The splenic marginal zone is a unique microenvironment where resident immune cells are exposed to the open blood circulation. Even though it has an important role in responses against blood-borne antigens, lymphocyte migration in the marginal zone has not been intravascularly visualized due to challenges associated with achieving adequate imaging depth in this abdominal organ. Here we develop a two-photon microscopy procedure to study marginal zone and follicular B-cell movement in the live mouse spleen. We show that marginal zone B cells are highly motile and exhibit long membrane extensions. Marginal zone B cells shuttle between the marginal zone and follicles with at least one-fifth of the cells exchanging between compartments per hour, a behaviour that explains their ability to deliver antigens rapidly from the open blood circulation to the secluded follicles. Follicular B cells also transit from follicles to the marginal zone, but unlike marginal zone B cells, they fail to undergo integrin-mediated adhesion, become caught in fluid flow and are carried into the red pulp. Follicular B-cell egress via the marginal zone is spheroid-1-phosphate receptor-1 (S1PR1)-dependent. This study shows that marginal zone B cells migrate continually between marginal zone and follicles and establishes the marginal zone as a site of S1PR1-dependent B-cell exit from follicles. The results also show how adhesive differences of similar cells critically influence their behaviour in the same microenvironment.

Marginal zone B cells are a unique B-cell subset that have a pivotal role in mounting antibody responses against systemic pathogens. Early studies of marginal zone B cells in rodents showed that they are non-recirculating and restricted to the spleen. Marginal zone B cells were later found to have elevated integrin expression and to depend on integrins to be retained in the marginal zone. These observations indicated that the cells were of limited motility. Yet, marginal zone B cells mediate the delivery of opsonized antigens from marginal zone to the follicle and recent studies provided indirect evidence that marginal zone B cells continually exchange between the marginal zone and follicle. However, this cellular behaviour has not been directly visualized. To permit real-time imaging of marginal zone B cells, we developed a way to label these cells. Follicular B cells can give rise to marginal zone B cells and marginal zone B cells, but not follicular B cells, are self-renewing in the absence of input from less committed precursors. We therefore asked whether follicular B cells could selectively reconstitute the marginal zone of CD19-deficient mice that have an empty marginal zone B-cell niche, but a normal follicle compartment. Transfer of GFP B cells into CD19-deficient mice for 8 weeks allowed substantial reconstitution of the marginal zone B-cell compartment. Moreover, typically ~90% of the transferred GFP B cells had a marginal zone B-cell phenotype and like their normal counterparts, the reconstituted marginal zone B cells were poised to respond to antigen and lipopolysaccharide. To determine if the reconstituted marginal zone B cells were positioned correctly, we labelled blood-exposed cells by intravenous (i.v.) injection of a fluorescently conjugated antibody 5 min before tissue isolation. This analysis, as well as immunofluorescence microscopy, indicated that 50–60% of the marginal zone B cells were in the marginal zone, whereas the remaining cells were located in the follicles (Fig. 1c, d), similar to their distribution in wild-type mice. Moreover, the reconstituted mice were rescued in their ability to deposit an opsonized antigen on follicular dendritic cells (FDCs) over a 16-h period (Fig. 1e and Supplementary Fig. 1). Consistent with a direct role of the marginal zone B cells in the delivery of opsonized antigen to FDCs, reconstitution with Ccr2+/− marginal zone B cells failed to restore antigen delivery (Supplementary Fig. 1).

For intravital two-photon laser-scanning microscopy (TPLSM), CD19 knockout mice reconstituted with a mixture of GFP and non-labelled B cells were injected with red fluorescent phycoerythrin-immune complexes (PE–ICs) 2 h before imaging. Tissue section analysis established that PE–ICs were concentrated on SIGN-R1+ (specific intracellular adhesion molecule-grabbing nonintegrin R1, also known as Cd209b) marginal zone macrophages in the first hours after injection of ICs in vivo. This analysis explained their ability to deliver antigens rapidly from the open blood-exposed cells by intravenous (i.v.) injection of a fluorescently conjugated antibody 5 min before tissue isolation. This analysis, as well as immunofluorescence microscopy, indicated that 50–60% of the marginal zone B cells were in the marginal zone, whereas the remaining cells were located in the follicles (Fig. 1c, d), similar to their distribution in wild-type mice. Moreover, the reconstituted mice were rescued in their ability to deposit an opsonized antigen on follicular dendritic cells (FDCs) over a 16-h period (Fig. 1e and Supplementary Fig. 1). Consistent with a direct role of the marginal zone B cells in the delivery of opsonized antigen to FDCs, reconstitution with Ccr2+/− marginal zone B cells failed to restore antigen delivery (Supplementary Fig. 1).

