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Chronic hyperglycemia drives alterations in macrophage effector function in pulmonary tuberculosis

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Diabetes mellitus (DM) alters immune responses and given the rising prevalence of DM in tuberculosis (TB) endemic countries; hyperglycemia can be a potential risk factor for active TB development. However, the impact of hyperglycemia on TB-specific innate immune response in terms of macrophage functions remains poorly addressed. We assessed macrophage effector functions in uncontrolled DM patients with or without TB infection (PTB+DM and DM), non-diabetic TB patients (PTB), and non-diabetic-uninfected controls. Phagocytic capacity against BCG and surface expression of different pattern recognition receptors (PRRs) (CD11b, CD14, CD206, MARCO, and TLR-2) were measured via flow cytometry. Effector molecules (ROS and NO) required for bacterial killing were assessed via DCFDA and Griess reaction respectively. A systematic dysregulation in phagocytic capacity with concurrent alterations in the expression pattern of key PRRs (CD11b, MARCO, and CD206) was observed in PTB+DM. These altered PRR expressions were associated with decreased phagocytic capacity of macrophages. Similarly, ROS was aberrantly higher while NO was lower in PTB+DM. These altered macrophage functions were positively correlated with increasing disease severity. Our results highlight several key patterns of immune dysregulation against TB infection under hyperglycemic conditions and highlight a negative impact of hyperglycemia with etiology and progression of TB.

Keywords: hyperglycemia · macrophages · tuberculosis · phagocytosis · diabetes mellitus

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

With the WHO 2019 report labeling diabetes and TB as co-epidemic and increased incidence of TB in diabetes has led to renewed interest in understanding the 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. Recent evidence has shown that DM

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patients tend to have a higher incidence, higher disease severity, including the development of drug-resistance TB, compared with those without DM. 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 the development of *M.Tb*. infection and persistence [3]. Recent evidence suggests that innate as well as adaptive immune responses might be affected [4]. Few mice model studies have suggested a defect in immune response in terms of immune cell recruitment at the site of infection and increased bacterial load in diabetic mice as compared to euglycemic mice [2, 5]. However, limited in vivo data are available in the context of TB-DM. In this regard, an understanding of alterations or disturbances in innate immune response in diabetic patients having TB infection is needed.

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 the 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. Several pattern recognition receptors (PRRs) like Toll-like receptor 2 (TLR2), Mannose receptor (CD206), Complement receptor 3, Macrophage receptor with collagenous structure (MARCO), and CD14 are involved in the 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 conditions like diabetes mellitus also lead to the production of ROS [12]. Overstimulated ROS production under this condition may modulate the inflammatory network in TB infection, leading to dysfunctional inflammatory responses and tissue remodeling having an adverse effect on the host.

Taking this available literature under consideration, we hypothesized an 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 the bacterial load, chronic inflammation, and central necrosis that would facilitate bacterial dissemination. Limited human studies are available in this area and none in diabetic patients having TB (PTB + DM) cohort.

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. Surface expression of different PRRs like TLR 2, Mannose receptor (CD206), Complement receptor 3, Macrophage receptor with collagenous structure (MARCO), and CD14 receptors in conjunction with functional properties such as phagocytosis, ROS and NO production, were studied in DM patients having TB. Understanding of defects in innate immune responses in diabetic conditions could help in the early identification of the active disease among diabetic individuals and future development of new treatment targets to limit the development of TB among them.

Results

Clinical and laboratory data of study participants

The present study included a total of 221 study participants who 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. The demographic and clinical characteristics are shown in Table 1.

All recruited pulmonary TB patients with or without type 2 DM underwent a sputum AFB test or gene Xpert test as a 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 1+ (23.07%). Since the phenotype of PTB + DMs might differ between study participants who were diabetic before the 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.

Chronic hyperglycemia variably affects phagocytosis capacity of monocyte-derived macrophages (MDMs)

To determine if chronic hyperglycemic state alters macrophage functions, differentiated macrophages were evaluated for the degree of phagocytosis of FITC labeled BCG. Phagocytic capacity was found to be higher in all patient groups as compared to healthy controls as shown in Figure 1a and 1b ($p < 0.0001$). In between patient groups, phagocytosis capacity was found to be significantly decreased in PTB + DM and DM patients compared to PTB patients ($P < 0.001$) suggesting a defect in the phagocytic

Table 1. Demographic and clinical characteristics of study participants

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)	NA	NA
AFB positive smear, n (%)	39(78)	57(87.69)	NA	NA
Sputum positivity			NA	NA
1+	9	34		
2+	9	11		
3+	21	12		

capacity of macrophages under chronic hyperglycemic milieu. PTB + DM and DM group had comparable phagocytosis capacity.

PTB and PTB + DM patients were subdivided into different sputum grades to check for alterations in phagocytosis capacity of macrophages with respect to disease severity. We 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 1c and 1d. However, no significant difference was found among both the groups (PTB and PTB+DM) within same severity levels (Figure 1e).

Chronic hyperglycemia leads to altered expression of different PRRs on MDMs

Surface expression of different PRRs namely CD11b, CD14, MARCO, TLR2, and CD206) was significantly altered in disease groups compared to healthy controls (Figure 2a–e). Complement receptor 3 or CD11b was found to be significantly decreased in PTB + DM patients as compared to PTB patients ($P < 0.01$). 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 was 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 the DM milieu in both PTB + DM and DM patients as compared to PTB ($P < 0.01$ and 0.001), suggesting a defect in bacterial uptake under chronic hyperglycemic milieu due to alterations in important pathogen recognition receptors.

Altered PRRs expression is associated with disease severity

Since we found a significant difference in levels of different PRRs in our patient group, we tried to evaluate whether there is any correlation 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 3a-3e and 3f-3j respectively. We 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 the 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 the lowest levels seen in 3+ sputum positive patients ($P < 0.0001$ and 0.001, respectively). Furthermore, levels of TLR2 were 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 the 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 ($P < 0.04$, altogether suggesting altered expression of these receptors affecting disease severity.

In order to further explore whether disease severity affects PRR expression, we also tried to compare PRRs levels in different patient groups within the same severity level. We observed that CD11b, MARCO, and TLR2 were significantly reduced in 3+ PTB+DM patients compared to 3+ PTB patients ($P < 0.02$, 0.04,

