Science Immunology

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Supplementary Materials for

Type I interferon suppresses virus-specific B cell responses by modulating CD8$^+$ T cell differentiation

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Published 21 October 2016, *Sci. Immunol.* **1**, eaah3565 (2016)
DOI: 10.1126/sciimmunol.aah3565

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SUPPLEMENTARY MATERIALS & METHODS:

Mice. C57BL/6J (B6), B6.129(Cg)-Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J (mTomato) (*66*), C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) (*45*), C57BL/6-Prf1^{tm1Sdz}/J (PRF$^{-/-}$) (*67*), and Gt(ROSA)26Sor$^{tm1(CAG-Brainbow2.1)Cle}$/J (Confetti) (*50*) mice were purchased from Jackson Laboratories. Blimp1-YFP (*68*) (provided by Dr. Michel Nussenzweig, Rockefeller University), actin-mCerulean (*69*), actin-mOrange (*69*), actin-YFP, IFNAR$^{-/-}$ (*70*) (provided by Dr. Jonathan Sprent, formerly at The Scripps Research Institute), KL25 H (*32*) (provided by Dr. Hans Hengartner, University of Zurich), CD45.1 KL25 H+L (provided by Dr. Matteo Iannacone, San Raffaele Scientific Institute), VI10YEN (*32*), P14 (*47*), Thy1.1^{+} P14, SMARTA (*71*), mCerulean^{+} SMARTA, mOrange^{+} SMARTA (all on a pure B6 background) were bred and maintained under specific pathogen–free conditions at the National Institutes of Health (NIH). The KL25 H+L mice were originally generated by Dr. Philip Greenberg at the University of Washington. All mice in this study were handled in accordance with the guidelines set forth by the NIH Animal Care and Use Committee.

Transgenic mouse generation. INS2-CMV-β-ac-GCaMP6s transgenic mice (actin-GCaMP6s) were generated in the National Institute of Mental Health (NIMH) Transgenic Core Facility. The GCaMP6s coding sequence was cloned from pGP-CMV-GCaMP6s (Addgene #40753) using primers (fwd: 5'ATCGGGATCCGCCACCATGGGTTCTCATCATCATCATCATC; rev: 5'ATCGACTAGTTCACTTCGCTGTCATCATTTGTACA) and inserted between BamHI and SpeI restriction sites in the INS2-CMV-β-ac plasmid (containing the CMV early enhancer/chicken β-actin promoter. The INS2-CMV-β-ac plasmid has been described previously (*72*). The resultant plasmid was digested with PacI and NotI, and a ~6 kb fragment containing the CMV early enhancer/chicken β-actin (CAG) promoter, GCaMP6s, and PolyA sequence, which are flanked by insulator sequences, was prepared for microinjection into the pro-nuclei of fertilized C57BL/6J mouse eggs. Following selection of transgene positive founder lines, all mice were backcrossed one additional generation onto the C57BL/6J background before intercrossing.

Generation of r3LCMV-iCre. Virus rescue was performed as described (*63*). Subconfluent

BHK-21 cells ($2x10^6$ cells/M6 well) were transfected for 5 hrs using 2.5 µl of Lipofectamine 2000 (Invitrogen) per µg of plasmid DNA. The cocktail plasmid included: pC-NP (0.8 µg) and pC-L (1 µg), together with plasmids pol-I L (1.4 µg), pol-I S1 (0.8 µg) and pol-I S2 (0.8 µg) that directed intracellular synthesis, via RNA pol-I, of the viral L, S1 and S2 genome RNA species. The pol-I S1 and pol-I S2 expressed the improved Cre (iCre) (*49*) gene from the NP and GPC, respectively, loci. For this, the iCre open reading frame was amplified using the PCR Extender System (5 Prime) and primers with either BsmB I or Bbs I sites for cloning into the GPC and NP, respectively, loci. The Bbs I site naturally found in LCMV Armstrong GPC sequence was removed by site-directed mutagenesis.

Virus infection. Mice were infected intravenously (i.v.) with $2.5x10^6$ plaque forming units (PFU) of LCMV clone 13 (CL13), LCMV CL13 M1 (*33*), or LCMV CL13 M2 (*33*) to generate a persistent viral infection. A state of acute infection was generated by infecting mice with low dose ($4x10^4$ PFU) LCMV CL13 M1. r3LCMV-iCre infections were achieved by intravenous injection of $3x10^4$ PFU. LCMV stocks were prepared by a single passage on BHK-21 cells.

