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Plasma cell $S1P_1$ expression determines secondary lymphoid organ retention versus bone marrow tropism

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After induction in secondary lymphoid organs, a subset of antibody-secreting cells (ASCs) homes to the bone marrow (BM) and contributes to long-term antibody production. The factors determining secondary lymphoid organ residence versus BM tropism have been unclear. Here we demonstrate that in mice treated with FTY720 or that lack sphingosine-1-phosphate (S1P) receptor-1 ($S1P_1$) in B cells, IgG ASCs are induced and localize normally in secondary lymphoid organs but they are reduced in numbers in blood and BM. Many IgG ASCs home to BM on day 3 of the secondary response and day 3 splenic ASCs exhibit S1P responsiveness, whereas the cells remaining at day 5 are unable to respond. $S1P_1$ mRNA abundance is higher in ASCs isolated from blood compared to spleen, whereas CXCR4 expression is lower. Blood ASCs also express higher amounts of Kruppel-like factor (KLF)2, a regulator of $S1P_1$ gene expression. These findings establish an essential role for $S1P_1$ in IgG plasma cell homing and they suggest that differential regulation of $S1P_1$ expression in differentiating plasma cells may determine whether they remain in secondary lymphoid organs or home to BM.

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Abbreviations used: ASC, antibody-secreting cell; KLF, Kruppel-like factor; NP-CGG, 4-hydroxy-3-nitrophenyl-acetyl coupled to chicken γ-globulin; PC, plasma cell; RP, red-pulp; S1P, sphingosine-1-phosphate; $S1P_1$, S1P receptor-1; SRBC, sheep red blood cell.

Although most antibody responses are initiated in secondary lymphoid organs, long-lived IgG-secreting plasma cells are located predominantly in the BM (1–3). The homing of IgG plasmablasts and plasma cells (hereafter referred to in combination as antibody-secreting cells [ASCs]) to the BM occurs only gradually and to a low level in the primary response but is rapid in the secondary response (1, 4). Studies with non-replicating antigens showed that ASCs were abundant in blood at day 3 of the secondary response but were few in number by day 7 (5, 6). Splenectomy at day 2 of the secondary response to sheep red blood cells (SRBCs) largely prevented ASCs from appearing in the BM, whereas splenectomy at day 4 had little impact on the number of BM ASCs (7). IgG ASCs in blood affinity mature in parallel with BM ASCs, supporting the view that blood ASCs are in transit to the BM (8).

Homing of ASCs to the BM is in part dependent on the chemokine receptor CXCR4 (9–11). The CXCR4 ligand, SDF-1 (CXCL12), is abundantly expressed in the BM and is also strongly expressed in the red-pulp (RP) of spleen and in the medullary cords in LNs (6, 12). ASCs that lack CXCR4 fail to localize appropriately in the splenic RP and LN medullary cords and fail to accumulate to normal numbers in the BM (6, 9). However, CXCR4-deficient ASCs are found in elevated numbers in the blood, indicating that this receptor is not essential for the cells to egress from secondary lymphoid organs (6).

Recent studies have identified an important role for sphingosine-1-phosphate (S1P) receptors in lymphocyte egress from secondary lymphoid organs. Initial studies with the immunosuppressant compound, FTY720, showed that it inhibits lymphocyte egress from LNs and Peyer's patches (13). The effects of FTY720

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on egress from the spleen have been difficult to assess because cell entry and exit both occur via the blood. After injection, FTY720 is rapidly phosphorylated and FTY720-P is a ligand for S1P receptors 1, 3, 4, and 5 (14, 15). SIP receptor 1 ($S1P_1$) is essential for blood vessel development, and mice lacking this receptor die at embryonic day 13.5–14.5 (16). Studies in fetal liver chimeras or in tissue-specific knockout mice showed that T cells lacking $S1P_1$ are unable to exit the thymus, and $S1P_1$-deficient B and T cells are inefficient in exiting secondary lymphoid organs (17, 18). S1P is abundant in circulation and low in secondary lymphoid organs, and lymphocytes are thought to egress in response to S1P (19). FTY720 treatment was not found to affect the antibody response to lymphocytic choriomeningitis virus or vesicular stomatitis virus (20), whereas in a recent study it reduced both the splenic and BM ASC response to 4-hydroxy-3-nitrophenyl-acetyl coupled to chicken γ-globulin (NP-CGG) in alum (21). However, the influence of FTY720 or $S1P_1$ on ASC egress from lymphoid organs has not been directly assessed. Since ASCs downregulate expression of CXCR5 and CCR7 and migrate to splenic RP and LN medullary cords—egress sites from these organs—in a CXCR4-dependent manner, it has been unclear whether they will use the same egress mechanisms as naive lymphocytes.

