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Supporting Online Material for

IL-21 Is Required to Control Chronic Viral Infection

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MATERIALS AND METHODS

Mice and virus: C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or the rodent breeding colony at The Scripps Research Institute. IL-21R$^{-/-}$ mice were initially provided by Warren Leonard at the National Institutes of Health (S*1*). The LCMV-GP$_{61-80}$-specific CD4^{+} TcR transgenic (SMARTA) mice and LCMV-GP$_{33-41}$-specific CD8^{+} TCR transgenic (P14) mice have been described previously (*2, 3*). All mice were housed under specific pathogen-free conditions. Mouse handling conformed to the requirements of the National Institutes of Health, The Scripps Research Institute Animal Research Committee and the University of California, Los Angeles Animal Research Committee guidelines. In all experiments the mice were infected intravenously with 2x10^{6} plaque forming units (PFU) of LCMV-Arm or LCMV-Cl 13. Virus stocks were prepared and viral titers were quantified as described (*4*). To deplete CD4 T cells for the day 9 IL-21 mRNA analysis, mice were treated with 500 µg anti-CD4 antibody (clone GK1.5) 1 day prior to infection. For the day 25 analysis, CD4^{+} T cells were depleted from total splenocytes ex vivo by positive selection using anti-CD4 magnetic beads (Miltenyi). In both experimental protocols CD4 depletion was confirmed by flow cytometry and was greater than >99%.

Isolation and adoptive transfer of virus-specific CD4^{+} (SMARTA) and CD8^{+} (P14) T cells: To specifically identify and isolate virus-specific CD4^{+} T cells, Thy1.1^{+} CD4^{+} T cells were purified from the spleens of naïve SMARTA mice by negative selection (StemCell Technologies). Following purification 5000 cells were co-transferred i.v. into Thy1.2^{+} C57BL/6 mice. P14 CD8^{+} T cells were similarly isolated from wt (GFP^{+}) P14 mice or IL-21R$^{-/-}$ (Thy1.1^{-}/Thy1.2^{+}) P14 mice. 1000 wt P14 or IL-21R$^{-/-}$ P14 cells were then transferred into separate IL-21R$^{+/+}$ mice (Thy1.1^{+} /Thy1.2^{+}). wt P14 cells were identified based on GFP expression and IL-21R$^{-/-}$ P14 cells were identified based on being Thy1.2^{+} and Thy1.1^{-}. To obtain IL-21R$^{-/-}$ P14^{+} mice and the IL-21R$^{+/+}$ mice used for recipients of these transgenic P14 cells, wt C57BL/6 P14^{+} IL-21R$^{+/+}$ (Thy1.1^{+} /Thy1.2^{-}) mice were crossed with IL-21R$^{-/-}$ (Thy1.1^{-} /Thy1.2^{+}) mice (that had been back crossed six generations with C57BL/6 mice) to generate (F1) P14^{+} IL-21R$^{+/-}$ mice and P14^{-} IL-21R$^{+/-}$ mice (the wt P14 mice are maintained as heterozygous breedings as a result yielding both P14^{+} and P14^{-} mice in this F1). Next, we used the F1 pups generated from the initial cross. The (F1) P14^{+} IL-21R$^{+/-}$ mice were crossed with the (F1) P14^{-} IL-21R$^{+/-}$ mice. This cross generated P14^{+} IL-21R$^{+/+}$, P14^{+} IL-21R$^{+/-}$, P14^{+} IL-21R$^{-/-}$ and P14^{-} IL-21R$^{+/+}$, P14^{-} IL-21R$^{+/-}$, P14^{-} IL-21R$^{-/-}$ mice. The P14^{+} IL-21R$^{-/-}$ mice were the source of cells for adoptive transfer. Finally, to obtain more P14^{-} IL-21R$^{+/+}$ mice for transfer recipients we crossed the (F2) P14^{-} IL-21R$^{+/-}$ mice with sibling (F2) P14^{-} IL-21R$^{+/-}$ mice. This cross generated the P14^{-} IL-21R$^{+/+}$ mice used for adoptive transfer recipients. The transfers enabled identification and isolation of LCMV-specific CD4 and CD8 T cells separately from endogenous responses. We avoided the problems associated with using large, non-physiologic numbers of transferred transgenic T cells (*5*) by only transferring low numbers of transgenic T cells. These wt transgenic T cells behave similarly to their endogenous (i.e., host derived) T cell counterparts, based on tetramer analysis and intracellular cytokine staining [(*6, 7*) and data not shown]. Mice were then infected with LCMV-Arm or LCMV-Cl 13 one to two days after cell transfer.

Bone-marrow chimera experiments: To perform mixed BM chimera experiments, wt CD45.2^{+} C57BL/6 recipient mice were lethally irradiated with 1100 rads to kill immune and stem cells. That same day irradiated mice were reconstituted with a mixture of BM cells from CD45.1^{+} wt (IL-21R$^{+/+}$) mice and CD45.2^{+} IL-21R$^{-/-}$ mice. Bone-marrow cells were isolated from femurs and tibia of donor mice and 20 million total cells were transferred into the irradiated recipient mice, i.v. Recipient mice were treated with antibiotics (Trimethoprim-Sulfa, supplied in the drinking water at Trimethoprim= 40mg per 5 ml water and Sulfamethoxazole= 200 mg per 5 ml water) for three weeks to prevent infection and allow immune reconstitution. Reconstitution was analyzed eight weeks after bone-marrow transfer and was determined to be 60:40 wt to IL-21R$^{-/-}$ bone-marrow derived CD8^{+} cells. Mice were then infected with LCMV.

