Supporting Information

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SI Materials and Methods

Mice. Female C57BL/6 (CD45.2/Thy1.2), B6.SJL (CD45.1/Thy1.2), and B6.PL (CD45.2/Thy1.1) mice (6–7 wk old) were purchased from The Jackson Laboratory and the National Cancer Institute. Female B6.SJL mice were bred with male B6.PL to generate B6.PL.SJL (CD45.1/CD45.2/Thy1.1/Thy1.2) mice. Vβ5 T cell receptor (TCR) transgenic mice were maintained on a B6.SJL background or bred with C57BL/6 mice to generate Vβ5/B6 × B6.SJL (CD45.1/CD45.2). All mice were maintained in specific pathogen-free conditions. All experimental procedures were approved through the University of Minnesota Institutional Animal Care and Use Committee, Minneapolis.

In Vivo Generation of "True" Memory Vβ5 CD8 T Cells. CD45.1 Vβ5 CD8 T cells (1×10^6) were negatively enriched from spleen and superficial lymph nodes, using a CD8α$^+$ isolation kit (Miltenyi Biotec) and injected i.v. into CD45.2 congenic C57BL/6 mice. Mice were immunized 24 h later by i.v. injection of 3×10^6 colony-forming units (cfu) of *Listeria monocytogenes* strain expressing ovalbumin (OVA) [*L. monocytogenes*-OVA (LM-OVA) *ActA* attenuated] (a kind gift of Hao Shen, University of Pennsylvania School of Medicine, Philadelphia). In vivo generated memory T cells were detected with fluorescent-labeled anti-CD45.1 (eBioscience) 30–60 d after priming.

Adoptive Transfer and Immunization. Combinations of CD45 and CD90 alleles were chosen to allow discrimination of cotransferred naïve, virtual memory (VM) and true memory (TM) populations. For Vβ5 CD8 T-cell experiments, spleen and superficial lymph nodes were harvested from unprimed Vβ5 transgenic (tg) mouse strains (CD45.1 or CD45.1/CD45.2), and/or mice carrying primed, memory Vβ5 CD8 T cells (as a source of TM cells). Collagenase digestion was performed on tissues, and CD8 T cells were negatively enriched by CD8α$^+$ isolation kit (Miltenyi Biotec). To avoid pre-TCR stimulation, cells were sorted by CD8, CD44, and relevant congenic marker antibodies without Ova/K^b-tetramer staining using a FACSAria (BD Biosciences). After the sorting, the number of antigen-specific cells within each population was determined using Ova/K^b-tetramer staining of an aliquot from the sorted samples. Next, 300–500 Ova/K^b-tetramer positive cells of the indicated phenotype were mixed 1:1 and cotransferred (i.v.) into naïve C57BL/6 (CD45.2/Thy1.2) hosts, which were infected with 3×10^6 cfu LM-OVA *ActA* 1 d later. To induce recall immune response, mice were infected with 1×10^5 cfu of virulent LM-OVA. For polyclonal CD8 T-cell transfer, VM and naïve CD8 T cells were sorted from unprimed C57BL/6 (CD45.2/Thy1.1) and B6.SJL (CD45.1/Thy1.2) mice (as described above), and 2×10^6 cells of each population were mixed 1:1 and cotransferred (i.v.) into naïve $(B6.PL \times B6.SJL)_{F1}$ hosts (CD45.1/CD45.2/Thy1.1/Thy1.2). The recipient mice were infected with 2×10^6 pfu of vaccinia virus (Western Reserve strain) (VV-WR) or a mixture of attenuated recombinant *L. monocytogenes* strains (*L. monocytogenes*-OVA-B8R, *L. monocytogenes*-OVA-HSVgB) 1 d later. Attenuated *L. monocytogenes*-OVA-B8R and *L. monocytogenes*-OVA-HSVgB strains were provided from Ross M. Kedl (University of Colorado, Denver) and SingSing Way (University of Minnesota, Minneapolis), respectively.

