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The sphingosine 1-phosphate receptor S1P2 maintains the homeostasis of germinal center B cells and promotes niche confinement

by

Jesse Allan Green

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

This dissertation is dedicated to my parents, David and Patricia Green,

who with all of their caring support never allowed a doctoral

degree in biology to seem out of reach

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Contributions to the Presented Work

All of the work presented in this dissertation was performed under the direct supervision of Dr. Jason G. Cyster. Additional contributions to specific chapters are described below.

Chapter 2 was published as Green JA, Suzuki K, Cho B, Willison LD, Palmer D, Allen CDC, Schmidt TH, Xu Y, Proia RL, Coughlin SR, and Cyster JG. (2011). *The sphingosine 1-phosphate receptor* $S1P_2$ *maintains the homeostasis of germinal center B cells and promotes niche confinement.* Nat Immunol. **12**(7): 672-680. Chris Allen and Ying Xu performed the initial gene expression analysis that identified S1P2 expression in germinal center B cells. Ying Xu also performed all of the quantitative PCR. Early experiments on the role of S1P2 in germinal center B cell migration and positioning were performed by Bryan Cho, who made the initial observations that S1P inhibited germinal center B cell movement, that S1P2-deficient B cells were mislocalized within mixed chimeric germinal centers, and that S1P2-deficient germinal centers were disorganized in CXCL13-deficient hosts. These observations prompted some of the experiments that led to data shown in Figures 4, 5, and 7, and Supplementary Figure 5. L. David Willison generated the *Gna12*-deficient mice and Daniel Palmer crossed them to *Gna13*$^{f/f}$ mice carrying the *Mx1-cre* transgene. He also treated the mice with polycytidylic acid to induce Cre expression and supplied them to me. Timothy Schmidt performed some of the Akt signaling experiments in Ramos cells, which generated data leading to Figure 2g-i. Kazuhiro Suzuki performed

the two-photon imaging experiments and analyzed the data, contributing to Figure 4g-h and Supplementary Figure 6. I performed all of the other experiments and analyzed the data. Jason Cyster and I wrote the paper. Other contributions are listed in the specific acknowledgements page for this chapter.

The sphingosine 1-phosphate receptor $S1P_2$ maintains the homeostasis of germinal center B cells and promotes niche confinement

By Jesse Allan Green

Abstract

The germinal center (GC) is a hallmark microenvironment for the T-dependent antibody response, in which activated B cells divide, mutate their antigen receptor genes, compete for antigen, and are selected on the basis of high-affinity antigen binding in order to produce protective antibodies. GCs form in the center of the B cell follicle and GC B cells remain both spatially confined to their niche and segregated from naive B cells in the surrounding follicle. In addition, GC size is tightly controlled, and it is not understood whether the spatial organization and physical restrictions play a role in controlling GC B cell growth and homeostasis. We set out to identify cues involved in positioning GC B cells in the center of the follicle and contributing to their confinement within the GC.

Sphingosine-1-phosphate receptor-2 (S1P2)-deficient mice develop diffuse large B cell lymphoma. However, the role of S1P2 in normal GC physiology is unknown. Here we show that S1P2-deficient GC B cells outgrow their wild-type counterparts in chronically-established GCs. We find that S1P2-,

G12-G13- and p115RhoGEF-mediated antagonism of Akt regulates cell viability and is required for growth control in chronically proliferating GCs. We also find that S1P2 inhibits GC B cell responses to follicular chemoattractants and helps confine cells to the GC. Moreover, S1P2 overexpression promotes centering of activated B cells within the follicle. We suggest that by inhibiting Akt activation and migration, S1P2 helps restrict GC B cell survival and localization to an S1P-low niche at the follicle center.

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Chapter 1

Introduction

T dependent antibody responses

The humoral immune response is characterized by the production and secretion of antibodies by B cells that have been activated by antigen. Secreted antibodies provide protection against pathogenic organisms in extracellular spaces in several ways, including binding and directly neutralizing pathogens, coating and inducing the uptake of pathogens by phagocytic immune cells, and binding to pathogens and activating the complement cascade. To elicit effective responses, the immune system relies upon the formation of distinct microenvironments in which different cell types interact efficiently to promote stimulation and differentiation. The hallmark microenvironment of the B cell immune response is the germinal center (GC), a structure consisting of a number of cell types that normally forms in the B cell follicle of secondary lymphoid organs during an antibody response (MacLennan 1994; Allen, Okada & Cyster 2007). Here GC B cells cluster and proliferate rapidly. The primary function attributed to the GC is the generation of large numbers of high-affinity memory B cells and isotype-switched antibody-secreting cells that disseminate to effector sites such as the red pulp of the spleen, the bone marrow, and the gut, where they secrete antibody important for the neutralization of pathogens.

One characteristic of the antibody response that has been recognized for several decades is known as affinity maturation, which refers to the phenomenon in which the affinity of serum antibody for a foreign antigen increases over time after immunization with that antigen (Eisen & Siskind 1964). The process of affinity maturation is strongly associated with the GC for several reasons. GC B

cells acquire increasing numbers of somatic mutations in their immunoglobulin genes which encode antibody receptors during the first two to three weeks after immunization (Jacob et al. 1991; Berek et al. 1991). These mutations are focused in antigen-binding regions of the antibody genes, and in addition specific mutations known to increase the affinity for antigen appear with increasing frequency over time in GC B cells, suggesting that an ongoing selection mechanism is taking place within the GC.

In addition, a physiological role for GCs has been shown by studies using mouse models in which GC formation is disrupted. Transgenic mice overexpressing soluble CTLA-4 exhibit normal T-cell priming but cannot form GCs (Lane et al. 1994), and mice lacking the gene encoding the lymphotoxin β receptor cannot form GCs (Fütterer et al. 1998). In each case, the absence of GCs correlates with low levels of class switching, reduced accumulation of somatic hypermutations, and low levels of antigen-specific antibodies. However, some affinity maturation can take place in experimental systems in which GC formation is disrupted but repeated immunizations or strong adjuvants are used to induce antibody responses (Matsumoto et al. 1996; Wang et al. 2000), suggesting that GCs are not absolutely required for the competition and selection that leads to improved antigen affinity to occur. However, the GC environment is likely optimized for the development of high affinity antibody responses.

B cell antigen encounter and early movements

Germinal centers form in the center of B cell follicles during T dependent antibody responses after a series of coordinated movements through lymphoid environments by antigen-activated B and T cells (Pereira et al. 2010). Prior to activation, naive B and T cells are compartmentalized into distinct regions within the spleen and lymph nodes, characterized by B cells follicles arranged around a central T zone. Both regions also contain distinct reticular networks of stromal cells, among which naive B and T cells move (Cyster et al. 2000; Mueller & Germain 2009). These stromal cells have long processes that present chemokines which help to guide the movements of B and T cells into and throughout their respective compartments. In the B cell follicle, follicular dendritic cells (FDCs) express CXCL13, which attracts B cells expressing the chemokine receptor CXCR5, and in the T zone fibroblastic reticular cells (FRCs) express CCL19 and CCL21, which promote migration of CCR7-expressing T cells. In the absence of activation signals, lymphocytes migrate within follicles and T zones for a period of several hours to a day, then leave the secondary lymphoid organ, entering the circulation by way of lymphatic sinuses in lymph nodes (Grigorova et al. 2009; Sinha et al. 2009) by using the sphingosine-1-phosphate receptor S1P1 (Schwab & Cyster 2007).

However, migration through follicles also promotes the encounter of B cells with their cognate antigen (Cyster 2010). Antigens can reach the follicle and be displayed to antigen-specific B cells through several different ways, which depend on the size and form of the antigen and the route of its entry into the body. Small soluble antigens can be drained from skin tissue by the flow of

plasma and lymph, and enter lymph nodes through afferent lymphatics where they flow through the subcapsular sinus (SCS) before reaching the medullary sinus. Some small antigens can access the follicle by diffusing through conduits that reach from the SCS to high endothelial venules (HEVs) where B cells enter the lymph node. SCS macrophages can also pick up antigen through a number of cell surface receptors and transfer it to B cells in the follicle. Large particles, on the other hand, may be taken up by dendritic cells (DCs) or other myeloid cells, which can migrate into the lymph node and display recycled antigen to B cells. In addition, B cells themselves can carry antigen in the form of antibody-bound immune complexes on their complement receptors or FcγRIIb, and can pass these immune complexes among each other or to FDCs in the center of the follicle. FDCs can bind and display immune complexes for long periods of time and display cognate antigen to B cells.

Upon encountering cognate antigen and undergoing activation through the B cell receptor (BCR), B cells upregulate CCR7, which directs them to the border of the T zone (Pereira et al. 2010). There, the likelihood of interacting with cognate T cells is increased. Antigen-engaged B and T cells undergo stable contacts at the B-T border (Okada et al. 2005). After 1-2 days, activated B cells migrate from the B-T border to interfollicular regions and the outer follicle (Coffey et al. 2009), a movement that is promoted by the recently identified chemoattractant receptor EBI2 (Pereira et al. 2009; Gatto et al. 2009), where they proliferate for an additional period of up to few days. Here activated B cells also make a cell fate decision, with some becoming plasmablasts and migrating

to extra-follicular sites to produce early antibody and others differentiating into GC B cell precursors and migrating into the center of the follicle, where they will interact with FDCs and form a GC cluster where they continue to proliferate. This movement is accompanied by the downregulation of EBI2, reducing the attraction to the outer follicle.

Spatial organization of the GC

The cues that guide GC B cell precursors to the center of the follicle are not fully understood. One important determinant is CXCL13, which is a critical follicular chemokine that plays a role in GC size and localization. Without CXCL13, B cells still gather into separate rings around T zones, but do not form polarized follicular clusters (Ansel et al. 2000). B cells from mice deficient in CXCL13 or its chemokine receptor CXCR5 can form GCs, but in the spleen GCs form in the periarteriolar lymphoid sheath (PALS) rather than the B cell follicle, suggesting that CXCR5 function is important for the localization of GC precursors (Voigt et al. 2000; Ansel et al. 2000). However, in the lymph nodes of CXCR5-deficient mice GCs are still localized to B cell areas, suggesting that additional factors can participate in the proper localization of GC precursor B cells to the center of B cell areas in lymph nodes. In addition, CXCL13 is not known to be particularly focused in the center of the B cell follicle, as it is made broadly by follicular stromal cells (Cyster et al. 2000), so while CXCL13 is important for follicular organization and attraction to the B cell area, it is likely that additional cues exist

to aid in the positioning of the GC B cell precursors that migrate to the center of the follicle.

GCs are seeded by a small number of activated B cells, which proliferate rapidly to form clusters of approximately 10,000 cells (MacLennan 1994; Allen, Okada & Cyster 2007). Interactions between GC B cells and FDCs that involve lymphotoxin (LT)-$\alpha1\beta2$ expression by GC B cells induce the maturation of the latter into GC-associated FDCs, which are characterized by increased expression of the integrin ligand VCAM-1 and FcγRIIb (Cyster et al. 2000). As GC B cells proliferate, some start to expand beyond the FDC network to form a separate region (Liu et al. 1991; Wang et al. 2005). The fully formed GC is divided into distinct zones known as the light zone and the dark zone. The light zone is made of GC B cells known as centrocytes, the FDC network, antigen-specific T cells that are known as follicular helper T cells, and tingible body macrophages. In contrast, the dark zone is made up primarily of GC B cells known as centroblasts, as well as some poorly defined stromal cells, T cells, and tingible body macrophages. The segregation of light and dark zones is mediated by the chemokines CXCL13 and CXCL12 (Allen et al. 2004). While CXCL13 is not concentrated in the GC relative to the surrounding follicle, within the GC CXCL13 is expressed by light zone FDC but not by stromal cells in the dark zone, and its receptor CXCR5 is important for the localization of GC B cells to the light zone. CXCL13 and CXCR5 are also important for the proper polarization of GC-associated FDC to the light zone. CXCL12 binds to the chemokine receptor CXCR4, which is expressed more highly on centroblasts than centrocytes, and

promotes the movement of centroblasts to the dark zone where CXCL12 is focused, though again CXCL12 is not known to be more abundant in the GC than outside the GC. The light zone of GCs is positioned towards the marginal sinus of the spleen and the subcapsular sinus of the lymph node, possibly to facilitate the transport of antigen from sites of entry into the lymphoid tissue to the FDC network of the light zone where it can be displayed to GC centrocytes (Allen, Okada & Jason G Cyster 2007).

Two-photon imaging experiments have shown that GC B cells migrate extensively throughout each zone and can cross from one zone to the other in either direction (Allen et al. 2007; Schwickert et al. 2007; Hauser et al. 2007; Victora et al. 2010). Recently the use of a photo-activatable fluorescent marker permitting the tracking of cells labeled specifically in one zone but not the other showed a faster movement from dark zone to light zone (Victora et al. 2010). Centroblasts in the dark zone were more often found to be cycling, while centrocytes expressed slightly higher levels of surface immunoglobulin (Victora et al. 2010), consistent with the classical model in which centroblasts divide, then move to the light zone and compete for antigen binding to undergo selection and differentiation or 'death by neglect' and apoptosis (MacLennan 1994).

In addition to the chemotactic cues that organize the GC dark and light zone, the GC is segregated from naive B cells in the follicle through a largely undefined mechanism. While CXCR5- and CXCL13-deficient GCs are small, often mislocalized, and have altered FDC polarization (Voigt et al. 2000; Ansel et al. 2000), they are still clustered. Naive B cells have been shown to briefly enter

and survey the GC light zone (Schwickert et al. 2007), but few enter the dark zone and GC B cells are strongly confined within the GC. Whether additional cues that attract GC B cells to the center of the follicle and the FDC network exist and what cells are the source of these cues, or whether other mechanisms exist that promote the confinement and segregation of GC B cells, is unknown.

Affinity maturation and selection within the GC

While proliferating rapidly, GC B cells are also undergoing two key genetic processes: somatic hypermutation (SHM) of their antibody gene V regions and class switch recombination (CSR), a genetic rearrangement in which GC B cells switch their immunoglobulin expression from IgM and IgD to another class with different effector functions, such as one of the IgG classes, IgA, or IgE. Both processes are dependent on the activity of activation-induced cytidine deaminase (AID), an enzyme that deaminates cytidines on DNA, introducing mismatches that are repaired (Maizels 2005). It is well established that the average serum affinity for antigen increases during the course of an antibody response, and that this increase depends on the addition of mutations to antibody V genes that occurs in the GC and can result in antibodies with higher affinity for their antigens.

However, the mechanisms through which GC B cells compete for survival, persistence, and differentiation into antibody-secreting cells are not fully understood. In the classical model of GC B cell selection, centrocytes compete for the binding of antigen retained on FDC processes, and those with the highest

affinity receive the strongest BCR signals (MacLennan 1994). There is evidence that BCR affinity for antigen affects GC competition and selection. Low-affinity B cells can accumulate and persist as GC B cells, but are outcompeted by B cells expressing a higher affinity BCR specific for the same antigen (Shih et al. 2002). However, there is little evidence that selection is based purely on the competition for antigen retained on FDC processes (Allen, Okada & Cyster 2007). Two-photon imaging experiments revealed few extended contacts between GC B cells and FDC processes. When transgenic B cells were engineered to be deficient in the secretion of antibody, there was undetectable immune complex deposition of FDCs, while GC kinetics appeared normal and there was some evidence of selection (Hannum et al. 2000). B cells lacking the tyrosine phosphatase CD45 exhibited defects in BCR signaling, but were able to undergo some antigen-driven selection within the GC (Huntington et al. 2006). In addition to competing for signals solely through the BCR, GC B cells could compete for signals from GC-associated follicular helper T cells by presenting antigen in major histocompatibility complex (MHC) class II (Allen, Okada & Cyster 2007). GC B cells depend on signals from T cells, such as CD40L, IL-4, and IL-21. In mice containing T cells disregulated by a mutation in the *Roquin* gene that leads to increased ICOS expression, GC responses are exaggerated (Vinuesa et al. 2005). Imaging experiments suggested that interactions between GC B and T cells were tightly regulated (Allen et al. 2007). Experiments have recently been performed in which T cell help was targeted towards a specific subset of GC B cells by using an antibody to DEC205 conjugated to the antigen recognized by

GC T cells (Victora et al. 2010). This allowed DEC205-expressing GC B cells to internalize and present cognate antigen at higher levels than DEC205-deficient GC B cells, and the result was that DEC205-expressing cells quickly outcompeted DEC205-deficient cells. Thus, T cell help is strongly implicated as a selection mechanism within the GC.

There is evidence that GC B cell apoptosis plays a role in affinity-mediated selection. GC B cells downregulate the anti-apoptotic protein Bcl-2 and are sensitive to apoptotic death *ex vivo* (Liu et al. 1989; Martinez-Valdez et al. 1996). Transgenic mice overexpressing anti-apoptotic Bcl-xL show reduced affinity maturation and persistence of lower affinity antibody-secreting cells, and mice either overexpressing Bcl-2 (Smith et al. 2000) or deficient in proapoptotic molecule Bim (Fischer et al. 2007) both exhibit an increased persistence of low affinity cells into the memory compartment, suggesting that GC selection was reduced, though in these cases stringent selection into the antibody-secreting cell compartment was retained. In addition, GC B cells express high levels of the Fas death receptor (Smith et al. 1995) which signals downstream to mediate caspase-dependent apoptosis (Krammer 2000). Antigen-driven clonal selection is reduced in Fas-deficient mice (Takahashi et al. 2001), again suggesting that when GC B cells are resistant to a form of apoptosis, selection mechanisms are not as effective.

In addition to the positive selection of B cells expressing high affinity BCR specific for foreign antigen, GC B cells are negatively selected against acquisition of affinity for self antigens. Point mutations in antibody region V genes due to

somatic hypermutation have the potential to result in antibodies that bind to self and are pathogenic (Radic & Weigert 1994). Potentially mimicking the binding of a ubiquitous soluble self-antigen, injection of a soluble antigen during the GC response results in GC B cell apoptosis and collapse of the GC response (Shokat & Goodnow 1995; Pulendran et al. 1995; Han et al. 1995). One model for the mechanism of apoptosis induced by the binding of soluble antigen by GC B cells is that a majority of BCRs bind antigen and are internalized during this process (Goodnow et al. 2010). This prevents tonic BCR activation of phosphatidylinositol-3-OH kinase (PI3K), which generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the plasma membrane. PIP3 regulates signaling by the prosurvival kinase Akt, and so a loss of PIP3 generation caused by internalization of most BCRs could quickly tilt GC B cells towards apoptosis. By extension, cells that receive intermediate levels of BCR signals such that PI3K is activated but enough BCRs are maintained on the surface or recycled back to the surface are able to persist and survive within the GC.

GC B cells in lymphoma

Coupled with their rapid proliferation, the mutagenic events involved in SHM and CSR put GC B cells in danger of acquiring genetic alterations leading to a loss of growth control and potentially lymphomagenesis (Klein & Dalla-Favera 2008). In fact, most B cell lymphomas contain somatically mutated V region genes and so come from a GC or post-GC origin (Kuppers et al. 1999). Ongoing mutation occurs within several types of B cell tumors, such as follicular lymphoma and

lymphoplasmacytoid lymphomas, as well as in certain diffuse large B cell lymphomas, MALT lymphomas, and Burkitt's lymphomas, suggesting that these tumor cells are of germinal center origin. Other types of B cell lymphomas, even if they are not actively undergoing mutation, may have acquired oncogenic mutations during the GC stage.

These oncogenic lesions in GC B cells include point mutations and small deletions derived from aberrant SHM as well as chromosomal translocations, in which the immunoglobulin locus is often juxtaposed with a proto-oncogene, causing dysregulated expression of a pro-survival or proliferation-inducing gene (Ulf Klein & Riccardo Dalla-Favera 2008).

