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Interferon-gamma and nitric oxide burst response in different categories of pulmonary tuberculosis patients

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ABSTRACT

Background: Anti-tuberculous cellular immunity involves the critical interplay of T lymphocytes, macrophages, and cytokines. The quantitative analysis of pro-inflammatory cytokine (interferon-gamma [IFN-γ]) release and nitric oxide (NO) production by *in vitro* challenged macrophages from tuberculosis (TB) patients of different clinical settings and healthy controls would enlighten our knowledge of macrophage efficiency at different age and status of disease, thereby paving the way for immunotherapy. **Aims and Objectives:** To study the pattern of release of IFN-γ and NO burst response by macrophages, from pulmonary TB cases compared to normal individuals, *in vitro* culture, on challenge with Bacillus Calmette-Guérin (BCG) and *Mycobacterium tuberculosis* H37Rv. **Materials and Methods:** Fifteen consecutive sputum smear-positive newly diagnosed TB and 15 relapsed TB cases were recruited. All cases were pulmonary with no BCG vaccination history. 15 tuberculin skin test (TST) positive, 15 TST negative healthy volunteer, and 5 newborn antigen naïve cases were included as control. Isolated macrophages were cultured and pretreated with BCG and H37Rv. IFN-γ and NO were measured from the cell free culture supernatant after 24 h. **Results:** The monocytes of the patients are functionally different than the healthy subjects toward the same antigenic stimulation. The level of NO release correlates with the extent of IFN-γ production. The presence of Th1 response and associated factors like NO burst are some of the main regulators of the outcome of the disease. **Conclusion:** Capacity to mount inflammatory response, effective NO generation determines the outcome of TB infection and may contribute to better understanding of the underlying immunopathology.

Key words: *Mycobacterium tuberculosis*; Monocytes; Cytokine; Interferon-gamma; Immune response; Nitric oxide

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INTRODUCTION

Mycobacterium tuberculosis (MTB) is the world's leading infectious cause of mortality. After infection with tuberculosis (TB), the lifetime risk of developing TB is approximately 10%, while 90% of infected people have latent infection with viable bacilli.[1] But little is known about the early interactions of microbes and immune cells that result in either restricted infection or dissemination and disease, nor of the reasons why some individuals reactivate latent infection. Anti-tuberculous cellular immunity involves the critical interplay of T lymphocytes, macrophages, and cytokines. It has been reported that the development of an *in vitro* human system may greatly facilitate studies to delineate immune cells, cytokines, and effector functions/genes critical in controlling TB.[2] *In vitro* human and murine *Mycobacterium* antigen (Ag)-specific CD4 T cells produce plentiful amounts of interferon-gamma (IFN-γ).

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The production of IFN-γ by CD4+T cells is thought to activate macrophages to control intracellular microbes by the expression of nitric oxide (NO) synthase (inducible NO synthase [iNOS], NOS2) and major histocompatibility complex class II Ag. In man, mutations of the IFN-γ receptor rendering it functionless are associated with fulminant TB and disseminated mycobacteremia after vaccination with Bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*.[3,4] Although induction of *in vitro* murine macrophage anti-MTB activity by IFN-γ has been observed, whether human macrophages can achieve anti-MTB activity *in vitro* via induction by IFN-γ remains controversial.[5,6] It has been recently reported that classical Ca^{2+}-dependent protein kinase-C is found to be related to the Th1 cytokines secretion.

The quantitative analysis of pro-inflammatory cytokine (IFN-γ) release and NO production by *in vitro* challenged macrophages from TB patients of different clinical setting and healthy controls would enlighten our knowledge of macrophage efficiency at different age and status of disease. The result will help us to make hypothesis of new treatment feasibility by immunotherapy.

