RESEARCH ARTICLE

Mycobacterium tuberculosis H37Rv is more effective compared to vaccine strains in modulating neutrophil functions: an *in vitro* study

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Abstract

Neutrophils are the primary cells contributing to initial defense against mycobacteria. Yet, little is known about the potential of various mycobacterial strains to stimulate neutrophils. This study was focused to compare the differential capacity of vaccine strains, *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) and *Mycobacterium indicus pranii* (Mw), and laboratory strain H37Rv to activate and enhance neutrophil functions. The expression of phenotypic markers like Fcγ receptor, toll-like receptor (TLR), and chemokine receptor; secretion of pro-inflammatory cytokines; and the rate of apoptosis were studied in infected neutrophils. Increased expression of CD32, CD64, TLR4, and CXCR3; increased TNF-α secretion; and downregulation of early apoptosis were observed in H37Rv-infected neutrophils. Among the vaccine strains, BCG increased the expression of only CD32 on neutrophils, while Mw was comparatively ineffective. To understand the paracrine role of neutrophils, the supernatants from infected neutrophils were used to stimulate monocytes and T helper cells. The secretory molecules from all infected neutrophils increased the expression of CCR5 on monocytes, whereas only H37Rv-infected supernatant increased the expression of CCR7 on monocytes and CD69 on T cells. Thus, H37Rv was more effective in activating neutrophils and in turn stimulating monocytes and T cells. By comparison, vaccine strains were less effective in modulating neutrophil functions.

Introduction

In response to *Mycobacterium tuberculosis* (MTB) infection, polymorphonuclear neutrophilic granulocytes (PMN) or neutrophils are among the first innate immune cells recruited to the site of microbial entry (Korbel *et al.*, 2008). Subsequently, activated neutrophils kill the bacteria and initiate innate and adaptive immunity by producing important pro-inflammatory cytokines, chemokines, and other granule products that can drive the recruitment of monocytes, T cells, and dendritic cells (DCs) (Scapini *et al.*, 2000; Yamashiro *et al.*, 2001; Alemán *et al.*, 2007; Sawant & McMurray, 2007; Mantovani *et al.*, 2011). The secretory products of PMN have also been shown to regulate antimicrobial activities in monocytes and macrophages (Soehnlein *et al.*, 2007).

The neutrophil cell membrane expresses a complex array of adhesion molecules and receptors for various ligands, including mediators, cytokines, immunoglobulins, and membrane molecules on other cells. The FCγ receptors namely CD32 and CD64, expressed on neutrophils, have been shown to promote phagocytosis and respiratory burst (Hoffmeyer *et al.*, 1997; Rivas-Fuentes *et al.*, 2010). Also, PMN infected with MTB undergo apoptosis, which is essential for the resolution of inflammation (Kasahara *et al.*, 1998; Alemán *et al.*, 2002). Neutrophils recognize microbial molecules through toll-like receptors (TLRs). In turn, TLR-stimulated neutrophils help in recruitment of innate, but not acquired, immune cells to sites of infection (Hayashi *et al.*, 2003). Thus, beside their key function as professional phagocytes, neutrophils influence both the induction phase and the effector phase of immunity.

A strong immune response enough to prime the innate immunity and in turn the adaptive immunity is sufficient to counteract subsequent infections. A vaccine administered

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with such vigor will thus be effective to the optimum level. *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) is the only vaccine available today for the protection against tuberculosis. Many human studies have been carried out to understand effective and protective immune responses post-BCG vaccination (Burl *et al.*, 2010; Smith *et al.*, 2010). However, very few studies have focused on the effect of BCG on the functions of granulocytic PMN.

Mycobacterium indicus pranii (MIP), also known as Mw, is another potent immunomodulator and shares antigens with MTB. Mw enhances T-helper1 response, resulting in the release of type-1 cytokines, predominantly interferon-γ, and thereby propagates cell-mediated immune responses (Nyasulu, 2010). In experimental models, Mw has shown a protective effect against tuberculosis in mice (Singh *et al.*, 1992). Clinical trials have shown significant benefits of Mw in leprosy (Zaheer *et al.*, 1993). Thus, Mw can be a successful vaccine candidate for tuberculosis (TB), and further clinical studies are planned in this direction.

There is an increasing support to the hypothesis that PMN are involved in early inflammatory host response during mycobacterial infections and hence might be involved in immune protection against them (Brown *et al.*, 1987). In our previous study, we have shown modulation of immune responses in normal and TB PMN by clinical strains of MTB (S7 and S10) (Pokkali *et al.*, 2009). However, very little is known about the interaction of PMN with vaccine strains of mycobacteria. As neutrophils are the first cells to get exposed to any antigen and generate early immune response, their interaction with vaccine strains will help us to understand the exact nature of protective immune response.