Quantitative RT-PCR: RNA from total splenocytes or purified cell populations was isolated with the RNeasy extraction kit (Qiagen). Immune cell subsets were FACS sorted based on staining with subset specific antibodies: CD4 T cells (CD4+, CD8-), virus-specific CD4 T cells (CD4+, Ly5.1+), NK T cells (CD45+, NK1.1+, CD3+), DC ($CD45^+$, $CD3^-$, $NK1.1^-$, $CD11c^+$), B cells ($CD45^+$, $CD3^-$, $NK1.1^-$, $CD11c^-$, $B220^+$), and macrophages ($CD45^+$, $CD3^-$, $NK1.1^-$, $CD11c^-$, $CD11b^+$). DNase-treated, RNA was amplified using the Qiagen One-step RT-PCR kit (Qiagen) using the manufacturers protocol. In initial experiments *IL-21* RNA expression was normalized to β-actin. Primer/probe pairs: IL-21 Fwd: 5'-AgAgggAAATCgTgCgTgAC-3'; IL-21 Rev: 5'-gCATTCgTgAgCgTCTATAgTgTC-3'; IL-21 Probe: 5'-TTCCCgAggACTgAggAgACgCC-3'; β-actin Fwd: 5'-AgAgggAAATCgTgCgTgAC-3', β-actin Rev: 5'-CAATAgTgATgACCTggCCgT-3'; β-actin Probe: 5'-CTgCgCATCCTCTTCCTCCC-3'. In other experiments *IL-21* RNA expression was normalized to HPRT using the Assays-on-Demand IL-21 and HPRT expression kit (Applied Biosystems). Amplifications were performed on the ABI7700 (Applied Biosystems) or the BioRad IQ5 (BioRad).

Intracellular cytokine analysis and flow cytometry: Splenocytes were stained directly ex vivo with LCMV-GP_{33-41} or LCMV-GP_{61-80} tetramers and for surface expression of CD8, CD4 and CD44. To analyze cytokine expression, splenocytes were stimulated for 5 hours with 5 μg/ml of the MHC class II restricted LCMV-GP_{61-80} or 2 μg/ml of the MHC class I restricted LCMV-GP_{33-41} peptide (all >99% pure; Synpep,) in the presence of 50 U/ml recombinant murine IL-2 (R&D Systems) and 1mg/ml brefeldin A (Sigma). Cells were stained for surface expression of CD4 (Pharmingen) and CD8 (eBioscience), then fixed, permeabilized and stained with antibodies to TNFα, IFNγ and IL-2 (Pharmingen) and/or IL-21R-Fc chimera (R&D Systems) followed by staining with PE-conjugated goat anti-human Fcγ antibody (Jackson ImmunoResearch Laboratories) as described (*8*). Splenic DC (CD45+, CD3-, NK1.1-, CD11c+), B cells (CD45+, CD3-, NK1.1-, CD11c-, B220+) and macrophages (CD45+, CD3-, NK1.1-, CD11c-, CD11b+) were simultaneously stained with antibodies to MHC class II (IA^b) and B7-2. Flow cytometric analysis was performed using the Digital LSR II (Becton Dickinson). Flow cytometric analysis was performed using the Digital LSR II (Becton Dickinson). The absolute number of virus-specific T cells was determined by multiplying the frequency of $tetramer^+$ or $cytokine^+$ cells by the total number of cells in the spleen.

LCMV-specific antibody ELISA and neutralizing antibody determination: Serum LCMV-specific IgG titers were determined by ELISA as previously described (*9*). LCMV-neutralizing antibody titers were determined by incubating 100 microliters of undiluted, heat-inactivated serum (30 minutes at 56^0C) with 200 PFU LCMV-Cl 13 (in 2 microliters) for 1 hour at 37^0C. 25 and 50 PFU equivalents were then plated for plaque assay. No plaques were observed in any of the samples when heat-inactivated serum was assayed alone.

Statistical Analysis: Student's *t*-tests and one-way ANOVA were performed using the SigmaStat/Plot 11.0 software (Systat Software Inc.). One-way ANOVA was used for direct comparison of three groups.

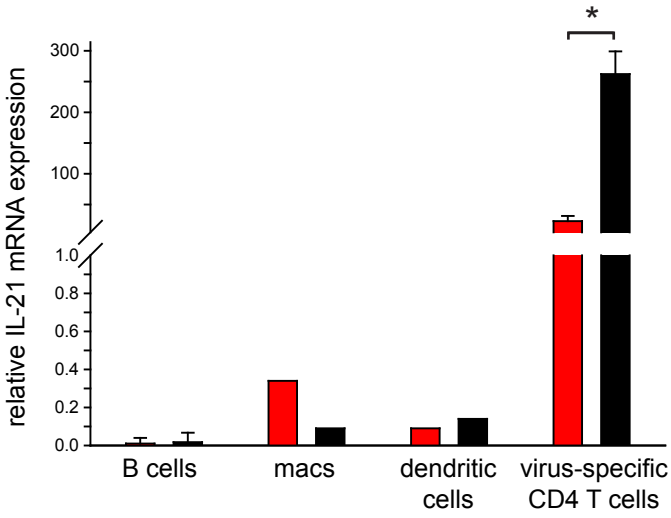

Fig. S1. IL-21 mRNA expression by antigen-presenting cells. Bar graphs indicate IL-21 mRNA expression in the indicated sorted cell population. Red Bars: cells sorted from LCMV-Arm infected animals; black bars: cells sorted from LCMV-Cl 13 infected animals. The level of IL-21 mRNA expression in virus-specific $CD4^+$ T cells is included for comparison to Figure 1B. Bar graphs represent the average ± SD of 4 mice per group and two independent experiments. *, $P < 0.05$.