Killing of ingested MTB would most likely take place within macrophage phagolysosomes. Toxic constituents found within this acidic vesicle include lysosomal hydrolases, reactive oxygen intermediates (ROI) such as H_2O_2 and O_2^-, and reactive nitrogen intermediates (RNI) such as NO and NO_2^-. The resistance of several strains of MTB to RNI *in vitro*, generated at an acidic pH, was found to correlate significantly with the virulence of the strain tested.[7] RNI production by murine macrophages is an important effector mechanism against a variety of pathogens.[8] In macrophages, NO and other RNI are derived from L-arginine via an enzymatic pathway controlled by an iNOS.[9] Cytokines are powerful modulators of murine macrophage RNI synthesis. While tumor necrosis factor (TNF)-α and IFN-γ are potent activators of iNOS, interleukin (IL)-4 and IL-10 suppress it.[10] Transforming growth factor b1 (TGF-b1) has also been reported to attenuate NO production and RNI-mediated antimicrobial functions.[11] Growth inhibition of mycobacteria by cytokine-stimulated murine macrophages strongly correlates with the generation of RNI.[12,13] IFN-γ-deficient mice infected with MTB are unable to restrict the growth of the organisms. These mice can develop granulomas but fail to produce RNI.[14]

The role of RNI in infected humans remains a matter of considerable debate.[15] Production of iNOS mRNA[16] and protein[17] by primary human macrophages has been reported, although efforts to demonstrate an L-arginine-dependent pathway for RNI production in human macrophages have generally produced inconsistent results. These disparate observations may simply reflect intrinsic differences between human and murine cells. One possible difference may be the requirement for the cofactor tetrahydrobiopterin, which may not be present in sufficient quantities in resting human macrophages.[18]

Alternatively, human macrophages may require additional induction signals for RNI production. One study showed that a combination of lipopolysaccharide (LPS) and the cytokines IL-1, TNF, and IFN-γ was required for human hepatocytes to produce RNI via an L-arginine-dependent pathway.[19] Furthermore, this iNOS activity was dependent on the co-induction of tetrahydrobiopterin synthesis.[20] Another possibility is that human macrophages may produce higher amounts of cytokines that suppress RNI production (e.g., IL-4, IL-10, and TGF-b1) compared with murine cells. Finally, the contribution of ROI to the defense against TB remains unclear. ROI alone may be insufficient to destroy MTB, but ROI combined with RNI can significantly enhance mycobacterial killing.[21] Several mycobacterial products, including sulfatides and lipoarabinomannan, can scavenge ROI or inhibit the respiratory burst that generates them.[22,23]

Aims and objectives

To study the pattern of release of IFN-γ and NO burst response by macrophages, from pulmonary TB (PTB) cases compared to normal individuals, in *in vitro* culture, on challenge with BCG and MTB H37Rv.

MATERIALS AND METHODS

The study recruited 15 consecutive sputum smear-positive newly diagnosed TB and 15 relapsed TB (RTB) cases fulfilling the inclusion and exclusion criteria. All the cases were pulmonary and had no BCG vaccination history.

15 tuberculin skin test (TST) positive and 15 TST negative healthy volunteer with matched age and sex were included as control. 5 newborn Ag naïve cases were also included in the study as control. Informed consent was procured from all participants and parents of newborn cases.

The study was approved by the Institutional Ethics Committee of the Institute of Postgraduate Medical Education and Research, Kolkata (Memo No. Inst./IEC/1020 Dated: January 07, 2011).

Recruitment of PTB cases and controls were from patients, referred to Department of Microbiology, Institute of Post Graduate Medical Education and Research (IPGMER), for isolation and antitubercular drug sensitivity tests and TST clinic of Department of Microbiology, IPGMER.

Newborn cases were recruited from the Obstetrics Department of IPGMER.

Inclusion criteria considered were as follows

i. Cases-PTB cases confirmed by acid–fast bacilli (AFB) positivity of sputum
ii. Age, sex, and economic condition matched persons.

Exclusion criteria taken into consideration were as follows

i. Diabetes
ii. Patients receiving immunosuppressives
iii. Malignancy
iv. Human immunodeficiency virus seropositive.

Sampling was done once a week and the period of sampling was for 3 months.

Isolation of macrophage-lymphocyte[24]

5 mL histopaque was taken in two tubes, and 5 mL blood was overlayed onto the histopaque. Then, the tubes were centrifuged at 1300 rpm for 40 min at room temperature. The buffy layer was collected in a fresh tube and added phosphate-buffered saline to the buffy layer containing tube to wash the cell and spinned at 1500 rpm for 10 min at room temperature. The supernatant was discarded.

The above-mentioned steps were repeated twice. Thereafter, the cell pellet was dissolved in 2 mL media (RPMI 1640). 10 μL was taken for hemocytometer count. The number of cells was counted in the hemocytometer chamber.

