Supplemental Information

Th17-derived memory cells are long-lived and retain a stem cell-like molecular signature

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Running Title: Th17 cells are long-lived

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Supplemental Experimental Procedures

Animals and cell lines

BwRAG1$^{-/-}$TRP-1 and Pmel1 TCR transgenic mice are bred at the National Cancer Institute, NIH animal facility, according to Six to eight week old C57BL/6, B6.PL (Thy1.1^{+}), *Tbx21*$^{-/-}$, *Ifng*$^{-/-}$ mice on C57/BL6 background were purchased from the Jackson Laboratory or bred at the NCI, NIH animal facility. Experiments were conducted with the approval of the National Cancer Institute Animal Use and Care Committee. *IL-17A*$^{-/-}$ mice backcrossed into C57BL/6 background were obtained from Dr. Rachel Caspi at the National Eye Institute, NIH with kind permission of Dr. Yoichiro Iwakura, University of Tokyo, Japan. IL-17F^{GFP} reporter mice were created as follows: murine bacterial artificial chromosome (BAC) was modified to introduce a *GFP* reporter gene into the *Il17f* gene using recombineering technology as described previously (Mazzucchelli et al., 2009) . By homologous recombination, the sequence of the signal peptide of *Il17f* in the BAC was disrupted and the GFP gene with polyA was inserted immediately after the ATG start site, replacing exon 1. Expression in transgenic reporter mice was validated by diverting CD4 T cells into the Th17 pathway and performing intracellular staining for IL-17F, showing correspondence with the reporter. BAC transgenic mice were made on a C57BL/6 background. Murine melanoma B16-F10 cells and splenocytes were maintained in culture media (CM) described previously (Muranski et al., 2008). Platinum Eco viral packaging cells (Cell Biolabs) were maintained in DMEM (Invitrogen) media with the same supplements as in CM.

In vitro polarization and stimulation

TRP-1 cells were stimulated with irradiated (30 Gy) C57BL/6 splenocytes pulsed with TRP-1 peptide (1μM) added at a 10:1 ratio. Th1 polarization was performed in presence of 3.33 ng/ml of IL-12 (Peprotech) and 10μg/ml of anti-IL-4. Th17 polarization was performed in the presence of IL-1 (10ng/ml, Peprotech), IL-6 (30ng/ml, R&D or Peprotech), IL-21 (100ng/ml), TGFβ2 (5ng/ml, R&D), anti-IL-2 (10μg/ml), anti-IL-4 (10μg/ml) and anti-IFN-γ (10μg/ml). After 72 hours, media containing IL-2 was added and the polarizing cytokines were repleted. Th17 cultures were also supplemented with

IL-23 (5ng/ml, R&D). TRP-1 cells were used for adoptive transfer experiments 7-8 days after the initial stimulation. Open repertoire $CD4^+$ cells or $CD8^+$ cells from indicated donors were stimulated under polarizing conditions using plate-bound anti-CD3 antibody (1μg/ml) and soluble anti-CD28 antibody (1μg/ml). All antibodies were purchased from eBioscience.

Flow cytometry and antibodies

Antibodies against IL-17A, IL-17F and RORγt were purchased from eBioscience. All other flow cytometry antibodies used in this report were purchased from BD Pharmingen. For intracellular cytokine staining, cells were stimulated for 3 to 4 hours with 50 ng/mL PMA (Sigma-Aldrich) and 750 ng/mL ionomycin (Calbiochem) or not at all. After 1 hour, GolgiStop (BD Pharmingen) was added per manufacturer's instructions. Cells were surface stained for 30 minutes at 4°C with anti-CD4 in PBS supplemented with 1% BSA and 0.2% sodium azide and Fixable Green viability stain (BD Pharmingen). Intracellular staining was performed using IC Fixation/Permeabilization Buffers (eBioscience) according to the manufacturer's instruction. For detection of intracellular cytokines in TRP-1 cells surviving *in vivo* bulk splenocytes were stimulated for 6 hours with TRP-1 peptide (1μg/ml) in presence of GolgiStop and further processed as above. Flow cytometry acquisition was performed on a FACS Canto II or FACSCalibur and analyzed with FlowJo 7.2 (TreeStar). The expression of Annexin V was measured upon overnight stimulation with soluble aCD3 and aCD28 monocolonal antibodies (1μg/ml) using Annexin V PE Apoptosis Plus Kit (Biovision) following the manufacturer's directions.

