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EBI2 positions naïve and activated B cells

Author
Kelly, Lisa

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EBI2 positions naïve and activated B cells

by

Lisa M. Kelly

DISSERTATION

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This dissertation is dedicated to Chris Jocius,

for always encouraging exploration, appreciation,

and wonderment of the world.

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schedules, during years when they were serving on many thesis committees, to mentor me as well. It has been refreshing over the years to hear the different perspectives of two great immunologists on my own work, and I only wish I had had more thesis committee meetings, as they were always encouraging me to do. Their positive feedback was always a pleasant surprise when I felt things were not going as well as I had hoped. I also have to thank Art for allowing me to rotate in his lab, thereby introducing me both to in-depth signaling pathways, which I choose not to pursue during my graduate career, and downhill skiing, which I have followed up on more.

The Cyster lab has always been a supportive environment. I have enjoyed my interactions with everyone over the years, from scientific discussions and experimental advice to lab ski trips and wine tastings. As the rest of this document shows, my graduate career would have been very different without João Pereira, a former postdoc in the lab. Joao's enthusiasm for science, and just about anything else, was often contagious, as was his perseverance through trying times. João is a strikingly generous individual, and I feel lucky to have collaborated with him while in the lab. I am also indebted to Taka Okada, a former postdoc, for his superhuman patience and wisdom during our overlapping time in the lab. I have always been impressed by Taka's elegance in everything from science to skiing. I still miss Taka but look forward to meeting up with him again in SF, outside Tokyo, or anywhere else. Alex Bankovich was an enormous asset to the lab, and not only because he kept us stocked with Goldfish and coffee. Alex was, and still is, unfailingly eager to help out with absolutely anything.

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My gratitude to Christopher Allen for bearing with me through this, and always, is immeasurable.

Contributions to presented work

All work presented in this dissertation was performed under the direct supervision and guidance of Dr. Jason G. Cyster. Chapter 2 was published as Pereira JP, Kelly LM, Xu Y, Cyster JG. (2009). EBI2 mediates B cell segregation between the outer and centre follicle. Nature. 2009 Aug 27;460(7259):1122-6. Epub 2009 Jul 13. The manuscript is reproduced here in accordance with the policies of the Nature Publishing Group. The research in this paper was designed and conceptualized by Dr. Jason Cyster and Dr. João Pereira, and they initiated generation of the EBI2 reporter used in this paper. The reporter construct was generated by Dr. Ying Xu. João conducted most of the work in this paper, as described here, and he was involved in the design, execution, and analysis of every experiment described here. I began working on the project after he had made many key observations and discussed all experimental design and interpretation I performed with him. Extensive initial characterization of the EBI2 KO under naïve and immuno-responsive conditions was performed mainly by João but also by Jason (Supplementary Fig. 2 and unpublished). João made the observation that EBI2 mediated B cell segregation between the outer and center follicle while examining mixed bone marrow chimeras he generated (Fig. 3 and Supplementary Fig. 3, 4), which was a primary observation of the paper and one of the findings that brought me into the project. João generated the EBI2 reporter expression data in Fig 1a, 1c, and 1e. Ying performed the qRTPCR in Fig 1b. I

performed experiments investigating the role of T cell help in EBI2 function, generating Fig 1d and 2d. João performed all immunizations and transductions to demonstrate the role of EBI2 in B cell positioning (Fig 2b), in the humoral immune response (Fig 2d), and in GC development (Fig 4, Supplementary Fig 6). Following the guidance of Jason and João, I performed the lymphotoxin-blocking experiments generating Fig 3c. The paper was written by Jason and the data assembled by João, Jason, and me. Other individuals who contributed to this study are listed in the acknowledgements section of that chapter.

Chapter 3 was originally published in *The Journal of Immunology* as Kelly LM, Pereira JP, Yi T, Xu Y, Cyster JG. 2011. EBI2 guides serial movements of activated B cells and ligand activity is detectable in lymphoid and nonlymphoid tissues. J. Immunol. Copyright © [2011] The American Association of Immunologists, Inc. The research in this paper was designed and conceptualized by Jason, João, and me. Many of the initial observations in this paper were generated from João's work described in Chapter 2. João had made the observation that EBI2-deficient B cells do not localize properly before receiving T cell help, providing the observation that was the basis of Fig. 2C and the impetus for Fig. 3. He generated the data for Fig. 1B as well as Fig. 2C at the 10 hour timepoint. João did all the work on the ligand activity in Fig. 5., including the method of measuring EBI2 activity by chemotaxis to spleen extract used in Fig. 6. Dr. Tangsheng Yi provided important technical advice in generating Fig. 6. Ying performed all qRT-PCR in the paper (Fig. 2A, 4B). I received advice from Jen Bando in Rich Locksley's lab regarding isolated lymphoid follicle preparation to

generate Fig. 1A. The paper was written by Jason and me; I generated a first version of the paper and we collaborated extensively on further revisions. João and I assembled the data. Other individuals who contributed to this study are listed in the acknowledgements section of that chapter.

EBI2 positions naïve and activated B cells

By Lisa M. Kelly

Abstract

The immune system is organized to allow lymphocytes to survey for antigen and rapidly respond to an infection. B lymphocytes reside in B cell follicles and, upon exposure to antigen, rapidly migrate to interact with T cells, and then later go on to quickly produce protective antibodies and more slowly generate a memory response to prevent later reinfection. The role of chemokines and their receptors in positioning naïve and activated cells is well appreciated; CXCR5 is required for B cells to home to B cell follicles, and CCR7 is required for migration toward T cells. However, the positioning of B cells is not completely explained by these two chemokine receptors. During activation, B cells migrate after T cell interaction to the outer follicle, and some then migrate toward the center of the follicle to form germinal centers. The cues positioning cells to these areas have been poorly understood. Here we describe the role of the G protein coupled receptor EBI2 in positioning B cells, both homeostatically and upon activation. EBI2 promotes naïve B cell localization to the outer follicle, and EBI2 deficient cells are found toward the center of the follicle in competition. EBI2 expression is increased after B cell activation, directing activated cells to the outer follicle before CCR7 brings them toward T cells. After receiving CD40 stimulation from T cells, activated B cells migrate again to the outer follicle in an EBI2-dependent manner. EBI2 downregulation is required for B cells to move into the follicle and

for germinal center development. Some cells remain at the outer follicle and produce antibody. EBI2 is required for efficient antibody production in a T-dependent immune response. These data demonstrate that EBI2 acts in coordination with the chemokine receptors CXCR5 and CCR7 to position naïve and activated B cells and contributes to the immune response.

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Chapter 1

Introduction

Secondary lymphoid tissue organization

The immune system has evolved to protect the organism from pathogens and is organized throughout the body. Primary lymphoid organs support the development of hematopoietic cells, while secondary lymphoid organs (SLO), including spleen, lymph nodes (LN) and Peyer's patches (PP), are distributed throughout the body and collect and concentrate antigens (Ag), which can then be displayed to rare recirculating antigen-specific T and B cells. The circulatory system makes possible the transport of both cells and Ag throughout the body. Naive lymphocytes circulate throughout the body in blood and can enter SLO to survey for Ag.

Lymphocytes also egress from lymphoid tissues via the lymph, or, when exiting the spleen, via the blood. Lymph is collected into the lymphatic ducts and then funnels back into the blood, allowing for continued recirculation of cells. While large Ag requires cellular transport to reach SLO, small particulate or soluble Ag can drain from sites of infection or immunization to the lymph, which in turn drains to LN. Ag reaches the spleen via the blood (Cyster, 2010). The site of infection or immunization will determine the site of Ag drainage.

Ag access to SLO is carefully moderated, allowing processing and display of Ag to proceed in order to launch a productive immune response to the Ag. Ag can arrive at the SLO in a sinus between the outer capsule of the SLO and a layer of cells surrounding a B cell follicle. In the LN, Ag arrives in the subcapsular sinus via the lymph, while in the spleen, blood drains through the marginal sinus (Figure 1). In PP, specialized epithelial cells, termed M cells, transcytose Ag from

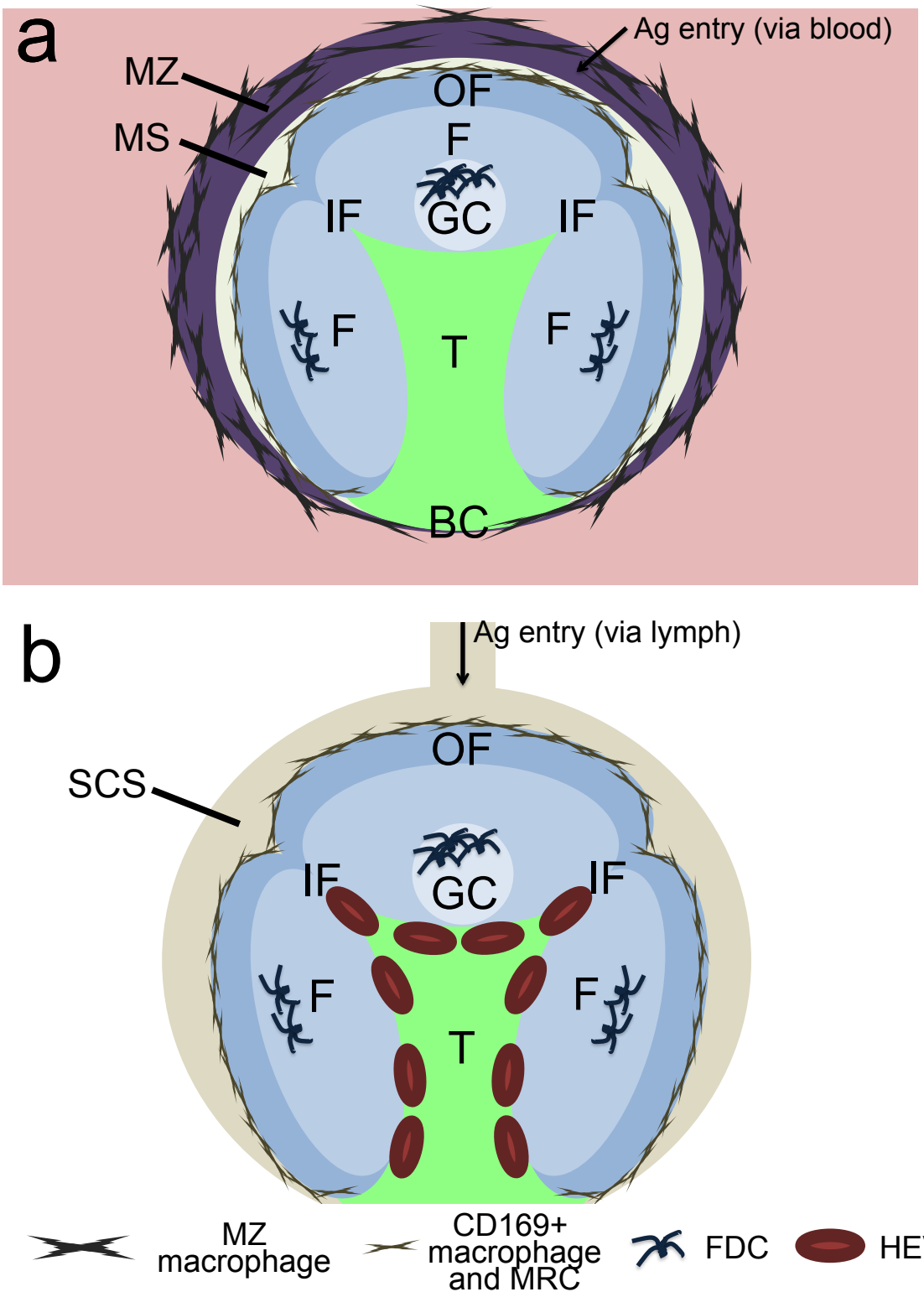

Figure 1: Schematic of splenic (a) and lymph node (b) architecture. See text for details. F, B cell follicle; OF, outer follicle (dark blue areas); IF, interfollicular areas; GC, germinal center; T, T cell zone; MS, marginal sinus; MZ, marginal zone; MRC, marginal reticular cell; BC, bridging channel; SCS, subcapsular sinus; FDC, follicular dendritic cell; HEV, high endothelial venule; Ag, antigen.

the gut lumen, to the subepithelial dome containing dendritic cells (DC) above the B cell follicle. While sufficiently small Ag can diffuse into the follicle, larger Ag must be actively transported across a layer of CD169+ macrophages. In the LN, CD169+ macrophages extend arms into the lymphatic sinus, allowing collection of Ag, which is translocated into the follicle (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007). In the absence of these macrophages, infection is not contained to the LN and may become systemic (Junt et al., 2007). In the spleen, the marginal sinus is lined by MadCAM1+ cells. The marginal zone contains SIGNR1+ MARCO+ marginal zone macrophages, while the white pulp contains CD169+ marginal zone metallophilic macrophages near the marginal sinus (Mebius and Kraal, 2005). Another cell type located among the macrophages is the marginal reticular cell (MRC), a stromal cell type found in SLO including the spleen, LN, and mucosal sites such as PP, isolated lymphoid follicles (ILF), and nasal-associated lymphoid tissue (Figure 1). MRCs are most easily distinguished by their staining for TRANCE (Katakai et al., 2008).

B cell follicle organization

Beneath the CD169+ macrophages and MRC in SLO is the B cell follicle. The B cell follicle is established via an interplay between stromal cells and lymphocytes. Stromal cells, including follicular dendritic cells (FDC), produce the chemokine CXCL13, which is recognized by cells expressing CXCR5, such as B cells and lymphoid tissue inducing cells (LTIC). These cells are recruited to nascent follicles and express lymphotoxin on their surface, which is recognized

by LTbR on FDC, leading to their maturation and further expression of CXCL13. This generates a positive feedback loop between CXCL13-expressing FDC and lymphotoxin-expressing B cells and LTIC, establishing B cell follicles in SLO (Ansel et al., 2000; Cyster, 2005; Ngo et al., 1999). A primary follicle consists of FDC centered in an area of naive B cells, in addition to a network of other stromal cells.

The B cell follicle itself is organized into different areas, the structure and function of which are still under investigation. The outer follicle (OF) is the area of the follicle closest to sites of Ag drainage; in spleen, the area of follicle closest to blood, in LN, the area closest to subcapsular sinus, and in PP, the area closest to gut epithelium. As multiple follicles exist within each SLO, there are areas where follicles are adjacent to each other, termed “interfollicular” (IF) areas (Figure 1). IF zones tend to be enriched for DEC205- CD11c+ cells in the spleen and LN (Steinman et al., 1997). In addition to containing selectively enriched hematopoetic cells, the IF zone is also stromally distinct from the follicle. While the follicle contains sparse reticular fibers, the IF zone has a higher density of reticular fibers, as stained with ER-TR7. The IF zone is the starting point of a “cortical ridge” of reticular fibers that extends around the B cell follicle, at the interface between the B and T cell zones. However, the cortical ridge is not observed in the spleen (Katakai et al., 2004). In the spleen, follicles can also be separated by bridging channels, formed where the T cell zone meets the red pulp (Figure 1). This area is often observed as a gap in the ring of CD169+ macrophages surrounding the white pulp and may have a higher concentration of

CD11c+ DEC205- DCs (Steinman et al., 1997). The IF zone may also contain a greater concentration of MRCs, which express CXCL13 and may thereby help recruit B cells to this area, although other cues may also bring cells to this area, as CXCR5-deficient cells localize to IF zones (Ansel et al., 2000; Katakai et al., 2008; Roozendaal and Mebius, 2011). This area has been proposed to be important for Ag capture and retention (Mueller and Germain, 2009). High endothelial venules (HEV) tend to be located in the LN between the B cell follicle and T cell zone and in IF areas, bringing newly arriving cells, possibly with Ag, to these areas. While subcutaneously injected, labeled Ag was observed in the subcapsular sinus 4 hrs after immunization, by 18 hr, it was enriched at IF zones of the draining LN (Ingulli et al., 2002).

The center of the B cell follicle contains FDC, radiation-resistant stromal cells that are important both for follicular development and the immune response. In a primary follicle, present in the absence of an immune response, FDC express the complement receptor CD35, allowing the binding of complement coated, opsonized Ag. During an immune response, a secondary follicle develops as a germinal center (GC) forms and the FDC mature, upregulating expression of the integrin ligand VCAM-1 and the Fc receptor FcγrIIb. FDC also express the stromal markers MadCAM-1, gp38 and BP-3 (Allen and Cyster, 2008). The germinal center has been recognized as a histologically distinct compartment of the follicle since its observation in 1884. Recent imaging of the GC has shown that B cells move dynamically within this structure (Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007).

Chemokines and lymphoid architecture

The organization of B cell follicles is mediated by chemokines and their receptors. CXCR5, expressed by B cells, recognizes the chemokine CXCL13 found throughout the follicle and made by stromal cells including FDC and MRC, as well as by hematopoietic cells (Ansel et al., 2000; Katakai et al., 2008). In the absence of either CXCL13 or CXCR5, neither splenic primary follicles nor peripheral LN form. In the spleen, B cells form rings around the T cell zone and the marginal zone is thicker than in WT (Ansel et al., 2000; Forster et al., 1996). However, B cells still do segregate in discrete areas from T cells, demonstrating that there is still organization of B cells in the absence of CXCL13/CXCR5. Naïve B cells express additional chemokine receptors including, at low levels, CCR7, which recognizes the chemokines CCL19 and CCL21. While these chemokines are found at high levels in the T cell zone, CCL21 extends into the B cell follicle adjacent to the T cell zone (Okada et al., 2005). CCR7 plays a role in positioning of naïve B cells, as CCR7-deficient B cells localize toward the T cell-distal area of the B cell follicle upon transfer (Reif et al., 2002). CCL19 and 21 are made by the T zone stromal cells, also known as fibroblastic reticular cells (FRCs). In addition, CCL19 is made by DC, and CCL21 by HEV. The mutant mouse strain plt lacks CCL19 and CCL21 in lymphoid tissues, resulting in decreased entry of T cells to LN via HEV and DC from lymphatics (Luther et al., 2000). Additionally, lymphoid organization is perturbed in plt mice, with smaller periarterial lymphoid sheaths (PALS) in the spleen, with few T cells residing in this area despite an increase in

T cell numbers in the spleen. In the LN, following immunization, T cells and DEC205+ DC can be observed prominently in IF zones (Mori et al., 2001). The CCR7 KO has similar perturbed architecture with T cells found in both the red and white pulp. Increased numbers of T cells are found in the spleen and blood, while numbers of T cells and DC are decreased in LN due to impaired homing, similar to the plt phenotype (Muller et al., 2003). CXCR5/CCR7 dKO have even more severely perturbed architecture, although B still cluster in the white pulp, and some T cells can be seen in the PALS. Interestingly, ER-TR7 is broadly distributed throughout the white pulp in these mice (Muller et al., 2003; Ohl et al., 2003b).

CCR7 was originally identified as EBI1 in a screen for genes upregulated by Epstein Barr virus infection in Burkitt's lymphoma cells in vitro (Birkenbach et al., 1993). The gene most highly induced in the study was identified as EBI2. Both genes were predicted to be GPCRs and have been confirmed as such. EBI2 shares homology with lipid and purine receptors (Rosenkilde et al., 2006). EBI2 was known to be highly expressed in naive B cells with increased expression in activated B cells, while GC B cells have decreased expression of EBI2 (Glynne et al., 2000; Shaffer et al., 2000). EBI2 has also been reported to be constitutively active (Rosenkilde et al., 2006). However, the role of EBI2 in B cell localization has been unclear. Interestingly, EBV-infected cells in human tonsils tend to localize to extrafollicular areas, outside the follicle or GC (Niedobitek et al., 1992).

The aforementioned receptors are important for homeostatic localization of lymphocytes. They also play important roles in relocalizing lymphocytes during an immune response, allowing cognate B and T cells to interact and generate a productive immune response.

Ag transport and recognition by B cells

B cells can recognize cognate antigen in SLO in different ways and locations. The Ag may diffuse into the LN follicle if it is small enough. This appears to occur within seconds to minutes after Ag administration, and within hours this Ag is no longer found in the follicles and instead is predominantly in the medullary sinus (Pape et al., 2007). Small Ag is not observed to freely diffuse into the T zone. Instead, a conduit system has been shown to transport Ag from the subcapsular sinus to DC. The conduits have a collagen core surrounded by FRC (Gretz et al., 1997; Gretz et al., 2000). These conduits are less dense in the B cell follicle, but a similar network has been suggested to distribute small Ag into this compartment (Bajenoff and Germain, 2009; Gonzalez et al., 2011; Roozendaal et al., 2009). While follicular conduits have been most extensively investigated in the LN, there is also evidence they exist and transport small Ag in the spleen (Nolte et al., 2003).

Larger, and/or opsonized, Ag is unable to reach the follicle by diffusion and instead must be actively transported to the LN follicle. As mentioned above, CD169+ subcapsular sinus macrophages may actively transport larger, opsonized Ag and virus, bringing Ag from the subcapsular sinus to the B cell

follicle, as has been recently imaged. Branches of the CD169+ macrophages extend membrane-attached Ag into the follicle (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007). Immune complexes containing Ag can be recognized by either cognate B cells, via the B cell receptor (BCR), or transported by non-cognate B cells via the complement receptor CR1/2 (Phan et al., 2007). These non-cognate B cells can bring opsonized Ag into the follicle to be deposited on FDC, due to the higher expression of complement receptors (Carroll, 1998). FDC in turn serve as a repository for opsonized Ag, as has been long observed, providing another site for B cell Ag encounter (Nossal et al., 1968; Nossal et al., 1964). FDC retain opsonized Ag longer than subcapsular sinus macrophages, providing a more enduring source of Ag for B cell encounter (Suzuki et al., 2009).

