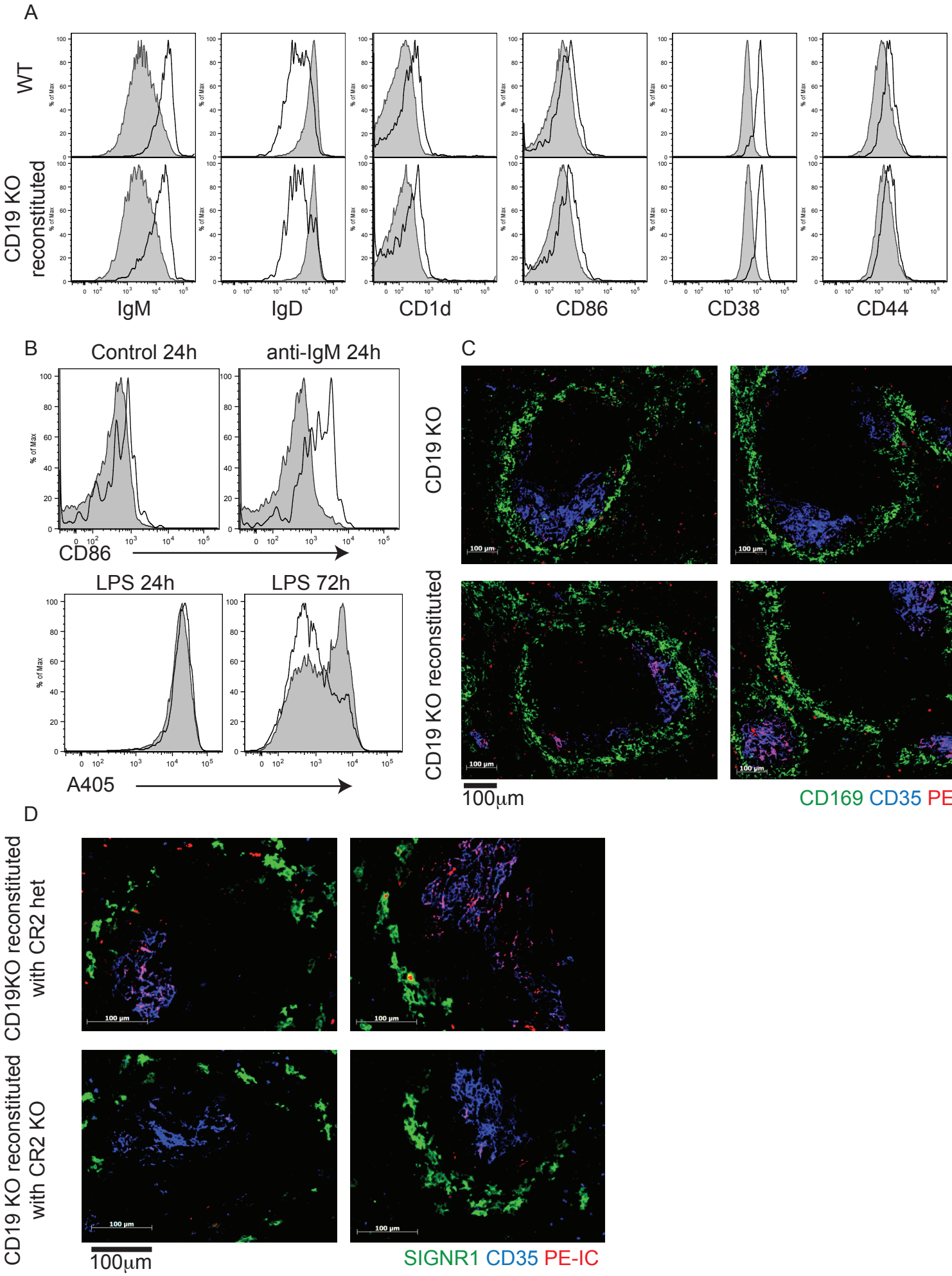
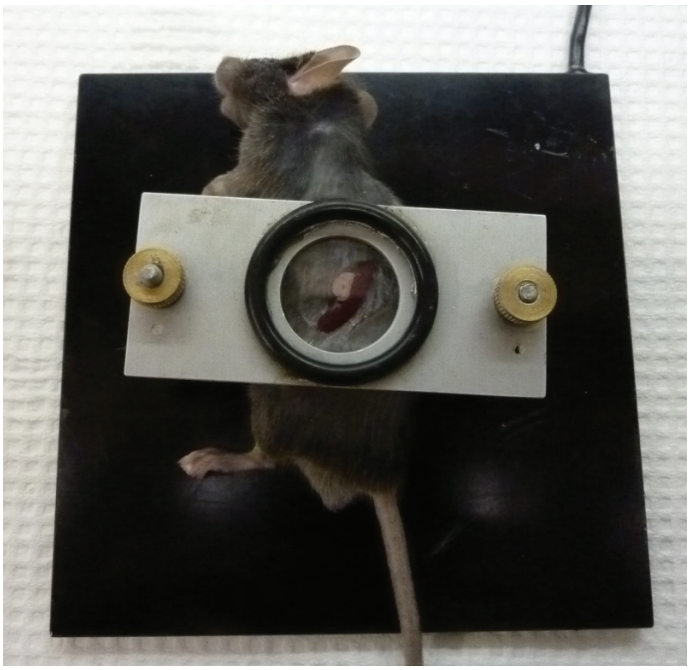


