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Mycobacterium tuberculosis strains modify granular enzyme secretion and apoptosis of human neutrophils

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ABSTRACT

Mycobacterium tuberculosis has evolved to employ multiple strategies to avoid an efficient host immune response. Accordingly, enzymes are important antimycobacterial elements and apoptosis decides the fate of any cell. Hence, we carried out this study to discern the amplitude of two clinical stains (S7 & S10) and a laboratory strain (H37Rv) of *M. tuberculosis* in modifying the release of lytic enzymes and apoptosis of neutrophils from healthy volunteers and pulmonary tuberculosis patients. We detected reduced levels of elastase in neutrophils from pulmonary tuberculosis patients. The laboratory strain H37Rv is found to increase the release of elastase and myeloperoxidase in neutrophils from both the groups. This strain is more efficient compared to clinical strains in inducing late apoptosis/necrosis in neutrophils. Our results proclaim the susceptibility of neutrophils in responding to infection with H37Rv. Also, at the functional level, neutrophils undergo changes related to release of enzymes after acquiring tuberculosis.

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1. Introduction

Neutrophils are a critical arm of defense against infection as they are the initial cells that encounter any microbe. They engulf and kill the pathogen during the process of phagocytosis with the help of proteolytic substances present within their granules. Once phagocytosis of the invader is over, the neutrophils have to be degraded to prevent the dispersion of these toxic proteolytic components. Neutrophil apoptosis, the process of programmed cell death and subsequent phagocytosis of these apoptotic cells by macrophages is thus central to the successful resolution of inflammation characterized during any infection (Fox et al., 2010) and tuberculosis (TB) is no exception. This prevents the release of neutrophil histotoxic contents, thereby limiting the destructive capacity of neutrophil products.

Neutrophils employ an arsenal of proteolytic enzymes present within their granules during the process of mycobacterial killing. Elastase, a major serine protease and myeloperoxidase (MPO), a peroxidase enyzme are such important enzymes stored in the granules of neutrophils. Neutrophil-derived elastase critically contributes to deceleration of pathogen replication during the early phase of antimycobacterial responses (Steinwede et al., 2012). Treatment of human neutrophils with elastase leads to cleavage of caspase 3 and 9 (Ginzberg et al., 2004), which are the main proteins of apoptotic pathway. MPO, another enzyme which is abundantly expressed in neutrophils, is generally associated with killing of bacteria and oxidative tissue injury. It suppresses neutrophil apoptosis in a mouse model of carrageenan-induced lung injury and delays spontaneous self-resolution of pulmonary inflammation (El Kebir et al., 2008). The formation of Neutrophil Extracellular Traps (NET), called NETosis is a self destructive process characterized by the release of cellular DNA, forming an extracellular trap to bind diverse pathogens. This process is also tightly regulated by elastase (Brinkmann et al., 2004) and MPO (Parker et al., 2011). Accordingly, we were interested in understanding the alterations occurring in the release of neutrophil enzymes namely neutrophil elastase, MPO and superoxide dismutase (SOD) during tuberculosis. Furthermore, neutrophils were infected with mycobacterial strains prior to assessing the enzyme release to understand whether mycobacterial strains influence enzyme release.

As neutrophils phagocytose *Mycobacterium tuberculosis* (MTB), destroy them by lytic enzymes in their granules and disintegrate by apoptosis, the release of neutrophil enzymes and apoptosis are inter-related. It is well established that apoptosis is crucial for

Abbreviations: MTB, *Mycobacterium tuberculosis*; HV, healthy volunteers; PTB, pulmonary tuberculosis patients; h, hours; UN, uninfected; AV, annexin V; PI, propidium iodide.

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the resolution of inflammation, failure of which may lead to tissue damage and pathophysiology. Also, MTB is believed to directly influence neutrophil apoptosis. For example, an earlier study states that infection of human neutrophils with MTB induces rapid cell death displaying the characteristic features of apoptosis such as morphologic changes, phosphatidylserine exposure, and DNA fragmentation (Perskvist et al., 2002). Hence we tried to figure out the differences in rate of neutrophil apoptosis upon infection with different MTB strains. This will be of immense help in understanding the role played by these strains in neutrophil life span modulations during tuberculosis.

TB being a chronic disease, neutrophils are not considered to play an active role in it since they are innate cells. Nevertheless, recent advances in the field of clinical research clearly emphasize a role for neutrophils in the initial immune response to MTB. *In vitro* studies suggest that human neutrophils are capable of inhibiting the growth of MTB (Morris et al., 2013) and show MTB induced activation in TB patients (Alemán et al., 2002). Given such importance to neutrophils, regulation of neutrophil functions could lead to the development of novel strategies to prevent and treat TB. Hence, this study was focussed to throw some light into the enzymatic and apoptotic functions of neutrophils. It is already disclosed that virulent strains of MTB employ several strategies to avoid the induction of macrophage cell death, and success in this process is clearly important for bacterial virulence (Briken and Miller, 2008). On that account, we examined the influence of different MTB strains on neutrophils since both neutrophils and macrophages occupy central position in innate immunity and share similar functions.

