Supporting Information

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SI Materials and Methods

Mice and Mouse Procedures. Mice carrying *Rosa26*^{YFP} (74), the P14 TCR transgene (75), and *Cd4*-cre (76), *CD2*-cre (29) or *Ox40*-cre (50, 51) (obtained from N. Killeen, University of California, San Francisco, via J. Zhu, National Institutes of Health, Bethesda) were previously described. CD45.1 and CD45.2 C57BL/6 mice were obtained from the National Cancer Institute Animal Production Facility. All transgenic mice were maintained heterozygous for the transgene. Mice were housed in specific pathogen-free facilities and analyzed between 6 and 16 wk of age. For LCMV infection, animals were injected intraperitoneally with 2×10^5 pfu of LCMV Armstrong (obtained from D. McGavern, National Institutes of Health, Bethesda). For bone marrow chimeras, T cell-depleted (Pan T Dynal kit; Invitrogen) bone marrow cells were prepared from CD45 disparate mice, mixed at a 1:1 ratio, and injected into lethally irradiated (900 rads) CD45.1 recipients. Eight weeks after reconstitution, transplanted animals were infected with LCMV.

Antibodies. Antibodies for the following specificities were purchased either from Affymetrix eBiosciences or BD Pharmingen: CD4 (RM4.4 or GK1.5), CD8α (53-6-7), CD40L (MR1), CD45.2 (104), CD45.1 (A20), TCRβ (H57-597), IFNγ (XMG1.2), IL-17a (eBio17B7), CD44 (IM7), and Thy1.1 (HIS51). Antibodies specific for granzyme B (GB12) were purchased from Invitrogen.

Protein Expression Analyses. Analysis of Runx protein expression was performed by Western blot as previously described (29), using anti-Runx antibody (EPR3099, Epitomics). Membranes were subsequently stripped and blotted with anti–β-actin (AC-15; Sigma) to control for lane loading.

Cell Preparation and Staining. Spleen and intestinal lymphocytes were prepared and stained as previously described (28, 77). Flow cytometry data were acquired on LSR II or LSR Fortessa cytometers (BD Biosciences) and analyzed with FlowJo (TreeStar) software. Dead cells and doublets were excluded by LiveDead staining (Invitrogen) and forward scatter height by width gating. Purification of lymphocytes by cell sorting was performed on a FACSAria (BD Biosciences). Analyses of intracellular cytokine or granzyme expression was performed as previously described (78). CD40L expression was assessed after 3 h in vitro stimulation as described (29). CD4$^+$ IL-17 and IFNγ-producing cells, referred to as Th17 and Th1 cells, were isolated from the siLP by cell sorting after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, as previously described (78).

In Vitro T-Cell Culture. Sorted naïve (CD44^{lo}) T cells were activated with anti-CD3 and anti-CD28 (BioXCell; 1 μg/mL and 3 μg/mL, respectively) in 96-well plates at 5×10^4 per well, in the presence of T cell-depleted irradiated WT splenocytes (2.5×10^5 per well). Cell were cultured for 3 d in the presence of IL-12 (10 μg/mL) and anti-IL-4 (10 μg/mL) (Tc1 and Th1 conditions), or of TGF-β (5 ng/mL), IL-6 (50 ng/mL), and antibodies against IFNγ, IL-4 and IL-12 (10 μg/mL each) (Tc17 and Th17 conditions), or in the absence of any additional cytokine or antibody (ThN conditions). Cultures were split (1:2), supplemented with IL-2 (100 ng/mL), and maintained for an additional day. Cytokines were from PeproTech and blocking antibodies from BioXCell.

In vitro cytotoxicity was determined using pan-T depleted WT splenocytes coated with relevant GP33 (KAVYNFATM) or irrelevant (SIIFNEKL) peptides (1 μg/mL; 3 h at 4°C and 1 h at 37 °C, 5% CO_2). GP33-loaded and control populations were incubated with distinct CFSE concentrations (0.1 μM and 2 μM, respectively), and cocultured with in vitro-derived CD8$^+$ effector T cells for 24 h. Specific killing was calculated as follows: $(1 - [R_{Target+Effector}/R_{Target}]) \times 100$, where R is the ratio of the percentage of GP33-loaded (CFSE^{low}) cells over the percentage of control peptide-loaded (CFSE^{high}) cells.

