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Follicular shuttling of marginal zone B cells facilitates antigen transport

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Abstract

The splenic marginal zone is a site of blood flow and the specialized B cell population that inhabits this compartment has been implicated in the capture and follicular delivery of blood-borne antigens. However, the mechanism accounting for this antigen transport has been unknown. Here we show that marginal zone B cells were not confined to the marginal zone but continuously shuttled between the marginal zone and follicular areas such that many of the cells visited a follicle every few hours. Migration to the follicle required CXCR5 whereas return to the marginal zone was promoted by $S1P_1$ and $S1P_3$. Treatment with an $S1P_1$ antagonist caused marginal zone B cell displacement from the marginal zone. Marginal zone-follicular shuttling of marginal zone B cells provides an efficient mechanism for systemic antigen capture and delivery to follicular dendritic cells.

The spleen is a major site for the induction of antibody responses against blood-borne antigens. Many B cells in the spleen are situated in follicles within the white pulp, where they migrate over the processes of follicular dendritic cells (FDCs) in search of antigen. FDCs are also a source of B lymphocyte chemoattractant (BLC or CXCL13), a chemokine that attracts B cells to follicles by engaging the receptor CXCR51. Marginal zone B cells constitute a second major B cell population in the spleen and are so-named because of their location in the marginal zone between the white and red pulp areas2,3. The marginal zone is separated from the white pulp by the marginal sinuses, sites where terminal arterioles open and release blood4. The outer borders of the sinuses are porous and blood can pass through the marginal zone before reaching the red pulp and returning to circulation via venous sinuses. These features ensure that cells situated in the marginal zone are readily exposed to blood-borne antigens2,3. Marginal zone B cells have a unique surface phenotype, expressing high amounts of the complement receptors CD21 (CR2) and CD35 (CR1) and the non-classical major histocompatibility complex (MHC) molecule, CD1d3. In rodents, marginal zone B cells are restricted to the spleen and do not recirculate2.

The pathways by which blood-borne antigens are delivered to splenic follicles have been under investigation for more than 30 years5-7. Several studies have shown that marginal

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AUTHOR CONTRIBUTIONS

G.C. and J.G.C. designed and conceptualized the research. G.C. performed the experiments. M.Z. was involved in several *in vivo* labeling experiments. O.M.L. helped with animal management and typing. F.W.F. generated VPC44116. G.C. and J.G.C. analyzed the data and prepared the manuscript.

zone B cells rapidly capture complement-opsonized antigens, such as Ficoll, via CD21 and CD35 (refs.5-7). Treatment with lipopolysaccharide (LPS) or pertussis toxin (PTX) causes displacement of marginal zone B cells from the marginal zone and this finding was correlated with an immediate deficit in the ability of injected antigens to become deposited on FDCs8-10. These studies implicated marginal zone B cells in antigen transport but did not exclude the alternative possibility that other LPS or PTX-sensitive cell types were required. The uncertainty about the contribution of marginal zone B cells to antigen transport has been amplified by the inability to detect reductions in marginal zone B cell numbers within the marginal zone following exposure to opsonized-antigens8,10.

Marginal zone B cell positioning in the marginal zone depends on the sphingosine-1-phosphate (S1P) receptor, $S1P_1$ (ref.11) encoded by endothelial differentiation gene-1 (*Edg1*). Marginal zone B cells that lack $S1P_1$ or have been exposed to the $S1P_1$ modulating drug, FTY720, fail to position in the marginal zone and are instead found inside the follicles11,12. S1P is abundant in blood and is thought to be present in much lower amounts in the follicles and T zones of lymphoid tissues13,14. However, some studies have questioned the view that the $S1P_1$ requirement directly implicates a role for S1P in cell positioning and have instead suggested that the role(s) of this ligand-receptor pair in lymphocyte function may be developmental15,16. Also complicating the picture are experiments with T cells showing that continual exposure to blood S1P causes down-modulation of $S1P_1$ (refs.14,17), making it unclear if marginal zone B cells could continually respond to plasma S1P in the marginal zone.

Here we provide evidence, using a small molecule antagonist, that engagement of $S1P_1$ by ligand is continually required for marginal zone B cell positioning in the marginal zone. Using mice treated with FTY720 or lacking $S1P_1$ in B cells we then show that marginal zone B cell positioning in the marginal zone was necessary for capture of blood-borne antigens and for their efficient deposition on FDCs. However, if these cells lacked CXCR5 and were not able to migrate into follicles, antigen was no longer transported to FDCs. Using an *in vivo* antibody labeling procedure, we find that in the resting state up to half of the marginal zone B cells were located in the follicle. By comparing the amount of antibody labeling after 5 and 20 min we obtained evidence that marginal zone B cells constitutively shuttled between marginal zone and follicle even in the absence of immunization and independent of B cell receptor (BCR) specificity or complement receptor expression. Follicle to marginal zone shuttling was regulated by the balance of CXCR5 and $S1P_1$ abundance. Finally, we demonstrate that $S1P_3$ contributed to the efficient positioning of marginal zone B cells in the marginal zone.

RESULTS

$S1P_1$ antagonists displace marginal zone B cells

To test whether $S1P_1$ engagement by ligand was a constitutive requirement for marginal zone B cell positioning we treated mice with the $S1P_1$ antagonist, VPC44116 (ref.18). Mice were treated with three doses of VPC44116 or equivalent volumes of carrier over 3 h and tissues were then isolated for histological sectioning or flow cytometric analysis. T cells from blood, spleen and lymph nodes of treated animals had upregulated $S1P_1$, establishing that sufficient antagonist was injected to reduce ligand engagement of the receptor (Fig. 1a).

The less complete upregulation of $S1P_1$ on cells in blood compared to lymphoid tissues most likely reflects incomplete antagonism of receptor engagement by the high amounts of S1P in blood13. Although the $S1P_1$ antibody preparation specifically stains wild-type but not S1P1-deficient T cells19, extensive efforts to stain B cells were unsuccessful due to high non-specific background staining on this cell type (data not shown). Analysis of tissue sections

from mice treated for 3 h with VPC44116 showed that marginal zone B cells had relocalized into the follicles (Fig. 1b). These data suggest that marginal zone B cell localization in the marginal zone is promoted by S1P signaling through $S1P_1$ in these cells.

Marginal zone positioning facilitates antigen capture

As an approach to test the importance of marginal zone positioning in capture of blood-borne antigen, mice were pretreated with FTY720 to cause displacement of marginal zone B cells into follicles11 and then injected i.v. with 5 or 50 μg of trinitrophenol (TNP)-Ficoll and analyzed after 30 min. In saline treated control mice, marginal zone B cells bound substantially greater amounts of TNP-Ficoll than follicular B cells and this binding was CD21-CD35 dependent (Fig. 2a and data not shown) as expected20. By contrast, in mice pretreated with FTY720, the amount of TNP-Ficoll bound to the majority of marginal zone B cells was minimal, similar to the amounts seen on follicular B cells (Fig. 2a). The inclusion of allelically marked *ex vivo* control B cells established that there was minimal *in vitro* capture of TNP-Ficoll complexes when mice had been injected with 5 μg of TNP-Ficoll and this amount was used in all subsequent experiments. To further test if the reduced TNP-Ficoll binding to marginal zone B cells was due to the redistribution of cells into follicles rather than an indirect effect of FTY720 we tested the impact of FTY720 on Ficoll binding in CXCL13-deficient mice. In these animals, FTY720 treatment does not cause displacement of marginal zone B cells from the marginal zone11 and it had minimal effect on Ficoll binding (Fig. 2b). Similarly, FTY720 did not affect TNP-Ficoll binding by circulating B cells in the blood (Fig. 2c).

