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Higher human CD4 T cell response to novel *Mycobacterium tuberculosis* latency associated antigens Rv2660 and Rv2659 in latent infection compared with tuberculosis disease

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ABSTRACT

One third of the world's population is infected with *Mycobacterium tuberculosis* (*M.tb*). A vaccine that would prevent progression to TB disease will have a dramatic impact on the global TB burden. We propose that antigens of *M.tb* that are preferentially expressed during latent infection will be excellent candidates for post-exposure vaccination. We therefore assessed human T cell recognition of two such antigens, Rv2660 and Rv2659. Expression of these was shown to be associated with non-replicating persistence in vitro. After six days incubation of PBMC from persons with latent tuberculosis infection (LTBI) and tuberculosis (TB) disease, Rv2660 and Rv2659 induced IFN-γ production in a greater proportion of persons with LTBI, compared with TB diseased patients. Persons with LTBI also had increased numbers of viable T cells, and greater specific $CD4^+$ T cell proliferation and cytokine expression capacity. Persons with LTBI preferentially recognize Rv2659 and Rv2660, compared with patients with TB disease. These results suggest promise of these antigens for incorporation into post-exposure TB vaccines.

1. Introduction

The success of *Mycobacterium tuberculosis* (*M.tb*) as a human pathogen may be ascribed to its ability to persist for long periods in asymptomatic people, a state known as latency [1]. Healthy latently infected individuals have approximately 10% lifetime risk of developing active TB disease [2]. This risk increases to approximately 10% per year in HIV-infected persons not on antiretroviral therapy [3]. Approximately one third of the world's population is infected with *M.tb*, 9 million develop TB disease and 1.8 million die annually [4,5]. To control this pandemic, an effective vaccine is urgently needed; however, this requires extensive insight into the mechanisms underlying protective immunity against *M.tb*.

The low bacterial burden associated with a latent infection has proven to be a major obstacle in characterizing the mechanisms by which *M.tb* persists and reactivates in the host [2]. In latent granulomatous lesions, *M.tb* is successfully contained and has to adapt to a hypoxic and nutritionally compromised environment [6]. It has recently been hypothesized that this immune pressure drives *M.tb* into a different metabolic state, compared with actively replicating organisms in lesions that characterize early *M.tb* infection or active disease [1]. This is supported by findings from *in vitro* studies which mimic the granuloma environment, where *M.tb* was shown to upregulate sets of genes that are distinct from those upregulated in actively replicating organisms; e.g., in nutrient deficient medium, a particular set of genes that were termed the "starvation stimulon" were upregulated [7]. We focused on 2 proteins of the starvation stimulon, Rv2660 and Rv2659, and hypothesized that these antigens will be preferentially recognized by persons with latent *M.tb* infection (LTBI), compared with persons with TB disease. Rv2660 and Rv2659 belong to the RD11 region encoded in *M.tb* [8], however the function of these proteins has not been determined [7].

The rationale for these studies is that latency-associated antigens might in the future be incorporated into novel post-exposure TB vaccine candidates, which could protect infected individuals from developing TB disease [1].

Abbreviations: *M.tb*, *Mycobacterium tuberculosis*; LTBI, Latent tuberculosis infection; OG, Oregon green.

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2. Materials and methods

2.1. Study participants

Participants were recruited from the Western Cape region of South Africa. Persons with LTBI were included if they had a positive QuantiFERON®-TB Gold In-Tube (QFT™) test, or a positive ELISPOT test for ESAT-6/CFP-10 (>17 spots/10^6 PBMC after background subtraction), or a positive Mantoux skin test (induration > 10 mm), for greater than 1 year at time of recruitment. Any clinical signs or symptoms suggestive of TB disease, a history of TB treatment, HIV infection, any other acute or chronic medical conditions, receipt of immunosuppressive medication, or a hemoglobin level < 9 g/dL resulted in exclusion.

Patients with TB disease were also included, if they had 2 positive sputum smears and/or a positive culture, with clinical features consistent with TB disease. Patients with extra-pulmonary TB disease were excluded, as were patients with exclusion criteria mentioned for LTBI above. Informed consent was obtained from all participants prior to study participation. The study was approved by the research ethics committees of the University of Cape Town and the University of Stellenbosch.