Here we have examined the possibility that differential $S1P_1$ expression contributes to promoting release versus retention of ASCs in secondary lymphoid organs. We show that FTY720 treatment inhibits IgG ASC egress from spleen and LNs. Using a mixed chimera approach we provide evidence that $S1P_1$ is required in B lineage cells for IgG ASC egress from the spleen and accumulation in the BM. In the secondary response, day 3 but not day 5 splenic ASCs and day 3 blood ASCs demonstrate responsiveness to S1P, and blood ASCs express higher amounts of $S1P_1$ than splenic ASCs. Blood ASCs are also found to have reduced CXCR4 expression compared with splenic ASCs, and they express higher amounts of Kruppel-like factor (KLF)2, a regulator of $S1P_1$ transcription. These findings suggest that differential $S1P_1$ expression is a control point for secondary lymphoid organ retention versus egress into circulation and subsequent BM homing of newly developing IgG ASCs.

RESULTS

FTY720 treatment inhibits IgG ASC entry into blood and homing to BM

To test the impact of FTY720 treatment on ASC homing during the primary response, mice were immunized with the T cell–dependent antigen, NP-CGG in the adjuvant alum, and then at day 4 and 7 with FTY720. The frequency of ASCs in spleen and BM was then determined at day 7 and 10 (Fig. 1 A). The FTY720 treatment was delayed until day 4 to limit effects on the initial recruitment of recirculating antigen-specific B and T cells. The IgG ASC frequency in the spleen was little affected by the FTY720 treatment (Fig. 1 A). In contrast, there was a decrease in the number of IgG ASCs in the BM at day 7 and 10 (Fig. 1 B). Similarly, s.c. immunization led to appearance of IgG ASCs in the draining LNs, and FTY720 treatment did not greatly affect this response. However, FTY720 largely inhibited the appearance of IgG ASCs in the BM after s.c. immunization (Fig. 1, A and B).

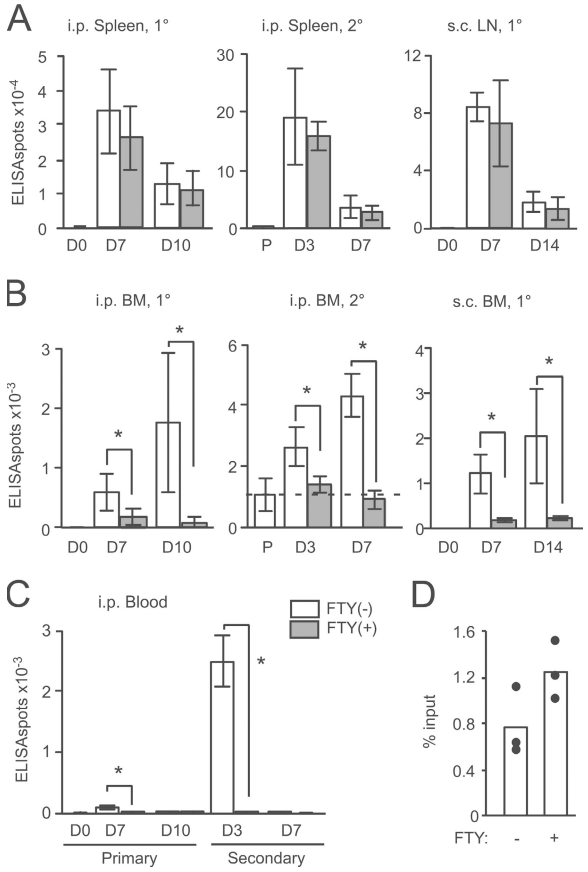

Figure 1. Antigen-specific IgG1 ASC numbers in spleen, LNs, BM, and blood after immunization and FTY720 treatment. Spleen or LN (A), BM (B), and blood (C) cells were isolated at the indicated days after primary (1°) or secondary (2°) i.p. or s.c. immunization with NP-CGG in alum. Secondary immunizations were given 3–4 wk after the primary. P indicates samples taken just before the secondary immunization. FTY720 (FTY(+)) or saline (FTY(−)) treatment was at day 4 and 7 after the primary or day 1.5 after the secondary immunization. ASC frequencies were measured by anti–NP-ELISPOT assay. The bars represent mean (± SD) number of ELISPOTs per organ or per ml of blood from at least three mice per group, and the data are representative of three experiments. The dashed line in the center panel of B indicates the baseline IgG1 ELISPOT frequency as a result of the primary immunization. (D) Percent of i.v. transferred ASCs that homed to the BM after 1 d in recipients treated 3 h before cell transfer with saline or FTY720. Donor cells were splenocytes from mice at day 3 of the secondary response. IgG1 ASC numbers determined by anti–NP-ELISPOT, and each point indicates an individual recipient. Student's *t* test was performed between the indicated groups. *, $P < 0.05$.