Western blot analysis

Cells were lysed in RIPA buffer (Cell Signaling Technology) with protease inhibitor. Protein concentration was quantified by Bio-Rad protein assay. 30 g of total protein was separated on a 4–12% SDS-PAGE gel followed by standard immunoblotting with antibody to β-catenin (BD Bioscience), antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon International), and horseradish peroxidase–conjugated goat antibodies to mouse IgG (Santa Cruz Biotechnology).

Quantitative gene expression analysis by RT-PCR

RNA isolation was performed with the use of QiaShredders and the RNeasy Mini Kit (Qiagen). cDNA was synthesized with the High Capacity RNA-to-cDNA Kit (ABI). qRT-PCR was performed on an ABI 7500 Fast machine following manufacturer protocols. Probes utilized include *Rorc*, *Tbx21*, *Bcl2* and *Tcf7*. All probes are commercially available (ABI).

Retroviral Production and Transduction

Retroviral Vector encoding TRP-1 TCR: The MSGV-1 retroviral vector encoding the TRP-1 TCR was constructed in two separate steps as described previously (Kerkar et al., 2011). In the first step, the alpha chain (Vα3.2) was placed upstream the GSG-linker P2A self cleaving peptide followed by the beta chain (Vβ14) by gene synthesis (Blue Heron Biotechnology) in a bacterial vector. This insert was flanked by the restriction sites 5' NcoI and 3' NotI. Endogenous NcoI sites present in the constant regions were mutated silently during the gene synthesis step. In the second step, the gene encoding the alpha-GSGP2A-beta TRP-1 sequences was subcloned into MSGV-1 retroviral vector by standard cloning techniques using NcoI/NotI restriction sites.

Retroviral Production: Platinum Eco 293 based cells were plated on poly-d-lysine coated 100mm plates (BD Biosciences) and transfected with 6 μg of pCL-Eco helper plasmid (Imgenex) and 9.3 μg of the MSGV-1 TRP-1 TCR vector with lipofectamine 2000 (Invitrogen) overnight in antibiotic-free CM. Viral supernatants were harvested 36-48 hrs post transfection.

Retroviral transductions: Purified $CD4^+$ T cells were cultured under Th1 or Th17 polarizing conditions as indicated in the presence of 1 μg/mL soluble anti-CD3 and anti-CD28 monoclonal antibodies (BD Biosciences). Two days later, splenocytes were collected and resuspended in retroviral supernatant with 60 IU/mL rhIL-2 and 10 μg/mL protamine sulfate (Abraxis Pharmaceutical Products), and spun at 1000g at 37° C for 90 minutes in 24 well plates. Cultured cells were adoptively transferred 3-5 days post transduction.

Microarray methods

RNA isolation and processing: Total RNA was isolated from cells using RNeasy Mini kits (Qiagen), following the manufacturer's instructions. Quality of total RNA was evaluated using RNA 6000 Nano LabChip (Agilent 2100 Bioanalyzer, Santa Clara, CA). All samples had intact 18S and 28S ribosomal RNA bands with RIN numbers from 8.1 to 10 and RNA 260/280 ratios between 1.9 and 2.0. Samples with insufficient mRNA quantity were rejected prior to analysis. Gene expression levels were determined using GeneChip Mouse Gene 1.0 ST arrays according to manufacturer's protocols (Affymetrix, Santa Clara, CA). 100 ng of tRNA was used as starting material for cDNA amplification and cRNA in vitro transcription (WT Expression Kit, Ambion). cDNA was fragmented labeled using Affymetrix GeneChip WT Terminal Labeling Kit. Fragmented and labeled cDNA was hybridized on the arrays for 18 h following the manufacturer's directions. Arrays were stained and washed in the Affymetrix Fluidics Station 400 and scanned on an Affymetrix GeneChip Scanner 3000 7G system.

Microarray statistical analysis: Gene expression analysis was performed using a one-way ANOVA with 8 treatment groups comprised of Th1 and Th17 cells, at Day 5 and Day 15, in both the Unstimulated and Stimulated arms of the study. Direct comparison of Th17 to Th1 cells was achieved through *post hoc* t-tests. False discovery rates (FDR) were generated only considering a subset of probesets excluding positive and negative controls. Contrasts at Day 0 are limited to fold-change estimation due to a lack of replication and are not subject to statistical analysis. Graphs of gene expression were plotted with the relative group means and standard errors.

Gene Set Enrichment Analysis: All of the genes differentially expressed by 2-fold or greater between primary and quaternary cells were selected from Table S4 in Wirth, *et al.* The genes were analyzed using Gene Set Enrichment Analysis (GSEA). Three GSEA tests were employed using default settings, testing the complete 2-fold gene set, the 2-fold up-regulated genes alone, and the 2-fold down-regulated genes alone. These three gene sets were analyzed in terms of the difference between Th17 cells at day 15 versus Th1 cells at day 15.

