TLR2-dependent eosinophil interactions with mycobacteria: role of α-defensins

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Abstract

Peripheral blood and tissue eosinophilia are a prominent feature in allergic diseases and during helminth infections. Eosinophil recruitment also frequently occurs upon mycobacterial infections, particularly in lung granuloma. However, the mechanism by which eosinophils interact with mycobacteria remains largely unknown. Since eosinophils have been recently involved in innate immune responses, we have investigated the direct interactions of eosinophils with *Mycobacterium bovis* BCG, as a study model. In the present work, we show that live BCG attracts human eosinophils and induces reactive oxygen species (ROS) synthesis, granule protein release and TNF-α secretion. Using anti-TLR2 neutralizing antibodies before eosinophil exposure of eosinophils to BCG, we showed a critical role of TLR2 signaling in ROS and eosinophil peroxidase release. BCG-induced eosinophil activation is mediated through the p38 MAP Kinase and NF-κB pathways. Additionally, a mycobacterial wall component, lipomannan, induced a TLR2-dependent eosinophil activation. In addition, we showed that eosinophils express and produce α-defensins upon stimulation with BCG and lipomannan and that α-defensins could inhibit mycobacterial growth in synergy with eosinophil cationic protein. These results suggest a role for human eosinophils as direct effectors in TLR2-mediated innate immunity against mycobacteria and confer to these cells potent cytotoxic functions through defensin and eosinophil cationic protein production.
Introduction

Tuberculosis is the most prevalent infectious disease worldwide with 3 million deaths annually. Control of Mycobacterium tuberculosis bacillus (MTB) infection requires the development of a Th1-type CD4 T cell response and activation of alveolar macrophages leading to the formation of lung granuloma. Initial recognition of mycobacteria by the innate immune system through pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) contributes to triggering this adaptive immune response. Indeed, cooperation between TLR2, TLR4 and TLR9 may contribute to resistance against mycobacteria and several studies have shown that mycobacterial components acted as TLR agonists. Indeed, lipomannans (LM) and lipoarabinomannans (LAM), lipoglycans ubiquitously found in the wall of mycobacteria, are involved in the release of proinflammatory or anti-inflammatory cytokines.

Whereas mycobacterial infections are rather associated to Th1 responses, the occurrence of Th2 cytokine responses has been also reported in human tuberculosis. It is suggested that these Th2 responses might depress macrophage immunity and lead to increased susceptibility to tuberculosis. Th2-driven immunity is associated with cell types seldomly studied during mycobacterial infections. Among them, eosinophils, which are now considered as multifunctional leukocytes, are involved in inflammatory processes as well as in modulation of innate and adaptive immunity. Indeed, besides the well known involvement of eosinophils in helminth infections, there are only few evidences for their role in defenses against other pathogens such as virus, fungi or bacteria. Eosinophilia has been associated with pulmonary tuberculosis in HIV-1 patients and in patients with concomitant intestinal helminth infections. Moreover, tissue eosinophilia has been also observed following the Bacillus Calmette-Guérin (BCG) vaccination at the site of tuberculin injection. Eosinophil recruitment within mycobacterial granuloma has been found in different animal models.
Eosinophils are potent effector cells through the release of several cytotoxic mediators upon activation. Cytoplasmic specific granule contain high amounts of cytotoxic proteins in particular cationic proteins [major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN)]\textsuperscript{19}. Cytotoxic potential of eosinophils also arises from their ability to mount a respiratory burst including superoxide and H\textsubscript{2}O\textsubscript{2} production, which in turn initiates production of more potent oxidants\textsuperscript{20,21}. Beside cytotoxic mediators, eosinophils not only express and secrete Th2 cytokines such as IL-4, IL-5 and IL-10 but also Th1 and pro-inflammatory cytokines such as interferon (IFN)-\(\gamma\)\textsuperscript{22} and TNF-\(\alpha\), which are typically released following MTB infection.

Although eosinophils accumulate at sites of mycobacterial infection, their activation and their involvement in the regulation of bacterial growth have not been investigated. In the present study, we used, the BCG strain derived from \textit{Mycobacterium bovis} as a model to delineate the potential interactions between eosinophils and mycobacteria.
Materials and Methods

Eosinophil and neutrophil purification

In accordance with French law for studies involving human biological samples, blood from healthy and eosinophilic donors was collected after informed consent from volunteers, in accordance with the Declaration of Helsinki, and approval from the Comité Consultatif des Personnes dans la Recherche Biomédicale de Lille (CCPRB) (Consultative Committee for Persons in Biomedical Research from Lille). Eosinophils were isolated from peripheral blood of healthy, allergic and hypereosinophilic donors. Eosinophils were separated from peripheral-blood mononuclear cells (PBMCs) by Percoll (Amersham GE Healthcare) centrifugation then purified by magnetic selection using immunomagnetic anti-CD16 (Miltenyi Biotec). Eosinophil purity was checked by cytocentrifugated preparations after RAL555 coloration and was found to be $> 98\%$. Neutrophils were collected in the positive fraction (purity was $> 99\%$).

*Mycobacterium bovis* BCG

*Mycobacterium* bovis BCG Pasteur strain was obtained from Dr C. Locht (Inserm U629, Institut Pasteur de Lille, France) and maintained at $37^\circ$C in Sauton’s liquid medium. Viable mycobacteria *M. bovis* BCG in logarithmic growth phase were used at concentrations indicated in figure legends. One absorbance unit at 600 nm for the BCG culture was calculated as $2 \times 10^7$ colony-forming units (CFUs). Heat-killing was carried out by culturing the BCG at $70^\circ$C for 1 hour.

Lipomannan and lipoarabinomannan from *M. bovis* BCG were purified, as previously described. Endotoxin contamination of each preparation was determined by the
chromogenic Limulus lysate assay (QCL1000; Cambrex) and were below 1 pg/mL in all experiments.