Even during a normal physiological response, GC B cells accumulate mutations in genes other than antibody V regions, such as the proto-oncogenic transcription factor *Bcl6* (Pasqualucci et al. 1998; Shen et al. 1998). These mutations appear to have the potential to cause pathogenesis. Diffuse large B cell lymphomas were found to have significant numbers of mutations in the 5' region of several proto-oncogenes, including *Bcl6*, *Myc*, *PIM1*, and *RhoH* (Pasqualucci et al. 2001). These mutations suggest that aberrant SHM could affect the regulatory and coding regions of these genes, possibly leading to their dysregulated expression and promotion of oncogenesis. Subsequently, many genes that are highly expressed in GC B cells were found to be mutated in an AID-dependent manner (Liu et al. 2008). Interestingly, when mice lacked Msh2/Msh6 and Ung, components of mismatch repair and uracil base excision repair pathways, the number of mutations in many non-immunoglobulin genes,

including *Myc*, was drastically increased, suggesting that AID can target a wide range of genes for deamination but that high fidelity DNA repair pathways normally prevent these mutations from persisting. Recently, pro-oncogenic mutations in NF-κB signaling components (Lenz et al. 2008; Compagno et al. 2009) and BCR signaling (Davis et al. 2010) have been found to contribute to diffuse large B cell lymphoma, as have mutations in the tumor suppressor and plasma cell differentiation factor Blimp-1 (Pasqualucci et al. 2006; Mandelbaum et al. 2010).

Translocations are commonly found in GC-derived lymphomas (Klein & Dalla-Favera 2008). Follicular lymphomas often harbor a translocation involving the IgH locus and the pro-survival gene *BCL2*. Translocations leading to dysregulated expression of the GC transcription factor *BCL6* are common in diffuse large B cell lymphomas, which could lead to a block in post-GC differentiation. BCL-6 drives a pro-proliferative program that also involves suppression of DNA damage recognition factors that allows GC B cells to maintain their rapidly dividing and mutating state but could also contribute to lymphomagenesis (Phan & Dalla-Favera 2004; Ranuncolo et al. 2007). Translocations involving another B cell lineage-specific transcription factor, PAX5, are found in lymphoplasmacytoid lymphomas, and *Myc* translocations are a hallmark of Burkitt's lymphoma. AID activity has been found to be involved in the generation of pathogenic translocations in mouse models. AID is necessary for the formation of DNA breaks at the Myc locus that lead to translocations (Robbiani et al. 2008), and AID deficiency conferred resistance to translocations

involving *Myc* in IL-6 transgenic mice (Ramiro et al. 2004). In Bcl-6-overexpressing B cells, AID deficiency prevented *Myc* translocations and B cell-derived lymphomas (Pasqualucci et al. 2008). In addition, AID overexpression led to frequent translocations in stimulated B cells, and when combined with p53-deficiency led to B cell malignancies in vivo (Robbiani et al. 2009).

GC homeostasis

Though GC B cells are dividing very rapidly (estimates range from 5-12 hours per division), GC size is normally tightly controlled even in conditions of chronic stimulation (Fagarasan et al. 2010). Though mice containing dysregulated T cell help have enlarged GCs (Vinuesa et al. 2005), there is incomplete understanding of B cell-intrinsic defects that cause a loss of ability to maintain GC homeostasis. GC B cell survival appears to play a role in GC size, as GC B cells are critically dependent on the pro-survival molecule Mcl-1 (Vikstrom et al. 2010). One mechanism that seems to control GC B cell survival and homeostasis is Fas-dependent apoptosis. T cells have been shown to cause death of B cells activated by a model self-antigen *in vivo* through interactions between FasL on T cells and Fas on B cells (Rathmell et al. 1995). GC B cell-specific ablation of Fas results in increased numbers of GC B cells, followed by eventual lymphoproliferative disorder (Hao et al. 2008). Possibly related is the accumulation of GC B cells deficient in the ubiquitin-modifying enzyme A20, which are somewhat resistant to Fas-mediated apoptosis (Tavares et al. 2010). Strong evidence exists that defective terminal differentiation of GC

B cells can result in their accumulation. Enforced expression of the transcription factor Bcl-6 in B cells led to increased GC formation, both before and after immunization (Cattoretti et al. 2005), likely through increasing the number of activated B cells that enter the GC and decreasing the terminal differentiation of GC B cells into antibody-secreting cells. Mice deficient in Blimp-1, a transcription factor required for the formation of plasma cells, form enlarged GCs upon immunization, also possibly due to defective differentiation of GC B cells into plasma cells and exit from the GC (Shapiro-Shelef et al. 2003). AID-deficient mice have enlarged GCs in gut-associated lymphoid tissues such as Peyer's Patches (PP) (Fagarasan et al. 2002). This was attributed to defective IgA production and control of bacterial flora in the gut, causing excessive antigenic stimulation of PP B cells, and antibiotic treatment reduced GC B cell numbers. However, AID-deficient GC B cells were found to outgrow wild-type GC B cells in competitive mixed chimeras, which don't suffer from increased bacterial load, and AID-deficient GC B cells had an increased resistance to apoptosis (Zaheen et al. 2009). This result suggested that in addition to an IgA deficiency causing increased bacterial load and antigenic stimulation, AID-deficient mice may contain GC B cells with an intrinsic defect in homeostatic control. A possible explanation for this would be reduced genotoxic stress, leading to reduced GC B cell apoptosis, due to the lack of AID-induced mutations formed in GC B cells. Interestingly, a mechanism in which AID-dependent genotoxic stress leads to DNA-damage response signaling and terminal differentiation of GC B cells has been proposed (Sherman et al. 2010), offering another possible explanation of

how AID deficiency could cause an increase in the number of GC B cells, in this case by preventing terminal differentiation.

One interesting possibility is that GC homeostasis and GC B cell survival is linked to spatial GC organization, in that the rapid proliferation and mutagenesis of GC B cells is confined to a specific niche designed to help maintain growth control. This could potentially be in the form of a specialized microenvironment in which GC B cells can survive, but if they stray out of this environment they would encounter cues that increase the likelihood of their apoptosis. As such, this could be a means of spatially restricting the size of the GC. One advantage of this system would be that it might confine apoptotic debris and potentially autoreactive cells to an area in which they could respectively be quickly phagocytosed or receive signals that cause negative selection. It would also allow for GC B cells to maintain their pro-proliferative, pro-apoptotic program within a permissive environment while still maintaining a restriction on the overall size of the niche. Whether factors exist that link the spatial organization of the GC with its homeostasis is unknown.

Sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) is a bioactive lipid signaling molecule that belongs to the sphingolipid family of lipids (Hannun & Obeid 2008). Sphingolipids include a number of related molecules interconnected by a complex network of metabolic enzymes. Sphingolipids are synthesized *de novo* from serine and palmitate, which are converted to ceramide in the endoplasmic

reticulum (ER) or ER-associated membranes through a series of reactions. Ceramide is also produced by the hydrolysis of sphingomyelin at the plasma membrane. Ceramide can be broken down by ceramidases into sphingosine, the immediate precursor to S1P.

S1P is produced by the phosphorylation of sphingosine by one of two sphingosine kinases (SphKs), SphK1 and SphK2 (Spiegel & Milstien 2003). It can then be exported by as yet poorly-defined transporters. S1P is degraded by the actions of several enzymes. S1P lyase cleaves S1P into hexadecenal and phosphoethanolamine. S1P is also dephosphorylated by two ER-localized S1P phosphatases (SPP1-2) or by 3 members of a family of non-specific lipid phosphate phosphatases (LPP1-3) that are found in the plasma membane and act as ecto-enzymes (Pyne et al. 2004). S1P has been shown to have physiological effects both through the binding of a family of cell surface receptors for S1P (S1P1-5) and through intracellular mechanisms independent of S1P receptors (Spiegel & Milstien 2011).

SphKs are primarily cytosolic, but SphK1 can be targeted to the plasma membrane and SphK2 to the nucleus or ER (Spiegel & Milstien 2007) where they act on their sphingosine substrate. SphK1 can be activated by external stimuli such as pro-inflammatory cytokines or growth factors (Spiegel & Milstien 2003), and has been implicated in promoting growth and proliferation, whereas SphK2 overexpression has been shown to promote apoptosis and suppress cell growth in certain contexts (Spiegel & Milstien 2007). Some of the activities of SphK seem to be mediated by intracellular actions of S1P that are independent of S1P

receptors on the cell surface. Interestingly, SphK1-mediated S1P production is involved in NF-κB signaling downstream of TNF-α stimulation (Alvarez et al. 2010), while SphK2 can associate with histone deacetylases in the nucleus and regulate the transcription of the cyclin-dependent kinas inhibitor *p21* (Hait et al. 2009), suggesting that differential localization can account for some of the different functions of SphK1 and Sphk2 that have been observed. However, there is also evidence that SphK1 and SphK2 have overlapping roles. While neither enzyme is required for survival, the double knockout is embryonically lethal due to defects in brain and cardiac development (Mizugishi et al. 2005).

S1P receptor functions

S1P binding to members of the family of five S1P receptors plays an important role in many cellular functions in different contexts. S1P1-deficient mice die in midgestation from hemorrhaging due to defects in vascular maturation (Y Liu et al. 2000). S1P1 and S1P3 are involved in endothelial barrier integrity and the formation of adherens junctions (Lee et al. 1999; Garcia et al. 2001). S1P and S1P1 promote cell migration in lymphocytes and control their egress from the thymus and secondary lymphoid organs (Schwab & Cyster 2007), as well as the positioning of marginal zone B cells within the spleen (Cinamon et al. 2004). S1P5 plays a role in the egress of NK cells from lymph nodes and bone marrow (Walzer et al. 2007; Jenne et al. 2009). S1P2-deficient mice are viable but occasionally suffer from seizures that can be lethal (MacLennan et al. 2001), and have also been found to be deaf due to vascular defects in the inner ear and a

defect in cochlear hair cell maintenance (Kono et al. 2007; Herr et al. 2007). Double deficiency in S1P2 and S1P3 is often perinatally lethal (Ishii et al. 2002), and is marked by vascular defects in the aorta and hemorrhaging (Kono et al. 2004). S1P2, unlike S1P1, has been shown to inhibit the migration of various cell types (Sugimoto et al. 2003; Takashima et al. 2008; H Okamoto et al. 2000; Lepley et al. 2005; Goparaju et al. 2005; Arikawa et al. 2003) and has recently been found to have roles consistent with this inhibition in the function of several types of immune cells. S1P2 deficiency results in increased numbers of macrophages homing to the peritoneum during peritonitis (Michaud et al. 2010). S1P2 has been proposed to promote the homing into and retention of macrophages in the arterial wall as well as the uptake of oxidized low density lipoproteins (LDLs) and the promotion of atherosclerotic plaques (Wang et al. 2010). S1P2 promotes the retention of osteoclast precursors in bone tissue, and S1P2-deficient mice had greater bone density (Ishii et al. 2010). Pharmacological inhibition of S1P2 was able to reduce the severity of an osteoporosis model. S1P2 is also involved mast cell responses, including degranulation, chemokine secretion, and anaphylaxis. S1P2 antagonism or genetic deficiency decreases the severity of histamine release and hypothermia during a mouse model of anaphylaxis (Oskeritzian et al. 2010). The studies of S1P2 in immune cell function are consistent with the idea that S1P2 is an inhibitory receptor that negatively regulates cell migration, in contrast to the pro-migratory functions of S1P1.

S1P receptor signaling

S1P receptors are G-protein-coupled receptors (GPCRs) that mediate diverse signaling events through coupling to distinct heterotrimeric G-proteins (Sanchez & Hla 2004). S1P receptors contain an extracellular N-terminus, seven transmembrane domains, and hydrophilic extracellular and intracellular loops in between those transmembrane domains. Signal transduction through S1P receptors, like other GPCRs, involves the dissociation of a heterotrimeric G-protein into Gα and Gβγ subunits, which can each transmit signals downstream. Binding to S1P activates signaling primarily through Gαi in the case of S1P1, but S1P2 can couple to Gα12 and Gα13 in addition to Gαq, thus providing an explanation for the different outcomes of signaling by the two receptors (Ishii et al. 2004). Gαi signals downstream to induce activation of the Rac GTPase through a mechanism that isn't fully understood in lymphocytes, but that involves the activation of guanine nucleotide exchange factors (GEFs) specific for Rac such as Dock2 (Fukui et al. 2001; Brugnera et al. 2002; Reif & Cyster 2002). Activation of Rac-GEFs leading to Rac stimulation and cell migration seems to be mediated both through PI3K-dependent and PI3K-independent mechanisms that may involve direct activation by Gβγ subunits. (Welch et al. 2002; Nombela-Arrieta et al. 2004). Rac-GEFs stimulate the exchange of GDP for GTP and activation of Rac, which can stimulate actin polymerization through WAVE and the Arp2/3 complex (Jaffe & Hall 2005). In this way, sensing of Gαi-coupled ligands at the leading edge of a migrating cell induces Rac activation and actin polymerization at the leading edge and movement towards the ligand.

In contrast to Gαi coupled signaling, GPCRs that couple to Gα12 and Gα13 such as S1P2 stimulate Rho activity through Rho-GEFs, including p115RhoGEF (Hart et al. 1998; Kjoller & Hall 1999). Rho has a long-appreciated role in mediating cell body contraction and rear end retraction in migrating cells (Raftopoulou & Hall 2004), but has also been shown to antagonize Rac activity through its substrate effector ROCK and the activation of GTPase activating proteins (GAPs) specific for Rac, such as FilGAP (Ohta et al. 2006) or ARHGAP22 (Sanz-Moreno et al. 2008). GAPs activate the GTP hydrolyzing activity of Rac, accelerating the transition of Rac to its inactive GDP-bound state. Rho-mediated antagonism of Rac has been shown to be important for S1P2's inhibitory effect on cell migration in several cell types (Sugimoto et al. 2003; Lepley et al. 2005). It is likely that the presence of Gα12- and Gα13-coupled ligands at the leading edge of a migrating cell leads to a local activation of Rho, which can antagonize Rac at the leading edge and allow for turning of the cell away from such ligands.

Regulation of S1P distribution

A sharp gradient of S1P levels is maintained between circulation and tissues, with much higher levels in lymph (high nanomolar range) and blood (micromolar range) than within tissues (Schwab et al. 2005; Pappu et al. 2007). Regulation of both S1P production and S1P degradation are critical for proper S1P distribution in vivo (Schwab & Cyster 2007). S1P is produced by SphKs intracellularly in all cell types, but the cell types important for the generation of secreted S1P in the

extracellular space seem to be more specific. Red blood cells are an important source of plasma S1P which contributes to thymic egress (Pappu et al. 2007), and non-hematopoietic sources have been suggested to contribute to plasma S1P as well (Venkataraman et al. 2008). Lymphatic endothelial cells produce S1P necessary for exit of lymphocytes from lymph nodes into lymph (Pham et al. 2010), suggesting that different cell types are important for S1P production in different compartments.

S1P lyase is critical for the maintenance of the S1P gradient, as it contributes to S1P degradation and low S1P levels within tissues, influencing the ability of lymphocytes to exit lymphoid organs (Schwab et al. 2005). Recent evidence shows that LPP3 expression on endothelial and epithelial cells in the thymus also contributes to the maintenance of low S1P levels that permit T cell exit from the thymus into circulation (Breart et al. 2011). In addition, plasma S1P has a half-life on the order of 15 minutes (Venkataraman et al. 2008), suggesting that tight spatial and temporal control of S1P levels is possible. The actual concentrations within lymphoid tissue have not been directly measured but are thought to be very low. S1P causes internalization of S1P1 receptor, and surface levels of S1P1 have been used as a proxy for the relative amounts of S1P within a tissue to show that they are likely in the low nanomolar range (Schwab & Cyster 2007). While S1P gradients have clearly been shown to play a role in lymphocyte trafficking in and out of lymphoid tissues, it has not been clear whether S1P distribution within the parenchyma of lymphoid organs can also play a role in the behavior of cells within the tissue.

Chapter 2

The sphingosine 1-phosphate receptor $S1P_2$ maintains the homeostasis of germinal center B cells and promotes niche confinement

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Abstract

Sphingosine-1-phosphate receptor-2 (S1P2)-deficient mice develop diffuse large B cell lymphoma. However, the role of S1P2 in normal germinal center (GC) physiology is unknown. Here we show that S1P2-deficient GC B cells outgrow their wild-type counterparts in chronically-established GCs. We find that S1P2-, Gα12-Gα13- and p115RhoGEF-mediated antagonism of Akt regulates cell viability and is required for growth control in chronically proliferating GCs. We also find that S1P2 inhibits GC B cell responses to follicular chemoattractants and helps confine cells to the GC. Moreover, S1P2 overexpression promotes centering of activated B cells within the follicle. We suggest that by inhibiting Akt activation and migration, S1P2 helps restrict GC B cell survival and localization to an S1P-low niche at the follicle center.

Introduction

Germinal centers (GCs) are induced in response to T-dependent antigens and support events necessary for antibody affinity maturation[1,2]. They arise in the center of B cell follicles and grow from small numbers of starting B cells to a size of ~10,000 cells in a matter of days. Despite the rapid growth rate, GC size is tightly regulated, even in mesenteric lymph nodes (mLNs) and Peyer's Patches (PPs) where the responses are chronically stimulated by gut flora[3]. Apoptosis is an integral part of GC growth control as GC B cells are highly prone to apoptotic cell death and are strongly dependent on CD40L and other trophic factors[4]. These factors act at least in part by maintaining expression of anti-apoptotic Bcl2-family proteins, including Mcl-1, that are critical for GC formation[5]. However, despite knowledge of key requirements for maintaining GC cell viability, the environmental cues involved in regulating GC size are not fully understood.

GCs are organized into dark and light zones by CXCL12 and CXCL13, respectively[6]. CXCL13 is present throughout the follicle and in the GC light zone; CXCL12 is present within the dark zone[6]. Despite the important roles of these chemokines, combined deficiency in the function of their receptors does not cause a complete loss of GC formation[6]. Another chemoattractant receptor, EBI2, is up-regulated in early-activated (pre-GC) B cells and functions in guiding these cells to the outer follicle[7,8]. EBI2 is down-regulated in GC B cells, a change that is important for GC B cells to access the follicle center[7]. However, in the

absence of EBI2, GCs form in their normal location indicating that additional cues must act to promote clustering of GC-precursors at the follicle center.

Sphingosine-1-phosphate (S1P) is a metabolic intermediate made by all eukaryotic cell types during sphingolipid metabolism through the action of sphingosine kinase-1 (sphk-1) and sphk-2[9]. S1P is secreted by some cell types. The extracellular lipid acts as a ligand for any of five G-protein coupled receptors, S1P1-S1P5[9]. Extracellular S1P is abundant (high nM to μM) in blood and lymph but has a half-life shorter than 15 minutes[10] and although no direct measurements of interstitial concentrations have been reported, indirect measurements indicate they are very low[11,12]. Red blood cells and endothelial cells are important sources of circulatory S1P[10,12,13]. Two S1P phosphatases, three lipid phosphate phosphatases (LPPs) and S1P lyase can degrade S1P and catabolism plays a critical role in maintaining the low interstitial concentrations[14]. S1P promotes egress of lymphocytes from lymphoid tissue into circulatory fluids. Whether sufficient amounts of interstitial S1P exist in the lymphoid parenchyma to regulate cell behavior has been unclear.

In this study we set out to define molecular cues involved in regulating GC size and GC B cell clustering. We found that S1P2 was expressed by GC B cells and was necessary to maintain control over the size of chronically-stimulated GCs. S1P2 and its downstream mediators $G\alpha12$, $G\alpha13$ and p115RhoGEF antagonized Akt signaling and cell viability. S1P2 also inhibited GC B cell

chemotaxis to follicular chemoattractants and helped to promote confinement of GC B cells to the GC. In addition, S1P2 overexpression in non-GC B cells promoted their localization to the follicle center. Based on these studies, we propose a model in which S1P signals through S1P2 to regulate GC B cell survival and positioning, thus promoting GC homeostasis through dual roles.

Results

Uncontrolled growth of S1P2-deficient GCs

Genome-wide comparison of gene expression between follicular and GC B cells identified S1P2 as one of the most strongly induced genes in GC B cells[15] (and data not shown), and this differential expression was confirmed by qRT-PCR (**Fig. 1a**). When 8-12 week old S1P2-deficient mice[16] were immunized with T-dependent antigens, they appeared to mount GC responses of normal magnitude. However, analysis of one year-old S1P2-deficient mice revealed expansion of GC B cell numbers in mLNs (**Fig. 1b**) as well as an increase in total B and T cell numbers. In about half the animals GC B cell numbers reached as much as 100x normal, and the architecture of the LN was effaced (**Fig. 1b, c**). We speculate that the bimodality of GC expansion in these mice is due to cooperation between S1P2-deficiency and secondary genetic events, resulting in a loss of GC homeostasis and development of GC-type lymphoma. Similar observations were made in another S1P2-deficient mouse line and the outgrowths were classified as diffuse large B cell lymphoma (DLBCL)[17]. These results demonstrate a requirement for S1P2 in maintaining GC B cell homeostasis.