Macrophage-lymphocyte co-culture[24,25]

3×10^6 cells were plated per 2 mL media (RPMI 1640 supplemented with 10% fetal calf serum). The cells were incubated overnight at 37°C in 5% CO_2 incubator. The non-adherent cells were washed off the next day and fresh media was added to incubate overnight.

The above-mentioned steps were repeated twice. After counting the cell BCG, MTB H37Rv were added in 1:10 ratio (10 bacilli per single cell) for the study. LPS was added as a positive control in the dose of 100 ng/mL.

BCG and MTB H37Rv culture

BCG and MTB H37Rv were inoculated in Lowenstein-Jensen medium and Kirchner medium and incubated at 37°C for 3 weeks. The cells were scraped and taken into normal saline. The cell suspension was then passed through 5 μm filter after vortexing. The cells were counted by Ziehl–Neelsen method for AFB and then used for the infection of the macrophage-lymphocyte cells.

Cytokine assay by sandwich enzyme-linked immunosorbent assay (ELISA)

The isolated macrophages were cultured and pretreated with BCG and H37Rv. After 24 h, the cell-free supernatant was collected and subjected to sandwich ELISA for IFN-γ. The kit was purchased from immunotools, GmbH, Germany (Catalog No.-31333539 for IFN-γ) and was performed according to the instruction of the manufacturer.

NO production assay from cell supernatants

NO generation was estimated by sampling culture supernatants for nitrite, which is a stable product of NO reaction. LPS (LPS, 100 ng/mL) was used as positive control for NO production. After 48 h, 100 μL volumes of cell-free supernatant were mixed with an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% N-1-naphtyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated for 10 min at 25°C. Optical densities of the samples were subsequently measured at 550 nm wavelength in a microplate reader (BIO-RAD). The nitrite concentration was determined by reference to a standard curve using sodium nitrite (10–80 μM/L) diluted in culture medium.

Analysis of data

Data generated were analyzed to determine whether there are any differences in the values of the estimated NO generation between the groups. Significance study will be done by calculating Chi-square, student t, and P-value.

RESULTS

The demographic and clinical characteristics of the patients and healthy controls are summarized in Table 1. The IFN-γ and NO released by the monocytes of the different study groups and the healthy controls were measured with and without the stimulation by BCG, live MTB (H37Rv) and LPS (Figures 1-8). The results showed that unstimulated (control) monocytes from the new TB patients release more IFN-γ and NO than the healthy controls.

As shown in Figure 1, there is a significant rise in IFN-γ level in TST-negative individuals on challenge with *Mycobacterium* spp. However, an increase in IFN-γ level with LPS challenge in all study groups validates the test results, i.e., macrophages are viable. Challenge with BCG yields more IFN-γ than H37Rv.

As shown in Figure 2, the unhindered Th1 response in TST-negative individual results in an increase in IFN-γ generation than TST-positive individuals and new TB and RTB patients. Challenge with BCG yields more IFN-γ than H37Rv.

Table 1: Demographic and clinical characteristics of TB patients and healthy controls

Parameters	TB patients		Healthy controls	
	New (n=15)	Relapsed (n=15)	TST positive (n=15)	TST negative (n=15)
Age (mean±SD)	42.9±14	31.8±7	43.8±12	33.7±9
Male: Female	3:2	7:3	7:4	3:2
Chest X-ray (%)				
TB Infiltration	100	80	0	0
Cavity	0	20	0	0
Miliary mottling	0	0	0	0
Pleural Effusion	0	0	0	0
Clinical findings: (mean±SD*)				
Weight, in kg	47.2±6	43.8±3	59.3±3	57.3±7
Body mass index	20.45±1.2	18.34±1.2	22.43±1.3	21.37±1.3
Hemoglobin, g%	11.7±0.7	11.3±0.9	12.7±0.5	12.5±0.7
Erythrocyte sedimentation rate	45.4±1.5	46.3±1.6	18.4±1.7	17.8±1.6

