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Coincident diabetes mellitus modulates Th1-, Th2-, and Th17-cell responses in latent tuberculosis in an IL-10- and TGF-β-dependent manner

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Type 2 diabetes mellitus (DM) is a risk factor for the development of active tuberculosis (TB), although its role in the TB-induced responses in latent TB (LTB) is not well understood. Since Th1, Th2, and Th17 responses are important in immunity to LTB, we postulated that coincident DM could alter the function of these CD4$^+$ T-cell subsets. To this end, we examined mycobacteria-induced immune responses in the whole blood of individuals with LTB-DM and compared them with responses of individuals without DM (LTB-NDM). T-cell responses from LTB-DM are characterized by diminished frequencies of mono- and dual-functional CD4$^+$ Th1, Th2, and Th17 cells at baseline and following stimulation with mycobacterial antigens-purified protein derivative, early secreted antigen-6, and culture filtrate protein-10. This modulation was at least partially dependent on IL-10 and TGF-β, since neutralization of either cytokine resulted in significantly increased frequencies of Th1 and Th2 cells but not Th17 cells in LTB-DM but not LTB individuals. LTB-DM is therefore characterized by diminished frequencies of Th1, Th2, and Th17 cells, indicating that DM alters the immune response in latent TB leading to a suboptimal induction of protective CD4$^+$ T-cell responses, thereby providing a potential mechanism for increased susceptibility to active disease.

Keywords: Diabetes · IL-10 · Tuberculosis · Th cells · TGF–β

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Introduction

Diabetes mellitus (DM) and tuberculosis (TB) continue to be two major scourges of public health worldwide and their synergistic role in amplifying disease morbidity has recently become a major topic of clinical and basic research [1]. Both tuberculosis and diabetes mellitus continue to be major disease burdens in developing countries and the dual burden of diabetes and TB clearly represents a growing global public health concern [2]. A growing surge of recent literature suggests that DM is an important risk factor for developing active pulmonary TB [3]. In addition to enhancing the risk for reactivating latent TB infection (LTB), there is also evidence that DM is associated with increased severity of TB disease, delayed sputum conversion, unfavorable outcomes, and greater risk of relapse [1, 4]. In addition, DM might predispose to the development of drug-resistant TB [5, 6]. However, despite the clinical and public health implications caused by the dual burden of TB and DM, very little is known about the immunological and cellular mechanisms of susceptibility.

Immunity to TB has been shown to be mainly dependent on CD4$^+$ T cells. More specifically, CD4$^+$ Th1 cells in primary immunity and Th17 cells in recall responses have been shown to play a

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Table 1. Demographics and biochemical parameters of the study population

Study demographics	LTB-DM	LTB-NDM	p value[b)]
No. of subjects recruited	22	22	
Gender (M/F)	12/10	11/11	
Median age (range)[a)]	44 (26–65)	35 (19–63)	NS
Height, m, median (range)	1.55 (1.4–1.69)	1.53 (1.39–1.71)	NS
Weight, kg, median (range)	54 (28–83)	56 (34–87)	NS
Body mass index	22.5 (16.2–37.3)	23.9 (17.6–33.3)	NS
Tuberculin skin test positive > 12 mm	>12 mm	>12 mm	
Interferon gamma release assay	Positive	Positive	
Random blood glucose, mg/dL	211 (143–537)	100 (64–163)	$p < 0.0001$
Glycated hemoglobin level, %	9.5(7–13.3)	5.08 (4.93–5.74)	$p < 0.0001$
Total cholesterol level, mg/dL	214 (148–259)	186 (134–251)	$p = 0.0069$
Serum triglyceride level, mg/dL	166 (84–339)	101 (47–222)	$p = 0.0007$
High-density lipoprotein cholesterol level, mg/dL	41 (30–82)	48 (34–86)	$p = 0.0142$
Low-density lipoprotein cholesterol level, mg/dL	132 (48–185)	109 (65–153)	$p = 0.0017$

a) Values represent the geometric mean or median (and range).
b) p values were calculated using the Mann–Whitney U test.

critical role in protection, in both animal models and human infections [7, 8]. Thus, IL-12, IFN-γ, and TNF-α (along with IL-17 and IL-23) all play important roles in the induction and maintenance of protective immune responses against TB disease [9–14]. Further, multifunctional T cells, which are estimated by their capacity to express two or more cytokines, have also been associated with resistance to infection in animal models [15] and in some human studies [16, 17]. These multifunctional T cells are generally considered superior effector cells when compared to those that produce a single cytokine [18]. We and others have shown that coincident DM in pulmonary TB is characterized by heightened systemic levels of proinflammatory cytokines and enhanced antigen-induced CD4$^+$ Th1 and Th17 responses [19–21]. In contrast, we have also shown that coincident DM is associated with depressed systemic and antigen-induced Type 1, Type 17, and other proinflammatory cytokine responses in LTB [22].

To study influence of DM on CD4$^+$ T-cell responses in LTB, we examined baseline, antigen-induced and polyclonal induction of mono-, dual-, and multifunctional cells of the Th1, Th2, and Th17 subsets in LTB individuals with coincident DM (LTB-DM) and compared them to those without diabetes (LTB-NDM). We show that those with LTB-DM have diminished frequencies of Th1, Th2, and Th17 cells, a response partially mediated by IL-10 and TGF-β. Thus, our data demonstrate that diabetes profoundly alters the CD4$^+$ T-cell response to TB antigens and possibly contributes to increased susceptibility to active disease.

Results

Study population characteristics

The baseline characteristics including demographics, clinical, and biochemical features of the study population are shown in Table 1. Compared to subjects without diabetes (LTB-NDM), those with diabetes and LTB (LTB-DM) had higher fasting blood glucose, glycated hemoglobin, alanine amino transferase (ALT), serum cholesterol, LDL, and triglycerides levels but lower HDL cholesterol levels. The groups did not differ significantly in age, sex, or body mass index. Ex vivo frequencies of Treg cells were determined by flow cytometry staining for CD4, CD25, Foxp3, and CD127 (Supporting Information Fig. 1). As shown, the frequencies of Treg cells(CD4$^+$, CD25$^+$, Foxp3$^+$, CD127dim) was significantly higher in LTB-DM compared to LTB-NDM individuals.

