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Rapid Communication

TLR stimulation of human neutrophils lead to increased release of MCP-1, MIP-1α, IL-1β, IL-8 and TNF during tuberculosis

J. Nancy Hilda, Sulochana D. Das *

Dept of Immunology, National Institute for Research in Tuberculosis, Chetput, Chennai 600031, India

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ABSTRACT

Neutrophils inform and shape immune responses. Toll-like receptors (TLRs) play an essential part in the perception of microbes and shape the complex host responses that occur during infection. The TLRs present on neutrophils play an indispensable role in neutrophil mediated pathogen recognition and elimination. This study was done to identify the role of significant TLRs in immune responses leading to differences in cytokine/chemokine release following stimulation. We evaluated the concentrations of various significant cytokines (IL-1β, TNF, MIP-1α, MCP-1 and IL-8) secreted by neutrophils from healthy donors and pulmonary tuberculosis patients following TLR ligand stimulation. TLR stimulation increased the release of such cytokines in both the groups. Thus it is noted that TLR stimulation of neutrophils definitely lead to increased cytokine response. Also, the release of all the studied cytokines are found to be greatly increased in patient neutrophils, affirming that neutrophils undergo secretory level modifications during tuberculosis infection.

1. Introduction

Neutrophils being one of the initial cells that provide early defence against invading microorganisms, their pathogen recognition machinery is of immense importance in any infection. TLRs have emerged as key components of the neutrophil mediated innate-immune system, activating multiple steps in the inflammatory reaction, eliminating invading pathogens, and coordinating systemic defences [1]. They play essential part in the perception of microbes and shape complex host responses that occur during infection [2] leading to a cascade of events resulting in the killing of the pathogen [3].

Toll like receptors (TLRs) are involved in cellular recognition of mycobacteria [4]. The cell surface TLRs are recognized to play important role in innate immune responses. In our preliminary work we used two clinical strains (S7, S10), well characterized epidemiologically [5] and immunologically [6,7] along with laboratory strain H37Rv to study TLR profile in neutrophils. We found that TLR1, TLR2 and TLR4 were induced on neutrophil surface upon *Mycobacterium tuberculosis* (MTB) infection (data to be published). Since all the MTB strains were able to influence these three receptors in some way, we decided to look into the specific TLR stimulated immune responses in neutrophils during tuberculosis infection. For this purpose, the TLR ligands Lipopolysaccharide (LPS) (TLR4 ligand), PAM_3CSK_4 (TLR 1/2 ligand) and FSL-1 (TLR 2/6 ligand) were used for stimulation of TLRs in neutrophils. Since TLR2 can either hetero dimerise with TLR1 or TLR6, two different ligands (PAM_3CSK_4-TLR1/2 ligand, FSL-1-TLR2/6 ligand) were chosen to stimulate this TLR. We decided to analyze the neutrophils at 4 h (h) post stimulation, as our early work proved neutrophils to have great vitality during early stages of their life [8].

Of the various cytokines/chemokines secreted by neutrophils, we studied the release of monocyte influencing chemokines and certain cytokines which were reported to be secreted in higher quantities during tuberculosis. Accordingly, Monocyte chemotactic protein-1 (MCP-1), Macrophage Inflammatory Protein-1α (MIP-1α), Interleukin-1β (IL-1β), IL-8 and (Tumor necrosis factor) TNF were chosen and their concentrations were analyzed in TLR stimulated healthy donor (HD)[1] and newly diagnosed pulmonary tuberculosis patients (PTB)[2] neutrophil supernatants. This was done to understand the effect of TLR stimulation on neutrophil mediated activation of other immune cells as cytokines serve as channels for cellular interaction. Comparisons were made between (a) unstimulated and TLR stimulated cells of the same group and (b) cells

* Corresponding author at: Department of Immunology, National Institute for Research in Tuberculosis (ICMR), 1, Mayor Sathyamoorthy Road, Chetput, Chennai 600031, India.
E-mail addresses: sulochanadas@gmail.com, dsulochana@nirt.res.in (S.D. Das).

[1] Healthy donor-HD.
[2] Newly diagnosed pulmonary tuberculosis patients-PTB.

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stimulated with same ligand in the two groups. Since LPS is the most potent stimulant and TLR4 is thought to be one of the important TLR in mycobacterial infections, the effect of LPS stimulation was also compared with the other two stimulants.

Cytokines serve as messengers, in turn stimulating other immune cells thereby establishing a string of immune cascade. The release of chief cytokines and chemokines by neutrophils were thus studied to perceive the idea of inter cellular signals activated in neutrophils as a result of TLR stimulation.

