Permanent Loss of Human Leukocyte Antigen E–restricted CD8$^+$ T Stem Memory Cells in Human Tuberculosis

To the Editor:

Host biomarkers can help diagnose tuberculosis (TB) state or are correlates of protection against active disease and may also direct the response to therapy. Specific CD8$^+$ (cluster of differentiation 8) memory T cells can be considered both correlates of protective immunity and potential biomarkers of infectious diseases, such as TB, particularly when there is a need to distinguish active TB (aTB) from latent TB infection (LTBi).

Functional CD4$^+$ and CD8$^+$ T cells differentially correlate to disease stage, mycobacterial load, and treatment (1–3), suggesting that certain subsets may serve as candidate biomarkers (4). Our previous studies showed that human leukocyte antigen E (HLA-E)–restricted and *Mycobacterium tuberculosis* (Mtb) peptide–specific CD8$^+$ T lymphocytes significantly expand in patients with aTB (5), particularly in patients coinfected with human immunodeficiency virus (HIV), compared with subjects with LTBi, but they normalize after anti-TB therapy. While further studying the functional relevance of discrete memory and effector subsets of HLA-E–restricted CD8$^+$ Mtb peptide–specific T cells, we found that patients with aTB disease lost CD8$^+$ T cells with a stem cell memory (T_{SCM}) phenotype. We analyzed 43 subjects divided into three groups according to their TB status: those with LTBi ($n = 15$; median age, 44 yr; 4 women); patients with aTB before therapy (TB-0) ($n = 17$; median age, 54 yr; 7 women); and those with TB with 6 months of therapy (TB-6) ($n = 11$; median age, 44 yr; 4 women), treated with a 6-month rifampicin-based regimen according to the World Health Organization guidelines for treatment of drug-susceptible TB and patient care (6). A control group of healthy subjects was also included ($n = 10$; median age, 36 yr; 5 women). LTBi was defined as QuantiFERON-TB Gold Plus positive, normal results on chest X-ray, and absence of clinical and microbiological signs of TB infection. All participants were HIV negative. The University of Palermo ethics committee approved the study, and written consent was obtained from participants. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll-Hypaque density centrifugation. Direct *ex vivo* binding of HLA-E tetramers (TMs) loaded with p44, p62, and p68 Mtb peptides to CD8$^+$ T cells was analyzed in PBMCs. HLA-E TM staining was performed for 15 minutes at 37°C. HLA-E TM preparation has been described previously (5, 7). After incubation, samples were washed with PBS/BSA 0.1% and stained with live/dead marker (Zombie NIR Fixable Viability Kit; BioLegend) according to the manufacturer's protocol. Samples were then incubated with the following fluorochrome-conjugated antibodies: CD3 (PerCP-Vio 700, clone REA613), CD8 (APC, clone REA734), CCR7 (VioBlue, REA546), CD45RA (VioGreen, clone REA562), CD62L (FITC, clone 145/15), and CD95 (PE-Vio 770, clone REA738) (all from Miltenyi Biotec). Data were analyzed using FlowJo software (version 10.5.3; Treestar Inc.). The analysis of each specific subset was performed using the gating strategy shown in Figure 1A in compliance with the Minimal Information About T Cell Assays guidelines (8) and CD8$^+$ T-cell subsets on the basis of the hierarchical model of human T-cell differentiation (9).

