

Dihydrolipoamide dehydrogenase-Lpd (Rv0462)-specific T cell recall responses are higher in healthy household contacts of TB: a novel immunodominant antigen from *M. tuberculosis*

Santhi Devasundaram and Alamelu Raja¹

Department of Immunology, National Institute for Research in Tuberculosis, Chennai, India

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ABSTRACT

The partial effectiveness against pulmonary tuberculosis (PTB), displayed by the existing tuberculosis (TB) vaccine, bacillus Calmette-Guérin (BCG), highlights the need for novel vaccines to replace or improve BCG. In TB immunology, antigen-specific cellular immune response is frequently considered indispensable. Latency-associated antigens are intriguing as targets for TB vaccine development. The mycobacterial protein, dihydrolipoamide dehydrogenase (Lpd; Rv0462), the third enzyme of the pyruvate dehydrogenase (PDH) complex, facilitates *Mycobacterium tuberculosis* to resist host reactive nitrogen intermediates. Multicolor flow cytometry analysis of whole-blood cultures showed higher Lpd-specific Th1 recall response (IFN- γ , TNF- α , and IL-2; $P = 0.0006$) and memory CD4⁺ and CD8⁺ T cells (CCR7⁺ CD45RA⁻ and CCR7⁻ CD45RA⁻) in healthy household contacts (HHC) of TB ($P < 0.0001$), which is comparable with or higher than the standard antigens, ESAT-6 and CFP-10. The frequency of Lpd-specific multifunctional T cells was higher in HHC compared with PTB patients. However, there is no significant statistical correlation. Regulatory T cell (T_{reg}) analysis of HHCs and active TB patients demonstrated very low Lpd-specific CD4⁺ T_{regs} relative to ESAT-6 and CFP-10. Our study demonstrates that the Lpd antigen induces a strong cellular immune response in healthy mycobacteria-infected individuals. In consideration of this population having demonstrated immunologic protection against active TB disease development, our data are encouraging about the possible use of Lpd as a target for

further TB subunit vaccine development. *J. Leukoc. Biol.* **102**: 135–151; 2017.

Introduction

TB continues to be a major public health problem in many countries of the world, despite extensive efforts taken to control it. BCG, the only available TB vaccine, was initially shown to be successful but later, demonstrated little or no protection in the United States and South India [1–4]. The variable efficacy afforded by BCG vaccination and the lack of protectivity in adult TB have been the primary incentives to develop new vaccine candidates as a replacement for BCG or as a subunit boosting vaccine. Effective TB vaccination requires the generation of a T cell-mediated immune response, as adequate T cell functioning is essential for protective immunity against TB infection [5].

The subunit vaccine approach is advantageous because of its increased safety, stability, and ability to boost prior BCG immunization [6, 7]. Potential TB subunit vaccines have been obtained by screening *M. tuberculosis* proteins that are capable of stimulating T cell responses in humans or mice during *M. tuberculosis* infection. Latently infected HHCs of TB, continuously exposed to *M. tuberculosis*, are able to control the infection, thereby considered to be a protective population against active TB disease, and are good experimental models for deciphering T cell reactivity to mycobacterial antigens [8]. Thus, the comparison of the immune response between the “protected population” and “susceptible population” would define the type of immune mechanism needed for efficient controlling of the disease [9]. The purpose of our study is to evaluate the mycobacterial antigen Lpd-specific immune responses to develop a TB vaccine. We hypothesized that assessment of circulating antigen-specific T cells in a protected population for their recall secretion of cytokines would indicate the dominant type of

Abbreviations: AFB = acid fast bacilli, BCG = bacillus Calmette-Guérin, CFA = culture filtrate antigens, CFP-10 = 10 kDa culture filtrate protein, ESAT-6 = 6 kDa early secretory antigenic target, FMO = fluorescence minus one, FoxP3 = forkhead box P3, IGRA = IFN- γ release assay, Lpd = dihydrolipoamide dehydrogenase-Lpd, PCV2 = porcine circovirus type 2, PDH = pyruvate dehydrogenase, PTB = pulmonary tuberculosis, QFT-IT = QuantiFERON-TB Gold In-Tube, ROC = receiver operating characteristic, TB = tuberculosis, TEM = terminal effector cell, T_{reg} = regulatory T cell

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: National Institute for Research in Tuberculosis (ICMR; formerly Tuberculosis Research Centre), No. 1, Sathyamoorthy Rd., Chetpet, Chennai-600 031, India. E-mail: alameluraja@gmail.com; Twitter: http://www.twitter.com/seraph_santhi_dev

protective cellular immune response, and therefore, it could be used as a rationale for antigen selection in potential vaccines [10].

Lpd (Rv0462), the *M. tuberculosis* sole Lpd [11], plays an essential role in intermediary metabolism as the E3 component of the PDH complex [12]. The potential of Lpd to induce dendritic cells, a key component in bridging innate and adaptive immune responses toward Th1 polarization, is already proven [13]. However, Lpd antigen-specific immune responses in *M. tuberculosis*-infected individuals and type of immune response have not been addressed so far. In silico analysis of T cell antigens from culture filtrate antigens of *M. tuberculosis* resulted in a higher percentage of class I and class II MHC alleles and higher population coverage by Lpd compared with standard mycobacterial antigens ESAT-6, CFP-10, and Ag85B. However, Lpd-specific T cell profiles were not explicitly analyzed [14]. Thus, we performed polychromatic flow cytometry for various T cell types, such as T_{regs}, memory T cells, Th1 cytokine-secreting cells, and Th17 subtypes against in vitro stimulation with Lpd at the single-cell level.

MATERIALS AND METHODS

Study participants

This study was approved by the Institutional Review Board of the National Institute for Research in TB, and informed, written consent was obtained from all participants. The study participants with diabetes mellitus, TB reinfection, a Mantoux skin test within <16 mo, renal disorders, neoplasm (particularly lymphoma), and HIV coinfection tested by a routine AIDS test and those undergoing immunosuppressive therapy were excluded from the study. Study participants with indeterminate QFT-IT results were also excluded from the present study.

A total of 35 study participants, 20 HHCs of TB and 15 PTB individuals, were recruited. The recruitment of HHCs was based on sharing the same living quarters with TB index case patients before commencing antitubercular therapy. HHCs had ≥ 10 h contact with a TB index case and were not treated for latent TB. Study subjects were identified by visiting the households of adult smear-positive PTB patients who were enrolled for treatment at the Government Thiruvattesar Hospital of Thoracic Medicine (Oteri, Chennai, Tamil Nadu, India). Their *M. tuberculosis* infection state was assessed by IGRA and scored positive for IGRA. Chest X-ray and sputum smear microscopy results confirmed no active disease in HHCs.

The criteria for a PTB individual's recruitment included collective positive sputum microscopy results from 3 sputum samples that were stained for AFB microscopy by the Ziehl-Neelsen method and were culture positive. PTB sputum smear was graded by microscopy analysis (10 \times eye piece) as scanty (1–9 AFB in 100 \times oil immersion fields), 1+ (10–99 bacilli per 100 fields), 2+ (1–10 bacilli after examination of 50 fields), and 3+ >10 AFB after examination of 20 fields), according to our institute standard procedure (<http://nirt.res.in/pdf/bact/SOP.pdf>). PTB patients were also positive for IGRA. These patients were enrolled for anti-TB treatment at the Government Thiruvattesar Hospital of Thoracic Medicine.

IGRA

Thirteen milliliters of blood was collected and 3 ml used for IGRA as 1 ml per antigen-coated tube. The QFT-IT kit (Cellestis; Qiagen, Germantown, MD, USA) contains 3 tubes, precoated with TB antigens (ESAT-6, CFP-10, and TB7.7) as a test, PHA as a positive control, and the nil antigen (coated with saline) as a negative control. These 3 tubes, along with 1 ml blood, were incubated for 16–24 h at 37°C in 5% atmospheric CO₂. The supernatants were

collected after centrifugation and stored at –80°C until further processing. Test results were interpreted as per-kit guidelines using software provided by the manufacturer.

In vitro stimulation of whole blood

The collected blood was diluted 1:1 with RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing penicillin/streptomycin (100 U/100 mg/ml), L-glutamine (2 mM), and HEPES (10 mM), followed by stimulation with *M. tuberculosis* antigens at a final concentration of 5 μ g/ml at 37°C. ESAT-6 and CFP-10 were received as recombinant plasmids from Colorado State University (Fort Collins, Colorado, USA), and the test antigen Lpd (Rv0462) was obtained by in vitro cloning and overexpression [14]. In brief, the encoding region of *lpd* was amplified from H37Rv genomic DNA with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) by standard PCR. During the PCR amplification, restriction sites (*Bam*HI and *Xho*I) were introduced in *lpd* gene-specific primers by site-directed mutagenesis for ligation (T4 DNA ligase) of *lpd* into the vector DNA pET30a (N-terminal 6-histidine tag). Positive recombinant plasmid was selected and transformed into an expression strain of *Escherichia coli* BL21 (DE3) cells for overexpression and purification of the Lpd antigen. The Lpd gene was cloned downstream to the T7 promoter and was induced by 1 mM isopropyl-D-thiogalactoside. Recombinant His-tagged Lpd was purified under denaturing (8 M urea) conditions using Ni-nitrilo triacetic acid metal ion affinity chromatography and quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The endotoxin (LPS) was removed during Lpd antigen purification by extensive washing with amidosulfobetaine-ASB-14 (Sigma-Aldrich). Endotoxin contamination was measured by the *Limulus* amoebocyte lysate assay (Lonza Group, Basel, Switzerland) and found to be at an acceptable level of ≤ 10 EU/mg Lpd antigen [15].

PHA was used at a final concentration of 1 μ g/ml and served as a mitogen control. Blood diluted with RPMI, without any antigen stimulation, served as a control and was used to assess a nonspecific background response. Purified costimulatory molecules CD49d/CD28 (BD Biosciences, San Jose, CA, USA) were added (0.5 μ g/ml) to all of the stimulations. Culture plates were then incubated for 16 h at 37°C, 5% atmospheric CO₂. Brefeldin A (10 mg/ml) was added after 4 h, and the plates were further incubated for 12 h. After 16 h of incubation, cells were harvested with PBS and centrifuged at 2600 rpm for 10 min. The pellet obtained was treated with BD FACS lysing solution (BD Biosciences) to lyse RBCs, per the manufacturer's instruction. The cells were fixed using BD Cytotfix/Cytoperm buffer and cryopreserved at –80°C until intracellular staining was done.