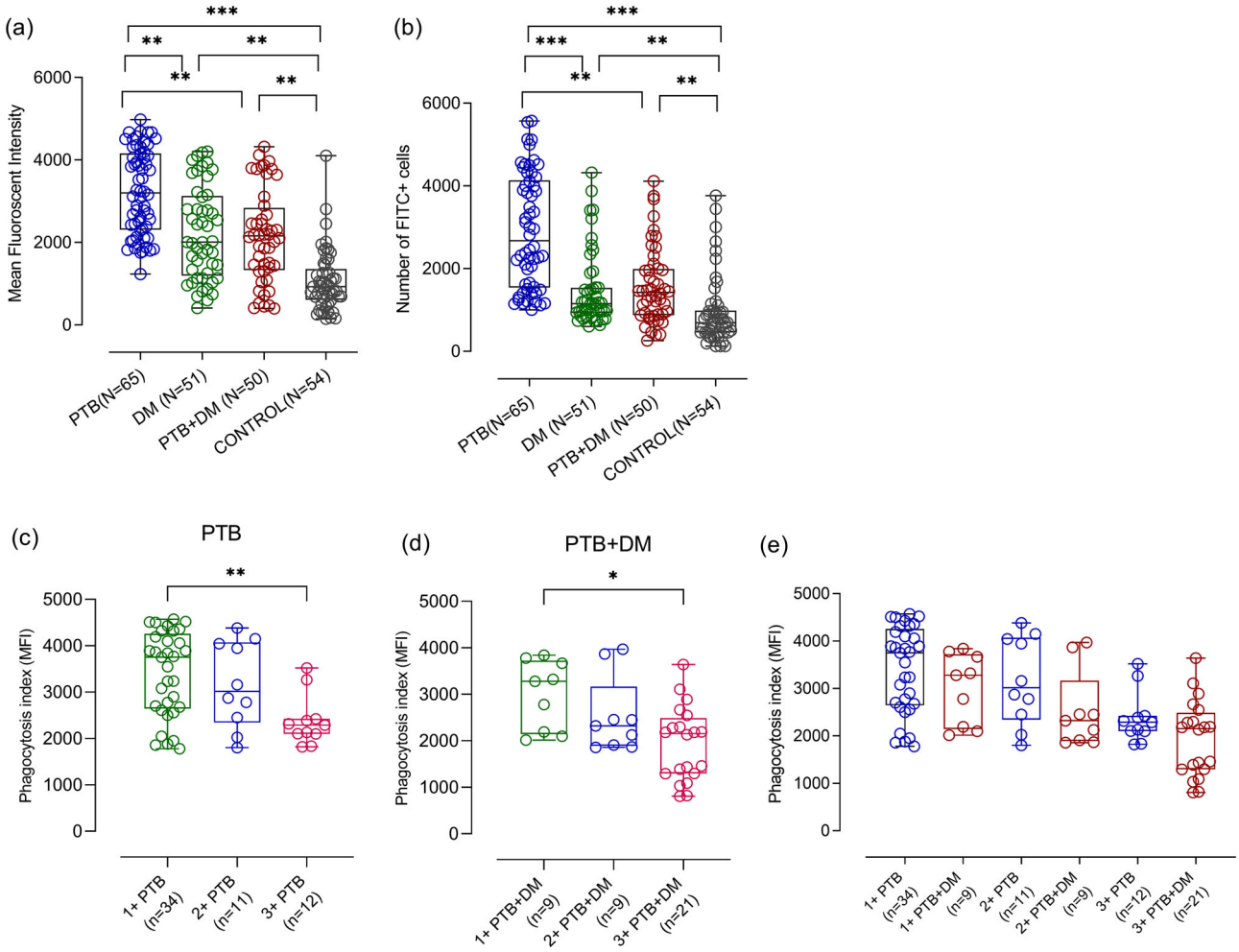

Figure 1. Phagocytosis capacity of monocyte-derived macrophages of study participants. Panels (a) and (b) represent the phagocytosis capacity of cultured macrophages after BCG infection in all study groups namely PTB, DM, PTB+DM, and controls in terms of MFI and frequency respectively. Panels (c) and (d) represent phagocytosis capacity of cultured macrophages after BCG infection in different sputum grade PTB and PTB+DM patients, respectively. Panels (e) represent comparison of phagocytosis capacity of cultured macrophages after BCG infection in PTB and PTB+DM patients of different sputum grades. Macrophages were incubated with FITC labeled BCG and phagocytosed bacteria were visualized using flow cytometry. Data are represented as fluorescent intensity and each point represents an individual sample value. Box plot represents median with interquartile range. Kruskal-Wallis testing with post hoc Dunn's multiple comparison testing was performed. *P*-values < 0.05 were considered to be statistically significant. One asterisk (*) indicates a *P*-value < 0.05; two asterisks (**) indicate a *P*-value < 0.01, three asterisks (***) indicate a *P*-value < 0.001 and four asterisks (****) indicate a *P*-value < 0.0001. PTB = Naïve active pulmonary TB; DM = Uncontrolled diabetic patients, PTB+DM = Uncontrolled diabetic patients with pulmonary TB, control = healthy controls with no history of TB and DM. 1+ PTB/ 1+PTB+DM = 1+ sputum positive PTB/PTB+DM; 2+ PTB/ 2+PTB+DM = 2+ sputum positive PTB/PTB+DM; 3+ PTB/ 3+PTB+DM = 3+ sputum positive PTB/PTB+DM.

and 0.05, respectively). Similarly, CD206 was found to be higher in 3+ PTB+DM patients compared to 3+ PTB patients ($P < 0.01$) (figure 4a-e)

Correlation of CD14 with its coreceptors, MARCO and TLR 2 in monocyte-derived macrophages (MDMs)

Since MARCO and CD14 act as a heterodimer to exert their function, we next assessed the correlation of surface expression of MARCO and CD14 present on macrophages of all the study groups. We found a 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 5a-5d suggesting a defect in bacterial uptake via CD 14-MARCO in the diabetic milieu.

Along with MARCO, CD14 is also required for TLR 2 activation and initiation of the downstream signalling pathway. Therefore, we correlated the surface expression of TLR 2 and CD14 present on macrophages of all the study groups. We found a strong positive correlation in levels of TLR 2 and CD14 in PTB and PTB + DM group (r = 0.72 and 0.69 respectively) compared to a weak correlation found in DM (r = 0.38) and healthy control group (r = 0.46) as shown in figure 5e-5h.

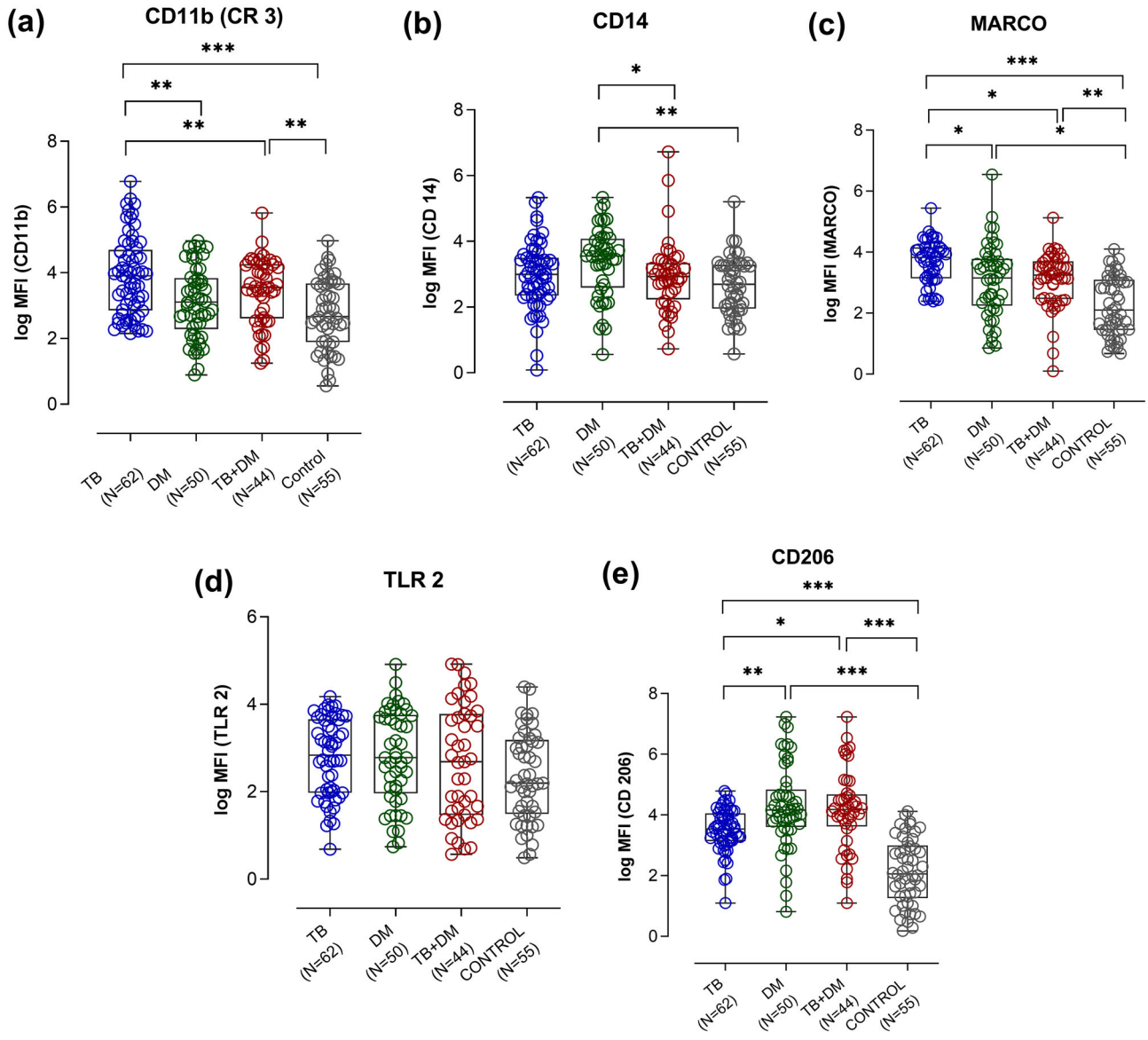

Figure 2. Surface expression of different pathogen recognition receptors on macrophages of study groups namely PTB, DM, PTB+DM, and controls. Surface expression was checked by flow cytometry using specific fluorochrome-tagged antibodies. Panel (a) shows MFI for CD11b, (b) shows MFI for CD14, (c) shows MFI of MARCO, (d) shows MFI of TLR 2, and (e) shows MFI of CD206. Data is represented as fluorescent intensity and each point represents an individual sample value. Box plot represents median with interquartile range. Kruskal–Wallis testing with post hoc Dunn's multiple comparison testing was performed to determine whether expression was statistically different among the different study groups. *P*-values < 0.05 were considered to be statistically significant. One asterisk (*) indicates a *P*-value < 0.05; two asterisks (**) indicate a *P*-value < 0.01, three asterisks (***) indicate a *P*-value < 0.001 and four asterisks (****) indicate a *P*-value < 0.0001. PTB = Naïve active pulmonary TB; DM = Uncontrolled diabetic patients, PTB+DM = Uncontrolled diabetic patients with pulmonary TB, control = healthy controls with no history of TB and DM