Growth advantage of *S1pr2*$^{-/-}$ cells in chronic GCs

To determine if the S1P2-requirement for GC B cell homeostasis was cell intrinsic we studied mixed bone marrow (BM) chimeras. In mice reconstituted with ~1:2 mixtures *of S1pr2*$^{-/-}$ and wild-type BM, S1P2-deficient GC B cells over-

accumulated relative to their wild-type counterparts in mLNs, whereas follicular B cells were represented in proportion to the BM chimerism (**Fig. 1d, e** and **Supplementary Fig. 1**). The outgrowth was even more evident in mice reconstituted with ~1:9 mixtures of *S1pr2*$^{-/-}$ and wild-type BM, and was evident in both mLNs and PPs (**Fig. 1f**). The outgrowth was not observed in chimeric mice immunized intra-peritoneally with sheep red blood cells (SRBCs) to induce splenic GCs at day 8 post-immunization (**Fig. 1e, f**) or in splenic or mLN GCs induced 14 days after NP-CGG immunization (**Supplementary Fig. 1**). However, a gradual outgrowth of S1P2-deficient GC B cells was noted in the endogenous splenic response occurring in unimmunized mixed BM chimeras (**Supplementary Fig. 1**). This suggests that the outgrowth may depend on the long-term persistence of the GCs rather than on unique properties of mucosal tissues. The advantage for S1P2-deficient cells was dependent on S1P derived from radiation-resistant cells, because it was reduced in polyinosine polycytidylic acid (polyI:C) treated *Mx1-cre*$^{+}$*Sphk1*$^{f/-}$*Sphk2*$^{-/-}$ hosts, animals that lack Sphk2 and are broadly deficient for Sphk1 in IFNα/β-responsive cells (**Fig. 1g**).

S1P2 can couple to Gα12 and Gα13[18]. To test the role of these G-proteins in GC B cells, we generated *Gna12*-deficient mice and intercrossed them with *Gna13*$^{f/f}$ mice[19] carrying the *Mx1-cre* transgene. Animals treated to induce Cre expression were used as a source of Gα12-Gα13-double deficient BM cells. In mice reconstituted with a mixture of wild-type and Gα12-Gα13-deficient BM, follicular B cells reflected the BM chimerism[20], while there was outgrowth of Gα12-Gα13-

deficient B cells in mucosal GCs (Fig. 1h). These observations suggest a selective role for Gα12 and Gα13 within GC B cells in mediating S1P2 signals. The small GC B cell accumulation seen at day 8 in splenic SRBC responses was similar in the control (Gα12-deficient) and Gα12-Gα13-double deficient groups and may reflect an effect of Gα12 single-deficiency or influences of genes from the 129 background. A major effector pathway of Gα12-Gα13 in lymphocytes is activation of p115RhoGEF (encoded by *Arhgef1*), leading to Rho activation[21-23]. 8-12 weeks after reconstitution with wild-type and *Arhgef1*$^{-/-}$ BM, p115RhoGEF-deficient GC B cells outgrew wild-type cells, although in this case the advantage was not focused in mucosal sites **(Fig. 1i** and **Supplementary Fig. 1**). These observations suggest that S1P2 signals through Gα12-Gα13 and Rho to maintain B cell homeostasis in chronically stimulated GCs.

S1P2 regulates cell survival in GC B cells

The rate of GC B cell proliferation, determined by measuring bromodeoxyuridine (BrdU) incorporation 0.5 and 6 hr after a single BrdU injection, was similar in S1P2-deficient and control GC B cells (**Supplementary Fig. 2**). By contrast, the rate of apoptotic cell death, examined *ex vivo* by measuring active caspase-3 and DNA fragmentation was reduced in S1P2-deficient cells (**Fig. 2a, b** and **Supplementary Fig. 2**) and in Gα12-Gα13-deficient cells (**Supplemental Fig. 2**). Because S1P2 and Rho were suggested to negatively regulate Akt in some contexts[24-27], we asked whether the activity of this pro-survival kinase was altered in the absence of S1P2. Intracellular flow cytometry for the threonine 308

phosphorylated form of Akt (pAkt T308) showed that S1P2-deficient GC B cells had higher amounts of active kinase than control GC B cells isolated from the same mixed BM chimeras (**Fig. 2c**). A similar elevation in pAkt T308 was observed after 30 min incubation of wild-type GC B cells with the S1P2 antagonist, JTE-013 (**Fig. 2d**) or when JTE-013 was added to the media used to prepare the cell suspensions (**Supplementary Fig. 2**). The cell preparation and incubation was performed in the absence of added S1P, indicating that cells had bound sufficient ligand at the time of isolation to promote S1P2 signaling. Co-incubation with the PI3K inhibitor, wortmannin, prevented the increase in pAkt (**Fig. 2d**). The S1P2-mediated inhibition of Akt activation was dependent on endogenous S1P, because pAkt was elevated in GC B cells from Sphk-deficient mice (**Fig. 2e**). GC B cells isolated from Gα12-Gα13 double-deficient and p115RhoGEF-deficient mice showed similar elevations of pAkt (**Fig. 2f**).
The propensity of wild-type GC B cells to undergo prompt apoptosis *in vitro* limits the biochemical measurements that can be performed in these cells. To assess the S1P2 downstream signals that regulate Akt we used the human GC B cell line, Ramos, which expresses S1P2 (data not shown). Ramos cells had high constitutive pAkt levels, which is not unusual for transformed cell lines (**Fig. 2g**). Exposure of these cells to S1P led to a reduction in pAkt, which was prevented by co-incubation with the S1P2 antagonist (**Fig. 2g**). Treatment with Y27632, an inhibitor of the Rho downstream effector ROCK, prevented S1P from causing the full reduction in pAkt levels (**Fig. 2h, i**). ROCK can inhibit Akt by activating PTEN, a phosphatidylinositol-3,4,5-triphosphate 3-phosphatase[28]. However, incubation

in the presence of the PTEN inhibitors bpV(pic) (**Fig. 2h**), bpV(phen) or VO-OHpic (not shown) did not prevent the S1P-mediated down-modulation of pAkt (**Fig. 2h**). These observations suggest that upon GC B cell encounter with S1P, S1P2 acts via Gα12-Gα13 and p115RhoGEF triggered activation of Rho and ROCK to antagonize Akt activation.

Akt activation confers an advantage in mucosal GCs

To address if elevated Akt activity was sufficient to give GC B cells a growth advantage, BM cells were transduced with a constitutively active *myr-Akt*[29] or control retrovirus and used to reconstitute wild-type mice. Stem cells harboring the *myr-Akt* construct were inefficient at generating follicular B cells, but there was enrichment for myr-Akt expressing cells within the mLN GC compartment (**Supplementary Fig. 3**). In animals receiving BM cells from *Cr2-cre* transgenic mice transduced with a flox-stop-flox modified version of the *myr-Akt* construct (containing a Thy1.1 reporter) resulting in mature B cell-restricted expression, follicular B cell reconstitution was more successful. The myr-Akt expressing cells were enriched within the GCs of mucosal lymphoid tissues (**Fig. 3a**) and GC B cells showed a reduced rate of apoptotic cell death (**Fig. 3b**). These results suggest that increased Akt activation is sufficient to confer a growth advantage on GC B cells.

Akt can promote cell viability by a number of pathways, including phosphorylation and inhibition of the pro-apoptotic molecule Bad, inhibition of Foxo transcription factors that induce pro-apoptotic molecules such as Bim and phosphorylation

and inhibition of the translation initiation inhibitor, 4E-BP1, which lead to small increases in cap-dependent translation of many proteins including some pro-survival molecules[30]. Analysis of mice reconstituted with a mixture of wild-type and Bad-deficient BM did not reveal a growth advantage for Bad-deficient GC B cells (**Supplementary Fig. 3**) and gene expression analysis of sorted wild-type and S1P2-deficient GC B cells failed to reveal differences in expression of Foxo target genes such as Bim (**Supplementary Fig. 3**). Intracellular staining revealed elevated amounts of phosphorylated 4E-BP1 in S1P2-deficient GC B cells (**Fig. 3c**). To directly test whether loss of S1P2 signaling promoted translation in GC-type B cells, we measured ^{35}S-cys/met incorporation in Ramos GC B cells. In the absence of S1P, or in the presence of the S1P2 antagonist, ^{35}S-cys/met incorporation by Ramos cells was increased (**Fig. 3d**). The translation inhibitory effect of S1P2 signaling was similar in magnitude to that achieved by rapamycin, an mTOR inhibitor (**Fig. 3d**). These findings suggest that S1P2 signaling in GC B cells normally suppresses Akt and thus mTOR activity, leading to reduced 4E-BP1 phosphorylation and a reduction in cap-dependent translation, which may affect the translation of a number of pro-survival molecules[30].

S1P2 regulates GC B cell migration and positioning

S1P2 coupling to Rho activation is associated with inhibition of migration in a number of cell types[31,32]. To test the impact of S1P2 on GC B cell migration to follicular chemo-attractants, we intercrossed S1P2-deficient mice with *Bcl2* transgenic mice to overcome the rapid *in vitro* death of GC B cells[6]. nM

concentrations of S1P inhibited GC, but not follicular B cell migration to CXCL13 and CXCL12 in a manner that could be reversed by co-incubation with S1P2 antagonist (**Fig. 4a**). The selective action of S1P via S1P2 in inhibiting GC B cell migration was confirmed using S1P2-deficient GC B cells (**Fig. 4b**). Although GCs continued to form in their normal location in the absence of S1P2, the boundary between the GC and mantle zone was often less defined (**Fig. 4c** and **Supplementary Fig 4**). In mixed BM chimeras, S1P2-deficient cells were segregated from wild-type cells and enriched at or beyond the GC perimeter (**Supplementary Fig. 5**). The segregation was largely reversed in Sphk-deficient hosts (**Supplementary Fig. 5**). G12-G13 double-deficient GC B cells showed a similar tendency to segregate from wild-type GC B cells (**Supplementary Fig. 5**). To enable a dynamic analysis of S1P2-deficient GC B cell behavior in the context of wild-type GCs, S1P2-deficient hen egg lysozyme (HEL)-specific Hy10 B cells and ovalbumin (OVA)-specific OTII T cells were transferred to wild-type hosts that were then immunized with the low affinity duck egg lysozyme (DEL)-OVA antigen complex to induce GC responses[33]. In frozen sections, S1P2-deficient Hy10 B cells were concentrated at the GC perimeter, often appearing to intermingle with IgD^{hi} follicular mantle cells (**Fig. 4d**). Two-photon microscopy of explanted LNs containing GFP^+ S1P2-deficient Hy10 B cells and CFP^+ wild-type Hy10 B cells revealed many more S1P2-deficient than wild-type GC B cells moving beyond the confines of the GC, as identified by the location of immune-complex laden follicular dendritic cells (FDCs) (**Supplementary Movies 1-3**). The S1P2-deficient GC B cells moved at higher velocities and with less turning

than the wild-type GC B cells (**Fig. 4e** and **Supplementary Fig. 6**). As a result, they traveled in straighter paths (**Fig. 4f** and **Supplementary Fig. 6**). When the GC surface was defined based on the distribution of naive B cells (**Fig. 4g, Supplementary Fig. 6** and **Movies 4-6**) it was apparent that S1P2-deficient GC B cells had similar velocities and turning angles whether located outside or inside the GC surface (**Fig. 4g, h** and **Supplementary Fig. 6**). In contrast, wild-type GC B cells showed a reduced velocity and increased turning when outside the GC surface (**Fig. 4g, h** and **Supplementary Fig. 6**). These observations suggest that as GC B cells move beyond the confines of the GC they receive S1P2-mediated signals that inhibit their migration and prompt their turning.

Despite the marked alteration in distribution of S1P2-deficient cells in the context of a predominantly wild-type GC, we did not observe effects on affinity maturation (**Supplementary Fig. 7**). This suggests that under the immunization conditions used here, S1P2-deficient cells continued to gain adequate access to antigen and helper T cells. This does not exclude the possibility that during the response to some antigen types, S1P2-deficient cells will exhibit defects in affinity maturation.

S1P2 promotes GC cell clustering

GCs continue to form in S1P2-deficient mice and although S1P2-deficient cells extend beyond the normal GC boundary, they do not disperse freely throughout the follicle, suggesting that additional cues promote their GC association. While the nature of these cues remains undefined, we considered the possibility that

removal of general follicular organizing factors might produce a hypomorphic state that could better reveal the confining activity of S1P2. CXCL13 is abundant in the follicle and GC light zone and immunized CXCL13-deficient mice have smaller, less organized GCs than wild-type mice but do retain these structures[6] (**Fig. 5a**). S1P2-deficient GC B cells failed to form GC clusters in immunized CXCL13-deficient hosts (**Fig. 5a**). Instead, the GL7^{hi} GC B cells were dispersed throughout the lymphoid areas (**Fig. 5a**). In another approach, mice were pre-treated with an antagonist of lymphotoxin (LT)-$\alpha1\beta2$ for 3 weeks to disrupt follicular stromal cell networks[34] prior to SRBC immunization. This treatment also led to a marked dispersal of S1P2-deficient compared to wild-type GC B cells (**Fig. 5b**). These data suggest that when GC organization is compromised by removing CXCL13 or altering follicular stromal cells, S1P2 plays a non-redundant role in promoting GC B cell clustering. The data also establish that S1P2 promotes GC B cell clustering by a mechanism beyond inhibition of the CXCL13 response.

B cells are capable of regulating S1P levels

The interstitial S1P concentrations in the lymphoid tissues are low compared to the high amounts in circulatory fluids but are not precisely defined and may vary between different parts of the tissue[9,35,36]. Although it is not possible to directly measure interstitial S1P, the extent of S1P1 down-modulation from the surface of cells has provided an indirect measure[11,12]. According to the staining levels of an anti-mouse S1P1 monoclonal antibody, wild-type follicular B cells express S1P1

at levels intermediate between blood B cells (that are exposed to μM S1P[9]) and B cells from S1P-deficient mice (**Fig. 6a**). By comparison with the amounts of S1P needed to cause partial down-modulation of S1P1 in lymphocytes *in vitro*[11], it can be approximated that the follicle contains interstitial S1P in the low nM range. These amounts are sufficient to reduce migration of S1P2-expressing cells to chemokines (**Fig. 4a**).

Analysis of S1P-degrading enzyme transcript abundance revealed that B cells expressed most of the enzymes that degrade extracellular S1P and, in comparison to T cells, they had especially high amounts of *Lpp2* and *Lpp3* (**Fig. 6b**), which have their active site in the extracellular region[14]. Incubation of S1P with purified B cells or T cells led to rapid degradation of the lipid by B cells (**Fig. 6c**). Because the source of extracellular S1P seemed to be radiation-resistant and thus likely stromal cells (**Fig. 1g**), we next asked whether FDCs, the main radiation resistant cell type present in the center of follicles and within GCs, were a necessary source of S1P. Deletion of *Sphk1* (in *Sphk2*-deficient mice) in FDCs using *Cr2-cre* did not prevent the growth advantage of S1P2-deficient mLN GC B cells and S1P2-deficient cells remained segregated from wild-type GC B cells (**Supplementary Fig. 5**). This indicated that FDCs are not a required source of S1P for mediating S1P2 functions in GC B cells. These combined findings suggest that extracellular S1P derives from stromal cells outside the follicle center and that it is rapidly degraded as it travels through the follicle, leading us to propose that interstitial S1P concentrations will be lowest in the follicle center.

S1P2 directs B cells to the follicle center

If our hypothesis regarding S1P distribution were correct, cells expressing S1P2 might be antagonized in their migration to chemoattractants in the outer follicle. In consequence, S1P2 expression should favor migration toward the center of the follicle. To test this possibility, we examined the distribution of transferred S1P2-expressing B cells in GC-containing follicles (**Fig. 7a**). Transduction of B cells with S1P2 led to their preferential clustering around GCs compared to cells transduced with a control vector (**Fig. 7a**). S1P2 overexpression was also sufficient to cause occasional clustering of the transferred cells in the center of primary follicles (that lack GCs), but this result was more variable. B cell activation, which is required for B cells retroviral transduction, results in upregulation of EBI2 (encoded by *Gpr183*), a receptor that guides B cells to the outer follicle[8]. We considered the possibility that increased EBI2 function might favor movement of the transduced B cells to the outer follicle and thus counteract the effect of S1P2 expression. *Gpr183*$^{+/-}$ B cells express about half as much *Gpr183* as wild-type B cells (**Supplementary Fig. 5**). Transferred *S1pr2*-transduced *Gpr183*$^{+/-}$ B cells showed a strong bias for migration to the follicle center (**Fig. 7b**). Importantly, vector-transduced *Gpr183*$^{+/-}$ cells did not show any bias in their distribution, demonstrating that EBI2 heterozygosity was not sufficient to cause a centering effect (**Fig. 7b**). Transfer of S1P2 expressing cells to hosts broadly deficient in Sphks did not result in a distribution bias (**Fig. 7c**), indicating that S1P was the signal required. However, the bias was still observed in hosts selectively lacking Sphks in FDCs (**Supplementary Fig. 5**). These

observations suggest that S1P2 upregulation favors cell movement to the follicle center, likely as a consequence of this being a low point in the S1P field. While considered less likely, the results do not exclude the possibility that S1P2-overexpressing cells preferentially die in the outer follicle compared to the center follicle as a result of differential S1P exposure.

Discussion

Here we demonstrate that S1P2 has a dual role in GC B cells, regulating survival and promoting clustering at the follicle center. We provide evidence that S1P2 acts through a signaling module involving Gα12-Gα13, p115RhoGEF, and most likely Rho and ROCK, to dampen Akt activation in GC B cells. When S1P2 is lacking, elevated Akt activity can lead to increased phosphorylation of 4E-BP1, a modification that releases this inhibitor from eIF4E, allowing for small increases in cap-dependent translation of a range of transripts[37]. 4E-BP1 regulates translation of transcripts with complex 5'UTRs, including those for a number of pro-survival molecules[38-40]. Our data do not exclude the possibility that Akt promotes GC B cell survival through additional pathways, such as regulation of GSK3 or Glut1[41]. S1P2 can signal via Akt-independent pathways [31,32] and it is possible that their reduction also contributes to the growth advantage observed in S1P2-deficient cells.

Although the *ex vivo* analysis suggests a strong prosurvival effect of S1P2-deficiency, the *in vivo* advantage only becomes evident over periods of weeks in

chronically stimulated GCs. This suggests that S1P2's contribution is relatively small during acute antigen-driven GC responses, while it is more strongly revealed under the conditions associated with cell isolation. This may be a consequence of GC B cell exposure to the higher amounts of S1P present outside the GC during tissue preparation as well as the stresses of in vitro culture. We suggest that S1P2's contribution *in vivo* may become most evident under conditions of elevated genotoxic stress arising due to chronic GC stimulation at mucosal sites. The survival advantage may increase the likelihood of cells acquiring and surviving secondary oncogenic hits, thereby setting the stage for progression to DLBCL. Gαq negatively regulates Akt activation in naïve B cells[42], indicating that multiple GPCRs exert a controlling influence over Akt activation in B cells. B cells with elevated Akt activity due to PTEN deficiency or Akt over-expression were reported to have reduced isotype switching as a result of decreased activation induced cytidine-deaminase (AID) function[43,44]. We did not observe defects in isotype switching or affinity maturation in S1P2-deficient Hy10 B cells, suggesting AID function is intact. These differences may reflect a lesser increase in Akt activity in S1P2-deficient compared to PTEN-deficient B cells, or they may indicate differences in the way distinct pools of activated Akt are integrated into downstream signaling networks[45].