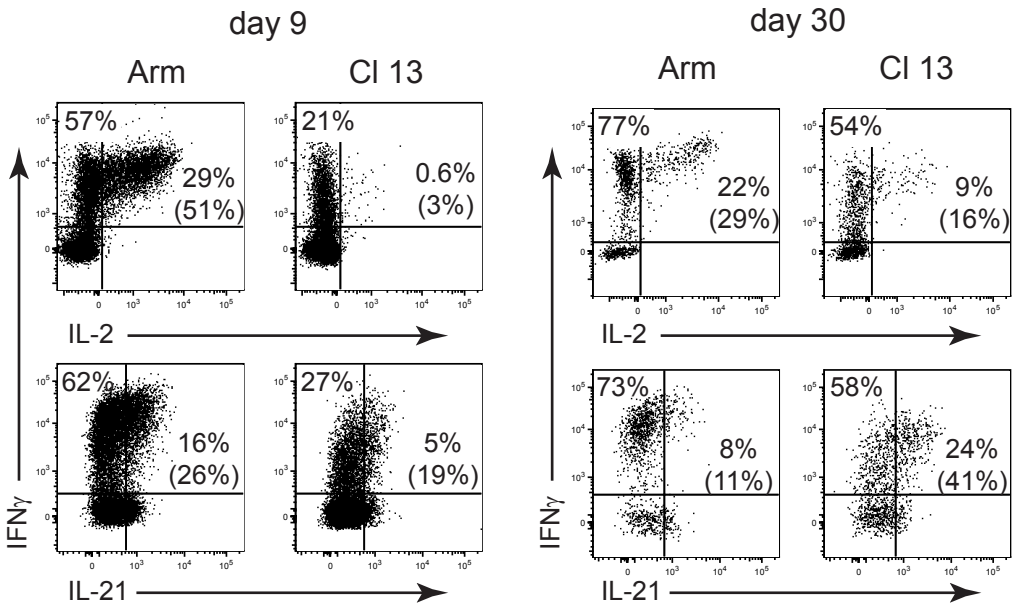

Fig. S2. IL-21 expression by $CD4^+$ T cells is increased during chronic virus infection. Flow plots are gated on virus-specific (SMARTA) $CD4^+$ T cells on day nine and thirty following LCMV-Arm or LCMV-Cl 13 infection. The number in the upper left quadrant of each plot indicates the total percentage of SMARTA cells that produced IFN-γ (y-axis) in response to LCMV-GP_{61-80} peptide stimulation. The top number in the right quadrant represents the total frequency of IL-2 (top plots) or IL-21 (bottom plots) producing SMARTA cells. The number in parenthesis indicates the -percentage of SMARTA cells that responded to stimulation (i.e., IFN-γ+) and simultaneously produced IL-2 or IL-21.