Splenocyte enumeration and flow cytometry. Spleens from infected animals were dissociated through a 100µm filter mesh in PBS containing 1% FBS and 5uM EDTA. Single cell suspensions were blocked with 1.5µg/ml anti-CD16/32 (2.4G2) and 15µg/ml mouse IgG (Jackson ImmunoResearch). Afterwards, splenocytes were stained with the following antibodies from BioLegend: anti-GL7 Alexa Fluor 488 (GL-7), CD138 Brilliant Violet (BV) 605 (281-2), CD19 APC-Cy7 (6D5), CD45.1 APC (A20), CD8 BV510 & APC-CY7 (53-6.7), CD4 BV-785 (RM4-5), Thy1.1 Alexa700 & PE (OX-7), Thy1.2 FITC (53-2.1), CD11c PeCy7 (N418). CD11b PE-Cy7 (M1/70) and IgM PE-Cy7 (II/41) were obtained from eBioscience. 7-AAD (BD Pharmingen) was used to identify and exclude dead cells, and 25µl of Flow Cytometry Absolute Count Standard™ (Bangs Laboratories) was added to each sample prior to acquisition to allow calculation of cells/sample. Intracellular cytokine staining was performed by treating single cell suspensions with Cytofix/Cytoperm (BD Pharmingen) and then staining intracellularly with anti-IFNγ PE/Cy7 (XMG1.2) and anti-TNFα FITC or Pacific Blue (MP6-XT22) (BioLegend). Intracellular granzyme B and LCMV were detected using anti–granzyme B PeCy7 (16G6; eBioscience) or anti-LCMV (VL4; Bio X Cell) antibodies, respectively. Anti-LCMV mAb was

directly conjugated to Alexa Fluor 647 or Alexa Fluor 488 using an antibody-labeling kit from Invitrogen. Samples were acquired using an LSR II digital flow cytometer (BD), and data were analyzed using FlowJo software version 10.0.7 (Tree Star).

Naïve B and CD8$^+$ T Cell Isolation. Single cell suspensions of lymphocytes from LNs and spleens were obtained as described above, and naïve B cells were isolated from naïve wildtype or transgenic mice using a mouse B cell negative selection kit (Stem Cell Technologies). Isolation of CD8$^+$ T cells from transgenic P14 mice performed using a mouse CD8$^+$ T cell negative selection kit (Stem Cell Technologies). The purity of the cells after isolation was determined to be greater than 95%.

In vivo depletion studies. All antibodies used for cell depletion and blocking assays were purchased from BioXcell. B6 mice were injected with 250 µg of anti-CD8α (YTS169), 500 µg anti-CD4 (GK1.5), 300 µg anti-NK1.1 (PK136), 1 mg Ly6G (1A8), or 1 mg anti-Gr1 (RB6-8C5) one day prior to infecting with LCMV-M1. One mg of anti-IFNAR (MAR1-5A3) and 200 µl of clodronate liposomes (Encapsula) were injected intravenously on day -1 and +2 post-infection.

ELISA. LCMV specific antibodies were quantified using Amplex mouse IgG detection ELISA (Invitrogen). 96-well non-treated black flat bottom plates (Corning) were coated with purified LCMV or LCMV-GP-Fc (generously provided by Dr. Daniel Pinschewer, University of Basel) and incubated overnight at 4°C. The following day plates were blocked with 1x PBS-BSA overnight at 4°C. The blocking media was replaced by hybridoma supernatant, and incubated overnight at 4°C with rat monoclonal antibody VL4 as the positive LCMV control. Plates were then washed and incubated with 50 ng/ml of goat anti-mouse IgG horseradish peroxidase (Rockland) for 1hr at room temperature. After washing the plates with 1x PBS three times, 50µg of the reaction mixture was added to each well and incubated at room temperature for 30 minutes. The plates were read using the Varioskan flash fluorometer using the following wavelengths: 530 nm (excitation) and 590 nm (emission).