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Figure 1 | Adoptive transfer system for GFP labelling marginal zone B cells. a, Frequency of CD35+CD23+ marginal zone B cells among B220+ cells in CD19+/− mice before (left) or 8 weeks after (right) transfer of GFP + B cells. Numbers indicate percentage of cells in each. b, Phenotype of CD19+ GFP + B cells from a, c, In vivo anti-CD19–PE labelling of marginal zone phenotype (CD35+CD23+) B cells. Number indicates percentage of labelled cells. d, Spleen section from mouse reconstituted with a 2:1 mixture of non-transgenic and GFP + B cells, stained with anti-GFP (green) and 4′,6-diamidino-2-phenylindole (DAPI, blue). Location of marginal sinus is indicated by the dashed white line. e, Spleen sections from the indicated mice that had received PE–IC (red) 16 h earlier, stained for CD169 (green) to label metachromatic marginal zone macrophages and CD35 (blue) to label FDCs. Scale bar, 100 μm.

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after injection, providing a means for locating this compartment (Fig. 2a). The spleen was surgically exposed, bathed in saline and stabilized by attachment to a platform placed over the mouse abdomen. Typically one or two white pulp cords per spleen passed sufficiently near the capsule to permit visualization (Supplementary Fig. 2). Marginal zone B cells were identified as being situated in the marginal zone or follicle based on whether their location overlapped with or was internal to the ring of PE–IC-labelled macrophages, respectively (Fig. 2b, c). Contours were drawn immediately internal to the PE–IC labelled cells in each z-plane and used to generate a three-dimensional surface (Fig. 2b) that approximated the position of the marginal sinus separating the marginal zone and follicle.

Marginal zone B cells within the marginal zone or follicle were migratory (Fig. 2c, d and Supplementary Videos 1 and 2). They travelled with similar velocities in both compartments, but cells within the marginal zone showed sharper turning angles and more deviation from movement in a straight direction in their migration paths (Fig. 2d), indicating a greater amount of confinement. Marginal zone B cells were larger than follicular B cells, as expected, and they exhibited a probing, dendritic morphology (Fig. 2e and Supplementary Videos 1 and 2) reminiscent of that seen for germinal centre B cells. In some cases (~20%) the marginal zone B cells exhibited trailing processes. A rate of 120–150 μm/min was observed for cells located in the marginal zone and follicle.

Treatments with FTY720 to disrupt S1PR1 function caused marginal zone B cells to leave the marginal zone and locate within the follicle. Analysis of this repositioning at four time points using CD19–PE labelling of blood-exposed spleen cells suggested that it was complete within 30 min (Fig. 2f and Supplementary Fig. 3). A similar rate of marginal zone B-cell relocation was observed by TPLSM (Fig. 2g, Supplementary Fig. 3 and Supplementary Video 3), indicating that the same behaviour also occurred during the intravitral imaging procedure.

To examine the rate of marginal zone B-cell movement between marginal zone and follicle in untreated mice, the tracks of cells that crossed between zones were manually annotated and counted (Fig. 3). Marginal zone B cells moving from the marginal zone across the boundary into the follicle were readily observed (Fig. 3a and Supplementary Video 4). Marginal zone B cells could also be seen migrating from the follicle to the marginal zone (Fig. 3b and Supplementary Video 5). Similar observations were made when the boundary between the follicle and marginal zone was defined using transferred follicular B cells rather than by PE–IC labelling (Supplementary Fig. 4), indicating that the migratory behaviour of marginal zone B cells was not a consequence of exogenous immune complex exposure. This analysis showed that at least 10% of the marginal zone cells that were tracked during a 30-min imaging session moved from the marginal zone to follicle, and a similar fraction of the marginal zone cells that started in the follicle moved to the marginal zone (Fig. 3c). On some occasions during passage across the boundary, the marginal zone B cells paused (Supplementary Video 4, example 1, and Supplementary Video 5) and sometimes they moved parallel to the surface before crossing (Supplementary Video 4, example 2). During the crossing event, some cells showed an obvious constriction of the cell body (Fig. 3a, Supplementary Fig. 4b lower panel, and Supplementary Video 4, example 2). We also observed several cells that remained tethered near the marginal zone–follicle interface by a