Efforts to determine whether there was an effect on ASC egress from lymphoid organs into the blood were hampered

by the low numbers of ASCs entering into the blood during the primary response. We therefore performed secondary immunizations, treating the mice with FTY720 at day 1.5 after secondary immunization since BM homing via the blood occurs by day 3 of the secondary response (7). Again the treatment had little effect on the IgG ASC number in the spleen (Fig. 1 A). A baseline of NP-specific IgG ASCs were detected in the BM because of the primary immunization, but the number was increased twofold by day 3 and four- to fivefold by day 7 in the control group. FTY720 treatment inhibited the increase in BM ASC number (Fig. 1 B). IgG ASCs were readily detectable in the blood of control mice at day 3 and were largely gone by day 7 (Fig. 1 C). In contrast, few if any IgG ASCs appeared in the blood of FTY720-treated mice (Fig. 1 C). We also asked whether FTY720 could affect BM homing after ASCs had entered the blood, by adoptively transferring spleen cells from mice at day 3 of the secondary response into unimmunized recipients that were either untreated or treated 1 d before with FTY720. ELISPOT analysis 1 d after transfer revealed similar numbers of donor-derived NP-specific ASCs in the BM of control and FTY720-treated mice (Fig. 1 D). Thus, FTY720 treatment inhibits the migration of IgG ASCs from spleen into blood but does not inhibit the homing of adoptively transferred IgG ASCs from blood to BM.

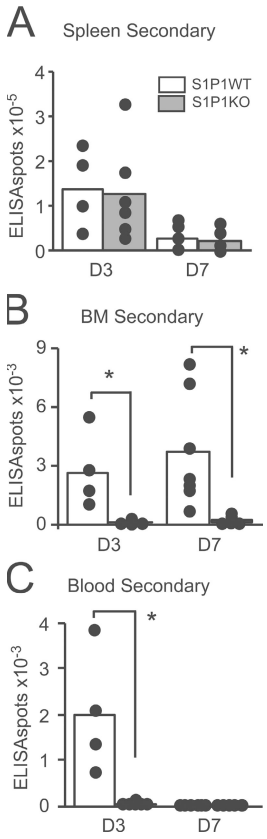

Figure 2. Intrinsic $S1P_1$ requirement for IgG1 ASC egress into blood. Igh^b mice reconstituted with a mixture of $S1P_1^{-/-}$ or $S1P_1^{+/+}$ Igh^a fetal liver and WT Igh^b BM were immunized with NP-CGG in alum, and $IgG1^a$ ASCs were measured 3 and 7 d later in spleen (A), BM (B), and blood (C). Data represent ELISPOTs per organ or per ml of blood in each animal, and bars show the mean ± SD from at least four mice per group compiled from three independent experiments. A Student's *t* test was performed between the indicated groups. *, $P < 0.05$.

Intrinsic $S1P_1$ requirement for BM homing

$S1P_1$ is abundant in naive lymphocytes and is required in these cells for egress from lymphoid organs (17, 18). $S1P_1$ is also abundant in endothelial cells (22). The ability of FTY720 to inhibit cell egress from lymphoid organs may be caused by agonistic effects on endothelial cells, down-modulation of receptors on immune cells, or disruption of S1P gradients (23–25). To test whether there was an intrinsic requirement for $S1P_1$ in ASCs for egress we reconstituted lethally irradiated Igh^b mice with $S1P_1^{-/-}$ Igh^a fetal liver cells and small numbers of Igh^b WT BM cells as a source of WT T cells. After a 6-wk reconstitution, the mice were primed and then boosted with NP-CGG in alum. In mixed chimeric animals that mounted similar day 3 splenic IgG ASC responses (Fig. 2 A), very few $S1P_1^{-/-}$ ASCs could be detected in the BM at day 3 or day 7 (Fig. 2 B). Consistent with $S1P_1$ being required for ASC egress from the spleen, very few IgG ASCs could be detected in the blood at day 3, whereas IgG ASCs could be detected in the blood of WT chimeras (Fig. 2 C).

Immunohistochemical analysis revealed that the distribution of IgG ASCs in FTY720-treated mice at day 5 of the secondary response was similar to their distribution in saline-treated controls (Fig. 3 A). Side-by-side comparison of the distribution of $S1P_1^{-/-}$ $IgG1^a$ and $S1P_1^{+/+}$ $IgG1^b$ ASCs in the spleen of mixed chimeras further demonstrated that this

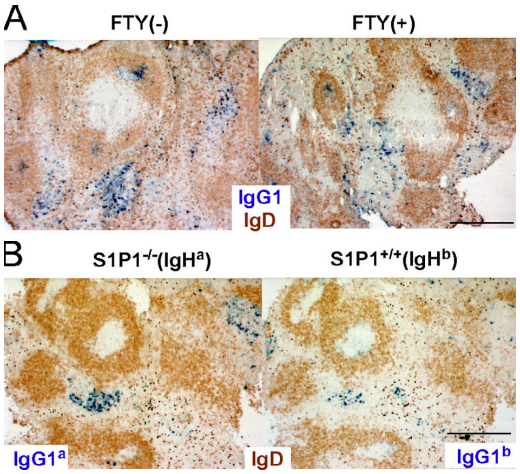

Figure 3. Normal ASC distribution in spleens of FTY720-treated WT mice and of $S1P_1^{-/-}$ plasma cells in mixed BM chimeras. Spleen sections from WT mice treated with or without FTY720 (A), and Igh^a $S1P_1^{-/-}$ fetal liver chimeric mice mixed with Igh^b WT BM (B) immunized 5 d earlier with NP-CGG in alum were stained with the indicated antibodies (labels are the same color as the reaction product for that marker). Bar, 340 μm.

pathway is not required for positioning of IgG ASCs in clusters within the RP (Fig. 3 B).

