Supporting Information

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SI Materials and Methods

Viruses. WT mouse cytomegalovirus (MCMV) refers to a bacterial artificial chromosome (BAC)-derived mouse cytomegalovirus (MCMV), MW97.01, previously shown to be biologically equivalent to the MCMV strain Smith (VR-1399) (1); the generation of the recombinant strain RAE-1γMCMV was described previously (2). MCMV-SIINFEKL, RAE-1γMCMV-SIINFEKL, and Δm152MCMV-SIINFEKL were constructed by orthotopic peptide swap on the WT MCMV, RAE-1γMCMV, or Δm152MCMV (3) backbone, respectively, as described previously (4). The genomes of the MCMVList and RAE-1γMCMVList strains were constructed by Red recombination in *Escherichia coli* as described (5). Briefly, a linear DNA fragment was generated using a kanamycin resistance marker (KanR) as a template, the primers List_swap_fw1 5′-*gactactgtcggacgtgggcgctgac*ggttacaaagatggaaatgaatatattaggatgacgacgataagtaggg-3′ and List_swap_rv1 5′-aatatattcatttccatctttgtaacc*gtcagcgccccacgtccgacagtagtc*caaccaattaaccaattctgattag-3′ for a first PCR, and the primers List_swap_fw2 5′-*gatcgagccggtggtaccggacgcggcggagccgttcggaaaggactactgtcggacgtggggcgctgac*-3′ and List_swap_rv2 5′-*cccaaggtctcctcgcccgctgccacgatggcctggttgttgacggcccagaa*aatatattcatttccatctttg-3′ for a second PCR.The underlined sequences mark the *Listeria monocytogenes* listeriolysin $O_{91–99}$ GYKDGNEYI-coding sequence, whereas the 5′-ends of the primers (shown in italics) were required for homologous recombination to replace the sequence for the D^d-restricted antigenic $m164_{167–175}$ peptide AGPPRYSRI with the sequence for the K^d-restricted listeriolysin O-derived peptide within the MCMV genome. The PCR product was electroporated into *E. coli* containing the respective MCMV-WT- or RAE-1γMCMV-BAC, and following insertion, the KanR was excised by a second recombination step following the *en passant* mutagenesis procedure (6) from the resulting BAC. Correct insertion of the listeriolysin $O_{91–99}$ epitope coding sequences into the m164 ORF was verified by sequencing. Deletion of the m152 ORF in the BAC MCMVList was performed as described previously (3), resulting in the MCMV genome Δm152MCMVList. Following characterization by restriction analysis, the recombinant BACs were used to reconstitute viruses by transfection of mouse embryonic fibroblasts (MEFs) (5).

High-titer virus stocks were produced by propagation on MEFs followed by sucrose gradient ultracentrifugation (7). The viral titers in organs were determined by standard plaque assay (7).

Lymphocyte Subset Depletion. Lymphocyte depletion was performed by i.p. administration of αCD8 T-cell [YTS 169.4 clone (8)] or αNK1.1 [clone PK136 (9)] antibodies. NKG2D blockade was performed by i.p. injection of 300 μg of anti-NKG2D antibody [C7 clone (10)].

***Listeria monocytogenes*.** The hemolytic EGD strain (serovar1/2a) of *L. monocytogenes* and recombinant *L. monocytogenes* that stably express the chicken ovalbumin (aa134–387) (kindly provided by D. Zehn, University State Hospital, Lausanne, Switzerland) were grown in brain heart infusion (BHI) broth (Difco Laboratories) at 37 °C for 24 h. The optical density of the bacterial suspension was estimated using a spectrophotometer at 600 nm, and the numbers of colony-forming units (cfu) of *L. monocytogenes* were extrapolated from a standard growth curve. The actual number of cfu in the inoculum was verified by plating on BHI agar. To determine organ *L. monocytogenes* burden, spleens and livers were removed from infected mice 4 d post-infection (p.i.) and homogenized separately in PBS, following incubation in distilled water. Serial 10-fold dilutions of suspensions were plated onto BHI agar, and cfu were determined after 24–48 h incubation at 37 °C.