Phagocytic capacity is associated with altered PRRs expression on MDMs under chronic hyperglycemia

The 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 the study groups. Phagocytosis capacity was associated with the PRRs namely CD11b, CD14, and CD206 in PTB + DM group (Table 2). Upon correlation analysis, in PTB + DM group, CD11b expression was found to be positively correlated with MARCO and TLR 2 while CD206 was negatively correlated with other receptors as shown in the correlation matrix (figure 6a). A similar pattern was observed in the DM group with weak correlation as shown in the correlation matrix (figure 6b). In PTB group, CD11b, MARCO, and TLR 2 were positively correlated among each other (figure 6c). A weak correlation was found among these PRRs in the healthy control group (figure 6d).

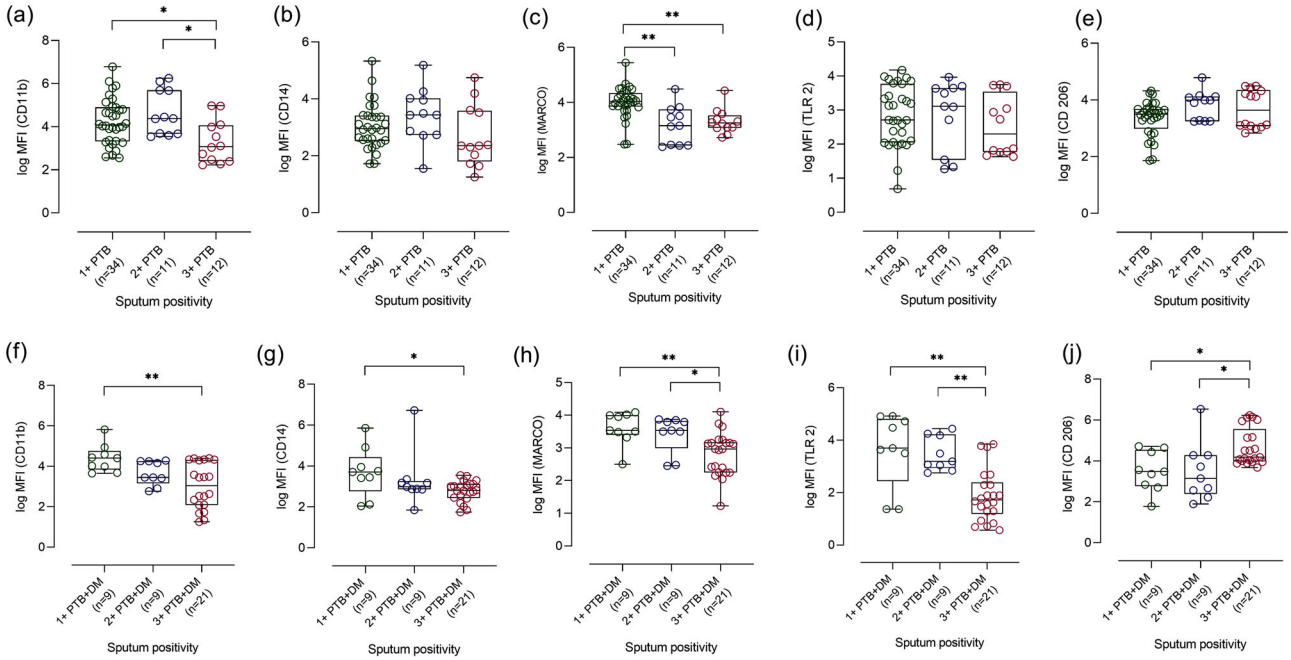

Figure 3. Surface expression of different pathogen recognition receptors on macrophages of different sputum grade PTB and PTB+DM patients. MFI for CD11b (a), CD14 (b), MARCO (c), TLR 2 (d) and CD206 (e) in PTB patients. MFI for CD11b (f), CD14 (g), MARCO (h), TLR 2 (i), and CD206 (j) in PTB + DM patients. Data is represented as fluorescent intensity and each point represents individual sample value. Box plot represents median with interquartile range. Kruskal–Wallis testing with post hoc Dunn's multiple comparison testing was performed to determine whether expression was statistically different among the different study groups. *P*-values < 0.05 were considered to be statistically significant. One asterisk (*) indicates a *P*-value < 0.05; two asterisks (**) indicate a *P*-value < 0.01, three asterisks (***) indicate a *P*-value < 0.001 and four asterisks (****) indicate a p-value < 0.0001. 1+ PTB/ 1+PTB+DM = 1+ sputum positive PTB/PTB+DM; 2+ PTB/ 2+PTB+DM = 2+ sputum positive PTB/PTB+DM; 3+ PTB/ 3+PTB+DM = 3+ sputum positive PTB/PTB+DM.

Similarly, the phagocytosis index was also found to be positively correlated with CD11b, MARCO, and TLR 2 and negatively correlated with CD206 in the patient group (figure 6a-6d).

Chronic hyperglycemia drives aberrant production of reactive oxygen species and nitric oxide

We have observed that ROS levels were significantly higher in PTB patients compared to healthy controls ($P < 0.001$) due to ongoing infection in these individuals. However, ROS levels were even higher in DM and PTB + DM patients as compared to PTB patients ($P < 0.01$ and 0.001 respectively) and healthy controls ($P < 0.001$) as shown in figure 7a suggesting overstimulation of ROS production under chronic hyperglycemic milieu. 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 7b-7c. A significant difference was also observed on comparing ROS levels in both the groups within same severity levels ($P < 0.01$ for 1+, $P < 0.05$ for 2+, $P < 0.0005$ for 3+) as shown in figure 7d.

Taking into consideration the fact that NO is an unstable molecule with a half-life of less than 5 seconds, we measured nitrites (stable serum metabolites of NO) as surrogates to measure the content of NO. The levels of NO were found to be higher in the 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 be statistically significant with higher levels in the PTB group as compared to DM and PTB + DM ($P < 0.001$ and 0.01) as shown in figure 8a. 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 8b-8c. However, no significant difference was found among both the groups (PTB and PTB+DM) within same severity levels (figure 8d).