Lower frequencies of antigen-induced mono- and multifunctional CD4$^+$ Th1 cells in LTB-DM

CD4$^+$ T cells play a key role in immune control of TB infection, and the frequency of multifunctional cytokine producing Ag-induced Th1 cells have been reported to be associated with control of infection [7, 8]. To determine the influence of DM on Th1 cells in LTB, we used multiparameter flow cytometry to define the frequencies CD4$^+$ T cells expressing IFN-γ, IL-2, and/or TNF-α at baseline and following stimulation with either mycobacterial antigens or PMA/Ionomycin (P/I). The gating strategy and a representative flow cytometry contour plot showing the baseline, early secreted antigen-6 (ESAT-6), and P/I stimulated Th1 cytokines are shown in Supporting Information Fig. 2. As shown in Fig. 1A, LTB-DM individuals exhibited significantly reduced frequencies of mono-functional Th1 (IFN-γ or IL-2 expressing) or dual-functional Th1 (IFN-γ/IL-2 or IFN-γ/TNF-α or IL-2/TNF-α coexpressing) cells at baseline. Similarly, in response to purified protein derivative (PPD) (Fig. 1B), ESAT-6 (Fig. 1C), and culture filtrate protein-10 (CFP-10) (Fig. 1D), LTB-DM individuals exhibited significantly decreased frequencies of mono- or dual-functional Th1 cells and in the case of ESAT-6 and CFP-10, multifunctional Th1 (IFN-γ/IL-2/TNF-α coexpressing) cells as well. In contrast, LTB-DM individuals did not exhibit any significant difference in the frequencies of

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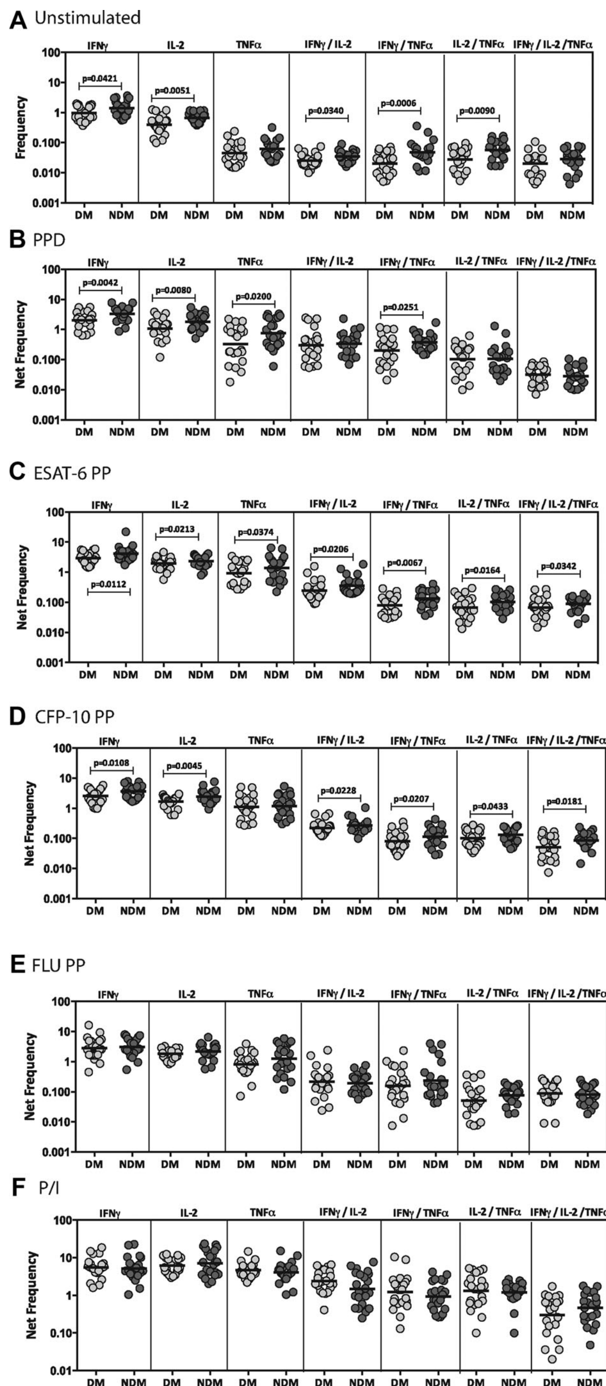

Figure 1. LTB-DM is associated with decreased spontaneously expressed and antigen-induced frequency of CD4$^+$ mono-, dual-, and/or multifunctional Th1 cells. Whole blood was cultured with media alone or mycobacterial or control antigens for 6 h and the baseline and antigen-stimulated frequencies of Th1 cells determined. The frequencies of mono-, dual-, and multifunctional CD4$^+$ Th1 cells in LTB-DM ($n = 22$) and LTB-NDM ($n = 22$) individuals (A) at baseline as well as in response to stimulation with (B) PPD, (C) ESAT-6 peptide pools, (D) CFP-10 peptide pools, (E) Flu peptide pools, and (F) PMA/Ionomycin was measured by flow cytometry. Each circle represents a single individual and the bars represent the geometric mean values. Net frequencies were calculated by subtracting baseline frequencies from the antigen-induced frequencies for each individual. p values were calculated using the Mann-Whitney test.

mono-, dual-, or multifunctional Th1 cells in response to control antigen (Flu peptides) (Fig. 1E) or P/I (Fig. 1F), indicating that the decreased frequency of Th1 cells present in LTB-DM individuals was relatively pathogen specific.

