SUPPLEMENTAL METHODS

Adoptive transfers

For P14 and STg adoptive transfer studies, $1\text{-}2\times10^3$ P14 or STg cells were transferred into recipient mice prior to infection, except in STg;10BiT *in vitro* peptide IL-10 induction experiments in which 10^4 cells were used. For polyclonal cell transfer experiments (Figure 7B), total splenocytes from a 10BiT mouse at day 8 p.i. with either LCMV-Arm or Cl.13 were transferred i.v. into an infection matched control.

Antibodies for Surface and Intracellular Staining

MHC I tetramers were generated as previously described (1) while the $GP_{66\text{-}77}$ MHC class II tetramer was obtained from the NIH tetramer core facility (Emory University, Atlanta, GA). Antibodies were purchased from eBioscience, BD Pharmingen or BioLegend. Cell types described in Figure 2 were defined as follows: Monocyte – $CD11b^+Gr\text{-}1^{int}SSC^{lo}$, Neutrophil – $CD11b^+Gr\text{-}1^{hi}SSC^{hi}$, B cell - $CD19^+$, NK cell – $CD3^-NK1.1^+$, $Foxp3^+$ Treg – $CD4^+Foxp3^+$. For DC staining, spleens were digested using collagenase and DNase prior to DC enrichment using CD11c microbeads (Miltenyi Biotec). DCs were defined as $NK1.1^-Thy1.2^-$ $CD19^-CD11c^{hi}MHCII^{hi}$ with pDCs defined as the $B220^+$ cells within this population, while cDCs were defined as the $B220^-$ DCs. Flow cytometry data was acquired on a BD LSRII with Diva software (BD Pharmingen) and analysed with FlowJo software (Treestar).

Retroviral knock-down

Knock-down efficiency was validated by quantitative RT-PCR of RV GFP+ cells sorted from P14 cells that were cultured in 10ng/ml peptide and 10ng/ml IL-2 for 24 hours, spin transduced, then expanded for another 2 – 4 days. Thymic retroviral knock-down was performed as described previously (2) with some modifications. Briefly, thymocytes isolated from $Ly5.1^+$ STg;10BiT mice were transduced by spin transduction in IL-7 and expanded in IL-7 overnight. RV

GFP+ thymocytes were isolated the following day by cell sorting and 1×10^5 RV GFP+ thymocytes were intrathymically injected into each recipient B6 mouse. Two weeks after intrathymic injection, mice were infected with LCMV-Cl.13 and Thy1.1 levels assessed within the expanded Ly5.1$^+$ RV GFP$^+$ STg;10BiT cells. Intrathymic injection of 1×10^5 RV GFP+ thymocytes yielded a STg response at day 8 p.i. that was equivalent to, or diminished, relative to a typical CD4$^+$GP66$^+$ response (typically ~1-5% of the total CD4$^+$ T cells). As in the original study (2), T cell differentiation in this system was indistinguishable from a normal STg response.

Cell isolation and stimulation

For *in vitro* IL-10 induction, cells were stimulated with 1µg/ml GP$_{66-77}$ peptide for 8 hours. For blocking antibody and inhibitor treatment, cells were pre-incubated with antibodies/inhibitors for 5 min prior to peptide addition, with antibodies/inhibitors left in the culture for the duration of the stimulation. Antibodies and inhibitors were used at the following concentrations: 1µg/ml IL-2 blocking antibody (Clone JES6-1A12), 10µg/ml ICOS blocking antibody (Clone 7E.17G9), 37µM PD98059, 1µM Cyclosporine A (CsA), 50µM LY294002, 5µM Triciribine, 10µM BAY-11-7082 and 20µM SP600125. For *in vivo* peptide injections, mice were given 50µg GP$_{66-77}$ i.v. 12 hours prior to analysis.

Statistical analyses

All graphing and statistical analyses were performed using the Prism graphing program (Version 5.0, GraphPad software). P values were calculated using a 2-tailed unpaired T test or a 2-tailed Mann–Whitney test when data failed normality tests. For multiple comparisons, a One-way ANOVA with a Tukey post test was used. For Figure 8, p values were calculated by paired T tests of the untreated control to treated cell values.

SUPPLEMENTAL REFERENCES

1.   Kaech, S.M., Tan, J.T., Wherry, E.J., Konieczny, B.T., Surh, C.D., and Ahmed, R. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4:1191-1198.

2.   Gerlach, C., van Heijst, J.W., Swart, E., Sie, D., Armstrong, N., Kerkhoven, R.M., Zehn, D., Bevan, M.J., Schepers, K., and Schumacher, T.N. 2010. One naive T cell, multiple fates in CD8+ T cell differentiation. *The Journal of experimental medicine* 207:1235-1246.

| Gene	Primer sequence (5' to 3')	
	Forward	**Reverse**
Rpl9	TGAAGAAATCTGTGGGTCG	GCACTACGGACATAGGAACT
Maf	CTGCCACAATCAAGCCTACA	TGGATGTCATGCGCTAAGTC
Prdm1	ACAGAGGCCGAGTTTGAAGA	GGCATTCTTGGGAACTGTGT
Il21	AGTTCTGGTGGCATGGAGAG	AAAACAGGCAAAAGCTGCAT
Il10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG
Nfil3	ATGGGGAAGACGAACAACAG	TTCCACCACACCTGTTTTGA
Ikzf1	CCAATGTGCTCATGGTTCAC	TCTTCTGCCATCTCGTTGTG
Rbpj	ATTTCAGGCCACTCCATGTC	CATCCCCAAACCACACTCTT
Bcl6	CCTGAGGGAAGGCAATATCA	CGGCTGTTCAGGAACTCTTC

