Supplemental Materials and Methods and Supplemental Figures

STIM1 and STIM2 control antiviral immunity by CD4 and CD8 T cells

Patrick J. Shaw[1#], Carl Weidinger[1#], Martin Vaeth[1], Kevin Luethy[1,3], Susan M. Kaech[2] and Stefan Feske[1]

[1]Department of Pathology, New York University School of Medicine, New York, NY 10016, USA;
[2]Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA;

[3] current address: Institute for Biology III, University of Freiburg, Germany.

both authors contributed to this study equally

Supplemental Materials and Methods

Intracellular Ca^{2+} measurements

Splenocytes were isolated from uninfected *Stim1*$^{fl/fl}$ *Stim2*$^{fl/fl}$ *Cd4-Cre* (DKO) or WT littermate mice and labelled with 2 µM Fluo-4 AM (Invitrogen). Cells were incubated with antibodies against CD4 (GK1.5), CD8 (53-6.7), CD11c (N418) and MHC-II (M5/114.15.2, all from eBioscience) for 30 min at 22–25°C, washed and kept in RPMI 1640 medium until use. Measurements of intracellular $[Ca^{2+}]_i$ were conducted using a LSR II flow cytometer (BD Biosciences). Baseline Ca^{2+} levels (F_0) were acquired in nominally Ca^{2+}-free Ringer solution containing (in mM) 155 NaCl, 4.5 KCl, 3 $MgCl_2$, 10 D-glucose, 5 Na-HEPES. After 30 s, cells were stimulated with 1 µM of the Sarco/endoplasmic reticulum ATPase (SERCA) inhibitor thapsigargin (EMD). After 300 s, Ringer solution containing 2 mM $CaCl_2$ was added to the cells (final extracellular Ca^{2+} concentration 1 mM). Data were analyzed using FlowJo software (Tree Star) by gating on $CD4^+$ and $CD8^+$ T cell populations or $CD11c^+$ $MHC\text{-}II^+$ $CD4^+$ dendritic cells. Intracellular Ca^{2+} levels were calculated as the F/F_0 ratio (F=Fluorescence intensity; F_0=Fluorescence intensity before addition of TG) over time in seconds (s). SOCE was averaged as the mean ± SEM of the F/F_0 ratio at 30 s after readdition of Ca^{2+} from 3 independent experiments performed in duplicates; data were plotted using Prism6 software (Graphpad).

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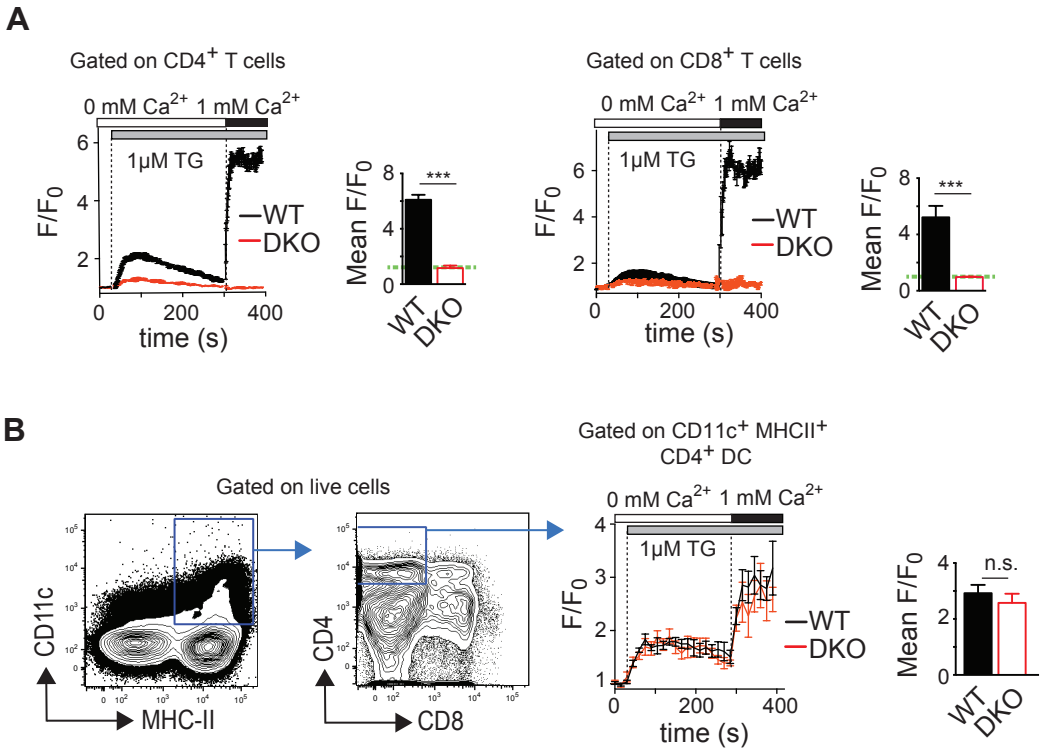
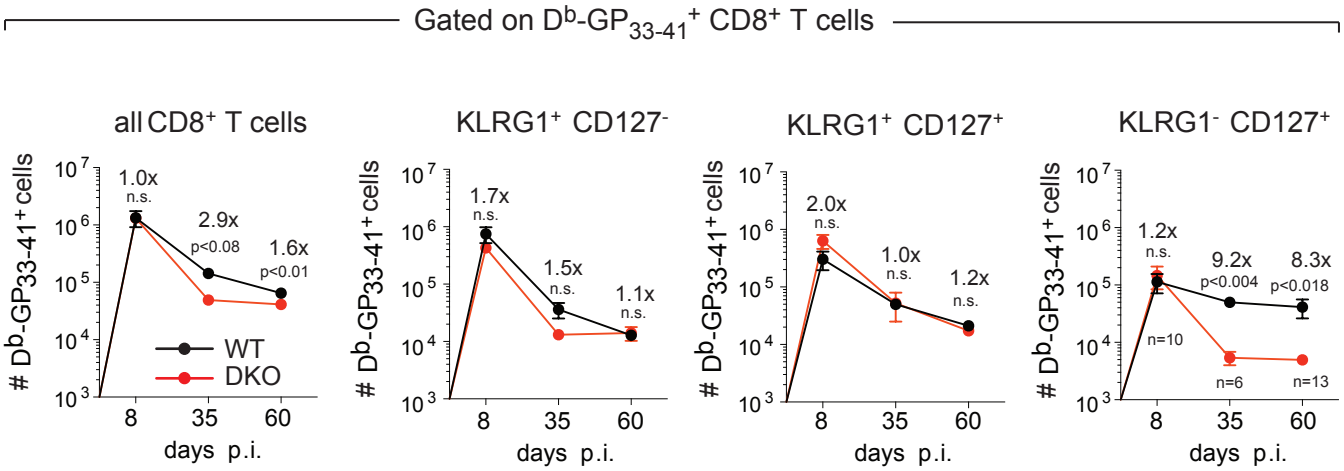


