Title

Chronic hyperglycemia drives alterations in macrophage effector function in pulmonary tuberculosis

Introduction

With WHO 2019 report labelling diabetes and TB as co-epidemic and, increased incidence of TB in diabetes have led to renewed interest in understanding pathophysiology of this co-epidemic (1). There is an urgent need to implement strategies for TB prevention among the millions of DM patients exposed to *Mycobacterium tuberculosis* (*M.Tb*) worldwide, but knowledge is limited on how and when DM alters the natural history of this infection. The increased incidence of TB in people with DM appears to be multifactorial. Chronic DM is associated with delayed innate immunity to *M.Tb.* due to late delivery of *M.Tb*-bearing antigen-presenting cells to the lung draining lymph nodes (2). Efficient phagocytosis and priming of the adaptive immune response are necessary to activate the cell-mediated immune responses that restrict initial *M.Tb.* growth and these delays likely contribute to the higher risk of DM patients for development of *M.Tb.* infection and persistence (3). Recent evidences suggest that innate as well as adaptive immune responses might be affected (4). Few mice model studies have suggested defect in immune response in terms of immune cell recruitment at site of infection and increased bacterial load in diabetic mice as compared to euglycemic mice (2,5). However, limited in vivo data is available in context of TB-DM. In this regard, understanding of alterations or disturbances in innate immune response in diabetic patients having TB infection is needed.

In the process of innate immunity, as the pathogen enters the host, it is recognized by innate cells like macrophages via pathogen recognition receptors followed by the phagocytosis of the pathogen and ultimately killing via formation of reactive oxygen and nitrogen species (6). Macrophages are key to the etiology of TB due to their dual role as a primary host cell reservoir for M. Tb, as well as being effector cells that control and eliminate *M.Tb.* (7,8). The examination of alveolar macrophages in TB-DM patients has revealed the presence of hypodense alveolar macrophages, which are less activated and are correlated with the severity of disease, implying that they might contribute to the increased susceptibility to *M.Tb.* infection (9). Little is known about the state of macrophage activation during the low-grade chronic inflammation linked to diabetes mellitus. According to our hypothesis, increased susceptibility of diabetic patients to TB could be due to the defects in macrophage effector functions like bacterial recognition, phagocytic activity, killing via reactive oxygen and nitrogen species and cellular activation due to hyperglycemia which could result in impaired or dysregulated immune response. Impaired immune response and killing of intracellular bacteria will then potentially increase bacterial load, chronic inflammation, and central necrosis that would facilitate bacterial dissemination.

Several pathogen recognition receptors (PRRs) like TLR 2, Mannose receptor (CD206), Complement receptor 3, Macrophage receptor with collagenous structure (MARCO) and CD14 receptors are involved in recognition of the *Mycobacterium tuberculosis* bacillus (10). After recognition, the bacteria are phagocytosed by the immune cells mainly by macrophages followed by phagocytic killing via nitric oxide (NO) and reactive oxygen species (ROS) (11). Chronic inflammatory condition like diabetes mellitus also leads to production of ROS (12). Overstimulated ROS production under this condition may also have adverse effect on host.

Taken these available literature under consideration, we postulated that hyperglycemia may affect the expression of different PRRs on macrophages which may alter the entry of *M.Tb.* ultimately affecting the phagocytosis and subsequent bacterial killing. Additionally, there are contradictory reports regarding production of ROS in diabetic milieu due to chronic inflammatory condition which may again lead to increased pathology in TB infection. So, there is a need to assess alterations in PRRs along with phagocytosis and ROS in macrophages to explain the host aspect in development of active tuberculosis under hyperglycemic condition. Limited human studies are available in this area and none in PTB+DM cohort. Therefore, with

this background, we hypothesize that under chronic hyperglycemia in type 2 DM there could be increased susceptibility of TB due to defect in innate immune responses in macrophages. The present study was designed to assess the alterations in macrophage effector function in terms of bacterial recognition followed by phagocytosis and bacterial killing via ROS/RNS in diabetic patients having TB. Therefore, surface expression of different PRRs like TLR 2, Mannose receptor (CD206), Complement receptor 3, Macrophage receptor with collagenous structure (MARCO) and CD14 receptors were studied in DM patients having TB. The change in phagocytic capacity along with ROS and NO production was also studied in these patients. Understanding of defect in innate immune response in diabetic condition could help in early identification of the active disease among diabetic individuals and future development of new treatment targets to limit development of TB among them.

Objectives

1. To study expression of pathogen recognition receptors (PRRs) namely TLR2, Mannose receptor (CD206), Complement receptor 3, MARCO and CD 14 on macrophages in four groups namely Type 2 DM with Active TB, Active TB without type 2 DM, only Type 2 DM with no history of TB and healthy individuals.
2. To study the phagocytic capacity of macrophages in all the study groups.
3. To assess the levels of ROS and NO in all the study groups.
4. To correlate phagocytosis index with different PRRs in all the study groups

Material and Methods

This was a cross sectional study which included treatment naïve pulmonary tuberculosis patients (PTB), uncontrolled type 2 diabetic patients with HbA1c levels >7.5 (DM), treatment naïve pulmonary tuberculosis patients along with uncontrolled type 2 diabetes with HbA1c levels >7.5 (PTB+DM) and healthy controls with no known history of TB and diabetes. Study participants were recruited from Department of Medicine, All India Institute of Medical Sciences, New Delhi and DOTS center, All India Institute of Medical Sciences, New Delhi and Vardhaman Mahavir Medical College, Safdarjung Hospital, New Delhi. A total of 221 individuals were recruited for the study based upon the inclusion and exclusion criteria which included 65 PTB, 51 DM, 50 PTB+DM and 55 healthy controls. Individuals were interviewed to assess risk factors for TB, history of DM and other factors that could affect their immune response. For PTB patients, individuals with previous history of TB were excluded from the study. For DM patients, individuals taking metformin, corticosteroids, aspirin or TNF blockers were also excluded to avoid the confounding factors which may alter the immune function. Height and weight were recorded to calculate body mass index. The study was approved by institutional ethics committee (IECPG-374/28.09.2017). Informed consent was taken from all the study participants.

PBMCs were isolated from whole blood taken from the study participants. Monocytes were isolated from PBMCs by plastic adherence method and were cultured into macrophages using 35 ng/ml GM-CSF for 9 days. The differentiated macrophages was then used in subsequent experiments after purity check by flow cytometry using CD11b and CD14 antibodies. Differentiated macrophages were $CD11b^{high}$ $CD14^{low}$.

Surface expression of different pathogen recognition receptors namely TLR2, Mannose receptor (CD206), Complement receptor 3, MARCO and CD 14 were studied on macrophages by flow cytometry using fluorochrome tagged antibodies. Briefly $1x10^5$ macrophages were stained with antibody cocktail for 30 min to 1 hour at 37°C followed by RBC lysis using 1X RBC lysis buffer (Bio legend). Cells were incubated in 500ul of red cell lysis buffer for 10 min followed by washing with 1X PBS. After staining, cells were acquired on BD LSR fortessa X-20 and median fluorescence intensity (MFI) values and percentage positivity were recorded. Analysis was performed on FlowJo V10.