Surface markers and intracellular cytokine staining

Fixed cells were thawed rapidly from –80°C and washed with PBS, and a single-cell suspension was made and stained for surface markers, as well as for intracellular markers in a different panel. Surface markers include PerCP 5.5-anti-CD3, allophycocyanin-Cy7-CD4, PE-Cy7-CD8, allophycocyanin-anti-CD69, FITC-CD197 (CCR7), allophycocyanin-CD45RA, FITC-CD127, and allophycocyanin-CD25 at a final concentration of 1 μ l/1 million cells. The following antibodies were used for intracellular staining: FITC-IFN- γ , PE-TNF- α , allophycocyanin-IL-2, PE-FoxP3, FITC-IL-17A, PE-IL-22, and allophycocyanin-IL-21. All antibodies were purchased from BD Biosciences or BioLegend (San Diego, CA, USA).

Surface staining was done at 4°C for 30 min in the dark, followed by washing with PBS. Cells were permeabilized with Perm/Wash buffer (BD Biosciences), and intracellular staining was performed overnight at 4°C in the dark. Acquisition was carried on a FACSCanto II flow cytometer with FACSDiva software, version 6 (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). All data are depicted as the percentage of CD4⁺ T cells expressing cytokine(s).

For all used antibodies, FMO controls were used to define positive and negative boundaries. Compensation was calculated with signals from fluorochrome mAb linked to CompBeads, purchased from BD Biosciences.

Data analysis

Statistical analyses were performed using GraphPad Prism, version 5 (GraphPad Software, La Jolla, CA, USA). Intergroup comparisons were performed using the nonparametric Mann-Whitney *U* test. The correlation between Lpd-reactive T cells (IFN- γ) and bacterial burden in PTB, in terms of sputum smear grade and age, was assessed by Spearman's rank correlation. We performed ROC curve analysis and determined cut-off value for Lpd antigen-specific IFN- γ based on Youden index (sensitivity + specificity - 1) to obtain maximum combination of sensitivity and specificity. For all analyses, differences were considered significant at $P < 0.05$.

RESULTS

The clinical characteristics of HHCs and active TB patients are summarized in **Table 1**. All study participants were positive (>3.5) for QFT-IT, confirming mycobacterial infection status. The response in QFT-IT mitogen tubes was similar in both HHC and PTB (≥ 0.5 IU/ml), showing no defects in immune responses in all study subjects. All PTB subjects were naive to anti-TB treatment at the time of study. There were no major differences in the percentage of CD4⁺ cells in both HHC and PTB, ranging from 20 to 40%, showing no defect in immune cells in all of the study subjects. The expression of early T cell activation marker CD69⁺ also did not vary between the two groups (Supplemental Fig. 1). FMO, for all results obtained (memory T cell, Th1, Th17, and T_{regs}), is provided in Supplemental Fig. 2A–D.

Increased antigen-specific CD45RA⁻ CCR7⁺ central memory phenotype in mycobacterial-infected healthy individuals

Many researchers have used CD45RA and CCR7 surface markers to define the memory T cell population [16–19]. Based on the two surface markers, CD45RA and CCR7, memory subsets were defined as naive (CD45RA⁺ CCR7⁺), central memory (CCR7⁺ CD45RA⁻), and effector memory (CCR7⁻ CD45RA⁻) cells and TEMs (CD45RA⁺ CCR7⁻). Their expression levels were measured at baseline (unstimulated control), followed by mycobacterial antigen stimulations. The specific response of mycobacterial antigens was obtained by subtracting unstimulated

control from test (Test - nil). Density plotting was followed to represent the memory T cell population in our stimulation of antigens (**Fig. 1**), as followed previously [18–20].

Our short-term T cell cultures predominantly consisted of the central memory-like phenotype, defined by the expression of CD45RA⁻ CCR7⁺ surface markers in all donors analyzed, followed by effector memory (CD45RA⁻ CCR7⁻) subtypes. As given in **Fig. 2A**, the percentage of central memory T cell subsets was invariably higher in HHC compared with active PTB. We observed significantly elevated levels of CD4⁺ central memory T cell frequencies with all antigen stimulations used, and the highest significance was obtained for Lpd stimulations in HHC when compared with PTB ($P < 0.0001$). The second-highest significance was observed with central memory subtypes for both ESAT-6 and CFP-10 ($P < 0.005$) stimulations. Based on these, we conclude that antigen-specific memory cells are readily detectable and higher in HHCs of TB. Predominantly, Lpd antigen-specific central memory cells were higher than the standard antigens, ESAT-6 and CFP-10.

The percentage of effector memory (CD45RA⁻ CCR7⁻) cells was also found to be higher in HHCs than PTB, and a significant difference was observed for ESAT-6 ($P < 0.05$) and Lpd ($P < 0.005$) but not for CFP-10 stimulation (**Fig. 2B**). The other two memory cell types—naive (CD45RA⁺ CCR7⁺) and TEM (CD45RA⁺ CCR7⁻) cells—were high in HHC when compared with PTB, except for CFP-10-specific naive cells. Mann-Whitney *U* test analysis showed no significant difference between HHC and PTB with any of the stimulations except for ESAT-6 for TEM types in HHC ($P < 0.05$; **Fig. 2C and D**).

CD8⁺ memory subtype analysis showed that central memory CD8⁺ cells were higher in HHC compared with PTB during stimulation with Lpd ($P < 0.005$), and no other stimulations (ESAT-6 and CFP-10) elicited a CD8⁺ central memory response in an antigen-specific manner (**Fig. 3A**). Effector memory CD8⁺ cells did not vary significantly between the two groups (**Fig. 3B**). Unlike CD4⁺ naive cells, CD8⁺ naive cells were higher in HHC, and the difference was significant against all antigen stimulations (**Fig. 3C**). Unlike CD4⁺ TEMs, CD8⁺ TEMs were higher in PTB with all antigen stimulations but with no statistical significance (**Fig. 3D**). A higher Lpd-specific memory response in HHCs

TABLE 1. Demographic details of all study participants

Characteristics of study participants	HHCs	Active PTB individuals
Sample size	20	15
Mean age, yr (range)	39 (27–55)	41 (25–56)
Sex		
Women, <i>n</i> (%)	11 (55)	7 (46)
Men, <i>n</i> (%)	9 (45)	8 (53)
Sputum smear positivity, <i>n</i> (%)	0 (0)	15 (100)
Smear grade, +++, <i>n</i> (%)	0 (0)	8 (53)
Smear grade, ++, <i>n</i> (%)	0 (0)	4 (26)
Smear grade, +, <i>n</i> (%)	0 (0)	3 (20)
QFT-GIT		
Positive, <i>n</i> (%)	20 (100)	15 (100)
Negative, <i>n</i> (%)	0 (0)	0 (0)
Indeterminate, <i>n</i> (%)	0 (0)	0 (0)
CD4, % range	20–65	21–62

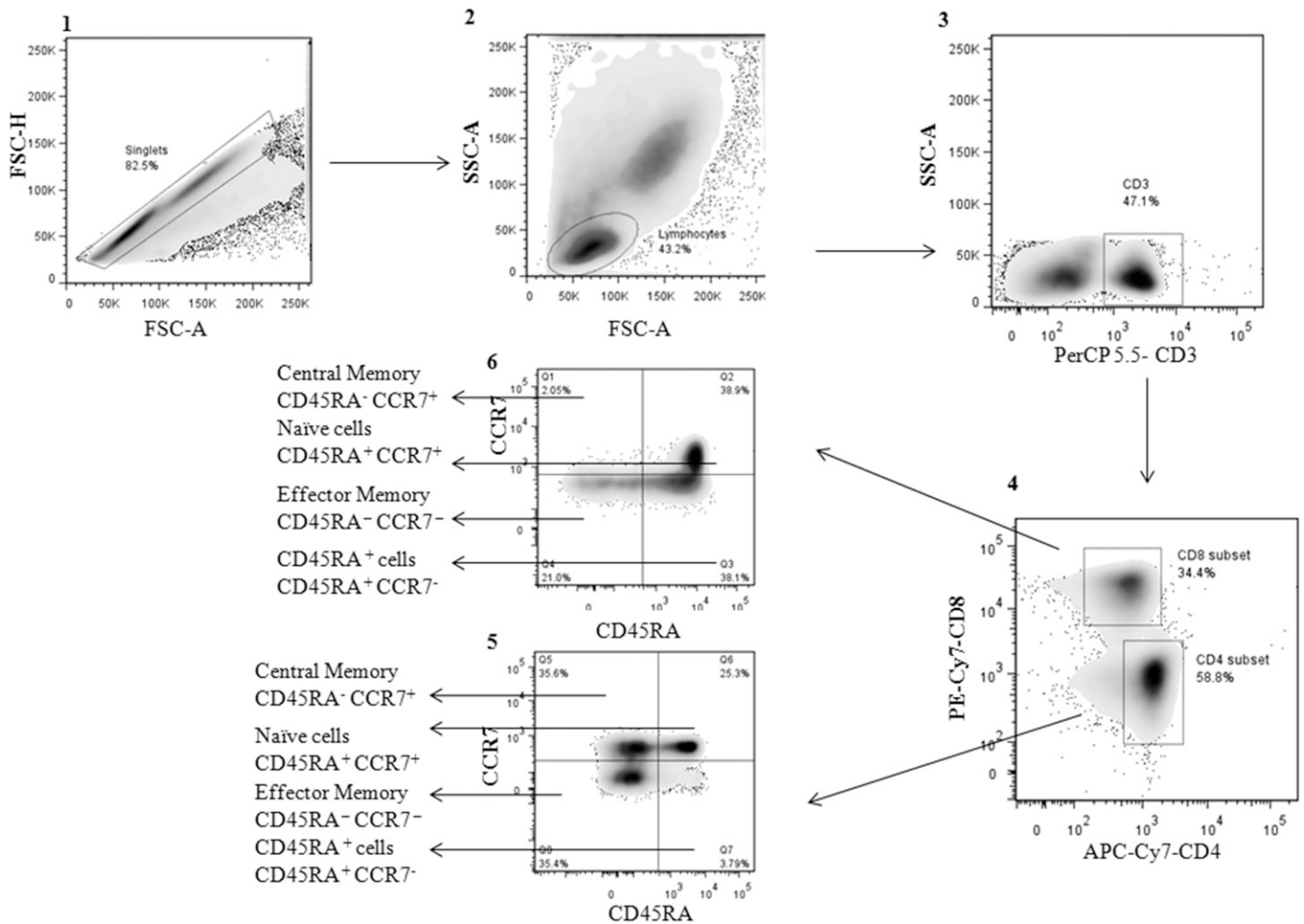


Figure 1. Gating strategy for memory T cell subtype classification based on CCR7 and CD45RA expression. (1) Cultured whole-blood cells were acquired and first gated for singlets [forward-scatter-area (FSC-A) vs. forward-scatter-height (FSC-H)]. (2) Lymphocyte population was gated based on the size (FSC) and the granularity [side-scatter-area (SSC-A)]. (3) Lymphocyte population was analyzed for expression of CD3, a highly specific T cell marker associated at all stages of T cell development. (4) CD3⁺ T cell were then gated based on the expression of CD4 (Th cells) and C8 (cytotoxic T cells). APC, Allophycocyanin. (5) These CD4⁺ and CD8⁺ T cells were then analyzed for the expression of memory T cell markers CD45RA and CCR7. (6) The staining pattern of CD45RA and CCR7 in our in vitro whole-blood culture and the 4 subtypes of memory T cells.

showed their possible association with protection against active TB disease development.