Microarray and Real-Time qPCR Analyses. Affymetrix Mouse Exon 2.0 ST array were analyzed at the NCI microarray facility from total RNA extracted from activated T cells using RNeasy Plus Mini columns (Qiagen), following the manufacturer's recommendation. For each condition or genotype, data are from three replicates generated from two T-cell preparations isolated from two distinct mice.

For real-time qPCR, cDNA templates were synthesized from total RNA using Thermoscript (Invitrogen). Quantitative PCR was performed with the QuantStudio 6 Flex System (Applied Biosystems) and the SYBR green dye (Invitrogen) detection protocol as previously described (31, 79). Within each experiment, mRNA expression values were normalized to the amount of *Actb*, encoding β-actin. Differences in gene expression were calculated using the $2^{-\Delta Ct}$ method. The following primers were used: *Actb* 5′-TGAGAGGGAAATCGTGCGTGAC-3′ and 5′-AAGAAGGAAGGCTGGAAAAGAG-3′, *Gzma* 5′-TGTGAAACCAGGAACCAGATG-3′ and 5′-GGTGATGCCTCGCAAAATA-3′, *Gzmb* 5′-TGCTCTGATTACCCATCGTCC-3′ and 5′-GCCAGTCTTTGCAGTCCTTTATT-3′, *Gzmk* 5′-GCCATTTATGGCGTCCATCC-3′ and 5′-TGATGTCATGCGATGCGGAA-3′, *Eomes* GCCTACCAAAACACGGATA-3′ and 5′-TCTGTTGGGGTGAGAGGAG-3′, *T-bet* 5′-GTTCCCATTCCTGTCCTTC-3′ and 5′-CCTTGTTGTTGGTGAGCTT-3′, *Prf1* 5′-CCCACTCCAAGGTAGCCAAT-3′ and 5′-GCTGTAAGGACCGAGATG CG-3′, and *Cd40lg* 5′-GGCAATTTGAAGACCTTGTCA-3′ and 5′-CTGCATTACTGTTGGCTTCG-3′.

