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Th1 and Th2 cytokine response of peripheral blood macrophages-lymphocyte co-culture on challenge with *Bacillus* Calmette-Guérin and *Mycobacterium tuberculosis* H37RV in different categories of tuberculosis patients

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ABSTRACT

Background: The quantitative analysis of Th1 and Th2 cytokines by *in vitro* challenged macrophages from tuberculosis (TB) patients of different clinical setting and healthy controls would enlighten our knowledge of macrophage efficiency at different ages and status of disease, facilitating new treatment feasibility by immunotherapy. **Aims and Objectives:** The aim of the study was to study the pattern of release of interferon-gamma (IFN-γ), interleukin-4 (IL-4), and IL-10 by macrophages, from pulmonary TB cases compared to normal individuals, in *in vitro* culture, on the challenge with *Bacillus* Calmette-Guérin (BCG) and *Mycobacterium tuberculosis* H37Rv. **Materials and Methods:** Fifteen sputum smear-positive newly diagnosed TB and 15 relapsed TB cases fulfilling the inclusion and exclusion criteria were recruited. Fifteen tuberculin skin test (TST) positive and 15 TST negative age- and sex-matched healthy volunteers were included as a control. Isolated macrophages were cultured and pretreated with BCG and H37Rv. After 24 h, the cell-free supernatant was collected and subjected to a sandwich enzyme-linked immunosorbent assay for IL-4, IL-10, and IFN-γ. **Results:** It was found that monocytes behave differently toward the virulent and avirulent strains of *Mycobacterium* in IFN-γ production. Th1 cytokine response was found to be higher by BCG challenge followed by H37Rv among all the study groups. However, Th2 cytokine responses with IL-10 and IL-4 were found to be higher in the patients as compared to healthy controls. **Conclusion:** Capacity to mount an inflammatory response determines the outcome of TB infection. Further study will enrich our knowledge in understanding the individual cell function and immunopathological mechanisms associated with the different clinical forms of TB.

Key words: *Mycobacterium tuberculosis*; Monocytes; Cytokine; Immune response

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INTRODUCTION

Mycobacterium tuberculosis (MTB) is the world's leading infectious cause of morbidity and mortality. The lifetime risk of developing tuberculosis (TB) is approximately 10%, while 90% of infected persons have latent infection with viable bacilli after being infected with MTB.[1] Neither much 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

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system may greatly facilitate studies to delineate immune cells, cytokines, and effectors functions/genes critical in controlling TB.[2] *In vitro* human and murine *Mycobacterium* Ag-specific CD4 T cells produce plentiful amounts of interferon-gamma (IFN-γ). The production of IFN-γ by CD4+ T-cells is thought to activate macrophages to control intracellular microbes by the expression of inducible nitric oxide synthase (iNOS, NOS2) and MHC Class II Ag. In human, 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* through induction by IFN-γ remains controversial.[5,6] It has been recently reported that classical Ca-dependent protein kinase-C (PKC) is found to be related to the Th1 cytokines secretion. It has been further reported that interleukin-12 (IL-12) is produced by phagocytic cells in response to stimulation by a variety of microorganisms as well as their products.[7,8] Further, the change in PKC level was found to be correlated with Th1 cytokine secretion change in murine macrophage cell line, a phenomenon not been studied in human macrophage.[9]

The quantitative analysis of Th1 and Th2 cytokines by *in vitro* challenged macrophages from TB patients of different clinical setting and healthy controls in this study 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.

Aims and objectives

To study the pattern of release of Interferon-gamma (IFN-γ), Interleukin-4 (IL-4), and Interleukin-10 (IL-10) by macrophages, from pulmonary TB cases compared to normal individuals, in in-vitro macrophages-lymphocyte co-culture, on challenge with Bacillus Calmette-Guérin (BCG) and Mycobacterium tuberculosis H37Rv.

MATERIALS AND METHODS

Study population

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

Fifteen tuberculin skin test (TST) positive and 15 TST negative healthy volunteer with matched age and sex were included as control.

Informed consent was obtained from all of the participants. 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: 07 January 2011).

