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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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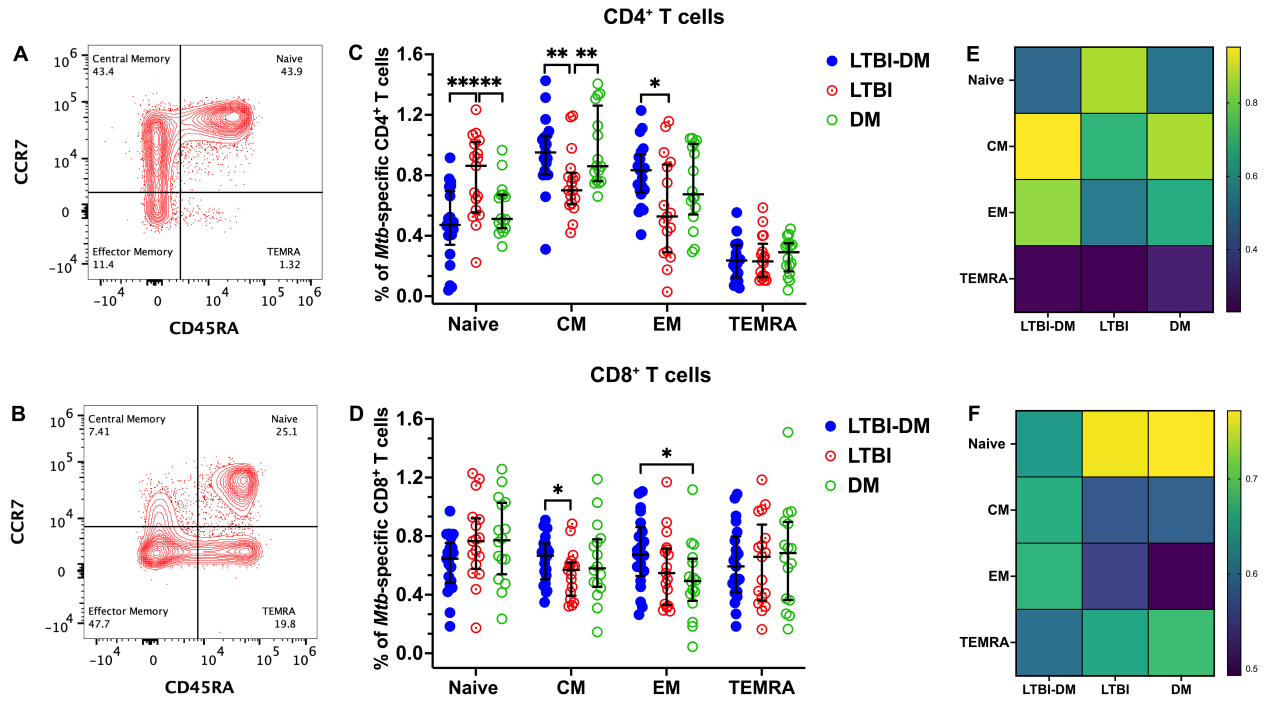

FIGURE 1
Type 2 DM alters the memory phenotype of *Mtb*-specific CD4$^+$ and CD8$^+$ T cells. **(A, B)** Representative flow cytometry plots are shown for CD4$^+$ and CD8$^+$ CCR7/CD45RA-defined T-cell memory subsets, respectively. PBMCs were stimulated and cultured for 18 hours with ESAT-6 and CFP-10 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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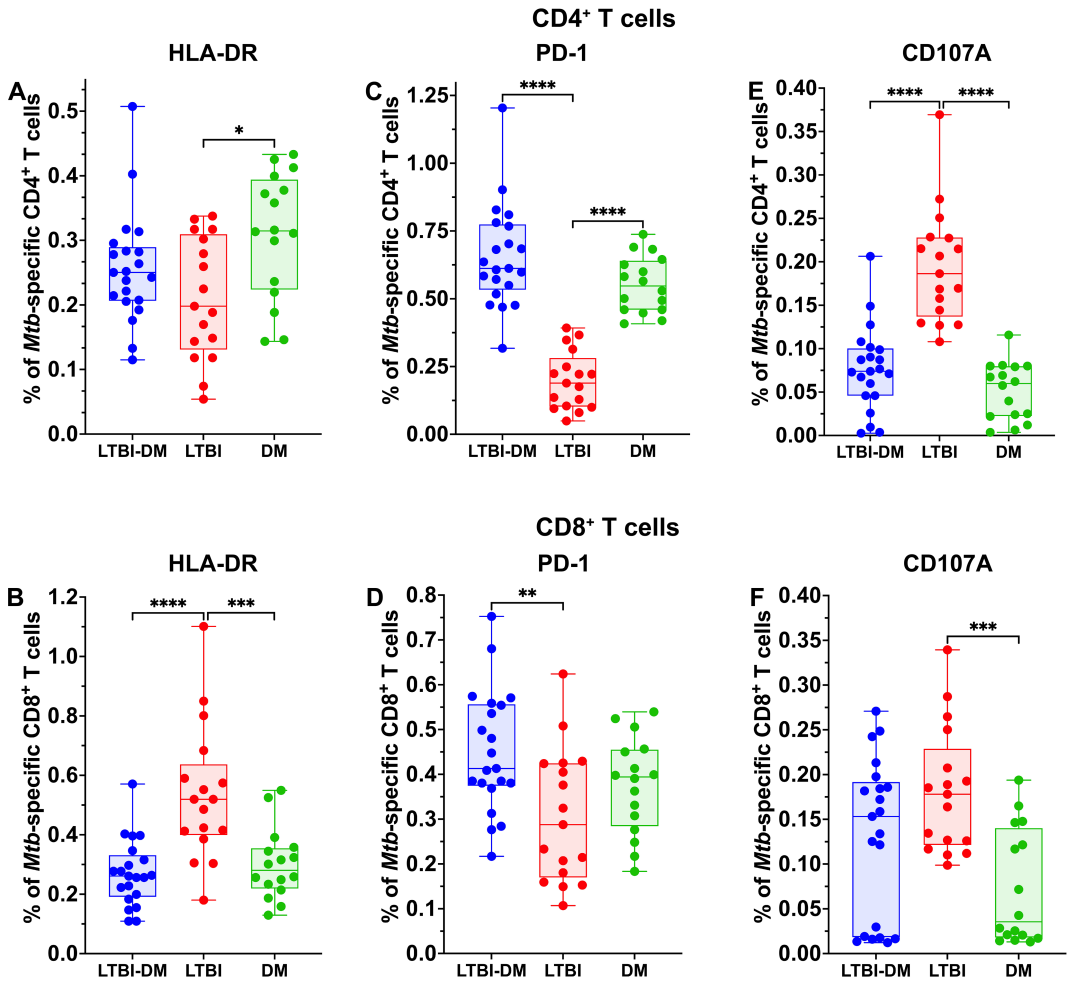