Antigen-specific, single-positive, Th1 cytokine-secreting cells were more frequent than multifunctional T cells in HHCs

In HHC, Lpd-specific, IFN- γ -secreting CD4⁺ cells were high when compared with PTB, showing the presence of antigen-specific, IFN- γ -secreting cells in the circulation of HHC (Fig. 4A). The percentage of CD4⁺ IFN- γ cells in the PTB group was also in the detectable range but significantly less than HHCs. An exception was the CFP-10 stimulation, which had approximately equal median values for IFN- γ in both HHC and PTB (Fig. 4B). The calculated level of significance by Mann-Whitney *U* test for Lpd is *P* = 0.0006. There was a negative correlation, but not significant (*r* = -0.4046, *P* = 0.1347), between Lpd-specific IFN- γ cells and sputum smear grade in PTB, showing a possible

reduction of Lpd-specific IFN- γ cells during active TB (Supplemental Fig. 3). We also performed ROC analysis and determined the cut-off value for Lpd antigen-specific IFN- γ based on the Youden index. The value above this cut-off point was considered positive in both HHC and PTB. ROC curve analysis showed the area under curve of 0.8467, with the *P* value of 0.0005 and confidence interval 95% of 0.7215–0.9719 (Supplemental Fig. 4). With a cut-off value of 0.618 for Lpd-specific CD4⁺ IFN- γ ⁺ T cells, 12 out of 20 HHCs (60%) and 1 PTB (smear grade 1+) out of 15 PTB (6%) patients were found to be positive for Lpd CD4⁺ IFN- γ ⁺ T cells.

The percentage of IL-2-secreting CD4 cells was higher in HHC than PTB (Fig. 4C), and the level of difference was statistically significant (*P* < 0.005) only with Lpd stimulation. No other stimulations gave this significance, despite having higher IL-2-secreting cells in HHC (Fig. 4D). Analysis for antigen-specific TNF- α analysis showed an increase in TNF- α -secreting CD4 cells in

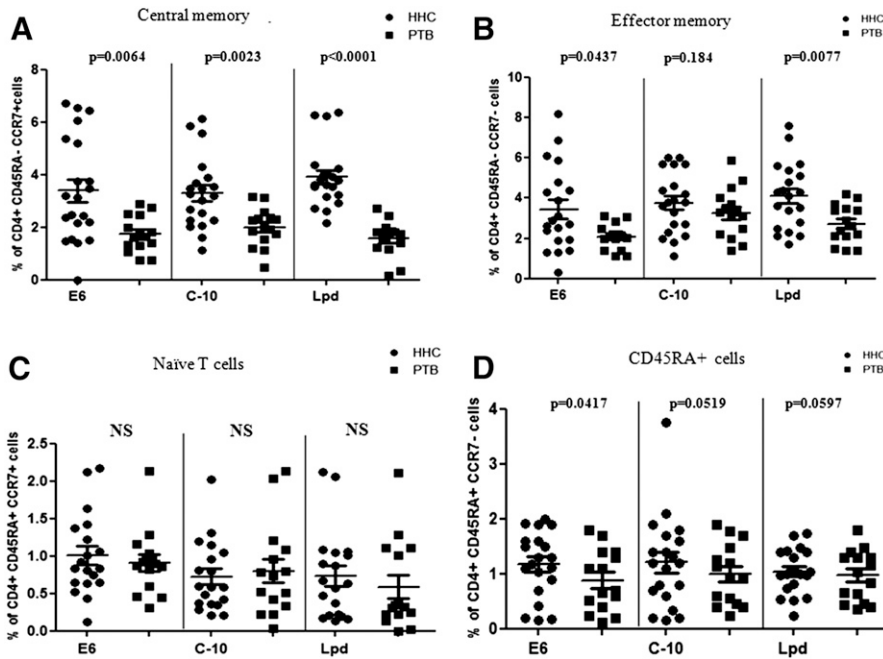


Figure 2. Antigen-specific CD4⁺ memory T cell subtypes in HHC and PTB. Scatter plot shows the percentage of antigen-specific (A) central memory (CD45RA⁻ CCR7⁺) CD4⁺ T cells, (B) effector memory CD4⁺ T cells (CD45RA⁻ CCR7⁻), (C) naive CD4⁺ T cells (CD45RA⁺ CCR7⁺), and (D) CD45RA-expressing CD4⁺ T cells or terminally differentiated CD4⁺ T cells in HHC (●) and PTB (■). Each dot on the plot represents individual study participants, and the middle line represents means with SEM. *P* values were calculated by Mann-Whitney *U* test, and *P* < 0.05 is considered statistically significant. NS, No significance in difference in values between HHC and PTB; E6, ESAT-6; C-10, CFP-10.

HHC with all antigen stimulations when compared with unstimulated cultures (Fig. 4E). A significant difference was found with Lpd stimulation (*P* < 0.05) between the 2 study groups that showed the presence of Lpd-specific TNF-α CD4 cells in the circulation of HHC, and recall responses were observed upon in vitro stimulation (Fig. 4F). In the case of the PTB study group, the baseline levels of TNF-α-secreting CD4 cells were approximately equal to HHC. Upon stimulation of ESAT-6 and CFP-10 antigens, specific elevation of TNF-α was observed, and the Lpd-specific TNF-α response in PTB was minimal.

Based on the coexpression of IFN-γ, TNF-α, and IL-2, the responding CD4⁺ T cells were classified as triple (3+), double (2+), or single (1+) cytokine-producing populations and their possible combinations obtained by using the Boolean gate platform in FlowJo analysis software. **Figure 5A** shows the gating strategy followed in analyzing the multifunctional cells. Although the proportion of triple (3+) T cells that secretes IFN-γ, TNF-α, and IL-2 was higher in HHC compared with PTB with all antigen stimulations, no statistical significance in difference was found. The only phenotype that was higher and statistically significant

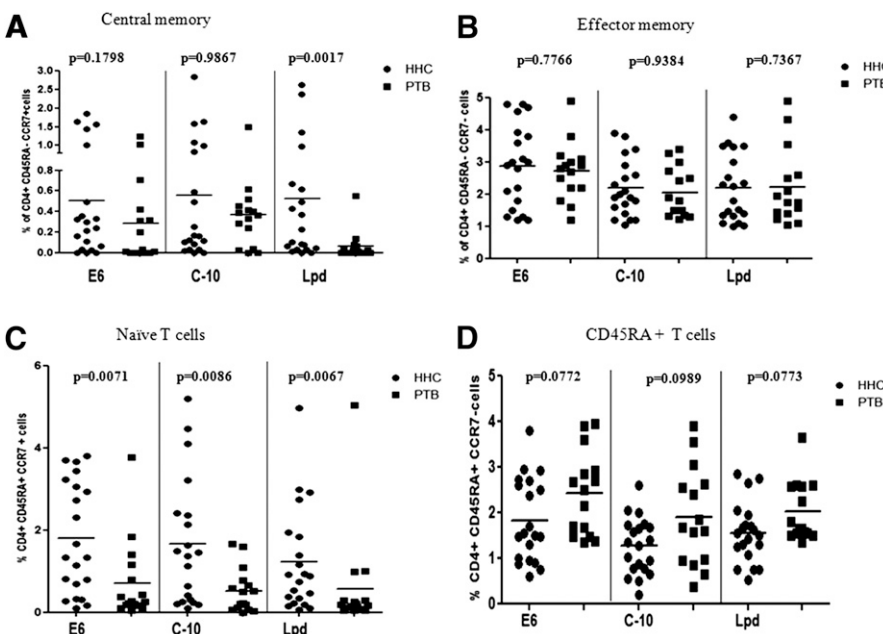


Figure 3. Antigen-specific CD8⁺ memory T cell subtypes in HHC and PTB. Scatter plot shows the percentage of antigen-specific (A) central memory (CD45RA⁻ CCR7⁺) CD8⁺ T cells, (B) effector memory CD8⁺ T cells (CD45RA⁻ CCR7⁻), (C) naive CD8⁺ T cells (CD45RA⁺ CCR7⁺), and (D) CD45RA-expressing CD8⁺ T cells or terminally differentiated CD8⁺ T cells in HHC (●) and PTB (■). Each dot on the plot represents individual study participants, and the middle line represents mean with SEM. *P* values were calculated by Mann-Whitney *U* test, and *P* < 0.05 is considered statistically significant.