A concern with the use of FTY720 to study access to a blood-borne antigen was the known activity of FTY720 in reducing vascular permeability21. We therefore performed further experiments using $S1P_1$-deficient fetal liver chimeras. As expected11, the majority of marginal zone B cells in $S1P_1$-deficient animals failed to locate in the marginal zone but instead could be found within follicles (data not shown). Similarly to the results obtained by FTY720 treatment, 30 min after intravenous TNP-Ficoll injection, marginal zone B cells in $S1P_1$-deficient animals bound little TNP-Ficoll (Fig. 2d). To confirm that the effect of $S1P_1$-deficiency was due to loss of $S1P_1$ in B cells, we performed experiments in *Edg1*$^{flox/-}$ mice22 that had been crossed with *Cd19-cre* mice23. Quantitative PCR analysis on sorted cells indicated that the Cre-mediated ablation of *Edg1* in marginal zone B cells was efficient and immunohistochemical staining showed that the lodgment of marginal zone B cells in the marginal zone was reduced to an extent similar to the reduction in *Edg1*$^{-/-}$ fetal liver chimeras (data not shown). Again, the marginal zone B cells bound little TNP-Ficoll (Fig. 2e). Immunohistochemical analysis of tissue sections showed substantial amounts of TNP-Ficoll overlapping with the B cell distribution in the splenic marginal zone of control mice (Fig. 2f). In the $S1P_1$-deficient animals there was still accumulation of TNP-Ficoll at the marginal zone, but in a more punctate pattern, perhaps reflecting association with marginal zone macrophages (Fig. 2f). Taken together, these observations suggest that marginal zone B cell localization at the marginal zone is essential for their ability to rapidly capture blood-borne antigen.

Marginal zone B cells transport antigen to follicles

Within 3 h of intravenous injection, Ficoll is found in large amounts on splenic FDCs. To test whether marginal zone B cells must be present in the marginal zone for this transport to occur, we assessed the extent of TNP-Ficoll deposition in follicles of mice pretreated with FTY720 or in $S1P_1$-deficient fetal liver chimeras. Both FTY720 pretreatment and $S1P_1$ deficiency were found to reduce, though not completely block, TNP-Ficoll deposition on FDCs, compared to the deposition seen in matched controls (Fig. 3a,b). TNP-Ficoll

deposition was similarly reduced in *Cd19-cre Edg1*$^{flox/-}$ mice (Fig. 3c), confirming that B cell expression of $S1P_1$ is required for efficient deposition of opsonized-Ficoll on FDCs.

While these experiments supported the conclusion that marginal zone B cells need to locate in the marginal zone to facilitate transport of a blood-borne antigen to FDCs, it remained possible that their role was solely in capture of the blood-borne antigen rather than in its transport. To further test this issue we used a mixed bone marrow chimera approach to generate mice in which follicles could develop normally but where marginal zone B cells would be unable to migrate from the marginal zone into follicles. In mice lacking CD19, follicles form, but marginal zone B cells fail to develop whereas in CXCR5-deficient mice, B cells develop normally but are unable to migrate into follicles and the B cell areas lack FDCs24-26. When mice that had been reconstituted with a mixture of CD19-deficient and CXCR5-deficient bone marrow were injected with TNP-Ficoll, very effective antigen-capture by CXCR5-deficient marginal zone B cells was observed but there was little or no deposition of this antigen on FDCs (Fig. 3d). Normal Ficoll deposition occurred in mixed bone marrow chimeric mice containing CXCR5-wildtype B cells (Fig. 3d). Staining with antibodies to CD35 confirmed the presence of FDCs in both types of mixed bone marrow chimeric mice (Fig. 3d). Taken together, these experiments provide strong evidence that marginal zone B cells not only capture systemic antigens in the marginal zone, but they also migrate in a CXCR5-dependent manner to follicles to facilitate antigen deposition on FDCs.

Folliclular shuttling of marginal zone B cells

Despite the efficiency of the TNP-Ficoll transport detected in wild-type mice, we were not able to observe a change in the number of B cells in the marginal zone following TNP-Ficoll injection (Fig. 4a and data not shown for earlier time points). However, we had repeatedly noted in CD1d-stained sections of control spleens that there were cells staining with the same intensity as marginal zone B cells located inside splenic follicles (Fig. 4a and data not shown). These combined observations led us to consider the possibility that there was constitutive migration of marginal zone B cells into follicles occurring in a manner independent of complement receptor CR1/2-mediated antigen capture. To examine this possibility further we needed a more quantitative method of assessing the number of B cells present in the marginal zone. Since this compartment is rapidly exposed to blood-borne antigen we asked whether an antibody pulse-labeling procedure developed to label cells in the vascular compartment (J. Pereira and J.G.C., manuscript in preparation) could be applied and tested whether a brief *in vivo* exposure to a CD21 antibody would selectively label marginal zone B cells amongst splenic B cells. Flow cytometric analysis of spleen cells from mice that had been treated with anti-CD21-phycoerythrin (PE) for 5 min revealed strong staining on a fraction of the marginal zone B cells (Fig. 4b). Microscopy analysis revealed intense staining on B cells in the MZ (Fig. 4c). Importantly, the flow cytometric analysis showed that the marginal zone B cell staining was bimodal with only ~50% of the cells becoming brightly labeled (Fig. 4b). We speculated that the poorly labeled cells corresponded to cells that had been shielded from the injected antibody because they were present within follicles during the 5 min treatment. Like lymph nodes, the white pulp cords of spleen are surrounded by stromal cells and associated extracellular matrix that together form a barrier restricting free diffusion of molecules the size of antibodies27-29. Follicular B cell staining was lower than on marginal zone B cells as expected though there was a tendency for some of the cells to show positive staining (Fig. 4b), consistent with the presence of recirculating follicular B cells in the splenic marginal zone and red pulp30. To test whether follicular localization shielded marginal zone B cells from exposure to the injected antibody, we treated $S1P_1$-deficient mice with anti-CD21-PE. In these animals, the majority of marginal zone B cells were poorly stained following the 5 min antibody injection (Fig. 4d,e). A similarly low fraction of cells became CD21-PE labeled in mice that

had been pretreated for 3 h with FTY720 (Fig. 4e). We then injected CXCR5-deficient mice with anti-CD21-PE and found that all the marginal zone B cells became labeled in 5 min (Fig. 4f). The staining intensity on these cells was lower than on the brightly stained subset in wild-type controls, perhaps because there are many more B cells located in the marginal zone of mice deficient in the CXCL13-CXCR5 axis compared to control mice26. To address the concern that white pulp organization is disturbed in CXCR5-deficient mice we also treated bone marrow chimeras that had been reconstituted with a mixture of CXCR5-deficient and $S1P_1$-deficient bone marrow. These mice have well-developed lymphoid follicles but the CXCR5-deficient B cells are unable to access these areas. In these animals, all the CXCR5-deficient marginal zone B cells became rapidly labeled whereas only few $S1P_1$-deficient marginal zone B cells became labeled (Fig. 4g) consistent with the conclusion that cells located within the marginal zone become heavily labeled whereas cells present within the follicle are shielded from immediate exposure to the CD21-PE.

