Supplementary Materials

Supplementary Text

rLCMV/INDG carrier splenocytes fail to transfer disease to naïve mice

To corroborate the conclusions drawn from the experiments presented in Fig. 6 we performed adoptive transfer experiments (donors and recipients schematically described in Fig S7A). Naïve C57BL/6 mice were irradiated (600 rad) and were transfused with splenocytes from either naïve ("naïve → naïve"), adult rLCMV/INDG i.c. immune ("immune → naïve") or rLCMV/INDG carrier mice ("carrier → naïve"). As a positive control for disease in the subsequent challenge experiment, irradiated rLCMV/INDG carrier mice were reconstituted with splenocytes from rLCMV/INDG i.c. immune mice ("immune → carrier"). Subsequently, all mice were challenged with ARM i.v.. Monitoring of the NP396-specific CTL response in blood by MHC class I tetramers revealed analogous kinetics as previously observed for the respective donor animals (Fig. S7B, compare to Fig. 1C). Only rLCMV/INDG i.c. immune splenocytes expanded to detectable levels within 4 to 5 days after infection, irrespective of the recipient ("immune → naïve" as well as "immune → carrier"). In "naïve → naïve" and in "carrier → naïve" recipients, NP396-specific $CD8^+$ T cells were not detected prior to day eight after ARM challenge, supporting the previous notion that virus-specific $CD8^+$ T cells of rLCMV/INDG carrier are inefficiently induced. Of note though, NP396-specific $CD8^+$ T cell frequencies on day eight after ARM challenge were significantly higher in "carrier → naïve" recipients than in "naïve → naïve" controls (analogous to the response of the respective donor animals, see Fig. 1C), providing additional support for a very low-level

expansion of virus-specific precursor CTLs in rLCMV/INDG carriers. As expected (45), irradiated recipients that had not been transfused with donor splenocytes failed to mount a detectable CTL response (0+/-0% NP396-specific $CD8^+$ T cells at all time points tested, n=5, not shown). Thus, the $CD8^+$ T cell responses of reconstituted mice originated indeed from the transferred donor cell populations.

By day 9 after ARM challenge "immune→carrier" recipients appeared moribund, forcing us to terminate the experiment. Histological analysis revealed that the disease of "immune→carrier" recipient was caused by severe inflammatory CNS disease, whereas the other groups, including "carrier→naïve" recipients, were free of brain infiltrates (Fig. S7C). Taken together, splenocytes from rLCMV/INDG carriers failed to home to the brain of ARM-challenged naïve recipients whereas the same cells caused severe CNS inflammation when triggered with ARM in the donor animals (Fig. S7C, compare to Figs. 3, 4A, 4C and 6D). Inversely, splenocytes of rLCMV/INDG immune mice caused severe CNS inflammation when triggered with ARM in rLCMV/INDG carrier recipients whereas the same cells failed to produce detectable disease when exposed to ARM in naïve recipients or in the donor animals themselves (Fig. S7C, compare to Figs. 3 and 4A,C). Thus, viral antigen in neurons was a necessary condition for disease in our model, and neonatal rLCMV/INDG i.c. infection did not lead to auto-reactive CTLs that could be triggered by adult ARM challenge.

Supplementary Methods

Antibody detection LCMV-NP binding serum antibodies were detected by ELISA against baculovirus-derived recombinant protein following established protocols (31, 46). The assay consisted of the following steps: 1) coating with baculovirus-derived LCMV-NP (1 µg/ml); 2) blocking with 2% BSA (Fluka, Switzerland) in PBS; 3) addition of 10-fold prediluted sera, titrated at three-fold dilutions over 12 dilution steps; 4) detection with IgG-specific HRP-labeled goat anti-mouse Abs (0.5 µg/ml; Southern Biotechnology Associates, Birmingham, AL); and 5) addition of substrate 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonate] (Roche, Germany) and H_2O_2 (Fluka). Plates were coated overnight at 4°C; all other incubations were conducted for 60–90 min at room temperature. Between incubations, the plates were washed three times with PBS containing 0.05% Tween 20. OD was measured at 405 nm in an ELISA reader, and antibody titers were determined as the highest dilution of serum yielding an optical density (OD) of twice background levels (naïve mouse serum) or higher. For the purpose of statistical analysis by one-way-ANOVA, these titer values were log-transformed (corresponding to the mode of presentation in Supplementary Fig. S8). Determination of a relative antibody titer value was chosen as readout because of the polyclonal nature of the antiviral antibody response in wild type mice, consisting of a wide spectrum of different antibody affinities with the potential to undergo affinity maturation with time. Hence, such antigen-specific serum antibodies cannot be accurately compared to a monoclonal antibody standard for determination of antibody concentrations as weight/volume values (e.g. ng/ml) as this is routinely done to determine total antibody or antibody isotype concentrations in serum.