**Figure 2 | Marginal zone B cells are migratory and exhibit long membrane processes.** a, Spleen section showing PE–IC (red), SIGN-R1 (blue) and CD169 (green) distribution 2 h after PE–IC injection. White dotted line indicates the location of the marginal zone sinus. Scale bar, 50 μm. b, Generation of surface corresponding to the interface between the marginal zone and follicle. Left image shows an example of a contour drawn ~10 μm internal to the PE–ICs to represent the boundary in a single x–y slice (3 μm). Middle images show contours drawn for each slice in the 60 μm z-stack. Last image on the right shows the final surface with overlaid PE–IC stain. Scale bar, 50 μm. c, TPLSM of GFP⁺ marginal zone B cells in reconstituted Cid19γ⁻/⁻ spleen. Left panel shows a 57 μm z-projection view. MZ, marginal zone. Middle panel shows a 30-μm slice from the centre of this region. FO, follicle. White dotted line indicates location of the marginal sinus. Right panel, representative classification of marginal zone B-cell tracks based on positioning with respect to surface. Pink, in marginal zone; blue, in follicle. d, Median velocity, distribution of turning angles and straightness of migration path of marginal zone B cells (n = 5 data sets from 3 mice). Straightness was calculated as a ratio of the total distance travelled divided by the displacement (difference between the initial and final position) for the first 5 min of each track. e, Time-lapse images of two marginal zone B cells (middle and bottom panels) compared with a follicular B cell (top panel). All cells are GFP⁺. Scale bars, 10 μm. f, Time in min indicated on the panels. g, Kinetics of marginal zone B-cell displacement into the follicle following FTY720 treatment. f, Frequency of in vivo anti-CD19–PE labelled marginal zone B cells at the indicated time after FTY720 injection (n = 6 mice), detected by flow cytometry. Circles indicate individual data points and the bar indicates the mean. g, Average distance (micrometres) over time of GFP⁺ marginal zone B cells from the most central point of the follicle during TPLSM imaging. First red arrow at 25 min, time of FTY720 injection; second red arrow, time of anaesthetic reinjection. In c, d and f, bars or lines indicate means, error bars are s.e.m., ***P < 0.0005 and NS, not significant (P > 0.05) by unpaired Student’s t-test.

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membrane process while the cell body moved back and forth between zones (Supplementary Video 2, yellow dashed circle).

As an independent approach to estimate the rate of marginal zone B-cell movement from the follicles to marginal zone, in this case at the level of the whole spleen, we took advantage of the essential role of integrins in mediating marginal zone B-cell retention in the marginal zone. Treatment with integrin-blocking antibodies causes selective loss of cells from the marginal zone while not displacing cells that are situated within follicles (Fig. 4a). Using this approach, loss of marginal zone B cells from follicles would occur over time as cells move from the follicle into the marginal zone. Mice were treated with integrin-neutralizing antibodies and the rate of marginal zone B-cell decay from follicles was determined by enumerating the in vivo CD19–PE unlabelled marginal zone B cells remaining in the spleen over time. The decay rate matched first order kinetics with a $t^{1/2}$ of around 2.5 h (Fig. 3d) consistent with the estimate from TPLSM of 20% exchange between the follicle and marginal zone per hour.

An exchange rate of 20% per hour indicates that some marginal zone B cells remain within the marginal zone for several hours. S1PR1 is required for marginal zone B cells to remain in the marginal zone and when it is down-modulated by treatment with FTY720 the cells relocalize into the follicle within around 30 min (Fig. 2f, g). The level of S1PR1 on marginal zone B cells in the spleen was higher than on marginal zone B cells that had been transferred into blood for 1 h (Fig. 3e) and was more similar to cells exposed in vitro to low nM sphingosine-1-phosphate concentrations (Supplementary Fig. 5). Red blood cells, the main source of sphingosine-1-phosphate in blood, were detectable in the marginal zone but were sparse compared to their density in blood vessels and in the red pulp (Supplementary Fig. 5). A lower interstitial sphingosine-1-phosphate concentration in the marginal zone may cause a more gradual or less complete S1PR1 down-modulation than occurs on cells in circulatory blood, enabling a dwell time of several hours in the marginal zone.