**Supplemental Table 1. Primer sequences used for quantitative RT-PCR.**
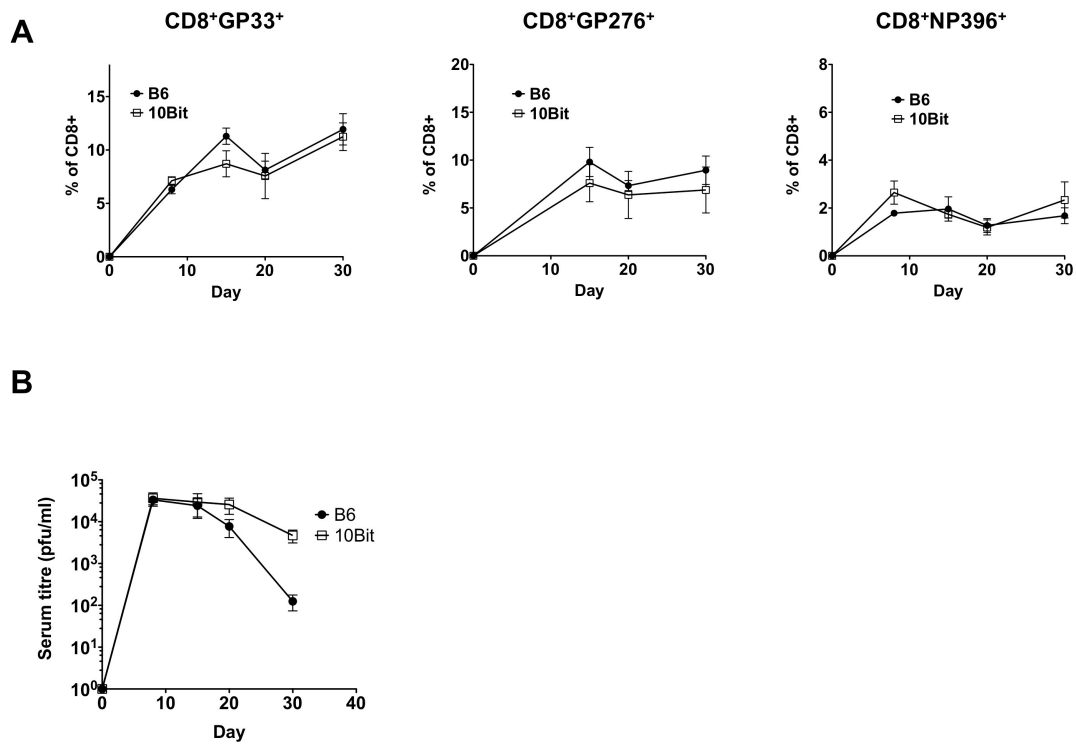
Supplemental Figure 1. Virus-specific T cell responses and serum viral titres in B6 vs 10BiT mice. B6 and 10BiT mice were infected with LCMV-Cl.13 and the virus-specific CD8$^+$GP33$^+$, CD8$^+$GP276$^+$ and CD8$^+$NP396$^+$ tetramer levels (A) and serum viral titres (B) were examined in the blood at the indicated time points. Pooled data from 2-3 experiments with 5-11 mice per time-point is shown.
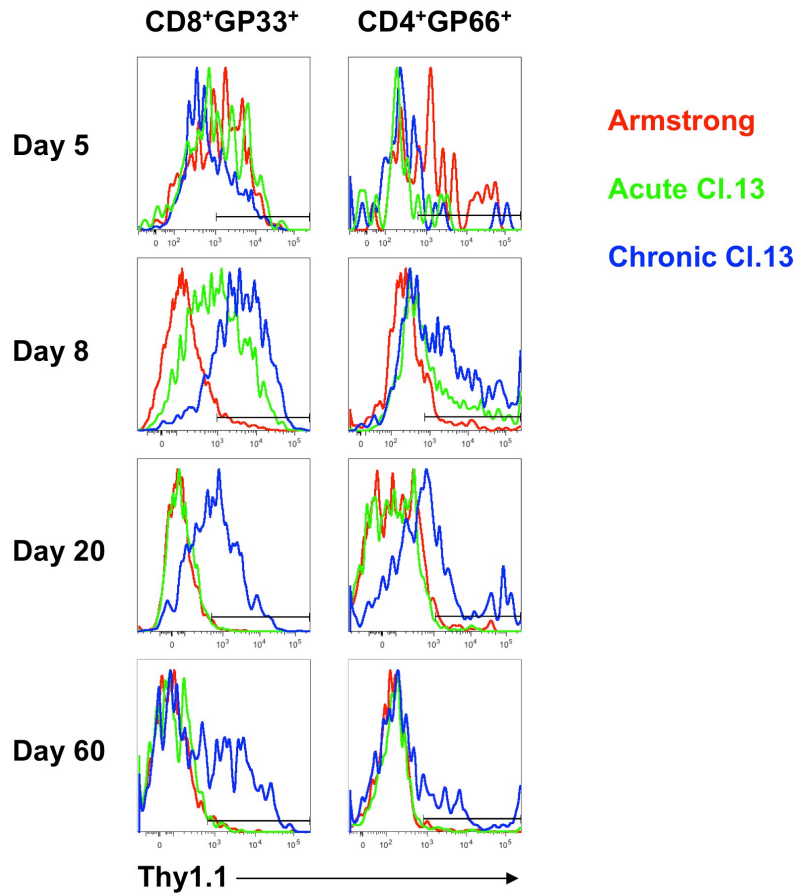
Supplemental Figure 2. IL-10 expression by virus-specific T cells over the course of acute and chronic LCMV infection. 10BiT mice were infected with either LCMV-Arm (Armstrong), Acute Cl.13 or Chronic Cl.13 as in Figure 1. Thy1.1 expression levels were then examined in tetramer stained splenic CD8$^+$GP33$^+$ or CD4$^+$GP66$^+$ T cells. Representative profiles from the graphs in Fig. 3A are shown. The gates shown were set using 10BiT transgene negative cells.

A

CD8+NP396+

Thy1.1 ⟶

CD8+GP276+

Thy1.1 ⟶

Armstrong

Chronic Cl.13

B
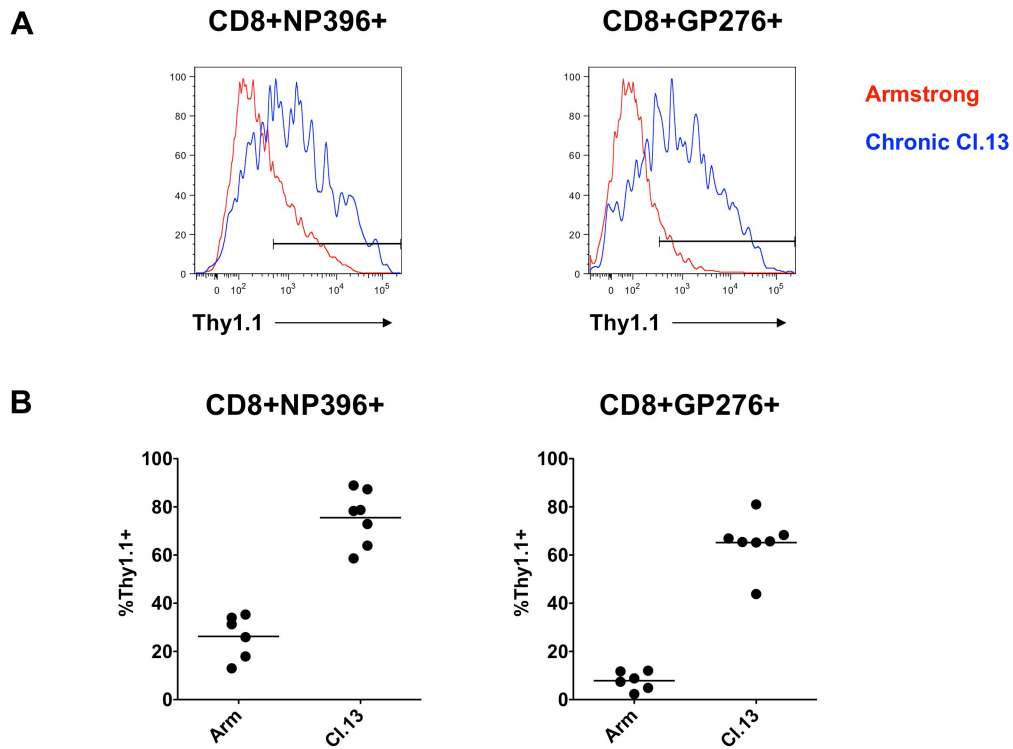
CD8+NP396+

CD8+GP276+

Supplemental Figure 3. IL-10 expression by CD8$^+$NP396$^+$ and CD8$^+$GP276$^+$ cells at day 8 p.i. 10BiT mice were infected with either LCMV-Arm (Armstrong) or Chronic Cl.13 as in Figure 1. Thy1.1 expression levels were then examined in tetramer stained splenic CD8$^+$NP396$^+$ or CD8$^+$GP276$^+$ T cells. Representative Thy1.1 profiles are shown in (A) while pooled data is graphed in (B). Pooled data from three independent experiments is shown.

