Supporting Information

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SI Results

Frequency of ESAT-6 system 1 (EspC)–Specific T Cells Compared with Frequency of T Cells Specific for 6-kDa Early Secretory Antigenic Target (ESAT-6) of *Mycobacterium tuberculosis* and 10-kDa Culture Filtrate Antigen (CFP-10). In active tuberculosis (TB), the frequency of EspC peptide-specific IFN-γ responses was high (Fig. 1*A*) and similar to the frequency of T cells recognizing ESAT-6 and CFP-10. There was no significant difference in the magnitude of response to EspC [median (interquartile range; IQR) spot-forming cells (SFC)/10^6 peripheral blood mononuclear cells (PBMC) 68 (20, 250)], to ESAT-6 [median (IQR) SFC/10^6 PBMC 70 (20, 150)] ($P = 0.51$), or to CFP-10 [median (IQR) SFC/10^6 PBMC 70 (20, 280)] ($P = 0.42$).

In LTBI, there was no significant difference in the frequency of IFN-γ–secreting T cells specific for EspC in subjects responding to EspC [median (IQR) SFC/10^6 PBMC 106 (58, 168)] compared with responders to ESAT-6 [median (IQR) SFC/10^6 PBMC 148 (72, 230)] ($P = 0.65$) or responders to CFP-10 [median (IQR) SFC/10^6 PBMC 78 (51, 182)] ($P = 0.51$).

SI Materials and Methods

Participants. Patients with TB and persons with risk factors for latent TB infection (LTBI) were recruited prospectively from Imperial College Healthcare National Health Service Trust, London. At venipuncture, patients were untreated or had received therapy for <2 mo. Risk factors for LTBI were predefined as recent exposure to active tuberculosis or birth in a high-incidence country (incidence >100 cases per 100,000 persons). Clinical, radiologic, and microbiological data were collected on standardized forms at recruitment, during follow-up, and at final case notes review. Ethical approval was granted by the St Mary's Research Ethics Committee. Our tuberculosis patient population has a very low (4%) prevalence of HIV coinfection, and no participants had any clinical or laboratory features suggestive of HIV infection.

Ex Vivo IFN-γ ELISpot Assays. Ex vivo IFN-γ ELISpot assays were performed as previously described (1). All peptides were 15mers overlapping their adjacent peptides by 10 amino acids. For each peptide, identity was confirmed by mass spectrometry, and purity exceeded 80%. Wells were scored by an automated ELISpot counter (AID-GmbH) as previously described (threshold, five or more above the mean of negative-control wells and more than two times the mean of negative-control wells). Our predefined cutoff of five SFCs is the standard threshold used with our assay in nine studies since 2001 comprising >2,500 participants and is robust and reproducible in our assay format (2–10).

CD4 and CD8 Phenotyping of IFN-γ and IL-2 Single- and Dual-Secreting Antigen-Specific T Cells. PBMC were stimulated with EspC peptides for 5 h and then were enriched for IFN-γ–secreting cells and stained with anti–CD4-fluorescein isothiocyanate– and anti–CD8-Pacific blue–conjugated antibodies. Dead cells were excluded by the 7-AAD dye. The frequency of $CD4^+$ or $CD8^+$ T cells secreting IFN-γ and/or IL-2 was calculated by dividing the number of postenrichment $CD4^+$ or $CD8^+$ T cells secreting IFN-γ and/or IL-2 by the number of $CD4^+$ or $CD8^+$ T cells before enrichment multiplied by 9 (to account for use of 90% of cells for enrichment). The lower limit of detection of antigen-specific cytokine-secreting cells using this enrichment protocol is 0.007% of $CD4^+$ or $CD8^+$ cells (1).

Bacteria and Culture for Mass Spectrometry. To increase the accuracy of quantitation in our quantitative proteomics strategy, we used metabolic labeling, which allowed us to measure like peptides from *Mycobacterium tuberculosis* wild-type strain H37Rv and the strain H37RvΔRD1with a mutation in region of difference 1 (RD1) at the same time. Wild-type H37Rv was fully metabolically labeled with ^{15}N by being passaged roughly 13 generations in modified Sauton's medium in which the only nitrogen sources were ^{15}N-asparagine and ^{15}N-ammonium sulfate (Cambridge Isotopes) for an estimated labeling efficiency of 99.5% or higher; H37RvΔRD1 was unlabeled. Culture filtrate proteins from the two strains were collected and pooled before processing and analysis. The metabolic label did not change the chromatographic properties of the peptides but did allow like peptides from each strain to be distinguished based on mass shift. Thus, coeluting peptides could be differentiated and quantitated at the same time. Collection of culture-filtrate proteins from labeled and unlabeled bacteria was performed as previously published (11).

