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Multifunctional T Cell Response to DosR and Rpf Antigens Is Associated with Protection in Long-Term *Mycobacterium tuberculosis*-Infected Individuals in Colombia

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Multifunctional T cells have been shown to be protective in chronic viral infections. In mycobacterial infections, however, evidence for a protective role of multifunctional T cells remains inconclusive. Short-term cultures of peripheral blood mononuclear cells stimulated with the *Mycobacterium tuberculosis* RD1 antigens 6-kDa early secretory antigenic target (ESAT6) and 10-kDa culture filtrate antigen (CFP10), which are induced in the early infection phase, have been mainly used to assess T cell multifunctionality, although long-term culture assays have been proposed to be more sensitive than short-term assays for assessment of memory T cells, which are essential for long-term immunity. Here we used a long-term culture assay system to study the T cell immune responses to the *M. tuberculosis* latency-associated DosR antigens and reactivation-associated Rpf antigens, compared to ESAT6 and CFP10, in patients with pulmonary tuberculosis (PTB) and household contacts of PTB patients with long-term latent tuberculosis infection (ltLTBI), in a community in which *M. tuberculosis* is endemic. Our results showed that the DosR antigens Rv1737c (*narK2*) and Rv2029c (*pfkB*) and the Rv2389c (*rpfD*) antigen of *M. tuberculosis* induced higher frequencies of $CD4^+$ or $CD8^+$ mono- or bifunctional (but not multifunctional) T cells producing interferon gamma (IFN-γ) and/or tumor necrosis alpha (TNF-α) in ltLTBI, compared to PTB. Moreover, the frequencies of $CD4^+$ and/or $CD8^+$ T cells with a $CD45RO^+$ $CD27^+$ phenotype were higher in ltLTBI than in PTB. Thus, the immune responses to selected DosR and Rpf antigens may be associated with long-term latency, correlating with protection from *M. tuberculosis* reactivation in ltLTBI. Further study of the functional and memory phenotypes may contribute to further discrimination between the different states of *M. tuberculosis* infections.

Upon *Mycobacterium tuberculosis* infection, T cell populations (including Th1, Th2, Th17, and T regulatory cells) are induced that display both proinflammatory and anti-inflammatory responses, which are finely coordinated by secreted cytokines. Among the cytokines, interferon gamma (IFN-γ), tumor necrosis alpha (TNF-α), and interleukin 2 (IL-2) are considered major players in the Th1 response. Targeting of the *ifng* gene in mice, leading to deficient IFN-γ production, resulted in increased susceptibility to *M. tuberculosis* infection, identifying this cytokine as critical for host defense (1, 2). Regulated levels of IFN-γ are also important for the control of mycobacterial infections in humans. Mutations in the *IFNGR* genes or in genes that control IFN-γ production or signal transduction, such as *IL12B*, *IL12RB1*, *IFNGR1*, *IFNGR2*, *STAT1*, *ISG15*, *IRF8*, *NEMO*, and *CYBB*, have been all associated with susceptibility to mycobacterial infections (3, 4). In addition to IFN-γ, TNF-α plays a significant role in the promotion of monocyte and macrophage effector mechanisms and the maintenance of granuloma integrity (5, 6), contributing to infection control. The increased incidence of tuberculosis (TB) in autoimmune disease patients treated with anti-TNF-α antibodies (7) underscores the importance of this cytokine. Finally, IL-2 is essential for T cell differentiation and survival, the maintenance of effector functions, cell renewal, and T cell memory (8). Low levels of IL-2 have been observed in patients with active TB, compared to healthy controls (9, 10), and restoration of normal levels upon anti-TB treatment has been reported (11).

T cells capable of simultaneously producing two (bifunctional) or three (multifunctional) cytokines have been described in recent years. In chronic viral infections such as HIV and hepatitis C virus (HCV) infections, such multifunctional T cells have been associated with protective immune responses (12, 13). These data were extended to murine models of leishmaniasis (14), suggesting that similar immune responses might be important in protection against mycobacterial infections. To date, however, results have been inconclusive. A higher frequency of multifunctional $CD4^+$ T cells, producing IFN-γ, TNF-α, and IL-2, in peripheral blood mononuclear cells (PBMCs) from patients with active pulmonary tuberculosis (PTB), compared to individuals with latent TB infection (LTBI), was reported (15–17) and decreased following anti-TB treatment (16, 17). However, others observed a lower frequency of multifunctional T cells in PTB, compared to LTBI (18–20), which increased after anti-TB treatment (18, 19). Furthermore, an increase in the frequency of bifunctional $CD4^+$ T cells producing IFN-γ and IL-2 was reported for PTB patients

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after anti-TB therapy (11). Thus, a consensus on this important issue of the immune response to *M. tuberculosis* infection in humans has not been reached to date.

Immunological memory is the hallmark of the specific recall response (21, 22). Central memory T (T_{CM}) cells are characterized by a long life and high proliferative potential upon antigen reencounter (22) and may originate from effector T (T_{EFF}) cells (22). On the other hand, effector memory T (T_{EM}) cells can rapidly proliferate upon antigen reencounter and produce effector cytokines (22). Different studies have shown that individuals with LTBI display greater frequencies of T_{CM} cells upon *in vitro* stimulation with RD1 antigens and purified protein derivative (PPD), compared to PTB patients, in long-term culture assays (23–25). In contrast, PTB patients display greater frequencies of T_{EM} or T_{EFF} cells (26–28). Thus, phenotypic and functional studies of T cell responses to particular mycobacterial antigens may help to define correlates of protection (29, 30). Most of the studies concerning *ex vivo* analysis of multifunctional T cell responses and T cell memory phenotypes have used the RD1 antigens 6-kDa early secretory antigenic target (ESAT6) and 10-kDa culture filtrate antigen (CFP10), while little research has been performed with antigens that are expressed at high levels during *M. tuberculosis* latency and reactivation.

Our laboratory and others have provided evidence showing that proteins encoded by the DosR regulon are preferentially recognized in LTBI (31–36). This regulon encodes 48 proteins and is expressed *in vitro* and *in vivo* under conditions that may exist in the lung granulomas of infected individuals, such as acidic pH, nutrient starvation, and hypoxia, among other conditions (37–39). Also, individuals with LTBI, compared to PTB patients, preferentially recognize resuscitation-promoting factors (Rpfs) (34, 36, 40–42), proteins known to participate in bacterial reactivation from a quiescent state and to be present in *M. tuberculosis* (43, 44).

In this study, we characterized the functions and phenotypes of $CD4^+$ and $CD8^+$ multifunctional T cells in response to PPD, the RD1 antigen ESAT6-CFP10 fusion protein, the DosR regulon-encoded antigens Rv1737c (*narK2*), Rv2029c (*pfkB*), and Rv2628, and the Rpf antigens Rv0867c (*rpfA*) and Rv2389c (*rpfD*) in patients with PTB and individuals with LTBI who had remained healthy for a long time (5 to 7 years) after initial exposure to the index case (i.e., long-term LTBI [ltLTBI]), in a community in the city of Medellín, Colombia, in which *M. tuberculosis* is endemic. Our results showed that NarK2, PfkB, and RpfD antigens induced higher frequencies of $CD4^+$ or $CD8^+$ mono- or bifunctional T cells producing IFN-γ and/or TNF-α in ltLTBI, compared to PTB, confirming and significantly extending previous results obtained with other populations. The frequencies of $CD4^+$ and/or $CD8^+$ T cells with a T_{CM} phenotype were also higher in ltLTBI, compared to PTB. A possible interpretation of these results is that the immune responses to the selected DosR and Rpf *M. tuberculosis* antigens are associated with latency maintenance and protection against *M. tuberculosis* reactivation in LTBI. Thus, the study of functional profiles and memory phenotypes may contribute to discriminating between different states of TB disease and identifying potential correlates of natural protection.