**Migration assays**

BCG ability to induce cell migration was measured by Transwell™ assay. Eosinophils were first labeled with 10 µM CFSE (carboxyfluorescein diacetate succimidyl ester, Molecular Probe) and suspended at 3 x 10^6 cells/ml in RPMI 1640 (Invitrogen) with 1% FBS. BCG, LAM or LM were placed in various concentrations in the lower wells and separated from eosinophils by a polycarbonate filter (pore size of 5 µm) (Corning). Migration toward IL-5 (10 ng/ml) (Peprotech) was used as positive control. Migration was carried out for 3h at 37°C in a humidified 5% CO2 incubator. The number of migrated eosinophils was determined on a FACSCalibur™ flow cytometer with the cellquest software (Becton Dickinson).

**Release of Reactive Oxygen Species and eosinophil degranulation**

A luminol-dependent chemiluminescence system was used to determine eosinophilic production of Reactive Oxygen Species (ROS). Briefly, 3 x 10^5 eosinophils resuspended in RPMI 1640 medium without phenol red (Invitrogen) were activated with stimulus such as BCG, Pam3CSK4 or ultrapure LPS from E. coli 0111:B4 (Invivogen). Luminol (25µg/ml in Tris HCl 0.01M pH7.4) (Sigma Aldrich) was added and chemiluminescence was immediately measured with a luminometer (Victor™ Wallac, Perkin Elmer). Kinetic measurement was performed at 37°C during 2h and chemiluminescence was counted for 5 seconds. To detect EPO, eosinophils (2 x 10^5 cells in 100µl) were incubated for 2 hours, in RPMI 1640 without phenol red, with stimuli at 37°C in 5% CO2. EPO activity in eosinophil supernatants (50 µl) was measured by oxidation of H2O2 by luminol (200 µg/ml in Tris HCl...
0.01 M pH 6). The reaction was amplified by the addition of D-luciferin (160 µM in Tris HCl 0.01 M pH 6) (Sigma-Aldrich) before measuring chemiluminescence.

For inhibition experiments, eosinophils were preincubated with blocking anti-TLR2 mAb (TL2.1) (Alexis, Switzerland); blocking anti-TLR4 mAb (HTA125) (eBioscience); total mIgG isotype control (Jackson ImmunoResearch Laboratories); MyD88 homodimerization inhibitory peptide (DRQIKIWFQNRRMKWKKRDVLPGT) or peptide control (DRQIKIWFQNRRMKWKK) (Imgenex); SP 600125, c-Jun N-terminal kinase (JNK) inhibitor (Alexis); PD 98059, ERK kinase inhibitor (Alexis), SB 203580, p38 MAP kinase (Invivogen) or BAY 11-7082, NF-kappaB inhibitor (Calbiochem) for 30 min at 37°C, before the addition of stimulus.

**ECP, TNF-α and α-defensin release**

Eosinophils (2x10⁶ cells/ml) were activated with the same stimuli as for ROS production. After 18h culture, supernatants were collected.

ECP, TNFα and α-defensin were measured by specific ELISA kits from MBL International Corp, Diaclone and HyCult Biotechnology, respectively. The lower detection limit was 0.125 ng/ml for ECP, 8 pg/ml for TNFα and 50 pg/ml for human α-defensins.

**Flow cytometry analysis**

Human eosinophils (2x10⁶/ml) were stimulated for 2 hours in RPMI 1640 with or without BCG as indicated in figure legends. Cell surface staining was done with PE-TLR2 (TL2.1), PE-TLR4 (HTA125) mAb (eBioscience), or the matched isotype control mPE-IgG₂a (Immunotech). For intracellular staining, purified eosinophils or neutrophils were first fixed with 2 % paraformaldehyde and then permeabilized with 0.01 % saponin in PBS. Non-specific binding was blocked with mouse serum for 10 min, and the cells were incubated with MAb; 0.01 M pH 6) (Sigma-Aldrich) before measuring chemiluminescence.
a mouse anti-HNP1-3 (D21) (Hycult biotechnology) or IgG1 isotype control (Diaclone) for 30 min in 0.01% saponin buffer. After washing, cells were incubated with a goat FITC-anti-mouse IgG1 secondary antibody (Southern Biotech) for 20 min. Cells were then immediately analysed on a FACSCalibur™.

**Immunofluorescence labeling**

Eosinophil cytocentrifugated preparations were performed and the cells were fixed in 4% paraformaldehyde, and rehydrated in 0.05 M Phosphate-buffered saline (PBS) pH 7.4. Cytospins were washed 3 times in PBS-0.1%BSA. Eosinophils were permeabilized in PBS-1% BSA-0.2% Triton X-100 for 5 min on ice. Endogenous fluorescence was blocked by 50mM NH₄Cl pH 7.4 for 15 min. Cells were then incubated with PBS-3% BSA for 30 min to prevent non-specific binding. Cytospins were then incubated overnight at 4°C with mouse anti-HNP1-3 (D21) (Hycult biotechnology) or a mouse IgG1 isotype control (Diaclone) in PBS-3% BSA-5%HSA. Cytospins were blocked using goat serum (30 minutes) and Alexa Fluor® 555 goat anti-mouse secondary antibodies (Invitrogen) was added in PBS-3% BSA-5% HSA for 2 hours. Slides were then incubated with biotinylated mouse anti-EPO or anti-MBP (BD Pharmingen) for 2 hours. Immunoreactivity against EPO or MBP was detected by staining slides with Alexa Fluor® 488 streptavidin (Invitrogen) for 2 hours. Cytocentrifugated preparations were mounted with Fluoromount G (Southern Biotechnology) and were examined using a Zeiss AxioImager Z1 microscope using AxioVision software (ZEISS). Fast iterative algorithm was used for deconvolution. Image editing and overlays were performed on Adobe Photoshop v. 7.0.