Phagocytosis activity of macrophages were studied using fluorescent detection method. Briefly, BCG were tagged with FITC by incubating 0.2ul of 5mg/ml FITC for one hour at room temperature with end-to-end rotation followed by washing with PBS to remove unbound FITC.

Macrophages were then incubated with FITC labelled BCG at MOI of 10 for one hour at 37^0C. The engulfed fluorescent targets were then detected using flow cytometry.

Reactive oxygen species in macrophages were estimated using Dichloro-dihydro-fluorescein diacetate (DCF-DA) assay. Briefly, 1-2 X 10^4 macrophages were taken in flow tube. Then 10^5 bacteria were added to the cells 1 hour prior to the treatment. 500ul of DCFDA solution was added in a concentration of 20 uM followed by 30 min incubation at 37^0C. Signal was read using flow cytometry. NO levels were measured in serum using colorimetric assay based on griess reaction (fig 6). Griess reagent is based on Griess reaction. The components include naphthylethylenediamine dihydrochloride suspended in water and sulphanilamide in phosphoric acid. This reagent reacts with nitrite in samples to form a purple azo product, absorbance of which is measured at 540 nm.

Statistical analysis

Data were managed and analysed using SPSS (version 13.0) and GraphPad Prism (version 6.0). Categorical variables were presented as counts and percentages. Continuous variables were reported as mean (SD) or median (interquartile range [IQR]) after assessment of normality. Non- parametric statistical analyses were performed throughout the study. Correlation between variables were assessed using Spearman correlation. Mann whitney U and Kruskal wallis test (with Bonferroni correction) were used for comparison between two groups and three groups respectively. Multiple linear regression was applied for association between multiple variables. Data was represented as Median ± Interquartile range. A 2-tailed $p < 0.05$ was considered statistically significant for all conducted analyses.

Results

Clinical and laboratory data of study participants

The present study included a total of 221 study participants from Delhi NCR population and were recruited under four groups namely pulmonary tuberculosis patients (PTB), uncontrolled Type 2 diabetes mellitus patients (DM), pulmonary tuberculosis patients having uncontrolled type 2 diabetes (PTB+DM) and healthy controls based upon the inclusion and exclusion criteria mentioned in the methodology section. Study participants were recruited from north Indian population after obtaining informed consent. The demographic and clinical characteristics are shown in Table 1.

All recruited pulmonary TB patients with or without type 2 DM underwent sputum AFB test or gene xpert test as confirmatory test for tuberculosis. Among 65 PTB patients, 87.69% were sputum positive, out of which 59.6% were 1+ grade, 19.29% were 2+ grade and 21.05% were 3+ grade. However, in PTB+DM group, 78% were sputum positive, out of which the frequency of 3+ grade was higher (53.84%) as compared to 2+ (23.07%) and 3+ (23.07%). Since the phenotype of PTB+DMs might differ between study participants who were diabetic prior to TB incident and those with an initial diagnosis of diabetes at the time of PTB incident, we restricted the recruitment criteria to participants with pre-existing uncontrolled diabetes with initial diagnosis of PTB.

Demographic characteristics	PTB+DM (n=50)	PTB patients (n=65)	DM patients (n=51)	Healthy controls (n=55)
Age (Mean ± SD)	40.6±5.5	44.2±6.3	49.5±6.9	39.2±3.4
Male n (%)	36 (72)	49 (75.3)	31 (60.78)	43(78.18)
Female n (%)	14 (28)	16 (24.6)	20 (39.21)	12 (21.81)
Region	North Indian	North Indian	North Indian	North Indian
Smoking n (%)	(11) 22	25 (38.46)	7 (13.72)	4 (5.4)

Body Mass Index (Kg/m^2) in mean±SD	22.2±4.2	19.6±4.1	25.1±3.6	18.9±2.6
HbA1c (%)	9.46±2.16	4.85±0.71	9.78±2.32	4.95±0.73
Gene expert, n (%)	11(22)	8(12.30)	Not done	Not Done
AFB positive smear, n (%)	39(78)	57(87.69)	Not done	Not Done
1+ sputum positivity (n) 2+ sputum positivity(n) 3+ sputum positivity(n)	9 9 21	34 11 12	Not done	Not done

Table 1 – Demographic and clinical characteristics of study participants

Surface expression of different pathogen recognition receptors on macrophages of study groups, namely PTB, DM, PTB+DM and controls

Significant alterations in PRRs levels were observed among all the study groups. All receptors were significantly higher in patient groups as compared to healthy controls (Figure 1). Complement receptor 3 or CD11b was found to be significantly decreased in PTB+DM patients as compared to PTB patients as shown in figure 3(a) ($p<0.01$). The levels were comparable in DM and PTB+DM patients. CD14 levels were comparable between PTB+DM and DM patients, however slightly higher than PTB patients ($p<0.05$). MARCO levels were significantly decreased in PTB+DM patients compared to PTB ($p<0.05$). The levels of TLR 2 were found to be higher in all patient group as compared to controls ($p<0.05$). However, levels were comparable between PTB, DM and PTB+DM. CD206 levels were found to be significantly higher in DM milieu in both PTB+DM and DM patients as compared to PTB ($p<0.01$ and 0.001).