FIGURE 2
Type 2 DM impairs the HLA-DR, PD-1, and CD107A expression of Mtb-specific CD4+ and CD8+ T cells. The PBCMs were surface stained with HLA-DR and PD-1 antibodies after 18 hours of incubation with ESAT-6 and CFP-10 peptide pools and brefeldin A. **(E, F)** For degranulation analysis of $CD4^+$ 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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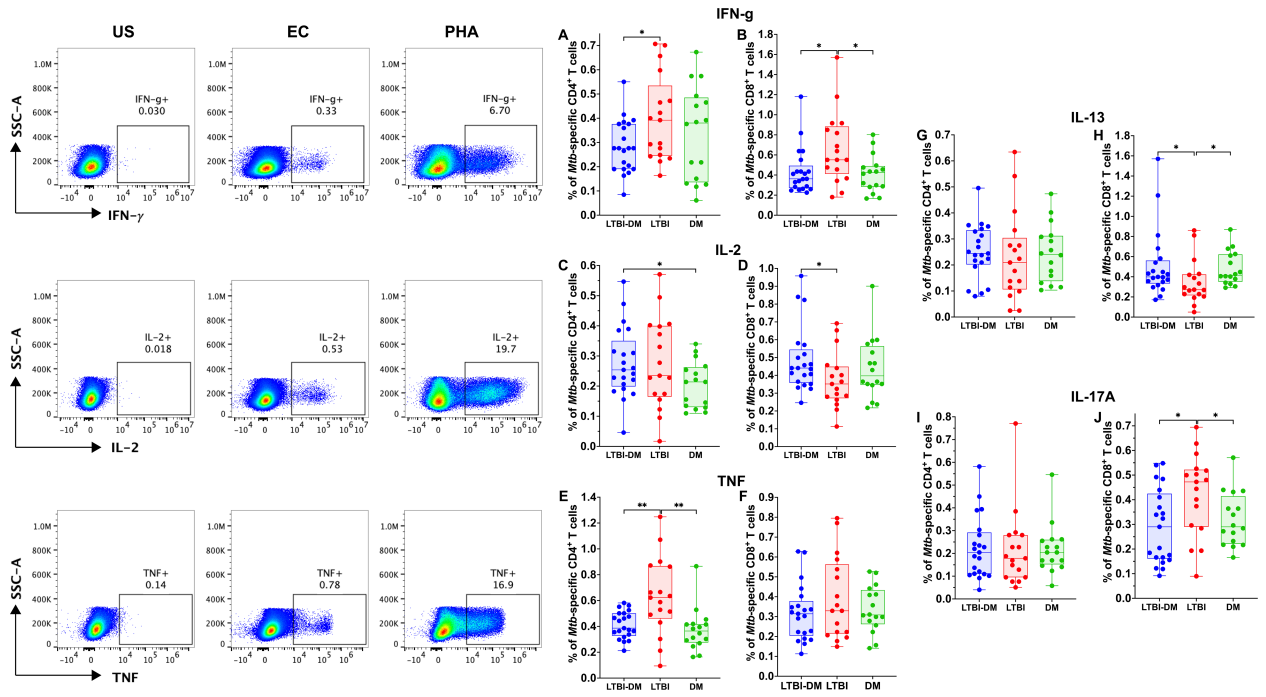

FIGURE 3