A

CD8⁺GP33⁺

CD8⁺NP396⁺

CD4⁺GP66⁺

B

Liver

Kidney
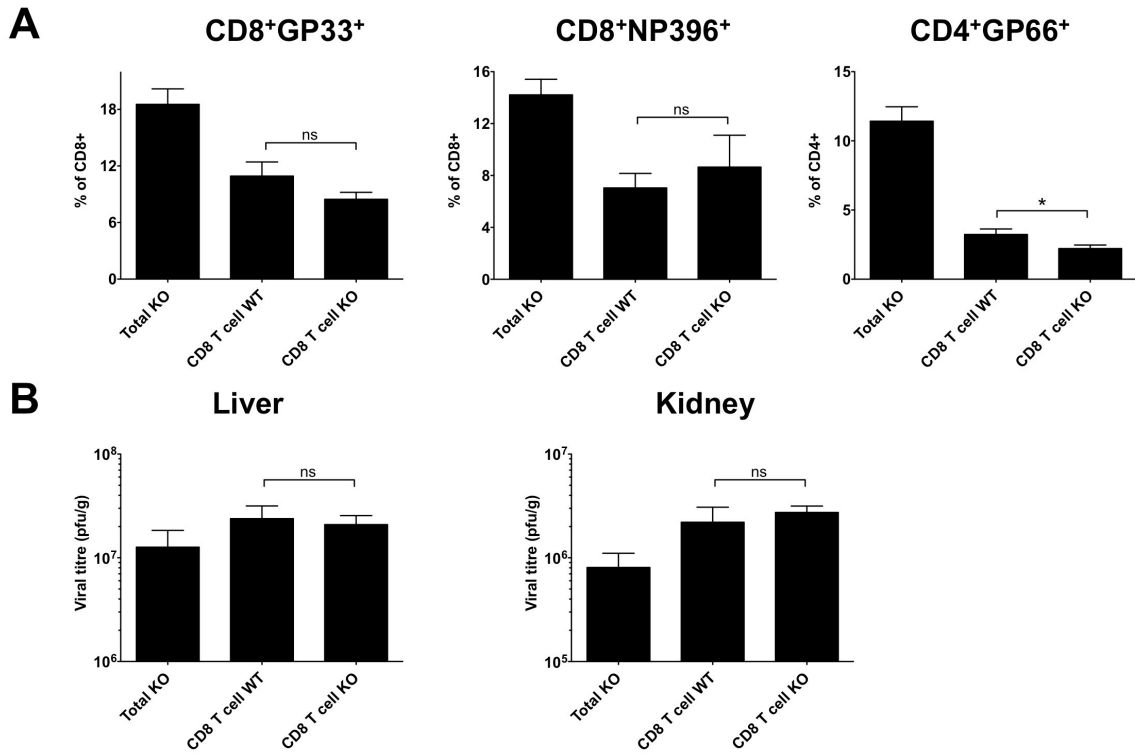
Supplemental Figure 4. IL-10 loss from CD8⁺ T cells has no effect on T cell responses or viral clearance. Irradiation BM chimeras were generated with either 100% IL-10$^{-/-}$ BM (Total KO), 75% CD8$\alpha^{-/-}$ + 25% B6 BM (CD8 T cell WT) or 75% CD8$\alpha^{-/-}$ + 25% IL-10$^{-/-}$ BM (CD8 T cell KO). BM chimeras were infected with 2×10⁶ pfu LCMV-Cl.13 and at day 8 p.i. the T cell response and viral load was measured. (A) Bar graphs show the percentage of IFN-γ producing CD8⁺ T cells in response to either GP33 (CD8⁺GP33⁺) or NP396 (CD8⁺NP396⁺) peptide stimulation (left two panels) or the percentage of IFN-γ producing CD4⁺ T cells in response to GP66 peptide stimulation (CD4⁺GP66⁺, right panel). (B) Viral titres in the liver (left) or kidney (right). Data are pooled from two independent experiments with 6–7 mice per group. All error bars depict SEM. * indicates p<0.05, ns indicates p>0.05.
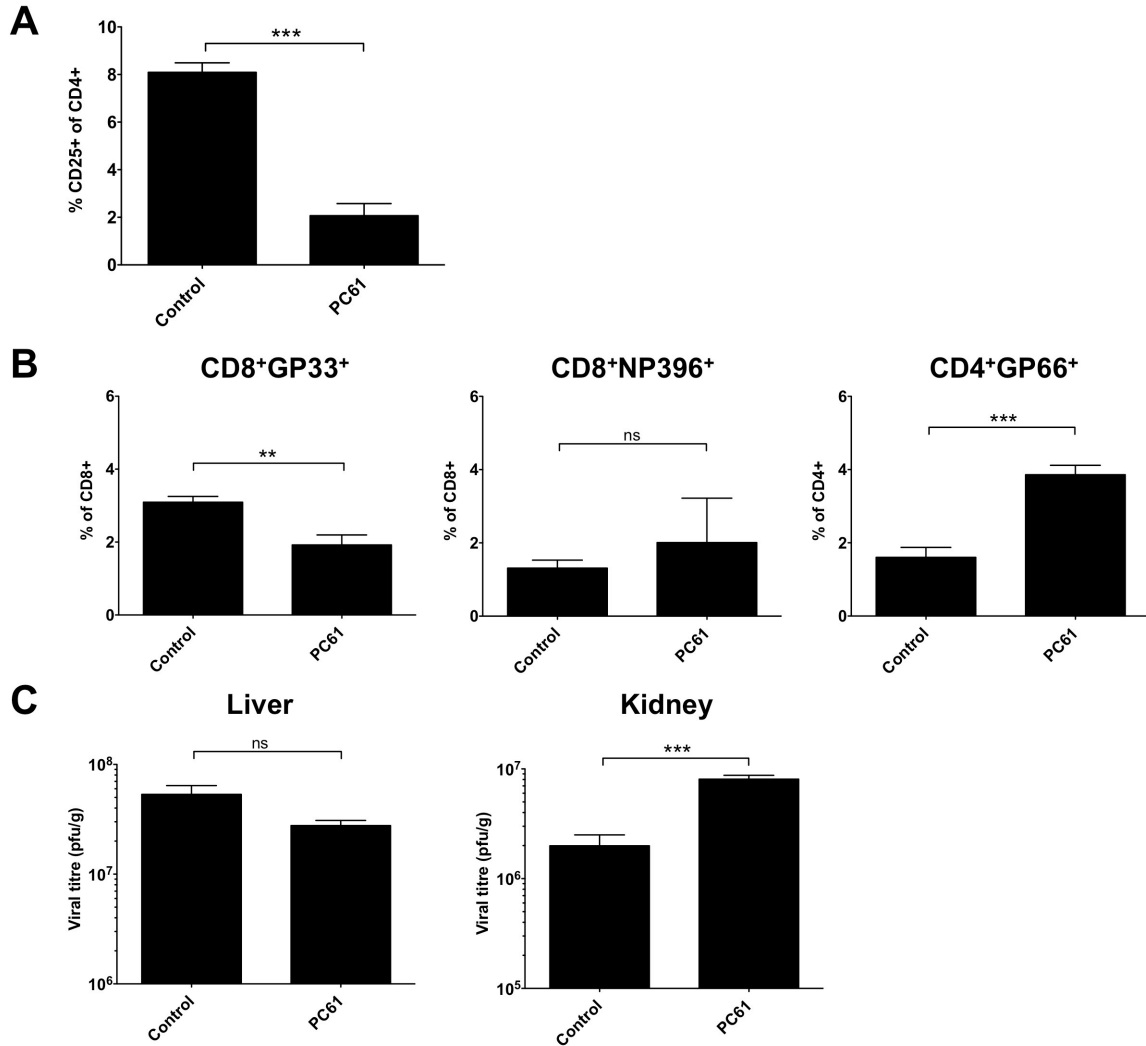
Supplemental Figure 5. Treg depletion has no effect on viral clearance. B6 mice were treated with 500µg of either control Ig or anti-CD25 (PC61) 3 days before infection with LCMV-Cl.13. At day 8 p.i. the T cell response and viral load was measured. (A) Graph showing splenic Treg depletion at day 8 p.i. (B) Bar graphs show the percentage of IFN-γ producing CD8$^+$ T cells in response to either GP33 (CD8$^+$GP33$^+$) or NP396 (CD8$^+$NP396$^+$) peptide stimulation (left two panels) or the percentage of IFN-γ producing CD4$^+$ T cells in response to GP66 peptide stimulation (CD4$^+$GP66$^+$, right panel). (C) Viral titres in the liver (left) or kidney (right). Data are pooled from two independent experiments with 6 mice per group. All error bars depict SEM. ** indicates $p<0.01$, *** indicates $p<0.001$, ns indicates $p>0.05$.