Flow Cytometry. For determining surface phenotype, cells were isolated from spleen and superficial lymph nodes and stained with the antibodies specific for the following molecules: CD3e (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD44 (IM7), CD69 (H1.2F3), KLRG1 (2F1), CD62L (MEL-14), CD122 (TMb1), CD127 (A7R34), CD45.1 (A20), CD45.2 (104), Thy1.1 (HIS51), Ly-6C (HK1.4), CD49d (R1-2), and CXCR3 (CXCR3-173). For detecting foreign antigen-specific CD8 T cells, fluorochrome (phycoerythrin or allophycocyanin) labeled Ova/K^b (SIINFEKL), B8R/K^b (TSYKFESV), and HSVgB/K^b (SSIEFARL) tetramers were generated as previously described (1, 2) and used to stain cells (30 min at 4 °C), simultaneously with other surface markers. In some experiments, tetramer binding cells were enriched by a MACS-based pull-down assay, as previously described in detail (3). For intracellular transcription factor staining, stained cells with surface antibodies were fixed and permeabilized with Foxp3 fixation/permeabilization solution (eBioscience), and stained with antibodies to T-bet (4B10) and Eomesodermin (Dan11mag) for 1 h at 4 °C in permeabilization solution. Flow cytometry was performed on LSRII or Fortessa instruments (BD) and analyzed using FlowJo analysis software.

In Vitro Stimulation and IFN-γ Production Assay. Naïve and memory Vβ5 tg splenocytes were incubated with various doses (10^{-6} M–10^{-10} M) of OVA peptide (SIINFEKL), and Golgi Plug (BD Biosciences) for 2–5 h. Cells were then surface stained, fixed, and permeabilized with BD Cytofix/Cytoperm (BD Biosciences) and intracellularly stained for IFN-γ (XMG1.2) and TNF-α (MP6-XT2.2) in BD Perm Wash Buffer (BD Biosciences), similarly to our previous studies (4).

Cell Cycle Analysis. Cell cycle analysis was performed using staining for DNA and RNA with DAPI and pyronin Y, respectively. Negatively enriched CD8$^+$ T cells were obtained using a CD8α$^+$ T Cell Isolation kit II, mouse (Miltenyi Biotec) from unprimed mice (for naïve and VM populations), and immunized animals (for TM cells). Cells were surface stained and then fixed/permeabilized with FoxP3/Transcription Factor Staining Buffer (eBioscience) overnight. The cells were then incubated with 5 μg/mL DAPI in 200 μL FACS buffer [2% (vol/vol) FCS, 0.1% NaN_3] for 1 h and, without further washing, an equal volume of 3 μg/mL pyronin Y diluted FACS buffer was added to each sample 5 min before flow cytometric analysis.

Quantitative Real-Time PCR. CD44^{high} memory phenotype, CD44^{low} naïve phenotype, and TM Vβ5 CD8 T cells were sorted on a FACSAria (BD Bioscience), as described above. RNA was isolated by RNeasy microkit (Qiagen), and cDNA was generated using SuperScript III Reverse Transcriptase (Life Technologies). Real-time RT-PCR was performed using the ABI 7700 sequence detection system, with SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences are available upon request.

Resident Memory CD8 T-Cell Determination. To exclude blood circulating CD8 T cells from tissue parenchymal resident memory T cell (Trm) populations, we performed an intravascular staining method with fluorescently labeled anti-CD8 antibodies as previously described (5). Briefly, mice were injected i.v. with fluorescently labeled anti-CD8α antibody on ~50–60 d postprimary infection of *L. monocytogenes*-OVA. Then, the mice were bled and killed 3 min later and perfused to remove residual blood. Spleen, kidney, and salivary glands were harvested, and single cell suspensions were prepared by collagenase treatment. Cells were subsequently staining with an anti-CD8β antibody conjugated to a different fluorochrome. CD8 T cells in the parenchyma were defined as those stained by the CD8β antibody but not by the i.v. administered CD8α antibody.

