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Phenotypic modulation in *Mycobacterium tuberculosis* infected neutrophil during tuberculosis

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***Background & objectives*: Polymorphonuclear leucocytes (PMN) or neutrophils infiltrate to the inflammatory sites and phagocytose mycobacteria thereby inhibiting the bacillary spread initially until the accumulated macrophages get activated. The present study was carried out to highlight the interaction of neutrophils with the two clinical isolates (S7 and S10) of *Mycobacterium tuberculosis* and the subsequent morphological changes.**

***Methods*: Dextran purified neutrophils from normal and TB patients infected with *M. tuberculosis* isolates were cultured for 3 and 18 h time points. At the end of termination, the cell surface expression of CD16, CD69, CXCR2 and induction of apoptosis were analyzed using flow cytometry. Cytokines and chemokines were estimated in supernatants by ELISA.**

***Results*: All infected PMN showed decrease in CD16 at both time points in normals while at 18 h in TB group. Interestingly, CD69 expression was significantly high at early time point in TB-PMN compared to normals. The high expression of CXCR2 was sustained in infected TB-PMN at both the time points. S7 and S10 infected neutrophils showed high phagocytic indices compared to H37Rv in both the groups. A significant increase in apoptosis was observed at both the time points in infected TB-PMN but only at 18 h in normals. Increased pro-inflammatory cytokine (TNF-α) and chemokine (IL-8) response was observed in infected neutrophils at 3 h in both the groups.**

***Interpretation & conclusions*: This study demonstrates the varying degree of modulation of neutrophil functions in both the groups. TB-PMN was more competent in amplifying the innate immune response and conferring protection at the early phase of infection. However, the response was not strain specific in either of these groups.**

Key words Apoptosis - chemokines - cytokines - *Mycobacterium tuberculosis* - neutrophils

The terminally differentiated polymorphonuclear leukocytes (PMN) or neutrophils are essential component of the human innate immune system and act as a first line of defense against invading microorganisms[1]. *Mycobacterium tuberculosis* evades the host killing mechanisms and survives in the infected phagocytes[2]. Infected PMNs secrete tumour necrosis factor (TNF) and interleukin-1 (IL-1) as an early inflammatory response[3]. These cytokine signals are amplified and propagated to elicit the expression of chemokines [interferon-inducible

protein-10 (IP-10), macrophage inflammatory protein-1α (MIP-1α) and IL-8)] which are critical in recruiting the PMN from the blood[4-6]. The timely localization of PMN to infected site is purely by the chemokine receptor expression predominantly the CXCR2. The chemokine gradient is sensed by the cognate receptor displayed on the neutrophils[7].

PMNs undergo rapid spontaneous apoptosis both *in vivo* and *in vitro*. The PMN containing ingested *M. tuberculosis* releases an arsenal of non specific cytotoxic compounds that hastens and exaggerates the inflammatory response leading to its own death by apoptosis[1]. This process appears to be important for the resolution of infection and inflammation at the infected site[8]. Various studies have highlighted the inverse relation of PMN apoptosis with increased shedding of CD16 molecule[9,10]. On the other hand, the activation inducer molecule or very early activation antigen (CD69) is induced *in vitro* on most cell types including the neutrophils. Though the stimuli for the CD69 expression and its role in neutrophil are still unknown, some studies have shown its role in regulating the cytokine production[11]. Thus, studying the apoptosis and inflammatory response of infected PMN would highlight their role during tuberculosis.

We had demonstrated the predominance of *M. tuberculosis* isolates harbouring the single copy of the insertion element IS6110 and their involvement in the active transmission of the disease in this area[12,13]. Immunological studies with these isolates exhibited differential immune response; the isolate S7 modulated the immune response towards Th2 by suppressing Th1 protective immune response while S10 showed potent induction of T-cell proliferation and also interferon- γ (IFN-γ) secretion in purified protein derivative (PPD) positive normal individual[14,15]. In this study we studied the potentiality of these two prevalent clinical isolates in modulating the neutrophil function and its apoptosis. Therefore, the early proinflammatory cytokine (TNF-α and IL-1β) response, modulation in the expression of cell specific markers (CD16 and CD69) and apoptosis of infected PMN were evaluated. The levels of chemokines (IL-8, IP-10 and MIP-1α) and chemokine receptor (CXCR2) expression were also analyzed on the infected PMN to understand their role in PMN and other mononuclear cell recruitment.