We then asked whether there was a change in the fraction of brightly labeled cells after 20 min and found at that time point ~70% of marginal zone B cells were highly labeled in wild-type mice (Fig. 4e, h). After longer time periods, all marginal zone B cells became antibody-labeled, consistent with a study that examined labeling 1 h after anti-CD21-biotin injection29. Importantly, in *Cd19-cre Edg1*$^{flox/-}$ mice the majority of marginal zone B cells remained poorly labeled at 20 and even at 40 min, indicating that the increased labeling was not a result of free antibody access to the follicle (Fig. 4e, i). The brightly stained cells that could be detected in these mice at 20 min appeared to correspond to a small number of cells that were residing within the marginal zone (Fig. 4c). Reciprocally, in CXCR5-deficient mice, all the marginal zone B cells were brightly labeled, consistent with all of these cells being in the marginal zone and accessible to the antibody (Fig. 4c,j). Together, these observations are consistent with the view that as many as 40% of the marginal zone B cells that were in the follicle of wild-type mice during the initial 5 min of labeling travel back into the marginal zone during the next 15 min. If this were the case, it would be anticipated that these cells would be replaced by CD21-PE labeled cells migrating into the follicle from the marginal zone. Microscopy analysis of splenic tissue sections taken 20 min after antibody injection showed that in addition to staining throughout the marginal zone there was now also labeling of a population of B cells within the follicle (Fig. 4c). By contrast, there were few CD21-brightly stained cells in splenic sections from 20 min treated $S1P_1$-deficient mice (Fig. 4c) ruling out the possibility that the brightly staining cells arose due to diffusion of the antibody. To exclude the possibility that the brightly staining cells appearing within follicles after 20 min were all recirculating B cells, we also treated *Cd19*$^{-/-}$ mice that lack marginal zone B cells but have normal numbers of follicular B cells23,24. This analysis indicated a lack of brightly stained cells (Fig. 4c). In wild-type mice, similar findings regarding marginal zone B cell labeling were made after injection of anti-CD1d-PE, B220-PE, CD35-biotin, CD21-fluorescein isothiocyanate (FITC), CD19-PE, and CD45.2-PE (data not shown) indicating that the shuttling was not dependent on engagement of CR1/2 by the CD21 antibody, nor were these observations notably affected by the nature of the antibody or the type of conjugation. The marginal zone B cell shuttling did not depend on expression of CR1/2 as similar labeling profiles were observed in *Cr2*$^{-/-}$ mice at 5 and 20 min after anti-CD45.2-PE injection (Fig. 4k). In addition, marginal zone B cell shuttling seems to be independent of their BCR specificity. In Ig-heavy chain knockin animals in which about half of the B cells are hen egg lysozyme (HEL) specific (VDJ mice), marginal zone B cells were similarly distributed between the marginal zone and follicles whether they were HEL specific or not (Fig. 4l). As a further approach to test for rapid migration of marginal zone B cells into the follicle we asked whether any marginal zone B cells that labeled with antibody during an initial pulse became protected from exposure to a subsequently injected antibody. Indeed, a fraction of the marginal zone B cells that had labeled with anti-CD35 during a 15-min pulse were protected from labeling by anti-CD21 in the ensuing 5 min (Fig. 4m, upper

left quadrant in right panel). The lower CD35 antibody staining on the protected (CD21-PE negative) cells was observed in repeated experiments and might indicate that after migration into follicles there is rapid loss of some antibody to surrounding B cells or FDCs. In control mice treated with both antibodies simultaneously, the labeled marginal zone B cells bound both antibodies equally, as expected (Fig. 4m, left panel). Taken together, these observations indicate that many marginal zone B cells are rapidly shuttling between marginal zone and follicle in a homeostatic manner that is independent of complement receptor or BCR engagement.

Actived C4 on marginal zone B cells

The antibody, FDC-M2, has been widely used as an FDC marker and is now known to bind an activated form of complement C4 (ref.31). In tumor necrosis factor (TNF)-deficient mice, animals that have poorly developed follicles and lack FDCs, there is an accumulation of FDC-M2 staining cells in the marginal zone32. Similar findings have been made for CXCR5-deficient mice33. We confirmed the marginal zone FDC-M2 staining pattern in CXCR5-deficient mice (Fig. 5a) and observed similar staining in CXCL13-deficient mice (not shown). Although it was suggested that these cells may be mislocalized FDCs or FDC precursors32,33 we speculated that in the absence of marginal zone-follicular shuttling, marginal zone B cells may be unable to off-load complement-coated antigens on FDCs and they might therefore accumulate C4-containing antigens over time. Indeed, by flow cytometric analysis we found that the marginal zone B cells in CXCR5-deficient mice had significantly higher amounts of active C4 on their surface (Fig. 5b,c). Interestingly, CXCR5-deficient marginal zone B cells also had slightly elevated surface CD21 expression (Fig. 5c). However, the fold difference in FDC-M2 staining was at least 3-fold greater than the difference in CD21 expression suggesting that the marginal zone B cells in unimmunized mice have a propensity to accumulate C4-containing immune complexes. Therefore, marginal zone B cell shuttling appears to have an homeostatic role in depositing complement opsonized antigens to FDCs.

$S1P_1$ and CXCR5 balance regulates shuttling

To test whether the balance of $S1P_1$ and CXCR5 activity was important in regulating the shuttling behavior observed above, we performed flow cytometric analysis of B cells from antibody injected $S1P_1$ or CXCR5 heterozygous mice. In *Edg1*$^{+/-}$ mice treated for 5 and 20 min with CD21-PE, fewer cells were brightly labeled than in controls (Fig. 6a,c) indicating a shift in the balance in favor of follicular positioning. Although we have not been able to quantitate $S1P_1$ surface protein amounts on B cells due to high background staining, *Edg1*$^{+/-}$ T cells have about half the normal amounts of surface $S1P_1$ of wild-type T cells17. Reciprocally, in mice heterozygous for the gene encoding CXCR5, *Blr1*, a greater fraction of the cells were CD21-PE labeled in 5 min than in wild-type controls and this was also the case at 20 min (Fig. 6b,c) indicating that a 50% reduction in CXCR5 protein abundance led to a shift in the balance of responsiveness in favor of S1P signaling and positioning in the marginal zone. These observations reveal a delicate balance of signaling by $S1P_1$ and CXCR5 and provide support for a model where the principal function of each receptor in marginal zone B cells is to attract or retain the cells in the reciprocal compartments.

$S1P_3$ is required for marginal zone B cell positioning

Although $S1P_1$ deficiency and FTY720 treatment led to a marked depletion of marginal zone B cells from the marginal zone and accumulation in the follicle, the *in vivo* CD21-PE labeling approach revealed that there was a residual population of B cells located in the marginal zone that were detectable by flow cytometric and immunofluorescence analysis (Fig. 4c,d). Previous work had shown that $S1P_3$, encoded by endothelial differentiation gene-3 (*Edg3*), is abundant in marginal zone B cells and supported strong *in vitro* S1P

chemotactic responsiveness11 though the distribution of marginal zone B cells in $S1P_3$-deficient animals appeared little affected11,34. Because the above findings indicated that many marginal zone B cells are rapidly shuttling between follicle and marginal zone, it seemed likely that the chemotactic function of $S1P_3$ may be involved in the return of marginal zone B cells from the follicles to the marginal zone. For these studies we used $S1P_3$-deficient bone marrow chimeras to exclude possible roles of $S1P_3$ in stromal cells34. When we quantified the $S1P_3$-deficient marginal zone B cells that became rapidly labeled following CD21-PE or CD35-biotin antibody treatment, the fraction was lower than in control animals (Fig. 6d,e). At 20 min the extent of labeling of $S1P_3$-deficient and control cells was similar (Fig. 6e) perhaps reflecting a less accurate quantitation of cell distribution at this time point due to the elevated staining on all B cells. These observations suggest that at homeostasis, a greater fraction of the $S1P_3$-deficient marginal zone B cells are located inside the follicle than in the marginal zone compared to wild-type mice. Moreover, when $S1P_3$-deficient bone marrow chimeras were treated with FTY720 and then tested for marginal zone B cells remaining in the marginal zone, a more complete displacement of the cells occurred (Fig. 6d,e). Interestingly, the treatment of $S1P_3$-deficient mice with FTY720 did not fully block marginal zone B cell shuttling; compared to the minimal labeling with the injected antibody at 5 min, more marginal zone B cells became labeled at 20 min (Fig. 6d). In summary, both $S1P_1$ and $S1P_3$ promote marginal zone B cell positioning in the marginal zone and an additional ligand-receptor system may exist that makes a similar contribution to marginal zone B cell positioning as $S1P_3$.

