Supplemental material

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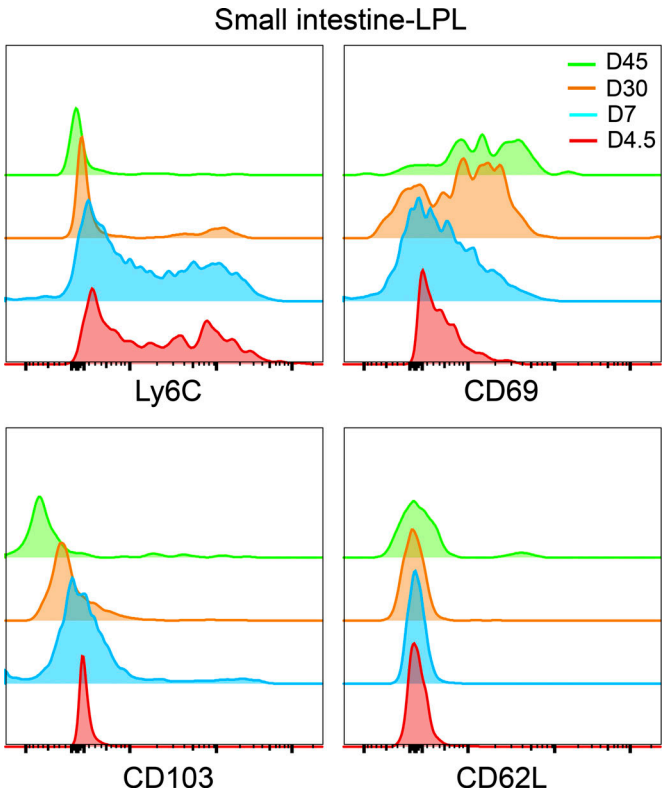

Figure S1. **Gradual acquisition of resident memory phenotype by CD4⁺ T cells in small intestine lamina propria (LPL).** CD45.1⁺ SMARTA CD4⁺ T cells were transferred to C57BL/6J mice 1 d before infection with LCMV-Armstrong. Ly6C, CD69, CD103, and CD62L expression on SMARTA cells from small intestine lamina propria was assessed at 4.5, 7, 30, and 45 d after infection. Data are representative of two separate experiments with three mice per experiment.