Flow Cytometry and Immune Assays. Custom $H2^d$-restricted listeriolysin O (LLO; ^{91}GYKDGNEYI^{99}) and $H2^b$-restricted MCMV-specific M45 (^{985}HGIRNASFI^{993}) and M38 (^{316}SSPPMFRV^{323}) peptides were synthesized to a purity of >95% by Jerini Peptide Technologies. Tetramers were synthesized by the National Institutes of Health tetramer core facility. The $H2^b$-restricted SIINFEKL pentamer was purchased from Proimmune. The following fluorescently labeled antibodies purchased from eBioscience were used: anti-B220 (PA3-6B2), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD3 (145-2C11), anti-CD8α (53-6.7), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti–IFN-γ (XMG1.2), anti-CD107a (LAMP-1) (eBio1D4B), anti-MHC II (M5/114.15.2), anti-NKp46 (29A1.4), anti–PDCA-1 (eBio927), anti–KLRG-1 (2F1), anti-CD127 (A7R34), and anti–TNF-α (MP6-XT22). For CD8 T-cell in vitro cytokine production, splenocytes were resuspended in complete cell culture RPMI 1640 supplemented with 10% (vol/vol) FCS and stimulated with 1 μg of MHC class I restricted peptides for 1 h, followed by an additional 4 h in the presence of Brefeldin A (eBioscience) at 37 °C. Subsequently, cells were surface-stained, fixed, and permeabilized, followed by intracellular IFN-γ and TNF-α staining. For NK cell IFN-γ production and anti-CD107 staining, splenocytes were incubated for 5 h in the presence of IL-2 (500 IU/mL; Becton Dickinson Biosciences) and Phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich) and Ionomycin (I; 500 ng/mL; Sigma-Aldrich) or IL12 (10 ng/mL; R&D Systems) and IL-18 (20 ng/mL, MBL International). Brefeldin A was added for the last 4 h at 37 °C. For natural cytotoxicity receptor 1 (NCR1) stimulation, 96-well plates were coated with 2 μg/mL of anti-NKp46 antibody, and 5×10^5 splenocytes were added per well and incubated for 5 h at 37 °C, with the Brefeldin A added for the last 4 h. For dendritic cell analysis, spleens were digested by collagenase D (Roche). For the in vivo cell proliferation assay, mice were i.p. injected with 2 mg of bromodeoxyuridine (BrdU) and killed 2 h later. Incorporated BrdU was detected according to the manufacturer's instructions (BrdU Flow Kit; BD Pharmingen). Flow cytometry was performed by FACSCalibur and FACSAria (Becton Dickinson Biosciences), and data were analyzed using FacsDiva (Becton Dickinson Biosciences) or FlowJo (Tree Star) software.

In Vivo Cytotoxicity Assay. Splenocytes from the uninfected BALB/c mice were loaded with 1 μg/mL of LLO peptide per 6×10^7 cells and labeled with 0.5 μM 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (Invitrogen). Unpulsed control targets were labeled with 5 μM CFSE. Differentially CFSE-labeled cells were mixed in equal ratios, and a total of 1×10^7 cells per mouse was transferred i.v. into MCMVList- or RAE-1γMCMVList–infected or uninfected mice. The survival of the transferred splenocytes was analyzed 6 h later by flow cytometry. Percentage-specific lysis of CFSE-labeled target cells was calculated as follows: [1 − (*r* uninfected control mouse/*r* infected test mouse)] × 100, where *r* = (frequency of unpulsed targets/frequency of peptide-pulsed targets).

Serum IFN-α Level Analysis. Serum levels of IFN-α were determined by an ELISA kit for IFN-α (PBL Biomedical Laboratories) according to the manufacturer's instructions.

Histopathology and Immunohistochemistry. Sections from formalin-fixed, paraffin-embedded spleens and livers were stained with

hematoxylin and eosin (both Thermo Scientific). αCD3 staining of lymphocytes on paraffin spleen sections was performed by anti-CD3 (SP7) (Abcam) followed by biotinylated goat anti-mouse IgG (IgG) antibody (BD Pharmingen) and avidin–biotin–peroxidase complex (Roche Applied Science). Counterstaining was performed with hematoxylin. Slides were analyzed on an Olympus BX40 microscope, and images were acquired by an Olympus digital camera (C-3030).