S1P responsiveness

As a read-out of $S1P_1$ function we examined the ability of ASCs to migrate in response to S1P. We compared the response of IgG ASCs from spleen at day 3 versus day 5 of the secondary response because previous studies have established that the majority of ASCs egress at day 3 with few cells continuing to egress into blood at day 5 (1). Day 3 but not day 5 splenic IgG ASCs showed a small but significant ($P < 0.05$) chemotactic response to S1P (Fig. 4 A). ASCs at both time points demonstrated a similar responsiveness to SDF-1 (Fig. 4 A). Consistent with previous demonstrations that high dose FTY720 treatment ablates S1P responsiveness in lymphocytes, cells from the treated mice had lost the ability to respond to S1P while continuing to respond to SDF-1 (Fig. 4 A). We also tested the ability of IgG ASCs from the blood to chemotax to S1P. Two technical difficulties with this experiment were the very small numbers of ASCs present in blood and the presence of large amounts of S1P in blood that down-regulate and desensitize $S1P_1$ (26). In preliminary experiments, we found it difficult to wash the cells free of blood S1P. However, when the CD45.1 blood cells were mixed with a 10-fold excess of CD45.2 spleen cells at the time of isolation and washing and then the spleen cells depleted using antibodies to CD45.2, it was possible to reveal an S1P response in the blood cells, and this response exceeded that of spleen ASCs isolated in parallel and through the same steps (Fig. 4 B).

An assessment of the migratory response to S1P was also performed using cells from immunized $Blimp1^{GFP}$ reporter mice (27). Blimp1 is up-regulated at an early step in ASC differentiation and is essential for plasma cell development and maintenance (28). As previously observed, the ASCs induced in the spleen at day 3 of the secondary response were predominantly Syndecan1$^+$ and GFP^{int} (Fig. 5 A), whereas there was an accumulation of Syndecan1$^+$ GFP^{hi} cells at later time points (27). The ASCs in the blood at day 3 were consistently Syndecan1$^+$ GFP^{int}, and very few Syndecan1$^+$ GFP^{hi} cells were detected (reference 27 and unpublished data). In migration assays, the splenic GFP^{int} cells showed a migratory response to S1P, whereas the GFP^{hi} cells showed little response (Fig. 5, A and B). The splenic GFP^{int} cells also showed a robust response to SDF-1 (Fig. 5, A and B). These findings confirm that the day 3 splenic ASC population contains S1P-responsive cells.

Analysis of ASCs that accumulate in E- and P-selectin–deficient mice showed that B220$^{int/lo}$ cells were more chemokine responsive than B220^{neg} cells (29). Our observations agree with these findings because the migratory GFP^{int} cells are B220$^{int/lo}$, whereas the poorly migratory GFP^{hi} cells express little if any B220 (Fig. 5 A, bottom and reference 27). In addition, within the GFP^{int} population, we saw a trend toward more S1P responsiveness in ASCs with higher amounts of B220 (Fig. 5 A, bottom). However, B220 levels were not markedly different between GFP^{int} cells in spleen and blood (unpublished data). In the course of these experiments we observed that CXCR4 was expressed at intermediate levels on the surface of blood ASCs compared with splenic ASCs (Fig. 5 C). Despite the lower CXCR4 expression, the magnitude of the blood and splenic ASC SDF-1 chemotactic responses appeared similar (Fig. 4 B). A lack of direct correlation between ASC CXCR4 levels and SDF-1 chemotactic responses has been observed previously (29–31).

$S1P_1$ and KLF2 are more abundant in blood versus spleen ASCs

To measure $S1P_1$ abundance in ASCs in blood versus spleen, $Blimp1^{GFP}$ reporter mice were primed and boosted with NP-CGG and Syndecan1$^+$ GFP^{int} and GFP^{hi} spleen cells or Syndecan1$^+$ GFP$^+$ blood cells were purified at day 3 of the response. The GFP^{int} subset in the spleen was further divided into CXCR4^{hi} and CXCR4^{int} to explore the possibility that the CXCR4^{int} population contained cells that were the most