In agreement with our previous results (5, 10), the frequency of HLA-E–restricted and Mtb peptide–specific CD8$^+$ T cells was significantly higher in the TB-0 and TB-6 groups than in subjects with LTBi (Figures 1B and 1C). Only samples with at least 50 TM$^+$ events were included for subsequent memory subset analysis to assess discrete populations. The majority of circulating HLA-E/Mtb peptide TM$^+$CD8$^+$ T cells had an effector memory profile, including terminally differentiated phenotypes ($P = 0.013$) marked by CD45RA expression. In contrast, percentages of HLA-E/Mtb peptide TM$^+$CD8$^+$ T cells with a T_{SCM} phenotype (defined by CCR7, CD45RA, and CD95 expression) were significantly higher in subjects with LTBi compared with those with TB-0 ($P = 0.0003$). Of note, the HLA-E/Mtb peptide TM$^+$CD8$^+$ T_{SCM} population seems to be permanently compromised in patients with TB, as it did not recover after successful antimycobacterial therapy (Figure 1B). Furthermore, to compare the CD8$^+$ memory T-cell compartment in different treatment stages, we used a semiautomated empowered work flow known as FlowCT (11) to analyze large data sets that include preprocessing, normalization, and multiple dimensionality reduction techniques (Figure 1C). Automated clustering was performed using FlowSOM on all CD3$^+$ live T cells, cluster annotation was achieved by visualizing expression degrees of each marker on uniform manifold approximation and projection, and lymphocytes were subclustered using phenotyping by accelerated refined community partitioning. Automated clustering yielded the identification of 20 subsets. Automated clustering using ~~phenotyping by accelerated refined community partitioning~~ uncovered the presence of two CD8$^+$ T_{SCM} cell clusters with (potentially) divergent polarization on the basis of differential expression of CCR7, CD45RA, and CD62L (Figure 1C). We called CD8$^+$ T_{SCM} cells and possible CD8$^+$ precursor of exhausted T (T_{PEX}) cells. On the basis of uniform manifold approximation and projection visualization, the CD8$^+$ T_{SCM} population was greater in LTBi compared with other TB conditions, and the CD8$^+$ T_{PEX} population was greater only after 6 months of TB therapy.

Recent studies have shown that patients with chronic HIV (12) or *Trypanosoma cruzi* (13) infection have a dramatically decreased frequency of CD8$^+$ T_{SCM} cells, which otherwise do not produce IFN-γ, TNF-α, and IL-2 in response to specific antigen stimulation. It is unclear whether this results from a T_{SCM} cell exhaustion program, marked by the upregulation of inhibitory receptors and decreased production of proinflammatory molecules, maintained under conditions of strong inflammation and high antigenic load (14, 15). In support of this, a recent study identified within the CCR7$^+$CD95$^+$ T_{SCM} pool a GZMK$^+$ (granzyme K) population of exhausted-like T cells (T_{PEX}) with memory features and critical elements of exhaustion (15).

As HLA-E–restricted and Mtb-specific CD8$^+$ T_{SCM} cells from TB patients very poorly expanded in response to peptide stimulation *in vitro* (Figure 2A), we performed a gene expression analysis in

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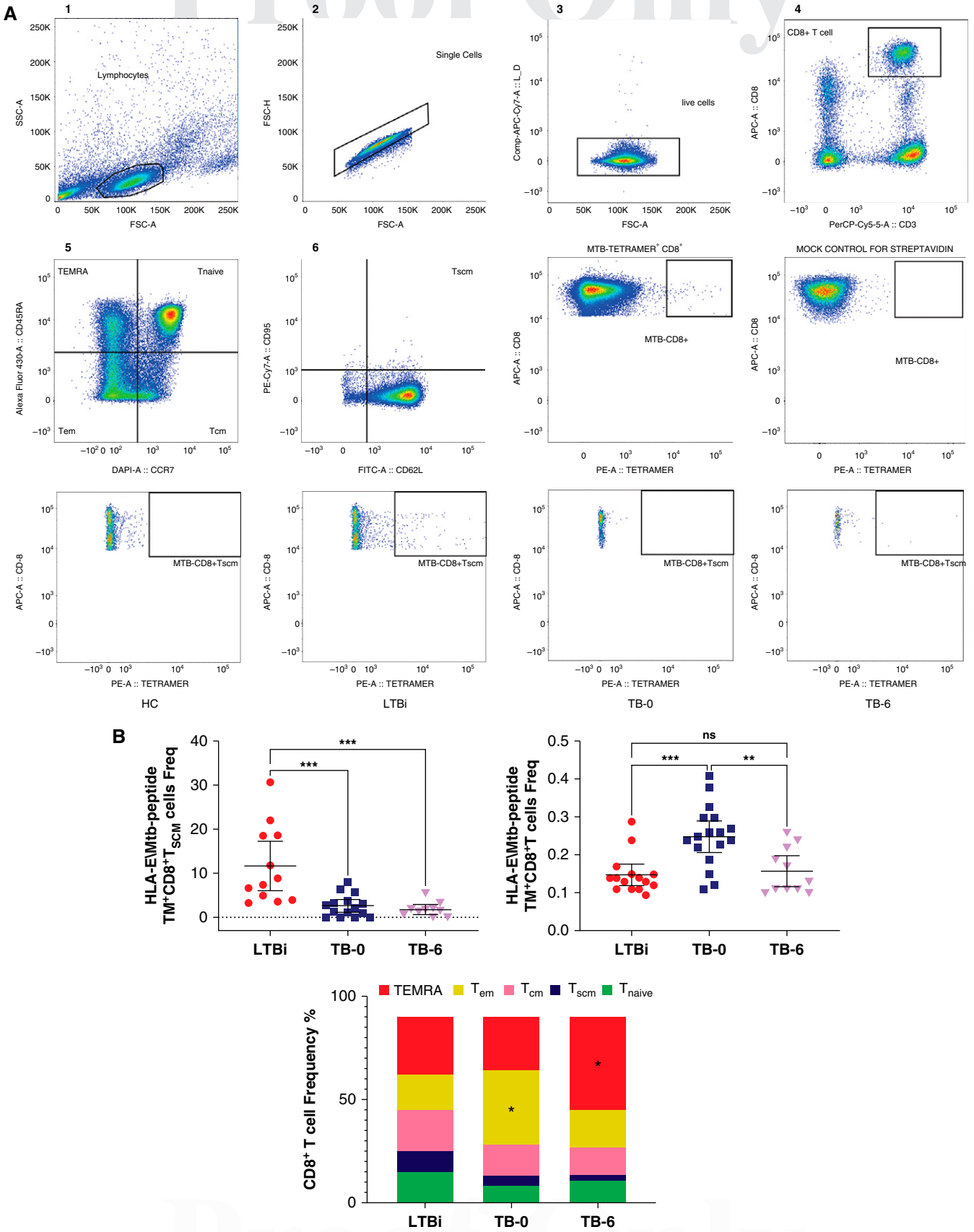