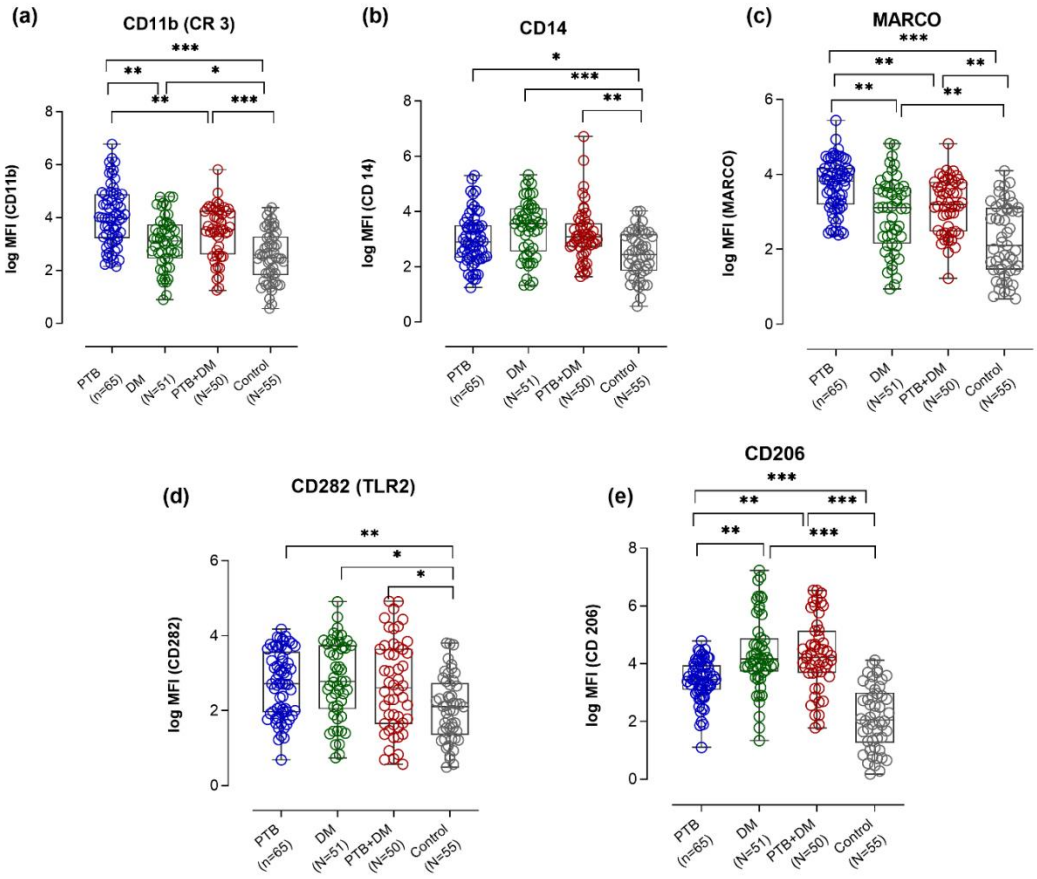

Figure 1 – Surface expression of different pathogen recognition receptors on macrophages of study groups namely PTB, DM, PTB+DM and controls

Since MARCO and CD14 act as heterodimer to exert their function, we tried to correlate the surface expression of MARCO and CD14 present on macrophages of all the study groups. We found positive correlation in levels of MARCO and CD14 in PTB and healthy control group (r=0.71 and 0.76). However, no correlation was found in PTB +DM and DM group as shown in Figure 2.

Along with MARCO, CD14 is required for TLR 2 activation and initiation of downstream signalling pathway. Therefore, we correlated the surface expression of TLR 2 and CD14 present on macrophages of all the study groups. We found positive correlation in levels of TLR 2 and CD14 in PTB and PTB+DM group (r=0.72 and 0.69 respectively). However, no correlation was found in DM and healthy control group as shown in figure 3.

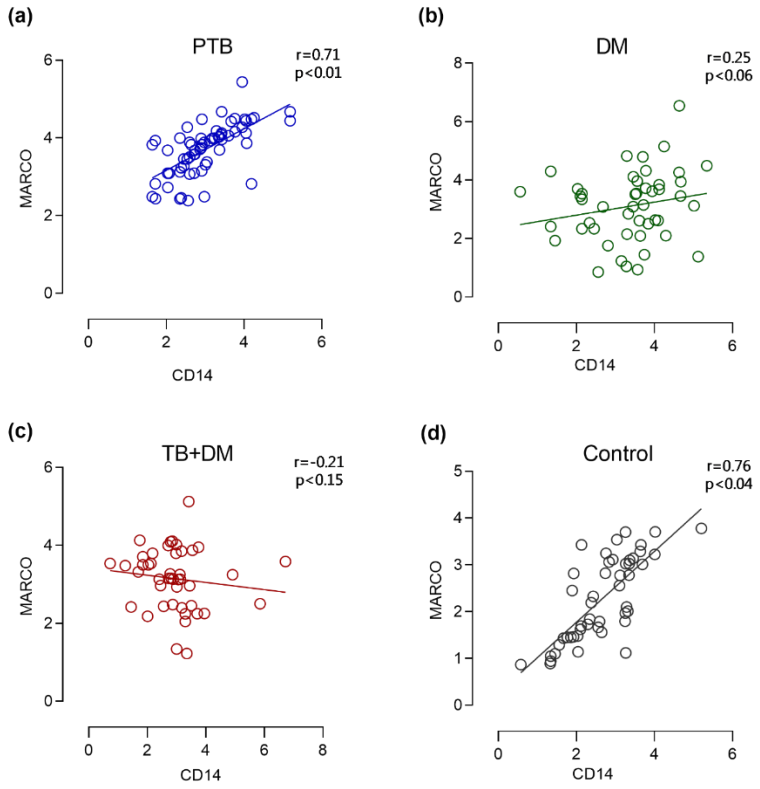

Figure 2 – Correlation between surface expression of MARCO and CD14 on macrophages of all the study groups namely PTB, DM, PTB+DM and controls.

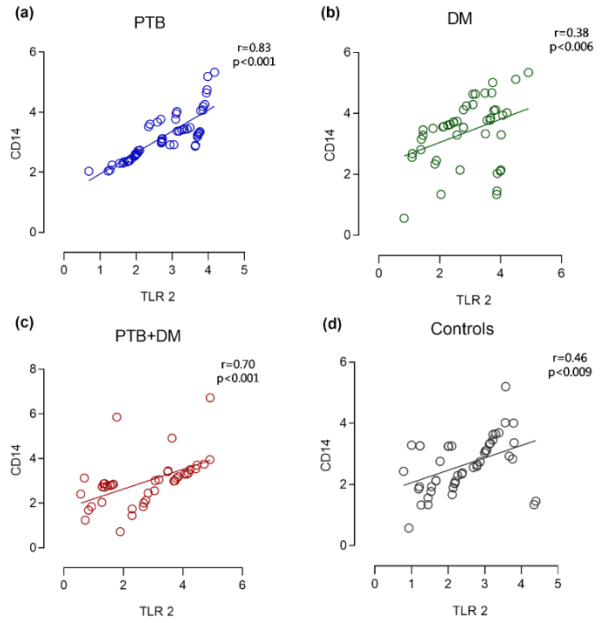

Figure 3 – Correlation between surface expression of TLR 2 and CD14 on macrophages of all the study groups namely PTB, DM, PTB+DM and controls.

Since we found significant difference in levels of different PRRs in our patient group, we tried to evaluate if there is alteration in these PRRs with disease severity. Therefore, we subdivided PTB and PTB+DM patients based upon their sputum positivity into 1+, 2+ and 3+ sputum

positive patients and assessed the levels of CD11b, CD14, MARCO, CD206 and TLR2 as shown in Figure 4 and Figure 5 respectively. We have observed significantly decreased levels of CD11b levels in 3+ sputum positive patients as compared to 2+ and 1+ sputum positive patients of both PTB and PTB+DM patients ($p<0.01$ and 0.06 respectively). In case of CD14, no difference was observed in different sputum positive PTB patients ($p<0.11$). However, the levels were significantly decreased in 3+ sputum positive PTB+DM patients as compared to 1+ patients ($p<0.02$). A similar trend of lower expression levels with increased disease severity was observed for MARCO in both PTB and PTB + DM patients with lowest levels seen in 3+ sputum positive patients ($p<0.0001$ and 0.001 respectively). Levels of TLR2 was also significantly decreased in 3+ patients as compared to 1+ and 2+ in PTB+DM patients ($p<0.0006$). However, no difference was found in PTB patients ($p<0.25$). Similarly, no significant difference was observed in CD206 levels of different sputum positive patients of PTB group. However, significantly higher levels of CD206 were observed in 3+ sputum positive PTB+DM patients as compared to 1+ and 2+ sputum positive patients ($p<0.04$).