MATERIALS AND METHODS

Study population. This study focused on a previously characterized community in the city of Medellín, Colombia, in which the disease is endemic, with a high incidence of *M. tuberculosis* infections (exceeding 70 cases per 100,000 population) (45, 46). In a previous study, 2,060 household contacts (HHCs) of 433 TB index cases were monitored for 3 years (2005 to 2008). According to IFN-γ levels in supernatants of long-term whole-blood cultures, HHC positive rates were 90.1%, 79.4%, 37.8%, and 31.6% for a culture filtrate protein preparation (CFP), CFP10, HspX, and antigen 85A, respectively (45). In the present study, 22 HHCs of recently diagnosed patients with PTB and 20 PTB patients from this community were included. An HHC (>18 years of age) was considered to be someone who had spent time regularly (weekly) in the same household as the index case for at least 1 month before the time at which the diagnosis for the index case was confirmed (45). The HHC group of individuals was initially selected from a previous cohort (45), based on having a positive IFN-γ response (≥22 pg/ml) to the specific *M. tuberculosis* antigen CFP10 at the initiation of the cohort study, remaining healthy for a long time (5 to 7 years) after initial exposure to the index case (i.e., ltLTBI), and continuing to live in the same area with endemic disease as at the time of original contact. All of the HHCs were healthy at the time of blood sample collection, and all were negative for HIV. In this study, the HHCs did not receive anti-TB treatment (according to the regulations of the Colombian Ministry of Health). PTB patients had received a recent diagnosis of PTB, which was confirmed microbiologically or by culture, with no more than 2 weeks of antibiotic treatment. *Mycobacterium bovis* BCG vaccination status was determined according to the presence or absence of the typical scar.

Ethical clearance. Blood samples were collected only after written informed consent was obtained. Study protocols were approved by the Ethics Committee of the Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia (Medellín, Colombia).

Reagents. RPMI 1640 medium and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Gibco (Grand Island, NY); Ficoll-Hypaque and penicillin-streptomycin solution from Bio-Whittaker (Walkersville, MD); dimethyl sulfoxide (DMSO), brefeldin A (BFA), bovine serum albumin (BSA), and sodium azide from Sigma-Aldrich (St. Louis, MO); pooled human serum (PHS) from Invitrogen (Eugene, OR); paraformaldehyde (PFA) from Mallinckrodt Baker (Phillipsburg, NJ); and Tween 20 from Promega (Madison, WI). The antibodies anti-CD4-phycoerythrin (PE)-Cy7 (clone OKT4), anti-CD27-peridinin chlorophyll (PerCp)-Cy5.5 (clone O323), anti-CD45RO-Pacific blue (clone UCHL1), anti-IFN-γ-PE (clone B27), anti-TNF-α-allophycocyanin (APC) (clone Mab11), and anti-IL-2 (clone MQ1-17H12) were obtained from BioLegend (San Diego, CA), and the antibody anti-CD8-APC-H7 (clone SK1) was obtained from Becton Dickinson (San Diego, CA).

Mycobacterial antigens. The RD1 ESAT6-CFP10 (E6-C10) fusion protein, DosR (Rv1737c, Rv2029c, and Rv2628) and Rpf (Rv0867c and Rv2389c) antigens used throughout this study were described previously (34, 47). Additionally, PPD (RT50) from Staten Serum Institute (Copenhagen, Denmark) was included in this study. The concentrations of the DosR, Rpf, and RD1 antigens tested were the same as those reported previously (34).

Isolation of PBMCs and culture conditions. PBMCs were collected from sodium heparin-anticoagulated venous blood (10 ml) and were separated by Ficoll-Hypaque density gradient centrifugation. PBMCs were washed twice in DPBS and counted in a hemocytometer, and cell viability was determined by trypan blue exclusion (>94% for all experiments). The cell culture protocol was described previously (34, 36). In summary, 1.5×10^5 cells/well were seeded in triplicate in 96-well U-bottom plates (Corning Costar Inc., Corning, NY), in a final volume of 200 μl/well of RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% PHS. Cells were cultured in the presence or absence of 5 μg/ml (final concentration) of PPD, the fusion protein ESAT6-CFP10, or the selected DosR and Rpf antigens. Cell cultures were incubated for 168 h (7 days) at 37°C in 5% CO_2 and 90% relative humidity.

Flow cytometric data analysis. To determine T cell phenotypes and cytokine production, cells were treated with 10 μg/ml of BFA 4 h before the end of culture, collected in polystyrene tubes, washed with DPBS,

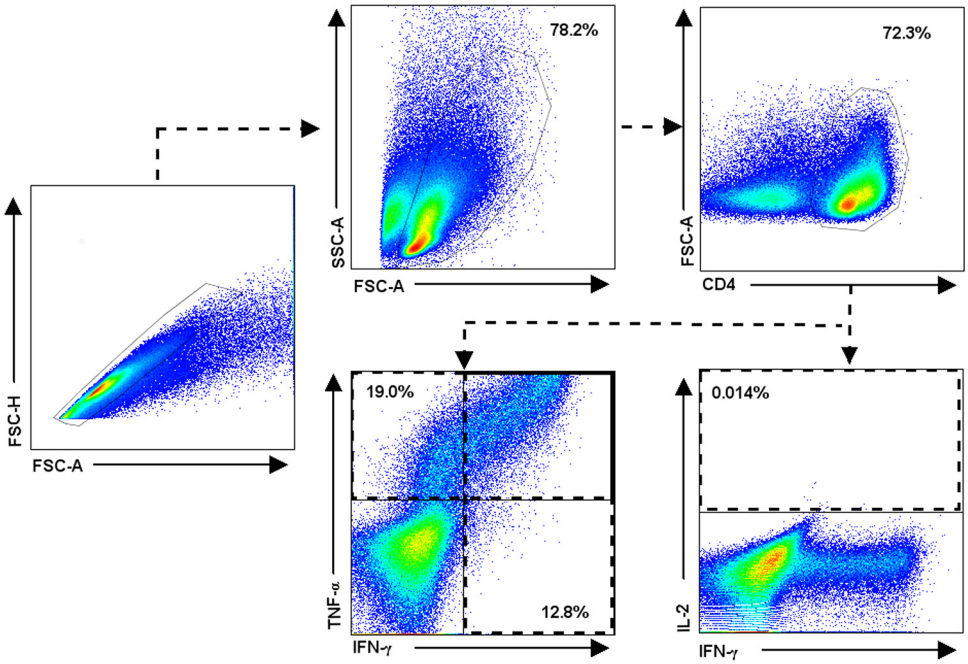

FIG 1 Representative flow cytometric analysis showing the gating strategy for identification of monofunctional and multifunctional $CD4^+$ and $CD8^+$ T cells. A total of 1.5×10^5 PBMCs were cultured in triplicate for 7 days in the presence or absence of PPD, E6-C10, and DosR and Rpf antigens. At the end of the cultures, the cells were stained with anti-CD4-PE-Cy7, anti-CD8-APC-H7, anti-CD45RO-Pacific blue, and anti-CD27-PerCp-Cy5.5, fixed, permeabilized, and stained with anti-IFN-γ-PE, anti-TNF-α-APC, and anti-IL-2-FITC. A representative experiment with a sample from a latently infected individual and PPD stimulation is shown. Analyses were similar for $CD4^+$ and $CD8^+$ T cells. Briefly, after exclusion of doublets, $CD4^+$ and $CD8^+$ T cells were gated versus FSC-A and analyzed for intracellular IFN-γ, TNF-α, and IL-2. Analysis was performed with FlowJo v7.6.1, using the combination gate tool in order to obtain the frequencies of single- and multiple-cytokine-producing cells.

incubated with blocking buffer (2% PHS, 0.05% NaN_3) for 20 min at 4°C, and then stained with anti-CD4-PE-Cy7, anti-CD8-APC-H7, anti-CD27-PerCp-Cy5.5, and anti-CD45RO-Pacific blue. The cells were then fixed, permeabilized, and stained with anti-IFN-γ-PE, anti-TNF-α-APC, and anti-IL-2-fluorescein isothiocyanate (FITC), using a commercial kit from eBioscience, following the manufacturer's recommendations. Data for 200,000 cells were acquired with a FACSCanto II flow cytometer (Becton Dickinson). The voltages were fixed using unstained cells within the lymphocyte gate, and then a compensation matrix was calculated using CompBeads (BD Biosciences). The compensation was verified using single staining of cells and checking that fluorescence from the singly stained samples did not overlap on any other channel.