S1P has a well-established role in promoting lymphocyte egress from lymphoid tissues[9,12]. The present findings establish that S1P also has a role in regulating cell behavior within lymphoid tissue. Follicular S1P is derived from radiation resistant cells other than FDCs and is rapidly degraded by B cells. S1P has a

half-life in plasma shorter than 15 minutes[10]. Given that the most frequent cells in blood (red blood cells) do not express S1P degrading enzymes[9] whereas B cells do, it seems likely that the S1P half-life in densely packed B cell follicles will be at least this short.

We propose a model where S1P concentrations are relatively high in the outer follicle and decay to a low point over the FDC network at the follicle center. We suggest that S1P2 induction in GC-precursor cells, by inhibiting their propensity to migrate towards CXCL13 and other attractants in the S1P^{high} outer follicle, helps to focus the cells to the follicle center. S1P2 may also act by promoting chemorepulsion[46], though we have not found GC B cells to move away from S1P in transwell migration assays. EBI2 down-regulation is also important in allowing GC cells to move away from the outer follicle[7]. Once a GC forms, S1P within this microenvironment may be kept at low levels by local degradation and by minimal carriage of S1P into the structure by newly arriving cells. When an S1P2-expressing GC B cell reaches the GC perimeter it likely encounters higher amounts of S1P, causing activation of Rho at the leading edge, prompting retraction of cellular processes[47] and cell turning. Because S1P2 signaling also causes Akt inhibition, this growth-regulatory effect may be strongest in GC B cells migrating near the GC perimeter. Thus, through dual growth regulatory and migration inhibitory activities, S1P2 may act as one of multiple factors that helps link GC size to the volume of the supportive niche at the follicle center. In future studies it will be important to develop techniques to measure interstitial S1P concentrations in order to have a better understanding of how S1P2 could be

controlling both processes. Nevertheless, we feel it reasonable to suggest, based on the existing data, that a general property of G12-G13-coupled receptors may be to help coordinate niche confinement and cell survival.

Acknowledgments

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Figure Legends

Figure 1. S1P2, Gα12-Gα13 and p115RhoGEF-deficient GC B cells have a growth advantage in chronic GCs. (a) Quantitative PCR analysis of *S1pr1* and *S1pr2* transcript abundance in follicular (FO) and GC B cells, shown relative to *HPRT*. (**b)** Number of GC B cells in spleen and mLNs and (**c)** immunohistochemical staining of mLNs from one year-old *S1pr2*$^{+/-}$ or *S1pr2*$^{-/-}$ mice. Scale bar, 500 μm. (**d)** Representative flow cytometric analysis of mLN FO and GC populations from 60% wild-type (CD45.1) and 40% *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ (CD45.2) mixed BM chimeras. Numbers indicate frequency of cells within gates. **e-f,** Contribution of CD45.2 cells to FO and GC populations in indicated tissues of mixed BM chimeras made with 60:40 (**e**) or 90:10 (**f**) ratios of CD45.1 and CD45.2 BM. In **e**, data are pooled from more than 5 experiments (*n* = 19-23); **f**, data are representative of 3 experiments (*n* = 5). (**g)** Relative

contribution of *S1pr2*$^{-/-}$ (CD45.2) GC B cells to GC/FO populations in mixed BM chimeras in *Mx1-cre*$^{+}$*Sphk1*$^{f/- \text{ or } f/f}$*Sphk2*$^{-/-}$ hosts. Data are from 3 experiments (*n* = 6). **h-i,** Contribution of CD45.2 cells to FO and GC populations in indicated tissues of mixed BM chimeras made with ~60:40 ratios of wild-type CD45.1 cells and CD45.2 cells from either littermate controls or mice deficient in Gα12 and Gα13 (**h**) or p115RhoGEF (*Arhgef1*) (**i**). Littermate controls in **h** are singly deficient in Gα12. Data are representative of 2 similar experiments. All chimeras in **d-i** were reconstituted for at least 8 weeks and had been immunized i.p. with SRBCs 6-8 days prior to analysis. * $P \leq .01$, ** $P \leq .001$, *** $P \leq .0001$.

Figure 2. Apoptosis resistance and increased Akt activation in S1P2, Gα12-Gα13, and p115RhoGEF-deficient GC B cells. Frequency of GC B cells with activated caspase-3 **(a)** or fragmented DNA detected by TUNEL assay **(b)** in chimeras reconstituted with mixtures of *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ (CD45.2) and wild-type (CD45.1) BM. The mice were immunized with SRBCs and analyzed after 6-8 days. Data are from 3 experiments (*n* = 8-9). (**c)** Flow cytometric analysis of P-Akt T308 in follicular and GC B cells from mixed chimeras. Right panel shows mean fluorescence index (MFI) of P-Akt T308 in the indicated GC B cell populations (*n* = 7). (**d)** P-Akt T308 analysis in wild-type GC B cells from spleen suspensions treated with JTE-013 or JTE-013 and wortmannin (WMN) for 30 min immediately *ex vivo* (*n* = 3). (**e**) Graph showing P-Akt T308 MFI of mLN GC B cells from *Mx1-cre*$^{+}$*Sphk1*$^{f/- \text{ or } f/f}$*Sphk2*$^{-/-}$ (S1P-deficient) mice, relative to the average of the controls (*n* = 12-13, pooled from 6 experiments). (**f)** P-Akt T308

analysis in GC B cells deficient in p115RhoGEF or both Gα12 and Gα13, compared to littermate control cells and wild-type (CD45.1) cells, from mixed BM chimeras. The Gα12-Gα13 DKO littermate control was G12-single deficient. Right panels show a summary of P-Akt T308 MFIs in GC B cells from mixed chimeras (data are representative of 2 experiments). (**g)** Western blot for P-Akt S473 in Ramos cells treated for 5 min with S1P in the presence or absence of JTE-013 (representative of 3 experiments). (**h**) P-Akt T308 analysis in Ramos cells treated for 5 min with S1P (10nm) alone or in the presence of the indicated inhibitors. Y27632, 10μm; JTE-013, 10μm; bpV(pic), 500nm. (**i**) Relative P-Akt T308 MFIs in Ramos cells treated with the indicated conditions, compared to untreated (dashed line) (*n* = 8, pooled from 8 experiments). $^{\#}$ $P \leq .05$, * $P \leq .01$, ** $P \leq .001$, *** $P \leq .0001$.

Figure 3. Akt activation confers an advantage in mucosal GCs and S1P2 regulates translation in GC cells. (a) BM from *Cr2-cre* transgenic mice was transduced with loxP-stop-loxP Thy-1.1 control retrovirus (Vector) or myristoylated Akt (*myr-Akt*) Thy-1.1 retrovirus and used to reconstitute irradiated recipient mice. Representative flow cytometric analysis showing representation of myr-AKT–Thy-1.1-expressing cells in IgD^{lo} Fas^{+} GC B cells relative to IgD^{hi} FO B cells of mLNs. Right panel shows summary of data as percent contribution of Thy-1.1^{+} cells to FO and GC B cell populations (*n* = 7, pooled from 2 experiments). (**b**) TUNEL assay of mLN GC B cells from mice of the type in a. (**c**) Flow cytometric analysis of P-4E-BP1 in FO and GC B cells from mixed BM

chimeras containing wild-type (CD45.1) cells and either *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ (CD45.2) cells. Graph on right shows MFI of P-4E-BP1 in the indicated GC populations (*n* = 4, from 4 experiments). (**d)** Relative incorporation of ^{35}S-labeled cysteine and methionine in 30 min by Ramos cells removed from S1P (No S1P) or treated with JTE-013, relative to cells cultured with S1P. Control samples were removed from S1P and treated with rapamycin. Triplicate measurements were obtained in each experiment and all data points were divided by the mean of the untreated group. $^{\#}$ $P \le .05$, * $P \le .01$, ** $P \le .001$, *** $P \le .0001$.

Figure 4. Cell migration and positioning of GC B cells are regulated by S1P2. (a) Transwell migration of wild-type *Bcl2*-transgenic FO and GC B cells to CXCL12 (0.3μg/ml) or CXCL13 (1μg/ml) in the presence or absence of S1P as well as JTE-013 (representative of at least 5 experiments). (**b**) Transwell migration of *S1pr2*$^{+/-}$ or *S1pr2*$^{-/-}$ *Bcl2*-transgenic GC B cells to CXCL12 in the presence or absence of S1P (representative of 2 experiments). (**c)** Immunohistochemical analysis of splenic GL7^{+} GCs in *S1pr2*$^{+/-}$ or *S1pr2*$^{-/-}$ mice immunized with SRBCs. (**d**) Lysozyme-specific *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$Hy10 (CD45.2) B cells were transferred into recipient (CD45.1) mice along with wild-type Hy10 (CD45.1) B cells and OVA-specific OT-II (CD45.1) T cells. Recipients were DEL-OVA immunized and at d14 LN sections stained to detect CD45.2^{+} Hy10 GC B cells (blue) and FO B cells (IgD, brown). Scale bar, 200μm. **(e)** Histograms showing velocities of *S1pr2*$^{+/+}$ and *S1pr2*$^{-/-}$ GC B cells determined by tracking migration of fluorescently labeled GC B cells using real-time 2-photon

microscopy of intact LNs (see also **Supplementary Movies 1-2**). (**f)** Confinement of wild-type and *S1pr2*$^{-/-}$ GC B cells, displayed as path length/displacement (data in e and f are representative of 4 GCs analyzed in 2 experiments). (**g**) Example views of GC surface (red) (see also **Supplementary Fig. 6**) showing tracks of wild-type (CFP^{+}, cyan) and *S1pr2*$^{-/-}$ GC B cells (GFP^{+}, green) (see also **Supplementary Movies 3-6**). (**h)** Velocities of GC B cells during migration inside or outside the GC surface. Downward arrows in e and h denote average values of their respective groups. * $P \leq .01$, ** $P \leq .001$, *** $P \leq .0001$, n.s. $P = 0.73$.

Figure 5. S1P2 cooperates with CXCR5 and FDCs to promote GC B cell clustering. (**a)** Immunohistochemical analysis of mLN sections from CXCL13-deficient mice reconstituted with BM from either *S1pr2*$^{+/-}$ or *S1pr2*$^{-/-}$ donors, showing GL7^{+} GC B cell and IgD^{+} B cell distribution. Right panel shows number of GC B cells per mLN in chimeras (*n* = 8, pooled from 3 experiments). (**b)** Immunohistochemical analysis of spleen sections from *S1pr2*$^{+/-}$ or *S1pr2*$^{-/-}$ mice treated with LTβR-Fc for 4 weeks. At the fourth injection, mice were immunized i.p. with SRBCs and analyzed 7 days later. Right panel shows numbers of GC B cells in spleen (*n* = 8, pooled from 3 experiments). Scale bars, 100μm.

Figure 6. B cells are capable of degrading S1P. (a) S1P1 surface abundance on follicular B cells from the indicated tissues of control (CTL), *S1pr1*-deficient (S1P1 KO), or *Mx1-cre*$^{+}$*Sphk1*$^{f/- \text{ or } f/f}$*Sphk2*$^{-/-}$ (S1P-deficient) mice. Data are representative of more than 3 mice of each type. **(b)** Transcript abundance of

S1P lyase (*Sgpl*), sphingosine-1-phosphate phosphatase-1 (*Sgpp1*), and lipid phosphate phosphatases (*Lpp*) 1-3 in B cells and T cells, shown relative to *HPRT*. **(c)** S1P was incubated in the presence or absence of B or T cells for the indicated number of minutes, supernatants were then collected and tested for remaining S1P by the extent of down-modulation of Flag-S1P1 on a reporter cell line. Data are plotted as MFI of Flag-S1P1 staining relative to reporter cells not exposed to S1P (data are from 3 experiments).

Figure 7. S1P2 directs activated B cells to the GC and the center of the follicle. (a) MD4 B cells retrovirally transduced with vectors encoding either control surface receptor (truncated *Ngfr*) or *S1pr2* as well as the hCD4 reporter were transferred into day 6 SRBC immunized recipient mice for 24 hours. Immunohistochemical staining of splenic sections shows localization of $hCD4^+$ transduced cells in GC-containing follicles. (**b**) *Gpr183*$^{+/–}$ MD4 B cells were retrovirally transduced as in **a** and transferred into naive recipients. Immunohistochemical staining shows localization of transduced cells in primary follicles. (**c**) Retrovirally transduced *Gpr183*$^{+/–}$ MD4 B cells were transferred into either littermate control (*Sphk2*$^{–/–}$) recipients or *Mx1-cre*$^{+}$*Sphk1*$^{f/– \text{ or } f/f}$*Sphk2*$^{–/–}$ mice (S1P-deficient). Immunohistochemical staining shows localization of transduced cells in primary follicles. Data in a-c are all representative of 3 independent experiments. Scale bars, 200 μm.