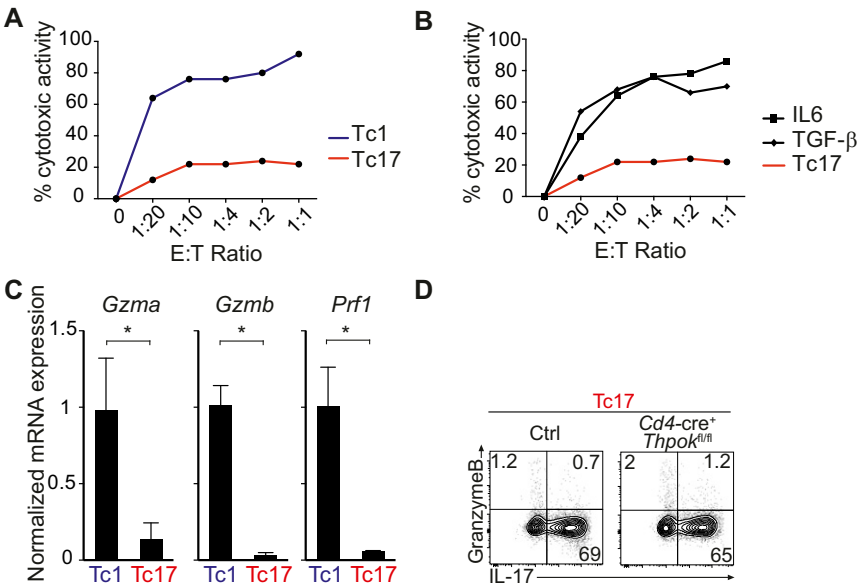

Fig. S1. The Tc17 transcriptional program represses cytotoxic functions independently of Thpok expression. (*A* and *B*) Percent death among target (T) cells (GP33 peptide-loaded splenocytes) cocultured with P14 TCR transgenic effector (E) $CD8^+$ T cells in the indicated conditions and at the indicated E:T ratios. Data are representative of two independent experiments for each condition. (*C*) Relative expression of *Gzma*, *Gzmb*, and *Prf1* mRNAs in Tc1 and Tc17 cells, evaluated by RT-qPCR. Data are expressed relative to values in Th1 cells, set to 1 for each gene. (*D*) Contour plots of IL-17 vs. granzyme B expression on $CD8^+$ T cells from *Cd4*-cre^- (Ctrl) and *Cd4*-cre^+ *Thpok*$^{fl/fl}$ mice expressing the P14 TCR transgene, cultured under Tc17 conditions. Similar results were obtained from MHC II-deficient *Thpok*$^{-/-}$ mice. Data are representative of three (*A*) or two (*B*–*D*) independent experiments. *$P < 0.05$.

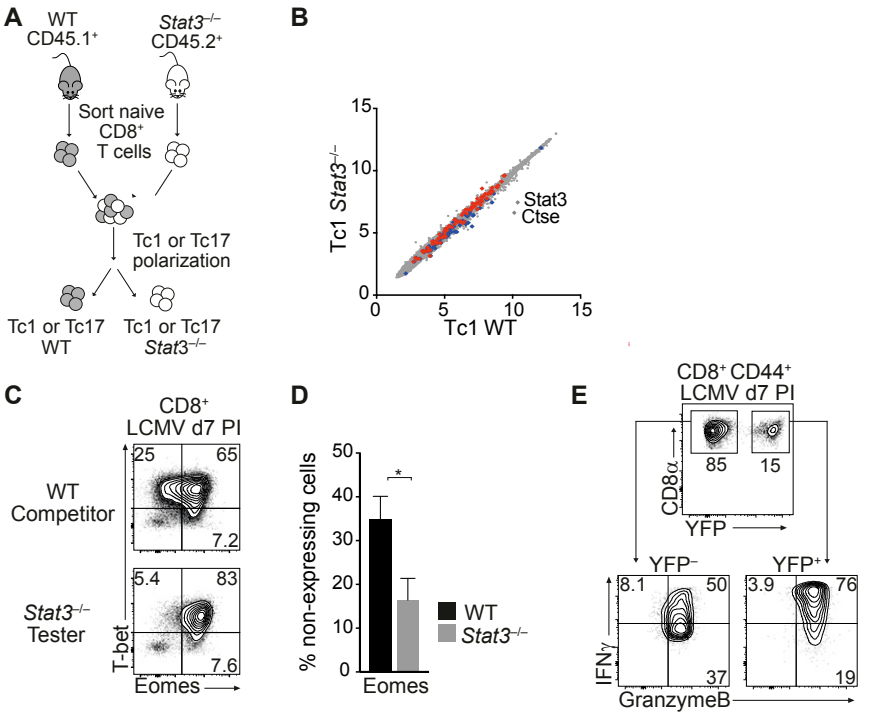

Fig. S2. STAT3 represses cytotoxic gene expression in CD8$^+$ T cells. (*A*) Schematic of experimental procedures. Naïve CD45.2$^+$ CD8$^+$ T cells from *Stat3*$^{-/-}$ (*Cd4*-cre$^+$ *Stat3*$^{fl/fl}$) mice were mixed at a 1:1 ratio with WT CD45.1$^+$ CD8$^+$ T cells, cultured under Tc1 or Tc17 conditions, and separated by cell sorting for CD45 allelism before analyses where indicated. (*B*) Scatterplots show microarray gene expression ($\log_2$ values, full gene set) in indicated cell populations after sorting from mixed cultures set as in *A*. Genes with 1.5-fold or greater expression change in wild-type Tc17 vs. Tc1 cells ($P < 0.05$), as identified in Fig. 2*B*, are shown in red and blue. Genes with reduced expression in STAT3-deficient Tc1 cells are indicated. Data are from three replicates. (*C* and *D*) Mixed bone marrow chimera (*Stat3*$^{-/-}$: WT; 1:1) were infected with LCMV Armstrong and analyzed 7 d after infection. (*C*) Contour plots show Eomes vs. T-bet intracellular expression in spleen WT or *Stat3*$^{-/-}$ CD44$^+$ CD8$^+$ T cells. (*D*) Percentage of CD8$^+$ CD44$^+$ T cells not expressing Eomes. Data are representative of eight mice analyzed in two independent experiments. (*E*) YFP$^-$ and YFP$^+$ CD44^{hi} CD8$^+$ T cells were sorted from the spleen of *Ox40*-cre$^+$ *Rosa26*^{YFP} mice 7 d after LCMV infection (*Top*) and analyzed for intracellular expression of granzyme B vs. IFNγ (*Bottom*). *$P < 0.05$.

