Supplemental Information

Materials and Methods

Flow cytometry

Exosome analysis: Aldehyde-sulfate bound exosomes were labeled with the following antibodies diluted in 0.05% BSA/PBS for 15 min on ice: 10 µg/mL anti-CD9 (clone KMC8; BD #553758), 2 µg/mL anti-CD19-phyoerythrin (PE; clone 1D3; BD #557399), 5 µg/mL anti-CD21-PE (clone 7G6; BD #552957), 5 µg/mL anti-CD24 (clone 30-F1; Biolegend #138501), anti-I-$A^{b,d,q}$/I-$E^{d,k}$-PE (clone M5/114; BD #557000) and donkey anti-mouse Ig-PE (Jackson ImmunoResearch #715-116-151). Unlabeled antibodies were detected with 5 µg/mL goat anti-rat Ig-PE (Southern Biotech #3052-09).

T cell proliferation: monoclonal antibodies were diluted in 0.1% BSA/PBS/2 mM EDTA and cells labeled for 15 min on ice. The following antibodies were used: 1 µg/mL anti-CD4-APC (clone RM4-5; BD #553051), 0.13 µg/mL CD8-Brilliant Violet 605 (clone 53-6.7; Biolegend #100743), 1 µg/mL CD45.1-PE (clone A20; BD #553776) diluted in 0.1% BSA/PBS/2 mM EDTA for 15 min on ice. The division index, defined as the average number of cell divisions (including the undivided peak) was calculated with FlowJo software.

Cytotoxicity (CTL) assays: Target survival was analyzed by flow cytometry and percent CTL killing determined using the following equation: 100 – [(ratio of mouse A pulsed targets to total targets / ratio of mouse A unpulsed targets to total targets) / (ratio of mouse PBS treated pulsed targets to total targets / ratio of mouse PBS treated unpulsed targets to total targets)] × 100.

ELISA for analyzing sucrose-cushion purified exosomes

Exosomes were detected by ELISA, in brief Nunc Maxisorp plates were coated with 100 µl/well of 2 µg/mL anti-mouse I-$A^{b,d,q}$/I-$E^{d,k}$ (clone M5/114; purified in house) in PBS overnight at 4°C. Plates were washed with 0.02% Tween-20/PBS and blocked for ten minutes at 20°C with 0.05% caseinate/PBS for ten minutes.

Unwashed sucrose fractions were serially diluted 1/4 with 0.05% caseinate/PBS and incubated for one hour at 37°C. After washing, biotin was detected with streptavidin-horse radish peroxidase (1/5000; DAKO #P0397) for one hour at 37°C. Plate was washed, developed in TMB and reaction stopped with 2N H_2SO_4. Optical density was measured using a Tecan Infinite M200 microplate reader at 450 nm.

Cold inhibition of biotinylated exosome binding

Naïve C57BL/6 spleen sections were blocked for ten minutes with 1% BSA/PBS, before incubation of 500 µg/mL unlabelled 'cold' B cell-derived exosomes for two hours at 37°C in a humid box. Biotinylated B cell-derived exosomes were then directly added to sections (without removal of unlabeled exosomes) for a final concentration of 50 µg/mL (10:1 ratio of cold exosomes to biotinylated exosomes) and incubated for a further two hours. The modified Stamper-Woodruff assay was then continued as described in the manuscript Materials and Methods.

Dendritic cell-derived exosomes

Bone marrow cells were cultured in R10 plus GM-CSF at 2×10^6 cells/mL, fed with additional culture medium on day three, matured overnight at day six with 200 ng/mL lipopolysaccharide (*Salmonella* Typhimurium; Sigma #L6511). For exosome isolation, DC were not treated with LPS and were cultured for a further two days in fresh R10 plus GM-CSF at 2×10^6 cells/mL. Exosomes were purified from culture supernatant using ultracentrifugation, as described for B cell-derived exosomes.