2.2. Whole blood incubation

Whole blood was collected in heparinised syringes. Blood was diluted in RPMI-1640 medium (Sigma) containing 1% L-glutamine (Sigma, final dilution 1:10), and incubated with an ESAT-6 peptide pool (17mers, overlapping by 10, final concentration 1 μg/mL/peptide), CFP-10 peptide pool (17mers, overlapping by 10, final concentration 1 μg/mL/peptide), TB10.4 protein (10 μg/mL), Rv2660 peptide pool (20mers, overlapping by 10, final concentration 1 μg/mL/peptide), or Rv2659 peptide pool (20mers, overlapping by 10, final concentration 1 μg/mL/peptide), in 200 μL volumes in 96-well U-bottom plates (Nunc, Cat No. 163320), for 6 days at 37 °C in 5% CO_2. All antigens were obtained from the Statens Serum Institute, Denmark. Blood incubated without antigen and blood incubated with phytohaemagglutinin (PHA, 5 μg/mL) were used as negative and positive controls, respectively. After incubation, 150 μL culture supernatant was removed and stored at −80 °C until further analysis.

2.3. PBMC incubation

PBMC were isolated by density gradient centrifugation and cryopreserved in 10% DMSO (Sigma–Aldrich) in heat-inactivated fetal calf serum (Adcock Ingram). PBMC were thawed in 12.5% AB serum/RPMI containing 2.5 μg/mL DNase (Sigma–Aldrich) and stained with 10 μg/mL of CellTrace Oregon Green 488 (Molecular Probes, Invitrogen) per 1×10^7 cells. The PBMC were resuspended in 12.5% AB serum in RPMI, and 200,000 cells in 200 μL medium were rested overnight at 37 °C, 5% CO_2 in a 96 well plate. Duplicate wells containing cells (i.e. 400,000 PBMC per condition) were stimulated with BCG (1331 Danish BCG) at an MOI of 0.01, PPD (0.5 μg/mL), ESAT-6/CFP10 peptide pool (2 μg/mL/peptide), TB10.4 peptide pool (15mers, overlapping by 10, final concentration 1 μg/mL/peptide), Rv2660 peptide pool (20mers, overlapping by 10, final concentration 1 μg/mL/peptide), Rv2659 peptide pool (20mers, overlapping by 10, final concentration 1 μg/mL/peptide), SEB (0.05 μg/mL, positive control, Sigma–Aldrich) or no antigen (negative control), for 6 days at 37 °C with 5% CO_2. The optimum concentrations of Rv2660 and Rv2659 peptide pools were determined in preliminary studies to be 1 μg/mL/peptide. All antigens were obtained from the Staten Serum Institute, Denmark. After incubation, plasma was collected and stored at −80°C until further use. PBMC were then incubated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (250 ng/mL), to induce cytokine production, and Brefeldin A (10 μg/mL) was added while incubation continued for a further 5 h. PBMC were harvested, stained for viability with LIVE/DEAD Fixable Violet Dead Cell Stain (ViViD, Invitrogen), fixed with FACS Lysing Solution (BD Biosciences), and cryopreserved until later analysis.

2.4. Flow cytometric analysis of processed PBMC

Fixed, cryopreserved PBMC were thawed and permeablised with Perm/Wash Solution (BD Biosciences) for 10 min. PBMC were washed with PBS and stained with the following antibodies: anti-CD3 Qdot605 (clone UCHT1, invitrogen), anti-CD8 PerCPCy5.5 (SK-1), anti-IFN-γ AlexaFluor700 (B27), anti-IL2 APC (MQ1-17H12) and anti-TNF-a Cy7.PE (MAb11) (all from BD Biosciences) for 1 h at 4 °C. After washing, cells were acquired on a LSRII flow cytometer (BD Biosciences), configured with 3 lasers and 10 detectors, using the FACS Diva 6.1 software. Compensation settings were defined using anti-mouse kappa Comp Beads (BD Biosciences) stained with each fluorochrome-conjugated antibody. Because the cellular dyes Oregon Green and ViViD do not bind Comp Beads, compensation of these dyes was done using FITC and Pacific Blue conjugated antibody-bound Comp Beads, respectively. Data was analyzed using Flowjo 8.8.4 (Treestar). Frequencies of proliferating and cytokine-expressing CD4 or CD8 T cells were determined after exclusion of dead cells ($ViViD^{high}$ events). Boolean gating was applied to generate combinations of cytokine expressing $CD4^+$ and $CD8^+$ T cell subsets.

2.5. IFN-γ measurement in supernatants

IFN-γ was quantified in culture supernatants by sandwich ELISA. The ELISA was performed as previously described [9], with the following modifications: OPD Fast (Sigma) was utilized as substrate, and plates were read at 405 nm on a Versemax ELISA plate reader using Softmax Pro software Version 4.7.1. The limit of detection of the ELISA was determined to be 15.6 pg/mL. A 1:2 dilution of supernatants was used, which was found optimal in preliminary experiments.