Proliferation assays

In vitro proliferation analysis of naïve purified TRP-1 cells was performed using carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) according to manufacturer directions. CFSE-labeled cells were stimulated in the presence of plate-bound anti-CD3 and soluble anti-CD28 monoclonal antibodies (1μg/ml) under Th1 and Th17-polarizing conditions. Cells were harvested and fixed at indicated timepoints. CFSE labeling was measured by flow cytometry on the final day of the experiment.

For ^{3}H-thymidine assays, *in vitro* generated Th1 and Th17 cells were re-stimulated with 1μg/mL plate bound anti-CD3 and 1ug/mL soluble anti-CD28 antibodies. At indicated times, cell numbers were equalized between groups, placed in 96-well round-bottom plates (Corning) and pulsed with 1 μCi 3H-thymidine and incubated for 18 hours at 37 degrees Celsius and 5% CO_2 in CM with IL2. Samples were harvested using a semiautomated sample harvester, and counts per minute were determined with a β-scintillation counter. Assays were performed in triplicates, and values are presented as mean ± SEM.

Adoptive cell transfer experiments

Tumor treatment experiments: C57/B6 mice 6 to 12 weeks of age were inoculated subcutaneously with $5x10^5$ B16-F10 melanoma cells. 7-10 days later $1x10^6$ TRP-1 $CD4^+$ T cells or $1.2x10^6$ TRP-1 TCR-transduced $CD4^+$ T cells derived from various donors, as indicated were adoptively transferred *via* tail vein injection. Recipient animals were sublethaly irradiated (5 Gy) prior to the adoptive cell transfer. Where indicated, a single dose of recombinant TRP-1 vaccinia virus vaccine (rVV TRP-1) and 3 daily doses of rhIL-2 (12 ng/dose; Chiron) were administered by intraperitoneal injection. Tumors were measured using calipers, and the products of the perpendicular diameters were recorded. All experiments were repeated independently at least twice, with similar results.

Persistence: A total of $1x10^6$ Th1 or Th17-polarized TRP-1 cells ($Thy1.2^+$), were transferred into 5 Gy irradiated B6PL ($Thy1.1^+$) mice bearing 4 day tumors with or without rVV TRP-1 and IL-2 as indicated in the figure legends. Spleens and inguinal lymph nodes were collected at indicated days and homogenized. Cells were enumerated

using trypan blue exclusion. Absolute numbers of transferred cells in the spleen and lymph nodes of mice were obtained by multiplying the percentage of Vβ14$^+$, CD4$^+$ and Thy1.2$^+$ cells by the total number of viable cells counted. For persistence experiments involving CD27^{high} and CD27^{low} subsets, Th1 and Th17 cells were stained for Thy1.2 and CD27 expression. Th17 cells were sorted by flow cytometry using Th1 cells as a positive control. Th1 cells and Th17 subsets were adoptively transferred to tumor-free B6.PL mice as described above. For experiments involving non-irradiated recipients 1x10^6 polarized cells were transferred into intact B6.PL mice. Blood was collected by tail vein incision. Frequency of CD4$^+$Thy1.2$^+$Vβ14$^+$ cells was analyzed by flow cytometry. For cytokine capture experiments *in vitro* polarized Th1 and Th17 TRP1 cells were stimulated for 3 hours with PMA and ionomycin and labeled using Mouse IL-17 Secretion Assay Cell Enrichment and Detection Kit (PE) (Mitenyi Biotec) following the manufacturer's instructions. IL-17-labeled Th17 cells were subsequently FACS-sorted using Th1 cells as negative control. Purification of cells derived from IL-17F^{gfp} reporter mice was performed in the same manner. A total of 4x10^5 Th1 and Th17 cells were transferred into B6.PL mice as described above. Frequency of CD4$^+$Thy1.2$^+$Vβ14$^+$ in peripheral blood collected by tail vein incision was analyzed by flow cytometry.

Adoptive cell transfer for serial gene expression profiling: *In vitro* polarized TRP-1 Th1 and Th17 cells were generated. Cells were enriched with magnetic bead separation (Miltenyi) by CD4 negative selection on the day of the transfer. Aliquots of the cells for the "Day 0" timepoint were further sorted for the expression of CD4 on a BD FACS Aria processed with a QiaShredder (Qiagen) to eliminate genomic DNA and frozen at -80 degrees Celsius. The remaining cells were transferred into B6.PL (Thy1.1$^+$) mice at a total dose of 1x10^6 per mouse. Four parallel transfers of cells were performed for each polarizing condition, yielding eight separate experimental groups. On days 5 and 15 post transfer, six mice from each group were sacrificed and their spleens and lymph nodes were harvested and pooled. CD4$^+$ T cells enriched with magnetic bead separation (Miltenyi) by negative selection, and CD4$^+$Thy1.2$^+$ T cells were sorted on a BD FACS Aria cell sorter. Recovered cells were shredded with a QiaShredder (Qiagen) and stored at -80 degrees Celsius for subsequent processing.