For confocal microscopy image were acquired using a DM-IRE2 inverted microscope with SP2-AOBS scan-head (Leica) at the Imaging Facility of Institut Pasteur de Lille.
Acquisitions were performed using a 100x 1.4 NA oil immersion objective. 3D pre-treatment, analysis and edition were performed with Leica Confocal Software.

RNA isolation and RT-PCR amplification

Total RNA was isolated using the RNeasy mini kit (Qiagen) from $10^7$ purified cells following the protocol recommended by the manufacturer. Reverse transcription was performed using SuperScript™-Reverse Transcriptase (200U/μl) as described in the manufacturer protocol (Invitrogen). cDNA were then amplified using specific primers (Proligo) (20 pmoles/μl) : MyD88 : (5’-GACTTTGAGTACTTGGAGATCCGG-3’ and 5’-GATGGTGGTGTTGTTGCTCTGATGA-3’), annealing temperature 56°C ; TIRAP (5’-CTGGCTCTCGGCGCTAAAGAA-3’ and 5’-CATCGCTGGAGGTGCTTTGCTTC-3’), annealing temperature 60°C ; TRAF6 (5’-GGTCCGGAATTTCCAGGAAA-3’ and 5’-CATTTTAGCAGTCGCTCCCG-3’), annealing temperature 56°C ; α-defensins (5’-CAGTGAGTGATGAGTCTGGAAACCGAAG-3’ and 5’-CATTCTGCCGCTGGAGGTGCTTTGCTTC-3’), annealing temperature 64°C. Polymerase Chain Reaction were run for 40 cycles (1 min at 95°C, 1 min annealing and 1 min at 72°C) using Taq polymerase (Bioprobe).

Western blot

Cell lysates were prepared from freshly isolated eosinophils or neutrophils (10 x 10^6 cells) in lysis buffer (20 mM Tris-HCl, pH 8.0 ; 120 mM NaCl ; 1 % Triton X-100 ; 10 mM EDTA ; 0.05 % 2 mercaptoethanol ; 1X protease inhibitors). Samples were analysed on 12 % SDS-PAGE under reducing conditions. The proteins were electrotransferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% milk in Tris-buffered saline with 0.05% Tween 20 for 1 hour at room temperature and probed with primary rabbit anti-human MyD88 antibody (Cell Signaling) at 4°C overnight. After washing, membranes were
incubated with secondary anti-rabbit antibody coupled to horseradish peroxidase (Jackson Immunoresearch for 1 hour at room temperature, followed by ECL Plus™ detection (Amersham Biosciences).

In assays determining phosphorylated forms of p38, eosinophils were incubated (2x 10^6 cells in PBS) with different BCG : eosinophil ratios for 5, 15, 30 min (37°C, 5% CO_2). After stimulation, cells were lysed and equal amounts of proteins were analysed with monoclonal rabbit anti-human p38 MAPK or anti-phosphorylated p38 MAPK antibodies (Cell Signaling) as described above.

For α-defensin detection, proteins were subjected to SDS-10 % polyacrylamide gel electrophoresis before blotting onto a nitrocellulose membrane as described above.

Phagocytosis

Eosinophils were labeled with 10 μM CFSE at 4x10^6 cells/mL in PBS. BCG were labeled with 10 μM PKH26 (Sigma-Aldrich) at a concentration of 10^8 bacteria/mL. On the day of each experiment, CFSE-labeled eosinophils are incubated with PKH26-labeled BCG at different BCG : eosinophil 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 RPMI to remove nonadherent mycobacteria and fixed in slide after cytospin for analyse. Cells containing fluorescent red mycobacteria were counted by fluorescence microscopy (Leica).

Bactericidal activity of eosinophils

Eosinophils were washed with RPMI without phenol red and diluted to 2.10^6/ml. Next, a bacterial suspension was diluted from the frozen aliquots in RPMI without phenol red to the appropriate concentration for use in experiments. BCG suspension was placed with the
eosinophil suspension at 37°C for different times. Numbers of CFU were determined as follow: dilutions of each sample were plated in duplicates on 7H11 (Difco) agar plates enriched with Oleic Acid Albumin Dextrose Complex (OADC) and incubated at 37°C and 5% CO₂. After 21 days, the number of CFU was determined on the plates and expressed in percentage of CFU control ((CFU with eosinophils/ CFU with medium) x 100).

For bactericidal activity against intracellular mycobacteria, BCG-infected eosinophils were washed three times with antibiotic-free medium. The low-speed centrifugation (300g) selectively pellets cells while extracellular bacteria remain in the supernatant. To measure intracellular mycobacterial growth, cells were lysed with 0.3% saponin (Sigma-Aldrich) to release intracellular bacteria. At all time points, an aliquot of unlysed infected cells was harvested and counted. This allowed an exact quantification of cells as well as the determination of cellular viability by trypan blue exclusion. Recovery of cells was 80% in all experiments, with cell viability regularly exceeding 90% of total cells. Lysates of infected cells containing intracellular mycobacteria were resuspended vigorously, transferred into screw-cap tubes, and sonicated in a preheated (37°C) water bath sonicator for 5 min. Aliquots were diluted in Sauton’s medium and plated on Middlebrook 7H11 (Difco) agar. CFU were counted after 21 days incubation at 37°C.

The bactericidal activity of purified α-defensins (Hycult biotechnology) and ECP (Diagnostics Development) against BCG was determined by measuring the effect on bacterial colony-forming activity as described above.

For inhibition experiments, eosinophils were preincubated with a blocking anti-α-defensin (NP-1) Antibody (MBL), anti-ECP (BD Pharmingen) or isotype control for 30 min at 37°C, before the addition of BCG.

Eosinophil granules
Purified eosinophils were lysed in ice-cold buffer (0.25 M sucrose, 200 U/ml heparin). The residual cells and debris were separated from the granules by centrifugation at 300 x G for 10 min. The eosinophil granules were isolated from the supernatant by centrifugation at 10 000 x G for 15 min.