Discussion

Diabetics are prone to develop various infections including tuberculosis, thus increasing the global TB burden. Despite its significance, the immunological and biochemical mechanisms of tuberculosis susceptibility in diabetes are not well understood. Therefore, the present study was designed to understand the mechanism of increased susceptibility to TB in DM patients focusing on the innate immune response. As macrophages are one of the first immune cells that mount immune response by phagocytosing the pathogen and subsequent activation of the adaptive immune response, we tried to understand any alteration in

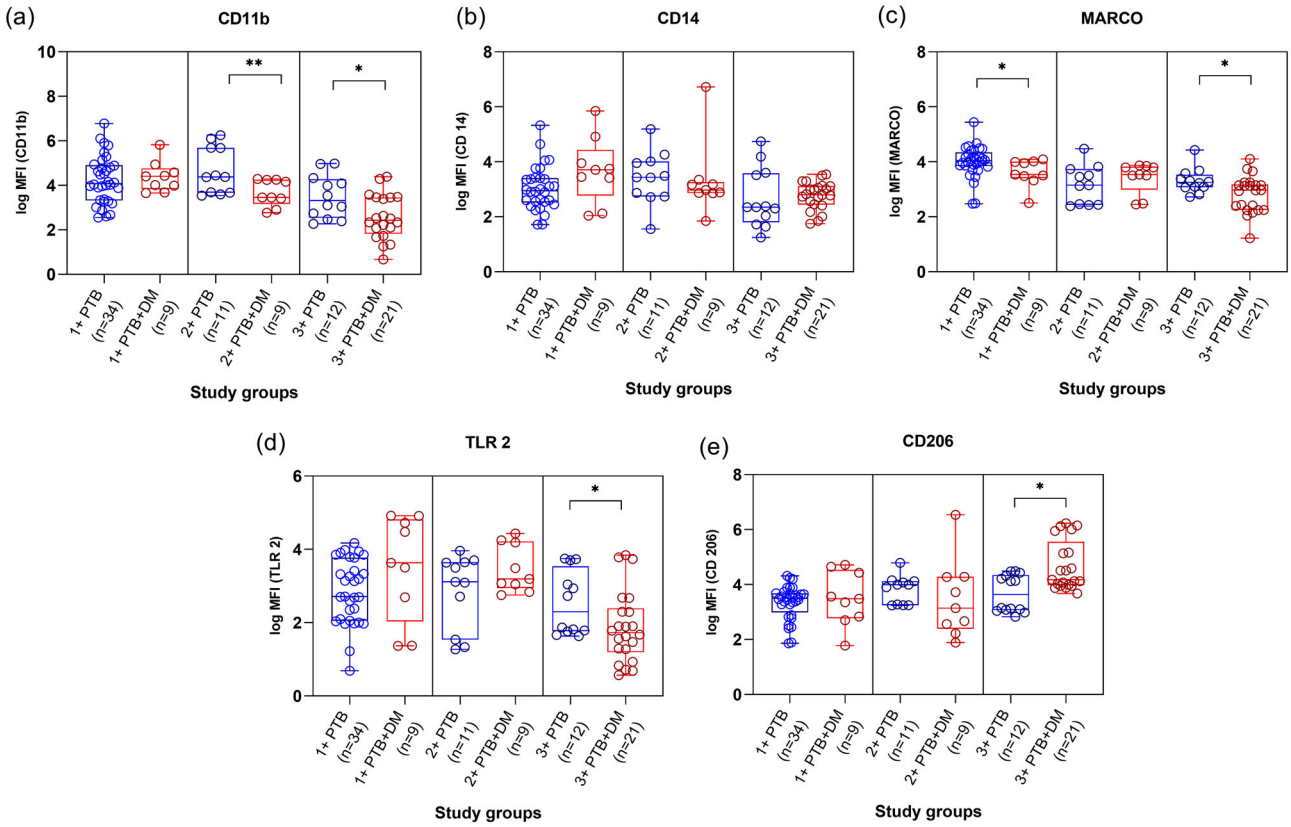

Figure 4. Surface expression of different pathogen recognition receptors on macrophages of different sputum grade PTB and PTB + DM patients. MFI for CD11b (a), CD14 (b), MARCO (c), TLR 2 (d), and CD206 (e) in different sputum positive PTB + DM and PTB patients. Data are represented as fluorescent intensity and each point represents an individual sample value. Box plot represents median with interquartile range. Kruskal–Wallis testing with post hoc Dunn's multiple comparison testing was performed to determine whether expression was statistically different among the different study groups. P-values < 0.05 were considered to be statistically significant. One asterisk (*) indicates a P-value < 0.05; two asterisks (**) indicate a P-value < 0.01, three asterisks (***) indicate a P-value < 0.001 and four asterisks (****) indicate a P-value < 0.0001. 1+ PTB/ 1+PTB+DM = 1+ sputum positive PTB/PTB+DM; 2+ PTB/ 2+PTB+DM = 2+ sputum positive PTB/PTB+DM; 3+ PTB/ 3+PTB+DM = 3+ sputum positive PTB/PTB+DM.

macrophage effector function under chronic hyperglycemic conditions. In this case-control 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. The majority of the individuals within the PTB + DM group had a high bacterial burden (3+ sputum positivity) which suggests a plausible association of diabetes with increased disease severity in pulmonary tuberculosis patients.

Macrophages play a critical role in phagocytosis of mycobacteria, production of chemical mediators, antigen presentation, and subsequent activation of adaptive immune responses in host-mycobacterial interactions [14]. We tried to evaluate the functional alteration in macrophages under chronic diabetic condition. We found significantly decreased phagocytic capacity within the macrophages of PTB + DM and DM patients plausibly due to diabetes-mediated intrinsic defect in these cells. Hyperglycemia can promote direct glycosylation of various proteins, including complements, and modify their tertiary structure, which can limit bacterial opsonization and thereby impair phagocytosis [15]. A subset of TB+DM patients with HbA1c levels of 7.5-8.0 showed better phagocytosis capacity as compared to others whereas patients having HbA1c levels more than 9.5 (9 in DM and 6 in PTB+DM group) showed a decrease in phagocytosis levels showing an adverse effect of hyperglycemia on macrophage function. Similarly, the phagocytic capacity was found to be decreased with increased disease severity, again suggesting adverse effects of hyperglycemia upon infection. Contrary to our findings, *Lachmandas et al* have shown that phagocytic capacity of macrophages remained unaltered under high glucose concentration. However, the phagocytosis was checked using beads instead of the bacteria. Similar to phagocytosis, they reported no differences in MTB killing or outgrowth between hyperglycaemic and euglycaemic macrophages. However, the study was done in a short-term in vitro hyperglycemia model with donor healthy monocytes differentiated into macrophages where donor comorbid conditions were not taken into account [16].

Reduction in phagocytosis is often influenced by concurrent alterations in pathogen recognition receptors that could be responsible for bacterial opsonization and internalization. Therefore, we next assessed the levels of different PRRs namely, CD11b (CR3), CD14, MARCO, TLR2, and CD206 in PTB, DM, and PTB + DM patients. Few of these receptors, such as CD14 and TLR2,

