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Supplementary Information for

Delineation of a molecularly distinct terminally differentiated memory CD8 T cell population

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This PDF file includes:

Materials and Methods

Antibodies, flow cytometry and cell sorting. CD8 T cell subsets were first gated on congenically distinct P14 cells or $CD44^{+}Tetramer^{+}$ cells (as indicated), and then defined as: terminal-TEM: $CD127^{lo}CD62L^{lo}$; TEM: $CD127^{hi}CD62L^{lo}$; TCM: $CD127^{hi}CD62L^{hi}$, conventional TEM: $CD62L^{hi}$; TE: D7 $KLRG1^{hi}CD127^{lo}$; MP: D7 $KLRG1^{lo}CD127^{hi}$. The following antibodies were used for murine surface staining: CD8 (53-6.7), CD27 (LG-7F9), CD43 (1B11), CD44 (IM7), CD45.1 (A20-1.7), CD45.2 (104), CD62L (MEL-14), CD127 (A7R34), CX3CR1 (SA011F11), KLRG1 (2F1), CXCR3 (CXCR3-173), Slamf6 (13G3), CD11a (M17/4), CD11b (M1/70), CD11c (N418), CD49d (R1-2). Cells were stained for 20min at 4°C in PBS supplemented with 2% bovine serum albumin and 0.1% sodium azide. Intracellular staining was performed with the Foxp3-transcription factor staining buffer kit (eBioscience) using the following antibodies: IL-2 (JES6-5H4), IFNγ (XMG1.2), TNFα (MP6-XT22), Bcl2 (3F11), GzA (CB9), GzB (GB12), Ki-67 (SolA15), T-bet (4B10), Foxo1(C29H4), Eomes (Dan11mag), TCF1 (C63D9). For cytokine staining, memory populations sorted from spleens at >30 days after LCMV infection were incubated for 4 hours at 37°C with 10 nM GP_{33-41} peptide, congenically distinct naive splenocytes, and CD107a (1D4B) antibody was included in the media for the entirety of the stimulation; protein transport inhibitor was added after the first hour of incubation. For analysis of human PBMCs, the following antibodies were used: CD8 (SK1), CD45RO (UCHL1), CCR7 (G043H7), CD62L (DREG56), CD127 (A019D5), CD27 (M-T271), KLRG1 (13F12F2), CD49d (9F10), GzA (CB9), GzB (GB11). Flow cytometry data were generated with an LSR Fortessa or LSR Fortessa X-20 (BD) and FlowJo Software (TreeStar) was used for analyses. Cells were sorted using FACS Aria, FACS Aria Fusion, or Influx (BD) instruments.

Collection of Human PBMCs. For mass cytometry, peripheral blood was obtained from patients undergoing colonoscopy at the University of California, San Diego and the San Diego

VA Healthcare System after obtaining informed consent. Healthy individuals were undergoing colonoscopy as part of routine clinical care for colorectal cancer screening/surveillance or non-inflammatory gastrointestinal symptoms that included constipation or rectal bleeding. Inclusion criteria included age over 18 years old and absence of significant comorbidities or colorectal cancer. Blood was collected in BD Vacutainer CPT tubes and centrifuged at 400 x g for 25 minutes. The buffy coat layer was removed, washed, and counted. Cells were resuspended in freezing buffer (10% DMSO, 40% complete RPMI [RPMI +10% fetal bovine serum (FBS) +100 U/mL penicillin/100 μg/mL streptomycin], 50% FBS), placed into a freezing container, and stored at -80°C. Cells were recovered, washed, filtered, and labeled with anti-human CD45 mAbs. $CD45^+$ immune cells were sorted on a FACSAria2 (BD Biosciences) or utilized for mass cytometry analysis. For flow cytometry analysis of intracellular and surface markers, buffy coats from healthy donors were purchased from Stanford Blood Center. PBMCs were isolated via Ficoll-Paque (GE Healthcare) density-gradient separation and flow cytometry analysis was performed immediately.