Suppl. Figure S1

Supplementary Figure 1. Phenotypic and functional characterization of reconstituted MZ B cells. CD19 KO mice were reconstituted with B cells from wild-type mice for a period of between 8 and 12 weeks. (A) Comparison of surface marker expression on unstimulated $CD19^{+}CD21^{hi}CD23^{lo}$ B cells (open histograms) from spleens of WT and reconstituted CD19 KO mice compared to $CD19^{+}CD21^{lo}CD23^{hi}$ FO B cells (shaded histograms). (B) Comparison of CD86 expression at 24 hr after anti-IgM stimulation (20ug/ml) and dilution of cell tracer violet label due to cell proliferation at 72 hr after LPS stimulation (1ug/ml) in MZ B cells ($CD19^{+}$ GFP^{+}, open histograms) versus FO B cells ($CD19^{+}$ GFP^{-}, shaded histograms) from CD19 KO reconstituted mice. (C) Additional examples of spleen sections from CD19 KO and reconstituted mice that had received intravenous PE-IC (red) 16hr earlier, stained with CD169 to detect marginal metallophilic macrophages (green) and with CD35 to detect FDCs (blue). (D) Analysis of PE-IC deposition on FDCs as in C but with CD19 KO mice reconstituted with control ($Cr2^{+/-}$) or $Cr2^{-/-}$ MZ B cells.