Recruitment of pulmonary TB (PTB) cases and controls were from patients, referred to Department of Microbiology, IPGMER, for isolation and antitubercular drug sensitivity tests and TST clinic of Department of Microbiology, 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 viruses seropositives.

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

Isolation of macrophage-lymphocyte[10]

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. Then, the buffy layer was collected in a fresh tube and to that phosphate buffered saline was added to wash the cell and spinned at 1500 rpm for 10 min at room temperature. Then, the supernatant was discarded and step was repeated twice. The cell pellet was dissolved in 2 mL RPMI 1640. 10 μL was taken for hemocytometer count. The number of cells was counted in the hemocytometer chamber.

Macrophage-lymphocyte co-culture[10,11]

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 at 5% CO_2 incubator. Next day, the non-adherent cells were washed off and fresh media was added to incubate overnight. The step was repeated. After counting the cell BCG, MTB H37Rv were added in 1:10 ratio (10 bacilli per single cell)[12] 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 LJ medium and Kirchner medium and incubated at 37°C for 3 weeks. The cells were scrapped and taken into normal saline. The cell suspension was then passed through 5 μm filter after vortexing. The cells were counted by AFB staining and then used for the infection of the macrophage-lymphocyte cells.

Cytokine assay by sandwich enzyme-linked immunosorbent assay

The isolated macrophages were cultured and were pretreated with BCG and H37Rv. After 24 h, the cell-free supernatant were collected and subjected to sandwich enzyme-linked immunosorbent assay (ELISA) for IL-4, IL-10, and IFN-γ. The kit was purchased from Immunotools, GmbH, Germany (Catalog No.-31333539 for IFN-γ, Catalog No.-31330109 for IL-10, Catalog No.-31330049 for IL-4) and was performed according to the instruction of the manufacturer.

Analysis of data

Data generated were analyzed to determine whether there are differences in values of the estimated cytokines (IL-4, IL-10, and IFN-γ) between the groups. A significance study was done by calculating Chi-square, student t, and P-value.

RESULTS AND ANALYSIS

After infection, cytokines were estimated by ELISA (in pg/mL) in the cell-free culture supernatant. Figures 1-3 show the level of IFN-γ among the different study groups. The demographic and clinical characteristics of different categories of TB patients and healthy controls are shown in Table 1.

Significant rise in IFN-γ level in TST negative individuals on challenge with *Mycobacterium* was noted.However, increase in IFN-γ level with LPS challenge in all study groups validates the test results, that is, macrophages are viable. Challenge with BCG yields more IFN-γ than H37Rv.

Unhindered Th1 response in TST negative individuals results in an increase of IFN-γ generation than TST positive individuals and new TB and RTB patients. Challenge with BCG yields more IFN-γ than H37Rv.

IFN-γ level is higher in new TB patients compared to RTB patients with or without mycobacterial and LPS challenge. The result shows that non-pathogenic *Mycobacterium* is potent inducer of proinflammatory cytokine than the pathogenic strain.

Figures 4-6 show the level of IL10 among the different study groups.

New TB patients show significant IL-10 level before *in vitro* antigenic challenge but no such preferential increase is noted in all three study groups in response to mycobacterial stimulation. Challenge with H37Rv yields more IL-10 than BCG. This fact shows the role of pathogenic *Mycobacterium* in the generation of anti-infammatory cytokine and helps survive the bacteria in the host.