DISCUSSION

Marginal zone B cells have long been thought to be a sessile cell population that localizes selectively in the splenic marginal zone. The above findings provide strong evidence that this is not the case and that instead the cells are shuttling continuously back-and-forth between marginal zone and follicle. The propensity to shuttle into the follicle is promoted by CXCR5 whereas shuttling to the marginal zone is mainly controlled by $S1P_1$ and $S1P_3$. The relative amount of these receptors is important in determining the propensity for the cells to dwell in one compartment versus the other. Our findings indicate that marginal zone B cell shuttling is important for the efficient capture and transport of complement-bound antigen from the blood to splenic FDCs.

Taking our observations together with findings in several other studies, we propose the following model to account for marginal zone-follicular shuttling of marginal zone B cells. The high abundance of S1P in blood and the blood-rich nature of the marginal zone make it likely that the marginal zone is an S1P-high environment. Our demonstration that antagonism of $S1P_1$ ligand binding rapidly displaces marginal zone B cells is consistent with this conclusion. By contrast, the lymphoid regions of lymphoid organs are S1P-low environments13. CXCL13 is abundant in follicles and transcript expression extends to the marginal sinus but not into the marginal zone35,36. Marginal zone B cells express 2-3 fold higher amounts of $S1P_1$ transcripts and similar amounts of CXCR5 transcripts to follicular B cells (11 and data not shown). Over-expression of $S1P_1$ in follicular B cells is sufficient to exclude them from migrating into splenic follicles in short term transfer experiments17. We propose that upon commitment to a marginal zone B cell fate and upregulation of $S1P_1$ and $S1P_3$, marginal zone B cells are attracted to the marginal zone where they can adhere and resist the shear forces associated with local blood flow due to their high expression of $\alpha_L\beta_2$ and $\alpha_4\beta_1$ (refs.37,38). However, we speculate that after a short period they are exposed to a sufficient amount of S1P to down-modulate $S1P_1$ and no longer be efficiently retained in the marginal zone. Although reagents are not yet available to stain mouse B cells for $S1P_1$, studies with T cells and transfected cell lines have shown that $S1P_1$ is highly sensitive to down-modulation after S1P exposure13,39-41. Following $S1P_1$ down-modulation the

marginal zone B cell response to CXCL13 dominates and the cells migrate to the follicle. Within the follicle, FDCs are thought to successfully compete for binding of opsonized antigens carried by the migrating marginal zone B cells due to their exceptionally high expression of CD21 and CD35. After a short period of migration within this CXCL13-high but S1P-low environment we suggest that marginal zone B cells regain sufficient $S1P_1$ surface expression to respond again to marginal zone S1P and now migrate back to this compartment. When T cells migrate from blood into lymph nodes they upregulate $S1P_1$ within 30-60 min17. The sensitive relationship between $S1P_1$ and CXCR5 surface abundance and propensity of marginal zone B cells to locate in the marginal zone or follicle suggests that even partial decreases or increases in receptor function might be sufficient to promote movement of the cell from one compartment to the other. Repeated cycles of this process would lead to constitutive marginal zone B cell shuttling between marginal zone and follicle. In future work it will be important to further test this model by disrupting desensitization motifs in $S1P_1$ and by developing approaches to image marginal zone B cell migration in real-time within the intact spleen.

The potential involvement of marginal zone B cells in the transport of opsonized antigens into follicles has long been under investigation5-7. Early electron microscopy studies identified rare examples of splenic (possibly marginal zone) B cells with labeled antigen at their uropod that may have been migrating in the direction of the follicle42. Correlative evidence that marginal zone B cells were needed for antigen transport was obtained by performing treatments that displace the cells and showing that this reduced subsequent deposition of injected antigen on FDCs8-10. An anti-CD21 injection study also observed a correlation between initial labeling of marginal zone B cells and subsequent antibody appearance on FDCs29. However, these studies could not exclude the possibility of indirect effects of the treatments and they did not establish how following immune complex or antibody capture the marginal zone B cells underwent repositioning into the follicle. Another experiment showed that CD19-deficient mice were defective in transport of immune complexes to splenic FDCs, consistent with a role for marginal zone B cells but not excluding the possibility that these cells performed only the capture and other cells performed the transport10. Our work builds from these findings to directly demonstrate that marginal zone B cells must be located in the marginal zone to efficiently capture opsonized antigen and that their transport of the antigen may then be a default feature of their shuttling behavior rather than being induced by complement receptor engagement.

Our finding that the fraction of marginal zone B cells labeled by anti-CD21 antibody injection increases from ~50% to ~70% in 15 min suggests that as many as 40% of the marginal zone B cells that were within follicles during the first 5 min redistribute back to the marginal zone in 15 min (corresponding to ~20% of total marginal zone B cells). At this rate of flux, the majority of marginal zone B cells would shuttle in and out of the follicle over a period of hours. Consistent with this, we find that compared to the heavy loading of the entire marginal zone B cell population with TNP-Ficoll 30 min after injection, the cells have all largely been cleared of surface complexes by 2-3 h. This clearance appears to depend on shuttling as marginal zone B cells in CXCR5- and CXCL13-deficient mice fail to become cleared of surface labeling over this time frame. Indeed, it would appear that constitutive shuttling contributes in an ongoing fashion to removal of opsonized antigens from the surface of marginal zone B cells since marginal zone B cells in CXCR5- and CXCL13-deficient mice have elevated amounts of activated C4 on their surface even in the absence of intentional immunization. It will be of interest in future studies to determine what fraction of these constitutive complexes contain autoantigens as this might implicate marginal zone-follicular shuttling and FDC loading with blood-borne antigen as a mechanism promoting self-tolerance of follicular B cells.

Although we found that there was a marked defect in antigen deposition on FDCs when marginal zone B cells were defective in shuttling to the marginal zone due to $S1P_1$-deficiency (or after FTY720 treatment), the antigen deposition was not completely prevented. This was also the case in FTY720 treated $S1P_3$-deficient mice, the conditions where we observed the most severe defect in marginal zone B cell shuttling. We speculate that the residual transport and deposition of opsonized antigen on splenic FDCs is mediated by recirculating follicular B cells. Blood B cells were able to rapidly capture low amounts of injected Ficoll and recent work has shown that follicular B cells in lymph nodes capture and transport immune complexes in a CD21-dependent manner to FDCs43.

