Supporting Online Material
Materials and Methods
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Materials and Methods

Mice. Mice were maintained and used in accordance with the University of Pennsylvania Institutional Animal Care and Use Guidelines. Mice were housed in specific pathogen-free conditions prior to use. Floxed *Eomes* mice were generated as described in Fig. S1. Mice carrying the floxed *Eomes* allele were backcrossed 8 generations to C57BL/6, bred to homozygosity, and crossed to mice transgenic for Cre under control of CD4 promoter/enhancer elements (*S1*). CD4 Cre-mediated excision of exon 1 of the floxed *Eomes* allele resulted in mice carrying a null allele of *Eomes*, specifically in T cells. Mice with T cells lacking both Eomes were, in turn, bred to homozygosity with *Tbx21*$^{-/-}$ mice, resulting in mice with combined deficiency for T-bet and Eomes in the T cell compartment.

Infections. Mice of indicated genotypes were infected with 2 X 10^5 plaque forming units (p.f.u.) of lymphocytic choriomeningitis virus (LCMV) Armstrong strain by intraperitoneal injection. For analysis of the endogenous CD8$^+$ T cell response, H-2D^b GP33-41 and H-2D^b NP396-404 tetramers were used to identify and/or sort LCMV-specific CD8$^+$ T cells. For CD4 and CD8 depletion experiments, mice received intra-peritoneal injection (0.5 mg in PBS) of GK1.5 or 2.43 antibody, respectively, 1 day before and 1 day after infection with LCMV, as well as every 3 days thereafter. Control-treated mice received PBS. For LCMV-derived peptide stimulations, splenocytes were stimulated for 6 hours with H-2D^b-restricted peptides GP33-41 (0.5 μg/ml) and NP396-404 (0.5 μg/ml), or the H-2A^b-restricted GP61-80 peptide (0.5 μg/ml) in the presence of brefeldin A (1 μg/ml). Surface staining, intracellular (cytokine, Ki-67, and active Caspase 3) staining, and flow cytometry were performed as previously described (*S2*). Antibodies used for flow cytometry were purchased from BD Biosciences.

Histology. Organs were harvested and fixed overnight in 10% formalin at 4^{o}C. After fixation, organs were progressively dehydrated in 70%, 95%, then 100% EtOH. Dehydrated organs were paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E). Images were acquired on a Leica 500 microscope.

Quantitative real-time RT-PCR. Total RNA was extracted using Trizol Reagent (Invitrogen). 1 microgram of RNA was reverse-transcribed using random hexamer primers and Maloney-MLV reverse transcriptase (Promega). Quantitative real-time polymerase chain reaction was performed using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The following intron-spanning primer and probe set was used for detection of HPRT: sense 5'-CTCCTCAGACCGCTTTTTGC-3', anti-sense 5'-TAACCTGGTTCATCATCGCTAATC-3', probe VIC-5'-CCGTCATGCCGACCCGCAG-3'-TAMRA. Pre-synthesized Taqman Gene Expression Assays (Applied Biosystems) were used to amplify the following sequences (gene symbols and Applied Biosystems primer set numbers in parentheses): IL-17A (*Il17a*, Mm00439619_m1), IL-21 (*Il21*, Mm00517640_m1), IL-22 (*Iltifb*,

Mm00444241_m1), IL-23R (*Il23r*, Mm00519942_m1), and RORγt (*Rorc*, Mm00441139_m1). Levels of test gene are expressed relative to HPRT, with the lowest value standardized at 1.

Cytotoxicity assay. Splenocytes from wild-type C57BL/6 mice were labeled with two different concentrations (5 μM = "dim" or 50 μM = "bright") of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes). CFSE bright splenocytes were incubated either without peptide or with LCMV-derived peptides GP33-41 or NP396-404 for 1h at 37^{o}C, while CFSE dim splenocytes were incubated without peptide 1h at 37^{o}C. After incubation, CFSE-labeled cells were washed and counted. 5x10^4 dim CFSE-labeled cells were mixed with 5x10^4 bright CFSE-labeled cells either pulsed with no peptide (control targets), GP33-41 ("GP33 targets"), or NP396-404 ("NP396 targets"). Percentages of GP33-specifc or NP396-specifc CD8$^+$ T cells from d8 LCMV-infected mice of different genotypes were determined by specific TNF-α production (intact in all genotypes) in response to 6 hr stimulation with GP33-41 or NP396-404. Increasing doses of GP33-specific or NP9396-specific CD8$^+$ T cells from different genotypes were added to wells containing various combinations of target cells. Unlabeled, wild-type CD57BL/6 splenocytes were added as necessary to equalize cell concentrations in each well. After 24hr incubation at 37^{o}C, CFSE-bright target cell destruction was assessed by flow cytometry. % specific lysis was calculated by subtracting % control cell lysis at a given dose of effector cells from % GP33 or NP396 target cell lysis at a given dose of effector cells.