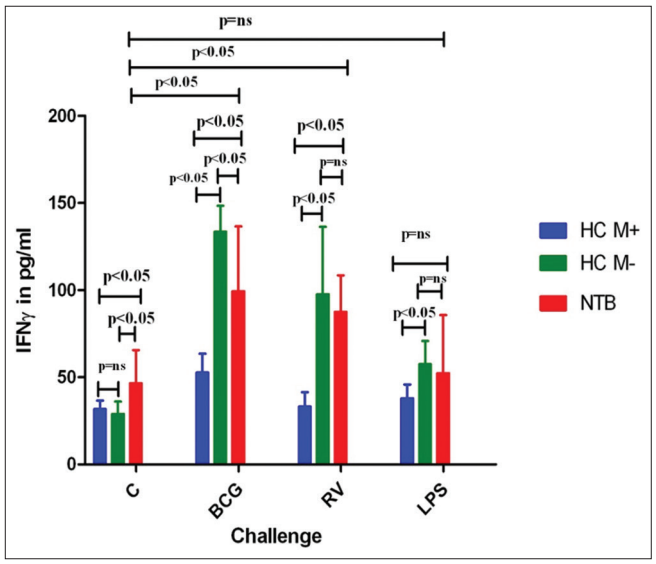

Figure 1: Interferon-gamma generation in healthy controls and new tuberculosis patients

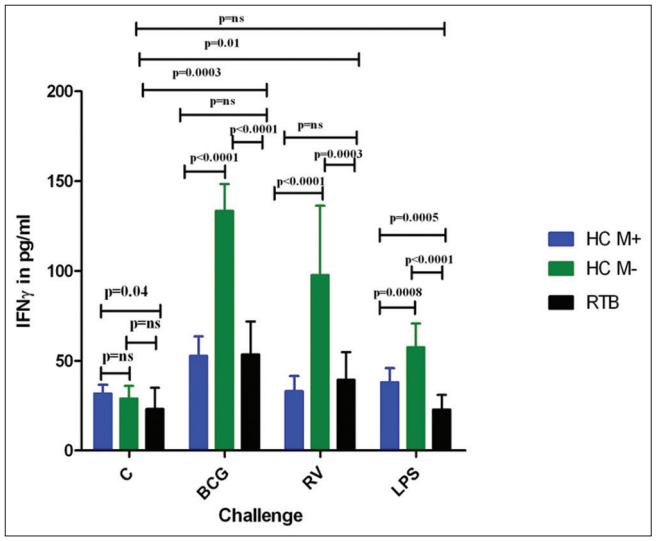

Figure 2: Interferon-gamma in healthy controls and relapsed tuberculosis patients

No preferential increase in IL-10 level is noted in all three study groups in response to mycobacterial stimulation. RTB patients show increased IL-10 level before challenge. The basal level of IL10 among the diseased categories is significantly high than the healthy controls. The challenge with H37Rv yields more IL-10 than BCG.

The response is more for the NTB cases as compared to other groups. High IL-10 response is inhibitory to Th1 response and subsequently overcomes the Th1 response in the diseased categories. Hence, IL-10 production was higher in anergic patients suggests that MTB-induced IL-10 production suppresses an effective immune response.

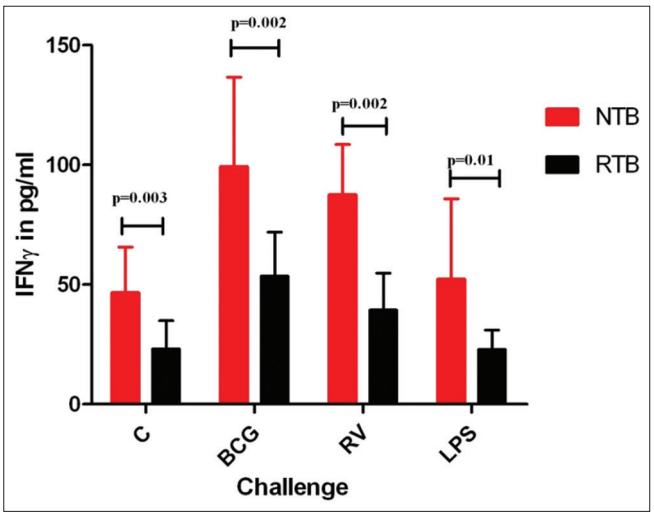

Figure 3: Interferon-gamma generation in new tuberculosis and relapsed tuberculosis patients