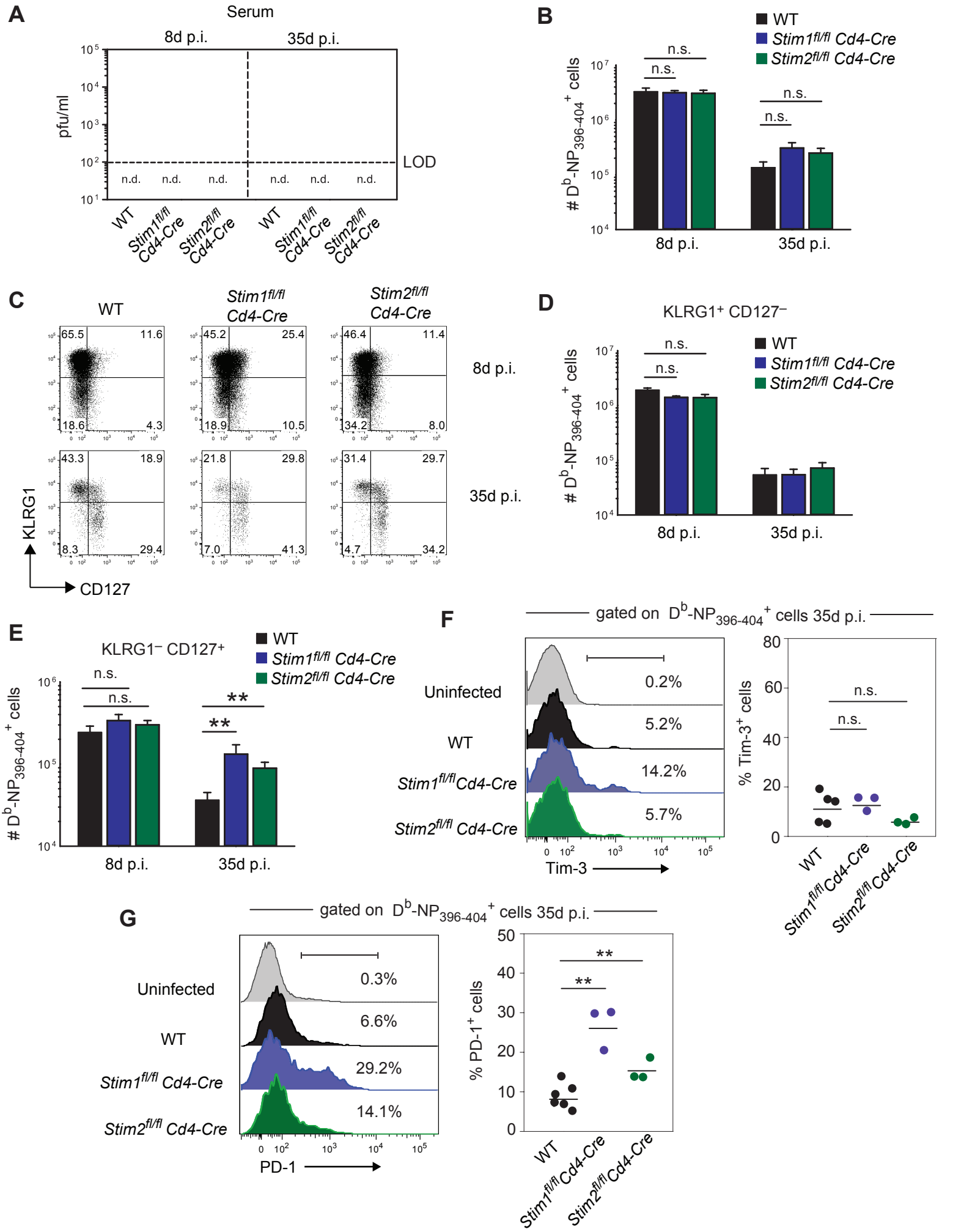
Supplemental Figure 1. Store-operated Ca^{2+} entry (SOCE) is impaired in T cells but not $CD4^+$ dendritic cells (DC) from *Stim1$^{fl/fl}$ Stim2$^{fl/fl}$ Cd4-Cre* mice.