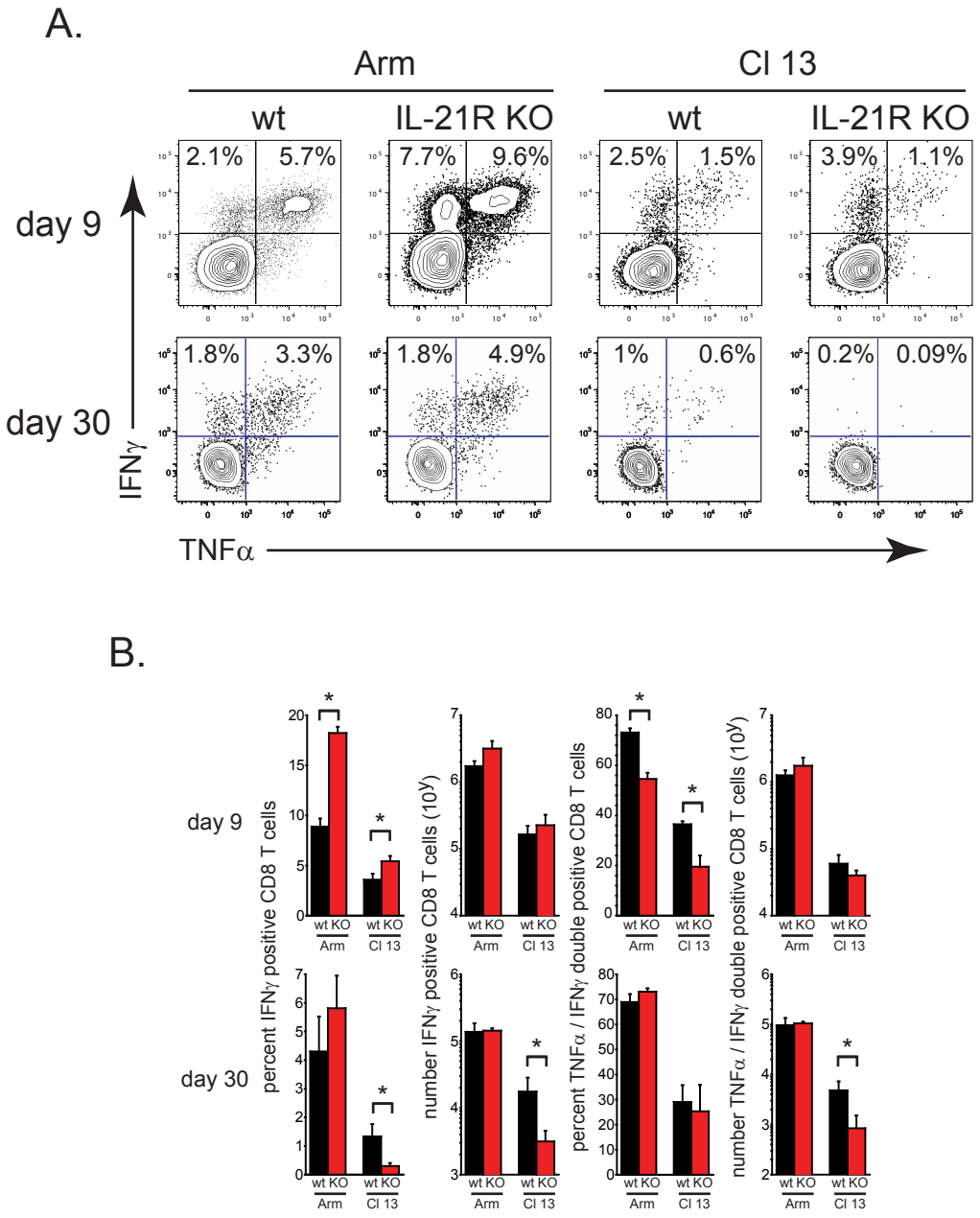

Fig. S3. Reduced virus-specific CD8$^+$ T cell function in the absence of IL-21R expression. **(A)** LCMV-GP$_{33-41}$ specific CD8$^+$ T cell responses were quantified on day nine and thirty after LCMV-Arm (left plots) or LCMV-Cl 13 (right plots) infection of wt or IL-21R$^{-/-}$ mice. Flow plots illustrate the frequency of IFN-γ and/or TNF-α producing CD8 T cells. **(B)** Bar graphs indicate the frequency of IFN-γ positive, the number of IFN-γ positive, the frequency of IFN-γ/TNF-α double positive and the number of IFN-γ/TNF-α double positive CD8 T cells from LCMV-Arm (left bars in each graph) and LCMV-Cl 13 (right bars in each graph) infected wt (black bars) or IL-21R$^{-/-}$ mice. Bar graphs represent the average ± SD. N=4 mice per group and represent 3 independent experiments. *, P <0.05.

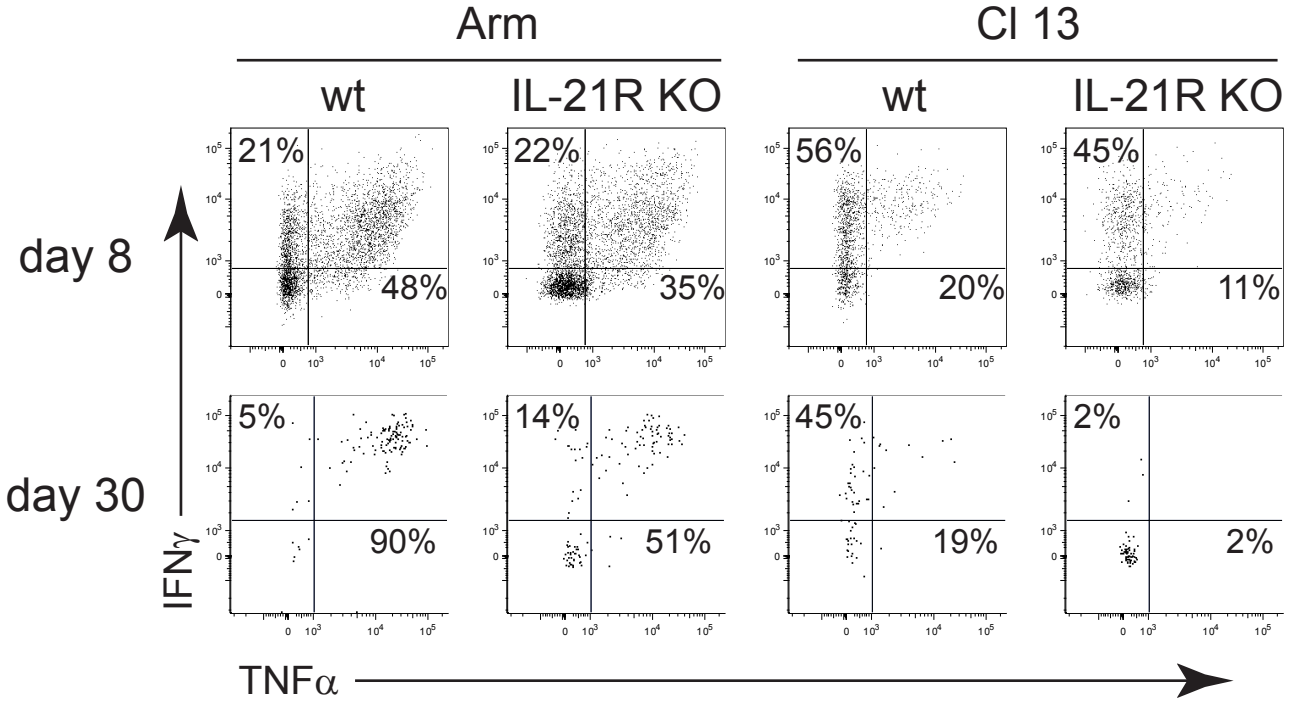

Fig. S4. IL-21 directly sustains virus-specific CD8$^+$ T cell function. wt or IL-21R$^{-/-}$ LCMV-specific TCR transgenic P14 CD8$^+$ T cells were transferred into IL-21R$^{+/+}$ mice and their functional responses were quantified on day eight and thirty following LCMV-Arm or LCMV-Cl 13 infection. Flow plots are gated on P14 T cells and the numbers in each quadrant illustrate the frequency of IFN-γ and IFN-γ/TNF-α dual producing P14 T cells. N=3-4 mice per group and represent 2 independent experiments. *, P <0.05.