A



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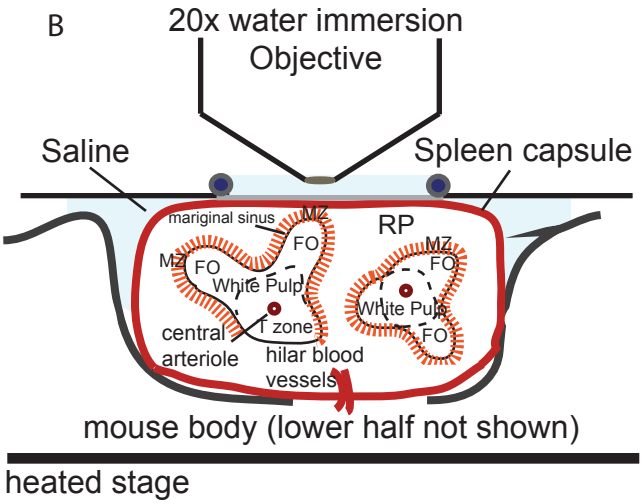
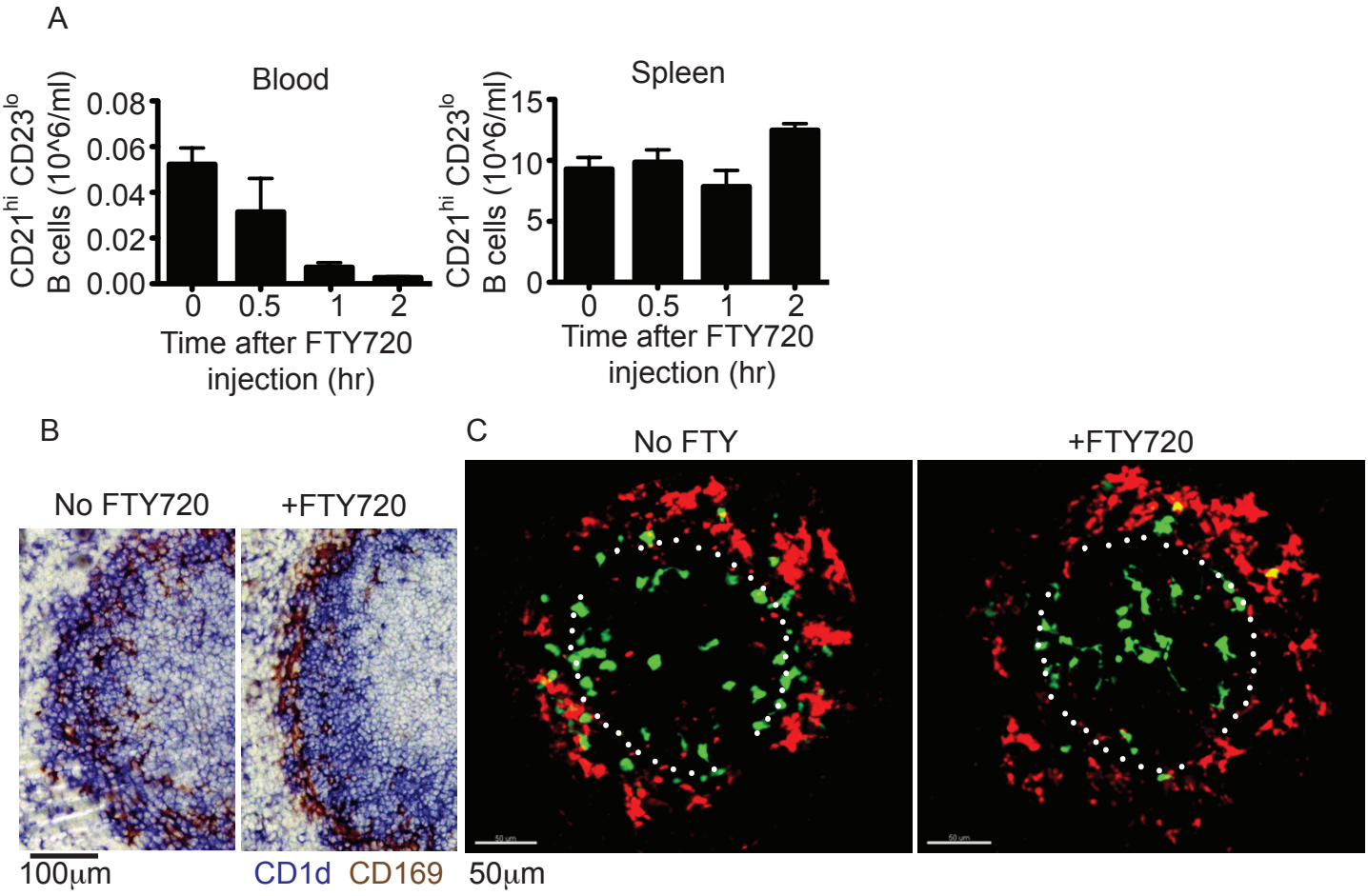
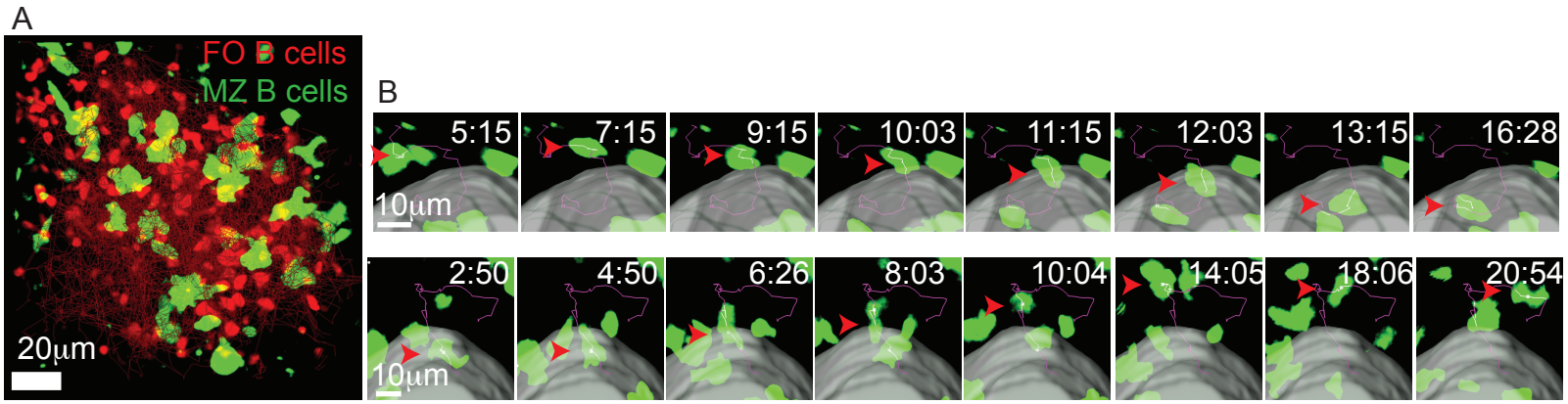