(**A)** SOCE in $CD4^+$ and $CD8^+$ T cells of DKO (*Stim1$^{fl/fl}$ Stim2$^{fl/fl}$ Cd4-Cre*) or WT (*Stim1$^{fl/fl}$ Stim2$^{fl/fl}$* Cre-negative littermate). Splenocytes were isolated from uninfected WT or DKO mice, labeled with the Ca^{2+} indicator dye Fluo-4 and stained with antibodies against CD4, CD8, CD11c and MHC-II. Endoplasmic reticulum (ER) Ca^{2+} stores were depleted with 1 μM thapsigargin (TG) in Ca^{2+}-free Ringer solution (0 mM Ca^{2+}) and SOCE induced by readdition of 2 mM Ca^{2+}. SOCE in $CD4^+$ and $CD8^+$ cells was calculated as the F/F_0 ratio (F=Fluorescence intensity; F_0=Fluorescence intensity before addition of TG) over time in seconds (s). Dotted green line in bar graphs represents F_0. ***, $p<0.001$, n.s.= non-significant. (**B**) SOCE in $CD11c^+$, $MHC\text{-}II^+$, $CD4^+$ DC from WT and DKO mice stimulated as in (A). Shown are representative graphs and mean ± SEM of Ca^{2+} influx levels after the readdition of Ca^{2+} (at 300 s) from 3 independent experiments performed in duplicates.



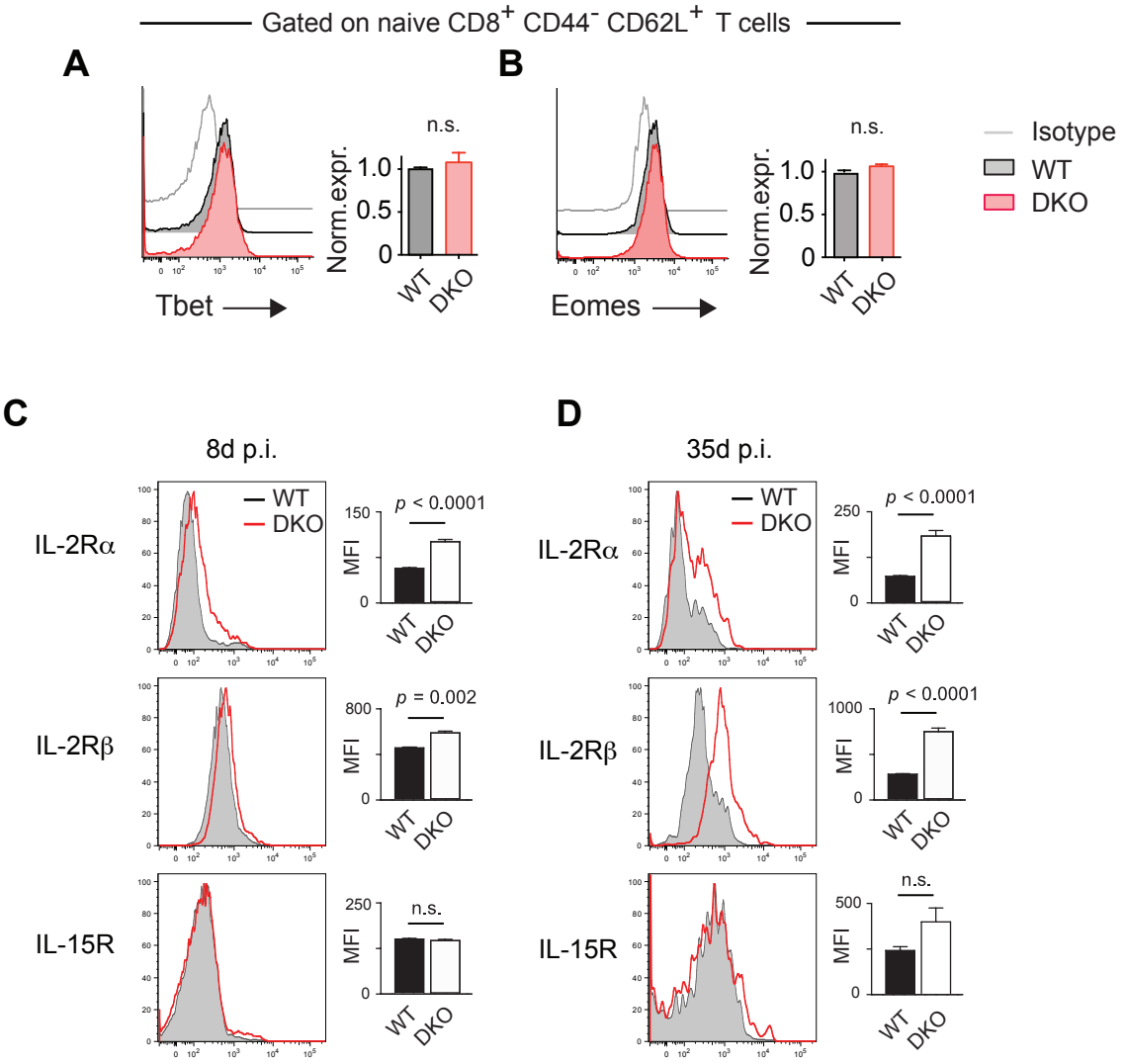
Supplemental Figure 2. Total number of LCMV-specific (D^bGP_{33-41} tetramer$^+$) $CD8^+$ T cells in WT and DKO mice.