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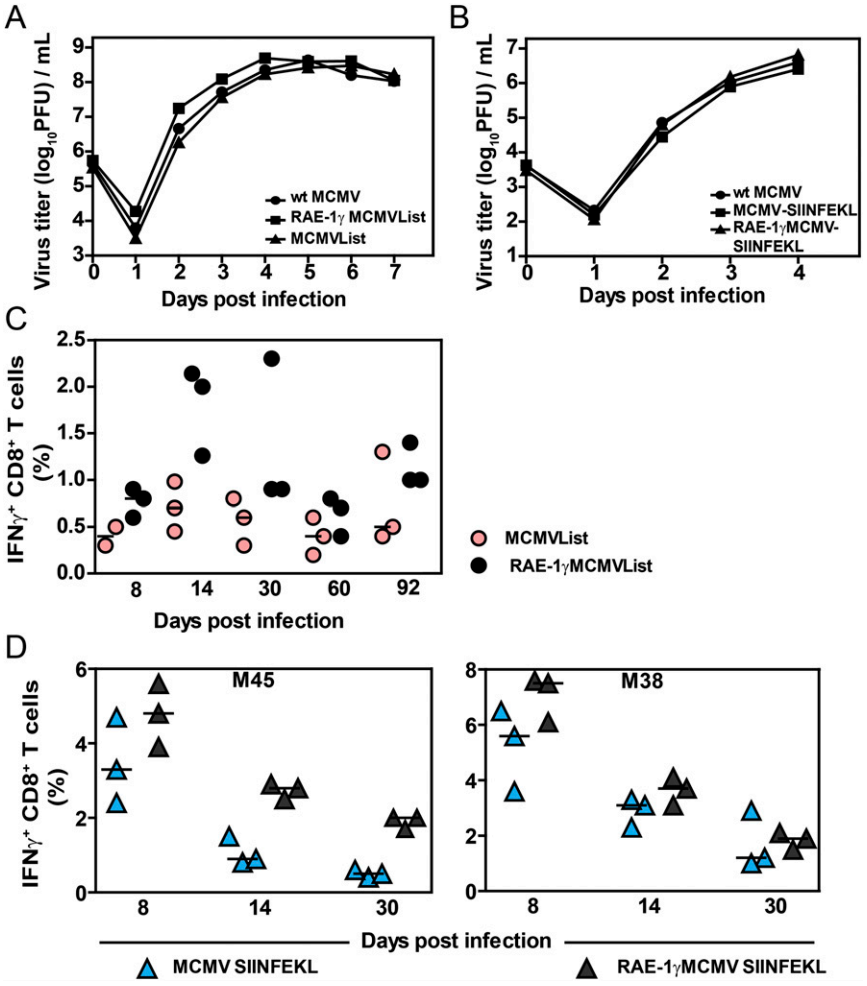

Fig. S1. Listeriolysin epitope expression in place of MCMV m164 epitope does not affect RAE-1γMCMVList replication in vitro but enhances epitope-specific CD8 T-cell response. (*A* and *B*) Murine embryonal fibroblasts were infected with 0.1 pfu per cell of indicated viruses. Supernatants were harvested at indicated time points, and viral titers were determined by plaque assay. (*C*) Splenocytes of MCMVList- or RAE-1γMCMVList–infected BALB/c mice were stained for LLO-specific IFN-γ production, infectious dose of 2×10^5 pfu per mouse via footpad (f.p.). (*D*) C57BL/6 mice were f.p. infected with 10^5 pfu per mouse of MCMV-SIINFEKL or RAE-1γMCMV-SIINFEKL. At indicated time points, splenocytes were isolated, stimulated with MCMV-specific (M38 and M45) peptides, and stained for the intracellular IFN-γ production. For *C* and *D*, individual animals (circles or triangles) and median values are shown.