Hybridomas. Hybridomas were generated using a ClonaCell-HY hybridoma kit (Stemcell Technologies). Briefly, Sp2/0 cells were mixed 1:2 with LCMV-infected splenocytes,

centrifuged, and then resuspended in polyethylene glycol. Newly fused cells were transferred into flasks overnight in non-selecting media. The following day, cells were washed and resuspended in HAT selecting semi-solid (methylcellulose) media and plated into petri dishes at 37°C. Nascent hybridomas became visible as colonies by ~10 days after which clones were identified visually, picked, and grown out in single 96 wells.

Immunohistochemistry. Spleens were fixed overnight in 2.5% buffered formalin, equilibrated for 2-4h in a 30% sucrose solution, and embedded in TBS mounting media (Triangle Biosciences). 20-30 μm cryosections were cut using a Leica CM1850 cryostat. Sections were stained in PBS containing 5 μM EDTA and 2% FBS. Sections were stained with the following antibodies: B220 APC (RA3-6B2), B220 PE (RA3-6B2), CD4 Alexa Fluor 647 (RM4-5), CD45.1 Alexa Fluor 647 (A20), IgD Pacific Blue (11.26.2a), anti-LCMV-Alexa Fluor 488 and 647 (VL4), Ki67-FITC (B56), and polyclonal anti-laminin (Abcam). All directly conjugated antibodies were from BioLegend, except Ki67 (BD Pharmingen) and anti-LCMV (BioXcell), which was conjugated to Alexa Fluor 647 or Alexa Fluor 488 using antibody-labeling kits from Invitrogen. Secondary antibodies included Alexa Fluor 488 conjugated polyclonal anti-GFP (Rockland), Alexa Fluor 405 conjugated polyclonal anti-GFP (Rockland), anti-FITC Alexa Fluor 488 (Invitrogen). Primary and secondary antibodies were incubated on cryosections at room temperature for 2-18 hrs. After staining, sections were washed 4-5 times in staining buffer, and 2-3 drops of FluorSave Reagent (Calbiochem) were added to each section before addition of a coverslip. Images were acquired using an Olympus FV1200 laser scanning confocal microscope equipped 4 detectors, 6 laser lines (405, 458, 488, 515, 559, and 635nm) and 5 objectives (4x/0.16 NA, 10x/0.4 NA, 20x/0.75 NA, 40x/0.95 NA, and chromatic aberration corrected 60x/1.4 NA).

Neutralizing antibody assay. Two-fold serial dilutions of serum (12μl) were incubated with ~40-50 focus forming units of LCMV-CL13 or LCMV-M1 in 96 well round-bottom plates for 90 minutes at 37°C in a 5% CO_2 incubator. After the incubation, $2.5x10^4$ Vero cells were added to each well in 60μl of 1x RPMI containing 2% FBS, 1% glutamine and 1% Pen/Strep. After ~4 hours, the liquid in each well was replaced with 0.75% methylcellulose in DMEM (containing 10% FBS, 1% glutamine and 1% Pen/Strep) and incubated for 44-48 hrs at 37°C in a 5% CO_2

incubator. Cells were subsequently fixed and stained for LCMV antigen as described (*64*). Neutralizing antibody titers were determined as 50% inhibition.

Infectious Center Assay: 10^7 CD45.1$^+$ KL25 H+L B cells were adoptively transferred i.v. into isotype-treated LCMV-M1 infected CD45.2$^+$ mice. Splenic KL25 cells were subsequently sorted (CD19$^+$CD11c$^-$Thy1.2$^-$CD45.1$^+$) from recipient mice at day 3 post-infection, and 5,000 cells were plated on Vero monolayers for 4 hours, after which LCMV foci were determined as for the neutralization assay. The liquid in each well was replaced with 0.75% methylcellulose in DMEM (containing 10% FBS, 1% glutamine and 1% Pen/Strep) and incubated for 44-48 hours at 37°C in a 5% CO_2 incubator. Cells were subsequently fixed and stained for LCMV antigen as described (*64*).

In vitro cytokine production: 5x10^5 P14 CD8$^+$ T cell containing splenocytes from d3-4 post-infection mice were plated in 96 well round bottom plates in RPMI complete media (RPMI, 10% FBS, 200 μM glutamine, 55 μM 2-mercaptoethanol) with 5μg/ml of Brefeldin A (Sigma) at 37°C for five hours with or without 2 μg/ml GP_{33-41} peptide *(*KAVYNFATC; Anaspec).