Hence, we studied *in vitro* modulation of neutrophil functions like phenotypic changes, apoptosis rate, and inflammatory cytokines after infection with vaccine strains (BCG and Mw) and compared with standard laboratory strain H37Rv. To understand the paracrine role of neutrophils and their influence on mononuclear cell recruitment, we also studied the expression profile of the activation markers and chemokine receptors on T cells and monocytes.

Materials and methods

Study subjects and specimen collection

The study protocol was approved by the institutional ethical committee 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 normal healthy volunteers ($N = 11$) (mean age 24 years, range 22–28 years) 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.

Strain selection and preparation

Two vaccine strains, namely BCG and Mw, available in India were used. The standard laboratory strain live H37Rv was used for comparison. Live, attenuated BCG vaccine was purchased from Serum institute of India, Chennai. Heat-killed Mw vaccine was purchased from Cadila pharmaceuticals Limited, Ahmedabad. Colonies of H37Rv from Lowenstein-Jensen-slopes were inoculated in Sauton's medium and grown as standing cultures at 37 °C. Log-phase cultures were centrifuged and 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^{-1} in sterile, endotoxin-free PBS and stored in aliquots at −70 °C until use. The viability of bacilli was enumerated by CFU values.

Neutrophil purification and infection

Human neutrophils were isolated by standard protocol (Böyum, 1968). Briefly, heparinized venous blood was layered over Ficoll-Hypaque (Amersham Biosciences) for gradient centrifugation followed by sedimentation in 3% dextran (Sigma Chemicals). The PMN rich supernatant was collected, and the residual RBCs were lysed by hypotonic lysis. The cells were washed and resuspended in RPMI 1640 (Gibco BRL, CA) supplemented with 1% fetal bovine serum (FBS) (Gibco BRL). The viability of the cells was assessed to be > 95% by the trypan blue exclusion test, and the purity was always found to be > 90%. The cell density was adjusted to 0.5×10^6 cells mL^{-1}. The cells were cultured in 5-mL falcon round-bottom tubes (BD Biosciences, San Diego, CA) and infected with the target strains (BCG, Mw and H37Rv) at the multiplicity of infection (MOI) 3 and incubated for 4 h at 37 °C in a humidified 5% CO_2 incubator. From our previous study (Pokkali *et al.*, 2009), an MOI of 3 was found optimum for infecting PMNs, and hence, same was kept as standard throughout this study. Because we aimed at observing the initial effect of mycobacterial vaccine strains on neutrophils, early time point of 4 h was chosen. Uninfected neutrophils (Control) served as negative control, and 10 nm phorbol myristate acetate (PMA) (Sigma Chemicals)–stimulated cells were used as positive control. After 4 h, the neutrophil culture supernatants (Nu sups)

were collected, centrifuged, and used to stimulate peripheral blood mononuclear cells (PBMCs), and the remaining was stored in aliquots at −70 °C until use. The cells were washed with PBS twice and used for fluorescence-activated cell sorting (FACS) staining protocol as given in the section 'cell phenotyping by flow cytometry'.

Culture of PBMCs with Nu sups

The buffy coat containing PBMCs was collected after Ficoll-Hypaque density gradient centrifugation. The cells were washed once with Hanks' balanced salt solution (HBSS) and suspended in RPMI 1640 medium supplemented with 1% FBS. The cell viability was always found to be > 95% through trypan blue exclusion test, and the cell density was adjusted to 1×10^6 mL^{-1}. The cells were stimulated with 200 μL of infected Nu sups and cultured in 12 Well Clear TC-Treated Multiple Well Plates (Corning Life Sciences) for 18 h at 37 °C in a humidified 5% CO_2 incubator. After 18 h, the cells were harvested and stained for FACS as given in the section 'cell phenotyping by flow cytometry'.

Cell phenotyping by flow cytometry

Cell surface expression of CD32, CD64, TLR-4, and CXCR3 on neutrophils (CD16+ve); CD69 and CXCR3 on T helper cells (CD4+ve); and CCR5 and CCR7 on monocytes (CD14+ve) was determined by staining the cells using the monoclonal mouse anti-human conjugated antibodies, i.e. CD16 (clone 3G8)–fluorescein isothiocyanate (FITC), TLR-4 (clone HTA125)–phycoerythrin (PE), CD32 (clone FL18.26), CD64 (clone 10.1), CD4 (clone RPA T4), CD14 (clone M5E2)–allophycocyanin (APC), CD69 (clone FN50)–phycoerythrin-cyanine5 (PE-Cy5) (BD Pharmingen), and CCR5 (clone 45549)–FITC, CCR7 (clone 150503), CXCR3 (clone 49801)–PE (R & D Systems), and their fluorescence emission was detected in FL-1 (FITC), FL-2 (PE), FL-3 (PE-Cy5), and FL-4 (APC) channels. The above specified clones were used throughout the study. Briefly, cells were incubated with PBS containing the combinations of antibodies at saturation for 20 min at 4 °C. Cells were washed and fixed with 1% paraformaldehyde (Sigma Chemicals) in PBS and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). An unstained sample and negative isotype immunoglobulin-stained control (mouse IgG) and single-stained samples for each conjugated dye were used as controls for setting up the flow cytometry initially. Fluorescence compensation on the flow cytometry was adjusted to minimize the overlap of the fluorochrome signals. For each sample, neutrophils were gated based on forward and side scatter parameters followed by gating CD16+ve cells, monocytes by gating CD14 +ve cells, and T helper cells by gating of CD4+ve cells, and totally 30 000 gated events were collected for each sample. Data were analyzed using Flowjo software (Three Star Inc.) and were expressed as median fluorescence intensity (MFI) for the cell phenotype markers.

