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Supplemental Information

B Cell Maintenance of Subcapsular Sinus Macrophages Protects against a Fatal Viral Infection Independent of Adaptive Immunity

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Supplemental Data Inventory

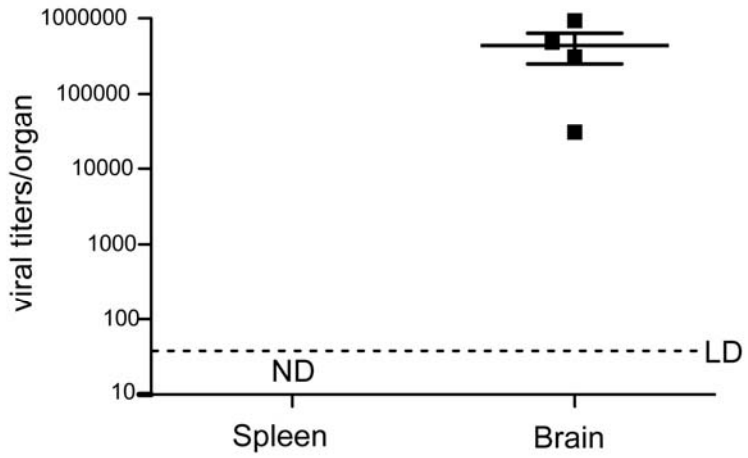

Figure S1. μMT mice die of CNS viral invasion without viremia. VSV titers in the brain and spleen of paralytic μMT, 7 days after infection. Results are representative of 2 experiments (*n* = 5 per experiment)

