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Supporting Information

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PLZF-expressing CD4 T cells show the characteristics of terminally differentiated effector memory CD4 T cells in humans

MATERIALS AND METHODS

Human samples

Fetal tissues were acquired from Advanced Bioscience Resources (Alameda, CA). Thymuses were procured from patients undergoing scheduled open-heart surgery via a median sternotomy as a routine part of their operation. Tonsils were obtained from children with sleep disordered breathing or recurrent tonsillitis. A human cord blood specimen was obtained from Women's Hospital Birth Center and fresh adult blood from healthy volunteers were provided by the Department of Pathology at the University of Michigan. We obtained normal spleen tissues from a site distant from a localized tumor of patients through the Tissue Procurement Core at the Department of Pathology, University of Michigan. All use of human samples was approved by Institutional Review Board at the University of Michigan.

Cell preparation and flow cytometry

Thymus, tonsils or spleen, tissues were minced and passed through 100 μm cell strainer. Cells were then resuspended in 30 ml PBS and layered on 15 ml Ficoll hypaque gradient mix and centrifuged at 970 x g at room temperatures with no brakes for 30 minutes. Non-parenchymal cells were collected at the interface of the two Ficoll layers and resuspended in FACS buffer. Adult blood and cord blood cells were isolated by Ficoll centrifugation as mentioned above. The isolated cells were stained for surface molecules antibodies in the presence of human FcγR-binding inhibitor (Affymetrix). For intracellular/intranuclear staining, the cells stained for surface molecules were fixed, permeabilized and stained with antibodies. Cell fluorescence was assessed using FACSCanto II (Becton Dickinson) and data were analysed using FlowJo software (Ver. 9.7, Tree Star).

All the antibodies were purchased from Affymetrix or BD Biosciences. The following antibodies conjugated to FITC-, PE-, PE-TxR, PerCP-Cy5.5, PECy-7, APC, Alexa fluor 700, APC-Cy7, Pacific Blue, Am-cyan and BV 605NC were used - CD4 (OKT4), CD8α (RPA-T8), TCR-β (IP26), CD27 (O323), CD28 (CD28.2), CD31 (WM59), CD57 (TB01), CD62L (DREG-56), CD69 (FN50), CD161 (HP-3G10), CD45RA (HI100), CD45RO (UCHL1), HLA-DR (LN3), IFN-γ (4S.B3), IL-2 (MQ-17H12), IL-4 (8D4-8), IL-17 (N49-653), PLZF (Mags.21F7), Eomes (WD1928), Bcl6 (K112-91), FoxP3 (PCH101), Ki-67 (20Raj1). PE-, APC- or Pacific Blue-conjugated human CD1d tetramers loaded with PBS-57 were kindly provided by the National Institutes of Health Tetramer Facility.

In vitro cytokine stimulation assay

Freshly isolated tonsillar lymphocytes (3 x 10^6 cells in 1ml) were stimulated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1.5 μM of ionomycin (Sigma-Aldrich), in complete RPMI media (10% FBS, β-mercaptoethanol, penicillin and streptomycin in supplement to RPMI 1640) for 5 hours. 3 μM of Monensin (Sigma-Aldrich) was added during the last 3 hours of stimulation followed by flow cytometry analysis as described above.