Data analysis was carried out using the FlowJo v7.6.1 (Tree Star Inc., Ashland, OR) software package. The numbers of negative and positive cells producing cytokines were determined by the fluorescence minus one (FMO) method (see Fig. S1 and S2 in the supplemental material). Dead cells were determined by staining with 7-aminoactinomycin D (7-AAD) (Thermo Fisher Scientific, Carlsbad, CA) and were excluded from the analysis. The percentage of viable cells at the end of the culture period was >80% for all experiments. Data analysis was performed as described previously (48). Briefly, doublets were excluded by using a forward scatter A (FSC-A)/forward scatter H (FSC-H) dot plot, followed by the selection of lymphocytes in an FSC-A/side scatter A (SSC-A) dot plot, and then $CD4^+$ or $CD8^+$ T cells were plotted versus FSC-A. Gated $CD4^+$ and $CD8^+$ T cells were evaluated for the frequency of IFN-γ-, TNF-α-, or IL-2-producing cells (Fig. 1). The frequencies of monofunctional, bifunctional, and multifunctional $CD4^+$ and $CD8^+$ T cells were evaluated using the combination gate tool in FlowJo v7.6.1. To identify the phenotypes of cytokine-producing cells, IFN-γ- and/or TNF-α-producing gated $CD4^+$ and $CD8^+$ T cells were evaluated for the expression of CD27 and CD45RO. Based on the presence of T cell surface markers, including CD45RO and the TNF receptor family member CD27 costimulatory molecule, T memory cells have been classified as central memory ($CD45RO^+$ $CD27^+$) and effector memory ($CD45RO^+$ $CD27^-$) cells (25, 34, 36, 49–51). In this context, early/naive T ($T_{E/N}$) cells display a $CD45RO^-$ $CD27^+$ phenotype, while effector T (T_{EFF}) cells display none of these markers ($CD45RO^-$ $CD27^-$).

Statistical analysis. A chi-square test was used to test for differences in gender and the presence or absence of a BCG scar. The frequencies of the different combinations of IFN-γ-, IL-2-, and TNF-α-positive cells following antigenic stimulation were calculated within the total population of $CD4^+$ and $CD8^+$ T cells. Net values were obtained by subtracting the background values (nonstimulated cells). Data normality was tested with the Shapiro-Wilk normality test. The significance of median differences in the frequencies of single- or multiple-producer cells and the memory phenotype between individuals with ltLTBI and PTB patients was determined with the nonparametric Mann-Whitney *U* test. All statistical analyses were performed using GraphPad Prism v6.0 (GraphPad Software, San Diego, CA). Statistical differences were considered significant for *P* values of ≤0.05.

RESULTS

Study population. This study focused on a previously characterized community in the city of Medellín, Colombia, in which TB is endemic, with a high prevalence of *M. tuberculosis* infections (45). HHCs for whom peripheral blood cultures stimulated with the RD1 antigen CFP10 produced ≥22 pg/ml IFN-γ in a 7-day culture assay were considered infected (79.4%) (45). In the present study, we included 22 previously identified HHCs who had remained healthy for at least 5 years, with no clinical signs of active TB, and were negative for HIV (individuals with ltLTBI) and 20 patients with confirmed (sputum and/or culture) PTB from the same community. The median age was 37 years (range, 18 to 65 years) in the

TABLE 1 Characteristics of the study population

Characteristic	ltLTBI ($n = 22$)	PTB ($n = 20$)
Age (median [range]) (yr)	37 (18–65)	29 (19–58)
Male/female (%)	54/46	60/40
BCG scar positive (%)	82	95

ltLTBI group; 54% of the subjects were male, and 82% had been vaccinated with *M. bovis* BCG. The PTB group showed a median age of 29 years (range, 19 to 58 years), 60% of the subjects were male, and 95% had been vaccinated with BCG. No significant differences in age and BCG vaccination rates were found between the ltLTBI and PTB groups (Table 1).

Multifunctional CD4$^+$ and CD8$^+$ T cell responses to DosR and Rpf antigens in ltLTBI and PTB. Most studies evaluating T cell responses to *M. tuberculosis* DosR and Rpf antigens in LTBI and PTB have been based on IFN-γ detection (31, 32, 34, 35, 40, 52–54). Here, we used a 7-day stimulation assay to optimize sensitivity to detect latent infection, since short-term cultures (25, 55–57) are less sensitive in settings of high levels of endemicity, where mixtures of recent and old infections are commonly found (55).

Figure 2 and Table 2 show the responses of CD4$^+$ T cells to E6-C10 and DosR and Rpf antigens. Individuals with ltLTBI displayed higher frequencies of monofunctional TNF-α$^+$ CD4$^+$ T cells ($P < 0.05$) and bifunctional IFN-γ$^+$ TNF-α$^+$ CD4$^+$ T cells ($P < 0.05$), compared to PTB patients; similar differences were observed for Rv2029c (PfkB) ($P < 0.01$ and $P < 0.05$, respectively). No significant differences in the frequencies of mono- and bifunctional CD4$^+$ T cells in the ltLTBI and PTB groups in response to Rv1737c (Nark2) and Rv2628 were observed, although there was a trend for increasing frequencies in the ltLTBI group, compared to the PTB group. The frequency of monofunctional TNF-α$^+$ CD4$^+$ T cells in response to the Rpf antigen Rv2389c ($P < 0.05$), but not Rv0867c, was higher for individuals with ltLTBI (Fig. 2). In response to PPD, individuals with ltLTBI displayed higher frequencies of monofunctional CD4$^+$ TNF-α$^+$ ($P < 0.05$) and CD4$^+$ IFN-γ$^+$ ($P < 0.05$) T