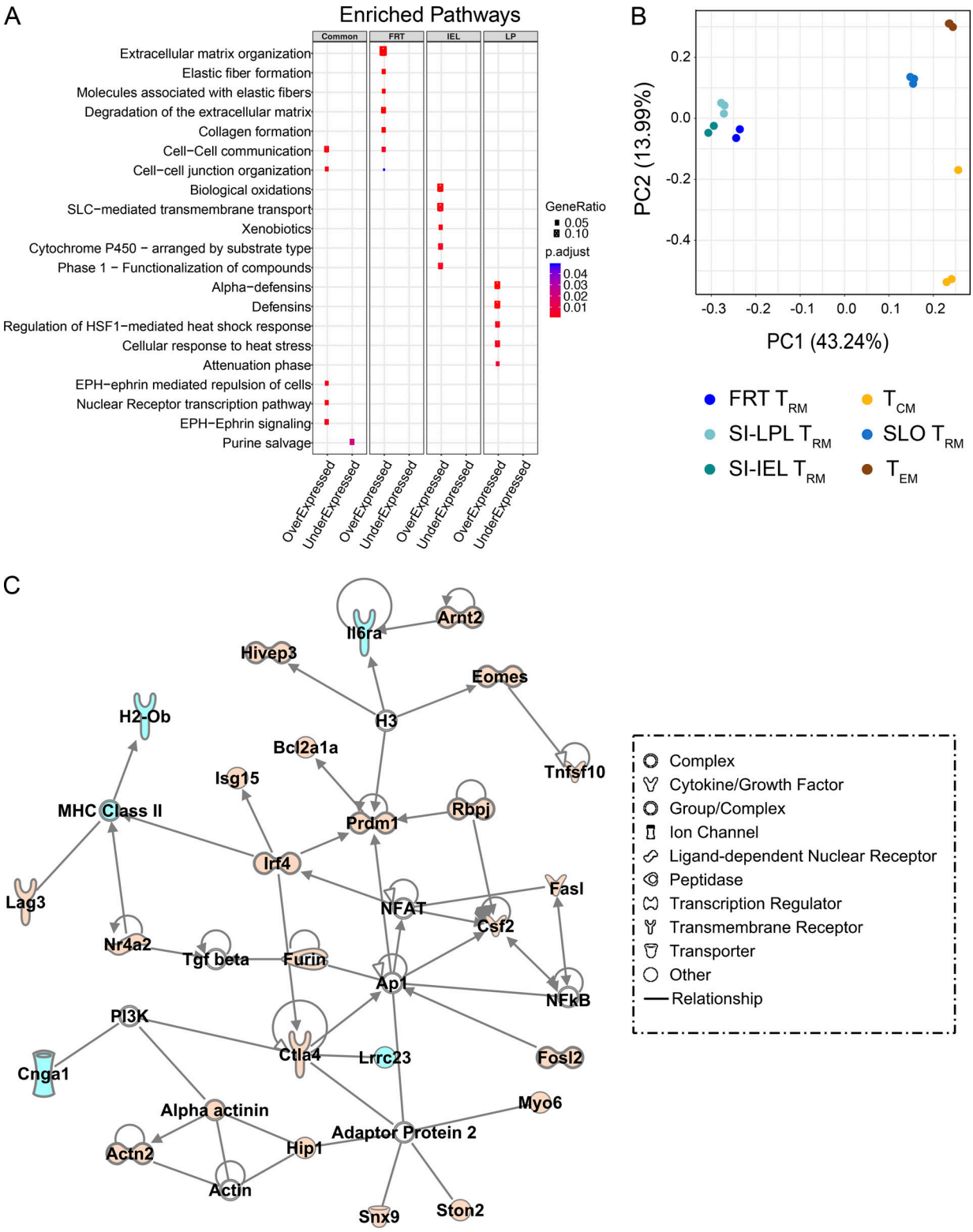

Figure S2. **Gene expression analyses. (A)** Enriched pathways. The clusterCompare R package was used to analyze and visualize enriched pathways (enrichPathways) for shared and unique T_{RM} DEGs identified in Fig. 5 A. Full lists of common and unique DEGs provided in Table S2. SLC, solute-carrier. **(B)** PCA. To remove unexpressed genes in the dataset, genes with a mean <1 across all of the samples were removed before analysis. The prcomp and autoplot functions in RStudio were used to perform the PCA and plot the first two principal components, respectively. **(C)** The second most enriched gene network. IPA was used to generate the network from the genes shared by both SLO T_{RM} and NLT T_{RM} (identified in Fig. 5 C and provided in Table S2) and the average fold change value obtained from averaging the absolute fold change from all comparisons of T_{RM} to circulating T_{CM} and T_{EM} cells. Edges (lines and arrows) represent direct interactions as supported by information in the IPA database. Genes that are included in the T_{RM} gene list have a colored node. Node color indicates up-regulated genes (orange) and down-regulated genes (blue) in T_{RM}. Node shapes represent functional classes of gene product.