Lower frequencies of antigen-induced mono- and dual-functional Th2 cells in LTB-DM

The role of Th2 cells in immunity to TB infection is not clear, although Th2 cells are thought to promote susceptibility to infection and/or disease [23]. To determine the influence of DM on Th2 cells in LTB, we used multiparameter flow cytometry to define the frequencies CD4$^+$ T cells expressing IL-4, IL-5, and/or IL-13 at baseline and following stimulation with either mycobacterial antigens or P/I. A representative flow cytometry contour plot showing the baseline, ESAT-6, and P/I stimulated Th2 cytokines is shown in Supporting Infomation Fig. 2. As shown in Fig. 2A, LTB-DM individuals exhibited significantly reduced frequencies of IL-4 expressing CD4$^+$ T cells at baseline. In addition, in response to PPD (Fig. 2B), ESAT-6 (Fig. 2C), and CFP-10 (Fig. 2D), LTB-DM individuals exhibited significantly decreased frequencies of mainly monofunctional Th2 (IL-4 or IL-5 expressing) cells but some dual-functional Th2 (IL-4/IL-5 or IL-4/IL-13 coexpressing) cells as well. However, this response does not appear to be mycobacterial—antigen induced since LTB-DM individuals also exhibited significantly decreased frequencies of monofunctional Th2 cells in response to control antigen (Flu peptides) (Fig. 2E) or P/I (Fig. 2F).

Lower frequencies of antigen-induced mono- and dual-functional Th17 cells in LTB-DM

CD4$^+$ Th17 cells are thought to play an important role in memory responses to TB infection [24], but IL-17 is also thought to contribute to pathology [25]. To determine the influence of DM on Th17 cells in LTB, we used multiparameter flow cytometry to define the frequencies CD4$^+$ T cells expressing IL-17A, IL-17F, and/or IFN-γ at baseline and following stimulation with either mycobacterial antigens or P/I. A representative flow cytometry contour plot showing the baseline, ESAT-6 and PMA/Ionomycin stimulated Th17 cytokines is shown in Supporting Information Fig. 2. As shown in Fig. 3A, LTB-DM individuals exhibited significantly reduced frequencies of mono-functional Th17 (IL-17A or IL-17F expressing) or dual-functional Th1 (IFN-γ/IL-17F or IFN-γ/IL-17A or IL-17F/IL-17A coexpressing) cells at baseline. Similarly, in response to PPD (Fig. 3B), ESAT-6 (Fig. 3C), and CFP-10 (Fig. 3D), LTB-DM individuals exhibited significantly decreased frequencies of mono- or dual-functional Th17 cells. In contrast, LTB-DM individuals did not exhibit any significant difference in the frequencies of mono -, dual-, or multifunctional Th17 cells in response to control antigen (Flu peptides, with the exception of IFN-γ/IL-17A and IL-17F/IL-17A double expressors) (Fig. 3E) or

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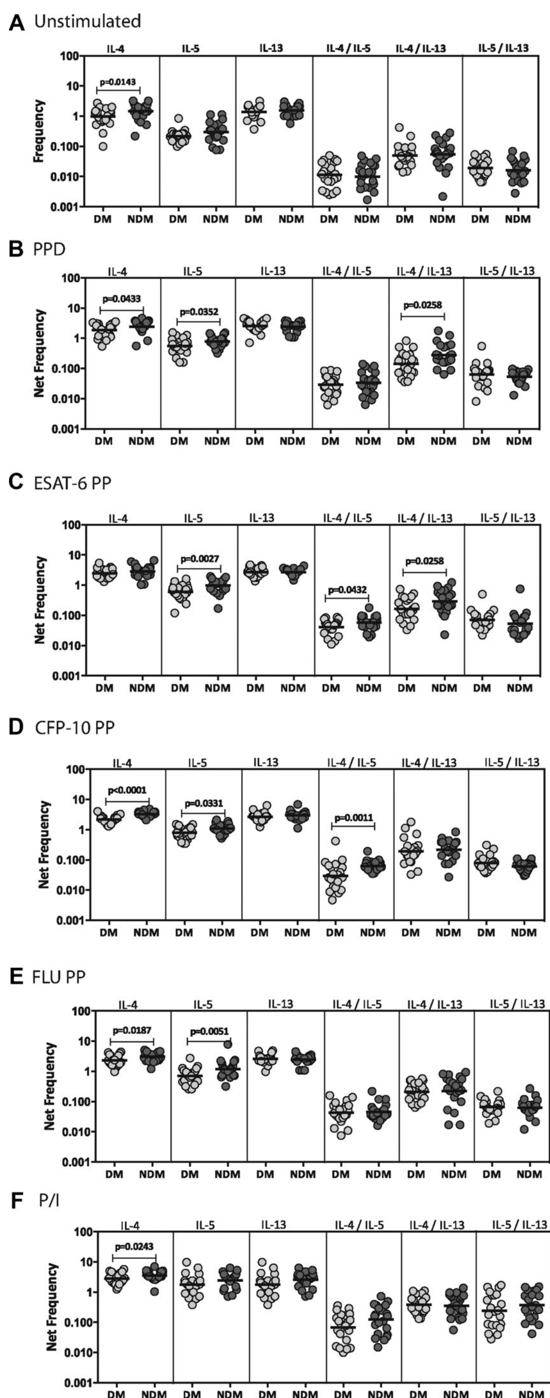

Figure 2. LTB-DM is associated with decreased spontaneously expressed and antigen-induced frequency of CD4$^+$ mono- and/or dual-functional Th2 cells. Whole blood was cultured with media alone or mycobacterial or control antigens for 6 h and the baseline and antigen-stimulated frequencies of Th2 cells determined. The frequencies of mono- and dual-functional CD4$^+$ Th2 cells in LTB-DM ($n = 22$) and LTB-NDM ($n = 22$) individuals at (A) baseline as well as in response to stimulation with (B) PPD, (C) ESAT-6 peptide pools, (D) CFP-10 peptide pools, (E) Flu peptide pools, and (F) PMA/Ionomycin was determined by flow cytometry. Each circle represents a single individual and the bars represent the geometric mean values. Net frequencies were calculated by subtracting baseline frequencies from the antigen-induced frequencies for each individual. *p* values were calculated using the Mann-Whitney test.