In vitro *CTL activity:* Thy1.1$^+$ P14 T cells from d3-4 post-infection mice were isolated via a Thy1.1 PE-selection kit (Stem cell technologies) to 75% purity and mixed 1:1 (75,000 of each) with Cell Tracker Violet (CTV; Invitrogen) labeled splenocyte targets and co-cultured in RPMI complete media. Targets were labeled in PBS with 1 μM CTV (Invitrogen) for 12 min at 37°C, then pulsed with 1 μg/ml GP_{33-41} peptide *(*KAVYNFATC) for 30 min at 37°C. Specific lysis was calculated as (non-peptide pulsed: peptide pulsed without CTL) / (non-peptide pulsed: peptide pulsed with CTL) x 100.

KL25 proliferation assay: Purified KL25 H+L cells were labeled with CTV as described above, transferred into B6 hosts, and infected the following day. Splenocytes were isolated 72 hours post-infection and analyzed by flow cytometry for CTV dilution.

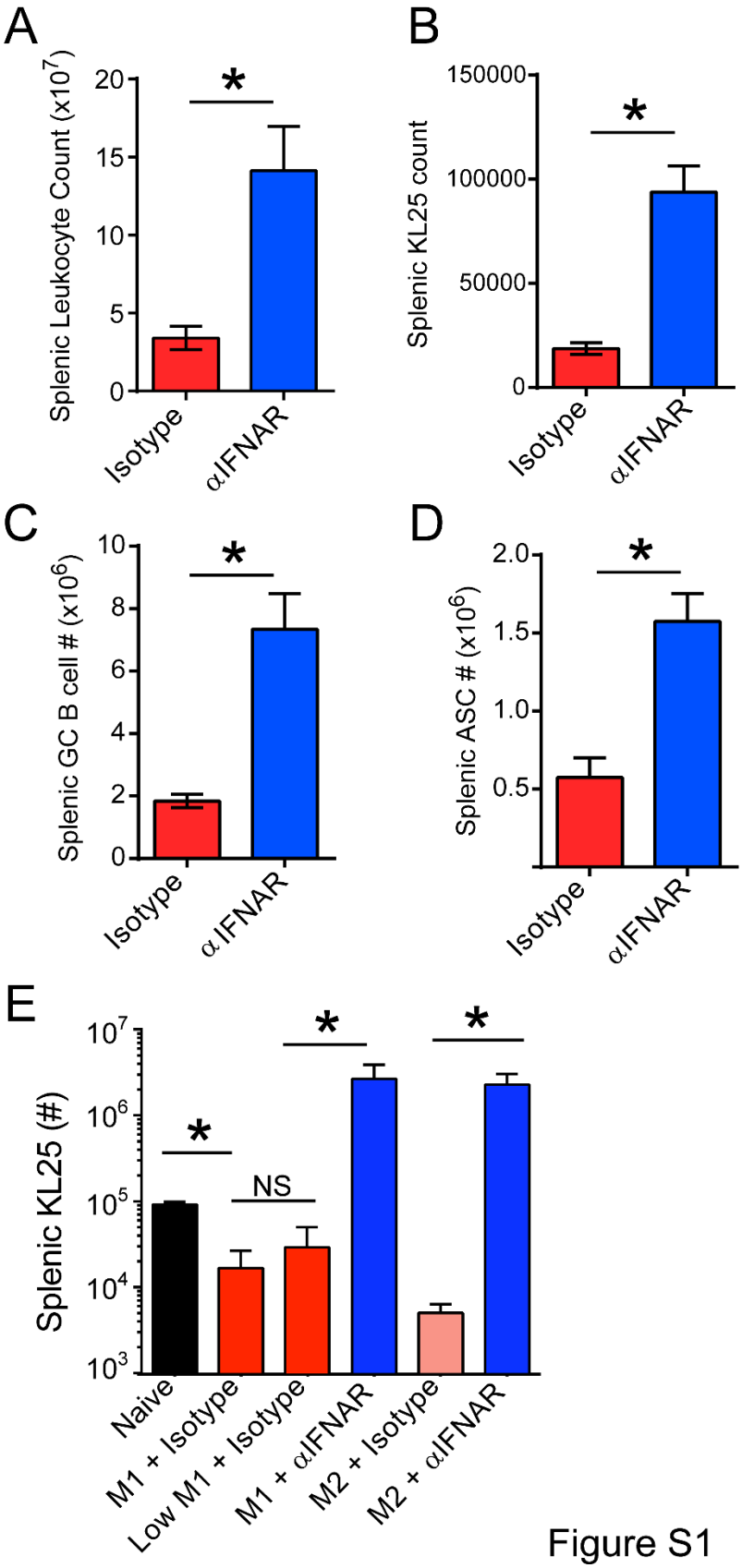
Adoptive transfers: For late transfer imaging studies, 1000-5000 naïve purified OFP$^+$CD4$^+$ SMARTA T cells and 1-2x10^6 mCerulean$^+$Blimp1-YFP$^+$KL25 H cells were injected i.v. into day