Mass Spectrometric Analysis. Culture filtrates from labeled wild-type bacteria and the unlabeled ΔRD1 deletion mutant were mixed together in equal volumes and concentrated roughly 100-fold on Vivaspin 5-kDa filter units (Vivascience). Concentrated protein samples were diluted with four volumes of GuHCl 100 mM ammonium bicarbonate and then were reduced, alkylated, and tryptically digested as previously described (11). Digested peptides were concentrated and desalted on Sepak 500-mg C18 cartridges (Waters Corp.) conditioned with 3 mL of 100% acetonitrile followed by 6 mL of 0.25% TFA water, and then eluted in 75% acetonitrile 0.1% formic acid.

Peptides were prefractionated off-line on a Whatman Partisphere SCX column (4.6-mm internal diameter × 25 cm) using a gradient of 0.1% formic acid, 25% acetonitrile to 350 mM ammonium formate, pH 2.7, 25% acetonitrile at 0.7 mL/min over 70 min. Fractions then were frozen to −80 °C and lyophilized to dryness. The samples were redissolved in 12 μL of 5% acetonitrile, 0.1% formic acid and separated on a 75 μm × 25 cm C18 column running at 250 nL/min flow with a gradient of 5–45% water 0.1% formic acid, acetonitrile 0.1% formic acid over the course of 180 min and analyzed on a LTQ-FT from Thermo Scientific.

Data Analysis for Quantitative Mass Spectrometry. Peptide identifications were made using the database search algorithm, SEQUEST (Thermo Scientific). Spectra were searched against a composite database containing the predicted ORFs annotated in the H37Rv genome (12) supplemented with those uniquely annotated in CDC1551 (13) and against this database amended for comprehensive ^{15}N labeling. A reverse-database strategy was used to estimate the false-discovery rate (FDR) (14). Peptides were filtered at 1% FDR and clustered into proteins using an Occam's approach, a strategy which produced protein identifications with probabilities of 0.98 or greater in parallel analyses using ProteinProphet (15). The number of spectral counts for all nonredundant peptides was pooled and used to compare protein abundance in culture filtrates from H37Rv and H37RvΔRD1. The difference in EspC secretion between these strains was confirmed manually by analyzing areas under the curve for the identified EspC peptides.

Mutation and Genetic Complementation of *Mycobacterium tuberculosis* Strains. To assess secretion of EspC in the presence and absence of the RD1 locus, we replaced the native copy of the EspACD locus with an ectopic copy of this locus in which EspC was epitope-tagged with an HA tag. Deletion of the EspACD locus from the H37Rv strain has been described previously (16). The same vector and two-step deletion strategy was used to delete the EspACD locus from the H37RvΔRD1 mutant (17). H37RvΔEspACD and H37RvΔRD1ΔEspACD then were complemented with pEspACD, an ectopic vector expressing EspACD under the control of a tetracycline-inducible promoter (18), or an empty vector, pEmpty, as a control as previously described (16). Secretion was assessed as previously described (11, 16). EspC was detected using antibody to the HA-epitope obtained from Novus Biologicals.

Statistical Analysis. Comparisons of the size of IFN-γ responses between antigens were analyzed using the Wilcoxon matched pairs test; $P < 0.05$ was considered significant. Analyses were performed using STATA v. 9.2 (STATACORP).