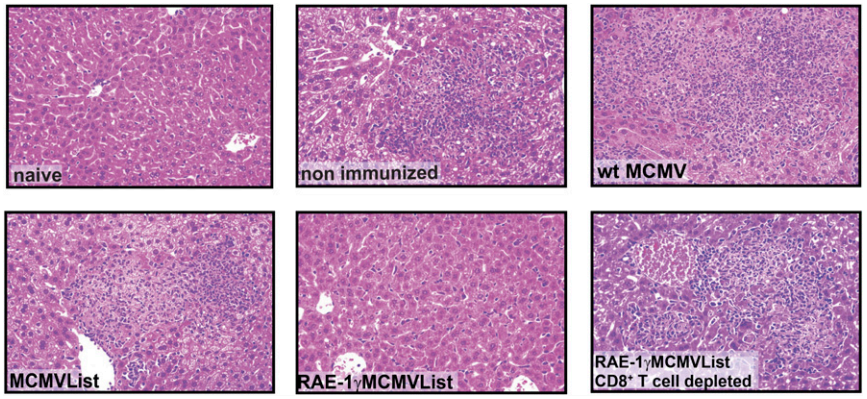

Fig. S2. CD8 T-cell depletion abolished the protective effect of immunization with RAE-1γMCMVList and results in liver pathology in *L. monocytogenes*-challenged mice. BALB/c mice were f.p. immunized with 10^5 pfu per mouse of indicated viruses. Three weeks postimmunization, mice were challenged with 10^4 cfu per mouse of *L. monocytogenes*. One day before the challenge, mice were depleted of CD8 T cells or left undepleted. Hematoxylin and eosin (HE) staining of paraffin embedded liver sections is shown. (Magnification: 20×.)

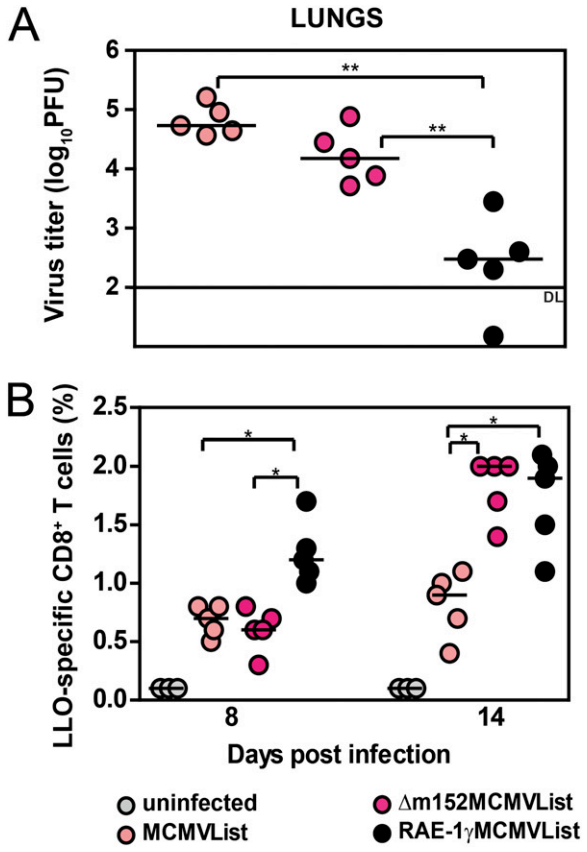

Fig. S3. RAE-1γMCMVList is superior in its capacity to induce CD8 T-cell response compared with the virus possessing only the deletion of *m152*. (*A*) BALB/c mice were infected i.v. with 2×10^5 pfu per mouse of the indicated viruses. Virus titer in lungs was determined on day 8 p.i. (*B*) Mice were infected with 10^5 pfu per mouse via f.p., and the frequency of LLO-specific CD8 T cells was determined on days 8 and 14 p.i. For both panels, individual animals and median values are shown.