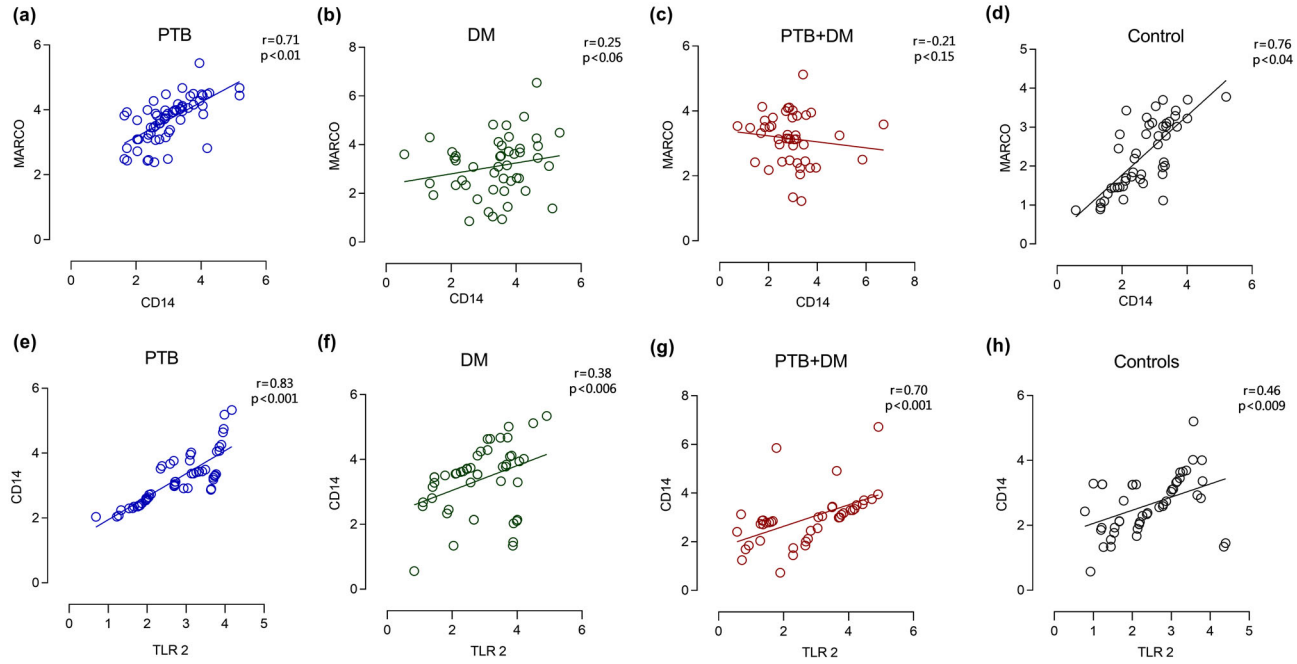

Figure 5. Correlation of CD14 with MARCO and TLR2 in all study groups. Correlation between surface expression of MARCO and CD14 on macrophages of all the study groups namely PTB (a), DM (b), PTB+DM (c), and controls (d), respectively. Correlation between surface expression of TLR2 and CD14 on macrophages of all the study groups namely PTB (e), DM (f), PTB+DM (g), and controls (h), respectively. Non-parametric Spearman correlation was used in correlation analysis. Spearman's correlation coefficient is displayed on the right. PTB = Naïve active pulmonary TB; DM = Uncontrolled diabetic patients, PTB+DM = Uncontrolled diabetic patients with pulmonary TB, control = healthy controls with no history of TB and DM.

Table 2. Variables (pathogen recognition receptors) associated with phagocytosis in study groups

Parameter estimates	Variable	Estimate	P-value	P-value summary	95% confidence interval
PTB + DM					
β1	CD11b	303.5	0.0172	*	56.45 to 550.6
β2	CD14	168.5	0.0409	*	7.282 to 329.7
β3	MARCO	216.9	0.1629	ns	-91.12 to 524.9
β4	TLR 2	118.1	0.1902	ns	-60.77 to 296.9
β5	CD206	-336.7	0.0022	**	-545.1 to -128.4
PTB					
β1	CD11b	285.8	0.0083	**	76.45 to 495.2
β2	CD14	89.09	0.4543	ns	-147.6 to 325.8
β3	MARCO	411.4	0.0095	**	104.1 to 718.7
β4	TLR 2	-6.721	0.9604	ns	-276.8 to 263.3
β5	CD206	113.5	0.4387	ns	-177.8 to 404.8
DM					
β1	CD11b	294.4	0.0183	*	52.17 to 536.5
β2	CD14	-124.2	0.2327	ns	-331.0 to 82.58
β3	MARCO	21.25	0.8217	ns	-167.6 to 210.1
β4	TLR 2	125.6	0.2274	ns	-81.13 to 332.4
β5	CD206	-464.2	0.001	***	-673.9 to -254.5
Controls					
β1	CD11b	183.1	0.0981	ns	-35.21 to 401.4
β2	CD14	46.90	0.7062	ns	-202.0 to 295.8
β3	MARCO	143.4	0.1932	ns	-75.22 to 362.1
β4	TLR 2	1.942	0.9872	ns	-239.9 to 243.7
β5	CD206	111.9	0.2507	ns	-81.72 to 305.5

Multiple linear regression analysis for association of phagocytosis (dependant variable) with different PRRs (independent variables) namely CD11b, CD14, MARCO, TLR 2, and CD206 in all study groups. Data are represented as 95% Confidence Interval. $P < 0.05$ was considered significant

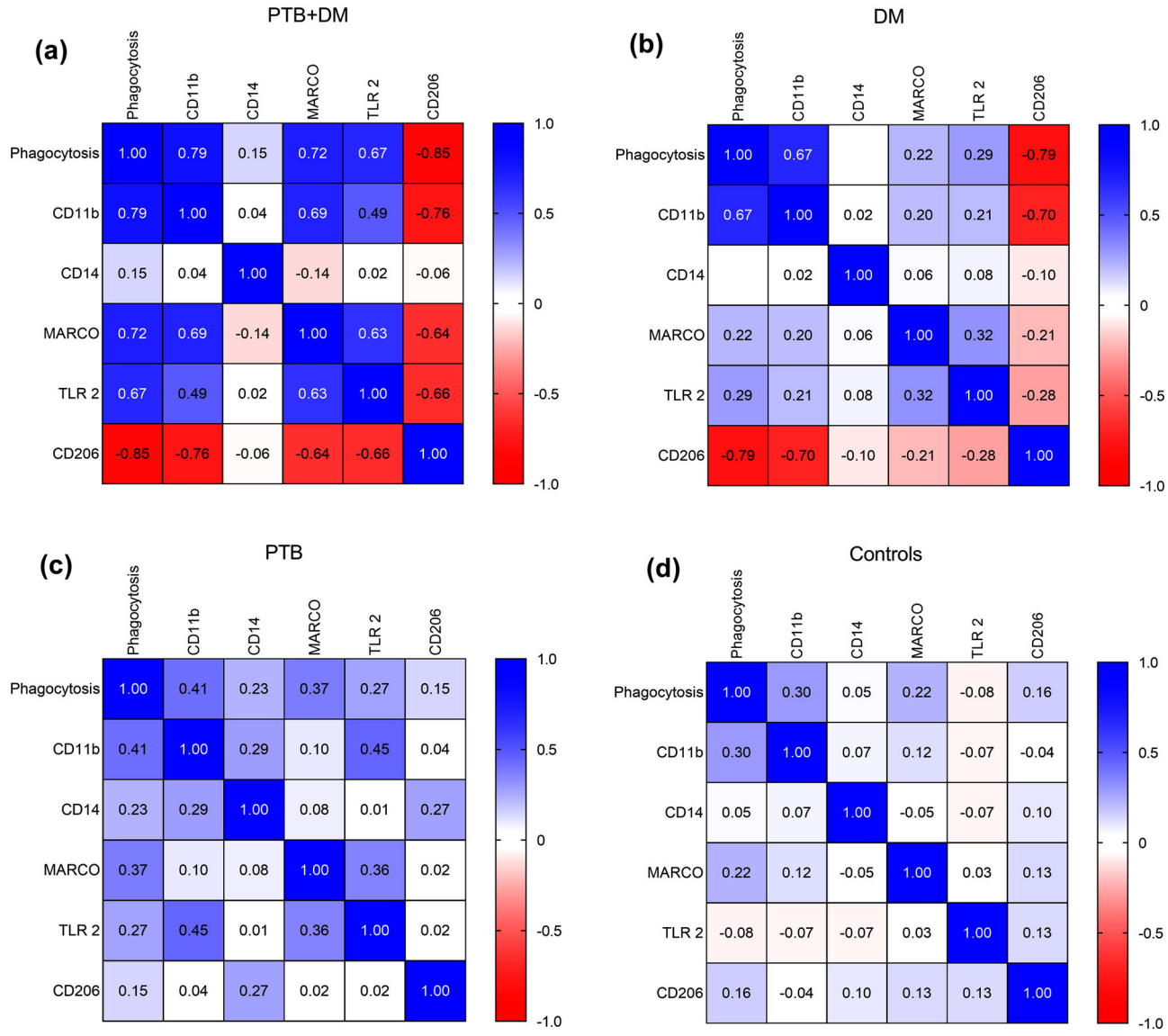

Figure 6. Correlation matrix surface expression of PRRs and phagocytosis index of macrophages in study groups. Values of PTB+DM (a), DM (b), PTB (c), and control group (d), respectively. Spearman correlation was used to correlate the variables. PTB+DM = Uncontrolled diabetic patients with pulmonary TB. Values in each block show Spearman's correlation coefficient.

induce pro-inflammatory cascades [17], while others, such as CD206, initiate an anti-inflammatory response [18], and are found to be altered in diabetic environment. However, till now, limited studies have been reported on assessing the levels of these PRRs in DM patients having active TB infection.