Irradiation and splenocyte reconstitution To assess LCMV-specific CTL responses of transferred splenocyte populations (Fig. S7) we followed established protocols (45). Recipient mice were irradiated with 600 rad one day prior to adoptive transfer of $5x10^7$ donor splenocytes i.v.. To limit adverse side effects of the irradiation-reconstitution procedure to the minimum possible, all recipients were given $2.5x10^6$ T cell-depleted bone marrow cells of naïve C57BL/6 animals simultaneously with splenocyte transfer. In addition, irradiated recipients were given Sulfadoxin/Trimethoprim in the drinking water (Borgal, Intervet, Switzerland). T cell depletion was carried out by magnetic cell sorting using anti-CD4 and anti-CD8a MicroBeads (Miltenyi Biotec, Germany) according to the manufacturer's instructions. Transferred bone marrow cells contained <0.05% $CD4^+Thy1.2^+B220^-$ and $CD8^+Thy1.2^+B220^-$ cells.

Supplementary Figure legends

Supplementary Figure S1: Neonatal but not adult rLCMV/INDG i.c. infection leads to viral persistence in brain.

Neonatal (A) or adult (B) C57BL/6 mice were inoculated with rLCMV/INDG i.c.. Fifty days later, brain sections were stained for LCMV-NP. Pictures are representative of at least seven individual brains analyzed. Magnification bar: 500 μm

Supplementary Figure S2: Lack of protective memory CTLs in rLCMV/INDG carriers.

Intracerebral infection of adult mice with LCMVwt results in lethal lymphocytic choriomeningitis. This immunopathological disease is mediated by CTLs that attack virus-infected cells of the meninges and chorioplexus, causing a fatal blood-brain-barrier breakdown (32, 47). In contrast, memory CTL protect against LCM by efficiently eliminating the virus from the CNS before the infection becomes too widespread (47). Intracerebral challenge infection with LCMVwt is therefore commonly used to test for protective LCMV-specific CTL memory.

On day –50, neonatal (■) or adult (▲) C57BL/6 mice were infected with rLCMV/INDG i.c., resulting in rLCMV/INDG carriers and rLCMV/INDG i.c. immune mice, respectively. Fifty days later (day 0), these mice and a control group of adult mice without rLCMV/INDG infection (●) were challenged with ARM i.c. to investigate the protective capacity of a putative memory $CD8^+$ T cell population in rLCMV/INDG carriers. The onset of lethal $CD8^+$ T cell-mediated choriomeningitis was monitored and

affected mice were sacrificed in accordance with the Swiss law for animal protection. Adult rLCMV/INDG i.c.-immunized mice were fully protected. But in accordance with the CTL kinetics (compare Figs. 1C, 7B), seven out of eight rLCMV/INDG carriers and all previously naïve mice developed terminal immunopathologic choriomeningitis. Two independent experiments comprising a total of 8 mice per group are summarized.

Supplementary Figure S3: Potent virus-neutralizing antibody response and viral CNS restriction in $A^{-/-}$ rLCMV/INDG carriers, but inefficient antiviral CTL induction in type I interferon competent µMT mice despite the lack of antibodies.

A: 129SvEv and $A^{-/-}$ mice were infected at birth with rLCMV/INDG. At 30 to 50 days of age, spleen and brain were tested for viral S segment and NP mRNA by Northern hybridization (ethidium bromide staining of 28S rRNA is shown as loading control). One representative of two independent experiments is shown. The blot was overexposed to detect any putative signal in spleen.

B: $A^{-/-}$ mice were infected at birth with rLCMV/INDG ("rLCMV/INDG carriers", n=2) or were left uninfected ("non-carriers", n=3). After weaning, all mice were challenged with $5x10^6$ PFU VSV-IND i.v. (>10'000-fold LD_{50} for A-/- mice) and the onset of lethal meningoencephalitis was monitored. Of note, in this experimental setting, protection is strictly antibody-mediated (31).

C: Neonatal (■) or adult (▲) C57BL/6 mice and neonatal B cell deficient µMT mice (◆) were infected with rLCMV/INDG i.c.. A control group of adult C57BL/6 mice without rLCMV/INDG infection (○) was also included. Mice from all groups were sacrificed three weeks later, and NP396-specific CTL activity was determined after in vitro

restimulation of splenocytes in the presence of rIL-2. Symbols represent the mean +/- SEM of 2 (○) to 3 (■,▲,◆) mice per group. Significant differences between groups are indicated (**: $p<0.01$; "n.s.": not significant).