Supplemental Figures

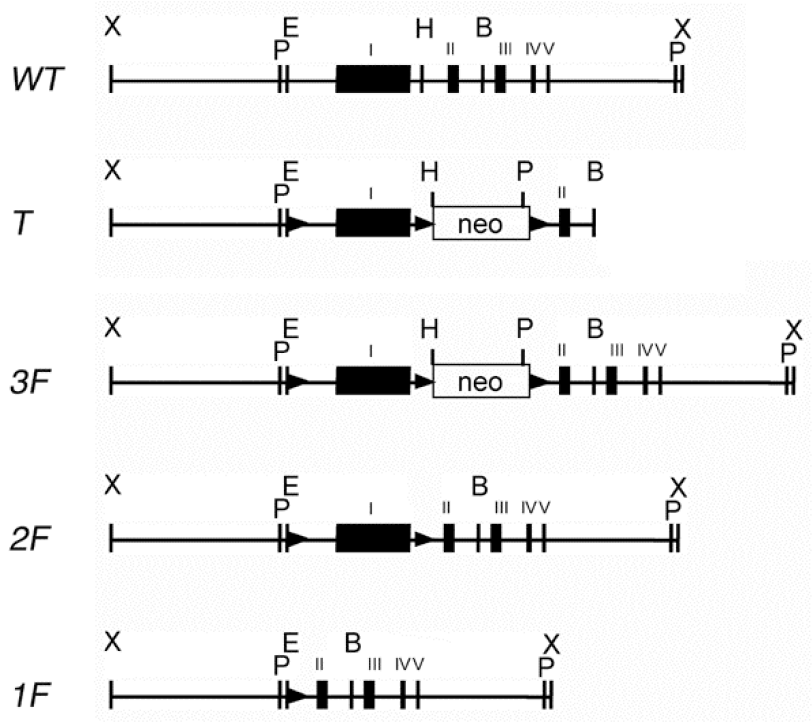

Fig. S1. Generation of floxed *Eomes* mice. A targeting construct ("T") containing a neomycin resistance cassette ("neo") flanked by *lox*P sites, was made where *lox*P sites flanked a 2.5 kb Eco RI-Hind III fragment containing exon I of the *Eomes* locus. The 3' end of the construct consisted of the 1.2 kb Hind III-Bam HI fragment containing exon II. This construct was electroporated into R1 ES cells (*S3*). Electroporation, selection and maintenance of ES cells have been previously described (*S4*). Southern blotting was used to identify clones which had undergone homologous recombination. Pst I digested DNA from selected clones was probed with an Eco RI-SacI probe, which generated an approximately 7.4 kb fragment in wild type mice ("WT"), but the presence of a Pst I site in the neomycin resistance cassette generated a 4.9 kb fragment for the homologously recombined floxed *Eomes* allele ("3F"). A partial restriction enzyme map is shown: Bam HI ("B"), Eco RI ("E"), Hind III ("H"), Pst I ("P") and Xba I ("X"). The *lox*P-flanked neomycin resistance cassette was deleted by Protamine Cre-mediated excision in germ cells of male mice ("2F") (*S5*), and PCR with selected primers was used to map deletions. Cre-mediated excision of exon I of the *Eomes* locus results in the "1F" null allele of *Eomes*.