6 LCMV-M1 infected B6 mice. For imaging of day 7 isotype vs. αIFNAR antibody treated mice, 1-2x10^6 mCerulean$^+$Blimp1-YFP$^+$KL25 H cells were injected i.v. into B6 mice 1 day prior to i.v. LCMV M1 infection. In vivo calcium imaging studies were set up by seeding B6 mice i.v. with 50,000 mTomato$^+$ GCaMP6s$^+$ P14 and 1x10^6 mCerulean$^+$ KL25 H+L cells, or 50,000 mCerulean$^+$ P14 and 2.5 x10^6 mTomato$^+$ GCaMP6s$^+$ KL25 H+L cells. All recipient mice were infected one day later with LCMV-M1.

Two-Photon Microscopy. Two-photon imaging was performed as described previously (*65*). Briefly, spleens and lymph nodes were glued to a plastic cover slip. The coverslip was then placed into a flow chamber perfused with 37°C high-glucose DMEM bubbled with a mixture of 95% O_2 and 5% CO_2. 3D time lapses were captured using a Leica SP5 two-photon microscope equipped with an 8,000-Hz resonant scanner, a 20× water-dipping objective (1.0 NA), a quad NDD4 external detector array, and Mai Tai HP DeepSee Lasers (Spectra-Physics) tuned to 905 and 990 nm. Stacks consisting of 15 to 30 planes (3 μm step size) were acquired at 30 sec intervals. Signal contrast was enhanced by averaging 8 video frames per plane in resonance scanning mode. Fluorescence emission was passed through customized dichroic mirrors (Semrock) in the NDD4 external detector. The following dichroic mirrors were used for most imaging studies: 458nm-LP, 484nm-LP, 562nm-LP. For Confetti imaging, a 509nm-LP replaced the 484nm-LP. For GCaMP6s and Confetti movies, stacks consisted of 6-12 planes (3 μm step size) and were acquired at 10-30 sec intervals using a Leica SP8 two-photon microscope equipped with an 12,000-Hz resonant scanner, a 25× color corrected water-dipping objective (1.0 NA), a quad HyD external detector array, a Mai Tai HP DeepSee Laser (Spectra-Physics) tuned to 905nm and an Insight DS laser (Spectra-Physics) tuned to 1050nm. Imaging data were processed with Imaris 7.6.1 software (Bitplane). To calculate the frequency of calcium fluxing in mTomato$^+$ GCaMP6s$^+$ P14 cells (Fig. 7C), we first identified and counted all P14 juxtapositions with mCerulean$^+$ KL25 B cells over 5 minute intervals in two-photon time lapses. We then calculated the frequency of these couplings that elicited a GCaMP6s$^+$ intracellular calcium flux within the P14 cell (n=237 pairings from 24 five minute intervals captured in 4 mice).

Statistical Analysis. Statistical significance ($P < 0.05$) was determined using a Student's *t* test (two groups) or a one-way ANOVA (more than two groups). An ANOVA on ranks was used for

datasets with a non-Gaussian distribution and more than two groups. All statistical analyses were performed using GraphPad Prism 6.0 or Sigma Plot 11.0.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M2 + Isotype
M2 + αIFNAR