Supplementary Figure S4: Restricted persistence of rLCMV/INDG in CNS neurons.
Brain sections of neonatally rLCMV/INDG-infected 5 to 7-week-old C57BL/6 mice were costained with cell type specific markers (green, left panel) and for LCMV-NP (red, center panel) as indicated. An overlay of the pictures presented in the respective left and center panels is shown in the corresponding right panel ("merge"). Note the colocalization of LCMV-NP antigen with neurons but not with any of the other cell types tested (astrocytes, oligodendrocytes, endothelia or microglia/macrophages). Magnification bar (only shown in bottom right panel, representative for all panels): 50 µm. The pictures included in this figure (compare also Fig. 2B) show neuron-restricted virus distribution as found in the entire CNS (n = 9 animals of three independent experiments with identical results: for each mouse ≥3 brain sections including cerebral cortex, hippocampus, thalamus and basal ganglia, 2 sections of cerebellum and brain stem, and ≥8 spinal cord sections of cervical, thoracic and lumbar level were analyzed).

Supplementary Figure S5: rLCMV/INDG persistence in the CNS of $A^{-/-}RAG^{-/-}$ mice is restricted to neurons, similar to wild type mice.
Brain sections of neonatally rLCMV/INDG-infected 5 to 7-week-old $A^{-/-}RAG^{-/-}$ mice were costained for LCMV-NP (red) and cell type-specific markers (green) as indicated. An overlay of the pictures presented in the respective left panels is shown in the right

panels at different magnification ("merge"). Note the colocalization of LCMV-NP antigen with neurons but not with any of the other cell types tested (astrocytes, oligodendrocytes or microglia/macrophages). The magnification bar shown in the top left panel (100 μm) is representative for all pictures taken at 20x magnification, the bar shown in the top right panel (20 μm) is representative for all pictures taken at 100x magnification. The pictures included in this figure show neuron-restricted virus persistence as found in the entire CNS (n = 5 animals from two independent experiments with identical results: for each mouse ≥3 brain sections including cerebral cortex, hippocampus, thalamus and basal ganglia, 2 sections of cerebellum and brain stem, and ≥8 spinal cord sections of cervical, thoracic and lumbar level were analyzed).

Supplementary Figure S6: Similar regional distribution of rLCMV/INDG in the brain of $A^{-/-}RAG^{-/-}$ and wild type mice.

Brain sections of neonatally rLCMV/INDG-infected 5 to 7-week-old $A^{-/-}RAG^{-/-}$ mice and C57BL/6 mice were stained for LCMV-NP (brown). Representative overview pictures from cortex, hippocampus and cerebellum are shown (n ≥ 5 animals from two independent experiments). The analysis of $A^{-/-}$ and 129SvEv mice revealed an analogous distribution of viral antigen (not shown). Magnification bars are 1000 μm in cortex and 200 μm in hippocampus and cerebellum.

Supplementary Figure S7: CNS immune disease depends on persistent rLCMV/INDG infection.

A: Schematic description of donors and recipient mice. Persisting virus in brain is indicated as red dots. B, C: groups of five recipient mice as outlined in panel A were irradiated (600 rad) on day –2 and were reconstituted with splenocytes of the indicated donor origin one day later. On day 0, all recipients were challenged with ARM i.v.. B: The frequency of NP396-specific cells amongst $CD8^+$ T cells were measured in the blood of the respective donor animals one day prior to collection of splenocytes (left part of panel B), and also in the recipients on the indicated time points after ARM challenge (right part of panel B). Symbols represent the mean+/- SD of five mice per group. Values differing significantly from those of naïve donors or from "naïve→naïve" recipients, respectively, are indicated (**: $p<0.01$). No antiviral $CD8^+$ T cell response was detectable in irradiated mice without splenocyte reconstitution that were challenged with ARM (0+/- 0% (mean +/- SD) NP396-specific $CD8^+$ T cells at days 4, 5, and 8; n=5, not shown), confirming that the responses measured originated from the transferred cell populations. C: Nine days after ARM challenge (when "immune→carrier" recipients were moribund) all mice were sacrificed and the brain inflammatory index was determined. Bars show the mean +/- SEM of five mice per group. Significant differences from the "naïve→naïve" control group are indicated (**: $p<0.01$; *: $p<0.05$; "n.s.": $p>0.05$).

