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Immune response in tuberculosis with comorbidities or coinfections

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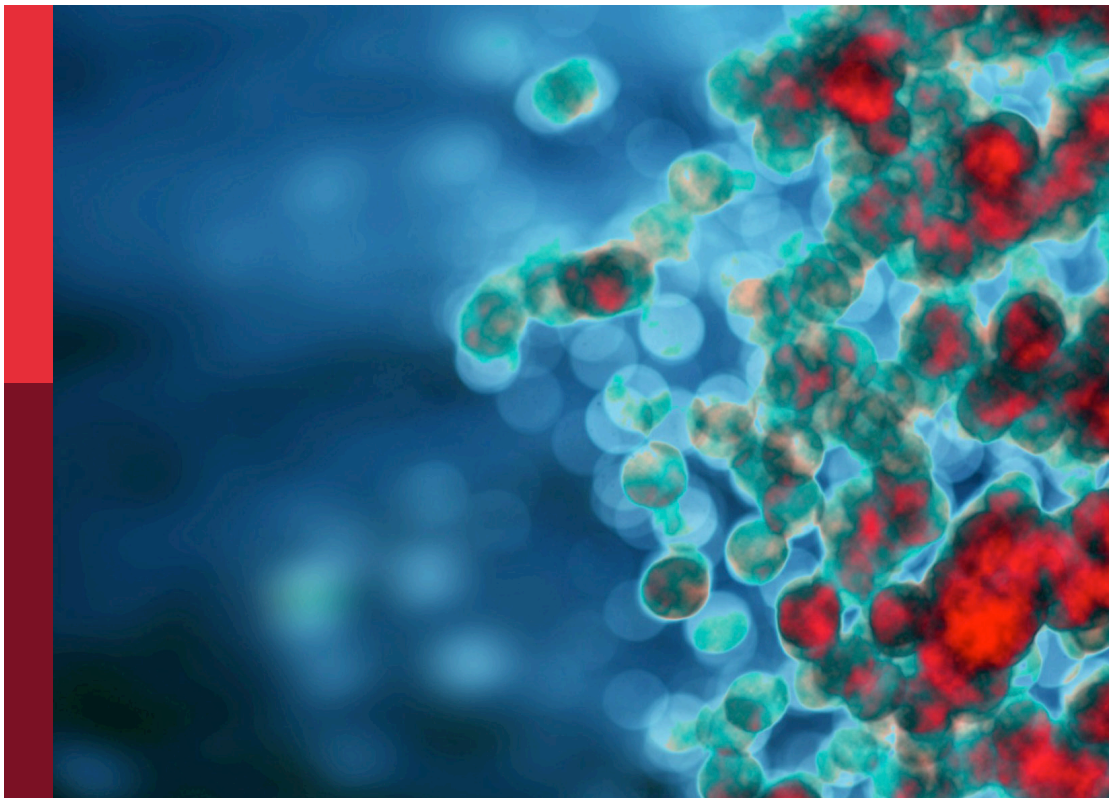

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Immune response in tuberculosis with comorbidities or coinfections

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Editorial: Immune response in tuberculosis with comorbidities or coinfections

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KEYWORDS

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Editorial on the Research Topic
Immune response in tuberculosis with comorbidities or coinfections

Tuberculosis (TB) remains a leading cause of infectious disease mortality globally. Its pathogenesis is now understood to be heavily shaped by immunometabolic perturbations and host comorbidities. In this context, the ten articles summarized in this Research Topic collectively deepen our understanding of how metabolic disorders, immune-mediated diseases, helminth infections, and viral coinfections converge to modulate TB immune response, whose profiles may be associated with disease susceptibility or post-treatment outcomes.

Several studies in this Research Topic illuminate the complex interplay between diabetes mellitus (DM) and TB. Araujo-Pereira et al. offer a comprehensive overview of TB-DM comorbidity, identifying chronic systemic inflammation and impaired immune regulation as pivotal mechanisms fueling susceptibility and complicating treatment. Complementing this, Ssekamatte et al. show how type 2 diabetes alters *Mycobacterium tuberculosis* (Mtb)-specific $CD4^+$ and $CD8^+$ T-cell phenotypes, reducing interferon gamma production and this occurs via an increasing of programmed cell death protein-1 expression, underscoring the impact of functional immune exhaustion in individuals with latent Mtb infection (LTBI). Rajamanickam et al. further demonstrate that TB with prediabetes is characterized by exacerbated profiles of pro-inflammatory cytokine and chemokine, linking immune dysfunction to glycemic dysregulation. Additionally, Ranaivomanana et al. reveal that DM skews longitudinal treatment monitoring biomarkers—specifically, the monocyte-to-lymphocyte ratio and the release of interferon gamma produced by T-cells specific to Mtb antigens—potentially limiting their interpretive value in diabetic TB patients.

A critical implication of these findings is the pressing need for immunomonitoring tools and personalized treatment strategies for TB-DM patients, especially in low-resource settings where this comorbidity is on the rise.

Beyond metabolic disorders, anemia emerges as an underappreciated but potent modulator of TB outcomes. Dasan et al. demonstrate that anemic TB patients

experience higher bacterial burdens, more extensive lung pathology, and worse treatment outcomes. These effects are mediated through an imbalanced cytokine milieu favoring pro-fibrotic and inflammatory pathways. This adds a new dimension to TB management, suggesting that addressing anemia could serve as an adjunctive intervention.

The immune landscape is further complicated by helminth coinfections, as shown by Pushpamithran and Blomgran. Their data indicates that *Ascaris lumbricoides* antigen exposure reprograms macrophage-derived extracellular vesicles (EVs) to enhance Mtb control while dampening Interleukin-1 betaproduction, via microRNA-mediated modulation of Phosphoinositide 3-kinase/Akt pathway and Mitogen-Activated Protein Kinase pathways. This illustrates the immunoregulatory potential of helminths and the therapeutic promise of EVs as immunomodulators in TB.

Autoimmune conditions and their treatments also significantly affect TB immunobiology. Farroni et al. report that patients with immune-mediated inflammatory diseases (IMID), such as rheumatoid arthritis, retain intact Mtb-specific immune responses production, and this occurs via via *in vitro* bacterial control, despite their underlying immune dysfunction and immunosuppressive therapies. However, Picchianti-Diamanti et al. warn that immunomodulatory biologics, particularly tumor necrosis factor inhibitors and Janus tyrosine kinase inhibitors, significantly increase TB risk, underscoring the importance of rigorous LTBI screening and prophylaxis in these populations. The discrepancy between preserved immune function and elevated TB risk raises important questions about the balance between systemic and compartmentalized immunity in the context of immunosuppressive biologic therapies.

Although the relationship between other viral infections and TB is not as strong as that of human immunodeficiency virus, some can affect immunity and increase the risk of developing the disease or worsening its course. However, due to its recent circulation in the population, little is known about the impact of Severe Acute Respiratory Syndrome Coronavirus 2 on patients infected with Mtb Peña-Bates et al. show that individuals with LTBI and mild coronavirus disease 2019 (Covid-19) exhibit enhanced $CD8^+$ T cell cytotoxicity, mitochondrial stability, and attenuated pro-inflammatory cytokine secretion compared to Covid-19-only patients, suggesting a potentially immune modulation conditioning by LTBI. Meanwhile, Kameni et al. reveal that polymorphisms in the angiotensin-converting enzyme 2 gene influence cytokine responses in TB-Covid-19 co-infection, suggesting that host genetics modulate disease severity in co-infected individuals.

Together, these studies underscore the necessity for an integrative view of TB pathogenesis, one that incorporates immunogenetics, comorbidity profiles, and host-pathogen-microbiome interactions. They also call for the development of stratified treatment and monitoring protocols that reflect the heterogeneity of TB hosts in real-world clinical settings.

However, gaps remain. While robust in design, several studies rely on small cohorts, lack longitudinal validation or confined to geographically distinct locations. Functional studies linking cytokine signatures to bacterial clearance or tissue pathology are needed to move from correlation to causation. Moreover, the interplay between metabolic control (e.g., glycated hemoglobin levels) and immune trajectory during TB therapy is insufficiently characterized, limiting translational applications.

In conclusion, the convergence of TB with metabolic, autoimmune, parasitic, and viral comorbidities demands a multidimensional approach to research and care. Immunological profiling, coupled with genetic screening and clinical biomarkers, holds promise for identifying vulnerable subpopulations and optimizing TB control strategies. As these findings integrate into practice, they may finally tilt the balance in the global fight against TB.

Author contributions

AG-S: Writing – review & editing. SB: Writing – review & editing. VCR: Writing – original draft.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cytokine and chemokine profiles in pulmonary tuberculosis with pre-diabetes

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Introduction: Tuberculosis (TB) remains a significant health concern in India, and its complexity is exacerbated by the rising occurrence of non-communicable diseases such as diabetes mellitus (DM). Recognizing that DM is a risk factor for active TB, the emerging comorbidity of TB and PDM (TB-PDM) presents a particular challenge. Our study focused on the impact of PDM on cytokine and chemokine profiles in patients with pulmonary tuberculosis TB) who also have PDM.

Materials and methods: We measured and compared the cytokine (GM-CSF, IFN-γ, IL-1α/IL-1F1, IL-1β/IL-1F2, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17/IL-17A, IL-18/IL-1F4, TNF-α) and chemokine (CCL1, CCL2, CCL3, CCL4, CCL11, CXCL1, CXCL2, CXCL9, CXCL10, and CXCL11) levels in plasma samples of TB-PDM, only TB or only PDM using multiplex assay.

Results: We observed that PDM was linked to higher mycobacterial loads in TB. Patients with coexisting TB and PDM showed elevated levels of various cytokines (including IFNγ, TNFα, IL-2, IL-17, IL-1α, IL-1β, IL-6, IL-12, IL-18, and GM-CSF) and chemokines (such as CCL1, CCL2, CCL3, CCL4, CCL11, CXCL1, CXCL9, CXCL10, and CXCL11). Additionally, cytokines such as IL-18 and GM-CSF, along with the chemokine CCL11, were closely linked to levels of glycated hemoglobin (HbA1c), hinting at an interaction between glycemic control and immune response in TB patients with PDM.

Conclusion: Our results highlight the complex interplay between metabolic disturbances, immune responses, and TB pathology in the context of PDM, particularly highlighting the impact of changes in HbA1c levels. This emphasizes the need for specialized approaches to manage and treat TB-PDM comorbidity.

KEYWORDS

pre-diabetes, tuberculosis, cytokines, chemokines, metabolism, immunity

1 Introduction

Tuberculosis (TB) remains a leading cause of death worldwide. Globally, million people were estimated to be newly diagnosed with TB with an estimated death due to TB being 1.3 million in 2022 (1). The intersection of TB and type 2 diabetes (T2D) significantly worsens health outcomes, increasing the risk of drug-resistant TB strains and elevating relapse and mortality rates during treatment (1, 2). In India, approximately 26% of TB patients also suffer from diabetes, highlighting a critical public health issue (3, 4).

Pre-diabetes (PDM), a condition of intermediate hyperglycemia characterized by impaired fasting glucose (IFG) or impaired glucose tolerance (IGT), affects nearly 298 million people worldwide—a figure projected to rise to 414 million by 2045 (5). PDM not only increases the likelihood of progressing to diabetes but also amplifies the risk of developing TB. This is attributed to an impaired immune response which is further compromised in the presence of active TB, potentially exacerbating dysglycemia (6–8).

Further, exacerbating the complexity, recent studies suggest that the immune systems of individuals with PDM are dysregulated, showing enhanced levels of pro-inflammatory cytokines including Type 1 and Type 17 cytokines and others like IL-1β, IFNβ, and GM-CSF, which are crucial in the pathogenesis of TB (9, 10). This cytokine profile is similar to that of patients with TB and diabetes, indicating a similar alteration in immune function that could exacerbate TB progression (3, 9, 11–21). Moreover, PDM has been shown to be associated with unfavorable treatment outcomes in TB (9, 14, 22, 23).

Given the increasing prevalence of PDM and its potential to progress to diabetes, understanding how PDM influences TB pathogenesis is crucial. This study aims to explore the impact of PDM on the cytokine and chemokine responses in patients newly diagnosed with active pulmonary tuberculosis (TB). By comparing cytokine levels in TB patients with and without PDM, we seek to unravel how even modest disruptions in glycemic control could influence the inflammatory milieu in TB, potentially informing better management strategies for this dual burden of disease.

2 Methods and materials

2.1 Ethics statement

This study was approved by the Ethics Committees of the Prof. M. Viswanathan Diabetes Research Center (ECR/51/INST/TN/2013/MVDRC/01) and NIRT (NIRT-INo:2014004). Informed written consent was obtained from all participants. All the methods were performed in accordance with institutional ethical committee guidelines. The study participants were recruited from the Effect of Diabetes in Tuberculosis Severity protocol conducted under the RePORT (Regional Prospective Observational Research for Tuberculosis) India consortium.

2.2 Study population

Individuals newly diagnosed with smear and culture-positive pulmonary TB, both with and without PDM, were enrolled between 2015 – 2018 in Chennai. This study analyzed baseline plasma samples from a cohort of 66 participants divided into three groups: 22 individuals co-positive for PDM and TB, 22 positive for TB and normoglycemic (NDM), and 22 PDM who were TB-negative. TB was diagnosed based on sputum smear and culture positivity, employing Ziehl-Nielsen smear microscopy and culture grading. In brief, before beginning anti-TB medication, all patients had three sputum samples (spot-morning-spot) taken after being instructed and shown how to provide high-quality sputum. Using the Ziehl-Neelsen (ZL) method, all of the sputum samples were stained for acid-fast bacilli (AFB). Individuals with pulmonary TB were diagnosed by positive solid cultures in Lowenstein–Jensen medium and were classified as 1+ (10–100 colonies), 2+ (>100–200 colonies) and 3+ (>200 colonies). Chest radiographs on enrollment were graded by two blinded readers using a validated severity score based on the percent area of lung involved with TB disease and the presence or absence of cavities. Smear grades were used to determine bacterial burdens and classified as 1+ (10–99 AFB in 100 fields), 2+ (1–10 AFB in each field) and 3+ (more than 10 AFB in each field) based on World Health Organization guidelines and according to the NTEP national laboratory guidelines (24). All standard test methods for smear, culture (solid and liquid), and Xpert MTB/RIF shall be carried out. Chest X-rays were used to determine cavitary disease and unilateral versus bilateral involvement. PDM was defined by a Glycated Hemoglobin (HbA1c) level of 5.7% to 6.4%, according to the American Diabetes Association criteria. PDM individuals were asymptomatic with normal chest X-rays and Quantiferon TB-Gold. Comprehensive assessments including anthropometric measurements (e.g., BMI) and biochemical parameters (e.g., HbA1c, random blood glucose, total cholesterol, serum triglycerides, HDL, and LDL cholesterol) were conducted. Additional clinical features, such as the presence of cavitary lesions and bilateral lung disease, were recorded.

2.3 Multiplex assay methodology

The levels of cytokines and chemokines were measured using the Bio-Rad, MAGPIX multiplex system (Bio-Rad, MAGPIX multiplex reader, xPONENT 4.2 acquisition and Bio-plex manager 6.1 software). Luminex Human Cytokines Magnetic Assay kit (R & D systems, USA) In brief, samples of plasma were purified and stored frozen at −80 °C prior to Luminex assays. The samples were thawed to room temperature and following the manufacturer's recommendations, the assay was performed to measure the levels of cytokines and chemokines. The lowest detection limits for cytokines were as follows: GM-CSF, 18.4 pg/mL; IFN-γ, 5.7 pg/mL; IL-1α/IL-1F1, 10.6 pg/mL; IL-1β/IL-1F2, 3.5 pg/mL; IL-2, 3.6 pg/mL; IL-4, 1.1 pg/mL; IL-5, 6.2 pg/mL; IL-6, 9.0 pg/mL; IL-10, 32.2 pg/mL; IL-12p70, 18.5 pg/mL; IL-13, 31.8 pg/mL; IL-17/IL-17A, 9 pg/mL; IL-18/IL-1F4, 2.5 pg/mL; TNF-α, 12.4 pg/mL and chemokines like CCL1, 1.57 pg/mL; CCL2, 31.8 pg/mL; CCL3, 90.9 pg/mL; CCL4, 103.8 pg/mL; CCL11, 21.6 pg/mL; CXCL1, 49.2 pg/mL; CXCL2, 49.2 pg/mL; CXCL9, 600.6 pg/mL; CXCL10, 2.88 pg/mL and CXCL11, 21.6 pg/mL.

2.4 Statistical analysis

Geometric means (GM) were calculated to describe the central tendency of data. Group comparisons among the TB-PDM, TB, and PDM were made using the Kruskal-Wallis test, with Dunn's multiple comparisons test applied to identify statistically significant differences. The Spearman rank correlation coefficient was used to assess correlations between variables. The cytokine and chemokine levels were correlated with HbA1c levels. The linear regression analysis was performed between the levels of cytokines and HbA1c, RBG and total cholestrol. Data analysis was performed using GraphPad Prism version 10.2.3 and JMP version 17.0.0.

3 Results

3.1 Characteristics of the study population

The demographic and biochemical baseline characteristics of the study population are presented in Table 1. Individuals with TB-PDM exhibited significantly higher levels of HbA1c (TB-PDM; Geomean (GM) 5.92, IQR 5.9–6.12 vs. TB, GM 5.6, IQR 4.12–5.35; PDM, GM 5.3, IQR 5.2–5.4; p = 0.0286), random blood glucose (RBG) (TB-PDM; GM 101, IQR 80–148 vs. TB, GM 86, IQR 74–116; PDM, GM 85, IQR 68–115; p = 0.0160), and total cholesterol (TB-PDM; GM 168, IQR 145–198 vs. TB, GM 146, IQR 128–190; PDM, GM 131, IQR 105–140; p = 0.0386) compared to those with TB alone and PDM alone. No significant differences were observed in age, sex, BMI, serum triglycerides, high-density lipoprotein cholesterol (HDL), or low-density lipoprotein cholesterol (LDL) among the TB-PDM, TB, and PDM groups. As detailed in Table 2, the subgroup with TB-PDM demonstrated significantly higher bacterial loads than the TB-only group, with mean scores of 3+ in acid-fast bacillus (AFB) staining and 4+ in culture results (p = 0.035 and p = 0.001, respectively). The prevalence of bilateral pulmonary involvement and occurrence of pulmonary cavities were comparable across the groups, with no statistically significant differences noted.

3.2 TB-PDM is linked to higher systemic levels of type 1 and type 17 cytokines

To explore the influence of PDM on the levels of type 1 and type 17 cytokines in individuals with active TB, we analyzed the circulating concentrations of these cytokines. Specifically, we measured type 1 cytokines (IFN-γ, TNF-α, and IL-2) and the type 17 cytokine IL-17 in groups with TB-PDM, TB alone, and PDM alone. As illustrated in Figure 1A, the levels of type 1 cytokines were substantially elevated in the TB-PDM group (IFN-γ: TB-PDM-median, 189.2 pg/ml; IQR, 177.6-200.5 pg/ml Vs TB, median 119.5 pg/ml; IQR, 105.0-142.5 pg/ml Vs PDM, median 101.8 pg/ml; IQR, 83.08-113.3 pg/ml: p<0.0001) compared to those with only TB or PDM. Similar patterns were observed for TNF-α (TB-PDM-median, 111.2 pg/ml; IQR, 109.3-118.8 pg/ml Vs TB, median 73.50 pg/ml; IQR, 71.28-81.15 pg/ml Vs PDM, median 41.40 pg/ml; IQR, 41.40 -44.49 pg/ml: p<0.0001) and IL-2 (TB-PDM-median, 95.20 pg/ml; IQR, 95.20 -97.23 pg/ml Vs TB, median

TABLE 1 Demographics and biochemistry profile of TB-PDM and TB and PDM individuals.

Parameter	TB -PDM n=22	TB n=22	PDM n=22	p value
Age	41 (18-65)	39.5 (20-65)	41.5 (22-65)	0.5647
Gender M/F	10/12	12/10	12/10	0.6821
BMI (kg/m^2)	22.5 (16.4 – 24.5)	20.4 (14.6 – 22.3)	24.5 (15.5 – 30.10)	0.4876
Smear Grade: 0/1+/2+/3+	0/12/4/6	0/9/10/3	NA	
Cavitary Disease (Y/N)	6/16	5/17	NA	
Lung Lesions (Unilateral/Bilateral)	15/7	16/6	NA	
Biochemical Parameters				
HbA1c (%)	5.92 (5.71–6.44)	5.6 (4.04–5.65)	5.3 (5.2– 5.4)	0.0286
RBG (mg/dl)	101 (78– 156)	86 (70–120)	85 (66–118)	0.0160
Total cholesterol (mg/dl)	168 (142–202)	146 (124–195)	131 (102-148)	0.0386
Serum triglycerides (mg/dl)	102 (64–424)	98 (56–384)	141 (50-179)	0.6382
HDL (ml/dl)	37 (29–59)	45 (25–66)	52.8 (30-96)	0.5926
LDL (ml/dl)	104 (44–180)	96 (52–146)	83. 5 (60-195)	0.6366

The values represent the geometric mean and the range (except for age where the median and the range) are shown.
Smear grades were used to determine bacterial burdens and classified as 1+ (10–99 AFB in 100 fields), 2+ (1–10 AFB in each field) and 3+ (more than 10 AFB in each field) based on World Health Organization guidelines.
Unilateral Lung Lesions: These are lesions that affect only one lung or one side of the chest. They may appear as localized abnormalities on imaging such as X-rays.
Bilateral Lung Lesions: These lesions affect both lungs simultaneously. They can manifest as widespread abnormalities throughout both lungs or as multiple discrete lesions scattered across both lung fields.

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TABLE 2 Clinical profile of TB-PDM Vs TB individuals.

	TB-PDM (n = 22)	TB (n = 22)	p-value
AFB			
1+	12	9	0.035
2+	4	10	
3+	6	3	
Culture			
1+	14	13	0.001
2+	1	9	
3+	7	0	
Cavity			
Yes	6	5	0.728
No	16	17	
Bilateral			
Yes	7	6	0.819
No	15	16	

AFB smear result with respect to the number of M. tuberculosis culture-positive specimens from three consecutive days of sputum samples.
Smear grades were used to determine bacterial burdens and classified as 1+ (10–99 AFB in 100 fields), 2+ (1–10 AFB in each field) and 3+ (more than 10 AFB in each field) based on World Health Organization guidelines. According to the NTEP national laboratory guidelines.
Chest X-rays were used to determine cavitary disease and unilateral versus bilateral involvement.

57.12 pg/ml; IQR, 57.12-61.98 pg/ml Vs PDM, median 38.08 pg/ml; IQR, 38.08-41.32 pg/ml: p<0.0001). Additionally, the level of the type 17 cytokine IL-17A was significantly higher in the TB-PDM group (TB-PDM-median, 53.31 pg/ml; IQR, 50.95-55.68 pg/ml Vs TB, median 44.34 pg/ml; IQR, 37.95-45.88 pg/ml Vs PDM, median 30.57 pg/ml; IQR, 30.57-33.41 pg/ml: p<0.0001) than in those with TB alone or PDM alone. The findings also revealed that the levels of both type 1 and type 17 cytokines were higher in individuals with TB compared to those with PDM. Therefore, TB-PDM is characterized by an increase in both type 1 and type 17 cytokines, indicating a more pronounced immune response in individuals with both TB and PDM.

3.3 TB-PDM exhibits elevated systemic levels of IL-10

In our investigation into the impact of PDM on type 2 and anti-inflammatory cytokines in individuals with active TB, we assessed the circulating concentrations of these cytokines. Specifically, we analyzed type 2 cytokines (IL-4, IL-5, IL-13) and the anti-inflammatory cytokine IL-10 in groups with TB-PDM, TB alone, and PDM alone. As depicted in Figure 1B, there were no significant differences in the levels of type 2 cytokines (IL-4, IL-5, IL-13) between the groups. However, the circulating levels of the anti-inflammatory cytokine IL-10 was notably higher in the TB-PDM group (TB-PDM-median, 62.28 pg/ml; IQR, 56.88 - 67.65 pg/ml Vs

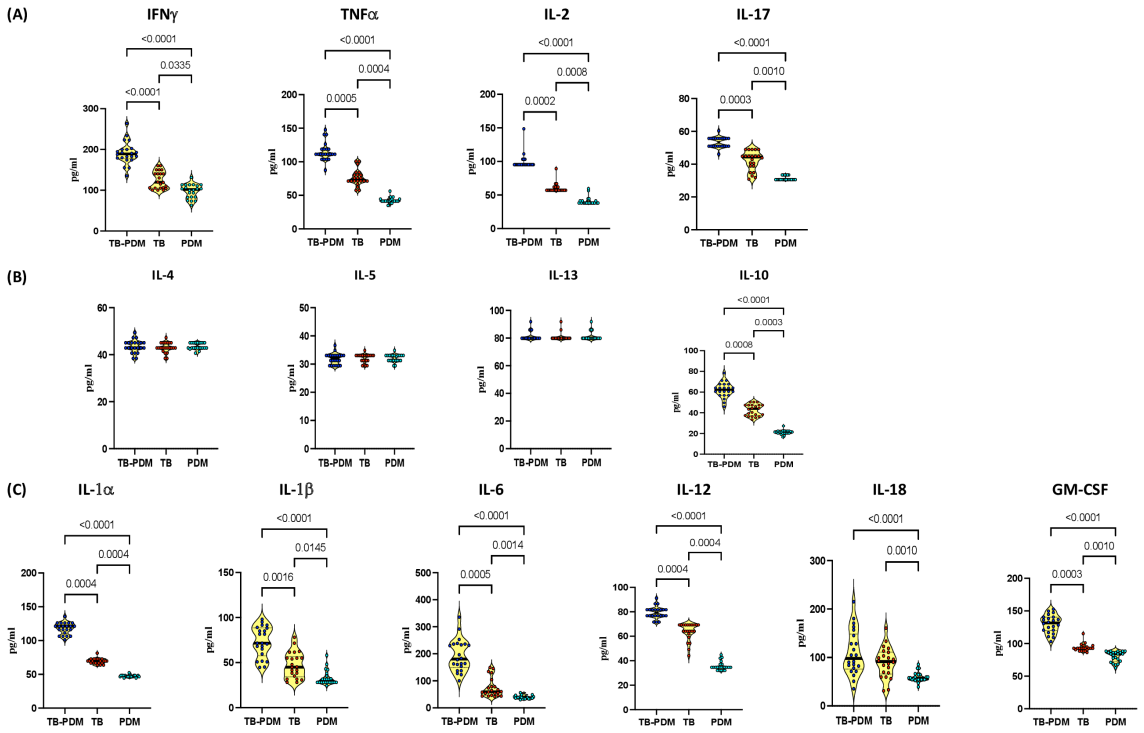

FIGURE 1
Elevated Levels of Type 1 and Type 17 Cytokines in TB-PDM. Plasma levels of **(A)** Type 1 (IFNγ, TNFα, IL-2) and Type 17 (IL-17) cytokines, **(B)** Type 2 (IL-4, IL-5, IL-13), and regulatory (IL-10) cytokines, **(C)** and other pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12, IL-18, and GM-CSF) were measured via Bio-Rad, MAGPIX multiplex system in individuals with TB-PDM (n=22), PDM (n=22), and TB (n=22). Data are presented as violin plots, with each circle representing an individual (Navy Blue – TB-PDM; Maroon – TB; Light Blue – PDM). p-values were calculated using the Kruskal-Wallis test with Dunn's multiple corrections.

TB, median 43.89 pg/ml; IQR, 37.14 – 47.40 pg/ml Vs PDM, median 21.36 pg/ml; IQR, 20.16 – 22.55 pg/ml: p<0.0001) compared to individuals with TB alone or PDM alone. Additionally, IL-10 levels were significantly elevated in the TB group compared to those with PDM alone. Therefore, TB-PDM is characterized by increased levels of the anti-inflammatory cytokine IL-10, suggesting a distinct immunological profile in individuals with both TB and PDM.

3.4 TB-PDM presents with heightened systemic levels of other pro-inflammatory cytokines and IL-1 family of cytokines

In our investigation into the impact of PDM on the IL-1 family and additional pro-inflammatory cytokines in individuals with active TB, we analyzed the circulating concentrations of IL-1α, IL-1β, IL-6, IL-12, IL-18, and GM-CSF. Comparing individuals with TB-PDM, TB alone, and PDM alone, we observed significant differences in cytokine levels, as depicted in Figure 1C. Specifically, the circulating levels of IL-1α (TB-PDM-median, 121.2 pg/ml; IQR, 110.1- 126.1 pg/ml Vs TB, median 69.80 pg/ml; IQR, 66.83 – 72.72 pg/ml Vs PDM, median 46.53 pg/ml; IQR, 46.53- 48.48 pg/ml: p<0.0001), IL-1β (TB-PDM-median, 71.60 pg/ml; IQR, 57.59- 88.51 pg/ml Vs TB, median 44.96 pg/ml; IQR, 34.16 – 56.64 pg/ml Vs PDM, median 29.84 pg/ml; IQR, 28.08 – 35.78 pg/ml: p<0.0001), IL-6 (TB-PDM-median, 180.6 pg/ml; IQR, 149.5 – 235.9 pg/ml Vs TB, median 59.42 pg/ml; IQR, 46.16 – 84.99 pg/ml Vs PDM, median 38.54 pg/ml; IQR, 35.18 – 41.16 pg/ml: p<0.0001), IL-12 (TB-PDM-median, 79.26 pg/ml; IQR, 76.78 – 81.73 pg/ml Vs TB, median 64.14 pg/ml; IQR, 61.37 – 69.26 pg/ml Vs PDM, median 34.63 pg/ml; IQR, 33.99 – 37.14 pg/ml: p<0.0001), and GM-CSF (TB-PDM-median, 131.7 pg/ml; IQR, 119.71- 152.7 pg/ml Vs TB, median 92.7 pg/ml; IQR, 90.06 – 95.34 pg/ml Vs PDM, median 84.14 pg/ml; IQR, 72.59 – 86.84 pg/ml: p<0.0001) were markedly elevated in individuals with TB-PDM compared to those with TB alone and PDM alone. Similarly, these cytokines were significantly increased in individuals with TB compared to those with PDM alone. These findings indicate that TB-PDM is characterized by heightened systemic levels of the IL-1 family of cytokines and other pro-inflammatory cytokines, suggesting an enhanced inflammatory response in individuals with both TB and PDM.

3.5 TB-PDM is associated with elevated systemic levels of CC and CXC chemokines

In our investigation to assess the impact of PDM on CC and CXC chemokines in individuals with active TB, we analyzed the circulating concentrations of various chemokines including CCL1, CCL2, CCL3, CCL4, CCL11, CXCL1, CXCL2, CXCL9, CXCL10, and CXCL11. Comparing individuals with TB-PDM, TB alone, and PDM alone, we observed notable differences in chemokine levels, as illustrated in Figure 2. As shown in Figure 2A, CCL2 (TB-PDM-median, 2105 pg/ml; IQR, 1820 - 2766 pg/ml Vs TB, median 839.5 pg/ml; IQR, 568 – 1160 pg/ml Vs PDM, median 458.6 pg/ml; IQR, 337.2 – 554.4 pg/ml: p<0.0001), CCL4 (TB-PDM-median, 245.4 pg/ml; IQR, 133.8 – 392.3 pg/ml Vs TB, median 172.7 pg/ml; IQR, 131.7 – 197.9 pg/ml Vs PDM, median 73.03 pg/ml; IQR, 59.36 – 94.31 pg/ml: p<0.0001), and CCL11 (TB-PDM-median, 253.9 pg/ml; IQR, 228.1 – 276.5 pg/ml Vs TB, median 186.6 pg/ml; IQR, 183.8 – 214.2 pg/ml Vs PDM, median 90.37 pg/ml; IQR, 85.66 – 102.1 pg/ml: p<0.0001) levels were elevated in TB compared to PDM individuals, indicating a distinct chemokine profile associated with TB-PDM. Regarding CXC chemokines, individuals with TB-PDM exhibited significantly higher levels of CXCL1 (TB-PDM-median, 97.79 pg/ml; IQR, 32.38- 119.2 pg/ml Vs TB, median 26.99 pg/ml; IQR, 24.52 – 37.34 pg/ml Vs PDM, median 19.17 pg/ml; IQR, 18.95 – 20.95 pg/ml: p<0.0001), CXCL9 (TB-PDM-median, 2.980 pg/ml; IQR, 2.940 – 3.383 pg/ml Vs TB, median 2.660 pg/ml; IQR, 2.520 – 2.660 pg/ml Vs PDM, median 2.200 pg/ml; IQR, 2.048 – 2.520 pg/ml: p<0.0001), CXCL10 (TB-PDM-median, 4336 pg/ml; IQR, 3588 - 5036 pg/ml Vs TB, median 3176 pg/ml; IQR, 2465 – 4179 pg/ml Vs PDM, median 1529 pg/ml; IQR, 1265 - 1912 pg/ml: p<0.0001), and CXCL11 (TB-PDM-median, 36.58 pg/ml; IQR, 28.75 – 43.64 pg/ml Vs TB, median 25.18 pg/ml; IQR, 16.21 – 29.06 pg/ml Vs PDM, median 16.95 pg/ml; IQR, 15.77 – 16.95 pg/ml: p<0.0001) compared to those with TB alone (Figure 2B). Additionally, circulating levels of CXCL1, CXCL9, CXCL10, and CXCL11 were significantly elevated in TB individuals compared to those with PDM alone. However, no significant differences were observed in the level of CXCL2 between the groups. These findings suggest that TB-PDM is characterized by markedly elevated systemic levels of both CC and CXC chemokines.

3.6 Examining the relationship between systemic cytokines/chemokines and HbA1c levels

Our study aimed to elucidate the connection between systemic cytokine and chemokine levels and the degree of glycemic control, as reflected by HbA1c levels, in individuals with TB-PDM. Elevated HbA1c values indicate poor blood sugar control. We assessed the correlation between HbA1c levels (%) and the circulating levels of various cytokines and chemokines. First, regarding cytokines, we observed a negative correlation between HbA1c levels and the systemic levels of IL-18 (r= -0.6335; p=0.0015) and GM-CSF (r= -0.6126; p=0.0024), as depicted in Figures 3A, B. However, we did not find significant correlations between HbA1c levels and other systemic cytokines in TB-PDM individuals. Furthermore, we explored the association between systemic chemokine levels and glycemic control in Figures 3C, D. We investigated the correlation between HbA1c levels (%) and the levels of CC and CXC chemokines, including CCL1, CCL2, CCL3, CCL4, CCL11, CXCL1, CXCL2, CXCL9, CXCL10, and CXCL11. Among the chemokines, CCL11 alone showed a positive correlation with the levels of HbA1c (r= 0.4354; p=0.0428).

The linear regression analysis revealed significant associations between specific cytokines and HbA1c levels. As illustrated in Figure 4A, IL-18 (R^2 = 0.4320; p = 0.0009), GM-CSF (R^2 = 0.3229; p = 0.0058), and CCL11 (R^2 = 0.3580; p = 0.0033)

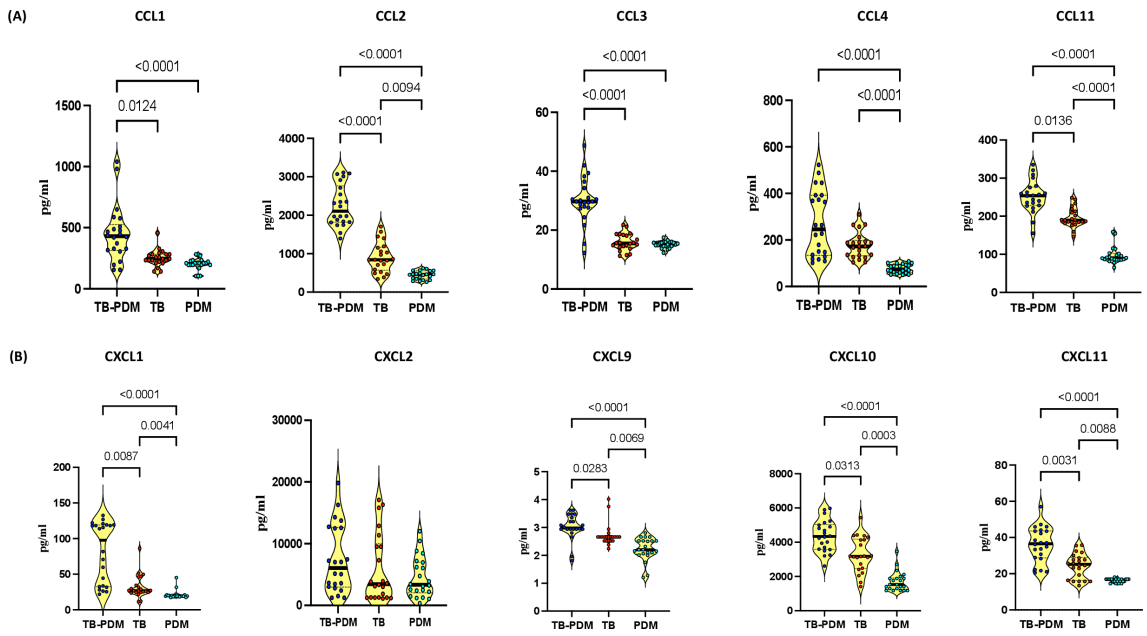

FIGURE 2
Association of TB-PDM with Increased Levels of CC and CXC Chemokines. Plasma levels of **(A)** CC chemokines (CCL1, CCL2, CCL3, CCL4, and CCL11) and **(B)** CXC chemokines (CXCL1, CXCL9, CXCL10, and CXCL11) were assessed via Bio-Rad, MAGPIX multiplex system in individuals with TB-PDM (n=22), PDM (n=22), and TB (n=22). Data are represented as violin plots, with each circle indicating an individual (Navy Blue – TB-PDM; Maroon – TB; Light Blue – PDM). p-values were determined using the Kruskal-Wallis test with Dunn's multiple corrections.

demonstrated substantial positive correlations. In Figure 4B, IL-18 (R^2 = 0.3656; p = 0.0029) and GM-CSF (R^2 = 0.2162; p = 0.0292) also exhibited significant positive relationships with RBG levels, while CCL11 (R^2 = 04.896e-005; p = 0.9753) did not show a significant association with RBG levels. Conversely, no significant relationship was observed between total cholesterol levels and cytokines or chemokines. Consequently, only specific cytokines and chemokines were found to be associated with HbA1c (%) and RBG levels.

4 Discussion

Diabetes mellitus (DM) and its precursor, PDM, are linked to immune system dysfunction, involving alterations in cytokine and chemokine levels, changes in immune cell types and activation status, and increased apoptosis and tissue fibrosis (25). PDM shares characteristics of glucose dysregulation and insulin resistance with DM, potentially affecting susceptibility to TB (10, 26). Although TB patients with PDM exhibit cytokines and chemokines profile akin to type 2 diabetes (T2D), the impact of PDM on TB severity remains unclear. In our study, TB patients with PDM demonstrated elevated levels of various cytokines (IFNγ, TNFα, IL-2, IL-17, IL-1α, IL-1β, IL-6, IL-12, IL-18, and GM-CSF) and chemokines (CCL1, CCL2, CCL3, CCL4, CCL11, CXCL1, CXCL9, CXCL10, and CXCL11). Our data also suggest that PDM is associated with increased bacterial burdens but not disease severity.

Cytokines play a crucial role in TB progression and host defense (27, 28). PDM and metabolic dysfunction are associated with mild inflammation, as evidenced by elevated levels of pro-inflammatory cytokines observed in TB patients with PDM (29). Key cytokines like IFN-γ, TNF-α, IL-17A, IL-1α, IL-1β, IL-18, IL-12, and IL-6 are vital in TB infections (30–33). IFN-γ and TNF-α play crucial roles in *M. tb* infections, IFN-γ, for instance, plays a crucial role in activating macrophages to combat intracellular mycobacteria, while TNF-α contributes to the formation of granulomas, essential for containing mycobacterial growth, while IL-17A mediates memory immune responses and appears to exacerbate inflammation in TB patients with conditions like diabetes, potentially worsening disease severity. IL-1 family cytokines, including IL-1α and IL-1β, are essential for resistance, and IL-18 and IL-12 are vital for immunity. IL-1α and IL-1β initiate and sustain inflammatory responses against mycobacteria, whereas IL-18 enhances IFN-γ production crucial for effective immune responses. IL-6 inhibits disease progression. IL-12 drives Th1 differentiation and IFN-γ production, pivotal in combating TB, and IL-6 has diverse effects including pro-inflammatory responses and B cell activation (30–33). Furthermore, IL-18 and IL-12 are crucial for immunity against *M. tb* infection (34, 35). Elevated systemic pro-inflammatory cytokines, common in T2D, are associated with increased TB risk (11, 12, 34–37). We have also previously shown that LTB with PDM is associated with alterations in cytokine production of NK cells, NKT cells, MAIT cells, and γδ T cells (38, 39). In our study, the TB-PDM group exhibited heightened levels of various cytokines compared to TB or PDM alone. Disease severity and bacterial burden were notably linked to this group, possibly due to chronic low-grade inflammation induced by insulin resistance or dysfunctional adipose tissue (11, 40). Elevated IL-17 levels in TB patients with diabetes may worsen inflammation and pathology, contributing to more severe TB disease in individuals

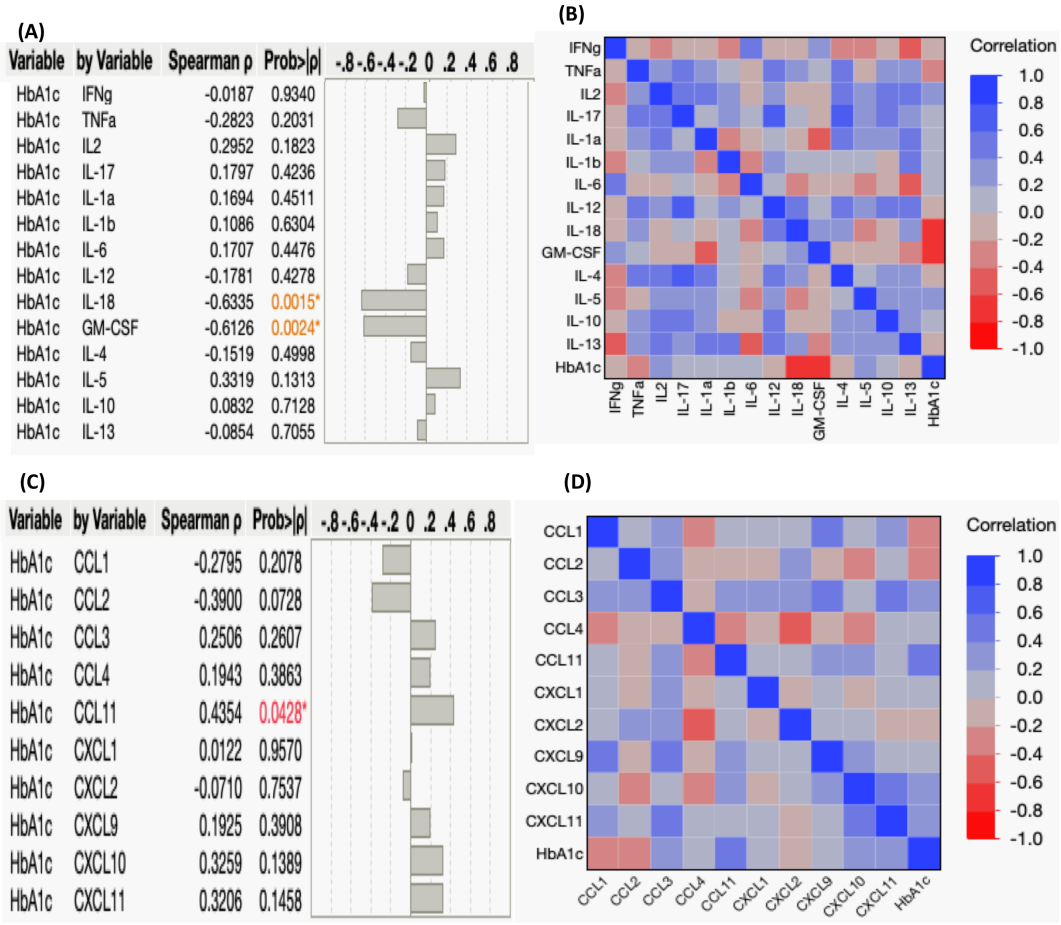

FIGURE 3
Correlation between Systemic Cytokines, Chemokines, and HbA1c Levels in TB-PDM. **(A)** A multiparametric matrix correlation plot depicts the relationships between plasma cytokines (Type 1, Type 17, Type 2, regulatory, and other pro-inflammatory) and HbA1c levels in TB-PDM patients. **(B)** Spearman's correlation coefficients are visualized, with blue indicating positive correlations and red indicating negative correlations. **(C)** A multiparametric matrix correlation plot depicting the relationships between plasma CC and CXC chemokines and HbA1c levels in TB-PDM patients. **(D)** Spearman's correlation coefficients are visualized, with blue indicating positive correlations and red indicating negative correlations.

with T2D. (13, 14, 41). The blood transcriptomic profiles of TB patients with pre-diabetes resemble those of TB patients with T2D more closely than those without dysglycemia, indicating that immune responses to *M.tb* are impaired in the early stages of dysglycemia in PDM (10). This is very similar to our previous findings in overt DM and PTB (9). Our data suggest that early stages of dysglycemia may contribute to the pro-inflammatory environment in PDM patients.

IL-1α and IL-1β are critical for TB resistance, as evidenced by studies in mice (31, 42). Elevated IL-10 levels in T2D patients with TB suggest its role in exacerbated immune dysregulation (31, 43–45) Our findings support previous studies, indicating intensified inflammatory reactions influenced by TB-induced immune dysregulation in the TB-PDM group. T2D may worsen TB severity by reducing alveolar macrophage activation via decreased IL-1β, IL-12, and IL-18 release (46, 47). Our findings suggest increased levels of pro-inflammatory cytokines and heightened responses from Th1 and Th17 cells and cytokines in patients with TB-PDM. Addressing cytokine imbalances in TB and PDM individuals could improve treatment outcomes.

In the context of TB and concurrent PDM, inflammation plays a crucial role, with chemokines emerging as key players (48–50). This inflammatory environment can activate cytokine signaling proteins, contributing to insulin resistance. Chemokines are vital for recruiting immune cells to the lung during early infection stages (50, 51). Notably, chemokines like CCL1, CCL2, CCL4, CCL5, CCL11, CXCL8, CXCL10, and CX3CL1 are implicated in T2D pathogenesis, affecting immunoregulation, inflammatory gene induction, and insulin signaling modulation (50). Chemokines act as signaling molecules in inflammation, activating pro-inflammatory mediators and inducing a variety of inflammatory factors. These factors trigger cytokine signaling proteins that impede insulin signaling receptor activation in pancreatic cells, thereby promoting insulin resistance (IR). This sequence of events is implicated in the progression from PDM to T2D (50). However, few studies have explored chemokine levels in TB and T2D comorbidity.

Animal models have shown that abnormalities in specific chemokine synthesis are linked to increased susceptibility to *Mycobacterium tuberculosis* (*M.tb*) infection (52). Animal models

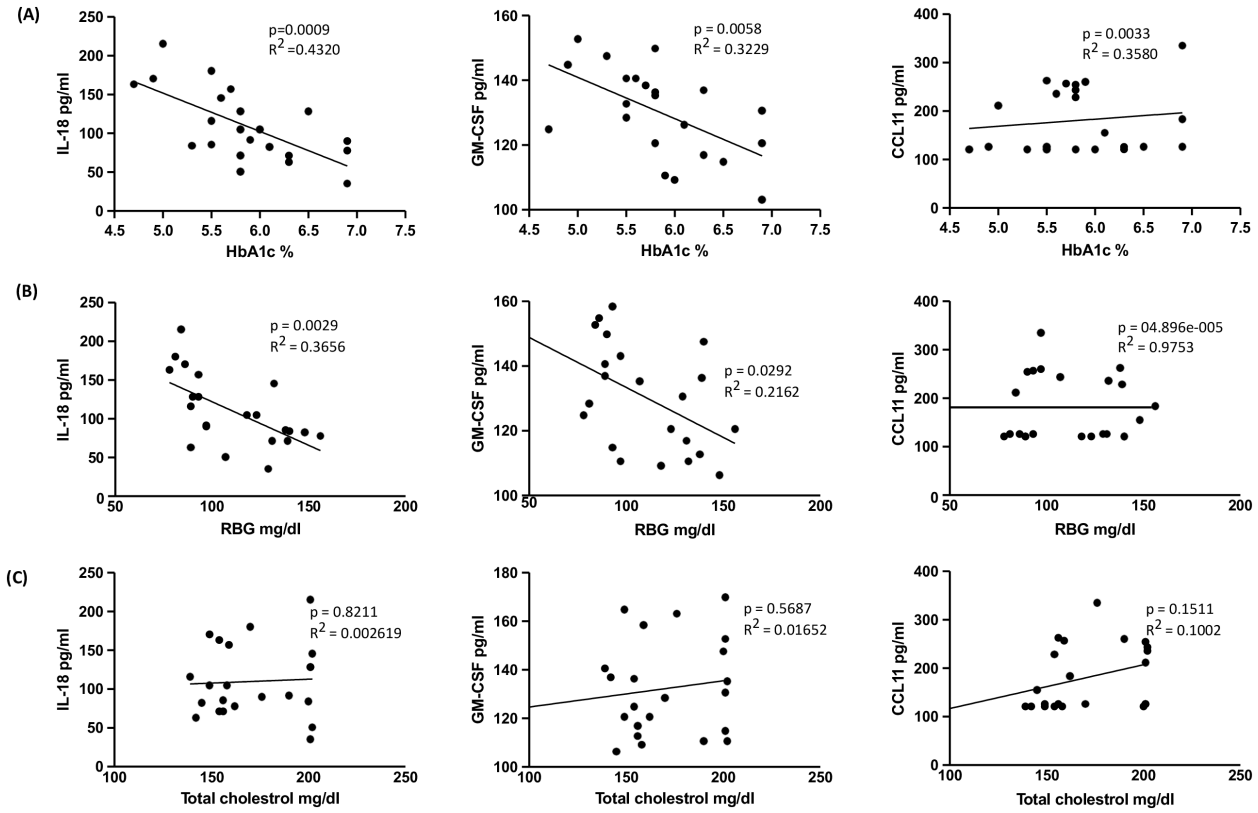

FIGURE 4
Relationship between IL-18, GM-CSF and CCL11 with HbA1c, RBG and Total cholesterol levels in TB-PDM. **(A)** Linear regression analysis depicting the relationship of cytokines IL-18 and GM-CSF and chemokine CCL11 with HbA1c%. **(B)** Linear regression analysis depicting the relationship of cytokines IL-18 and GM-CSF and chemokine CCL11 with RBG. **(C)** Linear regression analysis depicting the relationship of cytokines IL-18 and GM-CSF and chemokine CCL11 with the levels of Total cholesterol. R^2 values and p-values are indicated for each relationship, with statistically significant associations ($p < 0.05$).

of TB and diabetes exhibit exacerbated disease progression, with increased bacterial burden and dysregulated chemokine expression (53). CCR2 is a critical receptor involved in the development of T2D. Adipocytes secrete inactive CCR2, which, when activated, promotes the expression of inflammatory genes and reduces insulin-dependent glucose uptake. Adipocytes also release CCL2, which recruits macrophages to the site of inflammation. These mechanisms contribute significantly to the pathogenesis of T2D (50). CXCL10 plays a crucial role in initiating the destruction of β cells. Additionally, CXCL10 can impair insulin secretion and reduce the viability of β cells. The specific mechanism involves CXCL10-inducing dysfunction in β cells, which has been shown to be elevated in T2D patients (50). Our findings support these, showing elevated CCL2/MCP-1 and CXCL10 levels in the TB-PDM group, indicating increased bacterial burdens associated with dysregulated chemokine expression. Individuals who have both TB and PDM comorbidity exhibit elevated levels of pro-inflammatory cytokines. The interaction between TB and PDM potentially promotes pathology by enhancing the production of cytokines, potentially exacerbating the progression of diabetes mellitus (9).

Further, the linear regression analysis suggests that IL-18 and GM-CSF play significant roles in glucose metabolism as indicated by their associations with HbA1c and RBG levels. CCL11, while associated with HbA1c, did not correlate significantly with RBG levels, highlighting potential differences in its involvement in glucose regulation compared to IL-18 and GM-CSF. Further research into these cytokines' mechanistic roles and clinical implications could pave the way for targeted therapeutic approaches in managing pre-diabetes and preventing its progression to type 2 diabetes.

Our cross-sectional study lacks the ability to establish definitive cause-and-effect relationships between PDM and TB. Our study also suffers from the limitation of a small sample size. However, our findings suggest that individuals with both TB and PDM have elevated levels of pro-inflammatory cytokines and chemokines compared to those with either TB or PDM alone, potentially exacerbating TB pathogenesis in these patients. In TB-PDM comorbidity, these factors likely synergize, exacerbating inflammation and immune dysregulation, thus complicating disease progression. Understanding these mechanisms is crucial for developing effective strategies to manage TB-PDM comorbidity. Longitudinal studies are needed to determine causation and understand the complex processes underlying the relationship between PDM and TB. This study did not investigate the

responses following anti-TB treatment. However, future studies addressing the reversibility of PDM and the impact of anti-TB treatment on diabetes status would provide valuable clinical insights.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

This study was approved by the Ethics Committees of the Prof. M. Viswanathan Diabetes Research Center (ECR/51/INST/TN/2013/MVDRC/01) and NIRT (NIRT-INo:2014004). All the methods were performed in accordance with institutional ethical committee guidelines. The study participants were recruited from the Effect of Diabetes in Tuberculosis Severity protocol conducted under the RePORT India consortium. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. SK: Formal analysis, Methodology, Writing – original draft, Writing – review & editing. NK: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. VV: Data curation, Project administration, Resources, Writing – original draft, Writing – review & editing. SS: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. SH: Data curation, Project administration, Writing – original draft, Writing – review & editing. SN: Resources, Writing – original draft, Writing – review & editing. HK: Data curation, Funding acquisition, Writing – original draft, Writing – review & editing. SB: Conceptualization, Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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Impaired *Mycobacterium tuberculosis*-specific T-cell memory phenotypes and functional profiles among adults with type 2 diabetes mellitus in Uganda

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Background: Efforts to eradicate tuberculosis (TB) are threatened by diabetes mellitus (DM), which confers a 3-fold increase in the risk of TB disease. The changes in the memory phenotypes and functional profiles of *Mycobacterium tuberculosis* (*Mtb*)-specific T cells in latent TB infection (LTBI)-DM participants remain poorly characterised. We, therefore, assessed the effect of DM on T-cell phenotype and function in LTBI and DM clinical groups.

Methods: We compared the memory phenotypes and function profiles of *Mtb*-specific $CD4^+$ and $CD8^+$ T cells among participants with LTBI-DM (n=21), LTBI-only (n=17) and DM-only (n=16). Peripheral blood mononuclear cells (PBMCs) were stimulated with early secretory antigenic 6 kDa (ESAT-6) and culture filtrate protein 10 (CFP-10) peptide pools or phytohemagglutinin (PHA). The memory phenotypes (CCR7/CD45RA), and functional profiles (HLA-DR, PD-1, CD107a, IFN-γ, IL-2, TNF, IL-13, IL-17A) of *Mtb*-specific $CD4^+$ and $CD8^+$ T cells were characterised by flow cytometry.

Results: Naïve $CD4^+$ T cells were significantly decreased in the LTBI-DM compared to the LTBI-only participants [0.47 (0.34-0.69) vs 0.91 (0.59-1.05); (p<0.001)]. Similarly, $CD8^+$ HLA-DR expression was significantly decreased in LTBI-DM compared to LTBI-only participants [0.26 (0.19-0.33) vs 0.52 (0.40-0.64); (p<0.0001)], whereas $CD4^+$ and $CD8^+$ PD-1 expression was significantly upregulated in the LTBI-DM compared to the LTBI-only participants [0.61 (0.53-0.77) vs 0.19 (0.10-0.28); (p<0.0001) and 0.41 (0.37-0.56) vs 0.29 (0.17-0.42); (p=0.007)] respectively. $CD4^+$ and $CD8^+$ IFN-γ production was significantly decreased in the LTBI-DM compared to the LTBI-only participants [0.28 (0.19-

0.38) vs 0.39 (0.25-0.53); (p=0.030) and 0.36 (0.27-0.49) vs 0.55 (0.41-0.88); (p=0.016)] respectively. $CD4^+$ TNF and $CD8^+$ IL-17A production were significantly decreased in participants with LTBI-DM compared to those with LTBI-only [0.38 (0.33-0.50) vs 0.62 (0.46-0.87); (p=0.004) and 0.29 (0.16-0.42) vs 0.47 (0.29-0.52); (0.017)] respectively. LTBI-DM participants had significantly lower dual-functional (IFN-γ^+IL-2^+ and IL-2^+TNF$^+$) and mono-functional (IFN-γ^+ and TNF$^+$) $CD4^+$ responses than LTBI-only participants. LTBI-DM participants had significantly decreased dual-functional (IFN-γ^+IL-2^+, IFN-γ^+ TNF$^+$ and IL-2^+TNF$^+$) and mono-functional (IFN-γ^+, IL-2^+ and TNF$^+$) central and effector memory $CD4^+$ responses compared to LTBI-only participants.

Conclusion: Type 2 DM impairs the memory phenotypes and functional profiles of *Mtb*-specific $CD4^+$ and $CD8^+$ T cells, potentially indicating underlying immunopathology towards increased active TB disease risk.

KEYWORDS

latent tuberculosis infection, diabetes mellitus, T cells, memory phenotypes, functional profiles

Introduction

Despite significant efforts made to control tuberculosis (TB), the increasing burden of diabetes mellitus (DM) threatens the progress registered in reducing the global burden of TB, especially in low and middle-income countries (LMICs) (1). According to the 2021 International Diabetes Federation (IDF) estimates, approximately 537 million adults (aged between 20 and 79) live with DM. This figure is projected to rise to 783 million by 2045, with the most significant increase in Africa (2). Tuberculosis remains one of the leading causes of death from a single infectious agent, *Mtb*, worldwide (3). Globally, approximately 7.5 million people were newly infected with *Mycobacterium tuberculosis* (*Mtb*) or diagnosed with TB in 2022, with nearly 1.3 million deaths occurring (3). Epidemiologically, DM confers a 3-fold increase in the risk of developing TB disease and is associated with TB treatment failure and drug resistance (4). Indeed, it was recently reported that participants aged ≥ 40 years had increased odds of TB-DM comorbidity (5) and that Africans with DM have an increased latent TB infection (LTBI) risk (6). The risk for the development of active TB (ATB) is thought to be due to the immune-compromised status, but the underlying susceptibility mechanisms remain largely unknown.

The quality of the T-cell response is essential for *Mtb* immunity. $CD4^+$ and $CD8^+$ T cells are pivotal for immune control in *Mtb*-infected humans and murine TB models (7, 8). T-cell memory phenotypes are induced during LTBI and Bacillus Calmette-Guerin (BCG) vaccination that play a protective role in humans and in mice models (9–12). It is reported that LTBI is characterised by differential expression of functional markers, including decreased HLA-DR expression, a marker that distinguishes LTBI and ATB (13, 14), upregulated PD-1 expression, a marker that inhibits T-cell effector functions (15, 16), as well as downregulated Th1 (7) and Th17 (17, 18) cytokine production. Examining cytokine T-cell polyfunctionality is essential as these cells have been associated with resistance to infection (19, 20). Elevated frequencies of mono-functional and dual-functional $CD4^+$ Th1 cells are reportedly a hallmark of active TB and DM (TB-DM) comorbidity (21). This shows that type 2 DM modulates T-cell immune responses to *Mtb*, which could profoundly affect TB pathogenesis. However, the underlying immunological mechanisms for TB susceptibility during DM remain to be elucidated, specifically with phenotypes and functional markers during LTBI.

In this study, we hypothesised that type 2 DM modulates the *Mtb*-specific memory phenotype and functional profiles of T cells among participants with LTBI, leading to impaired responses and potentially promoting TB susceptibility, progression or reactivation. We aimed to assess the *Mtb*-specific $CD4^+$ and $CD8^+$ T-cell memory phenotypes and functional profiles. We compared the T-cell memory, activation, degranulation, exhaustion and cytokine polyfunctionality profiles among participants with LTBI-DM comorbidity.

Materials and methods

Study population and setting

Participants with LTBI and DM (LTBI-DM) and DM-only participants were enrolled from October 2018 to March 2019 at

the DM clinic at Kiruddu National Referral Hospital. This was part of the Tuberculosis and Diabetes (TAD) study (22), a longitudinal study which explored isoniazid prophylaxis outcomes among DM participants with LTBI and ATB. Participants with LTBI-only were enrolled in a TB household contact cohort [Kampala TB (KTB)] study from May 2011 to January 2012, Kampala, Uganda, at Kisenyi and Kitebi Health Centre IVs, as previously described (23). To get a proper negative control group, the study utilised LTBI-only PBMC samples from the KTB study, which did not collect DM-related parameters [weight, random blood sugar (RBS), blood pressure and HbA1c]. While LTBI-DM and LTBI-only are the main comparator groups, the DM-only group was included as a negative control to compare and assess how DM alone (without LTBI) might impact immune function.

Study methods

Peripheral blood mononuclear cell samples taken from 54 participants were assayed using flow cytometry (LTBI-DM [n=21], LTBI-only [n=17] and DM-only [n=16]). Diabetes Mellitus was diagnosed based on the American Diabetes Association (ADA) criteria (glycated haemoglobin [HbA1c] levels ≥ 6.5%), with normal ranges between 4% and 5.6% (24). Latent TB infection was diagnosed based on positive results for QuantiFERON TB-Gold (QFT)-Plus and QFT In-Tube assays. All participants were adults and HIV-negative.

Peripheral blood mononuclear cell isolation

Ten millilitres of heparinised blood collected by venepuncture was transported within 4 hours to the immunology laboratory at the College of Health Sciences, Makerere University and the MRC/UVRI and LSHTM Uganda Research Unit, Kampala, Uganda, for processing. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Histopaque density gradient centrifugation. The Cells were counted and resuspended in cold foetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO). Cells were then adjusted to a final concentration of $3x10^6$ cells/ml. Cells were transferred to a cold Mr Frosty™ freezing container overnight at -80°C and then moved to liquid nitrogen (-197°C) for long-term storage.

Cell stimulation and culture

Upon retrieval from liquid nitrogen, frozen cell vials ($6x10^6$ cells) were thawed at a 37°C water bath in R20 (RPMI with 20% FBS, 1% Penicillin/streptomycin, 2mM Glutamine, 25mM HEPES). The PBMCs were rinsed and rested in R10 (RPMI with 10% FBS, 1% Penicillin/streptomycin, 2mM Glutamine, 25mM HEPES) media in a humidified incubator at 5%CO_2, 37°C for 4 hours. The cells (200µl/$2x10^6$, resuspended in R20) were stimulated in a humidified incubator at 37°C, 5%CO_2 for 18 hours (overnight) with *Mtb*-specific peptide pools of early secreted antigenic target-6 kDa [ESAT-6 (21-peptide array; 10µg/ml)], and culture filtrate protein-10 kDa [CFP-10 (22-peptide array; 10µg/ml)], all from BEI Resources (Manassas, VA). The peptides consist of 15- or 16-mers peptides (overlapping by 11 or 12 amino acids) spanning the entire amino acid sequences for the ESAT-6 and CFP-10. Phytohemagglutinin-lectin (PHA-L [10µg/ml, Millipore, Sigma]) was used as a positive control, and unstimulated cells (R20 media) as a negative control. Stimulations were performed for 2 hours, after which Brefeldin A (5µg/ml, BioLegend) was added to all tubes. Cells were further incubated and stimulated for 16 hours. All experiments were performed in the presence of co-stimulatory antibodies, anti-CD28 and anti-CD49d (1µg/ml each, BD Biosciences) and CD107a brilliant violet (BV) 605 (H4A3, BioLegend) antibody for the 18 hours.

Cell staining

After stimulations, cells were washed with Dulbecco's phosphate buffered saline (PBS [1X, Sigma-Aldrich]), followed by staining with a fixable viability dye, zombie aqua (BioLegend) at room temperature for 20 minutes in the dark. Cells were then washed with cell staining buffer (BioLegend), blocked for Fcγ receptors using BD Fc block (2.5µg/ml, BD Biosciences) at room temperature for 10 minutes in the dark. Cells were surface stained at 4°C for 30 minutes in the dark with the following antibodies: CD3 FITC (UCHT1; BioLegend), CD4 PerCP-Cyanine5.5 (A161A1; BioLegend), CD8 BV650 (SK1; BioLegend), CCR7 PE-CF594 (2-L1-A; BD Biosciences), PD-1 BV785 (EH12.2H7; BioLegend), HLA-DR PE-Fire 640 (L243; BioLegend), and CD45RA APC-Cy7 (HI100; BioLegend). For intracellular cytokine staining, cells were washed, fixed using fixation buffer (4% paraformaldehyde, BioLegend), and permeabilised using working strength intracellular staining permeabilisation wash buffer (1X, BioLegend) according to manufacturer's recommendations. Fixed cells were intracellularly stained at room temperature for 20 minutes in the dark with the following antibodies: IFN-γ PE/Cy7 (4S.B3; BioLegend), TNF APC (MAb11; BioLegend), IL-2 PE (MQ1-17H12; BioLegend), BCL-2 BV421 (100; BioLegend), IL-17A APC-R700 (N49-653; BD Biosciences) and IL-13 Alexa Fluor (AF) 350 (32116; R&D Systems). The cells were immediately acquired on the CytoFLEX LX flow cytometer (Beckman Coulter). The flow cytometry antibody panel, including clone and catalogue number, is shown in Supplementary Table S1.

Data and statistical analysis

The flow cytometry data from this study was normalised to minimise batch effects across the two study PBMC T-cell responses using the ComBat algorithm from the "sva" package. The data was then analysed using FlowJo v.10.10.0 (BD Biosciences, San Jose, CA, USA) for Mac. Gating was standardised and set using Fluorescence Minus One (FMO) and compensation controls to correct for spectral overlap. Boolean combination gating was used to

calculate frequencies corresponding to seven different combinations of cytokines, including IL-2, TNF and IFN-γ. The gating strategy is shown in Supplementary Figure S1. The data was Arcsine transformed, and a linear regression model was fitted with age as a covariate in all groups using R(v.4.4.0). The linear regression results are reported in Supplementary Table S2. Statistical tests were performed using GraphPad Prism (v.10.1.1; GraphPad Software, La Jolla, CA, USA). To compare the memory phenotypes and functional profiles of *Mtb*-specific $CD4^+$ and $CD8^+$ T cells between participant groups, we used the Kruskal–Wallis with Dunn's tests for multiple comparisons for more than two participant groups. Mann-Whitney U test was used for two-group comparisons. The data was reported after background (unstimulated) subtraction. Unless otherwise stated, all data were reported for ESAT-6 and CFP-10 peptide stimulations. A p-value <0.05 was considered statistically significant.