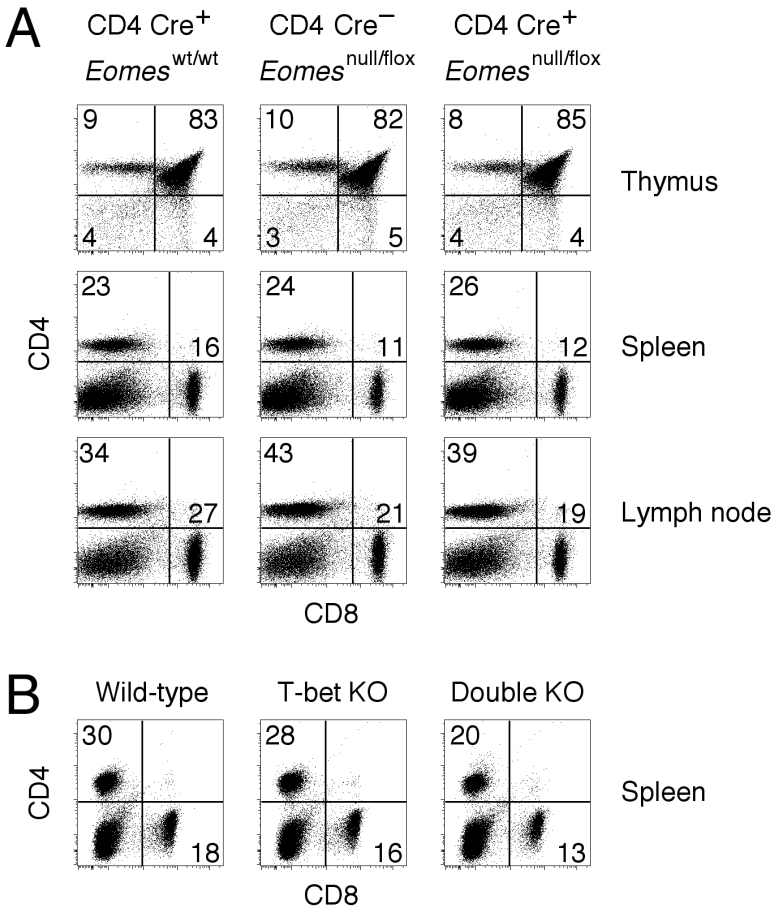

Fig. S2. T cell development in the absence of Eomes and/or T-bet. (**A**) Flow cytometry of T cells from indicated genotypes. Numbers represent % of cells from thymus, spleen, or lymph nodes expressing CD4 or CD8. *Eomes*$^{wt/wt}$ CD4 Cre$^+$ mice were similar to wild-type mice (*S6*). Eomes$^{null/flox}$ CD4 Cre$^+$ mice were indistinguishable from Eomes$^{flox/flox}$ CD4 Cre$^+$ mice (*S6*). (**B**) Flow cytometry of splenocytes from wild-type ("Wild-type"), *Tbx21*$^{-/-}$ ("T-bet KO"), or *Tbx21*$^{-/-}$ *Eomes*$^{flox/flox}$ CD4 Cre$^+$ ("Double KO") mice. Numbers represent % of cells expressing CD4 or CD8. Results are representative of at least 3 independent experiments.

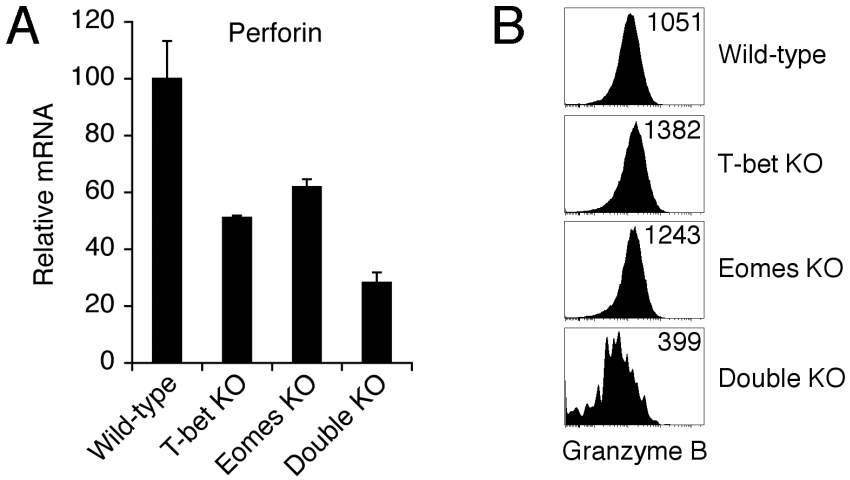

Fig. S3. Impaired cytotoxic differentiation of CD8$^+$ T cells lacking both T-bet and Eomes. (**A**) Perforin mRNA expression in LCMV-specific H-2D^{b}GP33$^+$ CD8$^+$ T cells 8d after infection determined by quantitative real-time PCR (Q-PCR). Values represent mean ±SEM of triplicate determinations normalized to HPRT. (**B**) Flow cytometry of granzyme B expression in H-2D^{b}NP396$^+$ CD8$^+$ T cells 8d after infection; numbers in histograms indicate mean fluorescence intensity (MFI) of granzyme B expression. All results are representative of at least 3 independent experiments.

