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Neutrophil extracellular traps are induced by *Mycobacterium tuberculosis*

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SUMMARY

Due to the intracellular nature of mycobacterial infections, little attention has been paid to the possible extracellular role that neutrophils might play in tuberculosis. The recent discovery of neutrophil extracellular traps (NETs), composed of DNA and antimicrobial proteins,[1] introduces a new perspective to our understanding of the mechanism used by the innate immune system to contain and kill microorganisms. In this study, we tested *in vitro* whether *Mycobacterium tuberculosis*, an intracellular pathogen, can induce NETs formation and if this newly discovered mechanism is involved in a control response during mycobacterial infection. We found that two different genotypes of *M. tuberculosis* exerted, *in vitro*, a cytotoxic effect and induced subcellular changes on infected neutrophils, leading to NETs formation in a time dependent manner. NETs trapped mycobacteria but were unable to kill them. NETs formation induced by *M. tuberculosis* could help understand the early stages of mycobacterial pathogenesis.

1. Introduction

Neutrophils constitute the first line of defense of the innate immune system, phagocytosing and killing microbes through oxygen dependent and/or independent mechanisms.[2] Recently, a new antimicrobial mechanism for neutrophils has been described: neutrophil extracellular traps (NETs). These are structures composed of chromatin decorated with granule proteins that bind and kill Gram-negative[1] and Gram-positive[1,3,4] bacteria as well as fungi.[5] Upon activation, NETs are released as a result of a cell death process, different from apoptosis or necrosis, which depends on reactive oxygen species generated by NADPH oxidase.[6]

Classically, neutrophils are viewed as phagocytes that clear the extracellular space of rapidly growing microorganisms. However, evidence has accumulated in the past few years clearly emphasizing the role of neutrophils in the control of intracellular pathogen infections.[7]

Tuberculosis is today, after AIDS, the second cause of death from an infectious disease worldwide.[8,9] The causative agent, *Mycobacterium tuberculosis*, is one of the most successful pathogens at evading the host immune response to establish infection.[10] *M. tuberculosis* pathogenesis is complex and is not completely understood. For several years neutrophils were underestimated in tuberculosis because of their short life-span and the fact that macrophages play the main role during infection. Recent evidence from *in vivo* studies revealed that the earliest immune response during mycobacterial infection is a migration of neutrophils to the site of infection, probably being important during the acute phase of tuberculosis.[11–13] Neutrophils are also essential for accurate early granuloma formation during chronic *M. tuberculosis* infection.[14] Despite this information, the role of neutrophils during *M. tuberculosis* infection remains controversial, particularly regarding the question of whether neutrophils are capable of killing *M. tuberculosis*, thus controlling the infection[15–18] or if they contribute to the development of the pathology.[19] Most studies of microbial killing by neutrophils are focused on phagocytosis and intracellular killing, in which the microorganisms are exposed simultaneously to antimicrobial peptides and reactive oxygen species (ROS) within intracellular phagocytic vacuoles. However, little attention has been paid to the possibility of an extracellular role played by

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neutrophils in tuberculosis, because this mechanism has been associated with host tissue damage related to inflammation observed in acute infections.[20] In this context, NETs represent a new extracellular mechanism produced by neutrophils to kill microbes and contain infection in a way that minimizes injury to surrounding tissue. Here, we showed that two strains of the *Mycobacterium tuberculosis* complex (MTC)[21] varying in virulence, *M. tuberculosis* H37Rv and *M. canettii*, induced subcellular changes leading to NETs formation in a time dependent manner, causing the death of infected neutrophils. NETs captured both mycobacteria, but were unable to kill them.

2. Materials and methods

2.1. Bacterial strains and cultures

Two strains of the MTC were used: *M. tuberculosis* H37Rv and 9600046 *M. canettii* genotype isolated at the Pasteur Institute, kindly provided by the National Institute of Public Health and the Environment of the Netherlands. Mycobacteria were cultured on Middlebrook-7H9 broth (BD-DIFCO, USA) supplemented with 10% OADC (BD-DIFCO, USA) at 37 °C in constant shaking at 150 rpm. *Listeria monocytogenes* strain ATCC 43249 was cultured in brain heart infusion broth (BD-DIFCO, USA) at 37 °C. All strains were grown to exponential phase. Inocula were prepared by suspending bacteria in D-MEM (Dulbecco's modified Eagle's medium, GIBCO™). The resulting suspension was vortex agitated and allowed to sit to let clumps settle. The top portion was adjusted to the McFarland nephelometer no. 1 standard, corresponding to 3×10^8 mycobacteria/ml.