Cytokine release assays

T cells were tested for secretion of IFN-γ, IL-17A, IL-17F, IL-21 and IL-2 in release assays using R&D Systems ELISA kits according to manufacturer's protocol. Irradiated splenocytes (30Gy) were pulsed with escalating doses of TRP-$1_{106-130}$ or 1 μM of an irrelevant peptide ($gp100_{25-33}$). Co-culture was performed with washed $1e^5$ TRP-1 $CD4^+$ T lymphocytes or TRP-1 TCR transduced $CD4^+$ cells and $1e^5$ TRP-1-peptide pulsed splenocytes in a 0.2-mL culture volume in individual wells of 96-well plates for 16 hours at 37°C. Data were fit with a standard curve against control protein of known concentration and analyzed. Cytokine secretion was measured in culture supernatants diluted to be in the linear range of the assay.

Ocular autoimmunity evaluation

Eyes were enucleated on D+58 from treated mice, pre-fixed in 4% phosphate-buffered glutaraldehyde solution for 30 minutes (to prevent artifactual retinal detachment), and then transferred to 10% phosphate-buffered formalin until processing. Fixed and dehydrated tissues were embedded in methacrylate, and 4- to 6-μm sections were stained with standard hematoxylin and eosin. Tissue sections were evaluated in a blinded fashion (C.-C.C.) as previously described (Palmer et al., 2008) with the following additions: the presence or absence of cataracts was scored as 0 or 1, respectively; retinal folds were scored as 1 and retinal degeneration was scored as 2. Values are presented as the mean +/- SEM for summation of pathology scores.

Statistical Methods

Tumor slopes were compared using Wilcoxon rank sum test. Single-measurement comparisons between two groups were tested using unpaired *t*-tests. Prism GraphPad software (GraphPad Software Inc.) was used for these analyses.