For detection of α-defensins by flow cytometer, eosinophil crystalloid granules were subjected to the same procedure described above for purified eosinophils. Eosinophil granules were identified by incubation with biotinylated anti-EPO or isotype control for 30 min and after a washing step, with streptavidin-APC (Molecular Probes) for 20 min in the presence of saponin.

**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 together with 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.
**Results**

*M. bovis* BCG attracts and activates human eosinophils

In various models of mycobacterial infections, eosinophils are recruited into developing lesions\(^{17,18}\). We have first tested the ability of BCG to induce the migration of purified eosinophils. Chemotactic activity of BCG for eosinophils was assayed using eosinophils alone (0:1) as negative control and rhIL5 as positive control. Compared to spontaneous migration, we found that eosinophils migrated significantly more towards live but not heat-killed BCG (Fig. 1 A). Eosinophil migration was dependent upon increasing BCG : eosinophil ratios. In addition, we observed that LAM and LM purified from *M. bovis* BCG, known as major cell wall lipoglycans, induced a migratory response in human eosinophils in a dose-dependent manner in the range of concentrations from 0.01 to 10 µg/ml (Fig. S1).

In response to activation, eosinophils are able to synthesize reactive oxygen species (ROS) and to mobilize preformed proteins stored within cytoplasmic-specific granules and to produce cytokines\(^6\). We have examined ability of BCG to induce ROS generation by eosinophils. The peak of eosinophil respiratory burst was reached about 30 min following BCG addition for eosinophilic patients and 60 min for normal donors (Fig. S2 A). At this time-point, ROS production by eosinophils was proportional to BCG number (Fig. 1 B). BCG-induced eosinophilic degranulation was also evaluated by EPO release in supernatants. Dose-dependent EPO release was observed when eosinophils were incubated with increasing BCG : eosinophil ratios (Fig. 1 B). Additionally, weak ROS and EPO release was observed when heat-killed BCG was added (data not shown). Similar results were found for ECP, confirming that BCG triggers eosinophilic degranulation in a dose-dependent manner (Fig. 1 C). Since several cytokines are also stored in eosinophil granules, we have evaluated BCG
capacity to induce TNF-α release, a key cytokine participating in the control of mycobacterial infection as well as in granuloma formation25,26. TNF-α was detected in supernatants from eosinophils incubated for 18 hours with different numbers of BCG (Fig. 1 D). Furthermore, several chemokines and cytokines, including IL-8, Migration Inhibition Factor (MIF), Macrophage Inflammatory Protein (MIP)-1α, MIP-1β, IL-13 and IL-16, were detected by protein microarray in cell-free supernatants of eosinophils following incubation with BCG (Fig. S2.B). Taken together, these results indicate that eosinophils can directly respond to BCG by degranulation and cytokine release.

Eosinophil expression of TLR2 and TLR4 is inducible

As reported for macrophages and dendritic cells, TLR2 and TLR4 have been identified as potential receptors for mycobacterial components2. Expression of TLRs by eosinophils has been recently described27 but their precise expression profiles and functions still remain unclear. Herein, we have compared TLR expression by eosinophils from normal donors (ND) and eosinophilic donors (ED). Surprisingly, TLR2 and TLR4 were not detected on the surface of eosinophils from normal donors, in contrast to eosinophils from eosinophilic donors which spontaneously expressed surface TLR2 and TLR4 (Fig. 2 A). Since TLR expression is known to be up-regulated in human monocytes treated with TLR agonists28, we investigated whether BCG could also modulate surface expression of TLRs on eosinophils. For 16 healthy donors and eosinophilic donors studied (Table S1), BCG significantly increased TLR2 and TLR4 expression on freshly isolated human eosinophils from most donors (Fig. 2A and B). These results show heterogeneous basal expression levels of TLR2 and TLR4 on human eosinophils and induction in vitro in the presence of BCG.

TLR2, but not TLR4, is essential in eosinophil activation by BCG
To further evaluate the functionality of TLRs on eosinophils, these latter were stimulated with either Pam3CSK4 (PAM) or lipopolysaccharide (LPS), respectively TLR2 and TLR4 specific ligands. As shown in Fig. S3 A, eosinophils from normal and eosinophilic donors released ROS and EPO in response to PAM and LPS and this release was inhibited by blocking anti-TLR2 and anti-TLR4 antibodies, confirming that TLR2 and TLR4 expressed by human eosinophils are functional.

Several reports have described TLR2- and TLR4-dependent activation of macrophages by mycobacterial ligands\textsuperscript{29}. To investigate the potential involvement of TLRs in eosinophil/BCG interactions, eosinophils were incubated with blocking anti-TLR2 and anti-TLR4 antibodies and their response to BCG was evaluated by ROS release. Production of ROS in response to BCG was reduced in a dose-dependent manner when eosinophils were preincubated with a blocking anti-TLR2 antibody (with a maximum of 47.3 ± 11.9 % inhibition) (Fig. 3 A), whereas no significant inhibition of ROS release was observed with a blocking anti-TLR4 antibody or an isotype control (Fig. 3 B). Similar levels of inhibition of EPO release was obtained with blocking anti-TLR2 antibody but not with anti-TLR4 antibody (Fig. 3 C). These data indicate that TLR2, but not TLR4 is involved in eosinophil activation by BCG.

LAM and LM have been widely described as key factors able to modulate the host immune response\textsuperscript{30}. No ROS production by eosinophils was observed following incubation with LM or LAM purified from BCG (data not shown). However, both molecules induced release of EPO by eosinophils, with LM being a more potent EPO inducer than LAM (Fig. S3 B). This prompted us to investigate the ability of LM to activate eosinophils. As shown in Fig. 3 D, EPO release by eosinophils in the presence of 1 µg/ml LM (an optimal concentration determined after dose-response preliminary experiments) was significantly reduced by 66.1 ± 20 % when eosinophils were pretreated with a blocking anti-TLR2 antibody but not with a blocking anti-TLR4 antibody or an isotype control. These results suggest that LM, a purified
component of BCG, could induce eosinophil degranulation mainly in a TLR2-dependent manner.