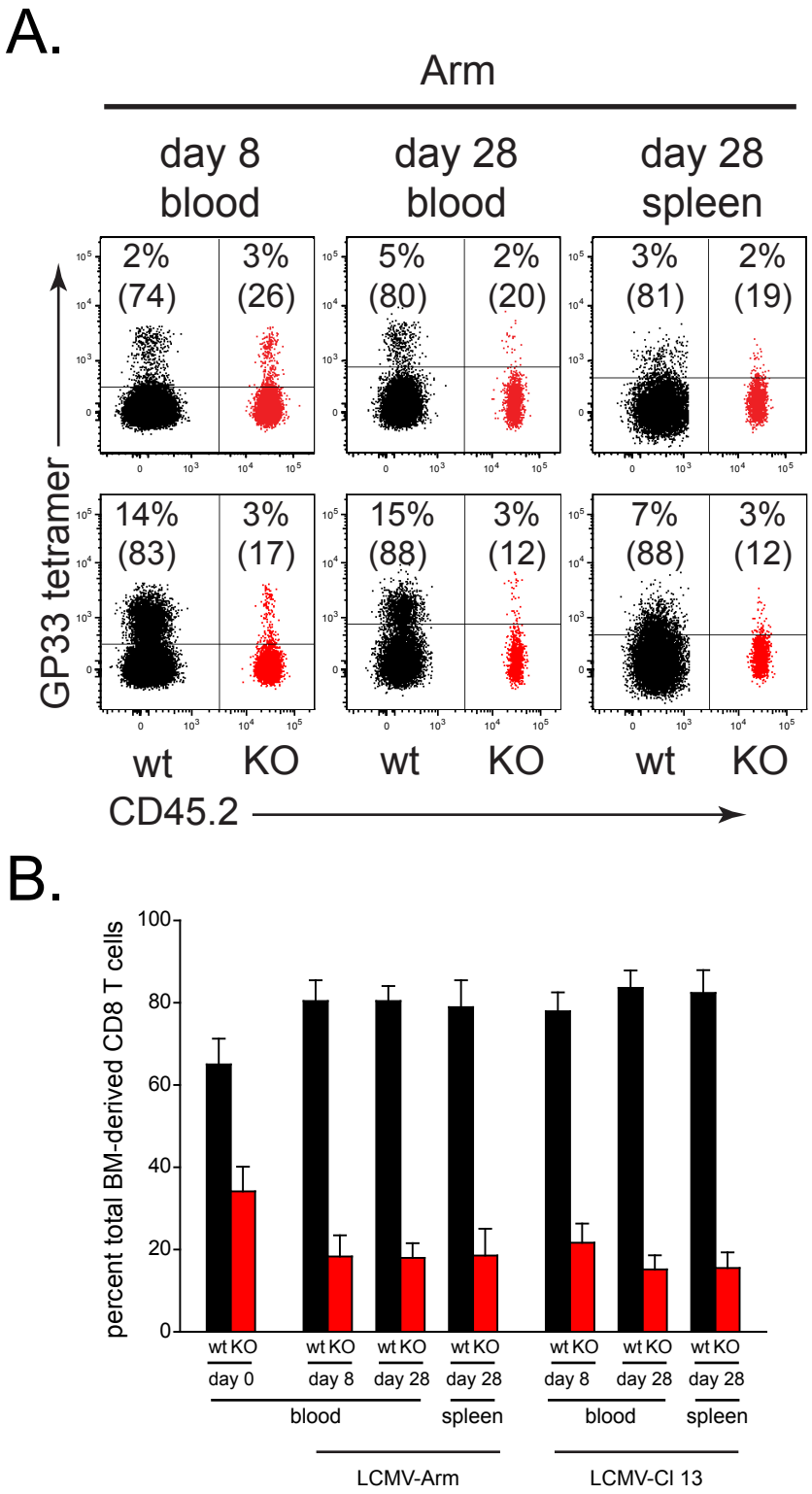

Fig. S5. IL-21R$^{-/-}$ virus-specific CD8^{+} T cell responses are not affected during LCMV-Arm infection in bone-marrow chimeric mice. **(A)** wt (CD45.1^{+}) / IL-21R$^{-/-}$ (CD45.2^{+}) bone-marrow chimeric mice were infected with LCMV-Arm and the frequency of LCMV-GP$_{33-41}$ specific tetramer^{+} CD8^{+} T cells was quantified on day eight and 28 in the blood and on day 28 in the spleen following infection. The figure shows the frequency of tetramer+ cells from two mice on each day. The number above each population indicates the frequency of tetramer^{+} CD8^{+} T cells. **(B)** Bar graph illustrates the ratio ± SD of wt to IL-21R$^{-/-}$ bone marrow-derived CD8 T cells prior to and following LCMV-Arm or LCMV-Cl 13 infection. Black bars: wt; red bars: IL-21R$^{-/-}$. *N* = Two-four mice per group and represents one-two independent experiments.