References

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Supplemental Figures

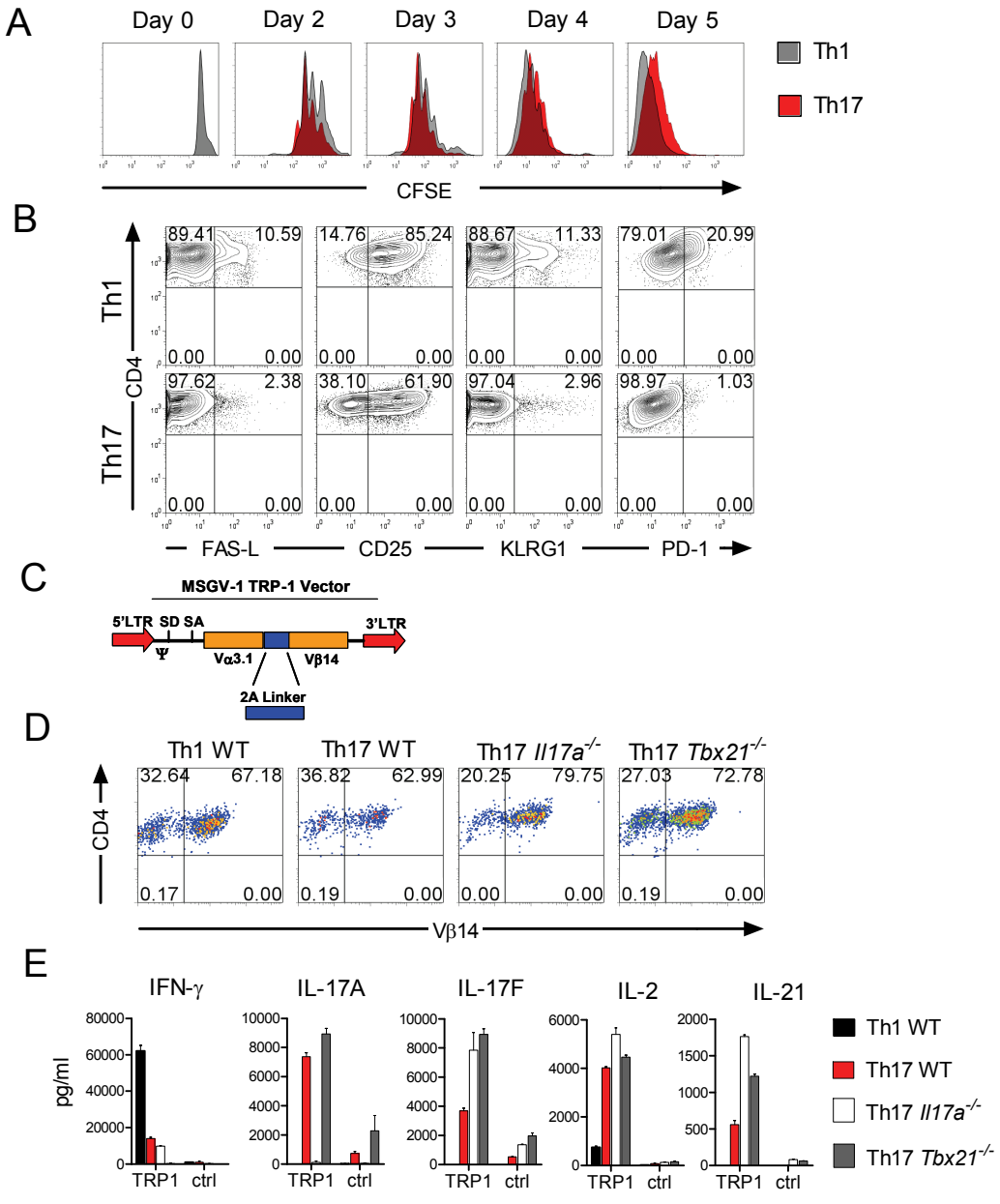


Figure S1. Characterization of *in vitro* generated Th1 and Th17 TRP-1 cells for adoptive immunotherapy.

(A) Proliferation of TRP1 cells cultured under Th1 and Th17-polarizing conditions. Naïve TRP1 cells were isolated by CD4 negative selection, stained with CFSE per manufacturer's instructions and stimulated *in vitro* with anti-CD3 and anti-CD28 monoclonal antibodies under Th1 and Th17 conditions. At indicated days cells were harvested and fixed. Samples were evaluated by flow cytometry at the end of the

experiment.
(B) Expression of indicated surface markers associated with memory differentiation state on *in vitro* polarized Th1 and Th17 cells cultured for 7 days.
(C) Graphical representation of the retroviral vector encoding TRP-1 TCR.
(D) Purified $CD4^+$ T cells from indicated donors were stimulated *in vitro* under indicated polarizing conditions for 48 hours and transduced with TRP-1-encoding retroviral vector. Vβ14 expression was measured on day 4 after transduction. Staining is representative of multiple experiments.
(E) The same TRP-1 TCR-transduced cells were tested for secretion of indicated cytokines upon 18hr stimulation with splenocytes pulsed with TRP-1 peptide or unrelated peptide (ctrl). Error bars indicate SEM (n=3).