2.6. Data analysis

For soluble IFN-γ analysis, antigen-specific cytokine levels were determined by subtracting levels from unstimulated conditions. A positive IFN-γ response was defined as 31.2 pg/mL, after background subtraction.

For the flow cytometric assay, proliferation and cytokine expression levels from the unstimulated condition (restimulated with PMA and Ionomycin) were subtracted from levels obtained after antigen-specific stimulation. Individuals were excluded from analysis for samples with <100 viable $CD3^+$ T cells, or if the positive control yielded a negative result. The Mann–Whitney *U* test was used to assess differences between persons in the two groups. The Fisher's exact test was used to assess differences between proportions of responders. *p*-Values < 0.05 were considered to be significant.

3. Results

3.1. Participant characteristics

We examined immune recognition of Rv2660 and RV2659, measured by IFN-γ expression in whole blood in a pilot study on 21 adults with LTBI. The median age was 32 years (interquartile range: 28–40); 90% were female. All participants had been vaccinated with BCG at birth, and all were from a mixed ethnic background.

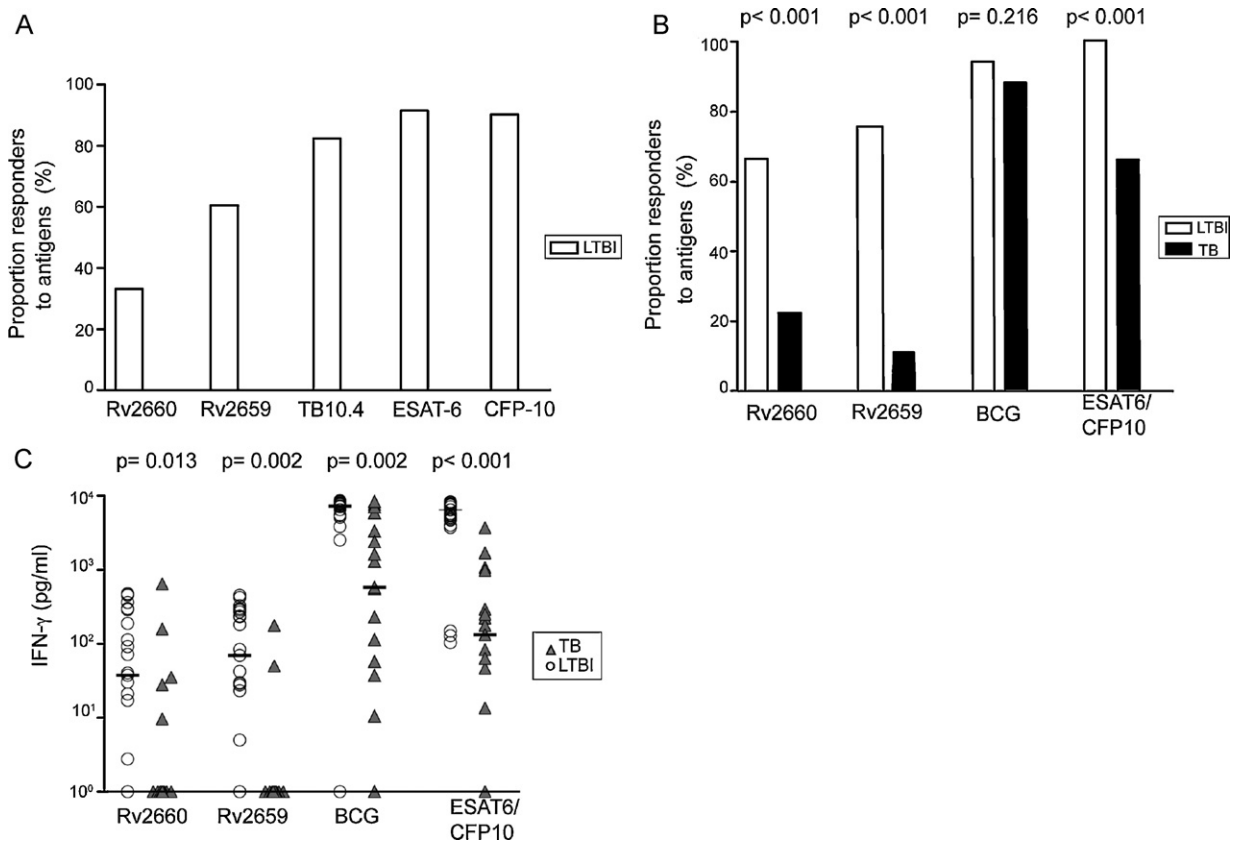

Fig. 1. Recognition of novel TB antigens in persons with LTBI and with TB disease. (A) Whole blood from persons with LTBI was incubated for 6 days with mycobacterial antigens and IFN-γ levels in supernatants measured by ELISA. The proportion of persons with a positive response, defined as an IFN-γ level above 31.2 pg/mL, following subtraction of the IFN-γ level from the unstimulated control, is shown. (B) PBMC from a different cohort of persons with LTBI or TB disease were incubated with the antigens for 6 days with mycobacterial antigens and IFN-γ levels in supernatants measured by ELISA. Proportion of persons with a detectable response, defined as mentioned above, is shown. The Fisher's exact test was used to assess differences between proportions of responders. (C) IFN-γ response (pg/mL) to the antigens indicated. The medians are represented by the grey horizontal bars. A positive response was defined as an IFN-γ level above 31.2 pg/mL after background subtraction. The Mann–Whitney *U* test was used to assess differences between the 2 groups.