Material & Methods

Study subjects and specimen collection: The study was conducted at Tuberculosis Research Centre (TRC), Chennai, during May 2004 - May 2006. The study protocol was approved by the institutional ethical committee and followed the ethical guidelines of the Government General Hospital, Chennai. Written informed consents were obtained from blood donors and 10 ml of heparinized blood was collected through venipuncture.

Two study groups consisted of the normal healthy lab volunteers (N-PMN) from TRC and active pulmonary tuberculosis patients (TB-PMN) from GGH with 25 subjects consecutively enrolled in each study group. All healthy volunteers (mean age 32 yr range 22-45 yr) received BCG vaccination in childhood, but their tuberculin skin test status was unknown. They presented with normal chest X-ray and showed no clinical signs and symptoms of tuberculosis or any other immunosuppressive diseases at the time of blood sampling.

The mean age of TB patients was 35 yr (range 23-58 yr). The inclusion criteria for pulmonary tuberculosis was based on the diagnosis of sputum positivity for *M. tuberculosis* smear, culture and polymerase chain reaction (PCR) positivity (IS6110 specific) together with the clinical picture of the chest X-ray. These patients showed positivity in at least any two of the above criteria and hence were categorized as TB group. Patients with other infections or underlying disease were excluded from the study. All these patients were first time diagnosed as TB and were not relapsed cases. They responded well to anti-tuberculous treatment (ATT) and were followed for first three months. Due to occasional cell culture contamination or the lower cell yield, data from only 20 subjects were included in each of the study groups.

Isolate selection and preparation: Two prevalent clinical isolates (S7 and S10) were chosen for the PMN infection. The standard laboratory strain H37Rv was used for comparison. Colonies of various *M. tuberculosis* isolates from Lowenstein-Jensen (LJ)-slopes were inoculated in Sauton's medium. The composition of this medium per litre was 0.5g $MgSO_4$ (Qualigens, India), 2g citric acid (Qualigens, India), 1g L-asparagine (Hi-media, India), 0.3g KCl (Qualigens, India), 1g glycerol (Qualigens, India), 0.5g Tween 80 (Hi-media, India), 320µl 0.5M $FeCl_3$ (Qualigens, India) and 100 µl of 1M NH_4Cl (Qualigens, India). Log phase cultures were grown as standing cultures at 37°C[16]. These cultures were centrifuged, washed with phosphate buffered saline (PBS) (Biowhittaker, Belgium) and finally the cell density was adjusted

to 2 at the optical density of 600 (OD_{600}). Single colonies obtained on 7H11 plates were further scaled up in Sauton's medium for higher yield for infection studies. The scaled up bacilli were harvested, washed 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 10 X 10^6 cells/ml in sterile PBS and stored in aliquots at -70°C until use.

PMN purification, culture and infection: Human PMN were isolated from the heparinized venous blood by Ficoll-Hypaque (Amersham Biosciences, USA) gradient centrifugation followed by sedimentation in 3 per cent Dextran[1] (Sigma Chemicals, USA). The PMN rich supernatant was collected and the residual RBCs were lysed by hypotonic lysis. The cells were washed and resuspended to 1 X 10^6 cells/ml in RPMI 1640 (Sigma Chemicals, USA) supplemented with 1 per cent fetal calf serum (FCS) (Gibco BRL, CA). The viability of the cells was assessed to be >95 per cent by the trypan blue exclusion test[17]. Totally 0.5X10^6 cells/ml of PMN were cultured in falcon 2063 tubes (BD Biosciences, San Diego, CA) and infected with the target strains (S7, S10 and H37Rv) at the multiplicity of infection (MOI) 3:1 and incubated for 3 and 18 h at 37°C in a humidified 5 per cent CO_2 incubator. Uninfected PMN served as control.

Cell phenotyping by flow cytometry: Cell surface expression of CD 16, CD 69 and CXCR2 were determined by staining the cells using the monoclonal mouse anti-human CD16-fluorescein isothiocyanate (FITC), CD69-CyChrome and CXCR2-allophycocyanin (APC) (BD Pharmingen, USA) conjugated antibodies in 1:5 dilution for 10^6 cells and their fluorescence emission was detected in FL-1, FL-3 and FL-4 channels respectively. Auto fluorescence was determined with unstained PMN sample[2]. Briefly, cells were incubated with PBS containing the combinations of antibodies at saturation for 20 min at 4ºC. Cells were washed and fixed with 4 per cent paraformaldehyde (Sigma Chemicals, USA) in PBS and analyzed on a FACS Calibur flowcytometer (Becton Dickinson, USA). Fluorescence compensation on the flow cytometry was adjusted to minimize the overlap of the FITC, PE, CyChrome and APC signals. For each sample, granulocytes were gated based on forward and side scatter parameters and totally 10,000 gated events were collected for each sample. Data were analyzed using CellQuest software (Becton Dickinson, USA), and were expressed as percentage positive cells for the cell phenotype markers.