We next examined the migration dynamics of follicular B cells. Intravital TPLSM of the spleen a day after intravenous transfer of follicular B cells showed a marked concentration of the cells within follicles (Fig. 4a). The follicular B cells migrated with a similar speed to marginal zone B cells and the two types of cell showed similar displacement over time while migrating within the follicle (Fig. 4b). As well as cells confined to follicles, transferred B cells could be visualized in the adjacent red pulp (Fig. 4c). In many cases the cells appeared to be moving in a straight path away from the follicle (Fig. 4c). B cells within the red pulp had a more rounded morphology than cells within the follicle and their axis ratio was approximately 30% reduced (Fig. 4d). In contrast to the active migratory behaviour of B cells within the follicle, many B cells within the red pulp failed to show evidence of active migration, but were instead intermittently stationary or fast moving (Fig. 4e and Supplementary Video 6). Occasionally during a fast-moving step the cell would disappear from view, possibly indicating that it had passed into a red pulp venule to be flushed from the spleen (Supplementary Video 6). Manual tracking of 550 red-pulp B cells showed that although many were stationary during the imaging period, threefold more cells appeared to move passively (fast and tangentially) versus actively (slow and meandering) (Fig. 4f).

The pathway by which follicular B cells exit from splenic follicles is not defined. One possible route these cells might take is by crossing the marginal zone sinuses, the path taken by marginal zone B cells. In agreement with this hypothesis, tracking follicular B-cell migration with respect to the surface generated using PE–IC-labelled marginal zone macrophages allowed the identification of cells migrating into the marginal zone (Fig. 4c, g and Supplementary Fig. 6a). Frequently upon entering this region, the follicular B cells underwent a rapid linear movement, perhaps a consequence of being caught in a region of flow (Fig. 4g, Supplementary Fig. 6a and Supplementary Video 7). The cells were usually retained moments later; some cells then appeared non-migratory during the rest of the imaging session (Supplementary Video 7, example 1), whereas others did continue to move (Supplementary Video 7, example 3). Axis ratio measurements of cells that crossed between zones showed that wild-type follicular B cells promptly became rounded after crossing into the marginal zone (Fig. 4h).

To test whether the striking difference in marginal zone and follicular B-cell behaviour within the marginal zone was a consequence of integrin-mediated adhesion, we examined the behaviour of GFP⁺ marginal zone B cells in reconstituted CD19 knockout mice in the first hours after treatment with integrin-neutralizing antibodies. Under these conditions, marginal zone B cells in the marginal zone frequently ceased active migration, became rounded and then moved fast and tangentially in the direction of the red pulp (Fig. 4i–l, Supplementary Fig. 6 and Supplementary Video 8). This change in behaviour was associated with a sharp increase in displacement over time (Fig. 4k, l). By contrast, marginal zone B cells within the follicle continued to migrate and they maintained their long membrane extensions (Supplementary Fig. 6), although their displacement over time was slightly reduced (Fig. 4l and data not shown), as observed for integrin-deficient cells in lymph nodes.