Figure 1

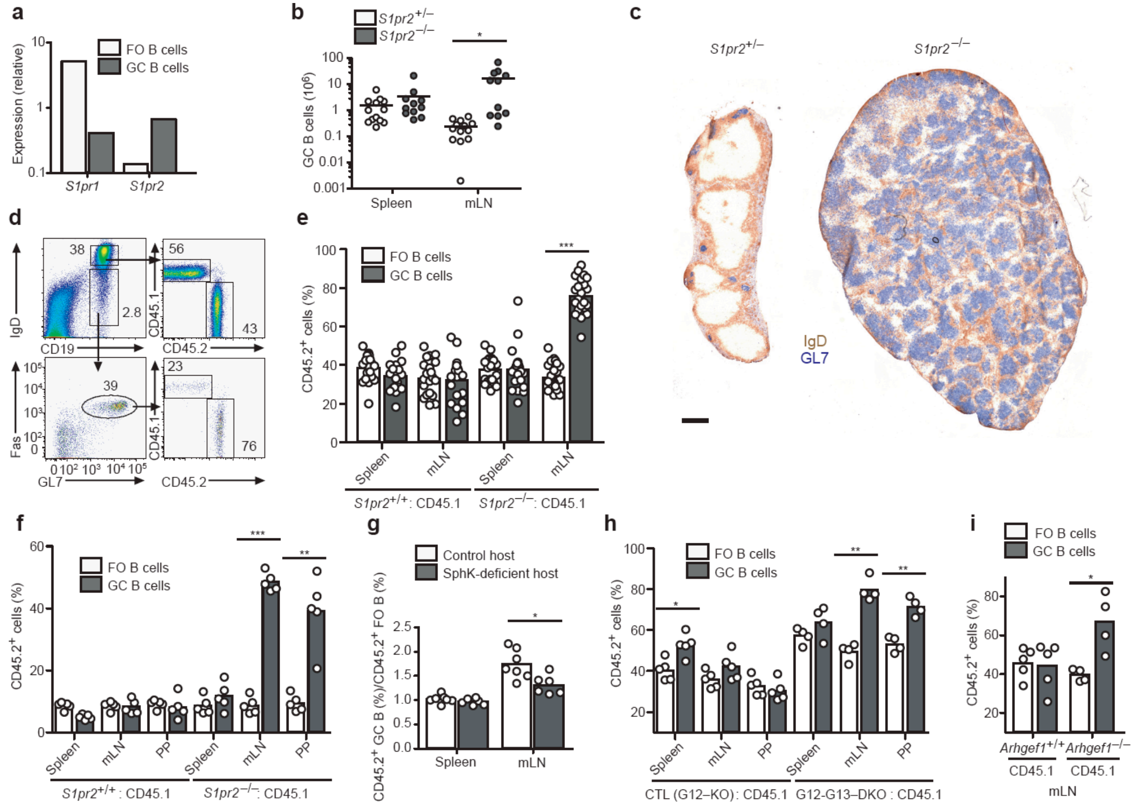


Figure 2

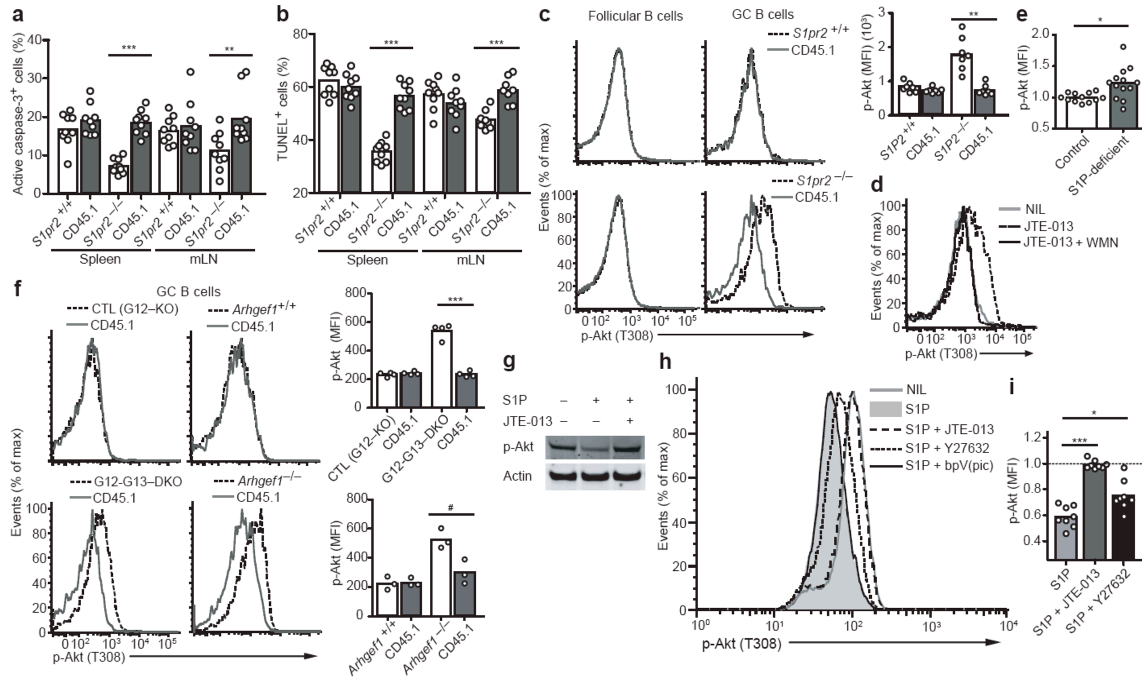


Figure 3

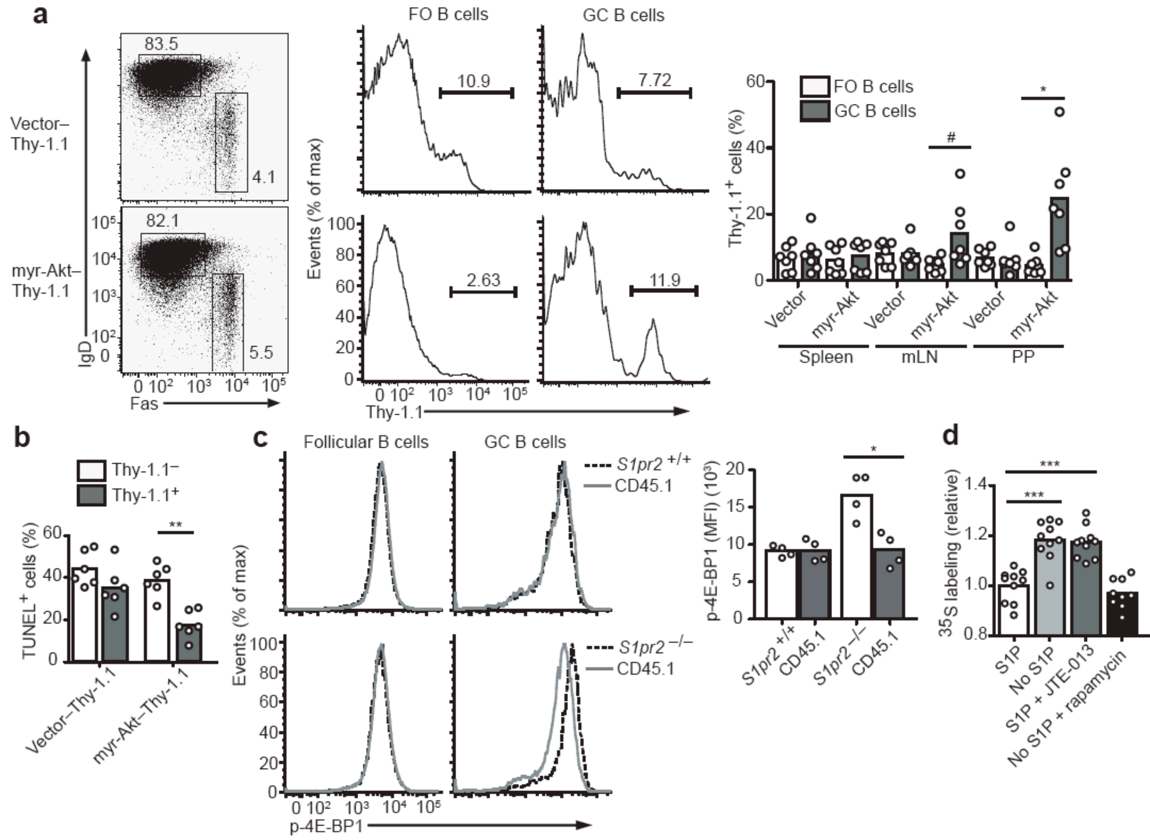


Figure 4

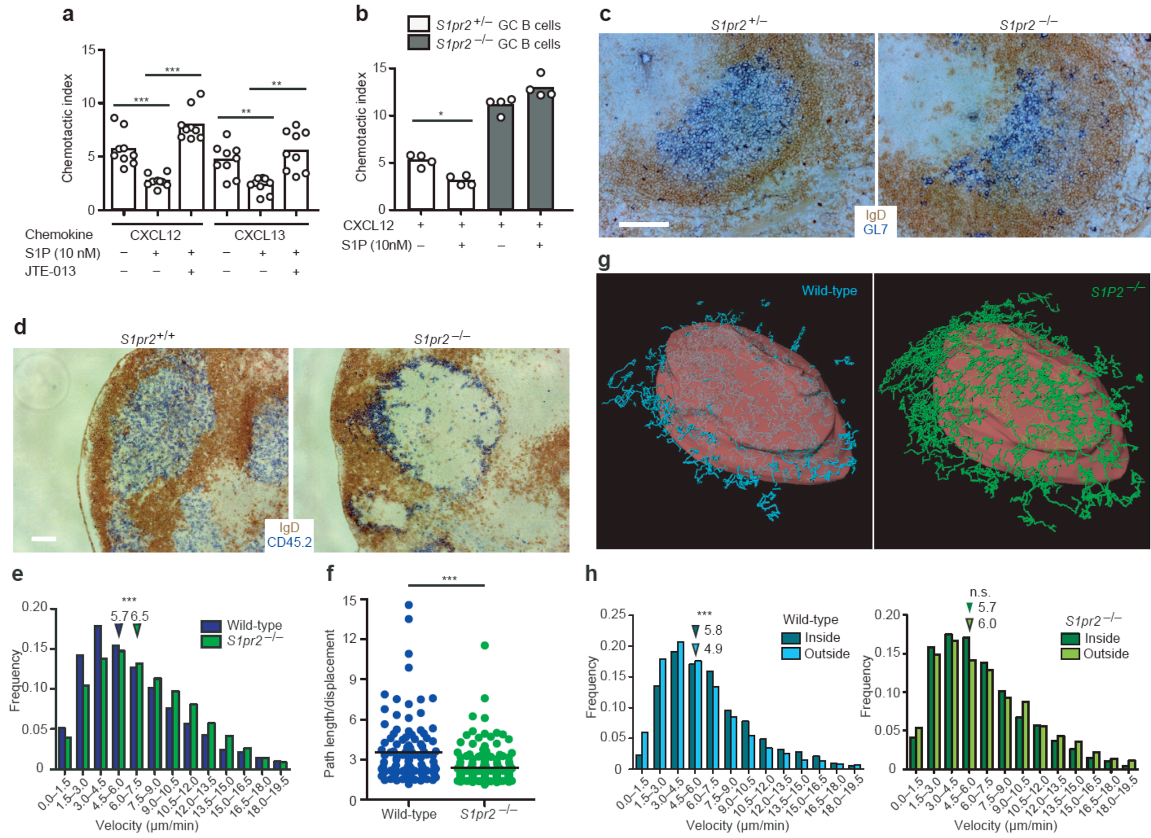


Figure 5

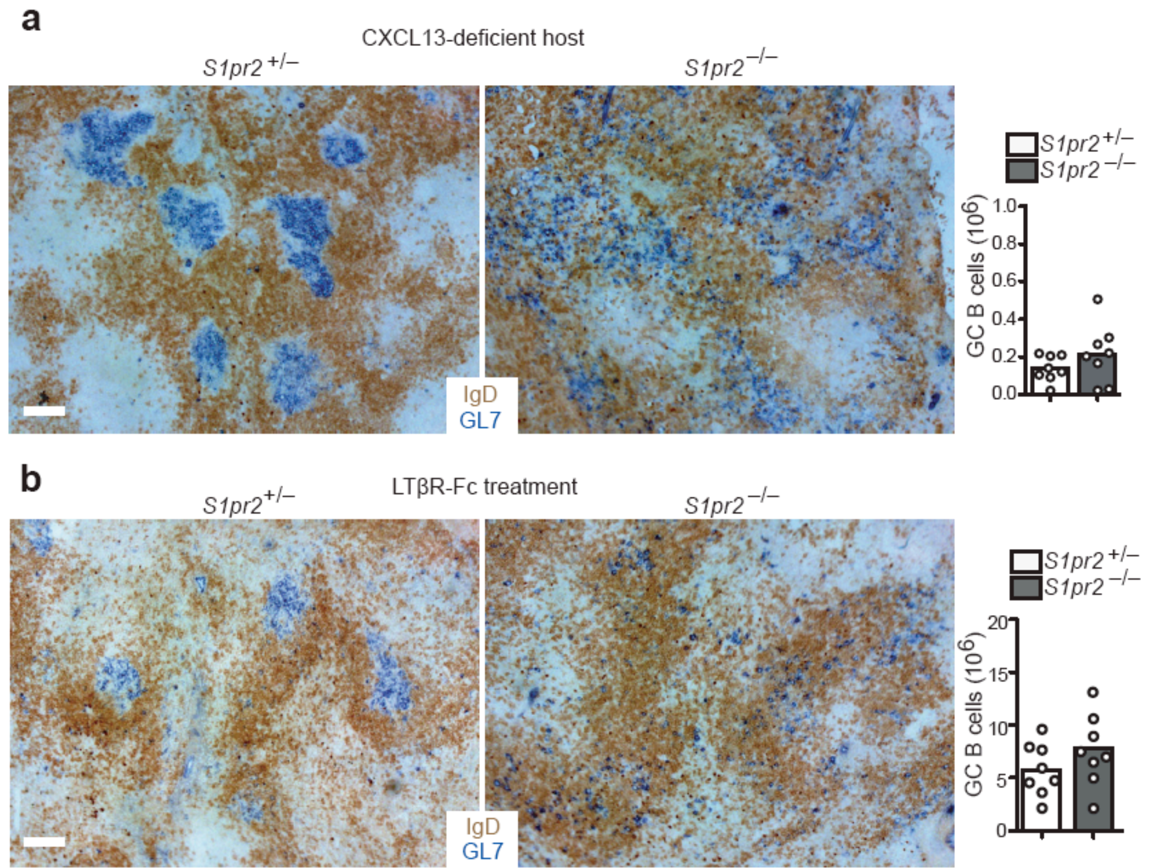


Figure 6

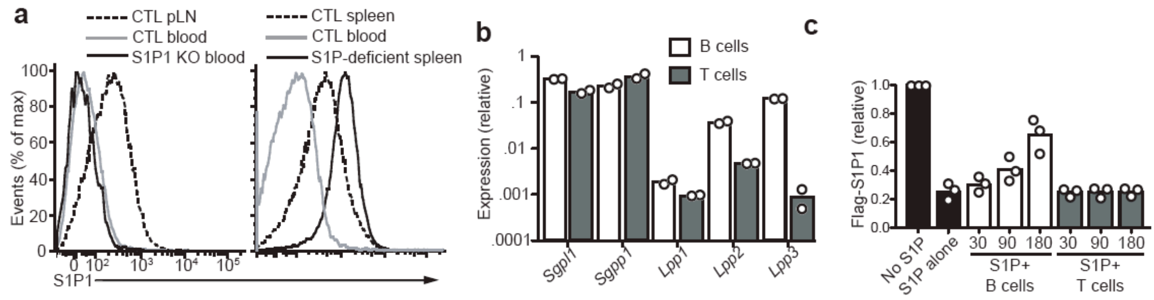
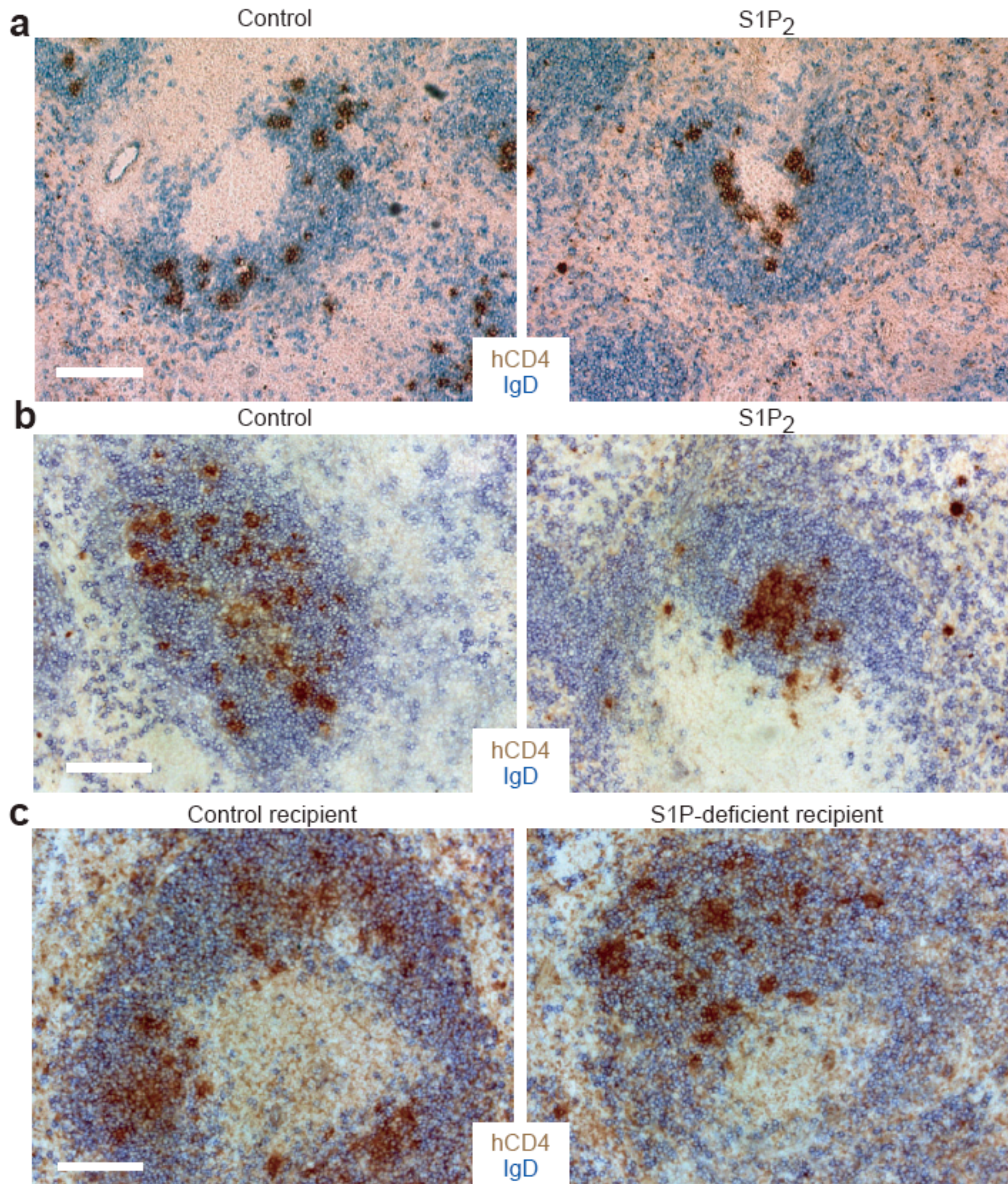
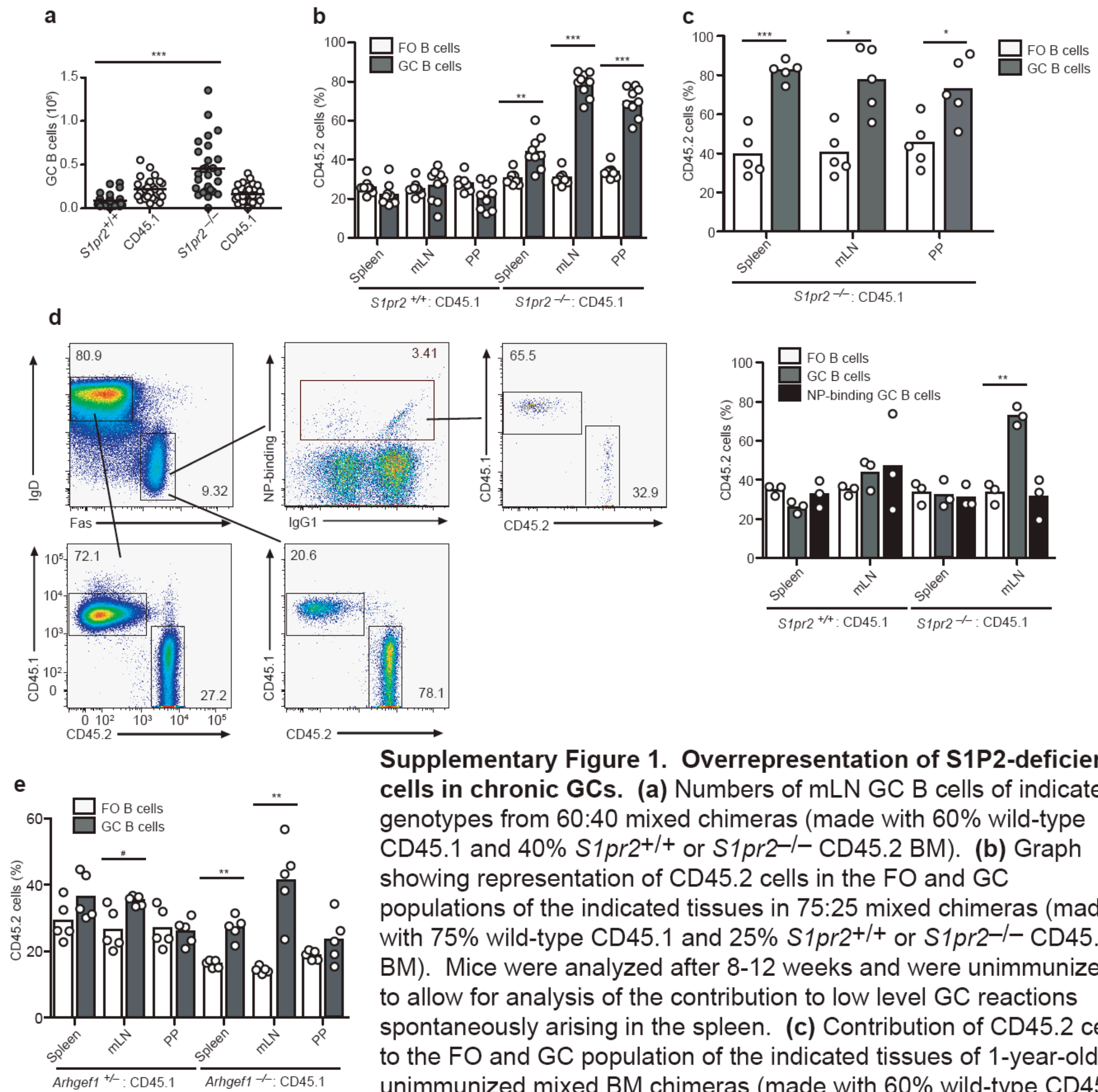