Figure S1

Supplementary Figure 1. Effects of IFNAR blockade and virus strain or titer on LCMV-specific B cells. (A-D) Total splenocyte count (B), total splenic KL25 count (C), total germinal center ($GL7^{+}FAS^{+)}$ B cell count (D), and total splenic ASC ($CD138^{+}$) count from isotype or αIFNAR mice 34 days post-LCMV M1 infection (*n*=4-5 mice per group; 2 independent experiments; *, $P < 0.05$). (E) Splenic KL25 counts from naïve, isotype, αIFNAR mice 7 days post-LCMV-M1 or LCMV-M2 infection (*n*=3-5 mice per group; 2 independent experiments; *, $P < 0.05$). One group of mice (low M1 + isotype) were infected with a low dose ($4x10^4$ PFU) of LCMV-M1 that results in acute clearance of the virus. All of the other groups of infected mice in this experiment received $2x10^6$ PFU of virus.

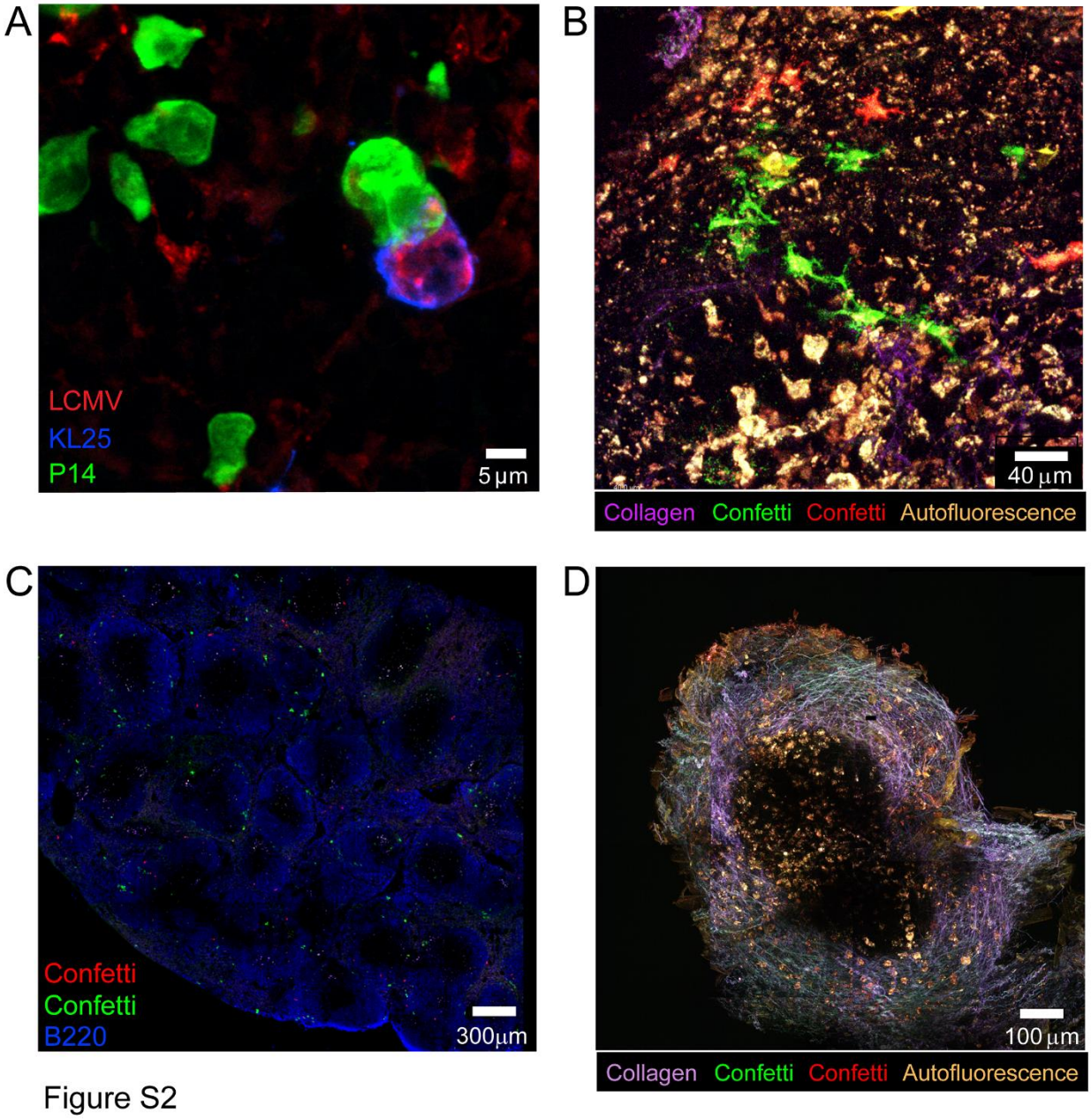

Figure S2

Supplementary Figure 2. CTL engagement of antiviral B cells and control LCMV-iCre–infected Confetti mice. (A) Representative confocal image from a day 3 spleen shows a YFP^+ P14 CTL (green) engaged with an LCMV M1–infected (red) $CD45.1^+$ KL25 B cell (blue). (B-D) Representative TPM (B,D) and confocal (C) images from LCMV-iCre infected isotype-treated Confetti mice on d30 post-infection. Images were captured in the spleen (B,C) and lymph node (D) (*n*=4 mice per group).

MOVIE LEGENDS

Movie S1. LCMV-specific B cells thrive upon late transfer into infected hosts. Representative time-lapses of 3D reconstructions from LCMV-M1 infected mice show mCerulean$^+$ (blue) Blimp1-YFP$^+$ (green) KL25 B cells interacting with OFP$^+$ SMARTA CD4$^+$ T cells (red) in splenic germinal center reactions. The KL25 and SMARTA cells were transferred into mice at 14-20 days post-infection and imaged 1-2 weeks later. Second harmonic signal corresponding to collagen appears in magenta.

Movie S2. IFNAR blockade prevents deletion of LCMV-specific B cells.
Representative time-lapses of 3D reconstructions from LCMV-M1 infected mice demonstrate examples of mCerulean$^+$ (blue) Blimp1-YFP$^+$ (green) KL25 B cells interacting with OFP$^+$ SMARTA CD4$^+$ T cells (red) in the spleen. Mice received either isotype control or αIFNAR antibodies one day prior to infection and were then imaged at day 7 post-infection. Second harmonic collagen signal appears in magenta.