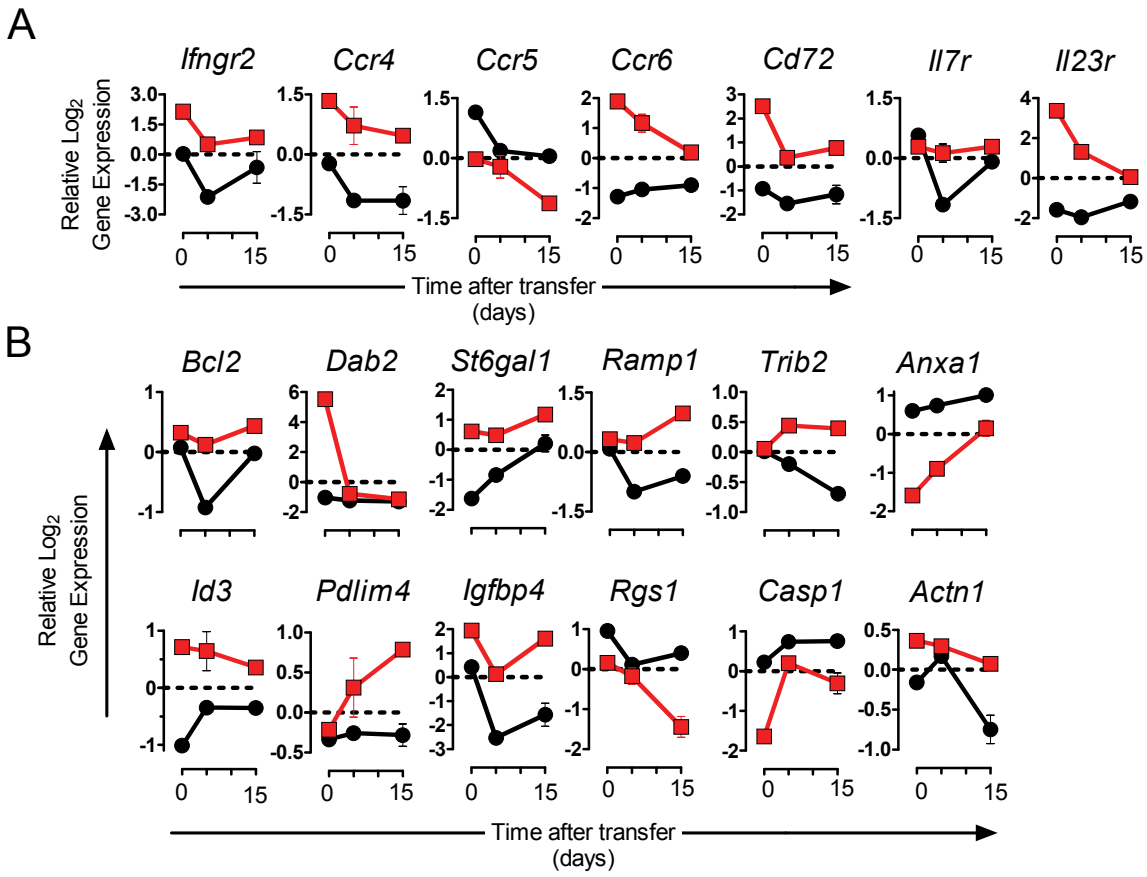
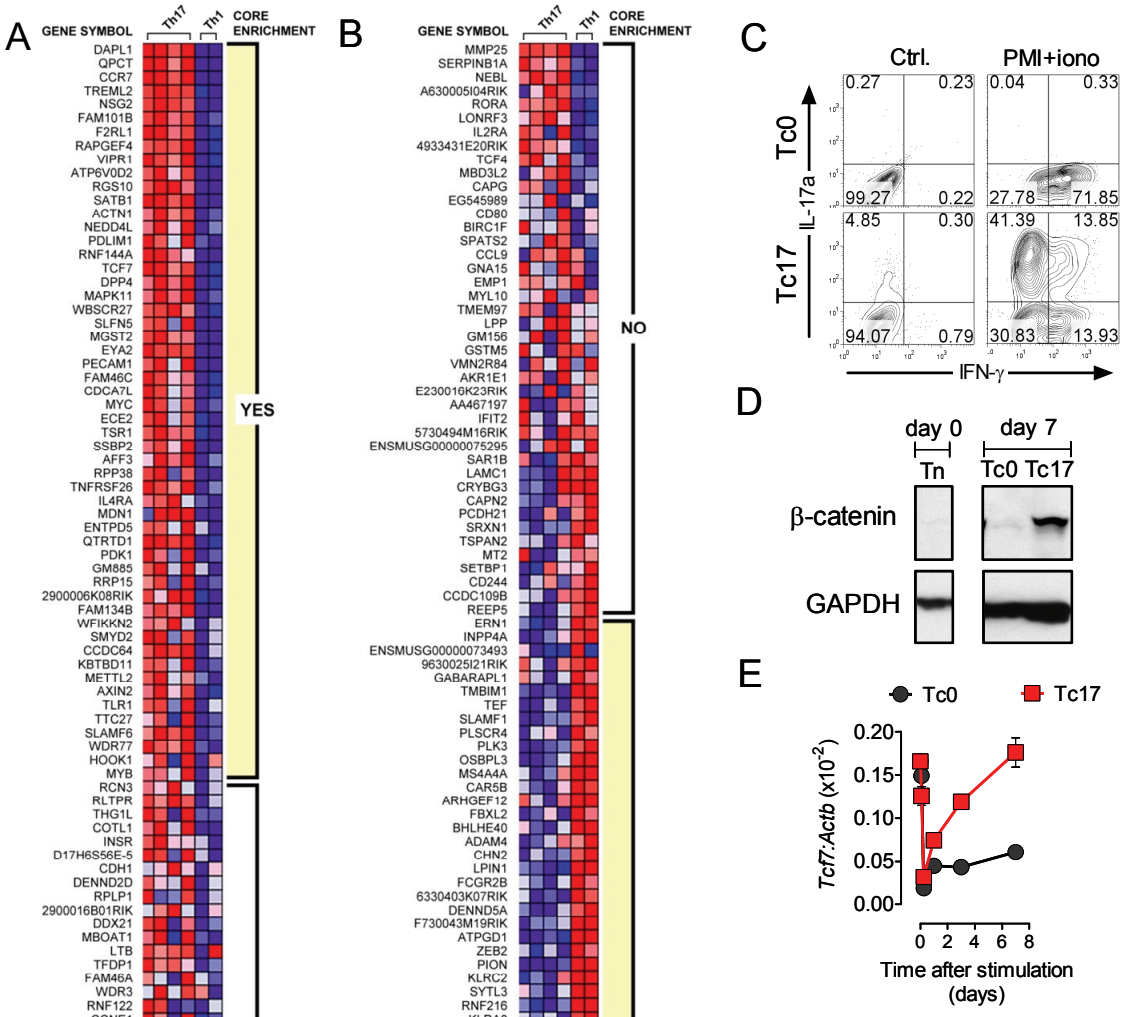