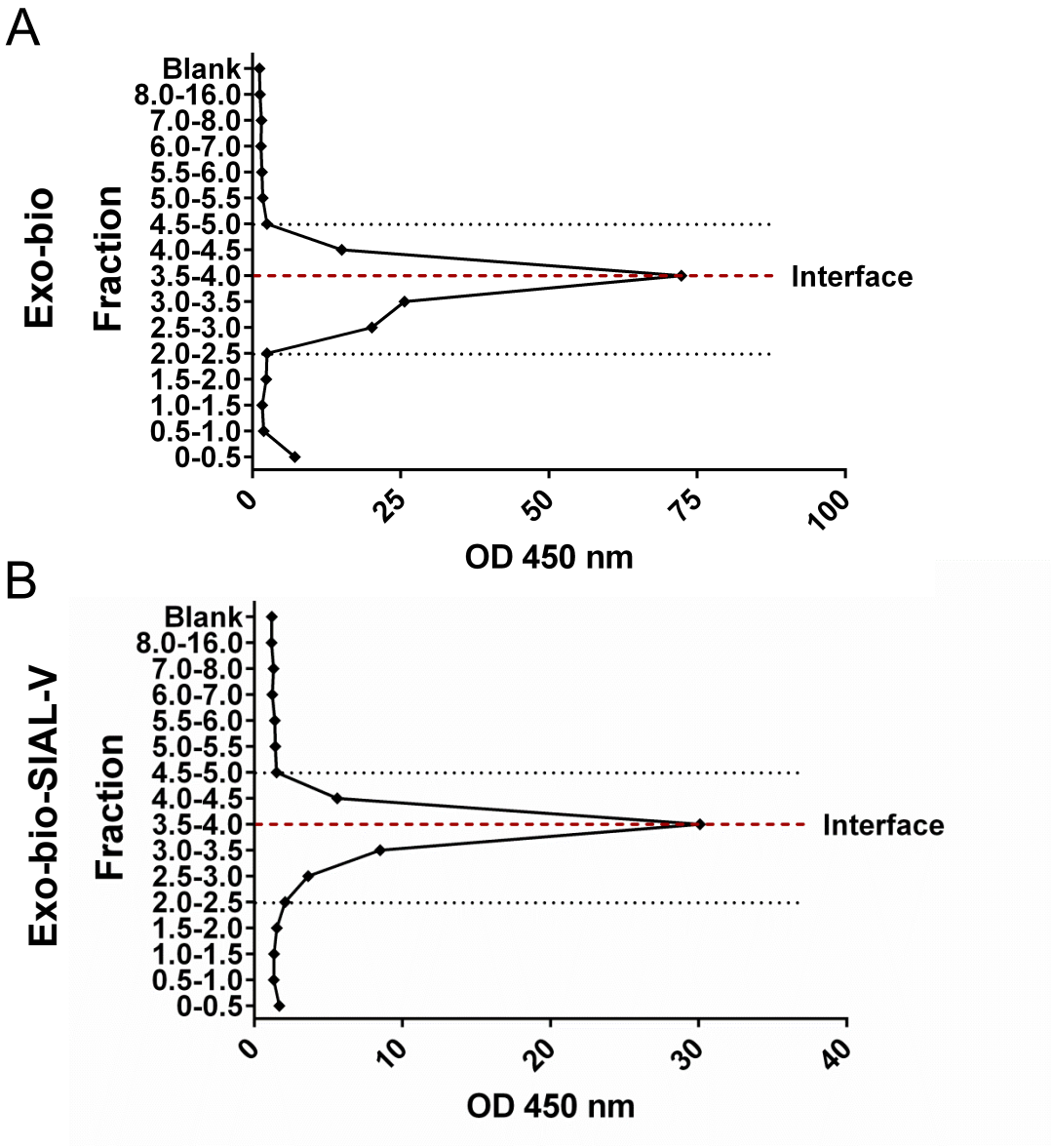

Figure S1: Sialidase treatment does not alter the buoyant density of exosomes. (A) Biotinylated or (B) biotinylated and SIAL-V treated exosomes were resuspended in 12 mL of PBS, overlaid onto 4 mL of 30% sucrose/Tris/D_2O cushion and ultracentrifuged for 75 min at 100 000 ×g (average). Fractions were removed as indicated and the pellet resuspended in the final 0.5 mL. Fractions were analyzed for MHC-II^+ exosome presence by ELISA. Briefly exosomes were captured with anti-MHC-II and biotin (on exosomes) detected with streptavidin-HRP. Following TMB addition, the optical density (450 nm) was measured. Red dashed line indicates the interface position; black dotted line indicates fractions above and below the interface that were pooled for use.