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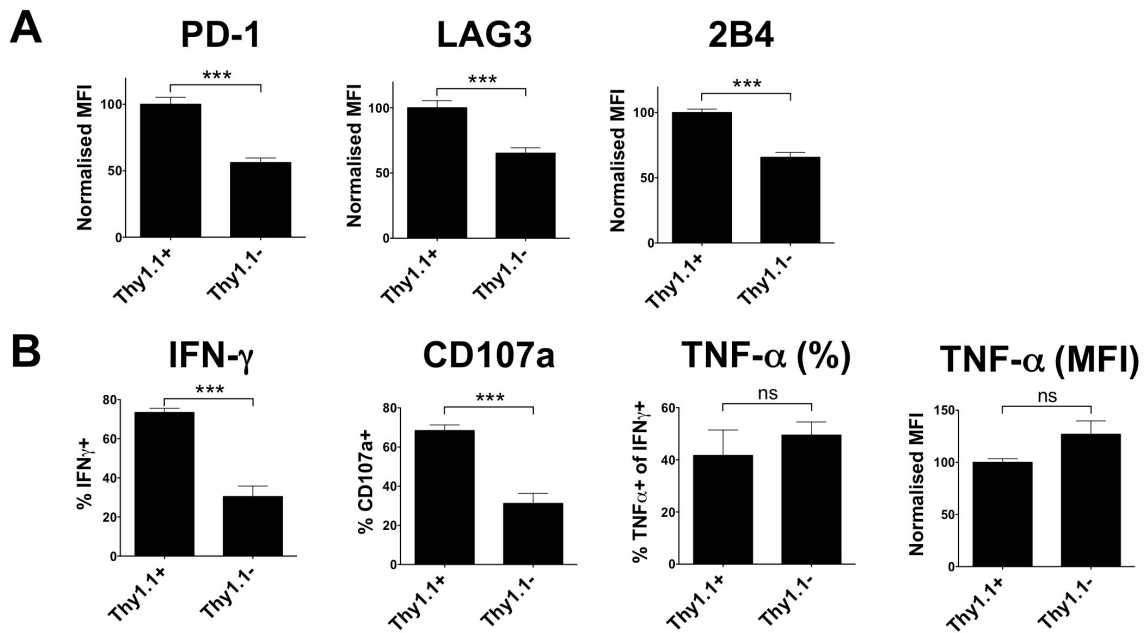
Supplemental Figure 6. Phenotype of IL-10 producing effector CD8$^+$ T cells. Quantitation of the markers shown in Figure 5A, B. (A) The MFI of PD-1, LAG-3 and 2B4 was calculated in the Thy1.1$^+$ and Thy1.1$^-$ splenic CD8$^+$GP33$^+$ tetramer stained cells. (B) (Left two panels) The percentage of IFN-γ^+ and CD107a$^+$ cells was calculated within the Thy1.1$^+$ and Thy1.1$^-$ cells of the total CD8$^+$ cell population after α-CD3 stimulation. (Right two panels) The proportion of TNF-α^+ cells and the TNF-α MFI was also calculated within the Thy1.1$^+$ and Thy1.1$^-$ cells of the IFN-γ^+CD8$^+$ cell population generated by α-CD3 stimulation. *** indicates p<0.001, ns indicates p>0.05. For normalised MFI values, data within each experiment was normalised to the average MFI of the Thy1.1$^+$ cell group.
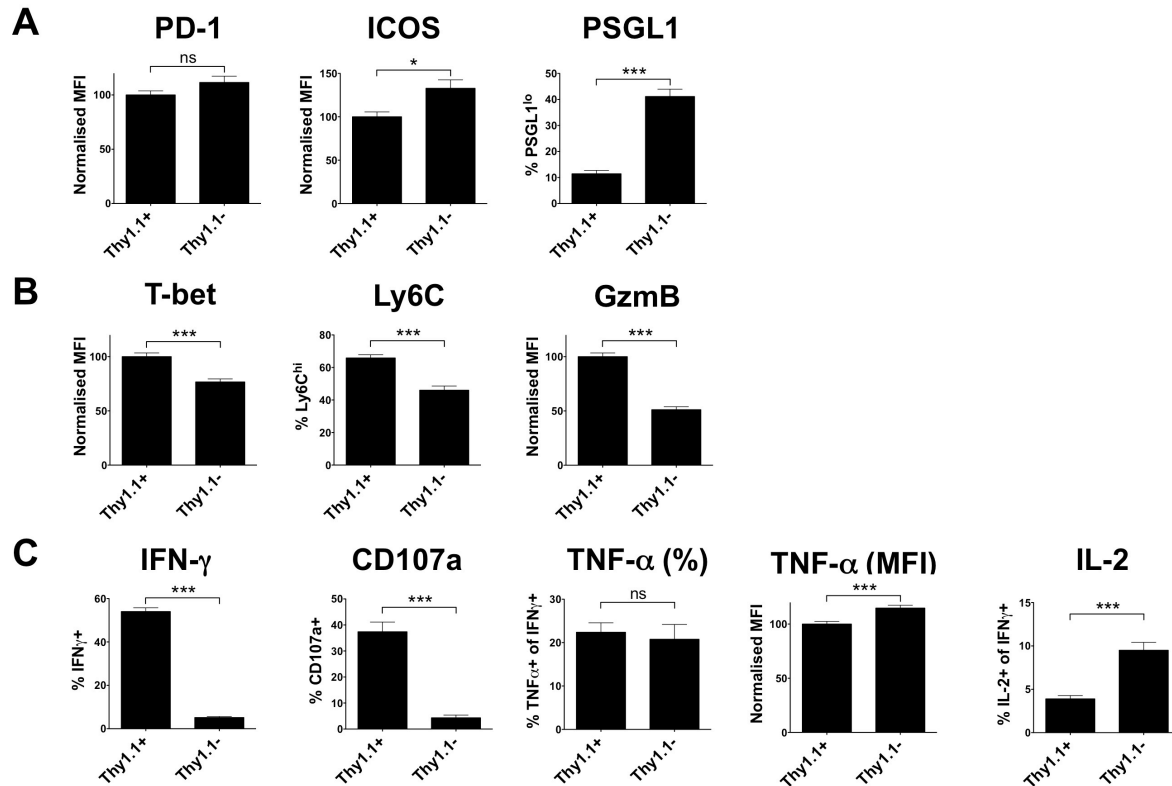
Supplemental Figure 7. Phenotype of IL-10 producing effector CD4$^+$ T cells. Quantitation of the markers shown in Figures 5C, E. (A) The MFI of the Tfh markers PD-1 and ICOS, and the % PSGL1^{lo} cells, was calculated in the Thy1.1$^+$ and Thy1.1$^-$ splenic CD4$^+$GP66$^+$ tetramer stained cells. (B) The MFI of the Th1 markers T-bet and GzmB, and the % Ly6C^{hi} cells, was calculated in the Thy1.1$^+$ and Thy1.1$^-$ splenic CD4$^+$GP66$^+$ tetramer stained cells. (C) (Left two panels) The percentage of IFN-γ^+ and CD107a$^+$ cells was calculated within the Thy1.1$^+$ and Thy1.1$^-$ cells of the total CD4$^+$ cell population after α-CD3 stimulation. (Right three panels) The proportion of TNF-α^+ or IL-2$^+$ cells and the TNF-α MFI was also calculated within the Thy1.1$^+$ and Thy1.1$^-$ cells of the IFN-γ^+CD4$^+$ cell population generated by α-CD3 stimulation. *** indicates p<0.001, * indicates p<0.05, ns indicates p>0.05. For normalised MFI values, data within each experiment was normalised to the average MFI of the Thy1.1$^+$ cell group.