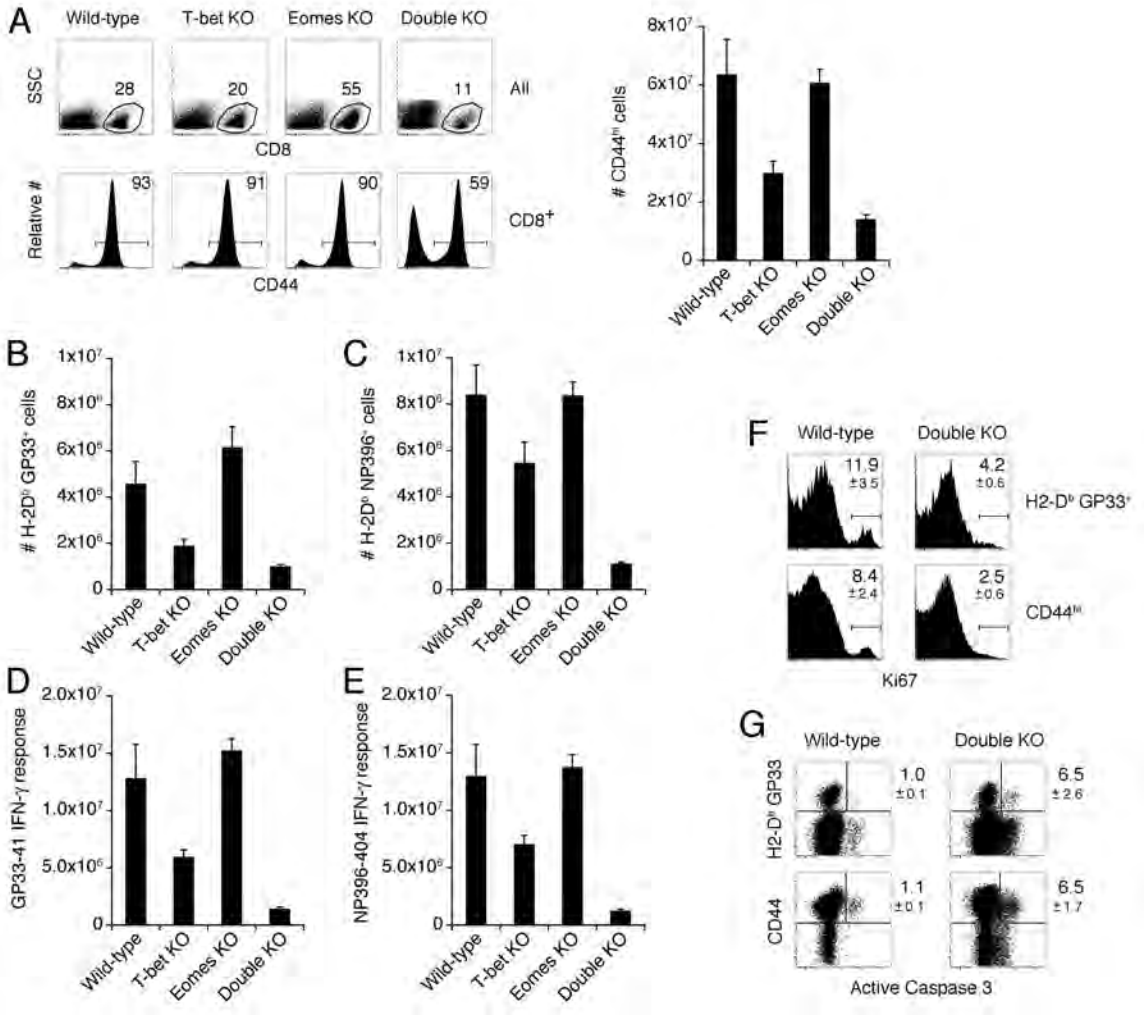

Fig. S4. Defective accumulation of virus-specific CD8+ T cells lacking both T-bet and Eomes. (**A**) Left, splenocytes from mice of the indicated genotype were examined by flow cytometry 8d after infection with LCMV. Top row shows % CD8+ T cells. Bottom row shows % CD44hi (activated) CD8+ T cells. Right, quantification of CD44hiCD8+ T cells in spleens of infected mice. Double KO cells accumulate 4.5-fold less than Wild-type cells. (**B**-**E**) Quantification of LCMV-specific CD8+ T cells 8d after LCMV infection, as assessed by (B) H-2DbGP33 tetramer staining or (C) H-2DbNP396 tetramer staining and IFN-γ production in response to stimulation with (D) GP33-41 peptide or (E) NP396-404 peptide. Results represent the mean ±SEM of mice from multiple experiments; wild-type, *n*=9; T-bet KO, *n*=7; Eomes KO *n*=4; Double KO, *n*=9. (**F**, **G**) Decreased proliferation and increased apoptosis of virus-specific Double KO CD8+ T cells. Splenocytes were isolated 7d after LCMV infection and stained for (F) Ki-67 to assess proliferation (*S7*) or (G) active Caspase 3 to detect dying cells (*S8*). For (F), upper histograms contain H2-DbGP33+CD8+ events while lower histograms depict all activated CD44hiCD8+ cells. Numbers represent % Ki-67 expression as mean ±SEM from 7 wild-type and 8 Double KO mice from two separate experiments. For (G), CD8+ events are depicted. Plots show Caspase 3 expression as a % of H2-DbGP33+ cells (upper row) or CD44hi (lower row) cells. Numbers represent mean ±SEM from 4 wild-type and 5 Double KO mice.