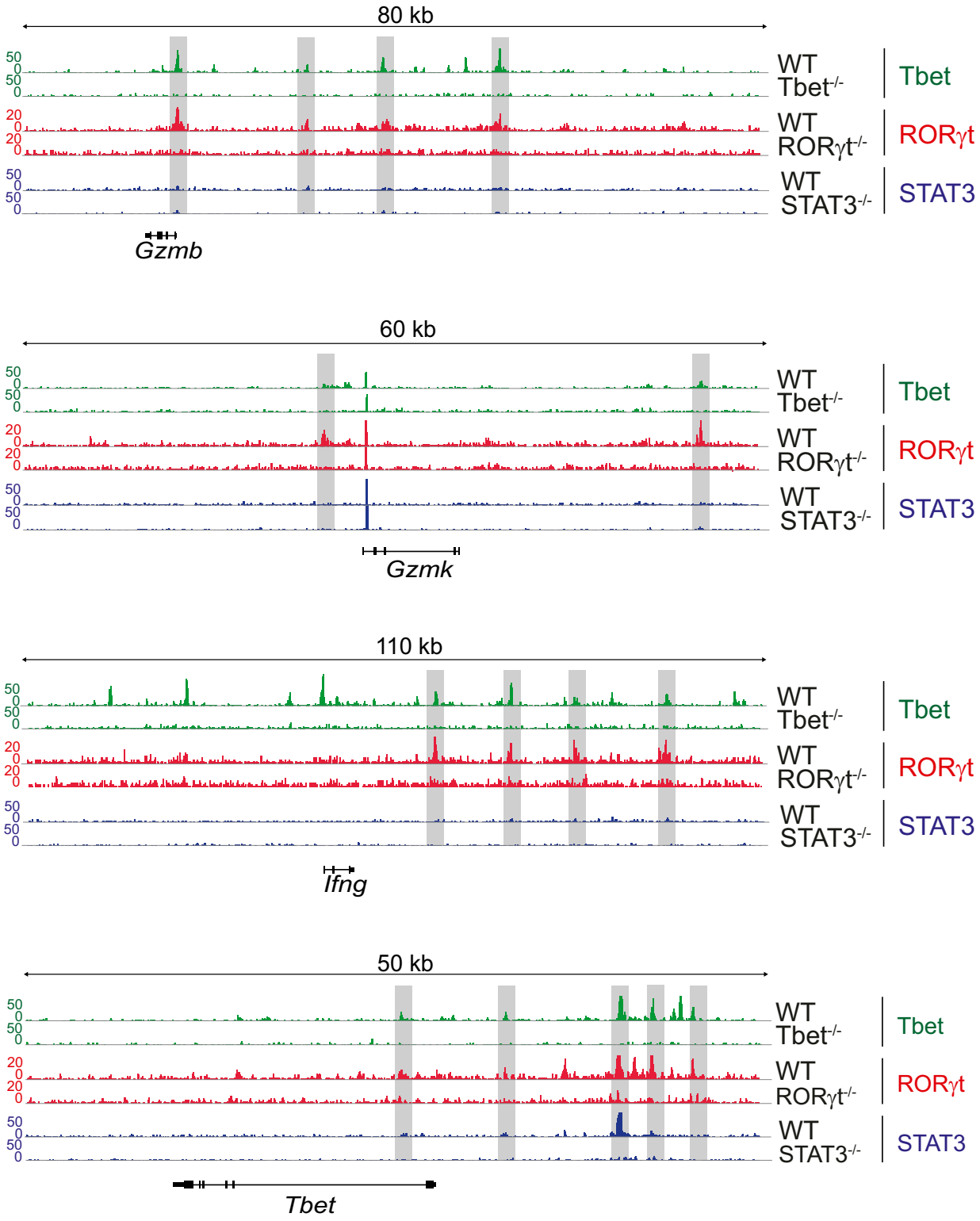

Fig. S3. STAT3 and RORγt bind genes associated with cytotoxic functions in T cells. Genome browser tracks show ChIP-seq signals (normalized sequence read numbers) of T-bet, RORγt, and STAT3 at *Gzmb*, *Gzmk*, *Ifng*, and *T-bet* (*Tbx21*) loci in $CD4^+$ T cells of the indicated genotypes. Relevant peaks are gray shaded. Original ChIP-seq data are from refs. 36 and 45.

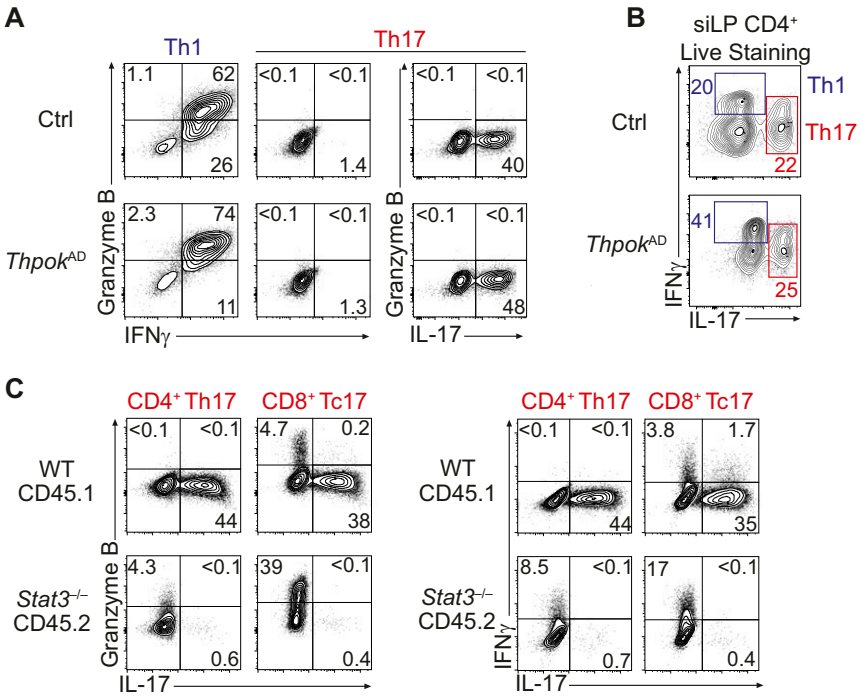

Fig. S4. Thpok is not required for Th17 differentiation. (*A*) Naïve CD4$^+$ T cell from *Ox40*-cre$^+$ *Thpok*$^{+/+}$ (Ctrl) or *Thpok*$^{fl/fl}$(*Thpok*^{AD}) were cultured under Th1 or Th17 conditions. Contour plots show intracellular expression of IFNγ (*Left*) or IL-17 (*Right*) vs. granzyme B in control or *Thpok*^{AD} effector CD4$^+$ T cells cultured in indicated conditions. (*B*) Contour plots of IL-17 vs. IFNγ expression are gated on TCRβ$^+$ CD4$^+$ CD44$^+$ Rosa26^{YFP+} live cells isolated from the lamina propria of control or *Thpok*^{AD} animals. Data (*A* and *B*) are representative of three mice per genotype analyzed in three independent experiments. (*C*) Contour plots show intracellular expression of IL-17 vs. granzyme B (*Left*) and IL-17 vs. IFNγ (*Right*) in *Stat3*$^{-/-}$ and wild-type CD4$^+$ or CD8$^+$ T cells, cultured in the indicated conditions and as described in Fig. S2*A*, with separate cocultures, each mixing both genotypes, for CD4$^+$ and CD8$^+$ effectors. Data are gated on WT CD45.1$^+$ or *Stat3*$^{-/-}$ CD45.2$^+$ cells and are representative of three mice per genotype analyzed in three independent experiments.