Phagocytic assay: To evaluate the phagocytic capacity of PMN, the infected PMN were washed with PBS and fixed on the cover slip with methanol, followed by the Kinyoun's staining[18]. Briefly, the fixed smear of cells was flooded with the carbol fuschin (Sigma Chemicals, USA) for 10 min and the excess stain was washed extensively with distilled water. The smear was then decolorized with 1 per cent acid alcohol, washed well and air-dried. Finally counterstained with 1 per cent methyl green (Sigma Chemicals, USA). The smear was observed under oil immersion objective (X100) of a light microscope (Olympus, Japan) and the percentage phagocytosis was determined by counting 200-300 stained PMN per slide.

Apoptosis assay: For determining the phenomenon of apoptosis, the infected PMN were stained with the TACS™ annexin V-FITC apoptosis detection kit (R & D Systems, USA) according to manufacturer's instruction. Briefly, cells were incubated in the binding buffer containing the annexin V (AV) and propidium iodide (PI) for 20 min at 4ºC in dark. Cells were washed and acquired immediately on the flow cytometer as mentioned above. The fluorescence emission of annexin-V FITC was detected in FL-1 channel and that PI in FL-2 channel. Isotype-control (BD PharMingen, CA) was used to determine the non specific background signals and auto fluorescence to the set quadrant markers for scatter plots. PI staining discriminates cells with intact cell membranes (PI^-) and permeabilized membranes (PI^+). The AV^-/PI^- population was regarded alive and AV^+/PI^- as an early apoptotic while AV^+/PI^+ represented the late apoptotic or the necrotic population of PMN.

Cytokine and chemokine assay: The cell free culture supernatants were harvested at the end of 3 and 18 h and kept frozen at -70°C until use. The concentrations of the inflammatory cytokines like TNF-α, IL-1β and the chemokines like IL-8, MIP-1α and IP-10 were measured using commercial ELISA kits (R & D Systems, USA) following the manufacturer's instructions. The cytokine and chemokine levels were expressed as pg/ml of the protein.

Statistical analysis: The data were subjected to statistical analysis using SPSS 15.0 (SPSS Inc. IL) and were represented as means ± SEM (standard error of mean). Both Student's t test and paired Student's t test were performed to compute the statistical significance. P <0.05 was considered statistically significant.

Results

Modulation of CD16 on infected neutrophils: The expression of CD16 (a low affinity Fc gamma RIII) which is expressed at high level on resting PMN, and its modulation after infection were analysed. Significant decrease ($P<0.05$) in CD16 expression was observed after *M. tuberculosis* infection in N-PMN at both time points while in TB-PMN only at later time point of 18 h (Fig.1A). The decrease was more pronounced at 18 h time point compared to 3 h in both N-PMN and TB-PMN after infection. There was no strain specific modulation of CD16 after infection.

Expression of CD69 on infected neutrophils: The N-PMN exhibited very low CD69 expression at 3 h with gradual upregulation at 18 h in all the infected conditions ($P<0.05$). In contrast, TB-PMN showed a high levels of CD69 at early time point of 3 h which significantly decreased at 18 h ($P<0.05$) (Fig. 1B). Both the N-PMN and TB-PMN demonstrated a significant increase in CD69 expression after infection when compared to the uninfected control ($P<0.05$) at both the time points. The increase at 3 h and decrease at 18 h in the CD69 expression was more prominent in TB-PMN when compared with N-PMN ($P<0.05$).

Expression of chemokine receptor CXCR2: A significant upregulation of CXCR2 ($P<0.05$) was observed on Rv and S7 infected N-PMN at both time points. In TB-PMN, a significant upregulation of CXCR2 was observed under all infected conditions and at both the time points ($P<0.05$). Further, TB-PMN showed a significant increase in CXCR2 expression in comparison to N-PMN at both the time points ($P<0.05$) (Fig. 1C).