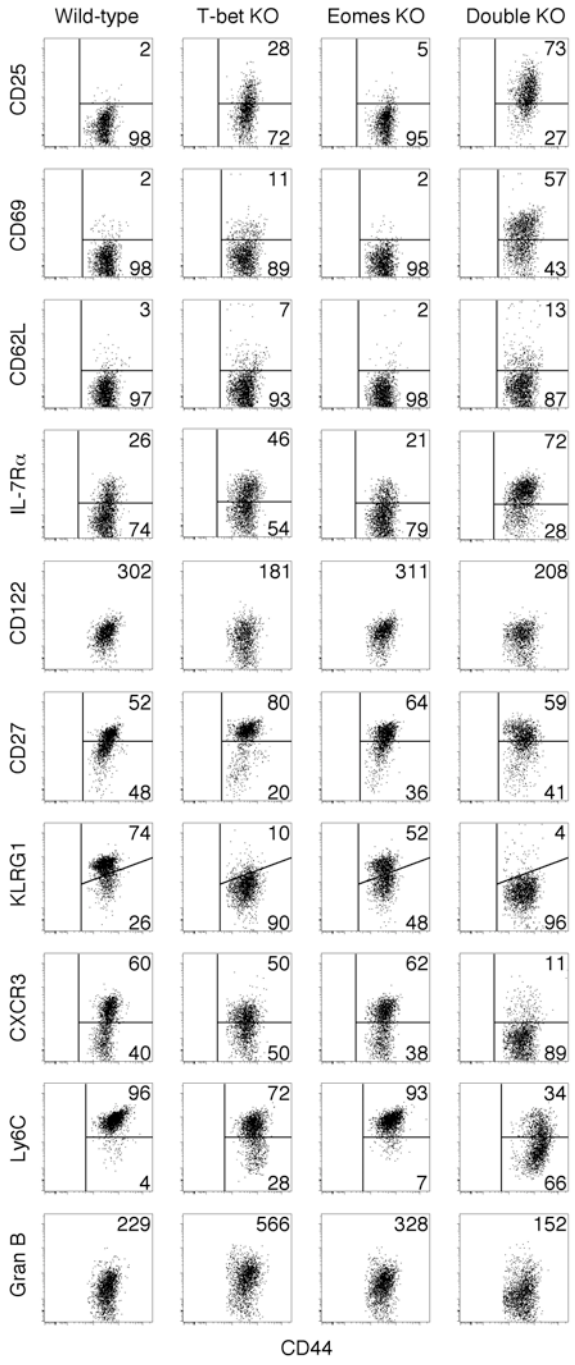

Fig. S5. Abnormal phenotypic markers of virus-specific CD8+ T cells from Double KO mice. Representative flow cytometry of GP33-specific CD8+ T cells from spleens 8d after LMCV infection. Plots show H-2D^{b}GP33+ events; numbers represent % of cells expressing indicated markers, except for CD122 and granzyme B ("Gran B") plots where numbers represent MFI. A similar pattern was observed with NP396-specific CD8+ T cells (*S6*). The elevated expression of activation markers such as CD25 and CD69 likely reflects continued antigen exposure (*S9*), while abnormalities in expression of CD27, KLRG1, CXCR3, Ly6C, and Granzyme B likely reflect the abnormal differentiation of DKO cells (*S10*). Results are representative of 5 independent experiments.