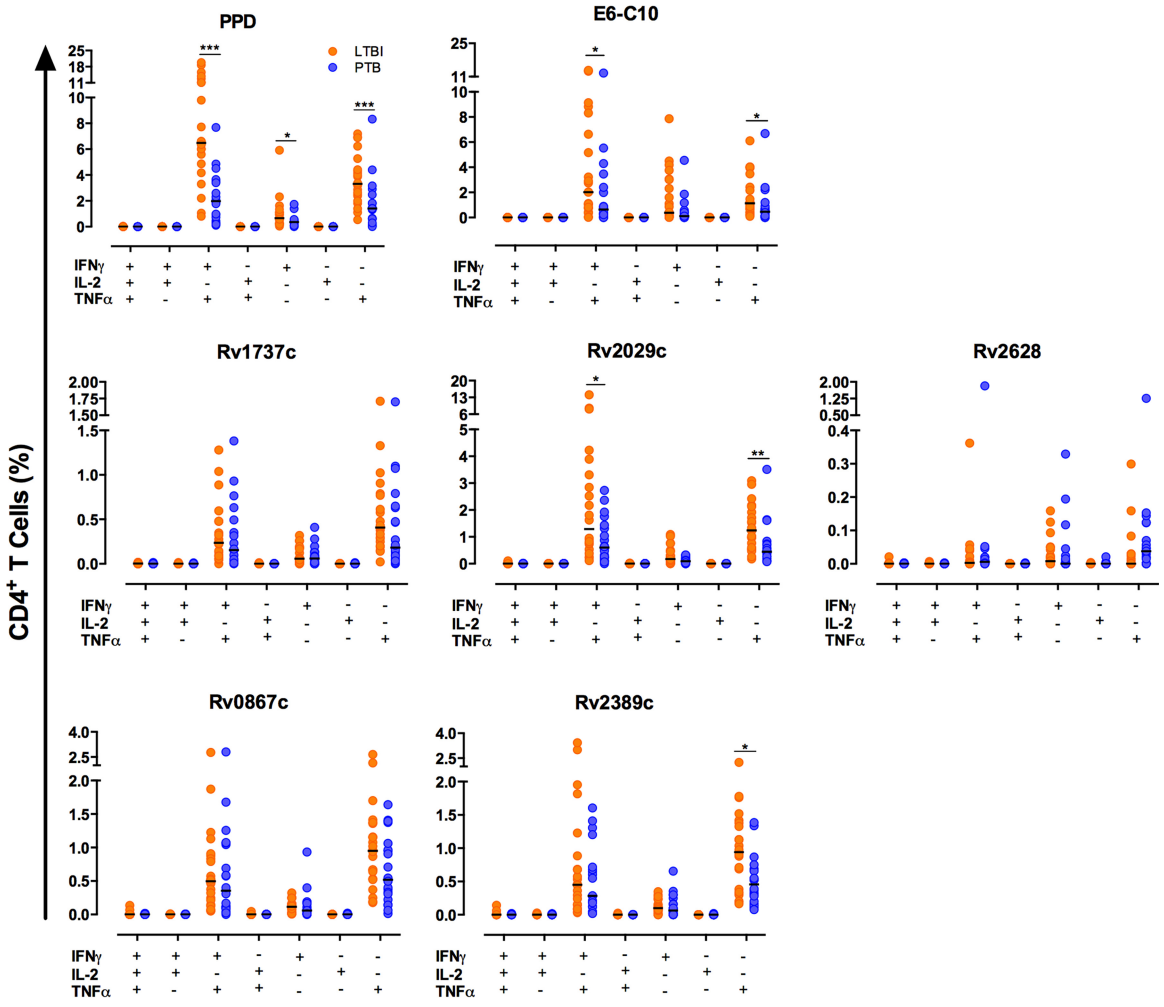

FIG 2 Frequencies of single- and multiple-cytokine-producing CD4$^+$ T cells in ltLTBI and PTB. A total of 1.5×10^5 PBMCs from individuals with ltLTBI or PTB were cultured in triplicate for 7 days in the presence or absence of PPD, E6-C10, and DosR and Rpf antigens. CD4$^+$ T cells were stained as described in Materials and Methods and then analyzed for intracellular production of IFN-γ, TNF-α, and IL-2. The frequencies of single- and multiple-cytokine-producing CD4$^+$ T cells were evaluated using the combination gate tool from FlowJo v7.6.1. Statistical differences between the groups were calculated with the Mann-Whitney *U* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 2 Frequencies of monofunctional (IFN-γ^+ or TNF-α^+), bifunctional (IFN-γ^+ TNF-α^+), and multifunctional (IFN-γ^+ TNF-α^+ IL-2^+) $CD4^+$ T cells in ltLTBI and PTB

	Frequency (median [interquartile range]) (%)							
	$CD4^+$ IFN-γ^+ T cells		$CD4^+$ TNF-α^+ T cells		$CD4^+$ IFN-γ^+ TNF-α^+ T cells		$CD4^+$ IFN-γ^+ TNF-α^+ IL-2^+ T cells	
Antigen	PTB	LTBI	PTB	LTBI	PTB	LTBI	PTB	LTBI
PPD	0.36 (0.09–0.40)	0.66 (0.25–1.13)[a]	1.41 (0.61–2.32)	3.32 (2.29–4.63)[b]	1.98 (0.52–3.61)	6.49 (4.69–11.59)[b]	0.00 (0.00–0.01)	0.00 (0.00–0.01)
RD1 antigens								
E6-C10	0.11 (0.03–0.46)	0.38 (0.08–3.03)	0.46 (0.13–0.85)	1.14 (0.60–2.69)[a]	0.63 (0.24–2.30)	2.02 (0.77–7.05)[a]	0.00 (0.00–0.00)	0.00 (0.00–0.00)
DosR antigens								
Rv1737c	0.06 (0.0–0.12)	0.06 (0.02–0.18)	0.18 (0.06–0.64)	0.41 (0.24–0.77)	0.15 (0.03–0.46)	0.24 (0.09–0.48)	0.00 (0.00–0.00)	0.00 (0.00–0.00)
Rv2029c	0.09 (0.05–0.18)	0.17 (0.07–0.46)	0.44 (0.18–0.73)	1.24 (0.59–1.97)[c]	0.60 (0.22–1.41)	1.29 (0.68–3.46)[a]	0.00 (0.00–0.00)	0.00 (0.00–0.00)
Rv2628	0.0 (0.0–0.02)	0.01 (0.0–0.04)	0.04 (0.01–0.11)	0.0 (0.0–0.03)	0.01 (0.00–0.02)	0.00 (0.0–0.01)	0.00 (0.00–0.00)	0.00 (0.00–0.00)
Rpf antigens								
Rv0867c	0.06 (0.01–0.16)	0.11 (0.02–0.16)	0.52 (0.24–1.04)	0.95 (0.53–1.36)	0.35 (0.10–1.06)	0.50 (0.20–0.88)	0.00 (0.00–0.00)	0.00 (0.00–0.01)
Rv2389c	0.06 (0.00–0.23)	0.10 (0.06–0.18)	0.46 (0.22–0.68)	0.94 (0.34–1.39)[a]	0.28 (0.12–0.71)	0.45 (0.13–0.97)	0.00 (0.00–0.00)	0.00 (0.00–0.00)

[a] $P < 0.05$.
[b] $P < 0.001$.
[c] $P < 0.01$.

cells and bifunctional IFN-γ^+ TNF-α^+ $CD4^+$ T cells ($P < 0.001$), compared to PTB patients (Fig. 2).

Figure 3 and Table 3 show the responses of $CD8^+$ T cells. In this case, individuals with ltLTBI displayed higher frequencies of monofunctional IFN-γ^+ T cells in response to Rv1737c and Rv2029c ($P < 0.05$), compared to patients with PTB. In addition, individuals with ltLTBI displayed higher frequencies of monofunctional TNF-α^+ T cells ($P < 0.05$) and bifunctional IFN-γ^+ TNF-α^+ T cells ($P < 0.001$) in response to Rv2029c, compared to PTB patients. The frequency of monofunctional TNF-α^+ $CD8^+$ T cells in response to the Rpf antigen Rv2389c was also higher in the ltLTBI group ($P < 0.01$). In response to PPD, individuals with ltLTBI displayed higher frequencies of monofunctional TNF-α^+ T cells ($P < 0.05$) and bifunctional IFN-γ^+ TNF-α^+ T cells ($P < 0.001$), compared to PTB patients. No significant differences in the frequencies of mono- or bifunctional T cells in response to E6-C10, the DosR antigen Rv2628, or the Rpf antigen RpfA (Rv0867c) were observed, although similar trends could be observed.

Memory phenotypes of mono- and bifunctional $CD4^+$ and $CD8^+$ T cells. Joint analysis of T cell functions and phenotypes may help establish whether a particular immune response is associated with protective immunity (29). Therefore, we evaluated the memory phenotypes of monofunctional (IFN-γ^+ or TNF-α^+) (Fig. 4 and 5) and bifunctional (IFN-γ^+ TNF-α^+) (Fig. 6) $CD4^+$ and $CD8^+$ T cells upon stimulation with PPD, E6-C10, and the selected DosR and Rpf antigens in the same 7-day-stimulated PBMC cultures.

Increased frequencies of monofunctional (IFN-γ^+ or TNF-α^+) $CD4^+$ T cells with a CD45RO$^+$ CD27$^+$ phenotype (T_{CM}) were observed for individuals with ltLTBI, compared to PTB patients, in response to E6-C10 ($P < 0.001$ and $P < 0.001$, respectively), the DosR antigens Rv1737c ($P < 0.05$ and $P < 0.001$, respectively), Rv2029c ($P < 0.05$ and $P < 0.001$, respectively), and Rv2628 ($P < 0.05$ and $P < 0.01$, respectively), and the Rpf antigens Rv0867c ($P < 0.05$ and $P < 0.01$, respectively) and Rv2389c ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 4 and 5); similar findings were observed upon stimulation with PPD ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 4 and 5). In addition, individuals with ltLTBI displayed higher frequencies of bifunctional (IFN-γ^+ TNF-α^+) $CD4^+$ T cells with a T_{CM} phenotype in response to PPD ($P < 0.001$), E6-C10 ($P < 0.001$), Rv1737c ($P < 0.01$), Rv2029c ($P < 0.001$), Rv0867c ($P < 0.05$), and Rv2389c ($P < 0.05$) (Fig. 6).

In contrast, PTB patients displayed higher frequencies of monofunctional TNF-α^+ $CD4^+$ T cells with a CD45RO$^+$ CD27$^-$ (T_{EM}) phenotype in response to PPD ($P < 0.001$) and the DosR antigen Rv2029c ($P < 0.05$) (Fig. 5). Also, PTB patients displayed higher frequencies of bifunctional IFN-γ^+ TNF-α^+ $CD4^+$ T cells with a T_{EM} phenotype upon stimulation with PPD ($P < 0.001$), E6-C10 ($P < 0.001$), Rv1737c ($P < 0.01$), Rv2029c ($P < 0.001$), Rv0867c ($P < 0.05$), and Rv2389 ($P < 0.05$), compared to individuals with ltLTBI (Fig. 6). Of note, high frequencies of monofunctional $CD4^+$ TNF-α^+ T cells with a CD45RO$^-$ CD27$^+$ ($T_{E/N}$) phenotype were observed for PTB patients in response to PPD ($P < 0.05$), E6-C10 ($P < 0.05$), Rv1737c ($P < 0.01$), Rv2029c ($P < 0.01$), Rv2628 ($P < 0.01$), Rv0867c ($P < 0.05$), and Rv2389 ($P < 0.01$) (Fig. 5).