Quantitative real-time PCR

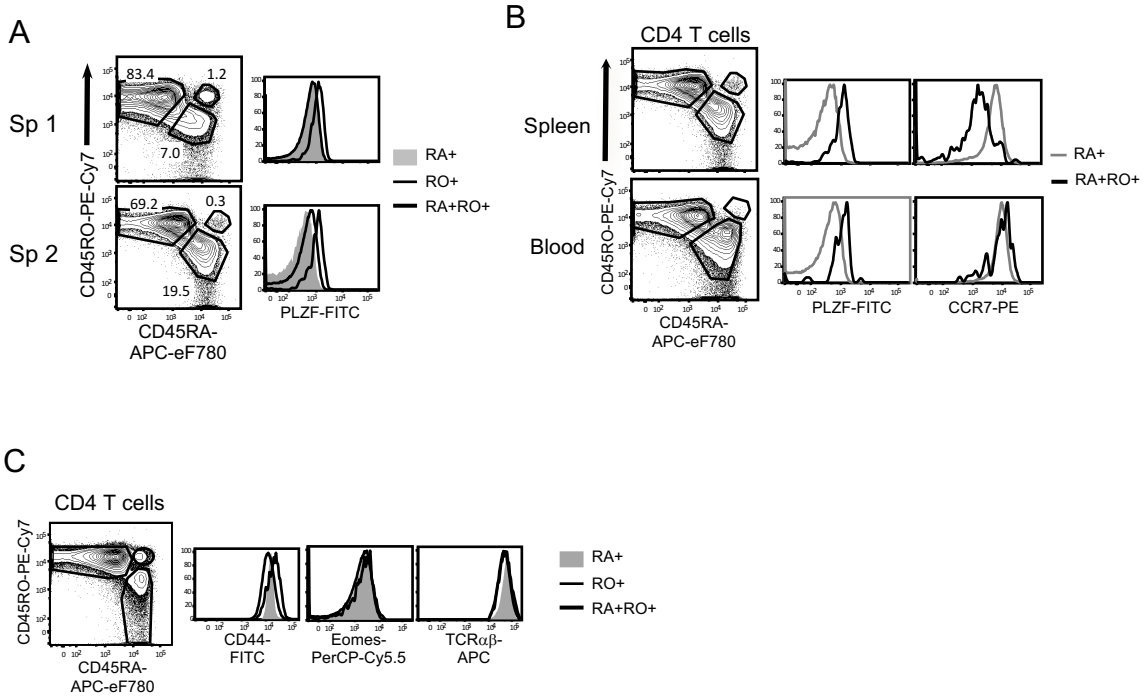
$CD45RA^+$, $CD45RO^+$ and $CD45RA^+RO^+$ CD4 T cells from the tonsil were sorted by flow cytometry. Total RNA was isolated from the sorted populations using RNeasy kit (QIAGEN) and 50 ng of RNA was reverse transcribed to cDNA using poly (dT) and MuLV RT Reverse Transcriptase (Applied Biosystems). RT-PCR was performed in the presence of cDNA, SYBR green mix and specific primers for the target gene on an Applied Biosystems 7400 PCR machine.

The primers (synthesized from IDT) used were as follows: PLZF forward 5'-TTC CTG GAT AGT TTG CGG CTG AGA-3' and reverse 5'-CCG TGT GGC TGG GGA AGG TG-3'. Gene expression of the target genes was normalized to the HPRT (forward 5'-GAA AGG GTG TTT ATT CCT CAT GG-3' and reverse 5'-CTC CCA TCT CCT TCA TCA CAT C-3') expression levels.