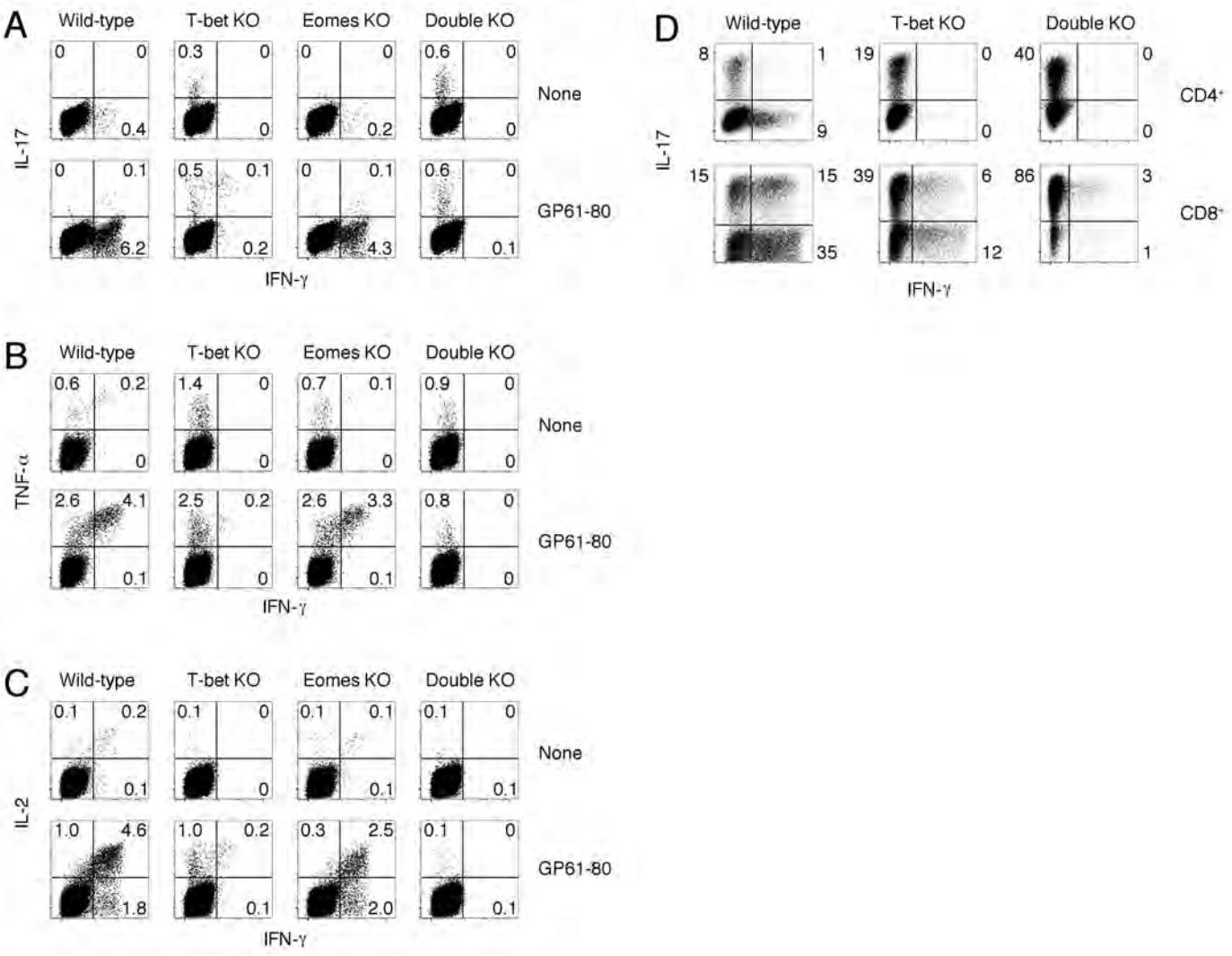

Fig. S6. Cytokine production by virus-specific CD4$^+$ T cells lacking T-bet and Eomes. (**A-C**) Splenocytes were isolated 8d after LCMV infection and cultured without (upper rows within pairs) or with (lower rows) the MHC class II H-2A^b-restricted GP61-80 peptide LCMV-derived peptide GP61-80 (bottom row). IFN-γ versus (A) IL-17, (B) TNF-α, and (C) IL-2 expression were assessed by intracellular staining. Only CD4$^+$ events are displayed. Numbers represent % of CD4$^+$ T cells expressing the indicated cytokine. Results are representative of 5 independent experiments. (**D**) Sensitivity of intracellular cytokine detection. Cytokine expression by CD4$^+$ and CD8$^+$ T cells from naïve mice stimulated in vitro for 3d in the presence of Type 17 inducing cytokines, TGF-β (2 ng/ml) and IL-6 (20 ng/ml). Although CD4$^+$ T cells from T-bet KO and Double KO mice have enhanced polarization toward IL-17 expression in vitro, the low frequency of virus-specific CD4$^+$ T cells from these genotypes in vivo (panel A) may reflect a role for T-bet in regulating differentiation, proliferation, or survival of virus-specific Type 17 CD4$^+$ T cells during LCMV infection.