Following the pilot study, PBMC proliferation and cytokine production were studied in 25 additional persons with LTBI, as well as 25 patients with TB disease. The median ages of the LTBI and TB diseased groups were 19 years (18.5–31.5) and 31 years (23.5–42), respectively ($p = 0.003$); 64% of individuals with LTBI were female, compared with 24% in the TB group ($p = 0.044$). Due to the difference in age and gender we compared the outcomes with respect to these co-variates. Only polyfunctional $CD4^+$ T cells, expressing IFN-γ, TNF-α and IL-2 were different in males and females. Participants excluded from analysis, with criteria for exclusion, are shown in Supplementary Fig. 1. Twenty-eight % of TB patients were excluded due either culture contamination or <100 viable $CD3^+$ T cells; 8% of LTBI participants were excluded due to a negative SEB result.

3.2. *Rv2660 and Rv2659 are recognized by T cells of persons with established latency*

To assess whether Rv2660 and Rv2659 are recognized during established human latent *M.tb* infection, diluted whole blood was incubated with the antigens for 6 days, and IFN-γ measured in the supernatant. Rv2660 and Rv2659 induced detectable IFN-γ production in a high proportion of persons with LTBI (Fig. 1A). The immunodominant control antigen, TB10.4, present in both BCG and *M.tb*, and the *M.tb* – specific antigens ESAT-6 and CFP-10, were recognized in substantially greater proportions of individuals (Fig. 1A). The positive control, SEB induced a response in all individuals (data not shown).

To compare immune recognition of Rv2660 and Rv2659 during established latent infection and TB disease, we incubated PBMC with these antigens for 6 days, followed by measurement of IFN-γ in supernatants. A greater proportion of persons with LTBI recognized Rv2660 and Rv2659, compared with patients with TB disease (Fig. 1B). A similar proportion responded to BCG; however, more persons with LTBI responded to ESAT-6/CFP-10, compared with patients with TB disease. Further, levels of IFN-γ in response to all antigens were higher in the LTBI group, compared with the TB diseased group (Fig. 1C).

3.3. *Decreased viability of $CD3^+$ T cells during TB disease*

We hypothesized that lower Rv2660 and Rv2659 recognition during TB disease is due to lesser capacity of peripheral blood T cells of TB patients to survive, compared with cells from persons with LTBI. After 6 days of incubation of PBMC in conditions as aforementioned, cell viability was assessed with flow cytometry following staining with the Violet amine reactive dye, ViViD (Fig. 2A). The LTBI group had significantly higher frequencies of viable $CD3^+$ T cells, for all conditions tested (Fig. 2B). The median number of viable $CD3^+$ T cells was 15,771 (IQR, 4,912-99,654; Supplementary Fig. 2).

3.4. *Increased frequency of proliferating Rv2660- and Rv2659-specific $CD4^+$ T cells during established latency*

We further characterized viable $CD4^+$ T cell responses to Rv2660 and Rv2659 during established latent infection and TB disease by