Figure 7

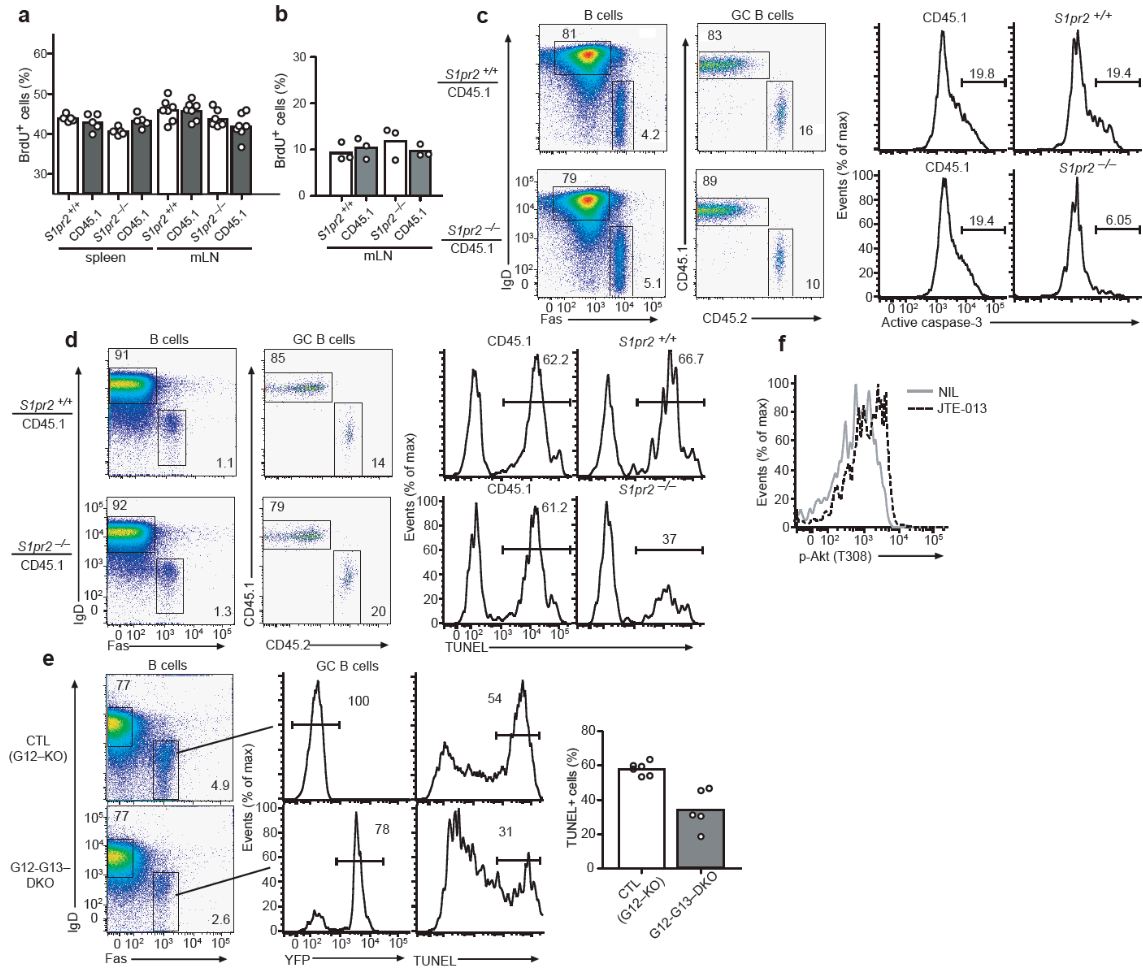


Supplementary Figure 1



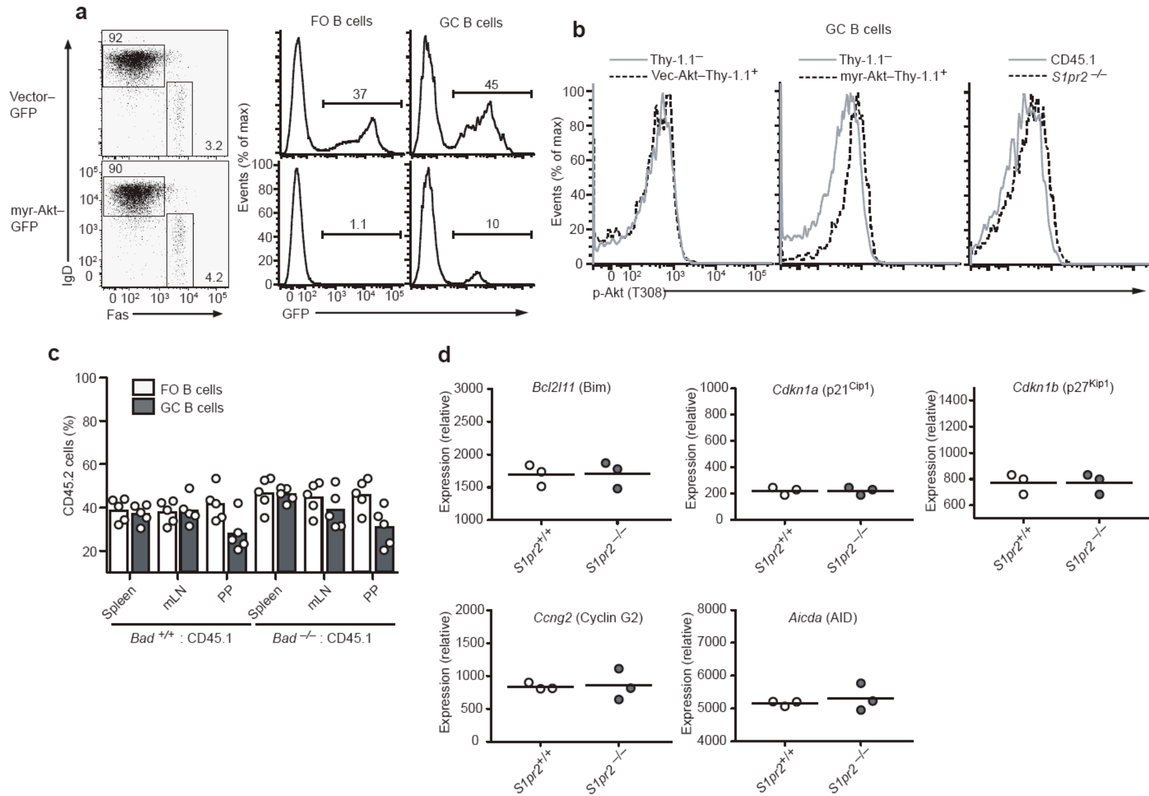
Supplementary Figure 1. Overrepresentation of S1P2-deficient cells in chronic GCs. (a) Numbers of mLN GC B cells of indicated genotypes from 60:40 mixed chimeras (made with 60% wild-type CD45.1 and 40% *S1pr2*+/+ or *S1pr2*−/− CD45.2 BM). **(b)** Graph showing representation of CD45.2 cells in the FO and GC populations of the indicated tissues in 75:25 mixed chimeras (made with 75% wild-type CD45.1 and 25% *S1pr2*+/+ or *S1pr2*−/− CD45.2 BM). Mice were analyzed after 8-12 weeks and were unimmunized to allow for analysis of the contribution to low level GC reactions spontaneously arising in the spleen. **(c)** Contribution of CD45.2 cells to the FO and GC population of the indicated tissues of 1-year-old unimmunized mixed BM chimeras (made with 60% wild-type CD45.1 and 40% *S1pr2*−/− CD45.2 BM). **(d)** Example of flow cytometric gating scheme for tracking the representation of CD45.2 cells in the NP-specific GC B cell population. Mixed BM chimeras were immunized i.p. with NP-CGG in alum and analyzed 14 days later. Graph on right shows the representation of CD45.2 cells in the FO, total GC, and NP-binding GC populations in the spleens and mLNs of mixed BM chimeras. **(e)** Contribution of CD45.2 cells to the FO and GC population of the indicated tissues in 75:25 mixed chimeras (made with 75% wild-type CD45.1 and 25% *Arhgef1*+/− or *Arhgef1*−/− CD45.2 BM). The *Arhgef1* heterozygosity may account for the increased representation of *Arhgef1*+/− CD45.2 cells in mLN GCs. # $P \leq .05$, * $P \leq .01$, ** $P \leq .001$, *** $P \leq .0001$.

Supplementary Figure 2



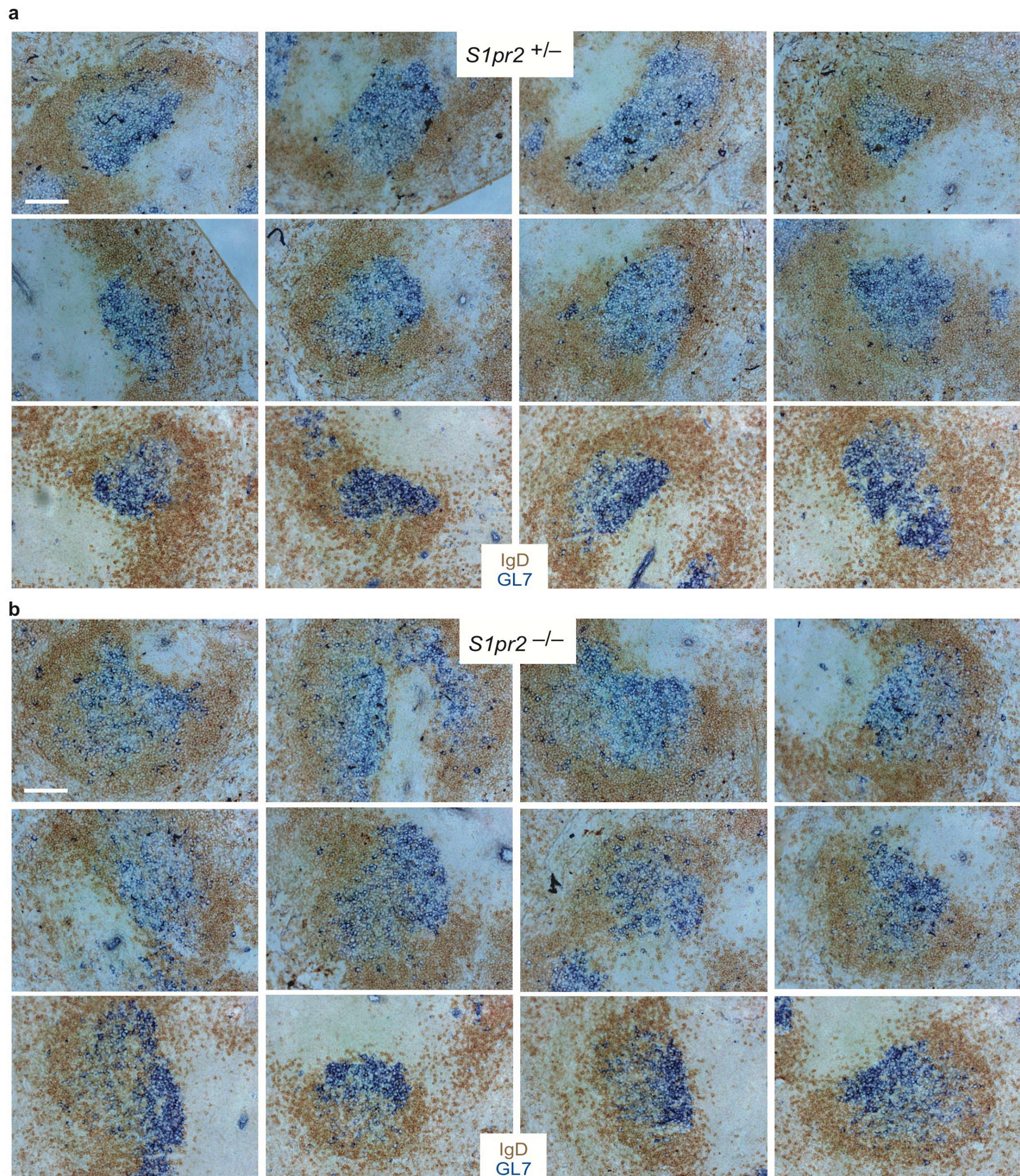
Supplementary Figure 2. Normal rate of BrdU incorporation but less caspase-3 activation and DNA fragmentation in S1P2-deficient GC B cells. (a) Graph showing percentage of BrdU+ cells in the GC population of the indicated tissues of mixed BM chimeras after a 6-hour labeling with BrdU. 60:40 chimeras (60% wild-type CD45.1 and 40% *S1pr2*+/+ or *S1pr2*–/– CD45.2 BM) were immunized i.p. with SRBCs, and at day 8 were injected i.p. with 2 mg BrdU and analyzed after 6 hours. **(b)** Graph showing percentage of BrdU+ cells in the GC population of mixed chimeras as described in a that were injected with BrdU and analyzed after 30 minutes. **(c)** Example of flow cytometric gating scheme to analyze the presence of active caspase-3 in GC B cells of mixed chimeras. Mixed chimeras containing wildtype CD45.1 and either *S1pr2*+/+ or *S1pr2*–/– CD45.2 cells were immunized with SRBCs and analyzed at day 8. Splenocytes were stained for GC markers (B220+, IgDlo, FAS+) and with FITC-DEVD-FMK. **(d)** Example of flow cytometric gating scheme to measure the percentage of TUNEL+ cells after a 37°C 3-hour in vitro incubation of splenocytes from mixed chimeras as described in c. After the incubation, cells were fixed and stained for GC markers as well as for TUNEL-positivity. **(e)** TUNEL assay of splenic GC B cells from mice deficient in G12 and G13. Doubly deficient cells are gated as YFP+ after Cre-mediated excision of a stop element upstream of *eYFP* as a reporter of *Gna13* excision. Graph shows pooled data of TUNEL assays on spleen samples containing G12-G13–DKO cells from 3 independent experiments. **(f)** Intracellular p-Akt staining of wild-type GC B cells harvested in cold S1P-free media in the presence or absence of JTE-013.

Supplementary Figure 3



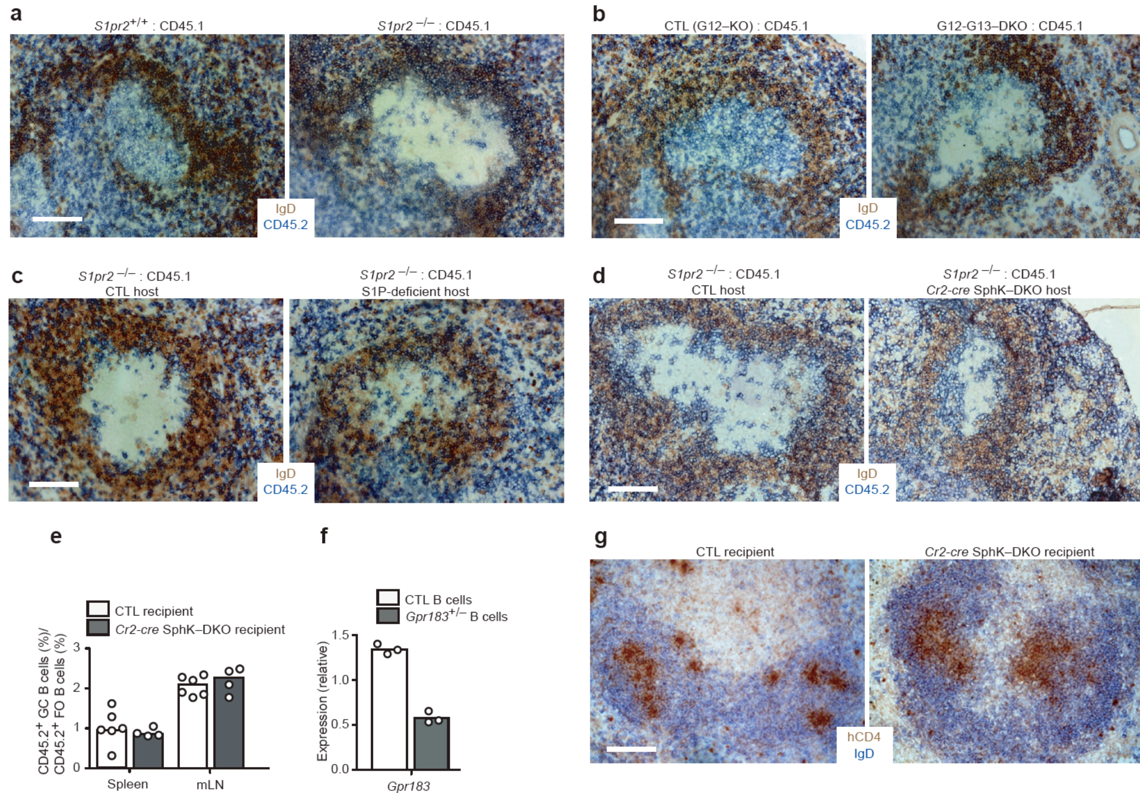
Supplementary Figure 3. Outgrowth of myr-Akt-expressing GC B cells, lack of outgrowth of Bad-deficient GC B cells, and normal expression of FOXO targets in S1P2-deficient GC B cells. **(a)** Wild-type BM was transduced with either GFP control retrovirus or retrovirus encoding GFP and myristoylated Akt (myr-Akt) and used to reconstitute irradiated recipient mice. Representative flow cytometric analysis showing increased representation of myr-AKT-GFP-expressing cells in IgD^{lo} Fas+ GC B cells relative to IgD^{hi} FO B cells of mLN (data are representative of 2 experiments). **(b)** Flow cytometric analysis of p-AKT T308 in mLN GC B cells expressing myr-Akt-Thy-1.1 or vector-Thy-1.1, or Thy-1.1^{-} control cells, in mice reconstituted with *Cr2-cre* transgenic BM transduced with the corresponding retroviruses. To detect the GPI-linked Thy-1.1 marker it was necessary to stain the cells with anti-Thy-1.1-biotin prior to the fixation and internalization steps. This necessitated the use of a directly conjugated secondary reagent for detection of p-AKT T308, accounting for the weaker staining in *S1pr2*$^{-/-}$ cells than in Figure 2. **(c)** Graph showing representation of CD45.2 cells in FO and GC B cell populations in the indicated tissues of 60:40 mixed chimeras (60% wild-type CD45.1 and 40% *Bad*$^{+/+}$ CD45.2 or *Bad*$^{-/-}$ CD45.2). Chimeras were immunized i.p. with SRBCs and analyzed at the peak of the response (d6-8). **(d)** Graphs showing relative expression of indicated FOXO target genes in wild-type and S1P2-deficient GC B cells sorted from mixed chimera mLNs and analyzed by Affymetrix Mouse Gene ST microarray.

Supplementary Figure 4



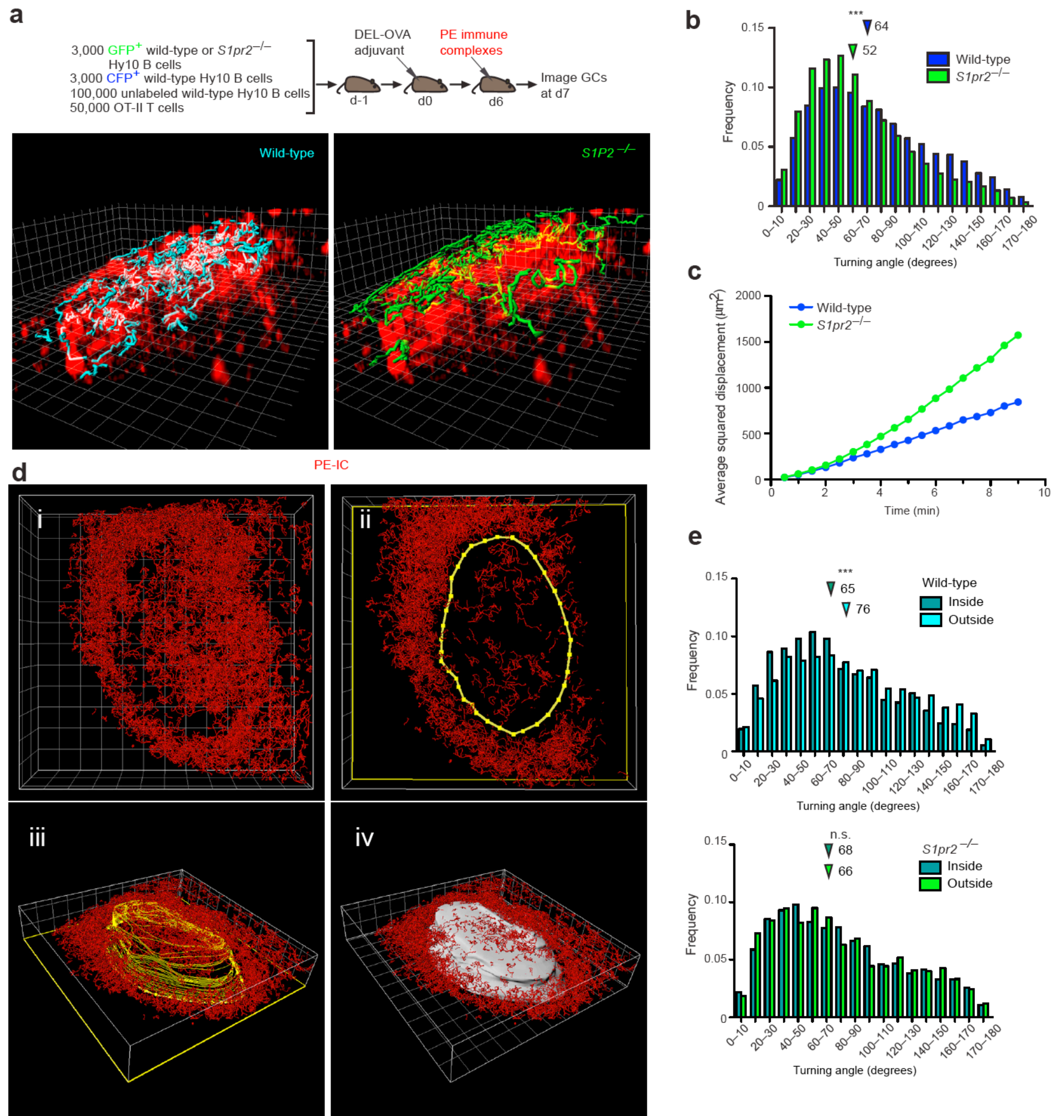
Supplementary Figure 4. Less well-defined GC boundary in S1P2-deficient mice. Immunohistochemical staining of splenic cryosections from SRBC-immunized *S1pr2+/–* (**a**) or *S1pr2–/–* (**b**) mice showing GL7+ GCs in B cell follicles. S1P2-deficient GCs tend to have increased mixing of GC B cells with naive B cells at the perimeter of the GC and a less clear border. Data are representative images from 3 mice of each genotype.

Supplementary Figure 5



Supplementary Figure 5. Localization of S1P2- and G12-G13-deficient cells to the perimeter of the GC. (a),(b) Immunohistochemical staining of splenic cryosections showing localization of CD45.2 cells within GCs of mixed chimeras. 60:40 chimeras were made with a mixture of 60% wild-type CD45.1 BM and 40% either *S1pr2*+/+ or *S1pr2*–/– CD45.2 BM **(a)**, or with a mixture of wild-type CD45.1 BM and either G12-G13-deficient or G12-deficient littermate control CD45.2 BM **(b)**. Although CD45.2 staining does not distinguish GC from FO B cells, it is evident that there is under representation of *S1pr2*–/– or G12-G13–DKO CD45.2+ cells in the center compared to the periphery of the GC (whereas flow cytometrically the representation of CD45.2+ GC B cells in the different chimeric mice is similar, as seen in Figure 1e and h). **(c),(d)** Immunohistochemical staining of splenic cryosections showing localization of CD45.2 cells within GCs of mixed chimeras containing wild-type CD45.1 and *S1pr2*–/– CD45.2 cells. In c, the irradiated hosts were either littermate controls or *Sphk2*–/– mice that lacked *Sphk1* in interferon-responsive cells and thus lacked S1P production by most radioresistant cells. In **d**, the irradiated hosts were either littermate controls or *Sphk2*–/– mice that lacked *Sphk1* in CD21Cre-expressing cells, which include FDCs. Mice were immunized i.p. with SRBCs and analyzed at day 8. **(e)** Graph of relative advantage (contribution to GC population/contribution to FO population) of *S1pr2*–/– GC B cells in spleen and mLN of mixed BM chimeras made in either littermate control or *Cr2-cre Sphk1*f/–*Sphk2*–/– hosts. **(f)** Transcript abundance of *Gpr183* in purified B cells from control or *Gpr183*+/– mice, shown relative to *HPRT*. **(g)** *Gpr183*+/– MD4 B cells were retrovirally transduced as in Figure 7b and transferred into *Cr2-cre Sphk1*f/–*Sphk2*–/– recipient or littermate controlmice for one day. Immunohistochemical staining shows localization of transduced (hCD4+) cells in naïve (IgD+) follicles. The 'halo' effect of the hCD4 staining is a consequence of the tyramide-based enzymatic amplification of the staining that was required to detect this marker.