Mass cytometry (CyTOF) of human peripheral blood mononuclear cells. Blood was pre-processed as above, washed with protein-free PBS (Rockland), and stained with Cell-ID cis-platin (Fluidigm) 5 minutes at room temperature, then washed with CyFACS buffer [PBS + 0.1%(w/v) bovine serum albumin (Sigma-Aldrich) + 2mM EDTA (Invitrogen) + 0.05%(v/v) NaN_3)]. Staining and fixing cells for CyTOF using 29 metal-conjugated antibodies in accordance with cell staining protocols (Fluidigm). Cells were analyzed on a Helios CyTOF 2 mass cytometer (Fluidigm) at the La Jolla Immunology Institute Flow Cytometry Core at approximately 300 events/s. All antibodies used for mass cytometry were purchased from Fluidigm or conjugated using Maxpar Antibody Labeling Kit (Fluidigm). FCS files created from mass cytometry were analyzed on FlowJo (BD Biosciences). UMAP (uniform manifold approximation and projection) for dimensional reduction was done on cells pre-gated on: Live cells, $CD45^+$, $CD3^+$, $CD4^-$, $CD8\beta^+$ cells, with down-sampling

to $5x10^4$ cells per patient, and subsequent concatenation of all individuals (6 individuals in total). UMAP utilized protein expression of CCR7, CXCR5, Eomesodermin, Perforin, Granzyme B, Integrin α4 (CD49d), Granulysin, and NKG2A (KLRC1) for analysis.

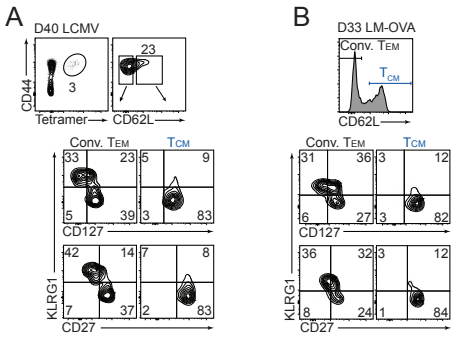

Fig. S1. Heterogeneity within conventional TEM during acute LCMV infection and *Listeria monocytogenes* infection. *(A)* Representative expression of KLRG1, CD127, and CD27 by TCM ($CD62L^{hi}$) and conventional (Conv.) TEM ($CD62L^{lo}$) on GP_{33-41}-$tetramer^{+}$ in PBL on day 40 of LCMV infection. *(B)* OT-I cells were transferred into congenically distinct recipient mice subsequently infected with *Listeria monocytogenes* expressing OVA (LM-OVA). Representative expression of KLRG1, CD127, and CD27 by TCM ($CD62L^{hi}$) and conventional (Conv.) TEM ($CD62L^{lo}$) on OT-I cells in PBL on day 33 of LM-OVA infection. All data are from one representative experiment of 2 independent experiments with n=3-4 per group.