Type 2 DM impairs the production of *Mtb*-specific Th-1, Th-2 and Th-17 cytokines by $CD4^+$ and $CD8^+$ T cells. The PBMCs were cultured and stimulated for 18 hours with ESAT-6 and CFP-10 peptide pools, brefeldin A, and intracellularly stained for cytokines. **(A–J)** Representative plots for $CD4^+$ 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

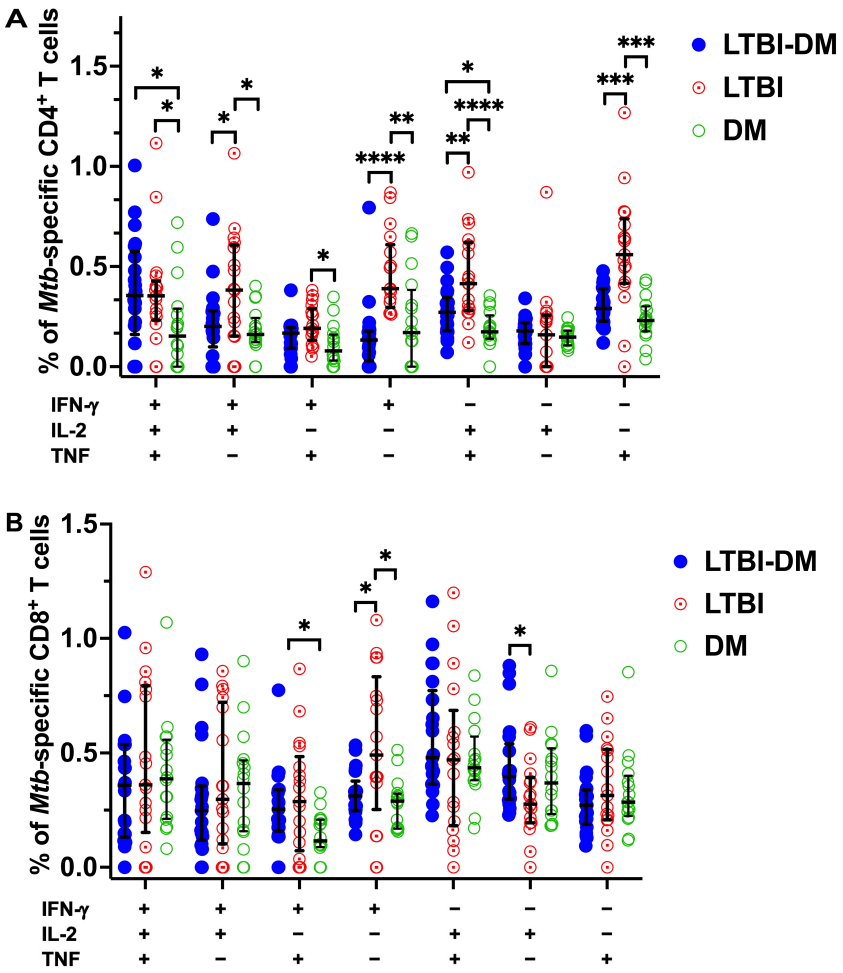

FIGURE 4
Type 2 DM impairs dual and mono-functional *Mtb*-specific $CD4^+$ and $CD8^+$ T-cell responses. **(A)** Polyfunctional *Mtb*-specific $CD4^+$ T-cell responses. **(B)** Polyfunctional *Mtb*-specific $CD8^+$ T-cell responses. The X-axis represents the frequencies