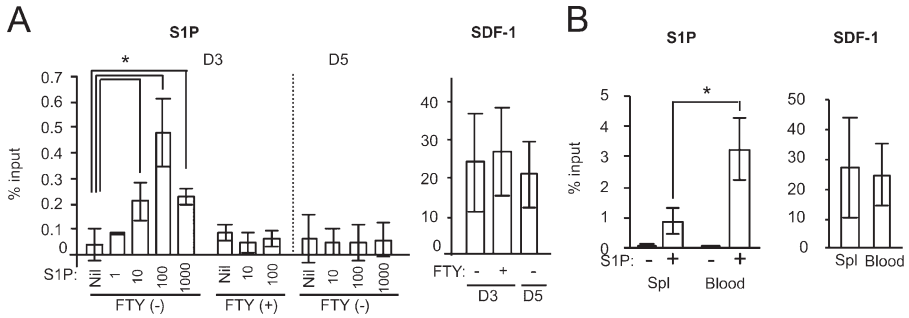

Figure 4. Chemotactic response of IgG1 ASCs to S1P and SDF-1 (CXCL12). (A) Spleen cells were prepared from mice 3 and 5 d after secondary immunization with or without FTY720 treatment. Input cells and cells that migrated to the lower well of a transwell chamber in the absence of chemokine (Nil) or in response to 1, 10, 100, or 1,000 nM S1P (left) or 0.3 μM SDF-1 (right) were analyzed by ELISPOT assay to detect IgG1-secreting cells. (B) Spleen and blood cells were prepared from mice 3 d after secondary immunization as described in Materials and methods. Cells that migrated to the lower well of a transwell chamber in response to 0 or 100 nM S1P (left) or 0.3 μM SDF-1 (right) were analyzed by ELISPOT assay to detect IgG_1-secreting cells. Bars represent means ± SD for six to eight samples from three experiments. A Student's *t* test was performed between the indicated groups. *, $P < 0.05$.

closely related to the blood ASCs (Fig. 5 C). Compared with naive B cells, GFP^{hi} splenic ASCs had ~20-fold reduced S1P$_1$ transcript levels (Fig. 6 A). Splenic GFP^{int}CXCR4^{hi} cells also showed a marked reduction in S1P$_1$ expression, whereas GFP^{int}CXCR4^{int} cells showed a trend toward increased expression (Fig. 6 A). GFP^{int} ASCs sorted from the blood had amounts of S1P$_1$ that were within twofold of the levels detected in splenic B cells (Fig. 6 A). Expression of S1P$_2$, S1P$_3$, S1P$_4$, and S1P$_5$ in blood ASCs was low or undetectable (unpublished data). We also quantitated Blimp1 transcripts and found that they were undetectable in splenic and blood B cells and abundant in the ASCs as expected (unpublished data). Finally, we examined the abundance of KLF2 (also known as lung KLF), a transcription factor that can promote S1P$_1$ transcription (32, 33), and found that levels were reduced in splenic GFP^{hi} and GFP^{int} CXCR4^{hi} ASCs (Fig. 6 B). As for S1P$_1$, KLF2 transcripts were more abundant in the splenic GFP^{int}CXCR4^{int} subset than in GFP^{int}CXCR4^{hi} cells and levels in blood GFP^{int} ASCs were similar to splenic B cells (Fig. 6 B).

DISCUSSION

The above findings demonstrate that egress of IgG ASCs from secondary lymphoid organs into blood and BM is sensitive to inhibition by FTY720 treatment and strongly dependent on intrinsic S1P$_1$ expression. S1P$_1$ abundance and S1P responsiveness is found to be low in most splenic ASCs but is detectable within a Blimp1^{int} CXCR4^{int} splenic ASC population and is most enriched in ASCs isolated from the blood. These findings establish an important role for S1P$_1$ in IgG plasma cell homing and they suggest that differential regulation of S1P$_1$ expression in differentiating plasma cells may be a key factor determining whether they remain in secondary lymphoid organs or exit these organs to home to other sites, such as the BM. We also find differential expression of KLF2 between splenic and blood ASCs, pointing to a possible role for this transcription factor in defining the gene expression program associated with the homing decision of differentiating plasma cells.

In a previous study, treatment with FTY720 from the day of primary immunization was found to reduce the