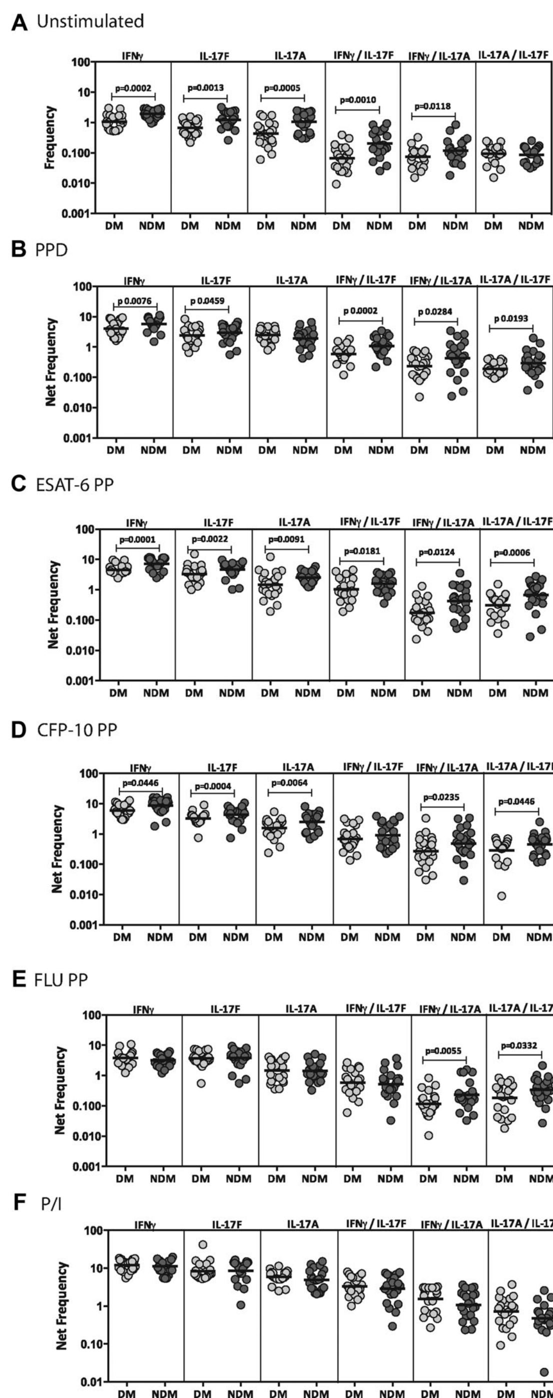

Figure 3. LTB-DM is associated with decreased spontaneously expressed and antigen-induced frequency of CD4$^+$ mono- and/or dual-functional Th17 cells. Whole blood was cultured with media alone or mycobacterial or control antigens for 6 h and the baseline and antigen-stimulated frequencies of Th17 cells determined. The frequencies of mono- and dual-functional CD4$^+$ Th17 cells in LTB-DM ($n = 22$) and LTB-NDM ($n = 22$) individuals (A) at baseline as well as in response to stimulation with (B) PPD, (C) ESAT-6 peptide pools, (D) CFP-10 peptide pools, (E) Flu peptide pools, and (F) PMA/Ionomycin was determined by flow cytometry. Each circle represents a single individual and the bars represent the geometric mean values. Net frequencies were calculated by subtracting baseline frequencies from the antigen-induced frequencies for each individual. *p* values were calculated using the Mann-Whitney test.

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P/I (Fig. 3F), indicating that the decreased frequency of Th17 cells present in LTB-DM individuals was relatively pathogen specific.

IL-10 modulates the frequencies of mono- and dual-functional Th1 and Th2 cells in LTB-DM

To determine the role of IL-10 in the modulation of Th1, Th2, and Th17 cells in LTB-DM, we measured the frequency of these $CD4^+$ T-cell subsets following stimulation with the PPD in the presence or absence of anti-IL-10 neutralizing antibody in LTB-DM ($n = 10$) and LTB ($n = 10$) individuals. As shown in Fig. 4A, IL-10 neutralization resulted in significantly increased frequencies of mono-functional (IFN-γ or IL-2 or TNF-α expressing) and dual-functional (IL-2/IFN-γ or IFN-γ/TNF-α coexpressing) Th1 cells. Similarly, as shown in Fig. 4B, IL-10 neutralization resulted in significantly increased frequencies of mono-functional (IL-4 or IL-5 or IL-13 expressing) Th2 cells. In contrast, as shown in Fig. 4C, IL-10 blockade did not result in any significant alteration in the frequencies of mono- or dual-functional Th17 cells. Finally, neutralization of IL-10 did not significantly alter the frequencies of PPD-specific Th1, Th2, or Th17 cells in LTB individuals (Fig. 4). To further confirm that IL-10 modulates cytokine expression on a per cell basis, we also measured the MFI of $CD4^+$ T cells expressing Th1, Th2, or Th17 cytokines. As shown in Supporting Information Fig. 3, IL-10 neutralization resulted in significantly increased MFI levels for Th1 and Th2 but not Th17 cytokines in LTB-DM individuals. Thus, IL-10 plays an important role in the modulation of Th1 and Th2 cells in LTB-DM.