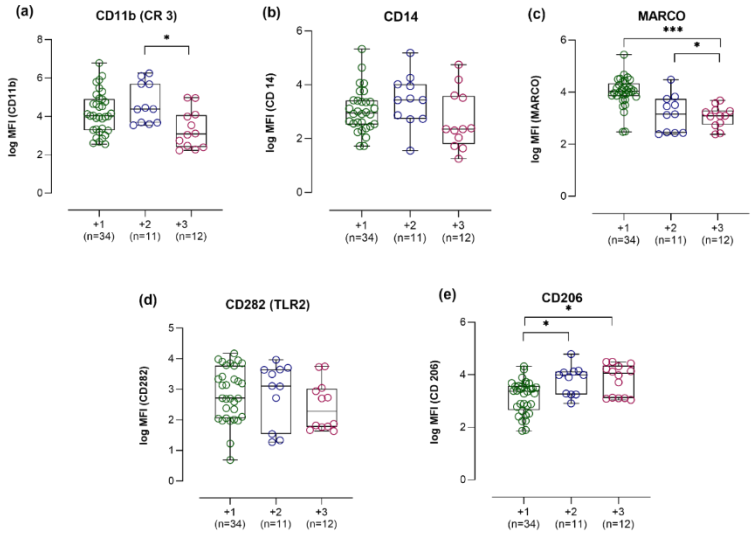

Figure 4 – Surface expression of different pathogen recognition receptors on macrophages of different sputum grade PTB patients.

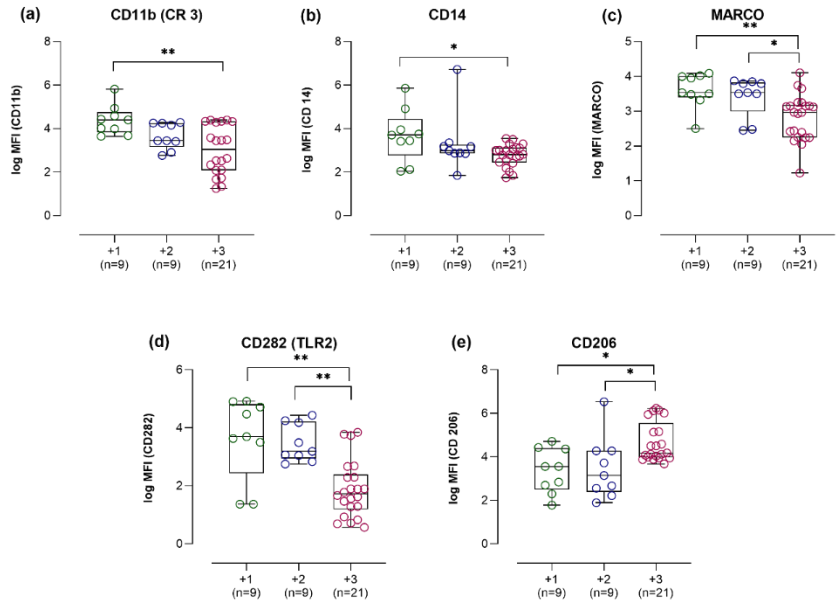

Figure 5 – Surface expression of different pathogen recognition receptors on macrophages of different sputum grade PTB+DM patients.

Phagocytosis capacity of macrophages of study groups, namely PTB, DM, PTB+DM and controls

M.Tb. entry through different PRRs have different fate on bacteria like proper opsonization and phagocytosis of bacteria. Therefore, phagocytosis capacity of macrophages was also studied in macrophages of study participants after invitro infection with BCG. Phagocytic capacity was found to higher in all patient group as compared to healthy controls as shown in Figure 6 ($p<0.0001$). In between patient groups, phagocytosis capacity was found to be significantly decreased in PTB+DM and DM patients compared PTB patients ($p< 0.001$). However, PTB+DM and DM group had comparable phagocytosis capacity.

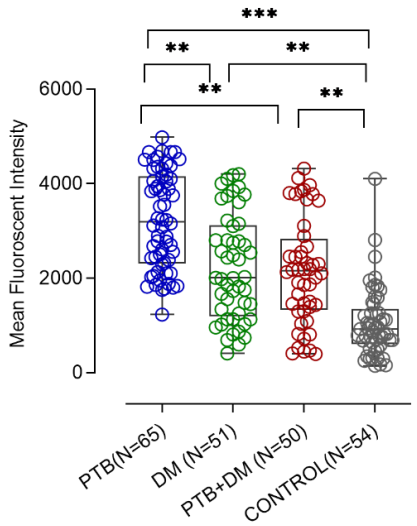

Figure 6 – Phagocytosis capacity of cultured macrophages after BCG infection in all study groups namely PTB, DM, PTB+DM and controls.

PTB and PTB+DM patients were subdivided into different sputum grades to check for phagocytosis capacity of macrophages in different disease severity. We have observed significantly decreased phagocytosis capacity with increased sputum positivity or increased disease severity in both PTB and PTB+DM patients ($p<0.007$ and 0.02 respectively) as shown in Figure 7

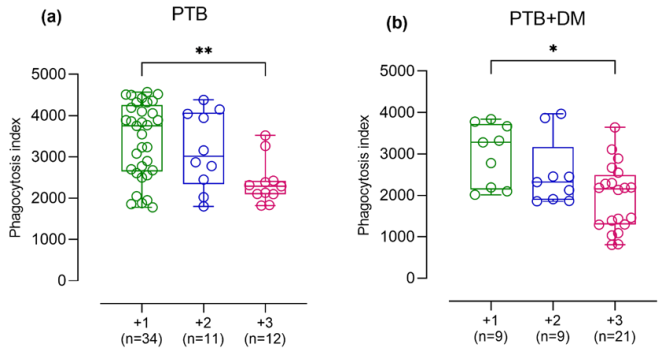

Figure 7 – Phagocytosis of cultured macrophages after BCG infection in different sputum grade PTB (a) and PTB+DM (b) patients.

Association among different pathogen recognition receptors and phagocytosis capacity with individual PRRs in study groups namely PTB, DM, PTB+DM and controls

Phagocytosis capacity of macrophages can be affected by the surface expression of different pathogen recognition receptors, therefore, the association of these PRRs (independent variable) with phagocytosis (dependant variable) was evaluated using multiple linear regression in all study groups. It was observed that phagocytosis capacity was associated with the PRRs namely CD11b, CD14 and CD206 in PTB+DM group (Table 2). Phagocytosis capacity was associated with the PRRs namely CD11b and MARCO in PTB+DM group and CD11b and CD206 in DM group (Table 2).