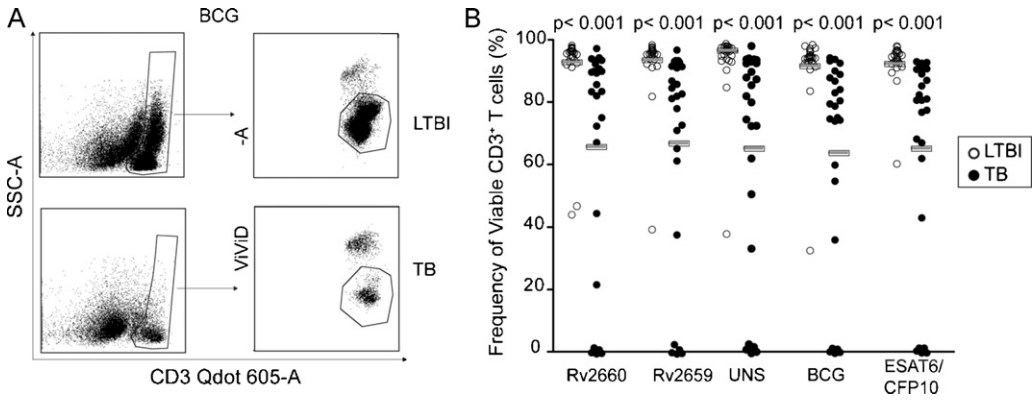

Fig. 2. $CD3^+$ T cell viability. Comparison of viable $CD3^+$ T cells in PBMC of LTBI and TB individuals stimulated with BCG and *M.tb*-specific antigens for 6 days. (A) Viable T cells were selected by gating on $CD3^+$ T cells against ViViD. (B) Frequency of viable $CD3^+$ T cells. The medians are represented by the grey horizontal bar. The Mann–Whitney *U* test was used to assess differences between the 2 groups.

assessing antigen-specific proliferation with a dye dilution assay, and the cytokine producing capacity of proliferating cells (Fig. 3A and B, respectively). Frequencies of proliferating antigen-specific $CD4^+$ T cells were higher in LTBI, compared with TB disease, for all antigens tested but the difference was clearly more pronounced for BCG and the ESAT6/CFP10 antigens (Fig. 4C–F, left panels).

The cytokine expression profile of proliferating $CD4^+$ T cells was assessed by adding a polyclonal stimulant for the last 5 h of incubation. Upon stimulation with Rv2660 and Rv2659 antigens, frequencies of proliferating $CD4^+$ T cells were higher in the LTBI group for all cytokine producing subsets, except for the total IL-2^+ population (Fig. 4C and D, right panels, respectively). In addition, Rv2660 and Rv2659 induced more polyfunctional (co-expression of IFN-γ^+, TNF-α^+ and IL-2^+ in the same cell) cytokine expression in persons with LTBI, compared with TB patients. In response to BCG- and to ESAT-6/CFP-10, specific (proliferating) $CD4^+$ T cells were also more likely to be polyfunctional in persons with LTBI, compared with patients with TB disease (Fig. 4E and F); total IFN-γ^+, total TNF-α^+ and total IL-2 were also higher in persons with latent infection (Fig. 4E and F, right panels).

$CD8^+$ T cell proliferation and cytokine expression in response to Rv2660 and Rv2659 was very low, and no difference was demonstrated between the 2 groups (Fig. 4A and B). However, persons with LTBI had greater frequencies of $CD8^+$ T cells that proliferated in response to ESAT-6/CFP10 and BCG, compared with frequencies in patients with TB disease. Proliferating cells were also more likely to be polyfunctional in persons with LTBI (Fig. 4C and D). The positive control, SEB induced a response in all individuals (data not shown).

4. Discussion

Mathematical modeling has indicated that an effective post-infection vaccine that targets latently infected individuals, would have a dramatic impact on the TB epidemic [10]. Our study suggests that Rv2660 and Rv2659 are good antigenic candidates for inclusion in such a vaccine; we show that T cells of persons with LTBI preferentially recognize these two latency-associated antigens, compared with T cells from patients with TB disease. This is the first clinical report of human immune recognition of the starvation stimulon gene products, Rv2660 and Rv2659.

Interestingly, the same pattern of preferential recognition in LTBI, compared with TB disease, was observed for the crude mycobacterial antigens BCG, PPD, and the immunodominant/early antigens ESAT-6/CFP-10 and TB10.4 (data for PPD and TB10.4 not shown). In agreement with our findings, Schuck et al. [11] recently reported higher T cell responses upon stimulation of PBMC with PPD and ESAT-6/CFP10, and with other latency antigens, such as Rv1733 and Rv0140, in individuals with LTBI, compared with TB patients. This decreased T cell response in peripheral blood may result from migration of specific T cells to the lung during active disease [12], or, with respect to Rv2659 and Rv2660, may relate to a preferential expression of these two antigens during latency. Alternatively, aberrant immune regulation during disease, mediated by regulatory T cells [11], anti-inflammatory cytokines such as IL-10 and TGF-β [13,14], or Th2 cytokines like IL-4 and IL-13 may be responsible for the differences noted [15]. Our observation that T cells from patients with TB disease were strikingly less likely to survive in a 6-day culture, compared with T cells from persons with LTBI, suggest that T cell exhaustion may also be responsible for lower responses in the diseased group. In chronic infections such as HIV and CMV, a persistently high antigenic load drives specific T cell exhaustion and dysfunction [16,17]. These cells upregulate markers like PD-1, and are more prone to apoptosis [18]. We postulate that this also occurs during active TB disease. We have recently shown that PD-1 expression is increased on *M.tb*-specific $CD4^+$ T cells in TB diseased patients, compared to persons with LTBI (C. Day, unpublished observations).