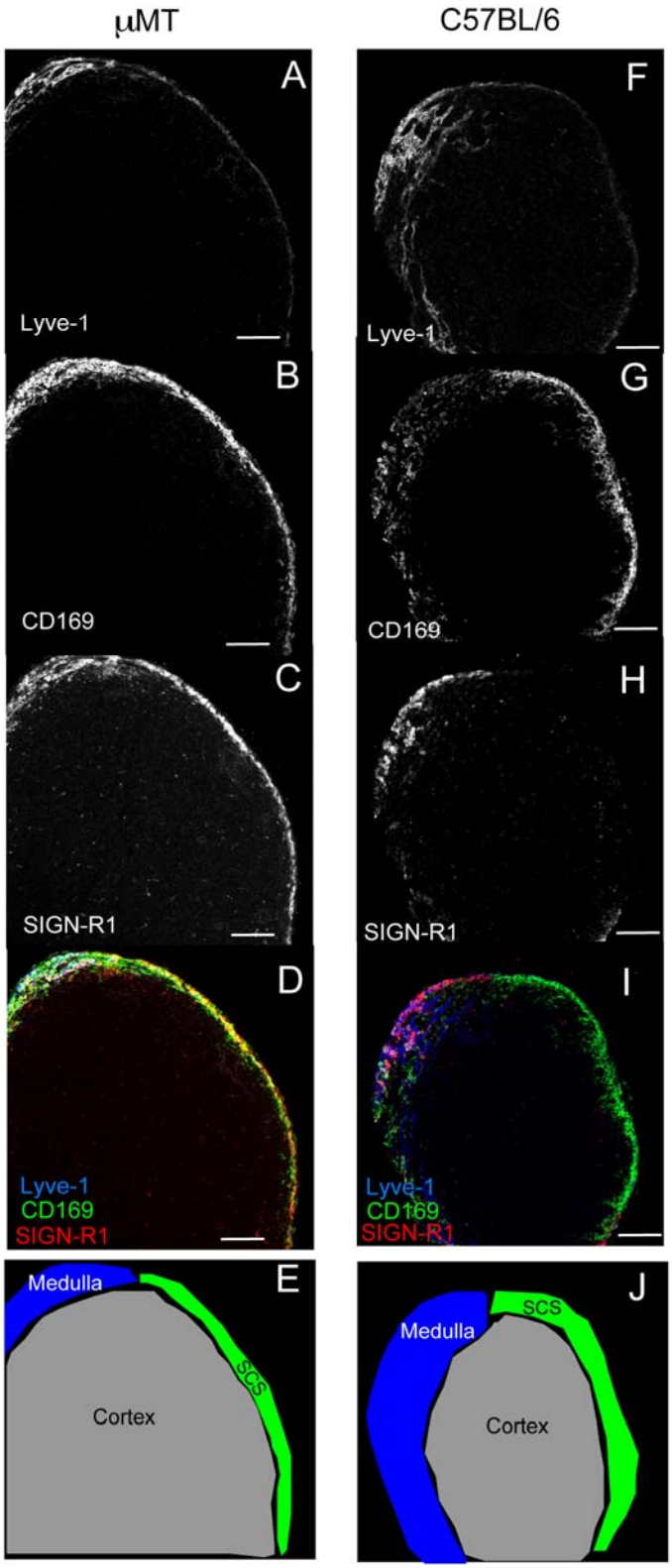

Figure S2. SIGN-R1 expression by SCS macrophages in μMT mice. Representative confocal micrographs of frozen LN sections from μMT (**A-D**), and C57BL/6 (**F-I**) mice stained with MAbs against CD169 (white in **B & G**, green **D & I**), SIGN-R1 (white in **C & H**, red in **D & I**), and Lyve-1 (white in **A & F**, blue in **D & I** blue). Schematic of representative LN sections from μMT (**E**) and C57BL/6 mice (**J**). Scale bars represent 100 μm.

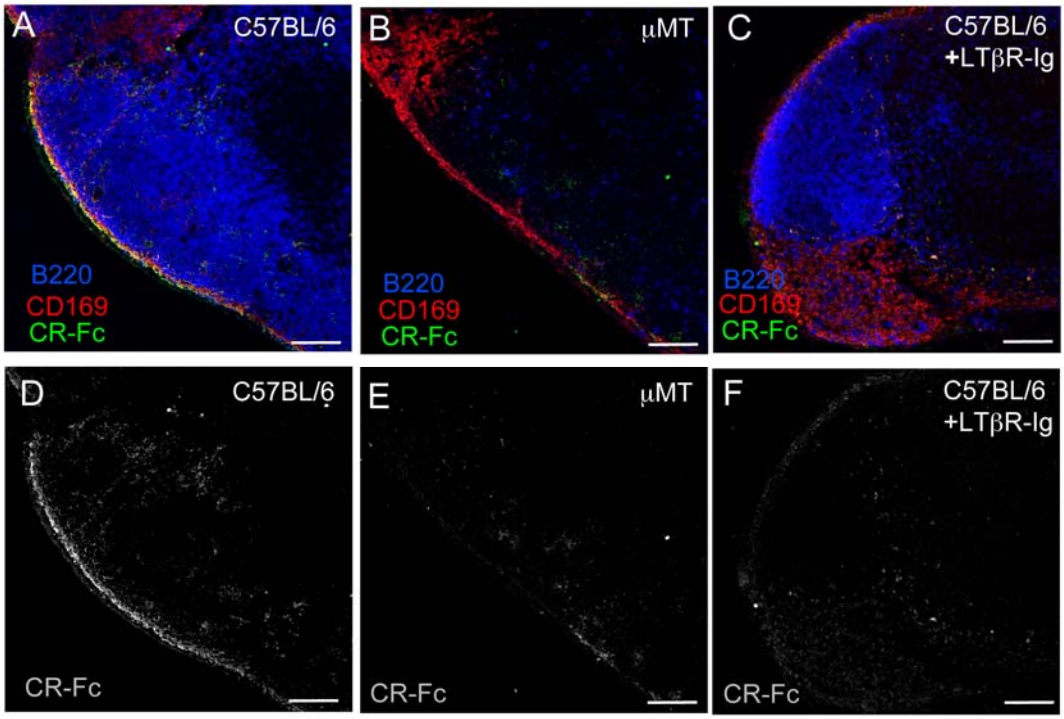

Figure S3. B cell-derived LTα1β2 is required for CR-Fc expression in LNs. Representative confocal micrographs of frozen LN sections from C57BL/6 (**A, D**), μMT (**B, E**), and LTβR-Ig treated C57BL/6 (**C, F**) mice stained for CR-Fc (green in **A-C**, gray in **D-F**), and with MAbs against CD169 (red, **A-C**), and B220 (blue, **A-C**). Scale bars 100 μm.