Parameter estimates	Variable	P value	P value summary	95% confidence interval
PTB+DM				
β1	CD11b	0.0172	*	56.45 to 550.6
β2	CD14	0.0409	*	7.282 to 329.7
β3	MARCO	0.1629	ns	-91.12 to 524.9
β4	TLR 2	0.1902	ns	-60.77 to 296.9
β5	CD206	0.0022	**	-545.1 to -128.4
PTB				
β1	CD11b	0.0083	**	76.45 to 495.2
β2	CD14	0.4543	ns	-147.6 to 325.8
β3	MARCO	0.0095	**	104.1 to 718.7
β4	TLR 2	0.9604	ns	-276.8 to 263.3
β5	CD206	0.4387	ns	-177.8 to 404.8
DM				
β1	CD11b	0.0183	*	52.17 to 536.5
β2	CD14	0.2327	ns	-331.0 to 82.58
β3	MARCO	0.8217	ns	-167.6 to 210.1
β4	TLR 2	0.2274	ns	-81.13 to 332.4
β5	CD206	0.001	***	-673.9 to -254.5
Controls				
β1	CD11b	0.0981	ns	-35.21 to 401.4
β2	CD14	0.7062	ns	-202.0 to 295.8
β3	MARCO	0.1932	ns	-75.22 to 362.1
β4	TLR 2	0.9872	ns	-239.9 to 243.7
β5	CD206	0.2507	ns	-81.72 to 305.5

Table 2– Linear regression analysis with phagocytosis as dependent variable.

Correlation of phagocytosis with different pathogen recognition receptors in study groups namely PTB, DM, PTB+DM and controls.

Different pathogen recognition receptors (PRRs) are engaged in phagocytosis during infection and may lead to either pro inflammatory or anti-inflammatory response. Therefore, the PRRs studied here were correlated with phagocytic capacity of the macrophages in all the study groups. It was observed that some of these PRRs affect the phagocytic capacity of macrophages in PTB, PTB+DM and DM group. CD11b was found to be positively correlated with phagocytosis index in PTB (r=0.51), PTB+DM (r=0.78) and DM (r=67) group (Figure 8). No correlation was found between CD14 levels and phagocytosis index in all the study groups (Figure 9). MARCO was found to be positively correlated with phagocytosis index in PTB (r=0.49) and PTB+DM (r=0.71) group (Figure 10). A positive correlation was found in TLR 2 and phagocytosis index in PTB (r=0.27), PTB+DM (r=0.67) and DM (r=0.41) group (Figure ure 11). In case of CD206, a negative correlation was found between CD206 and phagocytosis

index in PTB+DM (r=-0.85) and DM (r=-0.78) group (Figure 12). No correlation was found in PTB and healthy control group

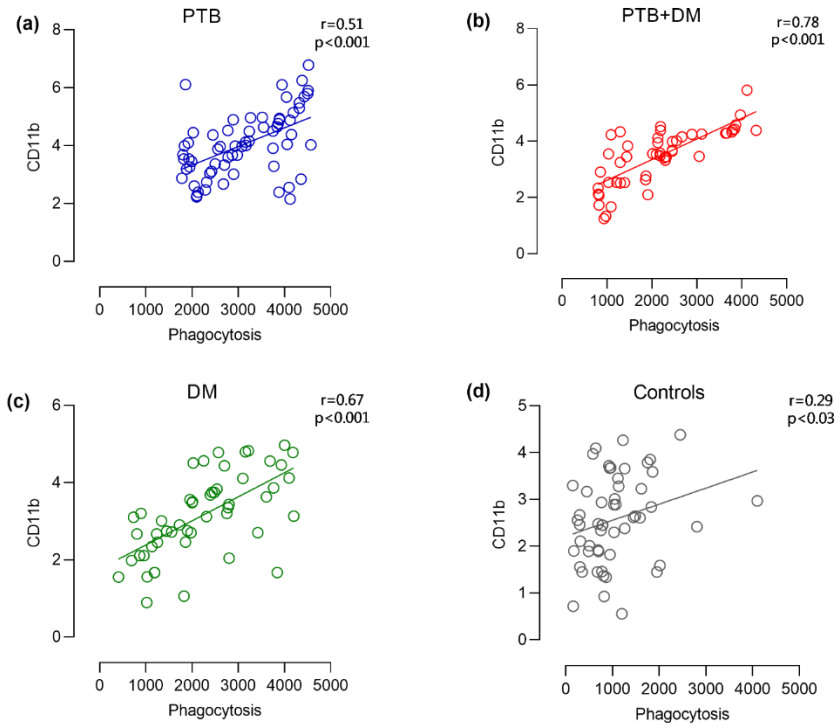

Figure 8 – Correlation between surface expression of CD11b and phagocytosis index of macrophages in all study groups (a) PTB, (b) PTB+DM, (c) DM and (d) controls.

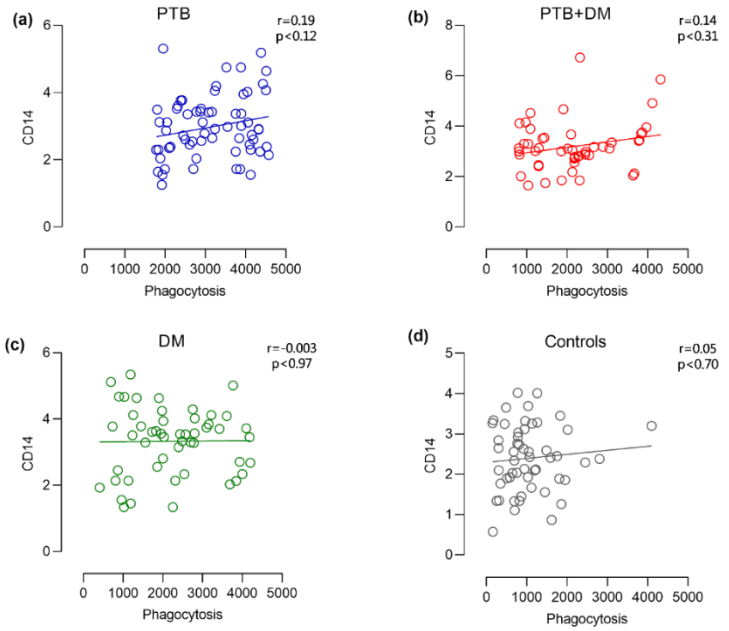

Figure 9 – Correlation between surface expression of CD14 and phagocytosis index of macrophages in all study groups (a) PTB, (b) PTB+DM, (c) DM and (d) controls.