Results

Baseline characteristics of the study participants

The baseline demographic and clinical characteristics of the study participants are summarised in Table 1. Age (p<0.0001) and systolic blood pressure (p=0.037) were statistically different between the study participants. Particularly, LTBI-only [24 (24–32)] participants had a lower median age compared to LTBI-DM [50 (47-56)] and DM [48 (39-54)] participants.

Type 2 DM alters the memory phenotype of *Mtb*-specific $CD4^+$ and $CD8^+$ T cells

We performed a memory phenotypic analysis of $CD4^+$ and $CD8^+$ T-cell subsets in participant PBMC samples with LTBI-DM, LTBI-only and DM-only. Flow cytometry was used to identify four categories of T-cell memory phenotypes based on the expression of CD45RA and CCR7 as a percentage of total $CD4^+$ and $CD8^+$ T cells. The T-cell memory phenotypes were defined as naïve ($CD45RA^+CCR7^+$), central memory (CM; $CD45RA^-CCR7^+$), effector memory (EM; $CD45RA^-CCR7^-$), and terminally differentiated effector memory (TEMRA; $CD45RA^+CCR7^-$) (Figures 1A, B). Naïve $CD4^+$ T cells were significantly decreased in the LTBI-DM compared to the LTBI-only participants (p<0.001), with naïve $CD8^+$ T cells being slightly decreased in the same participants (p=0.112) (Figures 1C, E, D, F). Additionally, central memory $CD4^+$ and $CD8^+$ T-cell frequencies were significantly increased in the LTBI-DM compared to the LTBI-only participants [(p=0.002) and (p=0.044)] respectively (Figures 1C, E, D, F). Compared to LTBI-only, participants with LTBI-DM had significantly increased effector memory $CD4^+$ T cells (p=0.012) (Figures 1C, E). No differences were observed for TEMRA $CD4^+$ and $CD8^+$ T cells.

Type 2 DM impairs *Mtb*-specific $CD4^+$ and $CD8^+$ T activation, exhaustion and degranulation

HLA-DR, an activation marker, is expressed on several cellular populations, including $CD4^+$ and $CD8^+$ T cells (Figures 2A, B). *Mtb*-specific HLA-DR expression on $CD8^+$ T cells was significantly decreased in LTBI-DM (Figure 2B) compared to LTBI-only participants (p<0.0001). Interestingly, *Mtb*-specific $CD4^+$ and $CD8^+$ T-cell PD-1 expression was significantly upregulated in the LTBI-DM compared to the LTBI-only participants [(p<0.0001) and (p=0.007)] respectively (Figures 2C, D). PBMCs were stained with CD107a (during incubation) to determine CD107a production. Compared to LTBI-only, participants with LTBI-DM had significantly impaired CD107a production by $CD4^+$ T cells (p<0.0001) (Figure 2E). Though non-significant, LTBI-DM participants had slightly impaired CD107a production by $CD8^+$ T cells compared to the LTBI-only participants (p=0.161) (Figure 2F).

TABLE 1 Baseline characteristics of study participants.

	Overall (n=54)	LTBI-DM (n=21)	LTBI (n=17)	DM (n=16)	p-value
Age, years (median [IQR])	43 (30-52)	50 (47-56)	24 (24-32)	48 (39-54)	<0.0001
Sex, n					0.287
Female (%)	35 (64.8)	11 (52.4)	13 (76.5)	11 (68.8)	
Male (%)	19 (35.2)	10 (47.6)	4 (23.5)	5 (31.2)	
Weight, Kg (median [IQR])*	71.8 (61.3- 87.3)	68.0 (58.2- 82.2)		75.2 (63.0- 91.2)	0.464
RBS, mmol/L (median [IQR])*	7.3 (3.5-13.1)	6.6 (0.0-9.0)		9.4 (5.5-14.2)	0.147
Systolic blood pressure, mm Hg (median [IQR])*	134 (125- 151)	147 (127-171)		129 (120- 138)	0.037
Diastolic blood pressure, mm Hg (median [IQR])*	83 (75-95)	90 (76-103)		81 (72- 85)	0.156
HbA1c, % (median [IQR])*	7.0 (6.0-9.1)	7.3 (6.2-9.1)		6.6 (5.5-9.3)	0.308

*Missing in the LTBI-only group.

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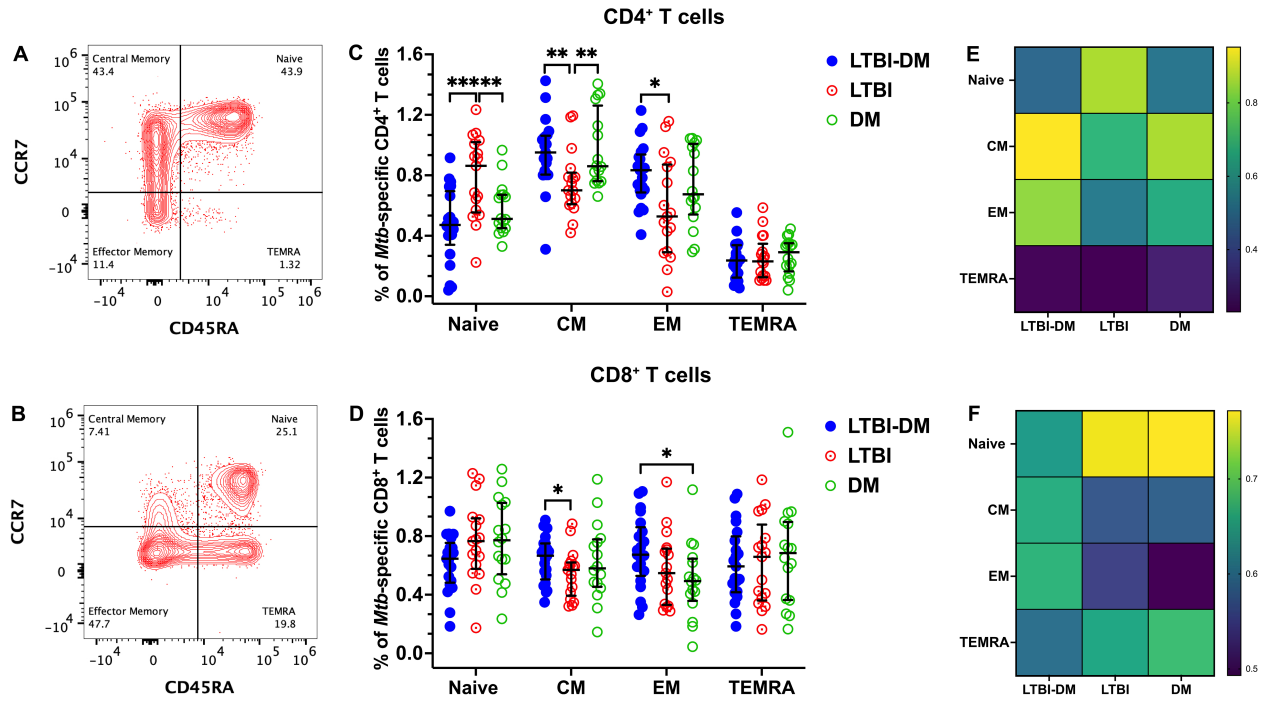

FIGURE 1
Type 2 DM alters the memory phenotype of *Mtb*-specific CD4$^+$ and CD8$^+$ T cells. **(A, B)** Representative flow cytometry plots are shown for CD4$^+$ and CD8$^+$ CCR7/CD45RA-defined T-cell memory subsets, respectively. PBMCs were stimulated and cultured for 18 hours with ESAT-6 and CFP-10 peptide pools plus brefeldin A and stained for surface markers. **(C, D)** Percentage expression of memory phenotypes in CD4$^+$ and CD8$^+$T cells, respectively. **(E, F)** Heat maps for the percentage distribution of all memory phenotypes in the three CD4$^+$ and CD8$^+$ T cell participant groups. Size of participant groups: LTBI-DM (n = 21), LTBI (n = 17), DM (n = 16). Data represent medians and interquartile ranges. The non-parametric Kruskal-Wallis and Mann-Whitney U tests were used to determine the statistical significance between the medians. p<0.05 (*), p< 0.01 (**), p<0.001 (***). Non-significant p-values were not shown.

Type 2 DM impairs the production of *Mtb*-specific Th-1, Th-2 and Th-17 cytokines by CD4$^+$ and CD8$^+$ T cells

To determine CD4$^+$ and CD8$^+$ T-cell functionality in terms of cytokine expression, PBMCs were stained with TNF, IFN-γ, IL-2, IL-13 and IL-17A (intracellularly) (Figure 3). Of the Th-1 cytokines, CD4$^+$ and CD8$^+$ T-cell *Mtb*-specific IFN-γ production was significantly decreased in the LTBI-DM compared to the LTBI-only participants [(p=0.030) and (p=0.016)] respectively (Figures 3A, B). Additionally, CD4$^+$ T-cell *Mtb*-specific TNF production was significantly decreased in participants with LTBI-DM compared to those with LTBI-only (p=0.004) (Figure 3E). Finally, CD8$^+$ T-cell *Mtb*-specific IL-13 and IL-17A production were increased and decreased in the LTBI-DM compared to the LTBI-only participants, respectively [(p=0.033) and (0.017)] (Figures 3H, J).

Type 2 DM impairs dual and mono-functional *Mtb*-specific CD4$^+$ and CD8$^+$ T-cell responses

To further analyse the quality of *Mtb*-specific CD4$^+$ and CD8$^+$ T-cell responses, we defined the polyfunctional potential of *Mtb*-specific CD4$^+$ and CD8$^+$ T-cell responses based on their capacity to co-express IFN-γ, IL-2 or TNF by applying the Boolean gating strategy to all samples using FlowJo and subtracting the non-specific polyfunctional responses (Figure 4). LTBI-DM participants had significantly lower frequencies of dual-functional IFN-γ$^+$IL-2$^+$ (p=0.018) and IL-2$^+$TNF$^+$ (p=0.006) CD4$^+$ T cells compared to LTBI-only participants (Figure 4A). Additionally, mono-functional IFN-γ$^+$ (p<0.0001) and TNF$^+$ (p<0.001) CD4$^+$ T-cell responses were significantly decreased in participants with LTBI-DM compared to those with LTBI-only (Figure 4A). Regarding CD8$^+$ T-cell polyfunctionality, only mono-functional IFN-γ$^+$ responses decreased significantly in participants with LTBI-DM compared to those with LTBI-only (p=0.033) (Figure 4B).

Type 2 DM impairs triple, dual, mono-functional *Mtb*-specific central and effector memory CD4$^+$ T cell responses

Following on from our previous result, Boolean gating strategy was further applied to all samples' CD4$^+$ T-cell central and effector memory responses to determine their polyfunctional capacity to produce *Mtb*-specific IFN-γ, IL-2 or TNF after non-specific polyfunctional cytokine production subtraction (Figure 5). With regards to central memory CD4$^+$ T-cell responses, LTBI-DM participants had decreased dual-functional IFN-γ$^+$IL-2$^+$ (p=0.002) and IL-2$^+$TNF$^+$ (p<0.001) frequencies compared to LTBI-only participants (Figure 5A). Additionally, mono-functional IFN-γ$^+$ (p=0.001), IL-2$^+$ (p=0.011) and TNF$^+$ (p<0.0001) central memory CD4$^+$ T-cell responses were significantly decreased in participants with LTBI-DM compared to those with LTBI-only (Figure 5A).

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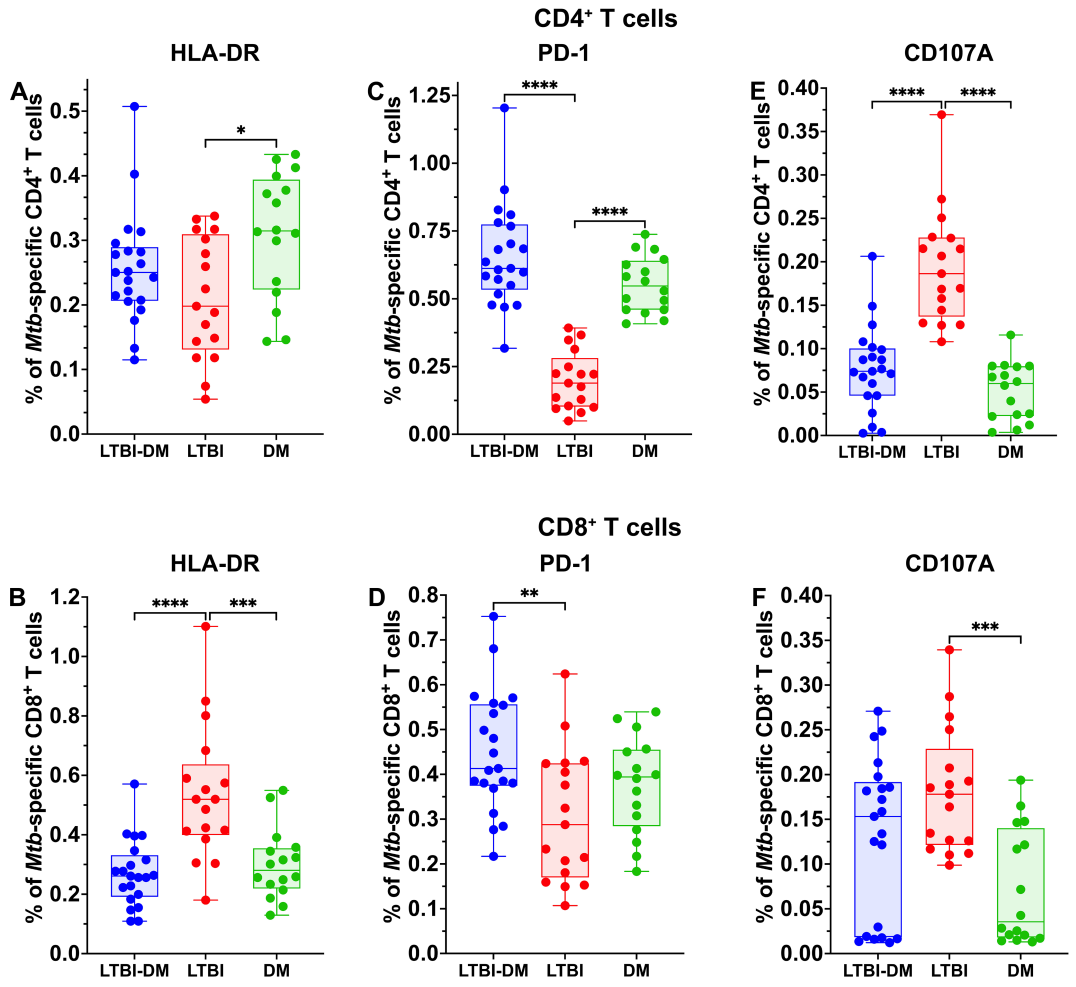

FIGURE 2
Type 2 DM impairs the HLA-DR, PD-1, and CD107A expression of Mtb-specific CD4+ and CD8+ T cells. The PBCMs were surface stained with HLA-DR and PD-1 antibodies after 18 hours of incubation with ESAT-6 and CFP-10 peptide pools and brefeldin A. **(E, F)** For degranulation analysis of $CD4^+$ and $CD8^+$ T cells, CD107a was added during stimulation. **(A-D)** Representative plots for HLA-DR and PD-1. Size of participant groups: LTBI-DM (n = 21), LTBI (n = 17), DM (n = 16). Data represent medians and interquartile ranges. The non-parametric Kruskal-Wallis and Mann-Whitney U tests were used to determine the statistical significance between the medians. $p< 0.05$ (*), $p< 0.01$ (**), $p<0.001$ (***) and $p< 0.0001$ (****). Non-significant p-values were not shown.

Regarding effector memory $CD4^+$ T-cell responses, LTBI-DM participants had decreased triple functional IFN-γ^+IL-$2^+$$TNF^+$ ($p=0.033$), dual-functional IFN-γ^+ TNF^+ ($p=0.004$) and IL-$2^+$$TNF^+$ ($p<0.001$) frequencies compared to LTBI-only participants (Figure 5B). Additionally, mono-functional IFN-γ^+ ($p<0.0001$) and TNF^+ ($p<0.0001$) effector memory $CD4^+$ T-cell responses were significantly decreased in participants with LTBI-DM compared to those with LTBI-only (Figure 5B).

Discussion

Immunological dysregulation is one mechanism that accounts for TB susceptibility and severity in DM, but it is not well elucidated and remains poorly characterised. We performed an extended analysis of the memory phenotypes and functional responses of *Mtb*-specific $CD4^+$ and $CD8^+$ T cells to identify immunological differences between LTBI-DM, LTBI-only and DM-only participants. Our study identified three key points: 1) Type 2 DM alters the memory phenotype of $CD4^+$ and $CD8^+$ T cells; 2) Type 2 DM impairs T-cell activation and degranulation but promotes T-cell exhaustion; 3) Type 2 DM impairs the $CD4^+$ and $CD8^+$ T-cell Th1, Th2 and Th17 cytokine responses, as well as the polyfunctional (triple, dual, mono) capacity of the $CD4^+$ T-cell, and central and effector memory $CD4^+$ T-cell subsets. We showed that type 2 DM is associated with profound impairment of *Mtb*-specific T-cell responses, which could increase TB susceptibility.

This study reveals that naïve $CD4^+$ T cells were decreased, whereas the $CD4^+$ and $CD8^+$ T-cell central and effector memory phenotypes were increased in the LTBI-DM compared to the LTBI-only participants. The reduction in naïve $CD4^+$ T cells is similar to a study by Kumar and colleagues, who reported decreased naïve CD4 T cells in active TB with DM participants (25). The decrease indicates a potential compromise towards delayed or insufficient immune responses against *Mtb* reactivation, allowing *Mtb* to potentially proliferate and increase susceptibility to active TB

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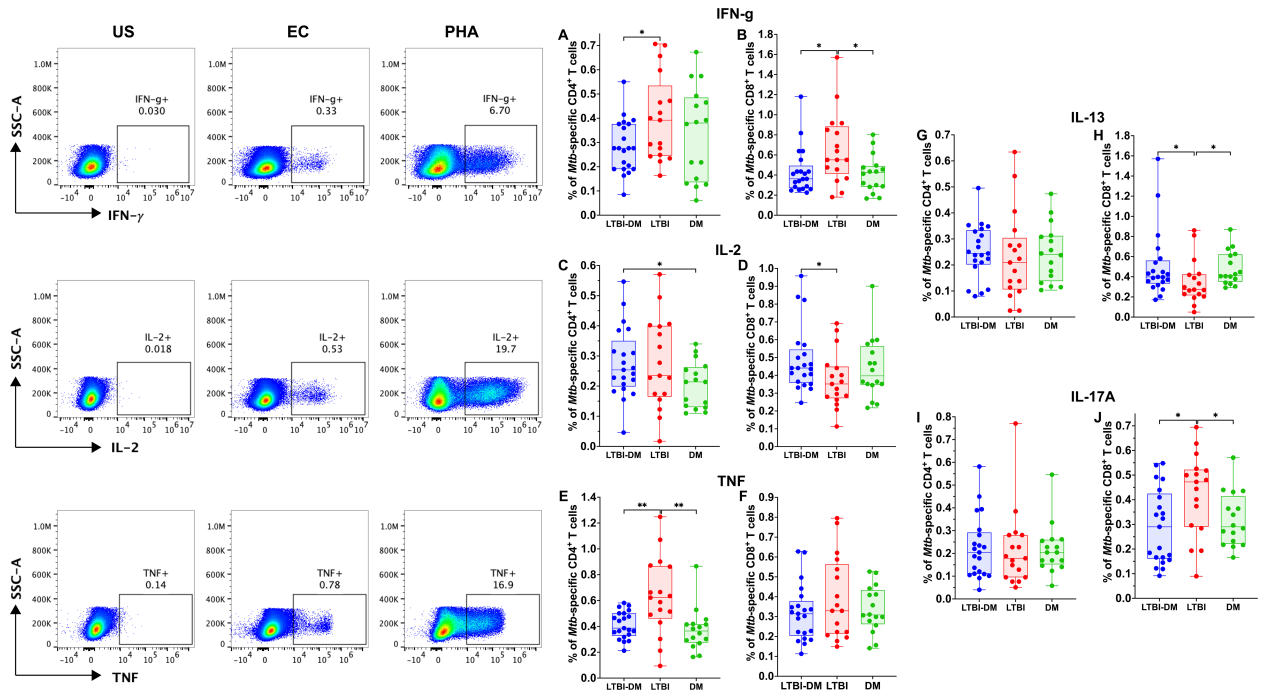

FIGURE 3
Type 2 DM impairs the production of *Mtb*-specific Th-1, Th-2 and Th-17 cytokines by $CD4^+$ and $CD8^+$ T cells. The PBMCs were cultured and stimulated for 18 hours with ESAT-6 and CFP-10 peptide pools, brefeldin A, and intracellularly stained for cytokines. **(A–J)** Representative plots for $CD4^+$ and $CD8^+$ T-cell producing TNF, IFN-γ, IL-2, IL-13, IL-17A cytokines. Size of participant groups: LTBI-DM (n = 21), LTBI (n = 17), DM (n = 16). Data represent medians and interquartile ranges. The non-parametric Kruskal-Wallis and Mann-Whitney U tests were used to determine the statistical significance between the medians. $p < 0.05$ (*), $p < 0.01$ (**). Non-significant p-values were not shown.

disease (26). The significant increase of central and effector memory T-cell frequencies in LTBI-DM participants implies a shift towards an activated memory T-cell phenotype. Memory T cells are crucial for long-term immune surveillance (27, 28) and rapid response upon re-exposure to *Mtb* (29). This increase may reflect an immune response to chronic *Mtb* stimulation or a compensatory mechanism in response to impaired naïve T-cell function. This could have implications for both TB protection and disease progression, as an increased T-cell memory phenotype could potentially contribute to *Mtb*-related chronic inflammation, resulting in T-cell memory cells with impaired immune function, including exhaustion, activation, homing and cytokine production (30). Type 2 DM orchestrated T-cell memory alteration may potentially decrease the overall robustness of the T-cell memory response, potentially increasing susceptibility to active TB disease.

The functional profiles and fitness of the T cells are significant factors to consider when assessing *Mtb*-specific responses in the face of DM. Our study reports significant upregulation of PD-1 on T cells in the LTBI-DM participants, a consensus to several studies that reported upregulation of PD-1 expression on T cells during *Mtb* infection and active TB disease (15, 16). PD-1 impairs T-cell proliferation during active TB disease (16) and Th1 immune function during *Mycobacterium bovis* BCG vaccination (31). Type 2 DM promoting increased PD-1 expression could have severe implications for other T-cell functional responses, including activation, degranulation and cytokine production. Interestingly, we report that type 2 DM impairs T-cell activation and degranulation. $CD8^+$ T-cell HLA-DR expression was decreased in the LTBI-DM participants compared to the LTBI-only group, an association with a lower activation state, and consistent with another human study that reported impaired HLA-DR expression on H37Rv-infected monocyte-derived macrophages of DM patients (32). HLA-DR is an activating receptor that binds and presents antigens to T cells, thereby activating immune responses, including cytokine and cytotoxicity functions to clear *Mtb*-infected cells (33). Its expression has also been characterized with effector T cells (34). The decrease in the $CD8^+$ T-cell activation state in the face of DM could impair their cytotoxic functions (33), leading to increased risk for LTBI acquisition and ATB progression. However, our study reports that fewer $CD8^+$ (but not $CD4^+$) T cells were activated. This needs a cautious interpretation, as TB immune impairment is often related to $CD4^+$ T-cell HLA-DR dysfunction (35). Interestingly, HLA-DR expression has previously been described as a biomarker that distinguishes LTBI from ATB (36). Whether HLA-DR expression could be used as a biomarker for identifying and distinguishing TB phenotypes in coincident DM remains to be assessed. In addition, our study reports that type 2 DM is associated with compromised $CD4^+$ and $CD8^+$ T-cell CD107a, a marker for degranulation and cytotoxicity function (37). Similar results have been reported for which type 2 DM compromises the cytotoxic effects of $CD8^+$ T and NK cells during active TB (38). $CD4^+$ and $CD8^+$ T cells have been reported to kill *Mtb*-infected monocytes directly by perforin and Fas/Fas Ligand independent pathways (39). It is important to note differences in the expression profiles of PD-1

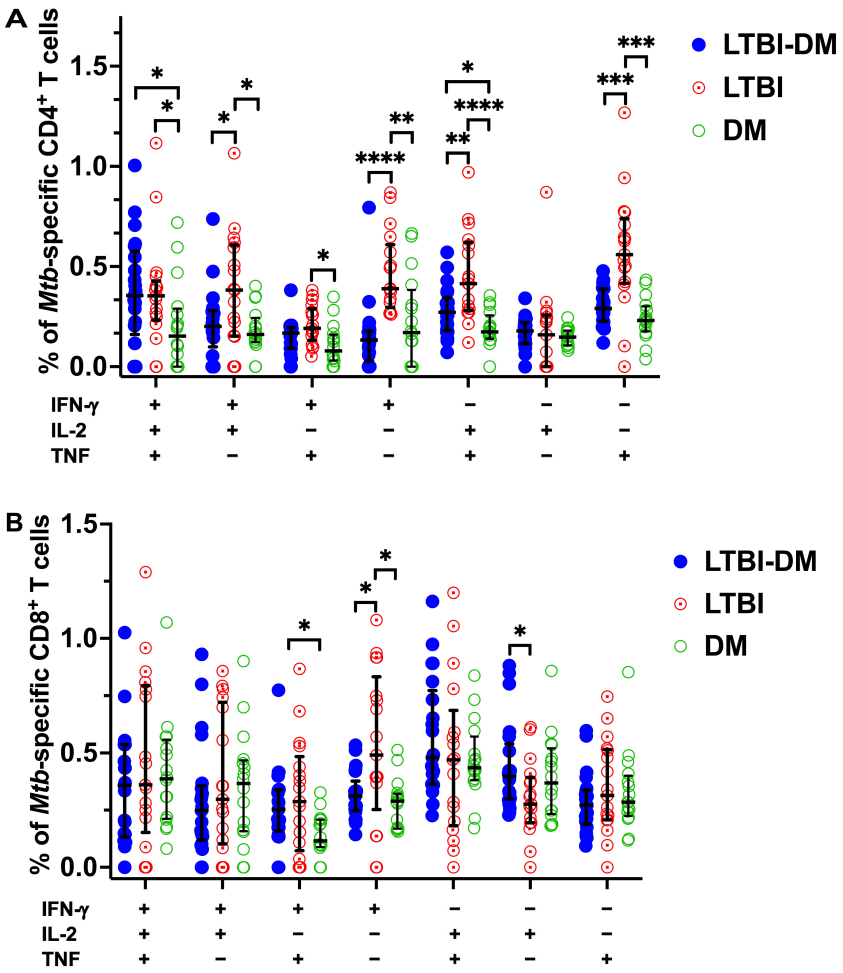

FIGURE 4
Type 2 DM impairs dual and mono-functional *Mtb*-specific $CD4^+$ and $CD8^+$ T-cell responses. **(A)** Polyfunctional *Mtb*-specific $CD4^+$ T-cell responses. **(B)** Polyfunctional *Mtb*-specific $CD8^+$ T-cell responses. The X-axis represents the frequencies of *Mtb*-specific $CD4^+$ T cells producing all possible IFN-γ, IL-2 and TNF combinations. Data represent medians and interquartile ranges. The non-parametric Kruskal-Wallis and Mann-Whitney U tests were used to determine the statistical significance between the medians. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

and HLA-DR in LTBI-DM and DM groups. These differences may reflect distinct mechanisms of immune activation in the DM group that are not directly related to *Mtb*-specific immune responses in the LTBI-DM group. PD-1 and HLA-DR can be influenced by various factors, including metabolic dysregulation caused by DM (40, 41). Taken together, impairment of HLA-DR expression and CD107a production by DM could promote heightened *Mtb* replication and increased TB risk.

Next, we assessed the effect of DM on $CD4^+$ and $CD8^+$ T-cell cytokine production, and we observed marked differences in cytokine expression profiles for IFN-γ, IL-2, TNF, IL-13 and IL-17A. $CD4^+$ T-cell IFN-γ and TNF, as well as $CD8^+$ T-cell IFN-γ and IL-17A production, were decreased, whereas $CD8^+$ T-cell IL-13 production was increased in the LTBI-DM participants compared to LTBI-only participants. $CD4^+$ and $CD8^+$ T-cell IFN-γ production mediates TB protection by controlling the *Mtb* burden and promoting host survival in mice (7) and humans (8). In addition, T-cell-derived TNF plays a crucial role in the early control of TB infection and promotes the formation of mature granulomas and the activation of infected macrophages in mice (42). Similarly, T-cell IL-17A, a Th17 family cytokine, recruits immune cells to *Mtb*-infected sites by upregulating chemokine expression, thereby contributing to granuloma formation and stability (43, 44). On the contrary, increased production of IL-13 is associated with lung damage and the formation of necrotic lesions in mice, which promotes and is consistent with human TB pathology (45, 46). Impairment of the $CD4^+$ and $CD8^+$ T-cell cytokine responses by DM in the face of TB infection could promote *Mtb* replication, thus promoting TB pathology.

Lastly, we assessed the effect of DM on combinations of polyfunctional Th1 cytokine co-expression profiles of $CD4^+$ and $CD8^+$ T cells, as well as $CD4^+$ T-cell memory phenotypes. Several studies that have profiled the role of polyfunctional $CD4^+$ T cells in producing multiple Th1 cytokines (IFN-γ, IL-2, TNF) during TB infection have associated polyfunctional $CD4^+$ T cells with protection against TB (47–51). It is conceivable that polyfunctional T cells are more effective at controlling infection than those producing single cytokines. Whether these can be used

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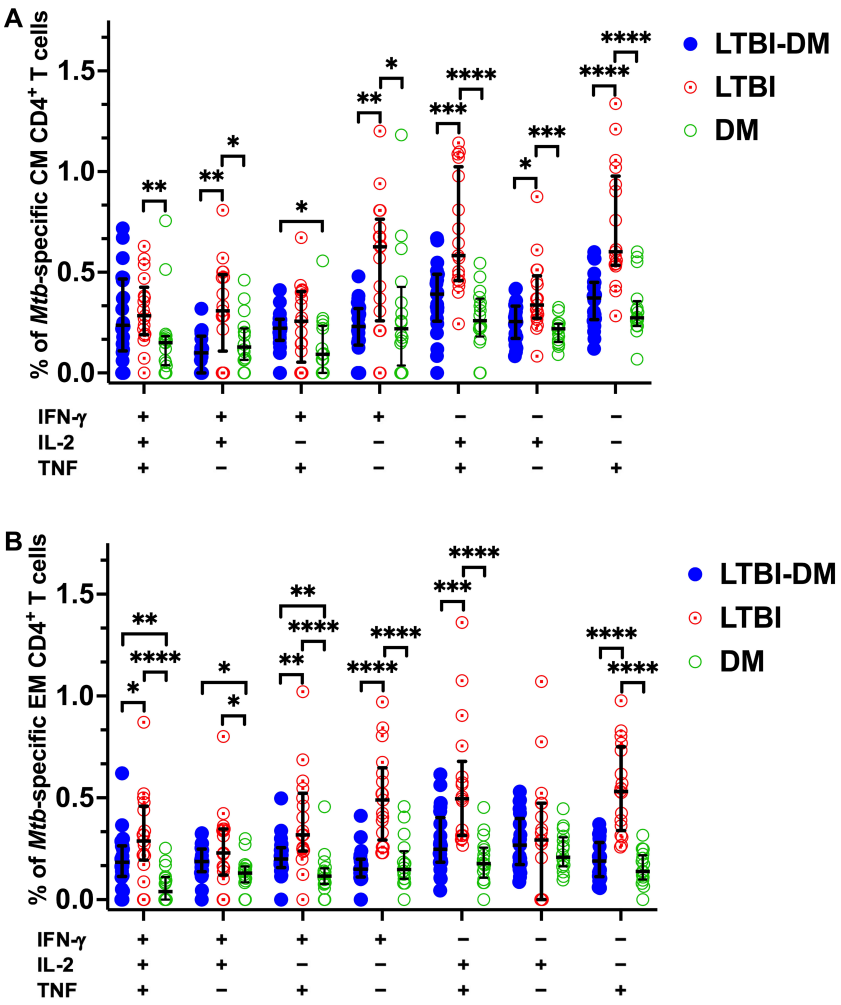

FIGURE 5
Type 2 DM impairs triple, dual, mono-functional *Mtb*-specific central and effector memory $CD4^+$ T-cell responses. **(A)** Polyfunctional *Mtb*-specific central memory $CD4^+$ T-cell responses. **(B)** Polyfunctional *Mtb*-specific effector memory $CD4^+$ T-cell responses. The X-axis represents the frequencies of *Mtb*-specific central and effector memory $CD4^+$ T cells producing all possible combinations of IFN-γ, IL-2 and TNF. Data represent medians and interquartile ranges. Kruskal-Wallis and Mann-Whitney U tests were used to determine the statistical significance between the medians. $p< 0.05$ (*), $p< 0.01$ (**), $p<0.001$ (***) and $p< 0.0001$ (****). CM, Central memory; EM, Effector memory.

as targets for TB vaccination in the face of DM remains to be assessed in more extensive studies. Our study is among the first to evaluate the impact of type 2 DM on $CD4^+$ and $CD8^+$ T-cell polyfunctionality, as well as the $CD4^+$ T-cell central and effector memory polyfunctionality. Interestingly, BCG vaccination in mice and humans has been reported to induce polyfunctional CD4 central and effector memory T cells that confer protective memory immunity against TB in a mice model (11, 12). Our data reveals that DM significantly impairs the dual (IFN-γ^+IL-2^+ and IL-2^+TNF$^+$) and mono (IFN-γ^+ and TNF$^+$)-functional capacity of *Mtb*-specific $CD4^+$ T cells in the LTBI-DM compared to the LTBI-only participants. Additionally, DM significantly impaired the triple (EM: IFN-γ^+IL-2^+TNF$^+$), dual (CM: IFN-γ^+IL-2^+ and IL-2^+TNF$^+$; EM: IFN-γ^+ TNF$^+$ and IL-2^+TNF$^+$), and mono (CM: IFN-γ^+, IL-2^+ and TNF$^+$; EM: IFN-γ^+ and TNF$^+$)-functional capacity of the *Mtb*-specific $CD4^+$ T-cell central and effector memory responses, contributing to first evidence of DM immune impairment on polyfunctional $CD4^+$ T-cell memory responses. The results are consistent with a study by Kumar et al. and colleagues (52) that reported diminished frequencies of dual- and mono-functional $CD4^+$ T cells in LTBI-DM participants. Moreover, Kamboj et al. (53) reported improved *Mtb* clearance after restoring dual functional IFN-γ^+TNF$^+$ $CD4^+$ T cells, further highlighting the importance of polyfunctional T cells as correlates of TB protection. This study demonstrates DM immune-modulatory effects and impairment of both *Mtb*-specific $CD4^+$ T cells and their central and effector memory polyfunctional responses during TB progression. This may promote increased TB disease risk and increase active TB progression.

This study faces limitations, including a limited sample size. It is also important to note that the data generated after *in vitro* culture may not represent what occurs *in vivo*. In addition, HbA1c and other DM-related parameters were not collected for participants in the LTBI-only group as these were from another control group comprised of household contacts of TB index patients (KTB study). As a result, our analysis could not adjust for HbA1c levels across all

groups. Hence, there remains a possibility of residual confounding related to diabetes severity, which could influence some of the observed immune differences between groups. Lastly, this focused exclusively on T-cell responses to peptides derived from ESAT6 and CFP10 peptides, representing only a subset of the numerous antigens expressed by *Mtb*. Consequently, the findings related to T-cell responses in this study may not be fully generalizable to the overall T-cell response to *Mtb*.

In summary, this study advances the understanding of immune impairment in the LTBI-DM comorbidity. Type 2 DM impairs the memory phenotype and polyfunctional profiles of *Mtb*-specific $CD4^+$ and $CD8^+$ T cells, which could influence the LTBI-DM immunopathology towards increased TB disease risk.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by School of Biomedical Sciences Research and Ethics Committee (SBS-REC), Makerere University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. RN: Methodology, Writing – review & editing. DS: Methodology, Writing – review & editing. MN: Writing – review & editing. BB: Writing – review & editing. DK: Writing – review & editing. AK: Writing – review & editing. DK: Writing – review & editing. SO: Conceptualization, Methodology, Supervision, Writing – review & editing. RC: Conceptualization, Methodology, Supervision, Writing – review & editing. SC: Conceptualization, Methodology, Writing – review & editing. IB: Conceptualization, Funding acquisition, Methodology, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1480739/full#supplementary-material

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Macrophage-derived extracellular vesicles from *Ascaris lumbricoides* antigen exposure enhance *Mycobacterium tuberculosis* growth control, reduce IL-1β, and contain miR-342-5p, miR-516b-5p, and miR-570-3p that regulate PI3K/AKT and MAPK signaling pathways

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Background: Helminth coinfection with tuberculosis (TB) can alter the phenotype and function of macrophages, which are the major host cells responsible for controlling *Mycobacterium tuberculosis* (Mtb). However, it is not known whether helminth infection stimulates the release of host-derived extracellular vesicles (EVs) to induce or maintain their regulatory network that suppresses TB immunity. We previously showed that pre-exposure of human monocyte-derived macrophages (hMDMs) with *Ascaris lumbricoides* protein antigens (ASC) results in reduced Mtb infection-driven proinflammation and gained bacterial control. This effect was entirely dependent on the presence of soluble components in the conditioned medium from helminth antigen-pre-exposed macrophages.

Methods: Our objective was to investigate the role of EVs released from helminth antigen-exposed hMDMs on Mtb-induced proinflammation and its effect on Mtb growth in hMDMs. Conditioned medium from 48-h pre-exposure with ASC or *Schistosoma mansoni* antigen (SM) was used to isolate EVs by ultracentrifugation. EVs were characterized by immunoblotting, flow cytometry, nanoparticle tracking assay, transmission electron microscopy, and a total of 377 microRNA (miRNA) from EVs screened by TaqMan array. Luciferase-expressing Mtb H37Rv was used to evaluate the impact of isolated EVs on Mtb growth control in hMDMs.

Results: EV characterization confirmed double-membraned EVs, with a mean size of 140 nm, expressing the classical exosome markers CD63, CD81, CD9, and flotillin-1. Specifically, EVs from the ASC conditioned medium increased the bacterial control in treatment-naïve hMDMs and attenuated Mtb-induced IL-1β at 5 days post-infection. Four miRNAs showed unique upregulation in response to ASC exposure in five donors. Pathway enrichment analysis showed that the MAPK and PI3K-AKT signaling pathways were regulated. Among the mRNA targets, relevant for regulating inflammatory responses and cellular stress pathways, CREB1 and MAPK13 were identified. In contrast, SM exposure showed significant regulation of the TGF-β signaling pathway with SMAD4 as a common target.

Conclusion: Overall, our findings suggest that miRNAs in EVs released from helminth-exposed macrophages regulate important signaling pathways that influence macrophage control of Mtb and reduce inflammation. Understanding these interactions between helminth-induced EVs, miRNAs, and macrophage responses may inform novel therapeutic strategies for TB management.

KEYWORDS

macrophage extracellular vesicles, miRNA, CREB1, MAPK13, SMAD4, tuberculosis, helminth-coinfection, inflammation

1 Introduction

Mycobacterium tuberculosis (Mtb), primarily targeting macrophages in the lungs, remains a global health challenge where a quarter of all humans are believed to have a latent tuberculosis (TB) infection and over 10.6 million yearly develop active TB disease (1). More than two billion people are infected with intestinal helminths, and particularly soil transmitted helminths affect more than 1.5 billion people in Africa, Asia, and Latin America (2). It has been indicated that the pooled prevalence of helminth coinfection among TB cases is approximately 30% (3) with increased prevalence in high-burden settings (4). As helminths can modulate the immune response to Mtb, helminth/TB coinfection may alter TB progression and treatment efficacy, complicate TB diagnosis, and affect vaccine effectiveness (3, 5). Understanding the interaction between helminths and TB would aid in alleviating the public health challenges associated with the management of TB. During latent TB infection, the bacterium is no longer believed to " lay" dormant, rather there is a constant immune battle to keep the bacteria in check where resident macrophages have an essential role in the containment of infection. Macrophages exhibit diverse functions and communication modalities, including the release of extracellular vesicles (EVs) containing biomolecules capable of modulating immune responses. Macrophages and dendritic cells are major producers of EVs (6). For instance, macrophage-released EVs during systemic candidiasis infection decrease the growth of *Candida* through the activation of ERK2 and p38 (7), and EVs from toll-like receptor (TLR) 3-activated macrophages confer anti-hepatitis C virus protection to hepatocytes (8).

In inflammatory diseases such as TB, EVs have emerged as important mediators recognized for regulating macrophage activation and polarization, where they have been shown to modulate the balance of M1/M2 macrophage polarization through alterations in glucose metabolism (9). The protective immune response against TB involves cell-mediated activation of macrophages by interferon-gamma-releasing CD4 T cells, as well as the efficient phagocytosis, autophagy, and other bactericidal traits of M1-polarized macrophages, which release proinflammatory cytokines such as TNF, IL-1β, and IL-6. During helminth/Mtb coinfection, there is an increase in regulatory networks that dampen Th1 responses and induce Th2-dominated immune responses, along with induction of regulatory T cells (10). Concurrently, there is an increased polarization of alternatively activated (M2) macrophages (11, 12), which are associated with tissue repair and anti-inflammatory functions that may have a reduced ability to control Mtb. However, it is not known whether helminth infection stimulates the release of host-derived EVs to induce or maintain their regulatory network that suppresses TB immunity.

By regulating gene expression, microRNAs (miRNAs) play a crucial role in modulating immune responses. miRNAs have been shown to influence innate immune responses, B-cell differentiation and antibody production, and T-cell development and function (13). For example, miR-146a, miR-21, and miR-155 have been

described as principal regulators of inflammatory pathways in myeloid cells (14). Major host defense mechanisms against Mtb found regulated by miRNA include the triggering of apoptosis, induction of autophagy, and stimulation of interferon-gamma and TNF (15–17). Mtb can, however, manipulate these regulatory miRNA expression patterns to evade host immune responses, and so miRNAs have been implicated as biomarkers or regulators of immunity during TB (17).

Exploring EV-mediated miRNA transfer for immune regulation holds promise for novel TB therapeutic strategies. Coinfection with helminths and other neglected tropical diseases can influence the immune response, potentially impacting the efficacy of novel TB treatments utilizing miRNA or their targets (18). Therefore, it is crucial to assess how helminth exposure modulates miRNA expression in EVs. Previous studies have demonstrated helminth species-specific effects on TB immunity in endemic settings with controls and TB patients (19), as well as in human monocyte-derived macrophages (hMDMs) exposed to helminth antigens (11). Our recent research showed that hMDMs exposed to *Ascaris lumbricoides* antigens could mitigate Mtb infection-induced inflammation (e.g., release of IL-1β and IL-6) and enhance intracellular Mtb growth control when conditioned medium from antigen-pre-exposed cells was reintroduced post-Mtb infection (20). Therefore, this study aims to investigate whether EVs in conditioned medium from helminth antigen-exposed hMDMs mediate similar effects on treatment-naïve hMDMs and explore potential miRNAs that regulate the immune response to Mtb during helminth coinfection. Besides the *A. lumbricoides* protein antigen, we also used the *Schistosoma mansoni* soluble egg antigen for the generation of hMDM-conditioned medium and the exploration of the effect of EVs derived by helminth antigen exposure, as we previously observed that helminth species-dependent variations are induced in macrophages and other immune cells *in vitro* (11, 20) and in TB patients (10, 19, 21, 22).

2 Materials and methods

2.1 Ethics statement

Normal human serum (NHS) and buffy coat preparations from whole blood, the source of peripheral blood mononuclear cells (PBMCs) and monocytes, were obtained from healthy volunteers from Linköping University Hospital Blood Bank and Jönköping Hospital Blood Bank. All donor samples were de-identified and anonymized before being provided to the researchers, ensuring complete confidentiality. All the work was carried out in accordance with the Declaration of Helsinki, not requiring a specific ethical approval according to paragraph 4 of the Swedish law.

2.2 Helminth antigens

Whole worm protein extracts of *A. lumbricoides* (ASC) from Allergen AB Thermo Fisher Scientific and *S. mansoni* soluble egg antigen (SM) donated by Professor Mike Doenhoff, Nottingham University, Nottingham, UK, were used. The protein concentration of each antigen was determined by Bradford assay and stored at −80°C until used. Concentration and pre-exposure time of helminth antigen was set based on our previous study (20), and that of the work with macrophage exposure by others (23).

2.3 Generation of EV-free cell culture medium

EV-free cell culture medium was prepared by ultracentrifugation of NHS pooled from five donors at 120,000 × *g* overnight at 4°C consecutively two times. The supernatant from double ultracentrifuged NHS was then added at 10% to DMEM medium with 10 mM HEPES and 1% L-glutamine used to prepare the medium, which was followed by filtration with a 0.22-µm Stericup quick-release vacuum-driven filtration system (Millipore, Darmstadt Germany). This EV-free cell culture medium was used for all incubations of mature macrophages (hMDMs), from generating conditioned medium, preparation of bacteria and infection experiments, and resuspending isolated EVs for functional experiments.

2.4 Generation of hMDMs

hMDMs were generated following the methodology outlined in previous studies (20). In brief, PBMCs isolated from buffy coats were plated and allowed to adhere for 1.5–2 h. Non-adherent cells were removed with warm Krebs-Ringer Phosphate buffer with glucose, and adherent monocytes were allowed to differentiate for 6 days with fresh complete DMEM (containing 10% NHS pooled from five donors, HEPES, L-glutamine, penicillin, and streptomycin, without specific addition of growth factors) that was replenished on the third day of culture. At day 6, mature macrophages (hMDMs) were detached using trypsin and plated in triplicate at 100,000 cells per well in 96-well plates in antibiotic-free EV-free cell culture medium and hMDMs were rested overnight before infected with Mtb. For generating cell culture supernatants, which were the source of conditioned medium used for further isolation and characterization of hMDM-released EVs, day 7 hMDMs were treated with or without 5 µg/mL of helminth antigens in EV-free cell culture medium for 48 h.

2.5 Bacterial preparation, infection, and luciferase measurement

Bacterial preparation and infection of hMDMs were performed according to our previous study (20). In brief, we used Mtb H37Rv (ATCC) carrying a luciferase construct cultured in Middlebrook 7H9 broth supplemented with 0.5% Tween 80 and 10% albumin-dextrose-catalase enrichment (ADC; Becton Dickinson, Franklin Lakes, NJ, USA) and 100 µg/mL hygromycin (Sigma) at 37°C to log phase. For infection experiments, Mtb was prepared in EV-free cell

culture medium and treatment-naïve hMDMs infected with a multiplicity of infection of four bacteria per cell for 1.5 h, before these hMDMs were washed and incubated for 5 days in EV-free cell culture medium with or without ultracentrifuge preparations of hMDM-derived EVs or conditioned medium depleted of EVs. Mtb luciferase was measured for quantifying Mtb replication, and the luciferase signal in both the supernatant and cell lysate were measured using decanal (Sigma-Aldrich, St. Louis, MO, USA) as the substrate. The luciferase signal from uninfected hMDMs was subtracted from the Mtb luciferase signal in infected samples to account for background noise. To determine the total Mtb growth in each well, the relative luminescence values from the lysate and supernatant were combined. The median value of each triplicate was then either expressed as the absolute value of relative luminescence units or normalized to the day 0 medians (day of infection) from the same donor and treatment, generating an Mtb-fold change relative to day 0.

2.6 Isolation of macrophage EVs

EVs were harvested from the cell culture supernatant of hMDMs stimulated with helminth antigen for 48 h. In brief, 48-h cell culture supernatants were centrifuged at 400 × *g* for 10 min at 4°C to clear cells and debris, named conditioned medium throughout. The conditioned medium was then centrifuged again at 2,000 × *g* for 20 min at 4°C to remove apoptotic vesicles, followed by ultracentrifugation with Beckman Coulter Optima L-80XP at 200,000 × *g* for 2 h at 4°C to isolate EVs. The pelleted EVs used in functional experiments were here resuspended in EV-free cell culture medium and added post-infection with Mtb. However, for characterization purposes, the pellets obtained after initial ultracentrifugation were additionally washed with EV-free PBS (DPBS filtered through a 0.22-µm pore size Stericup quick-release sterile vacuum-driven filtration system) at 200,000 × *g* for 2 h at 4°C. Subsequently, these pellets were resuspended in either EV-free PBS for characterization or QIAzol for isolation of total RNA/miRNA. For evaluating the functional role of EVs, a conditioned medium without EVs was simultaneously generated. In brief, supernatant collected from the initial ultracentrifugation was again centrifuged at 7,500 × *g* for 15 min at RT in 100-kDa Amicon Ultra concentrate filters to remove particles > 30 nm and used as EV-free conditioned medium.

2.7 Characterization of macrophage EVs with transmission electron microscopy

EV samples fixed with 1%–2% paraformaldehyde were analyzed using transmission electron microscopy at the Linkoping University Core Facility. EVs were identified and visualized using negative staining. In brief, 5 µl of samples was mounted to a hydrophilic formvar- carbon-coated, 300-mesh, copper electron microscopy grid (TED PELLA, Inc), and grids were washed, blotted, and negatively stained with 2% uranyl acetate. Electron micrographs were obtained using 80-kV transmission electron microscopy (JEOL JEM 1400 Flash, JEOL LTD, Tokyo, Japan).

2.8 EV characterization using the MACSPlex Exosome kit

Flow cytometric characterization of EVs was performed using the MACSPlex Exosome kit from Miltenyi Biotec (order no. 130-122-209), following the manufacturers instruction. In brief, each sample containing 6 µg of EVs was incubated with 15 µL of MACSPlex Exosome Capture Beads before being washed with MACSPlex buffer and subsequently incubated with 15 µL of MACSPlex Exosome Detection Reagent cocktail (consisting of anti-CD9, anti-CD63, and anti-CD81) in the dark at 4°C for 1 h. Then, samples were washed twice with MACSPlex buffer and acquired by a Gallios flow cytometer (Beckman Coulter), and data were analyzed using Kaluza 2.1 and presented after being normalized to the median signal intensity obtained from the buffer.

2.9 EV characterization with Western blot analysis

To confirm that the pellets from ultracentrifuged conditioned medium contained EVs and were free of cellular contamination, samples underwent Western blot analysis. EV samples were boiled for 5 min at 95°C with equal volume of 2× Laemmli sample buffer containing 5% 2-β-mercaptoethanol and freshly added dithiothretol (NuPAGE sample reducing agent). Proteins in EV lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Merck). Nitrocellulose membranes were blocked with 5% dry milk in PBS containing 0.075% Tween 20 for 1 h at RT. Membranes were immunoblotted overnight at 4°C with 1:2,000 diluted rabbit anti-human calnexin (Abcam, ab92573) and mouse anti-human flotillin-1 (BD Sciences, cat. 610820). After washing with PBS containing 0.075% Tween 20, the membrane was incubated with Alexa Fluor 680 goat anti-rabbit and Alexa Fluor 790 goat anti-mouse secondary antibodies (Invitrogen, 1:1,000 diluted) for 1 h. After washing, protein bands were identified with the Odyssey LI-COR CLX imaging system.

2.10 NTA measurement with Nanosight NS300

EV samples used for the nanoparticle tracking analysis (NTA) were diluted in PBS to a final volume of 1 mL. Ideal measurement concentrations were found by pre-testing the ideal particle per frame value (20–100 particles/frame). The following settings were used according to the manufacturer's software manual (NanoSight

NS300 User Manual, MAN0541-01-EN-00, 2017): camera level increased until all particles were distinctly visible not exceeding a particle signal saturation. The ideal detection threshold was determined to include as many particles as possible with the restrictions that 10–100 red crosses were counted while only <10% were not associated with distinct particles. Blue cross count was limited to 5. Autofocus was adjusted so that indistinct particles were avoided. For each measurement, five 1-min videos were captured under the following conditions: cell temperature: 21°C; syringe speed: 30 µL/s. After capture, the videos were analyzed by the in-build NanoSight Software NTA 3.4 with a detection threshold of 4. Hardware: embedded laser: 45 mW at 488 nm; camera: sCMOS. The count of finalized tracks consistently exceeded the suggested lower limit of 1,000 to minimize data skewing based on single large particles (24).

2.11 miRNA isolation

For isolation of miRNA, EV pellets from ultracentrifugation were resuspended and homogenized in QIAzol lysis reagent and stored at −80°C until RNA isolation. Total RNA was isolated using the miRNeasy Micro kit (cat. no. 217084, Qiagen) according to the manufacturer's protocol to include all miRNA. In brief, QIAzol-lysed samples were thawed slowly on ice and incubated at RT for 5 min. At this, time control oligos (cel-miR-39-3p and ath-miR1591) were added and mixed, followed by vigorous shaking with chloroform, and incubated for 3 min at RT. After 15 min of centrifugation, the upper aqueous phase was resuspended in 1.5 times the volume of 100% ethanol and transferred to RNeasy MinElute spin columns. Using RWT, RPE buffer, and 80% ethanol, spin columns were washed briefly under centrifugation. miRNA in the spin columns was eluted with 14 µL of RNase-free water by centrifugation at full speed for 1 min. The RNA concentration was determined with an Agilent 2100 Bioanalyzer using the RNA Pico chip.

2.12 Quantitative real-time PCR

miRNA expression profiling was conducted using the TaqMan Advanced miRNA Human A Card (A34714; Thermo Fisher Scientific) following the manufacturer's instructions. The assay was performed using the QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems). Data analysis was carried out using QuantStudio 3D Analysis Suite software version 3.1.6 (Life Technologies Corporation), a web-based tool that employs the comparative Cq (ΔΔCq) method for quantifying relative gene expression across samples. Relative quantification (RQ) or fold change (FC) was determined from the Cq values using the equation $RQ = 2^{-\Delta\Delta Cq}$. Endogenous controls provided in the TaqMan Advanced miRNA assay card were utilized for data normalization. hsa-miR-16-5p, which is a stable miRNA in cell line, was used as endogenous control (25).

2.13 Bioinformatics analysis

miRNA (RQ > 2) targets were extracted from mirWalk (miRWalk.umm.uni-heidelberg.de), based on the following conditions. Targets were selected if present in two of the three data bases, e.g., if targets were commonly present in TargetScan and miRDB, or if commonly present in mirTarBase (validated miRNA targets) and miRDB. These targets were pooled and used for functional enrichment analysis and GO (gene ontology) analysis by utilizing the web-based gene set analysis toolkit (WebGestalt, www.webgestalt.org) (26).

2.14 Statistical analysis

All statistical analyses were performed with Graph Pad Prism 8.4.3 (686). The data were presented as mean ± SEM and analyzed using ANOVA and Student's *t*-test. Graphs prepared using ggplot2 in R studio 2023.12.1 are indicated in figure legends.

3 Results

3.1 Characterization of macrophage-released EVs

Previously, we observed that cell-free culture supernatants from 48-h helminth antigen-exposed hMDMs can modulate intra-macrophage Mtb growth control and infection-driven proinflammation (20). In this study, we explored the role of EVs in mediating this effect. To generate the conditioned medium of helminth-exposed macrophages, similar to our previous work, hMDMs were exposed to *A. lumbricoides* protein antigen (ASC), *S. mansoni* soluble egg antigen (SM), or left unexposed (control) for 48 h. EVs isolated from the conditioned medium of macrophage culture supernatant using ultracentrifugation were verified through a combination of methodologies to ascertain the true identification of EVs, based on the MISEV2018 Guideline (27). Transmission electron microscopy was used for generating images of EVs at high resolution. The characteristic round double-membrane structures of EVs with different sizes were observed in all samples of macrophage-derived materials, and EV-free medium used for culturing of hMDMs showed no presence of vesicles (Figure 1). To confirm the biophysical features of EVs, further characterization of the EVs was performed using nanoparticle tracking assay (NTA) that utilizes light scattering properties for the determination of vesicle size. This demonstrated that EVs from all treatments exhibited an average size ranging from 50 to 150 nm (Figure 2), in line with them being EVs (27). The MACSPlex exosome kit in combination with flow cytometry analysis was additionally performed to evaluate the surface marker expression of EVs. This demonstrated the presence of canonical surface markers for EVs, including CD63, CD81, and CD9 on all isolated EVs (28) (Figure 3A). Notably, HLADR, a recognized indicator of antigen

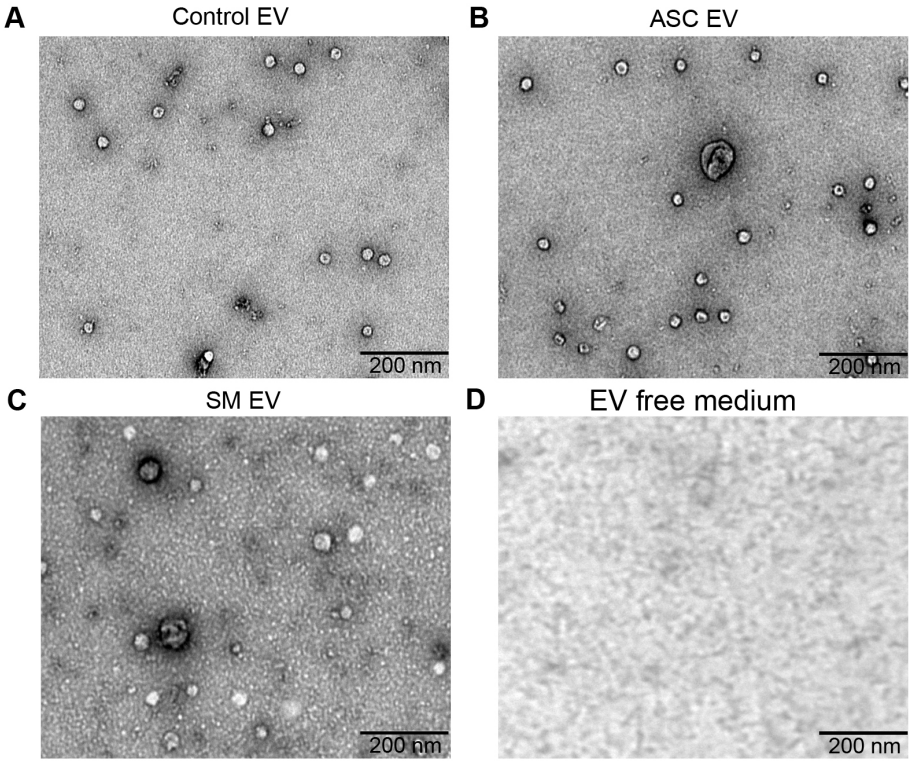

FIGURE 1
Confirmation of extracellular vesicles (EVs) released from human macrophages. Transmission electron microscopy (TEM) images of EVs released from healthy human monocyte-derived macrophages (hMDMs) that are unexposed **(A)**, 48 h 5 µg/mL *Ascaris lumbricoides* antigen exposed (ASC) **(B)**, 48 h 5 µg/mL *Schistosoma mansoni* antigen exposed (SM) **(C)**, or EV-depleted medium used for culturing of hMDMs **(D)**. Magnification 20,000×; the size of the scale bar is indicated in micrographs. Representative of n = 5 donors.

presentation and immune modulation, was highly expressed on the surface of macrophage-derived EVs. There was no difference in the marker expression on hMDMs EVs derived from the different treatments. To assess the purity of the isolated EVs, we further analyzed the presence of the typical EV marker flotillin-1 and the impurity marker calnexin by Western blotting (Figures 3B, C). Lysates from all isolated EVs expressed the specific EV marker flotillin-1, without having calnexin, indicating the high purity of isolated EVs.

3.2 Extracellular vesicles from helminth exposure enhance Mtb growth control in hMDMs

In our previous study, we found that adding back the conditioned medium from 48-h helminth antigen pre-exposed macrophages to the same cells after Mtb infection resulted in a 50% reduction of the total bacterial load compared to untreated on day 5 post-infection (20). Utilizing a similar experimental setup, we explored the effect of conditioned medium and capacity of isolated helminth-induced macrophage EVs to modulate the growth of Mtb within macrophages that had not previously been exposed to helminth antigens, i.e., treatment naïve. The addition of the conditioned medium resulted in an increased Mtb growth control at day 5 post-infection, which was only significant with the ASC conditioned medium (Figure 4A). More importantly, bacterial growth control was also significantly increased when isolated EVs from the ASC conditioned medium (ASC EVs) were added to unexposed macrophages. To substantiate our finding, we simultaneously used a conditioned medium that was depleted of EVs, which showed a total loss in Mtb growth control (Figure 4B). This shows that EVs from helminth-exposed macrophages indeed can affect the intra-macrophage growth of virulent Mtb. Conditioned medium or isolated EVs from preparations of SM-exposed hMDMs did not affect bacterial growth to the same extent, indicating a helminth species-dependent capacity in modulating Mtb growth control in macrophages. Similar to our previous finding of reduced Mtb infection-driven proinflammation provided by conditioned medium from the ASC pre-exposed hMDMs (20), we observed a significant reduction in IL-1β at day 5 post-infection when macrophage EVs from ACS exposure were added to treatment-naïve hMDMs after Mtb infection (Figure 4C). Conditioned medium from which EVs were isolated contained no

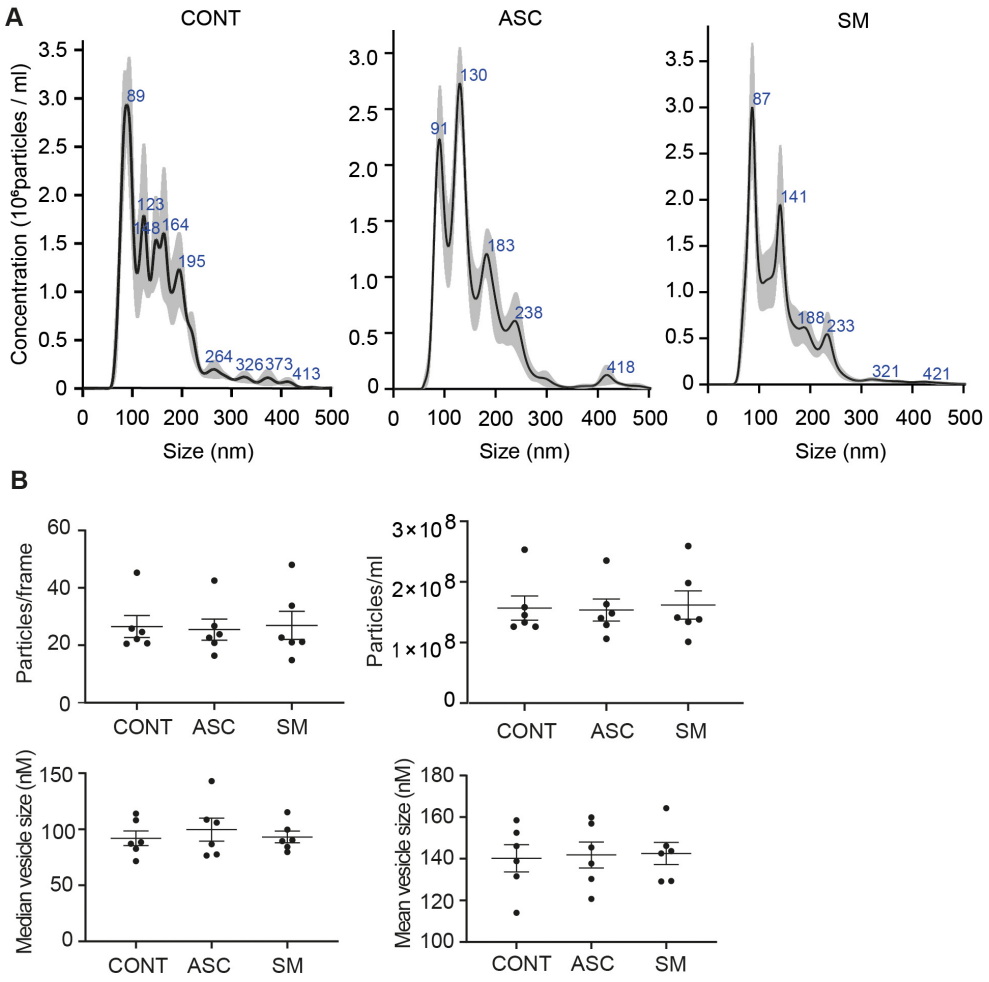

FIGURE 2
Characterization of hMDM-derived EVs using nano particle tracking assay (NTA). Five 1-min videos captured per sample, merged, and average reported. Representative NTA analysis **(A)**. Scatter plot graphs of concentration of EVs per frame, concentration of EVs per milliliter, median size, and mean size of released EVs **(B)**; no statistical difference was observed between the treatment using one-way ANOVA. Data expressed as means ± SEM from *n* = 6 independent donors.

IL-1β (Figure 4D). This indicates that EVs from helminth-exposed macrophages can regulate inflammation in TB.

3.3 Identification of differentially expressed miRNA in EVs of helminth-exposed macrophage

The TaqMan advanced miRNA human A card that detects the 377 most common mature human miRNAs was used to analyze the miRNA in isolated EVs of helminth antigen-exposed macrophages obtained from five donors. Out of these miRNAs, 214 and 213 were detectable in EVs from ASC and SM exposure, respectively. A total of 75 miRNAs were upregulated with ASC compared to control, and 70 were upregulated with SM compared to the control. miRNAs were deemed upregulated if their RQ-fold change was ≥ 2. Several miRNAs were overlapping between the two helminth antigen exposures, and 39 miRNAs showed unique upregulation in response to ASC exposure, while 34 uniquely expressed miRNAs were identified following SM exposure in the pooled data for the donors. Subsequently, we explored the presence of miRNAs commonly upregulated in all donors after ASC or SM exposure. Our analysis revealed that miR-342-5p, miR-516b-5p, miR-570-3p, and miR-188-3p were commonly upregulated in EVs from ASC-exposed hMDMs across all donors. For SM exposure, miR-296-5p and miR-452-5p were commonly upregulated across all donors (Table 1). The heat map for the differential expression of all 377 miRNAs following helminth antigen exposure displayed a vast donor variation (Supplementary Figure S1).