CD11b plays a critical role in mycobacterial infections in terms of cell adhesion, migration, and phagocytosis [19]. Decreased levels of CD11b under the 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 has been implicated in the recognition of LAM present on the mycobacterial envelope. While talking in terms of CD14 functions, very few studies have been conducted regarding the role of CD14 in mycobacterial infections, however, the findings were controversial. Our findings have shown increased surface expression of CD14 in a diabetic milieu which may have an adverse effect upon the outcome of infection as one of the studies has shown that during chronic *M.Tb*. infection, CD14 knock-out mice were shown to be protected from lethality caused by lung tuberculosis. This may be due to a reduction in an inflammatory response which suggests that CD14 contributes to the development of a chronic inflammatory response in the lung during tuberculosis that negatively influences the outcome of the infection [20]. CD14 with the help of its co-receptors can either be beneficial to the host by induction of an adequate inflammatory and immune response or harmful to the host due to excessive inflammation and/or dissemination of the pathogen depending

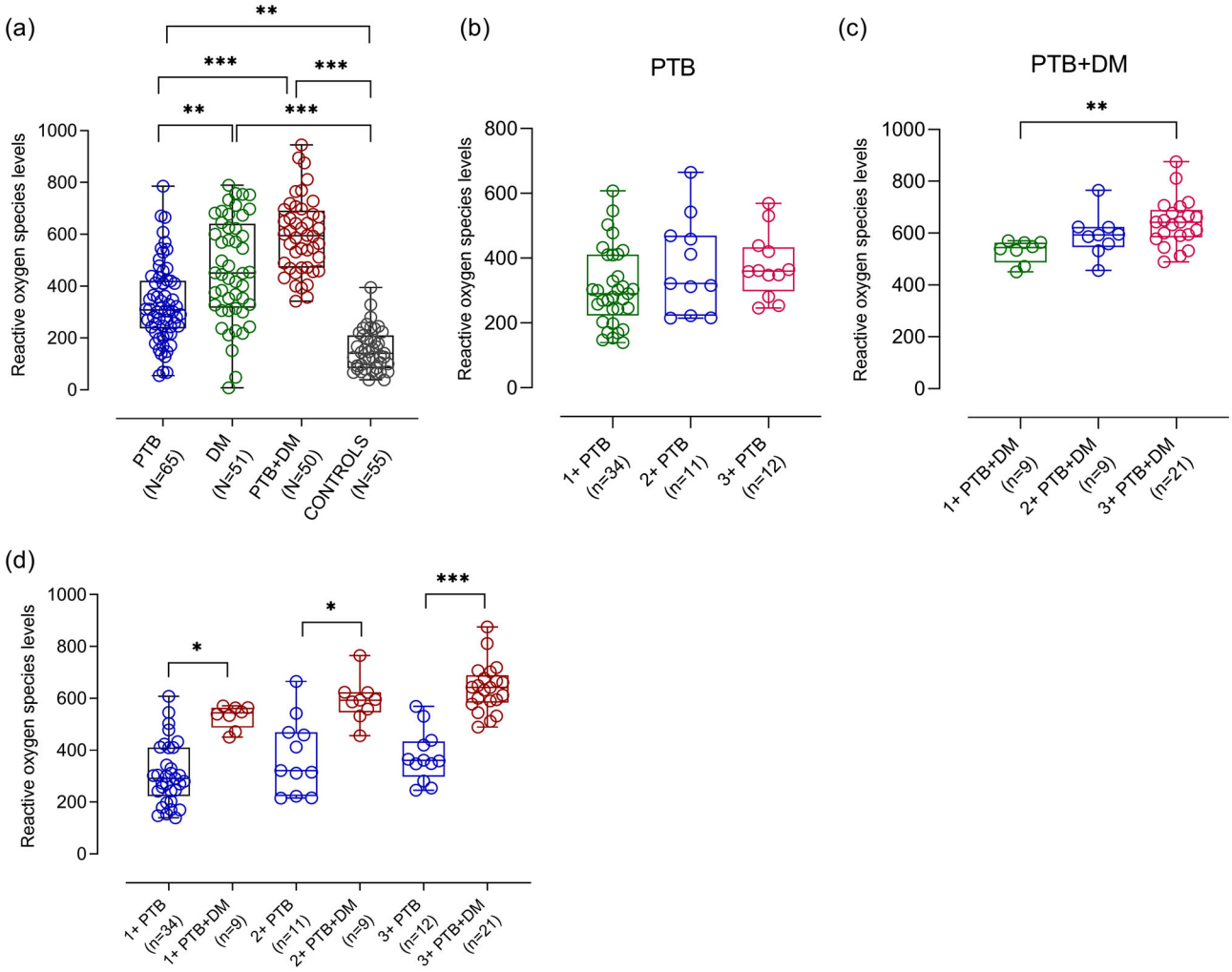

Figure 7. ROS levels in macrophages after BCG infection. After infection, cells were incubated with DCFDA for 30 minutes which will be converted to fluorescent compound DCF in the presence of ROS. The fluorescence signal was then read which corresponded to ROS levels. Panel (a) shows ROS levels in PTB, DM, PTB+DM, and controls. Panels (b) and (c) show ROS levels in different sputum grade PTB and PTB+DM patients respectively. Panel (d) shows ROS levels in PTB and PTB+DM within same severity levels. Data is represented as fluorescent intensity and each point represents an individual sample value. Box plot represents median with interquartile range. Kruskal–Wallis testing with post hoc Dunn's multiple comparison testing was performed. *P*-values < 0.05 were considered to be statistically significant. One asterisk (*) indicates a *P*-value < 0.05; two asterisks (**) indicate a *P*-value < 0.01, three asterisks (***) indicate a *P*-value < 0.001 and four asterisks (****) indicate a *P*-value < 0.0001. PTB = Naïve active pulmonary TB; DM = Uncontrolled diabetic patients, PTB+DM = Uncontrolled diabetic patients with pulmonary TB, control = healthy controls with no history of TB and DM. 1+ PTB/ 1+PTB+DM = 1+ sputum positive PTB/PTB+DM; 2+ PTB/ 2+PTB+DM = 2+ sputum positive PTB/PTB+DM; 3+ PTB/ 3+PTB+DM = 3+ sputum positive PTB/PTB+DM.

on the microbe and its co-receptors [21]. Therefore, we studied two co-receptors of CD14 namely MARCO and TLR2 which are required for *M.Tb*. recognition.

MARCO is a phagocytic receptor that has been implicated in host defense by recognizing trehalose 6,6′-dimycolate (TDM) which mediates potent inflammatory response via its coreceptor CD14 and TLR 2 [22]. MARCO-expressing macrophages show an increased propensity to phagocytose more BCG than neighboring macrophages that do not express MARCO in the splenic marginal zone. Decreased levels of MARCO observed in the PTB + DM group may suggest a disruption in sensing and clearing of the *M.Tb*. pathogen. Even though CD14 levels were higher in the diabetic milieu, lower levels of MARCO receptor may have led to defective recognition and hence, increased bacterial survival in the diabetic milieu. Positive correlation between CD14 and MARCO in PTB and control group is suggestive of an optimal expression of both the receptor and hence, increased bacterial uptake from CD14-MARCO receptor complex that is further supported by a lack of correlation between the two PRRs in diabetic patients with or without infection suggesting dysregulation in these receptors may have led to altered bacterial uptake and clearance.

TLR2 that is another coreceptor of CD14 was found to be higher in all the patient groups may be due to the trigger from active infection as well as inflammation (DM) that in turn can trigger various innate immune responses depending upon