L. *monocytogenes* Protection Assays. To assess protective immune function of VM, naïve, and TM Vβ5 CD8 T cells, each population was sorted as described above and an inoculum containing ~2×10^4 Ova/K^b-specific cells (determined by staining an aliquot of sorted cells with Ova/K^b tetramer) was adoptively transferred into an unprimed C57BL/6 host. In these experiments, populations were transferred singly, not cotransferred. One day later, mice were infected with 8×10^4 cfu of virulent *L. monocytogenes*-OVA or 1×10^4 cfu of virulent wild-type *L. monocytogenes* (the ~LD_{50} of each strain). On day 5 after infection, cfu of *L. monocytogenes* in the spleen and liver were measured as previously described (4, 6). For determining antigen-specific expansion of host and transferred CD8 T cells, splenocytes from the infected mice were counted and stained with K^b-OVA tetramer.

Statistics. A two-tailed, paired, or unpaired, Student *t* test was performed using Prism (GraphPad). In some figures, plotted data represent means ± SD, and *P* values are represented as follows: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, whereas NS, not significant, is used to denote *P* values >0.05.

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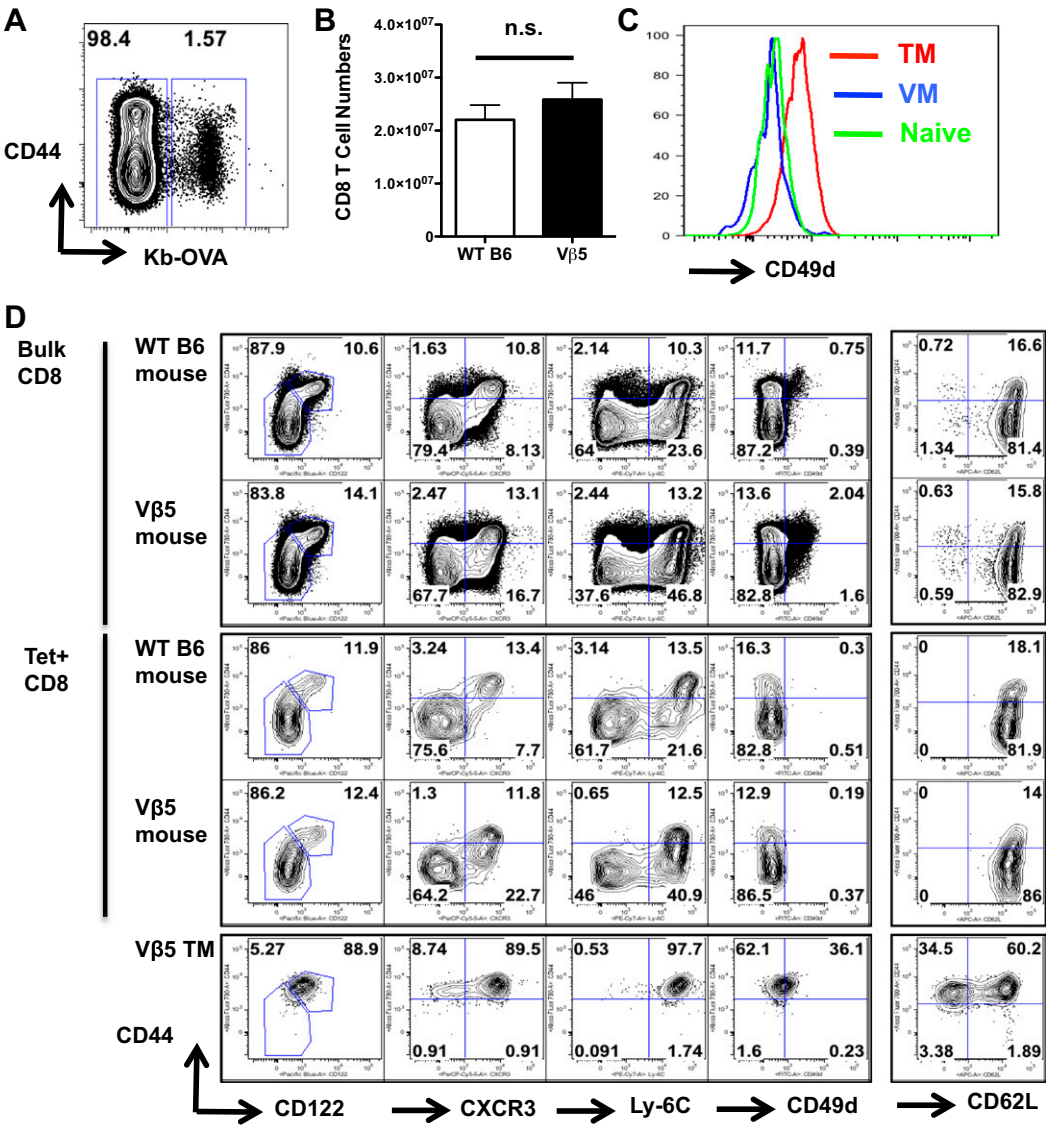