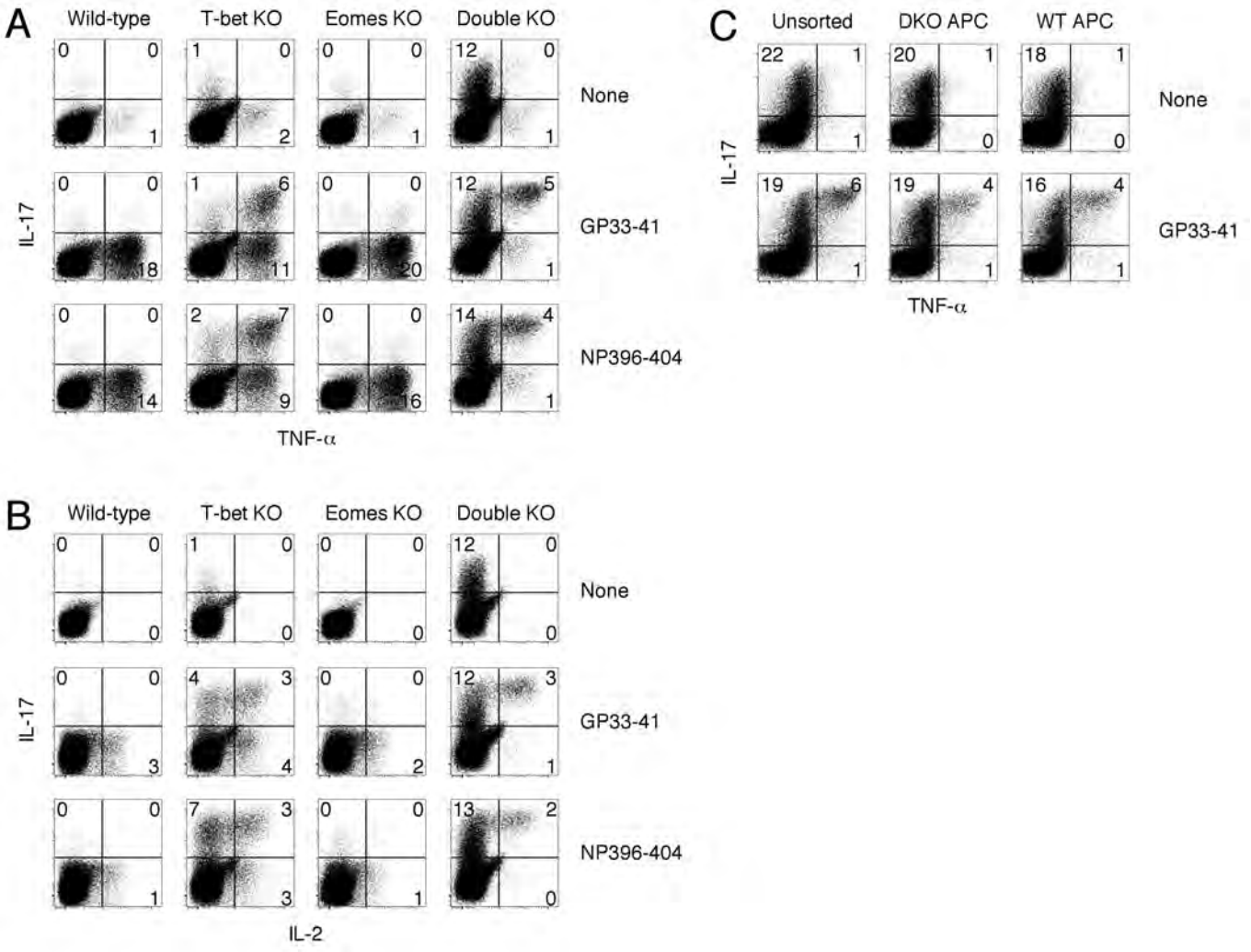

Fig. S7. TNF-α and IL-2 production by virus-specific CD8$^+$ T cells lacking both T-bet and Eomes. (**A** and **B**) Splenocytes were isolated 8d after LCMV infection and given no stimulation (top row) or stimulated with LCMV-derived peptides GP33-41 (middle row) or NP396-404 (bottom row); IL-17 and (A) TNF-α or (B) IL-2 production were assessed by intracellular staining; plots show CD8$^+$ events; numbers indicate % of CD8$^+$ T cells expressing the indicated cytokine. Results are representative of 3 independent experiments. (**C**) IL-17 production by Double KO CD8$^+$ T cells in the absence of specific peptide restimulation or infected APCs. CD8$^+$ T cells were sorted from spleens of Double KO mice 7d after LCMV infection. Purified Double KO CD8$^+$ T cells were cultured for 6hr with CD8-depleted splenocytes from the same LCMV-infected Double KO mice ("DKO APC") or CD8-depleted splenocytes from naïve wild-type mice ("WT APC") without or with LCMV-derived peptide GP33-41. Unsorted whole splenocytes from the same LCMV-infected Double KO mice are included for comparison (left column). Results are representative of two independent experiments. The expression of IL-17 by Double KO cells without specific re-stimulation could, therefore, be due to persistent virus in vivo and/or constitutive expression of this cytokine.

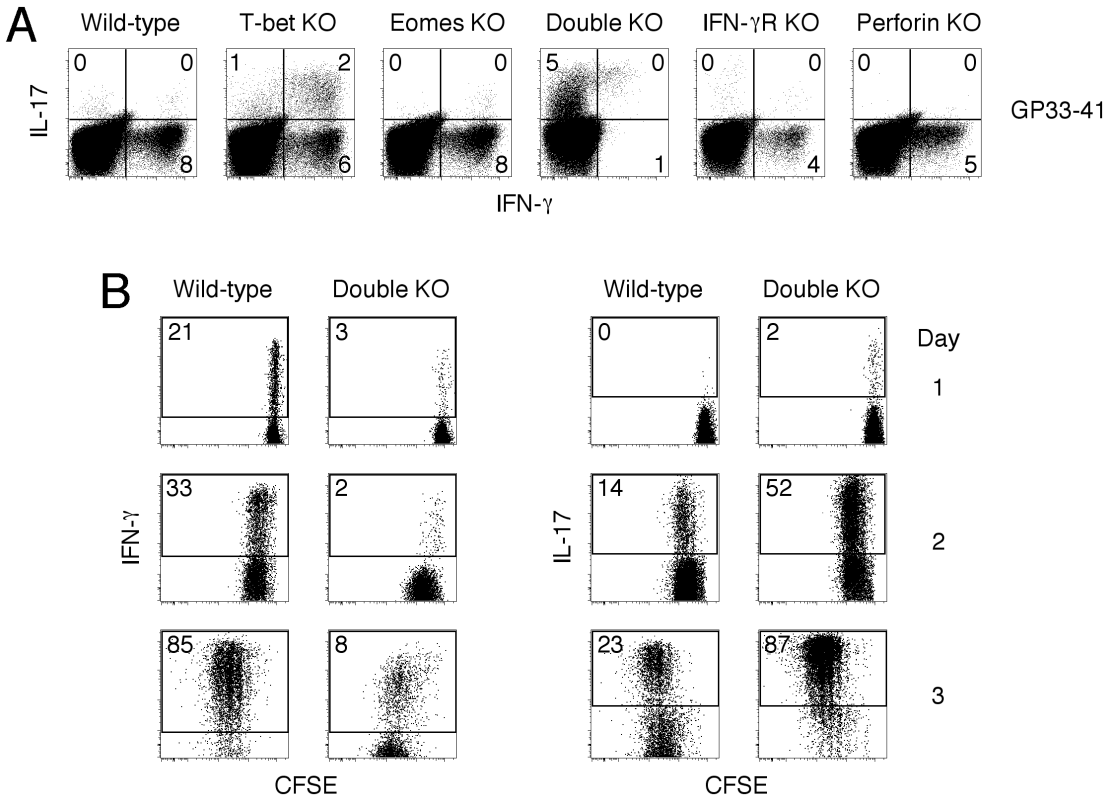

Fig. S8. Type 17 differentiation of Double KO CD8$^+$ T cells is not secondary to defective cytotoxic effector function or proliferation. (**A**) Splenocytes were isolated 7d after LCMV infection and stimulated with LCMV-derived peptide GP33-41. Cytokine production was assessed by intracellular staining. Plots show CD8$^+$ events; numbers represent % of CD8$^+$ T cells expressing the indicated cytokine. Similar pattern observed in response to stimulation with LCMV-derived peptide NP396-404 or PMA plus ionomycin (*S6*). Results are representative of 2 independent experiments. These results indicate that the deviation towards the Type 17 CD8$^+$ T cell fate is not dependent on impaired perforin expression or IFN-gamma signaling.
(**B**) Splenocytes from naïve mice were CFSE-labeled and stimulated in vitro in the presence of TGF-β (2 ng/ml) and IL-6 (20 ng/ml). Cytokine production by CD8$^+$ T cells as a function of CFSE dilution was analyzed at the indicated days. Numbers indicate % of cells expressing cytokine. These findings suggest that defective division by Double KO CD8$^+$ T cells is not required for their deviation towards the Type 17 fate.