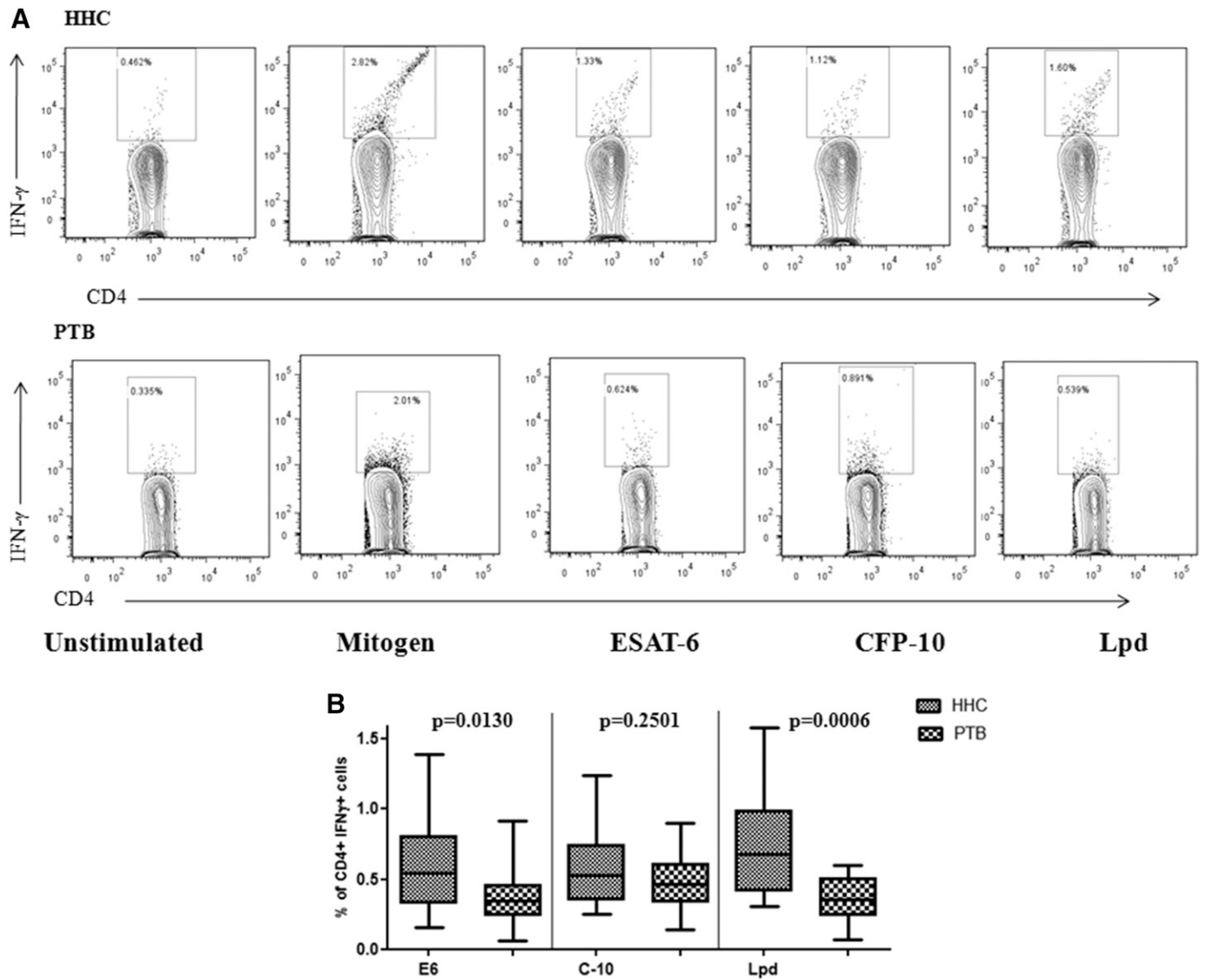


Figure 4. Th1 cytokine-secreting cell analysis in HHCs and PTB. Flow cytometry analysis of Th1 cytokine expression in CD4⁺ T cells after ESAT-6, CFP-10, and Lpd antigen stimulation. Stimulated cultures were analyzed for IFN- γ , IL-2, and TNF- α level. Representative contour plots from HHC and PTB for IFN- γ (A), IL-2 (C), and TNF- α (E) are given. Bar plots represent percentage of a single cytokine secreting CD4⁺ T cells IFN- γ (B), IL-2 (D), and TNF- α (F) with minimum and maximum value. The level of significance was analyzed by Mann-Whitney *U* test, and $P < 0.05$ was considered significant.

($P < 0.05$) in HHC when compared with PTB was IFN- γ ⁺ TNF- α ⁺ phenotype cells against Lpd antigen stimulations. A dual-positive cell that secretes TNF- α ⁺ and IL-2⁺ was slightly higher in PTB, but no statistical significance was obtained when compared with HHC (Fig. 5B). Lpd antigen-specific, multi-functional cells in percentages are shown in Fig. 5C. Elevated levels of an Lpd-specific Th1 response in healthy contacts reveal its possible association with successful containment of active TB disease.

Lpd-specific Th17 response did not vary significantly between HHC and PTB

Th17 cells are characterized by secretion of IL-17 (also called IL-17A), IL-17F, IL-21, and IL-22. Th17 cytokines were readily

detectable upon all stimulations used, primarily in CD3⁺ CD4⁺ T cells. Among the analyzed Th17 cytokines, IL-17 levels were almost equal in both HHC and PTB, except for CFP-10 stimulation. CFP-10 antigen-specific IL-17 was higher in PTB than HHC, with a P value of 0.0553 (Fig. 6A). The IL-21 level was also equal in both groups with all antigen stimulations, except for CFP-10, but encouraging statistical difference was not derived (Fig. 6B).

IL-22 levels were predominant over other Th17 cytokines measured in both HHC and PTB groups. IL-22 levels marginally increased upon ESAT-6 and Lpd stimulations, with a statistical significance of $P < 0.05$ obtained only for Lpd (Fig. 6C). An analysis of the coexpression of IL-17 and IL-22 (Th17⁺ 22⁺) cells showed a higher percentage in the circulation of HHC compared with PTB, with no significance difference. Notably, all Th17 dual

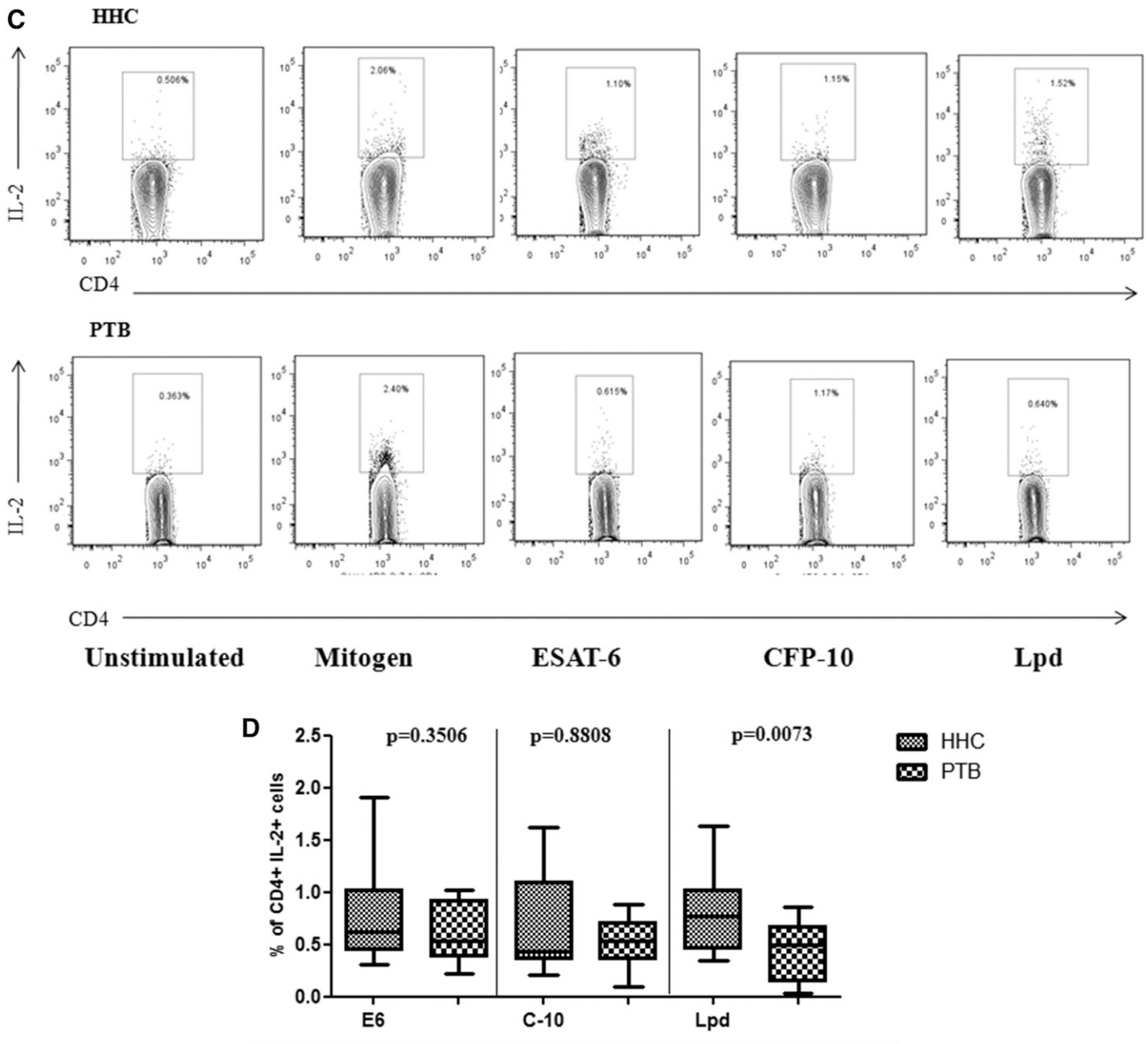


Figure 4. (continued)

cells (Th17+ 22+) showed the highest or equal percentage of IL-22-secreting phenotype compared with that of Th17 dual cells in healthy contacts, whereas in PTB, the IL-22-secreting phenotype was dominant over Th17 dual cytokine-secreting cells. In many PTB study subjects, the complete absence of dual-secreting Th17 cells was observed, but IL-22-secreting phenotype was still present in all stimulations (Fig. 6D).

PTB individuals have increased frequencies of circulating CD4⁺CD25^{high} CD127^{low} FoxP3⁺ cells

An inverse correlation between FoxP3 and CD127 (IL-7R) is well documented and used as a marker for T_{regs}, which were designated as CD4⁺ CD25^{high} CD127^{low/dim} FoxP3⁺ T_{reg}. The

gating criteria followed in defining T_{regs} is given in Fig. 7A. The baseline frequency of T_{regs} was 3-fold (median value of 0.039) higher in peripheral blood of PTB study subjects compared with HHCs (median value of 0.010), with a significance level of $P < 0.05$. We have divided the percentage of CD25^{high} CD127^{low/dim} FoxP3⁺ T_{regs} with the total CD4 count to get the net percentage of T_{regs}, and they are plotted in Fig. 7B.