TGF-β also modulates the frequencies of mono- and dual-functional Th2 cells in LTB-DM

To determine the role of TGF-β in the modulation of Th1, Th2, and Th17 cells in LTB-DM, we measured the frequency of these $CD4^+$ T-cell subsets following stimulation with the PPD in the presence or absence of anti-TGF-β neutralizing antibody in LTB-DM ($n = 10$) and LTB ($n = 10$) individuals. As shown in Fig. 5A, TGF-β neutralization resulted in significantly increased frequencies of mono-functional (IFN-γ or IL-2 or TNF-α expressing) and dual-functional (IL-2/IFN-γ or IFN-γ/TNF-α coexpressing) Th1 cells. Similarly, as shown in Fig. 5B, TGF-β neutralization resulted in significantly increased frequencies of mono-functional (IL-4 or IL-5 or IL-13 expressing) Th2 cells. In contrast, as shown in Fig. 5C, TGF-β blockade did not result in any significant alteration in the frequencies of mono- or dual-functional Th17 cells. Finally, neutralization of TGF-β did not significantly alter the frequencies of PPD-specific Th1, Th2, or Th17 cells in LTB individuals (Fig. 5). To further confirm that TGF-β modulates cytokine expression on a per cell basis, we also measured the MFI of $CD4^+$ T cells expressing Th1, Th2, or Th17 cytokines. As shown in Supporting Information Fig. 3, TGF-β neutralization resulted in significantly increased MFI levels for Th1 and Th2 but not Th17 cytokines in LTB-DM individuals. Thus, TGF-β plays an important role in the modulation of Th1 and Th2 cells in LTB-DM.

Frequency distribution of mono- and dual-functional Th1, Th2, and Th17 cells in LTB-DM

To determine the relative contributions of mono- or dual-functional cells to the composite Th1, Th2, and Th17 responses in LTB-DM and LTB-NDM individuals, we plotted the frequencies of each cell subset in response to different antigens. As shown in Supporting Information Fig. 4, the data are plotted as pie charts with each piece of the pie depicting the percentages of each subset (mono- or dual functional) in the total Th1, Th2, or Th17-cell population. Our data clearly reveal the changes in the frequencies of each major $CD4^+$ T-cell subset following stimulation with TB or non-TB antigens and P/I and also highlight the differences in the phenotype of these cells between DM and no diabetes mellitus (NDM).

Discussion

Susceptibility to developing active TB disease is influenced by many host factors, including coincidental infections and noncommunicable diseases [26]. HIV infection and DM are prime examples of coinfections/comorbidites that significantly impact immunity to TB [26]. DM increases the risk of active TB approximately threefold [3] and since there are currently 350 million estimated diabetes patients worldwide, the population attributable fraction of the TB burden due to DM is estimated to be as high as 15% [27]. In recent times, in developing countries such as India, the incidence of tuberculosis is maintained at a very high rate, while the prevalence of DM continues to increase alarmingly. In fact, a recent study examining the population of tuberculosis patients reporting to TB clinics in and around Chennai, has demonstrated that the prevalence of type 2 diabetes is approximately 25% and another 25% of these patients are prediabetic [28]. Despite this, very little is known about the effect of DM on the adaptive immune responses in individuals with latent infection. We have previously shown that LTB individuals with concomitant DM exhibit diminished circulating and TB-antigen induced levels of Type 1, Type 2, Type 17, and other proinflammatory cytokines [22], however, the role of T cells was not directly addressed in that study.

A deterministic factor in either control or spread of TB infection is the nature of the $CD4^+$ T-cell response that occurs following infection [8]. Thus, $CD4^+$ T cells typically producing more than one cytokine, especially of the Th1 type, are thought to be associated with a protective immune response and indeed, these multifunctional Th1 cells have been shown to be enhanced in latent infection compared to active disease [16, 17]. Moreover, these $CD4^+$ T cells have also been shown to be inversely associated with bacterial load, again implying a role for these cells as correlates of protective immunity [16]. However, not all studies are in agreement and the exact role of these cells remains

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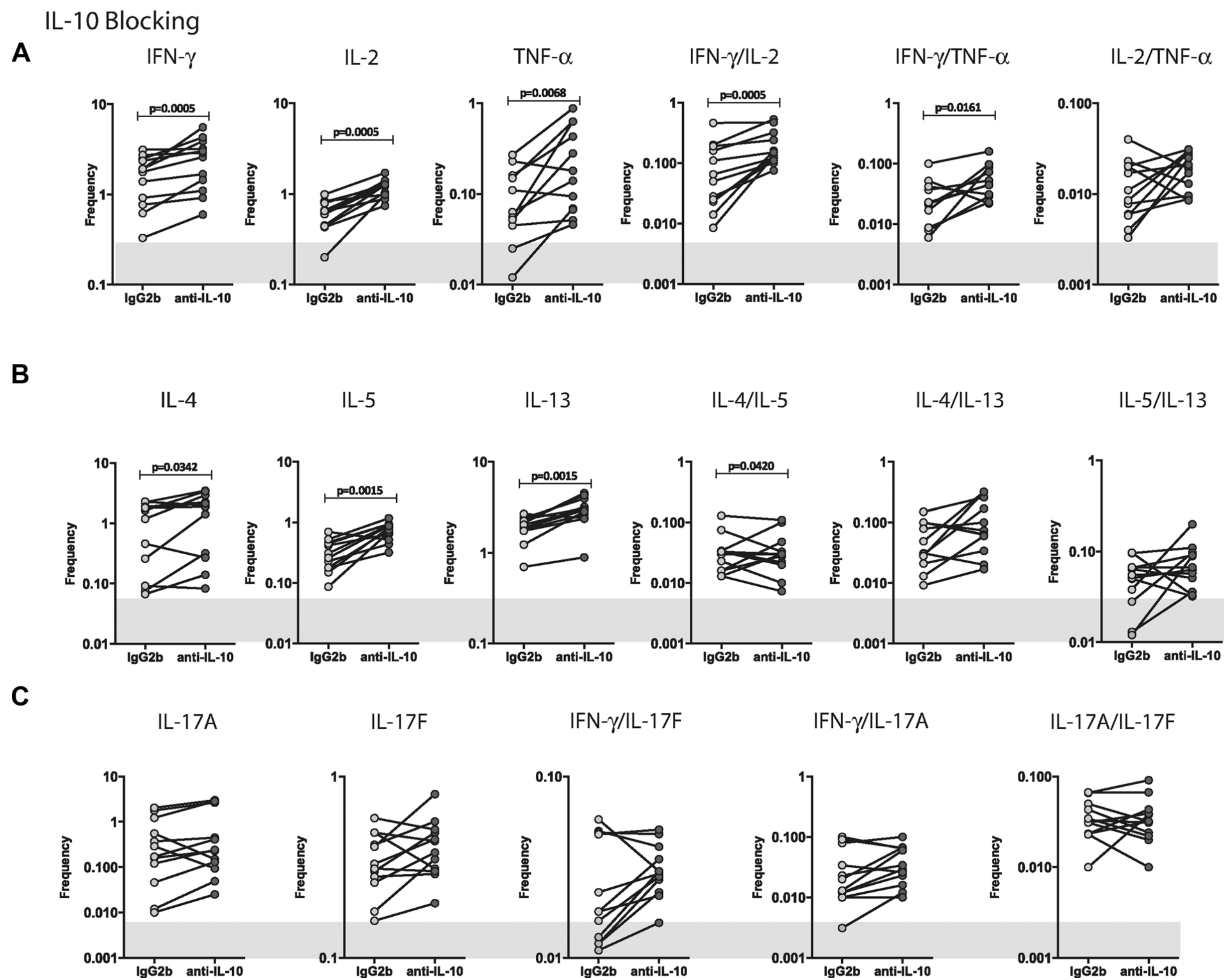