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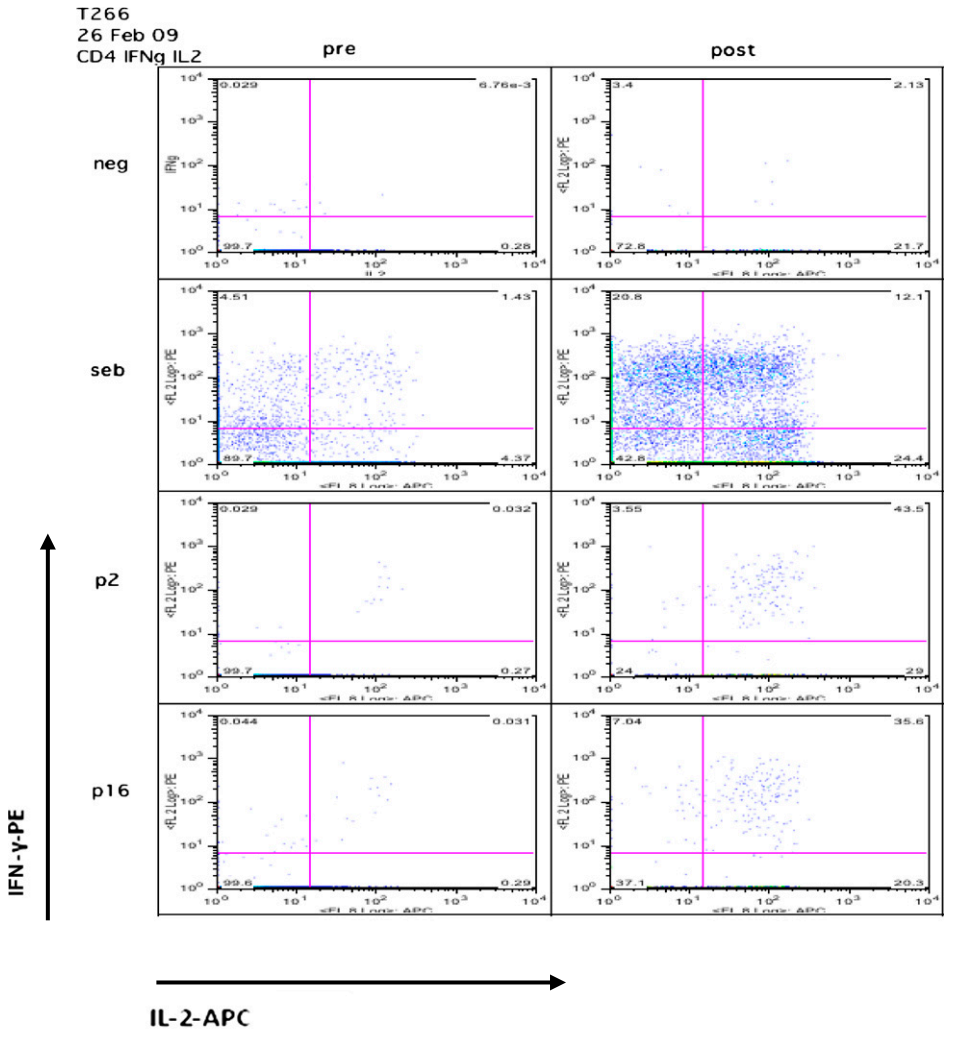

Fig. S1. Representative flow cytometric dot plots of cytokine secretion assay for EspC peptide-specific CD4 T-cell IFN-γ and IL-2 cytokine profiles, using IFN-γ-phycoerythrin (IFN-γ-PE) and IL-2-allophycocyanin (IL-2-PE), for donor T266 from Fig. 3. T266 is a patient who has active TB and who has undergone treatment for 2 mo. The dot plot shows T cells specific for EspC peptide 2 (EspC6-20) and 16 (EspC 76–90) stained after 5-h stimulation with EspC peptides 2 or 16, respectively. Dot plots pre- and positive-immunomagnetic enrichment (as described in *Materials and Methods*) are shown. Neg, unstimulated negative control; p2, peptide 2-stimulated; p16, peptide 16-stimulated; seb, staphylococcal enterotoxin B-stimulated positive control.

Table S1. Demographic and clinical characteristics of TB cases, LTBI, and healthy control subjects

	TB cases (n = 45)	LTBI (n = 27)	Controls (n = 27)
Demographic characteristics			
Mean age in y (range)	45 (18,79)	36 (22,65)	32 (21,44)
Sex male:female	26:19	16:11	11:16
Ethnicity			
Asian	26	6	3
Black African	11	10	0
Black Caribbean	1	2	1
White	7	9	23
Bacillus Calmette–Guérin scar	31/38 (82%)*	21/26 (81%)†	27/27
TST positive (≥10mm)	29/34 (85%)‡	22/25 (88%)§	0/9¶
Clinical characteristics of patients with TB**			
Location of disease			
Pulmonary	18	NA	NA
Extrapulmonary	11	NA	NA
Lymphatic	11	NA	NA
Disseminated	5	NA	NA
LTBI risk factors			
TB contact††	NA	15	NA
Born in endemic country‡‡	NA	12	NA

*The bacillus Calmette–Guérin scar status was unknown for seven cases.
†The bacillus Calmette–Guérin scar status was unknown for one person.
‡TST was not carried out in 11 cases.
§TST was not carried out in two cases.
¶TST was not carried out in 18 controls
**Twenty-seven cases had culture-confirmed TB, and 18 cases had highly probable TB. Histologic findings were available for 7 of 21 patients with highly probable TB and were supportive in four of these patients.
††LTBI suspects with TB contacts had been in contact with TB in the household (n = 6), in the workplace (n = 5), or socially (n = 4). All were still in contact with the index case when recruited into this study. Both of the LTBI suspects with social contacts, four of the LTBI suspects with household contacts, and four of the LTBI suspects with workplace contacts were exposed to pulmonary TB. The remaining LTBI suspect with a workplace contact was exposed to abdominal TB, and each of the two remaining LTBI suspects with household contacts was exposed to military or pleural TB.
‡‡Born in a country with an incidence >100 cases per 100,000 persons.