Considering $CD8^+$ T cells, individuals with ltLTBI displayed a higher frequency of monofunctional IFN-γ^+ cells with a T_{CM} phenotype in response to the DosR antigen Rv2628 ($P < 0.05$), compared to PTB patients (Fig. 4). In addition, the ltLTBI group displayed higher frequencies of monofunctional (TNF-α^+) and bifunctional (IFN-γ^+ TNF-α^+) $CD8^+$ T cells with a T_{CM} phenotype in response to PPD ($P < 0.01$ and $P < 0.01$, respectively), E6-C10 ($P < 0.01$ and $P < 0.05$, respectively), Rv1737c ($P < 0.05$ and $P < 0.01$, respectively), Rv2029c ($P < 0.05$ and $P < 0.05$, respectively), and Rv2389c ($P < 0.05$ and $P < 0.05$, respectively) (Fig. 5 and 6). In contrast, PTB patients displayed higher frequencies of bifunctional (IFN-γ^+ TNF-α^+) $CD8^+$ T cells with a T_{EM} phenotype upon stimulation with PPD ($P < 0.05$) and E6-C10 ($P < 0.05$), compared to individuals with ltLTBI (Fig. 6).

DISCUSSION

In this study, which was performed in a community in Colombia in which TB is endemic, we characterized the functions and phenotypes of $CD4^+$ and $CD8^+$ T cells (by flow cytometry) in re-

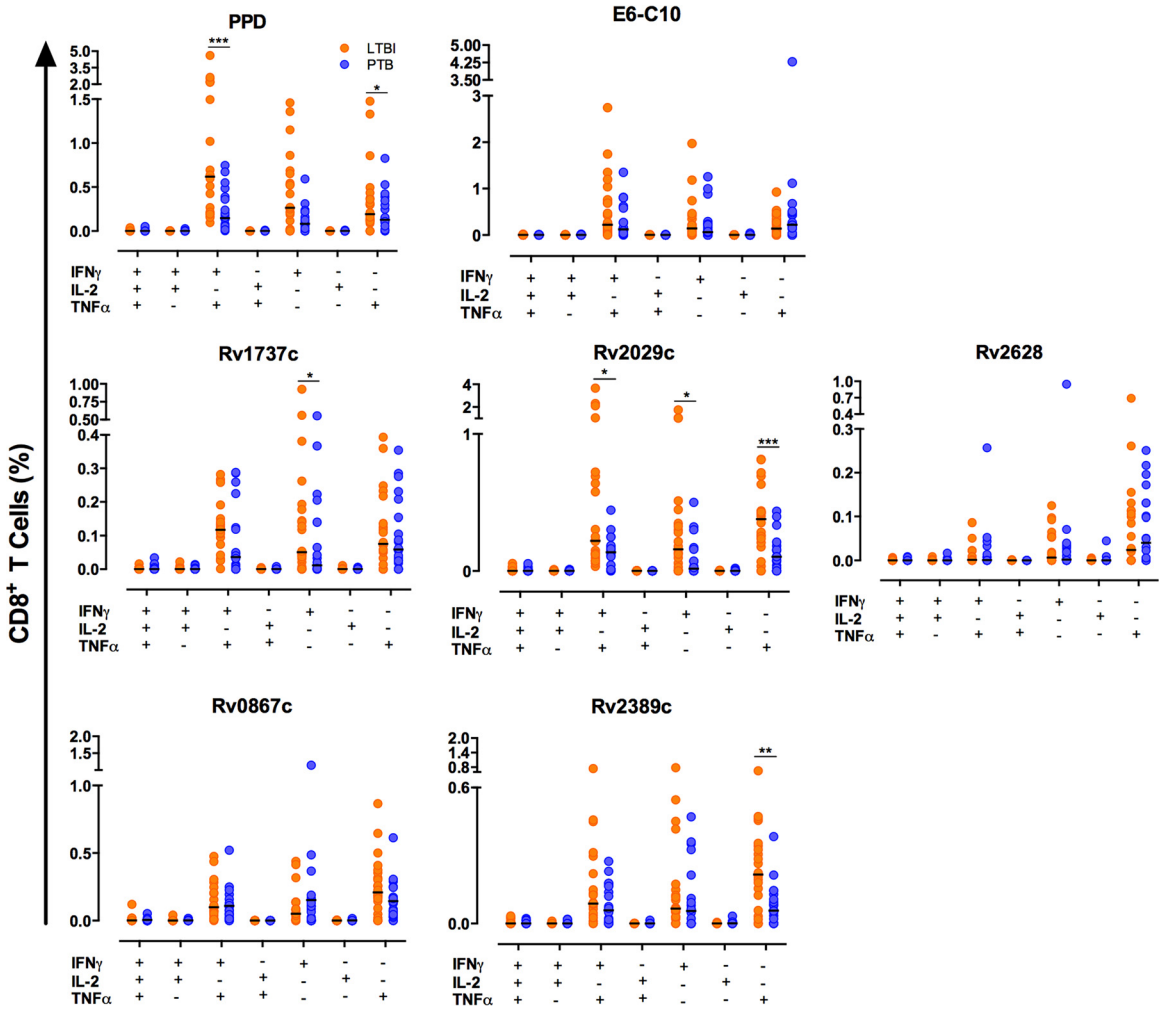

FIG 3 Frequencies of single- and multiple-cytokine-producing $CD8^+$ T cells in ltLTBI and PTB. A total of 1.5×10^5 PBMCs from individuals with ltLTBI or PTB were cultured in triplicate for 7 days in the presence or absence of PPD, E6-C10, and DosR and Rpf antigens. $CD8^+$ T cells were stained as described in Materials and Methods and then analyzed for intracellular production of IFN-γ, TNF-α, and IL-2. The frequencies of single- and multiple-cytokine-producing $CD8^+$ T cells were evaluated using the combination gate tool from FlowJo v7.6.1. Statistical differences between the groups were calculated with the Mann-Whitney *U* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

sponse to DosR regulon-encoded antigens and resuscitation Rpf antigens in individuals with ltLTBI and patients with PTB. We found that Rv1737c (NarK2), Rv2029c (PfkB), and Rv2389c (RpfD) antigens induced higher frequencies of $CD4^+$ or $CD8^+$ mono- or bifunctional T cells (producing IFN-γ and/or TNF-α) in ltLTBI, compared to PTB. In addition, higher frequencies of $CD4^+$ and/or $CD8^+$ mono- or bifunctional T cells with a T_{CM} phenotype ($CD45RO^+$ $CD27^+$) in response to RD1, DosR, and Rpf antigens were observed in ltLTBI, compared to PTB. Conversely, higher frequencies of bifunctional $CD4^+$ or $CD8^+$ T cells with a T_{EM} phenotype ($CD45RO^+$ $CD27^-$) in response to RD1, DosR, and Rpf antigens were observed in PTB, compared with ltLTBI. All of these data suggest that the response to *M. tuberculosis* DosR and Rpf antigens may contribute to mycobacterial control in latent infection and may help to discriminate further between the different states of *M. tuberculosis* infections.