Figure S3. Relative gene expression of selected genes in surviving Th17- and Th1-derived cells. Gene expression profile was analyzed in Th17 and Th1 cells at indicated timepoints.
(A) Transcriptomes encoding for selected surface markers, including cytokine and chemokine receptors are shown.
(B) Transcriptomes encoding selected genes, including transcriptional regulators with less known function in T cells. Some of the genes shown here have been associated with T cell memory subsets in literature. Error bars represent SEM for samples obtained on days 5 and 15 (n=2-4)


A
GENE SYMBOL
Th17
Th1
CORE ENRICHMENT
DAPL1
QPCT
CCR7
TREML2
NSG2
FAM101B
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RAPGEF4
VIPR1
ATP6V0D2
RGS10
SATB1
ACTN1
NEDD4L
PDLIM1
RNF144A
TCF7
DPP4
MAPK11
WBSCR27
SLFN5
MGST2
EYA2
PECAM1
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CD244
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TEF
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PLK3
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CAR5B
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BHLHE40
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PIK3AP1
CCR5
CMKLR1
FASL
CDC20B
SLAMF7
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KLRA9
GZMB
HAVCR2
EG433024
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KLRE1
KCNJ8
CX3CR1
S1PR5
KLRA10
KLRG1
NO
YES
C
Ctrl.
PMI+iono
Tc0
Tc17
IL-17a
IFN-γ
0.27
0.23
99.27
0.22
0.04
0.33
27.78
71.85
4.85
0.30
94.07
0.79
41.39
13.85
30.83
13.93
D
day 0
day 7
Tn
Tc0 Tc17
β-catenin
GAPDH
E
Tc0
Tc17
Tcf7:Actb (x10-2)
0.20
0.15
0.10
0.05
0.00
0 2 4 6 8
Time after stimulation (days)

Figure S3. Surviving Th17-derived cells display molecular signature associated with early memory CD8+ T cells. Type 17-polarized CD8+ T cells acquire stem cell-like molecular signature.

(A)(B)GSEA enrichment heat maps and core enrichment for gene sets representing molecular signature of early (A) and late (B) memory cells , generated as described in materials and methods and in Figure 3A and 3B.

(C) Purified Pmel1 $CD8^+$ T cells were stimulated *in vitro* with aCD3 and aCD28 monoclonal antibodies under neutral (Tc0) or type 17-polarizing conditions (Tc17). Representative intracellular staining demonstrating production of *in vitro* polarized IFN-γ and IL-17a by Tc0 and Tc17-polarized TRP1 cells generated upon 3 hour stimulation with PMA/ionomycin in the presence of brefeldin A. Resting cells were used as control.

(D) Presence of stable β-catenin was analyzed by Westen blot in Pmel1 CD8+ cells on indicated days. Tn represent freshly purified cells obtained from Pmel1 spleens..GAPDH was used as positive control.

(E) In the same experiment Tc0 and Tc17 cells were harvested at indicated times after initial stimulation. Expression of *Tcf7* was measured by RT-PCR and shown as expression relative to β-actin. Error bars represent SEM (n=3).