Finally, B cells can recognize unprocessed Ag presented on DCs near HEV present around the LN follicle and at IF zones. This may be relevant particularly for newly-arriving B cells which enter the LN from the blood via HEV (Qi et al., 2006). DC have been shown to retain some of the Ag they capture in unprocessed forms, allowing for recognition by B cells (Bergtold et al., 2005).

Ag can be transported to follicular B cells in the spleen by marginal zone (MZ) B cells (Cinamon et al., 2008). MZ B cells have high expression of CD21/35 and CD1d, allowing for capture of non-cognate Ag, and shuttle between the MZ and the follicle. Ag is cleared off MZ B cells within hours of exposure, provided that this shuttling is intact, and can be detected on FDC.

B cell activation

Once B cells recognize their cognate Ag, a signaling cascade is activated which will result in many changes within the cell, including migration. Activated B cells upregulate CCR7, promoting migration to the B:T boundary within hours, and interaction there with cognate T cells (Okada et al., 2005; Reif et al., 2002). Notably, CCR7 surface expression on activated B cells is lower than that of naive T cells, and it has been proposed that this, along with continued expression of CXCR5, prevents the activated B cell from migrating deep into the T zone (Okada and Cyster, 2006). Furthermore, activated B cells that lack CXCR5 relocalize to IF areas near the T zone, rather than spreading along the B:T boundary, and activated B cells that cannot respond to CCR7 signals (due to deficiency in the receptor or in the ligands) relocalize to OF and IF zones rather than to the B:T boundary (Reif et al., 2002). The signal directing these activated B cells to these locations has been unknown. Ablation of CD169+ macrophages leads to decreased ability of activated B cells to home to the IF zone, and much greater Ag dose, or more time, is required to position them at the IF zone. In the absence of subcapsular sinus macrophages, B cells may have decreased exposure to Ag, as measured by downregulation of surface BCR (Junt et al., 2007).

During the interaction at the B:T boundary, both B and T cells will produce and receive important signals. One of the most important signals an activated B cell receives is stimulation through CD40 via CD40L expressed on activated T cells, which is upregulated following their recognition of cognate Ag on DC. The

interaction between CD40/CD40L is crucial for GC development, affinity maturation, and isotype switching (Foy et al., 1996). When CD40 signaling is blocked for the duration of the immune response by repeated treatment with anti-CD40L, activated B cells do not expand (Garside et al., 1998). Humans with defective CD40/CD40L interactions have hyper-IgM syndrome, characterized by an excess production of IgM and inability to isotype switch. It has been suggested that increased CD40 signaling, from agonistic Ab, in activated B cells in a T-dependent response limits GC development but increases antibody secreting cell (ASC) development (Erickson et al., 2002). Additionally, B cells with constitutively active CD40 do not develop GC in response to a T-dependent immunization (Homig-Holzel et al., 2008). Both of these systems saw decreased GC formation relatively late in the response (d7-14). However, other systems with increased CD40 signaling in B cells have shown that while the early response is skewed toward ASC, GC development is initially normal, but is not sustained later in the response (Bolduc et al., 2010; Kishi et al., 2010). As B and T cells interact for long periods of time during the immune response, it is not clear at what point CD40L signaling is important or needs to be maintained, or what early signals CD40L delivers to activated B cells beyond survival.

Within the first days after recognizing cognate Ag, B cells begin to downregulate CCR7 and move toward the IF and OF (Chan et al., 2009; Coffey et al., 2009). This can be seen 1-2 days in the spleen and LN after immunization in BCR tg or knockin systems (Cyster and Goodnow, 1995; Garside et al., 1998; Pape et al., 2003). While the localization to these areas has been observed in

different systems, its role in the B cell response has not been well appreciated. Activated B cells proliferate in the OF during d3-4, as determined by Ki-67 staining, leading to an expansion of the cells in this OF location (Coffey et al., 2009).

B cell effector fates

From this point and subsequently, two B cell fates can arise: the GC B cell or the ASC. These fates are controlled by opposing programs orchestrated by the transcription factors bcl6 and blimp1. Bcl6 is highly expressed in GC B cells. In the absence of bcl6, germinal centers do not form, while ASC can still be generated and in some cases have increased development and Ab production (Dent et al., 1997; Tunyaplin et al., 2004; Ye et al., 1997). Blimp1 is expressed in ASC. B cell deficiency in blimp1 leads to severely decreased development of ASC and antibody response, with enlarged GCs (Shapiro-Shelef et al., 2003). Induction of blimp1 induces a decrease in proliferative capacity and increase in secretory capabilities, in addition to blocking GC development (Shaffer et al., 2002). Notably, blimp1's inhibition of proliferation is dose-dependent, as B cells expressing lower levels of blimp1 still are able to proliferate, as observed by BrdU incorporation. This suggests that blimp1 intermediate cells correspond to the short-lived ASC, while blimp1 high cells are long-lived ASC (Kallies et al., 2004). Bcl6 and blimp1 interact to repress each other (Shaffer et al., 2000; Tunyaplin et al., 2004). The cues that lead to the regulation of the blimp1 and bcl6 balance, and fate decision between GC and ASC, have been unclear.

Factors that may influence GC and ASC fate include BCR/Ag affinity and signals from T cells. Alternatively, the decision could be stochastic (Hasbold et al., 2004). Low affinity BCR are able to develop into both GC and ASC, so affinity does not automatically exclude cells from either compartment (Dal Porto et al., 1998; Shih et al., 2002). However, affinity can affect the likelihood of development into ASC or GC. In addition to the affinity of the Ag and BCR, the density of the Ag affects the avidity for the BCR, and may change its signaling properties. Experimentally reducing Ag density or BCR affinity leads to a reduction in contribution to the ASC, but not GC, compartment. In competition, high affinity B cells become ASC, while lower affinity B cells can enter the GC to undergo affinity maturation and generate increased affinity there (O'Connor et al., 2006; Paus et al., 2006). Lower affinity Ag leads to less expansion and survival of early ASC, despite normal generation of these cells (Chan et al., 2009). The differentiation is not over once cells enter the GC, as some cells will exit from the structure as ASC and some will remain as GC cells. Without competition, varying BCR affinities can contribute to a GC response, including low affinity BCR; however, low affinity BCR will lead to greater B cell death (Anderson et al., 2009). In competition, higher affinity BCR B cells dominate. This is due to increased proliferation of the high affinity cells prior to GC development, as they present more peptide:MHC to cognate T cells and preferentially receive limiting T cell help (Schwickert et al., 2011).

Activated B cell positioning

Much work regarding the ASC/GC decision has focused on strength of signal through the BCR, but positioning of activated cells may play a role. It has recently been proposed that in LN, activated B cells at the IF zone express bcl6 starting at d2 of the immune response, and by d4 these cells have moved toward the follicle, seeding the GC. Another population of activated B cells, residing at the OF, did not express bcl6 and instead may represent early PB development (Kerfoot et al., 2011). It is unclear both how these cells localize and if these cells develop different fates because of their localization, or if an earlier difference has directed them to these locations.

ASC downregulate CCR7 and CXCR5 and upregulate CXCR4. This chemokine receptor profile allows ASC to migrate to extrafollicular areas (the red pulp in the spleen and medullary cords in LN) (Chan et al., 2009; Hargreaves et al., 2001). When ASC lack CXCR4, they are unable to move as far into the red pulp, but instead reside close to the edge of the OF, near the MZ (Hargreaves et al., 2001). This suggests that ASC may remain attracted to follicular areas in the absence of CXCR4. The first wave of ASC are short-lived and rapidly secrete low affinity Ab, providing an initial means of defense while the immune response continues to evolve.

Other B cells will migrate from the outer- and inter- follicular areas toward the FDC at the center of the follicle, leading to the development of a GC. S1P2 has recently been shown to have a role in this process, as its expression is increased on GC cells relative to FB cells. It is hypothesized that a gradient of S1P exists in the B cell follicle, with the highest concentration at the edge of the

follicle, and the lowest at the center. S1P2 inhibits migration to CXCL13 and other chemoattractants, thereby promoting confinement of GC cells to the GC (Green et al., 2011). In the GC, B cells undergo affinity maturation and somatic hypermutation over days and weeks, leading to the development of long lived memory cells and ASCs that can produce high affinity Ab.

Isotype switching occurs in both ASC and GC cells. The earliest isotype-switched cells can be observed 2-3 d after immunization, in the B cell follicles, and may be enriched near IF or bridging zones. By d4, isotype switched cells are observed both extrafollicularly, in the red pulp near the MZ and bridging zones in spleen, and toward the center of the follicle, in the nascent GC (Pape et al., 2003). While the location where switching events occur has been tracked, it is not known how localization within lymphoid tissues may affect switching. It is possible that exposure to different cell types and cytokine milieus in microenvironments within lymphoid tissue may affect the B cell response.

While much insight about naïve and activated B cell localization has been gained, clearly many issues remain to be addressed. EBI2 is highly expressed on naïve and activated B cells, yet its role in positioning B cells has been unknown. CXCR5 and CCR7 have been shown to contribute to early activated B cell positioning, but movement of activated B cells to the OF by d2 of the immune response was not fully explained by these two receptors, nor was the migration toward the center of the follicle by GC B cells. Here we have examined the role of EBI2 in naïve and activated B cell localization and demonstrate its importance in the humoral immune response.

Chapter 2

EBI2 mediates B cell segregation between the outer and center follicle

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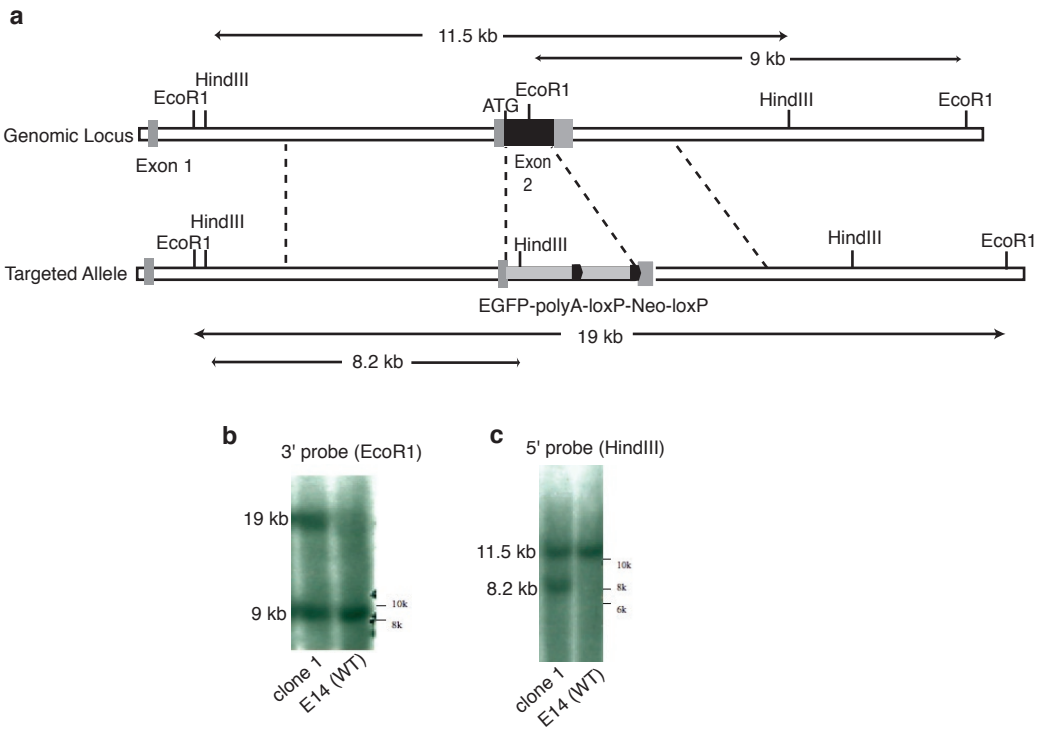
Abstract

B cell follicles are specialized microenvironments that support events necessary for humoral immunity (Allen and Cyster, 2008; Kelsoe, 1996; MacLennan, 1994). After antigen encounter, activated B cells initially seek T-cell help at the follicle–T-zone boundary and then move to IF and T-zone distal (outer) regions of the follicle (Coffey et al., 2009; Cyster and Goodnow, 1995; Garside et al., 1998; Jacob et al., 1991; Liu et al., 1988; Pape et al., 2003; Reif et al., 2002). Subsequently, some cells move to the follicle center, become GC B cells and undergo antibody affinity maturation (Allen et al., 2004; Brink et al., 2008; Kelsoe, 1996; MacLennan, 1994). Although GC within follicles were described in 1885 (Nieuwenhuis and Opstelten, 1984) the molecular cues mediating segregation of B cells between the outer and center follicle have remained undefined. Here we present a role for the orphan G-protein-coupled receptor, Epstein-Barr virus induced molecule-2 (EBI2, also known as GPR183) (Birkenbach et al., 1993), in this process. EBI2 is expressed in mature B cells and increases in expression early after activation, before being downregulated in GC B cells. EBI2 deficiency in mice led to a reduction in the early antibody response to a T-dependent antigen. EBI2-deficient B cells failed to move to the outer follicle at day 2 of activation, and instead were found in the follicle center, whereas EBI2 overexpression was sufficient to promote B cell localization to the outer follicle. In mixed bone marrow chimeras, EBI2-deficient B cells phenocopied GC B cells in preferentially localizing to the follicle center. When downregulation of EBI2 in wild-type B cells was antagonized, participation in the GC reaction was impaired.

These studies identify an important role for EBI2 in promoting B cell localization in the outer follicle, and show that differential expression of this receptor helps position B cells appropriately for mounting T-dependent antibody responses.

The propensity of B cells to migrate to the outer versus center follicle at different stages of the antibody response, together with the established roles of G-protein-coupled receptors (GPCRs) in controlling lymphocyte positioning events, led us to address whether new GPCRs differentially expressed between early activated and GC B cells may be involved in this subcompartmentalization. These criteria focused our attention on EBI2, a $G\alpha_i$-coupled orphan receptor (Birkenbach et al., 1993; Rosenkilde et al., 2006) abundantly expressed in Epstein-Barr virus (EBV)-infected and activated human B cells, and downregulated in GC B cells (Cahir-McFarland et al., 2004; Shaffer et al., 2001). To explore the expression pattern of mouse Ebi2, we generated a reporter mouse line carrying the enhanced green fluorescent protein (EGFP) gene inserted in place of the Ebi2 open reading frame (Supplementary Fig. 1). Analysis of $Ebi2^{GFP/+}$mice showed that EBI2 is upregulated during B cell maturation in the bone marrow, and is expressed in mature recirculating B cells in bone marrow, spleen and lymph nodes (Fig. 1a). The expression of the GFP reporter tracked closely with changes in Ebi2 transcript abundance (Fig. 1b). GFP levels were further upregulated after B-cell receptor (BCR) engagement with anti-IgM, or combined anti-IgM and anti-CD40 stimulation (Fig. 1c). To examine expression after receipt of T-cell help, we used an adoptive transfer system in which B cells from C57BL/6 (B6) $Ebi2^{GFP/+}$ mice were transferred to the coisogenic strain, bm12, that bears a three amino acid difference in the $I–A^b$ major histocompatibility complex (MHC) class II molecule and contains a high frequency of $I–A^b$ responsive helper T cells (Cyster and Goodnow, 1995; Mengle-Gaw et al., 1984). This approach permits



Supplementary Figure 1.Generation of Ebi2$^{GFP/+}$ mice.(a) Schematic of EBI2 genomic locus before and after gene targeting. The entire open reading frame (black box) is encoded in exon 2. The EGFP gene is inserted in place of the EBI2 open reading frame. Gray boxes in genomic locus indicate untranslated regions. Dashed lines indicate the approximate beginning and end of the 5' and 3' homology arms. Restriction enzymes sites used for Southern blot analysis, and size of predicted fragments are indicated. (b) Southern blot analysis of EcoR1-digested genomic DNA from a targeted ES cell clone and frm control E13 cells, hybridized with a probe external to the 3' homology arm. (c) Southern blot analysis of HindIII-digested genomic DNA from a targeted ES cell clone and from control E14 cells, hybridized with a probe external to the 5' homology arm.

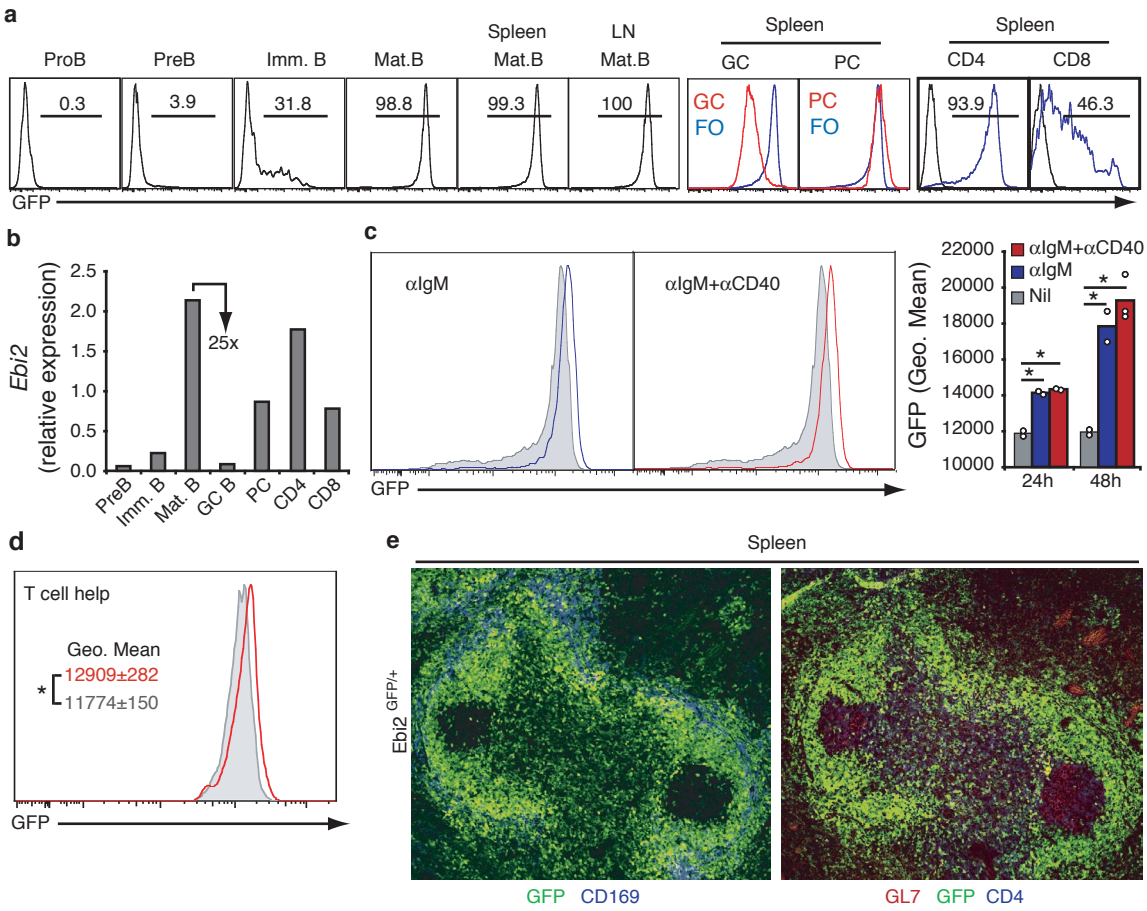
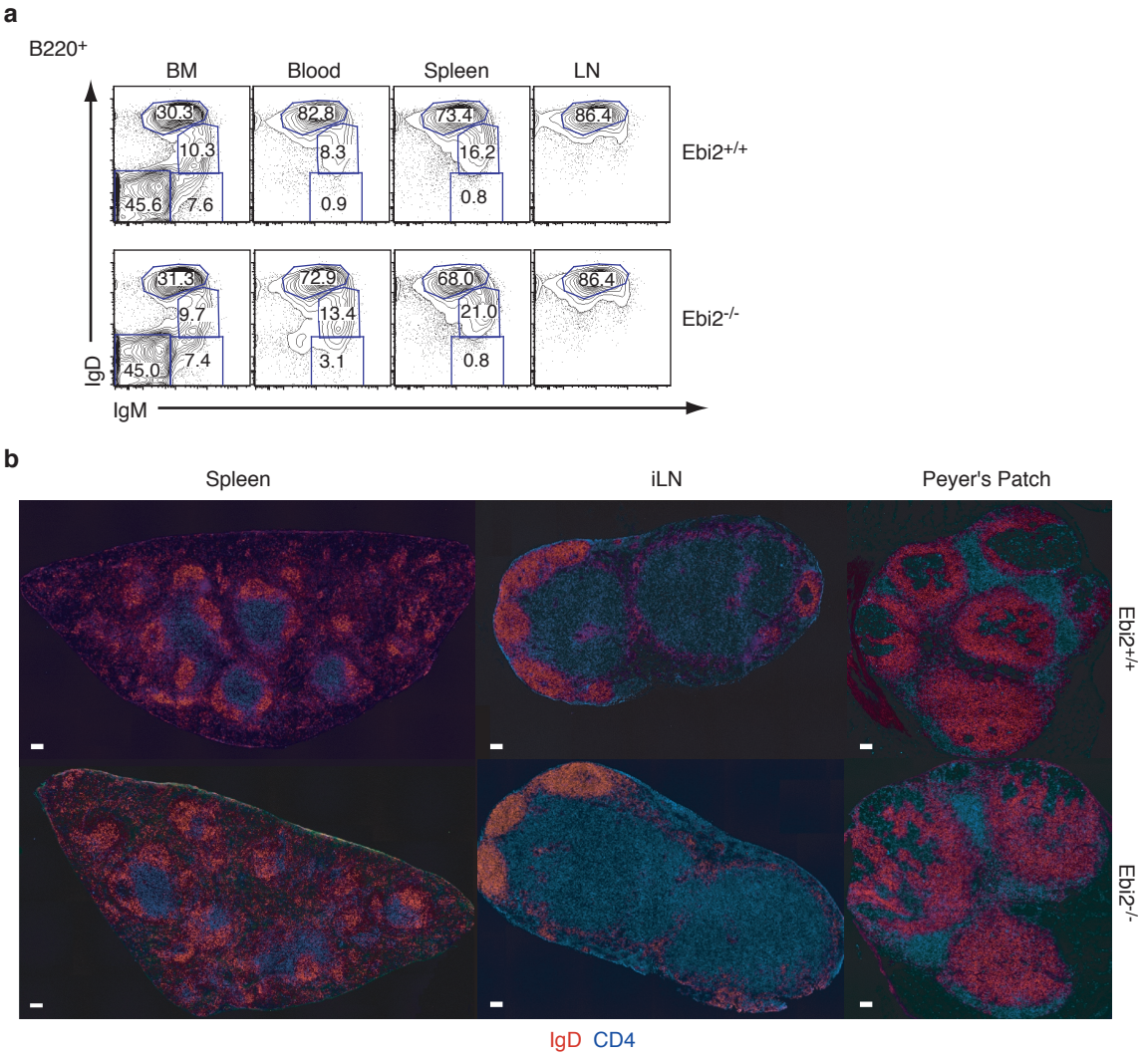