WT and DKO mice were infected with 2 x 10^5 pfu $LCMV^{ARM}$ (i.p.) and splenocytes were isolated at days 8, 35 and 60 p.i. Cells were stained with antibodies against CD8, KLRG1, CD127 and D^bGP_{33-41} MHC class I tetramer. The total number of all LCMV-specific $CD8^+$ T cells and those of effector ($KLRG1^+$ $CD127^-$) and memory ($KLRG1^+$ $CD127^+$ and $KLRG1^-$ $CD127^+$) subsets were analyzed in 6-13 mice per group. Numbers represent fold differences in LCMV-specific $CD8^+$ T cell numbers between WT and DKO mice. Statistical significance was calculated using Student's t-test. The analysis of D^bGP_{33-41} -specific $CD8^+$ T cells complements data shown in **Figure 1C-G**.


A
Serum
8d p.i.
35d p.i.
pfu/ml
LOD
n.d.
WT
Stim1fl/fl Cd4-Cre
Stim2fl/fl Cd4-Cre
B
WT
Stim1fl/fl Cd4-Cre
Stim2fl/fl Cd4-Cre
n.s.
Db-NP396-404+ cells
8d p.i.
35d p.i.
C
WT
Stim1fl/fl Cd4-Cre
Stim2fl/fl Cd4-Cre
8d p.i.
35d p.i.
KLRG1
CD127
D
KLRG1+ CD127−
E
KLRG1− CD127+
**
F
gated on Db-NP396-404+ cells 35d p.i.
Uninfected
0.2%
5.2%
14.2%
5.7%
Tim-3
% Tim-3+ cells
G
0.3%
6.6%
29.2%
14.1%
PD-1
% PD-1+ cells

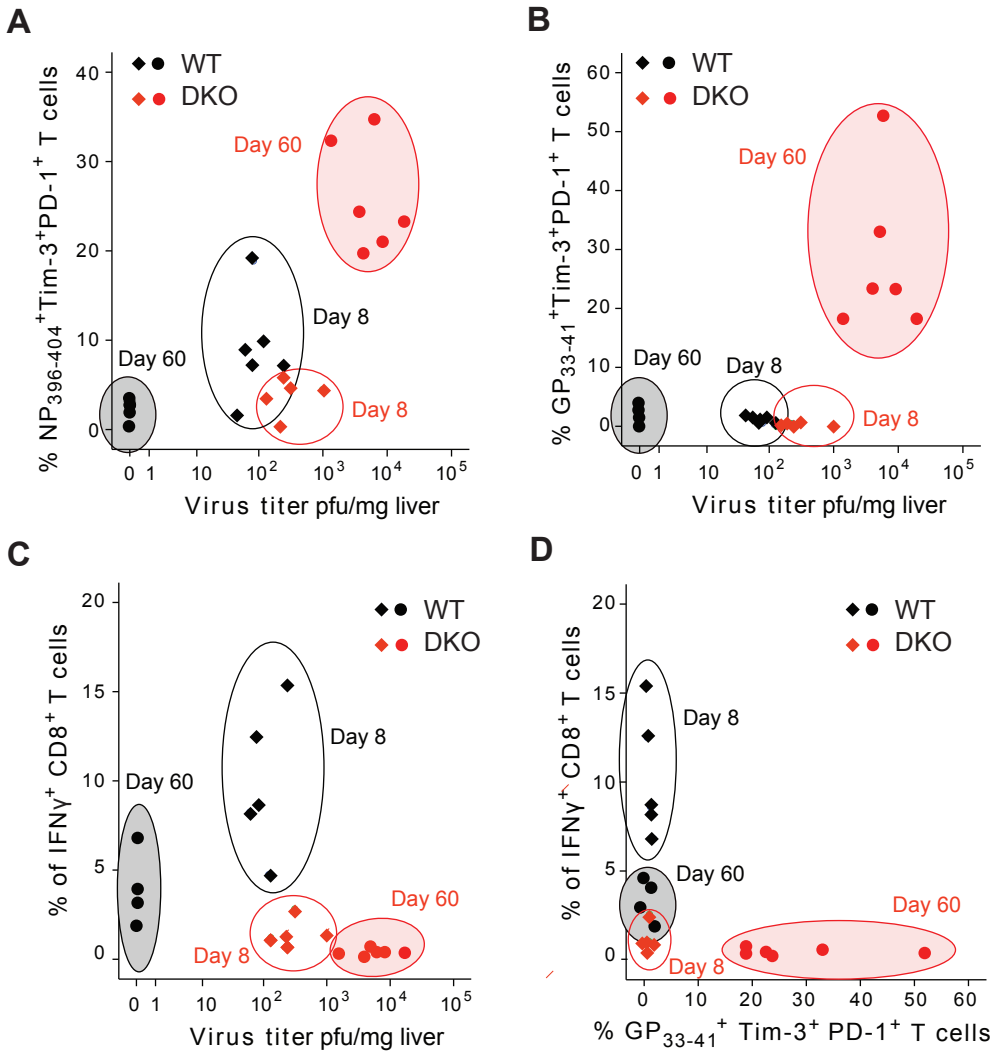
Supplemental Figure 3. Mice with T cell specific deletion of *Stim1* or *Stim2* genes alone mount and maintain normal antiviral immunity.