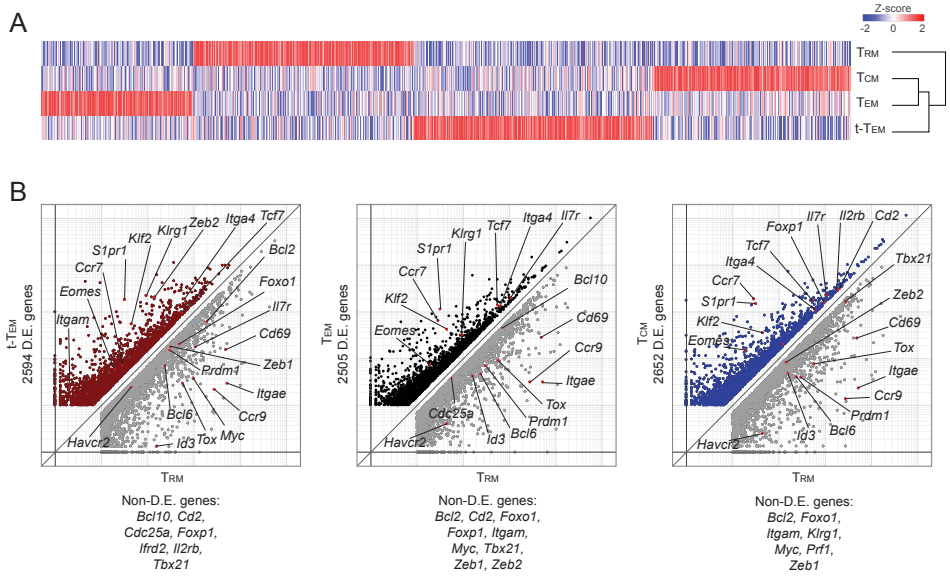

Fig. S2. Transcriptional relationship of terminal-TEM, TEM, TCM, and TRM. Transcriptional profile of terminal-TEM, TEM, TCM, and small intestine intraepithelial TRM P14 cells on day 55 of infection as in Figure 1E. *(A)* Heatmap illustrating differentially expressed genes (≥1.5-fold) ordered through k-means clustering between terminal-TEM, TEM, TCM, and TRM. *(B)* Comparison of gene expression between TRM and each memory subset with highlighted genes differentially expressed or select non-differentially (non-D.E.) expressed genes listed below. RNAseq samples consist of 2 biological replicates wherein each replicate is comprised of sorted cells pooled from 2 mice.

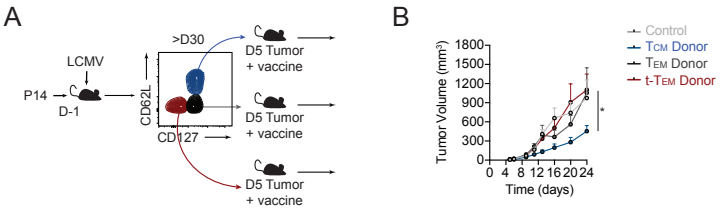

Fig. S3. Terminal-TEM confer minimal protection in a tumor vaccine model. P14 CD8 T cells were transferred into congenically distinct mice that were infected with LCMV the following day. *(A)* Experimental schematic demonstrating at >30 days of infection, terminal-TEM ($CD127^{lo}CD62L^{lo}$), TEM ($CD127^{hi}CD62L^{lo}$), and TCM ($CD127^{hi}CD62L^{hi}$) subsets were sorted and transferred to tumor-bearing mice. At the time of adoptive transfer, mice were immunized with 10 µg of GP_{33-41} and 2 µg of poly(I:C) in the ipsilateral flank. *(B)* Tumor volume was measured over time. Data are combined from two experiment with n=4 (control, no P14 transfer) or n=6-7 per group, *P<0.05 comparing t-TEM and TCM. Graphs indicate mean ± s.e.m.

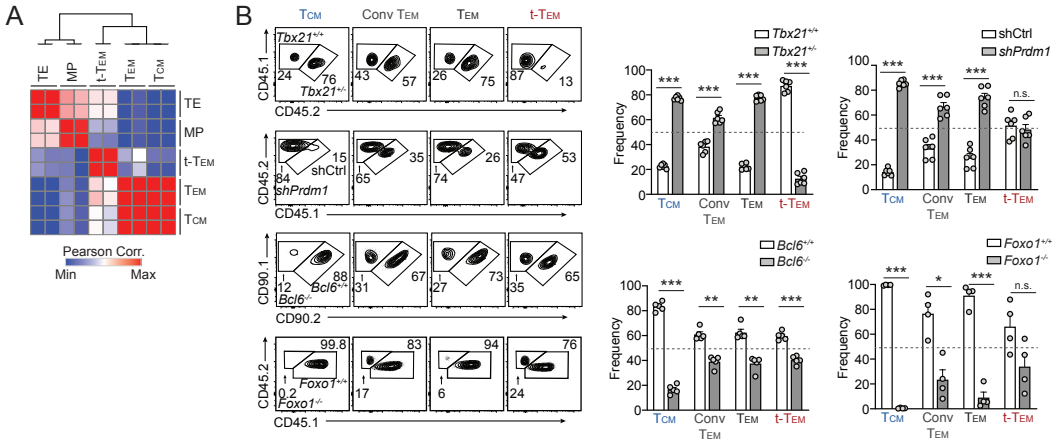

Fig. S4. Transriptional regulation of memory CD8 T cells. (*A*) Gene-expression similarity matrix (Pearson correlation) constructed based on differentially expressed genes between all populations of comparison from RNAseq data described in Figure 1. (*B*) Frequency of control or experimental donor cells in a mixed transfer experiment from Fig. 4*B-J*. All data are from one representative experiment of 2 independent experiments with n=4-6 per group *(C-I)*; *P<0.05, **P<0.01, ***P<0.005. Graphs indicate mean ± s.e.m, and symbols represent an individual mouse.