Figure 1: EBI2 upregulation in activated B cells and downregulation in GC. **a**, Flow cytometric detection of GFP fluorescence in the indicated bone marrow, spleen and lymph node (LN) cell subsets from Ebi2$^{GFP/+}$ mice. Numbers indicate the percentage of cells in the gate. FO, follicular B cells; GC, germinal center; Imm, immature; Mat, mature; PC, plasma cells. **b**, Quantitative PCR analysis of Ebi2 transcript abundance in the indicated cell populations. Expression is shown relative to Hprt1. **c**, **d**, Flow cytometric detection of GFP fluorescence in B cells stimulated for 1 day with anti-IgM or with anti-IgM and anti-CD40 (c), or that were stimulated for 1 h with anti-IgM and exposed in vivo for 2 days to T-cell help (red) or not provided with T-cell help (grey) (d). Grey histograms in c indicate unstimulated cells. The bar graph in c shows the geometric mean GFP fluorescence for 1 and 2 day cultures and summarizes three experiments. *P<0.05, unpaired, two-tailed Student's t-test. **e**, Immunofluorescence microscopy of fixed spleen tissue from an Ebi2$^{GFP/+}$ mouse, stained to detect GFP^{+} cells (green) and CD169^{+} marginal zone macrophages (blue, left panel) or GL7^{+} GC B cells and CD4 T cells (red and blue, respectively, right panel). Scale bar, 100 μm.

tracking of an activated B cell population in non-transgenic mice at early time points after receiving T-cell help. Two days after adoptive transfer of anti-IgM-stimulated B cells, we found higher GFP expression in B cells transferred to bm12 compared to B6 recipients, indicating that interaction with helper T cells promoted EBI2 upregulation (Fig. 1d). EBI2 expression was maintained in plasma cells but was markedly (~25-fold) downregulated in GC B cells (Fig. 1a, b). Most CD4 T cells and a smaller fraction of CD8 T cells also expressed EBI2, although at lower levels than B cells (Fig. 1a, b). In sections, GFP was detectable throughout the follicle and T zone, but was almost undetectable within GC, identified by their expression of GL7 (also known as LY77), making these structures appear as EBI2-deficient islands in a 'sea' of EBI2-expressing cells (Fig. 1e).

An initial analysis of lymphoid tissues from EBI2-deficient mice showed the presence of organized follicles and T-cell compartments, and the mice had normal numbers of B and T cells (Supplementary Fig. 2 and data not shown). Movement of activated B cells to the follicle–T-zone boundary within 6 h of BCR stimulation occurred similarly for EBI2-deficient and wild-type cells (Fig. 2a), suggesting that BCR-mediated EBI2 induction (Fig. 1) is not required for this CCR7-dependent relocalization event (Reif et al., 2002). We then asked whether the next stage(s) of activated B cell migration that occurs during T-dependent responses, movement to the OF and IF regions, were EBI2-dependent. To permit *in situ* tracking of activated B cells responding to T-cell help, we used the bm12 adoptive transfer approach introduced earlier. Littermate control B cells



Supplementary Figure 2. B cell development and B and T cell distribution in EBI2-/- mice. (a) Flow cytometric analysis of B cell subsets in bone marrow, blood, spleen and lymph nodes of adult EBI2+/+ and EBI2-/- mice. Plots show expression of surface IgM and IgD gated B220+ cells from indicated lymphoid organs. Numbers represent frequency of gated populations. (b) Distribution of B and T cells in the spleen, inguinal lymph nodes (iLN) and Peyer's patch of Ebi2+/+ and Ebi-/- mice. Cryosections were stained with anti-IgD (red) and with anti-CD4 (blue). Scale bar (white line) is 100 μm.

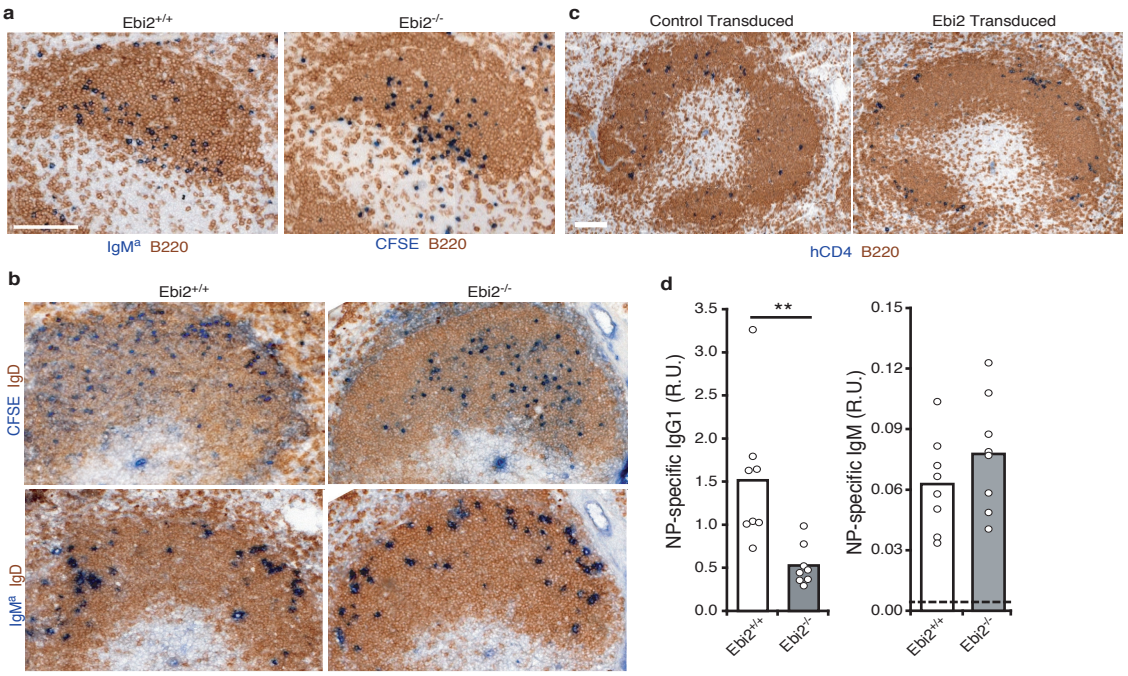


Figure 2: EBI2 promotes localization of activated B cells in the outer follicle. a-c, Immunohistochemical staining of spleen cryosections. **a**, Distribution of wild-type and EBI2-deficient B cells that had been stimulated with anti-IgM in vitro for 1 h, and analysed 6 h after being transferred to wild-type hosts. Ebi2$^{-/-}$ B cells were CFSE-labelled before transfer. Sections were stained with an antibody to detect CFSE (Ebi2$^{-/-}$) or co-transferred wild-type Igh^{a} (IgM^{a}, Ebi2$^{+/+}$) B cells (blue) and endogenous B cells (IgD, brown). **b**, Distribution of anti-IgM-treated wild-type and EBI2-deficient B cells (CFSE, blue), and internal control Igh^{a} B cells (IgM^{a}, blue), after 2 days exposure to T-cell help in bm12 hosts. Top and bottom panels are serial sections. Endogenous B cells were detected with anti-IgD (brown). **c**, Distribution of B cells transduced with control or Ebi2-expressing retrovirus (human CD4, blue), 1 day after transfer. Endogenous B cells were detected with anti-IgD (brown). Scale bars, 100 μm. **d**, Anti-nitrophenyl (NP) IgG1 and IgM serum titres in wild-type and EBI2-deficient mice on day 7 after immunization with NP-CGG in alum. RU, relative units. **P < 0.005, unpaired, two-tailed Student's t-test.

that had received anti-IgM stimulation were able to respond to T-cell help within bm12 recipients and relocalized to the outer follicle at day 2 (Fig. 2b), as did cotransferred wild-type Igh^a B cells (Fig. 2b), consistent with earlier studies using immunoglobulin-transgenic B cells (Cyster and Goodnow, 1995). In contrast, EBI2-deficient B cells were unable to localize to this region and instead favoured the central area of the follicle (Fig. 2b). To determine whether upregulation of EBI2 could be sufficient to control B cell localization to the IF and OF regions within lymphoid tissues, we transduced B cells with *Ebi2*-encoding or control retroviruses and transferred them to wild-type recipients. One day later, the EBI2-overexpressing cells, identified by expression of a human CD4 reporter, were situated in IF regions and in the outer follicle (Fig. 2c). This contrasted with the distribution of B cells transduced with the control retrovirus, where the cells distributed uniformly within the follicle (Fig. 2c). Thus EBI2 seems to be both necessary and sufficient to promote positioning of activated B cells in the OF and IF regions. Consistent with an essential role for EBI2 in the correct positioning of B cells during the early phases of T-dependent humoral responses, EBI2-deficient mice mounted a reduced day 7 IgG1 antibody response to nitrophenyl-chicken gamma-globulin (NP-CGG) in alum (Fig. 2d). The IgM response was not affected (Fig. 2d).

As another approach to examine the role of EBI2 in determining B cell localization, we examined cell distribution in 20:80 mixed bone marrow chimaeras that contained a minority of EBI2-deficient cells (20% Igh^b EBI2-deficient or littermate control and 80% Igh^a wild-type). Notably, EBI2-deficient B

cells selectively localized in a GC-like location at the center of follicles in spleen, LN and PP of the bone marrow chimeras (Fig. 3a). These foci of cells were not GC as they maintained high expression of IgD and lacked expression of GL7 (Fig. 3a and Supplementary Fig. 3). In contrast, EBI2-deficient and wild-type T cells appeared intermingled throughout the T zone (Supplementary Fig. 4). Analysis of 90:10 mixed bone marrow chimeras containing mostly EBI2-deficient (or littermate control) cells showed that EBI2-deficient B cells colocalized with the $CD35^+$ (also known as $CR1^+$) FDC network at the center of follicles, whereas the minor population of wild-type B cells in these mice was partially excluded from this area and enriched in IF regions or OF (Fig. 3b). Similar findings were made in LN and PP (Supplementary Fig. 5). It seems possible that in mice in which most B cells lack EBI2, there is increased EBI2 ligand availability and wild-type B cells predominate at the sites of ligand production. In control mixed bone marrow chimeras reconstituted with a minority (20:80) or majority (90:10) of *Ebi2*$^{+/+}$ bone marrow, the two types of wild-type B cells were intermingled in both the follicle center and periphery (Fig. 3a, b and Supplementary Figs 3 and 5). FDCs are dependent on lymphotoxin (LT) LTα1β2 for their maintenance (Fu and Chaplin, 1999). To test whether the segregation of wild-type and EBI2-deficient B cells was dependent on FDCs, we treated mixed bone marrow chimeras with LTβR-immunoglobulin, an LTα1β2 antagonist (Mackay et al., 1997), for 2–3 weeks. The $CD35^+$ FDC networks were depleted after treatment (Fig. 3c) as expected (Mackay et al., 1997; Ngo et al., 1999). Under these conditions, wild-type and EBI2-deficient cells no longer showed a segregated distribution in splenic B cell

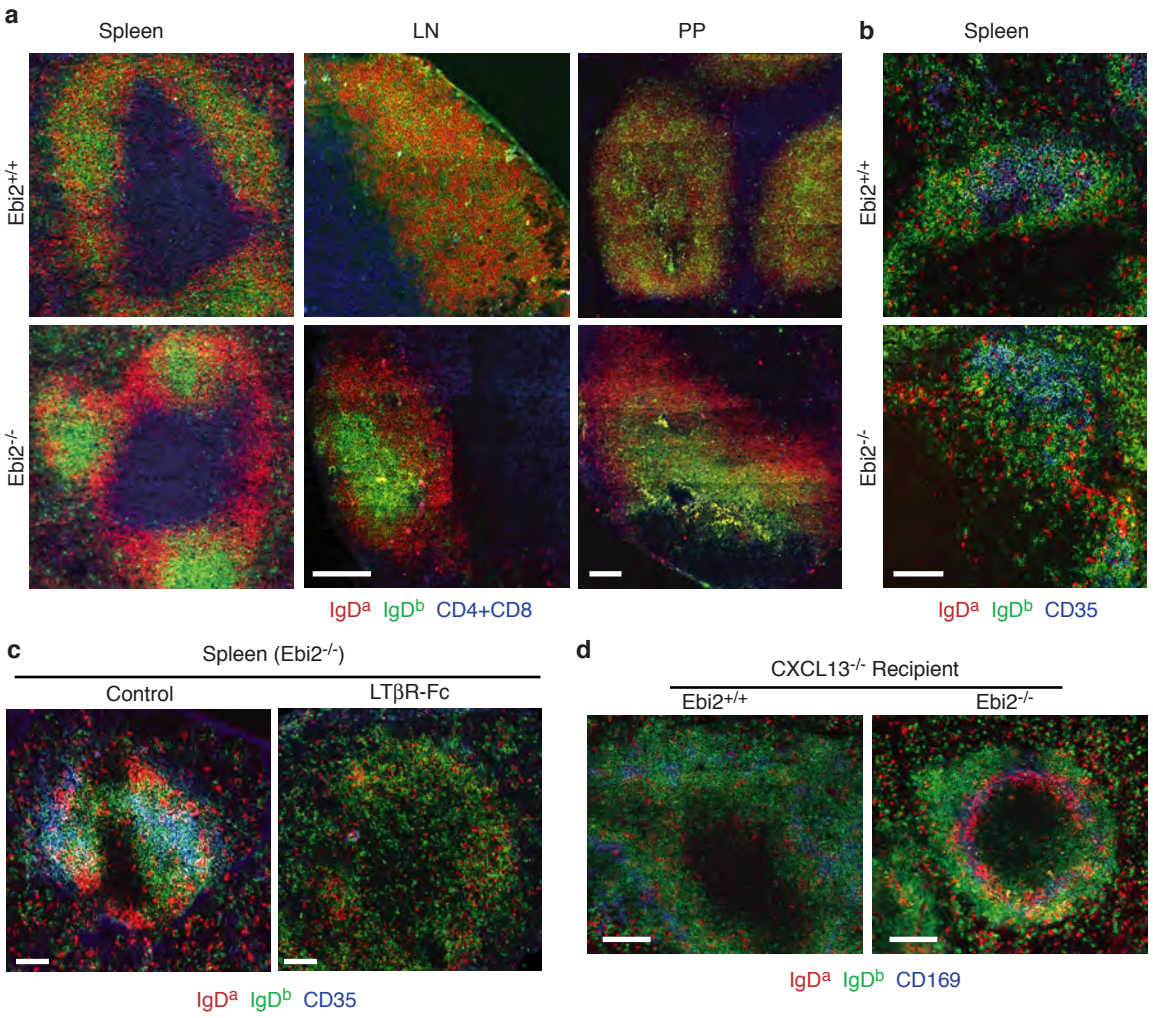
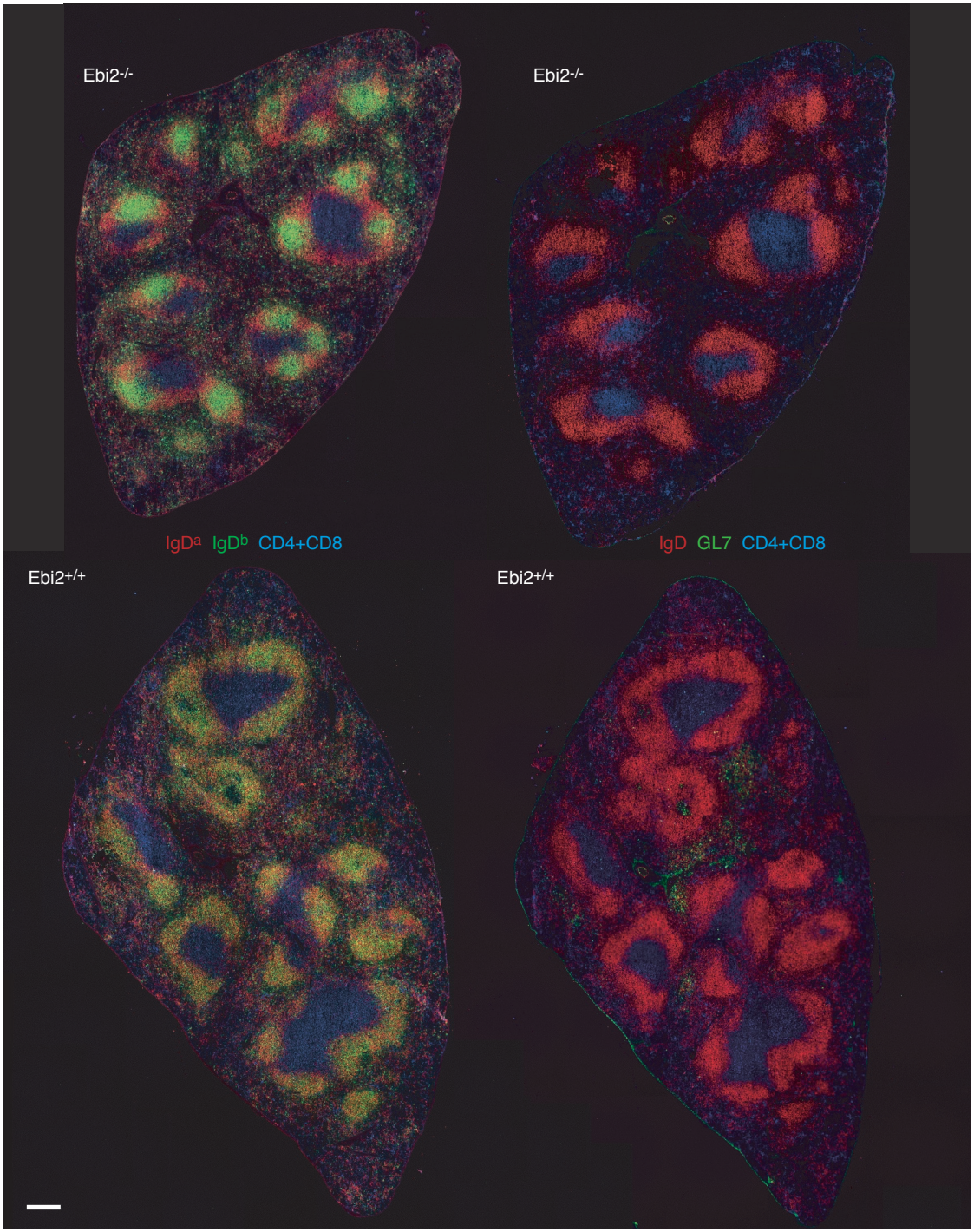
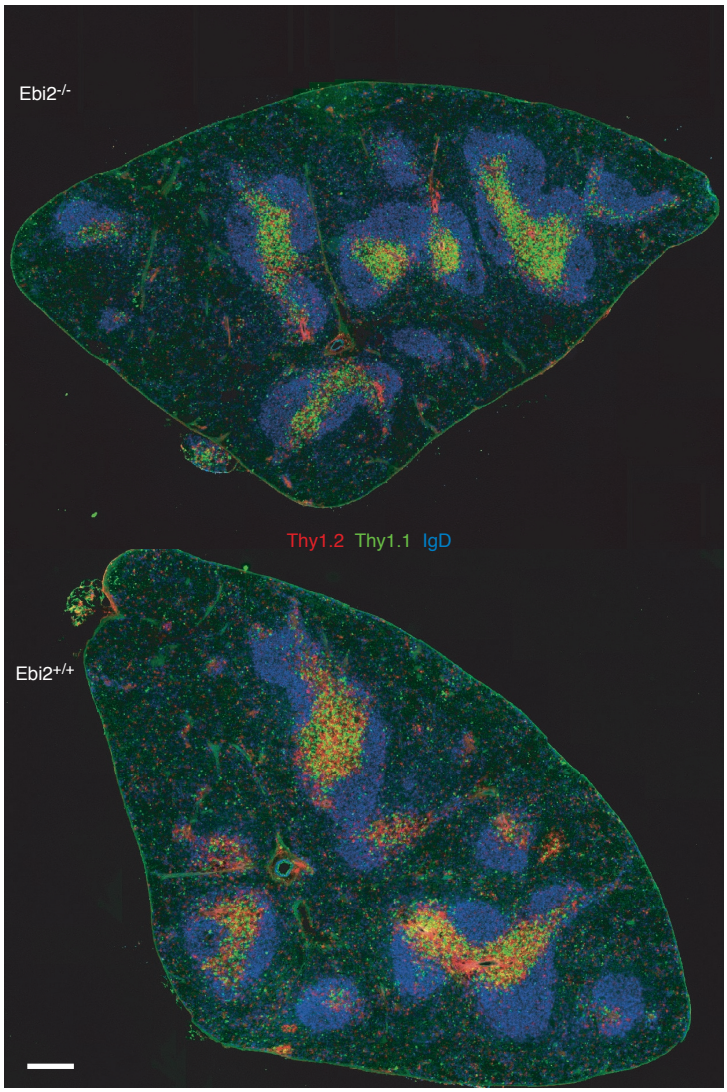