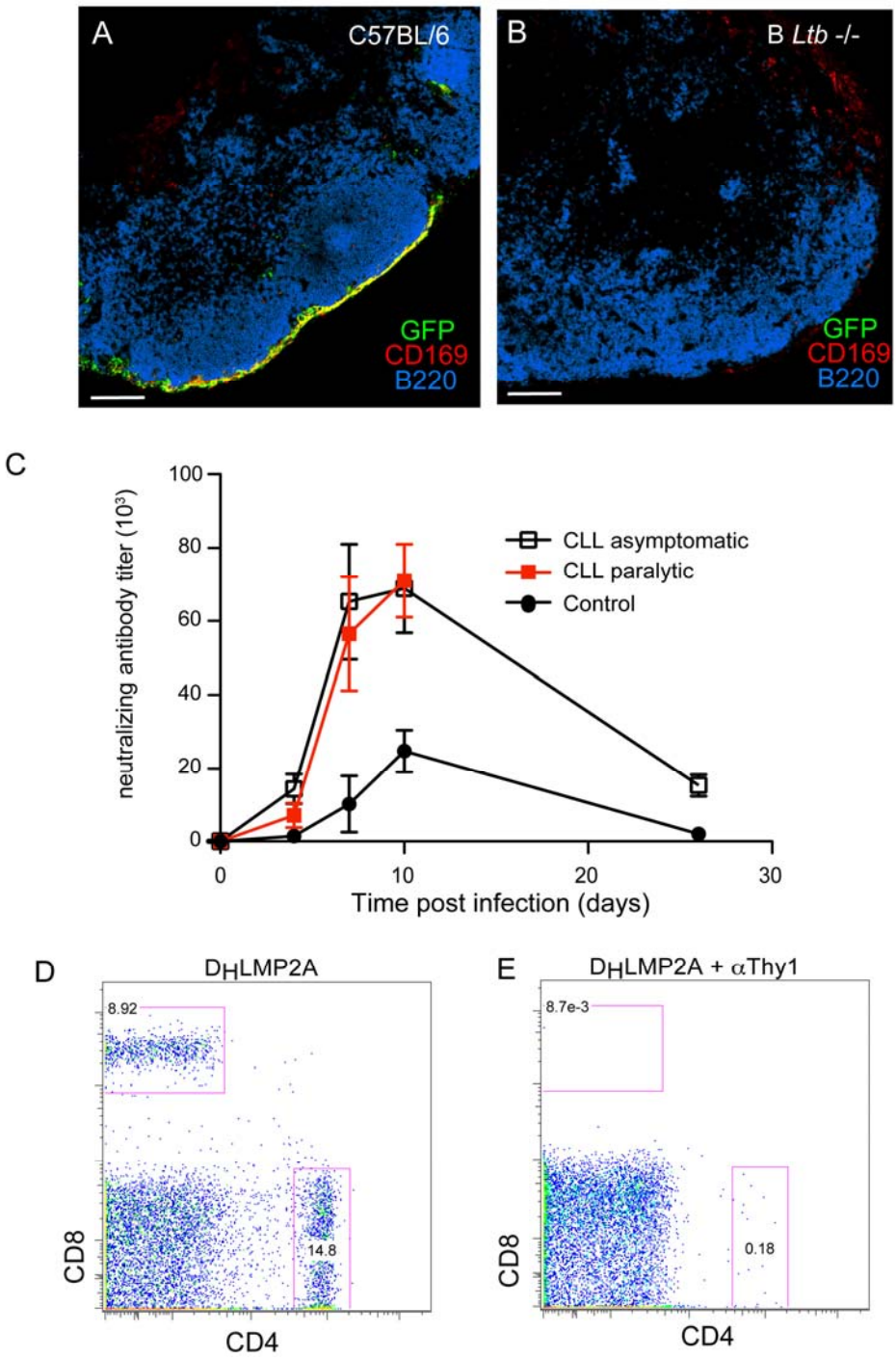

Figure S4. **Supplemental data for Fig. 7.** (**A, B**) B cell-derived LTα1β2 is required for VSV replication in SCS macrophages. Representative confocal micrographs of frozen popliteal LN sections from (**A**) C57BL/6 and (**B**) B *Ltb*$^{-/-}$ BM chimeras, 8 hours following subcutaneous VSV-eGFP infection. Sections were stained with MAbs against CD169 (red), B220 (blue) and GFP (green). Scale bars: 100 μm. (**C**) Humoral immunity is not sufficient for survival of subcutaneous VSV infection. Serum VSV-neutralizing antibody titers after subcutaneous VSV infection in control, asymptomatic CLL-treated and paralytic CLL-treated C57BL/6 mice. Results are representative of 5 experiments (n = 10 per experiment). (**D, E**) Intraperitoneal injection of anti-Thy1 antibody results in nearly complete depletion of circulating T cells from in D_HLMP2A mice. Representative FACS plots depicting CD4 and CD8+ cells in (**D**) untreated and (**E**) anti-Thy1 antibody treated animals on day 4 post infection.