TB: tuberculosis, TST: Tuberculin skin test. *SD: Standard deviation

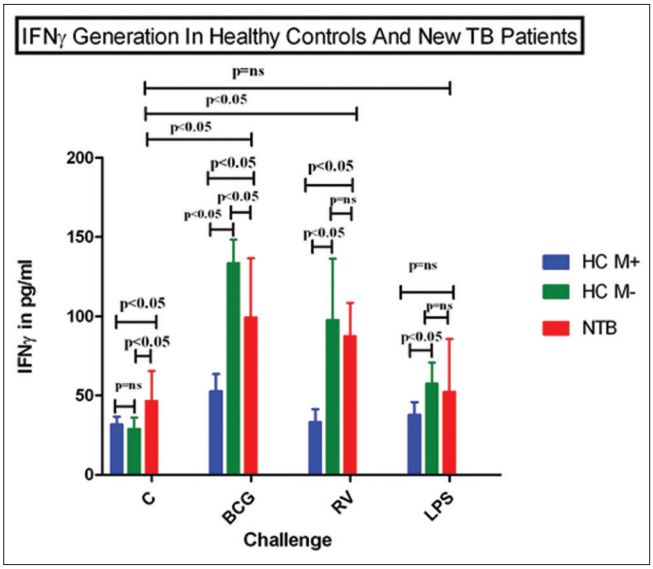

Figure 1: Interferon-gamma generation in healthy controls and new tuberculosis patients

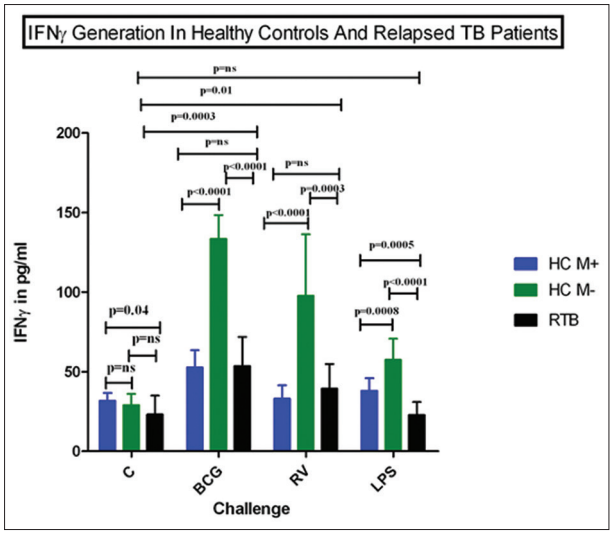

Figure 2: Interferon-gamma generation in healthy controls and relapsed tuberculosis patients

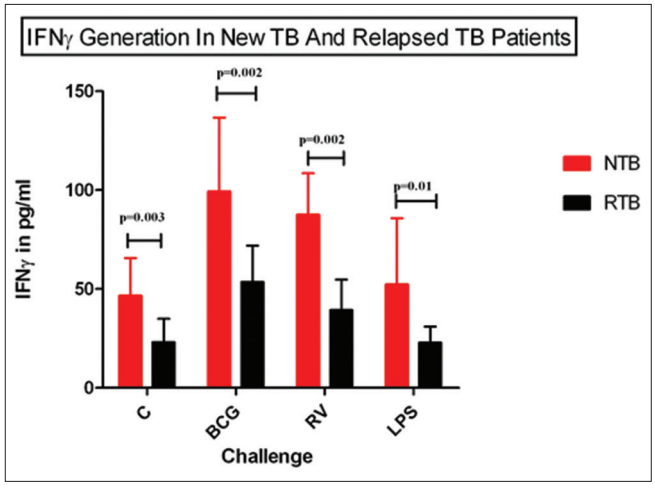

Figure 3: Interferon-gamma generation in new tuberculosis and relapsed tuberculosis patients

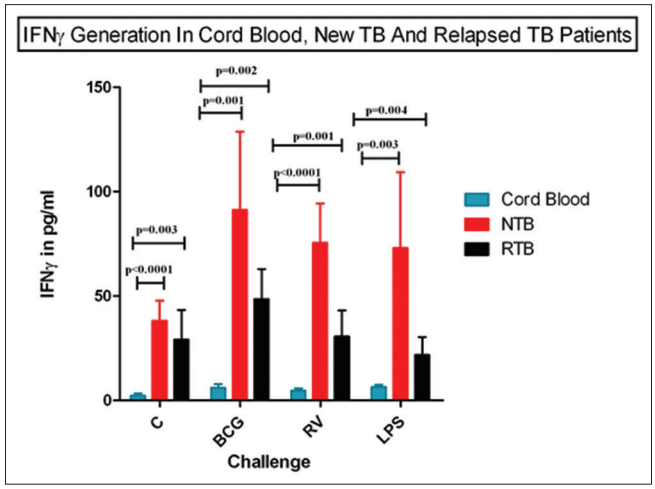

Figure 4: Interferon-gamma generation in cord blood, new tuberculosis, and relapsed tuberculosis patients