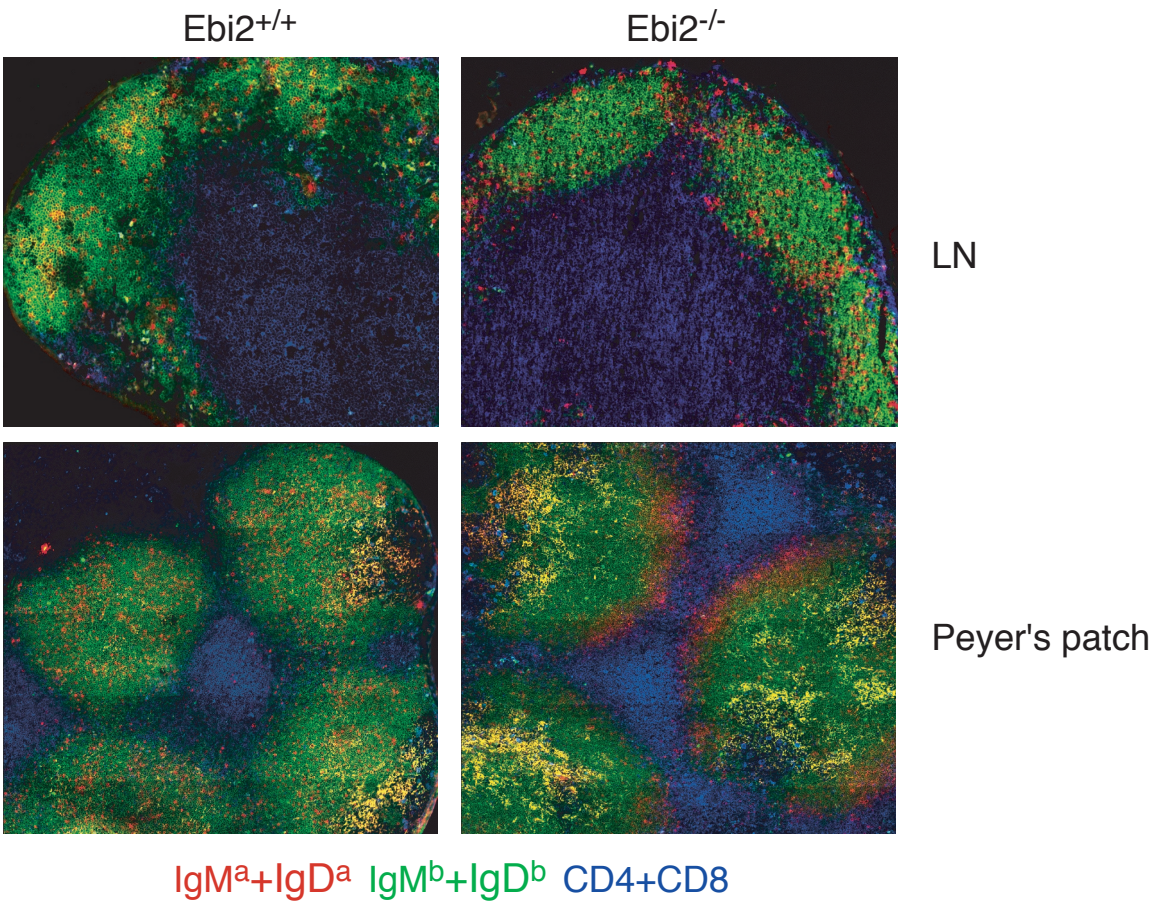
Figure 3: EBI2-deficient B cells localize to the follicle center in a LTα1β2- and CXCL13-dependent manner. a, Distribution of wild-type (*Ebi2*$^{+/+}$) and EBI2-deficient (*Ebi2*$^{-/-}$) B cells in spleen, lymph nodes (LN) and Peyer's patches (PP) of 20:80 mixed bone marrow chimaeras (20% Igh^{b} *Ebi2*$^{+/+}$ or *Ebi2*$^{-/-}$ and 80% Igh^{a} wild-type). Sections were stained to detect *Ebi2*$^{+/+}$ or *Ebi2*$^{-/-}$ B cells (IgD^{b}, green), Igh^{a} control B cells (IgD^{a}, red), and T cells (CD4+CD8, blue). **b**, Spleen sections from 90:10 mixed bone marrow chimeras (90% Igh^{b} *Ebi2*$^{+/+}$ or *Ebi2*$^{-/-}$and 10% Igh^{a} wild-type) stained to detect B cells as in **a**, and for CD35 to highlight FDC networks (blue). **c**, Similar analysis to **b** in control or 3 week LTβR-Fc treated 90:10 *Ebi2*$^{-/-}$ mixed bone marrow chimeras. **d**, Distribution of wild-type and EBI2-deficient B cells in *Cxcl13*$^{-/-}$ hosts reconstituted with 70:30 bone marrow mixtures (70% Igh^{b} *Ebi2*$^{+/+}$ or *Ebi2*$^{-/-}$and 30% Igh^{a} wild-type). Spleen sections stained to detect EBI2-deficient B cells (IgD^{b}, green), wild-type B cells (IgD^{a}, red) and marginal zone macrophages (CD169, blue). Scale bars, 100 μm.



Supplementary Figure 3: Distribution of Ebi2+/+ and Ebi2-/- B cells in spleen of mixed BM chimeras. Chimeras were prepared at a ratio of 20% Ebi2-/- or Ebi2+/+ (Igh^b) and 80% of Ebi2+/+ (Igh^a) as in Figure 3a. Serial cryosections were stained with anti-IgD^a (red), anti-IgD^b (green) and with anti-CD4+CD8 (blue, left panels) or with anti-GL7 (green), anti-IgD (red), and with anti-CD4+CD8 (blue, right panels). White bar depicts scale (200 μm).



Supplementary Figure 4. Distribution of Ebi2+/+ and Ebi2-/- T cells in spleen of mixed BM chimeras. Chimeras were prepared at a ratio of 20% Ebi2 -/- or Ebi2+/+ (Thy1.2) and 80% of Ebi2+/+ (Thy1.1) as in Figure 3a. Serial cryosections were stained with anti-Thy1.1 (red), anti-Thy1.1 (green) and with anti-IgD (blue). White bar depicts scale (200 μm). Flow cytometric analysis of B and T cell chimeriam showed that the hematopoietic reconstitution was approximately 20:80 for the Ebi2 -/- recipient and 40:60 for the Ebi2 +/+ recipient.



Supplementary Figure 5. Distribution of Ebi2+/+ and Ebi2-/- B cells in lymph nodes and Peyer's patch of mixed BM chimeras. Chimeras were prepared at a ratio of 90% Ebi2 -/- or Ebi2+/+ (Igh^b) and 10% of Ebi2+/+ (Igh^a) as in Figure 3b. Cryosections were stained with anti-IgM^a + IgD^a (red), anti-IgM^b + IgD^b (green) and with anti-CD4+CD8 (blue, left panels) or with anti-GL7 (green), anti-IgD (red), and with anti-CD4+CD8 (blue). The FDC network appears in yellow due to deposits of IgM.

areas (Fig. 3c), suggesting a role for FDCs or other LTα1β2-dependent cells in this process. In mice that lack CXCL13—a chemokine made broadly by the follicular stromal cell network (Gunn et al., 1998; Ngo et al., 1999)—B cell localization in the outer splenic white-pulp (where follicles would normally be located) is reduced but not completely blocked (Ansel et al., 2000)(Fig. 3d). In CXCL13-deficient mice reconstituted with a mixture of EBI2-deficient and WT bone marrow, EBI2-deficient B cells were selectively diminished within the white-pulp cords and instead accumulated in the MZ that surrounds the white pulp (Fig. 3d). These findings provide further evidence for EBI2-mediated attraction of B cells to the outer white-pulp.

These findings suggested that EBI2 downregulation in GC B cells may promote their positioning at the follicle center. To test directly the significance of EBI2 downregulation during GC development, we enforced constitutive EBI2 expression in hen egg lysozyme-specific immunoglobulin-transgenic B cells using retroviral gene transduction, and then tested their ability to participate in GC and plasma cell responses after short-term adoptive transfer to hen egg lysozyme-immunized hosts (Fig. 4a). The frequency of *Ebi2* or control-vector transduced B cells among the GC and plasma cell populations was tracked using the CD4 reporter. By flow cytometric analysis, *Ebi2*-transduced cells showed a reduced ability to participate in GC responses compared to vector-transduced cells, while participating with wild-type efficiency in the plasma cell response (Fig. 4a, b). Although some *Ebi2*-transduced cells could take on a GC phenotype (Fig. 4a), the cells were unable to position in GC (Fig. 4c and Supplementary Fig. 6).

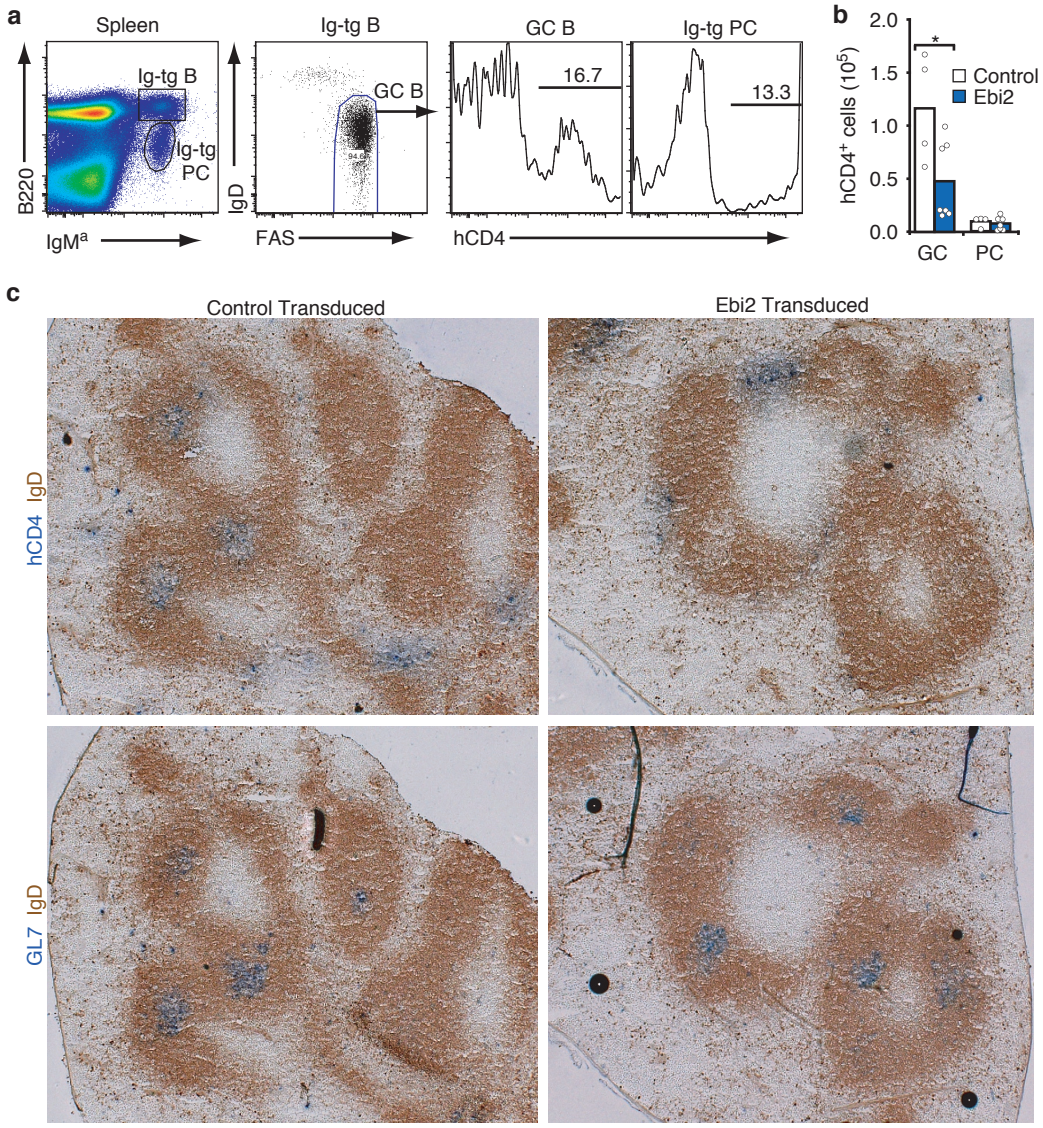
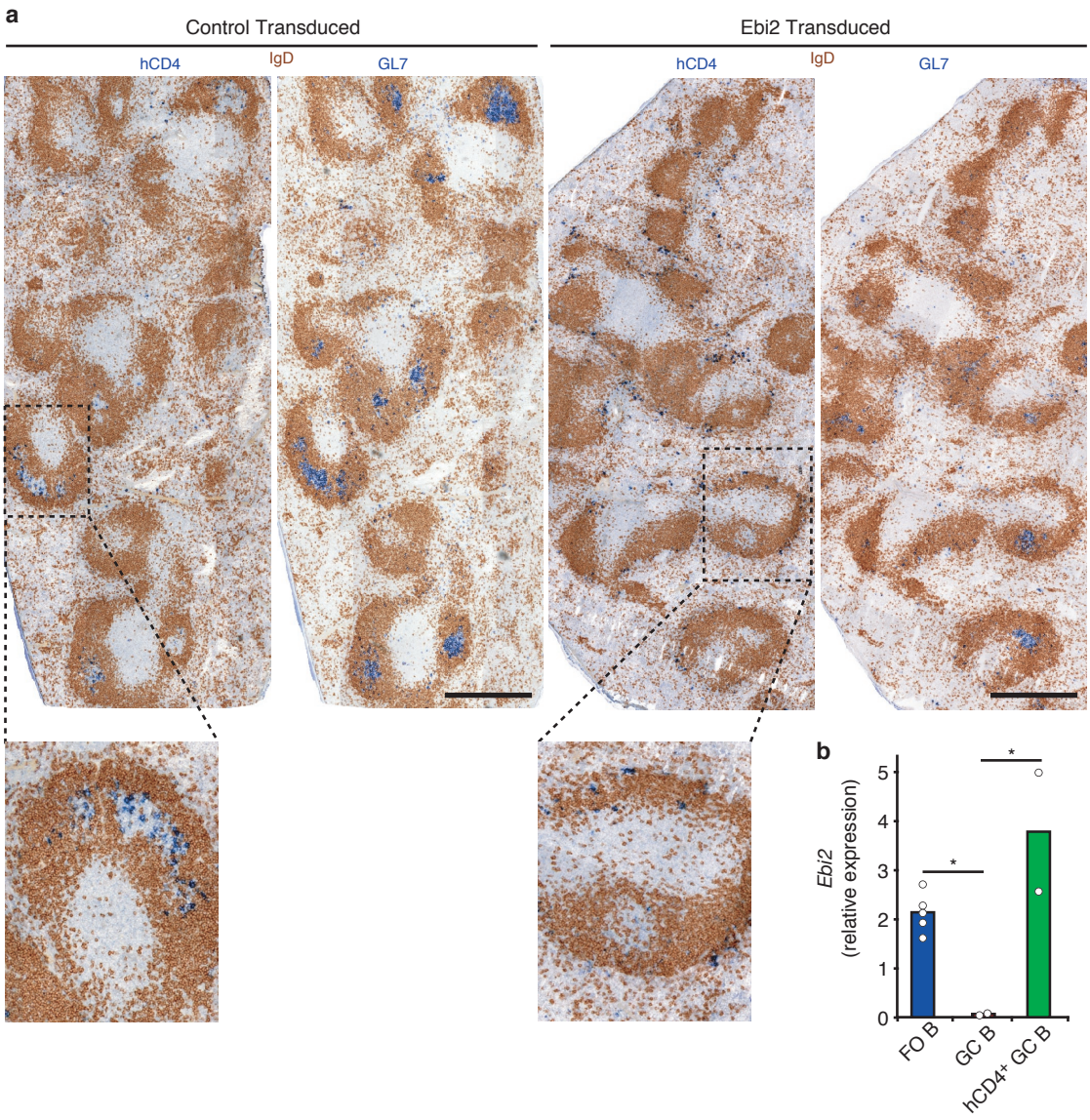


Figure 4: Maintained *Ebi2* expression impairs participation in GC response. a, Flow cytometric analysis of spleen cells from an immunized mouse receiving *Ebi2*-transduced Ig-transgenic (Ig-tg; IgM^a) B cells, 4 days after transfer, showing gating scheme to identify representation of transduced (human $CD4^+$) cells among GC B cells ($B220^+IgM^{a+}IgD^{lo}Fas^{hi}$) and plasma cells (PC; $B220^{lo}IgM^{a+}$). Numbers indicate frequency of cells in the indicated gate. **b**, Number of transduced (human $CD4^+$) B cells having a GC or plasma cell phenotype. **c**, Distribution of transduced B cells (human $CD4^+$, blue) in sections of spleen from mice receiving control vector or *Ebi2*-transduced B cells. Endogenous naive B cells are stained brown (IgD) and GC are detected in serial sections using GL7 (blue, lower panels). Scale bar, 100 μm.



Supplementary Figure 6. Quantitation of Ebi2 transcripts and further examples showing maintained Ebi2 expression prevents GC B cell positioning in GCs. (a) Immunohistochemical analysis of spleen cells from an immunized mouse 4 days after transfer of control (left panels) of Ebi2 transduced (right panels) Ig-transgenic B cells (as for the experiment described in Figure 4). Serial cryosections from the indicated mice were stained with anti-IgD (brown) and with anti-hCD4 (blue) or anti-GL7 (blue). Scale bar (black line) is 500 μm. Example GCs are shown in the enlargements. (b) Quantitative PCR analysis of Ebi2 expression in purified follicular B (FO B), GC B and Ebi2-transduced (hCD4+) GC B cells, presented relative to *Hprt1* expression. Bars indicated the mean; symbols indicate individual mice. *P<0.05.

Cells transduced with the control vector were readily detected within GC (Fig. 4c and Supplementary Fig. 6). *Ebi2*-transduced cells contained *Ebi2* transcripts in amounts within twofold of those present in follicular B cells, and at least 25-fold higher than in GC B cells (Supplementary Fig. 6). These experiments suggest that EBI2 downregulation is necessary for localization of developing GC B cells to the follicle centre.

In summary, we establish that EBI2 is upregulated in B cells after BCR and CD40 engagement and is necessary to promote the positioning of activated B cells to interfollicular regions and the outer follicle. Cognate B cells interact with helper T cells and undergo proliferation in these regions (Coffey et al., 2009; Garside et al., 1998). Defects in these processes are probably responsible for the diminished ability of EBI2-deficient mice to mount an early T-dependent IgG antibody response. Our findings suggest EBI2 is needed within B cells for these events, but we do not exclude that it also has a role in directing activated CD4 helper T cells and possibly other cell types to these regions. GC B cells markedly downregulate EBI2, a change that seems to be necessary to favor localization of activated B cells at the follicle center, in association with the antigen-presenting and GC-supportive (Tew et al., 1997) FDC network. BCL6, a transcription factor required for GC development, negatively regulates EBI2 expression (Shaffer et al., 2001). We suggest that by favoring appropriate niche occupancy, negative regulation of EBI2 represents an important component of the *Bcl6* gene expression program directing GC over plasma cell fate. Although the ligand for

EBI2 remains undefined, we speculate that it is more concentrated in the outer compared to centre follicle as well as in interfollicular regions. We propose that although CXCR5 is sufficient to promote B cell localization in follicles (Ansel et al., 2000; Forster et al., 1996), cells expressing EBI2 are more strongly attracted to the outer follicle compared to cells lacking this receptor. A further FDC-derived cue may favor positioning in the center follicle, and when cells lose responsiveness to EBI2 ligands, positioning in response to this cue is dominant. The activity of EBI2 provides a possible explanation for why CXCR5-deficient B cells continue to localize in IF areas and in regions corresponding to the outer follicle (Ansel et al., 2000; Forster et al., 1996). Our findings may also help explain the niche preferences of certain B cell lymphomas, particularly follicular center lymphoma (Lossos and Levy, 2003). Indeed, expression array studies demonstrate that EBI2 is downregulated in follicular and GC lymphomas (Alizadeh et al., 2000; Cahir-McFarland et al., 2004). Moreover, it seems possible that the marked EBI2 induction observed early after EBV infection (Birkenbach et al., 1993) serves as a mechanism used by the virus to promote positioning in niches that favor the survival of the infected B cells.