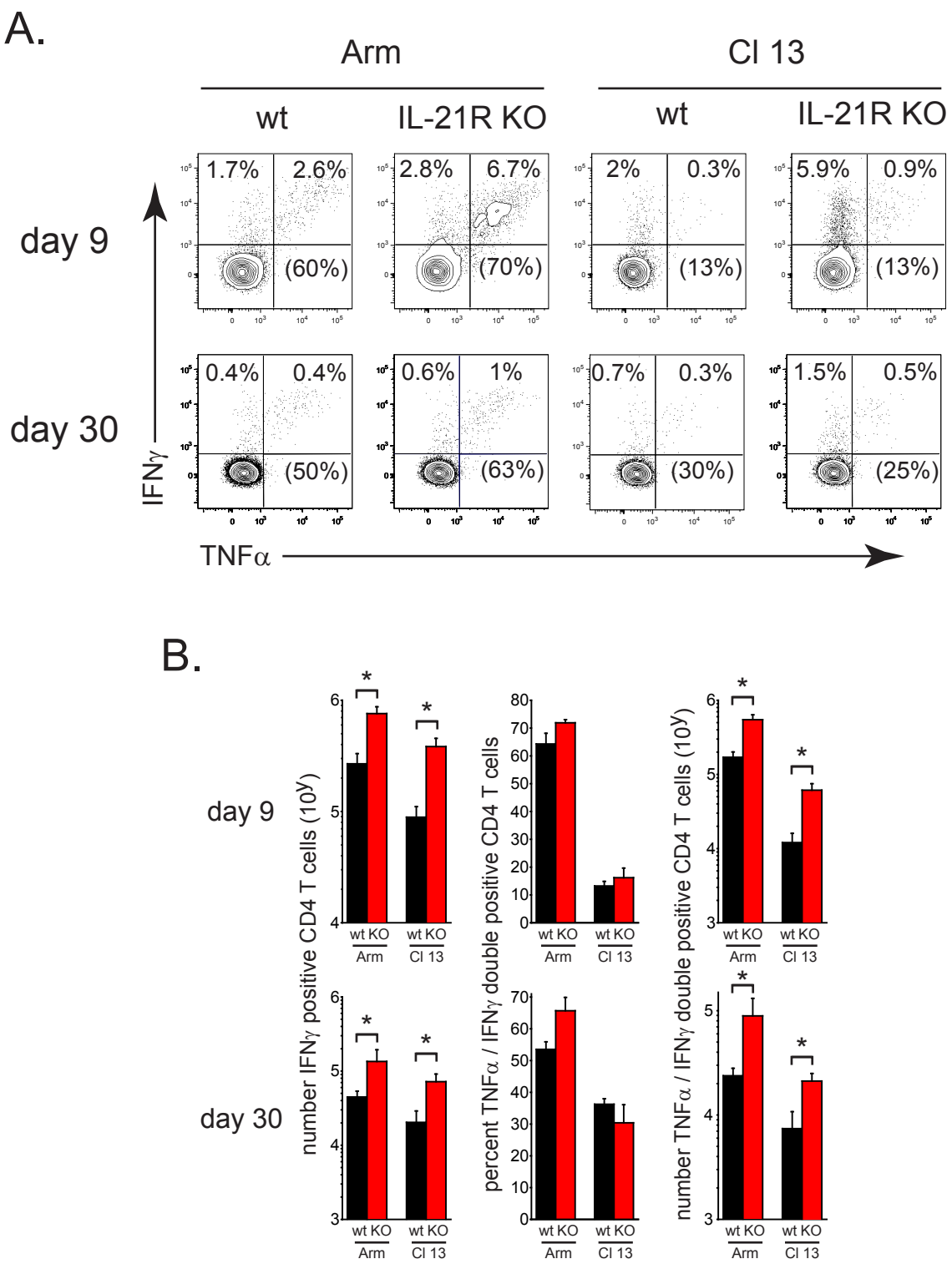

Fig. S6. Suppression of virus-specific $CD4^+$ T cell responses by IL-21 during chronic infection. **(A)** LCMV-GP_{61-80} specific $CD4^+$ T cell responses were measured on day nine (top) and thirty (bottom) after LCMV-Arm (left plots in each time point) or LCMV-Cl 13 (right plots in each time point) infection of wt or IL-$21R^{-/-}$ mice. Flow plots illustrate the frequency of IFN-γ^+ and TNF-α^+ $CD4^+$ T cells and the number in parenthesis indicates the frequency of IFN-γ^+ cells that simultaneously produce TNF-α. **(B)** The bar graphs indicate the average number and frequency ± SD of TNF-α/ IFN-γ double positive $CD4^+$ T cells on day nine (top) or thirty (bottom) after LCMV-Arm (black) or LCMV-Cl 13 (red) infection. Data are representative of four mice per group and four independent experiments. *, $P < 0.05$.