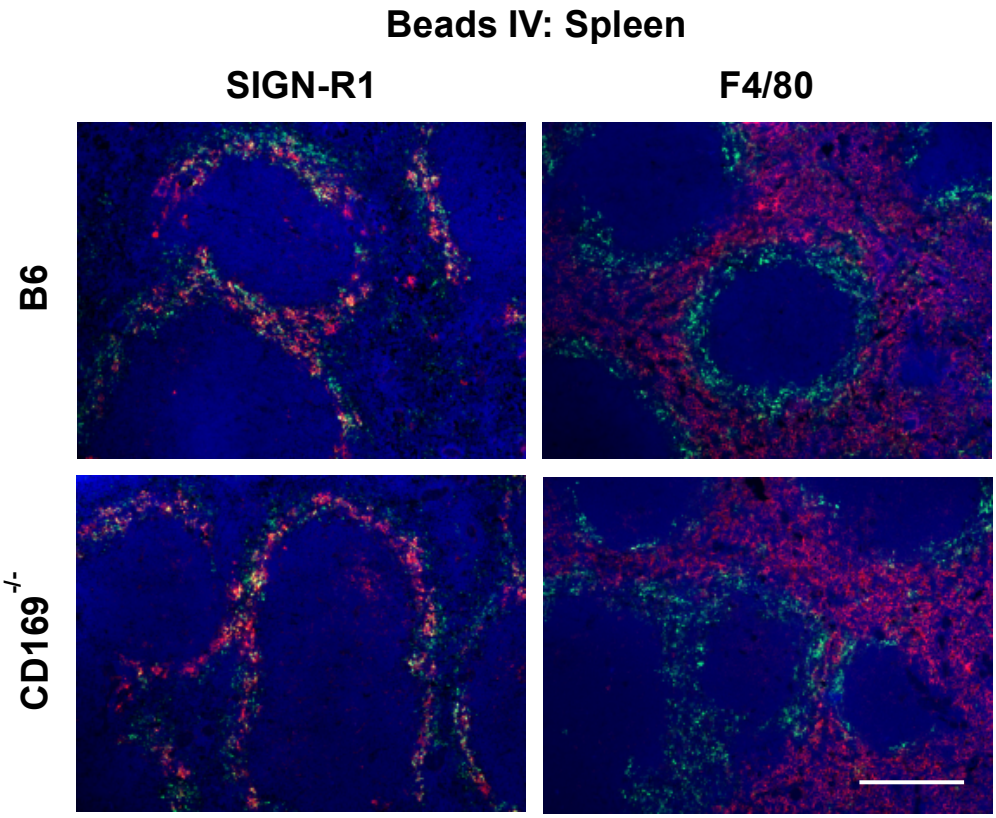

Figure S2: Non-sialylated beads are preferentially bound by SIGN-R1 macrophages in the spleen. C57BL/6 or CD169$^{-/-}$ mice were intravenously injected with 2 × 10^{11} 100 nm fluorescent microspheres (green), sacrificed at five minutes and spleens harvested. Sections were co-labeled for MZ or red pulp macrophages with anti-SIGN-R1 (ER-TR9) or anti-F4/80 respectively. Primary antibodies were detected with anti-rat IgG-Alexa-594 (red) and nuclei counterstained with DAPI (blue). Original magnification ×200. Bar represents 200 μm. Results representative of four mice per group.