Methods

EGFP was inserted in place of the *Ebi2* open reading frame within E14 (129) embryonic stem cells using standard procedures, and *Ebi2*$^{GFP/+}$ mice were backcrossed to C57BL/6 for six generations. Six-to-twelve-week-old C57BL/6 mice were from either the National Cancer Institute or Jackson Laboratories. B6(C)-H2-Ab1^{bm12}/KhEgJ (bm12) mice and B6.Cg-Igh^{a}Thy1^{a}GPi1^{a}/J (IgMa) mice were from Jackson Laboratories. MD4 mice (Goodnow et al., 1988) and CXCL13-deficient mice (Ansel et al., 2000) were from an internal colony. Bone marrow chimeras were generated as described (Allen et al., 2004) and analysed after 6–12 weeks. NP-CGG immunizations were performed using 50 μg NP-CGG (Solid Phase Sciences) in alum (Accurate Chemical & Scientific Corp.). The retroviral construct was made by inserting the mouse *Ebi2* open reading frame, with a preprolactin–Flag leader sequence (Ishii et al., 1993) in place of the ATG, into the MSCV2.2 retroviral vector containing cytoplasmic-domain-truncated human CD4 as an expression marker downstream of the internal ribosomal entry site (Reif et al., 2002). B cells were isolated and in some cases labelled with 2.5 μM 5(and 6)-carboxy-fluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) as described (Okada et al., 2005). For *in vivo* analysis of EBI2 expression, 5–10×10^{6} purified B cells as described previously (Allen et al., 2004) were transferred into bm12 recipients. Transduced cells were adoptively transferred 1 day after spin-infection for transfers to non-immunized hosts or immediately after spin-infection for transfers to immunized hosts. For GC experiments, B6 mice received 10^{5} MD4 B cells and 10^{5} OTII CD4^{+} T cells at day

-1, were intraperitoneally immunized with 50 μg HEL-OVA in RIBI adjuvant system (Sigma) at day 0, and received approximately 10^6 *Ebi2* or control-vector-transduced cells at day 1. Mice were analysed on day 5. Flow cytometry, ELISA, immunohistochemsitry and immunofluorescence microscopy were performed using standard techniques and are detailed in the Methods.

Mice

Six-to-twelve–week-old C57BL/6 were purchased from either the National Cancer Institute or Jackson Laboratories. B6(C)-H2-Ab1^{bm12}/KhEgJ (bm12) mice and B6.Cg-Igh^{a}Thy1^{a}GPi1^a/J (IgMa) mice were from Jackson Laboratories. MD4 mice[27] and CXCL13-deficient mice (Ansel et al., 2000) were from an internal colony. Bone marrow chimeras were generated as described (Allen et al., 2004) and analysed after 6–12 weeks. NP-CGG immunizations were performed using 50 μg NP-CGG (Solid Phase Sciences) in alum (Accurate Chemical & Scientific Corp.). Treatment with LTβR-Fc (provided by J. Browning, Biogen Idec) was as described (Ngo et al., 1999) using 100 μg once a week for 3 weeks. 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.

***Ebi2* gene targeting and retroviral constructs**

A 5′ homology arm (5.2 kilobases (kb)) and 3′ homology arm (3.7 kb) were generated from mouse genomic DNA by PCR and cloned using BD In-Fusion Dry-Down PCR cloning kit into vector EGFP-polyA-*loxP*-Neo-*loxP*-DTA-PL452

(provided by N. Killeen) to flank the EGFP-polyA-*loxP*-Neo-*loxP* insert. E14 (129) embryonic stem cells were transfected by standard techniques and 350 colonies were screened by long PCR (Roche Long Template PCR system), yielding 22 positive clones. Homologous recombination was confirmed by Southern blotting and three clones were used for microinjection into B6 blastocysts. Chimeras were bred to B6 mice and germline transmission was confirmed by allele-specific PCR and flow cytometric detection of GFP expression. *Ebi2*$^{GFP/+}$ mice were intercrossed with actin-Cre transgenic mice and deletion of the *loxP*-flanked neomycin-resistance cassette was confirmed by PCR. *Ebi2*$^{GFP/+}$ (neo^{-}) mice used in this study were backcrossed to B6 for at least six generations. The retroviral construct was made by inserting the mouse *Ebi2* open reading frame, with a preprolactin–Flag leader sequence (Ishii et al., 1993) in place of the ATG, into the MSCV2.2 retroviral vector containing cytoplasmic-domain truncated human CD4 as an expression marker downstream of the internal ribosomal entry site (Reif et al., 2002). The control vector contained cytoplasmic domain truncated human nerve growth factor receptor as an irrelevant insert.

Cell isolation, CFSE labelling, retroviral transduction and adoptive transfers

B cells were isolated and in some cases labelled with 2.5 μM 5(and 6)-carboxy-fluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) as described (Okada et al., 2005). For *in vivo* analysis of *Ebi2* expression, 5–10×10^{6} purified B cells as described (Allen et al., 2004), were transferred into bm12 recipients. *In vitro* analysis of*Ebi2* expression was performed by culturing 10^{5} splenocytes

from *Ebi2*$^{GFP/+}$ mice with 10–13 μg ml^{-1} anti-IgM (F(ab')2 goat anti-mouse IgM, Jackson Immunoresearch), 10 μg ml^{-1}anti-CD40 (clone FGK4.5, UCSF Hybridoma Core), or both, for 24 and 48 h. Retroviral supernatant was generated using Phoenix packaging cells. Retroviral transduction of activated B cells was performed as described[8] using MD4 Ig-transgenic B cells. Transduced cells were adoptively transferred 1 day after spin-infection for transfers to non-immunized hosts, or immediately after spin-infection for transfers to immunized hosts. For GC experiments, B6 mice received 10^5MD4 B cells and 10^5 OTII CD4$^+$ T cells at day -1, were intraperitoneally immunized with 50□μg HEL-OVA in RIBI adjuvant system (Sigma) at day 0 and received approximately 10^6 *Ebi2*- or control-vector-transduced cells at day 1. Mice were analysed on day 5.

Flow cytometry

Bone marrow B cell subsets were analysed as described previously (Pereira et al., 2009a). Spleen and LN cells were isolated and stained as described (Allen et al., 2004). For analysis of GC B cell differentiation, cells were stained with phycoerythrin (PE)–Cy5.5-conjugated anti-B220 (RA3-6B2; BD Biosciences), Pacific blue-conjugated anti-CD45.1, FITC-conjugated anti-IgD (11-26c.2a; BD Biosciences) and PE–Cy7-conjugated anti-Fas (Jo2; BD Biosciences). For analysis of EBI2 expression in T cells, spleens and lymph nodes were stained with allophycocyanin (APC)-conjugated anti-TCRβ (H57-597, eBioscience), PE–Cy5.5-conjugated anti-CD4 (RM4-5, Invitrogen), with PE-conjugated anti-CD8 (CT-CD8a, Invitrogen), and with biotin-conjugated anti-NK1.1 (PK136, BD Biosciences).

ELISA

IgG1 anti-NP ELISA was performed by coating 96-well plates (Immunolon) with 10 μg ml^{-1} NP(30)BSA (Solid Phase Sciences) in PBS for at least 2 h at 37°C, and blocked with 5% (w/v) BSA (Calbiochem) for 2 h at ~25°C. Serum samples were serially diluted (1:2) starting at 1:500 in PBS 0.01% Tween, incubated for 2 h at ~25°C, and NP-binding IgG1 was detected using biotin-conjugated anti-IgG1 (A85, BD Biosciences), followed by horseradish-peroxidase-conjugated streptavidin (Jackson Immunoresearch). Colour development was done using ABTS substrate (Southern Biotech) in 55 mM citrate buffer containing 0.03% H_2O_2. Absorbance was measured at 405 nm in a VERSAmax microplate reader using SoftMax pro 5.2 (Molecular Devices). The NP-specific IgG1 concentration was calculated by determining the dilution required to achieve an optical density of 0.5, 1.0 and 1.5 (across these values, the correlation coefficient was >0.99 in all serum samples), averaged, and displayed as relative units to a standard serum sample.

Immunohistochemistry and immunofluorescent microscopy

Cryosections of 5–7 μm were fixed and stained immunohistochemically as described[8] with combinations of the following antibodies: anti-IgD (11-26c.2a, BD Biosciences), anti-IgMa (DS-1, BD Biosciences), human CD4 (RPA-T4, BD Biosciences) and B220 (RA3-6B2, BD Biosciences). For immunofluorescence, staining with biotin-conjugated anti-IgD^a (AMS9.1, BD Biosciences) was detected with Alexa Fluor488-conjugated streptavidin (Invitrogen), PE-conjugated anti-IgD^b (217-170, BD Biosciences), and Alexa 647-conjugated anti-CD4, and anti-

CD8 (UCSF Hybridoma Core). The FDC network was stained using purified anti-CD35 (8C12, BD Biosciences) detected with APC-conjugated anti-rat IgG (Jackson Immunoresearch). Marginal zone macrophages were stained with an anti-Ser4 antibody (P. Crocker, University of Glasgow) conjugated to Alexa Fluor647. Analysis of T-cell distribution was performed with biotin-conjugated anti-CD90.1 (Thy-1.1, clone HIS51) detected with Alexa Fluor488-conjugated, and with PE-conjugated anti-CD90.2 (Thy-1.2, clone 30-H12). Sections were then blocked with 5% normal rat serum before staining with additional antibodies. FITC-conjugated anti T- and B-cell activation antigen (GL7; BD Biosciences) was used to detect GC. For detection of GFP, tissues were fixed in 4% paraformaldehyde and prepared as described[31]. Images were obtained with a Zeiss AxioObserver Z1 inverted microscope or a Zeiss AxioImager M1 upright microscope.

Chapter 3

EBI2 guides serial movements of activated B cells and ligand activity is detectable in lymphoid and non-lymphoid tissues

This chapter was published as

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Abstract

EBI2 was recently shown to direct the delayed movement of activated B cells to inter and outer follicular regions of secondary lymphoid organs and to be required for mounting a normal T-dependent antibody response. Here we show that EBI2 promotes an early wave of antigen-activated B cell migration to the outer follicle in mice. Later, when B cells have moved to the T zone in a CCR7-dependent manner, EBI2 helps distribute the cells along the B-T boundary. Subsequent EBI2-dependent movement to the outer follicle coincides with CCR7 downregulation and is promoted by CD40 engagement. Using a bioassay, we identify a proteinase K resistant, hydrophobic EBI2 ligand activity in lymphoid and non-lymphoid tissues. Production of EBI2 ligand activity by a cell line is sensitive to statins, suggesting production in an HMG-CoA reductase-dependent manner. CD40 activated B cells show sustained EBI2-dependent responsiveness to the bioactivity. These findings establish a role for EBI2 in helping control B cell position at multiple stages during the antibody response and they suggest EBI2 responds to a broadly distributed lipid ligand.

Introduction

B cells migrate into lymphoid follicles in a CXCR5-dependent manner, responding to the CXCR5 ligand CXCL13 that is abundantly displayed on follicular stromal cells (Cyster, 2010; Muller et al., 2003; Ohl et al., 2003a). Within the follicle B cells migrate at an average velocity of 6 μm/min in a 'random' walk, surveying for antigens displayed by sinus-associated macrophages, follicular dendritic cells (FDCs), conduits, or that have diffused into the follicle (Cyster, 2010). Within six hours after antigen encounter, antigen-engaged B cells move to the B-T zone boundary in a CCR7-dependent manner, responding to CCL21 and CCL19 made by T zone stromal cells, to interact with helper T cells (Reif et al., 2002). CXCR5 remains expressed by activated B cells and helps distribute cells along the B-T boundary. By day 2 of T-dependent responses, some activated B cells relocalize to the outer and inter-follicular regions (Coffey et al., 2009; Pereira et al., 2010). Plasmablasts then emerge, particularly in interfollicular regions, and germinal center (GC) B cells soon accumulate at the follicle center (Goodnow et al., 2010; Pereira et al., 2010).

EBI2 is an orphan GPCR that was identified during a screen for EBV-induced genes (Birkenbach et al., 1993). Transcript analysis and studies in an EBI2-GFP reporter mouse line showed that EBI2 is abundantly expressed in B cells and it is further upregulated following activation; expression was also found in some T cells and myeloid cells (Cahir-McFarland et al., 2004; Gatto et al., 2009; Glynne et al., 2000; Heinig et al., 2010; Pereira et al., 2009b; Shaffer et al., 2001). Studies in two EBI2-knockout mouse lines established that EBI2 was

required for B cells to correctly localize to inter- and outer-follicular niches at days 2-3 of the T-dependent antibody response (Gatto et al., 2009; Pereira et al., 2009b). As B cells differentiate into GC cells they downregulate EBI2 and this is important for the cells to participate in the GC response (Gatto et al., 2009; Pereira et al., 2009b). Deficiency in EBI2 leads to a reduction in the magnitude of the T-dependent antibody response, establishing a role for this receptor in humoral immunity (Gatto et al., 2009; Pereira et al., 2009b).

Based on sequence alignments, EBI2 has been clustered with a number of G-protein coupled receptor subgroups, most commonly with subsets of lipid receptors (Rosenkilde et al., 2006; Shimizu, 2009; Surgand et al., 2006). Although one study suggested EBI2 may be a constitutively active receptor (Rosenkilde et al., 2006), the *in vivo* studies provided strong evidence that EBI2 is responsive to an extrinsic ligand (Gatto et al., 2009; Pereira et al., 2009b).

Here we have further examined the kinetics of EBI2 induction and determined how the prompt upregulation of the receptor affects B cell behavior. We show that EBI2 helps early-activated B cells access the outer follicle but by 6 hours, CCR7 function dominates to shift cells to the B-T boundary. EBI2 continues to function at this stage by helping retain and distribute cells along the length of the B-T boundary. Subsequent EBI2-dependent movement of activated B cells back to the T-zone distal outer follicle and to inter-follicular regions is promoted by CD40 engagement. Finally, we employ a bioassay to provide evidence for EBI2 ligand activity in lymphoid tissues, and also multiple non-lymphoid tissues, and we suggest the ligand is a lipid.

Materials and methods

Mice. C57BL/6 (B6) and B6-CD45.1 mice were obtained from The Jackson Laboratory, the National Cancer Institute, or an internal colony. B6.Cg-Igh^{a}Thy1^{a}GPi1^{a}/J (IgMa) and bm12 mice were from The Jackson Laboratory. CXCR5-/- (MGI 2158677; (Forster et al., 1996)), *plt* (MGI 1857881; (Nakano and Gunn, 2001)), EBI2-/- (MGI 4399081; (Pereira et al., 2009b)), CD40-deficient (MGI 2182733), MD4 (MGI 2384162) and OTII (MGI 4836972) mice were from an internal colony. Mixed bone marrow chimeras were generated as described (Pereira et al., 2009b). Animals were housed in a specific-pathogen free environment in the Laboratory Animal Research Center at UCSF, and all experiments conformed to ethical principles and guidelines approved by the Institutional Animal Care and Use Committee.

Flow cytometry and cell sorting. Splenocytes were isolated and stained as described (Allen et al., 2004), except for CCR7 staining, in which cells were blocked with Fc block for 10 min at room temperature, and then stained with anti-CCR7 biotin (1:10 4B12, BioLegend) for 20 min at room temperature. The cells were washed twice and secondary staining occurred on ice. Flow cytometry analysis was conducted on an LSR II flow cytometer (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star, Inc). For cell sorting for *in vivo* analysis of *Ebi2* expression, cell suspensions were first prepared from spleens in HBSS (UCSF Cell Culture Facility) containing 0.5% FBS and 0.5% fatty acid−free bovine serum albumin (BSA; Calbiochem). Cells at a density of 4

$\times 10^7$ cells/ml were stained for 30 min on ice and then erythrocytes were lysed by centrifugation at 4 °C in a solution of Tris-buffered NH_4Cl. Cells were labeled on ice with B220, CD4, Ly5.1, and Ly5.2. Dead cells were excluded with DAPI. Cells were sorted on a FACSAria.

Cell isolation, CFSE labeling, immunizations and adoptive transfers. Mice were intraperitoneally immunized with 50 µg HEL-OVA in RIBI adjuvant system (Sigma) one day after receiving splenocytes containing 5-10x10^6 WT, EBI2 KO or CD40 KO MD4 B cells and 2.5-5x10^6 OTII T cells transferred intravenously. For CD40L-blocking experiments, mice were injected intravenously with 1 mg anti-mouse CD40L (clone MR1, BioExpress Inc.) 24 hours after immunization. Spleens were harvested and digested with 2 mg ml^{-1} collagenase type 2 (Worthington Biochemical Corporation) or frozen for sectioning. For positioning studies, splenocytes containing 10-40x10^6 MD4 cells were transferred intravenously and the following day mice were injected with 1 mg HEL (Sigma Aldrich) intravenously. Splenocytes containing 30x10^6 MD4 B cells were transferred into CD40-KO recipients, or lethally irradiated recipients reconstituted for at least 6 weeks with CD40 deficient bone marrow. The next day, 1 mg HEL was injected intravenously, followed 6 h subsequently by 250 µg anti-CD40 (clone FGK4.5, UCSF Hybridoma Core). Mice were analyzed 36 h later. Bm12 experiments were performed as in (Pereira et al., 2009b). Purified and labeled wild-type and CD40-deficient B6 B cells (20x10^6 each) were mixed and stimulated with anti-IgM for 6 hours, and then transferred to bm12 recipients for 2 days. B cells were isolated by negative selection using Dynabeads Mouse CD43,

following manufacturer's protocol. In vitro analysis of Ebi2 and Ccr7 expression was performed by culturing 10^5 purified B cells with 13 µg ml^{-1} anti-IgM (F(ab')2 goat anti-mouse IgM, Jackson Immunoresearch). For homeostatic positioning, $30x10^6$ B cells were isolated by negative selection as described and labeled with 2.5 µM CFSE (Molecular Probes).

Immunohistochemistry and immunofluorescent microscopy. Tissue was prepared and 7µm cryosections were fixed and stained immunohistochemically as described (Allen et al., 2004) with combinations of: goat anti-mouse IgD (Accurate Chemical and Scientific), biotin anti-IgM^a (DS-1, BD Biosciences), B220 FITC (RA3-6B2, Biolegend) and/or biotin anti-IgD^a (AMS9.1, BD Biosciences) followed by HRP-conjugated donkey anti-goat IgG (H+L), HRP-conjugated anti-FITC, AP-conjugated anti-FITC, and/or AP-conjugated SA (Jackson Immunoresearch). For immunofluorescence, staining was with FITC-conjugated anti-IgD^a (AMS9.1, BD Biosciences) and PE-conjugated anti-IgD^b (217-170, BD Biosciences). Images were obtained with a Zeiss AxioObserver Z1 inverted microscope or a Zeiss AxioImager M1 upright microscope.

EBI2 ligand bioactivity in mouse tissue extracts. Mouse tissue/organ interstitial fluid-enriched extracts were prepared as previously described (21). Briefly, organs were weighed and mashed in 10 volumes (assuming a density of 1mg/ml) of sterile chemotaxis media (RPMI + 0.5% fatty acid free BSA) through a 70 µm filter. Clean supernatants were collected after centrifugation and tested for bioactivity by transwell chemotaxis assays (Pereira et al., 2009b) of M12 B

cell line transduced with an EBI2-IRES-GFP retroviral construct (Pereira et al., 2009b) against chemotaxis media containing 10% of each tissue supernatant or 1% mouse plasma. SDF1-a (Peprotech) was used as a positive control for chemotaxis. PTX or oligomer B (List Biological Labs, Inc.) pretreatment of M12 cells was for 1 h at 100ng/ml.

EBI2 ligand bioactivity in mouse cell line supernatants, and inhibitors of Ebi2 ligand production. Supernatants from various cell lines including bone marrow stromal line OP-9, 3T3, WEHI, M12 and HEK293 cells, were obtained by incubating each cell line in chemotaxis media for 12 h at 37°C 5% CO_2. In some cases, HEK293 cells were cultured in chemotaxis media containing the indicated concentrations of cycloxygenase inhibitor (Ibuprofen, Sigma), cytosolic phospholipase A1 and A2 inhibitor ($AACOCF_3$, arachidonyltrifluoromethyl ketone, Biomol), cyclooxygenases and lipoxygenase inhibitor (ETYA 5,8,11,14-eicosatetraynoic acid, Biomol) or HMG-CoA reductase inhibitor (Atorvastatin and Mevastatin, Sigma) for 12 h at 37 5% CO_2.