Although S1PR1 and sphingosine-1-phosphate have been argued to have a role in lymphocyte egress from the spleen, this conclusion has been based on indirect assessments, and it has also been suggested that the spleen is distinct from other lymphoid organs in not being sensitive to egress inhibition by FTY720 (refs 22, 23). This lack of clarity arises in part because both cell entry to and exit from the spleen occur via the blood and because the S1PR1-dependent egress step has not been visualized. To test whether S1PR1 was required for follicular B-cell movement from follicle to marginal zone, we co-transferred fluorescently labelled wild-type and S1PR1-deficient B cells into wild-type recipients and performed TPLSM (Fig. 4m). Plotting the tracks of all
Figure 4 | Splenic follicular B-cell migration and S1PR1 requirement for exit. a, Fifty-four micrometre z-projection view showing transferred B cells (green-yellow) and their tracks (white lines) and PE-IC-labelled macrophages (red). Scale bar, 50 μm. b, Instantaneous velocities (left) and displacement versus square root of time (right) of follicle and marginal zone B cells in the follicle. Data from at least 5 or 6 experiments (3 or 4 mice). Forty-five micrometre z-projection view of follicular B cells (green) in follicle and red pulp (RP). Red line, marginal zone–follicle border; white lines, tracks of follicular B cells in the follicle; time-coded coloured lines (labelled as blue at the start and progressing to white at the end of the path), tracks of follicular B cells in red pulp; blue lines, follicular B cells in transition from the follicle to the marginal zone. Tracks of cells that were outside the follicle during the entire movie are highlighted with a white surface. Scale bar, 40 μm. d, Axis ratio of follicular B cells in the follicle and red pulp. e, Follicular B-cell movement in the red pulp. f, Percentage of cells in red pulp exhibiting stationary (static), rapid (fast) or migratory (slow) behaviour (5 experiments in 3 mice, n = 550 cells). g, Follicular B cell crossing from follicle to marginal zone. Grey surface, marginal zone–follicle interface. h, Axis ratio of follicular B cells migrating from follicle to marginal zone at the start and end of the track (n = 24 cells). i–l, Intravitral TPLSM of marginal zone B cells following integrin blockade. i, Marginal zone B cell crossing from follicle to marginal zone. j, Marginal zone B-cell movement from marginal zone to red pulp. k, Displacement versus square root of time (right) of marginal zone B cells in the marginal zone before (black) and two hours after (red) integrin blockade. l, Superimposed 10-min tracks of randomly selected marginal zone B cells. The tracks show the path of each representative cell relative to the point of origin (00) in the x–y plane. Units are in micrometres. Plus or minus indicates migration in a particular direction. Data for i–l were from eight movies obtained from three mice. m, Upper image, 90 μm z-projection view of wild-type (red) and S1pr1 knockout (KO) (green) follicular B cells in spleen. Lower image, automated tracks of transferred B cells (white). Tracks of wild-type cells (11 red lines) and S1pr1 knockout cells (single green line) leaving the follicle are shown. n, Follicle egress rate of wild-type and S1pr1 knockout B cells. Open circles, marginal zone–follicle interface determined based on PE–IC labelling; filled circles, interface determined based on follicular B cell tracks. In e, g, i and l, elapsed time is in mins, arrowheads point to tracked cells and scale bar denotes 10 μm. In d, f, h and n, bars or lines represent the mean (error bars in f denote ± s.e.m.). In d, h, n, **P < 0.005; ***P < 0.0005 by unpaired Student’s t-test.
them to follicular dendritic cells (FDCs) while migrating in the follicles. Remarkably, marginal zone B cells exhibit similar dynamics while resisting shear and migrating in an integrin-dependent fashion in the marginal zone and when moving in a largely integrin-independent fashion in the follicle. Precedent for a single cell type migrating with similar migration characteristics in an integrin-dependent and -independent manner is provided by findings using an in vitro system with dendritic cells44. The factors promoting formation of the long trailing cellular processes exhibited by many marginal zone B cells are unclear, but these membrane extensions may facilitate interactions with natural killer T cells25 or with follicular B cells scanning for surface-displayed antigens26. Follicular B cells have lower integrin-adhesive activity than marginal zone B cells6 and our data indicate that upon passage into the marginal zone they are unable to activate sufficient integrin activity to resist the local shear forces of blood flow and they become rounded and travel passively into the red pulp. As well as defining an S1PR1-dependent pathway of B-cell egress from the spleen, these results highlight how differences in the extent of adhesive interactions profoundly affect cell behaviour in the same microenvironment.

METHODS SUMMARY

Intravital imaging of marginal zone and follicular B cells in the spleen. B cells from Ub-GFP−/− (4 × 10^6) or non-transgenic (8 × 10^6) mice were co-transfered to Cd19−/− recipient mice for 8–12 weeks. For imaging follicular B cells, purified B cells (~40 × 10^6) from B6 or Sipri−/− × B6F1−/− mice were labelled with fluorescent dye and transferred 24 h before imaging. To label marginal zone macrophages, mice were injected with PE–IgC 2 h before imaging. All imaging experiments were done intravitaly using two-photon laser-scanning microscopy. To prepare for imaging, mice were anaesthetized17, a skin incision was made below the costal margin and the intravitally using two-photon laser-scanning microscopy. To prepare for imaging, mice were anaesthetized17, a skin incision was made below the costal margin and the intravitally using two-photon laser-scanning microscopy. To prepare for imaging, mice were anaesthetized17, a skin incision was made below the costal margin and the intravitally using two-photon laser-scanning microscopy.