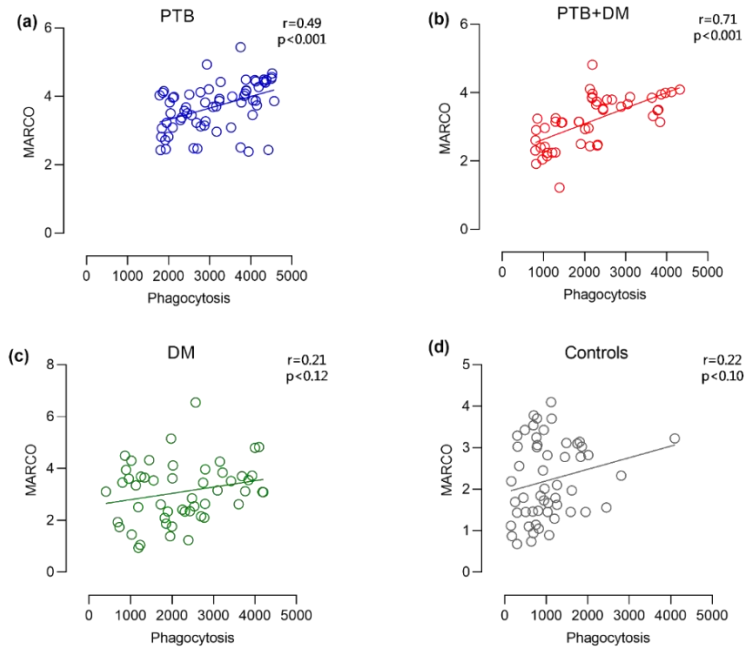

Figure 10 – Correlation between surface expression of MARCO and phagocytosis index of macrophages in all study groups (a) PTB, (b) PTB+DM, (c) DM and (d) controls.

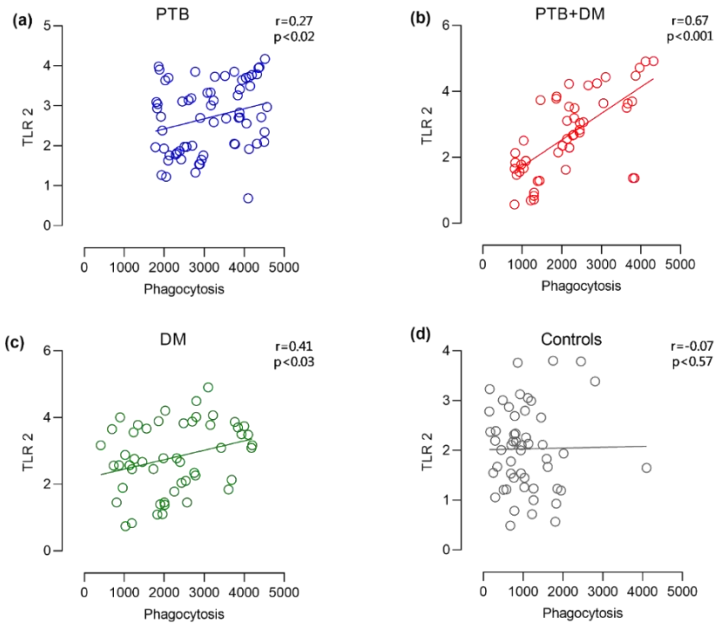

Figure 11 – Correlation between surface expression of TLR 2 and phagocytosis index of macrophages in all study groups (a) PTB, (b) PTB+DM, (c) DM and (d) controls.

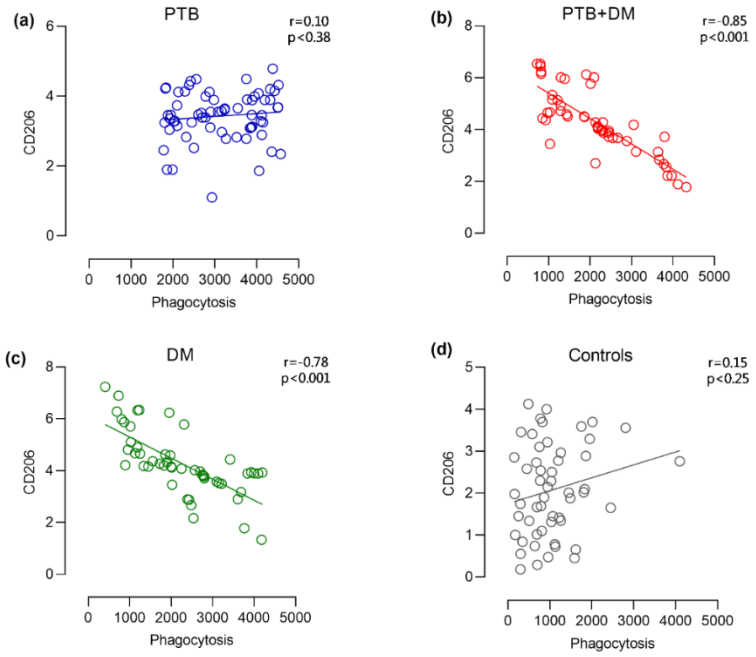

Figure 12 – Correlation between surface expression of CD206 and phagocytosis index of macrophages in all study groups (a) PTB, (b) PTB+DM, (c) DM and (d) controls.

Levels of Reactive oxygen species (ROS) in macrophages and serum nitric oxide (NO) of study groups namely PTB, DM, PTB+DM and controls

Reactive oxygen species (ROS) are antimicrobial agents generated by phagocytic cells in response to microbe recognition. However, ROS have been called double edge sword if its production is not regulated. Hyperglycemic state also leads to activation of NADPH oxidase and production of ROS leading to oxidative stress in diabetes. Therefore, we assessed the levels of ROS in PTB, DM and PTB+DM patients. We have observed that ROS levels were significantly higher in DM and PTB+DM patients as compared to PTB patients ($p<0.01$ and 0.001 respectively) and healthy controls ($p<0.001$). Also, PTB patients had higher levels of ROS as compared to healthy controls ($p<0.001$) as shown in Figure 13. Along with ROS, nitric oxide (NO) also plays bactericidal role in *M.Tb*. infection. Therefore, NO levels were also measured in all the study groups. Taking into consideration the fact that NO is an unstable molecule with half-life of less than 5 s, we aimed to measure the content of stable serum metabolites of NO that is, nitrites. The levels of NO were found to be higher in PTB group (27.32 ± 8.50 µmol/L) as compared to PTB+DM (22.76 ± 5.41 µmol/L), DM (19.44 ± 4.04 µmol/L) and healthy controls (20.60 ± 2.99 µmol/L). The difference was found to statistically significant with higher levels in PTB group as compared to DM and PTB+DM ($p<0.001$ and 0.01) as shown in Figure 13.