Phagocytosis of M. tuberculosis by neutrophils: At early time point of 3 h, there was an increase in phagocytic indices for the clinical isolates S7 and S10 when compared to H37Rv in both N-PMN and TB-PMN ($P<0.05$). At 18 h, no such change was observed. However, both the clinical isolates showed significant decrease in phagocytic indices in N-PMN while only S10 in TB-PMN when compared with their respective 3h indices ($P<0.05$) (Table I). The percentage phagocytosis was further divided into 4 categories *i.e.,* 0, 1-10, 10-20 and >20 bacilli per cell, and similar changes were found in lower infection categories of 1-10 and 10-20 bacilli per cell (data not shown).

Apoptosis of infected PMN: PMN tend to undergo spontaneous apoptosis and hence strong annexin V positivity was observed in both N-PMN and TB-PMN irrespective of time. At 3 h, in N-PMN only H37Rv while in TB-PMN all strains showed significant increase in apoptosis compared to uninfected control ($P<0.05$). At 18 h, all showed significant increase in apoptosis in both PMN compared to uninfected control ($P<0.05$) (Table II).

Pro-inflammatory cytokine release by infected neutrophils: At 3 h after infection, N-PMN showed

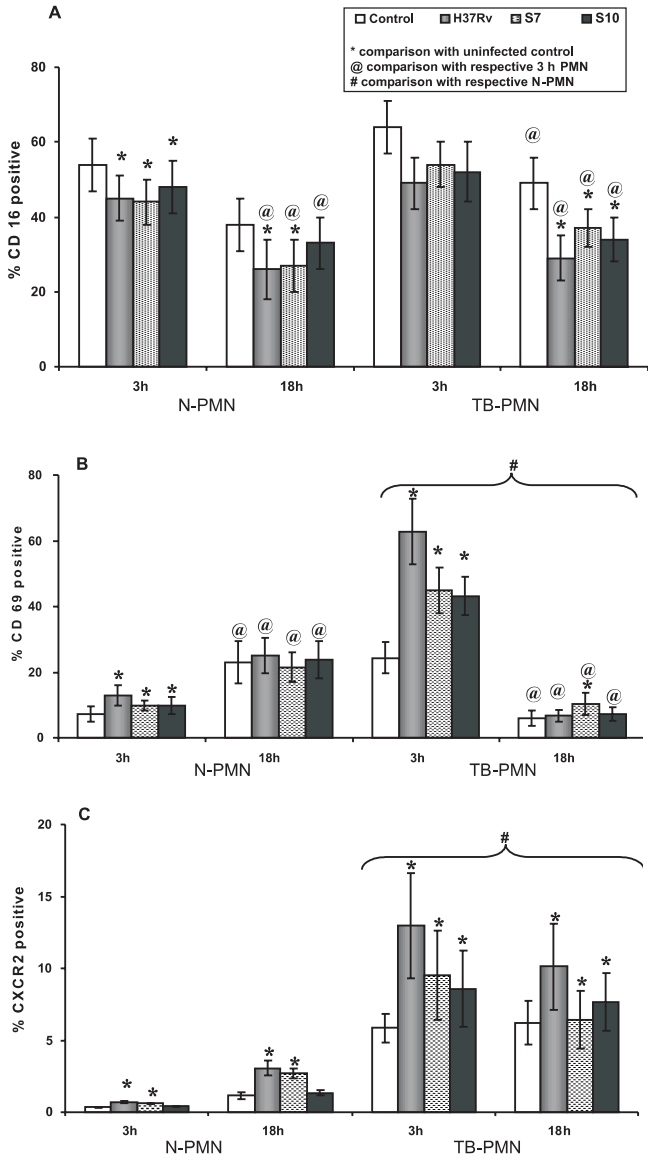

Fig. 1. Depicts the percentage expression of CD16 positive cells **(A),** the activation marker CD69 **(B)** and chemokine receptor CXCR2 **(C)** on CD16 positive cells upon infection with S7 and S10 and H37Rv laboratory strain infected of normal (N-PMN) (N=20) and tuberculosis neutrophils (TB-PMN) (N=20) at 3 and 18 h. Values are represented as mean ± SEM. *$P<0.05$ represents the statistical significance.