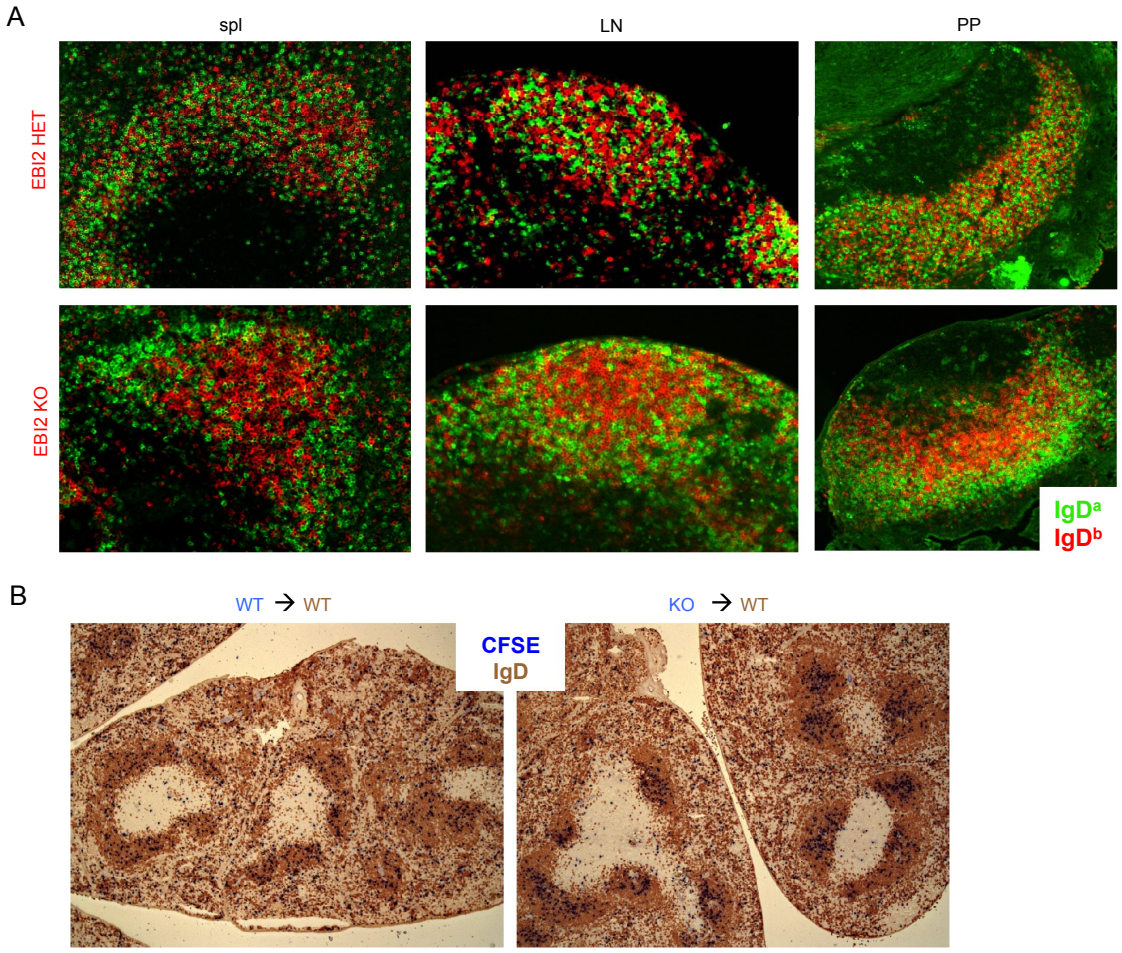
EBI2 ligand fractionations. Mouse tissue extracts and HEK293 culture supernatants were prepared for reverse-phase high pressure liquid chromatography (RP-HPLC) by adjusting trifluoracetic acid to 0.1% and CH3CN to 10%. Small precipitates were removed by centrifugation. Supernatants were fractionated with reverse phase C18 Sep-Pak columns (Waters) by serial washes with increasing concentrations of CH3CN +0.1%TFA. Semi preparative reverse

phase HPLC was performed on a Varian ProStar solvent delivery system equipped with a semi-preparative C18 Zorbax Stable Bond column (300Å Pore Size), Agilent Technologies (VWR) column and an analytical C18 Phenyl Zorbax Stable Bond column (80Å Pore Size), Agilent Technologies (VWR) using CH3CN (0.1% TFA)/H2O (0.1% TFA) gradient (10–100%) as the mobile phase and monitored by UV scan between λ=180 and 360 nm. One minute fractions were collected, lyophilized and tested for bioactivity by chemotaxis assay.

Results

EBI2 promotes movement of naïve B cells through the outer follicle. EBI2 is abundantly expressed in naïve B cells and when naïve cells lack this receptor they have a propensity to be enriched at the follicle center and underrepresented at the follicle periphery in spleen, LNs and Peyer's patches (Gatto et al., 2009; Pereira et al., 2009b) (Suppl. Fig. S1a). Isolated lymphoid follicles (ILFs) in the intestine are rudimentary B cell-rich aggregates that do not have all the features of secondary lymphoid organs (Fagarasan et al., 2010). However, here too we found that EBI2 favored access of naïve B cells to the outer follicle (Fig. 1a). In short-term transfer experiments, a bias in the distribution of EBI2-deficient naïve B cells between the outer and center follicle could also be detected though it appeared less marked than in mixed BM chimeras (Suppl. Fig. S1b) (Gatto et al., 2009). However, when the reciprocal experiment was performed and WT B cells were transferred to EBI2-deficient recipients, a striking bias in cell distribution to the outer follicle was observed in all the lymphoid tissues examined (Fig. 1b). We interpret this more obvious positional influence of EBI2 on the behavior of small numbers of WT cells to be a consequence of elevated availability of ligand in EBI2-deficient hosts.

EBI2 is rapidly upregulated after B cell activation and promotes early movement to the outer follicle. Transcript analysis showed that BCR engagement caused marked EBI2 upregulation within one hour, intermediate expression at 2 hours and a return to levels similar to naïve cells at 6 hours (Fig.



Supplemental Figure 1: EBI2 homeostatically promotes localization to outer and inter follicular areas: (A) Spleen (spl), lymph node (LN) and Peyer's patch (PP) sections from 50:50 mixed het or EBI2KO Igh^b (red) and WT Igh^a (green) BM chimeras, stained as indicated. (B) Distribution of CFSE-labeled WT and EBI2 KO B cells in the spleen of WT hosts one day after transfer. Transferred B cells were detected by staining for anti-FITC (blue) and endogenous B cells with antibodies to total IgD (brown).

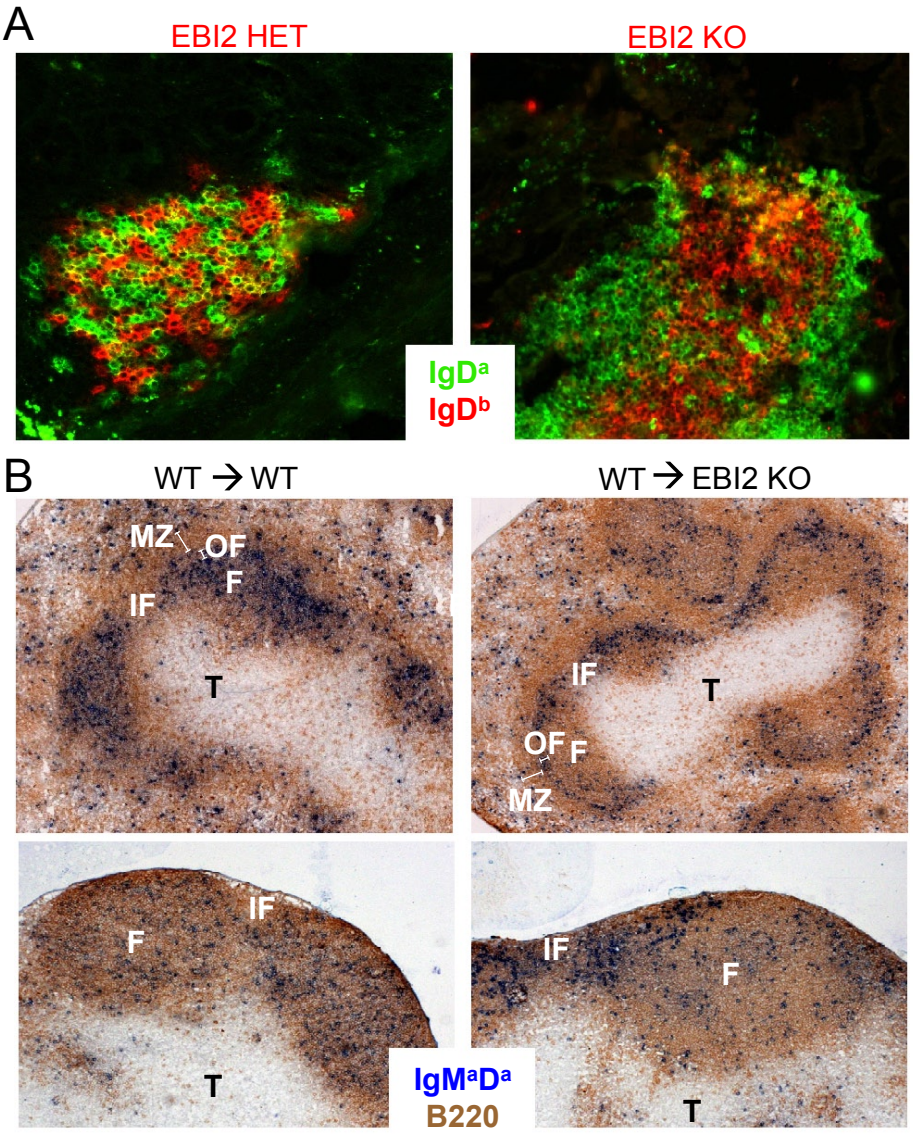


Figure 1. Naïve B cell access to the outer follicle is promoted by EBI2. (A) Isolated lymphoid follicles in the small intestine of 50:50 mixed het or EBI 2-/- Igh^b (red) and WT Igh^a (green) BM chimeras, stained as indicated. (B) Spleen and pLN sections from WT or EBI2 deficient mice that had received one day transfers of WT (Igh^a) B cells. Stained to detect the transferred B cells (IgM^aD^a, blue) and endogenous B cells (B220, brown). F, follicle (a single follicle is labeled); IF, inter follicular region; OF, outer follicle; T, T zone; MZ, marginal zone.

2a) in agreement with other studies (Cahir-McFarland et al., 2004; Gatto et al., 2009; Glynne et al., 2000; Shaffer et al., 2001). By contrast, CCR7 transcripts were not significantly upregulated in the first hours of activation under these stimulation conditions (Fig. 2b). CCR7 surface abundance did change, however, as anticipated (Reif et al., 2002), increasing only slightly by 2 hours but being significantly upregulated over control levels at 6 hours (Fig. 2b). The very rapid induction of EBI2 suggested it had a role in regulating B cell behavior in the first hours after activation, possibly before increases in CCR7 abundance had occurred. To examine this possibility, wild-type (WT) or EBI2-deficient hen egg lysozyme (HEL)-specific MD4 Ig-transgenic B cells were transferred to WT hosts and then the mice were systemically immunized with soluble HEL. Prior to antigen injection, EBI2-deficient B cells were distributed in follicles with a bias for the follicle center (Fig. 2c). Three hours after antigen injection, WT B cells were enriched in the outer follicle (Fig. 2c) whereas EBI2-deficient B cells failed to move to this region and instead had already arrived at the B-T boundary (Fig. 2c). By 6 hours after HEL injection, WT B cells were distributed along the B-T boundary. EBI2-deficient B cells also localized to the boundary at this time point (Fig. 2c) as previously observed (Gatto et al., 2009; Pereira et al., 2009b), though they tended to distribute more extensively into the T zone (Fig. 2c). Transcript abundance in 6 hour activated B cells was close to the levels in naïve cells (relative to *hprt*), amounts that generate sufficient EBI2 to influence B cell behavior (Fig. 1); direct assessment of EBI2 protein levels awaits generation of an antibody reagent. At 10 hours after transfer it was also evident that EBI2-

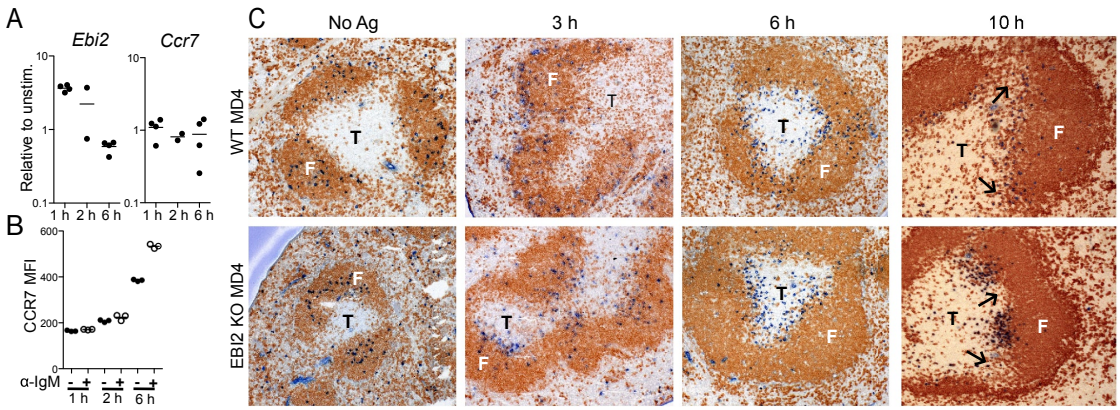


Figure 2. EBI2 is rapidly upregulated after B cell activation and promotes early movement to the outer follicle. (A, B) Ebi2 and Ccr7 transcript abundance (A), and CCR7 surface expression (B) in 1, 2 and 6 h anti-IgM stimulated B cells. In A, data are shown relative to unstimulated cells that had been incubated for the equivalent amounts of time. QPCR data were standardized against HPRT and data are pooled from four experiments. (C) Distribution of WT and EBI2 KO MD4 B cells in the spleen of WT hosts at the indicated time points after HEL antigen injection. Transferred MD4 B cells were detected by staining for IgM^a and IgD^a (blue) and endogenous B cells with antibodies to total IgD (1, 3 and 6 h) or B220 (10 h) (brown). Black arrows highlight interfollicular regions. Views are representative of at least two mice of each type.

deficiency caused the activated B cells to be more clustered at the midline of the follicle-T zone interface rather than being well-distributed along the length of the boundary (Fig. 2c). Increased dispersal of antigen-activated EBI2-deficient B cells into the T zone was also observed at day 1 of the response in a previous study (Gatto et al., 2009). Taken together, these observations suggest that EBI2 is upregulated in the first hours after antigen exposure, promoting early movement to the outer follicle, and that once cells have upregulated CCR7 and moved to the B-T boundary, EBI2 helps retain cells near and distributed along the boundary.

EBI2 functions with CCR7 and CXCR5 to distribute activated B cells along the B-T boundary. As a further test of EBI2 activity in B cells 6 hours after B cell activation, we examined the distribution of antigen-engaged B cells in *plt/plt* mice that are deficient in CCR7 ligand expression in lymphoid tissues (Luther et al., 2000; Nakano and Gunn, 2001). In these mice, 6 hour activated B cells fail to move to a location corresponding to the B-T boundary and instead accumulate in the outer follicle (Fig. 3a and (Reif et al., 2002)). Strikingly, 6 hour activated EBI2-deficient B cells failed to relocate to the outer follicle in *plt/plt* spleens and remained near the follicle center (Fig. 3a). These data provide further evidence that EBI2 is functional in 6 hour antigen activated B cells and they suggest that coordinated regulation of CCR7 and EBI2 function helps to direct B cell positioning during the early stages of activation.

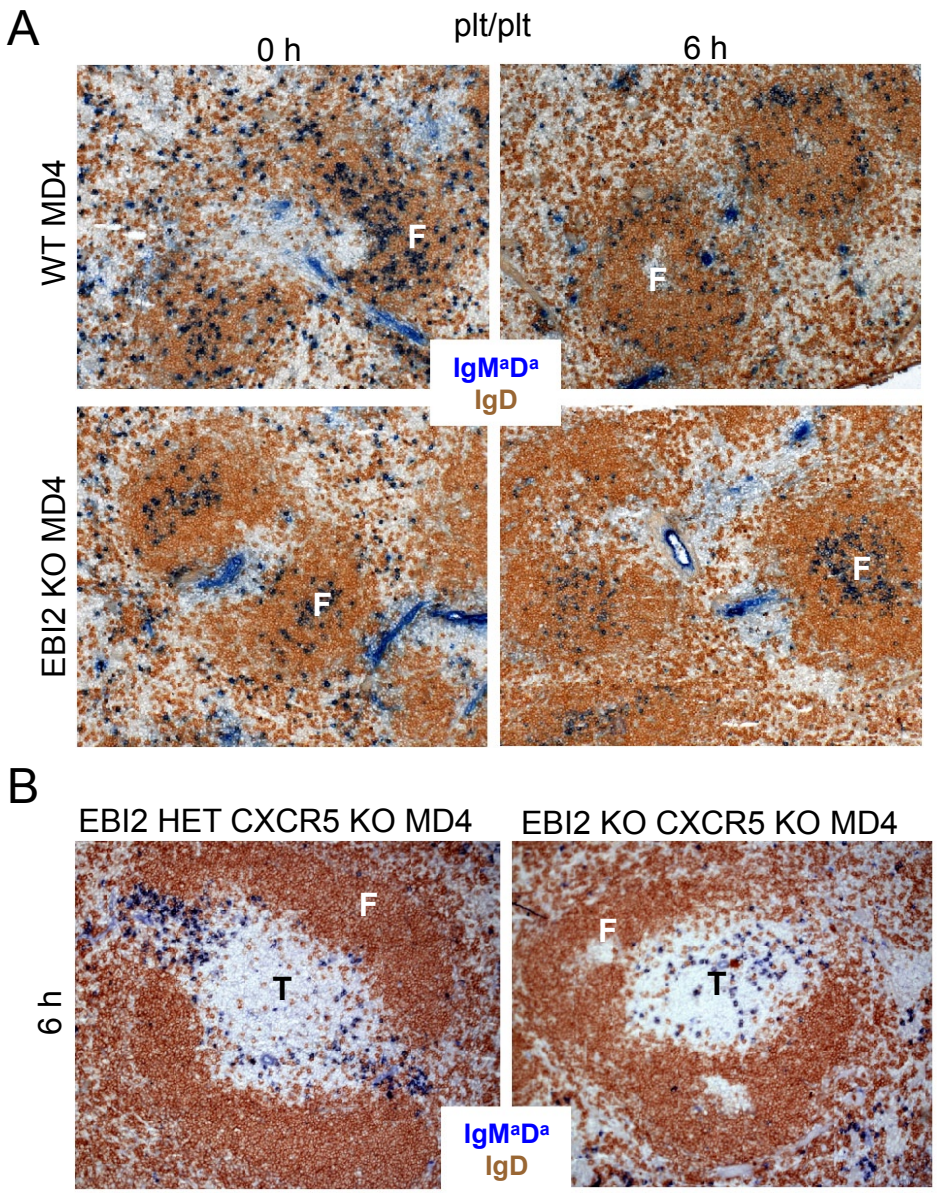
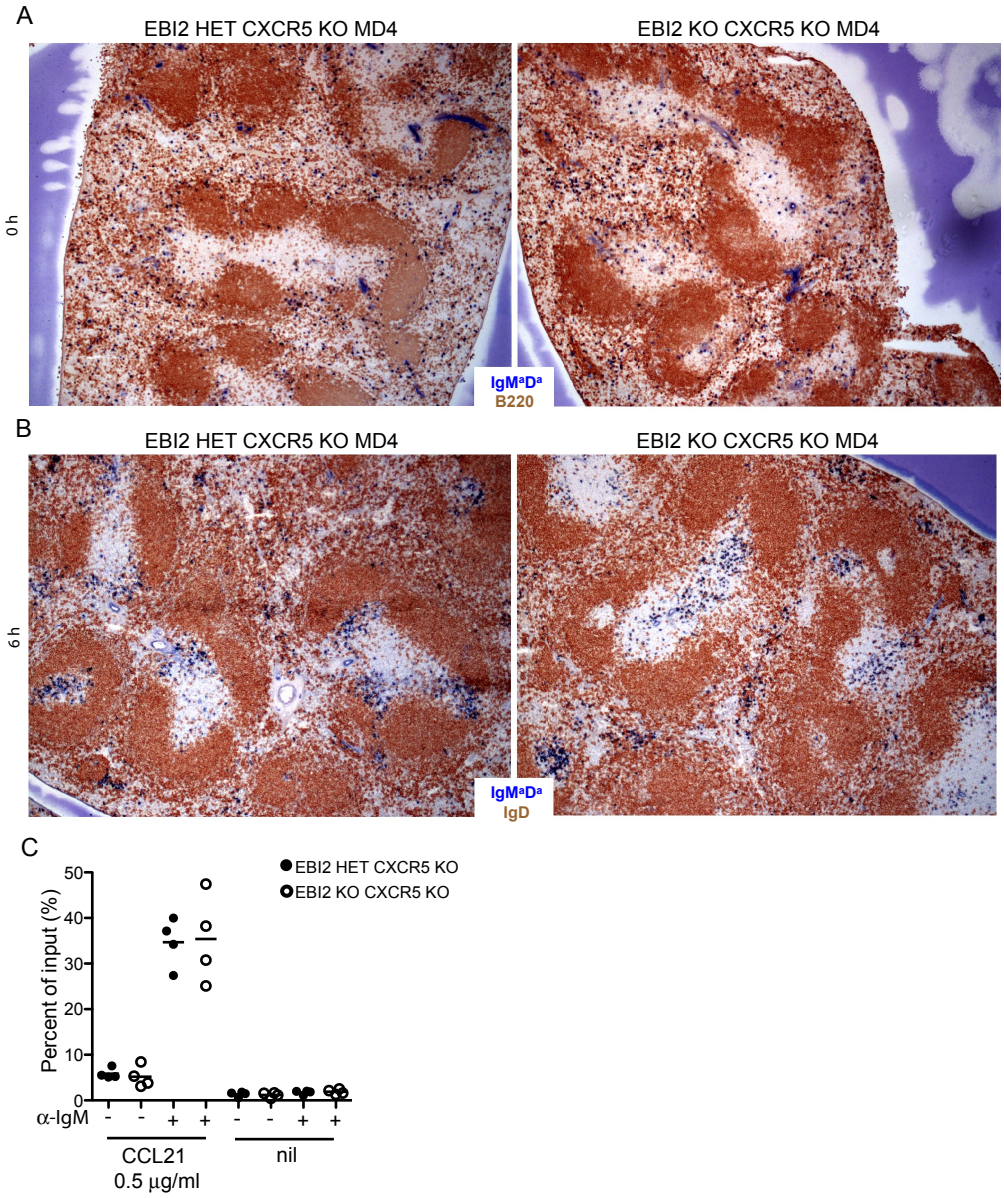


Figure 3. EBI2 functions together with CCR7 and CXCR5 to distribute activated B cells along the B-T boundary. (A) Distribution of WT and EBI2 KO MD4 B cells in CCR7-ligand deficient (plt/plt) recipient spleens at 0 or 6 h after HEL antigen injection. (B) Distribution of EBI2 het CXCR5 KO and EBI2 CXCR5 DKO MD4 B cells in the spleen of WT hosts 6 h after HEL antigen injection. Transferred MD4 B cells were detected by staining for IgM^a and IgD^a (blue) and endogenous B cells with antibodies to total IgD (brown). Views are representative of three mice.