Supplementary Figure 6



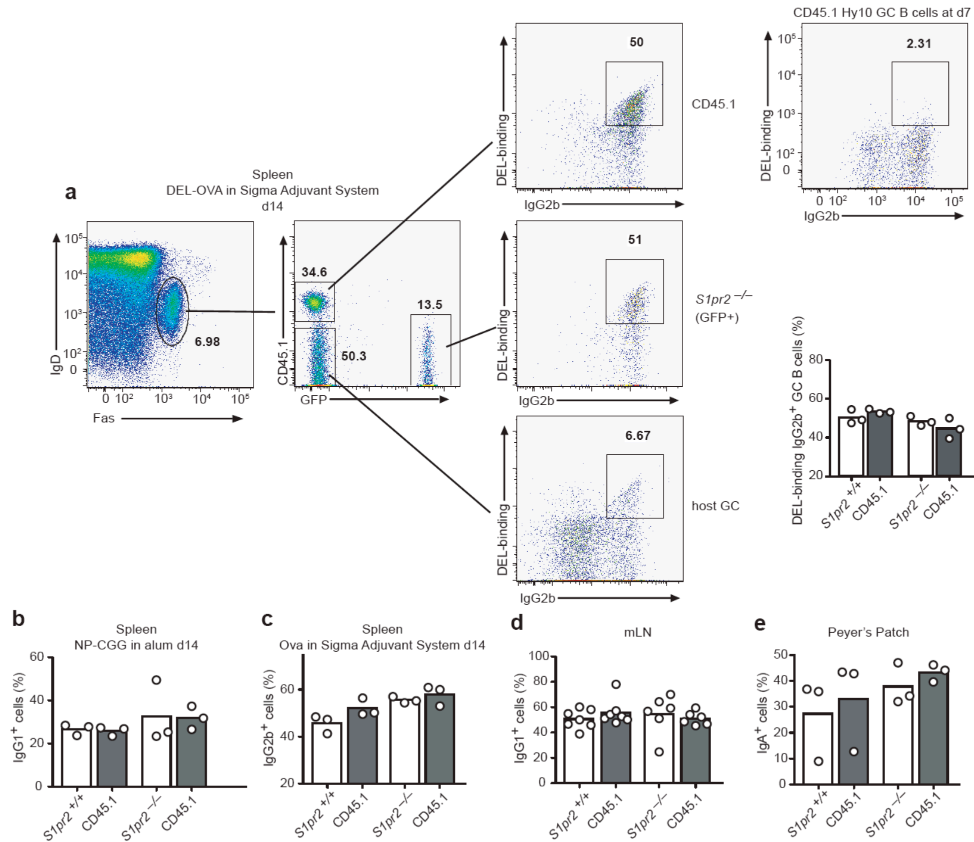
Supplementary Figure 6. Real-time 2-photon microscopy analysis of S1P2-deficient GC B cell migration. (a) Transfer protocol for induction of GCs containing 1-3% GFP+ *S1pr2*–/– and 1-3% CFP+ wild-type Hy10 B cells in the context of a majority of unlabeled wild-type Hy10 B cells and supported by OTII helper T cells. FDC networks are labeled using PE-immune complexes. Lower images show tracks of wild-type (blue) and *S1pr2*–/– (green) GC B cells in relation to the PE-labeled FDC network of a single GC. **(b)** Histograms showing turning angles of *S1pr2*+/+ and *S1pr2*–/– GC B cells determined by tracking migration of fluorescently labeled GC B cells as in a. **(c)** Displacement over time of wild-type and *S1pr2*–/– GC B cells. **(d)** Rendering of GC surface. Image i shows tracks of CMTMR labeled naïve B cells (transferred as in Figure 4g) in a projection view (114 μm in depth) through the GC. Image ii is a single x-y slice showing an example of the contour drawn to represent the follicle/GC boundary. Image iii

continued

Supplementary Figure 6, continued

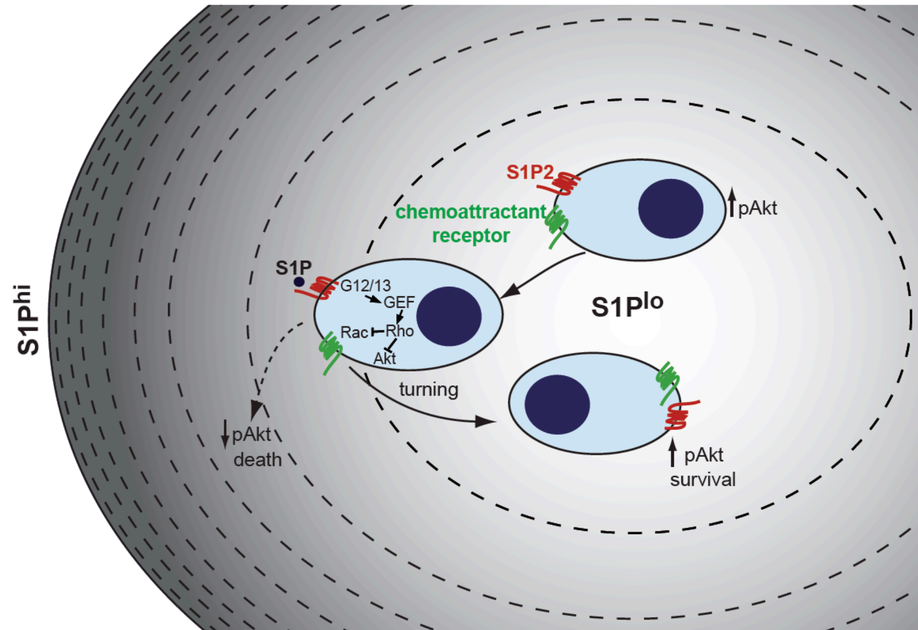
shows a stack of x-y slices of the type in image ii and image iv shows the results of the volume rendering using the 'contour surface' function of Imaris software. **(e)** Histograms showing turning angles of *S1pr2*$^{+/+}$ and *S1pr2*$^{-/-}$ GC B cells located inside or outside the GC surface. Data correspond to those shown in Figure 4h. Downward arrows indicate average values. *** $P \leq .0001$, n.s. $P = 0.88$.

Supplementary Figure 7



Supplementary Figure 7. Intact affinity maturation and isotype switching in S1P2-deficient GC B cells. (a) *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ GFP^{+} CD45.2 Hy10 B cells were co-transferred into CD45.1 recipient mice along with wild-type CD45.1 Hy10 B cells and CD45.1 OT-II T cells. Recipients were immunized subcutaneously with DEL-OVA in Sigma Adjuvant System and analyzed at d14. Shown is an example of a flow cytometric analysis of Hy10 GC B cells at d14, at which point both wild-type CD45.1 and *S1pr2*$^{-/-}$ cells have acquired the ability to bind DEL and have undergone switching to the IgG2b isotype. An example of Hy10 GC B cells at d7, prior to affinity maturation for DEL, is shown in the upper right panel. Graph on the right shows the percentage of gated Hy10 GC B cells of the indicated population that are DEL-binding and have switched to IgG2b at d14. **(b)** Percentage of GC B cells in spleens of mixed chimeras containing wild-type CD45.1 cells and either *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ CD45.2 cells that have switched to the IgG1 isotype at d14 after an i.p. immunization with NP-CGG in alum. **(c)** Percentage of GC B cells in spleens of mixed chimeras containing wild-type CD45.1 cells and either *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ CD45.2 cells that have switched to the IgG2b isotype at d14 after an i.p. immunization with Ova in Sigma Adjuvant System. **(d)** Percentage of GC B cells in mLNs of mixed chimeras containing wild-type CD45.1 cells and either *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ CD45.2 cells that have switched to the IgG1 isotype. **(e)** Graph showing percentage of GC B cells in Peyer's patches of mixed chimeras containing wild-type CD45.1 cells and either *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ CD45.2 cells that have switched to the IgA isotype.

Supplementary Figure 8



Supplementary Figure 8. Model to describe the follicular S1P gradient and how it helps define the GC niche. Contours and shading represent a decaying gradient of S1P from outer to center follicle, with the inner contour corresponding to the boundary of a GC. The gradient is suggested to arise due to S1P production by stromal cells that are abundant at the follicle perimeter, lack of production by FDCs at the follicle center, and rapid degradation by follicular B cells. A GC B cell expressing both S1P2 and a promigratory chemoattractant receptor is shown starting at the follicle center and having high pAkt levels. When the cell moves some distance from the center it enters a region of higher S1P, increasing activation of S1P2, triggering G12-G13 and p115RhoGEF (GEF) to activate Rho and antagonize Rac at the leading edge, resulting in cell turning back towards the follicle center. At the same time S1P2, acting again via G12-G13 and p115RhoGEF, and thus likely Rho, antagonizes Akt activation. As the cell turns and migrates back into the GC, S1P2-mediated antagonism of Akt is reduced and pAkt levels increase, promoting cell viability. Alternatively, if the cell strayed further away from the S1P low follicle center, pAkt levels may continue to drop, increasing the likelihood of cell death. S1P2 deficient cells would not be subject to this form of homeostatic regulation, increasing their chances of surviving at greater distances from the follicle center. Additional undefined cues are speculated to work with S1P-S1P2 to favor GC B cell clustering and survival at the follicle center.

Methods

Mice and immunizations. C57BL/6 and B6-CD45.1 mice were from the National Cancer Institute or Jackson Laboratories. *S1pr2*$^{-/-}$ mice[16] were either seven generations to B6 or on a B6/129 background. BM chimeras[7] were with B6 donors while experiments in *S1pr2*$^{-/-}$ mice were on the mixed background. To generate the *G*α_{12}*-lacZ* knockin allele, *Gna12* gene fragments were from BAC-4922.1. The 5' arm contained 2.8 kb of 5' sequence and the first coding 51bp of exon 1 joined in-frame to a *lacZ*-Sv40polyA cassette followed by a floxed neo cassette (*neo*^{r}). A 2.2 kb 3' arm comprised of exon 1 (26 bp) and intron 1 was inserted between the neo and thymidine kinase (*tk*) cassette. Mice were generated from a clone of targeted RF8 ES cells. The *neo*^{r} cassette was excised by crossing to β-actin-Cre mice. The G12-G13-deficient mice were *Gna12*$^{-/-}$, *Gna13*$^{fl/fl}$ [19], and *Mx1Cre-tg*$^{+/o}$ (on a 129SV/B6 mixed background, MGI 2176073), and were treated neonatally with poly(I:C). The *Rosa26-YFP* locus was introduced as a genetic marker of Cre activity. Sphk1/2-deficient mice[12] were *Sphk2*$^{-/-}$*Sphk1*$^{f/f\ or\ f/-}$ *Mx1-cre-tg*$^{+/o}$ mice that had been treated with poly(I:C) at day 3-5 after birth. *Arhgef1*$^{-/-}$ [48], *Gpr183*$^{+/-}$ [7], HEL-specific MD4 tg (MGI 2384162) and Hy10 mice (MGI 3702699), and *CXCL13*$^{-/-}$ (MGI 2384500) mice were from an internal colony. *Cr2-cre tg* mice (MGI 3047571) were from Klaus Rajewsky. BAD-deficient (MGI 2675966) BM was provided by Nika Danial (Dana-Farber). LTβR-Fc (provided by J. Browning, Biogen Idec) was injected i.v. once per week for 4 weeks in doses of 100 μg. NP-CGG (Solid Phase Sciences) immunizations were i.p. (50μg) in Alhydrogel (Accurate Chemical and Scientific Corp.). DEL-

OVA immunizations were as previously[33] in Sigma Adjuvant System. SRBC immunizations were i.p. injections of 2.5×10^8 SRBC in Hank's BSS. Animals were housed in specific pathogen-free environment in the Laboratory Animal Research Center at UCSF and all experiments conformed to ethical principles and guidelines approved by the UCSF Institutional Animal Care and Use Committee.

Cell isolation and flow cytometry. B cells from spleen, LNs and PPs were isolated and stained as previously[33]. For detection of surface S1P1, we used a rat monoclonal anti-mouse S1P1 antibody that is under development at R&D Systems. For detection of active caspase-3, 3×10^6 cells were stained for GC markers in 5mL polystyrene tubes, washed, and incubated for 40min at 37°C with FITC-DEVD-FMK (Biovision) in staining buffer (PBS 2% fetal bovine serum (FBS), 1mM EDTA, 0.1% sodium azide) then washed in Caspase Wash Buffer (Biovision). For TUNEL assays, 5×10^6 cells were incubated in RPMI1640 2% FBS for 3 hr at 37°C in 5mL polystyrene tubes. Cells were then fixed with 2% paraformaldehyde (Electron Microscopy Sciences) for 10min at 37°C, washed once in PBS, permeabilized in ice-cold methanol for 30min on ice, then washed and stained for DNA fragmentation as per the manufacturer's protocol (APO-BRDU Apoptosis Detection Kit; BD Biociences) and for GC markers. $3\text{-}5 \times 10^6$ cells prepared in RPMI1640 containing 0.5% fatty acid free (FAF) BSA (EMD Biosciences) were fixed and permeabilized as above, washed twice in staining buffer, and stained for pAkt T308 (9275 Cell Signaling Technology) or p4E-BP1 T37/46 (2855 Cell Signaling Technology) on ice for 45min followed by

biotinylated goat anti-rabbit IgG (BD Biosciences) and streptavidin-Qdot605 (Invitrogen) as well as GC markers. Ramos cells were stained similarly, sometimes using streptavidin-APC (Invitrogen). For intracellular analysis of cells expressing Thy1.1, cells were stained with biotinylated anti-Thy1.1 (HIS51; eBioscience) prior to fixation.

Cell culture, inhibitor treatments, metabolic labeling, and chemotaxis. For Akt activation, $3x10^6$ spleen or mLN cells in 5mL polystyrene tubes were resuspended in 200μL RPMI1640 containing 0.5% FAF BSA. 10 μM JTE-013 (Tocris Biosciences) and 200 nM wortmannin (Sigma) were added, and cells were incubated for 30min at 37°C before being fixed and stained as above. Ramos cells were washed twice and sensitized for 1 hr in 0.5% FAF BSA media. Cells were pretreated in 10μM JTE-013 or 10μM Y-27632 (Sigma) for 15 min and then with S1P (Sigma) for 5 min. For Western blots, cells were lysed in prewarmed 2X SDS lysis buffer (100 mM Tris pH 6.8, 4% SDS, 10% Glycerol, 2% betamercaptoethanol, and protease inhibitor cocktail (Roche)), ultracentrifuged, separated by SDS-PAGE, and probed with rabbit anti-phospho-Akt S473 (Cell Signaling Technology) and rabbit anti-actin (Sigma). For metabolic labeling, Ramos cells were washed twice, cultured for 90min in RPMI containing 0.5% FAF BSA with 10nM S1P and 10μM JTE-013 or 200nM Rapamycin (MBL International), washed once and plated in 24-well plates in the same conditions. After 45min, ^{35}S-met/cys labeling mix (EasyTag Express35S Labeling Mix; Perkin Elmer) was added. After 30min, cells were washed, lysed, and soluble lysate was loaded into scintillation fluid (EcoLume; MP Biomedicals)

and counts measured with a scintillation counter. Transwell assays were as described[6] with cells from Eμ-*Bcl2*-22 crossed mice (MGI 3052827). For measurement of S1P degradation, B cells were enriched from spleens using anti-CD43 or T cells using anti-B220 microbeads (Miltenyi Biotec). $5x10^6$ cells were incubated in 250μl RPMI containing 0.5% FAF BSA with 1μM S1P, spun down and 100μl of supernatants were tested for S1P concentrations by measuring downmodulation of Flag-S1P1 in WEHI231 cells[11].

Gene expression profiling. Total RNA was isolated from ~45,000 sorted wild-type CD45.1 and *S1pr2*$^{-/-}$ GC B cells from mixed chimeric mLNs with the RNEasy Micro Kit (Qiagen). RNA was amplified using the Ovation RNA Amplication System V2 (NuGEN) and cDNA fragments were labeled with the Encore Biotin Module (NuGEN). Samples from 3 experiments were then hybridized to the Affymetrix Mouse Gene ST array, stained, scanned, and normalized by the UCSF Gladstone Genomics Core facility. Normalized data were analyzed using dChip software (http://biosun1.harvard.edu/complab/dchip/). Foxo target genes were selected based on[49].

Immunohistochemistry. 7μm cryosections were cut and prepared as described[6]. For human CD4 staining, a tyramide kit (TSA Biotin System; Perkin Elmer) was used according. Images were captured with a Zeiss AxioOberver Z1 inverted microscrope.

Retroviral constructs and transductions. Mouse *S1pr2* or the control truncated *Ngfr* were inserted in a retroviral vector containing a cytoplasmic-domain truncated human CD4 reporter[50]. An insert containing a HA tag and the

14-amino acid myristoylation site of human *v-src* at the N-terminus of *Akt1* was inserted into the MSCV2.2 retroviral vector containing GFP downstream of the IRES. This construct was also inserted into a Thy1.1 reporter vector containing a loxP-eGFP-stop-loxP cassette upstream of the insertion site, and BM from *Cr2-cre* mice was transduced. MD4 B cells were transduced as previously[8]. For S1pr2, due to effects on cell viability or homing, ~3-fold more cells were activated and transduced. For BM transductions, donor mice were injected i.p. with 3 mg 5-Fluorouracil (Sigma). BM was harvested after 4 days and cultured in DMEM containing 15% FBS, antibiotics (50 IU/ml penicillin, 50 μg/ml streptomycin; Cellgro) and 10 mM HEPES (Cellgro), supplemented with IL-3, IL-6, and stem cell factor (SCF) (each at 0.1 μg ml^{-1}, Peprotech). Cells were spin-infected twice at day 1 and 2 and transferred to irradiated recipients on day 3.

Two-photon microscopy. Two similar experimental setups were used. In the first, 3000 *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ GFP^{+} Hy10 B cells were transferred with 3000 CFP+ Hy10 B cells, 100,000 unlabeled Hy10 B cells, and 50,000 OT-II T cells. In the second, *S1pr2*$^{+/+}$ or *S1pr2*$^{-/-}$ Hy10 BM was retrovirally transduced with a GFP-containing vector. After reconstitution of irradiated recipients, 3000 GFP^{+} and 15,000 GFP^{-} Hy10 *S1pr2*$^{-/-}$ B cells were transferred along with 3000 CFP^{+} Hy10 B cells and 85,000 unlabeled Hy10 B cells. At day 6 after DEL-OVA immunization, 2 mg of rabbit anti-PE (Rockland) was injected i.v. and 2 hours later 10μg PE was injected subcutaneously to label FDC networks with PE immune complexes. Alternatively, the mice received 10^{7} CMTMR labeled naïve B cells. On day 7, explanted LNs were prepared[33], and imaged with a custom two-

photon microscope equipped with a MaiTai Ti-Saphire laser (Spectra-Physics) or Zeiss LSM 7MP equipped with a Chameleon laser (Coherent). Images were acquired with Video Savant (IO industries) or Zen (Zeiss). Videos were processed with a median noise filter. Excitation wavelength was 910 nm. Emission filters were 570-640 nm for PE, 500-550 nm for GFP and 455-485 nm for CFP in the custom system, or 570-640 nm for PE, 500-550 nm for GFP, and 450-490 nm for CFP in the Zeiss system. Cell tracks were made with Imaris 5.7.2. x64 (BitPlane) and corrected manually. The velocities and turning angles of cells between each imaging frame were calculated with MatLab (MathWorks). Annotation and final compilation of videos were with Adobe After Effects 7.0. Video files were converted to MPEG format with AVI-MPEG Converter for Windows 1.5 (FlyDragon Software). The boundary of the GC was traced for each x-y slice according to the positioning of naïve follicular B cells, and then the GC surface was calculated by the 'Contour surface' function of Imaris software.

