experiments, eosinophils were preincubated with blocking anti-CR3 monoclonal antibody (mAb) (Bear-1; Santa Cruz, California, USA), blocking anti-CR4 mAb (BU15; Santa Cruz Biotechnology, California, USA), total anti-mouse (mIgG) isotype control (Jackson ImmunoResearch Laboratories, West Grove, PA), PI3K inhibitor (LY294002; Promega, Wisconsin, USA) for 30 min at 37 °C before addition of the stimulus without washing. Cell viability was tested using the trypan blue test after treatment.

2.4. Cytokine stimulation assay

After 18 h of eosinophil stimulation, the levels of TNF-α in the supernatants were determined by performing an ELISA (Diaclone) according to the manufacturer's recommendations.

2.5. Internalization assays

2.5.1. Carboxyfluorescein diacetate-succinimidyl ester (CFSE)-labeled eosinophils

Cells were resuspended at a concentration of 4×10^6 cells/mL in PBS. CFSE (Invitrogen, California) was added to a final concentration of 20 μM and incubated at room temperature for 10 min in the darkness. The cells were washed three times with DPBS.

2.5.2. PKH26-labeled mycobacteria

Mycobacteria were washed and resuspended at a concentration of 10^8 bacteria/mL in diluent C. Mycobacteria were incubated with 20 μM PKH26 (Sigma–Aldrich) for 15 min at room temperature. Reaction was blocked by decomplemented FCS for 1 min. The mycobacteria were washed three times with DPBS.

On the day of each experiment, CFSE-labeled eosinophils were incubated with PKH26-labeled mycobacteria at different mycobacteria:eosinophils ratios (5:1, 10:1, 20:1) in RPMI 1640 without phenol red and without serum at 37 °C in 5% CO_2. After selected incubation times, eosinophils were washed in cold RPMI to stop internalization and remove non-adherent mycobacteria. Trypan blue (5 mg/mL) was added on slides to quench adherent mycobacteria and those remaining in the extracellular medium [12]. The trypan blue-treated cells were washed three times with DPBS before fixation in 1% paraformaldehyde in PBS, pH 7.4, 30 min at room temperature and stored at 4 °C in the dark until analyze. Cells containing fluorescent mycobacteria were counted by alternately viewing them by phase contrast and fluorescence microscopy. For each condition, at least 100 cells were counted. Inhibitory experiments were performed as described above (eosinophils were preincubated with blocking anti-CR3 monoclonal antibody mAb or PI3K inhibitor before mycobacteria infection).

2.6. Flow cytometry analysis

Human eosinophils (2×10^6/mL) were stimulated for 2 h in RPMI 1640 with or without stimulus as indicated in figure legends. 2×10^5 cells were incubated in PBS containing 1% BSA with detection antibody or isotype control at 4 °C for 40 min. Cell surface staining was performed on cells with PE-anti-CD69 (BD Pharmingen), or the matched isotype control PE-mIgG1 (BD Pharmingen). The cells were then washed and were immediately analyzed on a FACSCalibur™.

2.7. Statistical analysis

All data were expressed as mean ± S.E.M. All statistical analyses were performed using SPSS software. Normality of data samples was assessed with the Normality test of Shapiro and Wilk. Parametric Student's *t*-test for paired experiments was employed to compare two variables. ANOVA followed by Dunnett's post-test were employed for comparisons of more than two data sets. A *P* value of less than 0.05 was used to indicate statistical significance. $^*P < 0.05$ and $^{**}P < 0.01$ are presented on the figures.

3. Results

3.1. Human eosinophils internalize M. bovis BCG

Eosinophils are characterized by several functional properties, including bacterial internalization [13]. First, the ability of eosinophils to ingest live *M. bovis* BCG was studied. Purified human eosinophils were incubated for different times (0, 5, 10, 20 and 40 min) in the presence of mycobacterial suspensions at a multiplicity of infection (MOI) of 10:1 (Fig. 1A). In order to discriminate between extracellular adherent *M. bovis* BCG and those that had been internalized, we used the trypan blue quenching technique (see material and methods). Adjusting the focus at different depths, we show that Human eosinophils contained one to four red fluorescent mycobacteria, which appeared clearly intracellular, by fluorescence microscopy (Fig. 1B). Interestingly, these data demonstrate the efficient internalization of *M. bovis* BCG by human eosinophils.

3.2. CR3 modulates eosinophil activation in response to M. bovis BCG

Macrophage phagocytosis of *M. tuberculosis* can be mediated by CR3 and CR4 [14]. Similarly, in this study, we investigated if *M. bovis* BCG internalization could be mediated by CR3 and CR4. We showed that *M. bovis* BCG internalization by eosinophils was significantly inhibited by the anti-CR3 antibody (73.3 ± 3.4% inhibition). No effect was observed with either the anti-CR4 antibody or the isotype control antibody (Fig. 2A). As we could not exclude the possibility that eosinophil activation might also be affected by the anti-CR3 antibody, we studied cytotoxic specific granule proteins, such as EPO or cytokine release, such as TNFα (Fig. 2B and C). We demonstrated EPO and TNFα releases by eosinophils in the presence of *M. bovis* BCG at a ratio 10:1. There was also a significant increase of EPO (by 33.3 ± 10.4%) and TNFα (51.13 ± 6.5%) when cells were pre-treated with the blocking anti-CR3 antibody. No increase was observed when the anti-CR4 or the isotype control was added.

To further understand the involvement of bacteria internalization in eosinophil activation, eosinophils were pre-treated with 5 μg/mL cytochalasin D (Fig. 2D). Indeed, cytochalasin D blocked