Supplementary Figure S8: LCMV-serology fails to differentiate rLCMV/INDG carrier status or "viral *déjà vu*" disease from antiviral immunity

At the time points indicated, neonatal and adult C57BL/6 mice were infected with rLCMV/INDG i.c. or with ARM i.v. or sequentially with both viruses as outlined in the chart and serum was collected at day 0. The resulting immunological status i.e. a virus

carrier status, CNS immune disease or antiviral immunity (in the absence of persisting virus) is indicated. ELISA was used to determine LCMV-NP-specific serum IgG in 10-fold prediluted serum. Pairs of groups that were not significantly different from each other are indicated (n.s.) whereas all other pairs of groups differed significantly ($p<0.01$, not shown). Symbols represent individual mice, with the mean of a group indicated as vertical line.

Supplementary References

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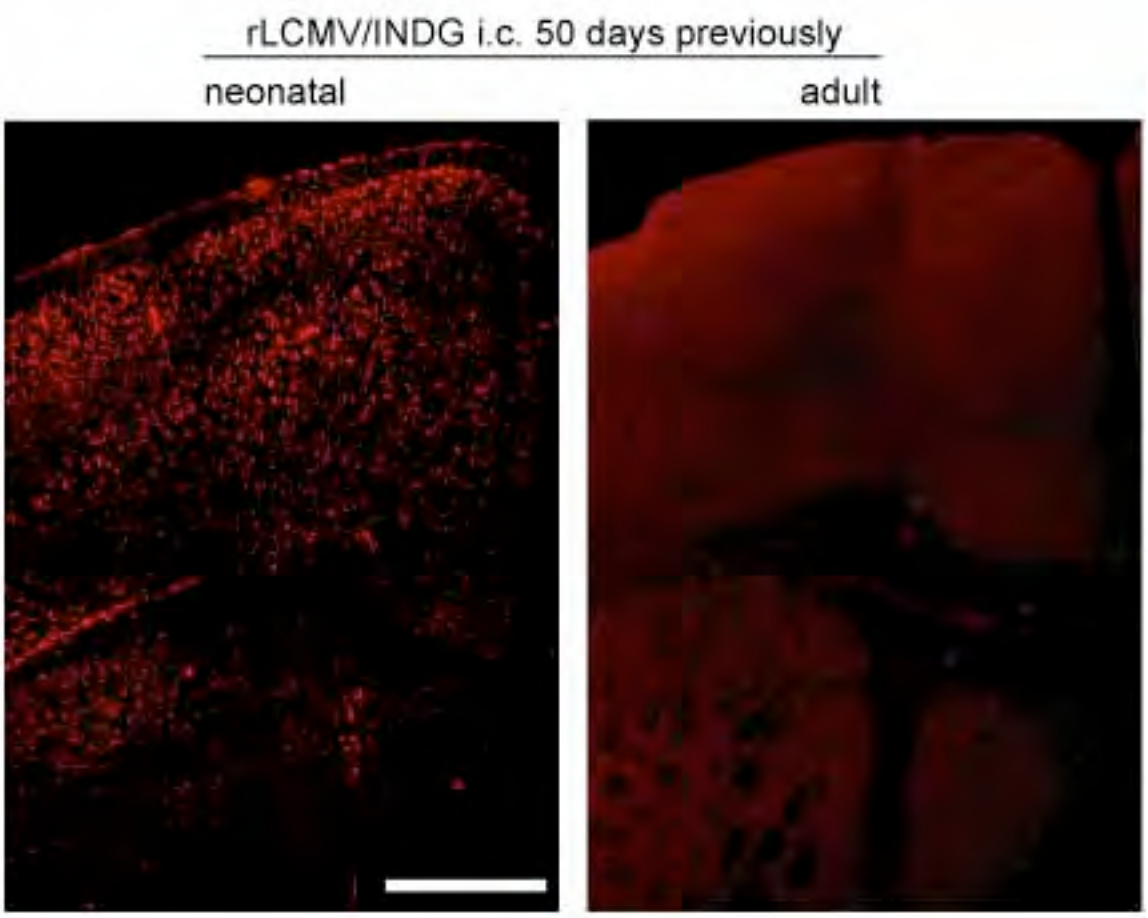
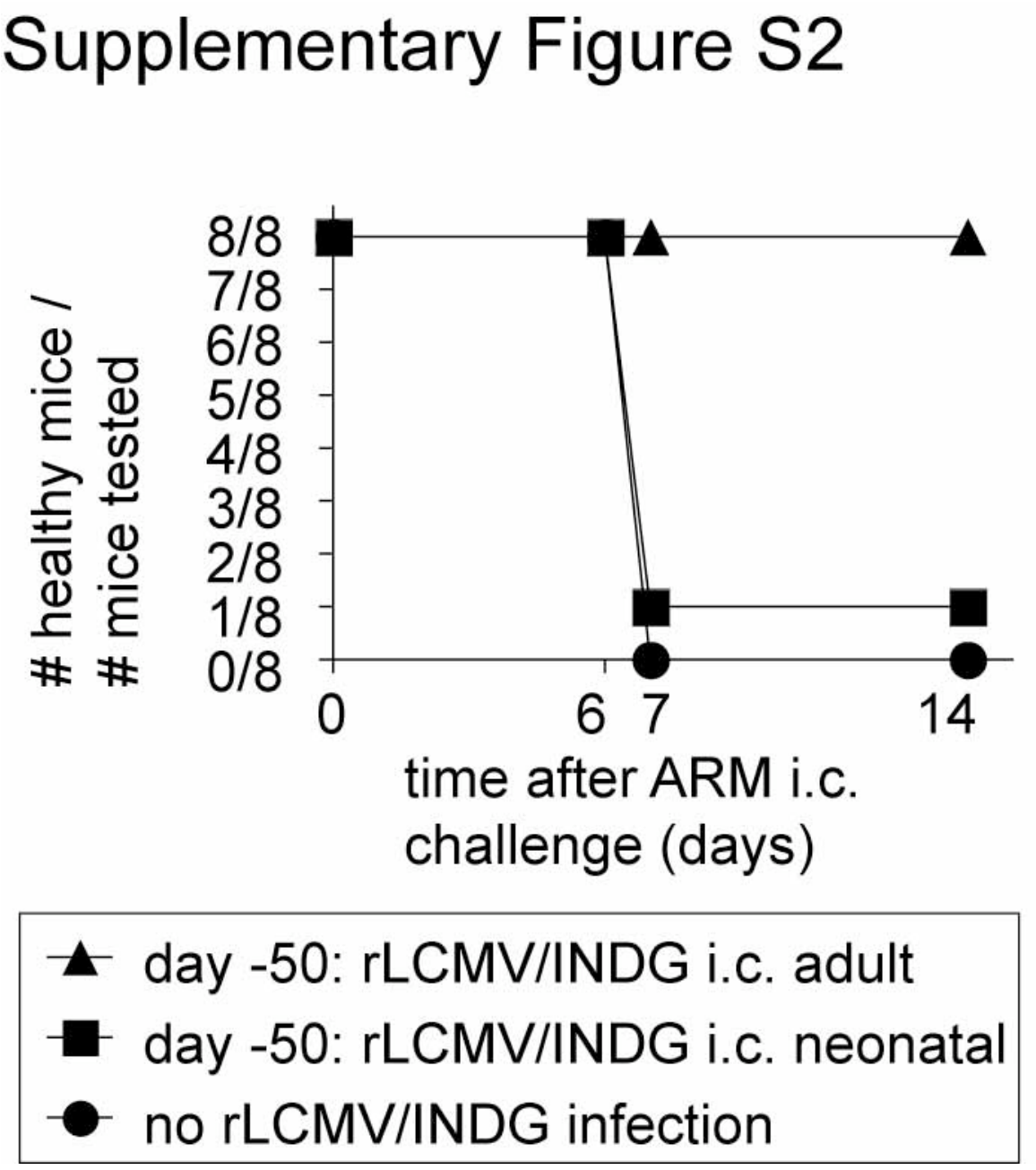
Supplementary Figure S1