IgM and IgG antibodies were injected together with fluorescent dyes. To study the distribution of the FDC network, fluorescently labelled and non-labelled FDCs were used in combination. For the analysis of IgM-containing immune complexes on follicular dendritic cells, Int. Immunol. 16, 1411–1422 (2004).

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METHODS

Mice. 6–12-week-old C57BL/6 (B6) mice were purchased from the National Cancer Institute. Some mice were provided by D. Erle. Antibodies were injected intravenously at 100 μg/kg26 24 h before28. Intravital two-photon laser-scanning microscopy (TPLSM) of spleen. Mice were anesthetized by intraperitoneal injection of 10 ml/kg sodium 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 approximately every 30 min. To expose the spleen, a skin incision was made below the costal margin in the flank overlying the spleen and extended inferiorty by ~1 cm 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 gastroepiploic 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 platform27 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 Biotronix stage warmer at 37 °C (Biocins) 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 3 μm z-spacing spanning a depth of 50–150 μm were collected every 15–30 s. Excitation wavelengths were 870–890 nm. Emission filters were 500–550 nm for CFDA-SE and GFP, and 570–640 nm for PE and CMTMR. The full extent of the spleen was surveyed in each animal at depths of ~50–100 μm and typically one or two white pulp cords were identified that passed sufficiently near the capsule to permit imaging of cells in the marginal zone and follicle. Videos were made and analysed with Imaris 7.4.2 × 64 (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 analysed using Imaris (Bitplane AG), MATLAB (MathWorks), and MetaMorph software. In Fig. 2d, graphs compare tracks that remained in the marginal zone or the follicle during 25–30 min of video acquisition. Marginal zone B cells that showed ‘ethered oscillation’ at the boundary (Supplementary Video 2) were not enumerated as crossing from one zone to the other as they did not travel a minimum of 10 μm into the opposite compartment. Statistical analysis was performed using Prism software (GraphPad Software). 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 (HyDragon Software).

Analysis of marginal zone B cells distribution before and after FTY720-treatment. Marginal zone B cells were imaged intravital for 25 min and then injected intravenously at 2 μg FTY720. Videos were made and analysed 2 h after treatment. The same region was resumed for an additional 50 min. Marginal zone B cells were identified and their positions were determined using automatic segmentation in Imaris software (BitPlane AG). 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 time frame, the centre 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 time frame centre point was calculated, and the mean of these distances for each time frame were transferred to Microsoft Excel. The frame numbers from the movie were adjusted to reflect overall time during the experiment.

Axis ratio calculation. The long and short axes 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 to the shorter axis. For axis ratio measurements of cells migrating from the follicle to the marginal zone, each data point reflects the mean axis ratio of a single cell measured in the first (start) or last (end) 3 frames of the track.

Follicular B-cell egress. Wild-type (CMTMR-labelled, red) and S1pr1 knockout (CISE-labelled, green) B cells were transferred into a wild type recipient 24 h before imaging. TPLSM. B-fractions were transferred via the tail vein in ~1 cm window that 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 gastroepiploic 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 platform27 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 Biotronix stage warmer at 37 °C (Biocins) 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 3 μm z-spacing spanning a depth of 50–150 μm were collected every 15–30 s. Excitation wavelengths were 870–890 nm. Emission filters were 500–550 nm for CFDA-SE and GFP, and 570–640 nm for PE and CMTMR. The full extent of the spleen was surveyed in each animal at depths of ~50–100 μm and typically one or two white pulp cords were identified that passed sufficiently near the capsule to permit imaging of cells in the marginal zone and follicle. Videos were made and analysed with Imaris 7.4.2 × 64 (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 analysed using Imaris (Bitplane AG), MATLAB (MathWorks), and MetaMorph software. In Fig. 2d, graphs compare tracks that remained in the marginal zone or the follicle during 25–30 min of video acquisition. Marginal zone B cells that showed ‘ethered oscillation’ at the boundary (Supplementary Video 2) were not enumerated as crossing from one zone to the other as they did not travel a minimum of 10 μm into the opposite compartment. Statistical analysis was performed using Prism software (GraphPad Software). 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 (HyDragon Software).

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