Apoptosis assay

For determining the phenomenon of apoptosis, the infected neutrophils were stained with the Annexin V: FITC Apoptosis Detection Kit I (BD biosciences) according to manufacturer's instruction. Briefly, cells were incubated in the binding buffer containing the annexin V (FITC) and propidium iodide (PI) for 15 min at RT in dark. Cells were washed and acquired immediately on the flow cytometer. The fluorescence emission of annexin V FITC was detected in FL-1 channel and that of PI in FL-3 channel. Totally, 50 000 gated events were collected for each sample. PI staining discriminates cells with intact cell membranes (PI−) and permeabilized membranes (PI+). The AV−/PI− population was regarded alive and AV+/PI− as early apoptotic, while AV+/PI+ represented the late apoptotic, and AV−/PI+ was regarded as the necrotic population.

Cytokine assay

The cell-free culture supernatants were harvested from the infected neutrophils at the end of 4 h and kept frozen at −70 °C until used for cytokine assays. The inflammatory cytokines like TNF-α and IFN-γ were measured in Nu sups using commercial ELISA kits (BD biosystems) following the manufacturer's instructions. The cytokine levels were expressed as pg mL^{-1}. The sensitivity of TNF alpha was 7.8 pg mL^{-1} and of IFN gamma 4.7 pg mL^{-1}.

Statistical analysis

The data were subjected to statistical analysis using GRAPH PAD PRISM software (V5.0 for Windows; GraphPad Software, Inc., San Diego, CA). Nonparametric Mann–Whitney *U*-test was performed to compute the statistical significance. $P < 0.05$ was considered statistically significant.

Results

Expression of Fcγ receptors CD32 (FcγRII) and CD64 (FcγRI) on neutrophils

Figure 1 shows representative histograms (a and b) and Box and Whisker plots (C and D) for CD 32 and CD64, respectively. As shown in Fig. 1c, expression of CD32 was significantly increased in BCG ($P = 0.04$)- and H37Rv ($P = 0.002$)-infected and PMA ($P = 0.01$)-stimulated neutrophils when compared to control. Although an increased