Fig. S1. Analysis of CD8 T-cell subsets derived from Vβ5 tg mice. (*A*) Representative data on the frequency and CD44 expression phenotype of OVA/Kb-specific CD8 T cells in unprimed Vβ5 tg mice. (*B*) Number of CD8 T cells in unprimed WT B6 mice and Vβ5 tg mice. Statistical significance between the number of CD8 T cells from WT B6 and Vβ5 mice is not significant (NS) ($P > 0.05$, Student *t* test). (*C*) CD49d expression of Ova/Kb tetramer$^+$ naïve, VM, and TM Vβ5 CD8 T cells. (*D*) Comparison of cell surface marker expression (CD122, Ly-6C, CXCR3, CD49d, and CD62L) between indicated populations of normal and Vβ5 CD8 T cells. Ova/K^b-specific unprimed and TM Vβ5 CD8 T cells were detected by an appropriate combination of congenic markers and Ova/Kb tetramer staining. Tetramer$^+$ CD8 T cells from wild-type B6 mouse were stained with a mixture of tetramers (Ova/Kb, B8R/Kb, and HSVgB/Kb) and enriched by tetramer pull-down assay. All of the data are representative of more than three experiments.

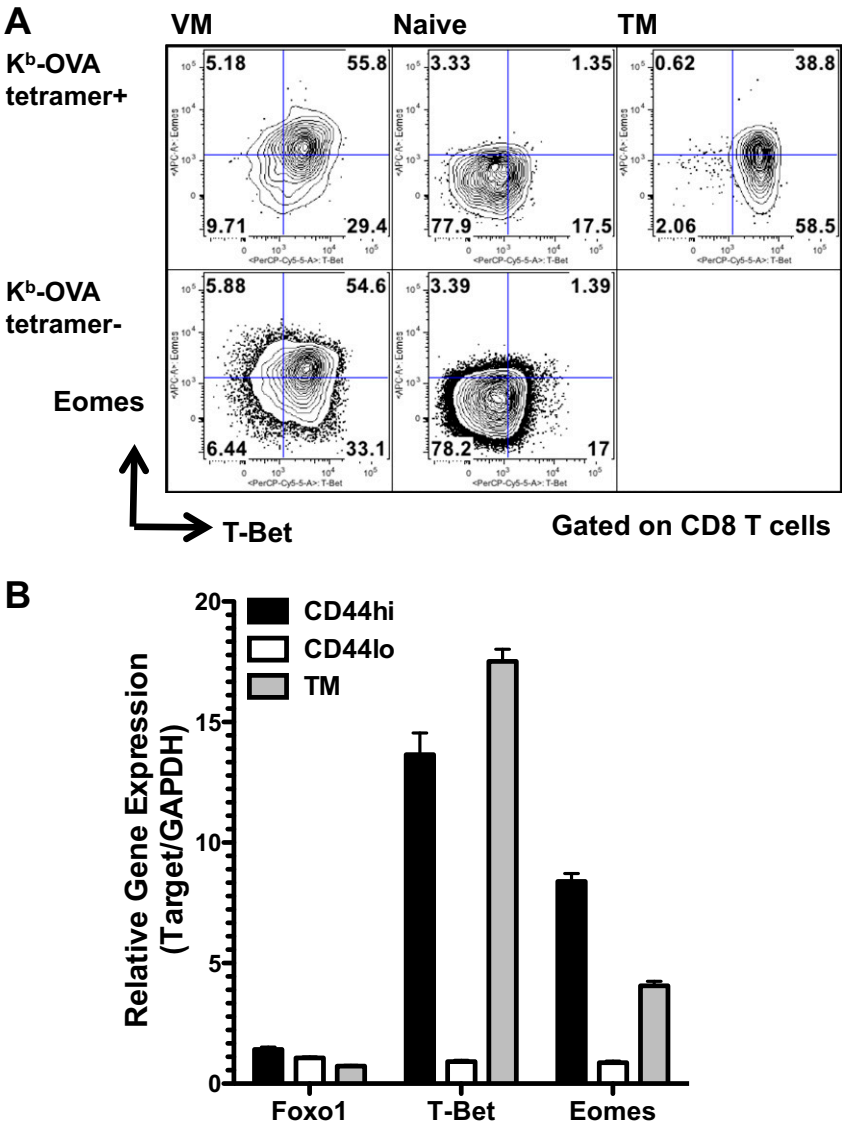

Fig. S2. Expression of T-box transcription factors on naïve, VM, and TM CD8 T cells. (*A*) Expression of T-bet and Eomes on Ova/K^b-specific VM, naïve, and TM Vβ5 CD8 T cells were determined by FACS. Data are representative of at least three independent experiments. (*B*) qRT-PCR analysis of