Supplemental Experimental Procedures

Mice

C57BL/6 and BALB/c mice, 6-8 weeks old, were purchased from Charles River or The Jackson Laboratory. D_HLMP2A mice (Casola et al., 2004) were provided by K. Rajewsky (Harvard Medical School)**.** *Lta*-/-, and μMT mice were purchased from The Jackson Laboratory. Bone marrow chimeras were generated by irradiation of C57BL/6 mice with 1300 rad in split doses and reconstitution with C57BL/6, B *Ltb* –/– (Tumanov et al., 2002), T *Ltb* –/– (Junt et al., 2006)or *Lta*-/- bone marrow and were allowed to reconstitute for at least 8 weeks prior to use. Mice were housed under specific pathogen-free conditions in accordance with National Institutes of Health guidelines. All experimental animal procedures were approved by the Institutional Animal Committees of Harvard Medical School and IDI.

Viruses and viral plaque assay.

VSV serotype Indiana (VSV-IND, Mudd-Summers derived clone, in vitro rescued and plaque purified(Whelan et al., 1995)) and VSV-eGFP(Chandran et al., 2005) were propagated at a MOI of 0.01 on BSRT7 cells and purified as described (Iannacone et al., 2010; Junt et al., 2007). Some batches were labeled with carboxylic acid succinimidyl ester of Alexa Fluor 488 (VSV488) ; Invitrogen-Molecular Probes), as described (Junt et al., 2007). Infectivity of VSV preparations and VSV titers from organs of infected mice were quantified by plaque assay on green monkey kidney cells (Vero), as described (Iannacone et al., 2010; Junt et al., 2007). Mice were infected with 10^4 plaque-forming units (pfu) of VSV Indiana into the right hind footpad (i.fp.), 10^6 pfu of VSV-eGFP i.fp., 10^7 pfu of VSV488 i.fp., or 10^6 pfu of VSV Indiana intravenously (i.v.). All infectious work was performed in designated BL2+ workspaces, in accordance with institutional guidelines, and approved by the Harvard Committee on Microbiological Safety.

Tissue digestion and flow cytometry

Single-cell suspensions of LNs for flow cytometry were generated as described (Iannacone et al., 2010). All flow cytometric analyses were performed in FACS buffer containing PBS with 2 mM EDTA and 2% FBS (Invitrogen-GIBCO) on a FACS CANTO (BD Pharmingen), and analyzed by FlowJo software (Treestar Inc., Ashland, OR). Antibodies used included APC-conjugated anti-CD4 (Clone RM4-5, Biolegend), PE-conjugated anti-CD8b (Clone 53-5.8, Biolegend), PE-Cy7-conjugated anti-CD11b (Clone M1/70, eBioscience), Alexa Fluor 647-conjugated SIGN-R1 (22D1, eBioscience), FITC-conjugated anti-CD169 (Clone 3D6, AbD-Serotec), Alexa Fluor 647-conjugated anti-CD11b (AbD-Serotec). The anti-CD169 antibody Ser4 (provided by Paul Crocker, University of Dundee, Dundee, UK) was purified from hybridoma supernatants according to standard methods, and biotinylated with a biotinylation kit from Pierce, or labeled with carboxylic acid succinimidyl esters of Alexa Fluor 488,

Alexa Fluor 568, Alexa Fluor 647 (Invitrogen-Molecular Probes) according to the manufacturer's instructions.

Confocal Microscopy

Popliteal LNs were harvested, processed, sectioned and stained as described previously (Iannacone et al., 2010; Junt et al., 2007). Antibodies used include Alexa Fluor 647-conjugated anti-B220 (Clone RA3-6B2, Caltag), biotinylated and Alexa Fluor 488-, 568- and 647-conjugated anti-CD169 (Clone Ser4, see above), Alexa Fluor 647-conjugated anti-SIGN-R1 (22D1, eBioscience) Alexa Fluor 568-conjugated streptavidin (Invitrogen), Alexa Fluor 488-conjugated anti-green fluorescent protein (Invitrogen), Alexa Fluor 488-conjugated anti-LYVE-1 (Clone ALY7, eBioscience), Alexa Fluor 488 anti-FITC (Invitrogen). CR-Fc, a fusion of the cysteine rich domain of the murine mannose receptor with Fc portion human IgG1, was a gift of Luisa Martinez-Pomares (Martínez-Pomares et al., 1996). CR-Fc was detected with FITC conjugated goat anti-human F(ab')2 (Southern Biotech) Samples were mounted in FluorSave reagent solution (EMD-Calbiochem) and stored at 4ºC until analysis. Images were collected with an Olympus Fluoview BX50WI inverted microscope and 10x/0.4, 20x/0.5, or 60x/1.42 objectives. Images were analyzed using Volocity software (Improvision), ImageJ (NIH) and Photoshop CS3 (Adobe).