of *Mtb*-specific $CD4^+$ T cells 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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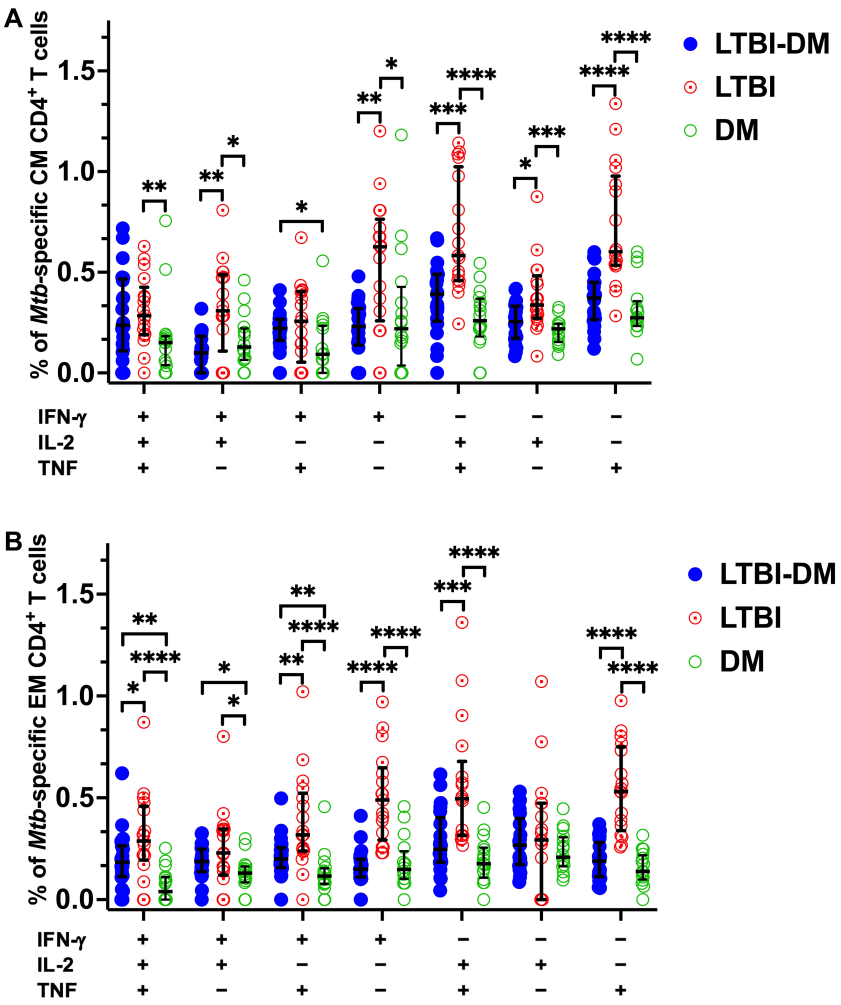

FIGURE 5
Type 2 DM impairs triple, dual, mono-functional *Mtb*-specific central and effector memory CD4$^+$ T-cell responses. **(A)** Polyfunctional *Mtb*-specific central memory CD4$^+$ T-cell responses. **(B)** Polyfunctional *Mtb*-specific effector memory CD4$^+$ T-cell responses. The X-axis represents 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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