indicated genes on sort purified $CD44^{high}$ and $CD44^{low}$ Vβ5 CD8 T cells from unprimed mice, in comparison with TM Vβ5 CD8 T cells (from immunized mice). Relative gene expression levels were normalized by GAPDH, and the levels in $CD44^{low}$ Vβ5 CD8 T cells were chosen as the baseline for comparison. Data are compiled from three independent experiments using independently generated cDNAs ($n = 3$) and graphs show mean ± SD.

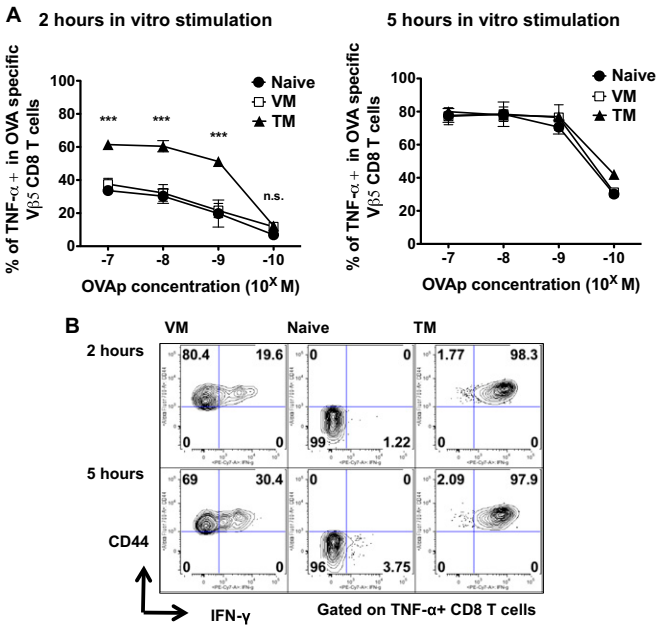

Fig. S3. Comparison of functional properties between naïve, VM, and TM CD8 T cells. (*A*) Percentage of TNF-α producing cells among Ova peptide-specific Vβ5 CD8 T cells upon in vitro stimulation of OVA peptide (10^{-7} M–10^{-10} M) for 2 or 5 h, corrected for the frequency of Ova/K^b tetramer$^+$ cells in an unstimulated sample (i.e., % of TNF-α producing CD8 T cells ÷ % of Ova/K^b tetramer-positive CD8 T cells). The graph shows compiled data from four independent experiments and lines show mean ± SD. Statistical significance between VM and TM is indicated (***$P < 0.001$; **$P < 0.01$; NS, not significant, is used to denote P values >0.05, Student t test). (*B*) IFN-γ production evaluated at 2 and 5 h after 10^{-7} M OVA peptide treatment and shown in comparison with CD44 expression levels. Data are representative of three independent experiments.