Supplementary Figure S2



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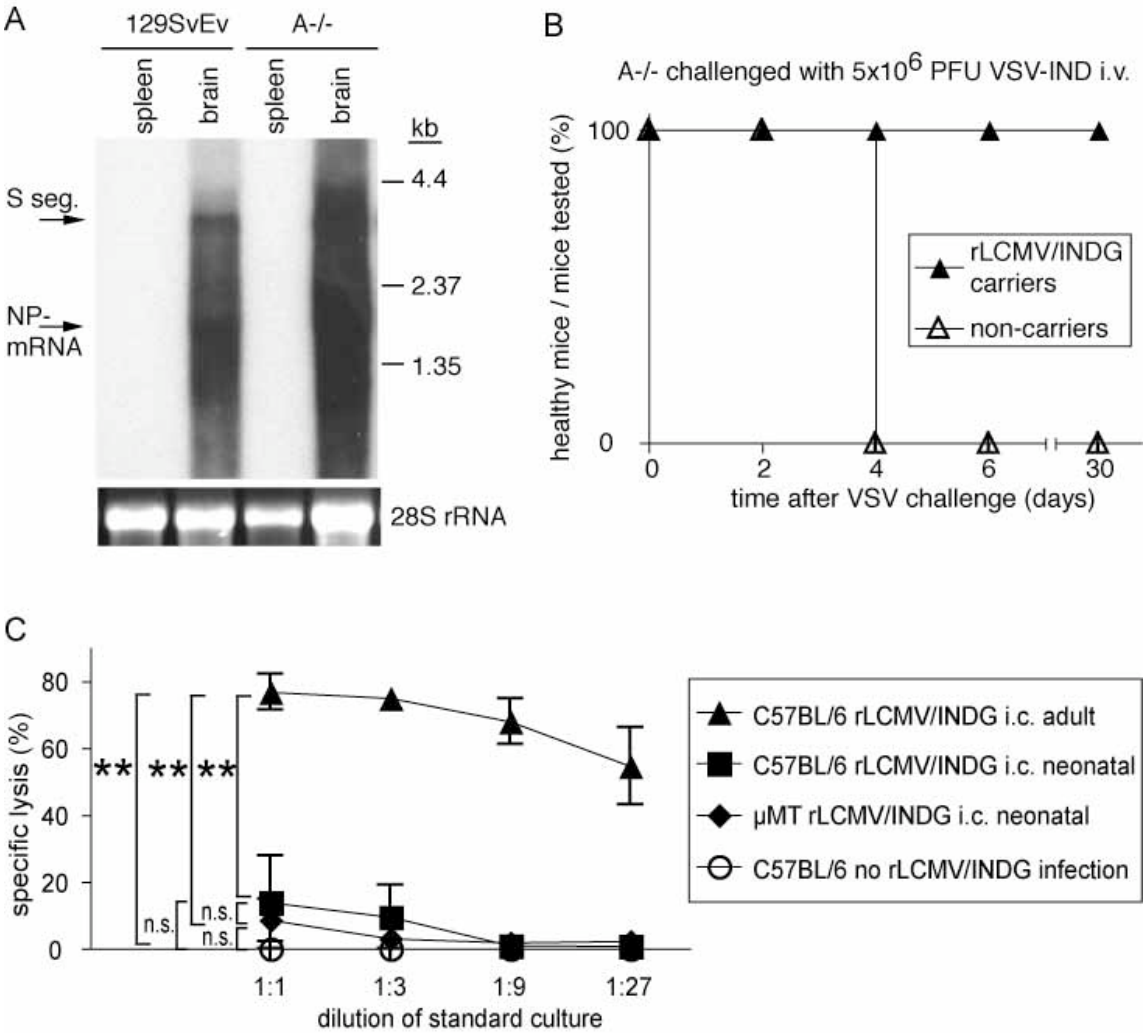

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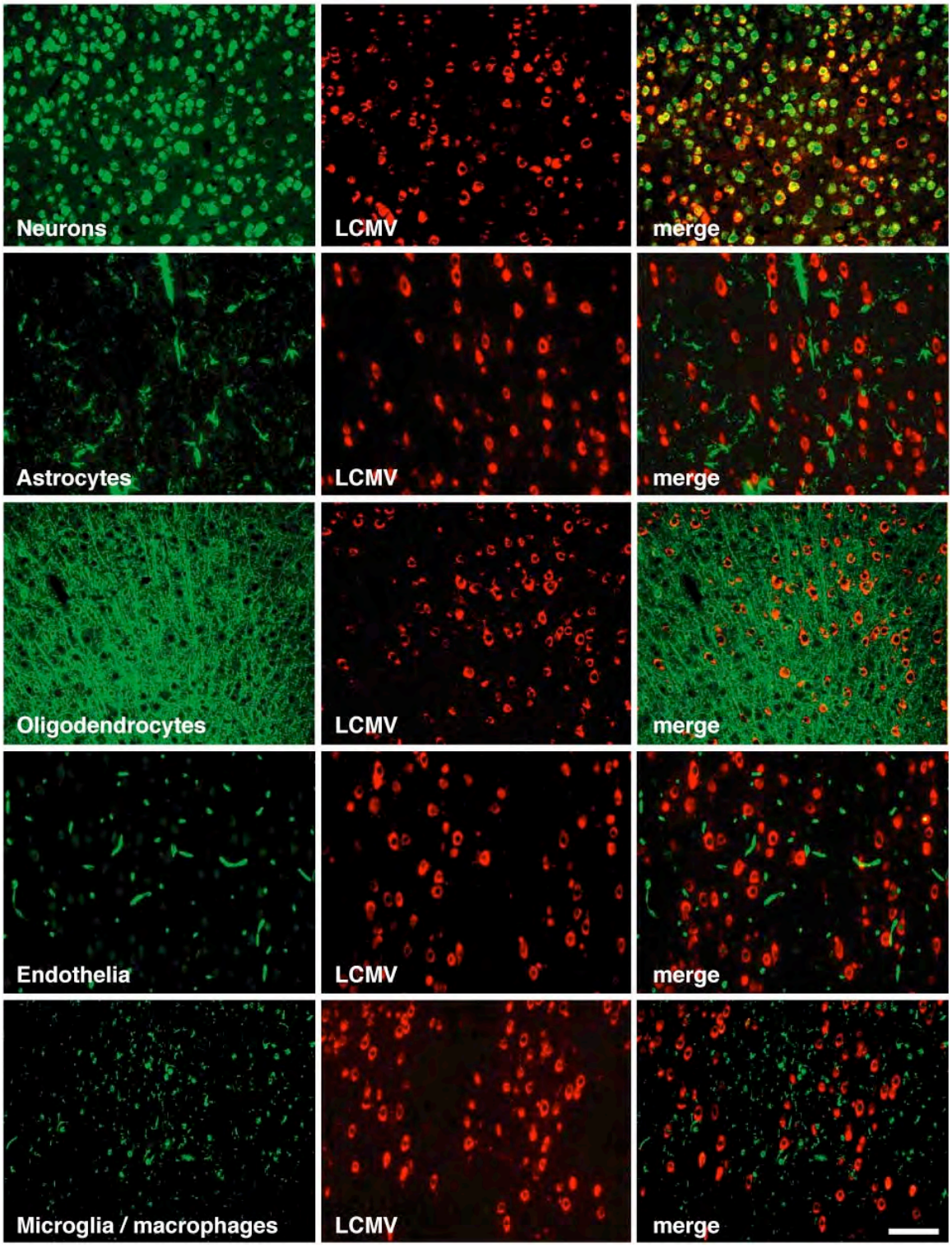