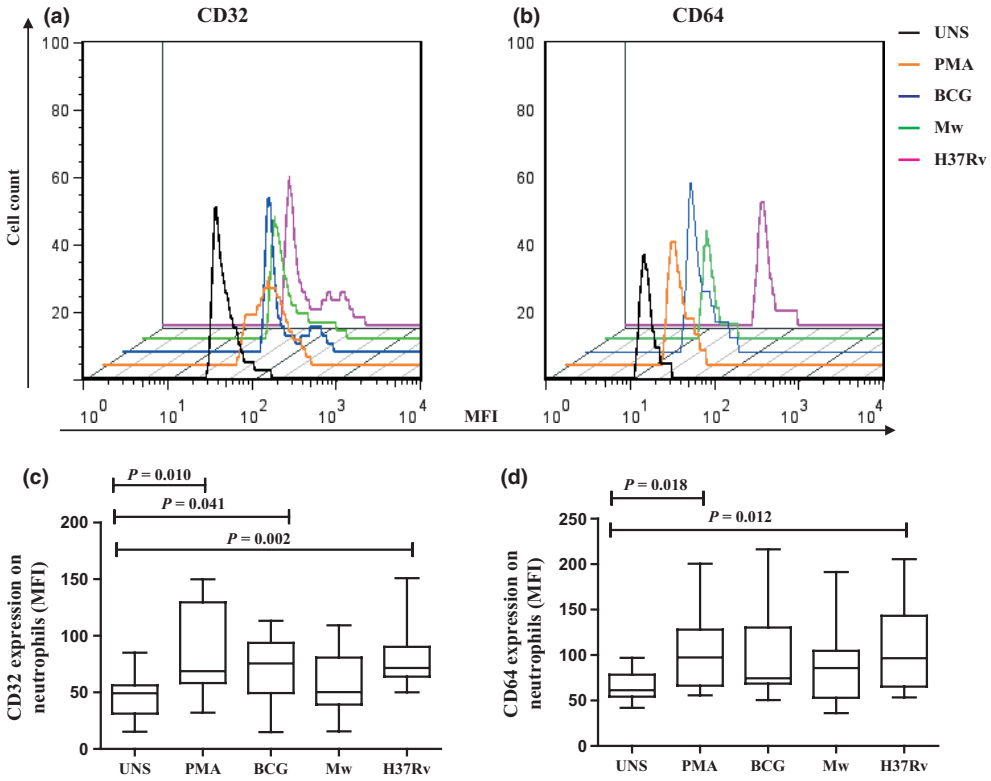

Fig. 1. Expression of Fcγ receptors: neutrophils were stimulated with PMA and infected with BCG, Mw, and H37Rv for 4 h at MOI of 3, and the expression of Fcγ receptors, CD32 (FcγRII) and CD64 (FcγRI), were analyzed by flow cytometry. (a) and (b) are representative histograms for CD32 and CD64, respectively. (c) and (d) show the MFI values of CD32 and CD64 for 11 individual donors in Box and Whisker plots. The line within the box indicates the median.

expression of CD32 was observed in Mw-infected neutrophils, the increase was not significant. As shown in Fig. 1d, expression of CD64 was significantly increased in PMA ($P = 0.01$)-stimulated and H37Rv-infected neutrophils ($P = 0.01$), but not in vaccine strains.

Expression of cell surface receptors (CXCR3 and TLR4) on neutrophils

Expression of CXCR3 and TLR4 is shown in Fig. 2 as representative histograms (a and b) and Box and Whisker plots (c and d). Expression of both these receptors was significantly higher in PMA-stimulated ($P = 0.02$, 0.01) and H37Rv-infected neutrophils ($P = 0.007$, 0.003) compared to control. BCG and Mw did not show any effect on the expression of these receptors (c and d).

Apoptosis of neutrophils

Apoptosis of neutrophils was significantly downregulated in its early stages by H37Rv ($P = 0.01$) when compared with the control. Other strains did not influence the rate of early apoptosis (Table 1). Considering late apoptosis, H37Rv ($P = 0.003$) and BCG ($P = 0.01$) induced significantly higher apoptosis when compared with Mw. When compared with control, there was an increasing trend in the rate of late apoptosis of H37Rv-infected neutrophils, but the change was not significant (Table 1). Similarly, PMA ($P = 0.001$), BCG ($P = 0.03$) and H37Rv ($P = 0.0005$) significantly increased the necrotic cell population when compared to control. Also, H37Rv ($P = 0.002$) was able to significantly increase the necrosis of neutrophils compared with Mw (Table 1). A representative scatter plot of apoptosis is shown in Fig. 3.

Secretion of pro-inflammatory cytokines by infected neutrophils

Figure 4 represents levels of pro-inflammatory cytokines in infected neutrophil supernatants. Significantly higher levels of TNF-α were observed in H37Rv-infected ($P = 0.01$) and PMA-stimulated ($P = 0.03$) neutrophils. Vaccine strains did not have profound effect on the release of TNF-α by neutrophils (a). None of the strains was able to modulate the secretion of the major pro-inflammatory cytokine IFN-γ by neutrophils (b).