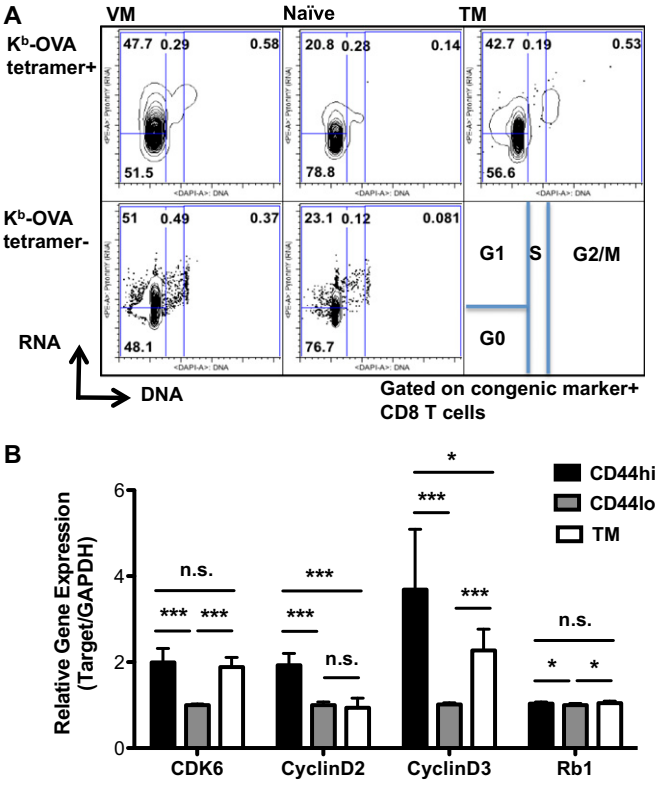

Fig. S4. Comparison of cell cycle status between naïve, VM, and TM CD8 T cells. (*A*) Cell cycle analysis of indicated Vβ5 CD8 T-cell populations. Numbers in boxed areas indicate percentage of cells in each. Data are representative of three independent experiments. (*B*) qRT-PCR analysis of cell cycle regulatory genes on sort purified CD44^{high} and CD44^{low} Vβ5 CD8 T cells from unprimed mice, in comparison with TM Vβ5 CD8 T cells (from immunized mice). Relative gene expression levels were normalized by GAPDH, and the levels in CD44^{low} Vβ5 CD8 T cells were chosen as the baseline for comparison. Data are compiled from three independent experiments using independently generated cDNAs ($n = 3$). Graphs show mean ± SD and statistical significance is indicated (***$P < 0.001$; *$P < 0.05$; NS, not significant, is used to denote P values >0.05, Student t test).