2. Materials and methods

2.1. Study subjects and specimen collection

The study protocol was approved by the institutional ethical committee (NIRT IEC No. 2011009) 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 HD ($N = 15$, Male-8, Female-7) and PTB ($N = 15$, Male-10, Female-5) individuals. The mean age of HD was 27 yr (range 22–38 yr) 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 yr (range 19–50 yr). The inclusion criteria for PTB patients was based on the diagnosis of sputum positivity for MTB smear, together with the clinical picture of chest X-ray. All these patients were freshly diagnosed for PTB (before the start of treatment) and were not relapsed cases. After proper screening, only HIV negative patients were chosen for the study. Patients with other infections or underlying disease were excluded from the study.

2.2. Neutrophil purification and stimulation

Human neutrophils were isolated by standard protocol [9] with some modifications as explained in Hilda et al., 2014. In each experiment, freshly isolated neutrophils were stained with anti-human CD16 (clone 3G8) – Fluorescein Isothiocyanate (FITC) (BD biosciences, USA) and analyzed through flow cytometer 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 stimulated with TLR ligands namely LPS (20 ng/ml), PAM3CSK4 (500 ng/ml) and FSL-1(20 ng/ml). Since we aimed at observing the immediate effect of TLR stimulation on neutrophils, early time point of 4 h was chosen. Unstimulated neutrophils (UNS) served as negative control and 5 nM phorbo myristate acetate (PMA) (Sigma chemicals, USA) stimulated cells were used as positive control. After 4 h, the neutrophil culture supernatants were collected, centrifuged and used to determine the concentrations of specific chemokines and cytokines present in them and the remaining was stored in aliquots at −70 °C until use. For determination of the concentrations of MCP-1, MIP-1α, IL-1β and TNF, undiluted supernatants were used. For measuring IL-8, the supernatants were diluted to 10× with assay diluent (10× Fetal bovine serum).

2.3. Enzyme Linked Immuno Sorbant Assay (ELISA) for measuring cytokines

The concentration of the cytokines such as MCP-1, IL-1β, TNF and IL-8 were measured using commercial ELISA kits from BD Biosciences, USA and MIP-1α was measured using commercial ELISA kit from R&D systems, USA following the manufacturer's instructions. Briefly, the plates were coated with capture antibody and left overnight at 4 °C. The plates were then washed and standards/sample were added and incubated at room temperature (RT) for 2 h. After washing, working detector 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 color developed. The reaction was stopped with 2 N H_2So_4. The plates were read at 450 nm/570 nm in Spectramax Plus 384 Microplate reader (Molecular Devices). The cytokine concentrations were expressed as pg/ml of the protein. The sensitivity of various parameters studied are given in Table 1.

Table 1
Sensitivity of parameters used.

Cytokine/chemokine	Specificity (pg/ml)
MCP-1[a]	7.8
MIP-1α[b]	46.9
IL-1β[a]	3.9
IL-8[a]	3.1
TNF[a]	7.8

We isolated human neutrophils from healthy donors (15 subjects) and PTB patients (15 subjects) and stimulated these cells with the TLR ligands-PMA, LPS, PAM_3CSK_4 and FSL-1 for 4 h. ELISA was carried out for detection of various cytokines in the supernatants of cultured neutrophils. Commercial ELISA kits were used and the sensitivity of each cytokine is specified in the given table.
[a] Commercial ELISA kits from BD Biosciences, USA.
[b] Commercial ELISA kits from R&D systems, USA.

2.4. Statistical analysis

The data were subjected to statistical analysis using Graph pad prism software (V5.0 for Windows; GraphPad Software, Inc., San Diego, CA, USA). Nonparametric Wilcoxon matched pairs test and Mann–Whitney U test were performed to compute the statistical significance. $P < 0.05$ was considered statistically significant.

3. Results

We stimulated neutrophils from healthy donors and PTB patients with specific ligands for TLR1, 2 and 4 for 4 h to assess the cytokine release strategy. The results obtained are explained below:

3.1. Effect of TLR stimulation on monocyte attracting chemokine release by neutrophils

The two important monocyte attracting chemokines-MIP-1α and MCP-1 were assayed in TLR stimulated neutrophils. Release of these two chemokines by neutrophils were significantly increased ($P < 0.05$) by LPS, PAM_3CSK_4 and FSL-1 compared to unstimulated cells in both the groups as seen in Fig. 1. The TLR 1/6 ligand FSL-1 induced significantly higher release ($P = 0.008$) of MCP-1 by HD neutrophils compared to the TLR4 ligand LPS (Fig. 1A). But inversely, FSL-1 significantly decreased the release of MIP-1α by neutrophils from HD ($P = 0.0017$) and PTB ($P = 0.0012$) groups compared to LPS (Fig. 1B).

3.2. Effect of TLR stimulation on pro inflammatory cytokine release by neutrophils

The pro inflammatory cytokines IL-1β and TNF are primary inflammatory cytokines that mediate local and systemic features of inflammation. Since neutrophils are major cells in any inflammation, the release of such cytokines by neutrophils after stimulation was studied and the results are given below.

Regarding IL-1β secretion, there was significantly increased level ($P < 0.05$) of the cytokine in supernatants stimulated with