3.4 miRNAs from EVs show distinct modulation of inflammatory pathways

To interpret the functional importance of the predicted miRNA target genes, an over-representation analysis of KEGG pathway and

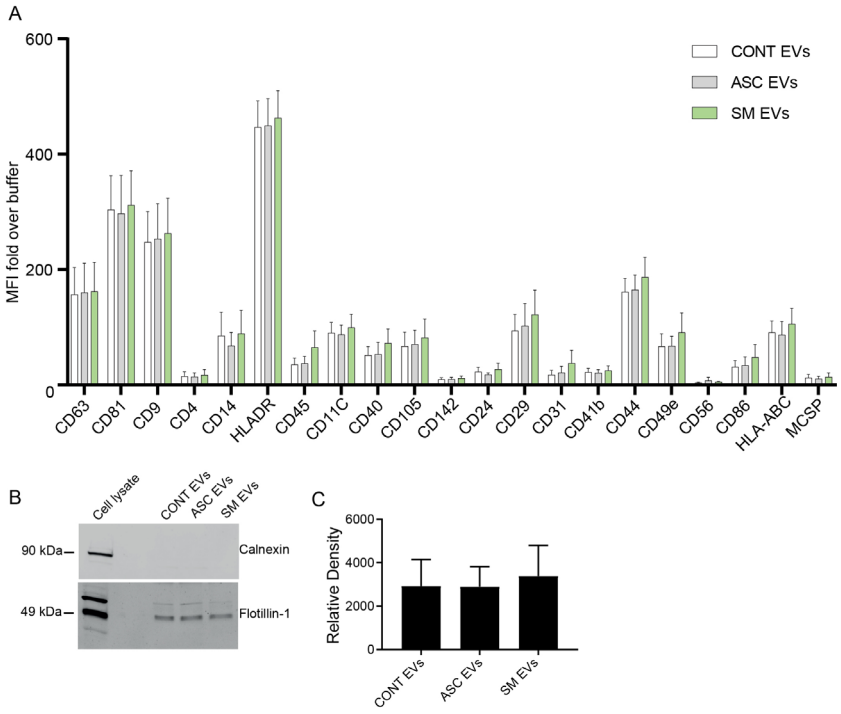

FIGURE 3
Characterization of EVs using the MACSPlex Exosome kit and Western blotting for flotillin-1. **(A)** Median fluorescence intensity (MFI) fold over buffer from different donors (n = 4) reported for markers twofold and above with the MACSPlex Exosome kit. No statistical difference was observed between the treatments using one-way ANOVA. Data expressed as means ± SEM. Isolated EVs carry flotillin-1 but not the impurity marker calnexin **(B, C)**. Isolated EVs were lysed in sample buffer and subjected to Western blot using anti-flotillin-1 (EV marker) and anti-calnexin (endoplasmic reticulum protein) **(B)**. Full scan of the entire original gel(s) (Supplementary Figure S5). Relative density of flotillin-1 **(C)**. Data expressed as means ± SEM from n = 8 independent donors.

GO for the biological processes was performed using a web-based gene set analysis toolkit. The analysis of the pooled miRNA targets from five donors of ASC exposure revealed modulation of autophagy, MAPK signaling pathway (Supplementary Figure S2) (29), ubiquitin-mediated proteolysis, endocytosis, and PI3K/AKT signaling pathway (Supplementary Figure S3) among the top 10 weighted pathways (Figure 5A). These pathways were also found modulated when analyzing each donor separately. Additionally, other FDR significant pathways of relevance for TB were found to be involved, such as the HIF-1 signaling pathway, TNF signaling pathway, and mTOR signaling pathway. Similarly, in response to SM exposure, modulation of the TGF-beta signaling pathway (Supplementary Figure S4), MAPK signaling pathway, endocytosis, ubiquitin-mediated proteolysis, and PI3K/AKT signaling pathway were among the top 10 weighted pathways (Figure 6A), along with several other FDR significant pathways including the RAS signaling pathway, mTOR signaling pathway, and Wnt signaling pathway.

Furthermore, GO analysis for biological functions of the pooled miRNA targets from five donors in ASC exposure demonstrated that the affected biological processes were closely matching with that found by the KEGG pathway analysis (Figures 5A, B). Conversely, the GO analysis for biological functions of the pooled miRNA targets of five donors in SM exposure demonstrated more distinct biological functions among the top 20 significant biological processes that did not intuitively cover the MAPK signaling pathway or the PI3K/AKT signaling pathway found targeted by the KEGG pathway enrichment analysis of the same data (Figures 6A, B). The biological processes found affected by ASC and SM exposure showed a strong variation across helminth species to induce a miRNA-dependent response. Collectively, these results demonstrate that miRNAs in EVs from helminth antigen-exposed hMDMs modulate inflammatory pathways.

Additional analysis using only the targets from the commonly overexpressed miRNA across all donors showed similar biological processes and pathways modulated with ASC exposure (Figure 7) as for SM exposure (Figure 8), with regard to MAPK signaling pathway and PI3K/AKT signaling pathway for ASC exposure and TGF-beta signaling pathway for SM exposure, respectively.

3.5 Key mRNA found targeted by commonly expressed miRNA in all donors

To focus on mRNAs relevant to TB, we further screened for important mRNA targets across donors and identified CREB1, MAPK10, MAPK13, and SMAD4 as significant targets. CREB1, a crucial component of the PI3K/AKT pathway and a key transcription factor within the CREB family, was found targeted

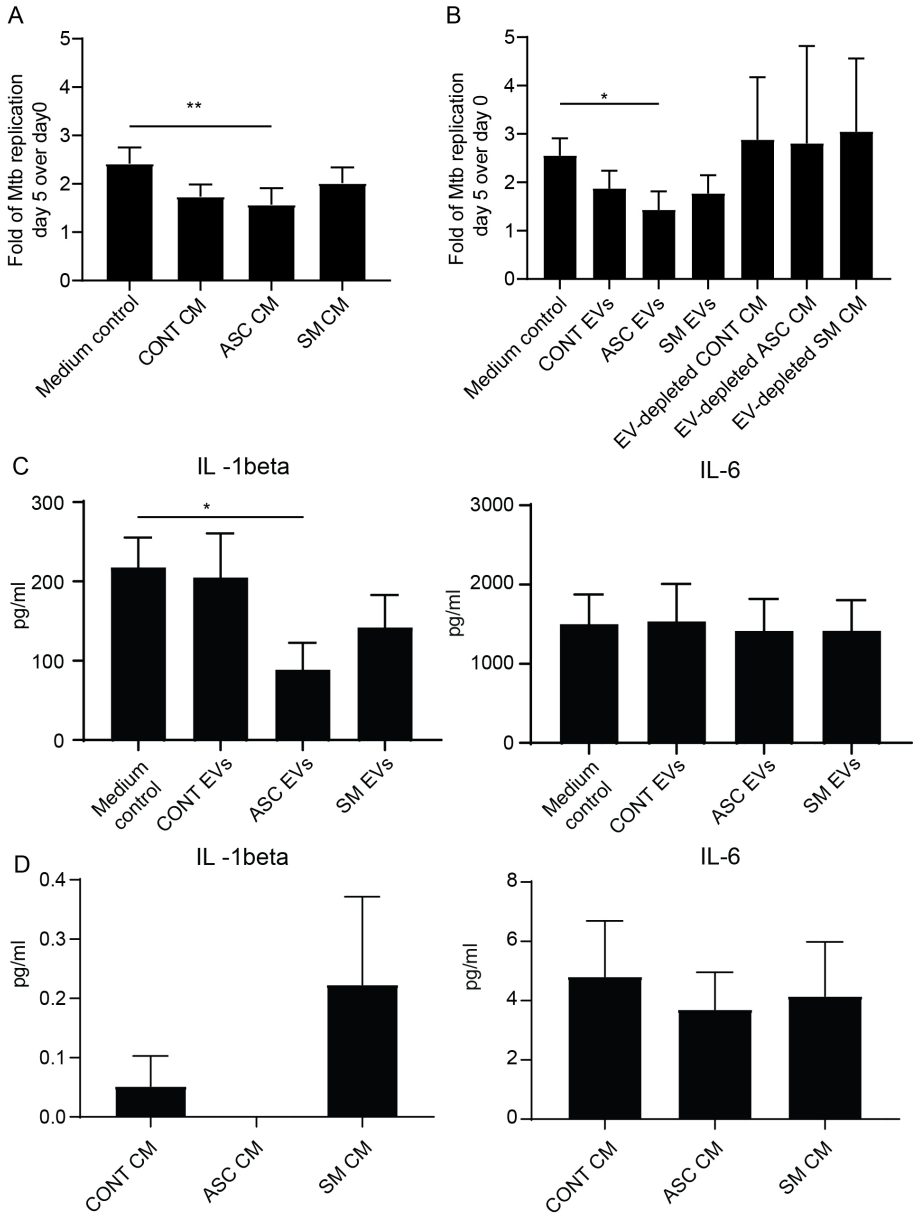

FIGURE 4
EVs from ASC-exposed hMDMs inhibit Mtb growth and lower infection-driven IL-1β. Treatment-naïve hMDMs were infected with luciferase-expressing H37Rv at a multiplicity of infection of four bacteria per cell for 1.5 h. Extracellular bacteria were removed and 50% of 48-h conditioned medium (CM) from different treatments was added, $n = 6$ **(A)**, or isolated extracellular vesicles from these CM, $n = 6$, were added **(B)**. Total luciferase values calculated by combining luminescence signal from supernatant and lysate are shown. Day 5 post-Mtb infection levels of IL-1β and IL-6 in cell-free culture supernatants of untreated hMDMs (Medium control) and extracellular vesicle (EV)-treated **(C)**, and pre-infection levels of IL-1β and IL-6 in the conditioned medium (CM) used to isolate EVs for B **(D)**. Data expressed as mean ± SEM from six independent donors with $*p < 0.05$ and $**p < 0.01$ using one-way ANOVA with Dunn's multiple correction. CONT EVs, isolated EVs from 48-h conditioned medium of unexposed hMDMs; ASC EVs, isolated EVs from 48-h conditioned medium of ASC-treated hMDMs; SM EVs, isolated EVs from 48-h conditioned medium of SM-treated hMDMs.

during ASC exposure. Ten miRNAs were found targeting CREB family, and 3 (miR-342-5p, miR-516b-5p, and miR-570-3p) out of 10 miRNA were found across all donors (Figure 9A). MAPK10 and MAPK13, also known as JNK3 and p38δ, respectively, belonging to the mitogen-activated protein kinase (MAPK) family and having a dominant role in cytokine production, phagocytosis, and antimicrobial response (23, 30), were also found targeted during ASC exposure. The same three miRNAs that targeted CREB were additionally found to target MAPK10 and MAPK13 (miR-342-5p, miR-516b-5p, and miR-570-3p) (Figure 9B). Of note, among the

TABLE 1 Commonly upregulated miRNA in EVs of helminth antigen-exposed hMDMs across all donors.

Commonly upregulated miRNA with ASC exposure			
miRNA	CONT RQ	ASC RQ	SM RQ
hsa-miR-342-5p 478044_mir	1	139.3821	1.427668
hsa-miR-516b-5p 478979_mir	1	12.75203	1.844326
hsa-miR-570-3p 479053_mir	1	70.40454	0.329303
hsa-miR-188-3p 477942_mir	1	9.70081	4.69554*
Commonly upregulated miRNA with SM exposure			
miRNA	CONT RQ	ASC RQ	SM RQ
hsa-miR-296-5p 477836_mir	1	13.39489*	547.9478
hsa-miR-452-5p 478109_mir	1	2.515781*	5.023398

ASC, miRNA in EVs from *Ascaris lumbricoides* antigen-exposed hMDMs; SM, miRNA in EVs from *Schistosoma mansoni* antigen-exposed hMDMs; RQ, RQ-fold. *RQ-fold below 2 in one or more donors.

differentially expressed miRNAs from all conditions, these three miRNAs were the only ones found to target MAPK10 and MAPK13. During SM exposure, there was induction of three miRNAs that target SMAD4, out of which one miRNA (miR-452-5p) was overexpressed in all donors (Figure 9C). SMAD4 was found to be involved in regulating the TGF-beta signaling pathway.

4 Discussion

Helminth coinfection with TB has been recognized to influence the immune response against Mtb. However, the precise impact of helminths or their antigens on host-derived EVs and miRNAs remains unknown. These EVs and miRNAs could potentially modulate the immune system and macrophage control of Mtb growth. In our previous work, we discovered that pretreatment with *A. lumbricoides* antigens significantly altered Mtb growth control and macrophage inflammatory capacity. Specifically, conditioned medium derived from helminth-exposed macrophages demonstrated improved control over Mtb growth, accompanied by a reduction of infection-driven proinflammatory cytokines. Our major finding in the present investigation is that *A. lumbricoides* antigen exposure stimulates macrophages to release EVs and that these EVs

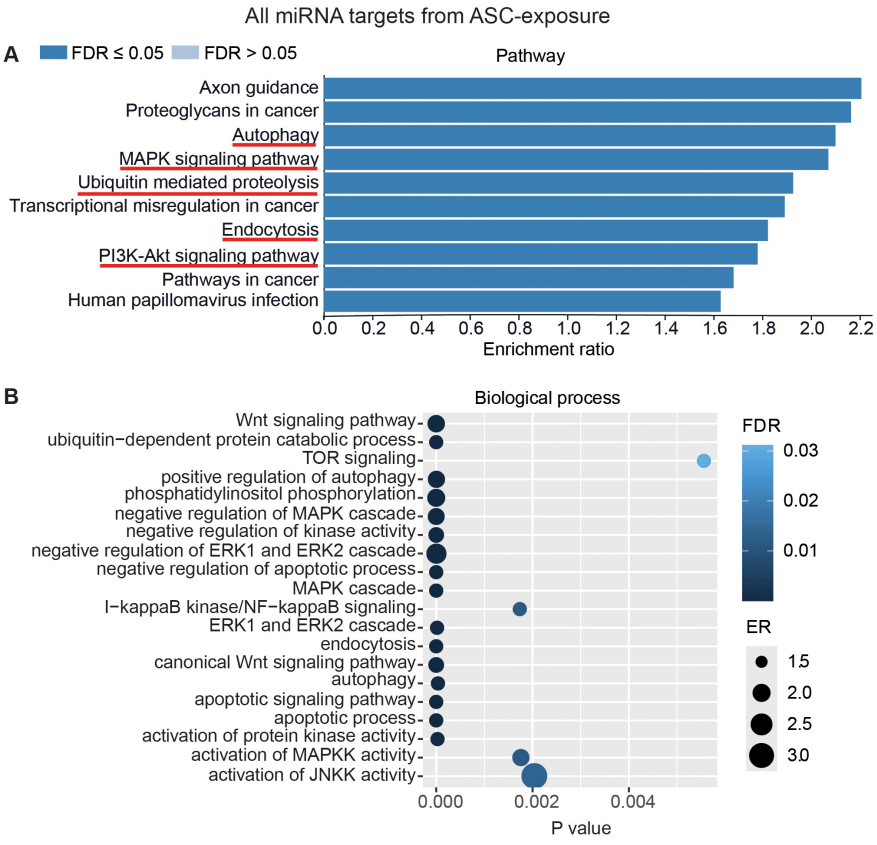

FIGURE 5
miRNAs target gene KEGG pathway enrichment analysis and GO analysis for ASC exposure. miRNAs with RQ-fold ≥ 2 from all donors were subjected to target prediction (miRWalk), and common targets across all donors were identified and used to detect pathways and biological processes that were affected. Top 10 enriched pathways using the KEGG pathway from Webgstalt **(A)**, and GO analysis of common target genes for 20 biological processes **(B)**. ER, enrichment ratio; FDR, false discovery rate. Underlined pathways in **(A)** were found modulated in all five donors when analyzing each donor separately. Graph in **(B)** was generated using ggplot2 in R.

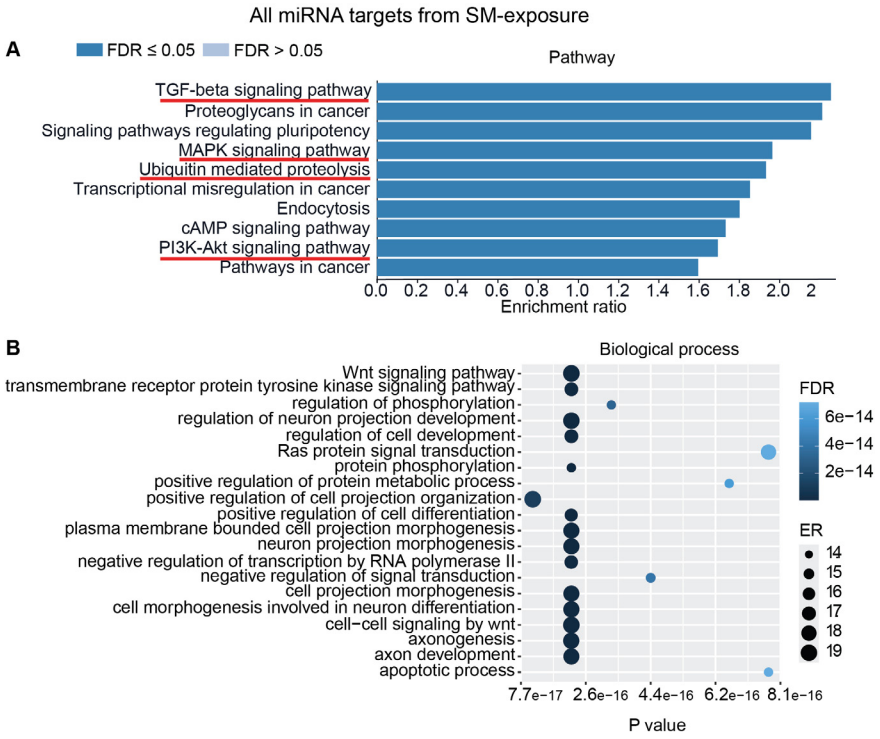

FIGURE 6
miRNAs target gene KEGG pathway enrichment analysis and GO analysis for SM exposure. miRNAs with RQ-fold ≥ 2 from all donors were subjected to target prediction (miRWalk), and common targets across all donors were identified and used to detect pathways and biological processes that were affected. Top 10 enriched pathways using the KEGG pathway from Webgestalt **(A)**, and GO analysis of common targets for 20 biological processes **(B)**. ER, enrichment ratio; FDR, false discovery rate. Underlined pathways in **(A)** were found modulated in all five donors when analyzing each donor separately. Graph in **(B)** was generated using ggplot2 in R.

were essential for enhancing macrophages' intracellular Mtb growth control. Although EVs, or rather CD9-, CD63-, CD81-, and flotillin-1-expressing EVs, were released from macrophages to the cell culture medium regardless of stimulation, only EVs from Ascaris exposure significantly improved Mtb growth control and reduced infection-induced IL-1β release in treatment-naïve hMDMs. Across all donors tested, EVs from Ascaris-exposed macrophages overexpressed miR-342-5p, miR-516b-5p, miR-570-3p, and miR-188-3p. In contrast, EVs from *S. mansoni* antigen exposure resulted in the overexpression of miR-296-5p and miR-452-5p. These miRNAs play a crucial role in modulating intracellular signaling cascades involved in Mtb growth control and inflammation, notably targeting MAPK, PI3K/AKT, and TGF-beta signaling pathways. Overexpressed miRNAs were found to target crucial mRNA molecules, including CREB1, MAPK10, MAPK13, and SMAD4. Understanding these interactions between helminth-induced EVs, miRNAs, and macrophage responses may inform novel therapeutic strategies for TB management.

miRNA has been recognized for its modulatory role in host immune responses, and in the response against Mtb particularly, processes such as autophagy and apoptosis are targeted. For instance, miR-155 has been shown to inhibit apoptosis in cells of TB patients (15) and to contribute to autophagy-mediated clearance of mycobacteria by targeting Rheb (31). Although the six commonly expressed miRNAs found herein, to our knowledge, have not been studied in TB, they have been linked to the same pathways we found through target prediction analysis. For example, miR-342-5p was found to suppress PI3K/AKT in classically active macrophages and act anti-inflammatory in an atherosclerosis plaque mouse model (32), and in an acute kidney injury model, exosomes loaded with miR-342-5p alleviated inflammation by targeting TLR9 to promote autophagy (33). Additionally, miRNA-296-5p, which was overexpressed in EVs after *Schistosoma* antigen exposure, has been suggested to induce inflammation by activating NF-kB (34). Similarly, the other miRNAs found expressed by helminth antigen exposure have been seen to modulate the predicted pathways, e.g., autophagy (35), MAPK (36), and TGF-beta pathway (37).

Our target analysis based on the miRWalk data base revealed important targets affected by our miRNAs. CREB1, or cAMP Response Element-Binding protein 1, is a significant target within the PI3K/AKT pathway (Supplementary Figure S3) that regulates the expression of immediate early genes and blocks the nuclear localization of NF-kB p65, a crucial transcription factor involved in immune responses against Mtb and inflammation. CREB1 is rapidly activated in hMDMs upon Mtb infection, generating a

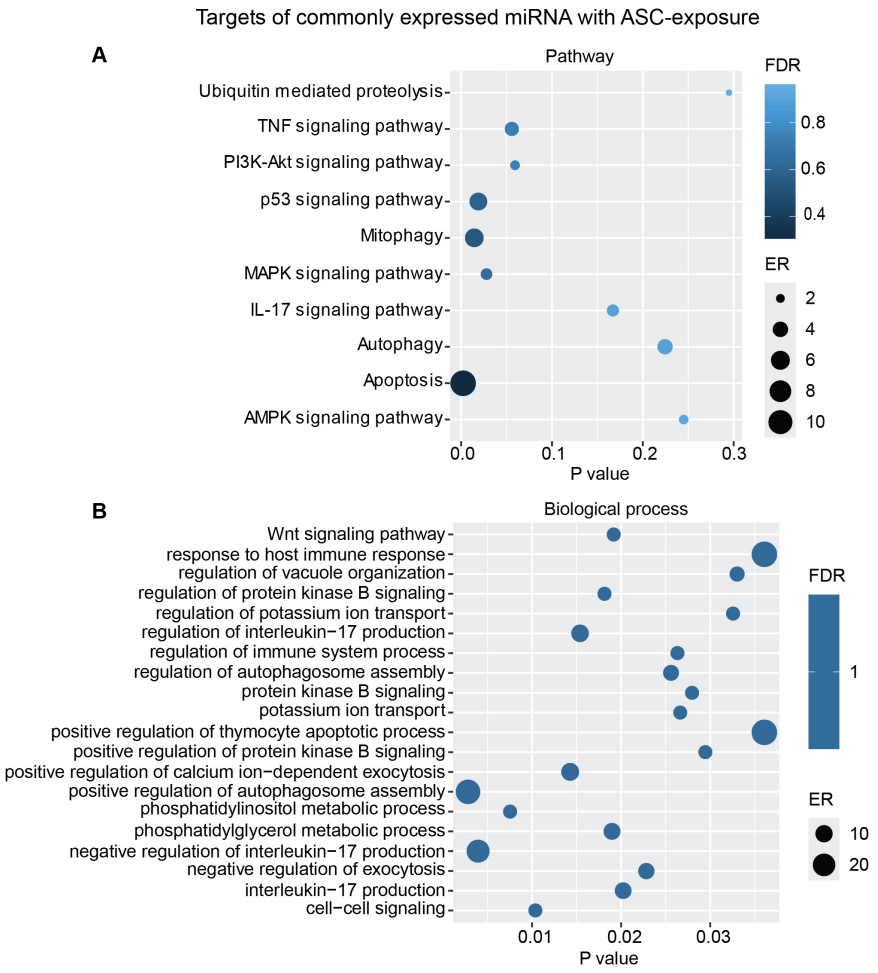

FIGURE 7
miRNAs target gene KEGG pathway enrichment analysis and GO analysis for the commonly expressed miRNA with ASC exposure. Commonly expressed miRNAs from all donors were subjected to target prediction (miRWalk), and common targets across all donors were identified and used to detect pathways that were affected using the KEGG pathway from Webgestalt **(A)**, and GO analysis of common target genes **(B)**. Graphs were generated using ggplot2 in R.

favorable environment conducive to Mtb growth through the blockade of phagolysosomal fusion and inhibiting the necroptotic pathway. Furthermore, inhibiting CREB1 resulted in intact nuclear localization of NF-kB and enhanced macrophage Mtb growth control (38). In line with this, it was reported that the Mtb-induced CREB1 activation and modulation of inflammation through NF-kB p65 blockade in RAW murine macrophage-like cells was reversed by siRNA silencing of CREB, leading to enhanced Mtb growth control (39). Based on these findings and the fact that CREB1 was a common target for three of the miRNAs expressed across all donors after stimulation with ASC, this is an important mechanism for the enhanced Mtb growth control that we observe with EV-stimulation of Mtb infected hMDMs. The CREB finding is further supported by the biological function of the targeted genes as the PI3K/AKT signaling pathway was found modulated in all donors with Ascaris exposure.

Other important targets modulated by miRNAs that were overexpressed in EVs of Ascaris-exposed hMDMs was MAPK10 (JNK3) and MAPK13 (p38δ) (Supplementary Figure S2). These targets belong to the family of MAPK that are major players during inflammatory responses, especially in macrophages. p38δ MAPK was identified as a novel regulator of NLRP3 inflammasome activation in primary human macrophages that mediates IL-1β cleavage and secretion (40). Additionally, IL-1β secretion was decreased in response to LPS in bone marrow-derived macrophages from p38δ- MAPK-deficient mice (41). Moreover, it has been shown that p38δ inhibition or deletion leads to a blockade of CREB, that p38δ is essential for mitogen- and stress-activated kinase 1 (MSK1) phosphorylation or activation in bone marrow-

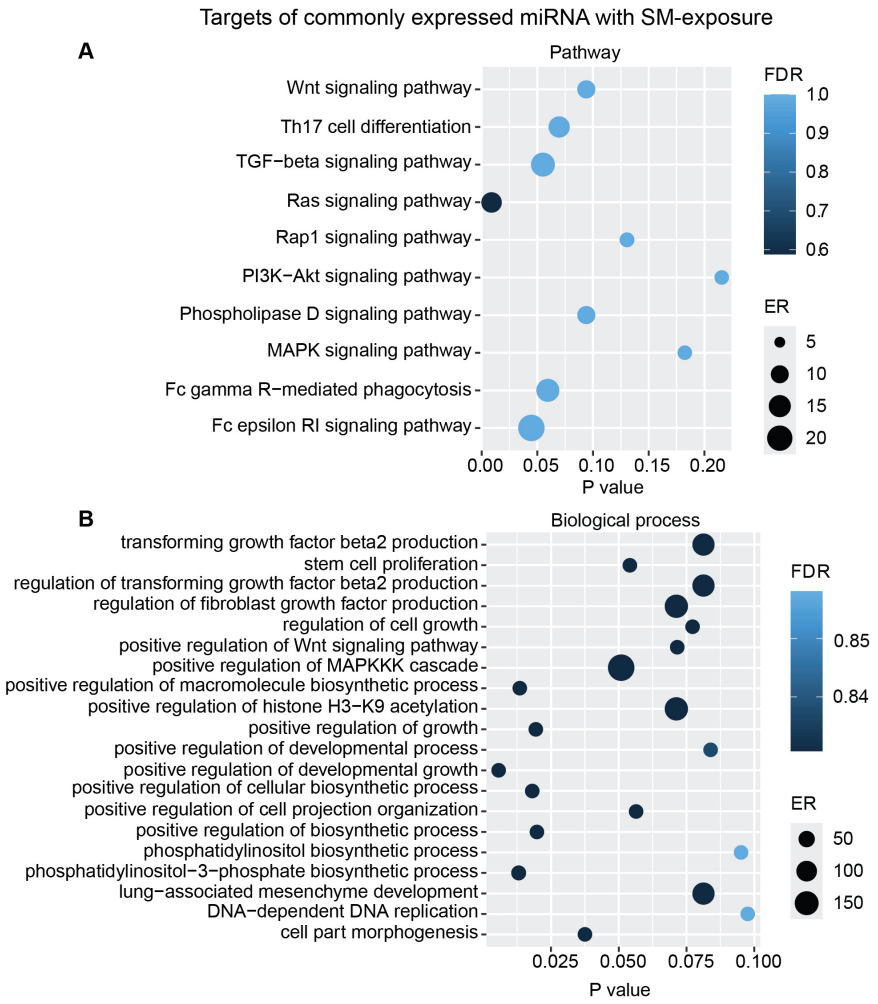

FIGURE 8
miRNAs target gene KEGG pathway enrichment analysis and GO analysis for the commonly expressed miRNA with SM exposure. Commonly expressed miRNAs from all donors were subjected to target prediction (miRWalk), and common targets across all donors were identified and used to detect pathways that were affected using the KEGG pathway from Webgestalt **(A)**, and GO analysis of common target genes **(B)**. Graphs were generated using ggplot2 in R.

derived macrophages, and that p38δ regulated MSK1 downstream targets that can limit inflammatory pathways downstream of TLRs (42). Thus, the observed decrease in Mtb infection-driven IL-1β release from hMDMs treated with EVs from Ascaris-antigen exposure aligns with the overexpressed miRNAs found targeting MAPK13 (p38δ). While IL-1β is generally associated with the restriction of intracellular Mtb growth (43) and IL-1β-deficient mice have higher bacterial loads when infected with Mtb (44), IL-1β signaling must operate within a narrow range as both excessive and defective IL-1β responses lead to lethal disease (45). EVs from Ascaris-exposed hMDMs thereby seem to lower the infection-driven IL-1β to an optimal range suitable for Mtb growth control. In fact, excessive IL-1β production and hyper-inflammation caused by various genetic polymorphisms have been associated with increased TB susceptibility, more severe forms of TB, including extrapulmonary TB, and a worsened treatment outcome (46, 47).

Schistosoma-induced miRNAs (miR-452-5p) were found to modulate the TGF-beta signaling pathway through the target SMAD4, which was common across all donors. TGF-β signaling is known to have an important function in macrophage polarization, immune regulation, and tissue homeostasis. SMAD-dependent TGF-β signaling pathways regulate M2 polarization, whereas SMAD-independent TGF-β signaling pathways regulate M1 polarization. SMAD4 is unexplored in the context of TB, but it was reported that SMAD4-dependant TGF-β signaling supressed TLR signaling, thereby interrupting pathogen recognition and induction of inflammatory responses (48). It was also previously reported that inhibition of SMAD4 resulted in significantly

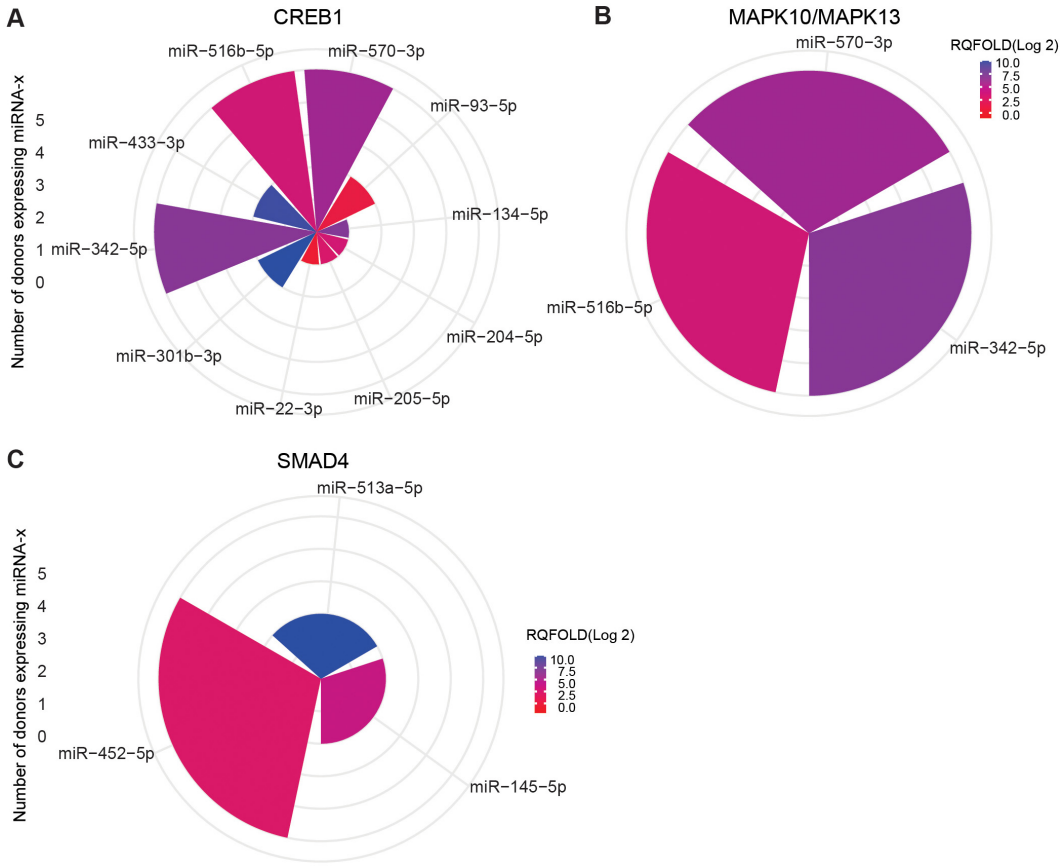

FIGURE 9
miRNA targeting CREB1 and MAPK10/13 in ASC exposure and SAMD4 in SM exposure of hMDMs. Commonly expressed miRNAs along with other miRNAs that target the same mRNA in the n = 5 donors analyzed. Target prediction using miRWalk showed CREB family **(A)** and MAP10/MAPK13 **(B)** targeted by miRNA upon ASC exposure and SMAD4 upon SM exposure **(C)**. Graphs were generated using ggplot2 in R.

enhanced renal inflammation (49). Our miRNA targeting SMAD4 induced by *S. mansoni* antigen exposure is therefore indicative of an interrupted SMAD-dependant TGF-β signaling that could promote an inflammatory environment. In line with this, we previously showed that *S. mansoni* antigen exposure increased the M1 marker CCR7 on hMDMs with decreased IL-10 secretion when infected with Mtb (11). However, the effect of a manipulated TGF-β signaling (incurred by SMAD4 inhibition) during Mtb infection could be argued to have the strongest effect on immune responses during chronic TB infection when TGF-β is substantially elevated (50). TGF-β1 is the strongest profibrotic cytokine discovered (51), and TB granulomas can bear signs of TGF -β-driven fibrosis (52), where TGF-β levels are significantly higher in post-TB patients with pulmonary fibrosis (53). Therefore, local miR-452-5p administration strategies that modulate SMAD-dependent TGF-β pathways could be promising for reducing lung fibrosis in chronic inflammatory diseases, including TB.

In conclusion, we found that helminth antigen exposure of hMDMs generates EVs containing miRNA that target important immune pathways involved in inflammation and Mtb growth control. These effects were found to be helminth species-specific. Further mechanistic studies are needed to evaluate these miRNAs or their targets as biomarkers or possible targets during inflammatory diseases.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies involving humans because All donor samples were de-identified and anonymized before being provided to the researchers, ensuring complete confidentiality. All the work was carried out in accordance with the Declaration of Helsinki, not requiring a specific ethical

approval according to paragraph 4 of the Swedish law. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from Normal human serum (NHS) and buffy coat preparations from whole blood, the source of peripheral blood mononuclear cells (PBMCs) and monocytes, were obtained from healthy volunteers from Linköping University Hospital Blood Bank and Jönköping Hospital Blood Bank. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

GP: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. RB: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1454881/full#supplementary-material

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Intersecting epidemics: deciphering the complexities of tuberculosis-diabetes comorbidity

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Within the global health landscape, tuberculosis (TB) presents an ongoing challenge, demanding innovative strategies for its control. This review spotlights the intersection of TB with diabetes mellitus (DM), recognized by the World Health Organization as a key risk factor in the TB epidemic. Particularly prevalent in low and middle-income nations, the TB-DM comorbidity drives up TB rates through a nexus of chronic inflammation. By delving into the epidemiological, clinical, and inflammatory dimensions, we elucidate the impact of TB-DM on patient prognosis and the multifaceted complications it introduces to disease transmission, diagnosis, and treatment protocols. Our synthesis aims to offer a fresh lens on TB-DM, fostering a nuanced understanding that could inform future healthcare policies and interventions.

KEYWORDS

tuberculosis, diabetes mellitus, epidemiology, inflammation, multi-omics

1 Introduction

In the contemporary global health landscape, tuberculosis (TB), a persistent challenge, intersects intricately with another widespread condition, diabetes mellitus (DM) (1, 2). Recognized by the World Health Organization (WHO) as a crucial risk factor in the TB epidemic, TB-DM comorbidity emerges as a significant concern, especially in low and middle-income countries (1). The coexistence of TB-DM presents a unique challenge in global health, demanding a nuanced understanding of their interplay, that affects TB disease progression and individual outcomes. An integrated approach, incorporating both traditional epidemiological methods and advanced molecular techniques, is essential to fully comprehend and effectively address the TB-DM comorbidity.

TB remains one of the major causes of death by a single pathogen worldwide, leading to a global health concern, with the disease affecting around 10 million people each year (1). The interplay between the *Mycobacterium tuberculosis* (Mtb) and the host, mediated by the inflammatory responses, determines a wide spectrum of clinical presentations. The challenge of reducing the disease burden arises from different factors, including the

influence of different comorbidities, mainly those that affect the quality of inflammatory responses, such as HIV (3), malnutrition (4) and DM.

DM is a chronic metabolic disorder that represents a dramatically high burden to healthcare systems worldwide. It has been reported that around half a billion people live with this disease (2), mainly in low- and middle-income countries. Additionally, DM is a significant contributor to global morbidity and mortality, leading to an array of severe complications, including kidney failure, stroke, and heart disease, and is directly responsible for a substantial number of deaths annually (2). DM affects the metabolism through multiple mechanisms, many of them related to the activation of poorly controlled pro-inflammatory pathways (5, 6), that influence disease progression and susceptibility to infectious diseases, such as COVID-19 (7) and TB (8).

In this context, the TB-DM comorbidity has garnered significant attention in the last years. Interestingly, some studies revealed regional disparities in the immune profile resulting from this interaction and emphasized the influence of socio-demographic and clinical factors on both diseases (9, 10). This article delves into the multifaceted relationship between TB and DM, exploring how this interplay exacerbates TB incidence. Our review compiles recent findings on the epidemiological, clinical, and inflammatory aspects of this nexus, highlighting its profound implications on patient outcomes and the broader challenges it poses in disease management. By scrutinizing the TB-DM interconnection, we aim to provide a comprehensive multiplatform perspective that not only sheds light on the complexities of this comorbidity but also suggests pathways for innovative healthcare strategies and policy formulations.

References for this review were identified through searches of PubMed for articles published from January, 1924, to January, 2024, by use of the terms "tuberculosis," "diabetes," "hyperglycemia," "epidemiology," "immune profile," "treatment outcomes," "clinical presentation," and "omics." Articles resulting from these searches and relevant references cited in those articles were reviewed, and the most relevant and recent papers published in English were included.

2 Epidemiological trends of DM among TB patients

The intersection of DM and TB poses a global health challenge, as DM is recognized by the WHO as a primary risk factor for new TB cases. Interestingly, many of the top 10 countries with the highest DM rates are also significant contributors to the global TB burden. Additionally, in these low and middle-income countries the prevalence of DM is increasing, creating a dual challenge that has serious public health implications (11). In a modeling study, conducted in 13 high endemic TB countries, the authors used dynamic tuberculosis transmission models to examine the impact of DM on TB epidemiology (12). The previous prevalence of DM in each of the 13 countries was accessed to simulate the future DM prevalence and a country-specific model calibrated to estimate the trend of TB incidence (12). Results show that lowering or stopping the rise of DM can avoid 6 million TB incident cases and 1.1 million deaths due to TB in the 13 countries for 20 years (12). Therefore, understand the epidemiological and clinical patterns of DM in TB patients is vital for delivering patient-centered care and managing these diseases to reduce the global TB impact (1).

The global prevalence of DM in TB cases exceeds 15%, surpassing the prevalence in the general adult population by over 50% in 2021 (13, 14). A recent metanalysis, including 2.3 million TB patients, identified that this trend is notably higher in North America (19.7%), Western Pacific (19.4%), Southeast Asia (19.0%), and Middle East and North Africa (17.5%) (13). While these variations in DM prevalence among TB patients appear to correlate with the general DM prevalence in each region, disparities within the same regions suggest more intricate underlying factors. Of note, these factors could be associated with the clinical management of the patients, that could vary according to the region. India and Sri Lanka, for example, possess a significantly higher burden of TB-DM than the other countries in the South Asia region (13, 15). This may be a reflection of the high coverage of DM screening in TB clinics, as well as points to the potential influence of regional epidemiological patterns, consumption habits, environmental factors, and genetic predispositions, now being explored through multiplatform studies, indicating a complex interplay of epidemiological elements in TB-DM comorbidity.

Despite well established relationships between the diseases, it is important to note that TB, as an infectious disease, could lead to hyperglycemia. Therefore, the use of screening algorithms for transient hyperglycemia displays pivotal role. In a case-control study conducted in Tanzania between July 2012 and June 2014, TB patients before treatment were paired with volunteers by sex and age. All participants underwent DM tests, as fasting capillary glucose, 2-h capillary glucose and HbA1c levels at enrollment (16). TB patients were submitted again to the tests after the therapy, and the authors found a significant decrease in DM prevalence (16), highlighting the relevance of DM confirmation after TB treatment to avoid an overestimation of the dual burden of diseases.

The prevention of progression from infection to disease is crucial to control the TB diseases burden worldwide (1). Currently, the identification of people infected with *Mtb* can be performed with tuberculin skin test (TST) or interferon-γ release assay (IGRA). Both tests are based on the host immune response against the bacilli, and could be affected by secondary morbidities that affect the inflammatory responses, such as DM. Some reports have associated DM with increases in the indeterminate IGRA results (17). However, the association has been controversial in other studies, where DM was not associated with indeterminate results (18). A Brazilian study performed with 553 participants from a high endemic area, highlight the QuantiFERON-TB Gold as a good tool to screening TB infection in DM (19), a susceptible population to TB infection and diseases. Of note, data suggests also a higher incidence of DM among those with TB infection (20). A retrospective cohort performed in the USA among US Veterans included patients who received TST or IGRA during 2000 to 2015. Patients with previous DM diagnosis were excluded, and the participants followed from TST/IGRA test date until date of DM diagnosis, death or 2015 (20). The results revealed a higher DM incidence among LTBI, when compared with those without positive TST/IGRA, 1.012 vs. 744; HR 1.4 [95% CI 1.3–1.4] (20).

Therefore, the investigation of TB infection in DM patients, as well as dysglycemia in TB infected people, is crucial as a cost-effective public health strategy to mitigate the impact of the epidemics.

The epidemiological factors associated with the TB-DM dynamicity have been the focus of several studies. Among the known risk factors, advanced age was consistently identified as a prominent risk factor for TB-DM comorbidity (21, 22). Lifestyle habits, such as sedentarism (23, 24) behavior, along with socio-demographic factors like urban living and high-income status (25, 26) have also been recognized as elements that increase the TB-DM prevalence. Clinical variables such as a family history of DM (24, 27, 28) further, contribute to an increase in the risk of TB-DM. However, the relationship between Body Mass Index (BMI) and TB-DM is unclear, with both low and high BMI values, as well as obesity and malnutrition, having been separately associated with TB-DM (24, 29). Furthermore, genetic factors at the population level may significantly influence the occurrence of TB-DM comorbidity. A study on Indian TB-DM individuals and their household contacts (HHC) observed that HHCs who developed TB, had a specific genetic pattern called the "GG genotype" of the interleukin(IL)-6−174G>C gene, indicating a genetic component in TB susceptibility (30). IL-6 is a cytokine essential for initiating and regulating the immune response to Mtb. Genetic variants affecting IL-6 can influence the immune system's ability to combat TB, impacting who may develop the disease after exposure (30). The insights from epidemiological and molecular studies on TB-DM comorbidity have significant implications for future healthcare policies and interventions. The relationship between clinical, lifestyle and genetic factors, such as the IL-6 GG encourage the design of molecular epidemiology projects to identify specific SNPs associated with increased disease risk, explain varied disease burdens across populations and inform tailored public health strategies, potentially reducing the incidence and improving the management of TB-DM comorbidity.

3 Dissecting the clinical interplay and implications of TB-DM comorbidity

The relationship between TB-DM represents a complex, bidirectional nexus significantly impacting clinical presentation, and disease dynamics and outcomes. DM is not only associated with the prevalence of TB but also exacerbates its progression (31, 32). By 2050, projections suggest that one-third of TB incidence and mortality within the Asia-Pacific region and similar environments, will be attributable to DM (33). This alarming trend underscores the need for integrated health strategies that address both TB and DM, particularly in regions with high prevalence rates.

Clinically, DM complicates TB management. In recent research spearheaded by our team, we uncovered a positive association between DM and increased mycobacterial loads, as well heightened Acid-Fast Bacilli (AFB) positivity, at diagnosis in TB-DM participants if compared with those without DM (32, 34). Furthermore, DM has been implicated in the presence of distinct lung lesions in chest radiographs of TB-DM patients in our Brazilian cohort (35). The presence of DM also influences the clinical presentation of TB, increasing the occurrence of TB symptoms such as hemoptysis, night sweats, and weight loss, as well as elevates the clinical severity (34–38). In contrast, TB-DM patients exhibited lower fever, reduced cough, and less sputum production, leading to delays in diagnosis and initiating appropriate treatment (39). The changes in clinical presentations and the consequences of a late diagnosis and start of TB treatment emerge as a clinical challenge in TB management and disease burden control.

In addition, the available evidence suggests that the prevalence of DM is higher among Drug Resistant (DR)-TB and Multidrug Resistant (MDR)-TB patients compared to the general population. A meta-analysis conducted in 2017 with 13 studies and 9,289 individuals shown a significant association between DM and MDR-TB (OR 1.7 [95% CI = 1.32–2.22]) (40). A more recent meta-analysis performed with 30 studies and including 225.812 patients also demonstrated a risk of MDR-TB among DM (HR 0.81 [95% CI: 0.60–0.96]) (41).

It is important to emphasize that DM also impacts anti-TB treatment, being associated with unfavorable outcomes, such as death, treatment failure and relapse cases (15, 42). Specifically, mortality and anti-TB treatment failure have been consistently linked with DM and higher glycated hemoglobin (HbA1c) levels (15, 43–46). Of note, treatment failure is more pronounced in TB-DM comorbidity within low- and middle-income countries (47), where DM conferred a 3.9 times increased risk of treatment failure in contrast with TB-only patients (48, 49). A pooled metanalysis also showed that TB relapse risk increases in TB-DM individuals (15, 50). Furthermore, DM was associated with poor outcomes in DTR-TB, with a risk of 1.56 times increased risk for unsuccessful outcomes (51). Following treatment completion, patients with TB-DM frequently require more extensive long-term care due to their challenges with managing blood sugar levels, elevated risk of cardiovascular disease, and potential for kidney damage (1).

DM also affects another important nexus to TB control: the transmission cascade. The key factors are the increased bacterial load, delayed diagnosis and unfavorable treatment outcomes in TB-DM patients, as abovementioned, which directly contributes to a higher transmission of Mtb. In a study from our group, conducted with a longitudinal multicenter cohort, it was observed that TB-DM patients shown an increased risk of Mtb transmission to their close contacts, if compared to TB-only patients (52). These factors interact in ways that enhance the transmission potential among TB-DM patients, thereby underscoring the need for timely diagnosis and targeted interventions to control the spread of TB in this vulnerable population.

On the other hand, antitubercular therapy also affects DM management. Due to drug interaction with rifampicin, a pivotal drug in TB treatment, and changes in food consumption and metabolic demand, with decreasing in systemic inflammation and consequently increasing in appetite, the targeted HbA1c level could be difficult to achieve in TB-DM (53). Additionally, despite metformin is not metabolized by P450 enzymes (53), the drug is a substrate for human transporters. Rifampicin could affect the expression of organic cation transporter (OCT1) leading to hepatic uptake of metformin, that could interfere in glucose levels in health participants (54). However, a pharmacodynamic study in TB-DM patients showed that rifampicin leads to changes in plasmatic

concentration of metformin, but do not lead to changes in glucose levels (55). Side effects in metformin use with rifampicin include gastrointestinal effects and rarely lactic acidosis (56). These effects could contribute to poor adhesion of both treatments, leading to a cascade of complications, highlighting the relevance of TB-DM to public health.

The challenges of TB-DM regarding clinical manifestations and, consequently, outcomes play pivotal role in the future direction of TB management. First, is necessary to expand the glycemic tests among TB patients, identifying and treating DM with better glucose control. On the other hand, among those DM patients, an active search for TB is fundamental to early diagnosis and treatment. In addition, it is important to focus on screening close contacts of TB-DM individuals, given the higher risk of TB infection. Finally, improving the understanding of the molecular mechanisms associated with the worse clinical presentation and unfavorable outcomes could help to tailoring effective and patient-centered interventions, improving treatment, and contributing with the burden control.

4 Cellular and molecular mechanisms underlying TB-DM comorbidity

The varying global incidences of TB-DM and the notable impact of DM on the clinical presentation, outcomes, and transmission of TB underscore a complex and intricate synergy between these conditions. In this landscape, multi-platform approaches are essential for dissecting the intricate cellular and molecular interactions in TB-DM comorbidity and provide a comprehensive view of the inflammatory processes involved. Such advancements have the potential to significantly enhance prognosis, follow-up, and contribute to a reduction in the burden of TB. In this context, several multimolecular biomarkers have been explored with the goal of enhancing diagnosis and clinical management of TB-DM patients.

4.1 Cellular immunology aspects

It becomes evident that the interplay between TB and DM significantly alters immune cell function and response. However, the mechanisms by which immune responses are impaired in individuals with TBM-DM are not fully understood, being complex and multifactorial. In TB infection, the effector functions of alveolar macrophages are crucial to containing the infection within the lungs (57). However, in DM patients, the functionality of these cells is decreased due to metabolic alterations associated with hyperglycemia (58, 59). DM impairs the functional activity of neutrophils (60) and reduces macrophage migration to sites of infection (57). Animal studies with diabetic mice have shown a delay in innate immune response initiation, which includes a compromise of nitric oxide production and phagocytic cell functionality, notwithstanding cytokine stimulation (57). In this same model, alveolar macrophages exhibited increased expression of CCR2, which potentially hampers the migration of monocytes to the lungs. Therefore, this may result in a compromised capability to kill intracellular Mtb, thus further contributing to both infection susceptibility and increased bacterial load (57, 61, 62). In addition, high blood glucose levels negatively impact the antigen presentation capabilities, which are vital for initiating adaptive immune responses against Mtb (63).

Similarly, the T cell response is notably affected, with DM patients often displaying dysregulated T cell responses. This dysregulation is hallmarked by imbalance in T helper (Th) cell subsets, with decreased Th1 responses and increased Th2 and Th17 responses. Such an imbalance can significantly alter the host ability to mount an effective response against TB. In a study comparing euglycemic and diabetic mice, it was observed that at the onset of infection diabetic mice exhibited a delayed activation of the adaptive immune system. This delay was indicated by decreased production of IFN-γ and fewer Mtb antigen (ESAT-6) presence compared to euglycemic mice (64, 65). Studies have shown that the frequencies of T cells producing type 1 and type 17 cytokines are significantly reduced in TB-DM patients compared to those without DM. This suggests a compromised ability to mount an effective immune response against Mtb due to altered cytokine signaling (66).

The interplay between TB-DM leads to significant impairments in both innate and adaptive immune responses. This results from a combination of altered cytokine production, diminished T cell functionality, innate immune cell dysfunction, phenotypic changes in immune cell populations, and metabolic influences due to hyperglycemia. These cellular alterations contribute to a weakened immune defense against TB in diabetic individuals, underlining the importance of targeted interventions that address these specific cellular immune challenges in TB-DM comorbidity.

4.2 Genomics

Multi-omics research has delved deeper into the layers of complexity in TB-DM comorbidity. Genomics, proteomics, and transcriptomics, each provide unique insights into the pathophysiological mechanisms in TB-DM. Genomic studies, for instance, have identified genetic variants that predispose individuals to TB-DM, revealing potential targets for personalized medicine approaches. Polymorphisms on IL-6 and IL-18 genes were associated with TB-DM comorbidity and the occurrence of TB in close contacts (30). Another study analyzed the interferon-gamma gene variants and found that the TACCCAGA haplotype was negatively associated with TB-DM. The frequency of this haplotype was high in healthy controls compared to TB-DM patients, ehich may denote the importance of genetic variation in TB-DM predisposition, as well as facilitate the identification of individuals at risk.

Currently, new approaches have been applied to better understand genetic variations and predispositions to TB in DM population. Using Mendelian randomization, a recent study selected 152 independent single-nucleotide polymorphisms (SNPs) as instrumental variables to evaluate genetic causality between type 2 DM and TB (67). Results reveal an increased risk of PTB among type 2 DM in the East Asian population (67). Evaluating the causal relationship between type 1 DM and TB, a Chinese group assessed SNPs of type 1 DM and PTB (68). Additionally, data from

Genome-Wide Association Study (GWAS) were utilized to explore clinical traits of DM, such as glycemic traits, lipids and obesity (68). Using inverse variance weighting method (IVW), weighted median method, and Mendelian randomization-Egger regression were used to evaluate the causal relationship, the group identified that type 1 DM and HDL-C were risk factors to PTB (68).

These findings collectively deepen our understanding of the genetic interplay between TB and DM, emphasizing the need for integrative approaches that consider genetic, metabolic, and environmental factors in addressing TB-DM comorbidity.

4.3 Transcriptomics

In the realm of transcriptomics, investigations in samples from TB-DM patients have illuminated the molecular pathways that may be dysregulated in this comorbidity. The superposition of TB-DM is marked by chronic inflammation, alongside qualitative and quantitative changes in immune activation characterized by distinct gene expression patterns. In a recent multi-center cohort study involving TB-DM individuals (69), pathway enrichment analysis had shown a notable trend toward heightened neutrophil and innate immune pathway activation in TB-DM participants even after anti-TB treatment commencement (69), that might reflect persistent inflammation. The findings of this same study unveiled that the genetic and immune responses may vary across different geographical regions (69), as discussed in the sections above. Additionally, the study dissect the correlations between the HbA1c levels and some biological pathways, highlighting positive correlation between HbA1c levels and pathways associated with insulin resistance, metabolic dysfunction, diabetic complications, and chromosomal instability (69). These correlations may play a pivotal role in the pathophysiology of TB-DM, contributing to a more severe clinical presentation and unfavorable outcomes.

Another multicentric study has found that DM amplifies the expression levels of genes related to the innate inflammatory response and reduces genes related to the adaptative immune response in TB individuals (70). A decreased type I interferon (IFN) response was identified in TB-DM participants if compared to TB-only patients. Despite IFN-γ be the most important IFN type against *Mtb*, with direct effect in macrophage activation, previous studies have identified an up-regulation in type I IFN in TB only patients when compared to controls (71–74). Therefore, type I IFN has also been associated with TB pathophysiology (74, 75). Although the excessive IFN responses in TB only has been associated with a deleterious activity (70). Additionally, a Chinese study revealed 952 differentially expressed genes (DEGs) in TB-DM, enriched in pathways associated with the cell cycle, homeostasis, and immunological processes, highlighting changes in several biological pathways induced by DM in TB patients (76). Expanding the scope of transcriptomic studies in TB-DM in a Brazilian cohort, the long non coding (lnc)RNA expression analysis led to the identification of a distinct lncRNA signature, which effectively distinguishes TB-DM from TB-only cases with an accuracy of 90%−94%. Notably, the lncRNAs included in the signature (LINCO2009, LINCO2471, ADM-DT, and GK-AS1) hold a critical role in the pathways related to inflammatory activation against Mtb (77). These studies demonstrate that transcriptomics has shed light on the field of TB-DM, revealing that there are consistently altered pathways in TB-DM patients.

4.4 Proteomics

Proteomic analyses, through the quantification of cytokines, chemokines, and other immune-related proteins, have significantly advanced in the understanding of immune responses in TB-DM. This approach has been essential in revealing how hyperglycemia-induced metabolic alterations in DM patients impair the functionality of the innate and adaptive immune responses in TB-DM. Proteomic data in TB-DM also has the potential to reveal key alterations in protein expression and point toward potential novel biomarkers. These insights are crucial in delineating the complex dynamics of immune dysfunction in TB susceptibility, transmission, and treatment outcomes (57, 64, 65, 78–81).

In TB-DM patients, proteomic data indicates an increase in proteins involved in the complement and coagulation cascade, as well as in cholesterol metabolism. This elevation suggests a potential link between lipid metabolism dysregulation and the heightened inflammatory state observed in TB-DM comorbidity (82). In another study, 18 differentially expressed protein spots were identified in TB-DM patients. These alterations were associated with potential metabolic complications specific to TB-DM and shifts in proteins governing cell cycle and growth regulation hint at disrupted processes like cell proliferation and apoptosis (83).

A recent mice study evaluates the changes induced by TBDM in tissue level. Specifically in liver, a set composed of 60 proteins shows deregulation in TBDM when compared to TB only mice. These proteins have been associated with small molecule catabolic process, retinol metabolism, polyol biosynthetic process, cysteine, and methionine metabolism (84). Functional analysis reveals perturbations in 20 functional modules, that include monocarboxylic acid metabolism, amino acids biosynthesis, cysteine and methionine metabolism, retinol metabolism, monocarboxylic acid metabolic process, polyol biosynthetic process, steroid hormone biosynthesis, small molecule catabolic, and biosynthetic process (84). Of note, cysteine and methionine are associated with glutathione metabolism, and consequently contributing with the balance of the oxidative stress. Is know that the imbalance in reactive oxygen species contributing with tissue damage in TB (85). Therefore, the study reveals that the alterations in livre proteomic induced in TBDM leads to progression of liver diseases (84). These findings not only provide a deeper understanding of TB-DM pathophysiology but also open avenues for new diagnostic, monitoring, and treatment strategies.

DM patients also present an altered cytokine milieu that favors immune dysregulation. In several studies, with TB and TB-DM individuals, it was observed that DM participants experienced higher levels of inflammatory activation than those without DM (81, 86). Patients with TB-DM have higher levels of pro-inflammatory cytokines such as IFN-γ, IL-1β, and IL-17, as well as lower levels of anti-inflammatory cytokines such as IL-10, compared to patients without DM. Additionally, throughout the

anti-TB treatment, these markers remained elevated for a longer period in TB-DM patients compared to non-DM individuals, which characterized persistent hyperinflammation in this group of individuals (86). In summary, the comorbidity of TB-DM is hallmarked by chronic and unbalanced inflammation, reflected in abnormal levels of proteins, and the superposition of these disorders leads to qualitative and quantitative changes in immune activation (69).

4.5 Lipidomic

Lipidomic changes have been studied in the context of TB-DM, and it is known that the association TBDM leads to an altered lipidomic (87). Some studies suggest that these altered lipids levels are associated with the increased TB risk in DM patients. In this context, glycerophospholipids were studied (88). The study identified 14 glycerophospholipids differentially regulated between TB and TB-DM, emerging as potentially biomarkers in the field (88). Using lipidomic approaches to identify persistent hyperinflammation by evaluating urinary lipid mediator profiles of participants with TB and TB-dysglycemia, it was observed that levels of a urinary metabolite of prostaglandin 2 (PGE-M) and leukotriene 4 (LTE4) were consistently higher during anti-TB treatment in the DM group compared to the normoglycemic group. These lipid mediators play a crucial role in modulating the immune response (89). Interestingly, in an adjusted multivariable model TB-DM was independently associated with increased concentrations of PGD-M, PGI-M, and LTE4 at baseline (89). This profile of higher metabolites expression in TB-DM patients if compared to TB-only patients helps explain why these individuals present severe symptoms and more enduring lung damage more often (86), which can be associated with unfavorable outcomes and Mtb dissemination.

4.6 Metabolomics

Metabolomics, by analyzing the small molecule metabolites present in TB-DM patients, offers insights into the metabolic disruptions caused by the interplay of TB and DM. In studies exploring metabolomics, specific metabolic changes induced by TB-DM were delineated (90). Plasma amine and acylcarnitine levels were measured in TB and TB-DM patients, with partial least squares discrimination analysis showing robust group discrimination (90). Notably, TB-DM exhibited lower levels of choline, glycine, serine, threonine, and homoserine compared to TB-only patients. Of note, the levels of these metabolites did not normalize during treatment (90). In a recent Korean study, plasma metabolic profiles of TB and TB-DM were investigated using metabolomics and lipidomics (87). TB-DM participants presented higher concentrations of bile acids and molecules related to carbohydrate metabolism, as well as the depletion of glutamine, retinol, lysophosphatidylcholine, and phosphatidylcholine (87). Arachidonic acid metabolism, crucial for eicosanoid production, emerged as a key factor in TB-DM pathophysiology (87). Eicosanoids, extensively studied in TB and TB-DM (89, 91), emerges as potential markers for diseases severity (80).

4.7 Integrative analysis

Multi-omics investigations have significantly advanced our comprehension of the complex interplay between these two diseases. By integrating data from various omics layers such as genomics, transcriptomics, proteomics, metabolomics, and epigenomics, it is possible to achieve a holistic understanding of the biological processes involved in TB-DM interaction and consequently prognosis. Funding multi-omic studies is fundamental to better understanding the pathophysiology of TB-DM and its impact on anti-TB treatment outcomes as well as in the identification of new targets to host directed therapies.

Using whole blood gene expression and plasma analytes (81), a groundbreaking study identified that DM in comorbidity with TB intensifies the neutrophilic inflammatory response, possibly indicative of a higher bacterial load or a distinct disruption in immune function. This heightened response was marked by increased plasma levels of cytokines and growth factors, as well as differentially expressed genes, that differentiate individuals with TB-DM from the majority of those with only TB or DM (81). Intriguingly, the expression patterns of TB-DM-exclusive genes were linked to critical biological processes and therapeutic targets. They were associated with endoplasmic reticulum stress, a vital cell stress response, and showed connections to the mechanisms of action of the antibiotic doxycycline and anti-cancer drugs such as 5-fluorouracil and semaxanib (81).

In another study, using a multi-platform approach to integrate clinical, transcriptomic, lipidomic, and proteomic data from a Brazilian TB-DM cohort, were identified several multimolecular baseline markers—MMP-28, LTE-4, 11-dTxB2, PGDM, FBXO6, SECTM1, and LINCO2009—that effectively differentiate between TB-DM, TB-only, DM-only, and healthy control groups (92). After anti-TB treatment onset, a notable decrease in these markers was observed, correlating with microbiological cure. Significantly, markers such as 11-dTxB2, SECTM1, and LINCO2009 not only emerged as indicators for new host-directed TB treatments but also as potential predictors of treatment outcomes (92). Furthermore, this integrated molecular signature demonstrated high accuracy in distinguishing TB-DM cases from TB-only, DM-only, and healthy control (without TB and DM) groups in Brazil and was validated in three external cohorts, outperforming signatures derived solely from transcriptomic data (69, 92). Crucially, these findings highlight that multimolecular signatures can be more predictive and impactful for precision medicine compared to single-omic approaches, underscoring the enhanced potential of multi-omic platforms in advancing our understanding of inflammatory and infectious diseases, as well in finding markers that can be implemented in the clinical practice.

5 Paving the path for future breakthroughs

The studies included in this review provide substantial evidence of the interplay between TB and DM and highlight the need for advanced research methodologies. Current evidence in epidemiology demonstrates a global prevalence of DM in TB cases, emphasizing age, lifestyle, socio-economic factors, family history, and hypertension as key risk factors. However, there

exists a knowledge gap that needs addressing to understand the regional disparities in TB-DM comorbidity. Investment in molecular epidemiology studies is crucial for this understanding and is pivotal for developing targeted public health strategies. This approach would not only elucidate regional differences but also aid in formulating more effective, region-specific interventions.

The clinical nexus of TB-DM presents a bidirectional impact, with DM complicating TB management and exacerbating disease progression. Research shows a positive association between DM and increased mycobacterial loads and distinct lung lesions, underscoring the need for integrated health strategies addressing both diseases. The next step in addressing TB-DM comorbidity in the clinical point of view would involve developing more targeted public health policies for individuals with both conditions. This could include enhanced TB screening in DM patients and the other way around, as well as expanding research into contacts of these patients to assess transmission dynamics. It is also important to highlight that further studies are needed to evaluate how DM multimorbidity (such as chronic kidney disease, fatty liver, and cardiovascular problems) affect the inflammatory profile of the TB-DM patients, making complex the potential identification of biomarkers or treatment targets. These strategies would improve individual patient care and contribute to broader public health efforts in managing and preventing the spread of TB-DM comorbidity.

It is also known that DM impacts immune cell function and response in TB, with specific genetic variations associated with TB susceptibility. This points to the potential of using advanced technologies like single-cell analysis to uncover new therapeutic targets and biomarkers. This review provides several cellular and molecular insights associated with TB-DM comorbidity. We discussed the altered immune cell function in DM patients which are crucial in containing TB infection, as well as the influence of genetic factors, such as polymorphisms, and the role of multi-omics in understanding molecular pathways disrupted in TB-DM. Building on this, the use of multi-platforms, as well as the addition cutting-edge technologies such as single-cell analysis could be instrumental. This technology can allow for a more granular understanding of cellular responses in TB-DM comorbidity at an individual cell level, potentially uncovering new pathways and therapeutic targets. In addition, the development, validation, and implementation of point-of-care testing for specific biomarkers already identified through these advanced methods could revolutionize early detection and monitoring of TB-DM comorbidity. This approach aligns with the development of predictive scores, integrating genetic, molecular, and clinical data to accurately assess disease progression and treatment outcomes. Additionally, considering the immune dis-function, a targeted TB vaccine could play crucial role in diseases prevention. Some TB vaccines has been developed, but anyone directed to population with impaired inflammatory responses. A better understanding of the nuances of immune activation and impairment in TB-DM could help the development of a new TB vaccine focused on DM patients.

Moreover, the creation and improvement of comprehensive risk scores, incorporating socio-demographic, lifestyle, and clinical variables, could greatly enhance the precision of public health interventions. These scores, derived from multi-omic and epidemiological data, could be tailored to specific populations, considering regional variations in TB-DM comorbidity. Figure 1 encapsulates the current state of knowledge and future directions in TB-DM comorbidity research. In essence, leveraging these innovative technologies and approaches could bridge the gap between current knowledge and the untapped potential in managing TB-DM comorbidity, leading to more effective, personalized treatment, and prevention strategies.