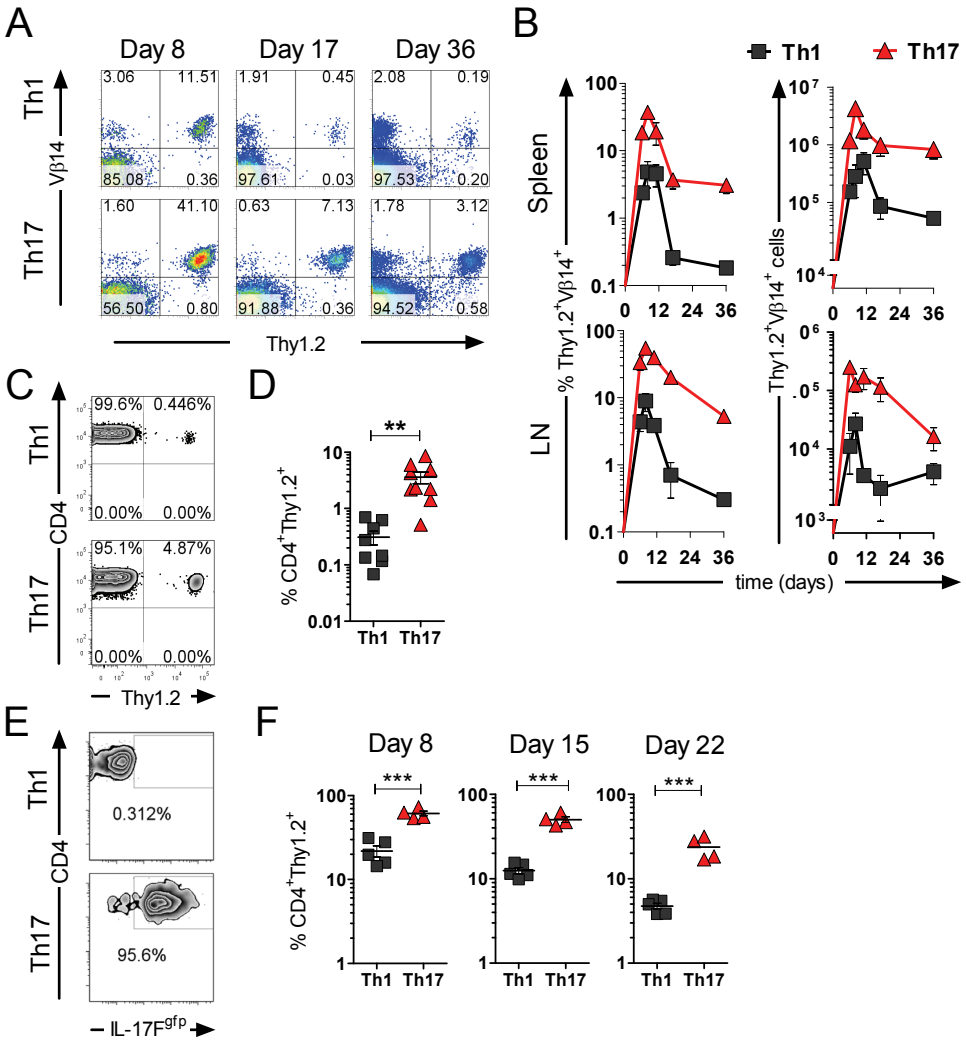

Figure S4. *In vivo* survival of Th1 and Th17-polarized TRP-1 cells upon adoptive cell transfer and co-administration of rVVTRP1 vaccination and rhIL-2. A total of $1x10^6$ Th1 and Th17 TRP1 cells ($Thy1.2^+$) were adoptively transferred to sublethaly (5Gy) irradiated B6.PL mice ($Thy1.1^+$). On the day of transfer mice received rVVTRP1 vaccine and three daily injections of rhIL-2. Spleens and inguinal lymph nodes were harvested and analyzed by flow cytometry at indicated time points for presence of $Thy1.2^+V\beta14^+$ cells.

(A) Representative contour plots shows surviving $Thy1.2^+V\beta14^+$ cells in spleens at indicated days after adoptive cell transfer.

(B) Mean frequency (left panels) and total number per mouse (right panels) of persisting Th1 or Th17 cells recovered at indicated days are shown. Error bars represent SEM (n=3-6).

(C) (D) *In vivo* persistence of TRP1 cells polarized into Th1 and Th17 subset following the transfer into intact (non-irradiated) B6.PL mice. Frequency of Thy1.2 cells (gated on $CD4^+$ population) was measured 10 days after transfer in peripheral blood. Representative stainings are shown in **(C)** and combined data from all experimental animals is shown in **(D)**. Error bar represents SEM (n=8-9); **=$p<0.01$.

(E) (F) CD4+ T cells were isolated from IL-17Fgfp BAC transgenic reporter mice and polarized *in vitro* into Th1 and Th17 subsets for 7 days. Cells were re-stimulated with PMA/ionymicin for 3 hours and cells expressing green florescent protein (GFP) were FACS sorted from the Th17 subset. Dot plots (E) show purity of sorted Th17 cells as compared to Th1 cells used as negative control. A total of 4x105 Th1 and purified Th17 cells were transferred into sublethaly irradiated B6.PL (Thy1.1+) animals. Frequency of transferred Thy1.2+ cells was measured in peripheral blood as in (C) and (D). Error bar represents SEM (n=4-5), ***=$p<0.001$.