Figure 1. *Ex vivo* analysis of CD8$^+$ ~~(cluster of differentiation 8)~~ T_{SCM} cells in different TB conditions. (*A*) Representative plot from 53 independent experiments showing the flow cytometry gating strategy for the detection of different memory phenotypes of human leukocyte

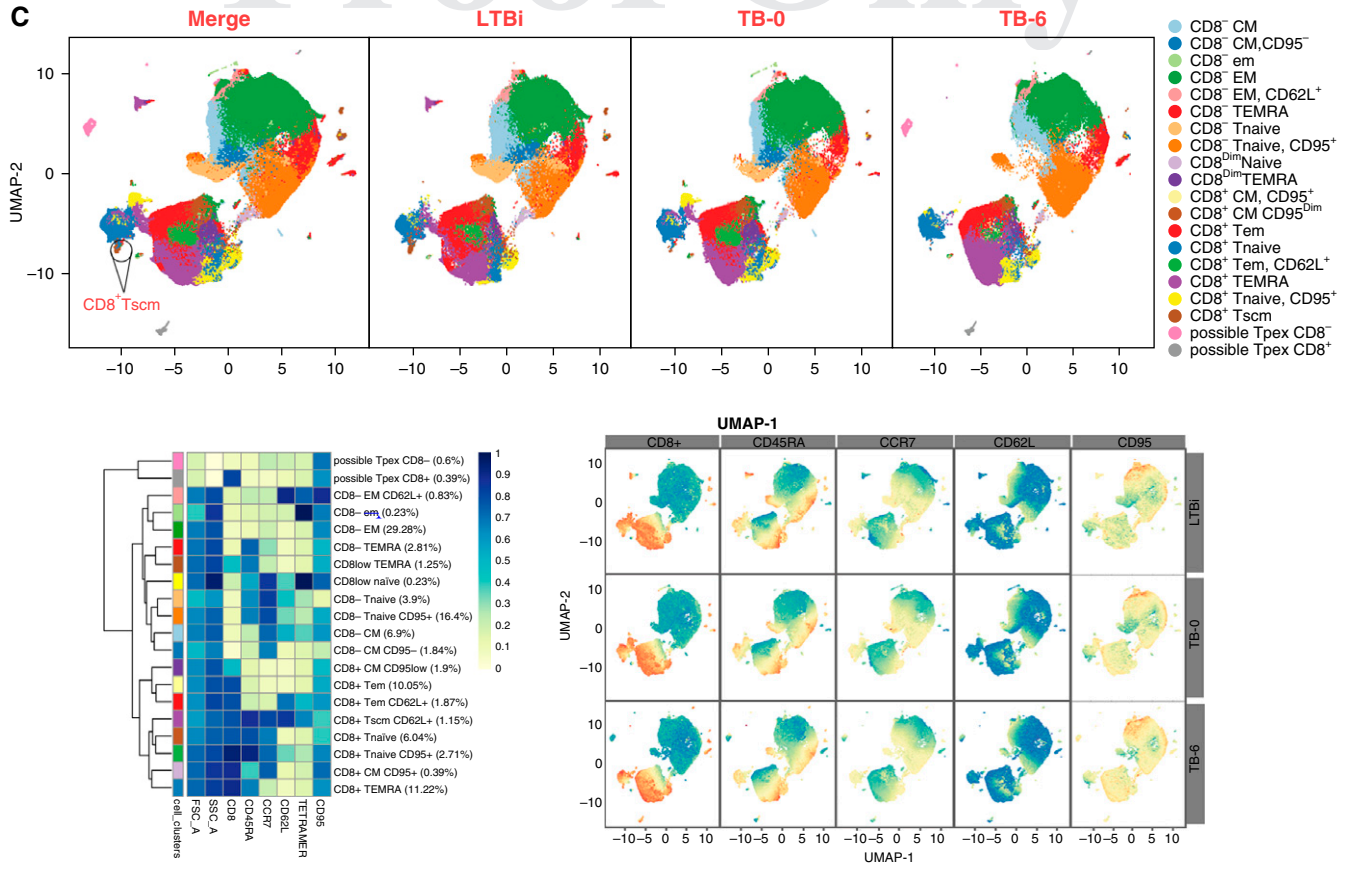

Figure 1. (*Continued*). antigen E (HLA-E)–restricted $CD8^+$ T cells. Lymphocytes were first gated (FSC-A/SSC-A), and duplicates were excluded (FSC-A/FSC-H). $CD8^+$ T cells were identified as $CD3^+CD8^+$ and memory phenotypes defined on the basis of $CCR7^+$ and $CD45RA^+$ expression. T_{SCM} cells were further identified on the basis of $CD62L^+CD95^+$ expression. Shown are pseudocolor plots of peripheral blood mononuclear cells from one representative subject per group. (*B*) The left panel and central panel show *ex vivo* frequencies of HLA-E/*Mycobacterium tuberculosis* (Mtb) peptide tetramer $(TM)^+$ total $CD8^+$ T cells and HLA-E/Mtb peptide TM^+ $CD8^+$ T_{SCM} cells, respectively. $^{**}P \leq 0.001$ and $^{***}P \leq 0.0001$ by the Mann-Whitney test. The right panel shows the phenotype distribution of HLA-E/Mtb peptide TM^+ total $CD8^+$ T cells in the different groups tested. $^{*}P \leq 0.05$ by two-way ANOVA. (*C*) A live $CD3^+$ subpopulation was used for nonlinear dimensionality reduction. A UMAP plot is shown of different T_{naive}, T_{SCM}, T_{CM}, T_{EM}, and T_{EMRA} clusters using FlowSOM in different TB conditions on the basis of $CD8^+$, CCR7, CD45RA, CD95, and CD62L expression. CM = central memory; EM = effector memory; Freq = frequency; FSC-A = forward scatter area; FSC-H = forward scatter height; HC = healthy control subjects; LTBi = latent TB infection; ns = not significant; SSC-A = side scatter area; TB = tuberculosis; TB-0 = patients with tuberculosis before therapy; TB-6 = patients with tuberculosis with 6 months of therapy; T_{CM} = central memory T; T_{EM} = effector memory T; T_{EMRA} = terminally differentiated effector memory T; T_{naive} = naive T; T_{pex} = precursor of exhausted T; UMAP = uniform manifold approximation and projection.

PBMCs of patients with TB and control subjects using whole-genome transcriptional microarrays (Gene Expression Omnibus: GSE54992) to study $CD8^+$ T_{SCM} cell functions.