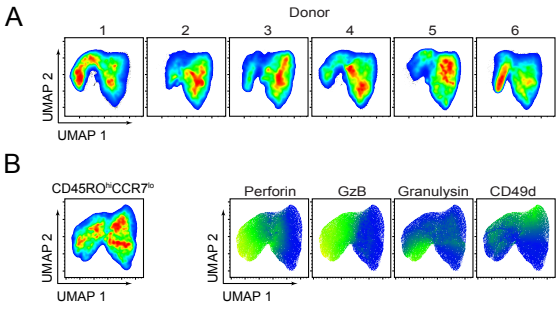

Fig. S5. Mass cytometry analysis reveals heterogeneity of conventional human TEM. Mass cytometry was performed on human PBMCs from healthy donors (pertaining to Figure 5*B*). *(A)* UMAP of $CD45^+CD3^+CD8\beta^+CD45RO^+$ cells constructed for each of the 6 donors. *(B)* $CD45^+CD3^+CD8b^+CD45RO^+CCR7^-$ (i.e conventional TEM) PBMCs from six donors integrated into one UMAP plot (left) and expression levels of highlighted molecules within this population (right).

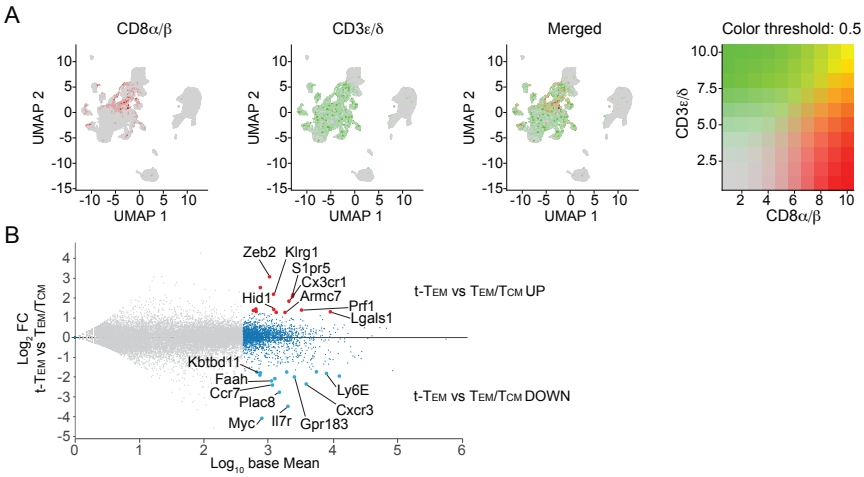

Fig. S6. Identification of human terminal-TEM through single cell RNAseq analysis of human PBMCs. Single-cell RNAseq analysis was performed on human PBMCs from healthy donors (pertaining to Figure 5). *(A)* Expression patterns of *CD8A, CD8B, CD3D,* and *CD3E* in total PBMCs. CD8 T cells were filtered from total single-cell datasets based on expression of *CD8A, CD8B, CD3D,* and *CD3E* for subsequent analyses (Figure 5). *(B)* Establishment of gene signatures upregulated in murine terminal-TEM compared to both TCM and TEM, as well as genes downregulated in terminal-TEM compared to TCM and TEM. Gene sets were used for Figure 5*D*.