The intricate relationship between TB-DM is a worldwide health threat, impacting treatment outcomes and mortality rates. The synthesis of epidemiological, clinical, genomic, transcriptomic, proteomic, and lipidomic studies is vital for understanding the complexities of TB-DM comorbidity. The study of multi-omic platforms emerges as an opportunity to gain insights into disease pathogenesis, given that it simultaneously explores several components of immune responses through multiple assay platforms. The identification of precise biomarkers for diagnosis and individualized treatment, along with public health

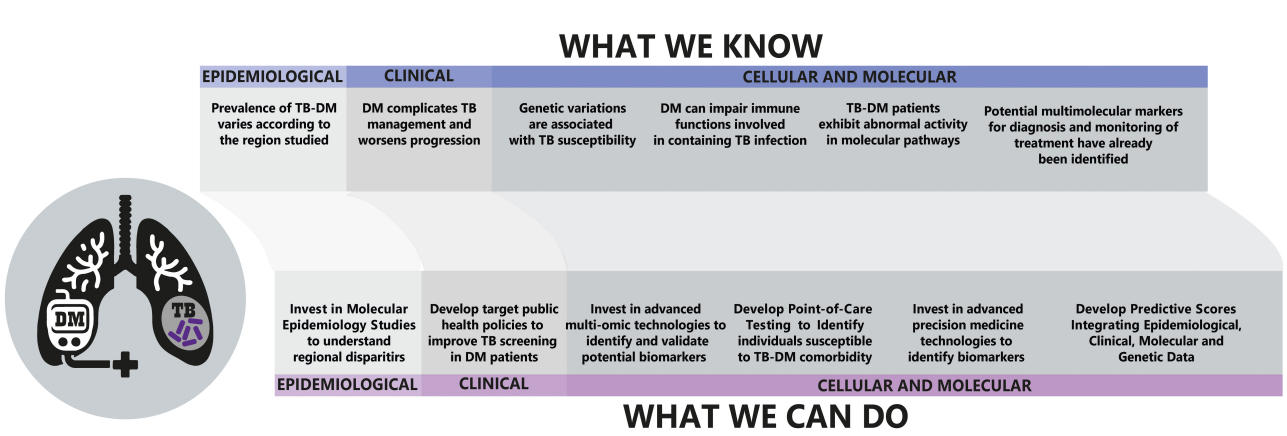

FIGURE 1
Overview of the interplay between Tuberculosis (TB) and Diabetes Mellitus (DM) and future directions. **Upper**: Current epidemiological, clinical, and cellular/molecular knowledge concerning TB-DM co-morbidity. **Down**: Prospective actions for advancing research and healthcare strategies.

strategies informed by molecular and epidemiological findings, is crucial. This area of research holds the promise of significant advancements, offering enhanced management of TB-DM comorbidity and contributing to global public health outcomes.

Author contributions

MA-P: Conceptualization, Writing – original draft, Writing – review & editing. CV: Conceptualization, Writing – original draft, Writing – review & editing. BB-D: Conceptualization, Writing – original draft, Writing – review & editing. KV-S: Conceptualization, Writing – original draft, Writing – review & editing. AQ: Conceptualization, Writing – original draft, Writing – review & editing. BA: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Specific immune response to *M. tuberculosis* and ability to *in vitro* control mycobacterial replication are not impaired in subjects with immune-mediated inflammatory disease and tuberculosis infection

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Background: Subjects with immune-mediated inflammatory diseases (IMID), such as rheumatoid arthritis, with tuberculosis infection (TBI), have a high probability of progressing to tuberculosis disease (TB). We aim to characterize the impact of IMID on the immune response to *M. tuberculosis* (Mtb) in patients with TBI and TB disease.

Methods: We enrolled TBI and TB patients with and without IMID. Peripheral blood mononuclear cells (PBMCs) were stimulated with Mtb-derived epitopes (MTB300). By flow-cytometry, we identified the Mtb-specific $CD4^+$ T cells as cytokine-producing T cells or as $CD25^+$ $CD134^+$ $CD4^+$ T cells. Memory and activation status of Mtb-specific T cells were assessed by evaluating: CD153, HLA-DR, CD45RA, CD27. Mycobacterial growth inhibition assay (MGIA) was used to evaluate the ability of PBMCs to inhibit mycobacteria growth. A long-term stimulation assay was used to detect a memory response.

Results: The IMID status and therapy did not affect the magnitude of response to Mtb-antigen stimulation and the number of responders. TBI-IMID showed a cytokine profile like TBI and TB patients. The Mtb response of TBI-IMID patients was characterized by an effector memory and central memory phenotype as in TBI and TB groups. This memory phenotype allowed the increased IFN-γ

production after 6 days of MTB300-stimulation. HLA-DR expression on Mtb-specific T cells was associated with TB, whereas CD153 was associated with TBI status. Finally, the TBI-IMID had an MGIA response like TBI and TB patients.

Conclusion: IMID condition does not affect key aspects of the immune response to Mtb, such as the cytokine response, memory and activation profile, and the ability to contain the mycobacteria replication. The immunological characterization of the fragile population of TBI-IMID patients is fundamental to understanding the correlation between protection and disease.

KEYWORDS

tuberculosis, rheumatoid arthritis, Th1, antigen-specific response, AIM assay, IFN-γ, MGIA, tuberculosis infection

Introduction

M. tuberculosis (Mtb), the etiological agent of tuberculosis (TB), is a leading cause of death from a single infectious agent with an estimated 10.8 million people falling ill with TB in 2023, and an estimated 1.25 million people died (1, 2). It has been estimated that a quarter of the world population has an immune response to Mtb defined as TB infection (TBI) (3). TBI can progress toward TB disease in 5–10% of TBI-infected subjects (4, 5). An immunological balance between the host and Mtb allows the pathogen persistence for years in a quiescent status continuously stimulating the immune system (6). Several conditions could affect this fragile equilibrium leading to a reactivation of Mtb replication and TB disease. Patients with immune-mediated inflammatory diseases (IMID) such as rheumatoid arthritis (RA) might have an increased susceptibility to infections, including TB, because the disease process already compromises their immune system. The risk ranges from 2.0 to 8.9 in RA patients with TBI not receiving IMID therapies and is lower in psoriatic arthritis (PsA), and ankylosing spondylitis (AS) (7–11). The relationship between immunity to Mtb and rheumatic disease is complex, primarily due to immunosuppressive therapies used in the management of IMID. Within the cells of adaptive immunity, T cells, particularly $CD4^+$ T cells (helper T cells), play a crucial role in fighting TB (12–16). However, IMIDs, such as RA, are characterized by an aberrant immune response, often involving autoantibodies and dysregulated T-cell responses leading to a higher risk of developing TB disease (8, 9).

Conventional synthetic Disease-Modifying Antirheumatic Drugs (csDMARDs), such as methotrexate, represent the first line immunosuppressive therapy in IMID patients. In patients affected by RA or PsA, biological (b-DMARDs) (17) and targeted synthetic DMARDs (ts-DMARDs) (18), are generally used after csDMARDs failure/intolerance, being highly effective in reducing disease activity and limiting disease progression. Although, this effectiveness can come at the cost of an impaired ability to fight infections (11). TB preventive therapy is mandatory for TBI-IMID patients undergoing treatment with bDMARDs and tsDMARDs such as TNF-α blockers, anti-IL-6, and JAKs inhibitors, considered drugs at high risk of TB reactivation (19). However, the highest TB risk is reported only in patients undergoing therapies with anti-TNF-α having a fourfold risk of developing TB disease (20), due to the known role of TNF-α in granuloma formation and integrity (21). However, following a principle of caution, TB preventive therapy is indicated as well as for other drugs targeting mechanisms of TB immunity (19), significantly down-modulating the immune function, and affecting T cells and macrophage function (7, 9–11) such as JAKs and IL-6 inhibitors.

Therefore, before initiating immunosuppressive therapy, RA patients are screened for TBI using tests such as the tuberculin skin test (TST) or interferon-γ release assays (IGRAs); if either of these tests is positive, a chest X-ray is performed to exclude TB disease (5, 11). If TBI is diagnosed, TB preventive therapy is proposed (7, 22).

In the last years, many studies characterized Mtb immunity to find new correlates of protection to have tools to monitor the immune response for designing TB vaccine. Polyfunctional CD4+ T cells simultaneously producing pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-2, have been deeply studied as a possible correlate of TB protection without a unique and definitive association with Mtb containment or Mtb replication (23, 24).

Despite this conflicting literature, BCG-based vaccine, the only licensed TB vaccine, and the novel TB vaccine candidates induce polyfunctional $CD4^+$ T cells with memory characteristics in both animal models and human studies (23). Therefore, significant focus remains on this specific subset of T cells, as they offer a viable means to assess the memory response induced by vaccines or Mtb infection.

An alternative tool to measure the Mtb-specific immunity is the Activation-Induced Markers (AIM) assay. The simultaneous expression of CD25 and CD134 identified the antigen-specific T cells, as described in response to Mtb-antigen stimulation in HIV-uninfected (25) and HIV-infected individuals (26). The CD134

(OX40) is a member of the TNFR whereas the CD25 is the IL-2 receptor a-chain, these markers are fundamental for survival, proliferation, and cytokine production upon antigen-specific stimulation (25).

Beyond cytokine production, the surface expression of memory and activation markers has been deeply studied. CD153, also known as 'CD30 ligand', is a costimulatory molecule member of the TNF superfamily (27). $CD4^+$ T cells expressing CD153 in response to Mtb antigens are associated with Mtb protection in both animal and human studies (28). They are inversely associated with the burden of TB disease in humans (29), providing a potential correlate of protection against pulmonary TB disease.

Similarly, other memory and activation markers have also been associated with different TB statuses. The CD27 downregulation (24, 30–32) and HLA-DR upregulation (28, 33–36) on Mtb-specific T cells, are associated with TB disease. Rigorous studies showed that the mycobacterial growth inhibition assay (MGIA) can be used to evaluate vaccine efficacy (37, 38), providing alternative standardized tools to evaluate the ability of the immune response to *in vitro* control the Mtb replication.

Currently, a great effort is underway to develop a new vaccine against TB disease (39–43). Since the TBI-IMID individuals represent an eligible population for TB vaccination, it would be important and clinically relevant to evaluate the status of the immune response to Mtb in these vulnerable subjects at higher risk of developing TB disease. However, few data are available on the Mtb-specific immunity of TBI subjects with IMIDs (42, 44–49).

Based on these premises, we aim to characterize the specific immune response to Mtb antigens in IMID patients with TBI and TB disease evaluating cytokine production, memory and activation markers, and MGIA. A control cohort of TB, TBI, and healthy control (HC) subjects without IMID was included. While TB patients serve as a model for Mtb replication, TBI-IMID subjects represent a model for Mtb containment. Additionally, TBI-IMID subjects are a vulnerable population that can be useful in dissecting the immunologically specific aspects of the TB spectrum.

Materials and methods

Study population

This study was approved by the Ethical Committee of the National Institute for Infectious Diseases Lazzaro Spallanzani-IRCCS (approval number 72/2015 and approval number 27/2019). Written informed consent was required to participate in the study. TB patients, TBI subjects and HC were enrolled from 2015 to 2023.

Since TBI in individuals with IMID is associated with a higher risk of progressing to TB disease (7), TB preventive therapy is offered before starting biological therapy. Note that in this study we enrolled 3 patients taking biologic drugs at the time of enrolment and TBI diagnosis. Note that these patients were not screened at the beginning of biologic therapy, although indicated by guidelines (50, 51), and the screening was prescribed only at the time of the therapy switching.

TBI diagnosis was based on a positive score to QuantiFERON (QFT)-Plus assay (Diasorin, Vercelli, Italy) without clinical, microbiological, and radiological evidence of TB disease.

TBI-IMID patients with a negative response to QFT-Plus showing radiological evidence of scars in the upper lung lobes and reporting a past exposure to TB cases (51), were considered TBI, and preventive therapy was proposed. Among the 9 subjects with TBI-IMID scored negative to QFT-Plus, 2 were taking anti-IL-6 drugs, 2 were taking csDMARDs and corticosteroids, 1 was under csDMARDs and 1 under corticosteroids. TBI and TBI-IMID cohorts were enrolled before starting the TB preventive therapy. TB disease diagnosis was based on microbiological and radiological signs of disease. TB patients were enrolled before starting treatment or within 7 days after therapy initiation. Among the TB-IMID patients, 3 were enrolled between 14 and 40 days of TB therapy and 3 within 7 days of TB therapy. Note that 3 TB-IMID were taking anti-TNF-α drugs at the time of TB diagnosis. Unfortunately, the type of IMID biologic therapy was unknown for one TB-IMID patient. As a control, we enrolled HC who scored negative for QFT-Plus. Demographic and clinical characteristics of all cohorts used in this study are reported in Table 1 and Figure 1. As this is an observational study with unpredictable outcomes, we selected a "convenient sample" of subjects, considering laboratory workflow, enrollment duration, patient flow in the hospital, and experimental protocol costs.

To perform this study, we followed the STROBE statement checklist for case-control studies (https://www.strobestatement.org/fileadmin/Strobe/uploads/checklists/STROBE_checklist_v4_case-control.pdf).

Stimulation and reagents

Withdrawing blood samples were collected in Heparin Blood Collection Tubes (BD Vacutainer® Blood Collection Tubes). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation with the SepMate™ tubes (StemCell; Cat.85460) within 4 hours from sampling. Cells were frozen in heat-inactivated fetal bovine serum (FBS) + 10% DMSO and stored in liquid nitrogen until further use. Thawed cells were cultured at a concentration of 0.5-1.0 x 10^6/mL in 96-multiwell plate for 24 and 48 hours at 37°C, 5% CO_2 in complete medium [RPMI-1640 (Gibco, CA, USA), 10% fetal bovine serum (FBS) (Gibco, Life Technologies Italia, Monza, Italy), 2mM L-glutamine, and 1% penicillin/streptomycin solution]. PBMCs were stimulated with a pool of 300 Mtb-derived peptides (MTB300, 1.5 μg/ml) (52). MTB300 peptide megapool contained a mixture of 300 Mtb-derived T-cell epitopes from 90 Mtb proteins (including ESAT-6 can CFP10) that target a large fraction of Mtb-specific $CD4^+$ T cells, which share epitopes with NTM species (52–54). As positive control cells were stimulated with staphylococcal enterotoxin B (SEB) (Merck Life Science Cat. S4881) at 200ng/mL. The costimulatory monoclonal antibodies α-CD28 and α-CD49 (1μg/mL each) (BD Biosciences, San Jose, USA) were added. BD Golgi Plug was added after 1 hour for cytokine detection, when appropriate. Unstimulated cells were incubated with costimulatory antibodies and Golgi Plug only.

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TABLE 1 Clinical characteristics of the enrolled patients.

	TBI	TB	TBI-IMID	TB-IMID	HC	TOTAL	p Value
N (%)	39 (29.5)	37 (28)	33 (25)	6 (4.5)	17 (12.9)	132 (100)	
Age median (IQR)	45 (32-55)	47 (32-55.5)	58 (48.5-66.5)	51 (41-62.25)	39 (36.5-49.5)	49 (36-57.75)	**0.0004***
Female N (%)	21 (54)	10 (27)	20 (61)	2 (33)	13 (76.5)	66 (50)	**0.0048****
Origin N (%)							
West Europe	25 (64)	11 (29.7)	21 (63.6)	2 (33.3)	17 (100)	75 (57.2)	
East Europe	6 (15.4)	13 (35.1)	5 (15.2)	3 (50)	0 (0)	27 (21)	
Africa	3 (7.7)	4 (10.8)	2 (6.1)	1 (16.7)	0 (0)	10 (7.6)	na**
Asia	2 (5)	7 (18.7)	1 (3)	0 (0)	0 (0)	10 (7.6)	
South America	3 (7.7)	2 (5.4)	4 (12.1)	0 (0)	0 (0)	9 (6.9)	
BCG-vaccinated N (%)	13 (33.3)	25 (83.3)	12 (36.4)	4 (66.7)	0 (0)	54 (41.2)	**<0.0001****
Type of IMID N (%)							
Rheumatoid arthritis	–	–	20 (60.6)	1 (16.7)	–		**<0.0001** [$]
Psoriatic arthritis	–	–	10 (30.3)	2 (33.3)	–		
Polymyalgia rheumatica	–	–	1 (3)	–	–		
Psoriasis	–	–	2 (6.1)	1 (16.7)	–		na[$$]
Crohn disease	–	–	–	1 (16.7)	–		
Ulcerative colitis	–	–	–	1 (16.7)	–		
Patients under IMID Therapy N (%)			20 (61)	5 (83)			0.2857 **
Type of IMID therapy N (%)							
B	–	–	3 (15)	2 (40)			0.1183 [$]
B+C	–	–	–	2 (40)			na [$$]
C	–	–	4 (20)	–			
cDMARDs	–	–	4 (20)	1 (20)			
cDMARDs +/- C +/-	–	–	9 (45)	–			
QTF-Plus N (%) at the time of enrolment[§]							
Positive	39 (100)	23 (76.7)	24 (73)	4 (66.7)	0 (0)	87 (66.4)	
Negative	0 (0)	11 (36.7)	9 (27)	0 (0)	17 (100)	39 (29.8)	na [‡]
Indeterminate	0 (0)	2 (6.7)	0 (0)	1 (16.7)	0 (0)	3 (2.3)	
Not available	0 (0)	1 (3.3)	0 (0)	1 (16.7)	0 (0)	2 (1.5)	

N, Number; TBI, TB infection; TB, tuberculosis; IMID, inflammatory mediated immune disease; HC, healthy control; BCG, bacillus Calmette-Guérin; QFT, QuantiFERON; IQR, interquartile range; B, Biological; C, Corticosteroids; cDMARDs, conventional DMARDs; na, not applicable, since Chi-square calculations are only valid when all expected values are greater than 1.0 and at least 20% of the expected values are greater than 5; *Kruskal- Wallis test; **Chi Square test; [$]Chi Square test among TBI-IMID patients; [$$]Chi Square test among TB-IMID patients; [‡]Chi Square test among TB, TBI-IMID and TB-IMID; Significant p values are reported in bold.

Intracellular staining assay and flow-cytometry analysis

To characterize the antigen-specific immune response to MTB300, we stained cultured PBMCs after 24h and 48h of incubation. Intracellular staining for cytokine evaluation was performed after 24h of incubation, using: Fixable Viability Stain 700, CD3 V450 (clone UCHT1), CD8 APC-H7 (clone SK1), CD27 BV605 (clone L128), CD45RA PE-Cy7 (clone L48), HLA-DR BV786 (clone G46-6), CD153 PE (R&D System, clone 116614), and CD4 ECD (Beckman Coulter, clone SFCI12T4D11), IFN-γ APC (clone B27), IL2 PerCP-Cy5.5 (clone MQ1-17H12) and TNF-α FITC (clone MAb11) (BD). Brilliant Stain Buffer (BD) and Cytofix/Cytoperm (BD) were used according to the manufacturer's instructions.

Activation-induced markers (AIM) were evaluated after 48h of incubation (25) using: Fixable Viability Stain 700, CD3 PE-Cy7 (clone SK7), CD8 APC-H7 (clone SK1), CD25 BV480 (clone 2A3), CD27 BV605 (clone L128), CD134 BV421 (clone ACT35), HLA-DR BV786 (clone G46-6) (all from BD), CD153 PE (R&D System, clone 116614) CD4 ECD (Beckman Coulter, clone SFCI12T4D11).

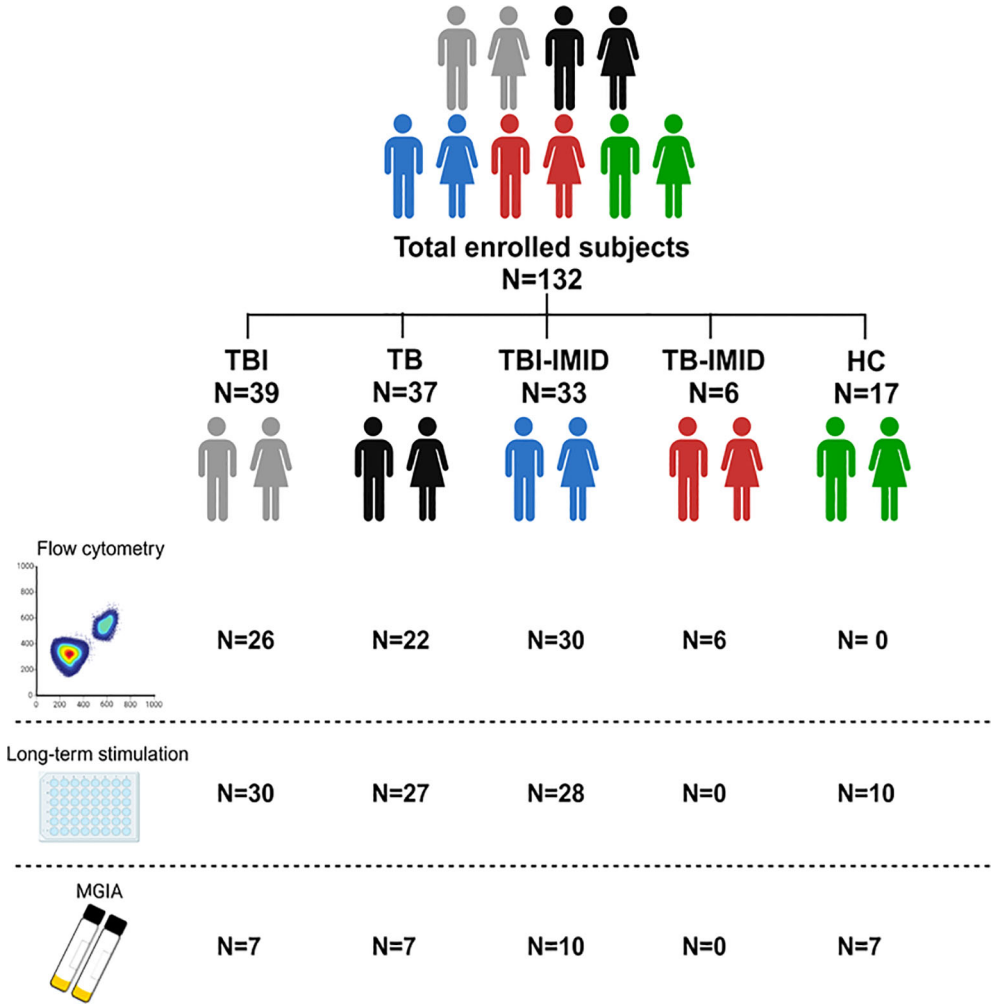

FIGURE 1
Scheme of subjects enrolled in the study. A total of 132 patients, with different TB statuses and with or without IMID, were enrolled for the study. The number of subjects used for each methodology is reported (Created in BioRender.com).

At least 100,000 lymphocytes were acquired using DxFLEX (Beckman Coulter) cytometer. Data were analyzed with FlowJo software (version 10.8.1), PESTLE and SPICE software [provided by Dr. Roederer (version for MacBook, Vaccine Research Center, NIAID, NIH, USA)]. Antigen-specific response was scored positive if the percentage of the stimulated cells were at least 2-fold higher compared to the unstimulated control and if the events gated were at least 10 (see Supplementary Figure S1 for the gating strategy). The analyses were conducted blindly by two different operators (EP and CF).

In vitro evaluation of the MTB300-specific antigen response after 6 days

The long-term assay was performed in a subgroup of the enrolled subjects with TBI, TB, TBI-IMID, and HC. Thawed PBMCs were seeded at $1x10^6$/mL in a 96-multiwell plate, stimulated with MTB300-peptides (1.5 μg/ml) and cultured for 6 days in a complete medium with α-CD28 and α-CD49 monoclonal antibodies (mAb) (1μg/mL each). At day three, recombinant human IL-2 protein (Bio-techne/R&D System) at 5 IU/mL was added (55). The supernatant was collected after 24h and after 6 days to evaluate the IFN-γ–specific response. The IFN-γ production was evaluated by ELISA assay (Diasorin, Vercelli, Italy) according to the manufacturer's instructions and the result was expressed as pg/ml (56).

Mycobacterial growth inhibition assay

The ability of the immune system to inhibit mycobacterial growth was assessed *in vitro* (57). Two million of PBMCs were seeded in 300μl in complete medium, without antibiotics, in a 48-well plate and 300μl of RPMI containing 300 CFU of Bacillus Calmette- Guérin (BCG) Pasteur. Infected PBMCs were incubated for 96 hours at 5% CO_2. Cells were lysed with sterile water and transferred into the MGIT tube supplemented with 800 μL of PANTA (antibiotics) and OADC broth (Becton Dickinson). MGIT tube were incubated in a Mycobacterial Detection System

(BACTEC MGIT 960) until the detection of positivity and growth. As control, the bacterial inoculum used was added directly to a MGIT tube: 300 CFU of BCG Pasteur without added cells were placed directly in the BACTEC MGIT 960 machine on day 0. Data were analyzed as time to positivity (TTP) and expressed in hours, subtracting the TTP of experimental control by the TPP of each experimental condition (ΔTTP).

Statistical analysis

Data were analyzed using Graph Pad Prism (Version 8.2.1) and SPSS software. The median and interquartile ranges (IQRs) were calculated for continuous measures. For pairwise comparison, Mann–Whitney U and Wilcoxon tests, were used, as appropriate. Friedman's test was used to compare paired data. Receiver Operator Characteristic (ROC) was used to determine the cut-off values and sensitivity/specificity of long-term stimulation with MTB300 at day 1 and day 6 in TB patients and HC individuals.

Results

Characteristics of the population

One hundred and thirty-two individuals with different TB status and with or without IMID, and HC were enrolled. Differences were found for age (p=0.0004) and proportion of females (p=0.0048). About 57% of the enrolled subjects were from Western Europe and 41% were BCG vaccinated. Most TBI-IMID patients had RA (60.6%) and 61% were under immunosuppressive therapy at the time of enrolment. None of TBI-IMID patients developed TB after one year from the end of TB preventive therapy. All the TB-IMID patients were under immunosuppressive therapy (Table 1). The characteristics of the specific cohorts for flow-cytometry, long-term and MGIA studies are described in Supplementary Tables S1-S3.

Evaluation of Mtb-specific CD4$^+$ T cells in TB and TBI subjects with and without IMID

To characterize the antigen-specific response, we evaluated both the cytokine-producing T cells and the AIM-positive cells (Figure 2, Supplementary Table S1). Unfortunately, we did not have sufficient CD8 responders to allow a robust analysis, so we focused only on the CD4$^+$ T-cell response. We performed the cytometry study on 26 TBI, 22 TB, 30 TBI-IMID, and 6 TB-IMID individuals (Supplementary Table S1). The cytokine-producing cells in response to MTB300 stimulation, hereafter referred to as Th1-response, were similar among groups (Figure 2A). Likewise, the frequency of IFN-γ^+, TNF-α^+, and IL-2$^+$ CD4$^+$ T cells was similar among groups (Figures 2B–D). Differently, after 48h of incubation, the frequency of antigen-specific CD4$^+$ T cells, identified as CD25$^+$CD134$^+$ (AIM assay), was higher in the TBI-IMID compared to the TBI individuals (p=0.034) (Figure 2E). In response to SEB, we observed a comparable distribution of Th1-response among groups and a significantly higher frequency of CD25$^+$CD134$^+$ CD4$^+$ T cells in TBI compared to TB (p= 0.033) and significantly lower frequency compared to the TBI-IMID cohort (p=0.024) (Supplementary Figures S2A–E). To better discriminate the effect of IMID therapy on the ability to respond to Mtb stimulation, we stratified the patients according to the type of IMID therapy (Tables 2, 3) Immunosuppressive therapy in the TBI-IMID and TB-IMID cohorts did not have an impact on the number of Mtb responders evaluated as Th1 CD4$^+$ T cells and CD25$^+$CD134$^+$ CD4$^+$ T cells (AIM assay) (Tables 2, 3).

Cytokine profile of Mtb-specific CD4$^+$ T cells in TB and TBI subjects with and without IMID

We investigated the functional cytokine profile of Mtb-specific CD4$^+$ T cells by applying a boolean gating analysis. All groups were characterized by the presence of IFN-γ^+ IL-2$^+$ TNF-α^+ CD4$^+$ T cells, IFN-γ^- IL-2$^+$ TNF-α^+ CD4$^+$ T cells, IFN-γ^+ IL-2$^-$ TNF-α^+ CD4$^+$ T cells, IFN-γ^- IL-2$^-$ TNF-α^+ CD4$^+$ T cells; whereas the IFN-γ^+ IL-2$^-$ TNF-α^- CD4$^+$ T cells were represented in all groups except for the TB. Note that the TB-IMID showed the highest proportion of IFN-γ^- IL-2$^-$ TNF-α^+ CD4$^+$ T cells compared to other groups (Figure 2F). We then assessed differences by analyzing the total polyfunctional or monofunctional proportion for each group (Figure 2G). We observed a similar proportion of polyfunctional and monofunctional Mtb-specific CD4$^+$ T cells among TBI, TB, and TB-IMID. Although not significant, TB-IMID patients were characterized by a predominance of monofunctional cytokine-producing CD4$^+$ T cells (Figure 2G). Moreover, in response to SEB, we found a comparable polyfunctional profile among cohorts with a similar proportion of the different T-cell subsets (Supplementary Figure S2F).

Activation profile of Mtb-specific CD4$^+$ T cells in TB and TBI subjects with and without IMID

Activation status of Th1-specific CD4$^+$ T cells in response to MTB300: we evaluated the surface expression of CD153 and HLA-DR on Th1-specific CD4$^+$ T cells after 24h of MTB300-stimulation. TB patients showed a higher frequency of Th1-specific HLA-DR CD4$^+$ T cells compared to TBI-IMID (p=0.0203); a similar, but not significant trend, was observed compared to TBI (Figure 3A). The HLA-DR expression on Th1 CD4$^+$ T cells agrees with previous studies (28). Differently from a previous study (28), the frequency of Th1-specific CD153+ CD4+ T cells was not modulated after 24h of MTB300-stimulation (Figure 3A).

Activation status of AIM$^+$ CD4$^+$ T cells in response to MTB300-stimulation: we evaluated the surface expression of CD153 and HLA-DR on AIM$^+$ CD4$^+$ T cells after 48h of MTB300-stimulation (Figure 3B). In this case, we observed an increased frequency of Mtb-specific CD153$^+$ CD4$^+$ T cells in TBI-IMID compared to TB (p=0.0014) and in TBI-IMID compared to TBI (p=0.0168). Note

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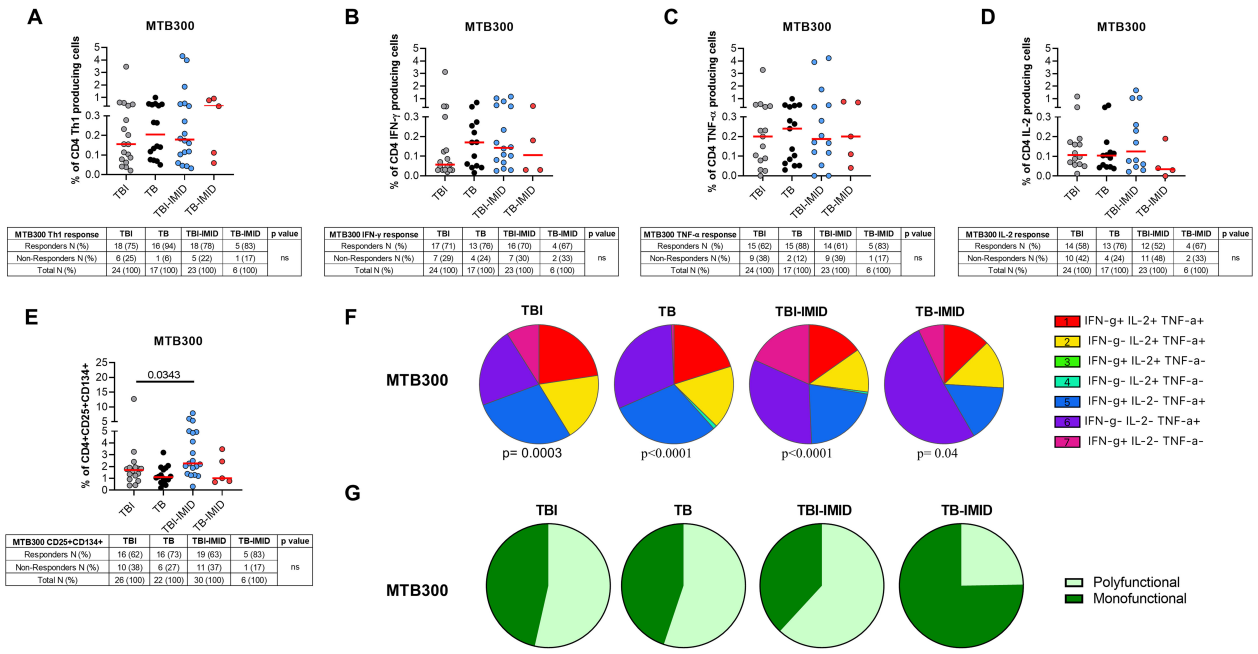

FIGURE 2
Evaluation of Mtb-specific CD4 T-cell response in TB and TBI subject with and without IMID. PBMCs were stimulated with Mtb-specific antigens (MTB300) for 24 or 48 hours and immune response was evaluated by flow-cytometry. All the analyses were performed only among the responders. **(A)** Antigen-specific response evaluated as total CD4 Th1 cytokine-producing cells after 24 h of stimulation. **(B)** Antigen-specific response evaluated as total IFN-γ^+ CD4 T cells after 24 h of stimulation. **(C)** Antigen-specific response evaluated as TNF-α^+ $CD4^+$ T cells after 24 h of stimulation. **(D)** Antigen-specific response evaluated as IL-2^+ $CD4^+$ T cells after 24 h of stimulation. **(E)** Antigen-specific response evaluated as $CD25^+$ $CD134^+$ $CD4^+$ T cells after 48h of stimulation. **(A–E)** Tables under the graphs report the number of $CD4^+$ T-cell responders to MTB300. Horizontal red lines indicate the median and each dot represents a single subject. Statistical analysis was performed using the Mann-Whitney test. **(F)** Pie charts representing the proportion of different cytokine-producing $CD4^+$ T-cell subsets. **(G)** Pie charts representing the proportion of monofunctional and polyfunctional antigen-specific $CD4^+$ T cells. **(F, G)** Boolean gate combination and Wilcoxon matched-pairs signed rank test were applied. TB, tuberculosis; TBI, tuberculosis infection; IMID, immune-mediated inflammatory disease; IFN-γ, interferon-gamma; IL-2, interleukine 2; TNF-α, tumor necrosis factor alpha.

TABLE 2 Flow-cytometry study: number of TBI-IMID subjects responding to MTB300-stimulation, stratified according to the IMID therapy.

Type of assay	Enrolled patients according to IMID therapy	Responders N (%)	Non-responders N (%)	p value
Th1	**Patients in therapy over total**	11/23 (48)	2/23 (87)	0.6175[§]
	Patients not in therapy over total	7/23 (30)	3/23 (13)	
	Type of therapy over all patients in therapy			
	B	0 (0)	1 (50) *	na
	C	3 (27.3)	0 (0)	
	cDMARDs	2 (18.2)	1 (50)	
	cDMARDs +/- C +/-	6 (54.5)	0 (0)	
CD25+ CD134+ (AIM)	**Patients in therapy over total (%)**	12/30 (40)	6/30 (20)	0.7116[§]
	Patients not in therapy over total (%)	7/30 (23)	5/30 (17)	
	Type of therapy over all patients in therapy			
	B	1 (8) *	2 (33) **	na
	C	3 (25)	1 (17)	
	cDMARDs	2 (17)	1 (17)	
	cDMARDs +/- C +/-	6 (50)	2 (33)	

N, number; TBI, TB infection; TB, tuberculosis; IMID, inflammatory mediated immune disease; B, Biological; C, Corticosteroids; cDMARDs, conventional DMARDs; AIM, Activation Induced Marker; n, Number; na, not applicable since Chi-square calculations are only valid when all expected values are greater than 1.0 and at least 20% of the expected values are greater than 5; [§]Fisher's exact test *anti-CD20; **anti IL-6.

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TABLE 3 Flow-cytometry study: number of TB-IMID subjects responding to MTB300-stimulation stratified according to the IMID therapy.

Type of assay	Enrolled patients according to IMID therapy	Responders N (%)	Non- responders N (%)	p value
Th1	**Patients in therapy over total**	4/6 (66.6)	1/6 (16.7)	>0.9999[§]
	Patients not in therapy over total	1/6 (16.7)	0/6 (0)	
	Type of therapy over all patients in therapy **B** **B+C** **cDMARDs**	 *2 (40) **1 (20) 1 (20)	 0 (0) **1 (100) 0 (0)	n.a
CD25+ CD134+ (AIM)	**Patients in therapy over total**	4/6 (66.6)	1/6 (16.7)	>0.9999[§]
	Patients not in therapy over total	1/6 (16.7)	0/6 (0)	
	Type of therapy over all patients in therapy **B** **B+C** **cDMARDs**	 *2 (40) **1 (20) 1 (20)	 0 (0) 1 (100) 0 (0)	na

N, number; TBI, TB infection; TB tuberculosis; IMID, inflammatory mediated immune disease; B, Biological; C, Corticosteroids; cDMARDs, conventional DMARDs; AIM, Activation Induced Marker; n, Number; na, not applicable, since Chi-square calculations are only valid when all expected values are greater than 1.0 and at least 20% of the expected values are greater than 5; [§]Fisher's exact test; *one patient was under anti-TNF-α therapy and one patient was under unknown biologic therapy; **patient was under anti-TNF-α therapy and corticosteroids.

that, even if not significant, the median frequency of Mtb-specific CD153^{+} CD4^{+} T cells in TBI individuals was higher than in TB patients. The analysis of the different CD153$^{+/-}$ HLA-DR$^{+/-}$ CD4^{+} T-cell subsets showed an increase of all subsets at 48h at the expense of the CD153^{-} HLA-DR^{-} CD4^{+} T cells (Figures 3C, D).

Activation status in response to SEB: we observed an increase of the different CD153$^{+/-}$ HLA-DR$^{+/-}$ CD4^{+} T-cell subsets in response to SEB stimulation compared to the response at 24h and 48h post *in vitro* stimulation (Supplementary Figure S3).

CD27, CD153, and HLA-DR evaluation on Th1-specific CD4^{+} T cells in response to MTB300: We evaluated the surface expression of CD27, CD153, and HLA-DR on Th1-specific CD4^{+} T-cells in response to MTB300 (Figure 4). The TBI and TBI-IMID subjects had a higher frequency of CD27^{+} CD153^{-} HLA-DR^{-} compared to the TB (p=0.0148 and p=0.0434 respectively). TB patients had a higher frequency of CD27^{-} CD153^{-} HLA-DR^{+} CD4^{+} T cells compared to TBI (p=0.0265) or TBI-IMID (p=0.0295) subjects. Despite the low number of subjects evaluated, the TB-IMID individuals showed an activation profile like the TB patients, with a higher frequency of CD27^{-} CD153^{-} HLA-DR^{+} CD4^{+} T cells compared to TBI (p=0.0429).

CD27, CD153, and HLA-DR evaluation on Th1 CD4^{+} T cells in response to SEB: we found a similar distribution of the activation markers among groups with a prevalent proportion of the CD27^{+} CD153^{-} HLA-DR^{-} CD4^{+} T-cell subset (Supplementary Figure S4).

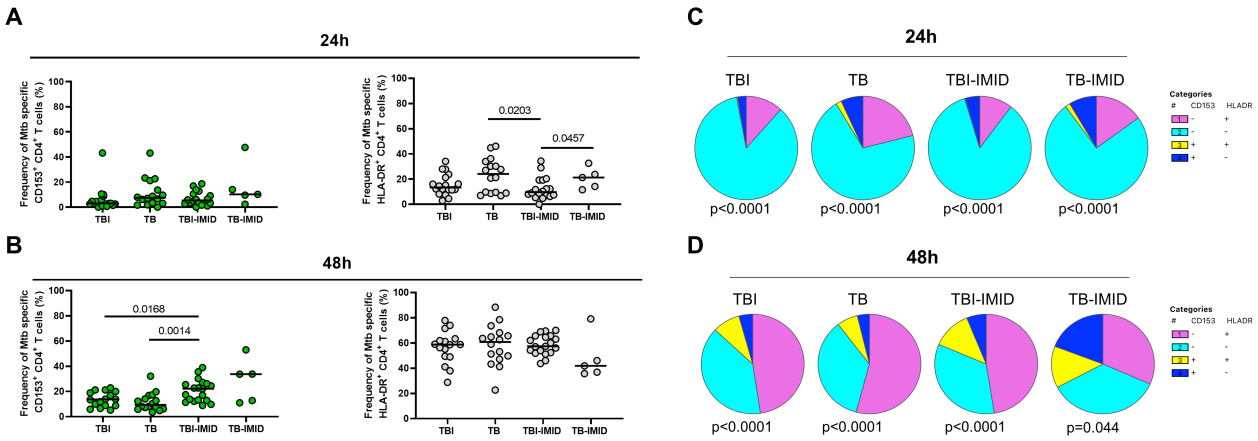

FIGURE 3
HLA-DR- and CD153 expression on Mtb-specific CD4^{+} T cells in TB and TBI subjects with and without IMID. PBMCs were stimulated with Mtb-specific antigens (MTB300) for 24 hours and 48 hours and the immune response was evaluated by flow-cytometry. The activation profile (CD153$^{+/-}$ HLADR$^{+/-}$) of Mtb specific T cells was evaluated only among the responders. **(A)** Activation profile of antigen-specific response, defined as total CD4 Th1 cytokine-producing cells; horizontal black lines indicate the median and each dot represents a single subject. **(B)** Activation profile of antigen-specific response, defined as CD25^{+} CD134^{+} CD4^{+} T- cell response; horizontal lines indicate the median and each dot represents a single subject. **(C)** Pie charts representing the proportion of different CD153$^{+/-}$HLADR$^{+/-}$ CD4^{+} Th1 cytokine-producing cells. **(D)** Pie charts representing the proportion of different CD153$^{+/-}$HLADR$^{+/-}$ CD25^{+} CD134^{+} CD4^{+} T cells. Statistical analysis was performed using the Mann-Whitney test and Wilcoxon test. TB, tuberculosis; TBI, tuberculosis infection; IMID, immune-mediated inflammatory disease; h, hours.

Memory profile of Mtb-specific CD4 Th1 cells in TB and TBI subjects with and without IMID

Then, we investigated if the IMID status may affect the specific response to MTB300, by characterizing the memory profile after 24h of incubation. To have enough events to be analyzed, we evaluated the expression of CD45RA and CD27 within the Mtb-specific T cells producing any Th1 cytokines (IFN-γ, TNF-α, or IL-2). We identified the naïve (N) T cells as CD45RA$^+$ CD27$^+$, the central memory (CM) as CD45RA$^-$CD27$^+$, the effector memory (EM) as CD45RA$^-$CD27$^-$ and the effector (E) as CD45RA$^+$CD27$^-$ (Figure 5) (24). In all groups, independently of the IMID status, the MTB300-specific T cells were mainly CD45RA$^-$CD27$^+$ (CM) and this subset was significantly higher in the TBI compared to the TB (p=0.024) (Figure 5B). Both TB and TB-IMID groups showed an increased frequency of the double negative CD45RA$^-$CD27$^-$ MTB300-specific CD4 T cells (EM), this difference was significant comparing TB and TBI-IMID subjects (p=0.0417). Note that TBI-IMID individuals showed a high frequency of CD45RA$^+$ CD27$^+$ T cells (N) compared to the others. In all groups, we found a low frequency of the CD45RA$^+$ CD27$^-$ T cells (E) (Figure 5B). Within each group, as shown by the pie charts, the distribution of the different memory subsets was statistically significant (Figure 5A). In response to SEB, we did not find significant differences among groups. However, we found an expansion of the CD45RA$^+$CD27$^+$ (N) and a reduction of the CD45RA$^-$CD27$^-$ T cells (EM) compared to the MTB300 response (Supplementary Figure S5). Overall, our data indicated that IMID status did not strongly affect the memory profile. Furthermore, we showed that TB patients, regardless of the IMID status, had a higher proportion of Mtb-specific CD45RA$^-$CD27$^-$ T cells (EM) than TBI subjects, as TB patients downregulated the CD27 expression (30, 58).

The long-term Mtb stimulation increases the IFN-γ production in subjects with different TB status independently of IMID

We next evaluated if the IMID subjects were able to improve their Mtb-specific T-cell response after 6 days of Mtb antigen stimulation. In a subgroup of individuals (Supplementary Table S2), PBMCs were long-term cultured and the Mtb-specific IFN-γ response was evaluated by ELISA. The supernatants were collected after 1 day and after 6 days of incubation (Figure 6). The IFN-γ production significantly increased after 6 days of stimulation (TBI: p<0.0001; TBI-IMID: p<0.0001; TB: p<0.0001) (Figure 6A). To select a cut-off of the MTB300-stimulation test, we performed a ROC analysis comparing HC and TB patients (day 1: AUC 0.85, p=0.0011; day 6 AUC 0.84, p=0.0016) (Supplementary Figure S6). We selected 0.2750 IU/mL as day 1 cut-off (73% sensitivity and 90% specificity) and 2.1750 IU/mL for day 6 (73% sensitivity and

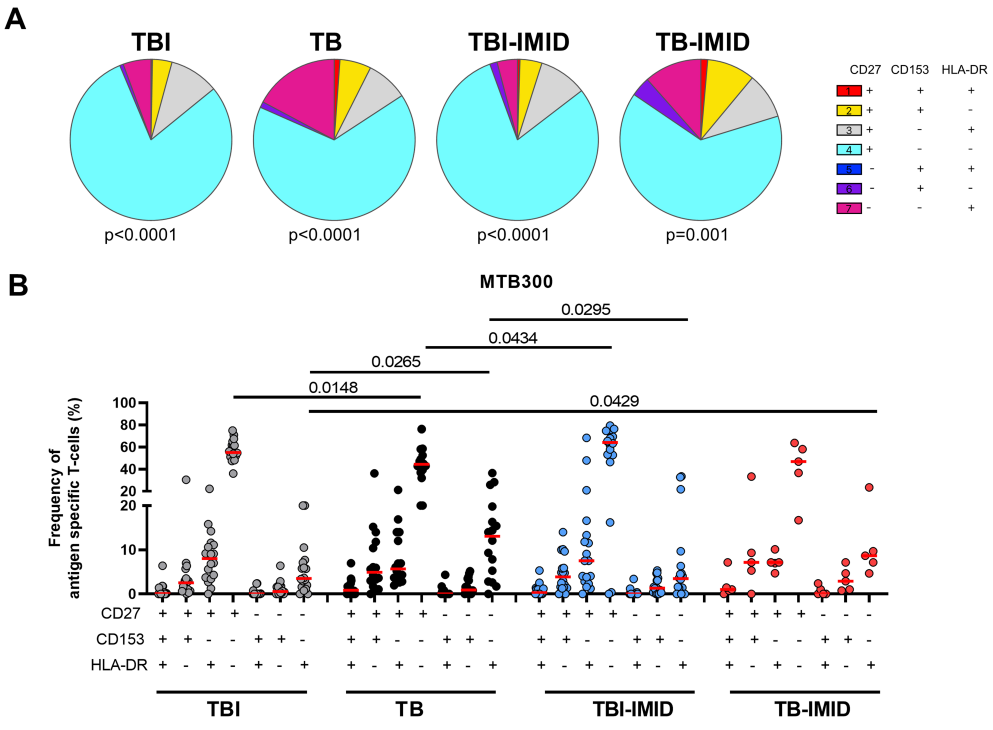

FIGURE 4
Activation profile of Mtb-specific CD4 Th1 cells in TB and TBI subject with and without IMID. PBMCs were stimulated with Mtb-specific antigens (MTB300) for 24 hours and the immune response was evaluated by flow-cytometry. Antigen-specific response was defined as total CD4 Th1 cytokine-producing cells and the activation profile was evaluated only among the responders. **(A)** Pie charts representing the proportion of CD27$^{+/-}$ CD153$^{+/-}$HLADR$^{+/-}$ CD4 T-cell subsets; Wilcoxon matched-pairs signed rank test were applied. **(B)** Frequency of antigen-specific CD27$^{+/-}$ CD153$^{+/-}$ HLADR$^{+/-}$ CD4 T-cell subsets. Horizontal red lines indicate the median and each dot represents a single subject. Statistical analysis was performed using the Mann-Whitney test. TB, tuberculosis; TBI, tuberculosis infection; IMID, immune-mediated inflammatory disease.

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100% specificity). Based on the selected cut-off we found that 46.7% of TBI, 70% of TB, and 50% of TBI-IMID scored positive to MTB300 at day 1, while 73% of TBI, 70% of TB, and 61% of TBI-IMID had a positive response after 6 days of stimulation (day 1 vs day 6: TBI p=0.0350; TB p>0.9999; TBI-IMID p=0.8064) (Table 4). Evaluating the rate of change, we found the highest increase in TBI subjects compared to the other groups (Table 4).

We then investigated whether the ability to retrieve a specific response was related to the memory T cells. We focused on patients with a positive long-term response after 6 days of stimulation and scored positive for an Mtb-specific Th1-response after 24 hours of stimulation. In all groups, we observed a significant predominant memory-subset responsible for the increased IFN-γ production reported at day 6 (p<0.0001) (Figure 6B).

The long-term Mtb stimulation increases the IFN-γ production in TBI-IMID patients scored QFT-plus positive and QFT-plus negative

We focused on the TBI-IMID cohort stratifying subjects according to the QFT-Plus results (Figure 7, Supplementary Table S2). The frequency of IFN-γ production increased in both TB-IMID QFT-Plus positive and QFT-Plus negative (p=0.0156; p=0.0023 respectively). Applying the selected cut-off, at day 6, 4/9 QFT-Plus negative (44%) had a positive long-term response, and 14/21 QFT-Plus positive (67%) had a positive long-term response. Both groups showed a high frequency of $CD45RA^{-}$ $CD27^{+}$ and $CD45RA^{-}$ $CD27^{-}$ Th1-specific CD4 T cells (p=0.0001) (Figure 7B). Subjects were also evaluated for their ability to produce Th1-specific cytokines in response to MTB300 (Figure 7C). The total Th1-specific response was comparable among groups.

MGIA response in TB and TBI subjects with and without IMID

To evaluate if the IMID status impaired the ability of PBMCs to control mycobacteria replication, we used the MGIA, using the vaccine strain BCG, in a subgroup of individuals with different TB statuses and with or without IMID (Figure 8). Clinical and demographical characteristics are reported in Supplementary Table S3. The MGIA response, expressed as the time to positivity (TTP) (59), was compared among different groups (Figure 8). No TTP differences were observed among groups, indicating that TBI-

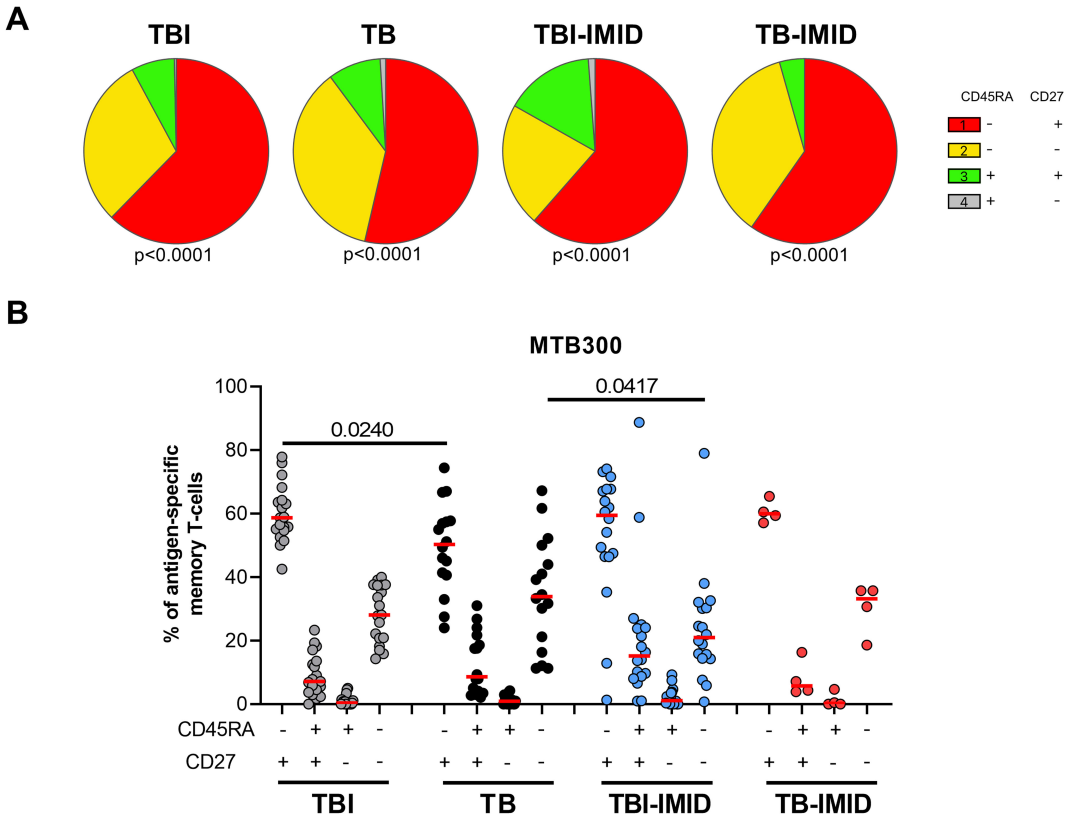

FIGURE 5
Memory profile of Mtb-specific CD4 Th1 cells in TB and TBI subject with and without IMID. PBMCs were stimulated with Mtb-specific antigens (MTB300) for 24 hours and the immune response was evaluated by flow-cytometry. Antigen-specific response was defined as total CD4 Th1 cytokine-producing cells and the memory profile was evaluated only among the responders. **(A)** Pie charts representing the proportion of $CD27^{+/-}$ $CD45RA^{+/-}$ CD4 T-cell subsets; Wilcoxon matched-pairs signed rank test were applied. **(B)** Frequency of antigen-specific $CD27^{+/-}$ $CD45RA^{+/-}$ CD4 T-cell subsets. Horizontal red lines indicate the median and each dot represents a single subject. Statistical analysis was performed using the Mann-Whitney test. N, number; TB, tuberculosis; TBI, tuberculosis infection; IMID, immune-mediated inflammatory disease.

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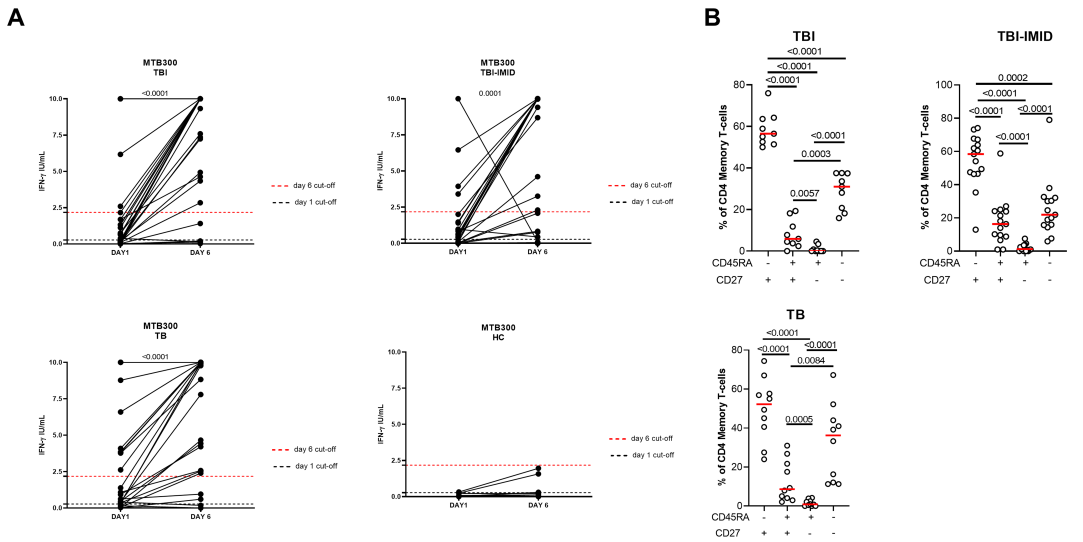

FIGURE 6
Long-term Mtb stimulation increases the IFN-γ production in subjects with different TB status with and without IMID and healthy controls. **(A)** PBMCs were stimulated with Mtb-specific antigens (MTB300) for 6 days in the presence of IL-2. IFN-γ was evaluated by ELISA on day 1 and day 6 on supernatants. **(B)** Memory profile of Mtb-specific CD4 Th1 cells. PBMCs were stimulated with Mtb-specific antigens (MTB300) for 24 hours and the immune response was evaluated by flow-cytometry. Antigen-specific response was defined as total CD4 Th1 cytokine-producing cells and the activation profile was evaluated only among the responders. The graph represents the frequency of MTB300-specific $CD27^{+/-}$ $CD45RA^{+/-}$ CD4 T-cell subsets of subjects tested in the long-term stimulation assay. Horizontal red lines indicate the median and each dot represents a single subject. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test. TB, tuberculosis; TBI, tuberculosis infection; IMID, immune-mediated inflammatory disease; HC, healthy control; IFN-γ, interferon-gamma.

IMID could control the mycobacteria replication like non-IMID individuals.

Discussion

Summary

In this study, we showed that the memory and activation status of individuals with TBI-IMID are similar to those of patients with TB or TBI without IMID. This finding suggests that TBI-IMID individuals possess effective Mtb-specific immunity and can control mycobacterial replication, as assessed by MGIA, just like non-IMID subjects. Additionally, TB-IMID patients exhibit a cytokine response and activation profile similar to TBI individuals without IMID. Thus, other immunological mechanisms might account for the impaired Mtb-specific immunity in this susceptible IMID group.

Mtb-specific T cells

To evaluate Mtb-specific immunity besides the response to ESAT-6 and CFP-10 (RD1 antigens) evaluated by QFT-Plus, we used a different Mtb-peptide pool, the "MTB300-peptide-megapool" containing a mixture of 300 Mtb-derived T-cell epitopes from 90 Mtb proteins (including ESAT-6 can CFP10) targeting a large fraction of Mtb-specific CD4+ T cells and sharing epitopes with NTM species (52–54). All groups' patients respond to MTB300-stimulation in terms of cytokine-production or AIM^+ $CD4^+$ T cells. TB therapy reduces the Mtb load in the host, leading to a decreased Mtb-specific immune response (60–62). In our experimental setting, half of TB-IMID patients have been enrolled during TB therapy. Although a limited number of patients enrolled, the TB-IMID cohort showed a similar level of cytokine-producing $CD4^+$ T cells or AIM^+ $CD4^+$ T cells compared to TB individuals enrolled before starting the TB therapy.

TABLE 4 Number of responders to *in vitro* long-term stimulation with MTB300.

Group	Number of responders				
	Day 1 (Cut-off=0.275)	Day 6 (Cut-off=2.175)	p*	Rate of change	p**
TBI [N/total (%)]	14/30 (46.7)	22/30 (73)	**0.0350**	1036.56	0.0772
TB [N/total (%)]	19/27 (70)	19/27 (70)	>0.9999	213.20	
TBI-IMID [N/total (%)]	14/28 (50)	17/28 (61)	0.8064	930.66	

N, number; TB, tuberculosis, TBI, tuberculosis infection, IMID, inflammatory mediated immune disease; * Chi-Square test; **Kruskal- Wallis test. Significant p values are reported in bold.

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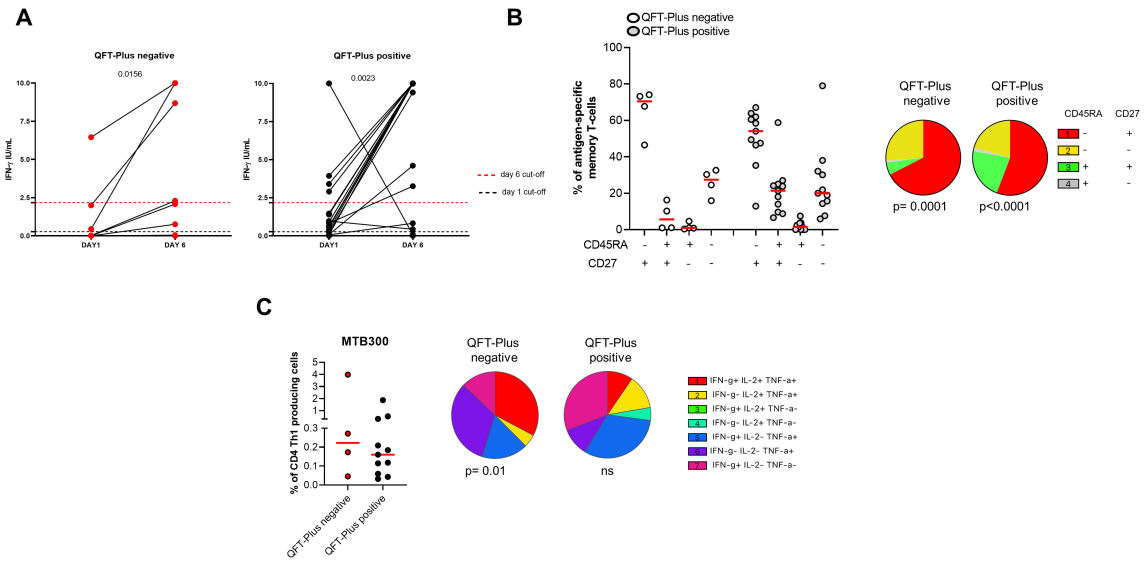

FIGURE 7
Long-term Mtb stimulation increases the IFN-γ production in TBI-IMID individuals stratified according to the QFT-Plus response. **(A)** PBMCs were stimulated with Mtb-specific antigens (MTB300) for 6 days in the presence of IL-2. IFN-γ was evaluated by ELISA at day 1 and day 6 on supernatants in TBI-IMID QFT-Plus negative and QFT-Plus positive. **(B)** Memory profile of Mtb-specific CD4 Th1 cells. PBMCs were stimulated with Mtb-specific antigens (MTB300) for 24 hours and the immune response was evaluated by flow-cytometry. Antigen-specific response was defined as total CD4 Th1 cytokine-producing cells and the activation profile was evaluated only among the responders. **(C)** Antigen-specific response evaluated as total CD4 Th1 cytokine-producing cells after 24 h of stimulation. Pie charts represent the proportion of different cytokine-producing $CD4^+$ T-cell subsets. Horizontal red lines indicate the median and each dot represents a single subject. Statistical analysis was performed using the Mann Whitney test and the Wilcoxon matched pairs signed rank test. TB, tuberculosis; TBI, tuberculosis infection; IMID, immune-mediated inflammatory disease; IFN-γ, interferon-gamma; IL-2, interleukine 2; TNF-α, tumor necrosis factor alpha.

All groups' patients, independently of IMID and TB status, had polyfunctional $CD4^+$ T cells producing at least two cytokines and monofunctional $CD4^+$ T cells for IFN-γ or TNF-α as previously demonstrated for MTB300 (52). According to the literature, the polyfunctional response is associated with infection control in the case of HIV and *L.major* infections (63, 64), whereas controversial data are available for TB (23). In our study, TBI-IMID showed a cytokine profile like TBI and TB patients, indicating that the Mtb-specific cytokine response is not altered by the IMID status. Differently, TBI individuals living with HIV infection had a lower percentage of cytokine-producing Mtb-specific $CD4^+$ T cells and a decrease of the double positive IFN-γ^+ IL-2^- TNF-α^+ $CD4^+$ T cells (65). These results indicate different mechanisms characterizing the groups at high TB risk such as the IMID subjects and the people living with HIV. A higher polyfunctional response associated with the TBI-IMID status and a monofunctional response characterized the TB-IMID patients, suggesting a loss of polyfunctional $CD4^+$ T-cell response in TB disease. Since half of TB-IMID patients have been enrolled during TB therapy, this status may lead to a monofunctional response switching. Our recent findings support these data, indicating that TB therapy did not significantly impact the cytokine response in TBI-IMID. However, it did result in a notable reduction of triple functional CD4 T cells in both TBI subjects and TB patients (44). Despite the small size of the TB-IMID cohort, we present these findings as describing such a rare group warrants further research to explore any potential link between Mtb-specific responses and TB outcomes. Lastly, we believe these findings could aid in developing new correlates of protection for patients with varying TB statuses.

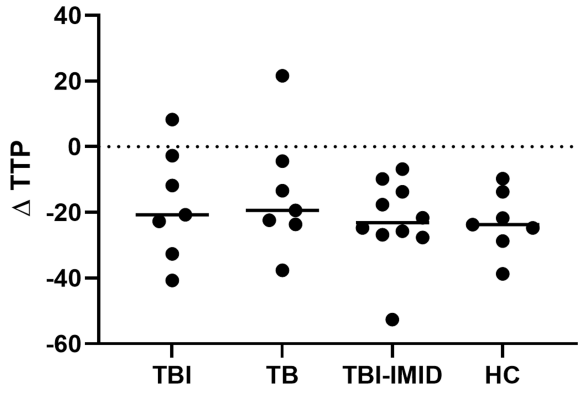

FIGURE 8
Comparison of MGIA response as TTP in TBI, TB, TBI-IMID, and healthy control individuals. TPP is expressed in hours. As an experimental control, the bacterial inoculum used was added directly to an MGIT tube. The TTP of experimental control was subtracted by the TPP of each experimental condition. Data are represented as the median. Mann-Whitney test was applied. TB, tuberculosis, TBI, TB infection; TTP, time to positivity; HC, healthy control.

According to the literature, the antigen-specific response can be investigated by the co-expression of CD25 and CD134 through the AIM assay (25). Our results indicated that IMID-therapy does not affect the count of either cytokine or AIM responders. The AIM assay reveals a stronger immune response than the intracellular

cytokine assay, making the differences between groups more evident. This finding is supported by an earlier study that demonstrated the AIM assay's superior capability in detecting antigen-specific T cells compared to cytokine-producing T cells (66).

Memory status of Mtb-specific CD4$^+$ T cells

Given the conflicting data on the relationship between cytokine profiles and TB protection, other elements of the immune response, such as the memory and activation status of antigen-specific T cells, were evaluated.