**BCG-induced eosinophil activation is dependent on both MyD88, p38 and NF-κB**

Activation of TLR2 signaling pathway involved recruitment of the intracellular adaptor protein MyD88 (Myeloid differentiation factor 88), TIRAP (Toll/IL-1R domain-containing Adaptor Protein), TRAF6 (TNF receptor-associated factor 6) and leads to the phosphorylation of mitogen activated protein kinases (MAPK) or NF-κB (nuclear factor kappaB) \(^3\). We first detected, by RT-PCR, MyD88, TRAF6 and TIRAP mRNA in human eosinophils (Fig 4 A). Western blot analysis of cell lysates confirmed the presence of the MyD88 protein in human eosinophils, similarly to neutrophils which were used as positive control (Fig. 4 B). To elucidate the signaling pathway underlying BCG-induced eosinophil activation, we demonstrated that a MyD88 inhibitory peptide, but not a control peptide, reduced ROS production in BCG-stimulated eosinophils, in a dose-dependent manner (Fig. 4 C).

We have then further assessed the role of MAPK and NF-κB by using various selective inhibitors of MAPK pathways, including SB 203580 (p38 MAPK inhibitor), PD 98059 (ERK inhibitor), SP600125 (JNK inhibitor) and BAY 11-7082. Whereas SB 203580 was able to significantly inhibit BCG-induced ROS release by eosinophils, PD 98059 and SP 600125 had only a marginal effect (Fig. 4 D). In addition, we obtained 51 ± 4.3% inhibition of ROS release by BCG-activated eosinophils with BAY 11-7082. Overall, these results suggest that p38 MAP kinase and NF-κB signaling pathways play a prominent role in BCG-induced ROS release by eosinophils. In order to confirm that intracellular signaling pathway of eosinophils was activated by BCG, we next investigated the p38 MAPK phosphorylation profile. As shown in Fig. 4 E, stimulation of eosinophils with BCG resulted in p38
phosphorylation after 5 minute stimulation. This p38 MAPK phosphorylation decreased after 30 minutes of BCG stimulation. In addition, LM-induced eosinophil activation involved a MyD88 and p38 MAP Kinase pathways, since LM-induced EPO release was inhibited by a MyD88 inhibitor (maximal inhibition 72.8 ± 6.2 %) and SB 203580 (maximal inhibition 49.6 ± 16.1 %) (Fig. S4). Of note LY294002, a Phosphoinositide Kinase-3 (PI3K) inhibitor, had not effect on ROS release by BCG-activated eosinophils (data not shown). Taken together, these findings indicate that BCG could activate eosinophils through MyD88-, p38 MAPK- and NF-κB- dependent pathways.

**Eosinophils control BCG growth**

Due to the release of cytotoxic mediators by eosinophils, we next addressed whether eosinophils may displayed phagocytic and antmycobacterial activity. Survival of BCG in the presence of human eosinophils was thus tested. A decrease in mycobacterial survival was observed for all BCG:eosinophil ratios (Fig. 5 A). Eosinophils were able to restrict BCG growth by 31.8 % and 56.9 % within 6 hours following BCG addition. Because BCG is an intracellular attenuated pathogen, we tested the ability of eosinophils to phagocytose PKH26-BCG by fluorescence microscopy (Fig. 5 B). Dose-dependent internalization was observed when eosinophils were incubated with increasing BCG:eosinophil ratios. Extracellular mycobacteria were removed by washing and only remaining eosinophils were lysed and plated at increasing intervals for assessment of viable bacilli. As shown in Fig. 5 C, for 5:1 ratio, number of colony forming units by intracellular BCG significantly decreased with incubation time, suggesting that eosinophil cytotoxicity could be due in part to release of cytotoxic mediators.

**Human eosinophils express α-defensins**
In order to investigate which mediators could contribute to eosinophil cytotoxicity towards BCG, we investigated the expression of defensins, major effector molecules in innate immunity against microbes. We did not detect any expression of mRNA β-defensins in unstimulated and BCG-stimulated eosinophils in contrast to neutrophils (data not shown). However, both neutrophils and eosinophils, but not Jurkat T cells, expressed α-defensin (HNP1-3) mRNA (Fig. 6 A). As shown in Fig. 6 B and C, α-defensins were also detected in eosinophils by flow cytometry and in cell lysates. The association of α-defensins immunoreactivity with crystalloid granules was shown by flow cytometry analysis on purified EPO+ eosinophil granules (Fig. 6 D). To determine the intracellular localization site of α-defensins, we carried out a double immunofluorescence staining with antibodies specific for MBP (localized in the granul crystalloid core) and EPO (localized in the granule matrix region) (Fig. 6 E). Immunoreactivity against MBP and α-defensins were mostly co-localized, while immunoreactivity against EPO and α-defensins only displayed minor co-localization. Taken together, our results showed a constitutive expression of α-defensins in eosinophils. These observations led us to hypothesize that eosinophils may respond to BCG by rapid release of α-defensins.