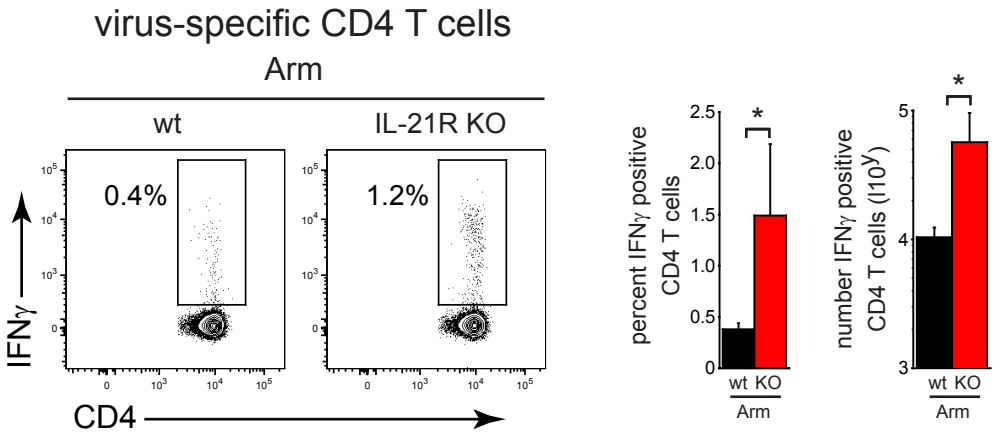

Fig. S7. Long-term virus-specific CD4$^+$ T cell responses following LCMV-Arm infection of IL-21R$^{-/-}$ mice. Flow plots illustrate the frequency and the bar graphs indicate the frequency and number of IFN-γ^+ CD4$^+$ T cells on day 100 after CMV-Arm infection of wt and IL21R$^{-/-}$ mice. *, P <0.05. Data are representative of three mice per group and three independent experiments.

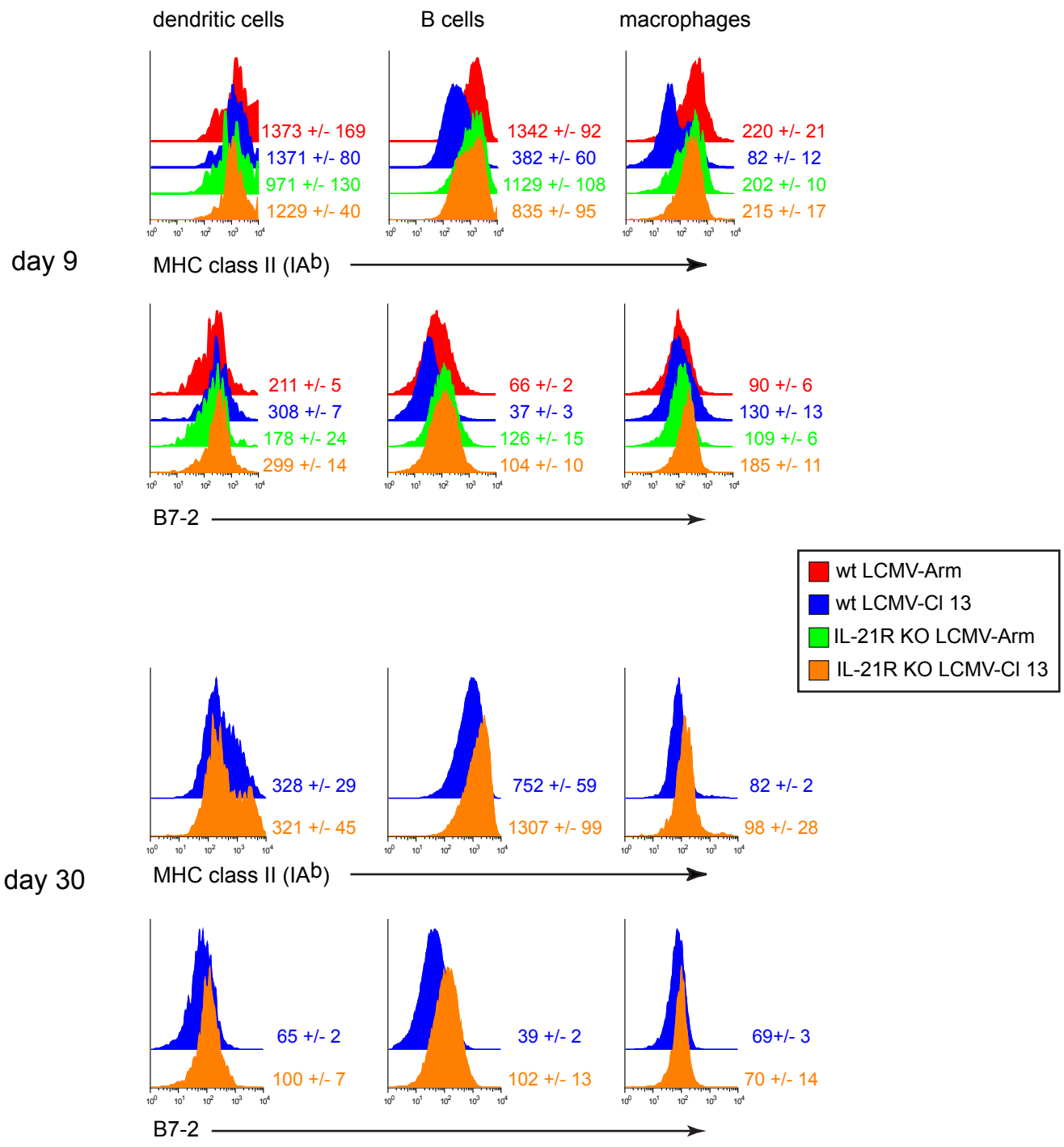

Fig. S8. Expression of maturation markers on antigen presenting cells. Expression of MHC class II (IA^b) and B7-2 was analyzed on day 9 (top set of plots) and day 30 (bottom set of plots) after LCMV-Arm infection of wt mice (red histograms), LCMV-Arm infection of IL-$21R^{-/-}$ mice (green histograms), LCMV-Cl 13 infection of wt mice (blue histograms) or LCMV-Cl 13 infection of IL-$21R^{-/-}$ mice (orange histograms). The numbers next to each histogram in each plot indicate the average geometric mean fluorescence intensity ± SD of four mice per group and two independent experiments.