After normalization and $\log_2$ conversion for the raw data of gene chips (Figure 2B), volcano plots showed significantly different expression of 9,461 genes ($P < 0.05$) between the LTBi and TB-0 groups and 7,806 genes between the TB-0 and TB-6 groups, but no significant differences were noted between subjects with LTBi and healthy control subjects (Figure 2C). Moreover, genes associated with T_{PEX} cells, such as *GZMK*, were significantly overexpressed in the TB-0 and TB-6 groups compared with subjects with LTBi and healthy control subjects. In contrast, genes associated with classic T_{SCM} cells, such as *CXCR3*, *CCR7*, *SATB1* (SATB homeobox 1), and *FASLG* (Fas ligand) (CD95), are overexpressed in subjects with LTBi and healthy control subjects compared with the TB-0 and TB-6 groups (Figure 2D and 2E). These data are in agreement with our *ex vivo* results and support the possibility that loss of T_{SCM} cells is the consequence of Mtb infection–induced exhaustion.

In summary, our results show that HLA-E–restricted and Mtb-specific $CD8^+$ T_{SCM} cells are irreversibly lost during TB infection, probably as a consequence of exhaustion. This finding may be valuable in defining new and robust biomarkers capable of distinguishing subjects with TB from those with LTBi. However, determining the consequences of this finding for the outcome of Mtb infection and memory generation will require additional studies. ■