For whole mount immunofluorescence analysis, popliteal LNs were removed and fixed in phosphate buffered L-lysine with 1% paraformaldehyde/periodate (PLP). LNs were then washed in PBS containing 1% Triton-X, and 0.2% BSA (WM buffer), before being stained overnight, slowly agitating at 4C. LNs were then washed with frequent changes of WM buffer for 8-12 hrs, after which the secondary antibodies were added to the WM buffer, and incubated again overnight. After 8-12 hours of washing the following day, LNs were analyzed on an Olympus confocal microscope (see above) or using a commercial Prairie Technologies Ultima Two Photon Microscope. For two-photon excitation and second harmonic generation, a Tsunami Ti:sapphire laser with a 10-W MilleniaXs pump laser (Spectra-Physics) with or without a Deepsee module was tuned to 900 nm. Alexa Fluor 488-conjugated anti-green fluorescent protein (Invitrogen).

Measurement of type I interferon

Popliteal LNs were harvested 8 hours p.i. and frozen in RNAlater (Qiagen) prior to disruption in QIAzol reagent (Qiagen) using the TissueLyser II (Qiagen). Total RNA was extracted following the miRNeasy kit's procedure (Qiagen), and sample quality was tested on a 2100 Bioanalyzer (Agilent). RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real time quantitative PCR reactions were performed on the LightCycler 480 system (Roche) with FastStart Universal SYBR Green Master Mix (Roche). Every reaction was run in triplicate and GAPDH

levels were used as an endogenous control for normalization. The following primers were used: Gapdh_L GGCAAATTCAACGGCACAGT; Gapdh_R AGATGGTGATGGGCTTCCC; Ifnb1_L2 CTGGCTTCCATCATGAACAA; Ifnb1_R2 AGAGGGCTGTGGTGGAGAA; Ifna2_L2 ACCTGAGAGAGAAGAAACACAGC; Ifna2_R2 GCAGCAAGTTGACTGAGGAA; Ifna4_L2 AGCCTGTGTGATGCAGGAA; Ifna4_R2 GGCACAGAGGCTGTGTTTCT.

VSV neutralization assay

VSV neutralization assay was performed as described. Briefly, serum of infected mice was prediluted 40-fold in MEM containing 2% FCS. Serial two-fold dilutions were mixed with equal volumes of VSV (500 pfu/ml) and incubated for 90 min at 37°C in 5% CO_2. 100 µl of serum–virus mixture was transferred onto Vero cell monolayers in 96-well plates and incubated for 1-2 h at 37°C. The monolayers were overlaid with 100 µl DMEM containing 1% methylcellulose and incubated for 24 h at 37°C. Subsequently, the overlay was discarded, and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as titer.

In vivo cell depletions In some experiments, LN macrophages were depleted by subcutaneous injections in the footpad of 30 µl clodronate liposomes (CLL), 7 days prior to infection, as described (Iannacone et al., 2010; Junt et al., 2007). CLL were prepared according to a previously published method(Van Rooijen and Sanders, 1994). In other experiments Thy1+ T cells and NK cells were depleted with an intraperitoneal injection of 500 µg of anti-mThy1.2 depleting antibody (30H12, Bioxcell) 4 days prior to infection. 100 µg of additional anti-mThy1.2 depleting antibody was injected on the day of infection and every 3 days subsequently.

Lymphotoxin signaling blockade

LTβR-mIgG1 fusion protein has been described (Fava et al., 2003). Mice received weekly intraperitoneal injections of 100 µg of LTβR-mIgG1 for 1, 2 or 3 weeks prior to infection, or tissue harvest.

Statistical analyses

Results are expressed as mean ± s.e.m. All statistical analyses were performed in Prism (GraphPad Software). Means between two groups were compared using two-tailed *t*-test. Means among three or more groups were compared using one-way or two-way analysis of variance with Bonferroni's post-test. Kaplan-Meier survival curves were compared using the Log-rank (Mantel-Cox) test.