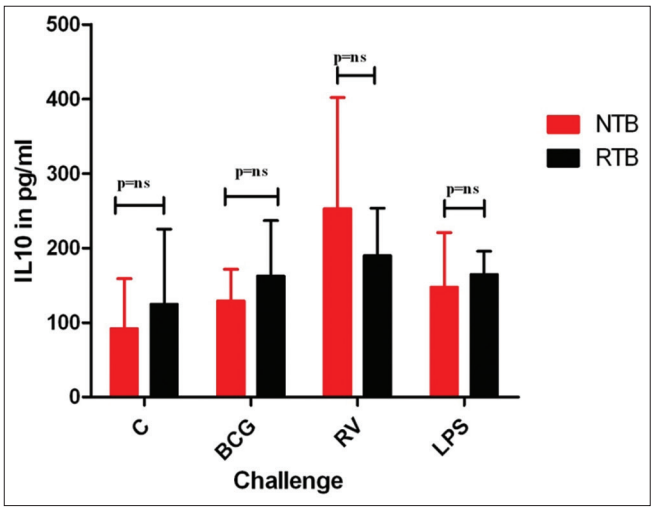

Figure 6: Interleukin-10 generation in new tuberculosis and relapsed tuberculosis patients

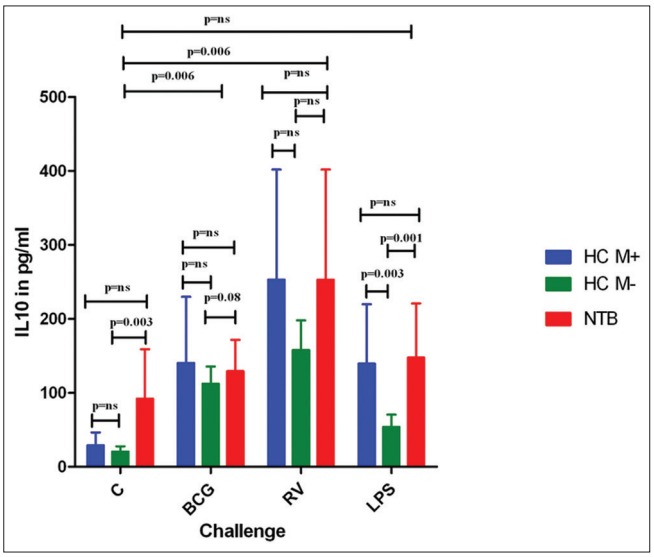

Figure 4: Interleukin-10 generation in healthy controls and new tuberculosis patients

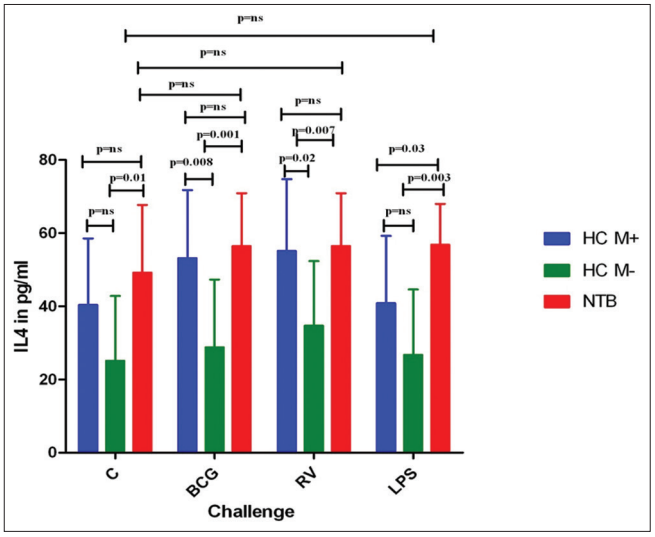

Figure 7: Interleukin-4 generation in healthy controls and new tuberculosis patients

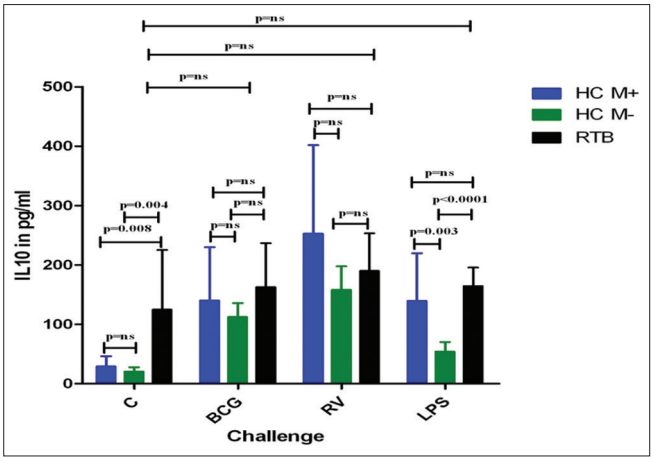

Figure 5: Interleukin-10 generation in healthy controls and relapsed tuberculosis patients

