(**h**) Axis ratio of FO B cells migrating from FO to MZ at start and end of track (n= 24 cells). (**i-l**) Intravital TPLSM of MZ B cells following integrin blockade. (i) MZ B cell crossing from FO to MZ. (**j**) MZ B cell movement from MZ to RP. (**k**) Displacement versus square root of time (right) of MZ B cells in the MZ before (black) and two hours after (red) integrin blockade. (**l**) Superimposed 10-min tracks of randomly selected MZ B cells, in the x-y plane. Units are in micrometers. Data for I-L were from 8 experiments (3 mice). (**m**) Upper, 90μm z-projection view of WT (red) and S1PR1 KO (green) FO B cells in spleen. Lower, automated tracks of transferred B cells (white). Tracks of WT cells (11 red lines) and KO cells (1 green line) leaving FO are shown. (**n**) FO egress rate of WT and S1PR1 KO B cells. Open circles, MZ–FO interface determined based on PE-IC labeling; filled circles, interface determined based on FO B cell tracks. In e, g, I, j, elapsed time is in min:sec, arrowheads point to tracked cells and scale bar indicates 10μm. In f, h, n, bars or lines represent mean (error bars in f, ±SEM).

Supplementary Materials

Methods

Mice. 6-12 week old C57BL/6 (B6) mice were purchased from the National Cancer Institute. S1PR1fl/- mice (Dr. Richard Proia, National Institutes of Health) were crossed with Mb1Cre/+ mice (Dr. M. Reth, Max-Planck Institute of Immunobiology, Freiburg, Germany) to generate S1PR1fl/−Mb1Cre/+. B6 mice expressing enhanced green fluorescent protein (GFP; 004353; Tg(UBC-GFP)30Scha/J) were from Jackson Laboratories. CD19-/- mice were on a B6 background and were generated by intercrossing CD19cre/+ mice27 to obtain CD19cre/cre mice. Cr2-/- mice28 were on a B6 background. Animals were housed in a specific pathogen-free facility and all experiments were in accordance with protocols approved by the University of California San Francisco Institutional Animal Care and Use Committee.

B cell isolation, adoptive transfer and selective reconstitution of labeled MZ B cells. B cells were isolated with an AutoMACS (Miltenyi Biotech) using MACS microbeads and antibodies to CD11c and CD43 (Miltenyi Biotech). Purity was typically over 95%. To selectively label MZ B cells, 4x10^6 B cells from a Ub-GFP^{+} donor and 8x10^6 B cells from non-tg B6 mice were mixed and transferred to CD19$^{-/-}$ recipient mice for 8-12 wks. The phenotype of the cells remained constant across this ~1 month analysis window. For imaging of FO B cells, purified B cells from B6 or S1P1$^{fl/-}$Mb1Cre/+ were labeled with 10μM 5-(and-6)-(((4 chloromethyl)benzoyl)amino)-tetramethylrhodamine (CMTMR; Invitrogen) or carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen). In some experiments B cells were purified from GFP+ donors and used. Cells (~40x10^6) were transferred 24 hrs before imaging.

Flow cytometry, in vivo labeling and immunofluorescence of cryostat sections. MZ B cells were gated as B220+ or CD19+ CD23lo CD21/35hi and follicular B cells as B220+ or CD19+ CD23hi CD21/35int. CD19, B220, CD23, IgM, IgD, CD1d, CD86, CD38 and CD44 antibodies were from Biolegend. CD35 was from BD Biosciences. In vivo labeling of MZ B cells was as described29,30. Immunofluorescence analysis of spleen sections was as described31. Marginal metallophilic macrophages were detected with CD169-FITC antibody (AbD Serotec) and marginal zone macrophages with SIGN-R1 antibody (eBioscience) followed by anti-Armenian hamster Cy5 (Jackson Immunoresearch). FDCs were detected with anti-complement receptor-1 (CD35) antibody (Biolegend). For spleen sections containing GFP+ cells, spleens were fixed in 4% paraformaldehyde (Sigma-Aldrich) and stained with GFP antibody (Invitrogen) and DAPI (Sigma-aldrich).

PE-IC deposition on FDCs. PE-ICs were induced *in vivo* by passively immunizing mice with 2 mg polyclonal rabbit IgG anti-PE (200-4199; Rockland) followed 2 hrs later by injection of 20μg PE (P-801; Invitrogen Molecular Probes) as described[32]. After 16 hrs spleens were collected and frozen in OCT for immunofluorescence analysis.

MZ macrophage labeling *in vivo* for intravital TPLSM. We attempted to visualize the boundary between the MZ and FO by intravital labeling of the marginal sinus-lining cells with MAdCAM1-specific antibody Meca379[33]. However, the intensity of marginal sinus labeling was insufficient to allow detection in the intact spleen by TPLSM (not shown). Instead, we found that when mice were injected with Meca379 antibody (BioXcell) that had been biotinylated using biotin X-NHS (EMD Chemical) and mixed with streptavidin-PE (Bio-Rad Laboratories) in a 4:1 ratio, and were examined 2-6 hours later, there was prominent labeling of MZ macrophages (that lack MAdCAM1), while the $CD169^+$ marginal metallophillic macrophages that are mostly positioned on the follicular side of the marginal sinus[2,34] were unlabeled. Since the labeling achieved by the biotinylated antibody–PE-streptavidin complexes was similar to that by immune complexes[8,32], for simplicity we refer to this method as labeling with PE-ICs. Mice were injected with the premade PE-ICs (100μg) 2 hrs before the beginning of imaging. While this approach allowed detection of heavily PE-IC decorated MZ macrophages, it was of insufficient sensitivity to permit TPLSM detection of PE-IC bearing B cells in the spleen (not shown).