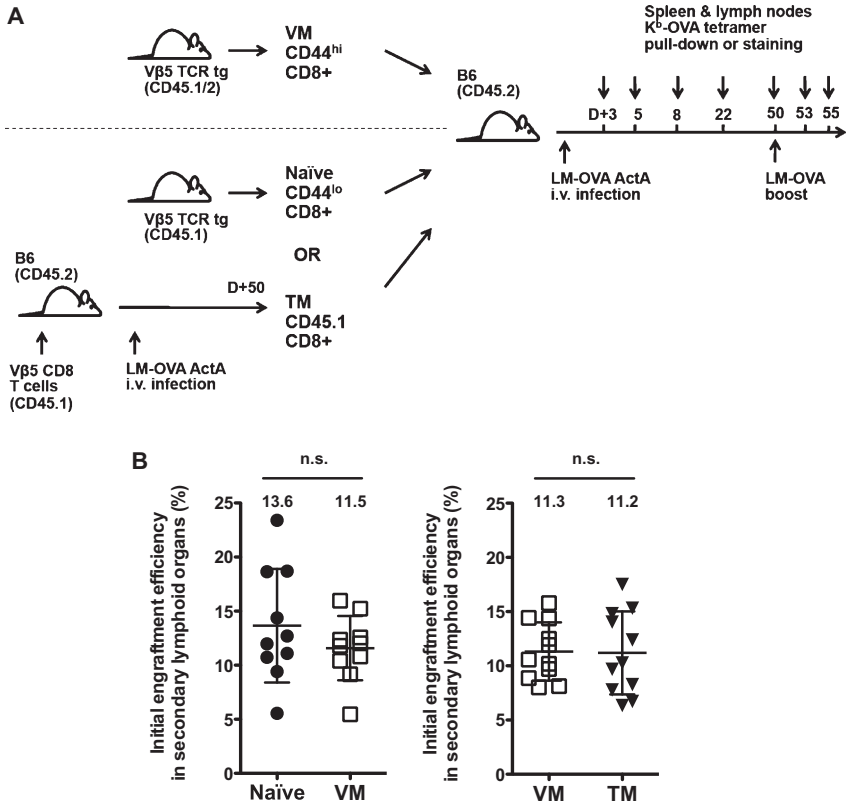

Fig. S5. In vivo adoptive transfer of Vβ5 VM CD8 T cells with naïve or TM. (*A*) Experimental schematics. To directly compare VM to naïve or TM during the cognate antigen-specific immune response in vivo, congenically distinct VM ($CD44^{high}$) and naïve ($CD44^{low}$) CD8 T cells were sorted from unprimed Vβ5 tg mice. Vβ5 TM CD8 T cells were generated in wild-type B6 recipients by adoptive transfer of unprimed total Vβ5 CD8 T cells and subsequent infection of attenuated (ActA) *L. monocytogenes*-OVA for at least 50 d, and sorted with congenic marker (CD45.1). Then, number of Ova/K^b-specific CD8 T cells was determined by Ova/K^b-tetramer staining, and VM CD8 T cells were cotransferred with naïve or TM CD8 T cells in 1:1 ratio (include 300–500 Ova/K^b-specific CD8 T cells in each population) into recipients, which were subsequently infected with *L. monocytogenes*-OVA ActA. Ova/K^b-tetramer and relevant congenic markers determined the Ova antigen-specific cells within each population. For inducing the secondary immune response, mice were infected with virulent *L. monocytogenes*-OVA at day 50 postprimary infection. (*B*) Initial engraftment efficiency of the transferred naïve, VM, and TM CD8 T cells in secondary lymphoid organs. To determine the efficiency of initial engraftment, the number of transferred Ova/K^b-specific CD8 T populations in unimmunized recipients was determined by Ova/K^b tetramer pull-down of cells from secondary lymphoid organs and then divided by the estimated input number of each population.