**BCG-induced α-defensin and ECP release contributes to eosinophil cytotoxicity**

Alpha-defensins immunoreactivity was further increased upon activation by BCG and eosinophil pretreatment with Brefeldin A (1 µg/ml) (Fig. 7 A). In addition, our results suggest that BCG-activated eosinophils also released α-defensins in a dose-dependent manner (Fig. 7 B). After stimulation with PAM or LM, but not LPS, α-defensins were detected in culture supernatants. Production of α-defensins in response to PAM or LM was significantly abolished when eosinophils were preincubated with a blocking anti-TLR2 antibody, whereas
no inhibition of α-defensin release was observed with an isotype control, indicating a TLR2-dependent α-defensin release (Fig. 7 C). Furthermore, preincubation with a blocking anti-α-defensin antibodies partially but significantly increased the BCG growth at the 5:1 ratio, suggesting that α-defensins indeed contribute to eosinophil cytotoxicity towards mycobacteria (Fig. 7 D). In addition, α-defensin killing activity against BCG was maximal in the presence of purified ECP (Fig. S5 A). Accordingly, we evaluated effect of a blocking anti-ECP antibody on BCG growth and showed increased survival upon inhibition of ECP release (Fig. S5 B). However, the presence of resorcinol and diphenyleneiodonium, the corresponding EPO and ROS inhibitors, did not significantly influence BCG growth (data not shown). These results thus suggest that LM is a BCG component accounting for the α-defensin release and that a killing mechanism used by eosinophils to inhibit intracellular BCG growth is α-defensin and ECP-dependent.
Discussion

Besides helminth infections, peripheral blood and tissue eosinophilia have been associated to mycobacterial infections. We have here investigated the mechanism of eosinophil-mycobacteria interactions in order to provide a molecular basis to this observation.

The present results indicate that BCG has the propensity to activate eosinophils and that TLR2 plays a major role in the activation process. Contradictory results have been reported regarding TLR expression by human eosinophils. The absence of TLR2 and TLR4 expression was reported in one study, whereas other reports evidenced TLR2 and TLR4 mRNA expression as well as protein expression of TLR1, 2, 4, 5, 6 and 9 on human eosinophils. We have shown an heterogenous donor/patient-dependent TLR2 and TLR4 expression by human eosinophils. Eosinophils from eosinophilic donors but not from healthy donors spontaneously expressed TLR2 and TLR4 at their surface. These observations might explain why eosinophils from eosinophilic donors display a quicker response to BCG than eosinophils from normal donors. Other receptors, such as CD14, complement receptors and Fcγ receptor expressed by eosinophils, could participate to BCG recognition and induce TLR expression on eosinophils purified from normal donors following incubation with BCG. Alternatively, it is also possible that TLR expression levels in normal donors are below detection threshold of flow cytometry and that this low expression is up-regulated upon ligand binding, as it is the case in allergic patients. Spontaneous TLR expression on eosinophils from eosinophilic donors could be explained by in vivo eosinophil priming. Such heterogeneity between eosinophil donors, already evidenced for other membrane receptors including IgE receptors, may account for discrepancies in earlier results, which did not discriminate between different clinical status of patients.
Live, but not heat-killed, mycobacteria were able, \textit{in vivo}, to attract eosinophils\textsuperscript{12}, indicating that metabolically active mycobacteria are required. We have shown that live BCG, but not heat-killed BCG, attracted human eosinophils. Our study also identified LM and LAM, two heat-stable cell wall-associated lipoglycans, as potent eosinophil migration-inducing factors. However, LAM secretion does require live bacteria in order to create a gradient allowing for eosinophil-chemotaxis. Furthermore, we can not exclude that other heat-sensitive secreted compounds are required for optimal chemotaxis. Nevertheless, whether eosinophils recognize, and are subsequently activated by mycobacteria remained unclear. Our experiments reveal an important immunostimulatory activity of LM on eosinophils, unlike LAM. These two lipoglycans have been reported to exhibit antagonistic activities and recent studies suggested that that LM displays stronger proinflammatory responses compared to LAM\textsuperscript{4}. In addition, we demonstrated a TLR2-dependent eosinophil activation effect by LM. In a murine model of BCG-induced pleurisy, eosinophil recruitment was enhanced by TLR2\textsuperscript{18}. Both TLR2 and TLR4 are essential to innate immunity against mycobacteria species and were the likeliest PRRs that would have endowed eosinophils with the capacity to be activated by mycobacteria. In the current work, only TLR2 appeared to play a functional role in eosinophil-mycobacteria interactions, since a blocking anti-TLR2 antibody partially suppressed BCG- or LM-induced eosinophils activation. This also suggests that other PRRs could be involved in mycobacteria-induced eosinophil activation such as TLR9\textsuperscript{40} or β-glucan receptor dectin-1\textsuperscript{41}. However, we cannot ruled out the possibility that other mycobacterial cell wall bioactive components, such as trehalose dimycolate (TDM) to participate in this process.

To date, one single study has examined the TLR signal transduction\textsuperscript{27}. Various TLR ligands differentially induced release of cytokines, chemokines, superoxides or granular protein release. Indeed, TLR7 ligand R837 and TLR2 ligand peptidoglycan activations were
dependent on the p38 or ERK MAP kinase pathway respectively and induced IL-1\(\beta\), IL-6, IL-8 and GRO-\(\alpha\) release by eosinophils. Surprisingly, expression and recruitment of MyD88, the central adapter shared by several TLRs, was never investigated in these previous studies. We have extended these observations by reporting that TLR-dependent activation of eosinophils through BCG activation required both MyD88, p38 MAP kinase and NF-kappaB molecules.