Stim1$^{fl/fl}$ *Cd4-Cre*, *Stim2*$^{fl/fl}$ *Cd4-Cre* and wild-type (WT, i.e. Cre-negative littermates) mice were infected with 2 x 10^5 pfu LCMV^{ARM} (i.p.). (**A**) Undetectable viral titers in the serum at days 8 and 35 p.i. (n.d., not detectable; LOD, level of detection). (**B**) Comparable numbers of $D^bNP_{396-404}^+$ $CD8^+$ T cells in the spleen of WT, STIM1- and STIM2-deficient mice at days 8 and 35 p.i. (**C**) Expression of KLRG1 and CD127 (IL-7R) on $D^bNP_{396-404}^+$ $CD8^+$ T cells at days 8 and 35 p.i. Numbers represent percentages of cells in gates. (**D,E**) Comparable numbers of $D^bNP_{396-404}^+$ terminal effector ($KLRG1^+$ $CD127^-$ in D) and memory ($KLRG1^-CD127^+$ in E) $CD8^+$ T cells in the spleen at days 8 and 35 p.i. (mean ± SEM). (**F,G**) Frequencies of Tim-3^+ (**F**) and PD-1^+ (**G**) $D^bNP_{396-404}^+$ $CD8^+$ T cells were determined in the spleens of WT, STIM1 or STIM2-deficient mice at day 35 p.i. 3-5 mice per group and time point were used. Horizontal lines in **F,G** represent mean percentages. n.s. = non-significant; ** $p<0.01$.



Supplemental Figure 4. Expression levels of T-bet, Eomes and cytokine receptors in STIM1/STIM2-deficient CD8$^+$ T cells.

(**A-B**) Splenocytes from uninfected *Stim1$^{fl/fl}$Stim2$^{fl/fl}$ Cd4-Cre* (DKO) and wild-type (WT) mice were isolated and the expression of T-bet (A) and Eomes (B) was determined in CD8$^+$ CD44$^-$ CD62L$^+$ T cells by flow cytometry. Representative histogram plots (left) and bar graphs (right) showing the means ± SEM of relative T-bet and Eomes expression from 3 independent experiments (one WT and DKO mouse per experiment performed in duplicates). To correct for variation in T-bet and Eomes levels between experiments, expression in DKO cells was normalized to that in WT cells for each experiment. (**C-D**) WT and DKO mice were infected with 2 x 10^5 pfu of LCMV^{ARM} (i.p.). LCMV-specific CD8$^+$ T cells were isolated from the spleen and analyzed 8 (C) and 35 (D) days p.i. for the expression of cytokine receptors required for memory CD8$^+$ T cell survival. Representative histogram plots (left) and bar graphs showing the means ± SEM of IL-2Rα, IL-2Rβ and IL-15R expression from 6 mice per group.



E

Spearman's rho correlation including WT and DKO mice

	Virus titer	$NP_{396-404}^{+}$ Tim-3⁺ PD-1⁺	GP_{33-41}^{+} Tim-3⁺ PD-1⁺
$NP_{396-404}^{+}$ Tim-3⁺ PD-1⁺	0.682*		
GP_{33-41}^{+} Tim-3⁺ PD-1⁺	0.769**	0.782**	
IFNγ⁺	- 0.684*	- 0.827**	- 0.65*

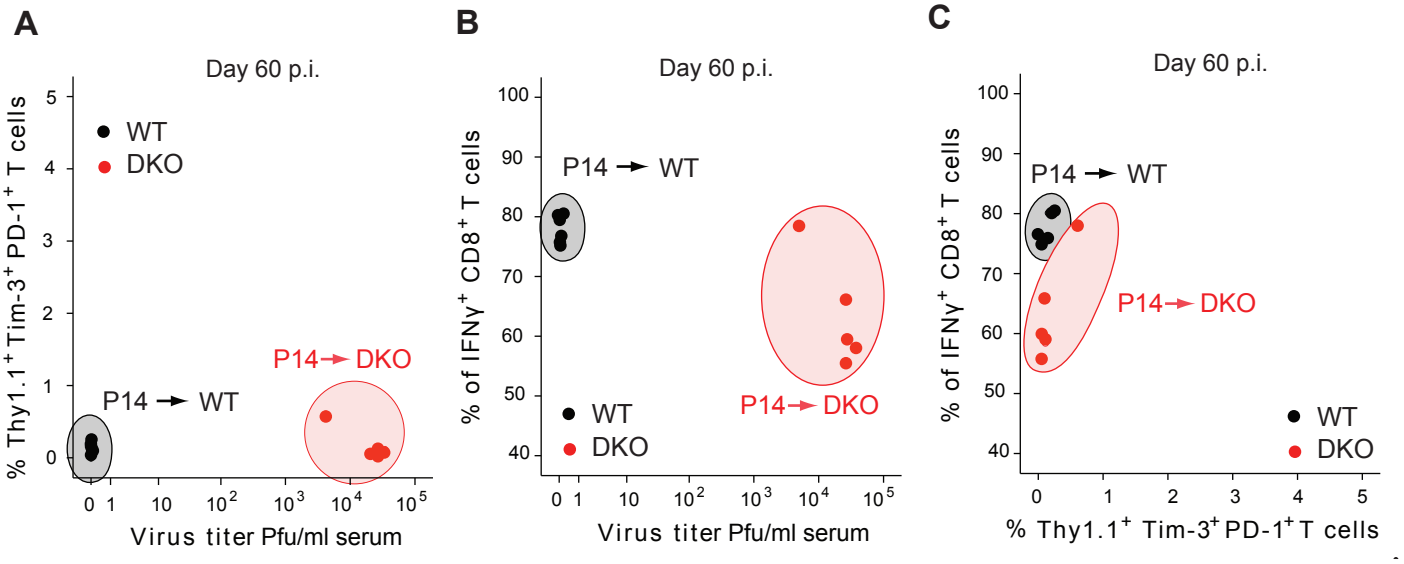
F

Spearman's rho correlation within the DKO cohort

	Virus titer	$NP_{396-404}^{+}$ Tim-3⁺ PD-1⁺	GP_{33-41}^{+} Tim-3⁺ PD-1⁺
$NP_{396-404}^{+}$ Tim-3⁺ PD-1⁺	- 0.314		
GP_{33-41}^{+} Tim-3⁺ PD-1⁺	0.086	0.029	
IFNγ⁺	0.319	- 0.232	0.49

Supplemental Figure 5. Correlation analysis of viral titers and CD8⁺ T cell exhaustion.