As shown in Figure 3, the IFN-γ level is higher in new TB patients compared to RTB patients with or without mycobacterial and LPS challenge. The result shows that

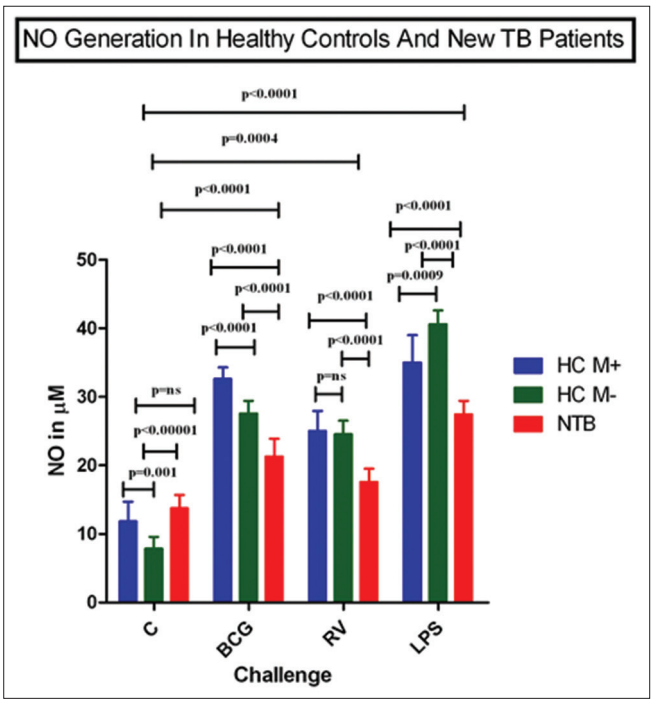

Figure 5: Nitric oxide generation in healthy controls and new tuberculosis patients

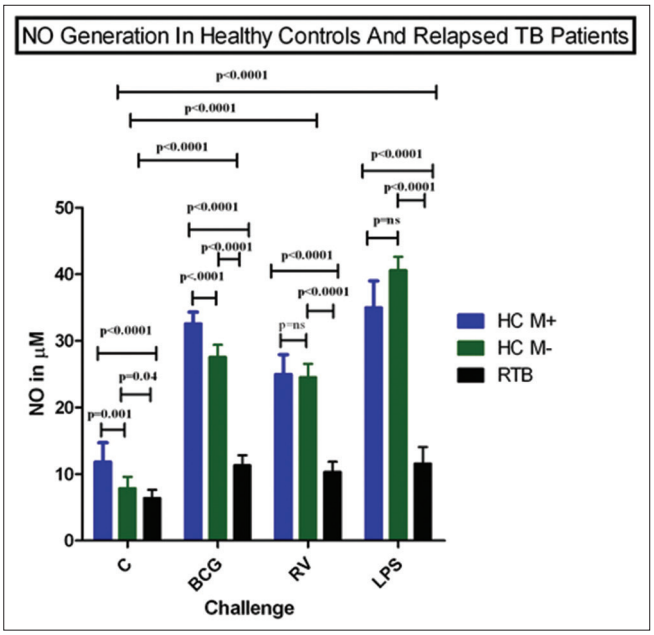

Figure 6: Nitric oxide generation in healthy controls and relapsed tuberculosis patients

nonpathogenic *Mycobacterium* spp. are potent inducers of pro-inflammatory cytokine than the pathogenic strain.

As shown in Figure 4, the cord blood shows muffled IFN-γ generation compared to all the other groups included in the study.