Here we showed that TBI-IMID patients were characterized by an Mtb-specific memory response CD45RA$^-$ CD27$^{+/-}$, as previously described in TB and TBI individuals (24, 67–69). In particular, the Mtb-specific CD45RA$^-$ CD27$^-$ CD4$^+$ T cells (effector memory) characterized the TB patients, whereas the Mtb-specific CD45RA$^-$ CD27$^+$ CD4$^+$ T cells (central memory) characterized the TBI individuals. These results highlight the role of the different T-cell subsets contrasting the Mtb replication. The presence of Mtb at a low replication rate during the TBI status constantly stimulated a central memory response fundamental to containing the Mtb load. Vice versa, during the TB disease, the frequency of Mtb-specific effector memory T cells increases to actively contrast the Mtb replication. These findings are consistent with earlier studies on patients with varied TB status, both with and without IMID (24, 44), and those with HIV (67).

The memory phenotype is fundamental for the increased IFN-γ-specific response after MTB300-long-term stimulation. It has been demonstrated that TBI-IMID subjects have a low IFN-γ response to QFT-Plus and a high proportion of results in the uncertain range (70). The long-term stimulation allows the recovery of the Mtb-specific response in TBI-IMID scored negative to QFT-Plus, suggesting this approach as an alternative diagnostic tool in this category of TBI subjects. Note that TBI-IMID had a high proportion of Mtb-specific CD45RA$^+$ CD27$^+$ CD4$^+$ T cells and this proportion was lower in TBI-IMID QFT-Plus negative compared to QFT-Plus positive. Since the CD45RA$^+$ CD27$^+$ T-cell subset could contain the stem cell memory T cells CD45RA$^+$ CD27$^+$ CCR7$^+$, as already demonstrated in TBI individuals (71), this subset may play a role in our TBI-IMID cohort modifying the phenotype of Mtb-specific T cells.

Activation status of Mtb specific CD4$^+$ T cells

Studies in animal models have demonstrated that Mtb-specific CD4 T cells expressing CD153 are protective against Mtb infection (28, 29, 72, 73). Moreover, the Mtb-specific CD153$^+$ CD4$^+$ T cells are inversely proportional to bacterial load and TB severity in patients with TB disease (29). In this study, we observed an increased CD153 expression only after 48 hours of MTB300-stimulation in TBI-IMID and TBI individuals. The TBI-IMID showed an increase of Mtb-specific CD153$^+$ CD4$^+$ T cells compared to TBI, supporting the role of autoimmunity in the CD153-expression (74). Age could be an important factor influencing this result, considering that our study population was older (Table 2) than the cohorts of previous studies describing the CD153-expression on Mtb-specific T cells (28, 29, 33). Since the expression of CD153 has been associated with senescence-associated T-cell impairment (75), we retain that age could be an important factor influencing this result. Based on this evidence, probably other mechanisms associated with immune-senescence affected the CD153 expression on Mtb-specific CD4$^+$ T cells. Also, the origin of our patients may have had an impact on CD153 expression. Our TBI population was mainly from low TB endemic countries, whereas the previous studies were performed in Africa (28, 29, 33), where repeated exposures to Mtb could affect the Mtb-immunity and modify the activation status.

The expression of HLA-DR on Mtb-specific T cells is increased in TB after 24 hours of MTB300-stimulation, as previously shown (28, 33, 34). Moreover, the HLA-DR expression increased in all groups after 48 hours of Mtb stimulation indicating a time-dependent differentiation of CD4$^+$ T cells (66). The TB-IMID patients seem to have a high proportion of activated Mtb-specific HLA-DR$^+$ CD4$^+$ T cells, like TB patients. Differently, in TBI-IMID, the proportion of Mtb-specific HLA-DR$^+$ CD4$^+$ T cells is like TBI, demonstrating a comparable activation status independent of the IMID comorbidity. Interestingly, the expression of HLA-DR and CD27 on Mtb-specific CD4$^+$ T cells demonstrated a dichotomous profile: the Mtb-specific CD27$^-$ CD153$^-$ HLA-DR$^+$ CD4$^+$ T cells associated with TB status and the Mtb-specific CD27$^+$ CD153$^-$ HLA$^-$ DR$^-$ CD4$^+$ T cells associated to TBI status, indicating a highly differentiated profile in patients with TB (24).

Mycobacterial growth inhibition assay

In this study we aimed to characterize the Mtb-specific immunity, however, we included the MGIA experiment to have a global evaluation of the immune defense against mycobacteria, looking simultaneously at the innate and adaptive compartments. Monocyte subsets are key cells of the innate immune response and are fundamental to stimulating adaptive immunity acting as antigen-presenting cells. It has been demonstrated that the monocytes/lymphocytes (M/L) ratio has a predictive value for TB disease, in particular, a high M/L is associated with the TB disease (76). MGIA is a largely used test to monitor the vaccine response and a powerful tool to indirectly test the presence of functional T cells and competence of the innate compartment (37, 38). Our results demonstrated that TBI-IMID had the same ability as TBI and TB to contain the mycobacteria replication evaluated by MGIA.

Translational application of the study

Since the BCG-based vaccine is the only TB vaccine licensed and has a low efficacy for protecting adolescents and adults from TB disease, the development of new vaccine strategies is the main goal of the TB control programs (77). The fragile population of TBI-IMID patients is a potential target of the new vaccine approach, and

its immunological characterization is fundamental to develop new biomarkers of protection and disease and to develop alternative therapeutic strategies based on host-direct therapy (78). Since few studies are available on Mtb-immunity in IMID patients (44, 48, 70), we contributed to build the immunological story characterizing the TBI-IMID subjects.

Limits of the study and future directions

The non-homogeneous IMID cohort may limit the solidity of data, however, the different types of ongoing IMID-therapy did not have an impact on the number of responders, as previously shown (44, 70). Due to the availability of cells, not all patients were characterized using the same methodology. Nonetheless, the statistically significant differences observed among the groups validate the consistency of our results. The immunological scenario of IMID is large and complex, however, we tried to summarize a particular aspect of the disease that increases the risk of TB development. Larger studies are needed to elucidate other features of the Mtb-induced immunity. In particular the investigation of specific mechanisms that increase the TB risk in patients undergoing therapy such as IL-6 or JAK inhibitors (19). Although the number of TB-IMID patients enrolled was low, we included this population because it was an opportunity to study Mtb immunity in a rare cohort, larger studies are necessary to eventually identify a correlation between the Mtb-specific immune response and TB outcome. Since TBI-IMID subjects had remote exposure to Mtb and TBI had a recent TB contact, future studies should include TBI with a remote TB exposure. It is known that only 5% of TBI individuals progress to TB disease within the first 5 years from the TB contact (79), whereas only 2% evolve to TB disease during their lifetime (80). Studying TBI individuals with different statuses of TB exposure is a useful approach to understand which type of immunity promotes the eradication or the efficient containment of Mtb. In low TB-endemic countries, TBI patients with remote exposure are not eligible for TB preventive therapy (22) and therefore the evaluation over time of their Mtb immunity could contribute to describing the mechanisms of Mtb containment.

Besides these limitations, we deeply characterized the immune response to Mtb of patients with different TB status in the presence or absence of IMID, showing that IMID status did not affect the Mtb immunity. Recently, it has been demonstrated that Mtb DNA can be detected in PBMC from subjects with TBI and TB disease. In particular, the $CD34^+$ cells represent a niche for Mtb (81). This approach could be applied to immunological studies to find a correlation between the Mtb-specific immune response and the presence of Mtb in the circulating reservoir.

Conclusions

We added a contribution to the knowledge of Mtb immunity in this fragile cohort to TBI-IMID subjects demonstrating that TB-IMID patients had a cytokine response, a memory and activation profile, and an ability to contain the mycobacteria replication similar to TBI individuals without IMID. The immune tools available do not completely explain the mechanisms of impairment of Mtb-specific immunity in this vulnerable population and new approaches are needed to overcome this limitation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The raw data are available in our institutional repository (rawdata.inmi.it), subject to registration. The data can be found by selecting the article of interest from a list of articles ordered by year of publication. No charge for granting access to data is required. In the event of a malfunction of the application, the request can be sent directly by e-mail to the Library (biblioteca@inmi.it).

Ethics statement

The studies involving humans were approved by Ethical Committee of the National Institute for Infectious Diseases Lazzaro Spallanzani-IRCCS. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CF: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Visualization. AGA: Methodology, Writing – review & editing. ASa: Methodology, Writing – review & editing. VV: Methodology, Writing – review & editing. GC: Data curation, Writing – review & editing. CA: Resources, Writing – review & editing. ASe: Resources, Writing – review & editing. GD: Resources, Writing – review & editing. IP: Writing – review & editing, Resources. SS: Methodology, Writing – review & editing. AA: Writing – review & editing, Methodology. AP-D: Writing – review & editing, Data curation. GG: Writing – review & editing, Data curation. FP: Writing – review & editing, Data curation. DG: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing, Investigation. EP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funding sources did not influence the study's design, data analysis, interpretation, or the writing of the manuscript.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1484143/full#supplementary-material

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Coexistent anemia modulates systemic inflammation and exacerbates disease severity and adverse treatment outcomes in tuberculosis

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Introduction: Anemia has been shown to be an independent predictor of disease progression and death in tuberculosis (TB) patients, significantly impacting TB in several ways. This dual burden poses significant challenges for TB control efforts. However, the mechanism by which anemia influences disease severity, bacterial burden, and TB treatment outcomes remains poorly understood.

Methods: In this study, we aimed to compare bacterial burdens, disease severity, and TB treatment outcomes in TB patients with or without anemia. Participants were recruited from Chennai, South India, as part of the prospective Effect of Diabetes on Tuberculosis Severity (EDOTS) study conducted from February 2014 to August 2018. Anemia was defined as hemoglobin (Hb) levels <13 g/dL and <12 g/dL for males and females, respectively. We employed chest X-rays to assess bilateral lung and cavitary diseases and sputum smear grades to measure bacterial loads in TB subjects. Treatment outcomes were defined as favorable or unfavorable. Cytokine profile was measured using multiplex ELISA.

Results: The study comprised of 483 culture-confirmed TB individuals, with 288 positives for anemia {Median Hb was 11.0 [interquartile range (IQR)], 10.3–12.3} and 195 negatives [Median Hb was 14.3 (IQR), 13.5–15.2]. The study revealed that TB patients with anemia had significantly higher bacterial loads [adjusted prevalence ratio (aPR), 4.01; 95% CI, 2.22–6.63; $p < 0.001$], cavitary lung lesions [aPR, 3.36; 95% CI, 1.95–5.68; $p < 0.001$] and unfavorable treatment outcomes [aPR, 1.61; 95% CI, 1.31–2.19; $p = 0.046$] compared to those without anemia. Our data also show that TB is associated with significantly lower levels of type-1 cytokines (IFNγ and IL-2) but significantly higher levels of pro-inflammatory cytokines (IL-6, IFNα, and IFNβ) and pro-fibrotic factors (VEGF, EGF, FGF-2, and PDGF-AB/BB) in anemic individuals compared to those without anemia.

Conclusions: These findings highlight a clear association between anemia and increased TB severity, elevated bacterial loads, and poor treatment outcomes. Our data also suggest that anemia might be associated with the modulation of cytokine responses, which could impart a detrimental effect on TB pathogenesis.

KEYWORDS

anemia, tuberculosis, cytokines, disease severity, treatment outcomes

Introduction

Tuberculosis (TB) remains a major global health threat, resulting in millions of new cases and fatalities annually (1). Anemia is a common risk factor and hematological abnormality associated with TB, with a prevalence of 20–94% in TB patients (2, 3). On the contrary, the likelihood of TB among anemic patients is higher than non-anemic patients (2–4). Anemia is defined as the insufficiency of erythrocyte mass to deliver adequate oxygen to peripheral tissues (5). TB is known to cause "anemia of inflammation" a condition in which systemic inflammation may change iron metabolism and lower red blood cell counts (6). The reduction of erythropoiesis by inflammatory indicators, malabsorption syndrome, and nutritional inadequacies are elucidated as the underlying pathophysiology of anemia in TB patients (7).

Anemia profoundly impacts the course and severity of TB in several ways and has been found to be an independent predictor of disease progression and fatality in TB patients (8, 9). TB patients with anemia have heavy sputum bacillary load and worsened pulmonary infection (10, 11). Studies indicate that anemia is associated with more severe forms of TB and unfavorable disease outcomes, including increased mortality rates and extended treatment periods (12–17). Anemia may increase the risk of complications like pulmonary dysfunction due to larger infectious zones in the lungs, further aggravating TB outcomes (18, 19). Additionally, anemia exacerbates the adverse impacts of TB medications, including gastrointestinal disorders and hepatotoxicity (20, 21).

Cytokines from the innate and adaptive immune systems play crucial roles in orchestrating the immune response to TB. Immune alterations favor the survival, multiplication, and dissemination of *Mycobacterium tuberculosis* (Mtb) and associated sequelae (22, 23). Cell-mediated Th1 immunity, coordinated by Interferon (IFN)-γ, is required to suppress Mtb inside macrophages at the infection site in the lung (24, 25). Th1 cytokines typically activate macrophages and cytolytic T cells to kill intracellular Mtb via the induction of reactive oxygen and nitrogen species, antimicrobial peptides, and autophagy (26). Conversely, Th2 cytokines, such as Interleukin (IL)-4 and IL-13, induce anti-inflammatory reactions that impede pathological inflammation while concurrently impeding macrophage and T cell capacity to efficiently eliminate Mtb (27). Pro-inflammatory cytokines such as IL-6 are multifunctional cytokines that play a crucial role in regulating the immune response, inflammation, and hematopoiesis and are key mediators of anemia of inflammation (24, 28). However, expression of these cytokines in immune responses to TB in anemic individuals have not been explored in detail, and clear data on their impact on bacterial burdens, disease severity, and treatment outcomes are lacking.

To address this knowledge gap, our study aimed to compare bacterial burdens, disease severity, and TB treatment outcomes in TB patients with or without anemia. Moreover, to explore the immunological underpinnings of the interaction between anemia and TB, we examined circulating plasma levels of a large panel of cytokines and pro-fibrotic factors in TB patients with or without anemia.

Materials and methods

Ethics statement

The study was approved by the ethics committees of the National Institute for Research in Tuberculosis (NIRT) and the Prof. M. Viswanathan Diabetes Research Center (MVDRC; ECR/51/INST/TN/2013/MVDRC/01).

Patient consent statement

Informed written consent was obtained from all participants, and study procedures adhered to institutional ethical guidelines.

Study population and data variables

Participants were recruited from Chennai, South India, as part of the prospective Effect of Diabetes on Tuberculosis Severity (EDOTS) study conducted from February 2014 to August 2018. Anemia was diagnosed based on WHO criteria (hemoglobin concentration <12 g/dL in women and <13 g/dL in men) (29). The study included adult individuals aged 25–73 who were newly diagnosed with positive sputum smears and culture. All the participants were screened for diabetes and nutritional indices. Smoking and alcohol consumption status were recorded. Exclusion criteria were previous TB episodes, prior TB treatment, drug-resistant TB, positive HIV status, use of immunosuppressive medications, pregnancy, and lactation. A complete blood count was done on all samples in a DxH 520 hematology analyzer (Beckman Coulter). Anthropometric measurements (height, and weight), and biochemical parameters were procured using standardized techniques. Low body mass index (LBMI) was described based on the American Heart Association/American College of Cardiology guidelines (LBMI $\leq$ 18.5 kg/m^2), overweight by body mass index (BMI) 25–29.9 kg/m^2, and obesity defined by BMI threshold of $\geq$30.0 kg/m^2. Diabetes was defined as an glycated hemoglobin (HbA1c) reading of 6.5% or greater and a fasting blood glucose of $\geq$126 mg/dl, according to the American Diabetes Association criteria. A sample of the individuals with a result of total cholesterol (TC) < 130 mg/dl, triglyceride (TG) < 90 mg/dl, low-density lipoprotein cholesterol (LDL-C) < 100 mg/dl, and high-density lipoprotein cholesterol (HDL-C) < 40 mg/dl were considered as hypolipidemic while individuals with the result of TC $\geq$ 200, TG $\geq$ 150, LDL-C $\geq$ 130 mg/dl, or HDL-C > 40 mg/dl were classified as hyperlipidemic. Vitamin D deficiency was defined as <30 ng/mL. High or low serum albumin were determined according to serum albumin level of $\geq$ or < 3.9 g/dl. Chest X-rays were utilized to assess the presence of bilateral lung disease and cavitary lesions and chest x-rays were read by two independent radiologists. Sputum smear grades were used to measure bacterial loads in individuals with TB and classified as 0, 1+, 2+, and 3+ with 0 being no bacteria in microscopy and 3+ the highest number of bacteria. The laboratory investigators were blinded to the chest x-ray and bacteriology results. All recruited TB patients

received anti-TB treatment through Directly Observed Treatment Short Course (DOTS) therapy as per WHO recommendations, monitored by the National Tuberculosis Elimination Program (NTEP). Follow-up extended through 6 months of treatment and 1-year post-treatment completion. Treatment outcomes were defined as favorable or unfavorable. Favorable treatment outcome (cure) was defined as negative results of sputum cultures at months 5 and 6 of treatment without recurrent disease during follow-up. Unfavorable treatment outcomes included treatment failure defined as positive sputum culture results at month 5 or 6, all-cause mortality, or recurrent TB within 12 months after initial cure. These participants did not receive any treatment for anemia.

Multiplex assays

Circulating plasma cytokines and pro-fibrotic levels were measured in a subset of anemic ($n = 288$) and non-anemic ($n = 195$) TB individuals using multiplex Luminex assay (Bio-Rad Laboratories, Inc.). The analytes measured included cytokines [Interferon (IFN)-γ, Interleukin (IL)-2, Tumor Necrosis Factor (TNF-α), IL-4, IL-5, IL-6, IL-13, IFN-α, and IFN-β] and pro-fibrotic factors (Vascular endothelial growth factor (VEGF), Epidermal growth factor (EGF), Fibroblast growth factor (FGF-2), and Platelet-derived growth factor (PDGF)-AB/BB). The experiment was conducted according to the manufacturer's instructions (R&D Systems).

Statistical analysis

Before analysis, the data was thoroughly checked for completeness and consistency. Continuous variables were examined for normality using the Shapiro-Wilks test and were found not to be normal. The data was then presented using frequency, percentages, median and quartiles. Measurements of central tendency utilized geometric means (GMs). Differences in continuous variables between the two groups were examined using the Wilcoxon rank sum test, while the relationship between groups and factors such as sputum smear grade, bilateral lung lesion, cavitary lesion, and TB treatment failure and relapse were examined using the Pearson chi-square test. Statistically significant differences between two groups were analyzed using the non-parametric Mann-Whitney *U*-test with Holm's correction for multiple comparisons. Generalized linear models with binomial regression and log-link functions were used to identify key factors. The selection of covariates for the regression model was determined based on data availability, a review of relevant literature, and the opinions of subject matter experts. Prevalence ratios (PR) and adjusted prevalence ratios (aPR) were calculated along with the corresponding 95% confidence intervals (CIs). Covariates with significant PR, were considered when adjusting for aPR. Data analysis was performed using STATA software, version 15.0 (StataCorp., Texas, USA), with all *P*-values considered two-sided and statistical significance set at the 0.05 α level.

Results

Study population characteristics

The study comprised 483 culture-confirmed TB individuals, with 288 positives (101 male, 187 female) for anemia and 195 negatives (90 male, 105 female). Median age was 45 years [interquartile range (IQR), 36.0–53.0] for participants with anemia and 47 years (IQR, 36.3–52.0) for participants without anemia. There were no statistically significant differences in age, BMI, smoking, alcohol use, and HbA1c between the TB subjects with anemia and those without anemia. However, significant differences were observed in gender ($p = 0.0175$; Table 1) and notable differences in certain hematological and biochemical parameters (Table 2). Individuals with anemia exhibited significantly lower levels of red blood cells (RBC; GM of 4.4 g/dL vs. 5.1 mg/dL; $p < 0.0001$), hemoglobin (Hb; GM of 11 vs. 14.3 g/dL; $p < 0.0001$), and hematocrit (HCT; GM of 34.9 vs. 42.4%; $p < 0.0001$), and elevated monocyte counts (GM of 708.2 vs. 620.1 cells/μL; $p = 0.0404$) compared to subjects without anemia. Additionally, biochemical parameters such as triglycerides (GM of 97.1 vs. 104.1 mg/dL; $p = 0.0310$), total cholesterol (GM of 157.3 vs. 168.2 mg/dL; $p = 0.0083$), LDL (GM of 89.6 vs. 95.4 mg/dL; $p < 0.0001$), total protein (GM of 7.9 vs. 8.2 g/dL; $p = 0.0048$), serum albumin (GM of 3.8 vs. 4.2 g/dL; $p < 0.0001$), and Vitamin D (GM of 15.4 vs. 17.4 IU; $p = 0.0269$) were significantly lower in individuals with anemia compared to subjects without anemia.

Association of clinical co-morbidities with anemia in TB individuals

No significant differences were observed in age, BMI, smoking, alcoholism, or HbA1c) between the two groups (Table 3). However, significant differences were noted in gender (female). The PR for female individuals with anemia was 3.21 (95% CI: 1.52–3.31; $p = 0.009$), and this association remained significant after adjusting for possible confounders (aPR 2.50, 95% CI: 1.90–2.40; $p = 0.028$).

Anemia is associated with increased radiographic TB disease severity and greater bacterial burdens

Anemia was significantly associated with an increased risk of cavitary disease (PR, 4.62; 95% CI, 3.04–7.08; $p < 0.001$) but not of bilateral lung lesions (PR, 2.21; 95% CI, 0.98–3.12; $p = 0.287$). After adjusting for confounding variables, anemia remained significantly associated with a higher risk of cavitation (aPR, 3.36; 95% CI, 1.95–5.68; $p < 0.001$), indicating increased TB disease severity in individuals with anemia. Additionally, anemia was significantly associated with an elevated risk of higher smear grades (PR, 5.51; 95% CI, 3.45–9.34; $p < 0.001$). This association persisted after adjusting for confounders, with anemia remaining significantly associated with increased smear grades (aPR, 4.01; 95% CI, 2.22–6.63; $p < 0.001$), indicating higher bacterial burdens in TB patients with anemia (Table 4).

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TABLE 1 Demographics and clinical characteristics of the study population.

Variable	Overall, $N = 483$[a]	Participants with anemia, $N = 288$ (59.6%)[a]	Participants without anemia, $N = 195$ (40.4%)[a]	p-value
Age in years, median (IQR)	45.0 (36.0 – 52.0)	45.0 (36.0 – 53.0)	47.0 (36.3 – 52.0)	0.649[b]
Age classification (in years), *n* (%)				0.787[c]
Up to 35 years	112 (23.2)	59 (24.1)	53 (22.3)	
36–45 years	136 (28.2)	64 (26.1)	72 (30.3)	
46–55 years	153 (31.7)	80 (32.7)	73 (30.7)	
>55 years	82 (17.0)	42 (17.1)	40 (16.8)	
Gender, *n* (%)				0.0175[c]
Female	292 (60.5)	187 (64.9)	105 (53.8)	
Male	191 (39.5)	101 (35.1)	90 (46.2)	
	20.0 (17.5 – 23.3)	20.3 (17.9 – 23.4)	19.9 (17.5 – 23.0)	0.356[b]
Body mass index (kg/m^2), median (IQR), and body mass index classification (kg/m^2), *n* (%)				
Normal (18.5–24.9 kg/m^2)	190 (39.3)	93 (38.0)	97 (40.8)	0.730[c]
Undernourished (<18.5 kg/m^2)	154 (31.9)	76 (31.0)	78 (32.8)	
Overweight (25.0–29.9 kg/m^2)	91 (18.8)	49 (20.0)	42 (17.6)	
Obesity (≥30 kg/m^2)	48 (9.9)	27 (11.0)	21 (8.8)	
	6.8 (5.7– 10.3)	6.7 (5.7 –10.4)	7.9 (5.8 – 10.4)	0.5014[b]
Glycated hemoglobin (HbA1c) %, median (IQR), and diabetes mellitus (DM) (HbA1c%), *n* (%)				
No, DM (<5.7%)	94 (19.5)	54 (22.0)	40 (16.8)	0.068[c]
Pre, DM (5.7–6.4%)	127 (26.3)	54 (22.0)	73 (30.7)	
DM (≥6.5%)	262 (54.2)	137 (55.9)	125 (52.5)	
Smoking status, *n* (%)				0.191[c]
Non-smoker	139 (28.8)	66 (26.9)	73 (30.7)	
Smoker	125 (25.9)	58 (23.7)	67 (28.2)	
Unknown	219 (45.3)	121 (49.4)	98 (41.2)	
Alcohol use, *n* (%)				0.464[c]
Yes	249 (51.6)	122 (49.8)	127 (53.4)	
No	75 (15.5)	36 (14.7)	39 (16.4)	
Unknown	159 (32.9)	87 (35.5)	72 (30.3)	
Cavitary lung lesions, *n* (%)				<0.001[c]
No, cavitary lung lesions	198 (41.0)	123 (50.2)	75 (31.5)	
Cavitary lung lesions	285 (59.0)	122 (49.8)	163 (68.5)	
Bilateral lung lesions, *n* (%)				0.004[c]
No, bilateral lung lesions	96 (19.9)	36 (14.7)	60 (25.2)	
Bilateral lung lesions	387 (80.1)	209 (85.3)	178 (74.8)	
AFB smear testing, *n* (%)				<0.001[c]
Smear –ve	63 (13.0)	47 (19.2)	16 (6.7)	
Smear +ve	420 (87.0)	198 (80.8)	222 (93.3)	
TB treatment outcome, *n* (%)				0.538[c]
Favorable outcome	448 (92.8)	229 (93.5)	219 (92.0)	
Treatment failure/relapse	35 (7.2)	16 (6.5)	19 (8.0)	

[a]Median (IQR) or frequency (%).
[b]Mann-Whitney test.
[c]Wilcoxon rank sum test; Pearson's Chi-squared test.
Anemia was defined as <12 g/dL for Female and <13 g/dL for Male.

TABLE 2 Hematological and biochemical parameters of the study population.

Parameters	Participants with anemia ($n = 288$) GM (range)	Participants without anemia ($n = 195$) GM (range)	p-value[a]
WBC count, x10^3cells/ul	97.4 (40–269)	94.1 (40–2000)	0.2656
Lymphocyte count, x10^6cells/ul	1,889 (500–4,015)	2062.3 (560–5,445)	0.0533
Neutrophil count, cells/ul	6,077 (1,632–16,020)	6512.8 (2,646–14,000)	0.5622
Monocyte count, cells/ul	708.2 (146–2,421)	620.1 (182–1,870)	0.0404
RBC, g/dL	4.4 (3–6.4)	5.1 (4–6.7)	<0.0001
Hb, g/dL	11 (6.3–12.9)	14.3 (13–20.1)	<0.0001
Hematocrit, %	34.9 (22–57)	42.4 (26–58)	<0.0001
Platelets, 10^3/uL	344.2 (90–800)	323.1 (123–817)	0.0719
FBG, mg/Dl	129.2 (74–516)	140.5 (62–417)	0.0560
HbA1C, %	6.7 (4.5–15.3)	7.9 (4.9–17.7)	0.5014
Triglycerides, mg/dL	97.1 (50–275)	104.1 (42–348)	0.0310
Total cholesterol, mg/dL	157.3 (80–294)	168.2 (91–330)	0.0083
HDL, mg/dL	35.5 (17–69)	37.5 (21–66)	0.7647
LDL, mg/dL	89.6 (33–187)	95.4 (35–223)	0.0411
VLDL, mg/dL	31.1 (13–157)	32.9 (10–166)	0.0796
Urea, mg/dL	16.6 (5–79)	17.5 (7–57)	0.0506
Creatinine, mg/dL	0.8 (0.5–1.7)	0.9 (0.5–2.1)	0.0540
Total bilirubin, mg/dL	0.5 (0.3–2)	0.6 (0.3–2.1)	0.0648
Total protein, g/dL	7.9 (5.7–10.4)	8.2 (6.1–10.1)	0.0048
Serum albumin, g/dL	3.8 (2.3–5.4)	4.2 (2.5–5.2)	<0.0001
Serum globulin, g/dL	4.0 (2.3–7)	3.9 (2.6–5.9)	0.0652
SGOT, U/L	18.0 (6–91)	17.9 (6–145)	0.9843
SGPT, U/L	14.6 (4–141)	15.6 (5–76)	0.0512
Alkaline phosphatase, U/L	269.9 (102–957)	259.4 (94–707)	0.9963
Vitamin D, IU	15.4 (3–47)	17.4 (3–67)	0.0269

[a]Mann-Whitney test.

GM, geometric mean; WBC, Whole blood cells; RBC, Red blood cells; Hb, Hemoglobin; FBG, Fasting blood glucose; HbA1c, Glycated hemoglobin; HDL, High density lipoprotein; LDL, Low density lipoprotein; VLDL, Very low-density lipoprotein; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, Serum Glutamate Pyruvate Transaminase.

TABLE 3 Association of clinical co-morbidities with anemia in TB individuals.

Variable	Anemia/ TB PR (95% CI)	p-value	Anemia/ TB aPR (95% CI)	p-value
Socio-demographic characteristics-Sex				
Male	Reference	1.009	Reference	0.028
Female	3.21 (1.52–3.31)		2.50 (1.90-2.40)	
Age, years				
18–34	Reference	0.879	Reference	0.981
35–44	1.61 (0.81–1.96)		1.60 (0.92–2.03)	
45–54	1.40 (0.76–2.21)		1.76 (0.87–2.01)	
≥55	1.10 (0.61–2.02)		1.11 (0.46–1.98)	
Smoking				
No	Reference	0.991	Reference	0.541
Yes	2.01 (0.89–3.25)		1.20 (0.76–2.54)	
Unknown	1.98 (1.74–2.87)		1.91 (0.82–3.10)	
Alcoholism				
No	Reference	0.928	Reference	0.571
Yes	2.50 (0.71–4.20)		1.80 (0.40–3.71)	
Unknown	2.81 (0.41–3.50)		1.30 (0.51–2.60)	
BMI (kg/m^2)				
Normal (18.5–24.9)	Reference	0.0781	Reference	0.211
Under nutrition (<18.5)	3.20 (0.51–2.80)		2.54 (0.61–1.71)	
Overweight (25.0–29.9)	2.68 (0.31–2.41)		1.52 (0.87–2.50)	
Obesity (≥30.0)	1.60 (0.86–2.71)		1.7 (0.71–4.51)	
HbA1c (%)				
NDM (<5.7)	Reference	0.741	Reference	0.438
PDM (>5.7–<6.4)	1.52 (0.75–2.01)		1.92 (0.73–0.90)	
DM (>6.4)	1.10 (0.61–1.47)		1.30 (0.61–0.10)	

PR, prevalence ratio; aPR, adjusted prevalence ratio; CI, Confidence interval; BMI, Body mass index; HbA1c, Glycated hemoglobin; NDM, Non-diabetes mellitus; PDM, Pre-diabetes mellitus; DM, Diabetes mellitus.

TABLE 4 Association of anemia with bacterial burden, disease severity, and treatment failure/relapse in TB.

Outcome variable	Anemia/ TB PR (95% CI)	*p*-value	Anemia/ TB aPR (95% CI)	*p*-value
Sputum smear grade	5.51 [3.45–9.34]	<0.001	4.01 [2.22–6.63]	<0.001
Bilateral lung lesions	2.21 [0.98–3.12]	0.287	1.96 [0.84–4.52]	0.354
Cavitary lung lesions	4.62 [3.04–7.08]	<0.001	3.36 [1.95–5.68]	<0.001
TB treatment failure/relapse	1.72 [1.11–2.61]	0.019	1.61 [1.31–2.19]	0.046

TB, tuberculosis; PR, prevalence ratio; aPR, adjusted prevalence ratio; CI, confidence interval.

Anemia is associated with increased risk of unfavorable TB treatment outcomes

Anemia was significantly associated with an increased risk of unfavorable treatment outcomes (PR, 1.72; 95% CI, 1.11–2.61; $p = 0.019$). This association persisted even after adjusting for confounding variables, with anemia remaining significantly associated with unfavorable treatment outcomes (aPR, 1.61; 95% CI, 1.31–2.19; $p = 0.046$). These findings indicate a heightened risk of treatment failure or TB recurrence in TB patients with anemia (Table 4).

Anemia is associated with altered levels of cytokines and pro-fibrotic factors in TB

The circulating levels of TNF-α, IL-4, IL-5, and IL-13 did not significantly differ between the two groups. However, pro-inflammatory cytokines [IFN-α (GM of 15.18 vs. 13.91 pg/ml, $p < 0.0001$), IFN-β (GM of 6.69 vs. 6.53 pg/ml, $p < 0.0001$), IL-6 (GM of 146.53 vs. 133.69 pg/ml, $p = 0.0032$)], and pro-fibrotic factors [VEGF (GM of 217 vs. 154.71 pg/ml, $p < 0.0001$), EGF (GM of 384.22 vs. 317.56 pg/ml, $p < 0.0001$), FGF-2 (GM of 2,584.82 vs. 2,000.96 pg/ml, $p = 0.0011$), and PDGF-AB/BB (GM of 1,758.71 vs. 1,601.31 pg/ml, $p = 0.0093$)] were significantly elevated in TB individuals with anemia compared to those without anemia. Conversely, the circulating plasma levels of type 1 cytokines [IFN-γ (GM of 275.56 vs. 313.81 pg/ml, $p < 0.0001$), IL-2 (GM of 104.75 vs. 121.96 pg/ml, $p = 0.0006$)] were significantly diminished in TB individuals with anemia compared to those without anemia (Figures 1, 2). Thus, anemia is associated with heightened levels of pro-inflammatory cytokines and pro-fibrotic factors and diminished levels of type 1 cytokines in TB individuals.

Discussion

To enhance TB management, targeted interventions must investigate the risk factors associated with disease progression and poor treatment outcomes (30). Anemia is a prominent comorbidity with TB (19). However, existing literature on the relationship between anemia and disease severity is scarce and inconsistent. While some studies suggest that anemia does not significantly predict TB risk (31–33), others identify it as a potential risk factor (34–36). This discrepancy underscores the necessity for robust, well-designed studies with larger sample sizes and standardized methodologies.

Consistent with prior research, our study revealed a substantial burden of anemia among TB patients in our cohort (37, 38). Notably, we observed higher rates of anemia among female TB patients compared to males, likely attributed to physiological differences, dietary habits, and variation in health-seeking behavior between genders (39). In contrast to non-anemic subjects, individuals with anemia exhibited a marked increase in monocyte levels and significant decreases in Hb, HCT, and RBC. Recent studies have linked elevated monocytes to poor prognosis and delayed pulmonary cavity closure in TB patients with anemia (10). Experimental evidence suggests that reduced Hb levels in anemic TB patients may result from the severity of TB infection and inflammation, impacting erythropoiesis and exacerbated by iron deficiency (40, 41). Hence, individuals with TB-related anemia may have a longer time for the proliferation and accumulation of Mtb, exposing them to inflammation for a longer time (12). The decreased production of RBC might result in reduced oxygen-carrying capacity and tissue hypoxia, which may have an impact on cytokine levels, leukocyte function, bone marrow function, and tissue destruction in TB (42, 43). Furthermore, our findings revealed that subjects with anemia had significantly lower levels of vitamin D and serum albumin compared to non-anemic subjects. Low serum albumin levels serve as a predictor of anemia and indicate the severity of inflammation (44, 45). The biological plausibility of lower vitamin D in anemia is supported by evidence suggesting that vitamin D regulates hepcidin production, thereby controlling iron homeostasis and erythropoiesis (46, 47).

Biomarkers for TB unfavorable treatment outcomes can play a major role in identifying novel TB intervention strategies (48–54). Cytokines are critical in the host defense against mycobacterial infections, serving as markers of disease severity and bacterial burden in active TB (55–57). Research shows that LBMI significantly impacts both acquired and innate host defense mechanisms, increasing susceptibility to TB (58–62). Our findings add to this knowledge by demonstrating that TB with coexistent anemia is associated with reduced levels of type 1 cytokines and increased pro-inflammatory and pro-fibrotic factors, potentially heightening TB risk. Our data indicate that TB patients with anemia have lower circulating levels of type 1 cytokines (IFNγ and IL-2), suggesting impaired protective immunity (63, 64). The reduced production of these cytokines in anemic individuals suggests a higher risk for severe TB due to weakened cell-mediated immunity, aligning with studies reporting lower type 1 cytokine levels in individuals with LBMI and TB compared to those with normal or high BMI (58, 62).

Loss of immune control in TB often results from excessive pro-inflammatory cytokine production, leading to neutrophil infiltration and pathological inflammation. This promotes granuloma remodeling and lung tissue destruction (65). We found significantly elevated pro-inflammatory cytokines (IL-6, IFNα, and IFNβ) in TB patients with anemia compared to non-anemic individuals. This aligns with previous research linking high

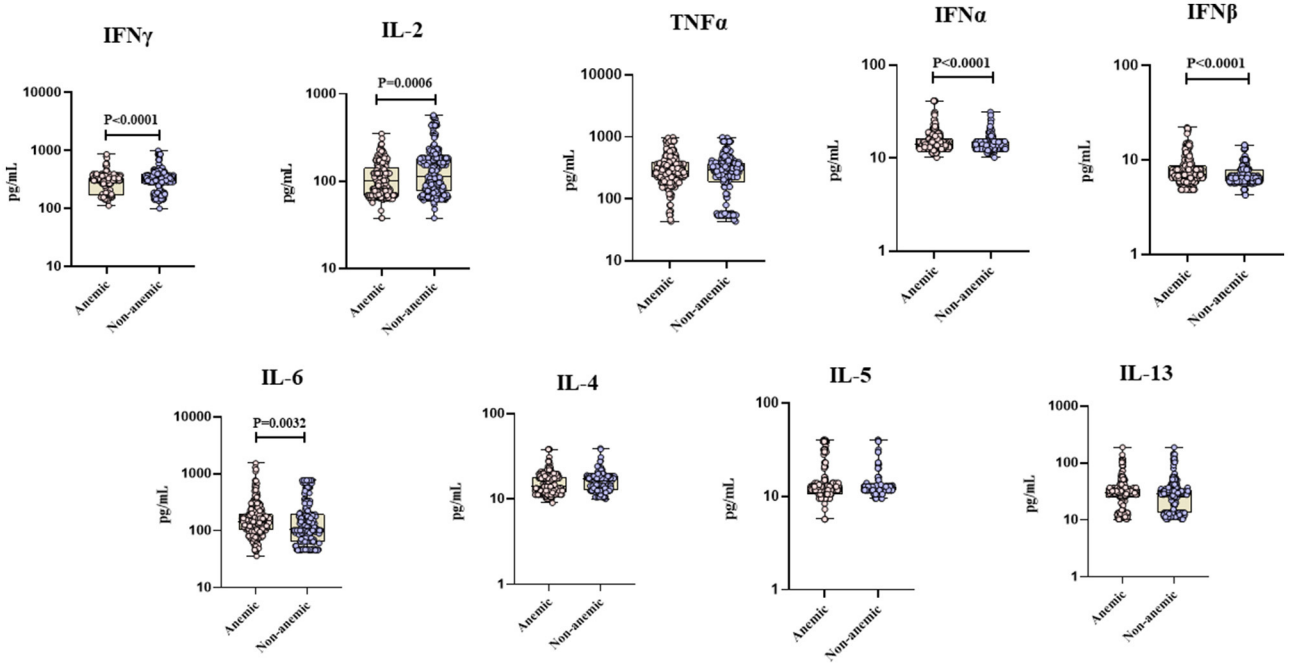

FIGURE 1
Anemia is associated with altered levels of cytokines in TB individuals. The figure illustrates the cytokine profile in anemic and non-anemic TB individuals. Circulating plasma cytokines including Interferon gamma (IFN γ), Interleukin-2 (IL-2), Tumor necrosis factor alpha (TNF-α), Interferon alpha (IFN α), Interferon beta (IFN β), Interleukin-6 (IL-6), Interleukin-4 (IL-4), Interleukin-5 (IL-5), and Interleukin-13 (IL-13) were measured. Each data point represents an individual subject, with the bar indicating the geometric mean (GM) cytokine level. Statistical analysis was performed using the Mann–Whitney *U*-test.

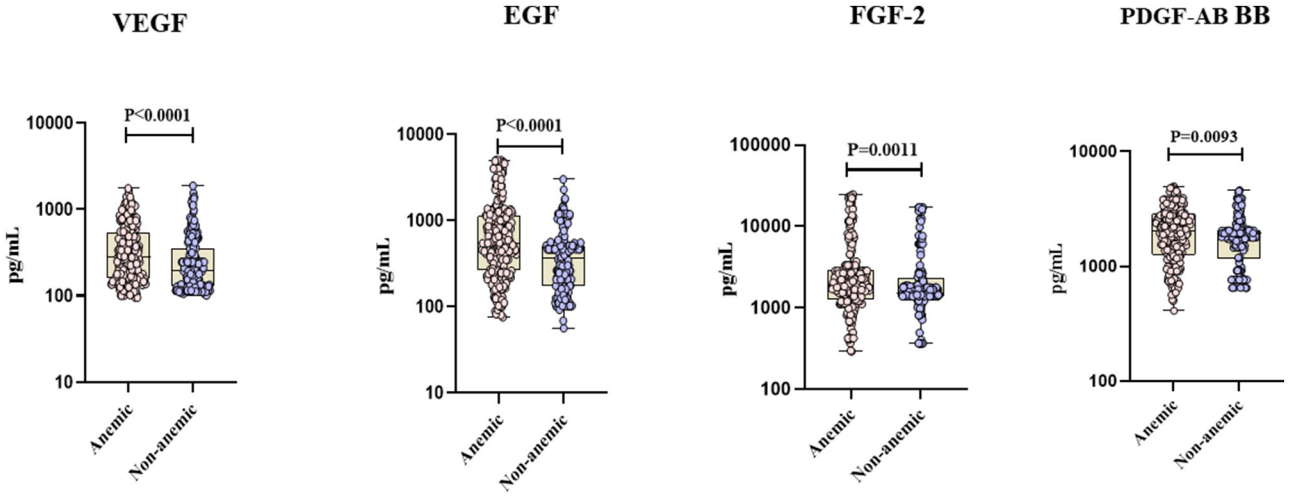

FIGURE 2
Anemia is associated with heightened levels of pro-fibrotic factors in TB individuals. The figure illustrates the pro-fibrotic factors profile in anemic and non-anemic TB individuals. Circulating plasma pro-fibrotic factors including Vascular endothelial growth factor (VEGF), Epidermal growth factor (EGF), Fibroblast growth factor 2 (FGF-2), and Platelet-derived growth factor (PDGF-AB BB) were measured. Each data point represents an individual subject, with the bar indicating the geometric mean (GM) cytokine level. Statistical analysis was performed using the Mann–Whitney *U*-test.

IL-6 levels to inflammation-related anemia, which inhibits iron absorption and exacerbates TB progression (66, 67). Additionally, high IL-6 concentrations are associated with anemia in TB/HIV co-infected patients (68). Pro-fibrotic factors are crucial in bacterial infection processes. Our study showed increased levels of pro-fibrotic factors (VEGF, EGF, FGF-2, and PDGF-AB BB) in anemic individuals compared to non-anemic individuals. VEGF, associated with pleural inflammation and fibrosis in TB patients, has been found at elevated levels in smear-positive and culture-positive TB subjects (69). Systemic VEGF levels also rise significantly in TB patients with cavitations and bilateral disease involvement (70).

In this study, rigorous control was exercised over several factors known to influence disease severity and bacterial burdens, such as age, BMI, diabetes, smoking status, and alcohol use. The findings of this study provide valuable insights into the association between anemia and TB disease severity. Notably, our study revealed several key findings that warrant further exploration. We observed that TB patients with coexistent anemia exhibit more severe disease manifestations, including lung cavitation, indicative of advanced TB disease. These findings align with previous research suggesting that such lesions negatively impact patients and may lead to poor treatment outcomes, relapses, and drug resistance (71). Our results revealed a strong correlation between anemia and elevated bacterial burdens in TB patients, a key indicator of transmission (10). Our data further confirm that TB individuals with anemia were at a significantly higher risk of experiencing unfavorable treatment outcomes, including treatment failure or TB recurrence. This finding aligns with previous research indicating that anemic patients with TB-HIV co-infection exhibit poor treatment outcomes and a heightened degree of inflammatory perturbation (72).

Our study suffers from the limitation of not measuring red cell indices (MCV, MCH, and MCHC) or biochemical measures (iron, ferritin, hepcidin, and transferrin) to assess the type of anemia. Another limitation of our study is that cytokine levels exhibit a great degree of overlap between groups and that there is variability in the responses of different individuals in the same group. It is theoretically possible that other factors not examined in this study could have contributed to the differential responses. Nevertheless, our study offers novel insights into the immunological underpinnings of the anemia-TB comorbidity.

Conclusion

Our study reveals intricate interactions between anemia and disease severity, bacterial burdens, and treatment outcomes in TB patients. Importantly, our data highlights the significant association of anemia with the cytokine milieu in TB, suggesting a plausible biological mechanism for the increased disease severity observed in TB individuals with coexistent anemia. Our findings highlight the critical need for further research and interventions aimed at addressing the complex interplay between anemia and TB to optimize patient outcomes and advance TB control efforts.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by National Institute for Research in Tuberculosis (NIRT) and Prof. M. Viswanathan Diabetes Research Center (MVDRC). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

BD: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. SM: Data curation, Formal analysis, Validation, Writing – review & editing. NP: Data curation, Formal analysis, Investigation, Project administration, Supervision, Validation, Writing – review & editing. KM: Data curation, Investigation, Methodology, Writing – review & editing. AP: Data curation, Investigation, Methodology, Writing – review & editing. SN: Resources, Validation, Writing – review & editing. VV: Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – review & editing. SS: Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – review & editing. SH: Resources, Supervision, Writing – review & editing, Conceptualization, Investigation, Project administration. KT: Data curation, Formal analysis, Writing – review & editing. HK: Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – review & editing. SB: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Assessing the effects of diabetes mellitus on the monocyte-to-lymphocyte ratio and the QuantiFERON-TB gold plus assays for tuberculosis treatment monitoring: a prospective cohort study

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Diabetes mellitus (DM) is an important risk factor for the development of active tuberculosis (TB). QuantiFERON-TB Gold Plus (QFT-P), white blood cell count (WBC) assays and monocyte-to-lymphocyte ratio (MLR) reflect the inflammatory reactions associated with TB and offer the potential to monitor TB treatment to allow a better management of the disease. The aim of this study was to assess the influence of DM on the respective performances of QFT-P and WBC assays in their capacities to monitor the treatment of drug-sensitive pulmonary TB (TBP). The QFT-P and WBC were prospectively compared between TB patients with and without DM at inclusion (D0), at the end of treatment (M6) and two months after the end of treatment (M8). After laboratory measurement of glycated hemoglobin (HbA1c), the patients were categorized into two groups: the TBP (n=43) and the TBDM (n=30) groups. The TBDM patients were characterized by an elevated *Mycobacterium tuberculosis*-specific QFT-P IFN-γ response after TB treatment compared to the TBP group ($p<0.001$ and $p<0.05$, respectively, after TB1 and TB2 antigens stimulation). A significantly higher proportion of positive QFT-P tests was observed in the TBDM group compared to the TBP group (91.3% vs 64.1%) at the end of the treatment ($p=0.03$). MLR analysis showed a decrease of MLR value after TB treatment for both diabetic and nondiabetic TB patients

(p<0.001 and p<0.05). These data reflected from immune-host based tests used to monitor the TB treatment, seemed to further suggest that TB with concomitant DM is associated with a persistent inflammatory response after TB treatment.

KEYWORDS

tuberculosis, treatment monitoring, diabetes mellitus, QuantiFERON-TB gold plus, white blood cell count, monocyte to lymphocyte ratio

1 Introduction

The World Health Organization (WHO) has identified diabetes mellitus (DM) as an important risk factor for tuberculosis (TB) and therefore recommends diabetes screening for active TB patients (1). Low- and middle-income countries account for approximately 80% of the global diabetes burden, and more than 90% of the global TB burden (2). For most patients, TB therapy provides a cure, but treatment failure and relapse can occur. It is now well established that DM is associated with an increased risk of progressing from latent TB infection to active TB disease. Moreover, TB patients with DM more frequently suffer from adverse TB outcomes, including delayed sputum conversion on treatment, TB treatment failure, death, and recurrent TB (1, 3–5). These outcomes are associated with moderate to severe adverse effects and long treatment durations that induce a lack of patient adherence to the treatment regimen and promote the emergence of drug resistance. Continuous monitoring and early identification of people with TB who are at risk of poor treatment outcomes could reduce the number of people who do not complete treatment. The WHO currently recommends sputum smear microscopy or mycobacteriological culture conversion at the end of the intensive phase of treatment for monitoring treatment response in adults with pulmonary TB (6). However, these microbiology-based methods rely on sputum samples, which are not readily available in all populations (e.g., pediatric TB, people living with HIV, extrapulmonary TB). Furthermore, while smear microscopy is related to poor sensitivity and specificity for outcome prediction, the TB culture has limited availability in primary care settings, and the delay in time to results constrains its clinical use.

There are novel tests and biomarkers in the pipeline that offer the potential to monitor TB treatment efficacy, predict outcomes, identify cure, and allow optimization of management. These potential tests include those using host characteristic assays, including assays for cytokines, transcriptomic profiles, and other biomarkers that are associated with the inflammatory reactions following TB infection. Some of these proposed tests, such as QuantiFERON-TB Gold Plus (QFT-P), white blood cell count (WBC) and the monocyte-to-lymphocyte ratio (MLR), are already commercialized to detect TB infection or used as routine basic laboratory measures for clinical practice. The QFT-P test, while primarily designed and validated for detecting *Mycobacterium tuberculosis* infection and guiding prophylactic treatment decisions in populations at higher risk of developing TB, has also been explored in research settings for its potential utility in detecting TB disease and monitoring TB treatment (7, 8). Patients with diabetes often have more severe inflammation at the time they are diagnosed with TB and experience higher risks for adverse TB treatment outcomes, with more severe lung damage in patients with pulmonary TB. This can have serious consequences like relapse or death, as frequently reported in TB patients with concomitant DM (TBDM) (4, 9). This inflammatory status can also alter the performance of host-immune-based assays' capacity to monitor TB treatment (3, 10–12). The aim of this study was to assess the influence of DM on the respective performances of QFT-P and WBC, two available tests already used in clinical practice, in their capacities to monitor the TB treatment of drug-sensitive pulmonary TB. Considering the significant worldwide occurrence of both TB and DM, integrating changes related to DM in TB immunodiagnostic and immuno-monitoring tests can enhance the care provided to TB patients.

2 Materials and methods

2.1 Study design and participants

We conducted a prospective cohort study from January to December 2019 to consecutively recruit newly confirmed active TB patients from individuals with presumptive pulmonary TB seeking diagnosis at the main anti-TB center in Antananarivo, Madagascar. The inclusion criteria for active TB disease were adult pulmonary TB patients (≥ 18 years old) who tested positive for conventional TB microbiological and molecular tests, and able to provide informed consent. For each included TB participant, sociodemographic information was collected, including age, sex, body mass index (BMI, calculated as weight in kilograms divided by height in meters squared), BCG vaccination status, alcohol consumption and smoking habits. Two control groups (≥ 18 years old) were randomly and simultaneously recruited, including i) community healthy volunteers (HC) without any clinical signs/symptoms of TB, recruited at the anti-rabic center of the Institut Pasteur de Madagascar, and ii) confirmed DM patients without any

clinical signs/symptoms of TB, recruited at the main Diabetes center of Antananarivo (AMADIA). Individuals who tested positive for HIV, children under 18 years of age, and individuals with other known comorbidities were excluded.

2.2 TB and DM diagnosis

Pulmonary TB is confirmed with sputum AFB smear microscopy using the auramine technique and/or the Loweinsten-Jensen (LJ) solid media culture and/or Xpert MTB/RIF at inclusion. The results were classified based on bacterial load as follows: AFB smear microscopy grades: 0 (no AFB observed), 1+ (10–99 AFB in 100 fields), 2+ (1–10 AFB per field in 50 fields), and 3+ (more than 10 AFB per field in at least 20 fields). Culture grades: 0 (no colonies), 1+ (1–100 colonies), 2+ (more than 100 discrete colonies), and 3+ (confluent growth or innumerable colonies).

DM was screened at the time of recruitment of the confirmed TB patients with an initial fasting plasma glucose (cut-off point at ≥ 6.1 mmol/L), and two points raised of glycated hemoglobin (HbA1c) were offered as confirmatory tests. Laboratory measurement of HbA1c with a diagnostic cut-off point ≥ 6.5% was used as the gold standard for the diagnosis of diabetes. To account for potential transient hyperglycemia, we made secondary analyses, defining diabetes by repeated raised HbA1c at the end of the TB treatment. HbA1c was measured in 1 mL of whole blood collected in ethylenediaminetetraacetic acid (EDTA) tubes and processed using immunoturbidimetry (Quest, Tucker, GA, USA). DM status was classified according to the American Diabetes Association guidelines (13) with a slight adjustment based on age-dependent HbA1c reference intervals (14).

2.3 TB treatment and follow-up visits

All patients were treated with the Directly Observed Treatment, Short Course (DOTS) and received the same TB treatment according to the WHO Drug-susceptible tuberculosis treatment recommendation (15) with a 6-month regimen composed of four first-line TB medicines: isoniazid (H, oral dose of 4-6 mg/kg/day), rifampicin (R, oral dose of 8-12 mg/kg/day), pyrazinamide (Z, oral dose of 20-30 mg/kg/day), and ethambutol (E, oral dose of 15-25 mg/kg/day). The regimen is a combination of those four drugs (HRZE) for 2 months followed by isoniazid and rifampicin (HR) for 4 months, administered daily. TB patients were followed up at the end of therapy after 6 months (M6) and at two months after the end of therapy (M8). At each of the three visits, a sputum sample and blood were collected for AFB smear microscopy, LJ culture, QFT-P assay, and WBC count.

2.4 QuantiFERON-TB gold plus assay

To perform the QFT-P and WBC count, 7 mL of whole blood was drawn in lithium heparin blood collection tubes. Four (4) mL were used for the QFT-P assay, and the remaining 3 mL were used for complete WBC, HbA1c, and the HIV test. The QFT-P assay was performed according to the manufacturer's instructions (Qiagen). Briefly, venous blood was collected in lithium-heparin tubes at the health centers, and then one mL of blood was dispensed into each of the four QFT-P assay tubes (Nil, TB1, TB2, Mitogen) where antigen stimulations were initiated within 8 hours from venipuncture and incubated at 37°C for 16 ± 24 hours (aiming at 18 hours' incubation time). After incubation, the tubes were centrifuged, and aliquots of the plasma supernatants were stored at -20° C. IFN-γ ELISA was performed in batches according to the QFT-P protocol (Qiagen). ELISA results were converted to international units per milliliter (IU/mL) and interpreted using the QFT-P software supplied by the manufacturer (TB Gold Plus Analysis Software v2.71). All IFN-γ concentrations were nil-corrected. The results were classified as positive, negative, or indeterminate according to the manufacturer's instructions, with a diagnostic IFN-γ cut-off of 0.35 IU/mL in either of the two antigen tubes. QFT-P conversion was defined as a change from negative to positive, and reversion as a change from positive to negative on serial testing.

2.5 White blood cells count assay

Complete WBC was performed with an XN 1000 automated hematology analyzer (Sysmex). The XN-1000 is a standalone, benchtop analyzer using a single XN-Series module. The XN-1000 automated analyzer provides a complete WBC and nucleated red blood cell (NRBC) count using the White Count and Nucleated Red Blood Cells (WNR) and the White blood cell differential count (WDF) channels. The blood cells are analyzed by flow cytometry-based optical measurement after red cell and platelet lysis, membrane permeabilization of the leukocytes, and introduction of a fluorochrome that binds to leukocyte nucleic acids. Scattergrams are generated after three-dimensional analysis of each cell signal according to cell volume (FSC: forward scatter light), cell structure (SSC: side scatter light), and cell fluorescence (SFL: side fluorescent light). The WNR channel evaluates the leukocyte and basophil counts and provides a systematic NRBC count. The WDF channel provides a count of the neutrophils, lymphocytes, eosinophils, monocytes, immature granulocytes, and a high-fluorescence lymphocyte count (HFLC). The monocyte-to-lymphocyte ratio (MLR) was determined by dividing absolute monocyte counts by absolute lymphocyte counts at each study time point.

2.6 Data collection and statistical analysis

Sociodemographic, clinical, and biological data were recorded in a dedicated RedCap® (Research Electronic Data Capture) software database (16). Statistical analysis was performed using GraphPad Prism (version 9 for Windows, GraphPad Software, Boston, Massachusetts USA). Categorical variables were analyzed using Fisher's exact test adjusted with Bonferroni's *post-hoc* test (17). Normal continuous variables were analyzed with Student's t-test and One Way ANOVA test. Non-normal continuous variables were analyzed with Mann-Whitney and Wilcoxon sum-rank tests for impaired and paired analysis respectively, and by One Way ANOVA

test with Dunn's Kruskal-Wallis rank sum-test (impaired) and Friedmann test (paired) for multiple comparisons *post-hoc* test (18).

2.7 Ethical considerations

This study was approved by the Ethical Committee for Biomedical Research of Madagascar (N° 099/MSANP/SG/AGMED/CERBM). Written informed consent was obtained prior to enrolment. All research was performed in accordance with relevant guidelines/regulations.

3 Results

3.1 Enrolment, clinical characteristics, follow-up visits, and sociodemographic data of study participants

Figure 1 shows the enrolment and follow-up visit flowchart of the study participants. Among the 92 eligible participants in the TB active group, a total of 73 newly confirmed pulmonary TB patients were included in the study. A total of 10 participants were excluded due to the following health conditions: high blood pressure (n=5), asthma (n=2), meningitis (n=1), measles (n=1), and gastritis (n=1). Additionally, 9 patients were excluded for having a negative TB test. Most patients were simultaneously positive for sputum smear microscopy (82%, 60/73) and/or culture (89%, 65/73) and/or Xpert (100%, 73/73). After laboratory measurement of HbA1c, these patients were categorized into 2 groups: the active pulmonary group without DM (TBP, n=43) and the active pulmonary with DM groups (TBDM, n=30) (Figure 1). Control groups including Diabetes only (DM, n=30) and healthy asymptomatic blood donors (HC, n=50) were enrolled. The clinical groups with DM (TBDM and DM) had a statistically significant higher age compared to HC groups ($p<0.0001$, Table 1). However, there was no statistical age difference between TBDM and TBP patients ($p=0.058$). A statistically significant lower body mass index (BMI) was observed with the TBP (17.6 Kg/m2) and TBDM (18.4 Kg/m2) groups compared to the DM group (23.1 Kg/m2) ($p<0.0001$). The frequency of underweight individuals (BMI<18.5) was significantly higher in both the TBP and TBDM groups compared to the DM group ($p<0.0001$), highlighting the pronounced impact of TB on nutritional status in affected individuals. Table 1 also shows that a statistically significant higher alcohol consumption ($p<0.003$) and higher smoker proportions ($p=0.008$) were observed within the TBP compared to TBDM. Then, in the downstream analysis, we decided to only perform with TB patients (TBP and TBDM) who simultaneously do not smoke and do not drink alcohol. Regarding the follow-up visits, 39 TBP and 16 TBDM patients had successfully achieved their TB

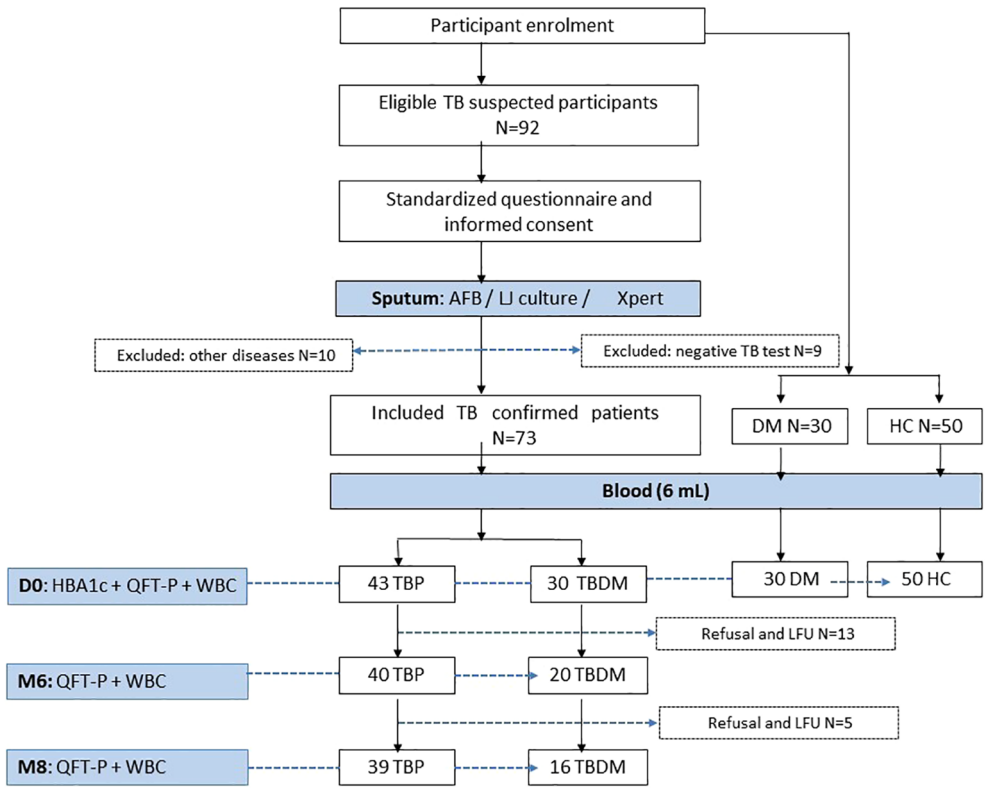

FIGURE 1
Flowchart of inclusion, follow-up visits and data collection process. AFB, acid fast bacilli; TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; DM, patient with diabetes mellitus; HC, healthy asymptomatic individuals; QFT-P, QuantiFERON-TB Gold Plus; WBC, White blood cell count; LFU, lost to follow up; HBA1c, glycated hemoglobin A1C; LJ, Löwenstein Jensen; Xpert, Xpert MTB/RIF Ultra.

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TABLE 1 Sociodemographic and clinical characteristics of study participant at inclusion.

Study demographics	TBP	TBDM	DM	HC	p-value TB vs TBDM	p-value All group
Age (Median, range)	31 (22-45)	43 (35-56)	59 (38-63)	34 (22-44)	0.058	<0.0001
Sex (N, %)						
Male	32 (74.4)	19 (63.3)	9 (30.0)	15 (30.0)	0.43	<0.0001
Female	11 (25.6)	11 (36.7)	21 (70.0)	35 (70.0)		
BMI (Kg/m^2) (Median, range)	17.2 (16.0-18.9)	18.0 (16.6-19.2)	22.9 (21.2-25.0)		0.18	<0.0001
BMI category						
Underweight	34 (79.0)	18 (60.0)	1 (3.3)		0.20	<0.0001
Normal weight	8 (18.7)	11 (36.7)	21 (70.0)			
Overweight	1 (2.3)	1 (3.3)	8 (26.7)			
BCG Vaccination (N, %)						
Yes	32 (74.4)	27 (90.0)	29 (96.7)	46 (92.0)	0.10	0.01
No	11 (25.6)	3 (10.0)	1 (3.3)	4 (8.0)		
HbA1c % (Median, range)	5.6 (5.4-5.9)	8.2 (7.0-9.1)	9.3 (7.6-11.1)	4.5 (3.4-5.1)	<0.0001	<0.0001
Alcohol (N, %)						
Yes	24 (55.8)	6 (20.0)	3 (10.0)		0.003	<0.0001
No	19 (44.2)	24 (80.0)	27 (90.0)			
Smoking (N, %)						
Yes	24 (55.8)	7 (23.3)	4 (13.3)		0.008	0.0003
No	19 (44.2)	23 (76.7)	26 (86.7)			
Total	43	30	30	50		

TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; DM, patient with diabetes mellitus; HC, healthy asymptomatic individuals; BMI, Body mass index; BCG, Bacille Calmette and Guerin; HBA1c, Glycated hemoglobin A1C; N, number.

treatment and completed their QFT-P and WBC count assays from the inclusion (D0), at M6 to the M8 follow-up visits (Figure 1). Respectively, 40 and 20 patients were followed until the end of the TB treatment for TBP and TBDM (Figure 1). All of them presented negative TB culture at the end of their treatment and were clear from any clinical TB signs at M6. Among the TBDM patient, only five individuals (n=5) did receive anti-diabetic treatment in parallel with their anti-TB treatment. Table 2 shows the degree of positivity of AFB smear microscopy and culture results within the TBP and TBDM groups at inclusion (D0). No significant difference was observed between the TBP and TBDM patients regarding the degree of positivity of AFB smear, culture and Xpert results.

TABLE 2 Comparison of AFB smear, culture and Xpert results between TBP and TBDM groups at inclusion (D0).

	Grade	TBP (n, %)	TBDM (n, %)	P-value
AFB Smear	0 1+ 2+ 3+	8 (18.6) 13 (30.2) 12 (27.9) 10 (23.3)	8 (26.7) 9 (30.0) 8 (26.7) 5 (16.6)	0.82
Culture	0 1+ 2+ 3+	5 (11.6) 10 (23.2) 13 (30.2) 15 (35.0)	6 (20.0) 5 (16.7) 13 (43.3) 6 (20.0)	0.9
Xpert	Not detected Low Medium High	2 (4.6) 5 (11.6) 9 (21.0) 27 (62.8)	3 (10.0) 6 (20.0) 6 (20.0) 15 (50.0)	0.55
Total		43	30	

TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; AFB, acid-fast bacilli.

3.2 QuantiFERON-TB gold plus results

In order to evaluate the impact of DM on IFN-γ production upon *Mycobacterium tuberculosis* antigen stimulation during TB

treatment, the plasma IFN-γ concentrations in response to TB1 and TB2 antigens were first measured with the QFT-P assay and compared between the TBP, TBDM, DM, and HC clinical groups. At inclusion (D0), no statistical difference in the proportion of QFT-P positive, negative, and indeterminate results was observed between the TBP vs TBDM nor between the other study groups (p=0.2) (Table 3). Furthermore, after quantitative analysis, no statistical differences in the baseline level of IFN-γ produced in QFT-P positive results after stimulation with TB1 (p=0.11) and TB2 (p=0.19) antigens were observed between the clinical groups (Supplementary Figure 1). Additionally, our results did not reveal any statistically significant differences in IFN-γ levels among the various smear and culture grades within the TBP and TBDM groups (p>0.05). The results revealed weak correlations between IFN-γ levels and both smear/culture grades and Xpert-derived bacillary load.