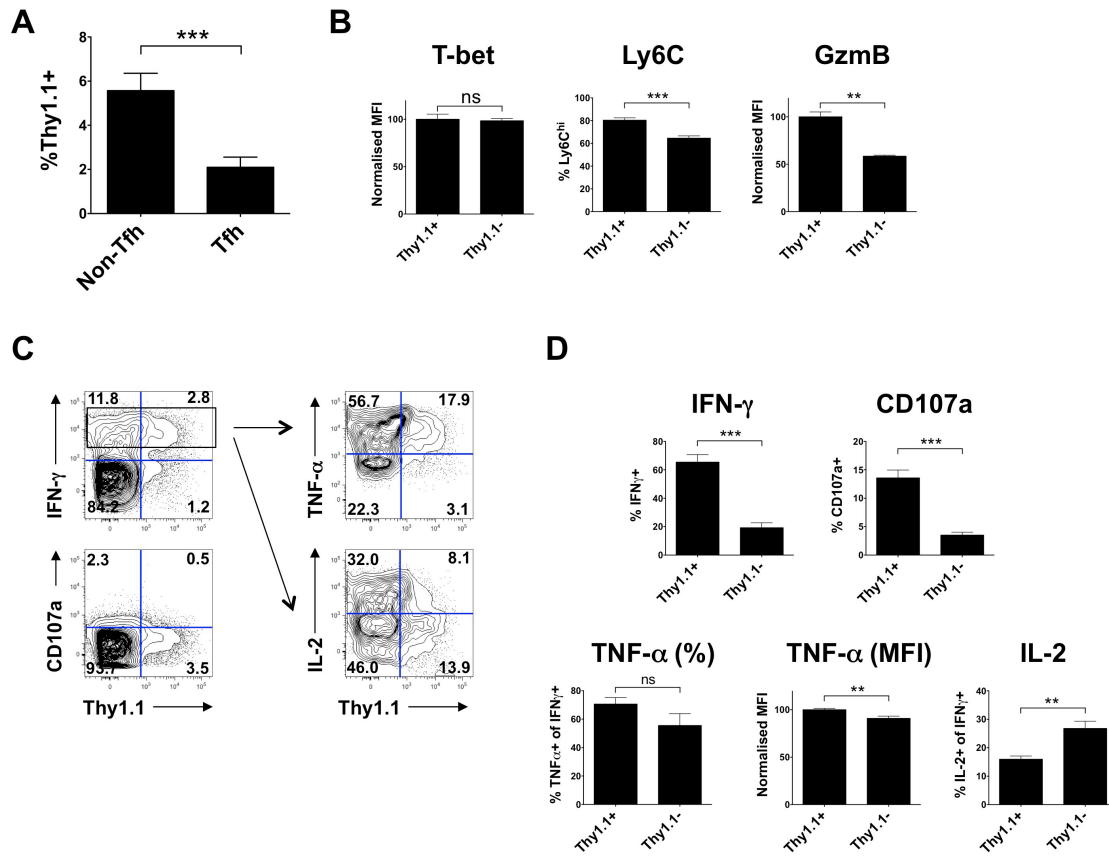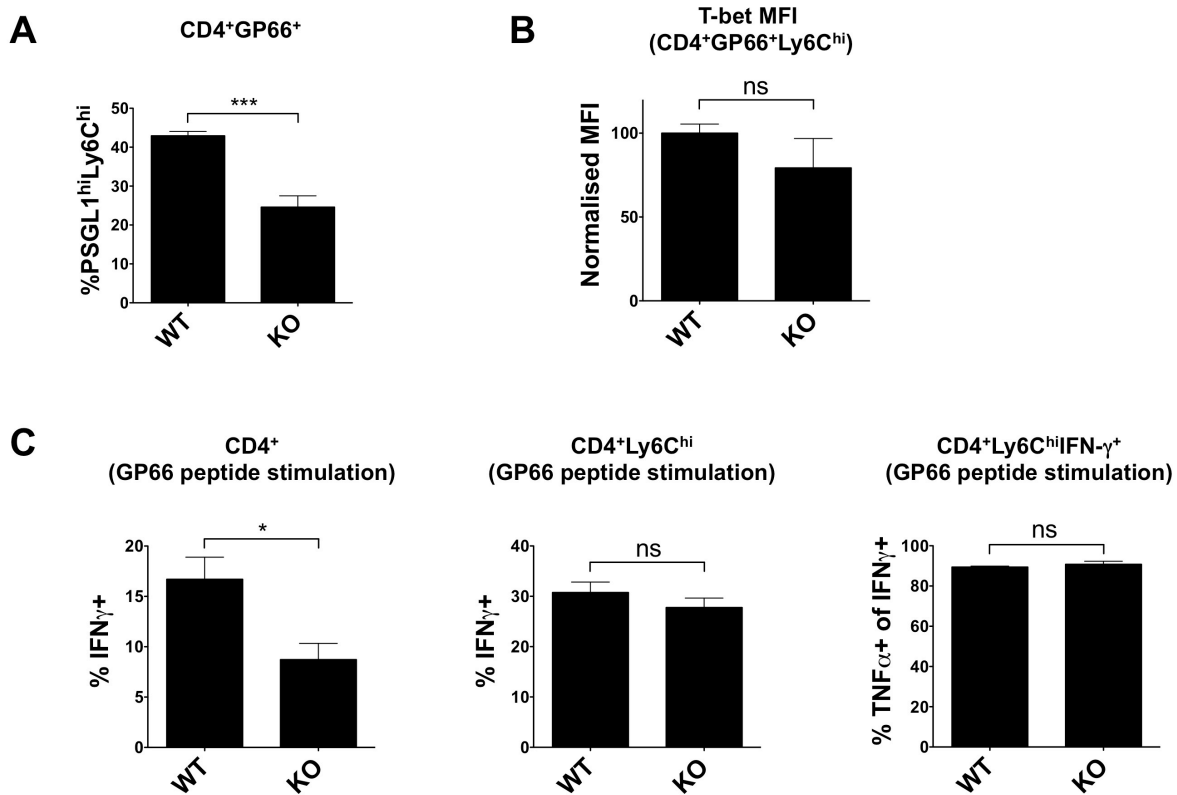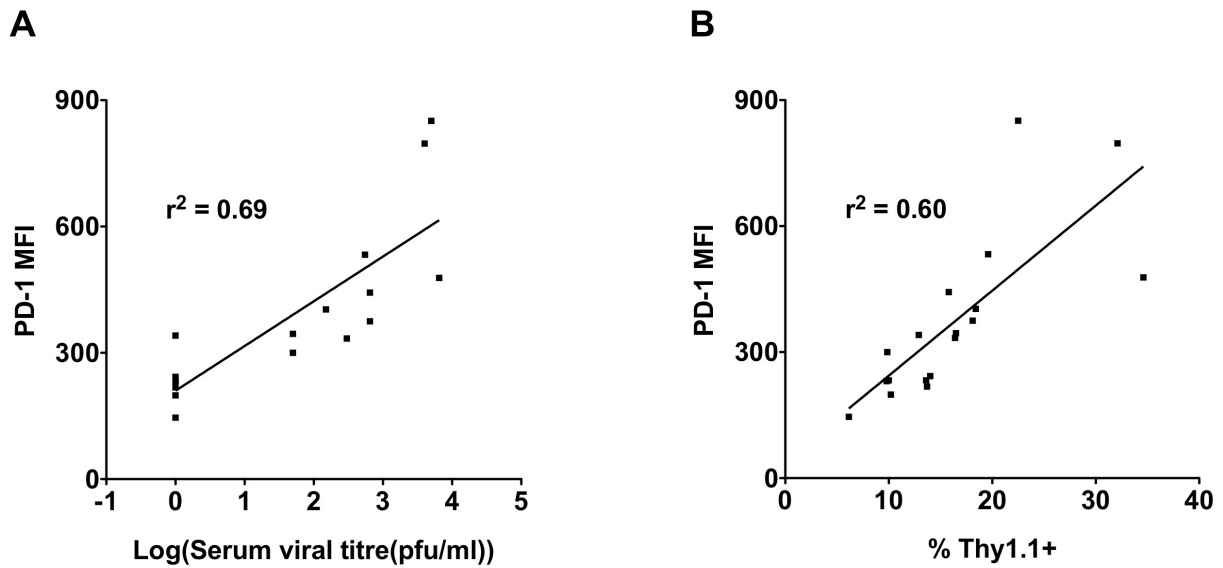
Supplemental Figure 8. Phenotype of IL-10 producing effector CD4$^-$ T cells during acute LCMV infection. (A) 10BiT mice were infected with 2×10^5 pfu LCMV-Arm and the proportion of Thy1.1$^+$ cells within the CD4$^+$GP66$^+$ tetramer-stained non-Tfh (PSGL1^{hi}CXCR5^{lo}) and Tfh (PSGL1^{lo}CXCR5^{hi}) populations was determined at day 8 p.i. Gating was as shown in Figure 5D. Data are pooled from 4 independent experiments (n=10 mice). (B) The MFI of the Th1 markers T-bet and GzmB, and the % Ly6C^{hi} cells, was calculated in the Thy1.1$^+$ and Thy1.1$^-$ splenic CD4$^+$GP66$^+$ tetramer stained 10BiT cells at day 8 p.i. with LCMV-Cl.13. Data are from 2-4 independent experiments (n=5-10 mice). (C) 10BiT mice were infected with 2×10^5 pfu LCMV-Arm and at day 8 p.i. the cells were polyclonally stimulated with plate-bound α-CD3 in the presence of Brefeldin A. Contour plots showing IFNγ and CD107a production (left) are gated on the total CD4$^+$ T cell population and those showing TNF-α and IL-2 production (right) are gated on CD4$^+$IFN-γ$^+$ cells. Data are representative of 2-4 independent experiments (n=6-11 mice total). (D) Quantitation of the data shown in (C). (Top two panels) The percentage of IFN-γ$^+$ and CD107a$^+$ cells was calculated within the Thy1.1$^+$ and Thy1.1$^-$ cells of the total CD4$^+$ cell population after α-CD3 stimulation. (Bottom three panels) The proportion of TNF-α$^+$ or IL-2$^+$ cells and the TNF-α MFI was also calculated within the Thy1.1$^+$ and Thy1.1$^-$ cells of the IFN-γ$^+$CD4$^+$ cell population generated by α-CD3 stimulation. *** indicates p<0.001, ** indicates p<0.01, ns indicates p>0.05. For normalised MFI values, data within each experiment was normalised to the average MFI of the Thy1.1$^+$ cell group.