CCR7 ligands are abundant throughout the T zone of WT mice and it has been unclear what factors restrain CCR7^{hi} activated B cells to the B-T boundary (Okada and Cyster, 2006). CXCR5-deficiency led to a less efficient distribution of cells along the boundary but did not allow their spread through the T zone (Reif et al., 2002). However, the finding of an increase in the number of 6-10 hours activated EBI2-deficient B cells extending into the T zone (Fig. 2c) led us to examine the impact of combined deficiency in CXCR5 and EBI2. Prior to activation, CXCR5-deficient B cells failed to access follicles (Suppl. Fig. S2a) consistent with earlier studies (Ansel et al., 2000; Forster et al., 1996). CXCR5 EBI2 double knockout (DKO) B cells also failed to access follicles, remaining mostly in the red-pulp though with small numbers of cells reaching the T zone (Suppl. Fig. S2a). At 6 hours after activation, CXCR5 KO cells were constrained to interfollicular regions and generally did not enter deeply into the T zone whereas CXCR5 EBI2 DKO cells often showed substantial penetration into the T zone (Fig. 3b and Suppl. Fig. S2b). EBI2 KO and EBI2 CXCR5 DKO cells had similar *in vitro* responsiveness to CCL21 suggesting that the differences in distribution were due to the loss of EBI2 function rather than indirect effects on CCR7 function (Suppl. Fig. S2c). These data provide further evidence that EBI2 ligand is present in interfollicular regions, and suggest that it extends along the B-T boundaries near these regions, whereas it is low or absent in the deep T zone, allowing EBI2 to help distribute activated B cells over the length of the B-T boundary.



Supplemental Figure 2: EBI2 and CXCR5 promote localization to the B cell follicle and interfollicular areas: (A,B) Distribution of transferred EBI2 HET CXCR5 KO MD4 and EBI2 KO CXCR5 KO MD4 B cells in WT recipient spleen at 0 (A) or 6 h (B) after HEL injection. Transferred MD4 B cells were detected by staining for IgM^a and IgD^a (blue) and endogenous B cells with antibodies to total IgD (brown). (C) Migration response of EBI2 HET CXCR5 KO and EBI2 KO CXCR5 KO B cells to CCL21 or medium alone (nil) before and after in vitro stimulation with anti-IgM for 6 h.

CD40 promotes movement of activated B cells to the outer follicle. At day 2 of the response to a T-dependent antigen, many activated B cells are redistributed to inter and outer follicular regions in a strictly EBI2-dependent manner (Fig. 4a and (Gatto et al., 2009; Pereira et al., 2009b)). Thus, although EBI2 transcripts are reduced in abundance (relative to HPRT) at this time point (Fig. 4b) the genetic studies indicate that the receptor continues to function. Previous studies have provided evidence that CCR7 can become downregulated on B cells by day 2 of the response (Chan et al., 2009; Coffey et al., 2009) and this was observed in our experiments (Fig. 4c). In the absence of T cell help, antigen-engaged B cells fail to relocalize from the B-T boundary at day 2 and many of the cells die in this location (Cyster and Goodnow, 1995). We speculated that CD40 engagement provides a key input from T cells that not only enhances B cell survival but also facilitates movement from the B-T boundary to the outer follicle. To test whether CD40 signaling was sufficient to promote movement of activated B cells to the outer follicle, we transferred WT or EBI2 KO MD4 B cells into CD40-deficient hosts, immunized with soluble HEL in the absence of adjuvant to activate the B cells but avoid recruiting helper T cells (Cyster and Goodnow, 1995) and then treated with or without anti-CD40. By using CD40-deficient hosts, we ensured that the CD40-activating signal was restricted to the transferred B cells. The WT MD4 B cells receiving CD40 stimulation were not only rescued from elimination but many were induced to relocalize to outer and inter follicular regions, while the EBI2 KO MD4 B cells did not relocalize to these areas (Fig. 4d). In the absence of CD40 signaling, many of

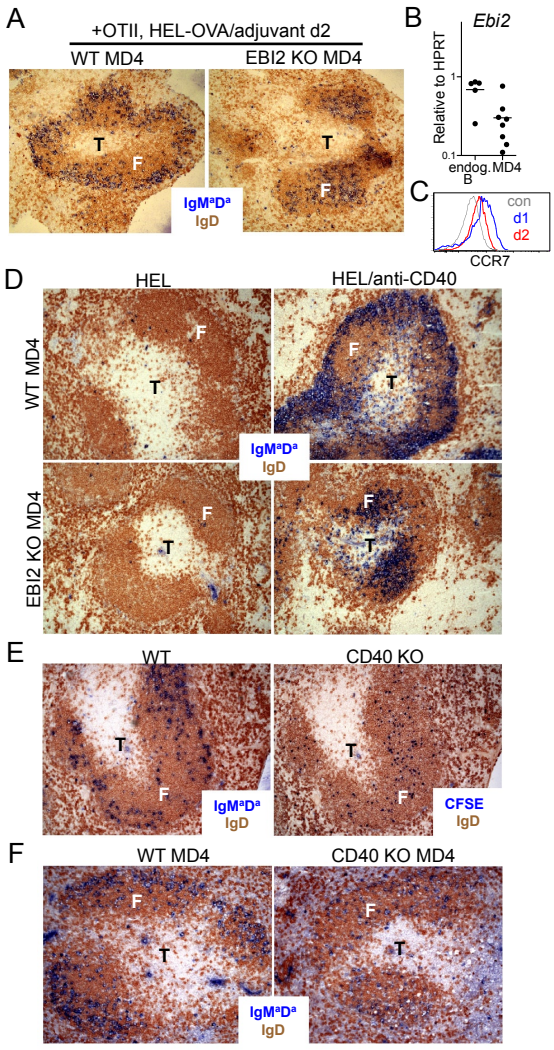
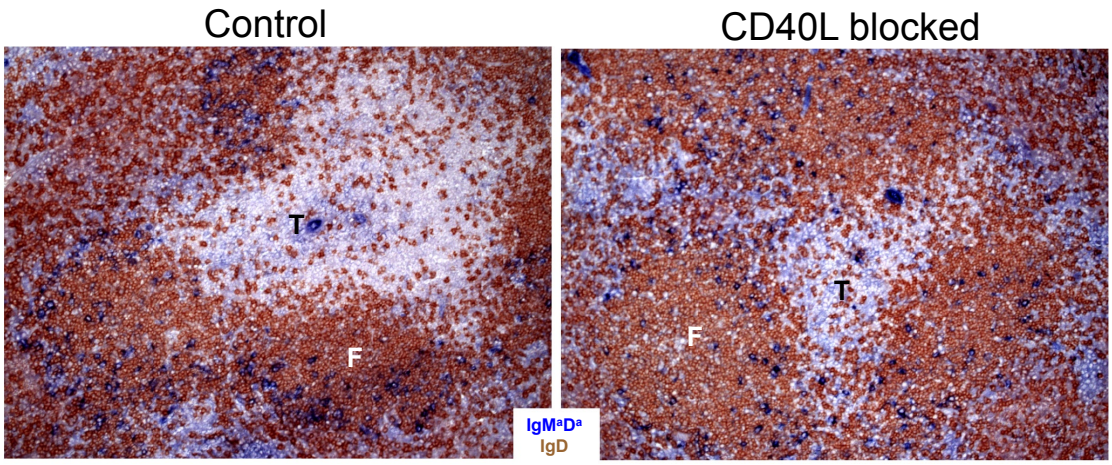


Figure 4. CD40 engagement promotes movement of antigen-activated B cells to the outer follicle. (A) Distribution of activated WT and EBI2 KO MD4 B cells in the spleen at day 2 of the response to HEL-OVA in adjuvant in the presence of OTII helper T cells. (B) EBI2 transcript abundance in sorted day 2 activated WT MD4 B cells (of the type in A) and endogenous B cells. Data are pooled from three experiments. (C) CCR7 surface abundance on activated B cells at day 1 and day 2 of the T-dependent response in mice of the type in A. Data are representative of two experiments. (D) Distribution of WT (upper panels) or EBI2 KO (lower panels) MD4 B cells in the spleen of CD40-deficient hosts, 2 days after immunization with HEL and treatment without or with anti-CD40. (E) Distribution of activated WT and CD40 KO B cells in the spleen at day 2 following co-transfer into bm12 recipients. Serial sections were stained to detect WT (IgM^aD^a) or CD40 KO (CFSE) transferred B cells (blue) and endogenous B cells (IgD, brown). (F) Distribution of WT and CD40 KO MD4 B cells in the spleen at day 2 of the response to HEL-OVA in adjuvant in the presence of OTII helper T cells. Transferred MD4 B cells in A, D and F were detected by staining for IgM^a and IgD^a (blue) and endogenous B cells with antibodies to total IgD (brown).

the antigen-engaged B cells were eliminated by 2 days of antigen exposure (Fig. 4d) as expected (Cyster and Goodnow, 1995). These findings suggest that CD40 engagement may be sufficient to augment EBI2 function in antigen-activated B cells, helping facilitate their movement to inter and outer follicular regions.

To test whether CD40 engagement during receipt of cognate T cell help was necessary for B cell movement to the outer follicle, we examined the distribution of activated CD40-deficient B cells in two T-dependent systems. First, BCR-stimulated WT or CD40-deficient B cells were adoptively transferred into coisogenic bm12 mice, which provide T cell help from I-A^b responsive T cells (Cyster and Goodnow, 1995). After 2 days, as expected, WT B cells became concentrated in the outer follicle. By contrast, CD40-deficient B cells did not become enriched in this region and instead were dispersed throughout the follicle (Fig. 4e). Second, the positioning of CD40-deficient MD4 B cells was analyzed at day 2 following T-dependent immunization. While the majority of WT B cells had relocalized to the outer follicle (Fig. 4f), CD40-deficient B cells were not uniformly positioned at this location, and instead were also found throughout the follicle. Finally, the role of CD40 signaling in promoting localization of activated B cells to the back of the follicle was investigated by blocking CD40L at day 1 following immunization (Suppl. Fig. S3). CD40L-blocking decreased the propensity of activated B cells to localize to the back of the follicle, and many remained localized near the B-T boundary. Together, these results suggest that, in addition to supporting activated B cell survival, CD40 transmits signals that promote localization to the outer follicle.



Supplemental Figure 3: CD40L blockade decreases activated B cell relocalization to outer and inter follicular areas: Distribution of activated WT MD4 B cells at day 2 of the response to HEL-OVA in adjuvant in the presence of OTII helper T cells in untreated (left) or 24 hour CD40L-blocked (right) WT recipient spleen.

Detection and properties of EBI2 ligand bioactivity. To test for the presence of EBI2 ligand within lymphoid tissues we generated tissue extracts using a procedure we had previously employed in our analysis of interstitial S1P concentrations (Schwab et al., 2005). To our surprise, extracts prepared from spleen, LNs and thymus showed a readily detectable attractant activity for EBI2-transduced but not control cells (Fig. 5a). Bioactivity was also detected in a number of non-lymphoid tissues including brain, kidney, liver and lung, but not plasma (Fig. 5a). Chemoattraction by this bioactivity was sensitive to pertussis toxin (PTX) pretreatment of the EBI2-expressing cells (Fig. 5b), providing evidence that EBI2 is a Gi-coupled receptor, in agreement with a previous report (Rosenkilde et al., 2006). We next tested if the bioactivity was proteinaceous in nature by treatment with proteinase K. While this treatment readily destroyed SDF1 (CXCL12) activity, it had no effect on the EBI2 ligand activity (Fig. 5c). The resistance of EBI2 ligand to digestion was not a consequence of inhibitory effects of the tissue extract because SDF1 could still be inactivated by proteinase K following mixing with tissue extract (Fig. 5c). The bioactivity bound to a C18 reverse-phase matrix and was eluted with 60% acetonitrile, providing evidence that it was hydrophobic in character (Fig. 5d), a property that was further established during HPLC-based purification efforts (Suppl. Fig. S4).

We also found that bioactivity was generated in the culture supernatants of a number of cell lines, including HEK293 cells (Fig. 5e). Given the protease resistant and hydrophobic nature of the activity, we tested whether treating cell cultures with inhibitors of lipid biosynthetic pathways altered ligand production.

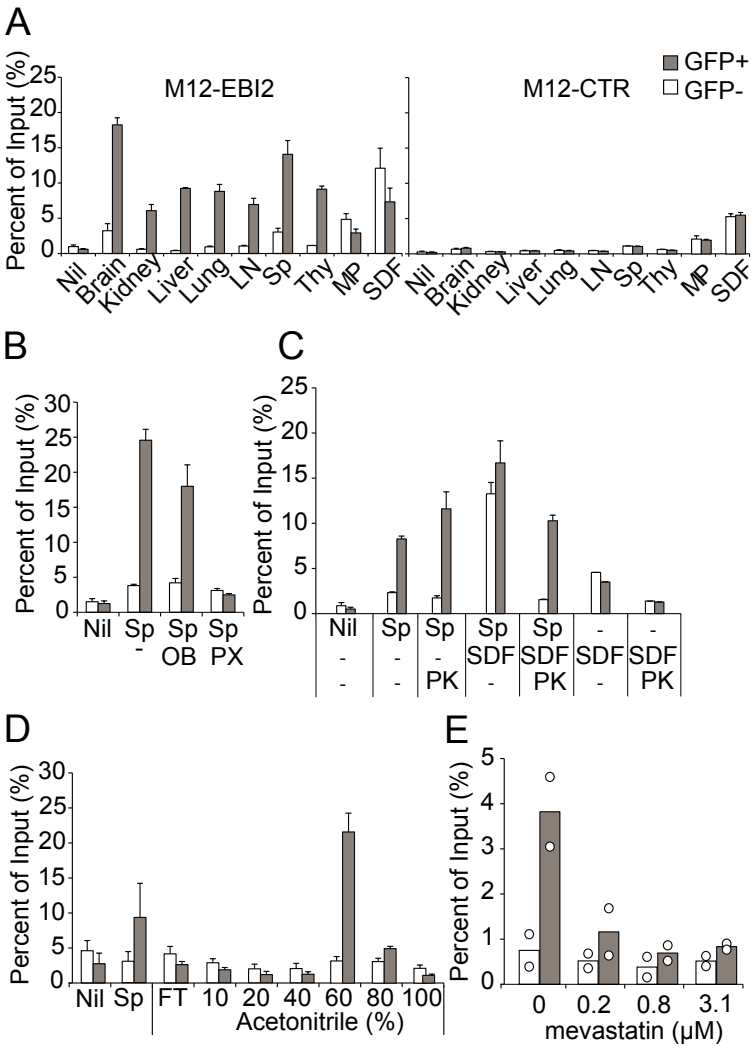
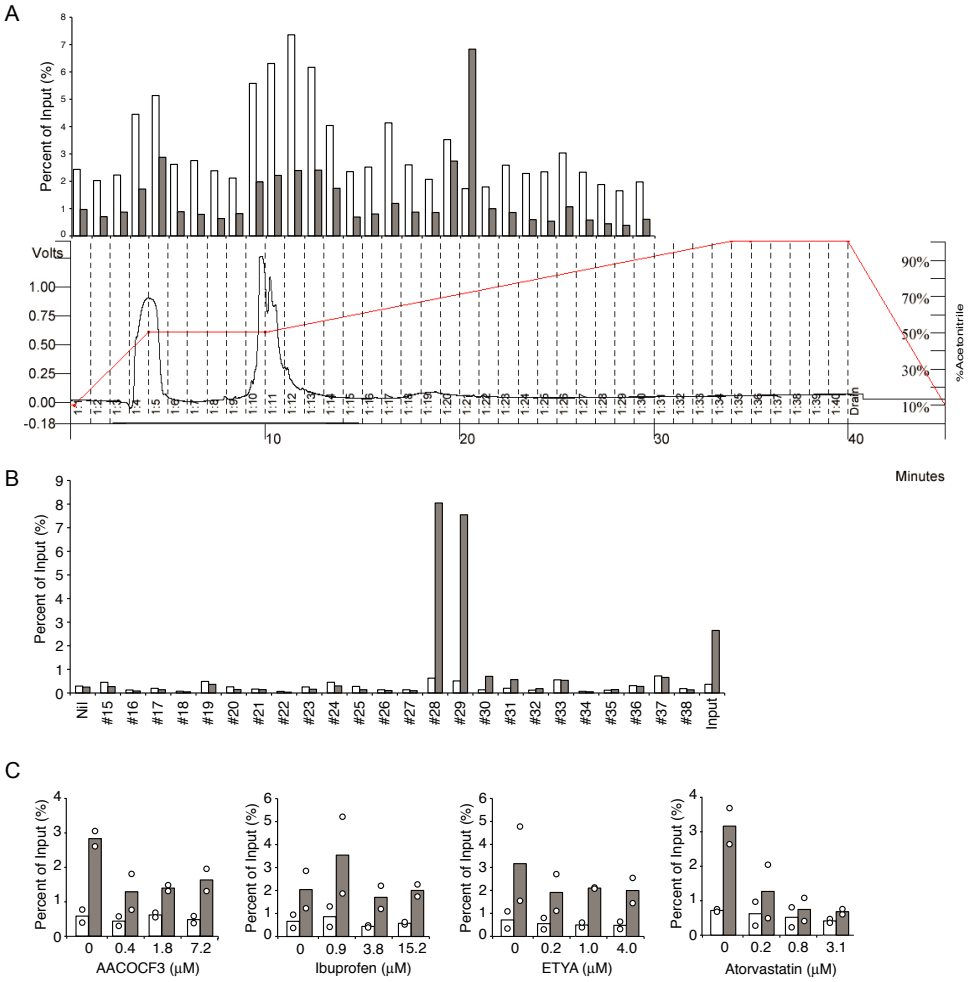


Figure 5. Detection of a proteinase K resistant, hydrophobic EBI2 ligand bioactivity in multiple tissues and statin-sensitivity of bioactivity generation by HEK293 cells. (A) Extracts from the indicated tissues were tested for their ability to attract EBI2-IRES-GFP transduced (GFP+, gray bars) compared to untransduced (GFP-, open bars) M12 cells in the same samples (left graph); control-IRES-GFP vector (CTR) transduced M12 cells were similarly analyzed (right graph). Sp, spleen; Thy, thymus; MP, mouse plasma. Nil indicates medium alone. (B) Migration response of control, PTX or oligomer B (OB, inactive PTX subunit) pretreated EBI2-IRES-GFP transduced M12 cells to spleen extract (Sp). Open bars, GFP- (EBI2-) and gray bars GFP+ (EBI2+) cells. (C) Migration response of transduced M12 cells to proteinase K (PK) treated spleen extract, SDF1 or spleen extract plus SDF1. (D) Migration response of transduced M12 cells to tissue extract fractions eluted from a C18 sep-pak column with the indicated amounts of acetonitrile. Sp, starting spleen extract; FT, flow through. (E) Migration response of transduced M12 cells to culture supernatants from HEK293 cells incubated in the absence or presence of the indicated concentrations of mevastatin. Data in C-E are plotted as in B.

Inhibitors of phospholipase A2 (AACOCF3), cyclooxygenase (ibuprofen) and lipoxygenase (ETYA) pathways had variable but not convincing inhibitory effects (Suppl. Fig. S4). However, treatment with either of two statins, inhibitors of HMG-CoA reductase, led to a decrease in migration of EBI2-transduced cells without affecting the background migration of the control cells (Fig. 5e and Suppl. Fig. S4). These observations provide evidence that EBI2 ligand biosynthesis depends on cells having an intact cholesterol biosynthetic pathway.