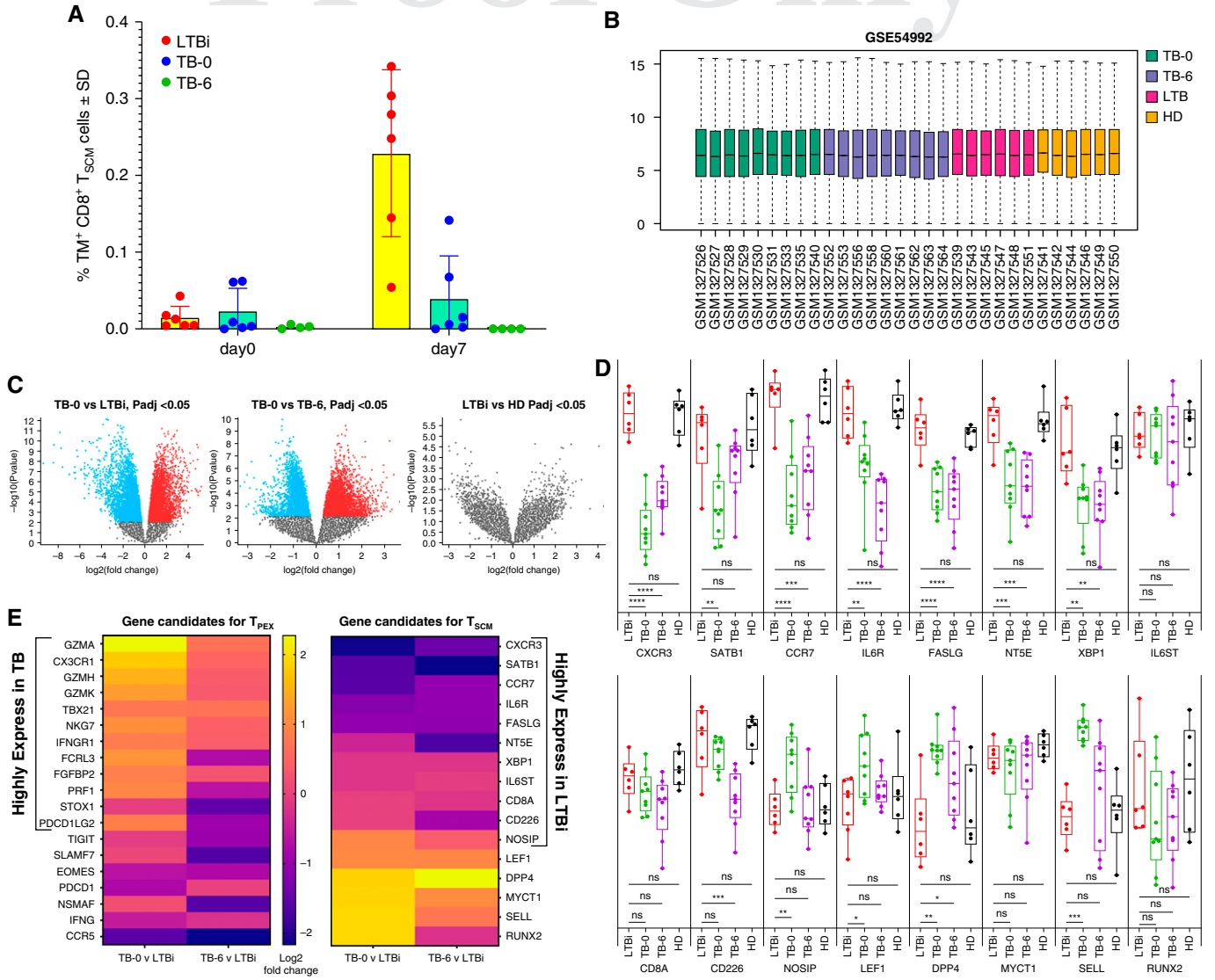

Figure 2. Gene expression analysis of PBMCs in patients with TB and control subjects. (*A*) Total human leukocyte antigen E–restricted Mtb-specific $CD8^+$ T cells and Mtb-specific $CD8^+$ stem cell memory T (T_{SCM}) cells were checked on the first day and after 7 days' *in vitro* expansion. PBMCs are derived from subjects at different stages of TB therapy ($N = 6$). (*B*) Quality control and normalization of all data. (*C*) Volcano plot of genes differentially expressed in PBMCs from the TB-0 and TB-6 groups and healthy control (HD) individuals. *P* values and adjusted *P* values (P_{adj}) were calculated using the Benjamini and Yekutieli adjustment test. Genes with significantly different degrees of expression were used for subsequent analysis. (*D*) Differentially expressed genes related to $CD8^+$ T_{SCM} T lymphocytes in PBMCs from the TB-0 and TB-6 groups, or HD subjects, at a significance cutoff of 0.05. Bars indicate mean ± SEM. $P = 0.0021$, $^{**}P = 0.0002$, and $^{***}P = 0.0001$ by the Holm-Šídák multiple-comparisons test. (*E*) Heat map of selected gene candidates ($P_{adj} < 0.05$) for T_{SCM} and T_{PEX} cells, where $\log_2$ FC was calculated on the basis of TB-0 versus latent TB infection (LTBi) and TB-6 versus LTBi (FC > 0 indicates upregulated in LTBi, and FC < 0 indicates downregulated in LTBi), in PBMCs from