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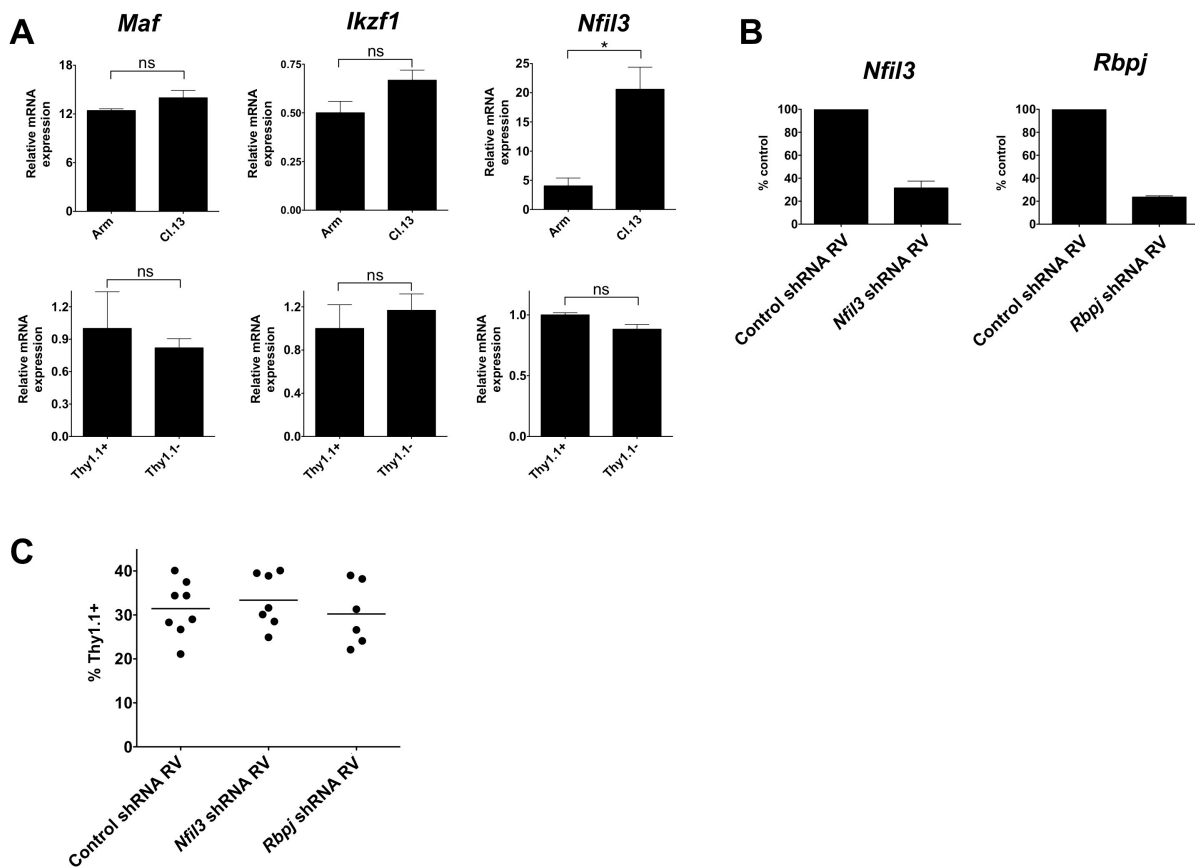
Supplemental Figure 9. CD4⁺GP66⁺ cell IL-10 production is independent of NFIL3 and Notch. (A) (Top graphs) Congenically marked Ly5.1⁺ STg cells were transferred into B6 mice subsequently infected with either LCMV-Arm or 2×10⁶ pfu LCMV-Cl.13. At day 8 p.i. the Ly5.1⁺ STg cells were isolated by cell sorting. (Bottom graphs) Ly5.1⁺ STg;10BiT cells were transferred into B6 hosts, infected with 2×10⁶ pfu LCMV-Cl.13 and sorted in Thy1.1⁺ and Thy1.1⁻ subsets at day 8 p.i. The amount of *Maf, Ikzf1* and *Nfil3* mRNA was assessed by quantitative RT-PCR in these sorted cell populations. * indicates p<0.05, ns indicates p>0.05. Pooled data from three independent experiments is shown. (B) P14 cells were activated *in vitro* with peptide and IL-2, spin transduced with either *Nfil3* or *Rbpj* shRNA RV 24 hours later, then expanded in peptide and IL-2 for a further 2 – 4 days. RV GFP+ cells were then sorted and *Nfil3* or *Rbpj* mRNA levels assessed by quantitative RT-PCR. Pooled data from two to three independent experiments is shown. (C) Ly5.1⁺ STg;10BiT thymocytes were transduced with shRNA retroviruses that express either a control (scrambled) shRNA or that target *Nfil3* or *Rbpj*. Transduced thymocytes were intrathymically injected into recipient mice that were infected with 2×10⁶ pfu LCMV-Cl.13 two weeks later. At day 8 p.i. the frequency of Thy1.1⁺ cells within the transduced Ly5.1⁺ STg;10BiT cells was determined. Pooled data from two independent experiments is shown. All error bars depict SEM.