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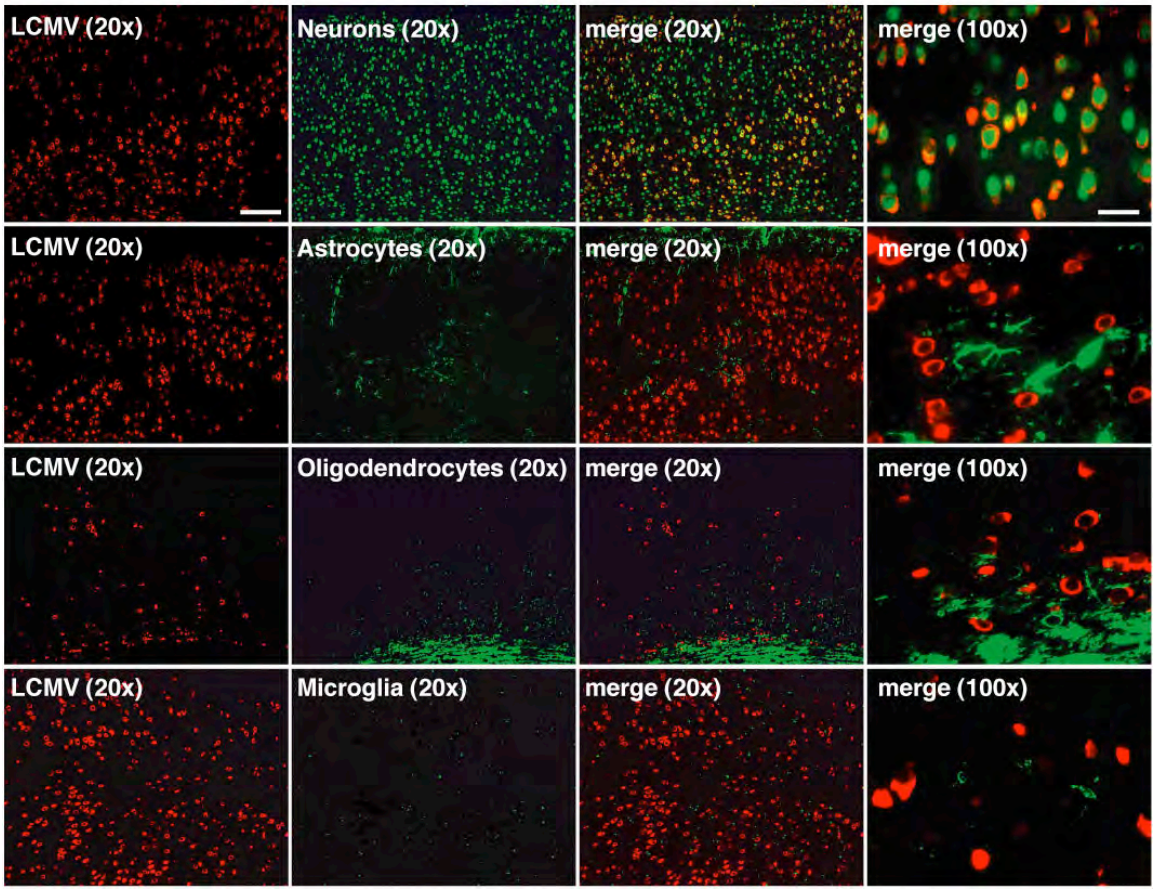