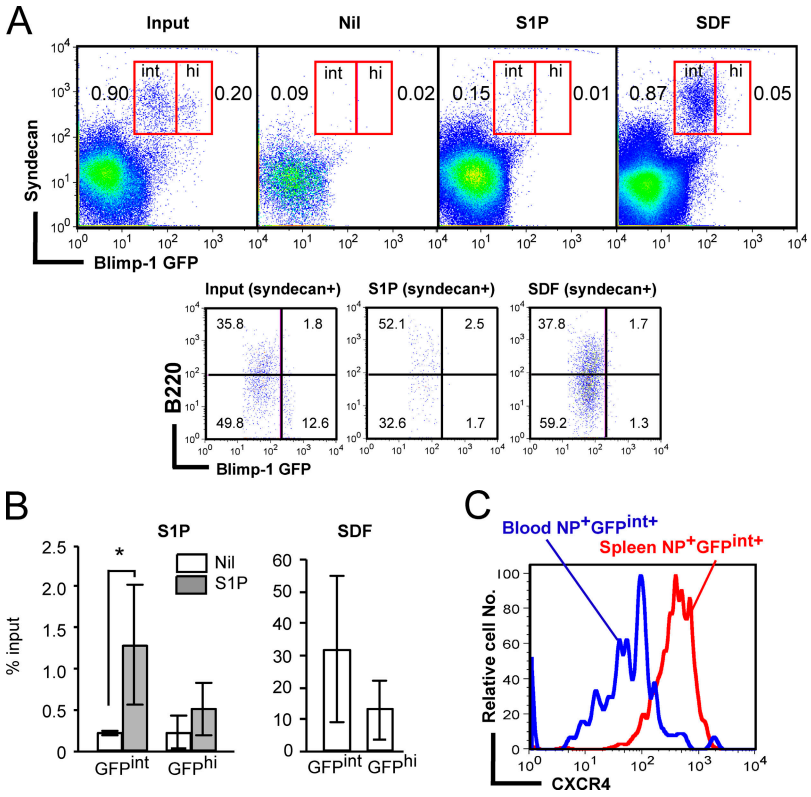

Figure 5. Chemotactic response of Blimp1$^{GFP/+}$ ASCs to S1P and SDF-1, and CXCR4 expression by spleen and blood ASCs. (a) Spleen cells were prepared from Blimp1$^{GFP/+}$ reporter mice 3 d after immunization and applied to transwell chambers. Input cells and cells that migrated to the lower well of a transwell chamber in the absence of chemokine (Nil) or in response to 100 nM S1P or 0.3 μM SDF-1 were analyzed by FACS. Top plots show division of Syndecan$^+$ GFP$^+$ plasma cell into GFP^{int} and GFP^{hi} subsets, and numbers indicate the percentage of total cells in each gate. Bottom plots are pregated for Syndecan$^+$ GFP$^+$ cells (the combined GFP^{int} and GFP^{hi} gates in A) showing B220 versus GFP, and numbers show the percentage of cells in each quadrant. (B) Chemotactic response of splenic GFP^{int} and GFP^{hi} ASC subsets to S1P (left) and SDF-1 (right) are shown as the percentage of input that migrated. Bars show the mean ± SD from three experiments. Student's *t* test was performed between the indicated groups. *, $P < 0.05$. (c) CXCR4 abundance on NP-specific GFP^{int} ASCs in the spleen and blood 3 d after secondary NP-CGG immunization. NP-specific GFP^{int} ASCs were identified as DAPI$^-$CD4$^-$CD8$^-$NP$^+$Syndecan$^+$GFP^{int}. The histogram plot shows the expression levels of CXCR4 on spleen and blood NP$^+$GFP^{int} ASCs.

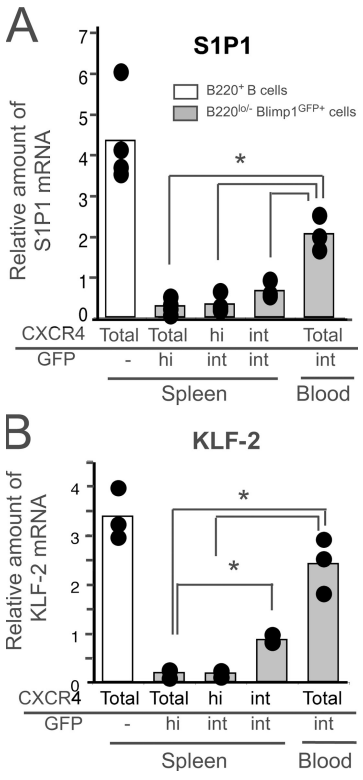

Figure 6. Quantitative PCR analysis of $S1P_1$ and KLF2 mRNA from B cells and plasma cells in spleen and blood. Spleen cells from Blimp-$1^{GFP/+}$ mice 3 d after immunization were sorted into $B220^{lo/-}$ $Syndecan^+$ GFP^{hi}, $GFP^{int}CXCR4^{hi}$, and $GFP^{int}CXCR4^{int}$ ASC subsets and $B220^+$ $Syndecan^-$ GFP^- B cells. The $CXCR4^{int}$ gate was assigned based on CXCR4 levels on blood GFP^{int} cells stained in parallel (see Fig. 5 C for an example). Blood $B220^{lo/-}$ $Syndecan^+$ GFP^{int} cells were from 3-d-immunized mice. The amounts of $S1P_1$ (A) and KLF2 (B) mRNA were expressed as relative amount of the indicated mRNA normalized to HPRT. CXCR4 "total" indicates the cells were isolated without gating on CXCR4 levels. Filled circles indicate individual samples, and bars represent the average. The data are pooled from three to four independent experiments. A Student's *t* test was performed between the indicated groups. *, $P < 0.05$.

magnitude of the IgG plasma cell and germinal center response (21). This may have been a consequence of reduced recruitment of antigen-specific T cells from other sites caused by the generalized block in secondary lymphoid organ egress (34). We delayed treatment of the mice for 4 d in the primary response or 1.5 d in the secondary response to provide an opportunity for recruitment of circulating antigen-specific B and T cells, and we found that the secondary lymphoid organ ASC responses were of similar magnitude in the treated and untreated groups, indicating that treatment did not severely affect the ability of the animals to mount an antibody response. In agreement with this conclusion, production of neutralizing antibodies after the first 3 wk of lymphocytic choriomeningitis virus and vesicular stomatitis virus infection were unaffected by low dose (0.3 mg/kg) FTY720 treatment (20). The ability of $S1P_1$-deficient B cells to mount antibody responses also demonstrates that B cell expression of this receptor is not essential for B cell activation and differentiation. However, because of limitations in the mixed fetal liver/BM chimera approach, our experiments have not examined the efficiency of each stage of the antibody and memory B cell response. Future experiments with mice carrying floxed alleles of $S1P_1$ (17) and B cell–selective Cre recombinase will be needed to more fully address the impact of ASCs and B cell $S1P_1$ deficiency on maintenance of long-term antibody production and on B cell memory.