A

B

Supplemental Figure 10. Blimp-YFP^{hi} and Blimp-YFP^{lo} expression marks Th1 cells and Tfhs. (A) 10BiT;Blimp-YFP mice were infected with 2×10^6 pfu LCMV-Cl.13 and at day 8 p.i. the Blimp-YFP phenotype of the CD4$^+$GP66$^+$ tetramer stained cells was examined against PSGL1, Ly6C and ICOS expression. Representative data is shown from 4 independent experiments with a total of 11 mice. (B) Blimp-YFP mice were infected with either 2×10^5 pfu LCMV-Arm or 2×10^6 pfu LCMV-Cl.13, and at day 8 p.i. the percentage of Blimp-YFP^{lo} Tfh were measured. Pooled data from two independent experiments are shown. *** indicates p<0.001.
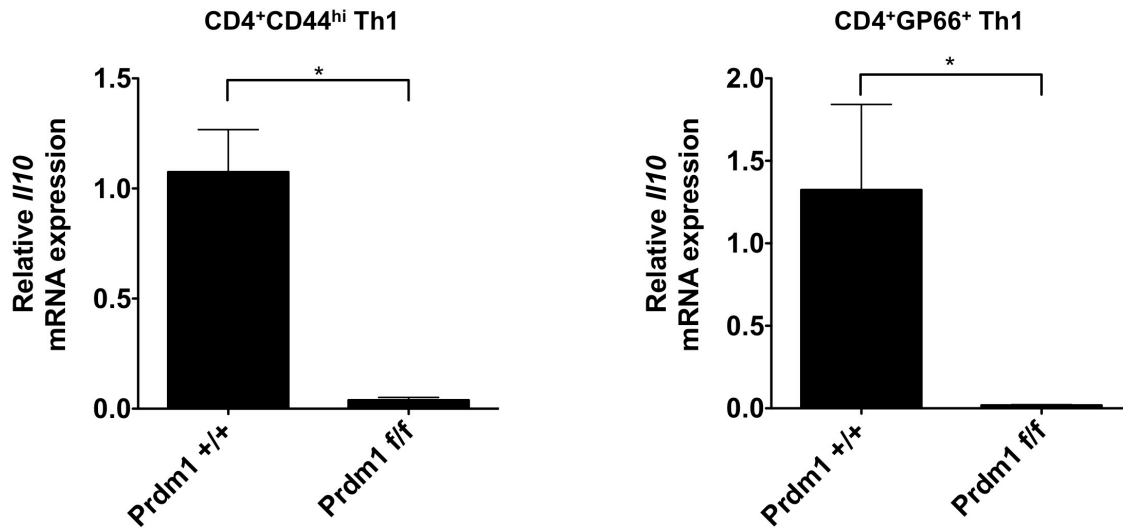
Supplemental Figure 11. Th1 cell differentiation in CD4-cre[+];Prdm1[f/f] mice during LCMV-Cl.13 infection. CD4-cre[+];Prdm1[f/f] (KO) mice or wild-type controls (WT; CD4-cre- or Prdm1^{+/+}) were infected with 2×10^6 pfu LCMV-Cl.13 and the phenotype of the Th1 cells was assessed at day 8 p.i. (A) The percentage of PSGL1^{hi}Ly6C^{hi} Th1 cells within the total CD4^{+}GP66^{+} tetramer^{+} population of WT and KO animals. (B) T-bet MFI within the CD4^{+}GP66^{+}Ly6C^{hi} tetramer^{+} Th1 cells of WT and KO animals. MFI values were normalised to the average of WT mice within each experiment. (C) Splenocytes were stimulated with GP66 peptide in the presence of Brefeldin A to analyse Th1 cytokine production. Graphs show the % of IFN-γ^+ cells within the total CD4^{+} cell population (left) and the CD4^{+}Ly6C^{hi} population (middle). Right graph depicts the % of TNF-α^+ cells within the CD4^{+}Ly6C^{hi}IFN-γ^+ Th1 cells. *** indicates p<0.001, * indicates p<0.05, ns indicates p>0.05. Pooled data from n=3-8 mice per group is shown.