The strong chemotactic activity of the $S1P_3$ receptor in marginal zone B cells made it surprising that $S1P_3$-deficient mice and bone marrow chimeras showed little evidence of impairment in B cell positioning in the marginal zone when examined non-quantitatively by immunohistochemistry11,34. However, using quantitative flow cytometric assessment we have now been able to reveal a contribution of this receptor to marginal zone B cell positioning in the marginal zone. It remains puzzling that in chemotaxis assays $S1P_3$ is more active than $S1P_1$ yet *in vivo*, $S1P_1$ plays a greater role in promoting cell positioning in the marginal zone. One possible explanation for this discrepancy is that *in vitro* assays are poor at reporting the *in vivo* promigratory activity of $S1P_1$, perhaps because of the high sensitivity of $S1P_1$ to down-modulation by ligand. In this regard, in preliminary experiments with transfected cells we find that $S1P_3$ is more resistant to ligand-mediated down-modulation than $S1P_1$ (data not shown). In contrast to the activity of FTY720 in down-modulating and promoting the degradation of $S1P_1$ (refs.40,41), cells taken from mice injected with FTY720 retained $S1P_3$ function11. Thus the more complete displacement of marginal zone B cells by FTY720 in $S1P_3$-deficient mice than in wild-type mice is consistent with FTY720 not inactivating $S1P_3$. It is notable that even $S1P_3$-deficient mice retain small numbers of marginal zone B cells in the marginal zone after FTY720 treatment, suggesting that at least one further mechanism functions to promote localization of marginal zone B cells in this zone. Finally, it was recently shown that shear forces are important for integrin activation of T cells by chemokines38. As the marginal zone is constantly flushed by blood and integrins are vital for marginal zone B cell lodgement at the marginal zone37 it will be important to study the possible role of shear forces in promoting marginal zone B cell adhesion as the cells shuttle into this zone.

METHODS

Mice, fetal liver and bone marrow chimeras

Wild-type C57BL/6 (B6) and congenic B6 CD45.1 mice 2-4 months old were from the National Cancer Institutes. *Edg1*$^{+/-}$ and *Edg1*$^{-/-}$ fetal liver (FL) chimeras were generated as described11. *Edg1*$^{+/-}$ mice44 were crossed for 11 generations or more to B6. *Cd19-cre* mice23 on a B6 background were crossed to *Edg1*$^{+/-}$ mice. Insertion of *cre* disrupts the *Cd19* coding sequence, leading to CD19 deficiency in the homozygous situation (*Cd19-cre/cre*, also referred to as *Cd19*$^{-/-}$). *Cd19-cre/cre Edg1*$^{+/-}$ mice were crossed to mice in which the *Edg1* alleles are flanked by *loxP* (*Edg1*$^{flox/flox}$)22. This resulted in *Cd19-cre Edg1*$^{flox/+}$ or *Cd19-cre Edg1*$^{flox/-}$ mice in which the B cell lineage has either one or no functional *Edg1* alleles, respectively. To generate bone marrow (BM) chimeras, lethally irradiated B6 CD45.1 mice were reconstituted with: BM from *Edg1*$^{+/-}$ FL chimeras that was mixed 95:5 with BM from CXCR5-deficient (*Blr1*$^{-/-}$) CD45.1 mice; BM from *Cd19*$^{-/-}$ mice that was mixed 90:10 with BM from B6 CD45.1 or *Blr1*$^{-/-}$ CD45.1 mice; BM from *Edg1*$^{+/-}$ B6 mice that was mixed 65:35 with BM from B6 CD45.1 mice; BM from $S1P_3^{-/-}$ mice that was mixed 50:50 with BM from B6 CD45.1 mice or the two BM types were used alone. *Cr2*$^{-/-}$45, *Cxcl13*$^{-/-}$46, *Blr1*$^{-/-}$47, *Edg3*$^{-/-}$48, and VDJ9-κ5 mice49 were previously described. Mice were housed in a specific pathogen-free environment in the Laboratory Animal

Research Center at the University of California, San Francisco, and all animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Treatments and TNP-Ficoll injection

For VPC44116 treatment18, the drug was first dissolved at 20 mM in a mixture of dimethylsulfoxide (DMSO) and 1N HCl at 95:5 ratio and immediately diluted to a final concentration of 1 mM in aqueous solution containing 2% 2-hydroxypropyl-β-cyclodextrin (Sigma). A carrier control solution was similarly prepared in the absence of VPC44116. Mice were injected i.p. with 10 mg/Kg of VPC44116, or the equivalent volume of carrier, or saline solution. Mice were injected at time -3, -2 and -1 h and were sacrificed at 0 h.

For FTY720 pretreatment, mice were injected *i.p.* with 1mg/Kg of the drug or an equivalent volume of saline for 3 h. For TNP-Ficoll deposition studies, mice were injected *i.v.* with different doses (see text) of TNP(89)- or TNP(65)-Ficoll (Biosearch Technologies Inc.) and were sacrificed 30 min or 3 h thereafter. Tissues were processed on ice to minimize further binding of the Ficoll *in vitro*.

In vivo labeling of B cells

For *in vivo* labeling of B cells, a method adapted from J. Pereira (manuscript in preparation) was used. Mice were *i.v.* injected for the times indicated in the text with the indicated amounts of the following antibodies: 1 μg anti-CD21-PE (7G6, recognizing both CD21 and CD35), 0.5 μg CD21-FITC (7G6), 1 μg CD35-biotin (8C12, recognizing selectively CD35), 1 μg CD19-PE (1D3) all from BD Bioscience, 5 μg CD1d-PE (1B1), 2 μg CD45.2-PE (clone 104) both from eBioscience, 1.5 μg B220-PE (RA-3-6B2) from Invitrogen, Caltag. Tissues were processed on ice to minimize further binding of the antibodies *in vitro*.

Flow cytometric and immunohistochemical analysis

Flow cytometric analysis was on a FACSCalibur or LSRII (both from Becton Dickinson). $S1P_1$ staining was previously described17. All other antibodies were from BD Bioscience. Marginal zone and follicular B cells were gated on CD19 or B220 positive cells. Marginal zone B cells were gated as $CD23^{lo}$ $CD21^{hi}$ or $CD23^{lo}$ $CD1d^{hi}$ and follicular B cells were gated as $CD23^{hi}$ $CD21^{med}$ or $CD23^{hi}$ $CD1d^{med}$. Since B cells may bind injected TNP-Ficoll or injected antibodies upon processing of the tissue *in vitro*, tissues from syngeneic non-treated mice were mixed with tissues from treated animals before further processing, and these cells were used as background controls. Immunohistochemical (IHC) and immunofluorescence (IF) analysis of spleen sections were performed as described11.

Statistical analysis—Data was analyzed using a two-sample student's *t*-test.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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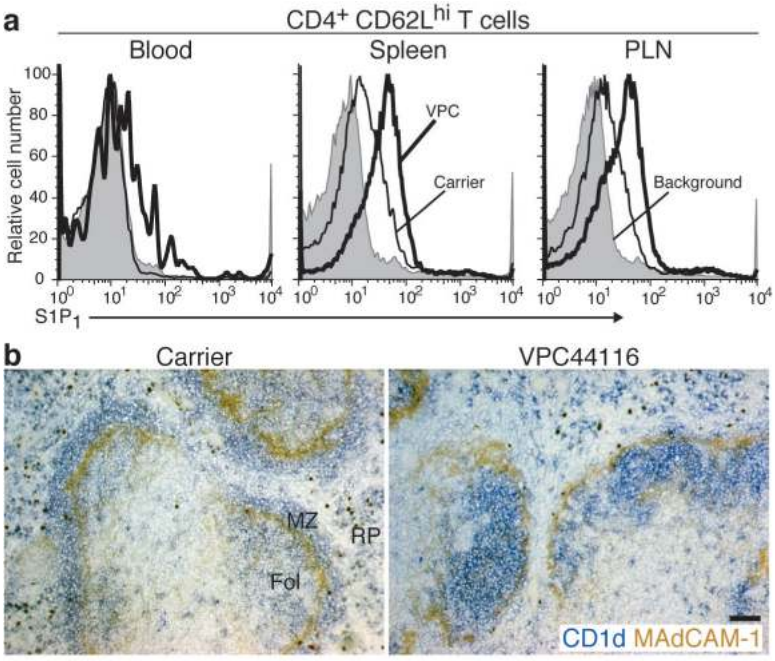

Figure 1.