(**A-D**) WT (n=4-6) and *Stim1*$^{fl/fl}$ *Stim2*$^{fl/fl}$ *Cd4-Cre* (DKO) (n=5-6) mice were infected with LCMV^{ARM} and analyzed at days 8 and 60 p.i. for viral titers in the liver as well as expression of exhaustion markers (PD-1, Tim-3) and IFNγ production by GP_{33-41} or $NP_{396-404}$ specific CD8⁺ T cells. The frequency of IFNγ producing CD8⁺ T cells was measured after restimulation with GP_{33-41} peptide for 6h in vitro. Each symbol represents one mouse. (**E,F**) Correlation coefficients were calculated for samples 60 days p.i. using Spearman's Rho test. (* $p<0.05$; ** $p<0.01$). (**E**) Both WT and DKO cohorts were included in the analysis. (**F**) Only DKO samples were included in the analysis. Correlation analyses were performed using primary data shown in **Figures 1, 2** and **3**.



D

Spearman's rho correlation including WT and DKO mice

	Virustiter	% Thy1.1$^+$ Tim-3$^+$ PD-1$^+$
IFNγ $^+$	-0.770 **	0.552
% Thy1.1$^+$ Tim-3$^+$ PD-1$^+$	-0.03	

E

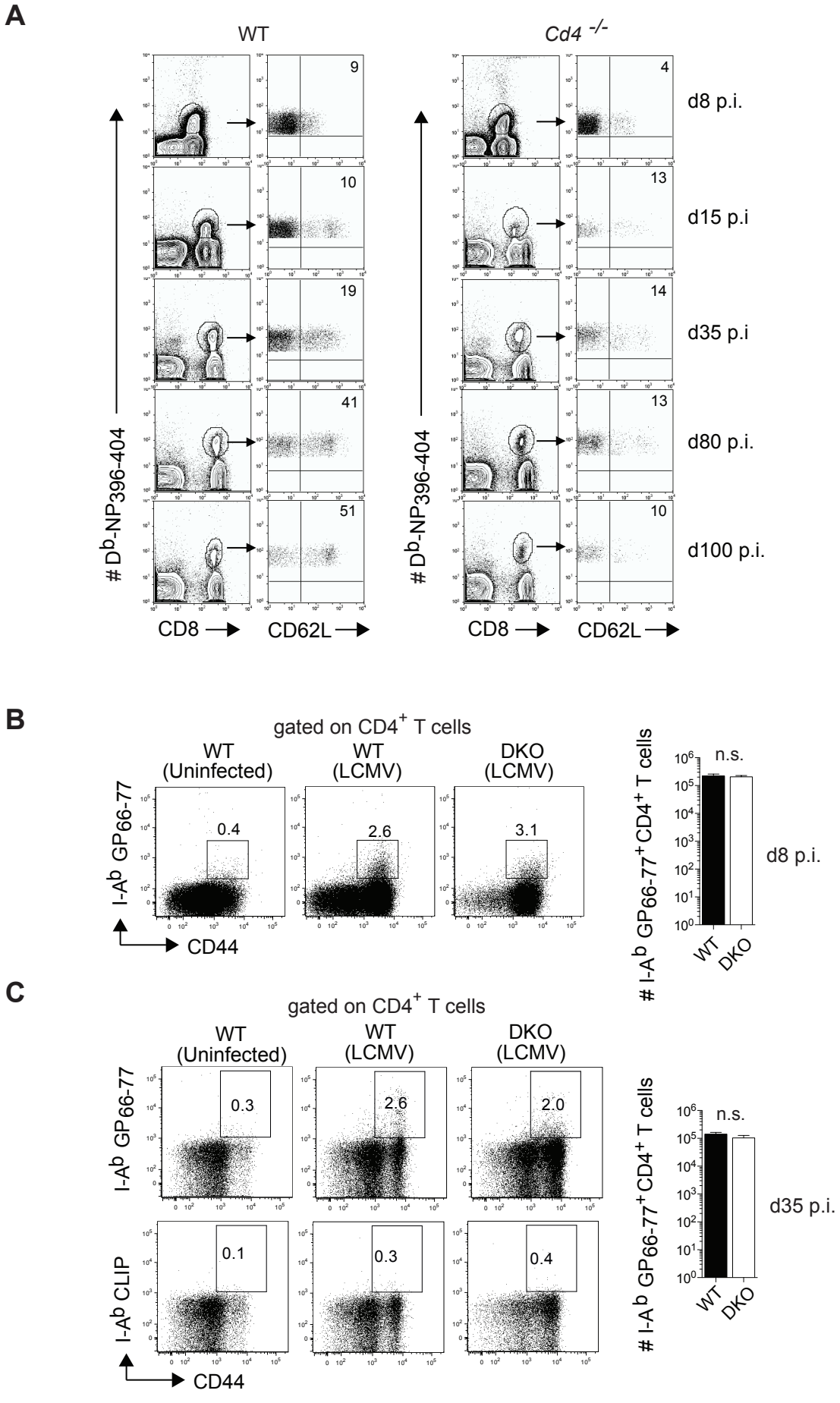
Spearman's rho correlation within the DKO cohort

	Virustiter	% Thy1.1$^+$ Tim-3$^+$ PD-1$^+$
IFNγ $^+$	-0.564	0.7
% Thy1.1$^+$ Tim-3$^+$ PD-1$^+$	-0.205	

Supplemental Figure 6. Correlation analysis of viral titers and CD8$^+$ T cell exhaustion in mice after transfer of WT P14 cells.