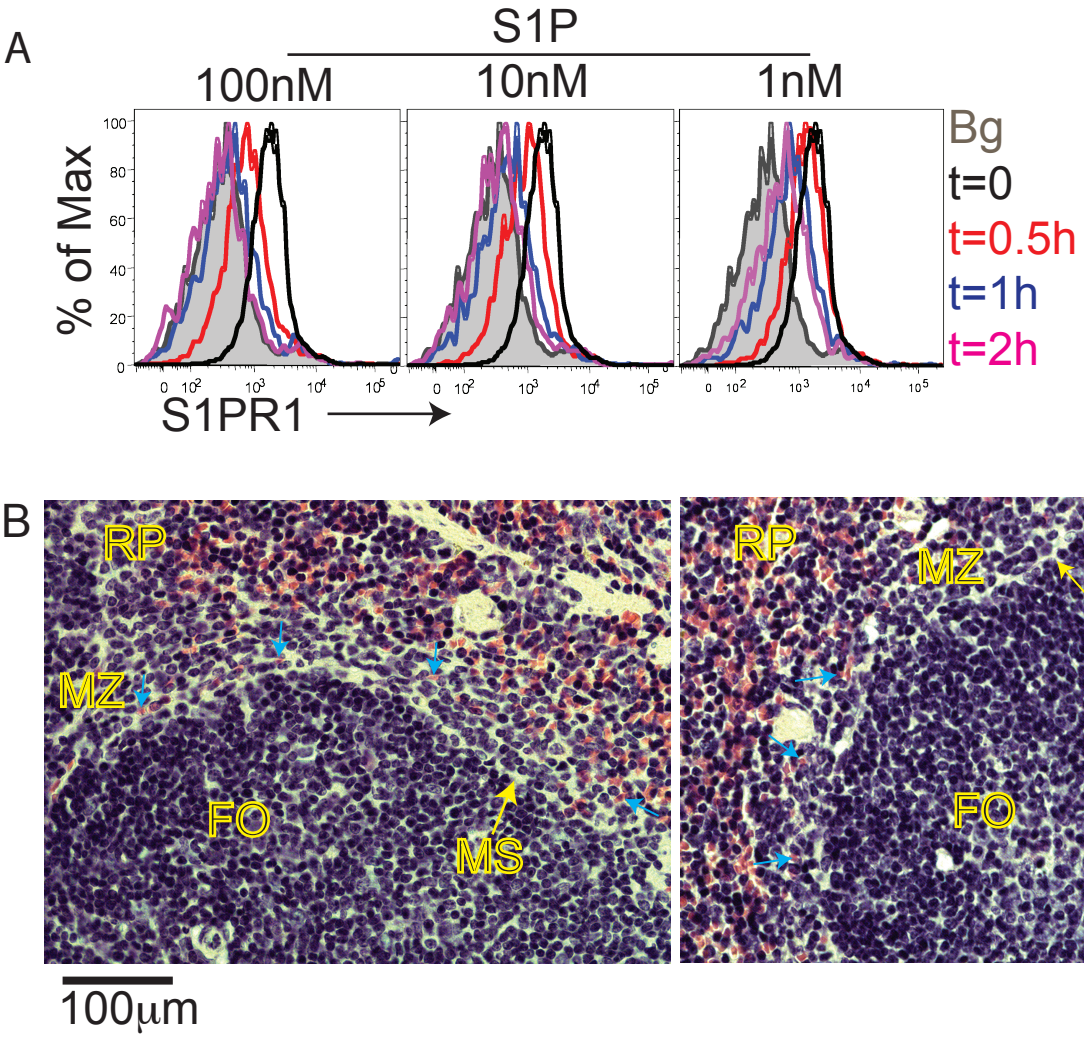
Supplementary Figure 2. Imaging platform and schematic cross-sectional view of imaged spleen region. (A) An anesthetized mouse is placed on a heated stage (37°C) and a skin incision is made below the costal margin in the left flank overlying the spleen. A small (~1cm) cut is made in the peritoneal cavity and the spleen is gently mobilized on its stalk exposing the splenic hilum. A spring-loaded platform containing an imaging chamber including a cover glass sealed with an O-ring is placed over the mouse and screwed down until the cover glass is in contact with the spleen capsule. A small amount of saline is trapped between the coverglass and mouse body, bathing the exposed spleen. (B) Cross-sectional view of the spleen as positioned for imaging. The exposed spleen is scanned at depths of ~50-100µm to identify white pulp cords that are sufficiently close to the capsule to allow imaging of marginal zone (MZ) and follicles (FO). The main structures and regions are labeled.