Treatment with $S1P_1$ antagonist VPC44116 for 3 h causes displacement of marginal zone B cells into follicles. (**a**) FACS histograms of $S1P_1$ on naive $CD4^+$ T cells ($CD4^+$ $CD62L^{hi}$) in blood, spleen, and peripheral lymph node (PLN) after treatment with VPC44116 (VPC, thick line) or carrier (thin line). $S1P_1$ amounts on T cells from carrier treated mice were similar to those of T cells from saline treated mice (not shown). Background histograms (shaded) represent control antibody staining of T cells from VPC44116 treated mice. (**b**) Cryostat sections of spleen from mice treated with carrier or VPC44116, stained for CD1d (blue) to detect marginal zone B cells, and MAdCAM-1 (brown) to detect marginal sinuses. Fol, follicle; MZ, marginal zone; RP, red-pulp. Scale bar, 50 μm. Data in a and b are representative of 3-5 experiments.

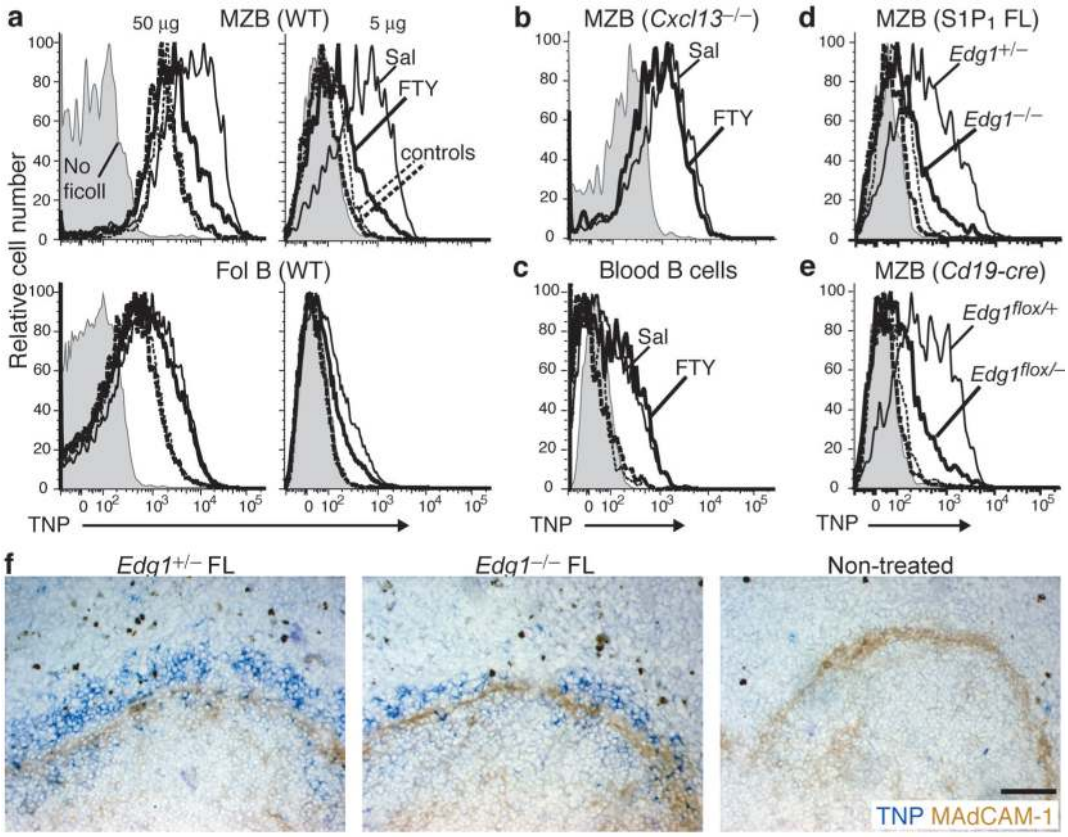

Figure 2.

Marginal zone B cell positioning in the marginal zone is required for efficient binding of blood-borne antigen. Mice were injected i.v. with TNP-Ficoll and 30 min later tissues were analyzed. (**a-e**) Flow cytometric analysis of TNP-Ficoll binding to splenic marginal zone B cells (MZB), follicular B cells (Fol B) or blood B cells, as indicated. MZ B cells were identified as $CD19^{+}CD23^{lo}CD1d^{hi}$ and follicular B cells as $CD19^{+}CD23^{hi}CD1d^{int}$. Panels include two staining controls: cells from non-treated mice (No Ficoll, shaded histogram) and cells from non-treated mice that were co-processed with cells of treated mice to account for TNP-Ficoll binding *in vitro* (controls, dashed lines). (**a**) Mice were pretreated with either FTY720 (thin line) or saline (thick line) for 3 h before being injected with 50 or 5 µg TNP-Ficoll. (**b**) *Cxcl13*$^{-/-}$ mice were treated with FTY720 or saline and injected with 5 µg TNP-Ficoll. *In vitro* controls are not depicted here. (**c**) TNP-Ficoll binding by blood B cells from mice injected with 5 µg TNP-Ficoll. (**d,e**) $S1P_1$-deficient (*Edg1*$^{-/-}$) FL chimeras (**d**) or *Cd19-cre Edg1*$^{flox/-}$ mice (**e**) and control mice injected with 5 µg TNP-Ficoll. (**f**) Cryostat sections of spleens from *Edg1*$^{+/-}$ and *Edg1*$^{-/-}$ FL chimeras that were treated with 5 µg TNP-Ficoll, or from a non-treated mouse, as indicated. Sections were stained for TNP (blue) and MAdCAM-1 (brown). Scale bar, 50 µm. All plots and micrographs represent data from at least three experiments, corresponding to at least three mice of each type.