Figure 4. IL-10 regulates the frequency of mono- and dual-functional Th1 and Th2 cells in LTB-DM. (A) The frequency of mono-functional or dual-functional CD4$^+$ Th1 cells following stimulation with PPD and IL-10 neutralization (with anti-IL-10 antibody) in a subset of LTB-DM individuals (n = 10). (B) The frequency of mono-functional or dual-functional CD4$^+$ Th2 cells following IL-10 neutralization. (C) The frequency of mono-functional or dual-functional CD4$^+$ Th17 cells following IL-10 neutralization. Antigen-stimulated frequencies are shown as net frequencies with the baseline levels subtracted. Each line represents a single individual. The shaded portion represents the upper threshold for the frequencies in LTB-NDM individuals. p values were calculated using the Wilcoxon signed rank test.

to be proven in TB infection [29]. Nevertheless, it is generally well accepted that CD4$^+$ Th1 cells and to a lesser extent Th17 cells are important in protective immunity in TB [7, 8]. Since the immunological basis for the susceptibility to tuberculosis among those with DM is not well understood, we postulated that impaired CD4$^+$ T-cell responses in latent TB would be a major facilitator for the increased risk of active TB development. It is already known from studies examining the innate and adaptive immune response to microbial antigens in diabetic patients, that T-cell responses are compromised, particularly in patients with chronic hyperglycemia [30–32]. Whether such a scenario is applicable to TB infection is not well known. Indeed, mice with experimentally induced diabetes mellitus exhibit deficient priming of the adaptive immune response resulting in a higher bacterial burden in the lung [33]. Therefore, in this study, we sought to determine the functional phenotype of CD4$^+$ T-cell subsets in LTB individuals with coincident DM.

Our findings clearly reveal that baseline frequencies of mono- and dual-functional Th1 cells is decreased in DM individuals compared to NDM individuals with LTB. Secondly, this impairment is augmented upon stimulation with TB antigens, especially ESAT-6 and CFP-10 peptides, where upon the impairment extends to multifunctional, triple-cytokine expressing Th1 cells. Finally, the reduced frequencies of mono- and dual-functional Th1 cells in LTB-DM individuals is relatively pathogen-specific since the differences in the Th1 frequency profiles between the two groups of LTB are almost completely abolished when stimulation with a control antigen or polyclonal stimulus was used. Our study, therefore, confirms an important role for Th1 cells in the pathogenesis of TB disease in DM and suggests that diminished frequencies of Th1 cells might actually reflect enhanced susceptibility to TB disease, although this remains to be proven in longitudinal studies. Although the role of CD4$^+$ Th1 cells in mediating cellular immunity to TB is well defined, it is also clear that these responses

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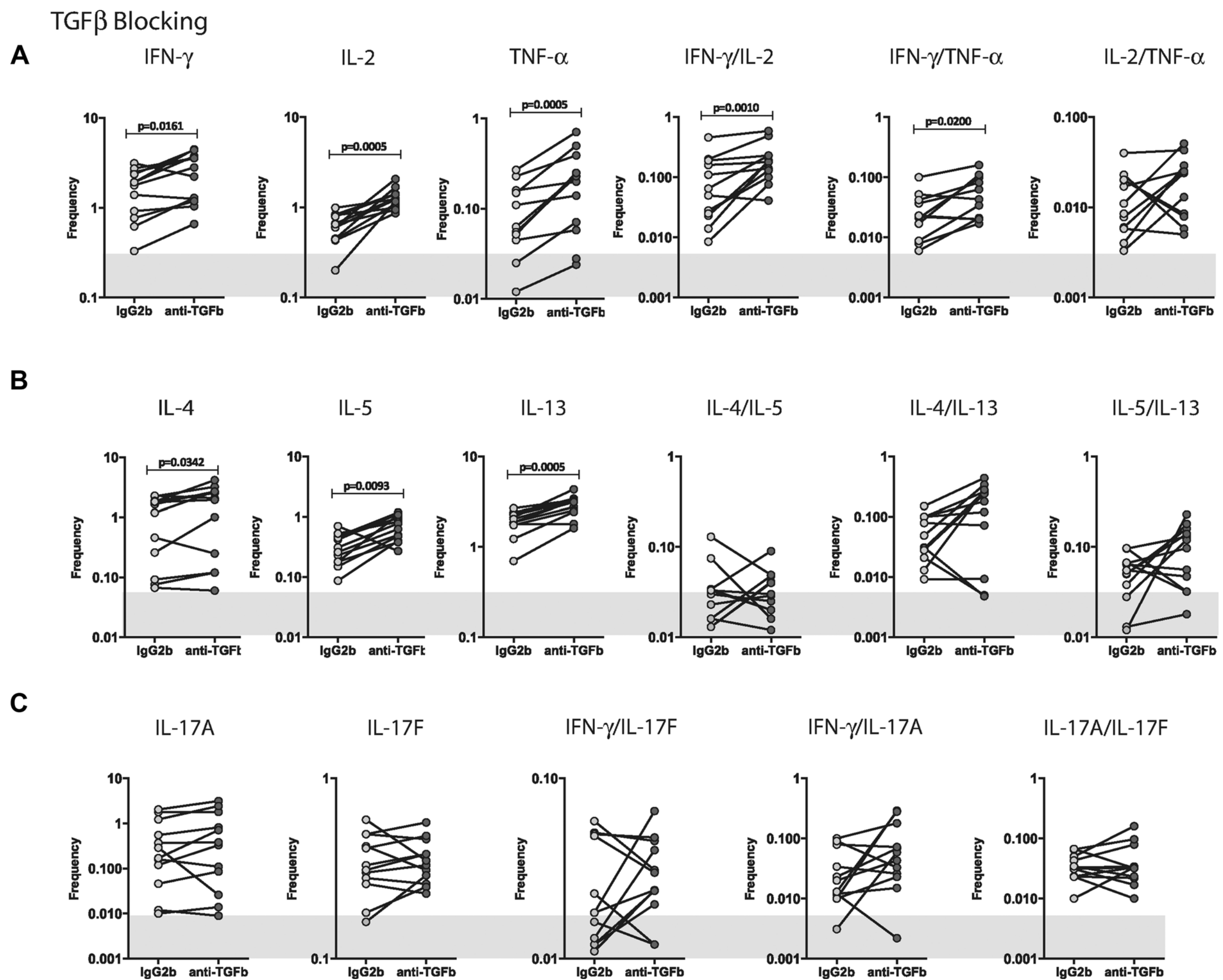