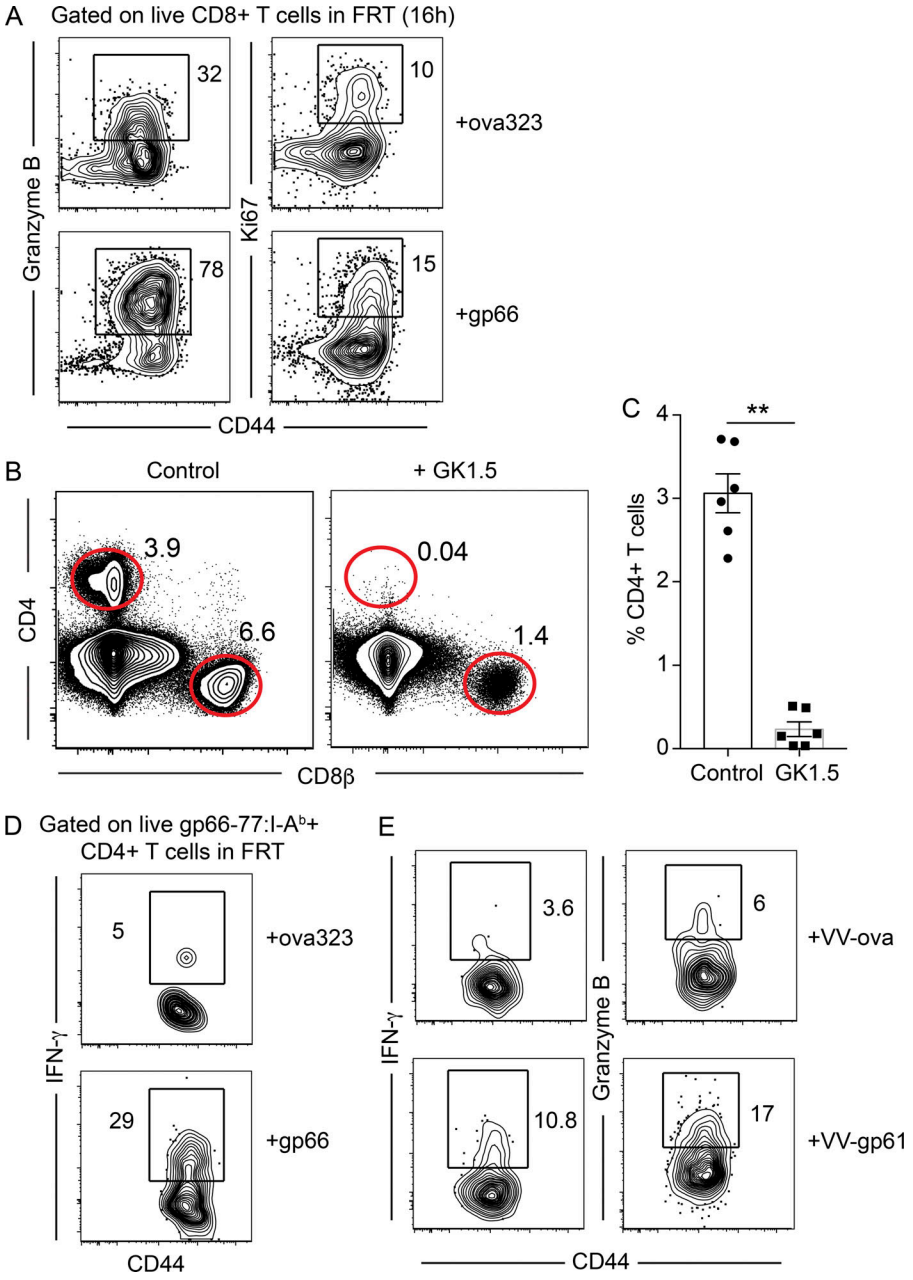

Figure S3. **Local CD4⁺ T_{RM} reactivation induces activation of other immune cells in the FRT. (A)** SMARTA memory immune chimeras were challenged transcervically with ova323 and gp66 peptide. CD8⁺ T cells within the FRT were assessed for IFN-γ production and Ki67 expression 16 h after challenge. **(B and C)** Verification of CD4⁺ T cell depletion following treatment with anti-GK1.5 antibody. **(D)** C57BL/6J mice were infected with LCMV-Armstrong. 63 d after infection, mice were challenged t.c. with gp66 or ova323 peptide. GP_{66-77}:I-A^b tetramer⁺ CD4⁺ T cells within FRT were assessed for IFN-γ production after 16 h. **(E)** Memory SMARTA immune chimeras were challenged t.c. with VV-ova or VV-gp61. SMARTA cells within FRT were assessed for IFN-γ and granzyme B production 24 h after recall. Bars indicate mean ± SEM. Mann–Whitney *U* test. **, P < 0.01. Data are representative of two separate experiments with three mice per experiment (A, D, and E) or three separate experiments with three mice per experiment (B and C).