CFP-10-specific T_{regs} were significant in PTB when compared with HHC ($P < 0.005$). Expansions of other antigen-specific (ESAT-6, Lpd) T_{regs} were not observed in HHCs, showing a lack of antigen-specific T_{regs} in circulation. In PTB, minimal expansion of T_{reg} for ESAT-6 and Lpd was seen compared with CFP-10. Like CD4⁺ T_{regs}, a 3-fold increase was observed with

E HHC

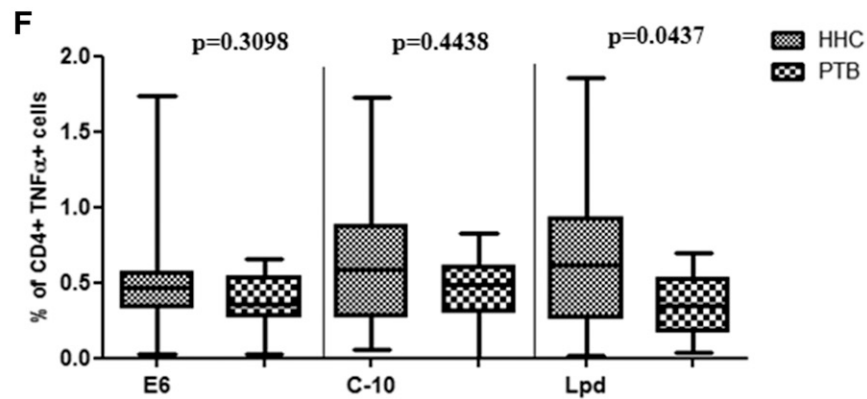
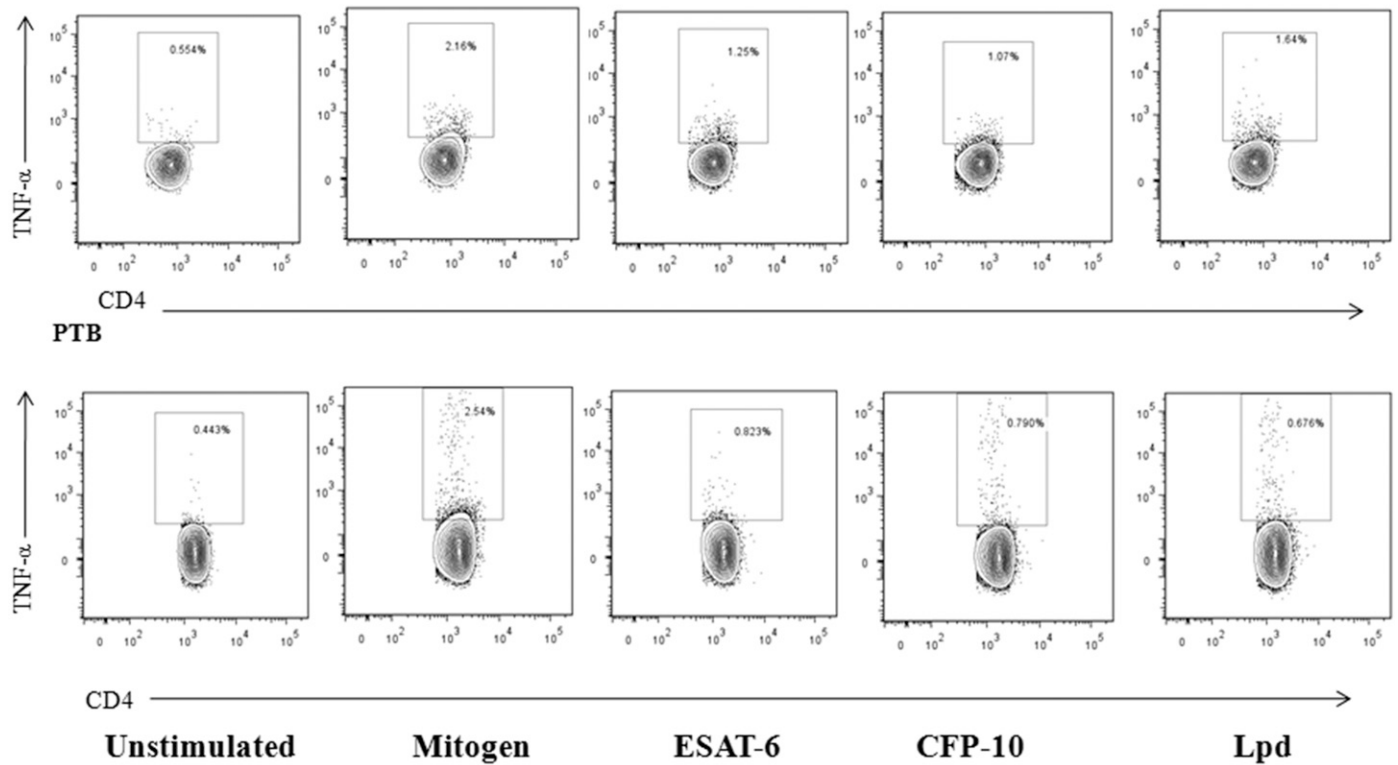


Figure 4. (continued)

circulatory CD8⁺ T_{regs} in PTB, with the same level of significance, but none of the stimulations showed an elevated increase of CD8⁺ T_{reg} in PTB or HHCs (Fig. 7B).

Antigen-specific T_{regs} are often characterized by high levels of IL-10 or IL-10 plus TGF-β production. In our study, we evaluated IL-10 levels against all 3 antigen stimulations. IL-10 levels were high in HHC for ESAT-6 and Lpd stimulations, in contrast to CFP-10, but this difference did not attain statistical significance (Fig. 7C). Low levels of Lpd-specific T_{reg} suggest that the dampening of an Lpd-specific immune response might not have occurred, as very less Lpd-specific T_{reg} expansion was observed. This hypothesis has to be confirmed by further experiments.

DISCUSSION

The quality of the memory response is often important to discern the real difference between protection and immunopathology and to design vaccination strategies [21]. In humans, an association between *M. tuberculosis*-specific central memory cells (high proliferative capacity that rapidly develops into effectors upon re-exposure to antigen) in peripheral blood and latent TB, instead of progression to active TB, has been reported [20]. Our results reiterate this finding that antigen-specific memory cells were higher in the latent population rather than active TB subjects. The Lpd-specific memory cell expansion was observed only in HHC, but not in PTB, indicating the absence or low number of Lpd-specific

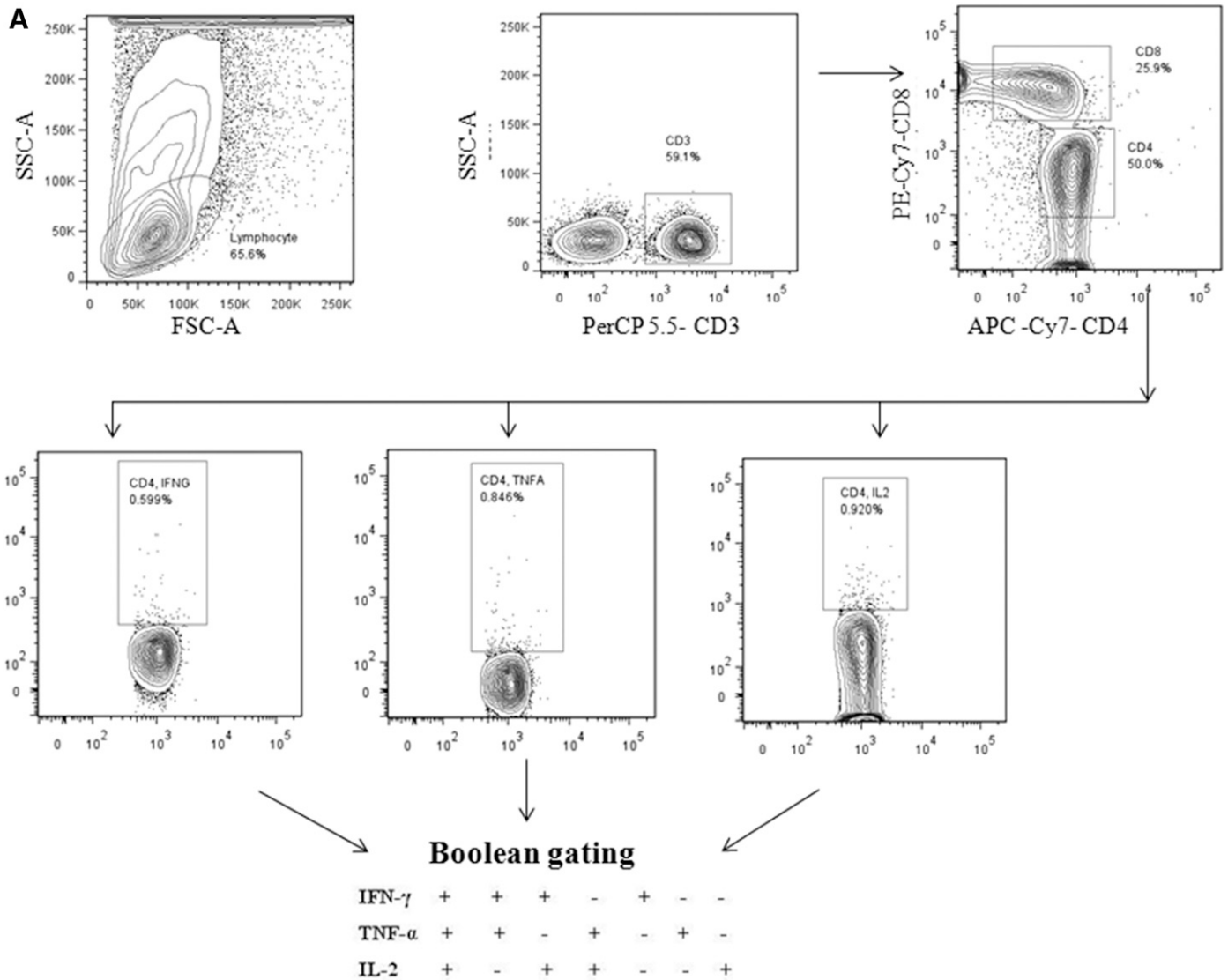


Figure 5. Percentage of triple-, double-, and single-positive Th1 cytokine-secreting CD4 T cells. (A) CD4⁺ T cells were analyzed for coexpression of Th1 cytokines. Gating criteria, followed in analyzing polyfunctional T cells (PFTs), are given. After acquiring, based on the size and granularity, the lymphocyte population was first gated and then analyzed for the expression of the CD3 marker. CD3⁺ cells were subgated according to the expression of CD4 and CD8. The IFN- γ , IL-2, and TNF- α -secreting CD4 cells were selected and submitted to Boolean gating, which automatically generate the combinations (triple, double, and single) of cells secreting IFN- γ , IL-2, and TNF- α . (B) The calculated percentage of triple- and double-positive PFTs against ESAT-6, CFP-10, and Lpd is represented as a bar graph. Triple- and double-positive PFTs were higher in HHC when compared with PTB, but the significance in difference was observed only for Lpd-specific IFN- γ ⁺ TNF- α ⁺ dual-secreting cells. (C) All combinations (triple, double, and single) of PFTs against the antigen Lpd with the pie chart are given and show dominant single cytokine-secreting cells.

memory cells in the circulation of the active TB population. Overexpression of Lpd during in vitro hypoxia in H37Rv and in most prevalent clinical mycobacterial strains as already observed (communicated manuscript). In contrast, ESAT-6 and CFP-10 antigens specific memory cells were comparatively higher in PTB when compared with Lpd-specific memory cells, showing that they are not only specific to latency, but also specific to active TB (Fig. 2A).