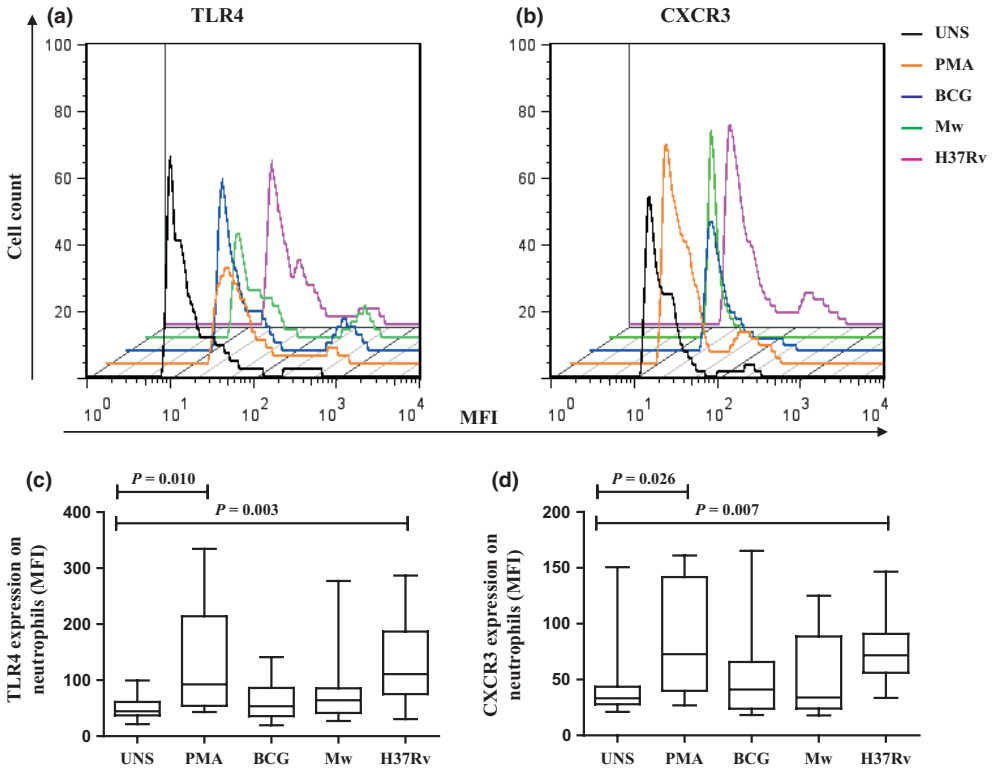

Fig. 2. Expression of cell surface signaling receptors: neutrophils were stimulated with PMA and infected with BCG, Mw, and H37Rv at MOI of 3 for 4 h, and the expression of CXCR3 and TLR4 on these neutrophils was analyzed by flow cytometry. (a) and (b) are representative histograms for CXCR3 and TLR4 expression; (c) and (d) represent the MFI values of CXCR3 and TLR4 in Box and Whisker plots. The line within the box indicates the median.

Table 1. Apoptosis of neutrophils: neutrophils were stimulated with PMA and infected with BCG, Mw, and H37Rv for 4 h, and rate of early apoptosis, late apoptosis, and necrosis was analyzed by flow cytometry

	Early apoptosis	Late apoptosis	Necrosis
UNS	5.3	0.4	0.4
PMA	3.5	1.0	2.1*
BCG	3.5	1.2†	1.6*
Mw	3.5	0.5	0.7
H37Rv	3.2*	1.4†	1.9*†

The table shows the median values of percentage positive cells for early apoptosis, late apoptosis, and necrosis. $P < 0.05$ was considered to be statistically significant.
UNS, unstimulated.
*Significantly different when compared to the respective UNS.
†Significantly different when compared to the respective Mw.

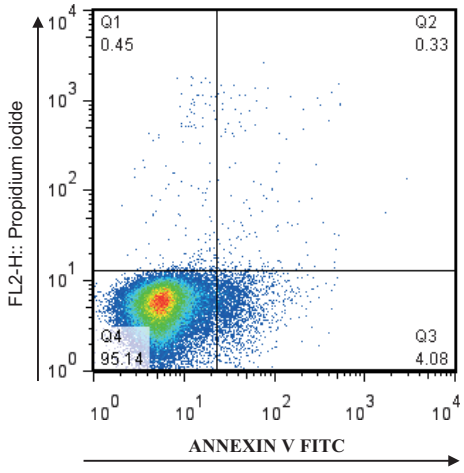

Fig. 3. Apoptosis of neutrophils: neutrophils were stimulated with PMA and infected with BCG, Mw, and H37Rv at MOI of 3 for 4 h, and rate of early apoptosis, late apoptosis, and necrosis was analyzed by flow cytometry. A representative scatter gram shows early apoptotic, late apoptotic, and necrotic cells from a single donor, in the quadrants Q3, Q2, and Q1, respectively.

Effect of neutrophil secretory products on monocytes

Figure 5 depicts the expression of chemokine receptors CCR5 and CCR7 in representative histograms (a and b) and Box and Whisker plots (c and d). The expression of

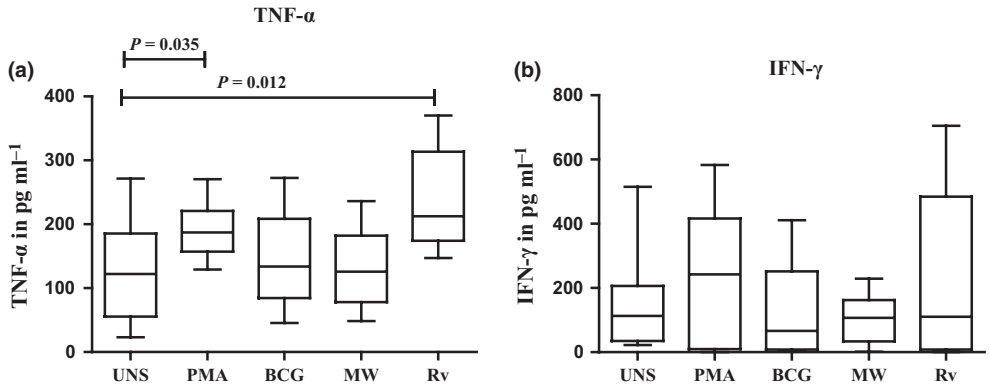

Fig. 4. Secretion of pro-inflammatory cytokines: neutrophils were stimulated with PMA and infected with BCG, Mw, and H37Rv at MOI of 3 for 4 h, and the culture supernatants were used to measure TNF-α (a) and IFN-γ (b) by ELISA. The values are represented in pg mL^{-1}.