2.2. Isolation of human neutrophils

Neutrophils were isolated from heparinized venous blood of healthy volunteers by percoll density-gradient centrifugation as previously described by Aga et al., 2002.[22]

2.3. Scanning electron microscopy

Resting neutrophils or neutrophils infected with *M. tuberculosis* H37Rv or *M. canettii* at an MOI of 10 (mycobacteria: neutrophil) for 2 h and 4 h, were fixed with 2.5% glutaraldehyde in phosphate buffered saline (PBS, pH 7.2) for 1 h at room temperature, postfixed with 1% osmium tetroxide and gradually dehydrated with increasing concentrations of anhydrous ethanol (50, 60, 70, 80, 90 and 100%). Then, specimens were critical point dried in CO_2 atmosphere in a Samdry®-780A apparatus (Tousimis Research, USA), gold coated in a Denton Vacuum Desk II (INXS. Inc. Florida) and analyzed in a JEOL JSM-5800 LV (JEOL Ltd. Tokyo, Japan) scanning electron microscope (SEM).

2.4. Transmission electron microscopy

2.4.1. Negative staining

Resting neutrophils or *M. tuberculosis* H37Rv and *M. canettii* infected neutrophils at a MOI of 10:1 (mycobacteria: neutrophil) were settled down on copper grids coated with a formvar plastic layer and incubated at 37 °C for 2 h in a humid chamber. After being rinsed with 25 mM HEPES, the grids were fixed with 2.5% glutaraldehyde for 10 min and then negatively stained with 0.2% uranyl acetate for 15 s. Specimens were analyzed in a JEOL 2000 EX (JEOL Ltd. Tokyo, Japan) transmission electron microscope (TEM).

2.5. Measurement of oxidative burst by chemiluminescence

Neutrophils were resuspended at 5×10^6/ml in D-MEM containing 10% heat inactivated fetal bovine serum (FBS) and combined with 20 μl of Luminol (1 mg/ml of dimethyl formamide). Immediately after, basal chemiluminescence was registered every minute for 20 min. At minute 20, neutrophils were infected with non-opsonized *M. tuberculosis* H37Rv or *M. canetti* at a MOI of 10:1. Activation with 20 nM phorbol mirystate acetate (PMA) was used as an activation control. Chemiluminescence was measured every minute for 20 min in a scintillation counter (Beckman LS 6000IC, USA).

2.6. Measurement of cytotoxic effect of M. tuberculosis genotypes on human neutrophils infected in vitro

Cytotoxic effect of *M. tuberculosis* or *M. canetti* on human neutrophils was determined according to the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche, Germany) protocol, which is based on a colorimetric assay for quantitating cytotoxicity/cytolysis by measuring LDH activity released from damaged cells. The colorimetric assay was performed by triplicate at each time point and samples were read in an ELISA reader using a 490–492 nm filter (Multiskan EX, Thermo, USA).

2.7. Fluorescence staining for actin filament cytoskeleton and immunofluorescence assays

Neutrophils were seeded on glass coverslips treated with 0.001% polylysine (Sigma-Aldrich, St. Louis, MO, USA), allowed to settle and either infected with mycobacteria at a MOI of 10:1, treated with 20 nM PMA or left unstimulated. Cells were incubated for 2 and 4 h at 37 °C in a 5% CO_2 atmosphere. After indicated time points, cells were fixed with 3.7% formaldehyde for 20 min, permeabilized with 0.04% Nonident-P40 (NP40) (Roche) and blocked with 1% FBS. For fluorescence staining of cytoskeleton, cells were first incubated for 1 h with 0.1 μg/ml phalloidin-FITC (Sigma-Aldrich, St. Louis MO, USA) to label actin filaments, followed by an incubation step with 4′,6-diamidino-2-phenylindole (DAPI) for DNA stain. Immunodetection of neutrophil elastase and the histone–DNA complex (H2A-H2B) was performed as previously described by Brinkmann et al., 2004,[1] but with the following modifications: after blocking, cells were incubated with primary antibodies, which were detected with secondary antibodies coupled to Alexa-Fluor-488 or Alexa-Fluor-568, followed by incubation with DAPI for DNA stain. Specimens were washed, mounted in Vectashield media (Vector Laboratories, Inc. Burlingame, CA, USA) and analyzed with an Axioscop 2 mot plus fluorescence microscope (Carl Zeiss, Mexico). Negative controls included cells only exposed to the fluorescent-secondary antibodies. None of these controls showed any conspicuous signal.

2.8. Isolation and quantification of NETs

NETs generated by neutrophils infected with *M. tuberculosis* genotypes, were digested with 500 mU/ml of micrococcal nuclease (Worthington Biochemical Corp., USA). The nuclease activity was stopped with 5 mM EDTA and the culture supernatants were collected and stored at 4 °C until further use. NET-DNA was quantified using Picogreen dsDNA kit (Invitrogen, USA) according to manufacturer's instructions. Twenty nanomolar PMA was used as a control of NETs induction.[6]

2.9. Fluorescence staining for mycobacteria

A mycobacterial suspension (*M. tuberculosis* H37Rv or *M. canetti*) adjusted to 1×10^8/500 μl in PBS 1X was labeled with 1 μl of Texas Red (Invitrogen-Molecular Probes, USA) for 1 h at room