The mechanism by which FTY720 reduces lymphocyte egress from lymphoid organs is under active investigation (18, 35), and it has been debated whether FTY720 impacts lymphocyte egress from the spleen (14). Our findings here provide evidence that egress of IgG ASCs from the spleen can be inhibited by FTY720 treatment. Although the pathway by which cells exit the spleen is not defined, it is likely that the cells need to reach RP sinusoids to return to circulation. Whether the ASCs that normally egress from the spleen correspond to cells that have first traveled into the RP is not yet clear. However, like spleen and LN ASCs, blood ASCs are found to have down-modulated CXCR5 and CCR7 (and up-regulated CXCR4), making it likely that these cells migrate out of the white-pulp before exiting into circulation (6). The lack of CXCR5 and CCR7 expression on these cells but the continued requirement for $S1P_1$ to permit efficient egress indicates that the intrinsic function of $S1P_1$ cannot solely be to overcome the retention function of these chemokine receptors. However, it remains possible that CXCR4 normally participates in retaining cells in the spleen and LNs, and one function of $S1P_1$ may be to overcome CXCR4-mediated retention. In this regard it is notable that the $Blimp1^{GFP+}$ cells in the blood were $CXCR4^{int}$ compared with the bulk of the $Blimp1^{GFP+}$ cells in the spleen. We propose that the reduced CXCR4 expression together with the increased $S1P_1$ expression in a subset of cells contributes to shifting the balance of signals in favor of $S1P_1$ and promoting egress. However, CXCR4 is only partially down-modulated, consistent with it also being required for lodgment of ASCs in the BM. Beyond this possible interplay, we anticipate that $S1P_1$ will be required during egress to overcome additional retention signals, provide directional information, and/or promote reverse transmigration across blood or lymphatic vessels. Although we have focused on the mobilization of IgG ASCs to the BM, we anticipate that similar requirements will exist for IgA ASC egress from mucosal lymphoid tissues before their homing to epithelial surfaces.

Our findings suggest that definition of the mechanism by which a subset of differentiating ASC up-regulate $S1P_1$ may illuminate how ASCs choose between the secondary lymphoid organ versus BM (or mucosa) tropic plasma cell fate. This cell fate decision may also encompass becoming short versus long lived, though some studies have indicated that long-lived plasma cells (PCs) are present in secondary lymphoid organs and BM (36) and, reciprocally, some of the PC homing to BM may be short lived. Although our studies show that $S1P_1$ is necessary, our studies have not established

that this receptor is sufficient to determine whether an ASC undergoes egress or is retained, and additional differences between the cells may be important. The finding that KLF2, a transcription factor shown to directly induce $S1P_1$ expression in T cells (32, 33), is increased in blood Blimp1^{GFP+} cells is consistent with a role for this factor in promoting $S1P_1$ expression in ASCs. KLF2 regulates the expression of additional genes in T cells (32, 33) and it may regulate expression of further genes involved in the commitment of differentiating B cells to becoming BM tropic ASCs. Previous studies have suggested that BM tropic ASCs are induced predominantly from germinal center B cells or memory B cells (5, 37–39). Indeed, the synchronized wave of ASCs traveling through the blood at day 3 of the secondary response most likely reflects rapid differentiation of memory B cells into BM tropic ASCs. Although it is thought likely that these ASCs contribute to the pool of long-lived PCs, we have not established that this is the case for the cells tracked in the present study. Finally, although we were able to enrich based on intermediate CXCR4 and Blimp1^{GFP} expression for splenic ASCs with an egress-related phenotype, this cell population had lower amounts of $S1P_1$ and KLF2 than the cells isolated from blood, suggesting that we had only achieved partial enrichment. The future development of reagents that permit tracking of $S1P_1$ expression at the level of single B cells and ASCs should provide a method to isolate and further characterize the earliest cells that are taking on the BM tropic ASC fate.

MATERIALS AND METHODS

Mice and fetal liver chimeras. C57BL/6 (B6) and B6 CD45.1 mice were from the National Cancer Institute. Blimp1$^{GFP/+}$ mice were generated as described (27). $S1P_1^{+/-}$ mice (16) were crossed for at least five generations to B6 or 129 mice, and then $S1P_1^{+/-}$ mice were intercrossed. $S1P_1^{+/+}$ or $S1P_1^{-/-}$ fetal liver cells were prepared at embryonic day 12.5 and genotyped by PCR (16). Each preparation was mixed with B6 BM cells (1.5×10^5 and 3×10^6 cells, respectively) and transferred to reconstitute three lethally irradiated B6 CD45.1 mice (6). Mice were immunized 6 wk after reconstitution. For FTY720 treatment, B6 mice were injected i.p. with 3.0 mg/kg of FTY720 or with an equivalent volume of saline 4 and 7 d after primary and 1.5 d after secondary immunization. FTY720 was from a custom synthesis performed at SRI International. Animals were housed in a specific pathogen-free facility and all experiments were in accordance with protocols approved by the University of California San Francisco Institutional Animal Care and Use Committee.