Recent advances in imaging have led to the observation that granulomatous lesions in persons with LTBI reflect a spectrum of differential ability to support or suppress the persistence of viable bacteria [19]. This spectrum is supported by our data of similar preferential recognition of latency-associated and immunodominant antigens in persons with LTBI, compared with active TB. A post-exposure vaccine would thus ideally contain both immunodominant and latency-associated antigens, respectively, to target replicating or persisting bacteria in progressing lesions and dormant bacteria in successful lesions.

LTBI individuals had elevated frequencies of Th1 cytokine expression among proliferating $CD4^+$ T cells, compared with those in TB patients. The pattern of cytokine production also differed: specific T cells from persons with LTBI were more likely to be "polyfunctional", i.e., able to co-express IFN-γ, TNF-α and IL-2. This characteristic has been proposed to be associated with long-term memory and efficient protection against intracellular infections like *M.tb* [20–23]. We therefore propose that robust polyfunctional responses to latency and immunodominant antigens may prevent LTBI individuals from progressing to TB disease. The lesser cytokine production in TB disease may also be due to T cell exhaustion, leading to a loss of function in particular for antigens like ESAT6/CFP10 that predominate during the early stages of infection, and where continued exposure may lead to exhaustion [16]. Therefore, because latency associated antigens would not be expressed in early stages of infection, T cells directed to these