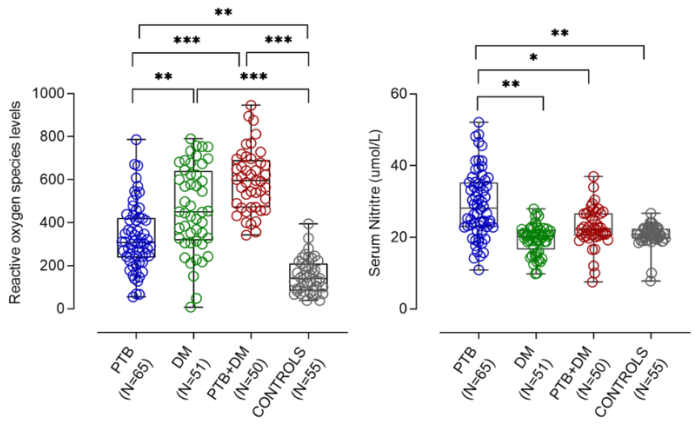

Figure 13 – ROS levels in macrophages after BCG infection and serum NO levels in study groups namely PTB, DM, PTB+DM and controls.

While comparing ROS levels in different sputum grade patients, no significant difference was observed in ROS levels of different sputum positive PTB patients ($p<0.08$). However, in PTB+DM patient group, higher levels of ROS were observed in 3+ sputum positive patients as compared to 2+ and 1+ ($p<0.005$) as shown in Figure 14. NO levels were found to be significantly decreased in 3+ sputum positive patients as compared to 2+ and 1+ sputum patients of both PTB and PTB+DM ($p<0.004$ and 0.005 respectively) as shown in Figure 14

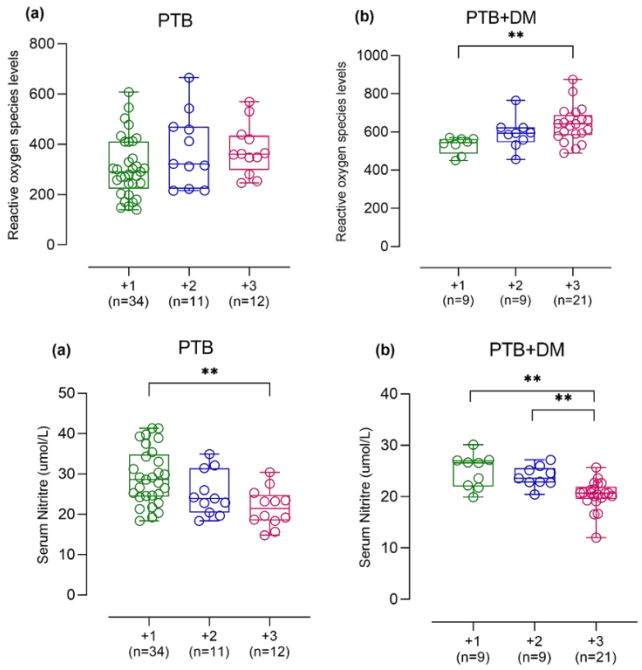

Figure 14 – ROS and NO levels in serum of different sputum grade PTB (a) and PTB+DM (b) patients.

Discussion

We conducted the first study aimed at identifying alterations in the innate immune response, with a particular emphasis on alteration in macrophage effector function under chronic hyperglycemic condition to understand the underlying mechanism of increased susceptibility to TB in DM patients. In the present study, newly diagnosed pulmonary TB patients (PTB), uncontrolled type 2 diabetic patients (DM) and patients with both PTB and uncontrolled

diabetes (PTB+DM) were recruited. Majority of the individuals within PTB+DM group had 3+ sputum positivity (high bacterial burden) which suggests a plausible association of diabetes with increased disease severity in pulmonary tuberculosis patients.

Upon *M.Tb*. infection, the first line of host defense starts with recognition of *M.Tb.* by binding of pathogen associated molecular patterns (PAMPS) to different pathogen recognition receptors (PRRs) present on innate cells like macrophages. There are various recognition markers required to identify and phagocytose the TB bacteria in order to neutralise it (Amaral et al., 2016). Depending upon the pathogen recognition receptors that are activated by the bacterial PAMPs, distinct pathways are initiated following bacterial phagocytosis, ultimately leading to either bacterial killing or latency or multiplication. Therefore, we assessed the alterations in levels of different PRRs involved in *M.tb*. recognition and uptake, namely, CD11b (CR3), CD14, MARCO, TLR2 and CD206 in PTB, DM, and PTB+DM patients. Till now, no studies have been reported on assessing the levels of these PRRs in the said study groups.

We have observed reduced levels of CD11b in PTB+DM patients as compared to only PTB patients. CD11b plays critical role in mycobacterial infections in terms of cell adhesion, migration and phagocytosis. Decreased levels of CD11b under hyperglycemic condition in our study group may have led to decreased complement-mediated opsonization of the bacteria and hence increased susceptibility to TB infection in diabetic patients. Another receptor, CD14 was found to be higher in diabetic milieu (PTB+DM and DM). CD14 with the help of its co-receptors can either be beneficial to the host by induction of an adequate inflammatory and immune response to eradicate the invading microbe, or harmful to the host due to excessive inflammation and/or dissemination of the pathogen depending on the microbe and the PAMPs it expresses. While talking in terms of CD14 functions, a very few studies have been conducted regarding the role of CD14 in mycobacterial infections, however the findings were controversial. CD14 is required for *M.Tb.* recognition and signalling when cooperated with other receptors like MARCO and TLR2. Therefore, we assessed levels of MARCO and TLR2 which are two co-receptors of CD14. MARCO is a phagocytic receptor which has been implicated in host defense. It recognizes trehalose 6,6′-dimycolate (TDM) which mediates potent inflammatory response via its coreceptor CD14 and TLR 2. Decreased levels of MARCO was observed in PTB+DM group which may suggest disruption in sensing and clearing of the *M.Tb*. pathogen and hence, increased susceptibility and disease severity. MARCO-expressing macrophages appear to phagocytose more BCG than neighbouring macrophages that do not express MARCO in the splenic marginal zone. Even though, CD14 levels were higher in diabetic milieu, lower levels of MARCO receptor may lead to defective recognition and hence, increased bacterial survival in diabetic milieu. Positive correlation between CD14 and MARCO in PTB and control group may suggest optimal expression of both the receptor and hence, increased bacterial uptake from CD14-MARCO receptor complex. However, no correlation was found in diabetic patients with or without infection suggesting dysregulation in these receptors thereby affecting bacterial uptake and clearance. TLR2 which is another coreceptor of CD14 was found to be higher in all the patient group which may be due to the trigger from active infection as well as inflammation (DM) which in turn can trigger various innate immune response depending upon its coreceptor as well as the ligand to which it binds. Initial higher expression of TLR 2 along with its co receptor CD14 may worsen the outcome of infection by destructive inflammation in pulmonary tuberculosis. It may also maintain dormant state of *M.Tb*. and help in surviving the bacteria in latent form. Although, increased expression of TLR 2 was observed in patient group, the downstream signalling and hence, bacterial fate, can be checked by looking upon the ligands and different TLR 2 coreceptors. In the present study, the levels of other TLRs in combination with TLR 2 and different ligands are not studied. However, CD14 is also one of the co-receptors of TLR 2, therefore while correlating the levels of CD14 and TLR 2, a positive correlation was found in PTB+DM patients, suggesting increased bacterial uptake from CD14-TLR 2 coreceptor rather than CD14-MARCO coreceptor (no correlation was found in PTB+DM patients). As discussed earlier, CD14-TLR 2 overstimulation may lead to dissemination of infection and hence