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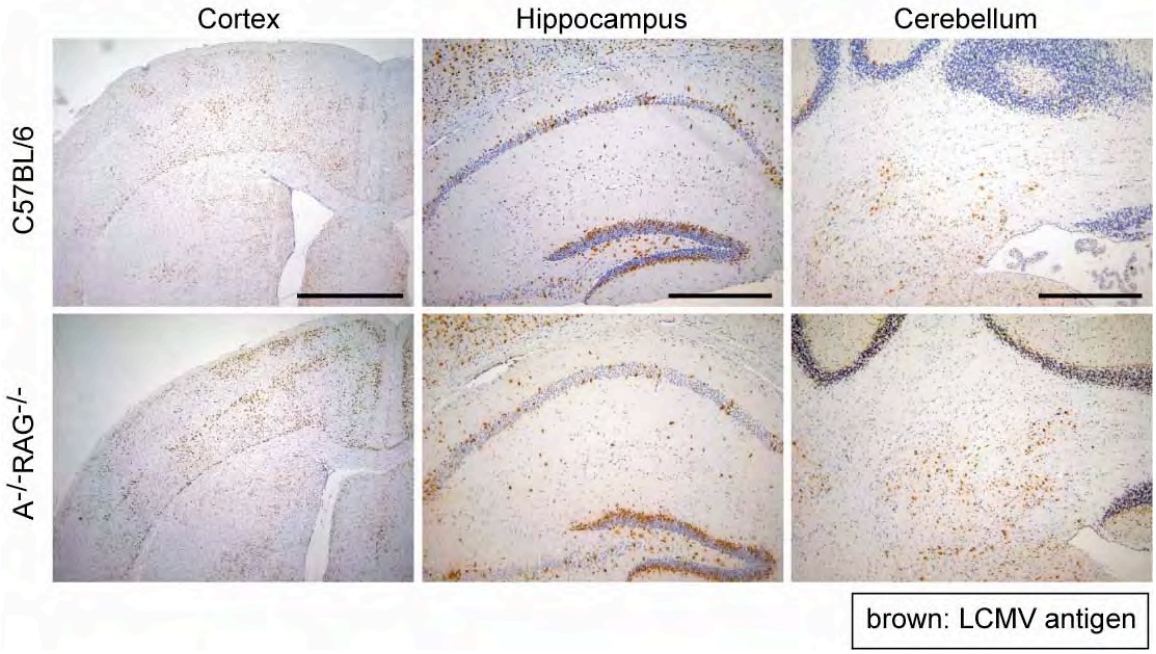
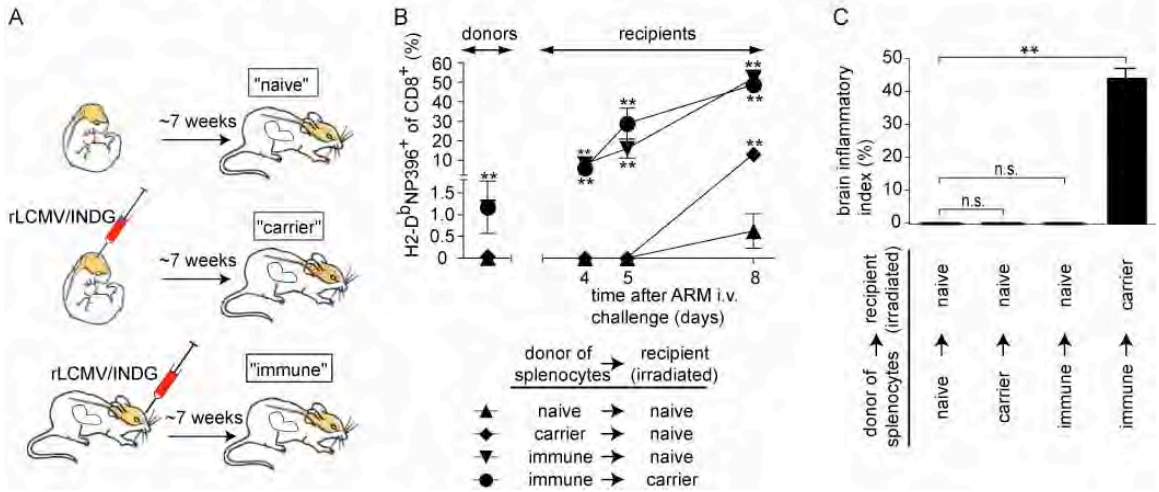


Supplementary figure S7



Supplementary Figure S8