TBP and TBDM had a follow-up visit at M6 from their TB treatment and two months after the completion of the TB treatment (M8) timepoints. TBDM group showed higher IFN-γ produced by the QFT-P after stimulation with both antigens, TB1 (p<0.001) and TB2 (p<0.05), at M6 compared to D0. In contrast no significant differences were observed in the TBP group at these timepoints or between M6 and M8 (Figure 2). When applying the manufacturer's recommendations for qualitative results, while no statistical difference of QFT-P results was observed between the two clinical groups at inclusion (D0) and M8, a significantly higher proportion of positive QFT-P results was observed for the TBDM group compared to the TBP group (91.3% vs 64.1%) (p=0.03) at the achievement point of the treatment (M6) (Figure 3). During follow up, there were no significant differences in QFT-P responses between TBDM patients receiving anti-diabetic treatment and those not receiving it (p>0.05).

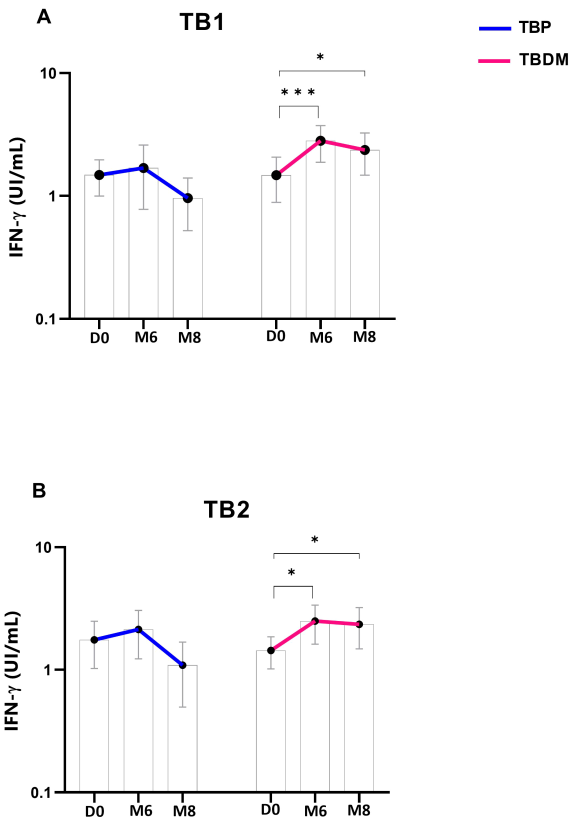

FIGURE 2
Dynamics of QuantiFERON-TB Gold Plus interferon-γ concentrations in TBP and TBDM groups throughout TB treatment: **(A)** Stimulation by TB1-specific antigen and **(B)** by TB2-specific antigen. Data represent median with 95% confidence interval. Data were analyzed using Friedman's multiple pairwise comparisons test. *: p < 0.05, ***: p < 0.001. TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; QFT-P, QuantiFERON-TB Gold Plus; D0, baseline before treatment initiation; M6, end of treatment, after six month; M8, 2 months after the end of treatment.

3.3 QuantiFERON-TB gold plus conversion/reversion rates

The QFT-P conversion and reversion rates during TB treatment showed that the TBDM patients had higher conversion rates (83.3% for TBDM vs 38.4% for TBP, p<0.05) and no reversions (0% for TBDM vs 21.7% for TBP, p<0.01) compared to the TBP at the end of the TB treatments (M6). Notably, five out of six TBDM patients with a negative QFT-P result at D0 had QFT-P conversion at M6, and 1/6 remained QFT-P negative at M6 (Figure 3B). Besides, none of the TBDM patients who had a positive QFT-P result at D0 reverted their QFT-P test as negative, and all remained QFT-P positive at M6 (Figure 3B). No statistical differences in QFT-P indeterminate proportion between the groups at the different follow-up visits were observed (Figure 3).

TABLE 3 QuantiFERON-TB Gold Plus results with the 4 study groups at inclusion.

QFT-P	TBP (n, %)	TBDM (n, %)	DM (n, %)	HC (n, %)	P-value
POS	25 (58.1)	17 (56.7)	14 (46.7)	25 (50.0)	0.2
NEG	15 (34.9)	10 (33.3)	16 (53.3)	23 (46.0)	
IND	3 (7.0)	3 (10.0)	0 (0.0)	2 (4.0)	
Total	43	30	30	50	

TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; DM, patient with diabetes mellitus; HC, healthy asymptomatic individuals; QFT-P, QuantiFERON-TB Gold Plus; POS, positive; NEG, negative; IND, indeterminate.

3.4 WBC, monocyte and lymphocyte counts

Before the initiation of TB treatment, the TB active groups (TBP and TBDM) had higher absolute WBC counts compared to the healthy control group (HC) (p<0.05 and p<0.001) (Figure 4A). TBDM also showed a significantly higher WBC count compared to its DM clinical counterpart group (p<0.05) (Figure 4A). Figures 4B, C show that TBDM and TBP patients displayed statistically significant higher monocyte (p<0.01 and p<0.05) with lower lymphocyte counts compared to HC and DM groups. The results did not reveal any significant associations between bacterial burden (by AFB smear, culture and Xpert) and leukocyte counts at inclusion within the two groups.

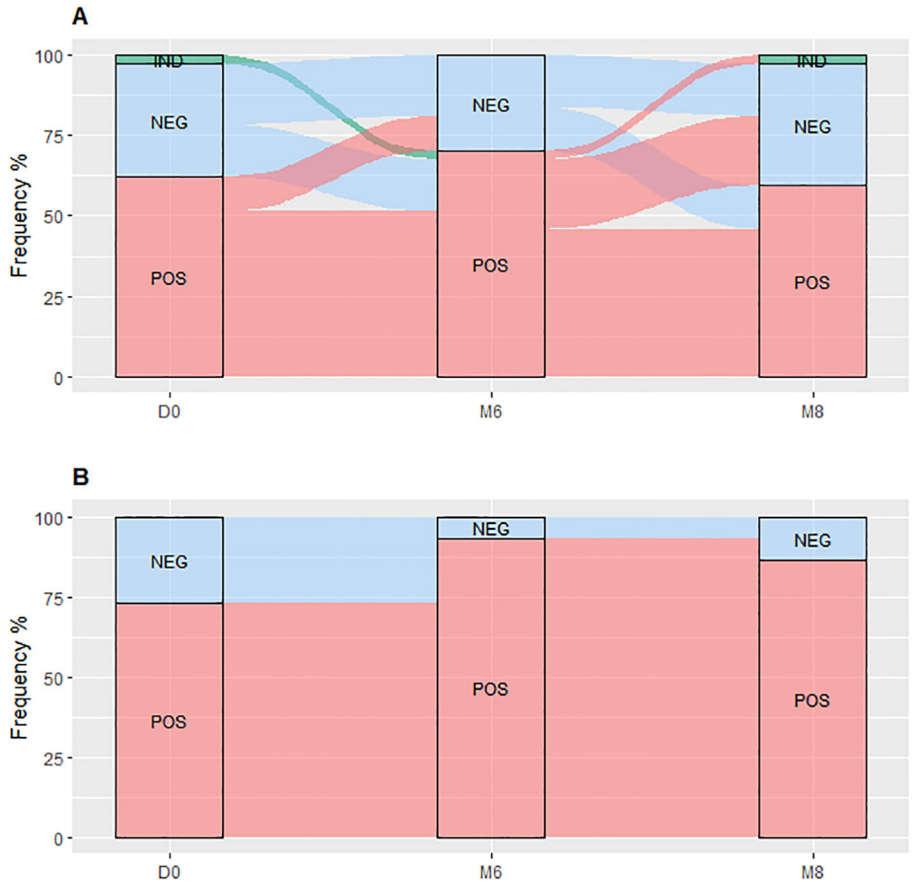

FIGURE 3
QuantiFERON-TB Gold Plus results dynamic proportion with the TBP **(A)** and TBDM **(B)** throughout TB treatment. TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; QFT-P, QuantiFERON-TB Gold Plus; POS, positive; NEG, negative; IND, indeterminate; D0, baseline before treatment initiation; M6, end of treatment, after six month; M8, 2 months after the end of treatment.

After their TB treatment, significant decreases in absolute WBC counts were observed in the TBP and TBDM clinical groups at both M6 and M8 (Figure 5A). Moreover, a significant decrease in monocyte absolute count within the TBP and TBDM group was observed after treatment compared to inclusion ($p<0.05$) (Figure 5B). While a statistically significant increase in lymphocyte absolute count was observed in the TBDM group after treatment both at M6 ($p<0.01$) and M8 ($p<0.0001$), no significant increase in lymphocyte absolute count was observed with the TBP after TB treatment (Figure 5C).

3.5 Monocyte-to-lymphocyte ratio analysis

After determining the MLR at inclusion, a significantly higher ratio was observed for both TBP and TBDM groups compared to their respective control counterparts, i.e., HC ($p<0.0001$) and DM groups ($p<0.01$ and $p<0.0001$, respectively for TBP and TBDM) (Figure 6). After the TB treatment, a significant decrease in the MLR was observed both at M6 and M8 in the TBP group ($p<0.05$, Figure 7). The same trend was observed with the TBDM at M6 and M8 but with a higher statistical difference compared to the trend observed with the TBP ($p<0.001$). A significant decline of the MLR level was observed with both TB active group after successful TB treatment. No statistical differences were observed regarding the MLR at M6 and M8 compared to HC (Supplementary Figure 2). The same trends were observed when analyzing the neutrophil-to-lymphocyte ratio (NLR) in both groups (Supplementary Figure 3). At baseline (D0), significantly higher NLR values were observed, which steadily declined following successful TB treatment at M6 and M8. During follow up, there were no significant differences in MLR and NLR responses between TBDM patients receiving anti-diabetic treatment and those not receiving it ($p>0.05$).

4 Discussion

Host characteristic assays, which are based on monitoring the host immune system, are alternative sputum-independent options for monitoring and predicting TB treatment outcomes. These assays can be performed in primary health care settings, where TB patients often first enter the health care system (6). Most of the technologies used for monitoring TB treatment are still in the early stages of development and are limited to central laboratories. These available TB monitoring tests have also faced challenges due to the host's heterogeneous variations, including co-morbidities associated with

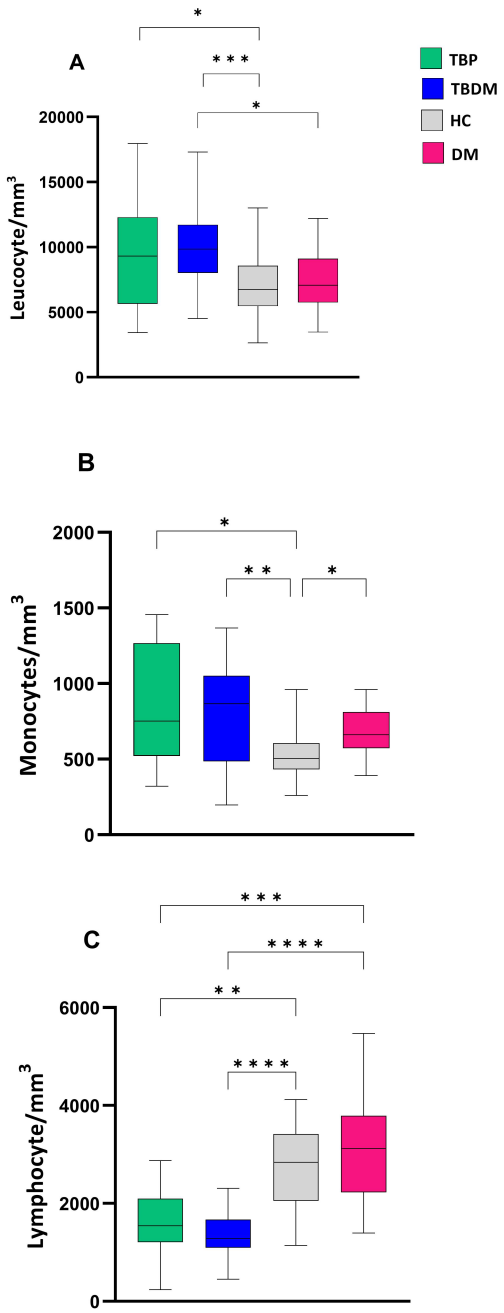

FIGURE 4
Comparison of baseline absolute white blood cell count between TBP, TBDM, HC and DM. **(A)** Leucocyte absolute count/mm^3 of whole blood. **(B)** Monocyte absolute count/mm^3 of whole blood. **(C)** Lymphocyte absolute count/mm^3 of whole blood. Box plots represent median and interquartile range (IQR) of the data. The upper and lower edges of the boxes represent the third and first quartiles (Q3 and Q1), respectively, while the line inside the box represents the median (Q2). Data were analyzed using Kruskal-Wallis with Dunn's multiple comparison test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; DM, patient with diabetes mellitus; HC, healthy asymptomatic individuals.

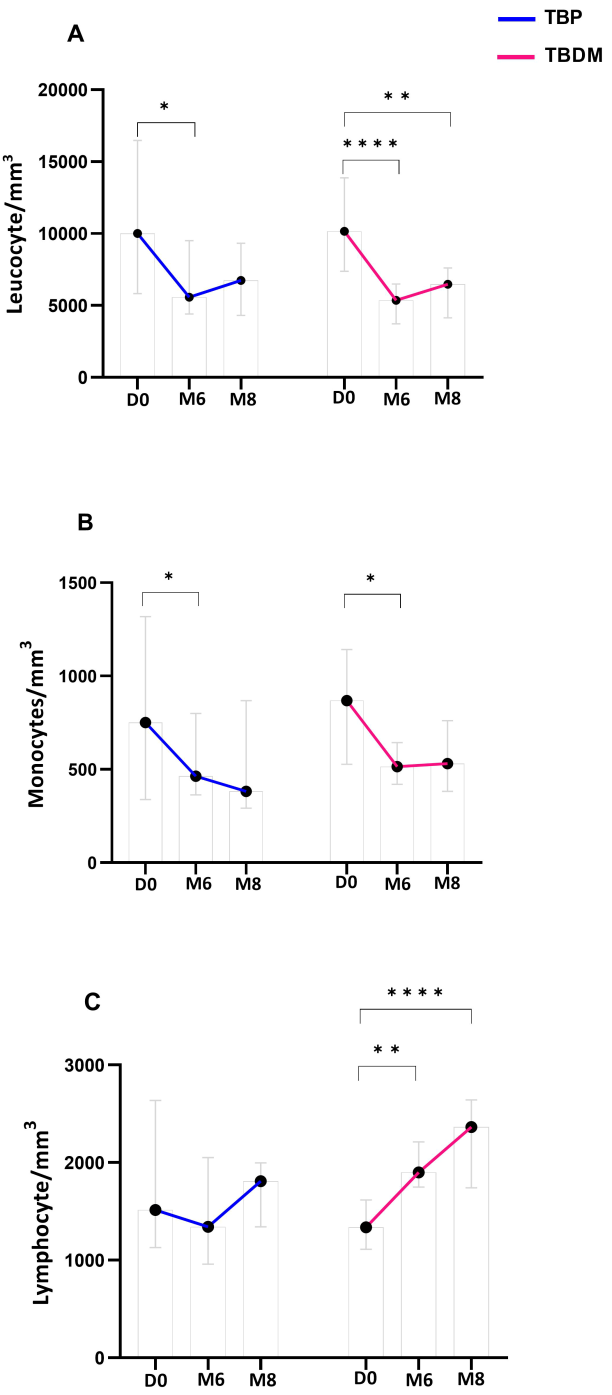

FIGURE 5
Dynamics of main white blood cell types proportion throughout TB treatment in TBP and TBDM groups. **(A)** Leucocyte absolute count/mm3 of whole blood. **(B)** Monocyte absolute count/mm3 of whole blood. **(C)** Lymphocyte absolute count/mm3 of whole blood. Data represent median with 95% confidence interval. Data were analyzed using Friedman's multiple pairwise comparisons test. *: $p < 0.05$, **: $p < 0.01$, ****: $p < 0.0001$. TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; D0, baseline before treatment initiation; M6, end of treatment, after six month; M8, 2 months after the end of treatment.

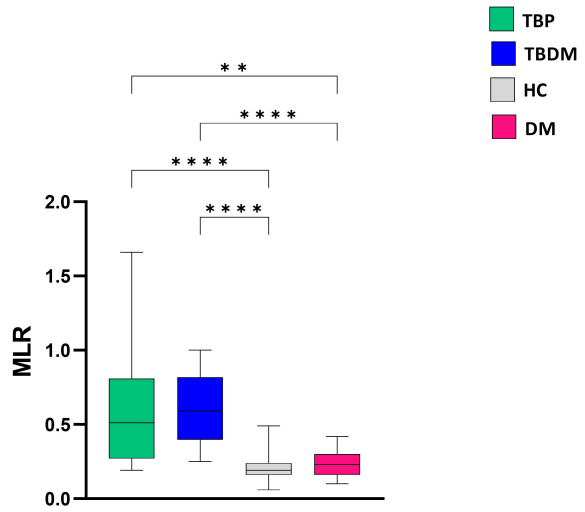

FIGURE 6
Comparison of baseline monocyte-to-lymphocyte (MLR) ratio between TBP, TBDM, HC and DM groups. Box plots represent median and interquartile range. The upper and lower edges of the boxes represent the third and first quartiles (Q3 and Q1), respectively, while the line inside the box represents the median (Q2). Data were analyzed using Kruskal-Wallis with Dunn's multiple comparison test. **: p<0.01, ****: p < 0.0001. TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; DM, patient with diabetes mellitus; HC, healthy asymptomatic individuals.

infection, as well as the practicalities of translating biomarkers into simple and rapid tests that are more suitable for low- and middle-income countries (LMICs) (8).

The aim of this study was to assess the influence of diabetes mellitus (DM) on the respective performances of QuantiFERON-TB Gold Plus (QFT-P) and white blood cell counts (WBC), two available tests already used in clinical practice, in their capacities to monitor the TB treatment of drug-sensitive pulmonary TB. While

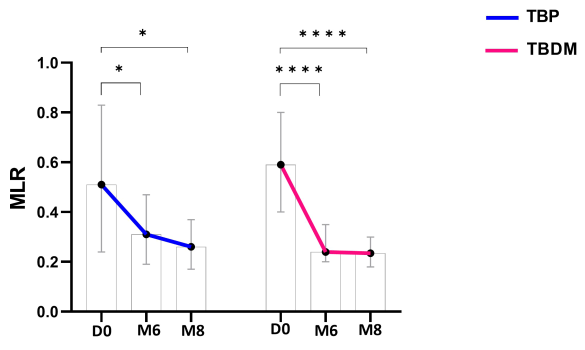

FIGURE 7
Dynamics of monocyte-to-lymphocyte (MLR) ratio throughout TB treatment with TBP (blue line) and TBDM (red line) groups. Data represent median with 95% confidence interval. Data were analyzed using Friedman's multiple pairwise comparisons test. *: p < 0.05, ****: p < 0.0001. TBP, tuberculosis patient without diabetes mellitus; TBDM, tuberculosis patient with diabetes mellitus; D0, baseline before treatment initiation; M6, end of TB treatment, after six month; M8, 2 months after the end of TB treatment.

IGRA from QFT-P has already been reported to vary depending on the TB treatment course (7, 19), the influence of DM on the QFT-P was mainly reported in cases of TB infection detections. Our study is consistent with previous studies that identified persistent inflammation with notably systemic type 1 and pro-inflammatory response during anti-TB treatment in TBDM patients (9, 20–22). In the present study, DM appeared to not influence the QFT-P qualitative result proportions at baseline, with identical performance in TBP, TBDM, and DM clinical groups, and no significant difference was observed in the quantitative responses to TB1 and TB2 antigens between diabetics and nondiabetic TB patients at inclusion. However, higher *Mycobacterium tuberculosis*-specific responses were reported in infected patients with prediabetes and may reflect pathological inflammatory and ineffectual responses to TB or active *Mycobacterium tuberculosis* replication in participants with diabetes (23). After TB treatment, a systematic review of about 30 studies found that quantitative IGRA responses generally decrease during treatment for TB (19). However, in the present study, after TB treatment, a significantly higher IFN-γ response was observed in the TBDM patients compared to those without DM (TBP). Moreover, a higher conversion rate (83.3%) and a low reversion rate (0.0%) were observed within the TBDM patients, as all treated TBDM patients with positive QFT-P at baseline remained positive until the end of treatment at M6. This observed heightened IFN-γ response in TBDM compared to TBP raises intriguing questions regarding the interplay between these two conditions and their impact on immune responses to TB treatment. This finding aligns with previous studies indicating that DM can exacerbate inflammatory responses and alter immune function, potentially influencing treatment outcomes in TB (22, 24). We suggest that following successful TB treatment, improved glycemic control may help restore immune function, enabling T cells to regain their capacity to produce IFN-γ. In other hand, this improved glycemic control following successful TB treatment might not only restore immune function but also help modulate the high concentrations of IFN-γ that persist post-treatment. This regulatory effect could play a crucial role in re-establishing immune homeostasis. While the interferon-gamma overactivation or dysregulation is known to play a role in the development or exacerbation of dysglycemia (25) by for instance promoting insulin resistance and contribute to dysglycemia. Hyperglycemia on the other hand can indeed impair the function of immune cells which can result in chronic inflammation and tissue damage (26). Hyperglycemia and inflammation create a feedback loop in which high blood glucose levels promote the release of pro-inflammatory cytokines and other inflammatory mediators like the interferon-gamma. These, in turn, contribute to insulin resistance and chronic inflammation, worsening dysglycemia. Over time, this feedback loop can lead to serious complications, especially in conditions like type 2 diabetes, where inflammation and insulin resistance perpetuate one another. Managing blood glucose levels and controlling inflammation are crucial to breaking this cycle and preventing the progression of metabolic disorders. Additionally, we anticipate that pulmonary impairment post-TB could be more pronounced in patients with concurrent diabetes due to the prolonged inflammatory state. Our

study may shed light on the heterogenous patterns of IGRA levels following treatment due to the high degree of variation between participants, which need to be adapted for DM and patients with similar dysregulated immune systems in order to be useful for monitoring anti-tuberculous treatment in clinical practice (27). The higher conversion rate and minimal reversion rate observed exclusively within the TBDM group suggest a distinct immune profile in these patients after treatment. The persistent positivity of QFT-P among all initially positive TBDM patients may simply imply a sustained immune activation despite treatment completion, which could reflect ongoing inflammation or impaired immune regulation in the context of DM (20–22).

Besides the QFT-P assay, we also performed WBC count analysis and monitored the monocyte to lymphocyte ratio (MLR) in the two groups (TBP and TBDM) prior to, during, and after completion of TB treatment. Prior to TB treatment, elevated WBC and monocyte counts with lymphopenia were observed in both groups of TB patients (TBP and TBDM). After TB treatment, WBC and monocyte absolute counts globally decreased for the two groups of TB patients treated (Figure 5A), indicating a systemic response to TB therapy, regardless of DM comorbidity. These findings suggest that while DM may predispose individuals to altered immune function and inflammatory responses (24), it does not significantly impact the overall dynamics of WBC levels in TB patients prior to treatment initiation. MLR has been observed to be associated with active TB and other studies have reported decreases in the MLR values after anti-TB treatment (28–31). Thus, the MLR assay can be used as a biomarker to identify TB and monitor the effectiveness of anti-TB therapy (30, 32). It has been reported that DM can modulate the dynamics of the immune cells, notably the monocytes and lymphocytes (33–36). In the TB field, DM could affect the basal activation state of some effector cells and their capacity to control *Mycobacterium tuberculosis* infection (37). However, in the present study, our results showed that DM does not globally affect the WBC dynamics in TB patients prior to treatment, and a MLR decrease was observed in both the TBP and TBDM groups after TB treatment. Thus, this reduction in MLR values following TB treatment in both groups reflects the efficacy of anti-tuberculosis therapy in modulating systemic inflammation, suggesting a restoration of immune balance and resolution of the inflammatory milieu associated with active TB infection (22). While further investigation should be conducted to assess the physical lung tissue damages and the persisting inflammation due to DM following TB treatment, it is worthwhile to notice that in the present study, like the TBP group, all the TBDM patients had successful TB treatment.

In this study, we did not show any differences in QFT-P or MLR results between TBDM patients receiving diabetes treatment and those not receiving it during TB follow-up. It suggests that diabetes management alone may not significantly alter immune response markers in TB treatment. This aligns with studies indicating that while diabetes can modulate immune function, glycemic control might not lead to measurable changes in TB-specific immune markers, such as IFN-γ release or lymphocyte reactivity. Chronic hyperglycemia in diabetic patients can induce a state of immune dysregulation that persists despite glucose-lowering interventions, affecting both innate and adaptive immunity without necessarily resulting in immediate changes in TB-specific immune responses during treatment (38). Additionally, diabetes is known to impact both pro-inflammatory and anti-inflammatory cytokine pathways, creating a complex immune environment that diabetes treatment alone may not fully normalize during the course of TB therapy (39). In this study, prediabetic patients were included in the TBDM group to enable a broader analysis of the spectrum of diabetes-associated TB. However, the relatively small number of participants in the prediabetic subgroup may have limited the statistical power to detect subtle immunological differences within this group. Based on the two points of HbA1c during the study period, none of the TBP patients changed to a diabetic status (TBDM), and none of the TBDM patients presented with transient DM.

In conclusion, our study provides insights into the complex interaction between TB and DM and their implications for treatment monitoring and immune response dynamics. Despite limitations in sample size, the limited number of TBDM patient receiving anti-diabetic treatment, the absence of treatment failure cases, and the prospective nature of our study, which does not account for retrospective biases or pre-existing health variations among participants, our findings highlight the distinct immune profiles observed in TB patients with and without DM, particularly regarding the performance of host immune-based assays such as QFT-P and MLR in monitoring treatment response. Our results suggest that, while DM may not globally affect WBC dynamics in TB patients prior to treatment, it can influence immune responses to TB treatment. This is evidenced by the heightened IFN-γ response and persistent QFT-P positivity observed in TBDM patient post-treatment, which may potentially contribute to immune-associated pathologies and poor clinical damage control. These findings highlight the importance of considering DM as a potential modifier of TB treatment outcomes and highlight the need for tailored monitoring strategies in this vulnerable population. To further investigate these dynamics, future studies should consider analyzing additional cytokines using the plasma samples stored in our biobank. Finally, while further research is needed to validate these findings in larger, more diverse patient cohorts and to explore the long-term implications of DM on TB treatment outcomes, our study emphasizes the significance of implementing comprehensive monitoring strategies and personalized approaches to TB management, particularly in regions with a high burden of both TB and DM.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethical Committee for Biomedical Research in Madagascar (Reference number: n°099-MSANP/CERBM). The studies were conducted in

accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PR: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. AR: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. MN: Formal analysis, Methodology, Writing – review & editing, Investigation. CR: Investigation, Data curation, Methodology, Supervision, Writing – review & editing, Project administration. HR: Writing – review & editing, Resources. PH: Investigation, Supervision, Writing – review & editing. MR: Investigation, Resources, Writing – review & editing. AHR: Conceptualization, Writing – review & editing. JR: Conceptualization, Writing – review & editing. JH: Conceptualization, Writing – review & editing, Methodology, Funding acquisition, Resources. RR: Investigation, Writing – review & editing, Formal analysis, Methodology, Supervision, Validation. NR: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1451046/full#supplementary-material

SUPPLEMENTARY FIGURE 1
IFN-γ value comparison between participants with positive QuantiFERON-TB Gold Plus among TBP, TBDM, DM and HC groups following stimulation with TB1 and TB2 antigens at inclusion (D0). Box plots represent median and interquartile range. The upper and lower edges of the boxes represent the third and first quartiles (Q3 and Q1), respectively, while the line inside the box represents the median (Q2).

SUPPLEMENTARY FIGURE 2
Monocyte-to-lymphocyte ratio (MLR) levels of TBP and TBDM at M6 and M8 compared to healthy controls (HC). Box plots represent median and interquartile range. The upper and lower edges of the boxes represent the third and first quartiles (Q3 and Q1), respectively, while the line inside the box represents the median (Q2).

SUPPLEMENTARY FIGURE 3
Comparison of neutrophil-to-lymphocyte ratio (NLR) levels after TB treatment (M6 and M8) among the TBP and TBDM groups, and healthy controls (HC). Box plots represent median and interquartile range. The upper and lower edges of the boxes represent the third and first quartiles (Q3 and Q1), respectively, while the line inside the box represents the median (Q2). Data were analyzed using Kruskal-Wallis with Dunn's multiple comparison test. ***: $p<0.001$, ****: $p < 0.0001$.

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Management of tuberculosis risk, screening and preventive therapy in patients with chronic autoimmune arthritis undergoing biotechnological and targeted immunosuppressive agents

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Tuberculosis (TB) is the leading cause of death in the world from an infectious disease. Its etiologic agent, the *Mycobacterium tuberculosis* (Mtb), is a slow-growing bacterium that has coexisted in humans for thousands of years. According to the World Health Organization, 10.6 million new cases of TB and over 1 million deaths were reported in 2022. It is widely recognized that patients affected by chronic autoimmune arthritis such as rheumatoid arthritis (RA), psoriatic arthritis (PsA), and ankylosing spondylitis (AS) have an increased incidence rate of TB disease compared to the general population. As conceivable, the risk is associated with age ≥65 years and is higher in endemic regions, but immunosuppressive therapy plays a pivotal role. Several systematic reviews have analysed the impact of anti-TNF-α agents on the risk of TB in patients with chronic autoimmune arthritis, as well as for other biologic disease-modifying immunosuppressive anti-rheumatic drugs (bDMARDs) such as rituximab, abatacept, tocilizumab, ustekinumab, and secukinumab. However, the data are less robust compared to those available with TNF-α inhibitors. Conversely, data on anti-IL23 agents and JAK inhibitors (JAK-i), which have been more recently introduced for the treatment of RA and PsA/AS, are limited. TB screening and preventive therapy are recommended in Mtb-infected patients undergoing bDMARDs and targeted synthetic (ts)DMARDs. In this review, we evaluate the current evidence from randomized clinical trials, long-term extension studies, and real-life studies regarding the risk of TB in patients with RA, PsA, and AS treated with bDMARDs and tsDMARDs. According to the current evidence, TNF-α inhibitors carry the greatest risk of TB progression among bDMARDs and tsDMARDs, such as JAK inhibitors and anti-IL-6R agents. The management of TB screening and the updated preventive therapy are reported.

KEYWORDS

tuberculosis disease, TB infection, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, biologic DMARDs, JAK inhibitors, preventive therapy

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1 Introduction

Tuberculosis (TB) is the leading cause of death in the world from an infectious disease. Its etiologic agent, the *Mycobacterium tuberculosis* (Mtb), is a slow-growing bacterium that has coexisted in humans for thousands of years. According to the World Health Organization (WHO), 10.6 million new cases of TB and over 1 million deaths were reported in 2022 (1).

As a respiratory pathogen, the transmission occurs through inhalation of aerosols or droplets containing bacilli expelled by a person with TB disease. An estimated quarter of the world population has been infected with Mtb (1). Most TB cases are reported in low- and middle-income countries. In particular, more than two-thirds of people with TB live in Bangladesh, China, India, Indonesia, Nigeria, Pakistan, Philippines and South Africa (1). This heterogeneous distribution is due to the differences between countries in terms of social and economic development and health-related factors, such as alcohol use disorders, diabetes, HIV infection, smoking and undernourishment, which are known to increase the risk of TB disease (2). In addition to health conditions, immunosuppressive therapies affecting the immune system, including those used for rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS), increase the risk of TB disease in Mtb-infected individuals (3).

Following infection, the majority (90%) control Mtb replication through innate and adaptive immunity establishing a state referred to as TB infection (TBI), and in the past called latent TB infection (4). On the other hand, 5-10% of the infected subjects can develop TB disease; half of them within the first 5 years, and half during their lifetime.

TB is traditionally classified as primary or secondary according to the time between the initial infection and the onset of the clinical disease. Primary TB occurs in previously uninfected subjects after *de novo* infection, whereas secondary TB develops in a previously sensitized host, and it may occur following reactivation of Mtb infection or reinfection from external source. Indeed, secondary TB usually, but not always, develops in a person with a weakened immune system (5).

In the context of Mtb infection, a dynamic equilibrium between the host and the microbe is present, with bacilli that can switch from a dormant state to intermittent or active replication depending on the capability of the host immune system to contain or not Mtb replication (6, 7). Therefore, TB is referred to as a "continuum process" characterized by different stages between TB infection and TB disease, as described elsewhere (8, 9).

In this review, we revised the current evidence from randomized clinical trials (RCTs), long-term extension studies (LTEs), and real-life studies regarding the risk of TB in patients with chronic autoimmune arthritis including rheumatoid arthritis (RA), psoriatic arthritis (PsA), and ankylosing spondylitis (AS), treated with biologic and targeted disease-modifying immunosuppressive anti-rheumatic drugs (bDMARDs; tsDMARDs).

2 Immunopathogenesis of TB

The immune response to Mtb infection is multifaceted and it involves both innate and adaptive immune response (4, 10). Upon infection, bacilli are phagocytosed by alveolar macrophages, which represent the first defense line against Mtb due to their antimicrobial mechanisms (11, 12). However, Mtb has evolved different mechanisms to avoid its elimination by inhibiting phagosome maturation and acidification, and escaping autophagy in macrophages (13–15), which become a permissive niche for Mtb replication.

As the infection progresses, macrophages migrate into the lung interstitium where they recruit other innate cells such as neutrophils, monocytes, macrophages, and dendritic cells due to the release of cytokines, including TNF-α, IL-1α, IL-6, IL-1β and IFN-γ, thus favouring the dissemination of mycobacteria to uninfected cells. Once activated, T and likely B cells are recruited to the site of infection contributing to the formation of the organized granuloma, a structure known as the hallmark of TB (10). The immune microenvironment within the granuloma influences the prognosis and outcome of TB disease leading to different scenarios: Mtb clearance, bacterial replication causing primary TB, bacterial dormancy, or reactivation of the infection (16–19).

$CD4^+$ Th1 cells producing cytokines such as IFN-γ and TNF-α have been identified as the most important cell subset to control Mtb infection. The differentiation of naïve $CD4^+$ T cells to Th1 cells is promoted by IL-12, a cytokine released by antigen presenting cells (APCs) (20). IFN-γ and TNF-α enhance the antibacterial activity of macrophages by increasing autophagy, promoting phagosome maturation, and inducing the production of antimicrobial peptides. Besides macrophages, IFN-γ and TNF-α activate B cells and the cytotoxic $CD8^+$ T cells. Both cytokines are of utmost importance for the formation and maintenance of a well-organized granuloma (21).

The role of Th17 cells, whose differentiation is induced by IL-23, is controversial. Th17 response seems to be involved in the early steps of protection from Mtb infection, and the recruitment of neutrophils, macrophages, and Th1 cells to the site of infection (22, 23). Th17 cells enhance the expression of cytokines (IL-17A, IL-17F, IL-21 and IL-22) and antimicrobial peptides that lead to phagocytosis of Mtb (23). IL-17 may be released by either innate lymphocytes of the ILC3 class or Th1/Th17, and it seems to be implicated in the maturation process of granulomas (24). However, an overproduction of IL-17 was also associated with exaggerated recruitment of neutrophils and inflammation leading to immunopathology (25, 26). As with IL-17, also the excessive production of other pro-inflammatory cytokines such as TNF-α, IL-1, IFN-γ may result in tissue damage and bacterial growth. A balance is crucial to control progression to TB disease (27).

The pivotal role of IL-12, IFN-γ, and TNF-α in controlling Mtb infection is corroborated by the higher susceptibility to TB disease of the individuals treated with immunosuppressive therapies like TNF-α inhibitors (28–30), or individuals with innate defects of the IL-12/IFN-γ axis (31–33), with HIV infection (34) or with primary immunodeficiencies associated with T-cell deficiency (35).

Mendelian susceptibility to mycobacterial disease (MSMD) is an inborn error of immunity associated with a selective predisposition to mycobacterial infections. MSMD involves specific mutations in 18 genes (*IFNG, IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, IL12RB2, IL23R, RORC, TBX21, IRF8, SPPL2A, ISG15, TYK2, JAK1, ZNFX1, NEMO, CYBB*), which are associated

with an impaired IFNγ/IL-12 response/production (35). Moreover, patients with defects of CD40 ligand (CD40L) and NF-kB signaling are more susceptible to mycobacterial disease, as this pathway is involved in the IL-12 production (36).

A number of distinct Mendelian disorders are also caused by inborn errors in components of the IL-6 family of cytokines and their signaling pathways (STAT3 and GP130) (37). The majority of patients with TYK2 defects, one of the three Janus kinases (JAKs) associated with GP130 signaling, shows defects in type I antiviral and mycobacterial immunity (38).

In addition to the use of TNF-α inhibitors, the inherited TNF deficiency has been identified as a genetic aetiology of recurrent pulmonary TB in adults observed within 1 year of the end of treatment. TNF deficiency seems to be responsible for the selective impairment of reactive oxygen species (ROS) production by alveolar macrophages. The ROS production is crucial for the phagocytic control of Mtb (39).

Regarding B cells and antibodies (Abs), initially there was some scepticism about their effective contribution to the host defense against Mtb due to the intracellular nature of the pathogen (40, 41). However, although B cells and Abs may not be able on their own to counteract Mtb, the accumulating evidence shows that they can favour and enhance cell-mediated immunity (42). Indeed, Abs binding to Mtb can mediate different processes such as antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, and complement activation, thus helping to reduce the mycobacterial burden (42). However, B cells are not limited to antibody production. They can act as antigen-presenting cells by presenting mycobacterial antigens to T cells, thereby inducing their activation. In addition, once activated, B cells can release cytokines, thus affecting the activity of different immune cells (43, 44). The role of B cells in controlling Mtb infection is corroborated by the association of TB disease with reduced B cell count and function (45, 46).

Considering the pivotal role played by the host immune response in controlling Mtb replication, risk factors for the progression to TB disease include immunosuppressive therapies (Figure 1).

3 Patients with chronic autoimmune arthritis and TB risk

Patients with chronic autoimmune arthritis such as RA, PsA and AS are at higher risk of infections and related complications, which are the main cause of mortality in these conditions (47).

It is widely recognized that patients affected by RA, PsA, and AS have an increased incidence rate of TB disease compared to the general population, primarily related to immunosuppressive therapy rather than the disease itself. As conceivable, the risk is associated with age ≥65 years and is higher in endemic regions, but the use of immunosuppressive therapy plays a key role also in low TB endemic countries (3, 48). There is evidence that corticosteroids (CCS) can increase the risk of TBI reactivation in a dose-dependent manner (49, 50).

Among csDMARDs commonly used for the management of RA, PsA, and AS, leflunomide and azathioprine have emerged as having the highest risk, whereas sulfasalazine and methotrexate appear to confer only low to moderate risk (50, 51). In the last 20 years, biologic DMARDs (bDMARDs) have significantly improved the treatment of rheumatologic diseases, including RA, PsA, and AS, contributing to introduce the ambitious target of remission/minimal disease activity.

Anti-TNFα agents were the first bDMARDs to be adopted and are still the most used worldwide. Currently, 4 anti-TNF monoclonal antibodies and one receptor fusion protein are available: adalimumab (ADA), etanercept (ETN), infliximab (IFX), golimumab (GOL), and certolizumab (CTP). Other bDMARDs are used exclusively for the treatment of RA, including the anti-IL6 receptors tocilizumab and sarilumab, as well as the anti-CD20 rituximab. In contrast, the anti-IL12/23 ustekinumab, anti-IL23 guselkumab and risankizumab, and IL-17 inhibitors secukinumab and ixekizumab are used only for PsA and AS. Finally, the CTLA4-Ig abatacept and JAK inhibitors (JAK-i) are approved for both RA and PsA/AS. The increased risk of TB in patients with chronic autoimmune arthritis undergoing anti-TNF agents is widely recognized (3, 52–55). Although the data are less robust, there are also substantial data available for other bDMARDs such as rituximab, abatacept, tocilizumab, ustekinumab, and secukinumab (56–58).

Conversely, data on anti-IL23 agents and JAK inhibitors (JAK-i), which have been more recently introduced for the treatment of RA and PsA/AS, are scarcer.

4 Specific biological therapy for autoimmune arthritis and TB risk

In the following section, we describe the mechanism of action of bDMARDs and tsDMARDs used for the treatment of RA, PsA, and AS, and analyze how inhibiting specific pathogenic pathways might affect the integrity of the TB granuloma. We then summarize the main data on TB risk associated with these therapeutic agents from RCTs, LTE, and real-world studies in these pathologies (Table 1). Indeed, it is important to underline that RCTs provide very reliable and complete data, but patients with TB disease were not included, and patients with TBI could be enrolled only after having received TB preventive therapy according to local guidelines. On the other hand, real-life studies (registries and observational studies) are affected by a higher risk of bias, but can also report cases of TBI patients who did not receive preventive treatment; thus, they are particularly relevant for inferring the impact of b/tsDMARDs on the natural course of TBI.

4.1 TNF-α inhibitors

TNF-α is a pivotal cytokine in the pathogenesis of RA, PsA, and AS, as it acts on different cells such as synoviocytes, macrophages, chondrocytes, and osteoclasts. It induces local inflammation and pannus formation, contributing also to cartilage degradation and bone erosions (59, 60). High TNF-α

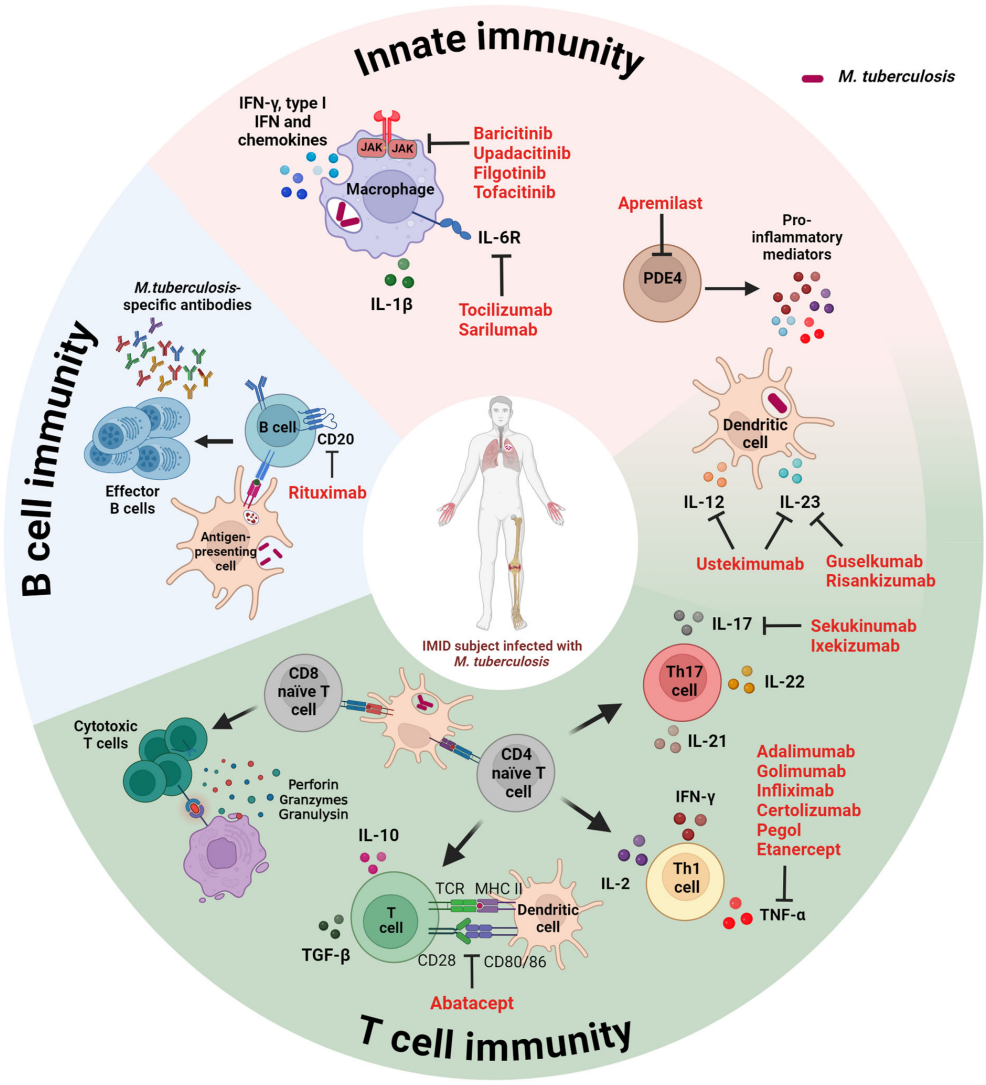

FIGURE 1
Schematic representation of the immune response targets of disease-modifying immunosuppressive anti-rheumatic drugs used for the management of IMID patients. Both innate and adaptive immunity (B and T cells) play a key role in controlling Mtb infection. Immunosuppressive therapies targeting host immune factors increase the risk for progression to TB. IMID, immune-mediated inflammatory disease; IFN, interferon; IL, interleukin; JAK, Janus kinase; MHC, major histocompatibility complex; PDE4, phosphodiesterase-4; TCR, T cell receptor; TGF, tumor growth factor. Created with BioRender.com.

levels have been observed in the synovial fluid and synovium of patients with RA and PsA (60).

As previously mentioned, TNF-α is also critical for the formation and maintenance of Mtb granulomas. TNF-α enhances the phagocytic capacity of macrophages, promotes the production of reactive nitrogen and oxygen species to kill intracellular bacteria, and facilitates the recruitment of immune cells at the site of infection (21).

TNF-α inhibitors neutralize TNF-α activity, by disrupting the immune response necessary for granuloma integrity, and leading to mycobacterial growth and dissemination with progression to TB disease.

There are remarkable differences among anti-TNF-α agents in their ability to inhibit TNF-α, that can explain the reported differences in TB risk. Anti-TNF-α mAbs target and neutralize both soluble and membrane-bound TNF-α with high affinity, and also have some cross-reactivity with Lymphotoxin (LT)-α (61). On the other hand, ETN, being a dimeric fusion protein, binds to the trimeric form of soluble TNF-α and, only to a lesser extent, to membrane-bound TNF-α and LT-α (62, 63).

The more comprehensive blockade of TNF-α activity and functions in immune defense mechanisms by anti-TNF-α mAbs may contribute to the observed higher risk of TBI reactivation.

The early clinical trials of IFX and ETN revealed a significant risk of TB, leading to the introduction of mandatory TB screening guidelines for patients starting anti-TNF-α therapy.

In particular, in the ATTRACT and ASPIRE RCTs there were 70 cases of TB disease among patients receiving IFX for an

TABLE 1 TB risk associated to the different biological drugs used for rheumatic patients.

Mechanism of Action	Biologic	Rheumatologic indications	TB risk	TB screening mandatory
TNF Inhibitors	Infliximab Adalimumab Etanercept Golimumab Certolizumab Pegol	RA, PsA, SpA	High High Medium/High Medium/High Medium/High	Yes
IL-6R Inhibitors	Tocilizumab Sarilumab	RA	Medium	Yes
JAK Inhibitors	Tofacitinib Baricitinib Upadacitinib Filgotinib	RA, PsA, SpA RA RA, PsA, SpA RA	Medium	Yes
CTLA4-Ig	Abatacept	RA, PsA	Low	Yes
IL-12/23 Inhibitor	Ustekinumab	PsA	Low	Yes
IL-23 Inhibitors	Guselkumab Risankizumab	PsA	Low	Yes
IL-17 Inhibitors	Secukinumab Ixekizumab	PsA, SpA	Low	Yes
CD20 Inhibitor	Rituximab	RA	Low	No
PDE4 Inhibitor	Apremilast	PsA	Low	No

*Risk based on mechanism of action and TB IR before the introduction of systematic TB screening.

incidence rate (IR) of approximately 0.5-1.0/100 patient-years (PY); the majority of these cases occurred within the first few months of therapy (64).

ETN showed a lower incidence of TB in its pivotal ERA trial; however, the risk was still significant enough to warrant concern with an IR of 0.02-0.1/100PY (65). By the early 2000s, both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) had issued guidelines recommending TB screening before starting treatment with anti-TNF-α agents.

The subsequent implementation of screening protocols has significantly reduced TB rates, contributing to the lower incidence observed in LTE studies and real-world applications of IFX, ADA, and ETN, as well as with newer anti-TNF-α agents like golimumab and certolizumab pegol (52). Indeed, the IR of TB reactivation in clinical trials and LTEs conducted after the introduction of TB screening, decreased to 0.2-0.3 for IFX and to 0.1-0.2/100PY for ETN and ADA (66–79).

Also, golimumab and certolizumab pegol trials reported relatively low rates of TB. Indeed, the GO-BEFORE and GO-FORWARD golimumab studies found an IR of 0.2/100PY, whereas the RAPID 1-2 certolizumab trials found an IR of 0.1/100PY (80–84).

Different registries evaluated the incidence of TB in patients receiving TNF-α inhibitors after the introduction of TB screening protocols with similar results. Among these, the RABBIT registry reported an IR of 0.14/100PY, the BIOBADASER found an IR of 0.05-0.1/100PY, while the ARTIS registry reported an IR of 0.15/100PY (85–87).

Data from previous systematic reviews and meta-analyses showed similar data, with an IR of 0.18/100PY in rheumatic patients receiving anti-TNF-α, 4 times higher compared to rheumatic patients not receiving these therapies (88). Rigorous screening and prophylactic treatment are required on the summary of product characteristics (SmPC) of anti-TNF-α agents.

4.2 Anti-IL6R

Tocilizumab (TCZ) is a humanized monoclonal antibody targeting the human IL-6 receptor (IL6R). It was approved by EMA in 2009 for the treatment of RA patients. Sarilumab is another fully human monoclonal antibody targeting the IL-6R, more recently approved for RA treatment.

IL-6 is a versatile cytokine with a wide array of functions, including modulation of acute phase reactant pathways, B and T lymphocytes, blood-brain barrier permeability, synovial inflammation, and hematopoiesis. This cytokine plays a crucial role in bridging innate and adaptive immune responses, and in facilitating the recruitment of macrophages. Dysregulation of the IL-6 axis is implicated in the inflammatory pathways of various autoimmune disorders, such as RA.

Previous studies on experimental mice models showed that IL-6 plays a significant role in the protection against Mtb, and the absence of IL-6 leads to an early increase in bacterial load with a concurrent delay in the IFN-γ induction. However, IL6 knockout mice contained and controlled bacterial growth and developed a protective memory response to secondary infection, demonstrating that while IL-6 is involved in stimulating early IFN-γ production, it is not essential for the development of protective immunity against Mtb. The role of IL-6 in human TB remains controversial, and the

specific functions of the IL-6 produced by B cells are still poorly understood, despite its abundance in TB-infected lungs. Some studies have reported increased concentrations of IL-6 in the sera of patients with advanced pulmonary TB compared to healthy controls, as well as elevated IL-6 gene expression in peripheral blood cells of TB patients, supporting a potential pathophysiological role (89, 90).

Furthermore, a recent study demonstrated that treatment with *in vitro* TCZ does not inhibit IFN-γ-specific response on whole blood from patients with TB disease stimulated with two different Mtb antigens, differently from the effects observed with ETN and IFX, both of which led to a reduced IFN-γ response (57, 91, 92).

A comprehensive safety analysis and systematic review published in 2014 assessed the incidence of TB in patients treated with TCZ from RCTs and LTE studies, and found no cases of TB disease among 15485 RA patients (29). A meta-analysis of RCTs trials and LTEs found 9 cases of TBI reactivation on 12509PY for an IR of 0.069/100PY (93). Data from the British Society for Rheumatology Biologics Register for Rheumatoid Arthritis (BSRBR-RA) showed one case of TB disease among 2171 RA patients treated with TCZ, resulting in an IR of 0.026/100PY (94).

Observational studies from European countries and a Japan nationwide study did not detect TB cases in TCZ users (95). Finally, a recently published nationwide observational study on RA patients from Korea, an intermediate TB burden country, reported 10 TB cases on 2185PY for an IR of 0.45/100PY (96).

The IR was similar to ETN and higher in TBI patients than those without TBI, indicating different effects between *de novo* infection and TB reactivation. Screening for TBI is mandatory in the SmPC of IL6R inhibitors.

4.3 Anti-IL-17

The IL-17 family encompasses six proteins (IL-17A to IL-17F) and five receptors (IL-17RA to IL-17RE). While IL-17A and IL-17F individually possess limited inflammatory potency, their robust inflammatory effects primarily stem from their ability to recruit immune cells and synergize with other pro-inflammatory cytokines like TNF-α, IL-1β, and IL-22. Through the recruitment and activation of neutrophils, IL-17A and IL-17F serve as pivotal components in the innate immune response against extracellular bacteria and fungi. Their protective role is particularly important on mucosal surfaces and skin, where they are rapidly released upon appropriate stimulation, thereby serving as a crucial link between innate and adaptive immune responses.

The involvement of IL-17 in TB pathogenesis has been debated, raising concerns and uncertainties about TB risk. Indeed, early granuloma formation may depend on IL-17A, but IL-17A-induced neutrophil recruitment may also increase pathological lesions and bacterial burden in chronic pulmonary infections. Notably, an *in vitro* study on a microgranuloma human model demonstrated that anti-TNF-α treatment could induce Mtb reactivation, whereas anti-IL17 treatment was comparable to control, indicating a lack of effect on Mtb dormancy. Moreover, mice lacking both IL-17RA and IL-22 pathways still managed to control TB, suggesting no compensatory relationship between these pathways. In contrast, TNF-α-deficient mice succumbed rapidly (97). In a series of studies by Khader and Cooper, low-dose aerosolized bacteria were delivered to the lower airways of the lungs in IL-17/IL-23 deficient mice (98, 99). Notably, the absence of IL-23 and IL-17 in the lung leads to more severe inflammation, suggesting that these cytokines help maintaining the granuloma integrity in later stages of Mtb-induced inflammation by limiting neutrophil death.

Secukinumab (SEK), a fully human monoclonal IgG1 antibody, specifically targets and inhibits IL-17A. Following the demonstration of its significant efficacy in phase 3 studies in 2015, it was approved for treating PsA and AS in 2016. Pooled data from 5 phase III trials on PsA (FUTURE program), and 4 phase III trials (MEASURE program) on AS, on a total of 2523 and 977 patients respectively, reported 5 cases of new TBI (one PsA and 2 AS patients), and no cases of TB disease (100). In LTE studies (1–5 years) in patients with PsA and AS, the safety profile was consistent with that of previous phase III studies, and no new TB infections or TB disease reactivations were observed (101). Notably, Liu et al. reported zero cases of reactivation among 3 PsA/AS patients with TBI who did not receive TB preventive therapy (102). Ngoc et al. in 2022 described a case of TB disease from Vietnam in a 19-years-old man affected by AS after two years of SEK treatment (103).

Ixekizumab (IXE) also neutralizes IL-17A but, differently from SEK, it is a humanized IgG4 monoclonal antibody. This structural variation contributes to its higher affinity for IL-17. Its approval for PsA and AS was granted in 2018. No TB disease cases from pooled analysis of 3 RCTs (SPIRIT program) on PsA patients were recorded. In the PsA studies, 32 (2.9%) patients resulted in TBI during the study, of whom 20 were discontinued per-protocol. Interestingly, among the remaining 12 patients continuing IXE treatment, no cases of TB reactivation were reported, even though only 7 patients received TBI therapy (104).

Finally, bimekizumab (BMK) represents a humanized IgG1 monoclonal antibody with dual neutralizing effects against both IL-17A and IL-17F. It has been very recently approved for the treatment of PsA and AS. A total of 267 PsA patients from BE COMPLETE trial and its open-label extension 1-yr follow-up (BE VITAL trial), reported no cases of TB disease (105). Data from two phase III BE MOBILES trials on AS, did not report TB disease cases at 1-yr follow-up. Finally, a total of 303 patients with active AS from BE AGILE trial and its open-label extension study reported no cases of TB disease (106, 107). We still need data from real-life studies and longer follow-ups. TB screening is only suggested in the SmPC of anti-IL17 agents.

4.4 Anti-IL-23 and IL-12/23

IL-12 and IL-23 are heterodimeric cytokines containing p35 and p40 subunits, and p19 and p40 subunits, respectively. IL-12 and IL-23 are produced by APCs, such as dendritic cells, macrophages, and monocytes. IL-12 plays a key role in the differentiation of naïve $CD4^+$ T cells into Th1 cells, whereas IL-23 is involved in the expansion and

maintenance of Th17 cells. A role for IL-12 and IL-23 dysregulation in the pathophysiology of PsA has been suggested (108).

Ustekinumab (UST) is a human monoclonal antibody that binds the p40 subunit shared by both IL-12 and IL-23, effectively suppressing their functions. It was approved for the treatment of PsA patients in 2013. No TB disease cases were observed neither in pivotal studies (PSUMMIT program) on a total of 1073 PsA patients, nor in their LTE data (109, 110). Few data from available real-life observational studies on PsA patients did not report cases of TB reactivation, and previous reviews have assessed the risk of TB reactivation as very low (111). A case of peritoneal TB in a PsA patient from Philippines, on UST treatment, with multi bio-failure, and after having been treated for latent TB, was observed (112). Screening and treatment of TBI are recommended in the SmPC supplied with UST.

Guselkumab (GUS) is a fully human IgG1λ monoclonal antibody, which specifically binds to the p19 subunit of IL-23. It stands as the first of its class to gain approval for the treatment of PsA patients.

Risankizumab (RSK) is a humanized immunoglobulin G1 monoclonal antibody that specifically inhibits IL-23 by binding to its p19 subunit, and it has been recently approved to treat PsA. Pooled data from DISCOVER 1 and 2 trials on 748 patients with active PsA for GUS reported zero cases of TB reactivation at 1-year follow-up (113, 114). RSK safety data sets from 4 phase II and III trials (KEEPsAKE program) in PsA on a total 1542 patients representing 2741.6PY, reported one case of TB disease in a patient from Taiwan previously treated with a 9-month course of isoniazid prophylaxis (115, 116). There is still very little real-life data on anti-IL-23 in PsA patients with RSK. Takeda et al. reported the case of a 64-year-old man affected by PsA who developed active pulmonary TB after two months of GUS therapy (the patient was negative at baseline TB screening) (117).

Notably, several real-world data are available for patients with psoriasis. A total of 68 and 25 TBI patients, who did not receive any or adequate TBI preventive therapy, were treated with RSK and GUS, respectively (118–122). Remarkably, there were no documented cases of TB reactivation, which corroborates the safety profile of anti-IL-23 agents in patients with TBI who did not receive prophylactic care. In the SmPC of anti-IL-23 agents is indicated that patients should be evaluated for TBI before starting treatment.

4.5 Anti-CD20

Rituximab (RTX) is a chimeric monoclonal antibody targeted against CD20, which is expressed on the surface of normal and malignant B lymphocytes. It was first approved by the FDA in 1997 for the treatment of malignancy, and in February 2006 for the treatment of patients with moderately to severely active RA, who did not adequately respond to one or more anti-TNF-α agents. RTX binds via its F(ab0)2 portion to the CD20 antigen expressed on B lymphocytes, whereas its Fc domain plays immune effector functions to mediate B cell lysis *in vitro*. RTX cytotoxicity is mediated by three different mechanisms including antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, direct disruption of signaling pathways, and triggering of apoptosis (123, 124).

RTX's targeted action on B cells, which spares the critical TNF-α pathways necessary for TB containment, likely explains its lower associated risk of TB reactivation compared to anti-TNF-α therapies as reported by several data (125–127). No cases of TB disease have been reported in patients receiving RTX in 9 RCTs conducted in 3623 RA patients. In two LTEs in RA patients, two cases of TB disease have been reported during a follow-up time of 9.5 years (IR 0.018/100PY) (29, 128). Data from real-life studies and registries found very few cases of *de novo* TB infection or TB disease reactivation during RTX treatment (129–132).

Data from observational studies and registries reported very few cases of TB disease. In particular, only one TB case was reported in 2484 RA patients treated with RTX in the German GENIRIS study, and 2 cases from the BSRBR-RA registry during 17154PY (0.012/100PY) (127, 133).

Finally, a meta-analysis including data from several clinical trials and LTEs reported that the IR of TB disease was high (>0.040/100PY) for patients treated with tofacitinib and all biologics but RTX (0.020/100PY) (93). Overall RTX emerged as having one of the lowest pooled IR of TB among bDMARDs. In line with this evidence, the summary of product characteristics of RTX does not specifically mandate routine screening for TB before initiating therapy.

4.6 CTLA-Ig

Abatacept (ABT) is a fully human recombinant fusion protein composed of the extracellular binding domain of human cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), fused to a modified segment of human IgG1. Its mechanism of action involves blocking the CD80/CD86 costimulatory pathways, thus preventing the activation of naïve T cells. ABT binds to CD80 and CD86 on APCs with higher affinity compared to CD28 on T cells, effectively interfering with the interaction between CD28 and CD80/CD86 (134). ABT has been approved by the EMA for the treatment of RA since 2007, 10 years later it received approval for the treatment of PsA patients.

Its peculiar mechanism of immune modulation, which avoids direct cytokine inhibition, seems to be a key factor that likely contributes to its safer profile compared to anti-TNF-α regarding the overall risk of infections and, in particular, TB reactivation.

Pooled data from 8 RCTs and LTE studies on RA patients revealed an overall IR of 0.0066/100PY (135). A recent 10-year international post-marketing study found a very low IR across different registries (ARTIS, FORWARD, RABBIT) with only one event on a total of 9652PY of exposure (136). TB rates were low but slightly higher in two Canadian and USA registries, with 9 cases on 1067PY and 17 cases on 3994PY, respectively (137). Finally, a recent meta-analysis of LTEs showed a low estimated pooled IR of 0.07/100PY (93). Despite these reassuring data, screening for TBI is recommended in the SmPC of abatacept.

4.7 Apremilast

Apremilast, an oral phosphodiesterase-4 (PDE4) inhibitor that effectively modulates various inflammatory mediators, has demonstrated efficacy with a favorable safety profile in several RCTs involving PsA patients with peripheral involvement (138).

These trials excluded patients with TB disease, but they did not require TBI screening for enrolment. This finding is noteworthy: patients with TBI treated with apremilast without preventive therapy showed no instances of reactivation (139).

Analysis of pooled data from the PALACE I–II–III studies involving a total of 1493 PsA patients, reported zero cases of TB disease (140). A comprehensive retrospective analysis using a large US-based claims database, including patients diagnosed with psoriasis and/or PsA who were administered at least one dose of apremilast between 2014 and 2018, identified only two cases of TB disease among 10074 patients (141).

Overall, available data highlight the minimal association of apremilast with TB reactivation. Notably, the prescribing information for apremilast does not mention the necessity for TB screening before initiation of treatment.

4.8 JAK inhibitors

Janus kinase inhibitors (JAK-i) are non-receptor tyrosine kinases associated with the cytoplasmic domain of type I and II cytokine receptors, which are activated after the engagement by their cognate ligands. Once phosphorylated, they phosphorylate signal transducers and activators of transcription (STATs), which then induce gene activation essential for cellular functions like signaling, growth, and survival (142).

The JAK family comprises four cytoplasmic non-receptor tyrosine kinases: JAK1, JAK2, JAK3, and TYK2. JAK-i are categorized into two generations. The first generation includes small molecules like baricitinib (BAR) and tofacitinib (TOF), which act as non-selective inhibitors of JAKs. In contrast, second-generation drugs such as filgotinib (FLG) and upadacitinib (UPA) exhibit more selective inhibitory activity against JAK1 than other JAK (143, 144). UPA and TOF are approved for the treatment of RA, PsA, and AS, whereas FLG and BAR are approved for RA only.

It can be speculated that the broad immunosuppressive effects exerted by the mechanism of action of JAK inhibitors, particularly through the downregulation of IFN-γ, TNF-α, and IL-6, could disrupt critical host defenses, including macrophage activation, granuloma formation, and Mtb containment.

Notably, in a BALB/c mice model, TOF was shown to diminish the control of Mtb, leading to increased bacterial replication in the lungs during chronic paucibacillary TB. This model is designed to replicate latency in a manner analogous to human TBI (145). Screening for TBI is recommended in the SmPC of all JAK-i.

Data on TOF and TB were pooled from 7061 patients across the completed 2 phase I, 10 phase II, 6 phase III, 1 phase IIIb/IV index studies, and 2 open-label LTE studies (total exposure 22875PY). TB disease was reported in 36 (0.5%) patients, with an IR of 0.2/100PY. Pulmonary and non-pulmonary TB occurred in 17 and 19 patients respectively, with most cases occurring in geographical regions endemic to TB (146). Twenty-six cases of TB disease were identified from a post-marketing surveillance analysis of TOF, on a total of 5671 RA patients for an IR of 0.21/100PY, most of which were from regions with high background IR for TB (147). Finally, a retrospective, single-center analysis from Western India reported 4 TB cases on a total of 102 RA patients treated with TOF (148).

Regarding baricitinib, an integrated study from 9 RCTs conducted over 20 countries in patients with RA and one LTE study with a follow-up period of up to 7 years, showed a total IR for TB disease of 0.2/100 PY (15 out of 3770 patients; 14744 PY) (149, 150). The IR did not increase with prolonged exposure and the events occurred mainly in endemic countries.

Data on TB risk in patients receiving UPA are pooled from 12 clinical trials (SELECT program) on 3209 patients with RA (9079.1PY), 907 patients with PsA (1872.3PY) and 182 with AS (320.1PY), showed only one case of TB reactivation (151–153). The long-term extensions analysis on 3209 RA patients for 11661.5PY, recorded one case of disseminated TB, one case of peritoneal TB, two cases of pulmonary TB, one case of female genital tract TB and 174 cases of TBI reactivation (154). No cases of TB disease were recorded in the RCTs and LTE studies conducted in PsA and AS patients, whereas a total of 51 cases of TBI reactivation were reported (155–157). We still have a few real-life data on UPA. In two recent prospective longitudinal multicenter Italian studies in RA patients enrolling 71 and 60 patients respectively, neither TB disease nor new TBI were detected during the 6 months follow-up (158, 159).

The FINCH programme, a 52-week phase 3 RCT evaluated FLG in 833 RA patients, recorded no cases of TB disease (160).