In previous studies in the same community in which TB is endemic, we showed that HHCs with LTBI displayed higher frequencies of $CD4^+$ IFN-γ^+ T cells with a T_{CM} phenotype ($CD45RO^+$ $CD27^+$) in response to *M. tuberculosis* DosR and Rpf antigens, compared to PTB patients (34). More recently, we monitored the T cell immune responses to RD1, DosR, and Rpf antigens in HHCs with LTBI over a 12-month period after TB index case diagnosis. At 12 months, E6-$C10^+$ HHCs displayed decreases in IFN-γ levels in response to E6-C10, DosR, and Rpf antigens and a generalized decrease in cytokine production. Conversely, E6-$C10^-$ HHCs at the end of the follow-up period (12 months) showed increases in the IFN-γ responses and cytokine levels in response to E6-C10 (36). The maintenance of $CD45RO^+$ $CD27^+$ $CD4^+$ T cells in E6-$C10^+$ HHCs and their increase in E6-$C10^-$ HHCs suggested that $CD45RO^+$ $CD27^+$ T cells may play a protective role in the immune response controlling *M. tuberculosis* infection and may be leading to a state of controlled latent infection (36). In viral infections, however, protective immune responses have been associated with the presence of multifunctional T cells, producing IFN-γ, TNF-α, and IL-2 (12, 13). Therefore, the presence and association of multifunctional T cell responses in TB have been examined by several groups, mostly

TABLE 3 Frequencies of monofunctional (IFN-γ^+ or TNF-α^+), bifunctional (IFN-γ^+ TNF-α^+), and multifunctional (IFN-γ^+ TNF-α^+ IL-2^+) $CD8^+$ T cells in ltLTBI and PTB

	Frequency (median [interquartile range]) (%)							
	$CD8^+$ IFN-γ^+ T cells		$CD8^+$ TNF-α^+ T cells		$CD8^+$ IFN-γ^+ TNF-α^+ T cells		$CD8^+$ IFN-γ^+ TNF-α^+ IL-2^+ T cells	
Antigen	PTB	LTBI	PTB	LTBI	PTB	LTBI	PTB	LTBI
PPD	0.08 (0.00–0.22)	0.26 (0.09–0.66)	0.13 (0.03–0.33)	0.19 (0.11–0.39)[a]	0.15 (0.06–0.38)	0.62 (0.20–2.19)[b]	0.00 (0.00–0.00)	0.00 (0.00–0.01)
RD1 antigens								
E6-C10	0.06 (0.0–0.24)	0.14 (0.03–0.43)	0.22 (0.06–0.48)	0.14 (0.03–0.43)	0.12 (0.05–0.61)	0.22 (0.06–0.83)	0.00 (0.00–0.00)	0.00 (0.00–0.00)
DosR antigens								
Rv1737c	0.01 (0.00–0.19)	0.05 (0.02–0.18)[a]	0.06 (0.02–0.19)	0.08 (0.01–0.22)	0.04 (0.00–0.12)	0.12 (0.04–0.14)	0.00 (0.00–0.00)	0.00 (0.00–0.00)
Rv2029c	0.02 (0.00–0.16)	0.16 (0.02–0.37)[a]	0.10 (0.03–0.18)	0.38 (0.20–0.49)[a]	0.14 (0.01–0.18)	0.22 (0.11–0.70)[a]	0.00 (0.00–0.02)	0.00 (0.00–0.01)
Rv2628	0.00 (0.00–0.03)	0.01 (0.00–0.06)	0.04 (0.00–0.13)	0.02 (0.00–0.11)	0.00 (0.00–0.01)	0.00 (0.00–0.01)	0.00 (0.00–0.00)	0.00 (0.00–0.00)
Rpf antigens								
Rv0867c	0.08 (0.00–0.16)	0.05 (0.01–0.13)	0.11 (0.04–0.23)	0.21 (0.08–0.36)	0.08 (0.02–0.15)	0.10 (0.03–0.22)	0.00 (0.00–0.01)	0.00 (0.00–0.01)
Rv2389c	0.06 (0.0–0.19)	0.07 (0.03–0.16)	0.06 (0.0–0.12)	0.21 (0.05–0.35)	0.06 (0.00–0.17)	0.09 (0.03–0.24)	0.00 (0.00–0.00)	0.00 (0.00–0.01)

[a] $P < 0.05$.
[b] $P < 0.001$.

in response to *in vitro* stimulation with ESAT6 and CFP10; as discussed above, however, results have been inconclusive (15–20).

It is now well established that, as a consequence of adaptation to the infected host cell intracellular milieu, *M. tuberculosis* changes its gene expression profile (37, 39). This implies that the immune response may develop a different specificity profile depending on the immunodominant antigens newly expressed by *M. tuberculosis* during its adaptation to the host. The DosR antigens become strongly expressed under stress conditions, including hypoxia, nutrient starvation, low pH, and high concentrations of reactive oxygen and nitrogen intermediates, all of which may mimic conditions inside granulomas (37–39). Also, expression patterns of *rpfA-E* genes have been observed during acute infection with *M. tuberculosis* (43, 44). Of note, the multifunctional T cell responses to DosR and Rpf antigens in communities in which *M. tuberculosis* is endemic have been poorly characterized.

With respect to the DosR antigens, our results showed that individuals with ltLTBI displayed higher frequencies of mono- and bifunctional $CD4^+$ and $CD8^+$ T cells producing IFN-γ and/or TNF-α in response to Rv2029c (*pfkB*), compared to PTB patients. Moreover, individuals with ltLTBI displayed a higher frequency of monofunctional $CD8^+$ IFN-γ^+ T cells in response to

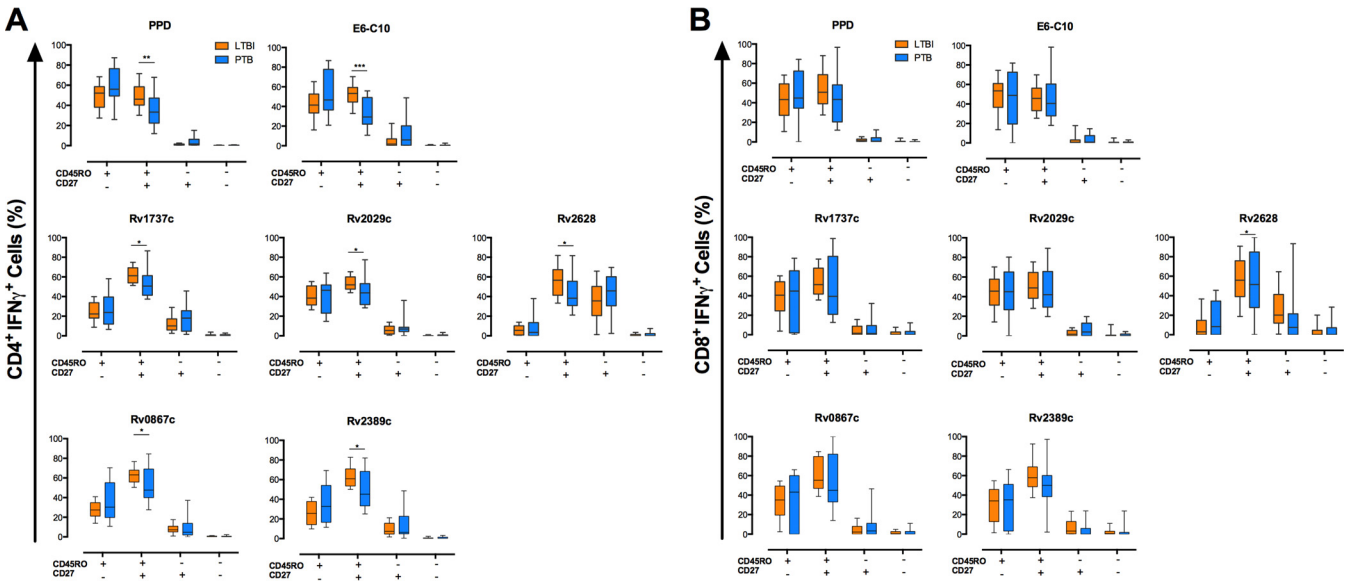

FIG 4 Memory phenotypes of monofunctional IFN-γ^+ $CD4^+$ and IFN-γ^+ $CD8^+$ T cells in ltLTBI and PTB. A total of 1.5×10^5 PBMCs from individuals with ltLTBI or PTB were cultured in triplicate for 7 days in the presence or absence of PPD, E6-C10, and DosR and Rpf antigens. $CD4^+$ and $CD8^+$ T cells were stained as described in Materials and Methods and then analyzed for intracellular production of IFN-γ, TNF-α, and IL-2. The frequencies of single- and multiple-cytokine-producing $CD4^+$ and $CD8^+$ T cells were evaluated using the combination gate tool from FlowJo v7.6.1. The memory phenotypes of monofunctional IFN-γ^+ $CD4^+$ (A) and IFN-γ^+ $CD8^+$ (B) T cells were evaluated by flow cytometry according the surface expression of CD45RO and CD27. Statistical differences between the groups were calculated with the Mann-Whitney *U* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