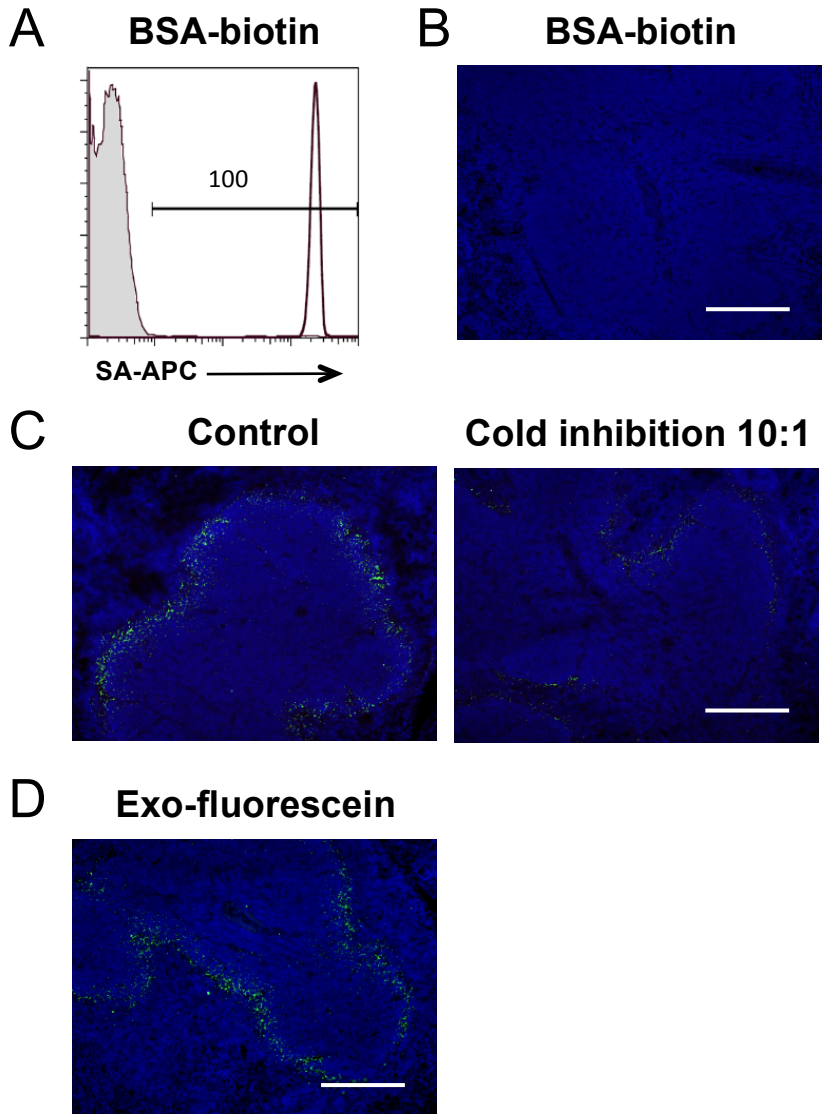

Figure S3. Biotin does not mediate *in vitro* binding of exosomes to the marginal zone. (A) BSA was biotinylated and the biotin level determined by binding to aldehyde-sulfate beads. Biotin was then detected with streptavidin-APC via flow cytometry (bold line). Shaded peak indicates negative control (non-biotinylated BSA). (B) 50 µg/mL biotinylated BSA was incubated with a naïve C57BL/6 spleen section using a modified Stamper-Woodruff assay. Biotin was detected with streptavidin-Alexa-488 (green) and nuclei counterstained with DAPI (blue). (C) Naïve C57BL/6 spleen sections were incubated for two hours with 0.1% BSA/IMDM (control) or 500 µg/ml unlabelled 'cold' exosomes prior to the direct addition of 50 µg/ml biotinylated exosomes (final concentration; 10:1 ratio of cold-exo:exo-bio) for a further two hour incubation. Biotin and nuclei detected as described for panel B. (D) Exosomes were conjugated to NHS-fluorescein (green), before 50 µg/mL exo-fluorescein was incubated with naïve C57BL/6 spleen sections and nuclei counterstained with DAPI. Original magnification ×200. Bar represents 200 µm. Representative of two to three experiments.

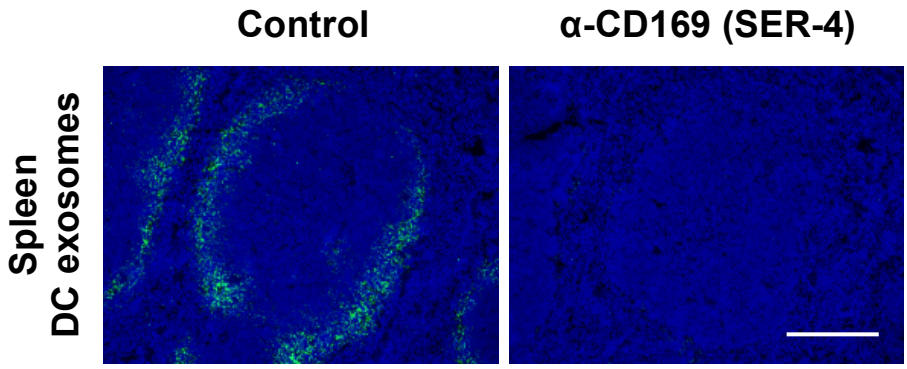

Figure S4: Dendritic cell-derived exosomes bind to the splenic marginal zone in a CD169 dependent manner. Biotinylated dendritic cell (DC) derived exosomes (50 μg/mL) were applied to naïve C57BL/6 spleen sections using a modified Stamper-Woodruff assay in the presence or absence of CD169 neutralizing antibody (SER-4). Biotin was detected with streptavidin-Alexa-488 and nuclei counterstained with DAPI. Original magnification ×200. Bar represents 200 μm. Representative of two experiments.