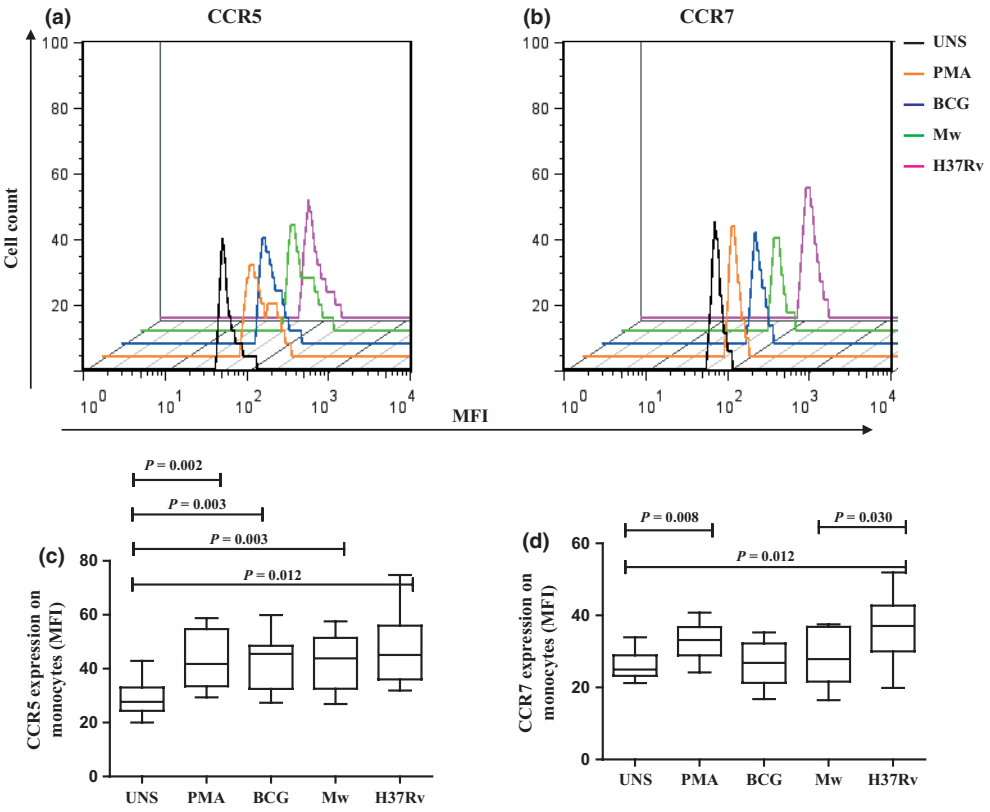

Fig. 5. Effect of neutrophil secretory products on monocytes: neutrophils were stimulated with PMA and infected with BCG, Mw, and H37Rv at MOI of 3 for 4 h, and the culture supernatants were used to stimulate autologous PBMCs overnight. The PBMCs were stained for flow cytometry analysis to check for the expression of various markers. The expression of chemokine receptors CCR5 and CCR7 by monocytes were analyzed through flow cytometry. (a) and (b) are representative histograms for the expression of CCR5 and CCR7, respectively, while (c) and (d) depict the MFI values of CCR5 and CCR7 of 11 individual donors in Box and Whiskers plots. The line inside the box is the median value.

CCR5 was significantly upregulated in all conditions (PMA: $P = 0.002$, BCG: $P = 0.003$, Mw: $P = 0.003$, H37Rv: $P = 0.01$) (c). With PMA-stimulated Nu sups, significantly increased expression of CCR7 ($P = 0.008$) was observed on monocytes. Similarly, CCR7 showed significantly higher expression on stimulation with Nu sups from H37Rv ($P = 0.01$) but not from BCG and Mw. Also, there was a significantly higher expression of CCR7 on monocytes stimulated with H37Rv-infected Nu sups ($P = 0.03$) when compared to Mw-infected sups (d).