Although eosinophilia has been evidenced in different mycobacteria-infection models, their contribution in controlling BCG growth is unknown. Our results show that human eosinophils expressed \(\alpha\)-defensins, localized within the eosinophil crystalloid granules. Eosinophils are among the few inflammatory cells capable of storing proteins, cytokines and chemokines in their secretory granules. These stored products may be rapidly mobilized from intracellular sites of storage and released locally during inflammation. In addition, eosinophils could release \(\alpha\)-defensins in response to BCG or LM through TLR2 ligation. Interestingly, \(\alpha\)-defensins can directly kill BCG \textit{in vitro} and \(\alpha\)-defensin-producing eosinophils play a key role in BCG growth inhibition. These results are quite surprising as human \(\beta\)-defensin-1 and 2 genes are usually considered to display strong anti-mycobacterial properties\textsuperscript{42,43}. Activity of \(\alpha\)-defensins towards mycobacterial species seems strain-dependent\textsuperscript{44}. The potent bactericidal activity of \(\alpha\)-defensins against virulent mycobacteria\textsuperscript{45}, is consistent with our data using BCG as a model. Because \(\alpha\)-defensin release is rapid, it is conceivable that when recruited to infection sites, eosinophils may directly contribute to bacterial killing. Other cytotoxic mediators might also be released by eosinophils since blocking of \(\alpha\)-defensin activity only partially prevented BCG growth. Indeed, it is worth pointing out that when eosinophils were activated with BCG release of both EPO, which has a lytic activity against \textit{M. tuberculosis}\textsuperscript{46}, and ECP, a ribonuclease which possesses antimicrobial activity against both Gram-negative and Gram-positive strains\textsuperscript{47} was observed. Our results indicate that \(\alpha\)-defensins and ECP have
to be considered as important components of the mechanisms by which eosinophils kill BCG. These results provide evidence that eosinophils harbour a diverse arsenal of cytotoxic components against mycobacteria.

The ability of eosinophils to interact and kill BCG challenges the concept that vaccination with BCG and resistance against acute tuberculosis is dependent on host ability to generate Th1 immunity. Although eosinophils are often intimately linked with Th2-driven immunity, they have been shown to express Th1 cytokines such as IFN-γ, upon stimulation by CD28. However, we failed to detect IFN-γ release by eosinophils in the presence of BCG (data not shown) whereas BCG induced TNF-α, IL-8, MIF, MIP-1α, MIP-1β, IL-13 and IL-16 release. Inflammatory granuloma responses are usually associated with angiogenesis, involving IL-8, and with chemokines, such as MIP, which facilitate the recruitment of inflammatory cells to the site of infection. It is noteworthy that LM, but not LAM, has been reported to be strong IL-8 and TNF-a–inducing factor. TNF-α is a key macrophage-activating cytokine for intracellular killing of *M. tuberculosis*. Both beneficial and deleterious effects of TNF-α have been observed in tuberculosis patients. These dual effects have also been attributed to tumor-associated eosinophils. On the other hand, an increased Th2 immunity has been associated with active tuberculosis. In many developing countries, patients infected with *M. tuberculosis* faced helminth infection, often associated with eosinophilia and Th2 response. Therefore the Th2-driven immunity could increase susceptibility to tuberculosis and could play a role in a persistent form of the disease. Extensive analysis of tuberculosis-associated eosinophilia and correlation with disease outcome would represent a further goal to better understand the role of eosinophils during tuberculosis infection and progression of the disease.

In conclusion, our results represent the first evidence of the involvement of α-defensins, released by human eosinophils upon TLR2 stimulation in immunity against an
intracellular pathogen. This study highlights a mechanism of direct pathogen recognition by human eosinophils, thereby broadening their functional importance as early direct sensors and effectors against bacteria.
Acknowledgements

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Authorship

V.D. performed research and wrote the paper; F.L. performed research and statistical analysis; E.H. analysed data and reviewed the drafts of the paper. S.L. was essential for helping with patient sample purifications; Y.G. and L.K. provided purified mycobacterial components; E.A. and G.W. critically reviewed the manuscript, D.D. provided intellectual input and reviewed drafts of the paper. M.C. designed and interpreted the experiments.

The authors declare no competing financial interests.
References


Legends

Figure 1. Eosinophil chemotaxis and activation mediated by *M. bovis*-BCG. (A) Eosinophil migration in response to different BCG:eosinophil ratios. Error bars represent Standard Error of the Mean (± SEM) from five independent experiments. (B) ROS generation and EPO release by eosinophils in cell supernatants. Results are expressed as ΔROS or ΔEPO cps (counts per second) values (values from medium stimulation are subtracted from values obtained with each ratio). (C) After 18h of incubation, ECP concentrations in the cell free supernatants were measured. Data are expressed as means ± SEM from the three independent experiments with different eosinophil donors. (D) Eosinophils were stimulated for 18h at different BCG : eosinophil ratios. TNF-α levels were quantified in the culture supernatants by ELISA. Results are represented as the mean of three independent experiments ± SEM. *, p < 0.05 ; **, p < 0.01.

Figure 2. Expression of TLR2 and TLR4 by human eosinophils. (A) Surface expression of TLR2 and TLR4 on human eosinophils incubated with or without BCG (10:1). Results correspond to one representative normal donor (ND, n°13 of Table S1) and eosinophilic donor (ED, n°5 of Table S1). Grey histogram and black line represent staining with specific and isotype control antibodies respectively. The data are displayed using a logarithmic scale and the ΔMean Fluorescence Intensity (ΔMFI: values from isotype control antibodies are subtracted from values obtained with each condition) are indicated in the plot. (B) Cell surface TLR2 and TLR4 expressions on purified eosinophils from 16 individual eosinophil donors stimulated with or without BCG (10 : 1). Group average expression for the indicated markers is represented (-). *, p < 0.05.
Figure 3. **Involvement of TLR2 in the activation of eosinophils by M. bovis-BCG.** (A) Kinetics of BCG-induced ROS release (ratio 10 : 1 ■) inhibited by an anti-TLR2 blocking antibody 20 µg/ml (▲), 10 µg/ml (★) or an isotype control antibody 20 µg/ml (□). Spontaneous ROS release by eosinophils is indicated (-). Results correspond to one representative of three experiments and are expressed in counts per second (cps). (B) BCG-induced ROS production (ratio 10 : 1 ■) inhibited by an anti-TLR4 blocking antibody 20 µg/ml (▲), 10 µg/ml (★) or an isotype control antibody 20 µg/ml (□). Spontaneous ROS release of eosinophils is indicated (-). Results are expressed as ROS cps values and one representative of three experiments was shown. (C) Inhibition of BCG-induced EPO release by anti-TLR2 and anti-TLR4 blocking antibodies (20 µg/ml) or an isotype control antibody. ΔEPO cps values (values from medium stimulation are subtracted from values obtained with each antibody). Results are expressed as mean ± SEM (n = 5-6). (D) LM (1 µg/ml)-induced EPO release inhibited by anti-TLR2 or anti-TLR4 blocking antibodies (20 µg/ml) or an isotype control antibody. Results are expressed as ΔEPO cps values and as mean ± SEM (n = 5). *, p < 0.05.