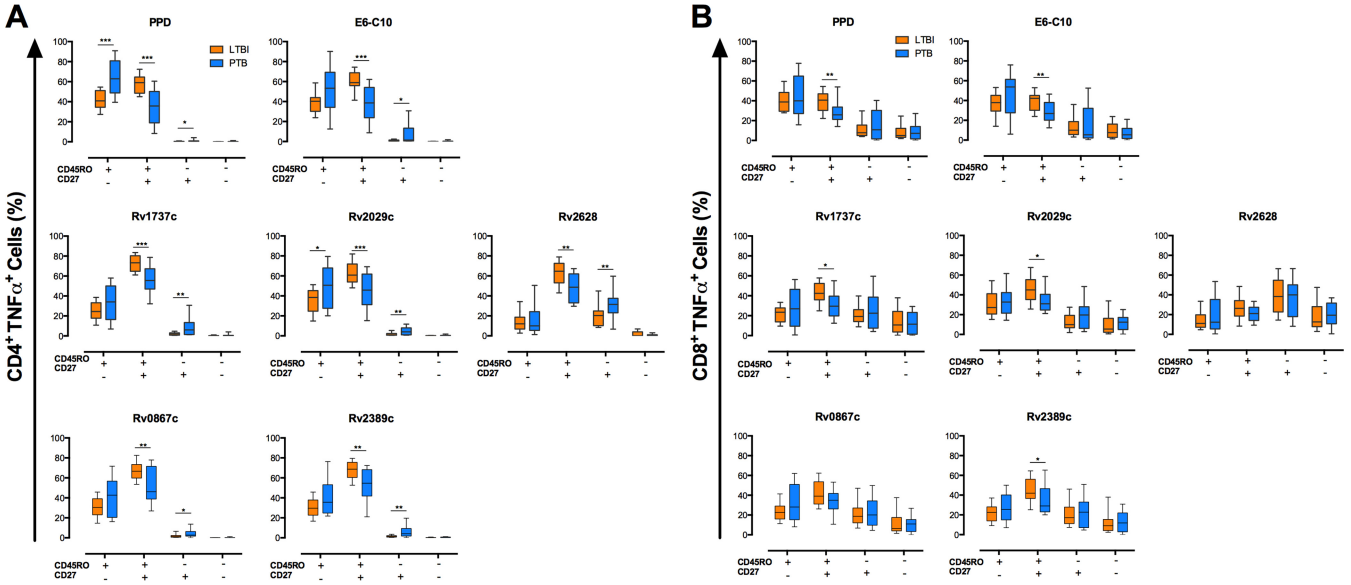

FIG 5 Memory phenotypes of monofunctional TNF-α^+ $CD4^+$ and TNF-α^+ $CD8^+$ T cells in ltLTBI and PTB. A total of 1.5×10^5 PBMCs from individuals with ltLTBI or PTB were cultured in triplicate for 7 days in the presence or absence of PPD, E6-C10, and DosR and Rpf antigens. $CD4^+$ and $CD8^+$ T cells were stained as described in Materials and Methods and then analyzed for intracellular production of IFN-γ, TNF-α, and IL-2. The frequencies of single- and multiple-cytokine-producing $CD4^+$ and $CD8^+$ T cells were evaluated using the combination gate tool from FlowJo v7.6.1. The memory phenotypes of monofunctional TNF-α^+ $CD4^+$ (A) and TNF-α^+ $CD8^+$ (B) T cells were evaluated by flow cytometry according the surface expression of CD45RO and CD27. Statistical differences between the groups were calculated with the Mann-Whitney *U* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Rv1737c (*narK2*), compared to PTB patients, suggesting that a protective immune response may develop in ltLTBI in response to DosR antigens. Indeed, greater immune responses to Rv1737c and Rv2029c in ltLTBI, compared to PTB, were reported previously for different human populations (31, 32, 34, 35). Furthermore, it has been reported that DosR antigens, including Rv2029c, induce predominant mono- and bifunctional (IFN-γ and/or TNF-α) $CD4^+$ and/or $CD8^+$ T cell responses in LTBI (33). Rv2029c (*pfkB*), a probable phosphofructokinase, is a key enzyme in glycolysis (58). A recent study suggests that, in *M. tuberculosis*, glycolysis leads to the accumulation of toxic metabolites, limiting *M. tuberculosis* survival under hypoxic conditions (58). Rv1737c

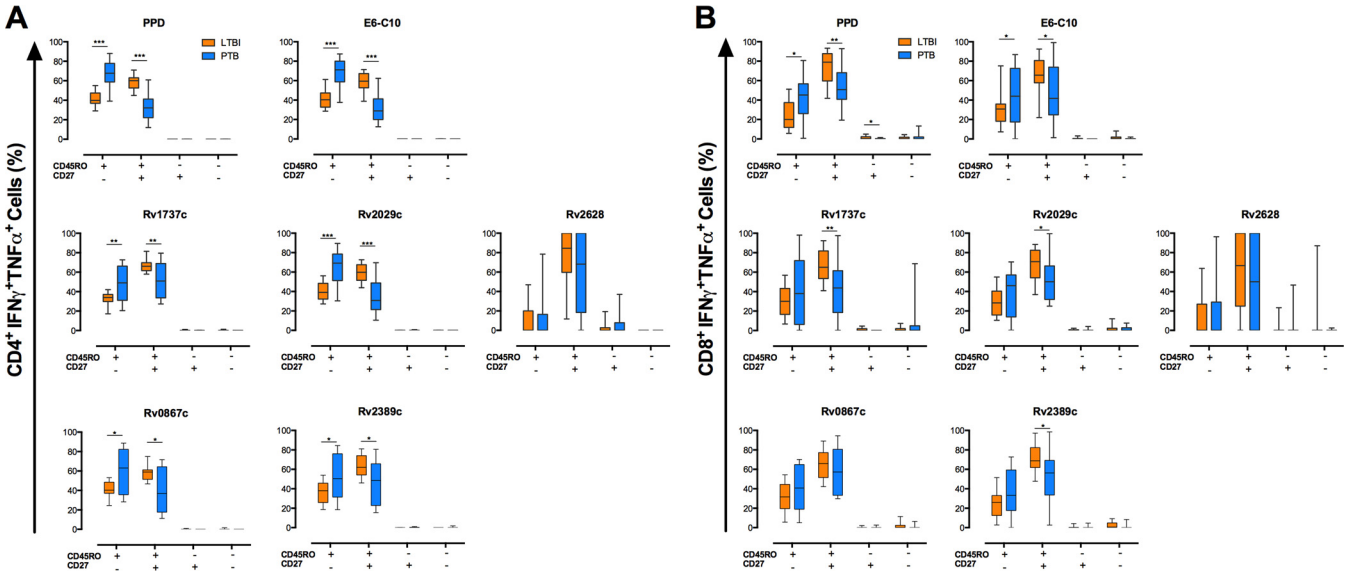

FIG 6 Memory phenotypes of bifunctional IFN-γ^+ TNF-α^+ $CD4^+$ and IFN-γ^+ TNF-α^+ $CD8^+$ T cells in ltLTBI and PTB. A total of 1.5×10^5 PBMCs from individuals with ltLTBI or PTB were cultured in triplicate for 7 days in the presence or absence of PPD, E6-C10, and DosR and Rpf antigens. $CD4^+$ and $CD8^+$ T cells were stained as described in Materials and Methods and then analyzed for intracellular production of IFN-γ, TNF-α, and IL-2. The frequencies of single- and multiple-cytokine-producing $CD4^+$ and $CD8^+$ T cells were evaluated using the combination gate tool from FlowJo v7.6.1. The memory phenotypes of bifunctional IFN-γ^+ TNF-α^+ $CD4^+$ (A) and IFN-γ^+ TNF-α^+ $CD8^+$ (B) T cells were evaluated by flow cytometry according the surface expression of CD45RO and CD27. Statistical differences between the groups were calculated with the Mann-Whitney *U* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(*narK2*) is a probable nitrite/nitrate transporter that participates in the regulation of the nitrate reductase activity of *M. tuberculosis* under hypoxic conditions (59, 60). It has been suggested that nitrate reduction may play an important role in *M. tuberculosis* survival during dormancy (61). Thus, the evidence presented in this paper and a previous paper (34), as well as others (62), reinforces the notion that *M. tuberculosis* DosR regulon-encoded antigens, including Rv1737c and Rv2029c, may be interesting biomarkers associated with a protective immune response and might be potential candidates for postexposure vaccines.