Immunizations. B6 mice were immunized with 50 μg of alum-precipitated NP-CGG (Solid State Sciences) i.p. or s.c. in a volume of 200 μl. In some experiments, mice received a secondary immunization of the same type 3 wk later. Spleen, LNs, blood, and BM were harvested from mice at the indicated time points as described previously.

Flow cytometry and immunohistochemistry. Flow cytometric data were collected on a FACS LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star). Antibodies used were: biotin-conjugated anti-CXCR4 (2B11; BD Biosciences), PE-conjugated anti-Syndecan (281–2; BD Biosciences), APC-conjugated anti-B220 (RA3-6B2; BD Biosciences) and NP (40), and APC-Cy7–conjugated anti-CD4 and CD8 (GK1.5, 53–6.7; BD Biosciences). Streptavidin-PE–Cy5.5 was from Caltag, and DAPI was used to exclude dead cells. Spleens of immunized B6 mice treated with or without FTY720 were frozen in Tissue-Tek OCT compound (Baxter Scientific). Cryostat sections (7 μm) were fixed in acetone and stained as described previously (41) with sheep anti-IgD (Binding Site, Inc.) and biotin-conjugated rat anti-IgG1, IgG1a, and IgG1b (MOPC-31C, 10.9, B68-2; BD Biosciences). Sheep antibodies were detected using HRP-conjugated donkey anti–sheep IgG (Jackson ImmunoResearch Laboratories). Biotinylated reagents were detected using the SA-AP (Jackson ImmunoResearch Laboratories). Enzyme reactions were developed with conventional substrates for peroxidases (diaminobenzidine; Sigma Chemical Co.) and alkaline phosphatase (Fast Blue; Sigma Chemical Co.).

Cell sorting and quantitative PCR. Spleen cells from B6 mice and Blimp1^{GFP} mice were stained, and ASCs and B cells were isolated with a MoFlo cell sorter (Cytomation). To isolate blood Blimp1^{GFP+} cells, blood from 15–20 mice at day 3 after secondary immunization was used for each experiment. In some cases, to generate enough mice bearing the Blimp1^{GFP} allele in their hematopoietic cells, BM chimeras were made by transferring CD45.2 Blimp1^{GFP} BM into irradiated CD45.1 B6 recipients and allowing 6 wk for reconstitution before primary immunization. The purity of the sorted cells was typically >95%. RNA was prepared from sorted cells with RNeasy (QIAGEN), and cDNA was used for quantitative PCR on an ABI 7300 sequence detection instrument (Applied Biosystems) using the following primer and probe sets with AmpliTaq Gold DNA polymerase (Applied Biosystems) CXCR4, forward, CGGCTGTAGAGCGAGTGTTG and reverse, TCTCCAGACCCCACTTCTTCA; probe FAM-CATGGAACCGATCAGTGTGAGTATATACACTTCTGA-TAMRA; Blimp1(Prdm1): forward, GGATCTTCTCTTGGAAAAACGTGT and reverse, AGCCGTGTAAAGTAGACTGCCTTG; probe FAM-TACGACCTTGCCAAAGGCTGCATTTTAAA-TAMRA; and HPRT, $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ as reported (18). KLF2 (and HPRT) was quantitated using SYBR Green (Bio-Rad Laboratories), and KLF2, forward, TATCTTGCCGTCCTTTGCCA and reverse, TTAGGTCCTCATCCGTGCC primers.

Chemotaxis assays. Cells were tested for transmigration across uncoated 5-μm transwell filters (Corning Costar Corp.) for 3 h to S1P (Sigma-Aldrich), SDF-1 (PeproTech), or medium in the bottom chamber and were enumerated by ELISPOT or, in the case of cells from Blimp1$^{GFP/+}$ mice, by flow cytometry (42). To prepare blood cells for chemotaxis, 2×10^6 Ly5.1 Igh^a RBC-lysed blood cells isolated by cardiac puncture were mixed with 10^8 Ly5.2 Igh^b RBC-lysed spleen cells, incubated for 30 min at 37°C in DMEM 0.5% fatty acid–free BSA, washed twice, and then the Ly5.1 and Ly5.2 cells were separated using Ly5.2 and AutoMACS. Because of the low frequency of ASCs, the blood cells that migrated across 8 transwells and the spleen cells that migrated across 4 transwells in response to S1P were pooled and tested in the ELISPOT or FACS assays.

ELISPOT assays. Freshly isolated spleen, blood, BM, and LN cells were used in ELISPOT assays as described previously (6). Plates were precoated with NP_{25}-BSA (Solid State Sciences). IgM, IgM^a, IgM^b, IgG1^a, IgG1^b, and IgG were detected with anti–mouse IgM biotin (Caltag), IgM^a biotin (DS-1; BD Biosciences), IgM^b biotin (AF6-78; BD Biosciences), IgG1 biotin (BD Biosciences), IgG1^a biotin (BD Biosciences), and IgG1^b biotin (BD Biosciences), followed by SA-HRP and True Blue (Kirkegaard & Perry Labs, Inc).

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