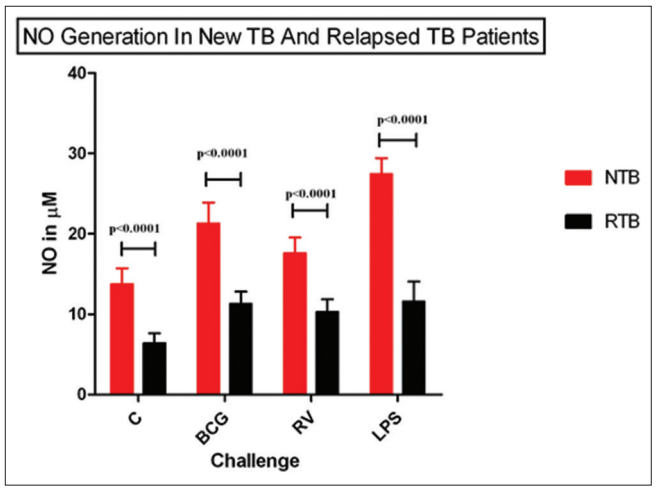

Figure 7: Nitric oxide generation in new tuberculosis and relapsed tuberculosis patients

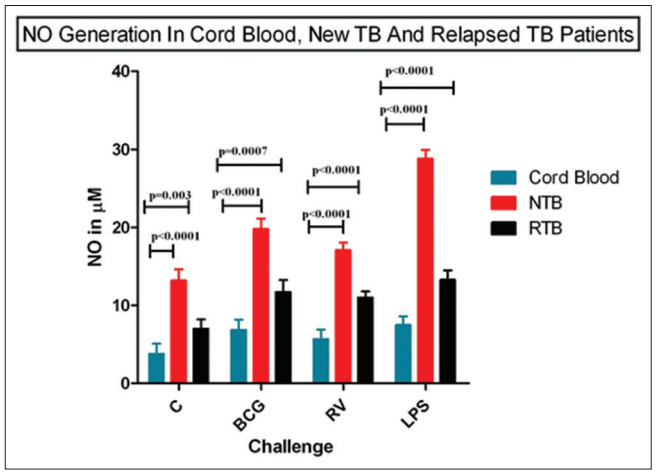

Figure 8: Nitric oxide generation in cord blood, new tuberculosis, and relapsed tuberculosis patients

As shown in Figure 5, there is a deficiency of NO generation in new TB patients compared to TST positive and negative individuals, though baseline NO is higher in TB patients. Challenge with BCG yields more NO than H37Rv.

As shown in Figure 6, there is further deficiency in NO generation in RTB patients compared to TST positive and negative individuals. Baseline level in RTB patients shows non-significant compared to healthy controls. Challenge with BCG yields more NO than H37Rv.

As shown in Figure 7, there is significant suppression of NO generation in RTB patients compared to new TB patients directly correlating to the chronicity and severity of the disease. Thus, the result of this study correlates with the well-established fact that the pathogenic *Mycobacterium* spp. downregulate NO production.

As shown in Figure 8, there is muffled NO generation in cord blood as compared to new and RTB patients.

DISCUSSION

Both innate and acquired immunity are involved in the protection to MTB infection. Lack of development of protective immunity against MTB infections is associated with monocyte dysfunction and favorable cytokine response.[26] To understand how monocytes respond toward the virulent and avirulent strains of *Mycobacterium* spp., IFN-γ and NO profile were studied from the diseased and healthy controls.

The protective role of IFN-γ in TB is well established.[27] Although the development of an immune response with abundant local production of IFN-γ is unable to eradicate the pathogen, it is a potent co-stimulator for iNOS.[28] *In vitro* studies with heat-killed MTB have been found to induce more IFN-γ production by the peripheral blood mononuclear cell (PBMC) from TB patients than TST-positive healthy controls. However, in our study, this cytokine response is found to be higher by BCG challenge followed by H37Rv among all the study groups, hence assumed that monocytes behave differently toward the virulent and avirulent strains of *Mycobacterium* spp. in IFN-γ production.

The release of NO by the macrophages is associated with IFN-γ production.[28] In our study, NO release is heightened after BCG challenge than H37Rv infection. Our results also confirm the poor NO response in relapsed PTB cases and correlate with the extent and condition of the disease. Simulating earlier studies, avirulent strain producing more NO than the virulent strains from human PBMCs was observed. In our study, the level of NO correlates with the extent of IFN-γ. A very high NO release was observed in the newly diagnosed cases. However, the generated RNI may not be sufficient to kill the invading organism. Numerous *in vitro* studies demonstrate that strains of *Mycobacterium* spp. vary about their susceptibility to ROI and RNI.