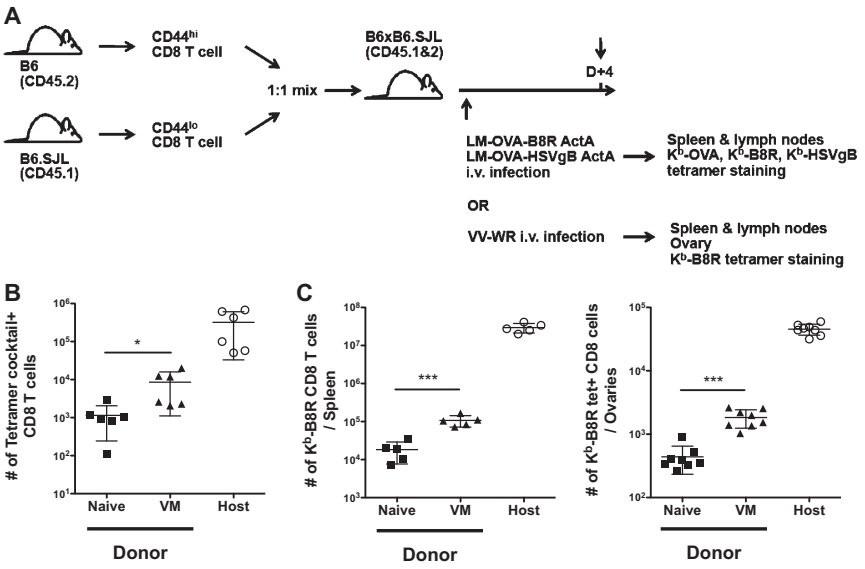

Fig. S6. VM CD8 T cells outcompete naïve counterparts for acute phase of immune response. (*A*) Experimental schematic. Congenically distinct polyclonal CD44^{high} and CD44^{low} CD8 T cells (2 × 106 cells of each population) from unprimed mice were cotransferred in 1:1 ratio into congenic wild-type host, which were subsequently immunized with attenuated *L. monocytogenes* strains (LM-OVA-B8R and LM-OVA-HSVgB) (*B*) or vaccinia virus (VV-WR) (*C* and *D*). (*B*) Number of tetramer mixture (Kb-OVA/Kb-B8R/Kb-HSVgB) positive CD8 T cells within each donor population (and host cells) in the spleen 4 d postinfection after *L. monocytogenes*-OVA ActA infection. Data are compiled from two independent experiments (three mice for each infection). (*C*) The numbers of donor and host Kb-B8R tetramer^{+} cells in the spleen (*Left*) and ovary (*Right*) of day 4 VV-WR infected recipients are shown. Data are compiled from three independent experiments and lines show mean ± SD.

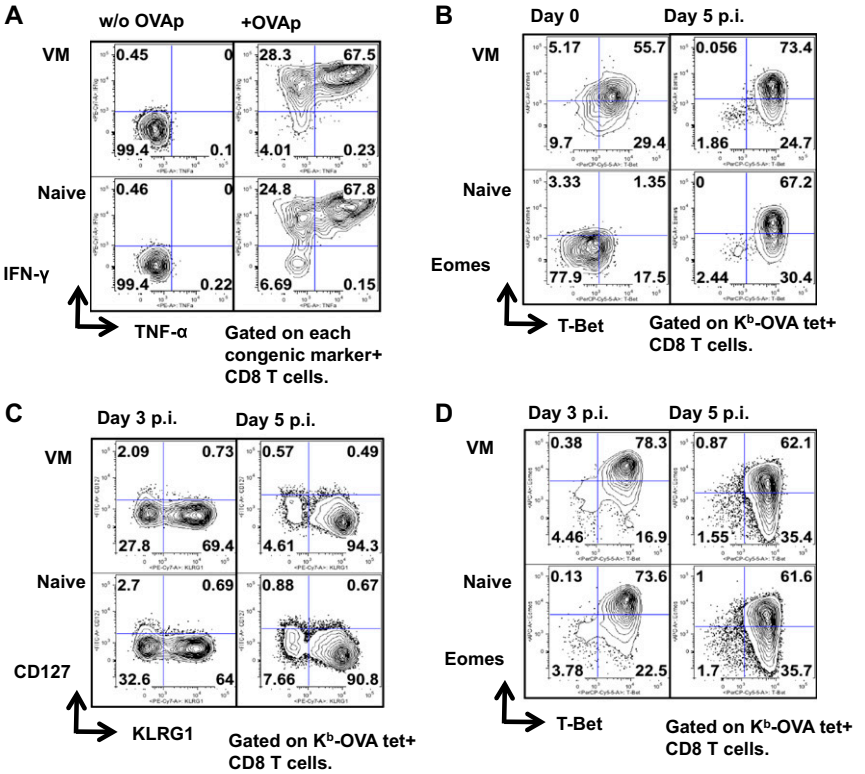

Fig. S7. Phenotypic and functional comparison between VM and naïve CD8 T cells during primary and secondary *L. monocytogenes* infection. (*A* and *B*) Responder cells derived from cotransferred naïve and VM Vβ5 CD8 T cells were assayed at day 5 following primary *L. monocytogenes*-OVA infection. Pro-inflammatory cytokine (IFN-γ and TNF-α) production was determined for donor populations (identified by congenic markers) (*B*), and expression of T-box transcription factors (T-bet and Eomes) was determined for OVA/K^{b} tetramer^{+} donor (*C*). Data are representative of two experiments (six mice total). (*C* and *D*) Phenotype of VM- and naïve-derived Vβ5 CD8 T cells at the indicated times during a recall response, induced by virulent *L. monocytogenes*-OVA infection. Data are representative of three independent experiments.