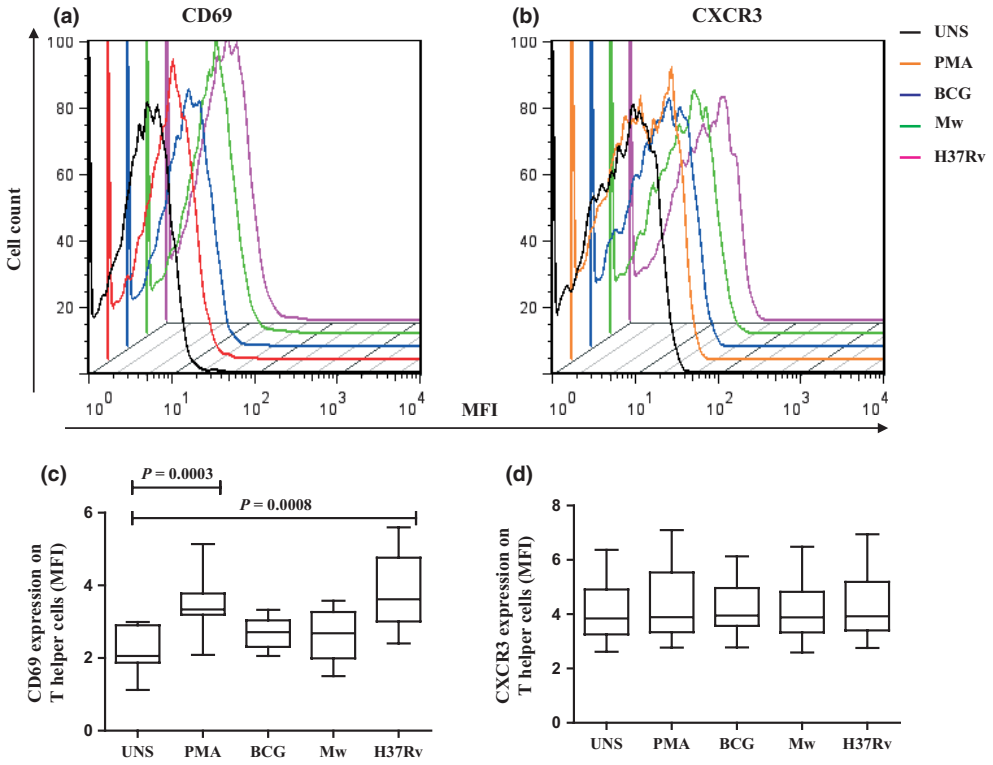

Fig. 6. Effect of neutrophil secretory products on T helper cells: Neutrophils were stimulated with PMA and infected with BCG, Mw, and H37Rv at MOI of 3 for 4 h, and the culture supernatants were used to stimulate autologous PBMCs overnight. After culture, the PBMCs were stained for flow cytometry analysis to check for the expression of CD69 and CXCR3 on T helper cells. (a) and (b) are representative histograms for CD69 and CXCR3, respectively, while (c) and (d) are box and whisker plots representing the MFI values of these receptors from 11 individual donors. The line inside the box is the median value.

Effect of neutrophil secretory products on T helper cells

Figure 6 depicts the expression of CD 69 and CXCR3 in representative histograms (a and b) and Box and Whisker plots (c and d). The activation marker CD69 was found to be significantly upregulated when stimulated with H37Rv ($P = 0.0008$)-infected Nu sups. PMA-stimulated Nu sup was also found to significantly increase the expression of CD69 ($P = 0.0003$) when compared with control (c). The expression of the chemokine receptor CXCR3 was not influenced on stimulation with any infected sup (d).

Discussion

The interaction of neutrophils with macrophages, as well as the downstream effects on T cell activity, could result in a range of outcomes from early clearance of infection to dissemination of viable bacteria together with an attenuated acquired immune response (Lowe *et al.*, 2012). Neutrophils are rapidly recruited to sites of mycobacterial infection, where they phagocytose bacilli and induce chain of responses through various receptors to initiate the immune response against MTB.

Among the receptors expressed by neutrophils, Fcγ receptors play an important role in adhesion and determining the phagocytosis potential of neutrophils. It is reported that different Fcγ receptors on neutrophils possess different phagocytosis capabilities, and CD32 (FcγRIIA) is the most efficient receptor among them (Rivas-Fuentes *et al.*, 2010). The affinity of human CD32 increases during neutrophil activation leading to CD32-dependent ligand binding and signaling (Nagarajan *et al.*, 2000). It has been documented that BCG has the capacity to increase the expression of CD32 (Suttmann *et al.*, 2003). Similarly, in this study, expression of CD32 was increased in BCG- and H37Rv-infected neutrophils indicating activation followed by functional upregulation of neutrophils. Another important FCγ receptor CD64 (FcγRI) that induces high respiratory burst (Hoffmeyer *et al.*, 1997) was also upregulated in H37Rv-infected neutrophils, which further indicates a physiological response to infection (Allen *et al.*, 2002).

Neutrophils recognize pathogens via TLRs and activate various pathways that contribute to the repertoire of defense mechanisms utilized by the immune system. Among TLRs, TLR2 is important in MTB infection and has been extensively studied. Another receptor TLR4, although important in innate immunity, has no direct role in protective immunity in mycobacterial infections (Reiling *et al.*, 2002). However, it mediates the signals responsible for the production of MTB-induced IL-17A response, which strongly relies on the endogenous IL-1 pathway (van de Veerdonk *et al.*, 2010). In another study, it was demonstrated that after Mtb infection neither TLR2, -4 and -9, nor MyD88 is required for the induction of adaptive T cell responses. Rather, MyD88, but not TLR2, -4 and -9, is critical for triggering macrophage effector mechanisms central to antimycobacterial defense (Hölscher *et al.*, 2008). In this study, an increased TLR4 expression was observed in H37Rv-stimulated neutrophils, which reflects the fact that TLR4 mediated activation of neutrophils occur during MTB infections; however, the activation does not necessarily lead to protective immune response.