Table I. Total phagocytic indexes for various *M. tuberculosis* strains by N-PMN and TB-PMN

	N-PMN (n=15)		TB-PMN (n=15)	
	3 h	18 h	3 h	18 h
H37Rv	80.5 ± 5.6	82.2 ± 3.4	62.3 ± 6.5	79.0 ± 3.2^{+}
S7	149.7 ± 33.8*	87.8 ± 3.2^{+}	90.8 ± 5.4*	82.4 ± 3.5
S10	119.1 ± 10.8*	87.6 ± 4.1^{+}	94.6 ± 6.8*	82.7 ± 3.0^{+}

$^{*}P$<0.05 compared with H37Rv
^{+}P<0.05 compared with respective 3 h
Calculation of phagocytic indexes: the ratio of the percentage of total cells counted with and/or without the bacilli to the number of cells with bacilli alone

Table II. Apoptosis of neutrophils induced by the *M. tuberculosis* strains

	N-PMN (n=20)		TB-PMN (n=20)	
	3 h	18 h	3 h	18 h
Control	13.4 ± 4.0	20.6 ± 5.8	21.5 ± 6.1	24.6 ± 6.2
H37Rv	19.6 ± 4.48*	25.6 ± 4.3*	26.65 ± 5.8*	29.2 ± 7.0*
S7	16.0 ± 4.9	26.0 ±4.6*	26.5 ± 6.28*	29.0 ± 5.8*
S10	17.0 ±5.1	26.2 ±5.2*	26.5 ± 5.1*	28.4 ± 7.0*

Data are mean ± SEM
$^{*}P$<0.05 compared with uninfected control

significant increase in IL-1β compared to their respective controls (P<0.05). Further significant increase in IL-1β levels was observed at 18 h in all N-PMN (P<0.05). No significant change in IL-1β levels were observed in TB-PMN at both time points (Fig. 2A).

All infected PMN from both groups (N-PMN and TB-PMN) showed significant increase in TNF-α levels at both time points when compared with their respective controls (P<0.05) (Fig. 2B).

Chemokine production by infected neutrophils: H37Rv and S7 showed a significant increase in the IL-8 concentration when compared to control (P<0.05) in the N-PMN at 3 h. The S7 and S10 showed a marked increase in IL-8 levels in the 3 h TB-PMN and only S7 at 18 h. In totality the 18 h TB-PMN was found to be significantly high for IL-8 levels when compared with the 3 h TB-PMN (P<0.05) (Fig. 3A).

Significantly high levels of MIP-1α were found in infected N-PMN at 18 h while in TB-PMN at 3 h (P<0.05) (Fig. 3B).

The levels of IP-10 were significantly high in infected N-PMN only at 18 h as compared to their respective controls (P <0.05) with no significant change in TB group for either time points (Fig. 3C).

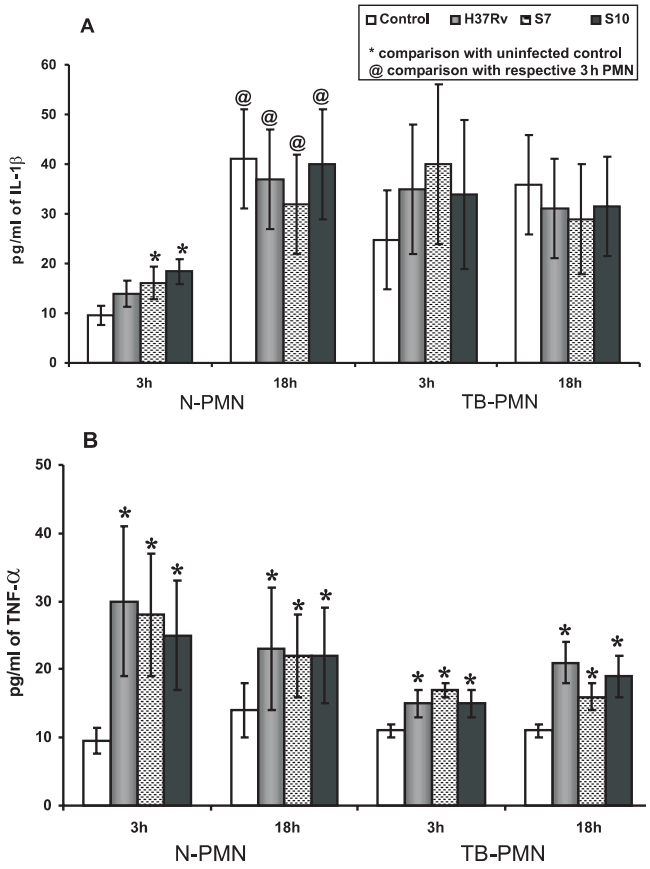

Fig. 2. Secretion pattern of pro-inflammatory cytokine IL-1β **(A)** and TNF-α **(B)** by N-PMN (N=20) and TB-PMN (N=20) post infection with S7, S10 and H37Rv at 3 and 18 h. Values are mean ± SEM. The statistical significance is shown as *P<0.05.