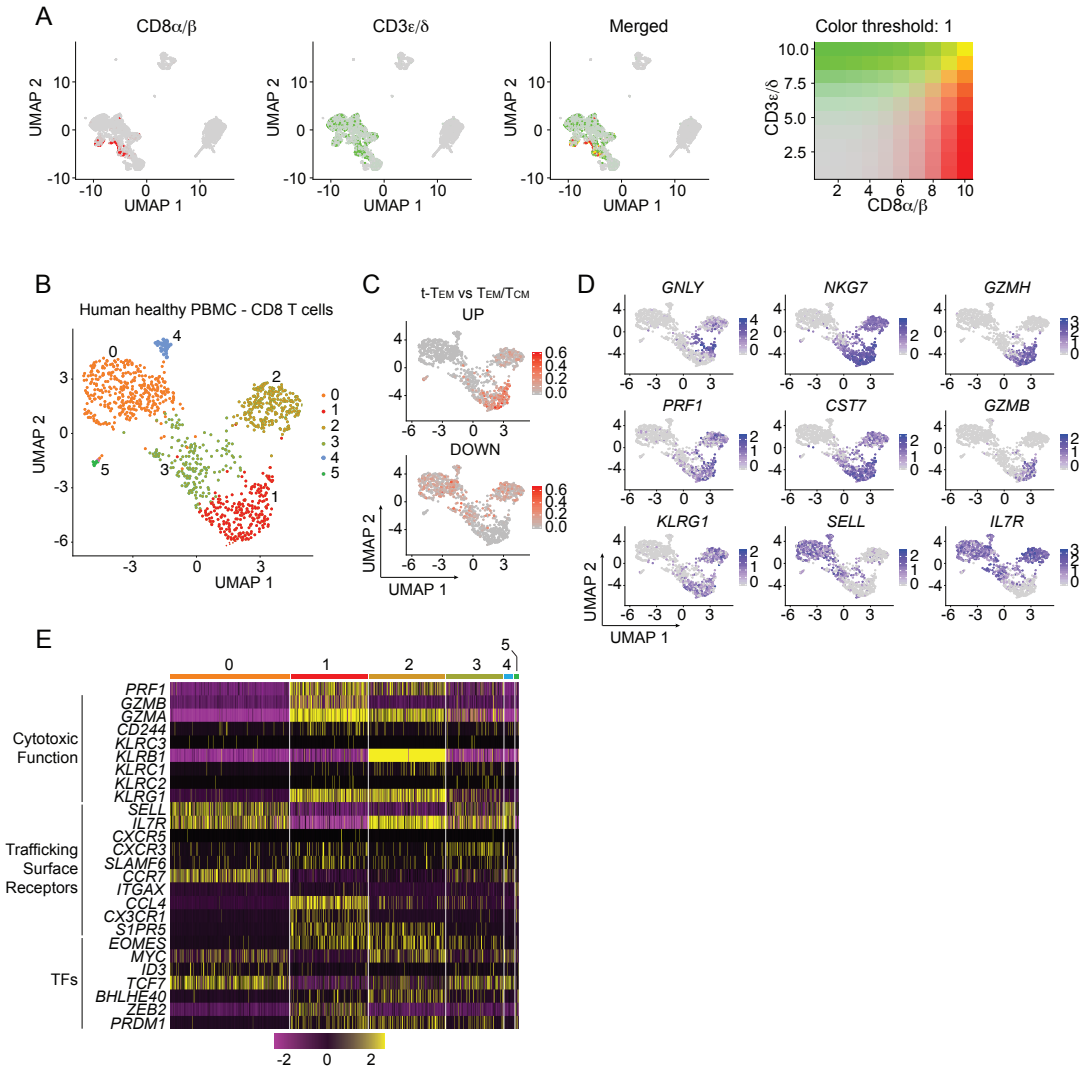

Fig. S7. Phenotype of terminal-TEM from a publicly available human PBMC single-cell RNAseq dataset. The publicly available "10k PBMC scRNAseq" dataset from 10x Genomics was analyzed for identification of a human terminal-TEM population. *(A)* Expression patterns of *CD8A*, *CD8B*, *CD3D*, and *CD3E* in total PBMCs. CD8 T cells were filtered from all cells based on expression of *CD8A*, *CD8B*, *CD3D*, and *CD3E* for subsequent analyses. *(B)* Unbiased UMAP analysis revealed 6 distinct clusters of CD8 T cells *(C)* Relative enrichment of the murine terminal-TEM gene-expression signature was evaluated within the human CD8 T cells. *(D)* Relative expression of key molecules between the 6 distinct clusters from *(B)*. *(E)* Expression levels of highlighted genes between the 6 distinct clusters.