The induction of memory T cells by vaccination against intracellular pathogens has definitely been a major challenge for the development of new subunit vaccines. It is also suggested that a better vaccine would generate an effector

memory cell population that is capable of migrating to the site of infection, such as the lungs [22]. Higher baseline levels of the effector memory cell phenotype from our latent study population indicate that the effector memory cell is also needed to control the infection, and its expansion against the Lpd antigen renders for its use in subunit vaccine formulation.

Researchers reported that the T cell responses, memory cell responses, and Th17 responses are altered over the age of 65 [23–25] in humans. Hence, study subjects <60 yr of age were preferred in our study. Our correlation analysis

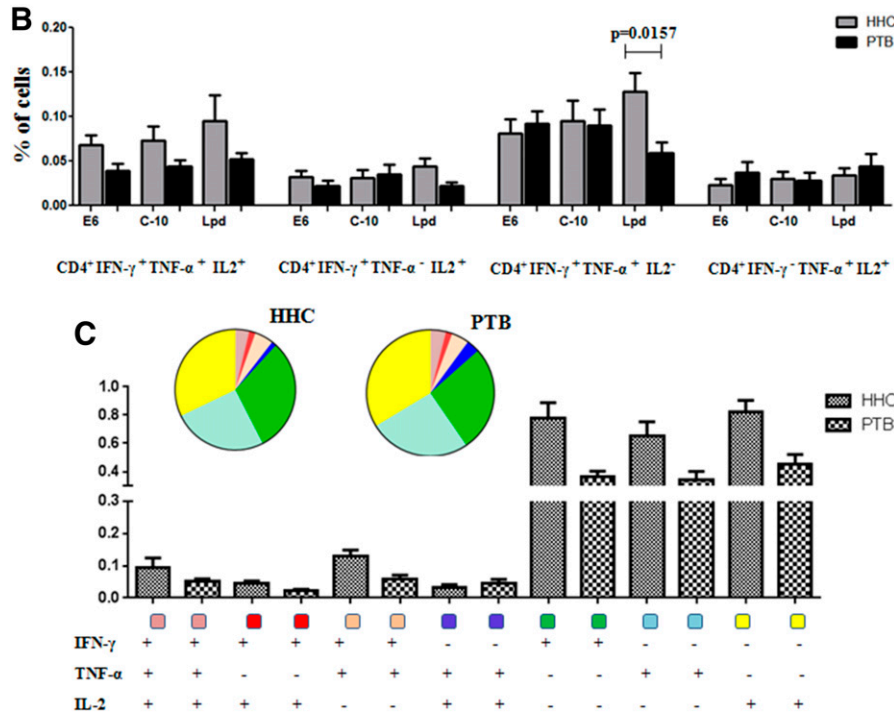


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between Lpd-specific T cells and age (<40 and >40) showed no correlation, indicating Lpd-specific immune responses might not have been influenced by the age in our study (Supplemental Fig. 5).

Optimum resistance to TB also requires CD8⁺ T cells, and the understanding of their role in host immunity to TB aids in designing an optimum TB vaccine [26]. Our data indicate that a recall CD8⁺ central memory response against the Lpd antigen was primarily observed in HHC when compared with PTB. In contrast, the specific CD8⁺ central memory recall response of the standard antigens ESAT-6 and CFP-10 was not only unique to HHC but also to PTB.

In general, production of IFN- γ as a response to *M. tuberculosis*-specific antigens is commonly used as a marker of potentially protective immunity against *M. tuberculosis* [27–29], despite a few contrary reports on IFN- γ roles [30]. Our earlier observation with Lpd showed significantly higher antigenic specificity with IFN- γ in HHC than PTB when measured by cytokine ELISA in whole-blood culture supernatant from d 6 culture [14]. Concordant to this, the present flow cytometry analysis of Th1 cytokines demonstrated higher IFN- γ -secreting cells in Lpd stimulation, particularly in the protected population of TB, and high when compared with ESAT-6 and CFP-10. The IFN- γ levels were less in PTB, and it is already known that an increased frequency of T_{regs} inhibits CD4 IFN- γ induction in PTB [31], which was also observed in our results. Positivity was calculated as number of study subject positive (reactive) to the antigen Lpd. The smear grade of a PTB subject who showed Lpd positivity was 1+, and also, the mean value of an Lpd-specific IFN- γ ⁺ T cell in 1+ sputum smear grade PTB patients (0.508) is

comparatively higher with 2+ (0.279) and 3+ (0.344) smear grade PTB patients. However, the number of PTB patients included in our study was 15 and might not be sufficient to derive such a conclusion on the association of Lpd-specific T cells with the lower bacterial load. Hence, further analysis on a larger study population is highly required to validate our observations.

A dominant TNF- α response is often observed in patients with active TB [32]. Although TNF- α is involved in both the pathophysiology and protection against TB, many emerging reports observe increasing TNF- α response in the latent population as markers of protection against TB [33–36]. In our observations, expansion of antigen-specific TNF- α cells was minimal in PTB with all antigen stimulations used except for CFP-10, showing the lack of these antigen-specific cells in PTB circulation. The high level of TNF- α -secreting cell expansion in HHC against CFA, ESAT-6, and Lpd stimulations probably facilitates effective control of disease progression, as TNF- α is known for enhancing the killing of mycobacteria by macrophages. TNF- α is also important for the long-term control of TB. It is important in orchestrating granuloma formation, which is the hallmark of TB and needed for TB control [31]. In recent times, many mycobacterial proteins have shown an equal or better Th1 response when compared with the standard antigens ESAT-6 and CFP-10 and serve as promising novel antigens for vaccine development and better diagnosis [36–38].

The dominant Th1 response, particularly TNF- α specific to *M. tuberculosis* antigens, was also observed in PTB patients and used for TB diagnosis [39] or as a marker of latent TB reactivation [40]. This contradicts the notion of correlating the antigen-specific Th1 response to protection against TB. Notably,

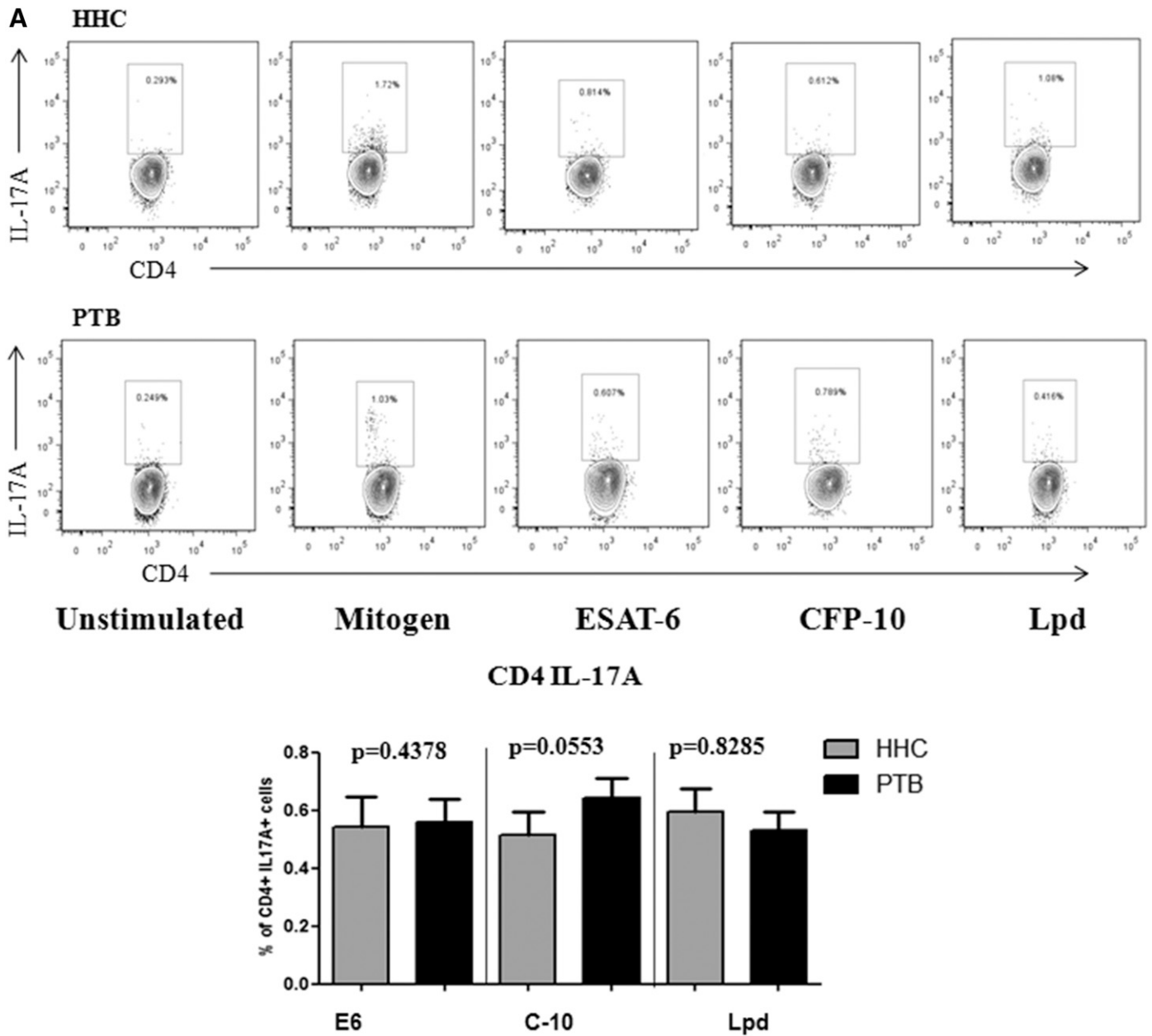


Figure 6. Antigen-specific Th17 response in HHCs and PTB individuals. Th17 cell cytokines IL-17A (A), IL-21 (B), IL-22 (C), and coexpression of IL-17A IL-22 (D) analyzed against all antigen stimulations in HHC and PTB. As shown in the figure, IL-22 levels were higher over other Th17 cytokine-secreting cells in PTB. Coexpression of IL-17A and IL-22 showed that these dual Th17 cytokine-secreting cells were less in PTB and were of IL-22-secreting cells in contrast to HHC. Bar graphs are plotted with means + SEM, and $P < 0.05$ is considered significant.

these reports used the standard antigens, such as ESAT-6, CFP-10, and Ag85A to assess the Th1 response in peripheral blood. In agreement with these reports, we have also observed an ESAT-6- and CFP-10-specific T cell response in TB. This shows that novel antigens from *M. tuberculosis* that are highly specific to latency are needed to study immune correlates of protection in healthy contacts of TB [41].