Discussion

Neutrophils constitute the bulk of early recruited leukocyte population that mediate and elicit immune response against *M. tuberculosis*. Though many reports highlighted the capability of various *M. tuberculosis* isolates modulating the monocyte or macrophage functions[19-23], very few studies demonstrated the importance of PMN during TB[24-26]. In this study, an attempt was made to highlight the functional aspects of PMN upon infection with prevalent clinical isolates of *M. tuberculosis*.

Certain anti-apoptotic factors, like cytokines and growth factors allow PMN to maintain a steady state CD16 expression and exhibit proper cellular function[1]. Interaction of bacillius or its antigen with PMN alters CD16 expression which is essentially required to prevent apoptosis and protect PMN against macrophage engulfment. A significant decrease in CD16 expression

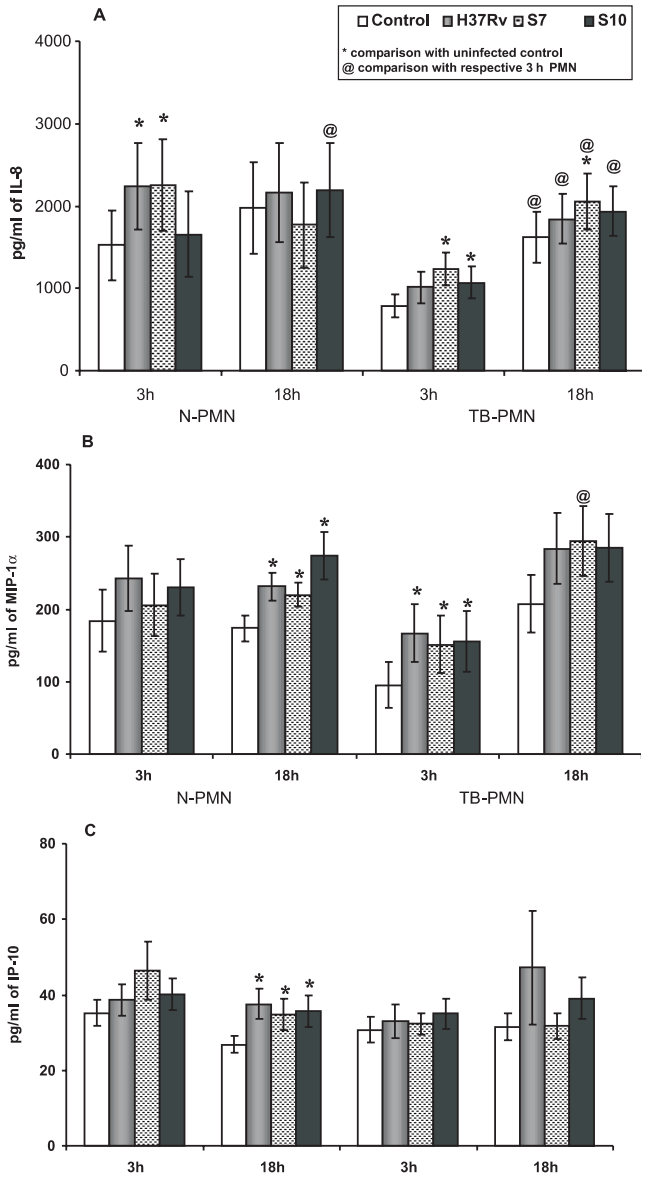

Fig. 3. The levels of IL-8 **(A)**, MIP-1α **(B)** and IP-10 **(C)** in the culture supernatants of N-PMN (N=20) and TB-PMN (N=20) post infection with S7, S10 and H37Rv at 3 and 18 h. Values are mean ± SEM. The statistical significance is shown as *@(P<0.05).

at 3 h of infection was observed only in N-PMN and not in TB-PMN. This could be due to *in vivo* priming of TB-PMN during tuberculosis[27]. The increased loss of CD16 at 18 h in both the groups indicated its shorter life span. Surprisingly, there was no strain related changes in shedding of CD16 on both PMN to help in delaying the apoptosis.