Neutrophils are traditionally known to express limited number of chemokine receptors; however, under inflammatory conditions, they undergo phenotypic changes, enabling them to expand their chemokine receptor expression pattern and respond to chemokines that are functionally inactive under resting conditions. The chemokine receptor CXCR3 that is normally inactive on neutrophils gets expressed when induced with TLR ligands (Hartl *et al.*, 2008). Here, the increased expression of CXCR3 on H37Rv-infected neutrophils indicates that H37Rv has the capacity to induce the expression of CXCR3, whereas BCG and Mw are not effective enough to stimulate its expression.

Neutrophils undergo spontaneous apoptosis that make them susceptible to engulfment by monocytes/macrophages. The induction of apoptosis in neutrophils also activates macrophages and DCs (Perskvist *et al.*, 2002; Alemán *et al.*, 2007). In the present study, early apoptosis was significantly decreased, whereas the late apoptosis showed an increasing trend in H37Rv-infected neutrophils. Such accelerated apoptosis of neutrophils after interaction with mycobacteria is essential for the resolution of inflammation (Alemán *et al.*, 2002; Hedlund *et al.*, 2010).

Apoptosis is also affected by the secretion of antiapoptotic or pro-apoptotic cytokines. TNF-α is one of the best known pro-apoptotic cytokine. The increased secretion of TNF-α in H37Rv-infected neutrophils suggests its role in inducing late apoptosis and necrosis of these cells. On the other hand, the pro-inflammatory cytokine IFN-γ is antiapoptotic for neutrophils (Colotta *et al.*, 1992) and gets secreted upon stimulation with appropriate agents (Ethuin *et al.*, 2004). However, in this study, only basal expression of IFN-γ was observed under all infected conditions. This indicates that none of the strains were effective in the release of IFN-γ by neutrophils within a short span of 4 h culture.

It is reported that TNF-α produced by infected neutrophils is also involved in the activation of alveolar macrophages in noncontact cultures (Sawant & McMurray, 2007). To determine whether TNF-α produced by infected neutrophils modulates monocyte functions, the expression of CCR5 and CCR7 on monocytes was studied. Usually, the expression of CCR7 by peripheral monocytes is low or negative, and little upregulation happens after differentiation into macrophages. Similarly, in this study, the expression of CCR7 was low and not significant on monocytes stimulated with BCG- and Mw-infected NU sups. However, increased expression of CCR7 was observed with H37Rv-infected Nu sup. This might be due to increased secretion of TNF-α in H37Rv-infected Nu sup; however, this requires further experimental proof.

On the other hand, CCR5 expression on peripheral monocytes is usually greater, and accordingly, its upregulation was observed under all infected conditions in this study. Although the exact mechanism for this upregulation is not known, it is sure to be neutrophil-mediated. In our previous report, we did not find any increase in the levels of MIP-1α (chemokine ligand of CCR5) at early time point of 3 h after infection of neutrophils with H37Rv (Pokkali *et al.*, 2009). This basal level of chemokine may not be sufficient to bind to CCR5 and downregulate its expression level; instead, it may act as a trigger for the monocytes to upregulate CCR5 expression. In another study, when mononuclear cells were stimulated with MTB antigen, CCR5 expression on monocytes was increased, but CCR7 was hardly detectable (Arias *et al.*, 2006). Interestingly, we observed increase in the expression of both the receptors on monocytes, supporting the fact that both CCR5- and CCR7-mediated monocyte signaling functions occur with the help of neutrophils.

Once exposed to inflammatory signals, activated neutrophils produce cytokines and chemokines that influence adaptive immune cells. Chemokines produced by neutrophils can direct T lymphocyte maturation and specifically attract Th17 cells (Pelletier *et al.*, 2010; Lowe *et al.*, 2012). To find whether the infected neutrophil secretions have the capacity to stimulate T helper cells, the expression of CD69 (an activation marker) on T cells was analyzed. The supernatants from H37Rv-infected neutrophils increased CD69 expression on T cells suggesting modulation of T helper cells through neutrophil-mediated signaling. This is in accordance with a previous study, where increased expression of CD69 was observed on T cells

from patients with TB (Wanchu *et al.*, 2009). It has been reported that expression of CXCR3 was increased on naïve T cells following activation and preferentially remains highly expressed on Th1 cells (Qin *et al.*, 1998). In this study, even though there was increased expression of the activation marker CD69, we did not find any modulation in CXCR3 expression on T cells when stimulated with infected neutrophil supernatants.

To conclude, the present study clearly indicates that H37Rv modulates neutrophils to the maximum followed by BCG, whereas Mw does not show any influence on the studied neutrophil parameters. This is evidenced from the upregulation in the expression of CD32, CD64, TLR4, and CXCR3; increased TNF-α secretion, and downregulation of early apoptosis in H37Rv-infected neutrophils, whereas only CD32 expression was increased in BCG-infected neutrophils. Also, secretory products from infected neutrophils were able to modulate T helper cells and monocytes to different extents. Further studies are required to understand whether these varied phenotypical changes induced by H37Rv and BCG on neutrophils are related to pathophysiology of these strains.

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