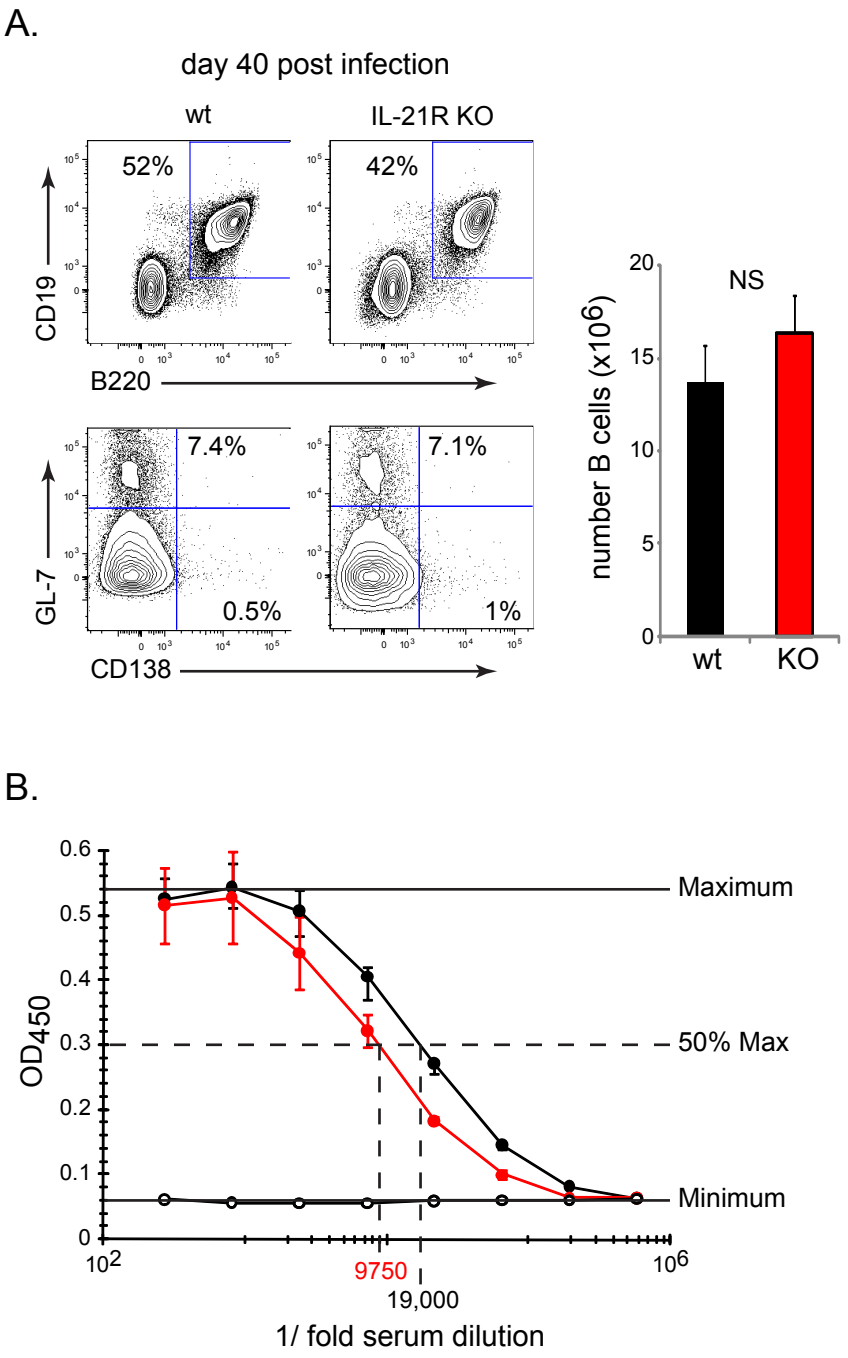

Fig. S9. Normal B cell numbers but decreased LCMV-specific antibody titers in IL-21R$^{-/-}$ mice. (A) The frequency of total B cells (upper flow plots) and germinal center versus plasma B cells (GL-7 versus CD138, respectively; lower flow plots) in the spleen of LCMV-Cl 13 infected wt (left plots) and IL-21R$^{-/-}$ (right plots) mice was analyzed on day 40 post infection. Numbers in each plot indicate the frequency of total B cells, germinal center B cells (top number in the lower plots) and plasma B cells (lower number in the lower plots). The bar graph indicates the total number ± SD of B cells in the spleen of LCMV-Cl 13 infected wt (black bar) and IL-21R$^{-/-}$ (red bar) mice. Data are representative of 4 mice per group and two independent experiments. NS, not significant. **(B)** Serum levels of LCMV-specific IgG antibodies ± SD were determined by limiting dilution ELISA in naïve mice (white circles) and on day 34 after LCMV-Cl 13 infection of wt (black circles) or IL-21R$^{-/-}$ (red circles) mice. Data are representative of 3-4 mice per group and two independent experiments.

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