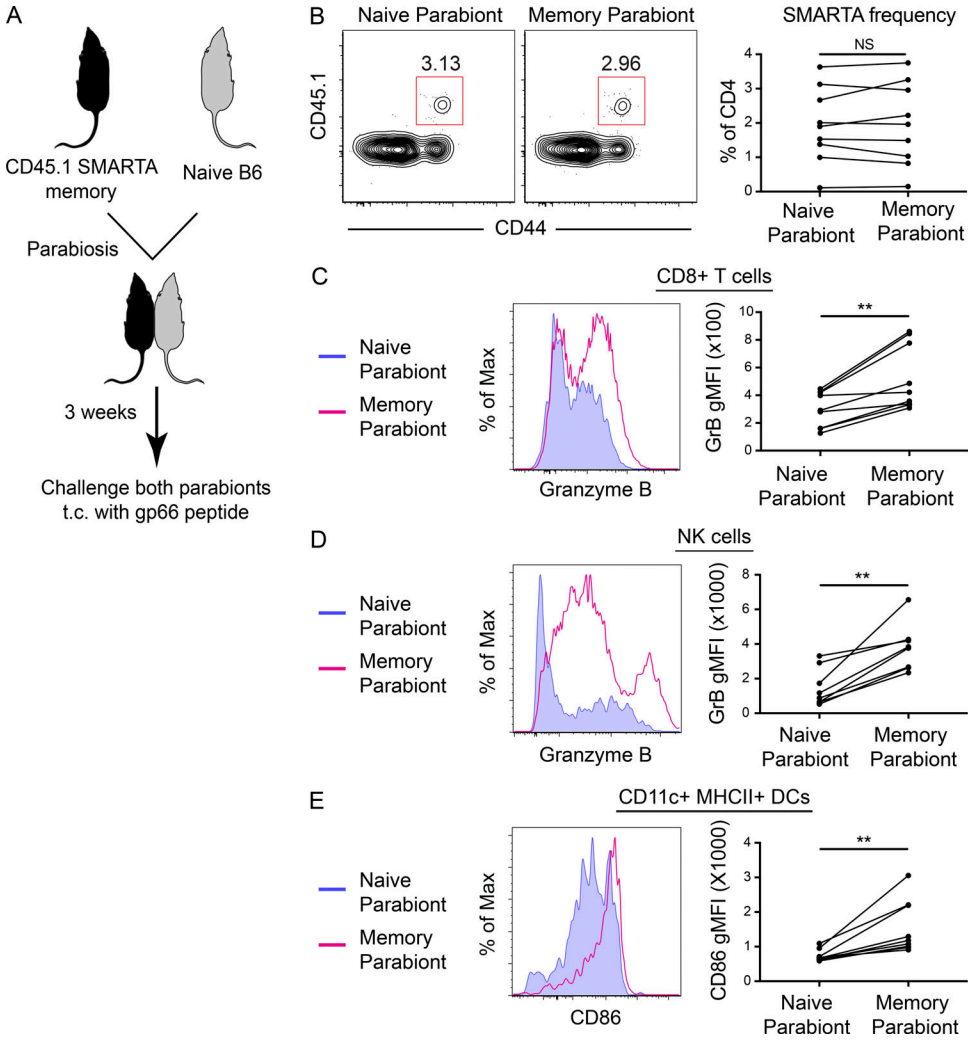

Figure S4. **Local CD4⁺ T_{RM} reactivation is responsible for activation of other immune cells in the FRT. (A)** Naive B6 and CD45.1⁺ SMARTA memory immune chimeras were conjoined via parabiosis 45 d after LCMV-Armstrong infection. 3 wk after parabiosis, parabiont pairs were challenged transcervically with gp66 peptide and analyzed 16 h following reactivation. **(B)** SMARTA CD4⁺ T cell frequencies within the blood of parabiont partners showing equilibration of circulating memory CD4⁺ T cells. Plots are gated on total live CD4⁺ T cells. **(C and D)** Up-regulation of effector function (measured by granzyme B production) in the FRT following antigen encounter. Plots are gated on total live CD8β⁺ T cells (C) and NK1.1⁺ cells (D). GrB, granzyme B. **(E)** Expression of the maturation marker CD86 on CD11c⁺ MHCII⁺ dendritic cells in the FRT following peptide rechallenge. All data are representative of three separate experiments with two to three parabiont pairs/experiment. Wilcoxon signed-rank test. **, P < 0.01.