The role of multifunctional T cells was already registered in the field of TB research in both aspects of protection [42] and immunopathology [43] of TB. As a result of these inconsistent results, the exact roles of multifunctional T cells remain unclear.

Our data showed higher levels of multifunctional T cells in healthy contacts, predictive of an association with protection that has to be confirmed by further evaluation. As it is already known that antigen dose influences the induction of antigen-specific multifunctional T cells [44], this could be a partial contributor to the poor statistical significance observed in our results. The antigen dose that was sufficient to analyze Th1 and memory recall response in our study might not be sufficient to detect the multifunctional recall response in our culture. Interestingly, dual cells that secrete IFN- γ /TNF- α were dominant against Lpd antigen stimulation in HHC. These IFN- γ /TNF- α -secreting cells

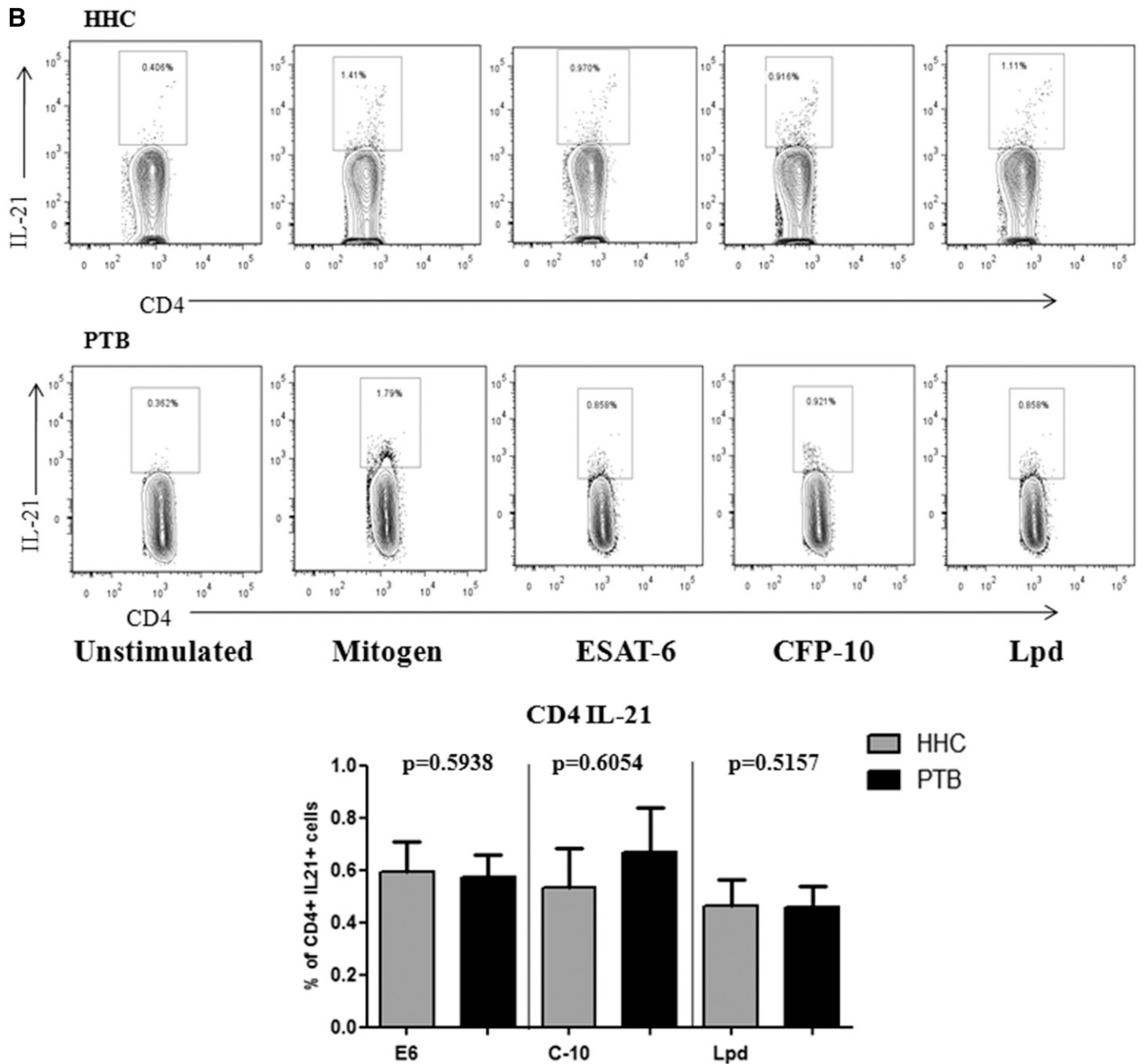


Figure 6. (continued)

were potentially correlated with protection during PCV2 vaccination given for PCV diseases. In addition, this report on PCV2 vaccination, highlighting the central and effector memory T cell phenotype induced by vaccination, confers protection [45]. The ability of the Lpd antigen to induce these memory phenotypes and IFN- γ /TNF- α -secreting cells strongly suggests that it could be used for further vaccine development against TB.

Involvement of Th17 cells that secrete IL-17, IL-21, and IL-22 during TB is emerging but with ambivalent context. Our results implied no difference in the IL-17A response between healthy contacts and the TB-diseased group. However, it was

detectable, unlike other studies, where insufficient, or absence of IL-17A was reported [46]. Regardless, our results are in agreement with other published findings of differential expansion of IL-17 and IL-22 in both the latent and active TB population after stimulation with mycobacterial antigens [47], and these cells might not necessarily be the mediator for TB protection [48].

The only Th17 phenotype that could be correlated with a possible role in protection based on the stimulations used in our study was Th17⁺ 22⁺ dual-positive cells in HHC, as their observed expansion occurs only in this group. In PTB, Th17 dual cells predominantly secreted IL-22, and an increased frequency of

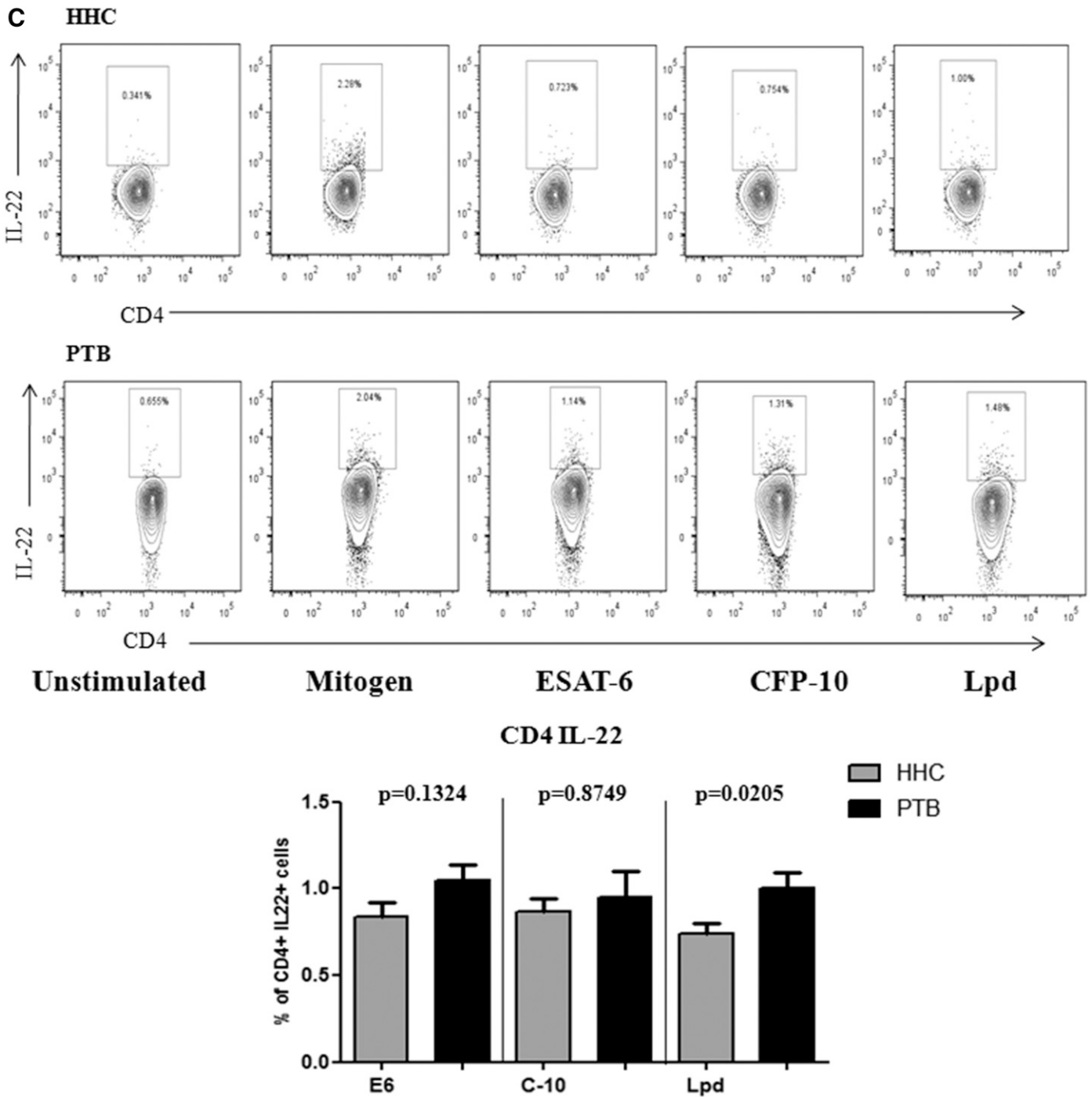


Figure 6. (continued)

IL-22 in bronchoalveolar lavage fluid in patients with active TB was already reported [46]. Based on our results, it is known that IL-17 and IL-22 are related to the same family of distinct responses found in healthy-infected and TB-diseased patients. However, IL-17 is involved in pathogenesis, whereas IL-22 is protective during autoimmune disease, suggesting existence of distinct cytokine mechanisms despite both being generated by Th17 cells [49].