Statistical analysis

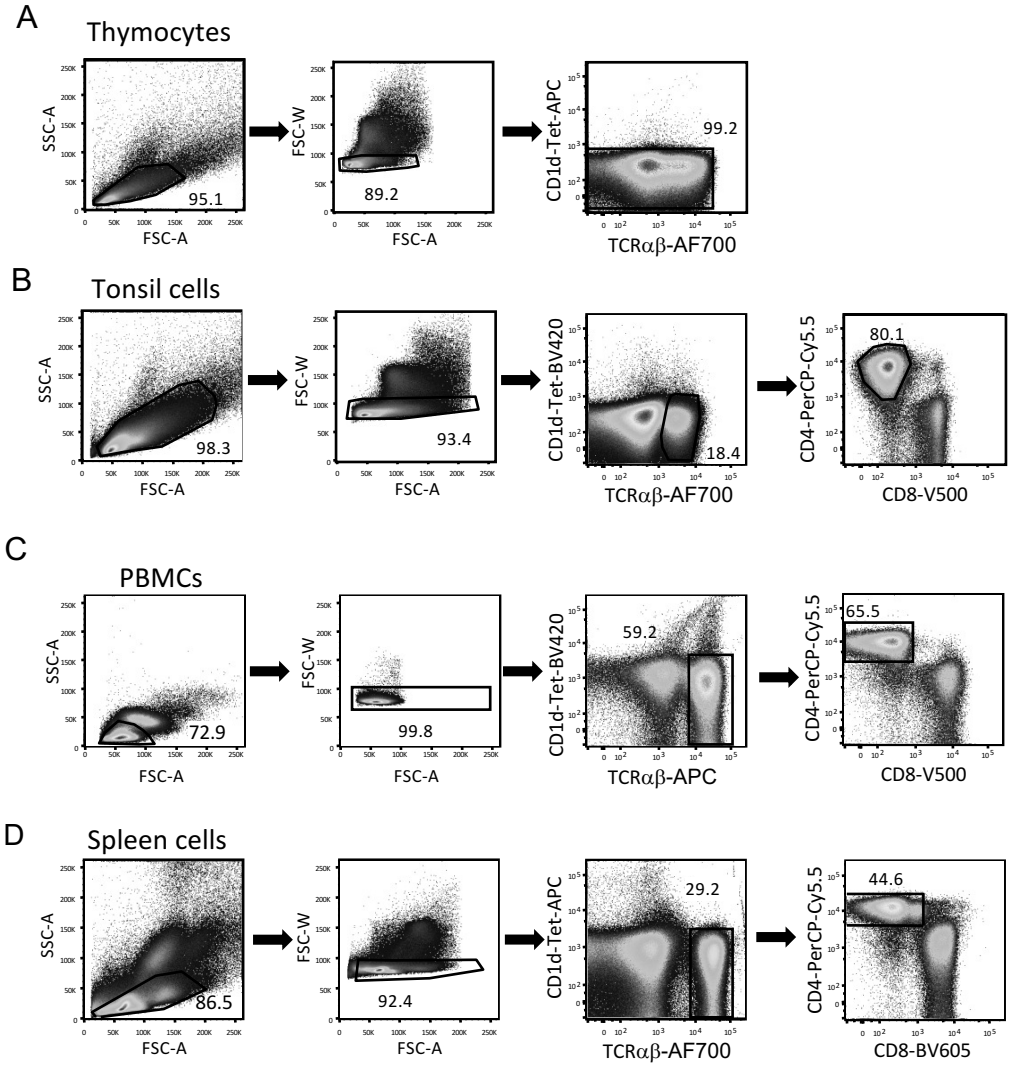
Differences were determined by using the two-tailed Student's *t*-test, two-tailed correlation test using GraphPad Prism version 6 (GraphPad software, LaJolla, CA). The following p values were considered statistically significant: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p<0.0001$.

Supplemental Figure 1



Supplemental Figure 1. Temra-like PLZF⁺ CD4 T cells are present in spleen but not blood. **(A)**Total lymphocytes from spleens were analyzed for PLZF expression by flow cytometry after staining with surface antibodies followed by intra-nuclear staining of PLZF in CD45RA⁺, CD45RO⁺ and CD45RA⁺RO⁺ CD4 T cells. Total 3 spleens were examined and two are shown. **(B)** Total splenocytes and PBMCs were stained as in (A) and gated based on CD45RA and CD45RO to compare PLZF and CCR7 expression levels. **(C)** CD45RA+, CD45RO+, CD45RA+RO+ CD4 T cells from spleen were analyzed for the expression of indicated molecules by flow cytometry after surface and intra-nuclear staining. 3 spleen samples were analyzed of which one representative is shown.

Supplemental Figure 2



Supplemental Figure 2. Gating strategy for flow cytometry analyses. Total cells from thymus (A), tonsil (B), peripheral blood mononuclear cells, PBMCs (C) and spleen (D) were analyzed by flow cytometry after staining with surface antibodies. Cells gated as shown in the preceding plot are shown for each dot plot.