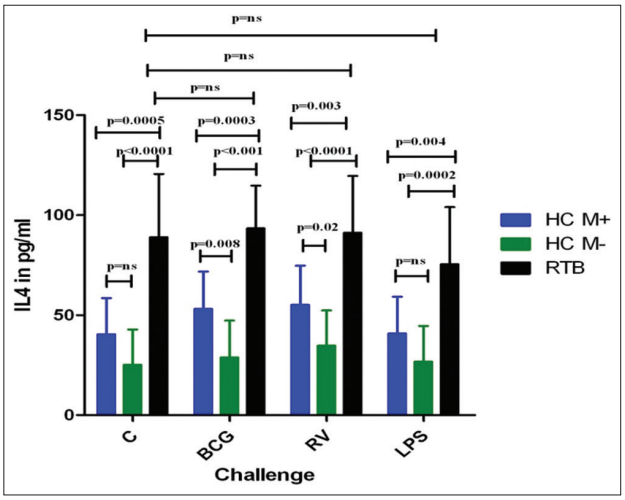

Figure 8: Interleukin-4 generation in healthy and relapsed tuberculosis patients

Figures 7-9 show level of IL4 among the different study groups.

The result shows that mycobacterial challenge hardly induces IL-4 production from peripheral blood mononuclear cells (PBMCs) except the healthy categories though deficient IL-4 production among TST negative individuals compared to the other two study groups. BCG and H37Rv yield almost the same level of cytokine in all the study groups, respectively.

Prominent baseline IL-4 production among RTB patients compared to the healthy controls; however, TST negative individuals show no significant increase in IL-4 level on mycobacterial challenge. BCG H37Rv yields almost the same level of cytokine in all the study groups, respectively.

In the relapsed categories, the basal level of IL-4 is significantly higher than the newly diagnosed cases. Further mycobacterial challenge did not show any change in this cytokine profile due to already existing Th2 cytokine in the milieu. The high IL-4 level in these diseased categories correlates with the chronicity of the disease and also parallels our findings.

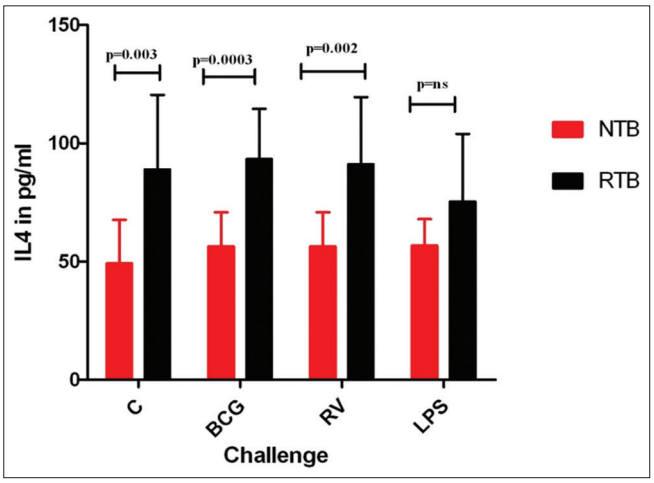

Figure 9: Interleukin-4 generation in new tuberculosis and relapsed tuberculosis patients

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 balance of pro- and anti-inflammatory cytokine response.[13] The Th1/Th2 cytokine balance is associated with the outcome as well as treatment responsiveness of the disease regardless the drug sensitivity pattern of the pathogen.[14] The protective role of IFN-γ in TB is well established.[15] *In vitro* studies with heat-killed MTB have been found to induce more IFN-γ production by the PBMC from TB patients than TST-positive healthy controls.[3] However, in our study, this cytokine response is found to be higher by BCG challenge followed by H37Rv among all the study groups. Hence, it is found that monocytes behave differently toward the virulent and avirulent strains of *Mycobacterium* in IFN-γ production.