Persistent muffled cytokine and NO burst response is seen on mycobacterial challenge of cord blood. This proves newborn macrophages are not competent enough to mount cytokine release and other killing mechanisms rendering them susceptible to infection.

The varied response among the individuals in the same study group may be due to polymorphism in toll-like receptor (TLR) or in the expression of proteins in the signal transduction pathway, as the bacterial factor and number of cells remain constant. In newborn, TLR system is not well developed, resulting in depressed response. Further experiment on TLR expression pattern and quantification along with signal transduction protein expression and quantification might throw light on polymorphism patterns.

PTB is a localized infection (except miliary TB) and no evidence of *Mycobacterium* spp. has been reported in the peripheral blood of the patients. Some monocyte populations of the bloodstream might have mycobacterial antigenic exposures. This proportion of the sensitized and memory cells will generate heightened responses after subsequent mycobacterial challenge. However, in a country like India, most healthy controls are exposed to environmental *Mycobacterium* spp. despite TST-negative results. It has been reported that the proportion of the memory cell along with other physiological status determines the protective capacity of vaccination or reactivation of the disease.

Overall, it appears that the monocytes of the patients are functionally different than the healthy subjects toward the same antigenic stimulation. Furthermore, the monocytes from the same groups produce different levels of cytokines toward the virulent and avirulent mycobacteria. Hence, the host factors as well as the pathogen factors create a complex immune-pathological response in TB.

From our limited results by *in vitro* experiments, it is found that mixed cytokine responses occur after mycobacterial infection. The presence of Th1 response and associated factors like NO burst are some of the main regulators of the outcome of the disease. More pertinent data using cell separators expanded by culture and subsequently measurement of cytokine on stimulation of mycobacteria will establish the fact in TB.

Pro-inflammatory response is essential in TB, but an unrestrained pro-inflammatory response may lead to tissue damage. Individuals genetically predisposed to higher production of these cytokines may display increased innate susceptibility to MTB. To date, such genetic predisposition has not yet been reported in humans.

More pertinent data subjecting clonal differentiation of Ag-specific cell, obtained by cell separators and expanded by culture, to stimulation of mycobacteria and subsequent measurement of cytokine and NO generation, would cast more insight about the knowledge of immunopathogenesis in TB.

Limitations of the study

This cross-sectional study showing capacity to mount inflammatory response, effective NO generation determines

the outcome of TB infection, in-vitro from a limited number of tuberculosis patients attending a tertiary care hospital. This may not represent the in-vivo scenario along with overall burden. However, the main objective of the study was to observe the cellular responses among different categories of tuberculosis patients, which may enrich our understanding of the pathogenesis of tuberculosis.

CONCLUSION

A unique of its kind, this study showcases the involvement of various categories of TB patients, their age and sex matched TST positive and negative healthy controls and newborn Ag naïve cases. Our objective was to measure Th1 (IFN-γ) cytokine and NO burst response in these groups. Unhindered Th1 cytokine response in the form of a significant rise of IFN-γ was seen in TST-negative individuals in comparison to TST-positive ones, new TB and RTB patients. IFN-γ release was more in new TB patients than RTB patients. Nonpathogenic *Mycobacterium* spp. are potent inducer of pro-inflammatory cytokines than pathogenic strains.

Significant suppression of NO generation in RTB patients compared to new TB patients directly correlates with the chronicity and severity of the disease. Downregulation of NO production by pathogenic *Mycobacterium* spp. was observed. Persistent muffled cytokine and NO burst response is seen in cord blood on mycobacterial challenge proving newborn macrophages are not competent enough to mount protective response making them susceptible to infections.

This is evident that in fibro-necrotic TB, response tilts toward pro-inflammatory side. Whether the host or parasite factors are responsible for the outcome of the disease remains inconclusive. Hence, it is concluded that capacity to mount inflammatory response, effective NO generation determines the outcome of TB infection.

Further investigation including comparison of anti-inflammatory cytokine response, increased study number in each subgroups generating a statistically significant data will enrich our knowledge in understanding the pathogenesis of TB.

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Authors' Contributions:
SS- Design of study, clinical protocol, data analysis, statistical analysis and interpretation, manuscript preparation, editing, revision and submission;
UC- Implementation of study protocol, data collection, literature survey, manuscript preparation, and revision; **AG-** Coordination and manuscript revision;
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