Integrin blockade assays. Anti-αL (clone M17/4, rat IgG2a) hybridoma was from American Type Culture Collection, and the anti-α4 (clone PS/2, rat IgG2b) hybridoma was provided by David Erle (University of California, San Francisco). Antibodies were injected i.v at 100μg per mouse for the indicated amounts of time followed by 5 min CD19-PE *in vivo* labeling as described before. In TPLSM experiments the antibodies were injected 15 min to 3 hr prior to image acquisition.

Surface expression of S1PR1 on MZ B cells in spleen and blood. Freshly harvested splenocytes from CD45.1+ donors (10x10^6 cells) were transferred into CD45.2+ recipients. S1PR1 expression on donor MZ B cells in the spleen was determined before transfer and in blood collected from recipients 1 hr after transfer. Expression of S1PR1 was detected by flow cytometry using a rat monoclonal antibody (mAb) (R&D Systems) followed by donkey anti–rat IgG

biotin (Jackson ImmunoReaserch) and streptavidin-APC (Invitrogen) as described[35,36]. Background S1PR1 stain refers to stained samples from mice that were intravenously injected with the S1PR1-modulating drug FTY720 (1mg/kg) 24 hr earlier[37].

Intravital two photon laser scanning microscopy (TPLSM) of spleen. Mice were anesthetized by intraperitoneal injection of 10ml/kg saline containing xylazine (1 mg/ml) and ketamine (5 mg/ml). Maintenance doses of intramuscular injections of 4 ml/kg of xylazine (1 mg/ml) and ketamine (5 mg/ml) were given every ~30 min. To expose the spleen, a skin incision was made below the costal margin in the left flank overlying the spleen and extended inferomedially. A ~1cm window was then made in the peritoneal cavity and the spleen was gently mobilized on its stalk with forceps and exteriorized without stretching or damaging the vessels in the hilum and the gastrosplenic ligament. After the spleen was exposed, the skin incision was partially closed with tissue glue (Vetbond) and the spleen was bathed in warm saline. A spring-loaded platform[38] was placed over the mouse and screwed down until the cover glass made contact with the spleen capsule. The spleen was kept almost sealed against the mouse body using the platform and attached coverslip, and the area around the spleen and in contact with the glass was kept filled with saline. The mouse was placed on a Biotherm stage warmer at 37°C (Biogenics) for the duration of the imaging. The temperature at the interface between the spleen and glass coverslip during and at the end of several imaging sessions was measured using a dual temperature controller (TC-344B, Warner Instruments) equipped with a CC-28 cable containing a bead terminator and was found to remain between 36-37°C. Images were acquired with ZEN2009 (Carl Zeiss) using a 7MP two-photon microscope (Carl Zeiss) equipped with a Chameleon laser (Coherent). For video acquisition a series of planes of 3μm z spacing spanning a depth of 50-150μm were collected every 15-30 sec. Excitation wavelengths were 870-890 nm. Emission filters were 500–550 nm for CFSE and GFP, and 570–640 nm for PE and CMTMR. The full longitudinal extent of the spleen was surveyed in each animal at depths of ~50-100μm and typically one or two white pulp cords were

identified that passed sufficiently near the capsule to permit imaging of cells in the MZ and FO. Videos were made and analyzed with Imaris×64 7.4.2 (Bitplane). To track cells, surfaces seed points were created and tracked over time. Tracks were manually examined and verified. Data from cells that could be tracked for at least 15 min were used for analysis. The velocities, turning angles, and displacement of cells between each imaging frame were analyzed using Imaris (Bitplane AG), MATLAB (MathWorks), and MetaMorph software. In figure 2d, graphs compare tracks that remained in the MZ or the FO during 25-30min of movie acquisition. MZ B cells that showed 'tethered oscillation' at the boundary (Movie S2) were not enumerated as crossing from one zone to the other as they did not travel a minimum of 10μm into the opposite compartment. Statistical analysis was performed using Prism software (GraphPad Software, Inc). Annotation and final compilation of videos were performed with After Effects 7.0 software (Adobe). Video files were converted to MPEG format with AVI-MPEG Converter for Windows 1.5 (FlyDragon Software).

Analysis of MZ B cells distribution before and after FTY720-treatment. MZ B cells were imaged intravitally for 25 min and then injected i.v. with 25μg FTY720. Five min after injection, imaging of the same region was resumed for an additional 50min. MZ B cells were identified and their positions were determined using automatic segmentation in Imaris software (BitPlane AG, Zurich). The coordinates of each cell were exported to a text file as comma separated values, and loaded into the R programming environment for analysis. For each timeframe, the center point of the population of cells was determined by taking the mean of the cell positions in x, y, and z dimensions. The distance of each cell position to its timeframe center point was calculated, and the mean of these distances for each timeframe were transferred to Microsoft Excel. The frame numbers from the movie were adjusted to reflect overall time within the experiment.

Axis ratio calculation. The long and short axis of the cells were measured in a single z plane via the line segment tool in Imaris software. Cells shape index was

then calculated as the ratio of the longer axis to the shorter axis. For axis ratio measurements of cells migrating from FO to MZ, each data point reflects the mean axis ratio of a single cell measured in the first (Start) or last (End) three frames of the track.

FO B cell egress. WT (CMTMR labeled, red) and S1PR1 KO (CFSE-labeled, green) B cells were co-transferred in to a WT recipient 24 hr prior to intravital TPLSM. Both B cell types were tracked and cells that travelled a minimum of 10μm into the MZ were scored. Each point corresponds to a single 30-60 min movie, open circles to experiments where the MZ–FO interface was determined based on PE-IC labeling (as in Figure 2) and filled circles to experiments where the interface was determined based on FO B cell tracks.

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