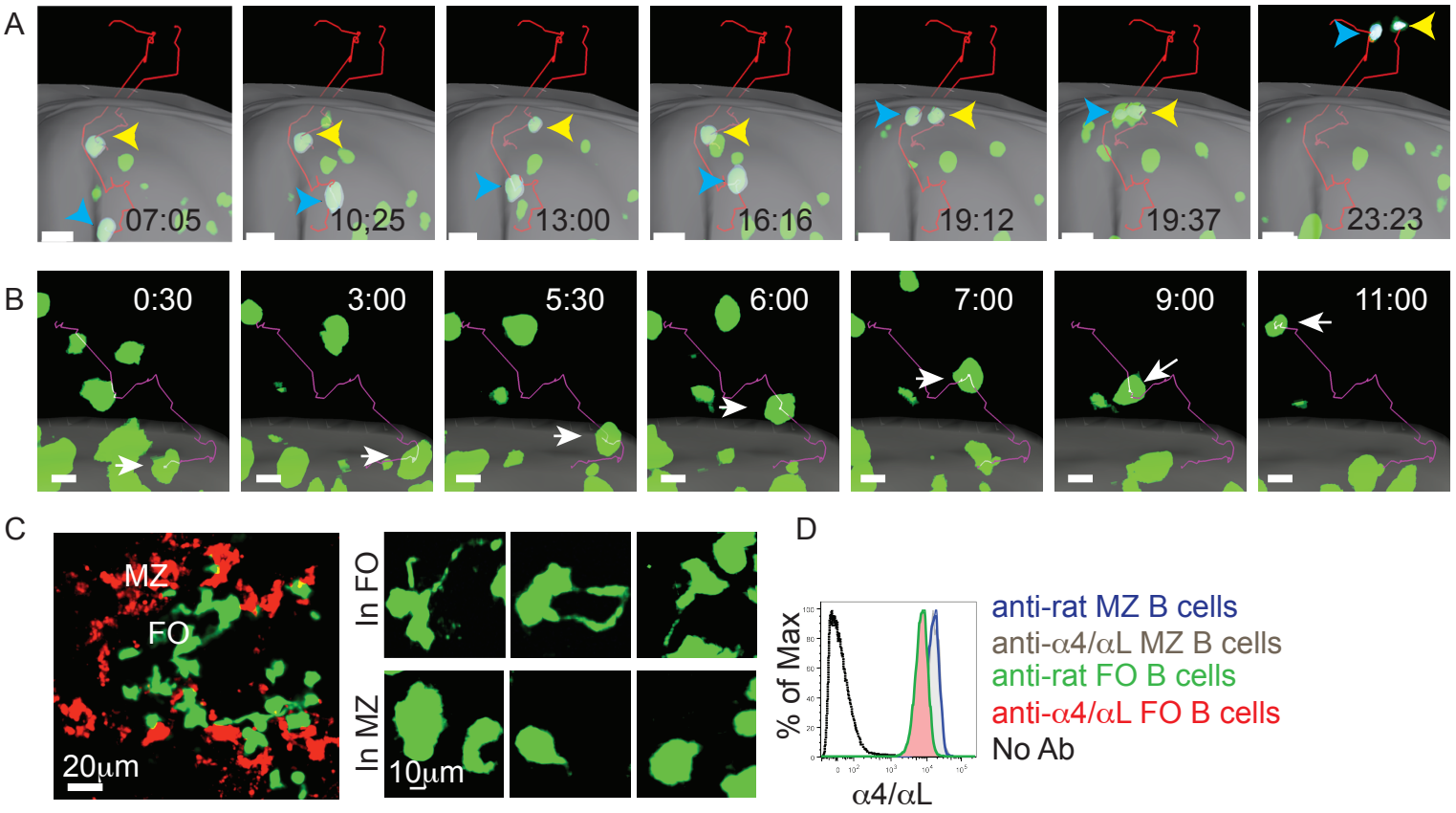
Supplementary Figure 3. Effect of FTY720 treatment on MZ B cell distribution. (A) Number of CD21^{hi}CD23^{lo} B cells in blood and spleen at the indicated time points after FTY720 treatment. Note that FTY720 treatment did not lead to o release of MZ-phenotype B cells into circulation or cause any change in their number within the spleen. (B) Distribution of CD1d^{hi} B cells in the spleen before and 12 hr after FTY720 treatment, detected by immunhistochemical staining. CD1d, blue; CD169, brown. Images show a single FO and MZ and are representative of multiple views from at least 3 mice. (C) TPLSM of MZ B cells and PE-IC labeled MZ macrophages in the spleen of GFP+ MZ B cell reconstituted CD19 KO mice, before (No FTY) or 1hr after treatment with FTY720. Panels show 33μm maximum intensity *z*-projection views of follicles of similar size. White dotted line indicates the approximate position of the marginal sinus.