the TB-0 and TB-6 groups ($n = 9$), compared with individuals with LTBi ($n = 6$), according to Galletti and colleagues (15). CD226 = CD226 molecule; CD8A = CD8a molecule; CX3CR1 = C-X3-C motif chemokine receptor 1; DPP4 = dipeptidyl peptidase 4; EOMES = eomesodermin; FASLG = Fas ligand; FC = fold change; FCRL3 = Fc receptor like 3; FGFBP2 = fibroblast growth factor binding protein 2; GZMA = granzyme A; GZMH = granzyme H; GZMK = granzyme K; IFNG = IFN gamma; IFNGR1 = IFN gamma receptor 1; IL6R = IL-6 receptor; IL6ST = IL-6 cytokine family signal transducer; LEF1 = lymphoid enhancer binding factor 1; MYCT1 = MYC target 1; NKG7 = natural killer cell granule protein 7; NOSIP = nitric oxide synthase interacting protein; NSMAF = neutral sphingomyelinase activation associated factor; NT5E = 5′-nucleotidase ecto; PBMC = peripheral blood mononuclear cell; PDCD1 = programmed cell death protein 1; PDCD1LG2 = programmed cell death 1 ligand 2; PRF1 = perforin 1; RUNX2 = RUNX family transcription factor 2; SATB1 = SATB homeobox 1; SELL = selectin L; SLAMF7 = SLAM family member 7; STOX1 = storkhead box 1; TBX21 = T-box transcription factor 21; TIGIT = T cell immunoreceptor with Ig and ITIM domains; TM = tetramer; XBP1 = X-box binding protein 1.

Author disclosures are available with the text of this letter at www.atsjournals.org.

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