Movie S3. LCMV-specific CTLs flux calcium upon contact with LCMV-specific B cells. Part 1. A representative splenic time-lapse depicts a mCerulean$^+$ (cyan) KL25 B cell contacting two different mTomato$^+$ (red) GCaMP6s$^+$ (green) P14 CTL at day 3 following LCMV-M1 infection. The KL25 B cell is motile and contacts two relatively sessile P14 CTL, which induces intracellular calcium signaling. White arrows depict the points of contact. **Part 2.** A higher magnification view of Part 1. **Part 3.** This time lapse captured at day 3 post-infection shows a region of extensive juxtaposition and interactions between P14 CTL and KL25 B cells in the splenic marginal zone. The border separating the white pulp (WP) and marginal zone (MZ) is demarcated with a dotted white line. Strongly autofluorescent cells are visible as orangish white, and collagen appears as magenta.

Movie S4. Antiviral CTLs productively engage LCMV-specific B cells.
Representative splenic time-lapses captured 3 days following LCMV-M1 infection show a low and high magnification view of an mTomato$^+$ (red) GCaMP6s$^+$ (green) P14 CTL interacting with a mCerulean$^+$ (cyan) KL25 B cell. This interaction denoted with a white arrow illustrates a long term interaction in which the B cell dictates CTL migration by dragging the engaging cell along

with it. Strongly autofluorescent cells are visible as orangish white and collagen as magenta.

Movie S5. LCMV-specific B cells are killed in vivo. Representative time-lapses captured in the lymph node 3 days following LCMV-M1 infection shows a low and high magnification view of mCerulean^{+} (cyan) P14 CTL engaging and killing mTomato^{+} (red) GCaMP6s^{+} (green) KL25 B cells. White arrows denote the interactions. Note that the first interaction induces a massive rise in intracellular calcium within the KL25 B cell that is followed by membrane blebbing. Collagen is shown in white.

Movie S6. LCMV-infected B cells contribute to clonal GCs in the absence of functional CTLs. Representative time-lapses were captured in the spleen and lymph nodes of r3LCMV-iCre infected Confetti mice treated with αIFNAR or αCD8 antibodies. Clonal germinal center B cells appear as accumulations of cells possessing the same combination of recombined fluorescent proteins. All germinal center reactions are highlighted with white dotted lines.