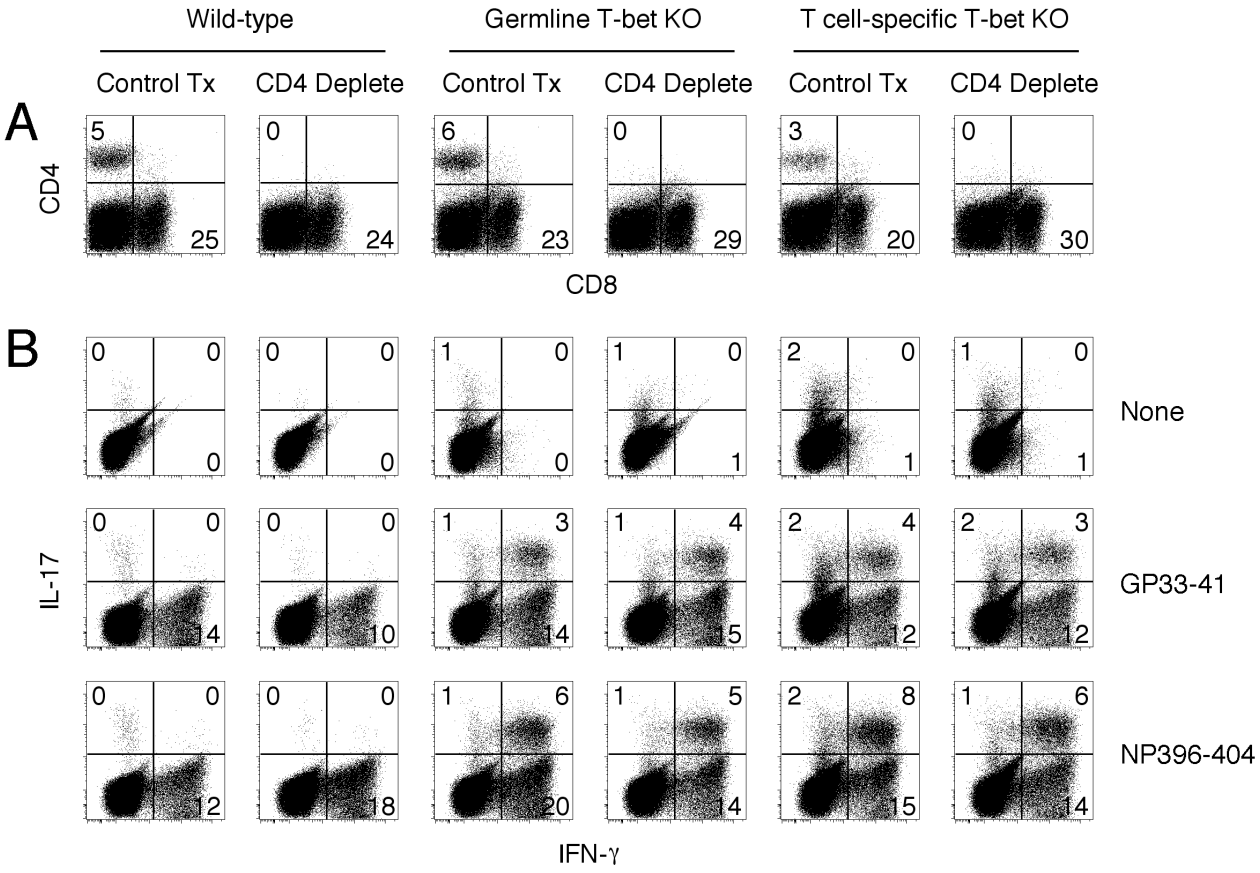

Fig. S9. Type 17 differentiation of virus-specific CD8+ T cells is not contingent on T-bet deficiency in innate immune cells or CD4+ T cells. T-bet can function in innate immune cells to shape interactions between host and pathogen (*S11*). Thus, T-bet deficiency in immune cells other than T cells might contribute to the initiation or propagation of the Type 17 CD8+ T cell response to LCMV in mice with germline deletion of T-bet. In order to address this possibility, we examined the immune response to LCMV in mice with T cell-specific deletion of T-bet. "T cell-specific T-bet KO" represents *Tbx21*$^{flox/flox}$ CD4 Cre+ mice (manuscript in preparation). Mice of the indicated genotype were treated with PBS ("Control Tx") or anti-CD4 ("CD4 Deplete", mAb GK1.5, 0.5 mg i.p.) 1d before, 1d after, and 5d after LCMV infection. Splenocytes were isolated 8d after LCMV infection. (**A**) Cells were stained for CD4 and CD8; numbers represent % expressing the indicated marker. (**B**) Cells were given no stimulation (top row) or stimulated with LCMV-derived peptides GP33-41 (middle row) or NP396-404 (bottom row); IFN-γ and IL-17 production were assessed by intracellular staining. Plots show CD8+ events; numbers represent % expressing the indicated cytokine. Results are representative of 2 independent experiments. These findings indicate that IL-17 expression by mutant CD8+ T cells is not contingent on T-bet deficiency in CD4+ T cells or cells of the innate immune system.

Supplemental References

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