2. Materials & methods

2.1. Study subjects and specimen collection

The study protocol was approved by the institutional ethical committee (NIRT-IEC No: 2011 009) and followed the institute ethical guidelines. Written informed consents were obtained from blood donors and 10 ml of heparinized blood was collected through venipuncture. The study group consisted of healthy volunteers (HV) ($N = 15$) and newly diagnosed pulmonary tuberculosis patients (PTB) ($N = 15$). The mean age of HV was 27 year (range 22–38 year) who received BCG vaccination in childhood, but their tuberculin skin test status was unknown. They showed no clinical signs and symptoms of tuberculosis or any other immunosuppressive diseases at the time of blood sampling. The mean age of PTB patients was 35 year (range 19–50 year). The inclusion criteria for PTB patients were the diagnosis of sputum positivity for MTB smear and the clinical picture of chest X-ray. All these patients were freshly diagnosed for PTB and were not relapsed cases. Patients with other infections or underlying disease were excluded

2.2. Strain selection and preparation

Two clinical strains (S7 and S10), well characterized epidemiologically (Das et al., 1995) and immunologically (Rajavelu and Das, 2003, 2007) were used in this study. The standard laboratory strain H37Rv was used for comparison. Colonies of MTB from Lowenstein–Jensen slopes were inoculated in Sauton's medium and grown as standing cultures at 37 °C. Log phase cultures were centrifuged, washed with phosphate buffered saline (PBS) (Biowhittaker, Belgium) and bacterial clumps were dispersed by passing them through 26-gauge needle. The bacterial suspension was centrifuged to remove the remaining clumps and the supernatant containing the single cell suspension was adjusted to 5×10^7 cells/ml in sterile, endotoxin free PBS and stored in aliquots at −70 °C until use. The viability of bacilli was enumerated by Colony Forming Unit (CFU) values.

2.3. Neutrophil purification and infection

Human neutrophils from healthy volunteers and PTB patients were isolated by standard protocol (Böyum, 1968). Briefly heparinized venous blood was layered over Ficoll-Hypaque (Amersham Biosciences, USA) for gradient centrifugation followed by sedimentation in 3% Dextran (Sigma Chemicals, USA). The neutrophil rich supernatant was collected and the residual RBCs were lysed by hypotonic lysis. After washing, the neutrophil pellet was subjected to Magnetic Assisted Cell Sorting (MACS) using CD16 microbeads (Miltenyi biotec, Germany) for increasing the purity of isolated neutrophils. The cells were washed and resuspended in RPMI 1640 (Gibco BRL, CA) supplemented with 1% fetal bovine serum (FBS) (Gibco BRL, CA). The viability of cells was assessed to be >95% by the trypan blue exclusion test. Since CD16 is constitutively expressed by all neutrophils, in each experiment, freshly isolated neutrophils were stained with anti-human CD16 (clone 3G8)-fluorescein isothiocyanate (FITC) (BD biosciences, USA) antibody and analysed through fluorescence activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson, USA) and the purity was always found to be >90%. The cell density was adjusted to 1×10^6 cells /ml. The cells were cultured in 5 ml falcon round bottom tubes (BD Biosciences, San Diego, CA) and infected with the target strains (S7, S10 and H37Rv) at the multiplicity of infection (MOI) 3 and cultured for 4 h (h) and 20 h at 37 °C in a humidified 5% CO_2 incubator. From our previous study, an MOI of 3 was found optimum for infecting neutrophils and hence same was kept as standard throughout this study (Pokkali et al., 2009). Since we aimed to study the initial enzyme modifications by different mycobacterial strains, early time point of 4 h was chosen as priority. But, apoptosis is crucial at any stage of neutrophil life span. Hence to understand the apoptosis rate of neutrophils during the different stages of life span, both early (4 h) and late time point (20 h) were chosen. Uninfected neutrophils (UN) served as negative control and 5 nM phorbo myristate acetate (PMA) (Sigma Chemicals, USA) stimulated cells were used as positive control. The use of PMA as positive control was based on our initial standardization experiments and our earlier studies (Hilda et al., 2012, 2014). After 4 h, the cells were harvested and supernatants were collected, centrifuged and stored in aliquots at −70 °C for estimating enzyme release using Enzyme Linked Immuno Sorbent Assay (ELISA). After 4 h and 20 h the cells were harvested and stained with fluorochrome conjugated antibodies and used for FACS staining.

2.4. ELISA for measuring neutrophil enzymes

The cell free culture supernatants were harvested at the end of 4 h culture period and kept frozen at −70 °C until use. The concentration of the enzymes like elastase and MPO were measured using commercial ELISA kits from Hycult Biotech (The Netherlands) and SOD was measured using commercial kit from USCN Life science Inc., (China) respectively, following the manufacturer's instructions. Briefly, the standards/sample were added to the plates and incubated. After washing, an enzyme conjugate solution was added and incubated for 1 h. Again the plates were washed and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to the plates and kept in dark till colour developed. The reaction was stopped with 2N H_2So_4. The plates were read at 450 nm/570 nm in Spectramax Plus 384 Microplate reader (Molecular Devices, USA). The enzyme levels were expressed as picograms per milliliter of the protein for SOD and Nanograms per Milliliter for elastase and MPO.

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