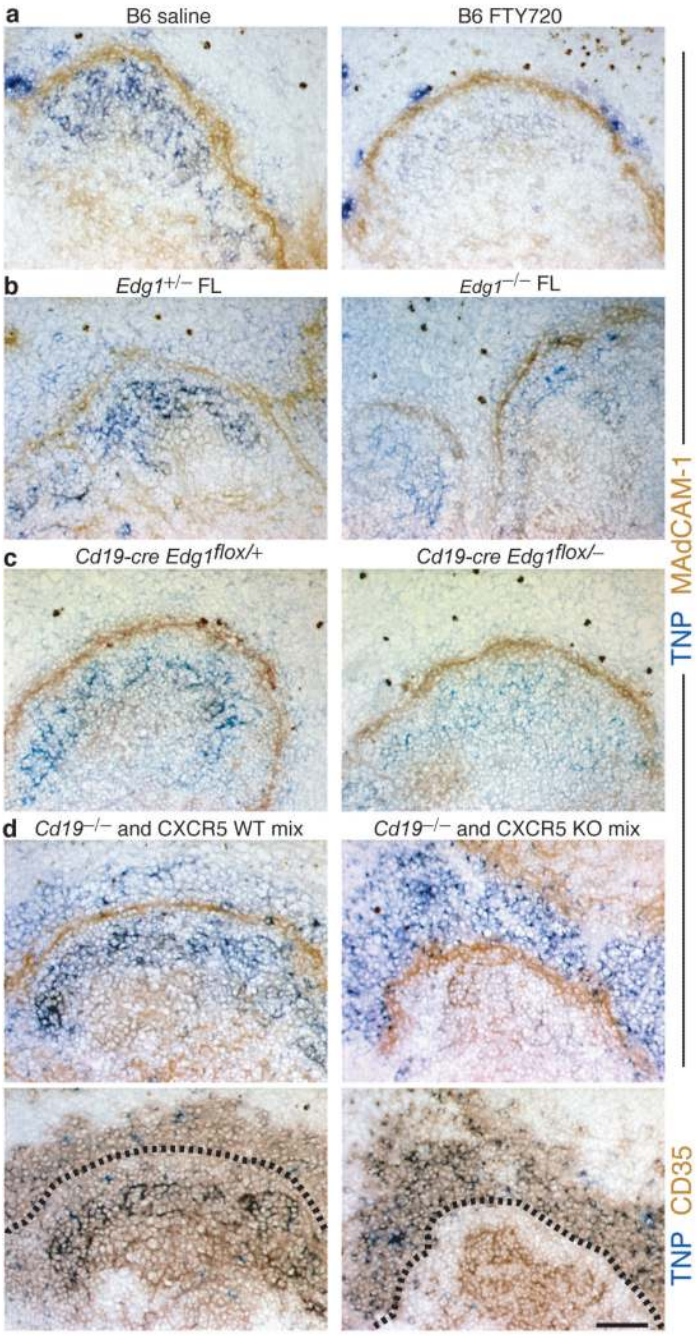

Figure 3.

Marginal zone B cell positioning in the marginal zone is required for efficient delivery of blood-borne immune complexes to FDCs. (**a-c**) IHC micrographs of spleens from mice that were injected with 5 μg TNP-Ficoll for 3 h. Sections were stained for TNP (blue) and MAdCAM-1 (brown). (**a**) Mice were pretreated with FTY720 or saline for 3 h before injection of TNP-Ficoll. (**b**) Chimeric mice that were reconstituted with either $S1P_1$-sufficient (*Edg1*$^{+/-}$) or -deficient (*Edg1*$^{-/-}$) FL. (**c**) Mice in which one or two alleles of *Edg1* were deleted in the B cell lineage only. (**d**) B cell CXCR5 expression is necessary for TNP-Ficoll deposition on FDCs. Chimeric mice that were reconstituted with a mixture of *Cd19*$^{-/-}$

and CXCR5 wildtype (WT) or CXCR5 knockout (KO) BM were treated as above. Spleen sections were stained for TNP (blue) and for MAdCAM-1 (brown, top panels) or CD35 (brown, bottom panels) to detect FDCs and MZ B cells. All micrographs represent data from at least three experiments. Scale bar, 50 μm.

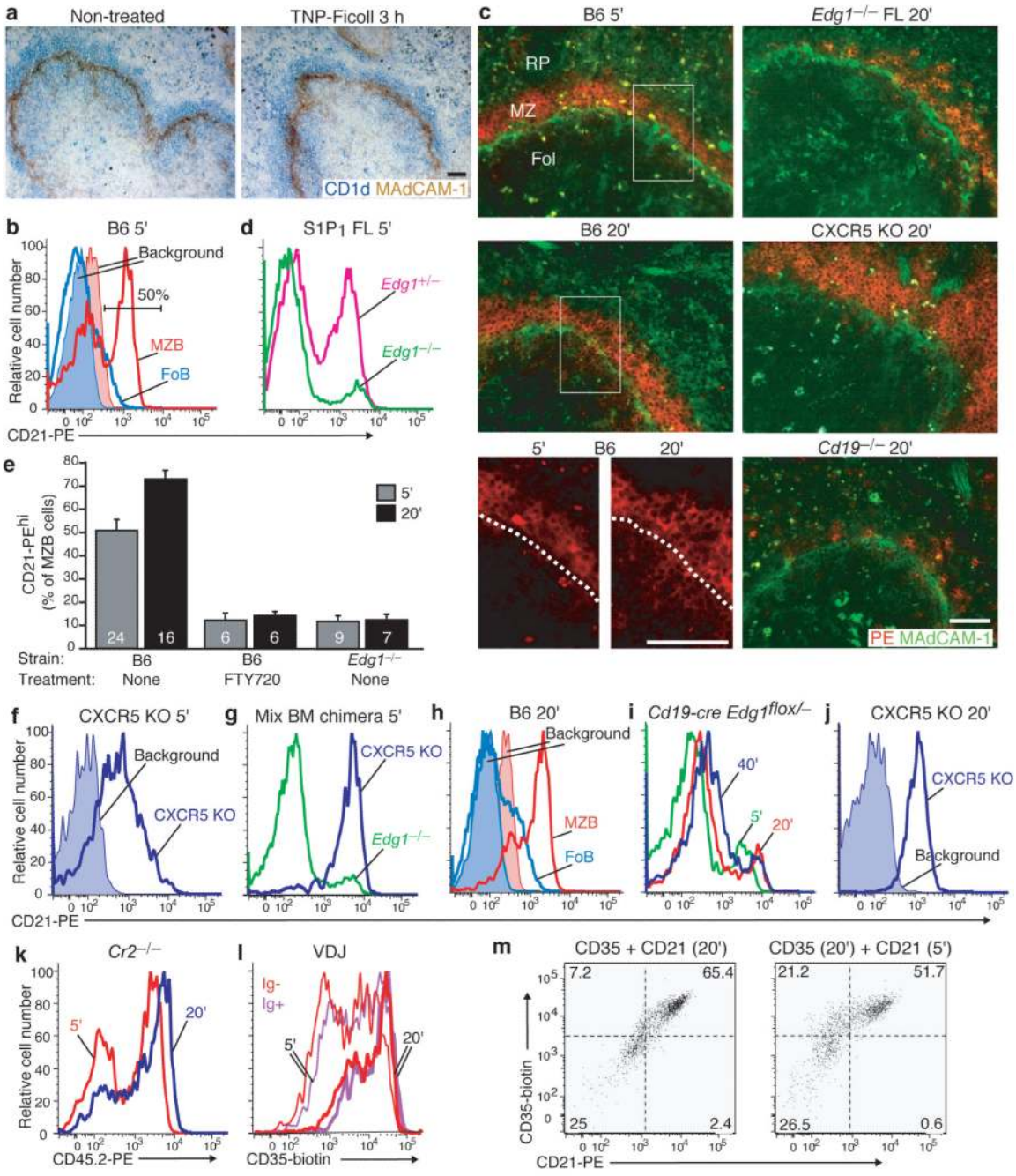

Figure 4.

In vivo antibody labeling provides evidence for marginal zone-follicular shuttling of marginal zone B cells in the absence of immunization. (**a**) IHC micrographs of spleen sections from non-treated mice, or mice that were immunized with TNP-Ficoll for 3 h, stained to detect CD1d (blue) and MAdCAM-1 (brown). Scale bar, 50 μm. (**b, d** and **f-l**) Flow cytometric analysis of splenic B cells isolated from the indicated mice 5 or 20 min after antibody injection, as stated. In **b**, antibody binding by both marginal zone B cells (MZB) and follicular B cells (FoB) is shown; in **d** and **f-I**, only the data for marginal zone B cells are shown. The injected antibody was anti-CD21-PE (**b, d** and **f-j**), anti-CD45.2-PE (**k**)

or anti-CD35-biotin (**l**). (**c**) IF micrographs of spleens from the indicated mice that were injected for 5 or 20 min with anti-CD21-PE (red) and stained with anti-MAdCAM-1-Alexa488 (green). The two lower left panels are 2x enlargements of areas corresponding to the white rectangles in the wildtype (B6) controls showing only the PE fluorescence. Dashed lines depict the location of the MAdCAM-1$^+$ sinus. Fol, follicle; MZ, marginal zone; RP, red-pulp. Scale bar, 50 μm. (**e**) Summary of marginal zone B cell data from accumulative experiments as described above. FTY720 pretreatment was for 3 h before the antibody injection. *Edg1*$^{-/-}$ data sum the results obtain from both FL chimera and *Cd19-cre* conditional KO animals. Bars indicate mean and error bars, s.e.m., of data from the indicated number of mice (white numbers within bars). (**m**) Flow cytometric analysis of marginal zone B cell from mice treated with non-competing anti-CD35-biotin and anti-CD21-PE antibodies either simultaneously for 20 min (left), or with anti-CD35-biotin for 20 min and anti-CD21-PE for 5 min (right). Cells from control spleens that were co-processed with the treated spleens were used as negative controls and the quadrants in each panel were set to confine these cells to the lower left quarter (not shown). The percentage of cells within each quadrant is indicated. Data are representative of three experiments.