Neutrophils can be induced to express activation marker, CD69 after stimulation. There is no specific ligand identified for CD69 activation, but studies indicate the importance of CD69 in signal transduction for the release of cytokine by PMN[28,29]. The isolates *M. tuberculosis* strains in this study were potent in driving more of CD69 expression on TB-PMN than N-PMN. The increased expression of CD69 on infected TB-PMN at 3 h, clearly indicated that these cells were prior activated *in vivo* and further *in vitro* infection magnified the expression of CD69, which diminished at 18 h priming the activated PMN to undergo apoptosis due to over bacillary load.

During chronic infections like TB, the PMN are persistently recruited to the sites of infection as an early immune response in which chemokines and their receptors play an important role. The CXCR2 which is expressed predominantly on neutrophils migrate in response to IL-8. We observed upregulation of CXCR2 at 3 h in all infected TB-PMN. In contrast to our results, Juffermans *et al*[30] have reported lower levels of CXCR2. This difference may be due to use of lypopolysaccharide (LPS), lypoarabinomannan (LAM) and not the whole live organisms in their *in vitro* experiments. Further, our observation of increased CD69 expression correlated well with increased CXCR2 and indicated that PMN gets activated and then migrates to the infection nodule.

Neutrophils after encountering mycobacteria, exhibit an early bactericidal responses including phagocytosis. The reduced viability of *M. tuberculosis* clinical isolates was observed in PMN and other short lived cells[23]. We observed significant increase in phagocytosis of clinical isolates by both N-PMN and TB-PMN at 3h which reduced at 18 h indicating that these isolates could not withstand host defense at the first few hours.

Infected PMN undergo rapid cell death displaying the characteristic features of apoptosis such as morphologic changes and phosphatidylserine exposure on the cell surface. A significant increase in apoptosis of infected PMN of both groups was seen at 18 h post infection. However, H37Rv and S7 infected TB-PMN showed increase in early apoptosis at 3 h after infection. It is likely that these isolates of mycobacteria may utilize multiple, distinct strategies to prevent host cell death by minimizing external signals that induce host cell apoptosis in healthy individuals during the period of early innate immune response[31]. Moreover, infection studies on monocyte-derived macrophages with attenuated strain of mycobacteria proved that it is apoptosis and not necrosis that reduces *M. tuberculosis* colony forming units (cfu) recovered during *in vitro* infection[32].

Neutrophils though encounter the mycobacteria, are not effective in scavenging. Thus the neutrophils play an important role in the transition from innate to adaptive immune responses by producing critical macrophage activating cytokines like the TNF-α, IL-1β and other immune cells attracting chemokines like IL-8, MIP-1α and IP-10. These chemokines and cytokines transduce a paracrine or an endocrine signals to the proximal lymph node to recruit the activated PMN, followed by monocytes and T-cells. The significant increase in cytokine and chemokine levels of infected PMN in this study confirmed their role in amplifying a protective cell mediated immune response to effectively curtail the infection[2,31-33]. The antigen presenting cells like monocytes, macrophages and dendritic cells would participate in the immune reaction for a longer period of time and exhibit strain specific modulation in the immune response. On the other hand, neutrophils being the first line of defense with lower life span could not mount strain specific immune response and hence no significant differences were observed in our study within the clinical isolates. However, neutrophils do help initially to decrease the bacillary load for the antigen presenting cells to elicit and build up an effective immune response.

To conclude, the present study showed that neutrophils shed CD16 irrespective of the infecting isolates thereby initiating the apoptosis of PMN during tuberculosis. Moreover, the expression of activation marker CD69 depicted the activation status of PMN upon encounter with bacterium. The decreased chemokine receptor CXCR2 expression highlighted the retarded migration of N-PMN at both the time points. The pro-inflammatory cytokine and chemokines responses were involved in amplifying the host immune response and conferring protection at the early phase of infection. Although, N-PMN and TB-PMN showed significant uptake of bacilli, there was no strain specific modulation in these cells. Moreover, the increased apoptosis of infected PMN from both the groups thus emphasize the initial encounter of these cells in immune response against *M. tuberculosis*. Further studies are needed to focus on the mechanisms of interplay of PMN with bacilli and antigen presenting cells.

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