Ninenty-one cases of TB disease reactivation and no new onset TB disease cases were reported in the DARWIN clinical trial and its LTE analysis on 739 RA patients (161). Overall, these findings suggest that JAK-i carry a risk of TB, particularly in endemic regions.

5 Screening strategies and preventive therapy for TB

It is estimated that approximately one-fourth of the global population has an immune response to Mtb, indicating previous exposure or infection with the bacterium (1). Since 2015, the WHO has recommended screening and treating TBI in populations at higher risk of progression to the disease, within preventive actions of the WHO End TB Strategy (3, 162).

Patients with autoimmune diseases candidates for biological treatment are considered at risk of progressing to TB. Therefore, International guidelines (163) recommend screening and TBI preventive treatment of those with a TBI diagnosis who are candidates for biological treatment. Patients are screened for TBI either using skin tests, or interferon-γ release assays (IGRAs) based on the national guidelines in place (6, 164). Skin tests are based on the intradermal inoculation in the forearm of the purified protein derivative (PPD) as in the tuberculin skin tests (TST), or on ESAT-6 and CFP-10, as in the new generation of skin tests (165).

IGRAs are blood tests based on *in vitro* stimulation with ESAT-6 and CFP-10; the read-out is based on IFN-γ or IP-10 detection that can be performed in automated or semi-automated ways (6, 164).

In countries with BCG vaccination, where TST may show false positives, ESAT-6 and CFP-10 based assays (IGRAs or skin tests) are preferred. If either the skin test or IGRA is positive, the patient is considered with TBI (162, 166) and will undergo a chest X-ray (CXR) to exclude TB disease (162). A baseline CXR can be useful also for those who score negative on skin tests or IGRA to evaluate lung apical scores compatible with spontaneously healed TB, such as non-calcified nodules with distinct margins and fibrotic linear opacity (167). If TBI is diagnosed from a positive skin test or IGRA without lung lesions, or based solely on lung apical scars, preventive treatment is offered to those at high risk to develop TB (162).

TB preventive therapy aims to eliminate the remaining replicating mycobacteria in the body, thus resulting in a lower risk of developing the disease. This has proven to be effective in preventing TB in several populations, including children (162, 168, 169).

6 TB preventive therapy drugs and drug regimens

TB preventive therapy comprises one or two antibiotics and is different from the therapy used for TB disease, in which four antibiotics are used to reduce the likelihood of acquired drug resistance. This assumes that in TBI the acquired drug resistance is unlikely, given the small number of viable bacteria present.

Drugs commonly used for TB preventive therapies may cause adverse reactions such as liver or neuro-toxicities (Table 2). Isoniazid (INH) is an oral antibiotic with intracellular and extracellular activity against Mtb. Isoniazid has been used globally, with an average protective effect for TB of 60% during the observation period (170). The duration is 6 months, although, in 1982, a randomized trial in subjects with fibrotic pulmonary lesions showed that the risk for developing TB disease compared with placebo was reduced by 21%, 65%, and 75%, respectively for 3-, 6-, or 9-months therapy, after 5 years of follow-up (171). However, a 6-month regimen was shown to be more cost-effective than 3 or 12 months for the reduction of side effects, regimen adherence and increased adherence (162, 172–174). Neuropathy can arise as an isoniazid side effect due to the inhibitory effect of isoniazid on the function of pyridoxine metabolites; therefore, pyridoxine (vitamin B6) supplementation is recommended especially in those with alcohol abuse, malnourished, and pregnant women (175).

Rifamycins, a group of oral antibiotics such as rifampin and rifapentine, inhibit bacterial RNA synthesis by binding to the DNA-dependent RNA polymerase. These antibiotics are used to treat TBI by themselves or in combination with isoniazid to limit the side effects and the poor adherence to the long treatment duration of isoniazid (162).

Rifampicin regimens, like the 4-month course of rifampicin (4R) (162) or even shorter regimens, like the 3-month course of isoniazid and rifampicin (3HR), showed good safety and completion rates, particularly among children, with dispersible fixed-dose combinations aiding administration (176–178). For adults, however, liver toxicity and completion rate are comparable to those of longer isoniazid preventive therapy (177, 179). Administering a once-weekly dose of isoniazid and rifapentine for 12 weeks (3HP) is associated with lower rates of hepatotoxicity and higher completion rates when compared with isoniazid, although it was linked with the incidence of a hypersensitivity systemic immune response (177). Conversely, a recent meta-analysis indicated an increased incidence of grade 3 and 4 adverse events as well as a greater rate of treatment discontinuation for the 3HP regimen when compared with the 6–9 month isoniazid preventive therapy (IPT) (180).

A regimen of one-month daily INH and rifapentine (1HP) is an alternative for HIV-infected patients, and WHO conditionally

TABLE 2 TB preventive therapies and their side effects.

Drug(s)	Dosage	Time	Major side effects	Pyridoxine supplementation
INH	5 mg/kg/die (max 300 mg/die)	6 months	Liver, peripheral neuropathy	Recommended
INH	5 mg/kg/die (max 300 mg/die)	9 months	Liver, peripheral neuropathy	Recommended
Rifampicin	10 mg/kg/die (max 600 mg/die)	4 months	Liver	
INH+ Rifampicin		3 months	Liver, peripheral neuropathy	Recommended
Rifapentine+ INH	Isoniazid 900 mg/weekly Rifapentine 900 mg/weekly	3 months	Liver, peripheral neuropathy, SDR*	Recommended
Rifapentine+ INH	Isoniazid 300 mg/day Rifapentine 600 mg/day	1 month	Liver, peripheral neuropathy	Recommended
Levofloxacin	<45 kg 750 mg/day; > 45 kg, 1g/day		Tenosynovitis, QT elongation, gastrointestinal symptoms	

SDR, systemic drug reaction, defined as either (1) hypotension, urticaria, angioedema, acute bronchospasm, or conjunctivitis; or (2) >4 flu-like symptoms, Age Impact on 3HP with >1 being grade 2 or higher.
The name of the drugs is highlighted in bold.

recommended it for people aged above 13 years, although additional evaluations of safety and efficacy are needed in people without HIV (162).

For contacts of people with MDR-TB, WHO recommends using levofloxacin daily for six months to protect contacts following exposure to MDR-TB (181, 182).

For isoniazid and rifamycin therapies, as liver damage and neurotoxicity are the main side effects, conditions such as diabetes mellitus or alcoholism predisposing to neuropathy development, or chronic hepatitis B and C predisposing to liver injuries need to be carefully evaluated. Therefore, especially in patients with rheumatological disorders that often experience a metabolic syndrome (183), at baseline before starting therapy, we need to evaluate fast glycemia, glycated hemoglobin, HBsAg/Ab, hepatitis C virus Ab, and transaminase levels. To evaluate the risk of side effects to preventive therapies based on rifapentine, new strategies based on Whole-Blood Gene Signature have been proposed (184).

In patients treated with biological therapies, few studies are available regarding the side effects of preventive TB therapy (3, 185–187). A moderate and transient increase of isoniazid-induced liver damage has been reported (186, 188). Similarly, in a large Italian study, it was shown that 95% (280/295) of rheumatological patients completed TB preventive therapy with isoniazid and 96% with rifampicin (27/28). Importantly, patients who stopped taking isoniazid due to side effects successfully finished their treatment with rifampicin, showing that switching medications can still provide a good option for completing TB preventive therapy (3, 186, 187).

Although the data available are limited, this evidence suggests that patients undergoing biological therapy generally tolerate preventive treatments and complete the full course.

7 Management of preventive therapy in rheumatological patients

Before initiating preventive therapy, the physician needs to conduct a thorough medical history asking for previous exposure to TB cases and for previous liver disease, alcohol use, and concurrent treatments (to identify potential drug interactions). Patients should be informed about the symptoms of potential liver damage and should also be advised on whom to contact if these symptoms appear.

Blood control intervals of blood count, transaminases, γGT, and bilirubin should be fixed at initially 2-weekly then 4-weekly.

Methotrexate, commonly used for RA and PsA, carries a risk of liver toxicity (189). During TB treatment, it is crucial to balance the risk of arthritis flares with potential liver damage in patients taking methotrexate (190). Although specific guidelines are lacking, switching to less hepatotoxic csDMARDs, such as hydroxychloroquine or sulfasalazine, may be advisable, particularly for patients with stable disease activity. Patients with pre-existing liver conditions require even greater caution due to their increased susceptibility to toxic effects.

If preventive therapy is not tolerated, the rheumatologist should consider prescribing anti-rheumatic treatment with a low risk of TB disease reactivation. This procedure must be written and shared with the patient. Afterward, both the rheumatologist and the patient need to carefully monitor the occurrence of possible symptoms of mycobacterial reactivation to promptly isolate the patient to avoid further transmission, diagnosis, and treatment.

Importantly, after a fully completed therapy for TB disease or TBI, no further TB therapy needs to be given. It is assumed that preventive therapy kills all mycobacteria, and therefore no further preventive TB treatment is needed. IGRA results can remain positive, even after preventive therapy (191, 192), because these tests indicate the presence of an immune response against Mtb, not the presence of Mtb itself (193). Few data are present on the importance of repeated annual TB screening in a non-endemic area (185, 194). Evidence suggests that serial IGRA testing among low-risk patients on DMARDs results in a very low incidence of newly diagnosed TBI. Consequently, it is recommended to conduct targeted TBI screening based on risk factors related to TB —such as geographical origin, comorbidities like diabetes, or travel to endemic areas— before IGRA testing, rather than implementing universal annual screening in non-endemic regions (185, 194).

8 Conclusions

In conclusion, based on the available evidence, patients with chronic autoimmune arthritis under immunosuppressive treatment have an increased risk for TB reactivation. Among bDMARDs, TNF-α inhibitors are associated with an increased risk of TB progression compared to other treatments; however, the risk is not negligible, especially for JAK-i and anti-IL-6R agents.

Based on the WHO recommendations, either skin tests or IGRAs are acceptable for TBI screening. Stratification of TB risk is important to drive the bDMARDs choice. The preventive treatment for TB is well tolerated in patients undergoing b and tsDMARDs.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Mutations in ace2 gene modulate cytokine levels and alter immune responses in *Mycobacterium tuberculosis* and SARS-CoV-2 co-infection: a Cameroonian cohort

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Introduction: SARS-CoV-2 and *Mycobacterium tuberculosis* (Mtb) share similarities in their modes of transmission, pathophysiological symptoms, and clinical manifestations. An imbalance in the immune response characterised by elevated levels of some inflammatory cytokines caused by tuberculosis (TB) and COVID-19 may increase the risk of developing a severe disease-like condition. It has been reported that TB increases the expression levels of Ace2 (angiotensin converting enzyme 2) and Tmprss2 (transmembrane protease serine 2) proteins, which are essential for COVID-19 pathogenesis. Single nucleotide polymorphisms (SNPs) variants of *ace2* and *tmprss2* genes can impact virus and host-cell interactions and alter immune responses by modulating cytokine production. This may modify the susceptibility and/or severity in COVID-19-infected people. The role of SNPs in *ace2* and *tmprss2* in relation to Mtb and SARS-CoV-2 co-infection is relatively underexplored.

Method: In this study, genotype frequency of 10 SNPs of *ace2* and 03 SNPs of *tmprss2* genes in a Cameroonian cohort consisting of COVID-19-positive (n = 31), TB-positive (n = 43), TB-COVID-19 co-infected (n = 21), and a control group (n = 24) were studied. The immune response was estimated by quantitating inflammatory cytokine levels alongside self-reported and clinically diagnosed symptoms. The relationship between specific genetic mutations in these ace2

gene SNPs and their impact on cytokine expression levels in Mtb and SARS-CoV-2 co-infected patients was investigated.

Results: We identified wild-type, heterozygous, and double-mutant genotypes in seven SNPs (rs2285666, rs6632677, rs4646116, rs4646140, rs147311723, rs2074192 and rs4646142) in *ace2* gene, which showed significant variations in distribution across the study groups. Our most significant findings include the association of double mutant alleles (AA) of rs4646140 and rs2074192 in the *ace2* gene with decreased IL-6 and IL-2 expression levels respectively in TB-COVID-19 participants. Also, the double mutant alleles (AA) of rs4646116 were responsible for increased expression level of IL-2 in TB-COVID-19 patients. Additionally, elevated serum levels of AST, urea, and D-dimer, as well as increased plasma concentrations of IL-10, IFN-γ, and TNF-α, have been associated with co-infections involving Mtb and SARS-CoV-2.

Conclusion: These biomarkers may reflect the complex interplay between the two pathogens and their impact on host immune responses and disease progression. This study highlights the critical role of genetic and immunological factors in shaping altered immune responses during co-infections involving Mtb and SARS-CoV-2. By elucidating these factors, the findings provide a foundation for a deeper understanding of host-pathogen interactions and their implications for disease progression and outcomes. Furthermore, this research has the potential to drive advancements in diagnostic approaches enabling more accurate detection and monitoring of co-infections.

KEYWORDS

***ace2*, *tmprss2*, SNPs, COVID - 19, tuberculosis, *Mycobacterium tuberculosis*, SARS-CoV-2, immune response**

Introduction

Tuberculosis (TB) is a global public health concern and is endemic in low middle-income countries, primarily those in sub-Saharan Africa and Asia. According to the Global TB Report 2024 by the World Health Organization (WHO), 8.2 million cases and 1.25 million deaths were reported in 2023, placing TB again as the leading infectious disease killer in 2023 (1). In 2020, the COVID-19 pandemic overtook TB-related deaths and incidences, making it the most contagious disease with the highest mortality. During this period, 3,931,534 cases and 100,952 deaths due to COVID-19 were reported in sub-Saharan Africa (SSA) (2). COVID-19, first reported in Hubei Province in China in December 2019, is caused by Severe Acute Respiratory Disease Syndrome Coronavirus 2 (SARS-CoV-2) (3). SARS-CoV-2 and *Mycobacterium tuberculosis* (Mtb) are major infectious causes of death. They do not cause similar diseases; however, they show few similarities in their modes of transmission, symptoms and manifestations (4). They transmit through respiratory tract secretions via aerosol mode and cause lower respiratory tract infections, pneumonia and associated lung fibrosis. Both diseases share similar clinical symptoms, such as cough, fever, weakness, and dyspnea. An imbalance of immune response, including significantly decreased absolute counts of T-cells and increased pro-inflammatory cytokines levels, may influence the risk of developing TB and COVID-19 diseases, among others (4). The cytokine storm causes a significant biological response identified in patients with severe COVID-19 due to the over-activation of the inflammatory cascade in the tissues exposed to harmful stimuli like injury, toxic chemicals or pathogens (5).

Coronavirus enters the host's cell by binding its spike protein to the angiotensin-converting enzyme 2 (Ace2) receptor at the surface of the host. The *ace2* gene is located on the long arm of human chromosome 17 and consists of 26 exons and 25 introns (17q23.3) with a mass of ~92.5 kDa (6). Ace2 catalyses the conversion of angiotensin I into angiotensin 1-9, and angiotensin II into the vasodilator angiotensin 1-7 (7)). It possesses a catalytic and collectrin-like domain that spans the membrane and makes it a surface protein. The spike protein of SARS-CoV-2 binds to the catalytic domain of Ace2, leading to a conformational change, thereby causing the viral entry into the host cell (8). Transmembrane protease serine 2 (Tmprss2) is a protein encoded by the *tmprss2* gene on autosomal chromosome 21q22.3, which regulates cell signaling and modulates host response to infection

(9). However, in SARS-CoV-2 infection, *tmprss2* primes the viral glycoprotein by cleaving the spike protein at the S1/S2 junction and the S2 site (10). Both *tmprss2* and *ace2*, expressed in the lung bronchial epithelial cells, are critical for viral entry of SARS-CoV-2 (11). Some studies have demonstrated that *tmprss2* activity is essential for viral spread and pathogenesis, enabling the entry of SARS-CoV-2 in *ace2*-expressing cells but not in cells without *ace2* (12, 13). Ziegler and colleagues have shown that Mtb infection increases *ace2* expression in the lung tissues through interferon stimulation (14). Single nucleotide polymorphisms (SNP), which represent a change in a single DNA building block, are one of the most common types of genetic variation amongst humans. It usually plays a role in altering disease severity and/or susceptibility by altering the structure and function of a gene which encodes a protein or by affecting the binding of enzymes that regulate cellular processes among several others. In the context of COVID-19, SNPs in *ace2* and *tmprss2* genes have been studied in several populations to elucidate the impact on the establishment of COVID-19 (6). Susceptibility to SARS-CoV-2 infection could be defined by age, gender, pre-existing comorbidities, genetic background, predisposing factors such as health status, immunological state of the host and lineage of the pathogen (15). The host *ace2* and *tmprss2* genes play a vital role in disease severity, viral replication, and inflammation, and variations at genetic levels, such as SNPs, may affect their function (16). Mutations in SNPs of *ace2* and/or *tmprss2* genes could influence immune response by modulating cytokine production and may contribute to immune imbalance in Mtb/SARS-CoV-2 co-infected patients. Limited literature is available on the effect of SNPs in *ace2* and *tmprss2* and their response to COVID-19 and TB association. In this study, 95 participants with either TB and/or COVID-19 alongside a control group of 24 participants who reported no history of either TB or COVID-19 were recruited from a Cameroonian cohort. Their biochemical and immunological parameters were analyzed, followed by genotyping to investigate the mutation patterns in the *ace2* and *tmprss2* genes. The findings shed light on specific mutations in these genes that may impact susceptibility, severity, or immune dynamics in the context of COVID-19 and TB co-infection.

Material and methods followed

Ethics statement, study participants recruitment and classification

A written formal consent was obtained from the Cameroon National Ethical Committee for Research in Human Health (N° 2020/07/1265/CE/CNERSH/SP) in Yaoundé for this study. This study was carried out in compliance with bio-ethical laws and data protection state and following good clinical practice. All study participants recruited presented signs and symptoms of a respiratory disease. These participants were enrolled at the Djoungolo District Hospital, Jamot Hospital, Ekoumdoum Baptist Hospital and Red Cross Hospital in Yaoundé during the COVID-19 pandemic from September 2020 to December 2023. This study was conducted in two phases: a prospective component spanning from June 2022 to December 2023 and a retrospective component covering the period from September 2020 to December 2022. The prospective study enrolled participants from Jamot Hospital, including individuals diagnosed with TB and a control group for comparative analysis. In the retrospective study, participants were selected from Djoungolo District Hospital, Ekoumdoum Baptist Hospital, and Red Cross Hospital, focusing on individuals who were either COVID-19 positive or co-infected with TB and COVID-19. Recruited participants were assessed for medical history, tobacco consumption and TB medication history. We assessed the following variables: i) socio-demographic factors such as age, sex, occupation, number of co-inhabitants, ii) COVID-19 and TB history, asthma history, HIV status, hypertension, diabetes mellitus, tobacco and alcohol consumption, iii) clinical characteristics such as cough, fever, headache, sore throat, asthenia, chest pain, loss of smell. The vitals were measured by clinicians at the time of patient enrolment using a thermometer for temperature and a sphygmomanometer for heart rate and blood pressure. Other symptoms viz, cough, sore throat, bloody sputum, rhinorrhoea, chest pain, myalgia, arthralgia, fatigue, loss of smell and headache were self-reported by participants and noted by clinicians at the time of enrolment. Demographic details and clinical characteristics are presented in Table 1. Every participant underwent clinical examination and laboratory assessment for COVID-19, TB and other infectious diseases endemic in the region, such as Influenza A, B, malaria, HIV and Hepatitis B. A group of symptomatic individuals who tested negative for COVID-19, TB, and the other infectious diseases mentioned above, who had been screened during the study, were enrolled as controls. Nasopharyngeal samples, which tested positive for only COVID-19 by RDT and real-time PCR, were considered COVID-19 positive; sputum samples that tested positive for only TB by microscopy and real-time PCR were included as TB-positive participants and those which tested positive for both infections only were considered as TB-COVID-19 co-infected. Exclusion criteria for the study groups were existing comorbidities such as malaria, Influenza A and B, HIV, Hepatitis and unwillingness to give signed informed consent. Written and signed informed consent was obtained from every recruit before the start of the study. Study protocol and consent forms were reviewed and approved by the Centre's regional ethical committee in Yaoundé. The study population was later classified into COVID-19 positive, TB positive, TB-COVID-19 association, and controls.

Nasopharyngeal sample collection and processing

Nasopharyngeal samples were collected from the participants by inserting the swab provided about 2 - 2.5 cm into the nostrils. The HIGHTOP Antigen Rapid Test device that Qingdao Hightop Biotech Company manufactured was used according to the manufacturer's instructions. The QIAamp viral RNA mini kit extracted coronavirus RNA from nasopharyngeal samples. A confirmatory COVID-19 diagnosis was later made using real-time PCR using the Logix Smart ABC (Cat #: ABC-K-001) test utilizing

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TABLE 1 Socio-demographic and clinical characteristics of study participants by study group (control, COVID-19, tuberculosis (TB), and TB-COVID-19).

Characteristics	Control (24)	COV (31)	TB (43)	TBCOV (21)	p-values
Age (mean ± SD)	30.5 ± 6.7	37.3 ± 12.8	36.8 ± 17.2	37.7 ± 14.5	ns
Male n (%)	11 (45)	17 (55)	32 (74)	13 (62)	N/A
Female n (%)	13 (55)	14 (45)	11 (36)	8 (38)	N/A
Temperature (°C)	37.2 ± 0.7	37.4 ± 0.7	37.6 ± 0.6	37.3 ± 0.8	ns
Heart Rate bpm (mean ± SD)	88.5 ± 16.4	87.5 ± 10.6	86.6 ± 10.5	87.5 ± 8.7	ns
Respiratory Rate bm (mean ± SD)	18.9 ± 1.3	16.8 ± 2.8	17.3 ± 3.1	17.9 ± 1.2	p<0.05
Systolic blood pressure BP/mmHg (mean ± SD)	117.8 ± 10.9	122.9 ± 19.0	125.3 ± 20.1	118.5 ± 8.4	ns
Diastolic Blood Pressure BP/mmHg (mean ± SD)	76.9 ± 8.8	80.7 ± 14.7	77.8 ± 13.8	79.8 ± 10.2	ns
O2 saturation % (mean ± SD)	98.0 ± 1.3	96.6 ± 1.8	97.9 ± 1.1	95.7 ± 3.2	p<0.05
Fever n (%)	11 (45.5)	22 (70.9)	20 (46.6)	15 (71.4)	P=0.05
Cough n (%)	12 (50)	23 (74.2)	23 (53.5)	18 (85.7)	ns
Sore throat n (%)	5 (22.7)	9 (29)	10 (23.3)	9 (45)	ns
Bloody sputum n (%)	0 (0)	1 (3.2)	7 (16.3)	2 (10)	p<0.05
Rhinorrhoea n (%)	10 (41.7)	12 (38.7)	7 (16.3)	7 (35)	ns
Chest pain n (%)	5 (22.7)	9 (29)	11 (25.6)	12 (57.1)	ns
Myalgia n (%)	5 (22.7)	12 (38.7)	9 (20.9)	8 (40)	ns
Arthralgia n (%)	12 (50)	6 (19.4)	9 (20.9)	11 (52.3)	ns
Fatigue n (%)	12 (50)	13 (41.9)	9 (20.9)	17 (80.9)	p<0.05
Loss of smell n (%)	5 (20.8)	9 (29.0)	6 (13.9)	6 (30)	ns
Headache n (%)	11 (45.8)	19 (61.3)	7 (16.3)	16 (76.1)	p<0.05

SD, standard deviation; ns, non-significant; bpm, beats per minute; bm, breathes per minute; °C, degree Celsius; O2, oxygen; COV, COVID-19; TB, Tuberculosis; TBCOV, Tuberculosis-COVID-19 association; NA, Not applicable.

the patented Co-Primer technology (17) according to the manufacturer's instructions. The Co-Primer triplex assay uses extracted viral RNA to detect Influenza A, B and SARS-CoV-2 (gene RdRp and E-gene) in upper respiratory tract samples and even saliva (Netongo et al., unpublished data).

Sputum sample collection and processing

Sputum samples were collected using plastic cups with 40 mL capacity. After collection, sputum microscopy was carried out. A slide was prepared for each sample, fixed, and later stained following the Zeihl-Nelseen staining technique. A confirmatory real-time PCR using the SARAGENE™ *Mycobacterium tuberculosis* test COSARA Diagnostics Ltd India and Logix Smart Mtb Kit (Cat #: MTB-K-007)-Co-Diagnostics inc, USA was performed on extracted bacterial DNA according to manufacturer's instruction. This test detects the presence or absence of IS6110 and MPB64 genes from *Mycobacterium tuberculosis*. The test kit includes an internal control to identify possible qPCR inhibition and verify the quality of sample extraction.

Blood sample collection and storage

5 ml of whole blood was collected into commercially available anticoagulant-treated tubes (EDTA) and dry tubes. The blood was centrifuged 5000 r.p.m for 10 mins. Plasma was obtained from blood collected in EDTA tubes, while serum was obtained from dry tubes. Both were then aliquoted and stored at -80°C for future use.

Evaluation of biochemical markers assays involved in TB and COVID-19 diseases

A set of serum biochemical markers aspartate aminotransferase (AST), alanine aminotransferase (ALT), Urea, creatinine (CREA), direct bilirubin (BIL-D), total bilirubin (BIL-T) and D- DIMER. AST, ALT, UREA and CREA were quantified using the PreciseMAX reagent kit according to the manufacturer's instructions and results read on the semi-automatic biochemistry analyzer. Serum levels of bilirubin were determined by the photometric detection of the azo derivatives obtained by the serum reaction with the diazonium ion of sulfamic acid. D-dimer was measured in serum using the dry fluoro-immunoassay analyzer (WWHS Biotech. Inc Shenzhen, P.R China).

Measurement of pro-inflammatory and anti-inflammatory cytokine levels involved in COVID-19 and TB

Serum cytokines (IL-6, IFN-γ, TNFα, IL-10, IL-2 and IL-1β) were assayed in serum using sandwich ELISA Origene kits (Origene Technologies, Inc, Rockville, MD 20850, US) according to manufacturer's instruction.

DNA extraction and single nucleotide polymorphism genotyping

Genomic DNA was harvested from the peripheral blood using the commercially available Quick-DNA™ Miniprep Kit (QIAamp DNA Blood Mini kit, Qiagen, Germany), according to the manufacturer's instruction and its quality was verified in agarose gels stained with ethidium bromide nucleic acid gel stain (Thermo Fisher Scientific, C.A, USA). Then, the DNA concentration was measured using nanodrop (Thermo Fisher Scientific, C.A, USA), and purity was determined by calculating the A260/280 ratio. The extracted genomic DNA was stored at -80°C until used in the genotyping reaction. SNPs were analyzed by real-time polymerase chain allelic discrimination technology using TaqMan SNP genotyping assay kit (Thermo Fisher Scientific, Waltham, MA, United States) on a Co-Dx Box Magnetic Induction Cycler qualitative Time Polymerase Chain Reaction (qPCR) machine (Co-Diagnostics Inc USA, Cat # MIC001355). The ten variants analyzed for the Angiotensin-converting enzyme gene (ACE 2) were rs2285666, rs4240157, rs4646142, rs4646116, rs6632677, rs4646140, rs147311723, rs2074192, rs35803318, rs4646179 and while three genotype variants were determined for the Transmembrane serine protease 2 Polymorphisms (TMPRSS2) gene, namely rs12329760, rs75603675 and rs61735791. Specifically, genotype variants were determined using the TaqMan™ SNP Genotyping Master Mix kit from Thermo Fisher Scientific, C.A, USA (Cat #: 4381656) that reveals Ace2 rs4646179 A>G, rs147311723 G>A, rs4646142 G>A, rs2074192 C>T, rs35803318 C>T, rs4646140 C>T, rs6632677 G>C, rs4646116 T>C, rs2285666 C>A, rs4240157 C>G and Tmprss2 rs12329760 C>G, rs75603675 C>A, rs61735791 C>A mutations.

The reaction mix of each sample was composed of 5 µL of 2X TaqMan Genotyping Master Mix, 0.5 µL of TaqMan assay (20X), and 4.5 µL RNase-free water. The thermal cycling protocol is optimized at 95°C for 10 min for AmpliTaq Gold, UP Enzyme Activation, followed by denaturation step at 95°C for 15 s and annealing/extension at 60°C for 1 min for 40 cycles. The qPCR was performed on a Co-Diagnostics PCR instrument (Co-Diagnostic, INC, Salt Lake City, USA), and the results were analyzed using Co-Diagnostic genotyper software. This software was used to plot the findings of the allelic discrimination data as a scatter plot of Allele 1 (VIC® dye) versus Allele 2 (FAM™ dye). Each well of the 96-well reaction plate was represented as an individual point on the plot.

Data analysis and management

The data was anonymized before analysis with numerical variables. All comparisons of cytokines and biochemical biomarkers data were analyzed using Graph Pad Prism 8.0. Arithmetic means, medians and standard deviation were also determined using built-in MS Excel 2016 Home Edition commands. Test techniques include independent student t-test and Oneway ANOVA test, one for comparing 2-independent groups and the other for more than 2-independent groups. SNP frequencies were expressed as numbers (%) in each group. The chi-square test was used to determine p-values and the association of genotypes with one of the groups.

Results

Socio-demographic and clinical characteristics of study participants

All study participants presented signs and symptoms of a respiratory disease. Participants were enrolled at the Djoungolo District Hospital (102), Jamot Hospital (n=83), Ekoumdoum Baptist Hospital (n=118) and Red Cross Hospital (n=96) in Yaoundé during the COVID-19 pandemic from the period of September 2020 to December 2023. The location of the study site in the Centre region of Cameroon is illustrated in Figure 1A. Every participant underwent clinical examination and laboratory assessment for COVID-19 and TB. Recruited participants were assessed for medical history, tobacco consumption and TB medication history. From 399 participants, 280 were excluded from the study due to the presence of other comorbidities such as Influenza A and B, HIV, malaria and Hepatitis (n=156), use of anti-TB treatment (n=9), missing consent and unconfirmed diagnosis (n=115). In total, only 119 participants were retained for this study. The study populations consisted of four groups: COVID-19 positive (n= 31), TB positive (n= 43), TB-COVID-19 positive (n= 21) and a set of controls (n= 24). The control group consisted of participants who tested negative for all the above-mentioned diseases screened at the time of the study. Patient recruitment workflow is illustrated as a flowchart in Figure 1B. The proportion of males to females was 60% and 40%, respectively. The most frequent symptoms in the COVID-19 group (n=31) were cough (74%), fever (70.9%) and headache (61.3%). The main clinical signs reported during TB-COVID-19 association (n=21) were cough (85.7%), fatigue (80.9%) and headache (76.1%). TB patients (n=43) presented predominant symptoms such as cough (53.5%), fever (46.6%) and chest pain (25.6%). Details of sociodemographic and clinical data are summarized in Table 1.

Serum AST, Urea and D-dimer levels are significantly higher in the TB- COVID-19 association

Every study participant's blood clotting factor and other biochemical parameters were evaluated to determine disease

severity in TB and/or COVID-19. To monitor participants' liver and kidney function, we measured the expression levels of those enzymes (ALT, AST, Urea, creatinine and bilirubin) reflecting these organs' function (Figure 2A). TB-COVID-19 co-infected patients had higher AST levels than TB mono-infected ($p<0.001$) and controls ($p<0.05$). AST levels were higher than the normal range (> 34 IU/L) in 50% (10) of participants with TB-COVID-19, 18% (8) with TB and 26% (8) with COVID-19 (Table 2). Urea levels in TB-COVID-19 co-infected patients were also significantly higher than in individuals with only TB ($p<0.001$) or COVID-19 ($p<0.01$) and controls ($p<0.001$). It was noticed that all TB-COVID-19 co-infected patients had urea levels exceeding the normal serum concentration (> 20 mg/dL). Conversely, 74% (18) of COVID-19-positive participants and 72% (19) of TB-positive patients exhibited abnormal urea levels (Table 2). The COVID-19 group showed significantly elevated ALT levels compared to both the TB ($p < 0.0001$) and TB-COVID-19($p < 0.001$) groups. ALT levels exceeding 36 IU/L were found in 8 COVID-19-positive participants and 2 TB-COVID-19 co-infected participants (Table 2). When comparing the AST and ALT profiles across the

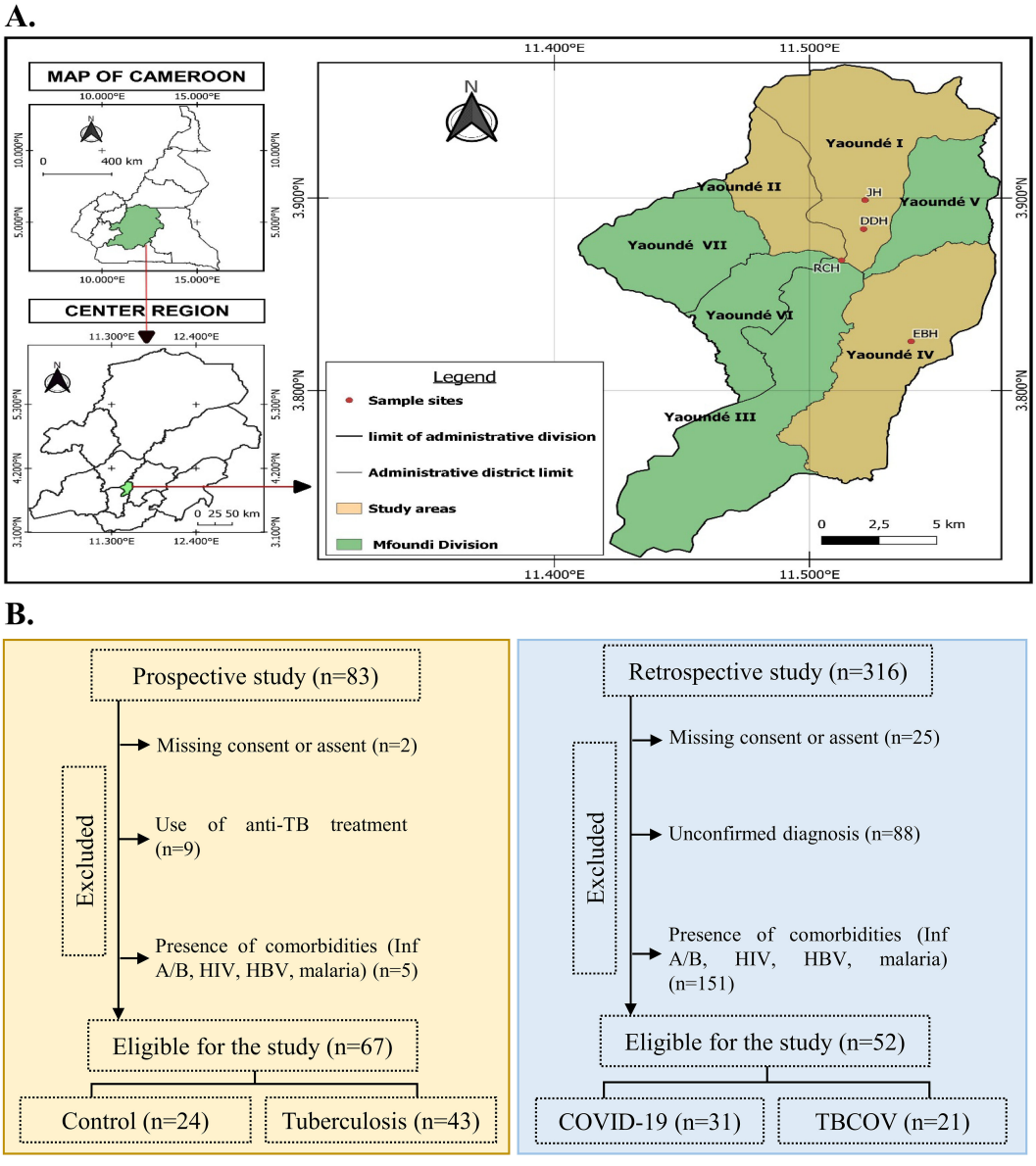

FIGURE 1
Study participant recruitment and classification. **(A)** Map showing the Centre region of Cameroon with different study sites. Samples for this study were collected at four hospitals: JH, DDH, RCH, and EBH, which are situated in Yaoundé. The sample sizes from each district were as follows: JH: 83; DDH: 102; RCH: 96; and EBH: 118. The map was created using QGIS version 3.32.3. **(B)** Based on COVID-19 and Tuberculosis infection status, participants were grouped into Controls: COVID-19 positive, Tuberculosis positive, and Tuberculosis and COVID-19 association. In total, our sample size consisted of 119 participants. JH, Jamot Hospital; DDH, Djoungolo District Hospital; RCH, Red Cross Hospital; EBH, Ekoumdoum Baptist Hospital.

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four study groups, we found that 26% (8 out of 31) of COVID-19-positive participants exhibited AST levels above the normal range. Interestingly, these same individuals also had ALT levels exceeding the normal range. Conversely, in TB-COVID-19 and TB, we found an increased expression level of AST but normal ALT levels in most participants. The D-dimer levels, which are essential for evaluating coagulation abnormalities in clinical settings, were measured among the study participants. In this study, over 53% (18) of TB-positive and 57% (12) TB-COVID-19 co-infected participants demonstrated elevated D-dimer levels (> 0.5 mg/dL) compared to just 25% (6) of control participants. Notably, a small subset of COVID-19 patients also exhibited abnormal D-dimer levels, highlighting the test's significance in this context. Serum creatinine levels were significantly higher in TB positive patients than those with COVID-19 ($p<0.001$) and TB-COVID-19 association ($p<0.05$) (Figure 2A). Elevated creatinine levels exceeding 1.4 mg/dL were noted in some of the patients: 16% (5) of those with COVID-19, 14% (6) with TB, and 19% (4) of those co-infected with TB and COVID-19 (Table 2). Plasma levels of total bilirubin were significantly higher in COVID-19 mono-infection compared to control ($P<0.05$), whilst no significant difference was observed between the COVID-19, TB and TB-COVID-19 association groups. Total serum bilirubin levels were abnormal (> 1.2 mg/dL) in three subgroups: 32% (10) COVID-19-positive

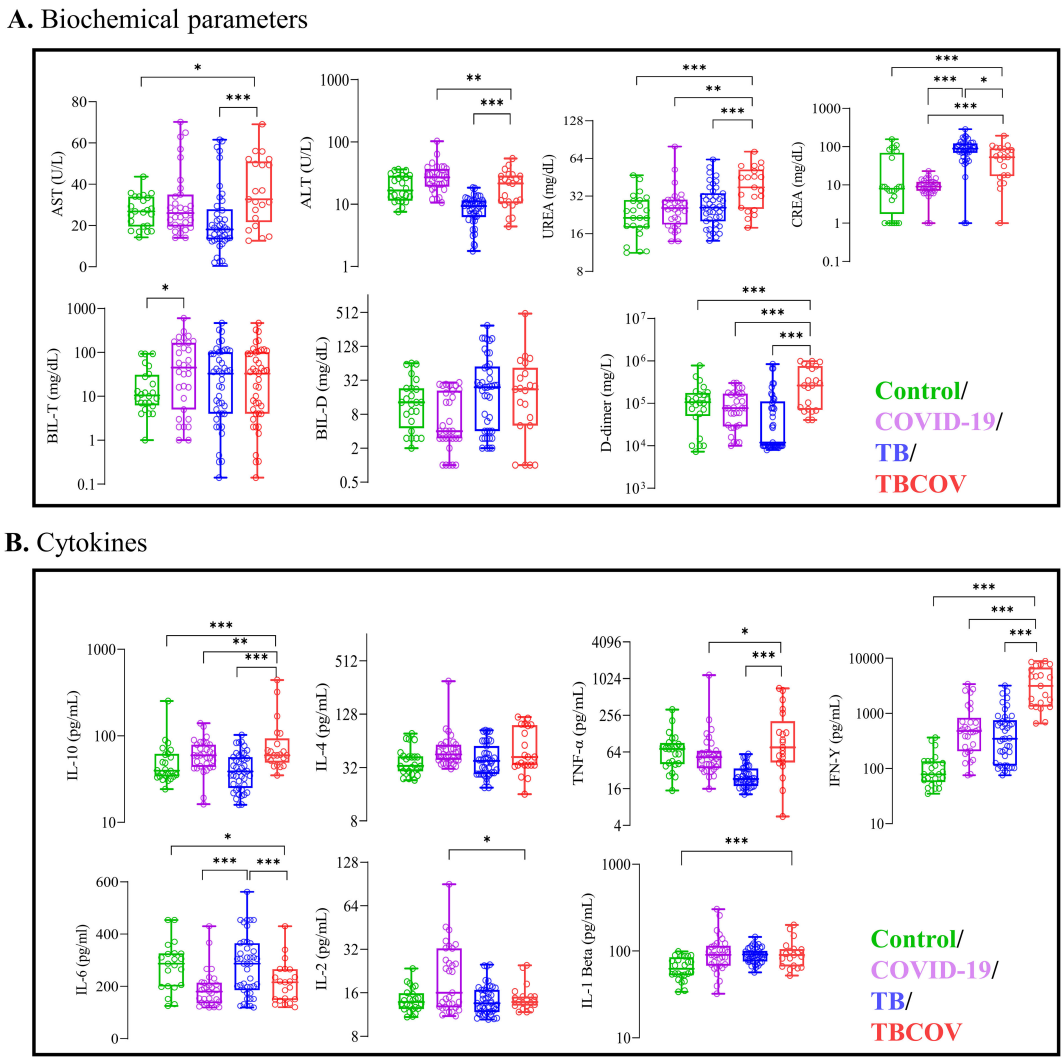

FIGURE 2
Increased serum levels of AST, UREA, D-dimer and plasma levels of IL-4, IL-10, TNF-α, IFN-γ showed association with Tuberculosis and COVID-19 co-infection. Each individual's data point is shown, with median values indicated by horizontal lines, the other lines above and below the median represent the interquartile range (IQR) and the minimum and maximum values as appropriate. **(A)** Biochemical parameters such as AST, ALT, D-dimer, Total-bilirubin, Direct-bilirubin, Urea and Crea were measured using spectrophotometry-based assays. **(B)** Plasma levels of anti-inflammatory cytokines are associated with TB/COVID-19association. Sandwich ELISA using Origene kits was employed to measure the systemic levels of anti-inflammatory cytokines (IL-10 and IL-4) and pro-inflammatory (TNF-α, IFN-γ, IL-6, IL-2 and IL-β) in plasma samples of the study population. Unpaired t-test was carried out between various combinations to determine statistically significant differences. AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; CREA, Creatinine; TNF, Tumour necrosis factor; IFN, Interferon; IL, interleukin; TB, tuberculosis; COV, COVID-19; TBCOV, Tuberculosis and Covid-19 association. *p <0.05, **p <0.01, ***p <0.001.

participants, 23% (10) of TB-positive participants, and 43% (9) of the TB-COVID-19 co-infected group (Table 2). Furthermore, direct bilirubin levels were elevated (>0.5 mg/dL) in 32% (14) of TB-positive participants and 24% (5) of TB-COVID-19 patients, in contrast to none of COVID-19 patients and 21% (5) of controls (Table 2). These results indicate liver and kidney function damage in TB-COVID-19 co-infected patients.

Higher plasma IL10, IFN-γ and TNF-α levels are associated with *Mycobacterium tuberculosis* and SARS-CoV-2 co-infections

Given the fact that disease severity can be determined by both tissue or organ damage and an exacerbated immune response, this study evaluated immune response involved during TB and/or COVID-19. To monitor the inflammation score, plasma IFN-γ, TNF-α, IL-6, IL-10, IL-4, IL-2 and IL-1β levels were observed between study groups. Plasma levels of two pro-inflammatory cytokines (TNF-α and IFN-γ) were found to be significantly higher in the TB-COVID-19 co-infected group when compared to the mono-infected groups; COVID-19 ($p<0.05$ and $p<0.001$) and TB ($p<0.001$ and $p<0.001$) positive groups respectively. In the COVID-19 group, plasma IL-2 levels were significantly higher than TB-COVID-19 ($p<0.05$) co-infected participants. Plasma IL-1β levels did not vary across the COVID-19, TB and TB-COVID-19 groups but were significantly lower in control participants ($p<0.001$) compared to TB-COVID-19 co-infected participants. TB-positive participants expressed significantly higher plasma IL-6 levels compared to TB-COVID-19 ($p<0.001$) and COVID-19 ($p<0.001$) positive participants. Among the two anti-inflammatory cytokines that were measured in this study, plasma IL-10 levels were significantly higher in TB-COVID-19 co-infected patients compared to the TB ($p<0.0001$) and COVID-19 ($p<0.01$) mono-infected patients. Plasma IL-4 levels expressed by COVID-19, TB and TB-COVID-19 patients did not vary significantly (Figure 2B). The above results indicate an imbalance in immune response during Mtb and SRAS-CoV-2 co-infection.

Wild-type, heterozygous, and double-mutant genotypes in 07 SNPs (rs2285666, rs6632677, rs4646116, rs4646140, rs147311723, rs2074192 and rs4646142) in the *ace2* gene showed significant variations in distribution across the study groups

To identify the common mutations in *ace2* and *tmprss2* genes present within a cohort of the Cameroonian population, we identified frequent SNPs of these genes which have been reported to be associated to COVID-19 susceptibility and/or severity. The mutations in allelic expression of 10 SNPs positions in *ace2* (rs2285666, rs4240157, rs4646142, rs4646116, rs6632677, rs4646140, rs147311723, rs2074192, rs35803318, rs4646179) and 03 in *tmprss2* genes (rs12329760, rs75603675, rs61735791) were profiled in every study participant (Figures 3A, B). The principal findings of our study, detailed in Supplementary Table 1, revealed a significant statistical association among the four groups concerning genotypes for seven SNPs, including rs6632677 ($p<0.05$), rs4646116 ($p<0.05$), rs4646142 ($p< 0.05$), rs2074192 ($p< 0.05$), rs147311723 ($p< 0.05$), rs4646140 ($p< 0.05$), and rs2285666 ($p=0.004$). The double mutant alleles of the *ace2* gene, specifically rs4646140 (GG) and rs6632677 (GG), were more prevalent in the TB-COVID-19 than in COVID-19 group (Figure 3).

TABLE 2 Comparison of liver, kidney, and coagulation test ranges across study groups: Control, COVID-19, TB, and TB-COVID-19.

		Control (n=24)		COVID-19 (n=31)		TB (n=43)		TB-COVID-19 (n=21)	
Biochemical parameters	Normal range	Within normal range n (%)	Above normal range n (%)	Within normal range n (%)	Above normal range n (%)	Within normal range n (%)	Above normal range n (%)	Within normal range n (%)	Above normal range n (%)
ALT	7-50 IU/L	24 (100)	0 (0)	23 (74)	8 (26)	43 (100)	0 (0)	19 (90)	2 (10)
AST	5-40 IU/L	23 (96)	1 (4)	23 (74)	8 (26)	35 (81)	8 (19)	11 (52)	10 (48)
Urea	5-20 mg/dL	10 (42)	14 (58)	8 (26)	23 (74)	12 (28)	31 (72)	1 (5)	20 (95)
Creatinine	0.7-1.3 mg/dL	23 (96)	1 (4)	26 (84)	5 (16)	37 (86)	6 (14)	17 (80)	4 (20)
Direct Bilirubin	<0.5 mg/dL	19 (79)	5 (21)	31 (100)	0 (0)	29 (67)	14 (33)	16 (76)	5 (24)
Total bilirubin	<1 mg/dL	23 (96)	1 (4)	21 (68)	10 (32)	33 (77)	10 (23)	12 (57)	9 (43)
D-dimer	0.2-0.5 mg/dL	20 (83)	4 (17)	30 (97)	1 (3)	20 (47)	23 (53)	9 (43)	12 (57)

This table displays the biochemical parameters for liver function, kidney function, and coagulation profiles for each participant, categorized into Control, COVID-19, TB, and TB-COVID-19 groups. The values are compared against established reference ranges from the literature, highlighting deviations and potential abnormalities associated with each condition.

Association between the seven (rs2285666, rs6632677, rs4646116, rs4646140, rs147311723, rs2074192 and rs4646142) *ace2* gene SNPs, which exhibited variations across the study groups, and cytokine expression levels in TB-COVID-19 individuals

This study evaluated whether the wild-type, heterozygous, and double-mutant genotypes in these SNPs in *ace2* gene impact variations in the expression levels of some pro-inflammatory (IL-6, IL-2, TNF-α and IFN-γ) and anti-inflammatory (IL-10) cytokines in individuals with both TB and COVID-19. Only statistically significant association were reported in Figure 4. Our most significant findings include the association of double mutant alleles of rs4646140 and rs2074192 in the ace2 gene with decreased IL-6 and IL-2 expression levels respectively in TB-COVID-19 participants. Additionally, the double mutant alleles of rs4646116 were linked to elevated IL-2 levels (Figure 4).

Discussion

Given the fact that TB and COVID-19 share similar signs and symptoms, misdiagnosis of disease due to clinical parameters alone is likely to be prevalent. All participants from the diseased group in this study had a high frequency of cough, fever, headache and fatigue, consistent with earlier reports (20–22). It has been documented that kidney and liver function could be altered during COVID-19 and TB infections through increased AST, ALT, urea, creatinine and bilirubin levels. In this study, increased serum AST, urea and D-dimer levels were associated with Mtb and SARS-CoV-2 co-infection. Significantly higher serum AST levels observed in the TB-COVID-19 patients in this study corroborated earlier reports (23–25). Higher AST levels in disease groups could be associated with worse outcomes (18). Higher serum urea levels in TB-COVID-19 co-infected patients in this study corroborated a previous report (26). Increased D-dimer levels in patients with concurrent TB and COVID-19 in this study may indicate elevated thrombotic risks. This is consistent with findings reported in Pakistani populations (27). The elevation of D-dimer, a fibrin degradation product, often correlates with inflammation and coagulopathy, critical considerations in managing patients with these comorbidities (28). An increase in expression levels of D-dimer reported in this study among TB-COVID-19 co-infected patients is generated and reported in severe inflammatory responses involving other diseases. Monitoring D-dimer levels could be essential for assessing thrombotic risk and guiding treatment strategies. As observed in various studies, elevated serum ALT levels in COVID-19 patients can suggest underlying liver dysfunction (23, 28, 29). COVID-19 has been associated with hepatic impairment, and increased ALT is often a marker of liver injury. Zhang et al. noted similar findings, highlighting the importance of monitoring liver function in COVID-19 patients (30).

One significant finding reported in this study was an imbalance immune response in TB-COVID-19 co-infected patients demonstrated by high levels of IFN- γ, TNF-α and IL-10. IFN-γ's role is partially reflected by the fact that it increases the production of proinflammatory cytokines via activation of the JAK-STAT pathway, leading to clinical manifestations of disease (31). Elevated IFN-γ levels in TB-COVID-19 may contribute to the abnormal systemic inflammatory responses that increase disease severity. While IFN-γ production shows subject-specific variations, reduced levels were reported in active TB patients (30, 32). Also, TNF-α, which is a key cytokine involved in inflammatory responses in TB and COVID-19, demonstrated higher levels in TB-COVID-19 positive patients. A recent study in mice models found that combining TNF-α and IFN-γ induced a cytokine-mediated inflammatory cell death signaling pathway via JAK-STAT1 (19). Increased TNF-α production could facilitate viral infection and exacerbate organ damage. Some studies suggest that blocking TNF-α with inhibitors can strongly modulate the balance between effector T cells and regulatory T cells, potentially enhancing immune regulation. In addition, inhibiting TNF-α has been shown to reduce levels of IL-6, IL-1, adhesion molecules, and vascular endothelial growth factor (VEGF) in rheumatoid arthritis (RA) patients, as reported in various clinical cohort studies on COVID-19 (31).Recent research has demonstrated that the synergy between TNF-α and IFN-γ is crucial for triggering robust cell death by activating the JAK/STAT1/IRF1 axis in human monocytic cells (THP-1) and primary human umbilical vein endothelial cells (19). Other pro-inflammatory cytokines such as IL-6, IL-2 and IL-1β showed varying expression levels among the diseased participants. Higher plasma IL-6 levels observed in TB patients discriminated efficiently from the other study participants (33–35). IL-6 levels associated with pro-inflammatory cytokine variants contribute to the cytokine storm, deteriorating COVID-19 outcomes (35, 36). The higher levels of IL-2 expressed during COVID-19 in this study corroborate other studies (37–40). A previous report demonstrated that IL-2 levels helped determine the prognosis of lung damage in influenza A patients (41). IL-1β, another pro-inflammatory cytokine, was expressed at higher levels in the TB, COVID-19 and TB-COVID-19 co-infection groups compared to the controls. This finding aligns with previous research on TB and COVID-19 patients (42, 43). IL-10, an anti-inflammatory cytokine, was found at significantly higher levels in TB-COVID-19 patients than other groups. Previous studies have also reported elevated IL-10 levels in TB-COVID-19 patients compared to those with either TB or COVID-19 alone, regardless of severity (27, 28). Plasma IL-4 levels in TB-COVID-19 co-infected patients were significantly higher than in the other diseased and control groups, corroborating earlier reports (44).

Despite data regarding variations in genotype frequencies of *ace2* and *tmprss2* during SARS-CoV-2 infection, limited information is available about their implication in TB and COVID-19 association. Our data adds considerable insight to the literature on mutations of *ace2* and *tmprss2* involved in TB-COVID-19 association pathogenesis. The present study sought to identify genotypic variations in human *ace2* and *tmprss2* genes,

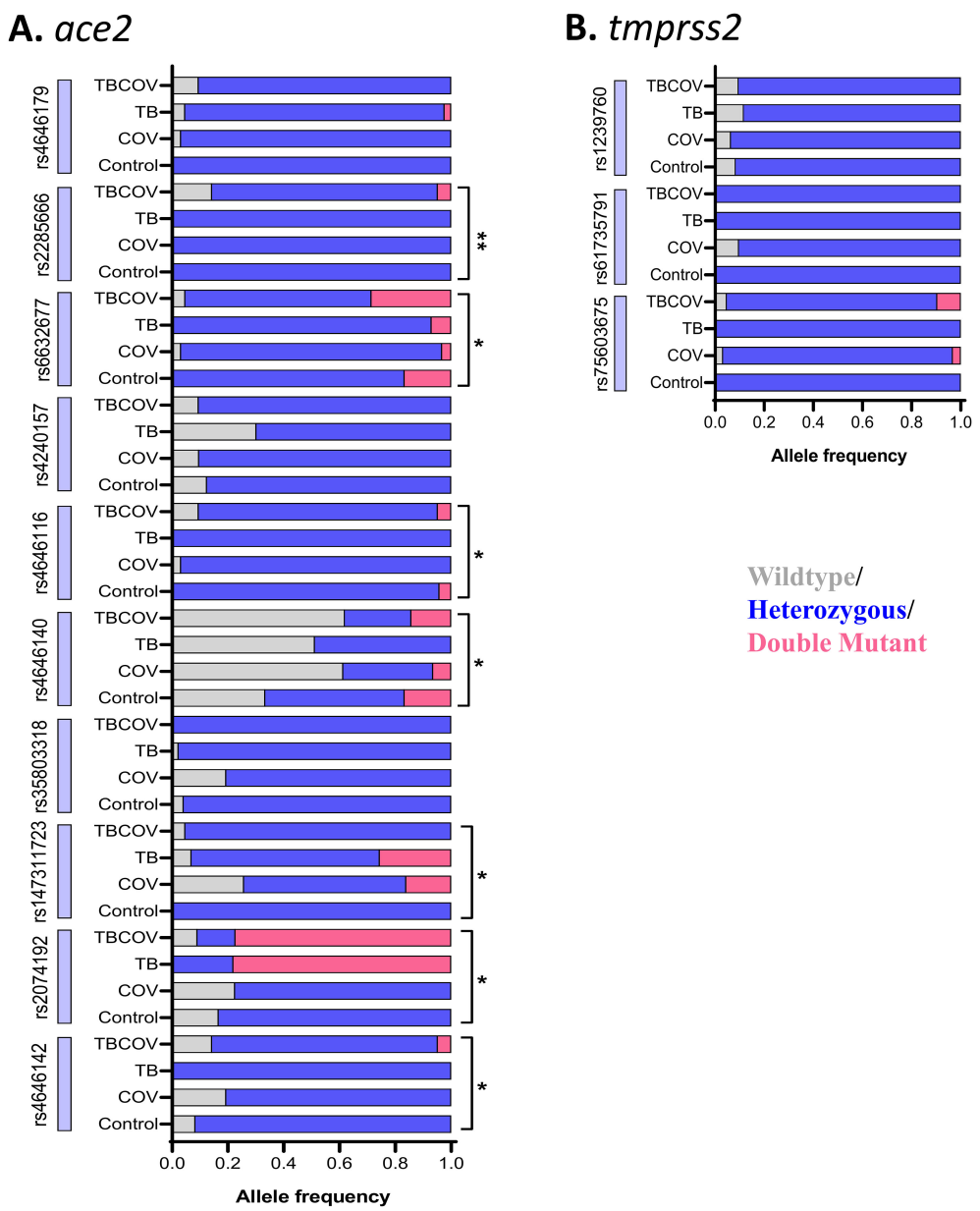

FIGURE 3
Wild-type, heterozygous, and double-mutant genotypes in seven SNPs (rs2285666, rs6632677, rs4646116, rs4646140, rs147311723, rs2074192 and rs4646142) in ace2 gene showed significant variations in distribution across the four study groups: Genomic DNA isolated using Zymo Human DNA Isolation Kit was subjected to quantitative real-time PCR with TaqMan probes. TaqMan SNP Genotyping Assays were employed to identify SNPs in *ace2* and *tmprss2* genes. **(A)** specific genotype frequencies in *ace2*. **(B)** specific genotype frequencies in *tmprss2*. Multigroup analysis was carried out using the Chi-square test, and SNPs having a p-value of less than 0.05 were considered to have a significantly perturbed presence between the groups (Control, TB, COV, and TBCOV). TB, Tuberculosis positive; COV, COVID-19 positive and TBCOV, Tuberculosis and COVID-19 association. Asterisks represent p-values determined after multivariate analysis that was done using the Chi-square test comparing the mutation patterns in the four groups, namely, control, TB, COVID-19, and TB-COVID-19.

associate them with immune response and correlate these with susceptibility to Mtb and SARS-CoV-2 co-infection. Numerous studies and reports have consistently demonstrated that the host Ace2 receptor plays a pivotal role as the primary entry point for the SARS-CoV-2 virus. At the same time, Tmprss2 has been identified as a crucial enzyme responsible for facilitating the activation of the viral spike protein, enabling its fusion with the host cell membrane and subsequent viral entry (10, 25, 45). Specific changes in the *ace2* and *tmprss2* gene sequences, which may increase the binding affinity and/or expression levels, may affect the entry of the SARS-CoV-2 (46). Populations with specific SNPs in the *ace2* and *tmprss2* genes have shown increased susceptibility to COVID-19 and TB-COVID-19 association (47–49). Reports by Chen and collaborators in the evaluation of the relationship between genetic variants of the *ace2* gene and circulating levels of Ace2 found several allele frequencies of *ace2* coding across different populations (South

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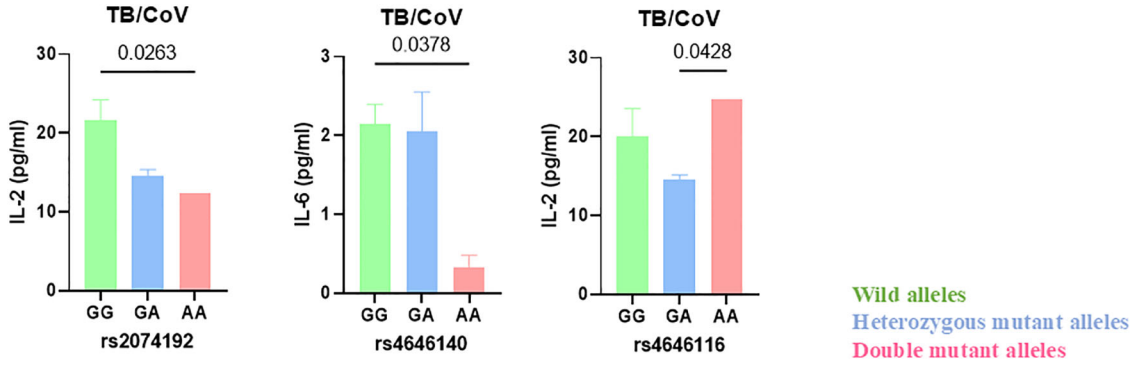

FIGURE 4
Reduced IL-6 and IL-2 levels linked to double mutant alleles of ACE2 gene polymorphisms rs4646140 and rs2074192 in TB-COVID-19 co-infected patients. The wild-type, heterozygous, and double-mutant genotypes in seven SNPs (rs2285666, rs6632677, rs4646116, rs4646140, rs147311723, rs2074192 and rs4646142) in ace2 gene were associated with expression levels of some pro-inflammatory (IL-6, IL-2, TNF-α and IFN-γ) and anti-inflammatory (IL-10) cytokines in individuals with both TB and COVID-19. Only statistically significant association are reported. The letters on the x-axis denote specific genotypes (wild-type, heterozygous mutant and double mutant). The bars represent the median values of cytokine levels, with error bars indicating the interquartile range (IQR). Horizontal lines and corresponding values above the bars indicate statistical comparisons, with p-values provided to highlight significant differences between groups.

Asian, East Asian, African, European, and mixed American populations). The allele frequencies of 11 of the 15 eQTLs (expression quantitative trait locus) that were linked to expression were more significant in East Asians (0.73–0.99) than in Europeans (0.44–0.65), which is indicative of the differential susceptibility to SARS-CoV-2 among different cultures (50). Such reports on the structural and regulatory variants of *ace2* and *tmprss2* conferring susceptibility to COVID-19 from the Cameroonian population are limited. Reports showed that African populations are genetically predisposed to low *ace2* and *tmprss2* expression, partly explaining the lower incidence of COVID-19 (18). On the other hand, allelic frequencies contributing to higher *ace2* and *tmprss2* expressions in South Asian, Southeast Asian, and East Asian populations reported higher infection rates (51, 52). In this study, we monitored polymorphism patterns in *ace2* and *tmprss2* to find a correlation with higher genetic susceptibility to TB-COVID-19. The high incidence rate of COVID-19 and TB-COVID-19 co-infected males could be potentially attributed to the presence of the *ace2* gene on the X-chromosome (53). A recent report demonstrated an inverse correlation between *ace2* expression levels and estrogen levels in SARS-CoV-2 patients (54). Estrogen may contribute to the suppression of *ace2* expression thus explaining and might partly explain the protective factors in females against COVID-19 (55). We observed the association of double mutant alleles of rs4646140 and rs2074192 in the ace2 gene with decreased IL-6 and IL-2 expression levels respectively in TB-COVID-19 participants. This may confer a protective effect against severe COVID-19 or worse outcome during these co-infections. Also, the double mutant alleles (AA) of rs4646116 were responsible for increased expression level of IL-2 in TB-COVID-19 patients. While IL-2 is crucial for T cell activation and expansion, persistently high levels can lead to T cell exhaustion, reducing their ability to effectively combat the virus hence prolonging infection. High IL-2 levels have been associated with severe COVID-19 cases, suggesting it could serve as a biomarker for disease severity (41). Monitoring IL-2 levels might help identify patients at risk of progressing to severe illness. The double mutant alleles (AA) of SNP rs2074192 in *ace2* were common in TB-COVID-19 co-infected patients. This implies that the AA allele may play a role in the susceptibility or pathophysiology of co-infection with these two diseases hence suggesting a potential genetic predisposition that could influence how these individuals respond to these infections. However, further research would be needed to fully understand the biological mechanisms and implications behind this finding.

It is essential to acknowledge the limitations of a small sample size, and the fact that participants were not monitored over an extended period as it can restrict the generalizability of the findings. The lack of long-term monitoring limits the ability to observe potential changes in biomarkers or disease progression over time. Future research with larger, longitudinal cohorts would be valuable to confirm and expand on the relationships between these SNPs and other research indices. Also, the SNP distribution evaluated in this study may be population-specific and may differ among different populations. The survey of polymorphisms from diverse genetic backgrounds might explain the vulnerability to diseases. This study has a limited scope of cytokine profiling due to the restricted number of cytokines analyzed. While several key cytokines were investigated, the study did not include a comprehensive panel of cytokines that could offer a more complete picture of the immune response. Furthermore, the cytokine levels measured were specific to certain time points, and longitudinal investigation could have provided more insights into cytokine dynamics during disease progression or treatment.

In summary, this study indicates that the double mutant alleles of rs2074192 and rs4646140 in the *ace2* gene reduced IL-2 and IL-6 production in TB-COVID-19 individuals which could potentially lead to milder disease outcomes in these individuals. Whilst the double mutant alleles of rs4646116 increased inflammatory

response through increase IL-2 production which may lead to deleterious outcomes in TB-COVID-19 individuals with time. These findings highlight the need for a human genetics initiative to understand better the genetic factors influencing susceptibility and/or severity during TB-COVID-19 association. This could inform prevention and treatment strategies during the future pandemic.

Data availability statement

All relevant data are within the manuscript and its Supplementary Information files that were uploaded during the review process.

Ethics statement

The studies involving humans were approved by Cameroon National Ethical Committee for Research in Human Health (No 2020/07/1265/CE/CNERSH/SP) in Yaoundé. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MN: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. EB: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing. SK: Formal Analysis, Methodology, Validation, Writing – review & editing, Visualization. NB: Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. AJ: Project administration, Supervision, Validation, Writing – review & editing. TN: Investigation, Methodology, Validation, Writing – review & editing. FR: Investigation, Methodology, Validation, Writing – review & editing. RN: Formal Analysis, Project administration, Supervision, Validation, Writing – review & editing. AA-A: Project administration, Resources, Validation, Writing – review & editing. JA: Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. PM: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing, Visualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Latent tuberculosis coinfection in mild COVID-19 is associated with a distinct immune cell phenotype marked by enhanced cytotoxic degranulation and mitochondrial alterations

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Introduction: The chronic nature of latent tuberculosis infection (LTBI) allows it to coexist with diverse pathologies. However, it remains unclear whether immune alterations associated with LTBI influence COVID-19 coinfection and patient outcomes. This study aims to compare the immune phenotype of patients with LTBI/COVID-19 to those with COVID-19 alone, in order to assess whether latent tuberculosis infection induces significant immune cell alterations.

Methods: Peripheral blood mononuclear cells were cultured and stimulated with the SARS-CoV-2 Spike protein and *Mycobacterium bovis* Bacillus Calmette-Guérin (*M. bovis* BCG) to evaluate cellular distribution and function.