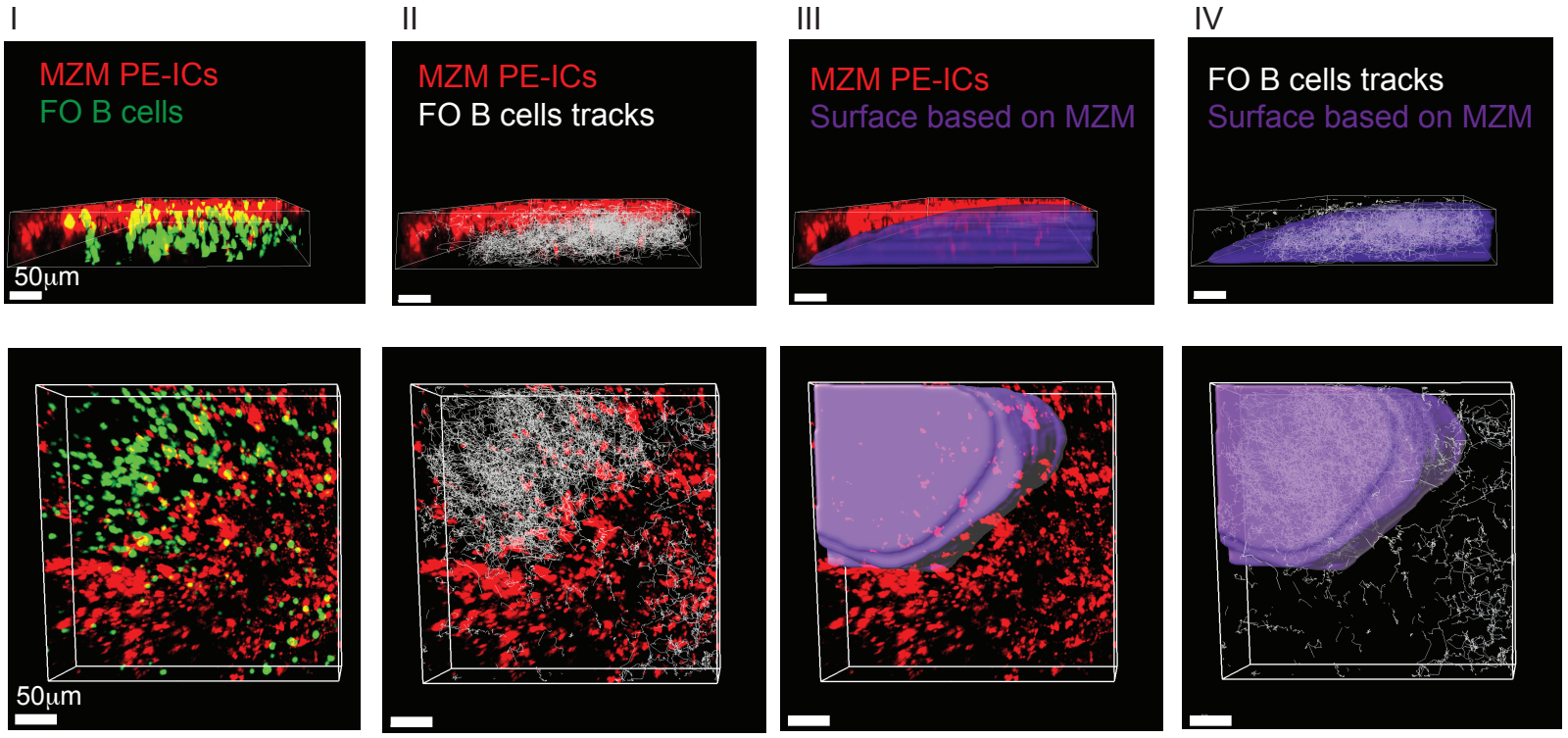
Supplementary Figure 4. MZ B cell movement between zones in the absence of PE-IC labeling. (A) 99μm maximum intensity *z*-projection view showing GFP-tg MZ B cells (green) in the spleen of a reconstituted CD19 KO mouse that received purified FO B cells (S1PR1 KO, CMTMR labeled, red) 24 hr prior to intravital TPLSM. ! utomated tracks of the FO B cells are shown in the red lines. These tracks were used to generate a surface (shown in B) corresponding to the FO–MZ interface. (B) Examples of MZ B cells migrating from MZ to FO (upper) and from FO to MZ (lower). Grey surface represents the MZ–FO interface determined based on the distribution of FO B cells as shown in A. Time lapse of each frame is shown (min:sec). Arrowheads point to the cell location at each time point and the thin pink line indicates entire tracked path of the cell.



Supplementary Figure 5. Surface internalization of S1PR1 on MZ B cells and red blood cell density in the MZ. (A) Surface S1PR1 internalization on MZ B cells exposed *in vitro* to 100nM, 10nM or 1nM S1P, as indicated, for 0.5, 1 or 2 hours. Shaded histograms show background (bg) staining of negative control. Figure shows that *in vitro* exposure of MZ B cells to 10nM S1P causes complete S1PR1 downmodulation within 2 hr whereas 1nM S1P causes only partial downmodulation. (B) Haematoxylin and eosin stained spleen sections showing the MZ and adjacent FO and red pulp (RP) and the density of red blood cells (red, several examples pointed out with blue arrows) in the MZ region. Marginal sinuses are indicated with yellow arrow (MS).