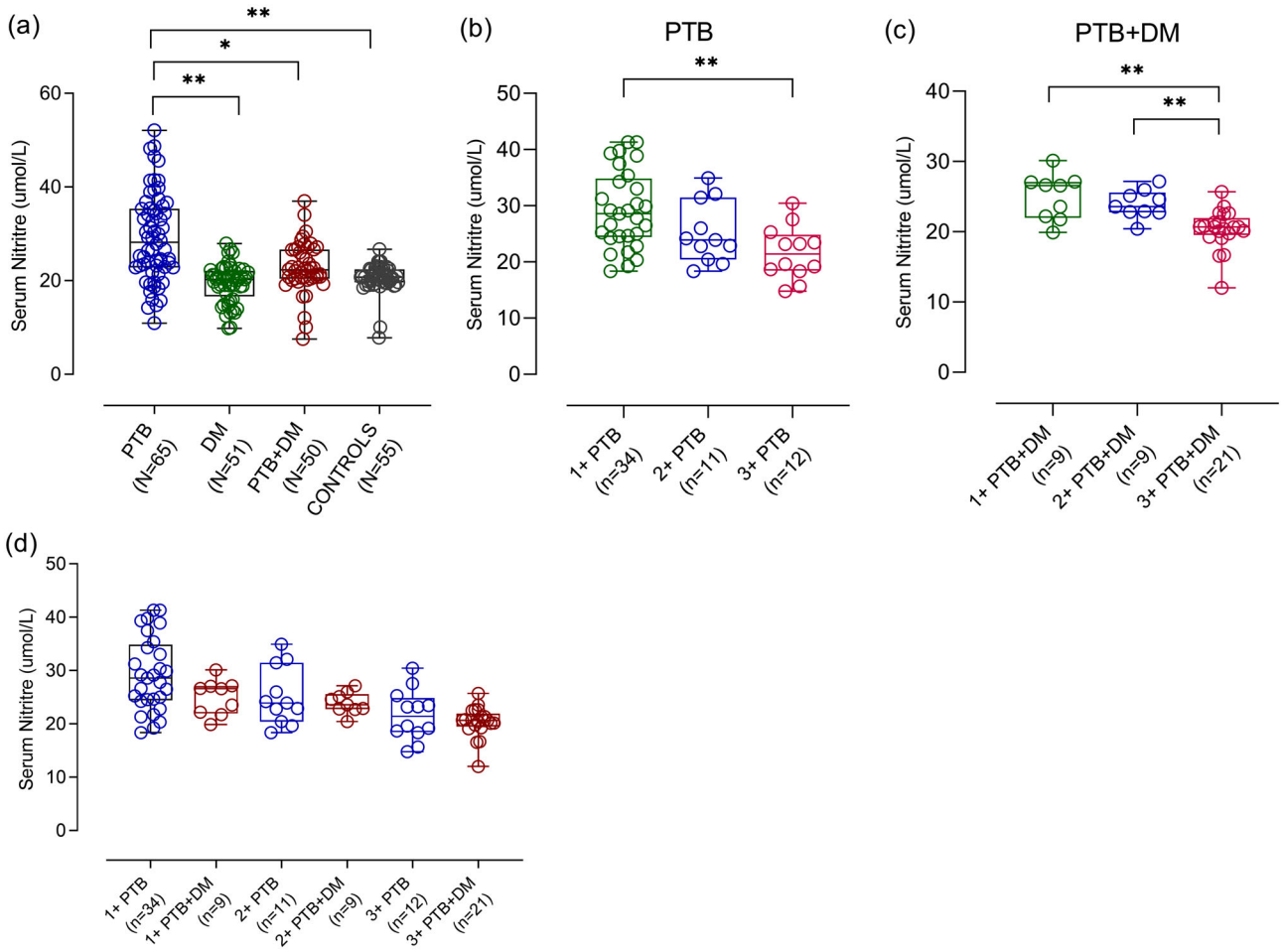

Figure 8. Serum NO levels in different study participants. NO was measured using Griess reaction. Panel (a) represents NO levels in PTB, DM, PTB+DM, and controls. Panels (b) and (c) represent NO levels in different sputum grade PTB and PTB+DM patients respectively. (d) shows ROS levels in PTB and PTB+DM within same severity levels. Data is represented as median with interquartile range and each point represents individual sample value. Box plot represents median with interquartile range. Kruskal-Wallis testing with post hoc Dunn's multiple comparison testing was performed. *P*-values < 0.05 were considered to be statistically significant. One asterisk (*) indicates a *P*-value < 0.05; two asterisks (**) indicate a *P*-value < 0.01, three asterisks (***) indicate a *P*-value < 0.001 and four asterisks (****) indicate a *P*-value < 0.0001. PTB = Naïve active pulmonary TB; DM = Uncontrolled diabetic patients, PTB+DM = Uncontrolled diabetic patients with pulmonary TB, control = Healthy controls with no history of TB and DM. 1+ PTB/ 1+PTB+DM = 1+ sputum positive PTB/PTB+DM; 2+ PTB/ 2+PTB+DM = 2+ sputum positive PTB/PTB+DM; 3+ PTB/ 3+PTB+DM = 3+ sputum positive PTB/PTB+DM

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 and spread of *M.Tb*. in pulmonary tuberculosis. A positive correlation between TLR2 and CD14 in PTB + DM patients suggests 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 have led to the dissemination of infection and hence persistence and increased severity of infection.

Mannose receptor (CD206) is another important receptor that we have studied and found that the levels were higher in the 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 [23]. However, this receptor is expressed in M2 macrophages that show anti-inflammatory effects during infection [24]. Our data showed increased expression of CD206 in the hyperglycemic condition that 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 that has an anti-inflammatory response. This may lead to persistence, bacterial multiplication, and a possible spread of infection.

Upon multivariate analysis, decreased phagocytosis capacity of macrophages in the diabetic milieu was found to be associated with lower levels of CD11b, CD14, and MARCO receptors which are required for internalization of the pathogen. This suggests that hyperglycemia alters the surface expression of these PRRs and hence affects the phagocytosis capacity of the macrophages. These alterations were also associated with disease severity as CD11b, CD14, MARCO, and TLR 2 were decreased and CD206 was increased in 3+ sputum positive PTB + DM patients. These

changes were also evident on comparing PRR levels in disease groups within same severity levels suggesting the association of hyperglycemia on PRR expression.

Once we studied phagocytosis and various PRRs 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 compared to controls suggest trigger from active bacterial infection to control the infection. Although ROS plays an important role in the mycobacterial killing, a recent study has shown that *M.Tb*. shows resistance against ROS during the chronic or persistent stage of infection [25]. The levels were much higher in the diabetic milieu (DM and PTB + DM) that may have an 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 that may lead to dissemination of the infection. Furthermore, if the ROS levels are overwhelmed by *M.Tb*. antioxidant systems, then the pathogen will continue to survive and replicate in the host [26]. Therefore, it is critical to emphasize that *M.Tb*. survival is largely dependent on the quantities of ROS produced by the host immune cells. It was also evident by our findings of increased ROS levels in 3+ sputum positive PTB + DM patients suggesting an increase in disease severity upon higher ROS production under hyperglycemic conditions. 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 the inducible form of nitric oxide synthase, iNOS. As expected, nitric oxide levels were higher in the 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 [27]. In the case of PTB + DM patients, decreased levels of NO under hyperglycemic condition suggests an alteration 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 [28]. Unlike ROS, NO levels were found to be significantly lower in 3+ sputum-positive patients suggesting the protective role of NO in *M.tb*. infection.

In conclusion, our findings reveal a dynamic link between diabetes mellitus and tuberculosis infection, with a complicated interplay between etiology and pathological progression. Modulation of anti-bacterial immune responses of macrophages in terms of defective bacterial uptake and clearance under chronic hyperglycemic conditions might be one of the plausible reasons for the increased susceptibility of TB in diabetics individuals. The present findings open up new research questions where a detailed mechanistic study for an in-depth understanding of the cellular basis of TB susceptibility in DM is required. This will further help in better understanding the mechanisms of hyperglycemia impairing host defenses in TB and thus lead to the rational development of therapeutic strategies to alleviate the dual burden of DM and TB.

Although the present study addresses the understanding of the potential mechanism behind increased susceptibility of tuberculosis infection in type 2 diabetes patients, there are a few limitations of the study. Although we added autologous serum in macrophage cultures, we did not maintain the same glucose concentration of the individuals in ex-vivo cultures. Chronic hyperglycemia can affect many cells and proteins in the body, so it would be interesting to see role of chronic hyperglycemia on other immune cells involved in tuberculosis pathophysiology. Mechanistic investigations need to be conducted to better understand the involvement of distinct pathogen recognition receptors (PRRs) in the diabetic milieu. Additionally, considering the diverse downstream signaling cascade that are influenced by the class of ligands binding to PRRs, assessment of the immunomodulatory roles of distinct PRR-ligand complexes should be investigated.

Materials and methods

Study subjects

The cross-sectional study was conducted in the Department of Biochemistry and Department of Medicine, All India Institute of Medical Sciences, New Delhi. Study participants were recruited under four groups namely pulmonary TB group (PTB), type 2 diabetes mellitus (DM) group, PTB + DM group, and healthy control group.