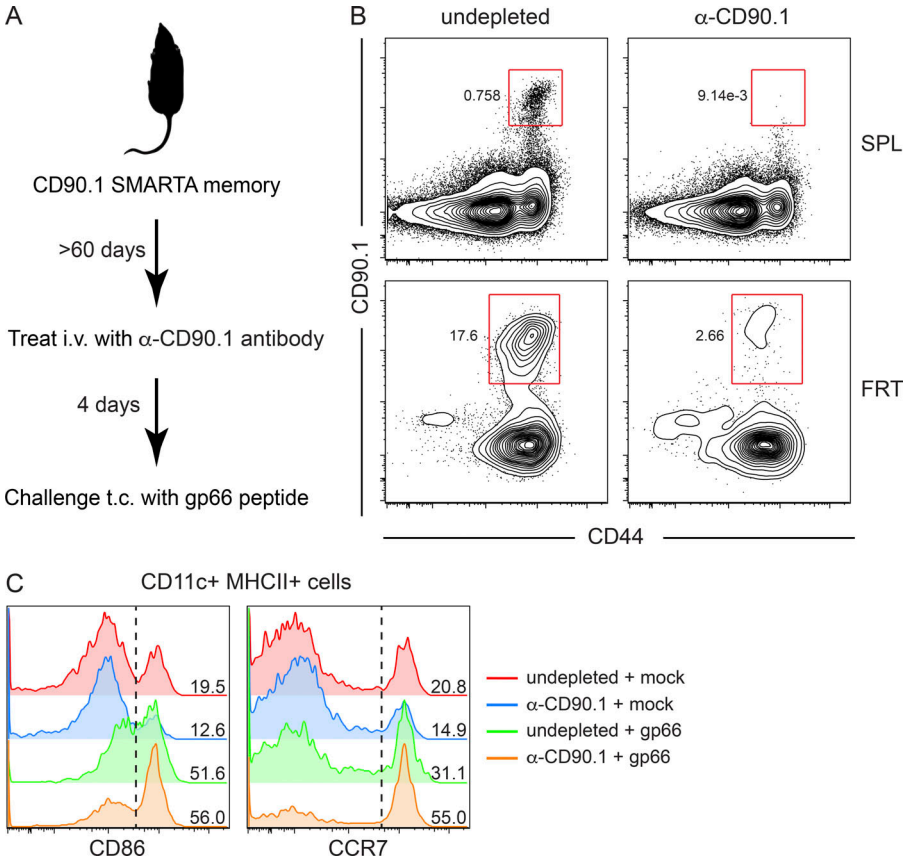

Figure S5. **FRT-resident CD4⁺ T cells are sufficient to induce local immune activation after TCR triggering. (A)** CD90.1⁺ SMARTA memory mice were treated with PBS or an anti-CD90.1 depleting antibody. Successful depletion of circulating SMARTA memory CD4⁺ T cells was checked in blood 4 d after antibody treatment. Then mice were challenged transcervically with mock (ova323) or gp66 peptide and analyzed 16 h following reactivation. **(B)** Representative flow plots showing SMARTA populations in the spleen and FRT following treatment with anti-CD90.1 antibody. Plots are gated on live CD4⁺ T cells. SPL, spleen. **(C)** Representative histograms showing CD86 and CCR7 up-regulation on CD11c⁺ MHCII⁺ DCs in the FRT following antigen challenge. All data are representative of two separate experiments with *n* = 2–3 animals/group.

Tables S1 and S2 are provided online as separate Excel files. Table S1 lists mapping statistics for each sample generated and used in this study. Table S2 lists shared and unique T_{RM} DEGs from pairwise comparisons.