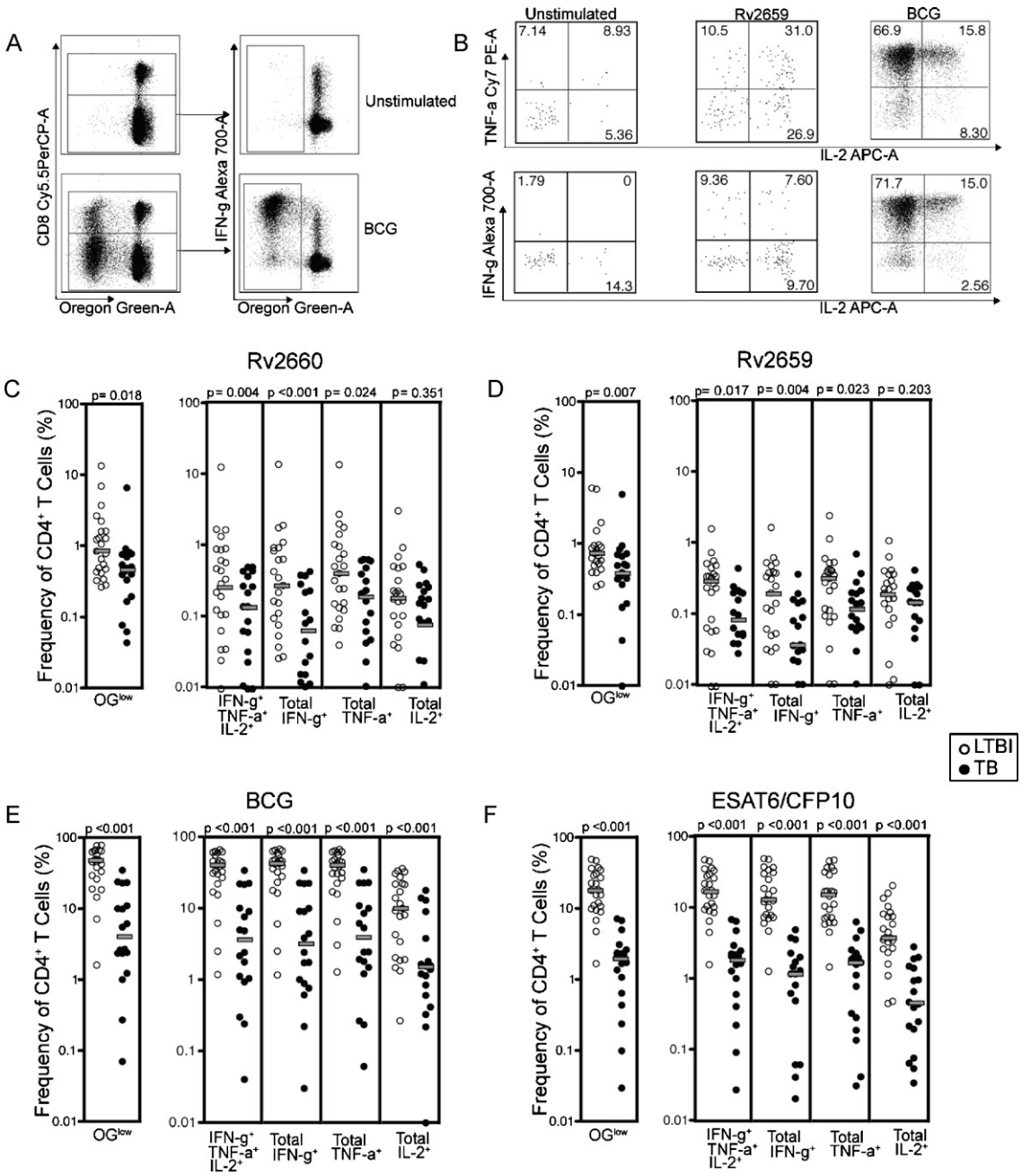

Fig. 3. Gating strategy to analyze the cytokine expression profiles of proliferating CD4⁺ and CD8⁺ T cells. PBMC were stained with Oregon Green and stimulated with mycobacterial antigens, SEB, or were left unstimulated for 6 days, and analyzed by flow cytometry. (A) Doublet cells were excluded by gating on forward scatter-height (FSC-H) against forward scatter-area (FSC-A), and viable T cells were then selected by gating on $CD3^+$ against ViViD. $CD3^+$ T cells were further differentiated into proliferating (OC^{low}) $CD8^-$ and $CD8^+$ T cell subsets by staining for $CD8^+$ ($CD3^+CD8^- = CD4^+$ T cells, $CD3^+CD8^+ = CD8^+$ T cells), and then cytokine expression patterns were analyzed in proliferating T cells. (B) Representative dot plots of cytokine co-expression patterns in unstimulated or antigen-specific proliferating $CD4^+$ T cells. (C–F) Comparison of T cell proliferation and intracellular cytokine expression in individuals with LTBI or TB disease. $CD4^+$ T cell antigen-specific proliferation upon incubation with Rv2660 (C), Rv2659 (D), BCG (E), and ESAT-6/CFP-10 (F), detected by gating on Oregon $Green^{low}$ $CD4^+$ T cells. Frequencies of $CD4^+$ T cell cytokine expression profiles; IFN-γTNF-α^+IL2^+, total IFN-γ, otal TNF-α^+ and total IL-2^+, of proliferating cells following incubation of PBMC with antigens (A–D, right panels), detected by intracellular cytokine staining. The medians are represented by the grey horizontal bar. The Mann–Whitney *U* test was used to assess differences between the 2 groups.

antigens may not be subjected to regulation by the established effector and/or regulatory T cells primed in the earlier stages of infection.

$CD8^+$ T cell responses to immunodominant antigens could readily be detected, and were higher in LTBI compared with TB disease. However, $CD8^+$ T cell responses to Rv2659 and Rv2660 were very low, which may be the reason why no difference could be demonstrated between the participant groups. We cannot exclude that differences in antigen presentation between $CD4^+$ and $CD8^+$ T cells could have affected these results.

A limitation of our study was the different age and gender ratios in the LTBI and TB groups. Ideally, participants in such groups should be matched for these co-variates. However, given the fact that only polyfunctional $CD4^+$ T cells and no other outcomes were different in males and females, the unequal gender ratio was unlikely to have markedly confounded our results.

The prophylactic TB subunit vaccines currently under development are almost exclusively based on early or immunodominant antigens secreted by replicating *M.tb*, which are recognized in the first stage of infection, and are designed as BCG boosters to prevent