As regard IL-10, H37Rv produced a higher response in all the study groups. This supports the fact that virulent H37Rv led to the higher anti-inflammatory response facilitating survival of MTB in the tissues, thereby carrying forward the infection process. Another interesting fact about IL-10 is that among the patient groups either new or relapsed, the base level IL-10 is significantly high as compared to healthy controls. Vankayalapati et al., showed the serum IL-10 level in PTB patients and TST-positive healthy individuals at the range of 70–80 pg/mL and 5±1 pg/mL, respectively.[16] This report also highlighted the IL-10 release by PBMCs increased in the same study groups, respectively, after MTB stimulation. In the patient group, the basal IL-10 level is significantly higher than the healthy controls. This might be due to the earlier antigenic exposure which already has increased the cytokine level. IL-

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

Demographic and clinical 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

10 level and favoring the survival of the pathogen toward disease progression.

Estimation of IL-4 shows significantly higher basal level of IL-4 in patient groups as compared to healthy controls. Similar *ex vivo* study report with PBMC is lacking. Rook et al., analyzed the existing data of different geographical locations and opined that IL-4 level is far high in patients from developing countries than the developed countries and correlated with chance of mycobacterial infection.[17] The serum level of IL-4 in far advanced TB patients is 1.2±2 pg/mL as revealed by Kart et al. In our study, base level IL-4 elaborated by PBMC ranges from 25 to 50 pg/mL and 60–90 pg/mL in the healthy and TB patients, respectively. Mycobacterial challenge has been found to increase IL-4 secretion in the healthy controls only. The reason perhaps the diseased individuals are inherently accelerated IL-4 producers facilitating the establishment of mycobacteria with proliferation and bring about pathological lesions. Moreover, it has already been reported by Hernandez-Pando et al., that a higher level of IL-4 in conjunction with tumor necrosis factor-alpha is toxic to the tissues causing degeneration and necrosis, a pathological phenomenon attended with TB lesions.[18] A further study by evaluation of various isoforms along with total IL-4 could have thrown more light to analyze the role of IL-4 in the pathogenesis of TB.

In this study, Th2 cytokine responses as explained with IL-10 and IL-4 are found to be higher in the patient groups as compared to healthy controls ($P<0.01$ and ≤ 0.0001, respectively, in all combinations) before our *in vitro* stimulation. Prior *in vivo* challenge of MTB among the patients has elevated the Th2 cytokine response in the system. Hence, in spite of a high Th1 response after mycobacterial challenge, the response is inhibited by the already existing Th2 cytokines, leading to tissue damage and expansion 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 produces 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. Although the presence of Th1 response is seen, the shift is more prominent toward the Th2 side for the diseased cases. More pertinent data subjecting clonal differentiation Ag-specific cell, obtained by cell separators and expanded by culture, to stimulation of mycobacteria and subsequent measurement of cytokine, would cast more insight about the knowledge of immunopathogenesis in TB.

Limitations of the study

This cross-sectional study showing the in-vitro immune response from a limited number of tuberculosis patients attending a tertiary care hospital 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 contribute towards understanding the immune response in tuberculosis.

CONCLUSION

The pattern of our data showed clear distinctions between the different clinical types of TB patients by their innate immune responses. This is evident that in new TB cases where fibronecrotic lesions were present, the response tilts toward proinflammatory side whereas, in RTB cases, the response is of anti-inflammatory type. Hence, it is concluded that the capacity to mount an inflammatory response determines the outcome of TB infection.

Further, investigation including more study number in each subgroups to generate a statistically significant data will enrich our knowledge in understanding the individual cell function and immunopathological mechanisms associated with the different clinical forms 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 the study protocol, data collection, literature survey, manuscript preparation, and revision; **AG-** Coordination and manuscript revision;
NKP- Concept, the definition of intellectual content, review manuscript.

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