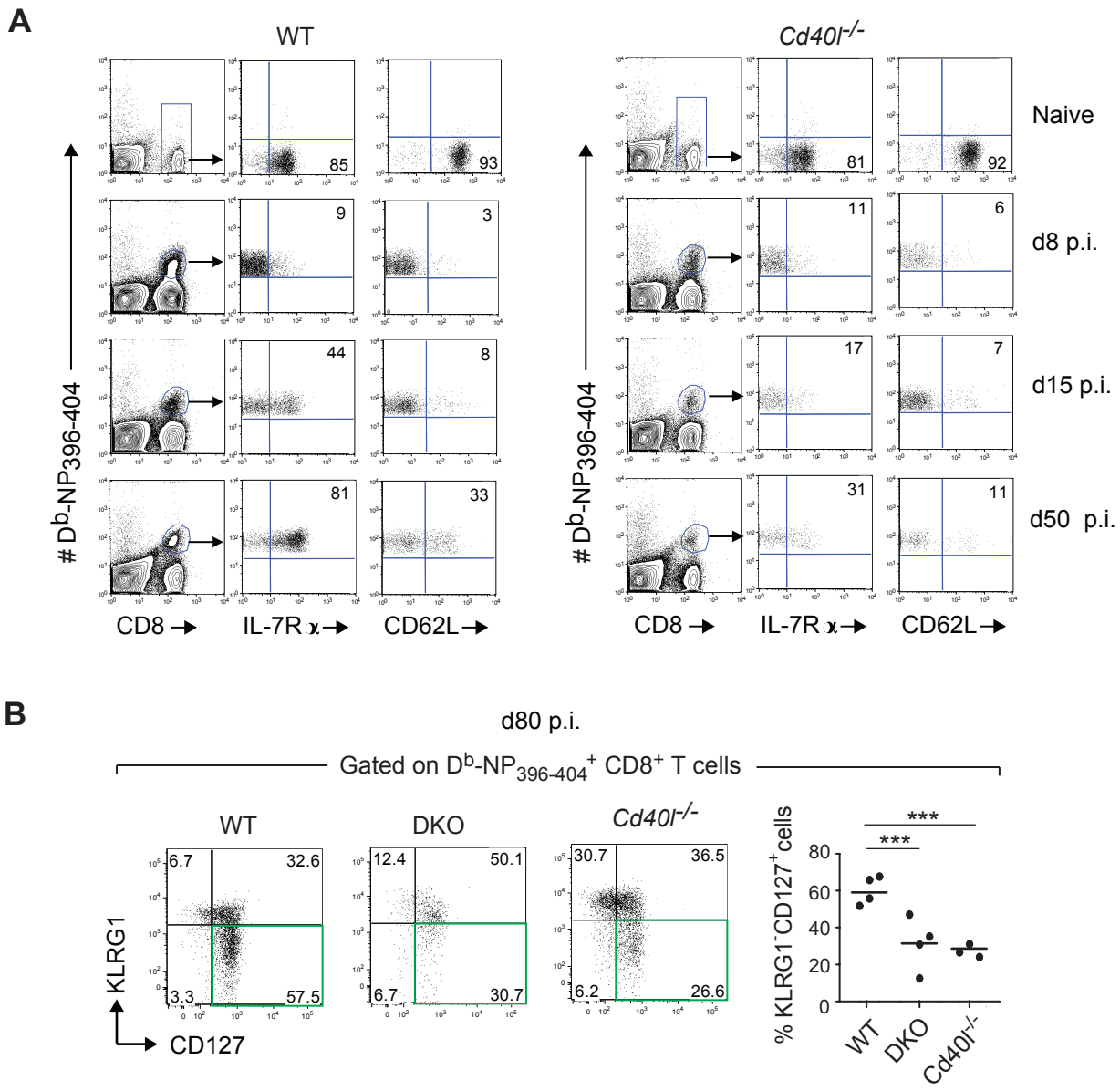
(**A-C**) WT (n=6) and *Stim1$^{fl/fl}$ Stim2$^{fl/fl}$ Cd4-Cre* (DKO, n=5) mice were infected with LCMV^{ARM} and at the same time injected (i.v.) with WT CD8$^+$ P14 cells that express a GP_{33-41} specific TCR. Mice were sacrificed 60 days p.i. and analyzed for viral titers in the serum as well as expression of exhaustion markers (PD-1, Tim-3) and IFNγ production by Thy1.1$^+$ P14 cells after restimulation with GP_{33-41} peptide for 6h in vitro. Each symbol represents one mouse. (**D–E**) Correlation coefficients were calculated for samples 60 days p.i. using Spearman's Rho test. * p<0.05; ** p<0.01. (**D**) Analysis including WT and DKO cohorts. (**E**) Analysis including only DKO samples. Correlation analyses were performed using primary data shown in **Figure 4E-L**.