Statistical analysis. All statistical analysis was with Prism software (GraphPad Software, Inc.). For comparison of two nonparametric data sets (Fig. 4f, g, and i), the Mann-Whitney U-test was used. Otherwise, means of two groups were compared with the two-tailed non-paired Student's *t*-test.

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Chapter 3

Conclusion

Our results show that S1P2 plays dual roles in GC B cells. First, S1P2 negatively regulates GC B cell migration to chemoattractants. This functions to promote movement to the center of the B cell follicle and confinement within the GC. Second, S1P2 limits the outgrowth of chronically-stimulated GCs by antagonizing signaling through the pro-survival kinase Akt, a downstream effector in the PI3K pathway. We propose a model that links these two functions, in which GC B cell localization, proliferation, and survival is restricted to a region of low S1P in the center of the B cell follicle. In this model, S1P exists in a decaying gradient throughout the follicle, with the important sources coming from stromal cells outside the center of the follicle, and with mechanisms of diffusion and active degradation maintaining low concentrations in the center of the follicle. GC B cells are clustered in the center of the follicle and migrate extensively within the GC, according to expression of chemokine receptors CXCR4 and CXCR5 and local gradients of their respective ligands CXCL12 and CXCL13. When GC B cells move toward the edge of the GC and the surrounding mantle zone, they begin to encounter higher levels of S1P. GC B cells express high levels of S1P2, and its interaction with S1P leads to signaling downstream through $G\alpha12$ and $G\alpha13$. This signals to cause activation of P115RhoGEF and Rho GTPase. This signaling cascade appears to have two effects on the migrating GC B cells. One is to reduce migration in the direction of the S1P, promoting the ability of the cell to turn in the opposite direction, move back into the center of the GC, and remain clustered with GC B cells. The other effect is to antagonize Akt signaling, a major effector hub of pro-survival PI3K signaling.

This antagonism becomes apparent through an increased resistance to apoptosis upon placing the cells into stressed conditions *ex vivo*, as well as through an *in vivo* outgrowth effect in GCs that are chronically stimulated by antigen. This restriction of Akt signaling and growth is critical to maintain GC homeostasis in mice, as aging S1P2-deficient mice often experience gross enlargements of their mesenteric lymph node GCs, with a loss of lymphoid architecture, which likely precedes the development of diffuse large B cell lymphoma (Cattoretti et al. 2009).

In the context of a wild-type GC, S1P2-deficient GC B cells are excluded from the center of the GC and intermingle with naive follicular B cells around the perimeter. Young S1P2-deficient mice are still able to form GC clusters with grossly normal appearance, but the boundary between GC B cells and naive follicular B cells is disrupted. This implies that other factors are involved in GC localization and clustering in cooperation with S1P2. In fact, chimeric mice lacking both CXCR5 (through CXCL13-deficiency in bone marrow chimera hosts) and S1P2 function had severely disorganized GCs, suggesting that S1P2 function is required in the absence of the important follicular organizing cue CXCL13. GCs were similarly disrupted when S1P2 deficiency was combined with antagonism of lymphotoxin (LT)-$\alpha2\beta1$ signaling, a treatment known to disrupt FDC networks (Allen et al. 2008), suggesting that FDC and S1P2 also cooperate to promote GC B cell clustering. LT$\alpha2\beta1$ antagonism reduces CXCL13 expression (Ngo et al. 1999), so it is not clear if the cooperative effect seen is solely dependent on the reduction of CXCL13 in the treated mice, or

whether FDCs also cooperate with S1P through an independent mechanism. While FDCs are thought to be an important source of CXCL13, and within the GC CXCL13 is concentrated on the light zone stroma (Allen et al. 2004), it isn't established whether CXCL13 is concentrated in the GC relative to the surrounding region. So, it is possible that FDCs contribute to GC clustering through an additional mechanism that is not yet discovered. This could be through the production of a chemoattractant that directs GC B cell migration, the degradation of an attractant for follicular B cells in order to keep them from moving into the GC, or even the production of a chemorepellant that repels follicular B cells.

The full set of cues regulating GC B cell positioning and clustering in the center of the follicle is not yet understood. Recent studies have revealed a previously ignored stage of B cell migration early during T dependent immune responses, where activated B cells migrate to interfollicular (IF) and outer follicle regions of the spleen after receiving T cell help, where they can undergo extensive proliferation before returning to the center of the follicle to initiate GC clustering (Coffey et al. 2009). This movement is directed by the B cell chemoattractant receptor EBI2 (Pereira et al. 2009; Gatto et al. 2009). Recently, activated B cells were shown to inhabit IF regions of lymph nodes and undergo interactions with cognate T cells, and in this experimental system GC commitment appeared to take place in the IF region (Kerfoot et al. 2011). Bcl6 expression was first observed in B cells in this region. While B cells also moved to the outer follicle, which in the lymph node is immediately beneath the

subcapsular sinus, B cells there did not express Bcl6, suggesting that they were not committing to the GC lineage, but rather expressed higher levels of intracellular immunoglobulin light chain, suggesting that they were differentiating into plasmablasts. The authors also observed a trend for B cells in the IF region to occasionally move into the center of the follicle, consistent with seeding of the GC. Downregulation of EBI2 takes place in GC B cells and is important for GC B cell positioning in the center of the follicle. We suggest that S1P2 upregulation also contributes to the movement of GC B cell precursors to the follicle center. S1P2 expression directs activated B cells to the center of the follicle, and S1P2-deficient B cells are excluded from the GC interior.

The mechanism by which S1P2 contributes to GC centering and clustering is not fully elucidated. While it is currently not feasible to directly measure S1P concentrations in tissue microenvironments, several findings imply that S1P is present in a decaying gradient in the follicle. The first is simply that S1P2 directs cells to the center of the follicle. S1P2 inhibits migration to a chemoattractant when S1P is present in a gradient, so by extension S1P2-expressing cells are most likely to move in the direction of lowest S1P. Second, the important source of S1P is stromal cells that are not the FDCs in the center of the follicle. So, much of the relevant S1P is being produced outside the follicle. S1P's half-life in plasma is on the order of 15 minutes, suggesting tight temporal and spatial control within tissues would be possible (Venkataraman et al. 2008). In addition, red blood cells in the plasma do not express S1P-degrading enzymes (Ito et al. 2007), while B cells in the follicle do and can efficiently degrade S1P in vitro,

suggesting that the S1P half-life in the follicle is likely very short and that B cells could actively maintain low S1P levels. Further, there is not extensive entry of naive B cells or other cell types into the GC throughout the GC response, suggesting that S1P on the surface of cells that recently arrived from circulation would not be carried efficiently into the GC and would likely be degraded by B cells in the follicle before it reached the GC. We found that wild-type GC B cells migrating within the GC slowed and turned after reaching the GC border more markedly than S1P2-deficient GC B cells, suggesting a sharp gradient of S1P at the GC border. It is possible that cells within the GC are even more active at degrading S1P than follicular B cells, or that the lack of cells entering the GC after arriving from circulation loaded with S1P contributes to this sharp gradient. As such, it is likely that the center of the follicle and the GC in particular is a region of low S1P, allowing S1P2 to contribute to maintaining GC confinement. It will be useful to determine if B cell-specific expression of enzymes with S1P-degrading potential is important for maintaining low levels of S1P in the follicle, and to narrow the source of relevant S1P to a specific subset of stromal cells.

Our results have also revealed a strong contribution of S1P2 to maintaining GC homeostasis. We first observed an outgrowth of S1P2-deficient GC B cells within chronically-stimulated mucosal GCs of mixed bone marrow chimeras, where knockout cells were over-represented relative to their contribution to the follicular B cell population. This outgrowth seemed to be specific to chronically-stimulated GCs, because when we tracked antigen-specific GC B cells in spleen or mucosal tissues after an immunization with a haptenated

protein carrier, S1P2-deficient cells and wild-type cells were represented equally in the GC according to their starting contributions to the follicular B cell population. In addition, in the spleens of aging, unimmunized mixed chimeras that had small GC reactions likely due to low-level chronic stimulation from microbial flora in our mouse colony, S1P2-deficient GC B cells were over-represented.

The outgrowth of S1P2-deficient GC B cells was coupled to increased resistance to apoptosis upon encountering stressful *ex vivo* conditions and increased activation of the pro-survival kinase Akt, suggesting increased PI3K signaling. As these effects were seen in S1P2-deficient GC B cells from any tissue analyzed, including the spleen, it is not entirely clear why the outgrowth of S1P2-deficient cells occurred only in chronically-stimulated GCs, or why it seemed to occur slowly over a period of weeks. One possibility for this difference is that the factors determining GC formation and GC B cell survival could be regulated differently by acute immunization than by chronic exposure to microbial flora, and that S1P2's regulatory actions affect specifically the latter. The inductive signals leading to GC formation and persistence in mucosal tissues are not entirely understood. A mouse model was generated in which the BCR has been replaced with the Epstein-Barr virus (EBV)-encoded latent membrane protein (LMP) 2A, which contains an immunoreceptor tyrosine activation motif (ITAM) homologous to that of Igα and Igβ of the BCR complex (Casola et al. 2004; 2006). This allows for signals that mimic tonic BCR signaling, allowing for B cell survival while preventing specific BCR recognition and stimulation by

antigen. These B cells were able to form GCs in mucosal tissues, suggesting that antigen-specific recognition was not required for GC formation in tissues chronically exposed to microbial antigens. From these experiments it seems possible that when GCs are driven by signals other than affinity for specific antigen, the conditions regulating survival and selection could be different than in a newly-generated GC in which GC B cells are competing for antigen and undergoing affinity-mediated competition.

Consistent with this idea, CD19-deficient mice, which lack induced GCs in spleen and peripheral lymph nodes, contain GCs within Peyer's Patches (Gärdby et al. 2000). The defect in splenic GC formation in CD19-deficient mice is thought to be primarily due to a lack of recruitment and activation of PI3K signaling downstream of BCR stimulation, because PTEN deficiency rescues the ability of CD19-deficient mice to form GCs upon immunization (Anzelon et al. 2003). In addition, transgenic mice expressing a form of CD19 containing mutations affecting PI3K recruitment exhibit defective GC maturation in peripheral tissues (Wang et al. 2005). Interestingly, mice deficient in or containing a catalytically inactive form p110δ (Clayton et al. 2002; Jou et al. 2002; Okkenhaug et al. 2002), a catalytic subunit of PI3K, are unable to form GCs even in mucosal tissues. The requirement for p110δ activity may be specific to T cells for GC formation (Rolf et al. 2010), but several pieces of evidence suggest that redundant contributions of p110 isoforms could be critical in B cells. Both p110δ and p110α can associate with CD19 upon B cell stimulation (Vigorito et al. 2004), p110α rescues B cell survival after BCR

deletion (Srinivasan et al. 2009), phenotypes in PTEN-deficient B cells are only partially reversed upon p110δ deletion (Janas et al. 2008), and a loss of both p110δ and p110α completely blocks B cell development (Ramadani et al. 2010). This suggests that PI3K activity is likely required for GC formation and/or persistence but that in conditions of chronic stimulation, there may be signals leading to sufficient PI3K activation that are independent of BCR signaling and CD19 function.

There could be several explanations for the specificity of the S1P2-deficient outgrowth in chronic rather than antigen-driven GCs. One is that PI3K signaling downstream of the BCR is modulated separately from the PI3K signaling regulated by S1P2. This could mean that S1P2's growth-control effect is not revealed in situations in which BCR-driven PI3K signaling is the determining factor in GC B cell selection but is more important in the context of non-limiting amounts of microbial stimulation. PI3K signaling downstream of the BCR utilizes the catalytic subunits p110δ and p110α, but the subunits p110γ and p110β signal downstream of GPCRs (Guillermet-Guibert et al. 2008). It isn't entirely clear how differential usage or regulation of catalytic subunits of PI3K plays out in different cellular contexts. PI3K signaling has been found to suppress Foxo-dependent AID expression and class switching (Omori et al. 2006; Dengler et al. 2008). However, we did not observe that increased Akt activation in S1P2-deficient cells led to decreased class switching, suggesting that PI3K signaling can be context-dependent and could vary depending on upstream inputs or the location of cellular pools of PIP3 and Akt. This is

consistent with a recent finding that Akt activity can be substrate-specific depending on context (Schenck et al. 2008). Thus, it is possible that when BCR activation of PI3K signaling is mediating selection, S1P2's effects on survival are masked. It is also possible that the increase in pro-survival Akt signaling in S1P2-deficient GC B cells is very small *in vivo* and only occasionally alters the balance between survival and death for a GC B cell. One possible mechanism by which GC B cell fate would be determined in this scenario is through accumulating genotoxic stress due to off-target mutational activity of AID. The decision to induce apoptosis could take place, for instance, after recognition of double strand breaks by ATM or ATR and activation of a p53-dependent apoptotic response. As discussed in the introduction, AID-deficient GC B cells have an intrinsic survival advantage, likely due to the lack of accumulated mutations (Zaheen et al. 2009).

Another possibility that isn't mutually exclusive with this explanation is that S1P and S1P2 act to dampen Akt signaling in only a small minority of GC B cells at any point in time. Evidence from our imaging data suggests a sharp increase in S1P levels at the edge of the GC, so it is likely that GC B cells in the center of the GC are proceeding relatively uninfluenced by S1P2 signaling. The few that venture outside into the periphery, mingle with naive follicular B cells, and encounter higher S1P levels may experience S1P2 signaling and a dampening of Akt activation, but normally turn back into the center of the GC and survive. This dampening of pro-survival signaling would only rarely cause the cell to die, a consequence to which S1P2-deficient GC B cells would be resistant. Over the

lifetime of the organism, resistance to this form of homeostatic regulation clearly has consequences, as S1P2-deficient mice often harbor large outgrowths of GC B cells in mucosal tissues. This seemingly sudden loss of homeostasis suggests that secondary oncogenic hits could be taking place in S1P2-deficient GC B cells, possibly due to increasing resistance to apoptosis upon the acquisition of DNA breaks and genotoxic stress.

The mechanism by which S1P2 signaling leads to dampened Akt activation is not clearly understood. Downstream effectors Gα12 and Gα13, as well as p115RhoGEF, had the same effect on Akt activation, and inhibition of the Rho effector kinase ROCK partially recapitulated the effect, so it is likely that Rho activation is involved in suppression of PI3K signaling. Rho and ROCK can activate the lipid phosphatase PTEN (Li et al. 2005), but our experiments with PTEN inhibitors didn't reveal a role for PTEN in S1P2-mediated Akt inhibition in GC B cells. A second possibility is that the relevant PI3K signaling and Akt activation is downstream of Rac, as constitutive Rac activity has been shown to activate PI3K in several contexts (Genot et al. 2000; Gonzalez et al. 2006; Higuchi et al. 2001). In this case, S1P2-mediated Rho activation could lead to Rac antagonism and dampening of Akt activation. A third possibility is that S1P2 signaling leads to adenylate cyclase activation by Gα12 and Gα13, and that the subsequent cAMP production inhibits Akt activation through PKA (Michaud et al. 2010). Gα12 and Gα13 have been shown to activate adenylate cyclase (Jiang et al. 2008), and PKA has previously been suggested to mediate cAMP's inhibitory effect on Akt activation (Mei et al. 2002). It will be interesting to determine how

various inputs control Akt activation in GC B cells and how S1P2 signaling is integrated into this pathway.

Finally, the downstream mechanism by which S1P2 deficiency and increased Akt activation is leading to an outgrowth of GC B cells is not yet fully explained. Akt can promote cell survival through a number of signaling pathways (Manning et al. 2007). One potential mechanism is through direct phosphorylation and inhibition of the pro-apoptotic BH3-only protein BAD, which normally inactivates the pro-survival molecules Bcl-2, Bcl-xL, and Bcl-w (Chen et al. 2005). Of these molecules, Bcl-xL is highly expressed in GC B cells (Vikstrom et al. 2010). However, BAD deficiency did not recapitulate the outgrowth of S1P2-deficient GC B cells. Consistent with this result, Bcl-xL-deficient B cells did not have a defect in GC formation (Vikstrom et al. 2010). A second possible mechanism downstream of Akt is phosphorylation of FOXO transcription factors, leading to their displacement from target genes and export from the nucleus. FOXO proteins can induce transcription of the BH3-only protein BIM. We did not detect transcriptional changes in FOXO targets in S1P2-deficient GC B cells, suggesting that Akt inhibition of FOXO proteins is not the mechanism for increased survival. It is possible that a minority of GC B cells is experiencing S1P2 signaling at the edge of the GC at any one point in time, making transcriptional changes in bulk populations difficult to detect. However, the *ex vivo* survival advantage of S1P2-deficient cells became apparent more rapidly than would be expected if transcriptional regulation were behind differences in resistance to apoptosis. The third possibility we were able to investigate involved

Akt's positive regulation of mTOR complex 1 (mTORC1), which regulates translation initiation and ribosome biogenesis. Akt phosphorylates the tuberous sclerosis complex 2 (TSC2), an upstream inhibitor of mTORC1 that mediates this inhibition by acting as a GAP for Rheb, which when bound to GTP activates mTORC1. So, through inhibiting TSC2, Akt signaling leads to increased Rheb activity, which stimulates mTORC1 (Manning et al. 2007). When activated, mTORC1 can phosphorylate and inhibit the translational repressor 4E-BP1, allowing the eukaryotic translation initiation factor eIF4E to recruit other initiation factors and initiate translation of a subset of mRNAs (Ma et al. 2009). The target specificity of this form of translational regulation is not fully understood, but it is thought to induce translation of mRNAs that contain complex 5' untranslated regions with secondary structures and rely on eIF4A helicase activity (Koromilas et al. 1992; Hsieh and Ruggero 2010). It has been found to lead to increased expression of pro-survival molecules including Mcl-1 (Wendel et al. 2007; Hsieh et al. 2010). We found that S1P2 deficiency increased the level of phosphorylated 4E-BP1 and that S1P2 signaling suppressed protein synthesis in the GC-like cell line Ramos, suggesting that S1P2 regulates the mTORC1-eIF4E pathway of translation in GC B cells and that dysregulation of this pathway is a potential mechanism for the increased survival of S1P2-deficient GC B cells.

Mcl-1 is an attractive possibility for acting as a downstream mediator of increased Akt signaling. It has recently been shown to be highly expressed in GC B cells and to be critical for their survival (Vikstrom et al. 2010). In addition, Mcl-1 is independent of regulation by the pro-apoptotic molecule BAD, which we

found did not have an effect on GC B cell outgrowth. Multiple pathways downstream of Akt promote expression of Mcl-1, one example of which is the translational regulation by mTORC1 described above and that we found to be regulated by S1P2 signaling.

Akt phosphorylation of GSK3 isoforms has also been found to prevent GSK3-dependent targeting of Mcl-1 for degradation (Maurer et al. 2006). A third possibility for an Akt-dependent increase in Mcl-1 is due to Akt's phosphorylation of MDM2, inhibiting its ability to act as a negative regulator of p53 function. Upon recognition of DNA damage, p53 can be activated to upregulate the pro-apoptotic molecules Puma and Noxa (Manning et al. 2007). Noxa has been found to have a strong specificity for Mcl-1 (Chen et al. 2005). Because of the amount of genotoxic stress that GC B cells experience, it would be interesting to ask whether p53-dependent regulation of apoptosis by Noxa plays a role in the homeostasis of chronically-stimulated GCs. In initial experiments, we were not able to detect large differences in Mcl-1 amounts between populations of wild-type and S1P2-deficient GC B cells. However, this could be because Mcl-1 levels are only varying in a small proportion of cells at any given time, so that differences in bulk populations would be very small.

It is possible that contributions from multiple pathways downstream of Akt are contributing to the survival and outgrowth of S1P2-deficient GC B cells. It will be very interesting to learn more in future studies about how S1P levels within the tissue are controlled, how S1P2 signaling affects GC B cell survival, and how S1P2 regulation of Akt signaling is integrated with BCR regulation of Akt

signaling. Not only will this help us understand how the control of S1P levels within tissues regulates GC homeostasis, but it will also increase our understanding of how GC B cells maintain their delicate balance between proliferation, survival, and mutation on one side, and apoptosis and differentiation on the other.

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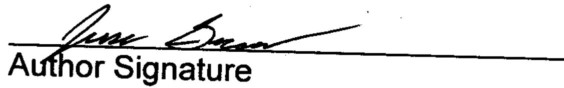

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