Supplementary Figure 6. Intravital TPLSM of MZ B cells in the spleen following blockade of α4 and αL integrins. (A) Further examples of FO B cells migrating from FO to MZ with the MZ-FO interface (grey) determined using PE-IC labeling of MZ macrophages. Time lapse of each frame is shown (min:sec). Arrowheads point to the cell location at each time point and the thin pink line indicates entire tracked path of the cell. The images correspond to Movie 7 example 3. (B) Blocking antibodies to α4 and αL were injected i.v before TPLSM as in Figure 4i-l . Shown is a further example of a MZ B cell crossing from FO to MZ two hours after integrin blockade. Surface represents the MZ–FO interface using PE-IC labeling. Elapsed time shown in min:sec. Arrowheads point to tracked cells at each time point and thin pink lines indicate entire tracked path of each cell. Scale bars represent 10um. The images correspond to Suppl. Movie S8, example 1. (C) Distribution and morphology of GFP+ MZ B cells located in MZ and FO four hours after integrin blockade. (D) B cell saturation by integrin neutralizing antibodies injected *in vivo*. Spleen cells from mice treated as described in Figure4i-l (or untreated control) were stained with anti-rat IgG alone to detect amounts of anti- α4/ αL antibodies bound on the surface of MZ (blue) and FO (green) B cells 1hr after *in vivo* antibody treatment, or after additional incubation *in vitro* with saturating amounts of anti-α4/ αL (MZ B cells, grey histogram, FO B cells, red histogram) 'anti-rat' indicates cells that were exposed to α4 and αL *in vivo* only whereas anti- α4/ αL indicates additional exposure to these antibodies *in vitro* prior to addition of the anti-rat IgG. Note that MZ B cells have higher integrin surface levels than FO B cells.



Supplementary Figure 7. Comparison of FO B cells tracks with MZ-FO boundary surface generated using PE-IC labeling. Image I shows y-z (top) and x-y (bottom) projection view of FO B cells (CFSE, green) transferred into mice injected with PE-IC (red) 2 hours earlier. Image II shows FO B cells tracks (white lines), bordered by the PE-IC stain. Image III shows a surface (purple) generated in Imaris as described in Fig. 2b, drawn based on labeling of MZ macrophages with PE-IC. Image IV shows an overlay the drawn surface with FO B cells tracks. Note that the tracks fill up most of the drawn surface area.

Supplementary Movie Legends

Movie S1. Intravital TPLSM of MZ B cell migration in the spleen (~25 min). Time lapse image sequence (57μm thick z stack) showing MZ B cells (GFP+, green) migrating in the spleen. MZ is identified by labeling MZ macrophages (MZM) with PE-IC (red). Example 1, MZ B cells are large and motile in both MZ and FO compartments and some show a dendritic morphology. Example 2, movie zooms on MZ B cells migrating between MZM and in the FO. Many MZ B cells are in close contact with MZM. The volume image is displayed using normal shading to show the 3D shape of the cells. Elapsed time is shown as hrs:min:sec. Data represents more than seven experiments.

Movie S2. Intravital imaging of MZ B cell migration in the spleen 2 (~25 min). Time lapse image sequence (39μm thick z stack) of spleen showing three typical behaviors of MZ B cells (GFP+, green) with respect to the MZ (MZM coated with PE-IC, red). White circle and arrow highlight a cell moving from the FO towards the MZ. Blue circle shows a cell that moves from the MZ towards the FO. Yellow dashed circle shows an example of a cell that remains confined to the MZ-FO interface by a membrane process (yellow arrow) while the cell body stretches back and forth between zones. The three cells are further highlighted by a white shadow. Many of the cells show long tails (~40μm). Image is displayed using normal shading. Elapsed time is shown as hrs:min:sec. Data are representative of more than seven experiments.

Movie S3. Intravital imaging of MZ B cells migration before and after FTY720 treatment (~21min). Time lapse image sequence (30μm thick z stack) of spleen showing MZ B cells (GFP+, green) migrating within the same follicle before (left image) or after (right image) injection with FTY720. Movie starts with a still image showing MZ B cells distribution in relation to MZM (PE-ICs, red). White dotted line indicates approximate MZ-FO interface. The image is displayed

using normal shading to show the 3D shape of the cells. Elapsed time is shown as hrs:min:sec. Data represents one out of two experiments.