Figure 5. TGF-β regulates the frequency of mono- and dual-functional Th1 and Th2 cells in LTB-DM. (A) The frequency of mono-functional or dual-functional CD4+ Th1 cells following stimulation with PPD and TGF-β neutralization (with anti-TGF-β antibody) in a subset of LTB-DM individuals ($n = 10$). (B) The frequency of mono-functional or dual-functional CD4+ Th2 cells following TGF-β neutralization. (C) The frequency of mono-functional or dual-functional CD4+ Th17 cells following TGF-β neutralization. Antigen-stimulated frequencies are shown as net frequencies with the baseline levels subtracted. Each line represents a single individual. The shaded portion represents the upper threshold for the frequencies in LTB-NDM individuals. *p* values were calculated using the Wilcoxon signed rank test.

alone are not sufficient to confer protection [7]. Th17 cells, defined by the production of IL-17A and IL-17F, have been shown to be major players in protection against TB infection in animal models and also been found to be essential in protective immunity against hyper-virulent TB strains [34]. Our data on the examination of Th17 subsets reveals that mono- and dual-functional Th17 cells are also present at decreased frequencies in LTB-DM. Therefore, a diminished Th17 response, similar to the Th1 response, occurs in LTB individuals with diabetes and possibly contributes to increased risk of susceptibility as well. It would be interesting to investigate how a metabolic condition like DM could potentially influence antigen-induced responses to microbial antigens.

One potential mechanism for the decrease in baseline frequencies of Th1 and Th17 cells in LTB-DM individuals would be a concomitant increase in the baseline frequencies of Th2 cells, since Th2 cells are known to antagonize the differentiation of Th1 and Th17 cells [35]. However, our study reveals that the frequency of CD4+ T cells expressing Th2 cytokines is actually decreased in LTB-DM individuals, suggesting that DM is associated with a global diminution in the ex vivo and antigen-specific frequencies of most CD4+ T-cell subsets, irrespective of their cytokine status. Another important mechanism that could account for the diminished Th1, Th2, and Th17 response is the heightened frequencies of regulatory T cells in TB disease [36–39]. In this study, we show that Treg frequencies ex vivo are significantly higher in DM compared to NDM individuals and provide evidence for the role of two major mediators of effector Treg function. IL-10 and TGF-β are regulatory cytokines with a broad spectrum of activity, predominantly anti-inflammatory and suppressive and both cytokines are known to suppress the immune response in a variety of settings [40]. Previous studies (both human and animal) have clearly shown an important role for these cytokines in regulating T-cell responses in TB infection and disease [41]. In this study, we provide the first direct evidence for a role for both IL-10 and TGF-β in the down modulation of mycobacterial-antigen specific Th1 and Th2 responses in the setting of LTB with coincident DM. Interestingly,

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neither IL-10 nor TGF-β appear to have a significant role effect in reversing the downregulation of Th17 responses in the same setting. Thus, our data clearly reveal an important regulatory feature in LTB-DM, with IL-10 and TGF-β as key but not the only players, that results in a global impairment in the ability to mount adequately effective protective immune responses. This inability to mount effective $CD4^+$ T-cell responses could therefore potentially underlie the increased risk of susceptibility to TB disease in this instance, but could also potentially affect other bystander infection in the context of poorly controlled hyperglycemia and attendant metabolic abnormalities (dyslipidemia). In this study, we are unable to attribute the effect of diabetes on LTB to one particular metabolic abnormality but instead suggest that is the complex interplay of hyperglycemia and dyslipidemia, which is typically characteristic of diabetes, that is the major factor.

Our study thus provides important insights into the influence of Type 2 DM on the pathogenesis of TB infection and disease. Our data support evidence that $CD4^+$ T-cell responses are critical to protective immunity to TB disease and that deficiency in this arm of the immune system could predispose the development of active disease. Our study also provides an impetus to perform longitudinal studies in LTB-DM individuals to identify those at highest risk of disease and using these studies to characterize predictive immunological biomarkers of disease. Finally, our data support the prediction that diabetes could significantly impede the control measures to eliminate TB in the community to a significant degree and therefore provides a rationale for treating latent TB in the diabetic population in India.