Figure 4. **MyD88, p38 MAP kinase and NF-κB pathway activation after stimulation of eosinophils with M. bovis-BCG.** (A) MyD88, TIRAP and TRAF6 expression on eosinophils was analyzed by RT-PCR. (B) Total proteins were extracted from eosinophils and neutrophils and equal protein amounts were analysed using Western blot for MyD88. The gel from one of two similar experiments is shown. (C) Measurement of ROS release by eosinophils preincubated with serially diluted MyD88 inhibitor 1 µM (X), 10 µM (△) or 100 µM (●) or with peptide control (100 µM) (○) at 37°C for 30 min, further activated with BCG (10 : 1). ROS release by BCG (1:10 ■)-activated eosinophils without MyD88 inhibitor and by unstimulated eosinophils (-) were determined. Results are expressed as ROS cps (counts per
(D) Purified eosinophils were pre-treated with inhibitors to JNK1/2 (SP 600125, 0.1 µM), ERK1/2 (PD98059, 0.1 µM), p38 MAP kinase(SB 203580, 0.1 µM), or NF-κB inhibitors (BAY 11-7082, 1µM) for 30 minutes at 37°C, before the addition of BCG (10 : 1). ROS release by eosinophils was analysed by chemiluminescence. Bar graph represents the percent ROS release inhibition. Results are expressed as mean ± SEM (n = 3-4). (E) Purified eosinophils were stimulated with different numbers of BCG for the times indicated, and total cell extracts were analysed by Western blotting using Abs against phosphorylated or nonphosphorylated forms of p38 MAP kinase. The results are representative of two independent experiments. *, p < 0.05.

Figure 5. **BCG growth inhibition by eosinophils.** (A) BCG growth in the presence of eosinophils for the indicated times. Data are shown as the mean of three independent experiments ± SEM, expressed in percentage of CFU control ((CFU with eosinophils/ CFU with medium) x 100). (B) Quantitative detection of internalized PKH26-BCG by fluorescence microscopy. Eosinophils were incubated, or not, with BCG for 40 min at 37°C. (C) Intracellular BCG growth inhibition with different BCG : eosinophil ratios (5 : 1 (■); 10 : 1 (△); 20 : 1 (●)). Results are expressed as mean ± SEM (n=3). Significant differences between conditions studied and controls are indicated as followed: *, p < 0.05, **, p < 0.01.

Figure 6. **Expression of α-defensins by human eosinophils.** Intracellular α-defensin (HNP1-3) expression on eosinophils (eosino) and neutrophils (neutro) was analysed using RT-PCR (A), flow cytometry (B) and western blot (C). (D) α-defensins expression on EPO+ crystalloid granules purified from human eosinophils. Staining with control isotype antibodies is represented. Results correspond to one representative eosinophil donor. (E)
Immunofluorescence and confocal (*insets*) analysis of human eosinophils with Alexa Fluor® 555 goat anti-mouse secondary antibody (red) to detect the HNP1-3 immunoreactivity, or Alexa Fluor® 488 streptavidin (green) to detect immunoreactivity against MBP or EPO.

Figure 7. **Alpha-defensins synthesis and release by human eosinophils in the presence of BCG.** (A) α-defensin expression after 2 hours of eosinophil stimulation (grey histogram) or unstimulation (dotted line) with BCG (10:1) in the presence of brefeldin A. Black line represents staining with isotype control. (B) α-defensin levels released by eosinophils activated by BCG were quantified by ELISA in cell culture supernatants. Results are expressed as mean ± SEM (n=3). (C) After incubation of eosinophils, pre-treated by blocking anti-TLR2 antibody or isotype control, with PAM (0.5 µg/ml), LPS (0.1 µg/ml) or LM (1 µg/ml), α-defensin levels were quantified in the culture supernatants by ELISA. Results are represented as the mean of three independent experiments ± SEM. (D) Intracellular BCG growth inhibition. Eosinophils were treated or not treated with a blocking anti-α-defensin antibody (△) or an isotype control (-) and were infected with BCG (5 : 1 ■). Values are mean ± SEM of three independent experiments. Significant differences between conditions studied and controls are indicated as followed: *, p < 0.05, **, p < 0.01.
Figure 2
Figure 3

A and B: Graphs showing ROS (relative optical signal) over time (in minutes) for TLR2 and TLR4, respectively, with different conditions indicated by different symbols.

C: Bar graph showing ΔEPO (change in EPO) for different conditions: 20:1, isotype control, anti-TLR2, and anti-TLR4.

D: Bar graph showing ΔEPO for LM, isotype control, anti-TLR2, and anti-TLR4 conditions.

* indicates statistical significance.
Figure 4

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**Figure 5**

A. Bar graph showing colony forming units (%) over time (min: 0, 30, 120, 240, 360) for different ratios (5:1, 10:1, 20:1).

B. Bar graph showing phagocytosis (%) over time (min: 0, 5, 10, 20, 40) for different ratios (5:1, 10:1, 20:1).

C. Line graph showing colony forming units (%) over time (min: 0, 30, 120, 240, 360).
Figure 6
TLR2-dependent eosinophil interactions with mycobacteria: role of \( \alpha \)-defensins

Virginie Driss, Fanny Legrand, Emmanuel Hermann, Sylvie Loiseau, Yann Guerardel, Laurent Kremer, Estelle Adam, Gaetane Woerly, David Dombrowicz and Monique Capron