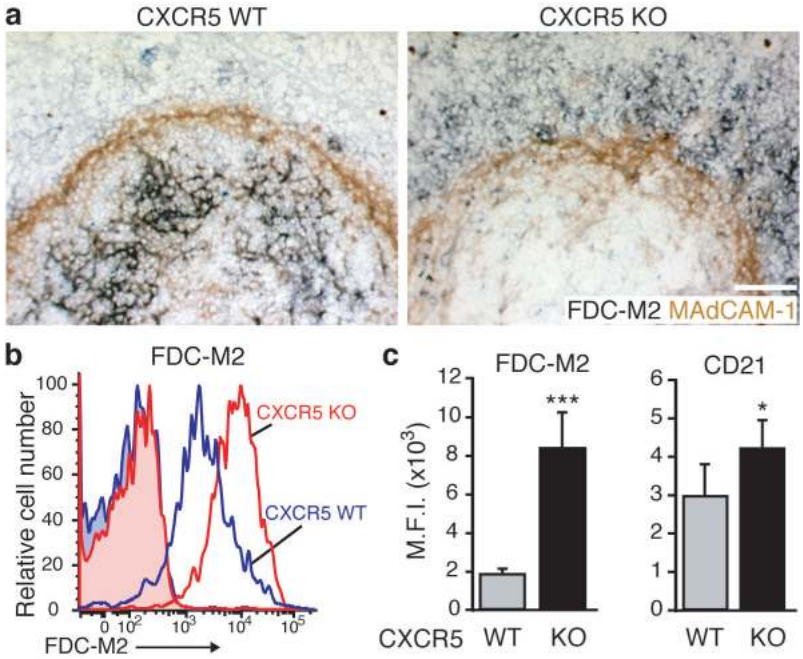

Figure 5.

FDC-M2 accumulation on marginal zone B in CXCR5-deficient mice. (**a**) IHC micrographs of FDC-M2 staining in CXCR5 wild-type (WT) and CXCR5-deficient (KO) mice. Spleen sections were stained for FDC-M2 (dark blue) and MAdCAM-1 (brown). Scale bar, 50 μm. Micrographs represent data from three untreated mice. (**b**) Flow cytometric analysis of FDC-M2 staining on marginal zone B cells from CXCR5-deficient and -sufficient mice. Background staining (shaded histograms) is that of the secondary antibody alone. (**c**) Summary of the mean fluorescence intensity (MFI) of FDC-M2 ($n = 10$) and CD21 ($n = 8$) staining (left and right, respectively) by flow cytometric analysis on MZ B cells of CXCR5 wild-type and CXCR5-deficient mice. Bars indicate mean and error bars, s.e.m. *, $p < 0.02$. **, $p < 0.001$.

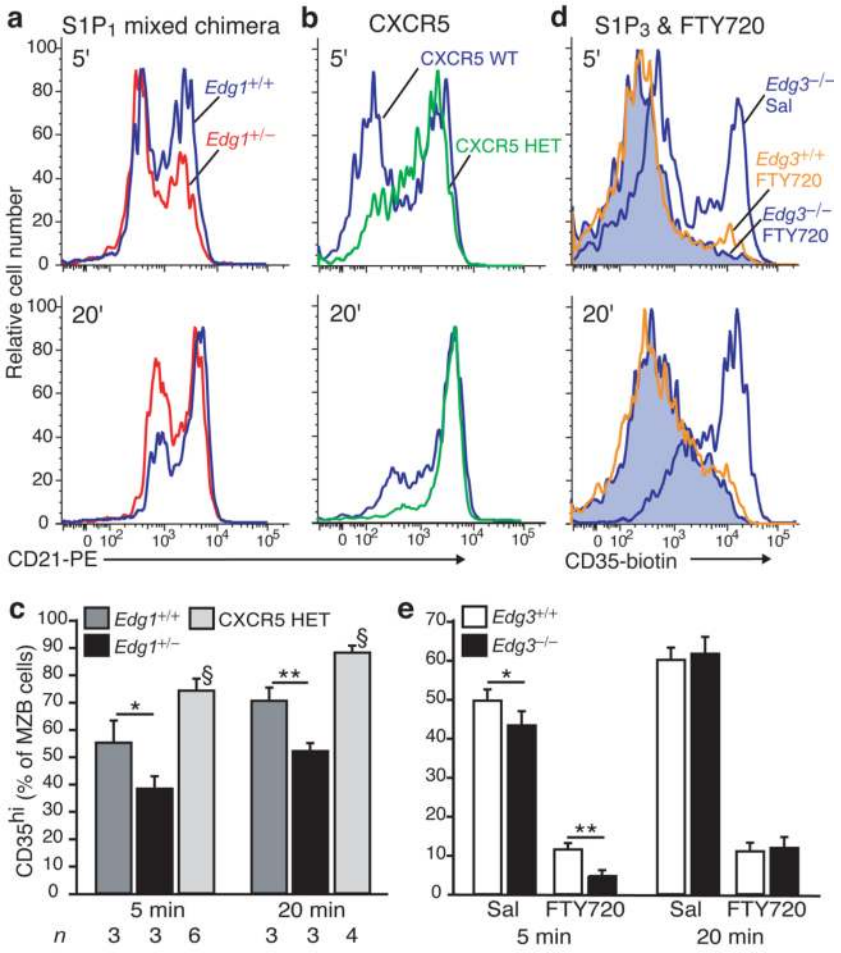

Figure 6.

Balance of $S1P_1$ and CXCR5 responsiveness, and $S1P_3$, control marginal zone B cell shuttling. (**a, b**) Flow cytometric analysis of marginal zone B cell labeling by injected antibodies. (**a**) Chimeras reconstituted with *Edg1*$^{+/+}$ and *Edg1*$^{+/-}$ BM, and (**b**) CXCR5 wildtype (WT) or CXCR5 heterozygote (Het) mice were injected with anti-CD21-PE antibody for 5 or 20 min, as indicated. (**c**) Summary of the data depicted in **a** and **b**. Bars indicate mean, and error bars, s.e.m. *, $p < 0.05$. **, $p < 0.01$ between *Edg1*$^{+/+}$ and *Edg1*$^{+/-}$ cells. §, $p < 0.01$ relative to CXCR5 WT controls. (**d**) FACS histogram of CD35-biotin labeling of marginal zone B cells after 5 or 20 min, as indicated, in *Edg3*$^{+/+}$ and *Edg3*$^{-/-}$ BM chimeras following saline or FTY720 pretreatment for 3 h. (**e**) Summary of the data depicted in **d**. Bars indicate mean, and error bars, s.e.m., and $n \geq 4$. *, $p < 0.05$. **, $p < 0.01$.