Materials and methods

Study population

We studied a group of 44 individuals with LTB—22 with diabetes and 22 without. All individuals were screened as part of a natural history study protocol conducted in a rural population outside Chennai, South India. LTB was diagnosed on the basis of being positive for both the tuberculin skin test (>12 mm) and for Quantiferon TB Gold-in-tube assay (Qiagen) with absence of pulmonary symptoms and normal chest radiographs. They were not known contacts of active TB cases. DM was diagnosed on the basis of random blood glucose and glycated hemoglobin (HbA1c) levels, according to the American Diabetes Association criteria (random blood glucose > 200 mg/dL and HbA1c > 6.5%). All non-DM individuals has HbA1c levels <5.7% to exclude prediabetics. All the individuals were HIV negative. All individuals were anti-TB treatment naive. They were also all newly diagnosed diabetics not on treatment. Biochemical parameters, including plasma glucose, lipid profile, urea, creatinine, aspartate amino transferase, ALT, and HbA1c were obtained using standardized techniques. All individuals were examined as part of a natural history study approved by the Institutional Review Board of the National Institute of Research in Tuberculosis (NCT00375583), and informed written consent was obtained from all participants.

Antigens

TB antigens used were PPD (Serum Statens Institute), ESAT-6 peptide pools and CFP-10 peptide pools (both from BEI resources, NIAID, NIH). Influenza virus (Flu) peptide pools (BEI resources, NIAID, NIH) was used as a control antigen. Each peptide pool comprised of 10 overlapping peptides (15 amino acid long with 11 overlaps) and was used at 1 μg/mL of each peptide in the pool. Final concentrations were 10 μg/mL for PPD, ESAT-6, CFP-10, and Flu peptide pools. Phorbol myristoyl acetate (PMA) and ionomycin, at concentrations of 12.5 ng/mL and 125 ng/mL (respectively), were used as the positive control stimuli.

In vitro Culture

Whole blood cell cultures were performed to determine the intracellular levels of cytokines, as previously described [20]. Briefly, whole blood was diluted 1:1 with RPMI-1640 medium, supplemented with penicillin/streptomycin (100 U/100 mg/mL), L-glutamine (2 mM), and HEPES (10 mM) (all from Invitrogen) and distributed in 12-well tissue culture plates (Costar). The cultures were then stimulated with PPD, ESAT-6, CFP-10, Flu, or P/I or media alone in the presence of the costimulatory molecules, CD49d /CD28 at 37°C for 6 h. Brefeldin A (10μg/mL) was added after 2 h. After 6 h, centrifugation, washing, and red blood cell lysis was performed. The cells were fixed using cytofix/cytoperm buffer (BD Biosciences) and cryopreserved at –80°C. For cytokine neutralization experiments, whole blood from a subset of LTB-DM ($n = 10$) and LTB ($n = 10$) individuals was cultured in the presence of anti-IL-10 (5 μg/mL) or anti-TGF-β (5 μg/mL) or isotype control antibody (5 μg/mL) (R& D Sytems) for 1 h following which PPD and brefeldin A was added and cultured for a further 23 h.

Intracellular cytokine staining

The cells were thawed, washed, and then stained with surface antibodies for 30–60 min. Surface antibodies used were CD3, CD4, and CD8. The cells were washed and permeabilized with BD Perm/Wash buffer (BD Biosciences) and stained with intracellular cytokines for an additional 30 min before washing and acquisition. Cytokine antibodies used were IFN-γ, TNF-α, IL-2, IL-17F, IL-17A, IL-4, IL-5, and IL-13. The details of the antibodies used are shown in Table 2. Ex vivo frequencies of Tregs were determined using CD25, Foxp3, and CD127 staining. Eight-color flow cytometry was performed on a FACSCanto II flow cytometer with FACSDiva software v.6 (Becton Dickinson). The lymphocyte gating was set by forward and side scatter and 100 000 lymphocytes events were acquired. Data were collected and analyzed using Flow Jo software (TreeStar Inc). All data are depicted as frequency of $CD4^+$ T cells expressing cytokine(s) or as MFI of $CD4^+$ T cells expressing

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Table 2. Antibodies used in this study

No	Antibodies	Clone	Company
1	IFNγ PE	4S.B3 (RUO)	BD Pharmingen
2	IL-2 APC	MQ1-17H12	eBioscience
3	TNFα FITC	64D1-1111	BD Biosciences
4	IL-4 FITC	MP4-25D2 (RUO)	BD Biosciences
5	IL-5 APC	TRFK5 (RUO)	BD Pharmingen
6	IL-13 PE	JES10-5A2	BD Pharmingen
7	IL-17A FITC	CZ8-23G1	Miltenyi Biotech
8	IL-17F APC	197301	R & D systems
9	CD3 Amcyan	SK7	BD Biosciences
10	CD4 Pecy7	SK3	BD Pharmingen
11	CD8 APCH7	SK1	BD Biosciences
12	CD127 FITC	eBioRDRS	eBioscience
13	FoxP3 PE	236A/E7	eBioscience
14	CD25 APC	M-A251	BD

cytokine(s). Baseline values following media stimulation are depicted as baseline frequency while frequencies following stimulation with antigens are depicted as net frequencies (with baseline values subtracted). The T-cell assays are compliant with the MIATA guidelines.

Statistical analysis

Data analyses were performed using GraphPad PRISM (GraphPad Software, Inc.). Geometric means (GM) were used for measurements of central tendency. Statistically significant differences between two groups were analyzed using the nonparametric Mann–Whitney U test. Multiple comparisons were corrected using the Holm's correction. Statistically significant differences following cytokine blockade were analyzed by Wilcoxon signed rank test.

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Abbreviations: **ALT**: alanine amino transferase · **DM**: diabetes mellitus · **CFP-10**: culture filtrate protein-10 · **ESAT-6**: early secreted antigen-6 · **LTB**: latent tuberculosis · **NDM**: no diabetes mellitus · **PPD**: purified protein derivative · **P/I**: PMA/ionomycin · **TB**: tuberculosis

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