A few reports demonstrated that BCG-specific expansion of T_{regs} can dampen proinflammatory responses and contribute to

its insufficient protection [50–52]. The induction of both $CD4^+$ and $CD8^+$ T_{regs} was observed with BCG in immunized infants and adults [53]; however, the most suppressive T_{regs} coincide with the $CD4^+$ T cells. Standard antigen (ESAT-6 and CFP-10)-specific T_{regs} are known to be correlated with impaired protection against TB [54, 55]. It was also demonstrated that an immune response to predicted mycobacterial-protective antigens was dampened by T_{regs} [56]. Interestingly, Lpd-specific T_{regs} , both $CD4^+$ and $CD8^+$, were not expanded during in vitro stimulation of peripheral

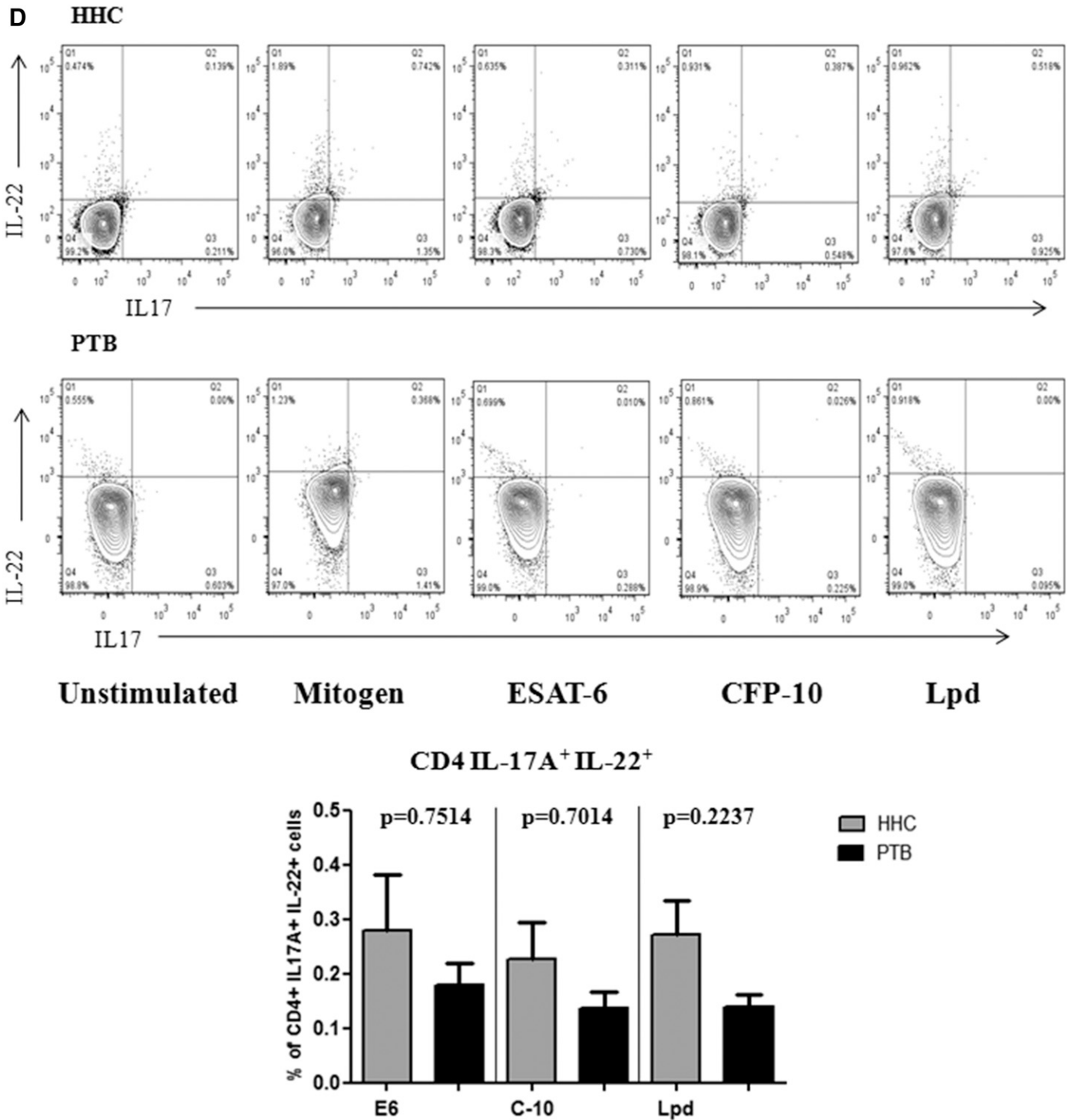


Figure 6. (continued)

blood collected from HHC, showing their possible use as a vaccine target.

Although antigen-specific T_{regs} are characterized by IL-10 or IL-10 plus TGF- β [57–59], an earlier study showed that neutralization of TGF- β had no effect on a purified protein derivative (PPD)-induced IFN- γ response. This suggests that TGF- β might play only a minor role in PTB, unlike IL-10, in

endemic settings. Lower levels of IL-10 were observed in PTB when compared with healthy contacts and control in endemic settings, and the reason is yet to be explored [60, 61].

Collectively, our results showed higher T cell recall response in HHCs from TB endemic regions against the novel T cell antigen Lpd. This is comparably better than proven standard antigens, such as ESAT-6 and CFP-10. Lpd holds promise for developing a

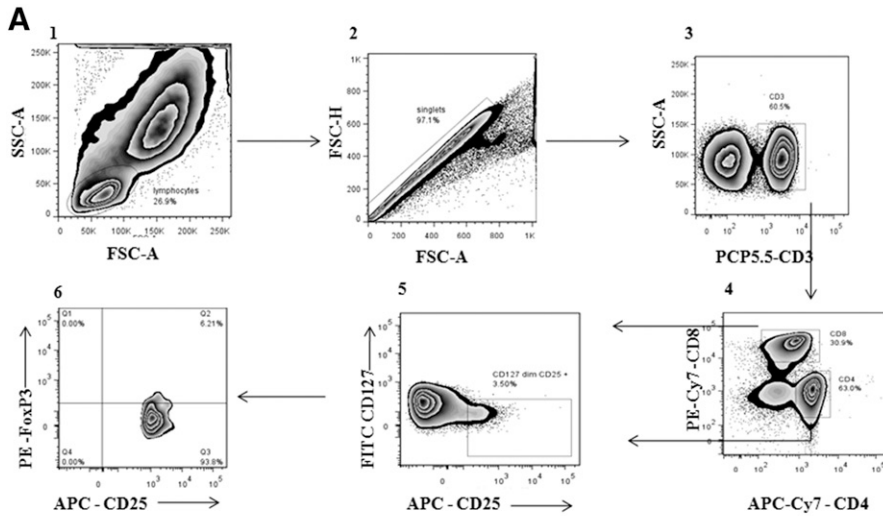
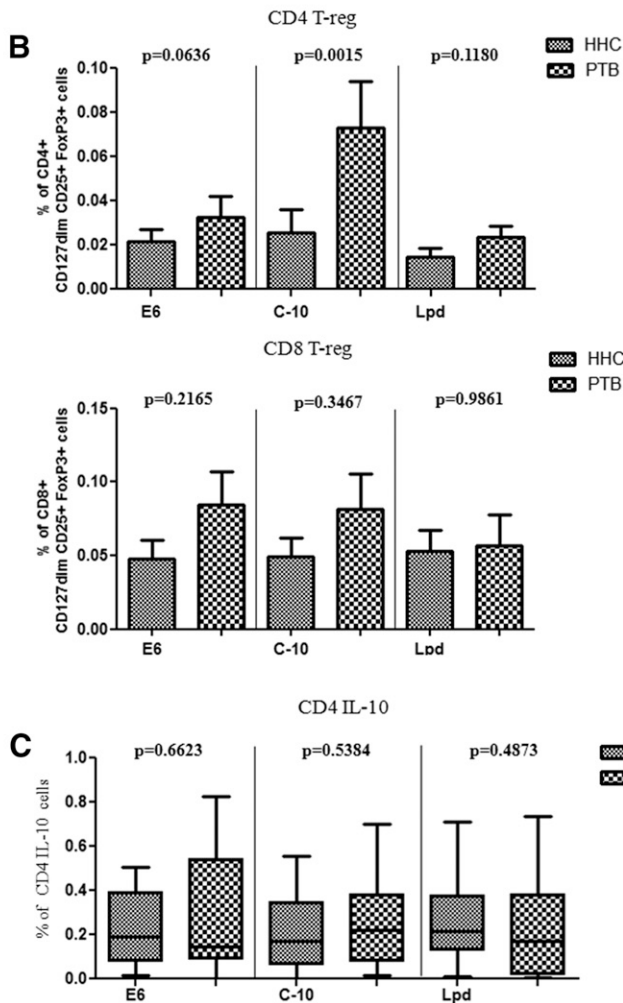


Figure 7. Antigen-specific T_{reg} gating and analysis in HHC and PTB. (A) Gating criteria to analyze the expression of T_{regs} in HHC and PTB against all antigen stimulation are represented as a zebra plot. Initial gating for CD3, CD4, and CD8 (1–4) was similar, as follows for memory cell and Th1 cell analysis. (5) CD4⁺ cells were first analyzed for CD25, a conventional marker, and CD127, whose expression is down-regulated on T_{regs}. Hence, T_{regs} are considered as CD25⁺ CD127^{dim} or ⁻, and these CD25⁺ CD127^{dim} or ⁻ cells were analyzed for the expression of FoxP3, which is a specific marker of T_{regs} (6). PCP5.5, PerCP 5.5. (B) The bar graph represents antigen-specific CD4⁺ and CD8⁺ T_{regs} in circulation of HHC and PTB, and the lines above the bars show means with SEM. *P* values were calculated by Mann-Whitney *U* test, and *P* < 0.05 is considered significant. No significant difference was observed for CD8⁺ T_{regs}. (C) The box and whisker plot represents antigen-specific CD4⁺ IL-10 levels in HHC and PTB with minimum and maximum values. *P* < 0.05 is considered significant.



new subunit vaccine, as earlier reports showed its ability to induce dendritic cells, the most potent APCs known to trigger T cell immunity. One of the reasons for BCG failure is variable efficacy among different ethnicities, but Lpd has a higher population coverage, and MHC binding might overcome ethnicity-based

variations if included in vaccine formulation. The highest T cell memory recall response—dominant Th1 response and minimal T_{reg} response in healthy-infected individuals from an endemic region, such as South India—further supports research on the evaluation of Lpd antigen as a subunit vaccine.

AUTHORSHIP

S.D. and A.R. conceived the study. S.D. performed experiments and analyzed the results. S.D. and A.R. wrote the manuscript.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

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KEY WORDS:

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