A

PD-1 MFI (y-axis), Log(Serum viral titre(pfu/ml)) (x-axis)

$r^2 = 0.69$

**B**

PD-1 MFI (y-axis), % Thy1.1+ (x-axis)

$r^2 = 0.60$

Supplemental Figure 12. Correlation between CD8$^+$GP33$^+$ cell exhaustion and serum viral titre or CD4$^+$GP66$^+$ IL-10 production at day 60 p.i. Correlation between CD8$^+$GP33$^+$ tetramer$^+$ cell PD-1 MFI and either serum viral titre (A) or % Thy1.1$^+$ cells within the CD4$^+$GP66$^+$ tetramer$^+$ cell population (B). Data are from 10BiT mice at day 60 p.i. with LCMV-Cl.13 from the experiment depicted in Figure 7D. Each point represents an individual mouse.
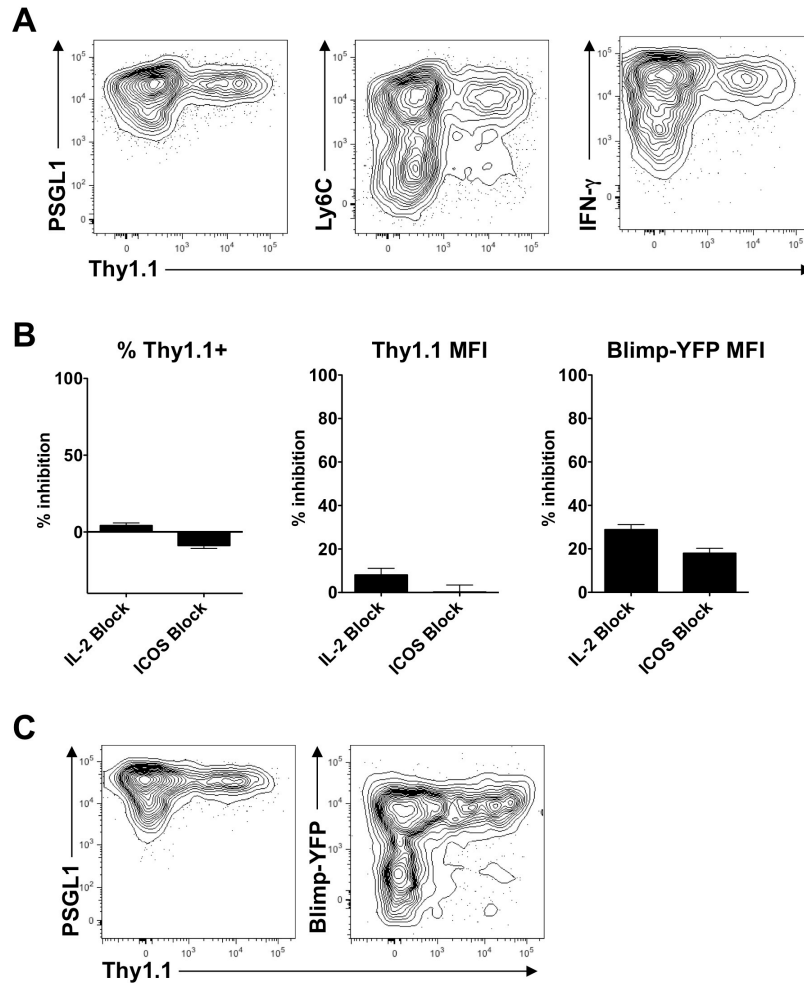
Supplemental Figure 13. TCR engagement converts CD4$^+$GP66$^+$ Th1 cells from acute viral infection into Blimp^{hi} IL-10 producers. (A) Phenotype of peptide stimulated cells shown in Figure 7A, B. Note, for the IFN-γ stain, cells were stained intracellularly for both IFN-γ and Thy1.1 as Brefeldin A inhibits cell surface Thy1.1 expression. (B) STg;10Bit;Blimp-YFP cells were isolated after LCMV-Arm infection and peptide stimulated as in Figure 7A, B. Cells were treated with IL-2 or ICOS Blocking antibody during peptide induction of Thy1.1 and Blimp-YFP. Left graph shows inhibition of the peptide-induced increase in % Thy1.1$^+$ cells, middle graph shows inhibition of peptide-mediated Thy1.1 MFI induction within Thy1.1$^+$ cells and right graph shows inhibition of peptide-mediated Blimp-YFP MFI induction within YFP^{hi} cells. Pooled data from 3-5 independent experiments with 11-21 mice per group is shown. (C) Phenotype of Thy1.1 expressing CD4$^+$GP66$^+$ cells shown in Figure 7C. All error bars depict SEM.

Supplemental Figure 14. Stimulation induced IL-10 production in Th1 cells from acute infection is Blimp-1 dependent. CD4-cre[+];Prdm1[f/f] (KO) mice or wild-type controls (WT; CD4-cre- or Prdm1^{+/+}) were infected with 2×10^5 pfu LCMV-Arm. At day 8 p.i., either total polyclonal (CD44^{hi}) or antigen-specific (GP66 tetramer+) CD4^{+} Th1 (Ly6C^{hi}PSGL1^{hi}) cells were sorted and restimulated for 4 hours with PMA and Ionomycin, and the amount of *Il10* mRNA was assessed by quantitative RT-PCR. * indicates p<0.05. Data are pooled from 3-5 independent sorts. Error bars depict SEM.
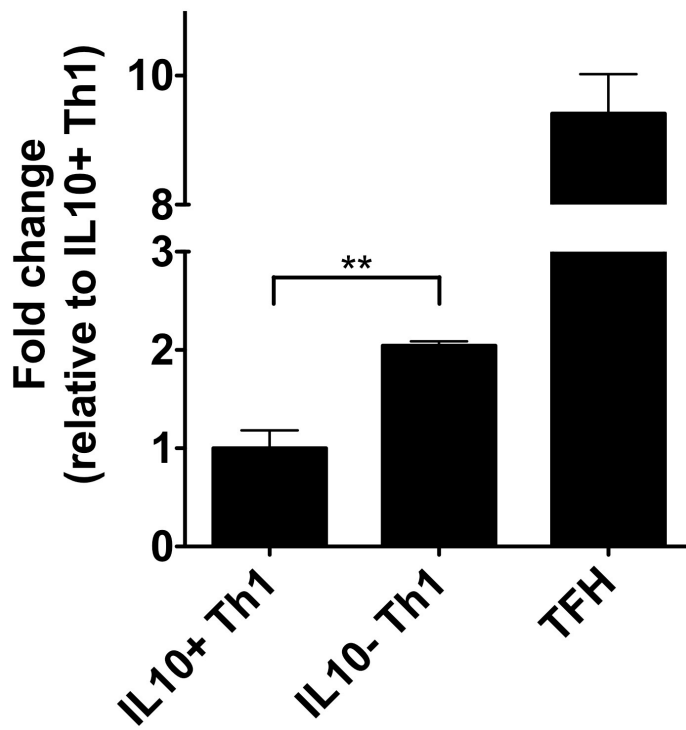
Supplemental Figure 15. *Bcl6* **mRNA expression within IL-10$^+$ and IL-10$^-$ Th1 cells.** Congenically marked Ly5.1$^+$ STg;10BiT cells were transferred into B6 mice subsequently infected with 2×10^6 pfu LCMV-Cl.13. Donor PSGL1^{hi}Thy1.1$^+$ (IL10+ Th1), PSGL1^{hi}Thy1.1$^-$ (IL10- Th1) and PSGL1^{lo}(Thy1.1$^-$) (TFH) STg;10BiT cells were purified by FACS at day 8 p.i. and the amount of *Bcl6* mRNA was assessed by quantitative RT-PCR. ** indicates p<0.01. Data are pooled from three independent experiments. Error bars depict SEM.