Results: the LTBI/COVID-19 group exhibited a narrower range of symptoms and required less complex treatment regimens than the COVID-19 group. The cellular evaluation revealed that individuals with COVID-19 displayed a distinct immune profile, characterized by a predominance of monocytes expressing pro-inflammatory and regulatory markers, including TNFR2, HLA-DR+TNFR2, and CD71. While CD4+ T cell subpopulation distribution and function were similar across groups, LTBI/COVID-19 and COVID-19 exhibited similar frequencies of CD8+perforin+ and CD8+Granzime B+ T cells. However, LTBI/COVID-19 displays lower soluble levels of granzyme B and perforin in culture supernatants and perforin, granulysin, and sFas in plasma compared to COVID-19. Notably, CD8+ T cells from LTBI/COVID-19 showed higher antigen-specific

degranulation than COVID-19. Moreover, LTBI/COVID-19 individuals predominantly displayed CD4+ and CD8+ T cells with highly polarized, compact mitochondria at baseline, which remained unchanged under stimulation. In contrast, COVID-19 had T cells with highly polarized, fragmented mitochondria at baseline, a profile that persisted under stimulation.

Conclusion: The findings reveal significant alterations in monocytes and T cells of individuals with LTBI/COVID-19, suggesting that co-infection may induce changes in the cellular phenotype and cytotoxic function of CD8 T cells.

KEYWORDS

latent tuberculosis, COVID-19, coinfection, T lymphocytes, mitochondrial changes

1 Introduction

The World Health Organization (WHO) reported that tuberculosis (TB) caused 1.25 million deaths worldwide in 2023 and noted 10.8 million active TB cases (1). Those with active TB typically have a cough lasting three weeks or more, and they can spread the infection (2). Most people exposed to the bacteria *Mycobacterium tuberculosis* (Mtb) have an immune response that controls but does not eliminate it, leading to latent TB infection (LTBI). Individuals with LTBI show no symptoms and cannot spread the disease; although not a source of infection, they are considered a reservoir for future active TB cases. At least one-fourth of the world's population has LTBI, making it a significant global health issue (3).

An individual with LTBI can remain in this state throughout his or her life without developing TB. Although it may coexist with various comorbidities and infections, these are at high risk for developing pulmonary TB (4, 5). It has been reported that CD4+ T cell depletion associated with severe COVID-19 and corticosteroid-based treatments could induce the activation of pulmonary TB in individuals with LTBI (6, 7). However, there is very little data on the immunological consequences of LTBI in co-infection with COVID-19 (8).

The SARS-CoV-2 infection, the causative agent of COVID-19, induces a wide clinical spectrum ranging from asymptomatic to severe disease (9). Patients who develop respiratory complications and severe disease exhibit significant immune dysregulation, including cytokine release syndrome, exhausted NK and CD8+ T cells, mitochondrial metabolic alterations in T cells, and decreased secretion of type I interferons (9, 10). Collectively, these abnormalities often result in fatal outcomes.

The introduction of SARS-CoV-2 vaccines significantly reduced the number of individuals with severe disease and associated mortality rates (11). Experimental studies have demonstrated that SARS-CoV-2 vaccines, including AZD1222/Covishield and BV152/Covaxin, elicit a memory response in CD4+ and CD8+ T cells that persists over time, providing long-term protection through T cell-induced immune pathways (11–13). A murine model has proposed that prior exposure to M. tuberculosis (Mtb) may confer protection against SARS-CoV-2; similarly, it has been suggested that vaccination with Bacillus Calmette-Guérin (BCG) might provide comparable protection (14–16). However, the results of these studies remain controversial and vary depending on the population and the type of vaccine administered during early childhood (17).

Data indicate that aging patients with LTBI/COVID-19 coinfection exhibit modulation of their immune response, which has been associated with the presence of Mtb infection (18, 19). Conversely, another study found that patients infected with Mtb (whether LTBI or active TB) have a limited SARS-CoV-2-specific response, notably reduced IFN-γ release, suggesting a potential detriment to an adequate immune response during LTBI/COVID-19 coinfection (20).

Additionally, studies have examined the impact of SARS-CoV-2 vaccination on individuals with active TB who have received SARS-CoV-2 vaccination. These cells were exposed to SARS-CoV-2 antigens, and notable immunological alterations were identified, including a reduction in CD8+CD69+ and CD8+TNF+ T cells and an increase in CD4+IL-10+ T cells, suggesting that Mtb infection promotes an anti-inflammatory profile (21).

Despite efforts to elucidate the impact of LTBI during acute COVID-19, several questions about Mtb and SARS-CoV-2 coinfection remain unanswered. This study analyzes the immunologic phenotype during mild COVID-19 in a group of individuals who have received a mixture of SARS-CoV-2 vaccines, were previously vaccinated with BCG in early childhood, and have been identified as having latent tuberculosis infection (LTBI) to assess its influence on patient outcomes.

2 Materials and methods

2.1 Ethics statement

This study was approved by the Institutional Ethics Committee of the Instituto Nacional de Enfermedades Respiratorias Ismael

Cosio Villegas (INER, protocol number B23–23). All participants signed a written informed consent. All procedures in this work followed the ethical standards indicated in the Helsinki Declaration.

2.2 Study populations

From October 2023 to May 2024, healthcare workers from the Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas (INER) were invited to participate in a study. The medical staff evaluated all participants in the Occupational and Preventive Health Department for clinical evaluation. 110 healthcare workers (over 18 years) were recruited.

All individuals have received the SARS-CoV-2 vaccination, and 94% received the BCG vaccination in early childhood. Eight subjects were excluded from the study due to their decision to discontinue participation or insufficient sample size. Furthermore, subjects who had been diagnosed with Human Immunodeficiency Virus (HIV) infection, active TB, cancer, chronic obstructive pulmonary disease, solid organ transplant recipients, or those who had been prescribed immunosuppressive or anticoagulant therapy were excluded from the study.

A reverse transcriptase polymerase chain reaction for SARS-CoV-2 (qRT-PCR, BioFire Diagnostics, LLC, USA) and QuantiFERON-Gold plus (QFT, Qiagen, HD, Germany) to identify Mtb infection were performed in all participants. They were classified into four groups: healthy donors (HD, n=20), latent TB infection (LTBI, n=15), mild COVID-19 (COVID-19, n=52), and coinfection LTBI/mild COVID-19 (LTBI/COVID-19, n=15) (details in Figure 1). This study only included mild COVID-19 patients, and according to the WHO definition and the institutional clinical staff, mild COVID-19 is considered when patients have no viral pneumonia and hypoxemia and present an oxygen saturation >92%; consequently, no patient required hospitalization or supplemental oxygen. Additionally, showed at least 2 of the following symptoms: cough, fever, or headache, accompanied by at least 1 of the following signs or symptoms: difficulty breathing, anosmia, joint or muscle pain, throat pain, and nasal congestion (22, 23).

2.3 Peripheral blood mononuclear cells

20 ml of EDTA blood samples were collected from healthcare workers into BD Vacutainer tubes (BD Biosciences, CA, USA). Then, peripheral blood mononuclear cells (PBMC) were isolated by a density gradient (Lymphoprep™, Accurate Chemical-Scientific, NY, USA). The trypan blue (Gibco™, NY, USA) exclusion assay

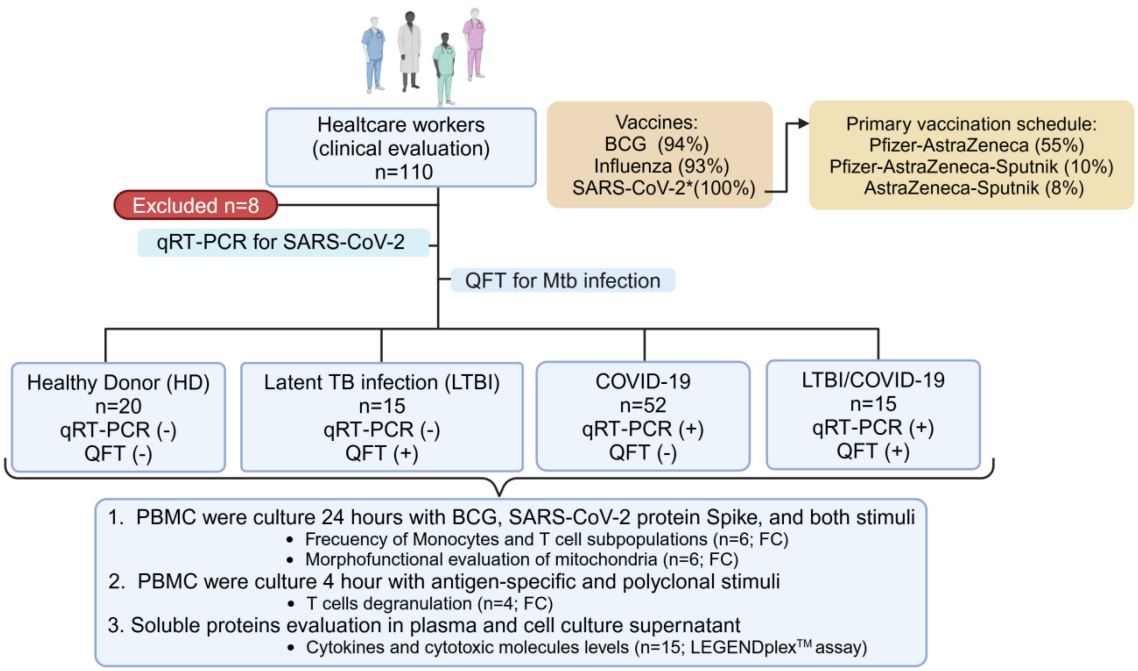

FIGURE 1
Workflow of recruited individuals. 110 healthcare workers (over 18 years) were recruited. All have received SARS-CoV-2 vaccination. Eight subjects were excluded from the study due to their decision to discontinue participation or insufficient sample size, and 102 were classified according to the reverse transcriptase polymerase chain reaction (qRT-PCR) and QuantiFERON-Gold plus (QFT) tests, to identify the SARS-CoV-2 or *Mycobacterium tuberculosis* (Mtb) infection, respectively. The study population was divided into four groups: healthy individuals (HD, n=20), individuals with latent TB infection (LTBI, n=15), individuals with confirmed mild SARS-CoV-2 infection (COVID-19, n=52), and individuals with latent TB infection and confirmed mild SARS-CoV-2 infection (LTBI/COVID-19, n=15). The cells of these individuals were evaluated to measure monocyte and T cell subpopulations' parameters (n=6 per group), determine the degranulation capacity of CD8+ and CD4+ T cells (n=4 per group), and assess cytokines in plasma (n=15 per group) and culture supernatant (n=6 per group) under specific stimuli. FC, Flow Cytometry; PBMC, peripheral blood mononuclear cells. Created in https://BioRender.com.

was used to determine the number of viable cells, and a minimum of 90% viability was required to process the use of the cells. Plasma was obtained and stored at −70 °C until use. To note, cells used for the *in vitro* studies were selected from those patients that did not present comorbidities to avoid variables.

2.4 Culture of BCG and *in vitro* infection assay

A suspension of BCG Pasteur strain 1172 P2 was prepared in the Middlebrook 7H9 broth medium BD (Becton Dickinson, USA) supplemented with oleic albumin dextrose catalase (OADC) (24). After a 21-day incubation period at 37°C, the mycobacterial stock solution was harvested. Aliquots of disaggregated mycobacterial stock cultures were prepared and stored at −70°C until required for *in vitro* infections. The mean concentration of the frozen BCG stock suspensions after disruption of mycobacterial clumps was determined by counting colony-forming units (CFU) on 7H10 agar plates in triplicate serial dilutions of the disaggregated stock suspensions.

For infection assay, a suspension of bacteria was prepared; briefly, an aliquot of bacteria was thawed and centrifuged at 6000 x g for 5 min. The bacterial pellet was resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamine,1M HEPES (Gibco™, NY, USA), and 10% fetal bovine serum (Gibco™) and shaken in the presence of sterile 3 mm glass beads. The resulting mycobacterial suspension was centrifuged to remove residual large lumps, and single-cell suspensions of bacteria were used for the infection of PBMC. Then, PBMC (1×10^6/mL) were infected with BCG Pasteur strain at a multiplicity of infection (MOI) of 1 (1 cell per 1 bacteria). The infected PBMC were incubated at 37°C for 4 hours, and no phagocytosed bacteria were eliminated by washing. Then, cells were stimulated, as described below.

2.5 PBMC stimulation

1×10^6 of PBMC/mL of HD (n=6), LTBI (n=6), COVID-19 (n=6), and LTBI/COVID-19 (n=6) were plated in RPMI-1640 medium supplemented with 2 mM L-glutamine,1M HEPES (Gibco™), and 10% fetal bovine serum (Gibco™).

Three different stimuli conditions were performed: a) PBMC infected with BCG Pasteur strain (MOI 1), b) PBMC stimulated with the recombinant SARS-CoV-2 Spike (S1+S2) protein, here after called as only spike (Biolegend, CA, USA) at 1 µg/mL concentration and, c) PBMC were infected with BCG (MOI 1) plus the spike (1 µg/mL). The culture was maintained for 24 hours (h) at 37°C in a 5% CO_2 humidified atmosphere. After the incubation, the supernatants were recovered and frozen at -20°C. The recovered cells were prepared for flow cytometry staining. Unstimulated PBMC (mock control) were cultured in the same conditions. Supernatant cultures were recovered and stored at -20°C until use.

2.6 Flow cytometry: staining and analysis strategy

$1x10^6$ PBMC of HD (n=6), LTBI (n=6), COVID-19 (n=6), and LTBI/COVID-19 (n=6) were recovered at the end of the culture, and extracellularly stained with monoclonal antibodies (mAb) against CD2, CD3, CD14, CD16, HLA-DR, CD120a, CD120b, CD71, CD274, CD8, CD4, CD69, CD98, CD279, TIM3 (Biolegend, CA, USA), for 30 min at 4°C in the dark, washed with cell staining buffer (BioLegend) and fixed.

For intracellular staining, a mAb cocktail against T-bet, GATA-3, perforin, and granzyme B was used. Briefly, after extracellular staining, cells were washed with cell staining buffer and permeabilized with BD Cytofix/Cytoperm™ buffer (BD Biosciences) for 20 min at 4°C. Following, it was incubated with the intracellular mAb cocktail for 30 min at 4°C in the dark. Finally, the cells were washed and acquired.

For mitochondria evaluation, briefly, PBMC were stained for 15 min 37°C with mitotracker green FM (500 nM) and mitotracker Deep Red (250 nM) probes. Following, cells were washed, and an extracellular stain was done. MDIVI1 (Sigma-Aldrich, USA), a mitochondrial fusion inductor, allowed us to distinguish compacted from fractionated mitochondria, and FCCP (Sigma-Aldrich), an uncoupler of oxidative phosphorylation, allows us to obtain a polarized/depolarized mitochondrial mass control, were used as controls.

Data were acquired using a LSRFortessa™ BD flow cytometry with FACSDiva 6.1.3 software (BD Biosciences). Fluorescence Minus One (FMO) condition was stained and acquired in parallel to identify background levels of staining, and dead cells were excluded using the viability staining Zombie Red Dye solution (BioLegend). In each condition, at least 50,000 events per sample were acquired. The flow cytometry (FCS) data file was analyzed using Flow Jo (Flow Jo) ™ v10.10.1 (Flow Jo, LLC, OR, USA). More details of the antibodies used can be found in Supplementary Table S1.

The analysis strategy involves selecting live cells employing the viability plot (Zombie Red negative). Subsequently, the limitation of the single T cells through forward scatter (FSC-A vs. FSC-H) was performed. Subsequent PBMC were chosen through side scatter and forward scatter (FSC-A versus SSC-A), and a second singlets events plot was made (SSC-A versus SSC-H) (Supplementary Figure S1A).

Subsequently, PBMC was selected, and further analysis was conducted on CD2- or CD2+ cells, monocytes (CD2-CD14+), and T cells (CD2+CD3+), along with their CD4+ or CD8+ subpopulations. For monocytes, activation markers such as HLA-DR and CD71, as well as death or survival receptors TNFR1 and TNFR2, respectively, were evaluated. Moreover, classical monocytes (CD14+CD16-) and non-classical monocytes (CD14+CD16+) were assessed. In the T cell gate, activation (CD69) and exhausted-like phenotype (PD-1, and TIM-3) markers were assessed. In addition, into the T cells CD4+ gate, GATA-3 and T-BET were evaluated to define the Th1 or Th2 profile, while at the CD8+ gate, granzyme

and perforin were analyzed to assess the presence of cytotoxic molecules (Supplementary Figure S1A).

The CD4+ and CD8+ gates were evaluated, utilizing MDVI1 and FCCP controls to delimit the cutoffs for mitochondrial fragmentation and polarization. Employing Mitotracker Green FM (a mitochondrial mass indicator) and Mitotracker Deep Red (a mitochondrial membrane potential-dependent indicator), cells were divided according to the presence of fractionated or compacted mitochondria and high or low polarized (Supplementary Figure S1B).

2.7 CD8+ T cell degranulation

For the polyclonal stimuli, 1×10^6 PBMC/mL of HD (n=4), LTBI (n=4), COVID-19 (n=4), and LTBI/COVID-19 (n=4) were plated in RPMI-1640 medium supplemented and maintained at 37°C. A mAb anti-CD107a (Biolegend) (5 µL/mL) was added, and after 30 minutes, a mixture of phorbol-12-myristate-13-acetate with ionomycin (PMA/IO, Thermo Fisher Scientific, CA, USA) at 1X of was added. The cultures were incubated for 4 h, but 2 h before the end of the culture, monensin (0.002 mM, Biolegend) was added to the cell culture. Kinetics of CD8+ T cell degranulation was performed, including four time points: basal, 1, 2, and 4 h.

For antigen-specific stimuli, 1×10^6 PBMC/mL of HD (n=4), LTBI (n=4), COVID-19 (n=4), and LTBI/COVID-19 (n=4) were plated in RPMI-1640 supplemented. Briefly, after 30 min of culture assay, anti-CD107a was added and stimulated with the spike and BCG for 4 h at 37°C. Two hours before the end of the culture assay, monensin was added. After polyclonal or antigen-specific stimuli, extracellular staining was done to identify CD3, CD4, and CD8.

Data were acquired using a FACSCanto II™ flow cytometer with FACSDiva 6.1.3 software (BD Biosciences). In each condition, at least 50,000 events per sample were acquired. The flow cytometry (FCS) data file was analyzed using Flow Jo (Flow Jo) ™ v10.10.1 (Flow Jo, LLC, OR, USA).

2.8 Soluble molecules evaluation

Following instructions provided by the manufacturer (BioLegend), IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α, soluble Fas, soluble FasL, Granzyme A, Granzyme B, Perforin, and Granulysin were measured in plasma samples and supernatants from culture assays using the LEGENDplex human CD8/NK panel (kit' details in Supplementary Table S1). Data were collected using a FACSAccuri C6™ with CFlow software (BD Biosciences).

2.9 Statistical analysis

The D'Agostino-Pearson test was used to test the normality of data. Non-normally distributed variables are shown as median value and interquartile range (IQR, 25–75). Kruskal–Wallis test with Dunnett's post-test was used for multiple comparisons. In relation to the clinical laboratory data, they presented a normal distribution, so they were analyzed by means of the One-Way Anova test, the data were expressed as the mean and the respective maximum and minimum values. $p<0.05$ were considered statistically significant (GraphPad Software, V9.0.2). Details on size sample are provided in the Supplementary Material.

The Heat map was created in the interface of Morpheus, in which a matrix of values is mapped to a matrix of colors. The values are assigned to colors using the minimum and maximum of each row independently. Versatile matrix visualization and analysis software (Morpheus, https://software.broadinstitute.org/morpheus).

3 Results

3.1 Demographic and clinical characteristics

Demographic characteristics were compared between groups (Supplementary Table S2). In summary, the cohort consisted of young adults, predominantly female, who had received at least two doses of the SARS-CoV-2 vaccine. Most patients received a combination of vaccines, with the following combinations standing out: Pfizer-AstraZeneca (55%), Pfizer-AstraZeneca-Sputnik (10%), and AstraZeneca-Sputnik (8%). Approximately 70% received their last dose in December 2022, 20% in February 2022, and 10% received their last vaccine in November 2021. All subjects received the last doses of the vaccine in similar data before being enrolled.

Overweight and obese were the most common comorbidities in the COVID-19 and LTBI/COVID-19 groups, respectively. 49% reported contact with TB patients (because of the nature of their work). With respect to SARS-CoV-2 exposure, the majority of individuals had been ill and diagnosed at least once with COVID-19, although in the HD and LTBI groups, it was not possible to know the time of their last SARS-CoV-2 infection (Supplementary Table S2).

No significant differences between the groups regarding hematological and biochemical parameters (Supplementary Table S3). In contrast, LTBI/COVID-19 exhibited a narrower range of symptoms compared to COVID-19; symptoms such as asthenia, adynamia, abdominal pain, fatigue, dyspnea, ageusia, and anosmia were not reported (Supplementary Figure S2, upper panel). As a result, treatment schedules differed between the groups: 73% of LTBI/COVID-19 cases were managed adequately with paracetamol, levodropropizine, and fexofenadine, whereas the COVID-19 group required more complex treatment regimens (Supplementary Figure S2, lower panel).

3.2 Circulating TNFR1+ monocytes are predominant in LTBI/COVID-19, whereas COVID-19 has TNFR2+ monocytes

Within the monocyte gate (CD2-CD14+), the expression of HLA-DR was evaluated, and no significant differences were

observed (Figure 2A, up). However, compared to HD, the frequency of monocytes expressing the transferrin receptor (CD71), an indirect activation marker, was increased in COVID-19 individuals without stimulation ($p<0.05$), in response to spike ($p<0.01$) and to BCG ($p<0.05$). Meanwhile, LTBI/COVID-19 showed an increase in the frequency of CD71+ monocytes in response to spike and BCG+spike (Figure 2A, up).

Subsequently, the frequency of classical (CD14+) and non-classical (CD14+CD16+) monocytes was evaluated. It is observed that COVID-19 shows a decreased frequency of classical monocytes compared to LTBI/COVID-19 ($p<0.05$), and it is maintained even with Spike and BCG stimulus (Figure 2A, mild). In contrast, COVID-19 has increased the frequency of non-classical monocytes compared to LTBI/COVID-19 ($p<0.05$), and it was observed without stimuli or with spike (Figure 2A, mild).

The expression of TNFRs was evaluated due to their involvement in COVID-19 severity (25, 26). Compared to HD and COVID-19, LTBI/COVID-19 had an increased frequency of TNFR1+ monocytes without stimulation ($p<0.05$), and this increase was sustained upon stimulation with spike ($p<0.05$) and BCG/spike ($p<0.05$). Conversely, compared to HD and LTBI/COVID-19, COVID-19 exhibited an increased frequency of TNFR2+ monocytes without stimulation ($p<0.05$), which persisted after stimulation with a spike ($p<0.05$), BCG ($p<0.05$), and BCG/spike ($p<0.05$) (Figure 2A, down).

Given that TNFR1 and TNFR2 mediate opposing functions, we investigated their expression specifically on antigen-presenting monocytes (HLA-DR+). We found that the frequency of HLA-DR+TNFR1+ monocytes was not significantly altered. However, at baseline, the frequency of HLA-DR+TNFR2+ monocytes was elevated in COVID-19 compared to HD, LTBI, and LTBI/COVID-19 ($p<0.05$). This increase persisted under stimulation in COVID-19 individuals compared to LTBI and LTBI/COVID-19 ($p<0.05$) (Figure 2B).

3.3 Activation and Th1/Th2 profile of CD4+ T cell is not modified during LTBI/COVID-19 coinfection

Data showed that the frequency of CD4+ and CD8+ T cells and the CD4/CD8 ratio were not different between groups (Supplementary Figure S3). Activation of T cells did not show important differences, only to note that HD increased the frequency of CD4+CD69+ T cells after BCG stimulation, whereas the frequency of CD8+CD69+ T cells was lower in HD at baseline compared to other groups (Supplementary Figure S4).

Next, we evaluated whether CD4+ T cell function was altered by assessing Th1 (T-bet) and Th2 (GATA3) profiles. Despite COVID-19 individuals having a discreet increase in the frequency of CD4+T-bet+ T cells following spike stimulation, compared to HD, the GATA3/T-bet ratio was not modified (Figure 3A). We measured pro- and anti-inflammatory cytokines in the supernatant to confirm the inflammatory profile. At baseline, compared to HD and LTBI/COVID-19, COVID-19 had elevated levels of pro-inflammatory cytokines IFN-γ ($p<0.01$) and TNF ($p<0.05$). However, COVID-19 individuals also showed increased levels of the anti-inflammatory cytokine IL-10 at baseline ($p<0.05$) and after spike stimulation ($p<0.05$) (Figure 3B); all together suggested that the Th1/Th2 profile is not altered even under the coinfection context. Plasmatic levels of selected pro- and anti-inflammatory cytokines did not differ significantly between groups (Supplementary Figure S5A).

3.4 CD8+ T cells of LTBI/COVID-19 show similar behavior to those of COVID-19, but soluble cytotoxic molecules are decreased

Compared to HD, COVID-19 has increased the CD8+Perforin+ T cells' frequency at baseline ($p<0.05$). In response to spike stimuli, both COVID-19 and LTBI/COVID-19 showed an increased frequency of T CD8+Perforin+ cells ($p<0.05$) compared to HD. Additionally, LTBI/COVID-19 maintained a high frequency when cells were stimulated with BCG/spike compared to HD and LTBI ($p<0.05$). The frequency of CD8+Granzyme B+ T cells did not differ significantly between groups or stimuli. However, the frequency of T CD8+ cells double-positive for Granzyme B and Perforin increased in both COVID-19 and LTBI/COVID-19 compared to HD upon spike stimulation ($p<0.05$), and COVID-19 maintained this high frequency with BCG/spike stimulation ($p<0.05$) (Figure 4A).

Following, soluble cytotoxic molecule levels were evaluated in the culture supernatant. Compared to COVID-19, LTBI/COVID-19 produced lower levels of perforin and granzyme B at baseline ($p<0.05$), a pattern that persisted after stimulation with spike and BCG/spike ($p<0.05$). Granzyme A levels did not differ between groups (Figure 4B). Furthermore, plasmatic levels of perforin and sFas ($p<0.01$), were lower in LTBI/COVID-19 compared to COVID-19 (Figure 4C). Granulysin levels were also lower in LTBI/COVID-19 compared to COVID-19, LTBI, and HD ($p<0.05$, $p<0.01$) (Figure 4C). However, systemic levels of granzyme A, granzyme B, and sFasL did not differ significantly between groups (Supplementary Figure S5B).

3.5 CD8+ T cells from LTBI/COVID-19 have not an exhausted-like phenotype and exhibit high antigen-specific degranulation capacity

Given the discrepancy between intracellular and soluble cytotoxic molecules in LTBI/COVID-19, we assessed PD-1 and TIM-3 expression to determine whether T CD8+ cells exhibited an exhausted-like phenotype. The frequency of CD8+PD-1+ T cells was higher in LTBI compared to HD in response to stimuli ($p<0.05$), though no significant difference was observed between COVID-19 and LTBI/COVID-19. Conversely, the frequency of CD8+TIM-3+ T cells was higher in COVID-19 than in LTBI/COVID-19 following stimulation with spike or BCG/spike ($p<0.05$), although LTBI still displayed a higher frequency compared to HD ($p<0.05$) (Figure 5A).

To confirm cytotoxic function, degranulation capacity was evaluated by CD107a expression. T CD8+ cells from all groups

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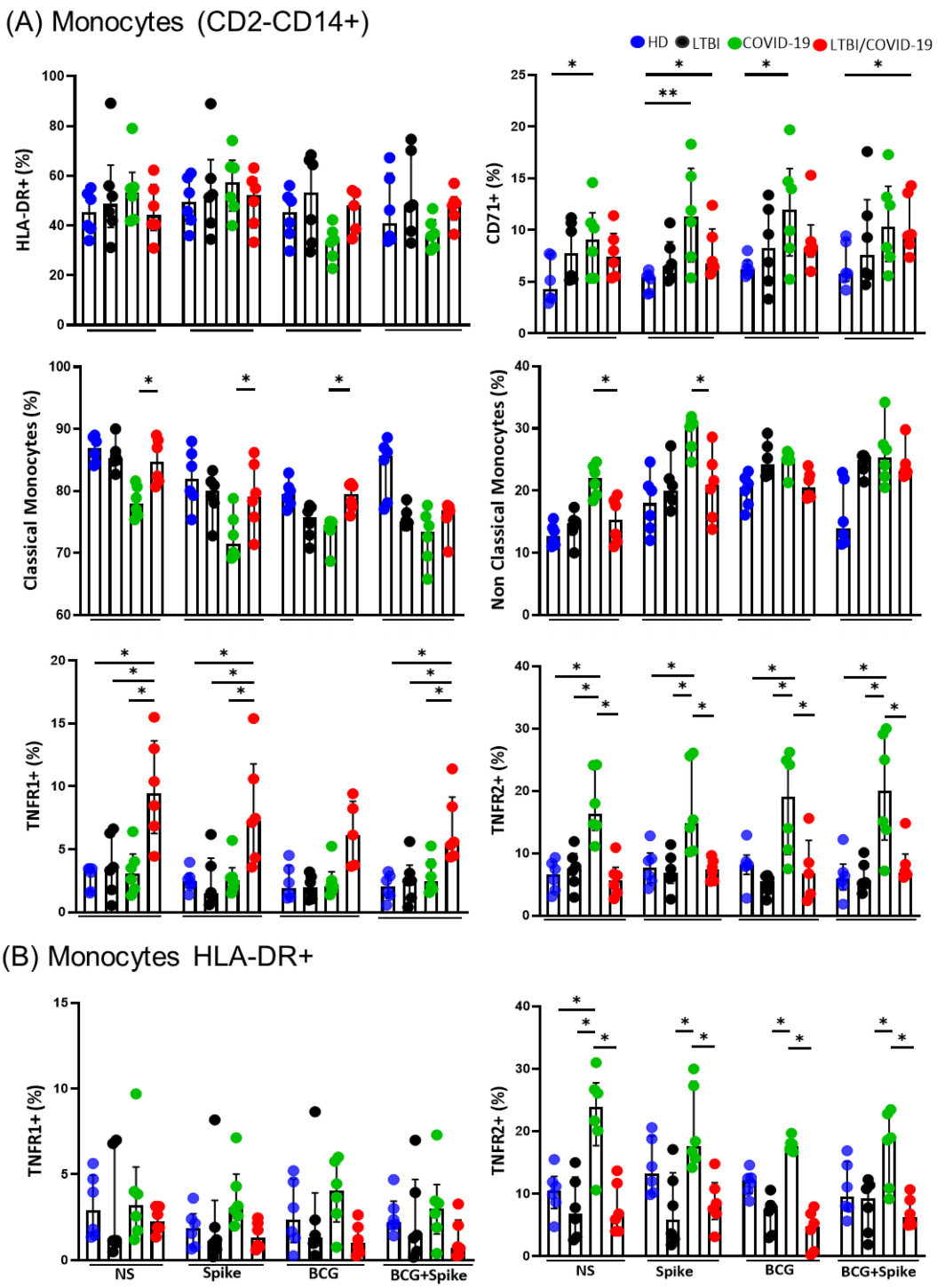

FIGURE 2
The expression of the receptor on monocytes is different in the LTBI/COVID-19 vs COVID-19 group. Peripheral blood mononuclear cells stimulated with BCG (MOI 1:1) and S protein (1µg/mL) were cultured for 24 hours, recovered, and evaluated by flow cytometry, n=6 per group. Into the gate CD2-CD14+ (monocytes), the frequency of monocytes HLA-DR+, classical monocytes CD14+, Non-Classical monocytes CD14+CD16+, CD71+, and TNFR1+ and TNFR2+ **(A)** was evaluated. Into the gate monocytes HLA-DR+, the frequency of TNFR1+ and TNFR2+ was evaluated **(B)**. Data are represented as medians with an interquartile range (IQR, 25-75), and each point represents individual data. The statistical comparison was performed using Kruskal-Wallis's test, *p<0.05, **p<0.01. NS, not stimulated; HD, healthy donor; LTBI, latent tuberculosis infection; COVID-19, individual with COVID-19; LTBI/COVID-19, individual with latent tuberculosis and COVID-19 coinfection.

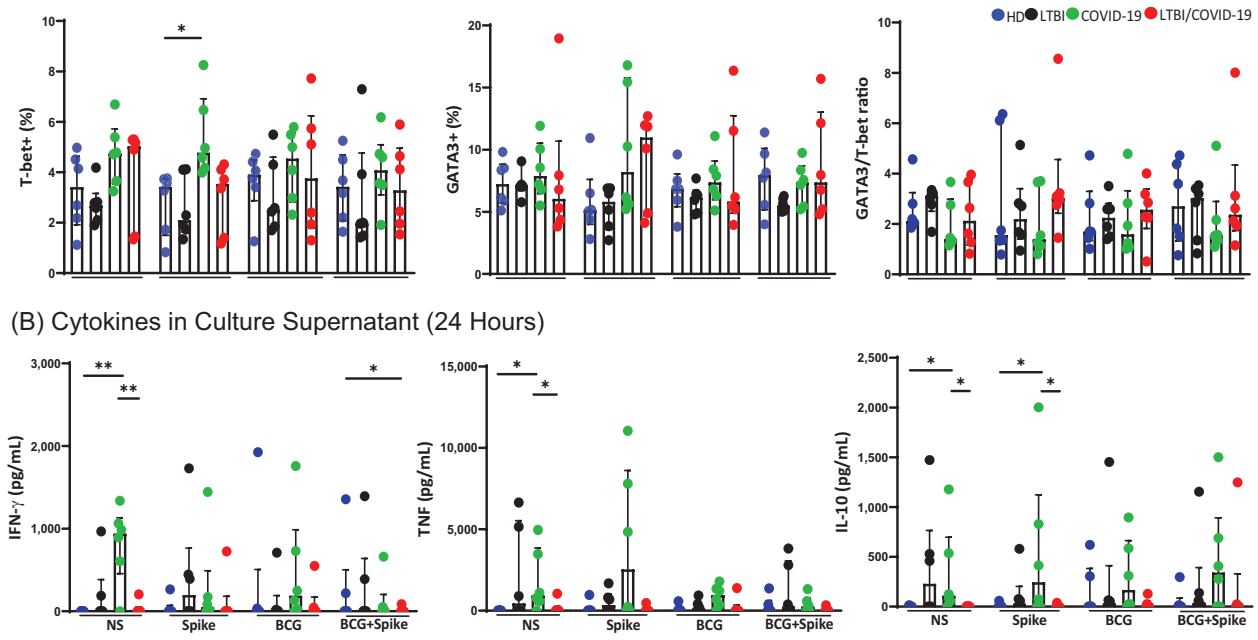

FIGURE 3
Th1 and Th2 responses are at comparable levels, with no evident predominance of one over the other. Peripheral blood mononuclear cells stimulated with BCG (MOI 1:1) and spike (1µg/mL) were cultured for 24 hours, recovered, and evaluated by flow cytometry, n=6 per group. Into the gate, CD2+CD3+ (T cells), the frequency of T CD4+ nuclear markers T-bet (Th1) or GATA-3 (Th2) **(A)**, and assessment of cytokines in culture supernatant by LEGENDplex™ **(B)** were evaluated. Data are represented as medians with an interquartile range (IQR, 25-75), and each point represents individual data. The statistical comparison was performed using Kruskal-Wallis's test, *p<0.05, **p<0.01. NS, not stimulated; HD, healthy donor; LTBI, latent tuberculosis infection; COVID-19, individual with COVID-19; LTBI/COVID-19, individual with latent tuberculosis and COVID-19 coinfection.

showed similar degranulation capacity following polyclonal stimulation. Degranulation began at 1 hour and peaked at 4 hours post-culture. The frequency of CD8+CD107a+ T cells was higher in LTBI/COVID-19 than in other groups but without statistically significant (Figure 5B, left). When evaluating antigen-specific degranulation at 4 hours post-culture, LTBI/COVID-19 exhibited a higher frequency of T CD8+CD107a+ cells compared to COVID-19 when stimulated with BCG ($p<0.05$), and higher than LTBI when it was stimulated with spike ($p<0.05$) (Figure 5B, right).

Recently, it was highlighted the presence of cytotoxic CD4+ T cells to compensate for exhausted CD8+ T cells in TB patients (27). First, we evaluated the exhausted-like phenotype in CD4+ T cells, and our data showed that the PD-1 expression on CD4+ T cells was unaltered. However, the frequency of CD4+TIM-3+ T cells was elevated in COVID-19 compared to HD at baseline ($p<0.01$). Under stimulus conditions, LTBI ($p<0.01$), COVID-19 ($p<0.001$), and LTBI/COVID-19 ($p<0.01$) groups exhibited higher TIM-3+ frequencies than HD (Figure 5C).

We also observed CD4+ T cells with degranulation capacity (CD107a+) across all groups. The maximum frequency of CD4+CD107a+ T cells at 4 hours post-culture was lower than CD8+CD107a+ T cells (10% vs. 30%, respectively). To note, with polyclonal stimulus, all groups showed a maximum of 10% of CD4+CD107a+ T cells (Figure 5D, left); interestingly, under antigen-specific stimuli, the top of degranulation was similar to the observed with polyclonal stimulus, suggesting that most of cytotoxic CD4+ T cells are antigen-specific (Figure 5D, right).

3.6 The LTBI/COVID-19 coinfection modulates the morpho-functionality of the mitochondria of T cells

The mitochondrial morpho-functionality in T cells was evaluated. Into de CD4+ and CD8+ T cells, the regions evaluated were high-polarized compacted mitochondria (HCM); high-polarized fractionated mitochondria (HFM); low-polarized compacted (PCM) and low-polarized fractionated (PFM) (Figure 6A).

The data demonstrated that compared to COVID-19, LTBI/COVID-19 has predominantly CD4+ T cells with high-polarized compacted mitochondria (HCM) at baseline ($p<0.0001$) and a similar profile is maintained even with stimulus (Figure 6B). On the contrary, compared to LTBI/COVID-19, COVID-19 has predominantly CD4+ T cells with high-polarized fractionated mitochondria (HFM) at baseline ($p<0.01$) and a similar profile is maintained even with stimulus (Figure 6B).

A similar behavior was observed in CD8+ T cells, where LTBI/COVID-19 has predominantly HCM CD8+ T cells at baseline ($p<0.01$) and maintained with stimulus, whereas COVID-19 has predominantly HFM CD8+ T cells at baseline and maintained

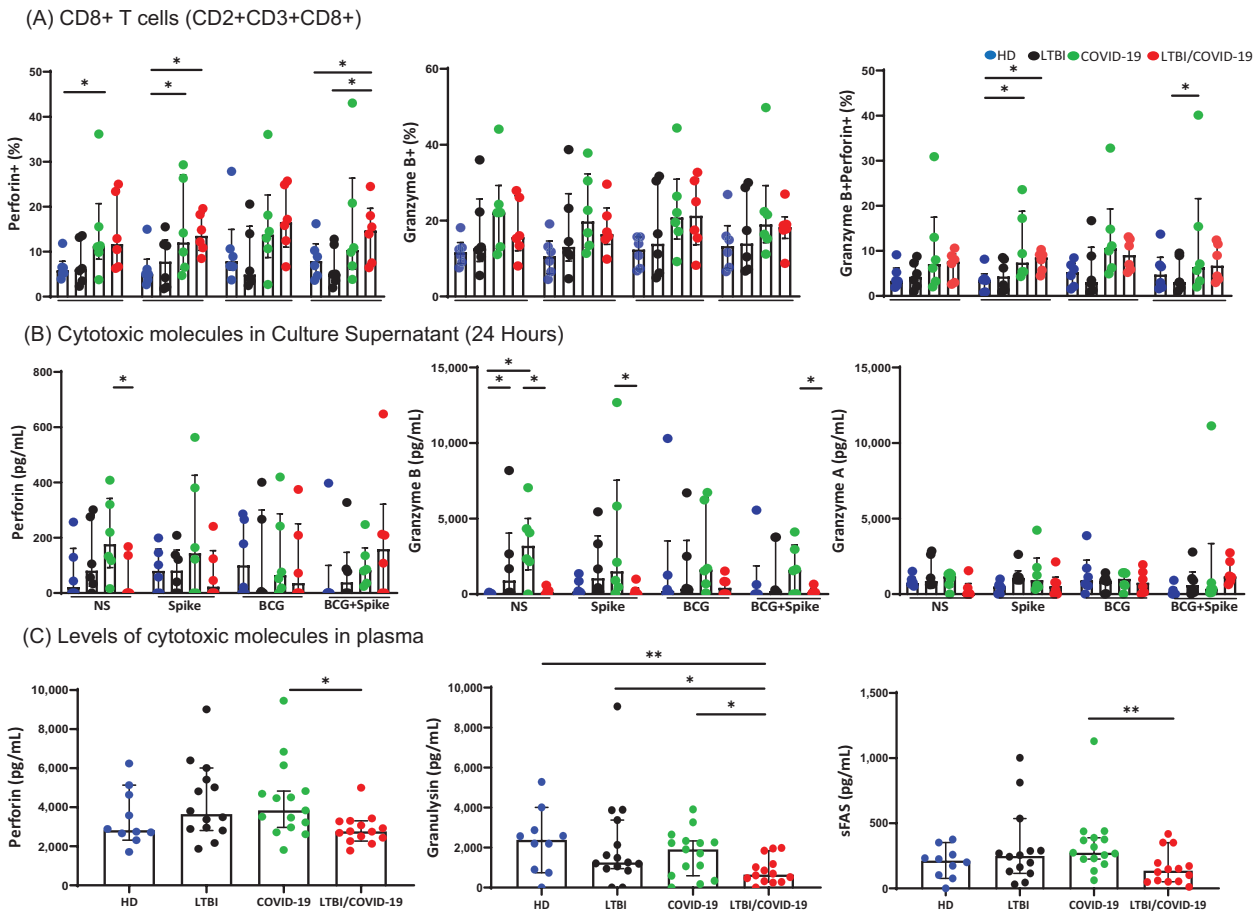

FIGURE 4
Secretion of cytotoxic molecules differ in LTBI/COVID-19 vs COVID-19 individuals. Peripheral blood mononuclear cells stimulated with BCG (MOI 1:1) and spike (1µg/mL) were cultured for 24 hours, recovered, and evaluated by flow cytometry. Frequency of CD8+Perforin+, CD8+ Granzyme B+, and CD8+ Granzyme B+Perforin+ T cells (CD2+CD3+ gate), n=6 per group **(A)**. Levels of cytotoxic molecules in the culture supernatant evaluated by LEGENDplex assay, n=6 per group **(B)**. Plasma levels of cytotoxic molecules were evaluated by LEGENDplex assay, n=10 (HD) and n=15 (LTBI, COVID-19, and LTBI/COVID-19) **(C)**. Data are represented as medians with an interquartile range (IQR, 25-75), and each point represents individual data. The statistical comparison was performed using Kruskal-Wallis's test, *p<0.05, **p<0.01. NS, not stimulated. HD, healthy donor; LTBI, latent tuberculosis infection; COVID-19, individual with COVID-19; LTBI/COVID-19, individual with latent tuberculosis and COVID-19 coinfection.

under stimulus (Figure 6C). T cells with low-polarized compacted (PCM) mitochondria were not altered (Figures 6B, C).

3.7 LTBI/COVID-19 individuals exhibit distinct receptor profiles and unique immunological features compared to COVID-19 individuals

Our results show that LTBI significantly impacts the immune cell profile, and the set of symptoms also differs from those of COVID-19 individuals with or without LTBI. To simplify the molecular immune landscape between groups, a heatmap was made representing the groups evaluated on the vertical axis and the experimental conditions applied for each of them. On the horizontal axis, the molecules and receptors evaluated for the different cell groups are shown. The blue colors indicate lower values, the white colors indicate medium values, and the red colors represent high values.

Figure 7A shows that the profile of monocyte subsets from individuals with LTBI/COVID-19 is more similar to HD and LTBI than to COVID-19. Similarly, LTBI/COVID-19 has predominantly CD4+ and CD8+ T cells with highly polarized, compact mitochondria like HD and LTBI, whereas T cells from COVID-19 show highly polarized, fragmented mitochondria (Figure 7A). Finally, LTBI/COVID-19 has lower plasmatic levels of cytotoxic molecules compared to COVID-19 (Figure 7B), and it is similar to that observed *in vitro* under stimuli (Figure 7A).

The data evaluated suggest that the profile of an individual with LTBI/COVID-19 generates a unique immunological signature compared to individuals with COVID-19, which could point to a regulatory role of Mtb in this viral infection, however, further evaluation is needed to determine the phenomenon.

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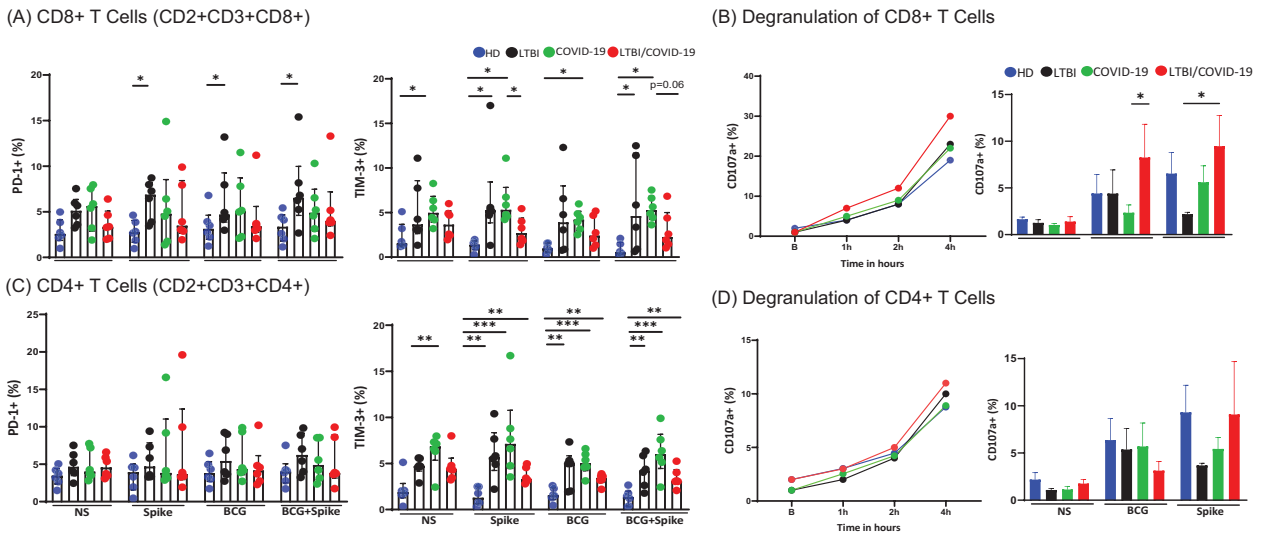

FIGURE 5
CD8+ T cell degranulation and cytotoxic regulation remain unaltered in LTBI/COVID-19 individuals. Peripheral blood mononuclear cells stimulated with BCG (MOI 1:1) and spike (1µg/mL) were cultured for 24 hours, recovered, and evaluated by flow cytometry (n=6 per group). Then, into the gate, CD2+CD3+ (T cells), the frequencies of CD8+PD-1+, CD8+TIM-3+ **(A)** and CD4+PD-1+, CD4+TIM-3+ were evaluated **(C)**. Peripheral blood mononuclear cells stimulated with PMA/IO (1X) as a polyclonal stimulus or BCG (MOI 1:1) and spike (1µg/mL) were cultured for 4 hours, recovered and evaluated by flow cytometry. The CD107a expression as a marker of degranulation (n=4 per group). Then, into the gate, CD2+CD3+ (T cells), the frequencies of CD8+CD107a+ **(B)**, and CD4+CD107a+ were evaluated **(D)**. Data are represented as medians with an interquartile range (IQR, 25-75), and each point represents individual data. The statistical comparison was performed using Kruskal-Wallis's test, *p<0.05, **p<0.01, ***p<0.001. NS, not stimulated. HD, healthy donor; LTBI, latent tuberculosis infection; COVID-19, individual with COVID-19; LTBI/COVID-19, individual with latent tuberculosis and COVID-19 coinfection.

4 Discussion

Clinical studies have indicated that COVID-19 can induce the progression of LTBI to active TB in previously infected individuals (6, 8, 28). However, limited studies have highlighted the immunological alterations in LTBI that modulate the immune response during co-infection with COVID-19.

Based on cross-sectional studies, patients with asymptomatic COVID-19 and LTBI showed elevated levels of serum cytokines, chemokines, growth factors, and immunoglobulins compared to patients with COVID-19 alone. Despite not being vaccinated against SARS-CoV-2, these findings suggest a stronger immune response against the virus (18). Similarly, other reports indicated that patients admitted with COVID-19 and LTBI exhibited higher counts and proportions of neutrophils, monocytes, and lymphocytes than those with COVID-19 alone (29).

This study investigates the impact of LTBI in individuals vaccinated against SARS-CoV-2 and with mild COVID-19. Given the nature of our cohort (healthcare workers), all enrolled subjects reported receiving the SARS-CoV-2 and BCG vaccines. It is important to note that the HD control group used to evaluate the phenotypic profile and functional capacity in response to Spike and BCG proteins intentionally included individuals who had previously experienced COVID-19. This choice reflects the current context in the world and allows us to assess immune alterations potentially arising from memory responses generated by prior SARS-CoV-2 infection and/or vaccination, as previously reported (30); in this way, observed differences include the background generated only by the memory. Our data revealed that individuals with COVID-19 displayed a distinct immune profile characterized by a predominance of monocytes expressing pro-inflammatory and regulatory markers, including TNFR2, co-expression of HLA-DR/TNFR2, and CD71. In contrast, the monocyte subsets in LTBI/COVID-19 individuals were more similar to those of healthy donors (HD) and LTBI individuals. Furthermore, compared to COVID-19 individuals, LTBI/COVID-19 individuals showed an increased degranulation capacity of CD8+ T cells, along with similar frequencies of CD8+perforin+ and CD8 +Granzime+ T cells, and reduced levels of soluble cytotoxic molecules regardless of the stimuli. Similarly, the levels of soluble cytotoxic molecules in the peripheral circulation were reduced. LTBI/COVID-19 individuals exhibited T cells with highly polarized, compact mitochondria, whereas COVID-19 individuals had highly polarized, fragmented mitochondria.

Previous studies indicated that monocytes from vaccinated mild COVID-19 patients do not show significant alterations (31–33), which is in concordance with our data on HLA-DR+ monocytes. On the other hand, LTBI/COVID-19 exhibited monocyte frequencies more similar than HD, suggesting that LTBI modifies the cell's presence, probably by a chronic activation. It has been reported that monocytes from LTBI do not modify CD16 and HLA-DR expression after LPS stimulus (34). In our study, we observed a reduction in classical monocytes and an increase in non-classical monocytes in COVID-19, a pattern not observed in LTBI/COVID-19, suggesting a modulatory role of LTBI on monocytes (35). Further research is needed to evaluate their functionality,

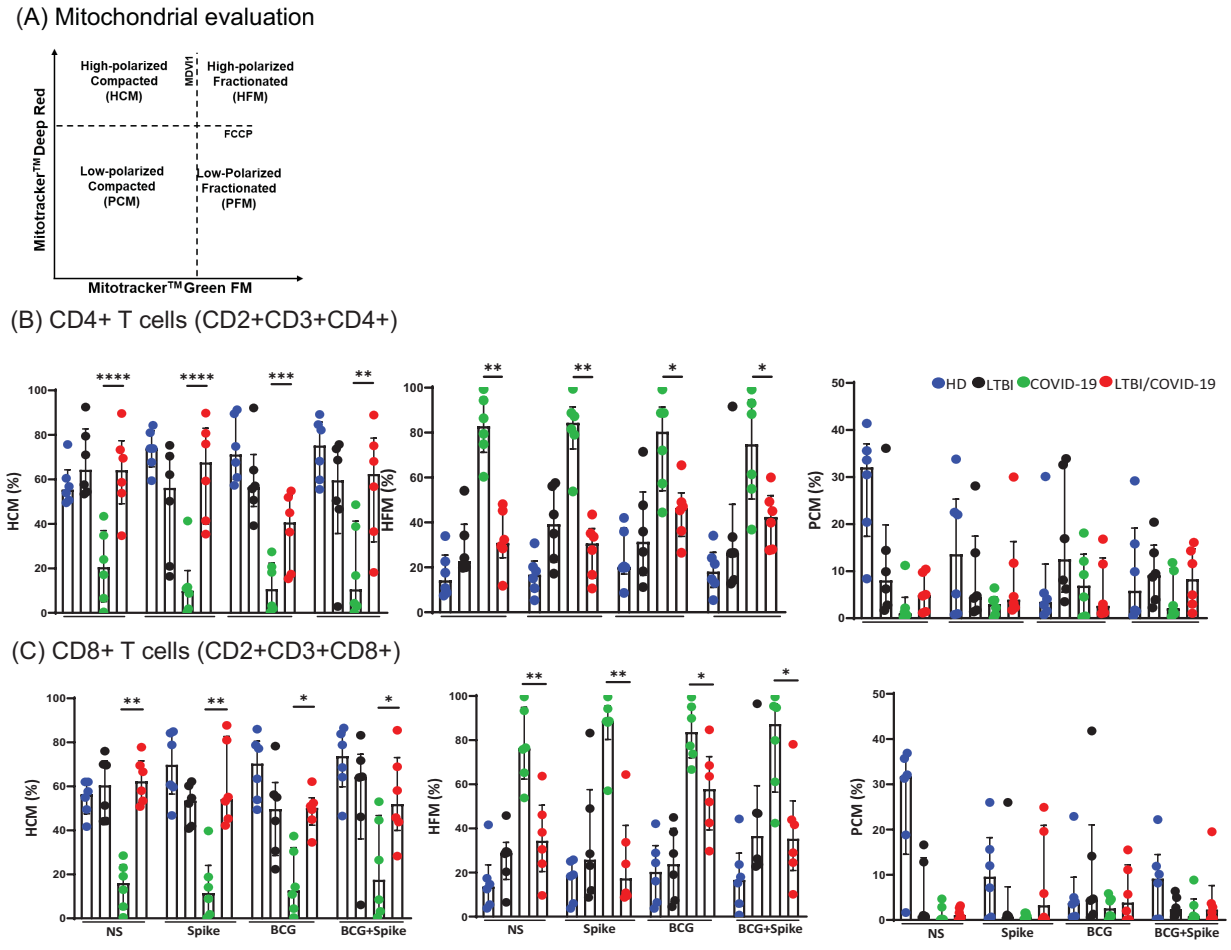

FIGURE 6
LTBI/COVID-19 coinfection and antigenic stimuli modulate T cells mitochondrial function. Peripheral blood mononuclear cells stimulated with BCG (MOI 1:1) and spike (1µg/mL) were cultured for 24 hours, recovered, and evaluated by flow cytometry (n=6 per group). Into the gate, CD2+CD3+ (T cells), the mitochondrial region selection **(A)**, the frequency of CD4+HCM, CD4+HFM, and CD4+PCM **(B)**, and the frequency of CD8+HCM, CD8+HFM, and CD8+PCM were evaluated. Data are represented as medians with an interquartile range (IQR, 25-75), and each point represents individual data. The statistical comparison was performed using Kruskal-Wallis's test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. NS, not stimulated; HD, healthy donor; LTBI, latent tuberculosis infection; COVID-19, individual with COVID-19; LTBI/COVID-19, individual with latent tuberculosis and COVID-19 coinfection. HCM, High-polarized compacted mitochondria; HFM, High-polarized fractionated mitochondria; PCM, Low-polarized compacted mitochondria.

metabolism, and migration in response to specific antigens to determine whether these differences in monocyte frequencies between COVID-19 and LTBI/COVID-19 aid in better regulation of the inflammatory status.

Numerous studies have associated elevated levels of soluble TNFR1 and TNFR2 with the severity of COVID-19 (25, 36, 37). The COVID-19 individuals enrolled in this study did not present severe or critical illness. Consequently, soluble forms of these receptors were not evaluated. Nevertheless, LTBI/COVID-19 individuals displayed an increased frequency of TNFR1+ monocytes, whereas COVID-19 individuals exhibited TNFR2+ monocytes, primarily within the HLA-DR+ monocyte subset. TNFR1 has been linked to mediating cell death, while TNFR2 is associated with cell survival. Their roles in mycobacterial infections have been extensively studied (38, 39). These results raise new questions, such as whether TNFR1 mediates monocyte subset regulation during LTBI/COVID-19 through cell death promotion, as reported in other contexts (40, 41). On the contrary, TNFR2 may promote monocyte survival, favoring a specific monocyte profile (42).

We did not identify differences in blood parameters, including lymphocyte frequency, between groups. Interestingly, LTBI/COVID-19 individuals exhibited less severe symptoms. Other studies have suggested that LTBI enhances innate and adaptive immunity due to prior Mtb exposure, which may provide a protective effect in COVID-19 co-infection by preventing lymphopenia, a condition associated with higher mortality rates (43). Additionally, we did not observe an altered Th1/Th2 profile, consistent with the findings of Song HW et al. (44).

It is essential to highlight that there are differences between COVID-19 and LTBI/COVID-19, even without stimulation. It is suggested that LTBI/COVID-19 exhibits these immunological changes as a characteristic associated with LTBI, with only minor changes related to stimulation. The Spike protein has been

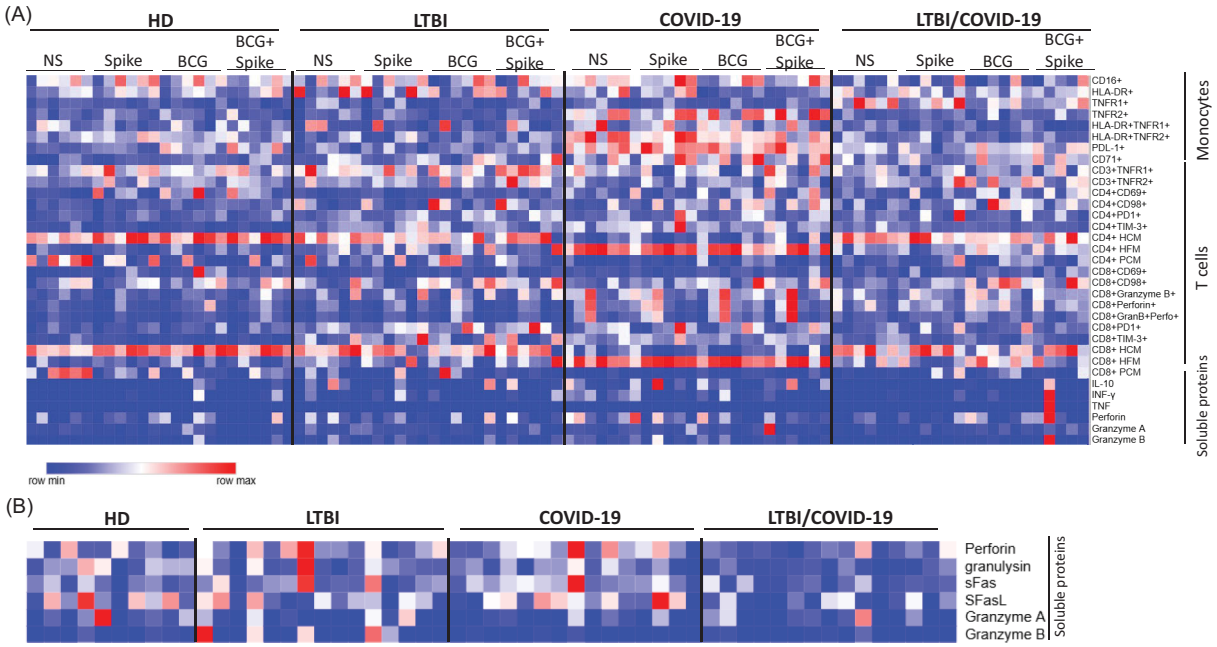

FIGURE 7
Distinct receptor profiles and immunological features in LTBI/COVID-19 vs. COVID-19 individuals. Heatmap displaying the expression levels of all cell markers evaluated in this study. In this dataset, red indicates increased marker expression, while blue represents decreased expression relative levels. Each column corresponds to one of the six individuals analyzed, categorized by their respective stimulus conditions and classification group. This visualization highlights inter-individual variability and stimulus-specific effects on the immunophenotypic profiles of the evaluated cell populations **(A)**. An evaluation of the plasma soluble proteins was carried out, classifying them according to the group to which they correspond **(B)**, n=10 (HD) and n=15 (LTBI, COVID-19, and LTBI/COVID-19). HD, healthy donor; LTBI, latent tuberculosis infection; COVID-19, individual with COVID-19; LTBI/COVID-19, individual with latent tuberculosis and COVID-19 coinfection, NS, not stimulated. Created in Morpheus, https://software.broadinstitute.org/morpheus.

implicated in cytokine release syndrome, favoring severe or critical COVID-19 (45), which was probably not observed in our study because it included only individuals with mild COVID-19. Moreover, all groups reported a mixture of vaccines based primarily on the Spike protein, and previous reports have indicated that immunization influences the response to the Spike protein (11–13). A recent study showed that BCG vaccination does not affect the immune response to COVID-19 or SARS-CoV-2 vaccines such as AstraZeneca or Pfizer (46).

In our study, we cannot determine whether the low response to the Spike protein is influenced by prior BCG vaccination (received in childhood by 94% of the enrolled subjects). It should also be considered that the subjects are healthcare workers with a high risk of exposure to Mtb and other non-tuberculous mycobacteria. This raises a new question about whether exposure to mycobacteria is sufficient to modify the immune response to SARS-CoV-2 antigens.

Unlike CD4+ T cells, CD8+ T cells from LTBI/COVID-19 individuals exhibited increased degranulation capacity upon antigen-specific stimulation. However, levels of soluble cytotoxic molecules were reduced both *in vitro* and *in vivo*. Previous studies have reported that COVID-19 patients experience diminished cytotoxic responses, with CD4+ T cells acquiring a cytotoxic profile to compensate for CD8+ T cell dysfunction (47). It has also been suggested that CD8+ T cells with low perforin levels during the acute phase of severe SARS-CoV-2 infection may predict long COVID (48). Moreover, decreased levels of sFas and sFasL have been linked to compromised clearance of SARS-CoV-2-infected cells, adverse outcomes, and increased risk of organ failure (49, 50).

Previous studies have demonstrated that exhaustion markers, such as CD39 and TIM-3, are elevated on CD8+ T cells during COVID-19 and active TB, while PD-1 is not prominently expressed (27, 51–53). However, in our study, we did not observe an exhausted profile of T cells. Although the expression of the regulatory molecule TIM-3 was altered on both CD4+ and CD8+ T cells, this alone does not indicate an exhausted profile. Instead, it may be associated with the discrepancy between intracellular and soluble cytotoxic molecule levels observed in LTBI/COVID-19 individuals.

Regarding the increased degranulation capacity of CD8+ T cells in LTBI/COVID-19 individuals, it has been previously reported that CD8+ T cells from individuals living with HIV and with an undetectable viral load exhibit a potent cytotoxic response following SARS-CoV-2 vaccination (54). Given the low levels of soluble cytotoxic molecules, an important question arises: How do these cells effectively control mycobacterial growth or viral replication? We speculate that, although cells from LTBI/COVID-19 individuals display efficient degranulation in response to stimuli, their cytotoxic capacity may not be optimal, as suggested by the low levels of cytotoxic molecules. This finding aligns with the impaired

cytotoxic function observed in TB patients, likely due to chronic antigen exposure (27), which could potentially compromise pathogen control. Alternatively, it might represent a regulatory mechanism to prevent excessive immune activation and tissue damage. However, further studies are needed to respond to this speculation and determine the true consequences of this observation.

Although the presence of CD4+ T cells with a cytotoxic phenotype has been reported in both COVID-19 and TB (27, 48), our study specifically identified that LTBI/COVID-19 individuals possess these unconventional CD4+ T cells, which appear to be entirely antigen-specific.

Mitochondrial fragmentation has been associated with altered metabolism, characterized by decreased cellular energy levels, increased apoptosis, excessive production of toxic molecules, and reduced immune response efficacy (10, 55). Our data demonstrated that LTBI/COVID-19 individuals predominantly exhibit T cells with a mitochondrial profile distinct from that observed in COVID-19 individuals. These findings suggest that SARS-CoV-2 induces mitochondrial stress, consistent with several studies highlighting this process in viral infections (55–57).

However, pre-existing chronic infections such as LTBI appear to condition a different mitochondrial response, which could be crucial for maintaining diverse cellular mechanisms. In the context of LTBI/COVID-19, lower mitochondrial fragmentation does not necessarily indicate cellular well-being; instead, it may reflect a more balanced state of mitochondrial dynamics, potentially influenced by LTBI. A study on acute COVID-19 demonstrated that increased mitochondrial mass could prevent apoptosis, creating an intracellular environment favorable for virus propagation in infected cells (10, 55).

Further research, including specific metabolic investigations, is needed to determine whether this phenomenon is driven by mitochondrial fusion or fission mechanisms. Additionally, these studies may aim to elucidate the potential long-term immunometabolic consequences in the affected individuals.

Our study is not free from limitations. First, we cannot clarify the timing of the last COVID-19 diagnosis in the reference groups (HD and LTBI). Second, we are unable to determine if there is any immunological involvement of the BCG vaccine and prolonged exposure to Mtb respiratory disease in the recruited individuals. Finally, we cannot ascertain with QuantiFERON how long the patient was infected with Mtb, thus it is also not possible to determine whether individuals with LTBI still harbor viable bacilli that could induce subsequent Mtb development or if these bacilli trigger ongoing immunologic changes.

5 Conclusion

The findings reveal significant alterations in monocytes and T cells of individuals with LTBI/COVID-19, suggesting that coinfection of both pathologies may induce changes in the immune phenotype, impacting cytotoxic function and cellular activation. However, further investigation is necessary to elucidate the mechanisms underlying these responses and to ascertain their impact on individuals' clinical progression.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Institutional Ethics Committee of the Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas (INER, protocol number B23–23). All participants signed written informed consent. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CP-B: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – original draft. LR-L: Methodology, Writing – original draft. JF-G: Methodology, Writing – original draft. EE: Resources, Writing – original draft. MM-M: Methodology, Writing – original draft. KM-Q: Resources, Writing – original draft. MV-H: Resources, Writing – original draft. NT-N: Data curation, Writing – original draft. FS-G: Data curation, Writing – original draft. EB-V: Data curation, Writing – original draft. MS: Data curation, Writing – original draft. LC-G: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1566449/full#supplementary-material

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