An immune response to Rpf antigens of *M. tuberculosis* has been preferentially associated with individuals with LTBI (34, 36, 40, 42). In a previous study, we reported a higher frequency of $CD4^+$ IFN-γ^+ T cells in HHCs with LTBI, compared to PTB, in response to RpfD (Rv2389c) (34). The higher frequencies of monofunctional $CD4^+$ TNF-α^+ and $CD8^+$ TNF-α^+ T cells in ltLTBI that we observed in the present study are concordant with the observation of the presence of monofunctional $CD4^+$ TNF-α^+ and $CD8^+$ TNF-α^+ T cells in *M. tuberculosis*-infected nonprogressors of Norwegian origin (41). Thus, monofunctional $CD4^+$ TNF-α^+ T cells may play an important role in controlling *M. tuberculosis* reactivation in latently infected individuals. We previously observed a lower frequency of IFN-γ^+ T cells and lower levels of IFN-γ production in response to Rv0867c (RpfA), compared to RpfD (34, 36). It has been reported that the *rpfA* and *rpfD* genes are differentially expressed during *M. tuberculosis* growth and under stress conditions (63). It is tempting to speculate that differences in the expression and/or function of RpfA and RpfD in *M. tuberculosis* may lead to different immune responses during latency.

Our results showed that HHCs with ltLTBI displayed significant increases in the frequencies of monofunctional (IFN-γ^+ or TNF-α^+) or bifunctional (IFN-γ^+ TNF-α^+) $CD4^+$ and $CD8^+$ T cells in response to stimulation with the RD1 and PPD antigens. Both IFN-γ and TNF-α play important roles in the protective immune response against *M. tuberculosis* infection, participating in the activation of effector mechanisms of monocytes and macrophages and in granuloma integrity (5, 6). Thus, our observations may suggest that mono- and bifunctional $CD4^+$ and $CD8^+$ T cells producing IFN-γ and/or TNF-α can contribute to effective mycobacterial growth control in individuals with LTBI (34, 48, 64). Although in other studies higher frequencies of mono- and bifunctional $CD4^+$ T cells producing IFN-γ and/or TNF-α in response to RD1 were found in PTB, compared to LTBI (18, 19, 27, 28), the difference in the lengths of the *in vitro* cultures might explain this difference. Short-term cultures (24 h) have been mainly associated with the detection of a T cell effector memory phenotype, while long-term cultures (5 to 7 days), as used in this study, have been mainly associated with the detection of a T central memory phenotype (25, 36, 56, 57, 64). An alternative explanation for the observed reductions in the frequencies of mono- and bifunctional $CD4^+$ T cells in peripheral blood samples from PTB patients may involve the previously reported sequestration of $CD4^+$ T cells at the site of infection (65, 66).

T cell memory generation is critical for specific immune responses. Studies in viral models of chronic infection showed that effector T cells expanded during viral replication, while memory cells were detected upon virus control (30). Memory generation is also critical for protective immunity to *M. tuberculosis* (67). PBMCs from tuberculin skin test-positive and cured TB patients that were stimulated with RD1 antigens displayed a higher frequency of $CD4^+$ T cells with a T_{CM} phenotype, compared to patients with moderate or severe TB, who displayed a preferential T_{EM} phenotype (23). In a previous study, we found a higher frequency of $CD4^+$ T cells with a T_{CM} phenotype ($CD45RO^+$ $CD27^+$) in LTBI, compared to PTB, in response to the fusion protein ESAT6-CFP10 (34). Furthermore, BCG vaccination induces the expansion of a $CD4^+$ T cell population with a T_{CM} phenotype (68), although it is not clear whether this expansion may result in long-term protection. In the present study, we found that individuals with ltLTBI displayed higher frequencies of mono- and bifunctional $CD4^+$ and/or $CD8^+$ T_{CM} cells that produced IFN-γ and/or TNF-α, compared to PTB patients. Conversely, higher frequencies of bifunctional $CD4^+$ and $CD8^+$ T cells with a T_{EM} phenotype ($CD45RO^+$ $CD27^-$) that produced IFN-γ and TNF-α were found in PTB patients, compared to individuals with ltLTBI. Overall, these results suggest that mono- and bifunctional T cells with a T_{CM} phenotype may play an important role in *M. tuberculosis* infection control in latently infected individuals, while T_{EM} cells may be associated with the presence of replicating mycobacteria in PTB patients and may represent biomarkers of the mycobacterial load. Commandeur and colleagues reported higher frequencies of mono- and bifunctional T cells with a T_{EM} phenotype in response to stimulation with DosR and Rpf antigens in LTBI (33, 41). This difference from our results may be explained by the length of the *in vitro* cultures. While we used long-term cultures (7 days), Commandeur and colleagues used short-term cultures (24 h) (33, 41). It has been argued that long-term cultures (5 to 7 days), in contrast to short-term cultures (24 h), select potentially long-lived T cells, particularly central memory T cells (55–57). This may also explain the absence (or lower levels) of effector T cells ($CD45RO^-$ $CD27^-$) for single-cytokine-producing $CD4^+$ and $CD8^+$ T cells. Under our experimental conditions using long-term cultures, we have observed enrichment of the T_{CM} phenotype ($CD45RO^+$ $CD27^+$ T cells) (25, 34, 36, 64).

Recent studies demonstrated that RD1 antigen stimulation induces greater proportions of multifunctional $CD4^+$ T cells in individuals with LTBI, compared to patients with active TB (18–20). In this study, we found that mono- and bifunctional $CD4^+$ and $CD8^+$ T cells producing IFN-γ and/or TNF-α were observed more frequently than monofunctional IL-2^+ or multifunctional (IFN-γ^+ TNF-α^+ IL-2^+) T cells, independent of the antigen and disease status, as observed by others (48). A similar observation in the macaque model was recently published (69). The low frequency of antigen-specific T cells uniquely producing IL-2 may be a consequence of the long-term culture used in our study, as reported previously (40, 48). In a recent study, Han and colleagues, using a short-term stimulation assay, reported that the T cell multifunctional response is the result of sequential production of cytokines for short times, during which T cells simultaneously secrete multiple cytokines (70). More recently, it has been shown that some methodological factors, including the source of T cells (fresh whole blood, fresh PBMCs, or frozen PBMCs), the length of the culture (short term versus long term), and the use of costimulatory antibodies, can affect the sensitivity of intracellular cytokine assays (71). These results may explain the contrasting results of the T cell cytokine profiles observed in our study.

Another potential limitation of the present study is that we analyzed the immune responses of a relatively small number of

subjects in each group, which is why this constitutes a pilot study. Although larger populations and longitudinal studies are needed to confirm these observations, the results generated in this study are consistent with results observed in other human populations.

In this study, PTB patients are defined as having received a recent diagnosis of PTB, which was confirmed microbiologically or by culture, with no more than 2 weeks of antibiotic treatment. There is some evidence in the literature of changes to the transcriptome and cell populations as early as 1 week posttreatment, which may affect the composition of the T cell population responding to the infection (72). However, it has been reported that significant changes in the immune response and cell populations in the host are principally observed 1 to 3 or 6 months after the initiation of TB treatment (73–75), indicating that changes at a transcriptomic level are not translated immediately into protein and cellular changes in the host. We think that our results may not be affected by the anti-TB treatment. Also, some reports have presented evidence suggesting that the immune responses of patients with multidrug-resistant (MDR) TB may be different from those of patients with drug-sensitive TB (76, 77). Since all of our PTB samples were collected within the first 2 weeks of treatment, no testing for antibiotic resistance was performed. However, the frequency of MDR TB in our population is low (estimated rate, 2.4%) (78), and thus our results may not be affected by this factor.

In conclusion, we have shown that individuals with ltLTBI display prominent mono- and bifunctional (IFN-γ^+ and/or TNF-α^+) T cell responses, with a CD45RO$^+$ CD27$^+$ phenotype, to *M. tuberculosis* DosR and Rpf antigens, which we hypothesize are associated with maintenance of immune control of latent *M. tuberculosis* infection and protection from disease reactivation. To our knowledge, the present data represent the first description of the multifunctional T cell responses to DosR and Rpf antigens in individuals with ltLTBI and PTB in a community in which TB is endemic. Our results may contribute to a better understanding of latency and the definition of predictive biomarkers of latency and reactivation.

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