Movie S4. MZ B cells move from the MZ into the FO. Movie shows two example (24min) time lapse image sequences (57μm thick z stacks) from intravital TPLSM of spleen showing MZ B cells (GFP, green) moving from the MZ to the FO. Grey surface was generated in Imaris using PE-IC labeled MZM and the red dashed line represents the MZ-FO interface. In example 2, cell is shown to crawl for a short time across the MZ-FO boundary before making a sharp turn and entering the FO. The cell body appears to stretch at the time of crossing. Pink lines indicate entire tracked paths of the cells. White arrows point at the crossing cell. Elapsed time is shown as hrs:min:sec. Data are representative of more than seven experiments.

Movie S5. MZ B cell moves from the FO to the MZ. Two example time lapse image sequences (69μm thick z stacks) from intravital TPLSM of spleen showing examples of MZ B cells (GFP, green) moving from the FO to the MZ. Grey surface was generated in Imaris using PE-IC labeled MZM and the red dashed line represents the MZ-FO interface. In example 1 the MZ B cell pauses at the FO boundary for ~10 min before crossing to the other side. In example 2, the cell pauses for ~5 min at the FO boundary before crossing to the MZ. In the MZ, the cells continue to migrate in parallel to the FO-MZ interface. Pink lines indicate entire tracked paths of the cells. White arrows point at the crossing cell. Elapsed time is shown as hrs:min:sec. Data are representative of more than seven experiments.

Movie S6. FO B cells migration in the red pulp (RP) (27min). Time lapse image sequence (78μm) from intravital TPLSM of spleen showing FO B cells

(green) migrating in a FO surrounded by PE-IC coated MZM (red) and an adjacent RP. In contrast to the FO, in the RP most cells alternate between stationary and fast movement. White dashed circles show examples of cells that either pause or move fast in a directional path away from the FO. White circled cells are further highlighted with light blue surfaces. Green dashed circles show examples of cells that remain stationary for the duration of the movie. Blue circle shows a relatively rare example of a cell that shows migratory behavior similar to cells in the FO. Elapsed time is shown as hrs:min:sec. Data are representative of more than eight experiments.

Movie S7. FO B cells exit via the MZ. Movie shows three example time lapse image sequences of intravital TPLSM of spleen showing FO B cells (green) moving from FO to MZ. The movie begins with a time lapse image sequence (51μm thick z stack) showing both the FO B cells (GFP, green) and the PE-IC labeled MZM (red). The white dashed circle shows a FO B cell that migrates in the FO for few minutes before jumping across the FO-MZ boundary. After crossing the cell becomes immobile for the last 8 min of the movie. White line indicates entire tracked path of the cell. In the next two image sequences (54μm and 60μm thick z stacks, respectively) FO B cells (CFSE labeled, green) are shown moving from FO to MZ, with the FO-MZ boundary shown by a grey surface. The red dashed line represents the MZ-FO interface. In example 1, the blue dashed circle shows a cell that jumps across the FO-MZ boundary. Example 2 shows two FO B cells (yellow and blue dashed circles) that reach the boundary of the FO at approximately the same time and appear to jump across the MZ-FO interface simultaneously. After crossing, the cells continue to either move fast or pause similarly to the movement observed in the RP. Red line indicate entire tracked path of the cell. Circled cells are further highlighted by a light blue surface. Elapsed time is shown as hrs:min:sec. Data are representative of more than eight experiments.

Movie S8. MZ B cell movement after integrin blockade. Three time lapse image sequences (51μm thick z stacks) from intravital TPLSM of spleen 2 hr after treatment with integrin blocking antibodies (as in Figure 4i-l) showing examples of MZ B cell (GFP, green) movement in the spleen. The first two examples show MZ B cells moving from FO to the MZ after integrin blockade. Red dashed line represents the MZ-FO interface. In both examples the MZ B cells show a meandering migration within the FO but upon crossing into the MZ they show fast tangential movements, consistent with having been caught by flow. Pink lines indicate entire tracked paths of the cells. White arrows point at the crossing cell. Third time lapse image sequence shows GFP+ MZ B cells movement in the MZ (PE-ICs, red) 2 hrs after integrin blockade. White arrow highlights a MZ B cell that was initially adherent but gradually becomes displaced and rapidly disappears from view. Blue arrow highlights another MZ B cell that exhibits mixed adhesive and jumping movements and travels into the red pulp. The volume image is displayed using normal shading to show the 3D shape of the cells. Elapsed time is shown as hrs:min:sec. Data are representative of more than five experiments.

Movie S9. S1PR1 is required for FO B cells to egress from FO (49min). Time lapse image sequence (90μm thick z stack) from intravital TPLSM of spleen showing WT FO B cells (CMTMR labeled, red) and S1PR1 KO FO B cells (CFSE, green) migrating in the FO. Follicles are identified by the high density of WT and KO B cell tracks (white lines). Blue lines show tracks of WT B cells that were observed leaving the FO. Green line shows a single S1PR KO B cell that was detected moving away from the FO. Elapsed time is shown as hrs:min:sec. Data are representative of more than eight experiments.