Supplemental Figure 7



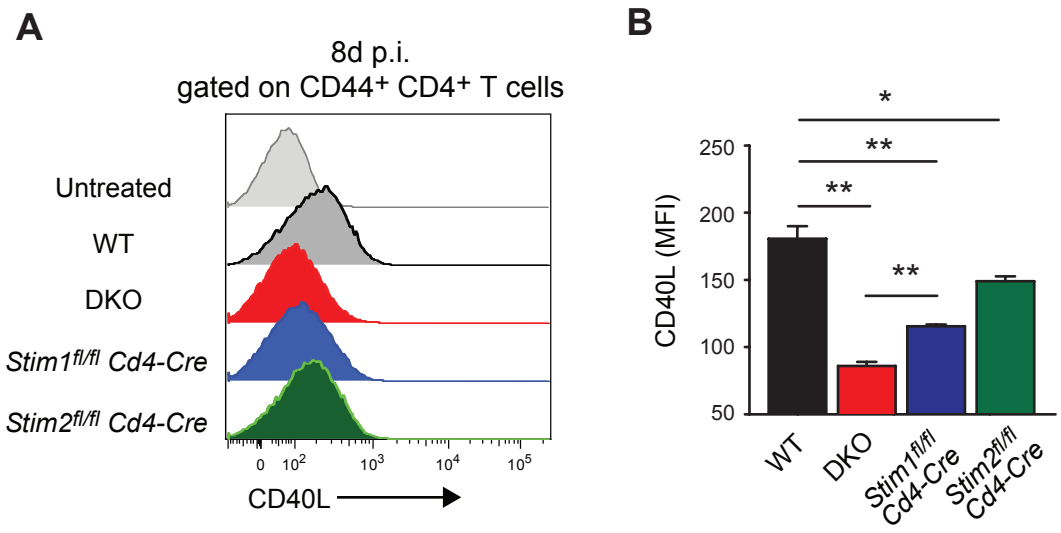
Supplemental Figure 7. $CD4^+$ T cells are required to maintain memory $CD8^+$ T cells but the development of LCMV-specific $CD4^+$ T cells is independent of STIM1 and STIM2.

(**A**) Impaired maintenance of LCMV-specific memory $CD8^+$ T cells in the absence of $CD4^+$ T cell help. WT and *Cd4*$^{-/-}$ mice were infected with 2 x 10^5 pfu of $LCMV^{ARM}$ (i.p.) and analyzed at the indicated time points post-infection (p.i.) for the frequencies of $D^bNP_{396-404}$ tetramer$^+$ $CD62L^+$ memory $CD8^+$ T cells. (**B,C**) WT and DKO mice were infected with 2 x 10^5 pfu of $LCMV^{ARM}$ and splenocytes were stained with I-A^b GP_{66-77} MHC class II tetramers and analyzed for the total number of LCMV specific $CD4^+$ T cells 8 days (**B**) and 35 days p.i. (**C**). Representative dot plots and the mean ± SEM absolute number of LCMV-specific $CD4^+$ T cells from 4 WT and 4 DKO mice per time point are shown. Controls included $CD4^+$ T cells from non-infected mice and $CD4^+$ T cells stained with APC-conjugated I-A^b tetramers loaded with CLIP peptide. Statistical significance was calculated by Student's t-test. n.s., non-significant. Numbers in FACS plots in A-C represent percentages of cells in gates.



Supplemental Figure 8. Impaired maintenance of virus-specific memory CD8$^+$ T cells in *Cd40l*$^{-/-}$ and DKO mice.

(**A)** WT and *Cd40l*$^{-/-}$ mice were infected with LCMV and analyzed at the indicated days post-infection (p.i.) for the frequencies of LCMV-specific ($D^bNP_{396-404}$ tetramer$^+$) memory cells expressing CD62L and IL-7Rα (CD127). (**B**) WT, *Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$ *Cd4-Cre* (DKO) and *Cd40l*$^{-/-}$ mice were infected i.p. with 2 x 10^5 pfu of LCMV^{ARM} and analyzed for memory CD8$^+$ T cell and antibody responses 80 days p.i. Plots show the expression of KLRG1 and CD127 on $D^bNP_{396-404}$ tetramer$^+$ CD8$^+$ T cells. Green boxes emphasize KLRG1$^-$ CD127$^+$ memory CD8$^+$ T cells. The scatter plot (right panel) shows frequencies of KLRG1$^-$ CD127$^+$ memory CD8$^+$ T cells from 3-4 mice per group. Horizontal lines represent mean percentages. Statistical significance was calculated using Student's t-test. ***, $p<0.001$. Numbers in FACS plots in A,B represent percentages of cells in gates.



Supplemental Figure 9. Partial decrease of CD40L expression in CD4$^+$ T cells lacking either STIM1 or STIM2.

WT, *Stim1$^{fl/fl}$ Cd4-Cre, Stim2$^{fl/fl}$ Cd4-Cre* and *Stim1$^{fl/fl}$Stim2$^{fl/fl}$ Cd4-Cre* (DKO) mice were infected with LCMV^{ARM} and sacrificed 8 days p.i. Total CD40L expression was measured by flow cytometry after restimulation of splenocytes with with LCMV GP_{61-80} peptide in the presence of anti-CD40L antibody LCMV for 2 h. Representative histogram plots (**A**) and mean ± SEM of the mean fluorescence intensity (MFI) of CD40L expression on CD4$^+$ CD44$^+$ T cells from 4 mice per group (**B**). Statistical significance was calculated using Student's t-test. * $p<0.05$; ** $p<0.01$.