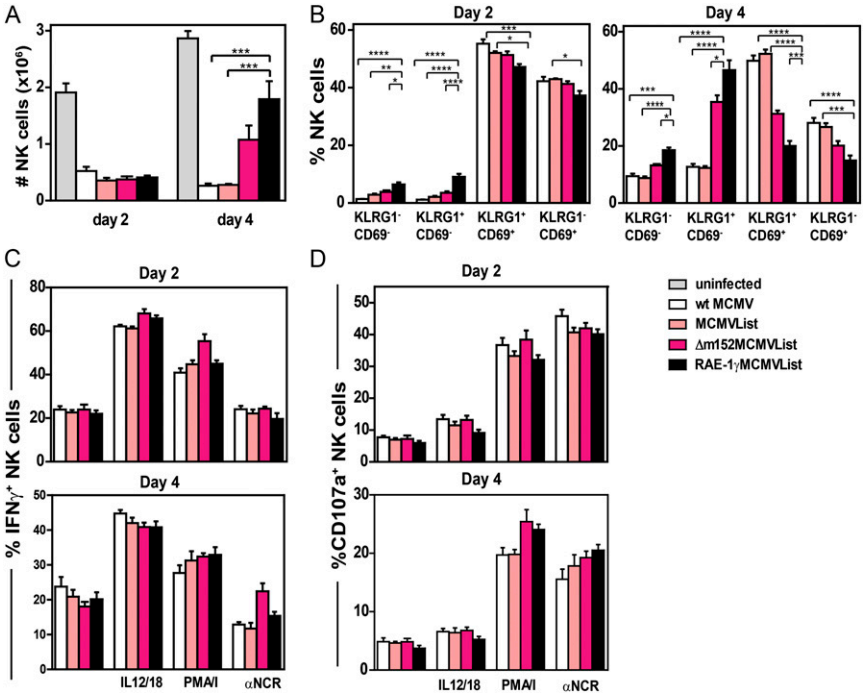

Fig. S4. NK cells derived from RAE-1γMCMVList–infected mice are responsive and functional. BALB/c mice were infected i.v. with 2×10^5 pfu per mouse of the indicated viruses. On days 2 and 4 p.i., NK cells in the spleen were analyzed for (*A*) frequency, (*B*) KLRG1 and CD69 expression, (*C*) IFNγ secretion, and (*D*) CD107a expression. $n = 5$. For all panels, mean ± SEM is shown.

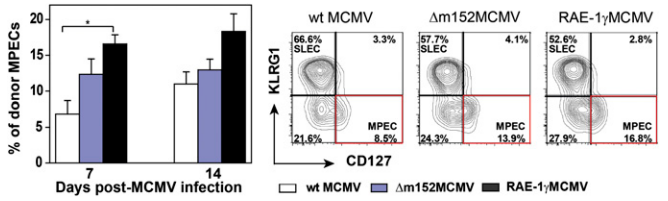

Fig. S5. Accelerated transition of early-memory to late-memory CD8 T cells in RAE-1γMCMV–infected mice. Naive recipients (CD45.2) were transferred with 10^4 CD8 T cells from M38 TCR transgenic mice and infected with 10^4 pfu per mouse of either WT MCMV, Δm152MCMV, or RAE-1γMCMV 24 h later. On days 7 and 14 p.i., donor cells in spleen were analyzed for the frequency of memory precursor effector cells (MPECs) ($KLRG1^-CD127^+$ CD8 T cells) ($n = 3$–4). Representative FACS plots of donor MPECs expansion are shown; $n = 3$–4.

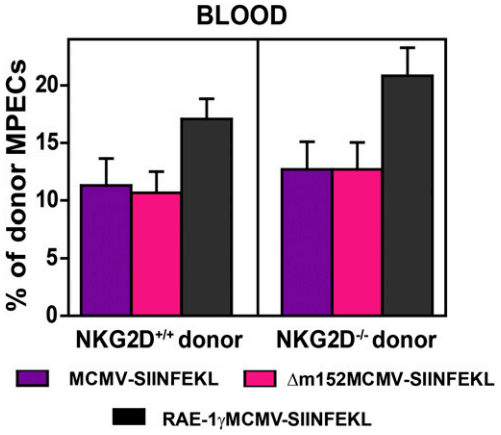

Fig. S6. SIINFEKL-specific CD8 T-cell priming and enhanced MPECs formation in RAE-1γMCMV vector infection are NKG2D-independent. Splenocytes were isolated from OT-$1^{+/-}$Ly5.1/2 and $NKG2D^{\Delta/\Delta}$Ly5.2 donors and mixed in a 1:1 ratio of CD8 T cells. In total, 5,000 CD8 T cells were transferred to $NKG2D^{-/-}$Ly5.1 recipients. After 24 h, mice were infected with 10^4 pfu of MCMV-SIINFEKL, Δm152MCMV-SIINFEKL, or RAE-1γMCMV-SIINFEKL. On day 7 p.i., donor CD8 T cells were analyzed for the frequency of MPECs ($KLRG1^-CD127^+$ CD8 T cells) in the blood. Mean ± SEM is shown; $n = 4$–5.