increased susceptibility to the infection. However, mechanistical studies are required to validate these findings. Mannose receptor (CD206) is another important receptor which we have studied and found that the levels were higher in diabetic milieu (PTB+DM and DM) as compared to PTB only. CD206 plays a role in immune recognition of pathogens, following antigen internalization and presentation. However, this receptor is expressed in M2 macrophages which has anti-inflammatory effect during infection. Our data showed increased expression of CD206 in hyperglycemic condition which may suggest increased *M.Tb.* uptake via CD206 receptor which in turn may help in bacterial survival by inhibiting phagosome-lysosome fusion and shifting the macrophages to M2 phenotype which has anti-inflammatory response. This may lead to persistence, bacterial multiplication and a possible spread of infection. However, mechanistic studies need to be conducted to see the effect of CD206 receptor inhibition on bacterial killing.

We also assessed the levels of these PRRs in different sputum grades of PTB and PTB+DM patients. Interestingly, we observed significant decreased expression of CD11b, CD14, MARCO and TLR 2 in 3+ sputum positive which is more severe disease condition than 1+ sputum positive PTB+DM patients. As expected, CD206 levels were higher in 3+ as compared to 1+ and 2+. These findings again suggest the adverse effect of hyperglycemia on alterations in these pathogen recognition receptors which correlates with disease severity. These findings altogether may suggest alterations in different pathogen recognition receptors which ultimately affect the downstream signalling pathway like defective chemotaxis due to decrease expression of CD11b and altered route of entry of the pathogen through CD206 in DM patients which may influence the downstream activation of signalling pathways in macrophages and survival of the bacteria. However, mechanistical studies are required for validating these results. An alteration in these PRRs under hyperglycaemic condition could also lead to defect in macrophage function like phagocytosis and killing mechanism which was studied next.

Upon *M.Tb.* infection, macrophages uptake the bacteria through various PRRs as described above and lead to phagocytosis and subsequent bacterial clearance. We found significantly decreased phagocytic capacity of macrophages of PTB+DM and DM patients. One of the plausible reasons for decreased phagocytosis is that hyperglycemia can cause direct glycosylation of several proteins including complements and alter their tertiary structure which may inhibit opsonization of bacteria and thus decrease phagocytosis. Findings of decreased phagocytosis capacity of macrophages in diabetic milieu corroborated with lower levels of CD11b, CD14 and MARCO receptors which are required for internalization of the pathogen. Similarly, the phagocytic capacity was found to be decreased with increased disease severity as 3+ sputum positive patients showed decreased phagocytosis.

Once we studied recognition markers and phagocytosis capacity of macrophages in our study groups, we then checked for the *M.tb.* killing mechanism of macrophages via levels of reactive oxygen species (ROS) and nitric oxide (NO) in macrophages. In our study group, we observed significantly higher levels of ROS in PTB+DM patients as compared to PTB only, however PTB patients had higher levels than healthy controls. Increased levels of ROS in PTB suggests trigger from active bacterial infection to control the infection. Although ROS plays an important role in mycobacterial killing, a recent study has shown that *M.Tb*. shows resistance against ROS during the chronic or persistence stage of infection. The levels were much higher in diabetic milieu which may have adverse effect on infected tissue as overproduction of ROS can lead to necrosis of granuloma due to increased oxidative stress in the immune cell and hence, disruption of granuloma which may lead to dissemination of the infection. Therefore, it is important to point out that the survivability of *M. tuberculosis* is highly dependent on the levels of ROS produced by the host immune cells. It was evident by our findings of increased ROS levels in 3+ sputum positive PTB+DM patients suggesting increase in disease severity upon higher ROS production under hyperglycemic condition. Therefore, higher ROS levels in PTB+DM could be counterproductive in terms of bacterial killing. Mechanistically, among all the ROS and RNS, nitric oxide (NO) is known to be one of the major contributors as an anti-TB agent and it is synthesized by inducible form of nitric oxide synthase, iNOS. As expected,

Nitric oxide levels were higher in PTB group suggesting active infection and induction of NO via iNOS. NO can however, have a ying-yang effect on the clearance of infection and the inflammatory response depending upon the mycobacterial strain. In case of PTB+DM patients, decreased levels of NO under hyperglycemic condition may suggest alterations in iNOS activity as it was shown previously that high glucose condition leads to glycation of several proteins including iNOS which may affect their activity. Unlike ROS, NO levels were found to be significantly lower in 3+ sputum positive patients suggesting protective role of NO in *M.tb*. infection.

Taken together, our data shows there is a dynamic relationship between TB infection and DM disease where there is a complex interplay between the pathogenesis and progression of the pathology in them. The present study has observed alterations in macrophage function in terms of recognition, phagocytosis and killing of *M.Tb.* under hyperglycemic state which may be one of the plausible reasons for increased susceptibility and co-morbidity of diabetes and TB. In the present study, 4 TB+DM patients having HbA1c levels of 7.5-8.0 showed better phagocytosis capacity as compared to others. Patients having HbA1c levels more than 9.5 (9 in DM and 6 in TB+DM group) showed decrease in phagocytosis levels showing adverse effect of hyperglycemia on macrophage function. Based upon this finding, we have planned to study innate immune response in terms of macrophage and other immune cell effector functions at different cut offs of HbA1c levels so as to point out the hyperglycemic state at which macrophage dysregulation occurs. A detailed mechanistic study for an in depth understanding of the immunological basis of TB susceptibility in DM will help in the rational development of therapeutic strategies to alleviate the dual burden of these diseases. Also, a better understanding of the mechanisms of hyperglycemia impairing host defense against pathogens is crucial for the development of novel strategies to treat infections in diabetic patients, thus improving treatment outcomes.

Impact of the research in the advancement of knowledge or benefit to mankind

The risk of developing active tuberculosis is a two-step process, beginning with initial exposure to and infection by Mycobacterium tuberculosis followed by subsequent progression to disease. Being an immunocompromised state DM is a risk factor for development of tuberculosis. However, there is no clear understanding of how Type 2 DM is associated with increased susceptibility of acquiring TB. It is absolutely essential to determine the mechanism by which this phenomenon occurs and has to be prevented thereby reducing the burden of disease in the already potentially immune compromised hyperglycemic state.

Since macrophages are the first line of defense against *M.Tb.* infection, the present study has explored the role of defective macrophage function if any under conditions of hyperglycemia in the pathogenesis of TB. These findings would further help in early identification of the active disease among diabetic individuals and new treatment targets to limit development of TB among them.

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