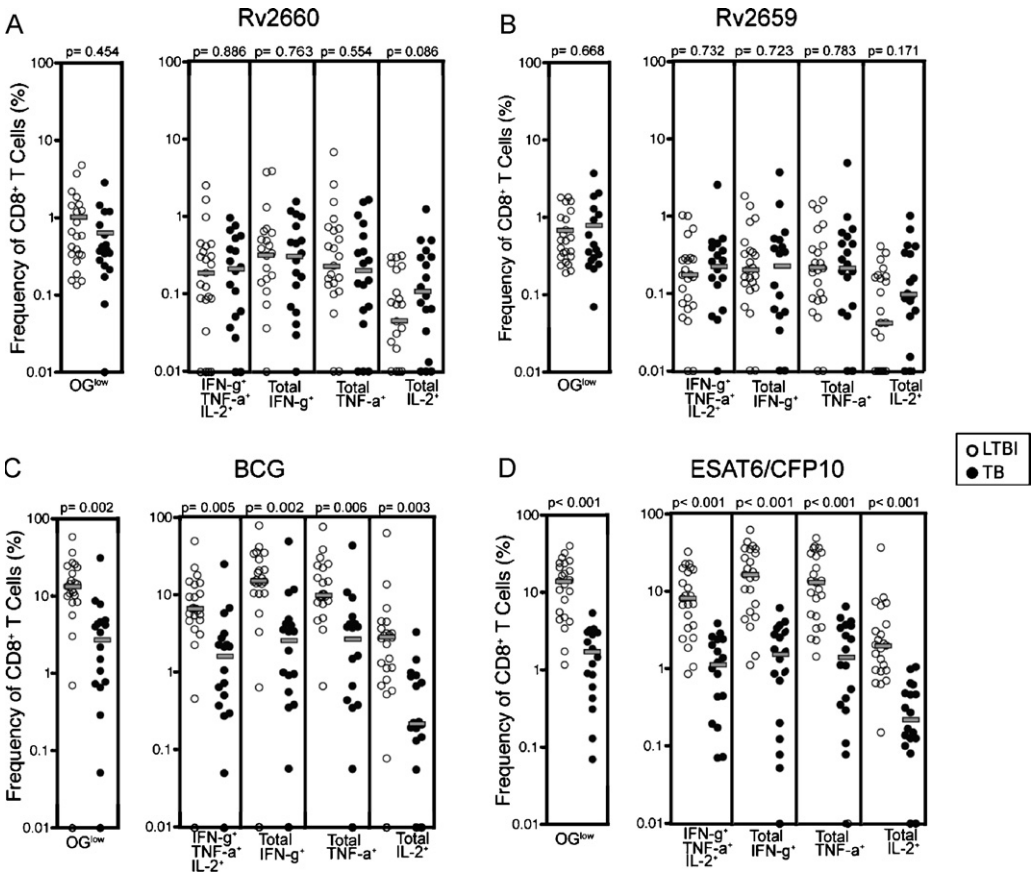

Fig. 4. Rv2660- and Rv2659-specific $CD8^+$ T lymphocyte proliferation and cytokine expression profiles. Comparison of T cell proliferation and intracellular cytokine expression in individuals with LTBI or TB disease. $CD8^+$ T cell antigen-specific proliferation upon incubation with Rv2660 (A), Rv2659 (B), BCG (C) and ESAT-6/CFP-10 (D), detected by gating on Oregon $Green^{low}$ $CD4^+$ T cells. Frequencies of $CD8^+$ T cell cytokine expression profiles; IFN-γTNF-α^+IL2$^+$, Total IFN-γ, Total TNF-α^+ and Total IL-2^+, of proliferating cells following incubation of PBMC with antigens (A–D, right panels), and detected by intracellular cytokine staining. The medians are represented by the grey horizontal bar. The Mann–Whitney *U* test was used to assess between the 2 groups.

primary TB disease [24]. Addition of latency associated antigens to well-established prophylactic vaccines (i.e., the vaccine candidates under clinical testing today) has been suggested as the basis for a future generation of multi-stage TB vaccines with activity against all stages of infection [1]. Our finding of immune recognition of these novel antigens in persons with LTBI has contributed to the development of a new subunit vaccine, H56, by the Statens Serum Institute. This vaccine is based upon the H1 vaccine currently in clinical trials, which contains Ag85B and ESAT-6, and the Rv2660 antigen, and will be the first to enter clinical trials as a post-infection vaccine candidate [25]. We hypothesize that inducing robust T cell responses to latency and immunodominant antigens may enhance immunological control of latent TB, and prevent reactivation. Much more research is needed to determine whether candidate latency-, starvation-, or resuscitation-associated antigens other than Rv2659 or Rv2660 would be needed for inclusion in an optimal post-infection vaccine.

Ultimately, a combined strategy that includes primary vaccination with a live attenuated vaccine followed by a multistage subunit boost vaccine; may have maximum impact on the TB epidemic.

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Conflict of interest: P.A. is the inventor of TB vaccines that incorporates the RV2659/60 antigens. All rights have been assigned to the Statens Serum Institute. The rest of the authors declare no financial or commercial conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.10.022.

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