Sixty-five newly diagnosed treatment naïve pulmonary TB patients (PTB) were enrolled under PTB group. A PTB case was defined as a patient with a clinically diagnosed case of TB affecting the lungs, having symptoms of fever or cough and sputum smear that showed acid-fast bacilli or culture positive for *M.Tb*. or Gene Xpert. Any other conditions like extra-pulmonary and drug-resistant tuberculosis, HIV, diabetes, hypertension, significant organ dysfunction of heart, liver, and kidney, abnormal hematologic function, inflammation like autoimmune disease, atopic dermatitis, pregnant or lactating women, or any other uncontrolled concurrent illness were excluded.

Fifty-one uncontrolled type 2 diabetic patients with HbA1c levels >7.5 (DM) without any other disorders as mentioned above including tuberculosis were recruited under the diabetes (DM) group. Individuals taking metformin, corticosteroids, aspirin, or TNF blockers were also excluded to avoid the confounding factors which may alter the immune function.

Fifty newly diagnosed treatment naïve pulmonary TB patients who had uncontrolled type 2 diabetes mellitus with HbA1c > 7.5% (PTB + DM) were also recruited for the study with the above-mentioned inclusion and exclusion criteria.

Fifty individuals with no known history of TB and diabetes or any other disorders were recruited as healthy controls. All study participants were age (18–50 years) and sex matched. Height and weight were recorded to calculate body mass index. The

study was approved by the institutional ethics committee (IECPG-374/28.09.2017). Study participants were recruited from North Indian population after obtaining informed consent.

Culture of *M. bovis* BCG and labeling with FITC

M. bovis BCG was used and grown to the log phase in 7H9 Middlebrook medium supplemented with oleic albumin dextrose catalase (OADC). The bacteria were then harvested, washed, and frozen at −80°C in PBS plus 10% of glycerol. Bacterial load was determined by plating serial 10-fold dilutions on 7H10 Middlebrook agar (supplemented with OADC (M0678)) and counting colonies after incubation for at least 3 weeks. ZN staining was performed to rule out contamination by other bacteria (Figure S1).

Generation of monocyte-derived macrophages (MDMs)

PBMCs were isolated from whole blood using Ficoll gradient separation. For monocyte isolation by plastic adherence method, 1–2 million PBMCs were plated into 12-well plates (Nunc, Thermo Fisher) at 37°C and 5% CO_2 and allowed to adhere for two hours in 1 mL of RPMI 1640 (Hyclone) supplemented with 10% fetal bovine serum (Himedia) and 1% penicillin/streptomycin. After 2 h, non-adherent cells were removed and adhered monocytes were then cultured into macrophages using 35 ng/ml GM-CSF for 9 days along with the addition of autologous serum every third day. After 9 days, cells were differentiated into macrophages with larger sizes and protruding appendages (Figure S2). The purity was checked by using CD11b and CD14 by flow cytometry using fluorochrome tagged antibodies [PE-Cy7-CD11b (557743) and PerCP-Cy5.5-CD14 (562692)]. Cells with $CD11b^{high}$ $CD14^{low}$ were taken as differentiated macrophages (Figure S3). The cell viability was checked by trypan blue and more than 90% of cells were found to be viable. Differentiated macrophages were then taken forward for further experiments.

Phagocytic activity of macrophages using FITC labeled BCG

Phagocytosis activity of differentiated macrophages was studied using the fluorescent detection method. Briefly, BCG was tagged with FITC by incubating 0.2 μl of 5 mg/ml FITC for 1 h at room temperature with end-to-end rotation followed by washing with PBS to remove unbound FITC. Macrophages were then incubated with FITC labeled BCG at MOI of 10 for 1 h at 37°C. After incubation, 1 ml of ice-cold 1× PBS was added next to stop phagocytosis. Cells were scraped out and washed with 1× PBS (3 times) to remove free bacteria. Cells were then stained with CD11b and CD14 for macrophage purity. To distinguish cells that have phagocytosed bacteria from those simply binding the bacteria at the surface, 100 μl of trypan blue was added to the cells and incubated for one minute to quench surface FITC fluorescence. Cells with phagocytosed bacteria were then analyzed using flow cytometry and median fluorescence intensity (MFI) values and percentage positivity were recorded.

Surface expression of different pathogen recognition receptors on macrophages

Surface expression of different pathogen recognition receptors namely Toll-like receptor 2 (TLR2), Mannose receptor (CD206), Complement receptor 3, MARCO, and CD 14 were studied on macrophages by flow cytometry using fluorochrome tagged antibodies [PE mouse anti-human TLR 2 (565349), APC mouse anti-human CD206 (550889), Mouse anti-human MARCO antibody (HM2208) + FITC conjugated goat anti-mouse secondary antibody (Ab97259), PE-Cy7-CD11b (557743), PerCP-Cy5.5-CD14 (562692)]. Briefly, 1×10^5 macrophages were stained with the titrated volume of antibody cocktail for 30 minutes at 37°C. Cells were then washed with FACS buffer (PBS + 0.5% BSA). 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. FMO (fluorescence minus one) was used for gating the signals. Single-stained compensation controls were used for compensation in multicolor flow experiments. Unstained controls were used as negative controls to control background autofluorescence. The experiment and analysis were performed according to the guidelines provided by Cossarizza et al. used for flow cytometry and cell sorting in immunological studies [13]. The representative histograms of different PRRs are shown in Figures S4 and S5.

Levels of cellular Reactive oxygen species (ROS) and serum nitric oxide (NO)

Reactive oxygen species in macrophages were estimated using Dichloro-dihydro-fluorescein diacetate (DCF-DA) assay (ab113851). Briefly, $1–2 \times 10^4$ macrophages were taken in a flow tube. Then 10^5 bacteria were added to the cells 1 hour before the treatment. Five hundred microliters of DCFDA solution was added at a concentration of 20 μM followed by 30 min incubation at 37°C. Signal was read using flow cytometry.

NO levels were measured in serum using a colorimetric assay based on Griess reaction. Griess reagent includes naphthyl ethylenediamine dihydrochloride suspended in water and sulphanilamide in phosphoric acid. This reagent reacts with nitrite in samples to form a purple azo product, the absorbance of which is measured at 540 nm. Serum and Griess reagent was added in 1:1 ratio. The mixture was incubated for 10 min followed by reading absorbance at 540 nm. The concentration of nitrite in the sample was determined from a sodium nitrite ($NaNO_2$) standard curve

Statistical analysis

All statistical analyses were performed on GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) or R statistical software version 4.1.1 (R Project for Statistical Computing) within RStudio statistical software version 1.4.1717 (R Studio). Categorical variables were presented as counts and percentages. Continuous variables were reported as mean (SD) or median (interquartile range [IQR]) after the assessment of normality. Non-parametric statistical analyses were performed throughout the study after checking for normality. Correlation between variables was assessed using Spearman correlation. Mann–Whitney *U* and Kruskal–Wallis tests were used for comparison between two groups and three groups respectively. Linear regression analyses were used to determine factors (PRRs) associated with phagocytosis and were reported as beta coefficients and 95% CIs. A 2-tailed $p < 0.05$ was considered statistically significant for all conducted analyses.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

Author contribution: A.S. and S.P. conceptualized and designed the study. S.P. drafted the manuscript. S.P. and D.M.S. carried out recruitment of patients under the guidance of A.S., A.M., N.K.V., and N.K.G. S.P. and D.M.S. carried out sample collection, standardization, and execution of experimental work along with data acquisition and interpretation of data under the guidance of A.S., K.L., J.K., L.R.S.F., and A.A. executed some of the experimental work along with data acquisition. A.S. critically reviewed and contributed to the final version of the manuscript. A.S. gave the final approval of manuscript submission and supervised the project.

Ethics approval: The study was conducted adopting the ethical principles stated in the latest version of the Helsinki Declaration as well as the applicable guidelines for good clinical practice (GCP). Ethical approval was obtained from the Institutional Ethics Committee of All India Institute of Medical Sciences, New Delhi (Ref NO: IECPG-374/28.09.2017).

Patient consent statement: Informed consent was obtained from all individual participants included in the study. The authors affirm that human research participants provided informed consent for the publication of data.

Data availability statement: Data available on request from authors. The data that support the findings of this study are available from the corresponding author upon reasonable request

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