CD40 engagement promotes sustained EBI2-dependent responsiveness to bioactivity. We took advantage of the identification of EBI2 ligand activity in tissue extracts to test whether EBI2-dependent chemotactic function was detectable in day 2 activated B cells, a time point when EBI2 transcript levels were slightly reduced but the *in vivo* positioning data showed EBI2 was highly functional (Fig. 4). Indeed, chemotaxis assays with cells harvested at day 2 of the T-dependent response showed migration to spleen extracts that was EBI2-dependent (Fig. 6a). Endogenous (naïve) B cells did not show an EBI2-dependent response, likely because the extracts contained only low amounts of ligand (Fig. 6a). Moreover, stimulation of HEL-antigen exposed B cells for 2 days with anti-CD40 led to an EBI2-dependent migratory response to spleen extracts (Fig. 6b). Extracts prepared from CXCL13-deficient spleens and thus lacking this efficacious B cell attractant revealed even more clearly the EBI2-dependent migration of cells activated by antigen plus anti-CD40 (Fig. 6b), whereas cells exposed to antigen only did not demonstrate an EBI2-dependent migratory



Supplemental Figure 4: Detection of hydrophobic EBI2 ligand bioactivity and statin-sensitivity of bioactivity generation by HEK293 cells: (A) Migration response of EBI2-IRES-GFP transduced (GFP+, grey bars) and untransduced (GFP-, white bars) M12 cells to tissue extract fractions eluted with a 10-90% acetonitril gradient by HPLC. (B) Migration response of EBI2-IRES-GFP transduced (GFP+, grey bars) and untransduced (GFP-, white bars) M12 cells to tissue extract fractions 15-38. (C) Migration response of EBI2-IRES-GFP transduced (GFP+, grey bars) and untransduced (GFP-, white bars) M12 cells to culture supernatants from HEK293 cells incubated in the absence or presence of the indicated inhibitors of lipid biosynthetic pathways.

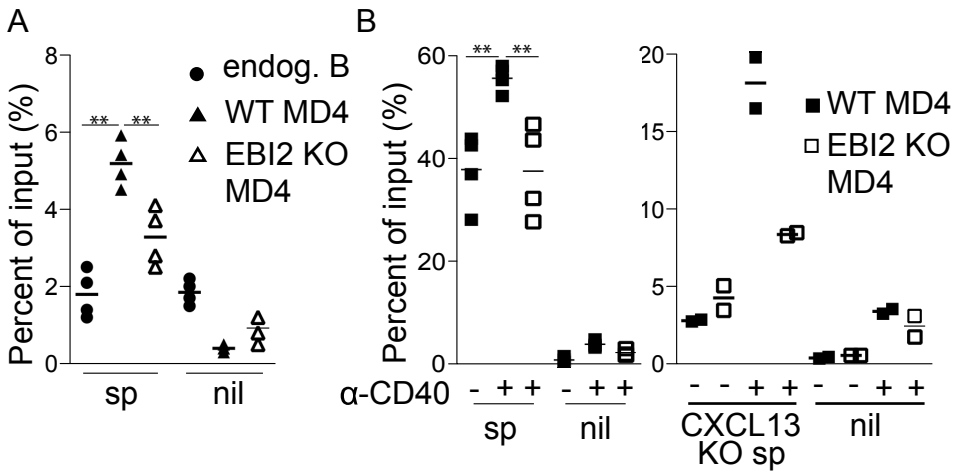


Figure 6. EBI2-dependent migration response of day 2 activated B cells. (A) Migration of day 2 activated WT and EBI2 KO MD4 B cells, and endogenous B cells, to spleen extract (sp) or medium alone (nil). Data are from two mice. (B) Migration of WT and EBI2 KO MD4 B cells activated by exposure to HEL antigen and *in vitro* culture with anti-CD40 to spleen extracts from WT (left panel) and CXCL13-deficient (right panel) mice (**P<0.01, unpaired, two-tailed Student's t- test).

response in this assay (Fig. 6b). The EBI2-independent bioactivity present in CXCL13-deficient extracts likely reflects the presence of other chemoattractants of activated B cells such as SDF1 and CCL21. These findings provide strong evidence that despite the slight reduction in mRNA abundance, EBI2 function is elevated in antigen-exposed B cells by CD40 engagement.

Discussion

The above studies demonstrate that in the first hours after BCR engagement EBI2 is transcriptionally upregulated and mediates attraction of B cells to the outer (T-zone distal) follicle. At 6-10 hours, CCR7 upregulation dominantly influences cell location but EBI2 functions together with CXCR5 to distribute the activated cells along the length of the B-T boundary. Subsequent movement of activated B cells to inter and outer follicular regions is promoted by CD40 engagement and is associated with sustained high EBI2 function. Finally, we demonstrate that EBI2 functions as a Gi-coupled chemoattractant receptor and provide evidence that EBI2 ligand is a lipid and is present not only in lymphoid tissues but in many non-lymphoid tissues. The widespread distribution of ligand is consistent with our finding that EBI2 is active in intestinal ILFs. These observations coupled with the presence of EBI2 in multiple hematopoietic cell types and the recent genetic evidence that EBI2 may regulate an inflammatory gene network (Heinig et al., 2010) suggest a broad role for this receptor in the immune system.

The propensity of WT B cells to localize to the outer follicle of EBI2-deficient mice demonstrates that the receptor is active in naïve B cells. However, naïve B cell migration to the outer follicle can take place in the absence of EBI2. Since the CXCR5 ligand, CXCL13 is abundant in the outer follicle (Cyster, 2010), the sufficiency of CXCR5 in supporting cell movement to this region is not surprising. It will be important in future studies to determine whether EBI2 influences the dynamics of naïve B cell migration in the outer follicle even in the presence of

CXCR5. The basis for WT cells preferentially accumulating in the outer follicle in EBI2-deficient mice is not yet clear but might indicate that EBI2-expressing cells contribute to local depletion of ligand or provide a feedback signal that modulates local production. Analysis of EBI2 ligand bioactivity in EBI2-deficient mice has not revealed elevated production at the whole organ level indicating that any such alteration must be local. Alternatively, differences in the strength of attraction to the outer follicle of WT versus EBI2-deficient B cells might somehow lead to a competitive 'sorting out' of the cells.

The tight temporal coupling of EBI2 induction to BCR signaling suggests an important role for EBI2 during the early hours of B cell activation. Our studies suggest that at least part of this role is to promote a transient increase in migration to the outer follicle, prior to CCR7 upregulation and redirection to the T zone. The outer follicle in all lymphoid tissues is the most proximal region to sites of antigen (Cyster, 2010). Recent studies have highlighted a role for LN subcapsular sinus macrophages, located between the incoming lymph and the outer follicle, in presenting antigens to B cells (Cyster, 2010). It seems possible that B cells that have encountered low amounts of antigen in the follicle (or while entering the tissue from circulation) initially relocalize to the outer follicle to survey for further incoming antigen on such macrophages, improving their chance of internalizing sufficient antigen to later interact productively with helper T cells. Attraction to the outer follicle might also increase exposure to IFNa/b and other cytokines or innate stimuli reaching the tissue from sites of infection, helping instruct appropriate differentiation of the cells.

CCR7 and CCR7 ligands are critical for movement of 6 hour activated B cells to the B-T boundary. Our finding that the reverse movement in the absence of CCR7 function – to the outer follicle (Reif et al., 2002) – is EBI2 dependent provides *in vivo* evidence that EBI2 is highly active in 6 hour activated B cells. Thus, CCR7 normally comes to dominate over the EBI2-dependent outer-follicle tropism by 6 hours, and the time course of CCR7 upregulation is consistent with this delayed effect. The activity of EBI2 in helping to retain and distribute activated B cells along the length of the B-T boundary may contribute to ensuring efficient B-T interaction. These observations indicate EBI2 ligand is present at the B-T boundary as well as in inter and outer follicular regions, a suggestion supported by the circumferential distribution of WT naïve B cells around follicles in spleens of mixed bone marrow chimeras (Suppl. Fig. 1 and (Pereira et al., 2009b)). The propensity of EBI2 over-expressing cells to travel selectively to the outer follicle (Pereira et al., 2009b) could indicate that ligand concentration is highest in this region but might also reflect the outcome of the concerted action of EBI2 and CXCR5 relative to CCR7 in the activated B cells used in such retroviral transduction experiments.

Although EBI2 transcripts appear to be reduced in B cells that have been activated for 2 days in the presence of helper T cells, our *in vivo* data show that EBI2 is active in positioning the cells at this time and our *in vitro* studies provide evidence that EBI2 has elevated chemotactic function in these cells. We provide evidence that a key T cell-derived signal promoting high EBI2 function is CD40L engagement of CD40 on the B cell. Determining the basis for this augmenting

effect of CD40 signaling will require development of tools to study EBI2 protein abundance on the cell surface and within the cell. Additionally, while CD40-deficient B cells have reduced access to the outer follicle following T cell help, they were not excluded from this area to the extent of EBI2-deficient B cells, suggesting that further T-cell derived signals promote EBI2-mediated positioning during an immune response.

The widespread distribution of EBI2 ligand activity, including production by HEK293 cells, might explain why a previous study concluded EBI2 had constitutive activity (Rosenkilde et al., 2006); HEK293 cells were one of the cell types used in that study. The properties of the EBI2 ligand bioactivity from tissue extracts and the sensitivity of ligand production by HEK293 cells to statins suggests that it is a lipid whose synthesis depends on an intact cholesterol biosynthetic pathway. Consistent with these data, a recent patent publication reported identification of 7α,25-dihydroxycholesterol and 7α,27-dihydroxycholesterol as EBI2 ligands present in inflamed sheep and pig liver (patent # WO/2010/066689). It will be important in future studies to test whether these oxysterols are physiological EBI2 ligands in lymphoid and non-lymphoid tissues. It will also be important to determine the key cell types producing EBI2 ligand within lymphoid tissues. The detection of EBI2 ligand bioactivity in multiple organs suggests that EBI2 will have functions beyond regulating B cell responses. Consistent with this prediction, genetic studies in rats recently linked polymorphisms in the EBI2 promoter to differences in the inflammatory state of a number of organs including the kidney, liver and pancreas (Heinig et al., 2010).

Polymorphisms in human EBI2 were also associated with type I diabetes and other inflammatory diseases (Heinig et al., 2010). EBI2 is expressed in a range of myeloid cells as well as some T cells (Heinig et al., 2010; Pereira et al., 2009b) and the rat studies suggested EBI2 may regulate IRF7-mediated gene expression in macrophages (Heinig et al., 2010). We can therefore anticipate a broad role for EBI2 in influencing cell migration and immune function during innate as well as adaptive immune responses.

Acknowledgments

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Chapter 4

Conclusion

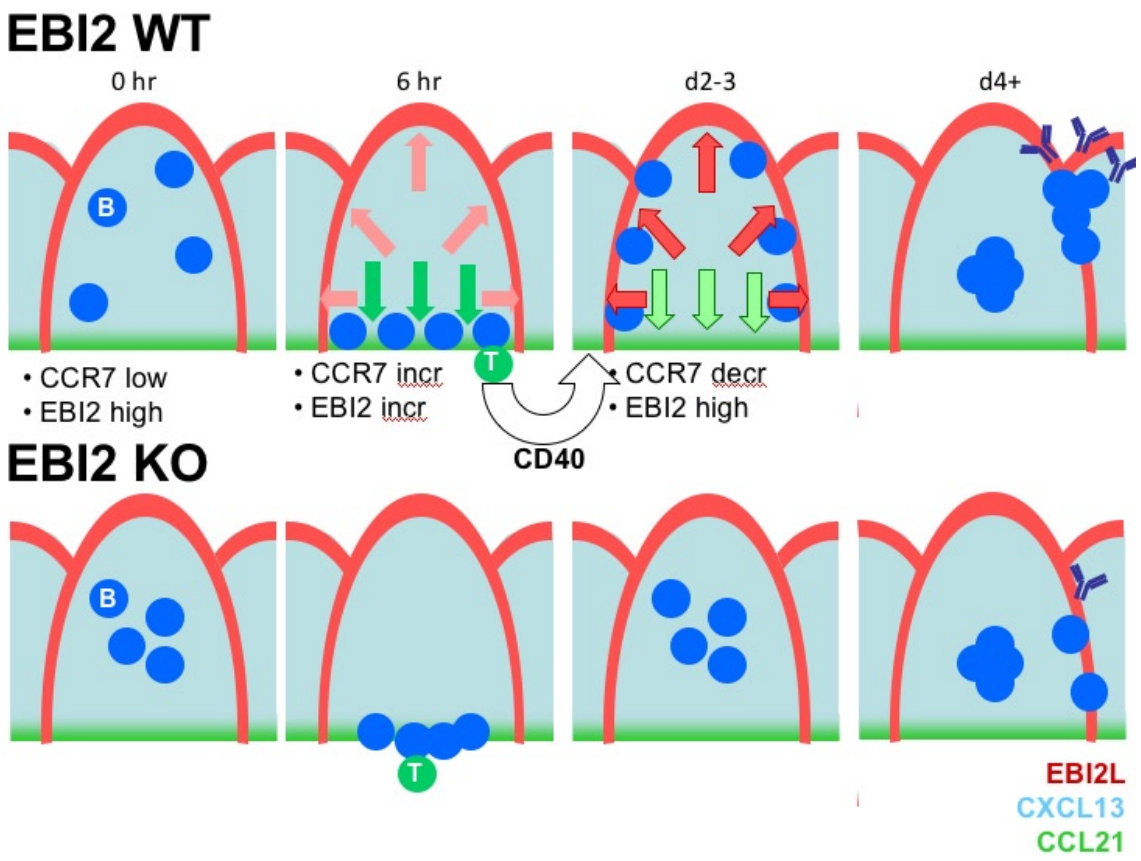

Figure 1: Model of B cell positioning by EBI2 during homeostatis and an immune response. Naïve WT B cells (top) express CXCR5 and EBI2, which allow them to localize to and migrate throughout the follicle, in which CXCL13 is displayed (light blue). In the absence of EBI2, B cells still localize to the follicle in a CXCR5-dependent manner, but tend to be found near the center of the follicle (bottom). Within 6 hours of B cell activation by cognate Ag, both EBI2 and CCR7 have increased activity, first positioning activated B cells to the OF in an EBI2-dependent manner (pink arrow), and then bringing them to the B:T boundary in a CCR7-dependent manner (green arrows), drawn by CCL21 in the T zone (green). EBI2 KO B cells also migrate to the B:T boundary, but are not evenly distributed toward IF regions and sometimes extend further into the T zone. By d2-3, activated B cells interact with T cells and receive CD40 stimulation. CCR7 surface abundance is decreased (light green arrows), while EBI2 functionality increases (red arrows), leading to positioning of B cells at the OF and IF. Without EBI2, B cells are unable to reach the OF and IF (bottom). By d4, some B cells have downregulated EBI2 and migrated to the center of the follicle to become GC B cells, while other B cells remain at the OF and IF and become ASC (top). EBI2 KO B cells are preferentially found toward the center of the follicle and have decreased propensity to become ASC, leading to decreased Ab production (bottom). Based on these observations, we propose EBI2L (red) is present in the OF and IF, and low in the center of the follicle.

Dynamic B cell positioning

We have shown a role for EBI2 in the positioning of naive and activated B cells, and demonstrated that it is necessary for efficient PB generation and Ab production (Chapter 2). However, there are many questions that remain regarding the role of EBI2 in B cells. We do not know how EBI2 contributes to real-time B cell migration dynamically and rapidly. It will be interesting to determine if EBI2 plays a role in B cell movement, both homeostatically and during an immune response, using two-photon microscopy. EBI2 could play a role in determining B cell velocity and turning angles, especially as cells approach the OF. Future analysis needs to determine to the distribution of ligand in the follicle. We have used indirect evidence to posit that the concentration of EBI2L is low at the center of the follicle and deep T zone, and relatively high at the edges of the follicle. CXCL13 is distributed throughout the follicle, including the outer follicle, so we would expect EBI2 KO B cells to still gain access to the OF normally without competition. They might have different migration kinetics at the outer follicle, however. Additionally, naïve B cells require EBI2 to efficiently access the outer follicle when in competition with other B cells (Chapter 2); it would be interesting to determine if EBI2 KO B cells simply slow down when migrating toward the OF or if they actively turn around. It would also be interesting to determine if EBI2 KO B cells actually interact with FDC more than WT B cells. EBI2 KO B cells seem to reside toward the center of the follicle in static sections, but whether they interact more with FDC, or just migrate near them is unknown. Additionally, we have observed the same effect noted by

another group, that EBI2 KO B cells home to the center of the follicle, but do not enter into GC (Gatto et al., 2009). This may be due to lack of expression of S1P2, which is expressed on GC B cells and helps confine them to the GC (Green et al., 2011). It would be interesting to observe this migration pattern dynamically as well to determine how EBI2 KO B cells travel around the GC and if they have an increased probability of entering it compared to the rare WT naive B cells that have been observed to do so. The function of homeostatic access to the outer follicle is unknown. It is possible this allows more rapid access to Ag arriving from outside the follicle, although this has been technically difficult to demonstrate ex vivo. Using intravital microscopy could be a useful step in preserving the architecture of the LN itself as well as the lymphatic structures and subcapsular sinus. Ag could then be administered subcutaneously and Ag capture by EBI2 WT or KO B cells monitored in real-time to determine if EBI2 plays a role in this process; additionally, EBI2 may act in conjunction with other GPCRs in these processes, which would also warrant investigation.

Early activation

EBI2 is induced early after B cell activation, and we have shown that this induction repositions activated B cells to the outer follicle within hours, before later migration to the B:T boundary (Chapter 3). The importance of this movement is unknown, although it also appears to be present soon after viral infection (Scandella et al., 2007). It is possible that a BCR signal induces EBI2 to promote the search for further Ag or other stimulation in the outer follicle. This

could increase B cell exposure to Ag and the chance for B cells to encounter the highest affinity Ag possible. We have shown that EBI2 is required for efficient PB generation, but it is unclear at what point between initial activation and PB development is affected. B cells that lack EBI2 do not localize to IF/OF areas at d2 of the immune response, but is it this positioning defect that leads to decreased PB production, or is interaction with the cognate T cell affected? We have attempted to look at early activation markers of B cells and have not observed a difference between WT and EBI2 KO B cells. Another group has observed that EBI2-deficient B cells have reduced proliferation early in the immune response, although we have not reproducibly seen this effect (Gatto et al., 2009). It would be interesting to observe dynamic interactions by two-photon microscopy between EBI2 deficient B cells and WT T cells to see if there is a defect in the B:T interaction. Alternatively, the B:T interaction could occur normally, but the cells simply may not localize to the IF/OF. If it were necessary for B cells to localize to this area to become PB, then this may be responsible for the defect.

EBI2 and IF/OF

The nuances of the B cell follicle anatomy clearly require further exploration. We have shown that EBI2 can direct cells to the OF and IF zones by d2 of the immune response, but these areas, and the signals they may convey to B cells, are still ill-defined (Chapter 2). The nature of the OF and IF zones will likely also differ depending on the lymphoid tissue. As discussed above, the

spleen is exposed to Ag from the blood into the marginal sinus, whereas Ag drains to the subcapsular sinus in the LN, so the OF in each location may be exposed to different Ag and process them differently. The marginal zone is only present in the spleen, and plays a role in Ag capture from the blood. Additionally, the IF zones in the spleen may be near bridging channels, which do not exist in LN. One group has observed that activated B cells migrate more extensively to IF zones in the LN, while in the spleen they are sometimes less congregated to these areas and instead more evenly distributed along the B:T boundary (Kerfoot et al., 2011). The IF area has been observed as the first site of bcl6 induction (Kerfoot et al., 2011; Kitano et al., 2011). Bcl6 has observed in B cells at the IF, but not OF, early in the immune response, suggesting that GC B cells arise from activated B cells positioned at the IF, while PB may arise from activated cells at the OF (Kerfoot et al., 2011). How these distinct, although potentially interconnected, populations might arise is unknown, as is whether the fate decision might occur before or after localization to these areas. Ag exposure and T cell help may be different at these two locations. It is also unknown whether this same pattern is observed in the spleen.

We have shown that EBI2 promotes activated B cell localization to the outer follicle and is required for full development of early, isotype switched PB (Chapter 2). We hypothesize that cues seen in outer and interfollicular areas promote PB development and isotype switching, but the nature of these cues remains unknown.

Despite initial reports that did not observe T cells at the OF (Coffey et al., 2009) possibly owing to a lack of clearly trackable T cells, cognate T cells also localize to the IF with B cells, and could continue to provide signals to activated B cells (Kerfoot et al., 2011; Kitano et al., 2011). This could provide a location for B and T cells to undergo continued interaction. However, it remains to be determined if EBI2 deficient B cells still colocalize with cognate T cells, despite the mispositioning of activated B cells to the follicle center by d2.

CD11c+ staining is clearly observed in IF spaces (Steinman et al., 1997), but the role of DCs here is not clear. It has been suggested that these DCs interact with B cells in T-independent responses, but ablation of DCs has not affected the early T-independent response to virus or NP-Ficoll (Hebel et al., 2006; Scandella et al., 2007). Some of the dendritic cells in these areas in the spleen that present Ag carry it in from the blood (Balazs et al., 2002). It is possible that DCs interact with B cells during T-dependent immune responses, as they have been proposed to do in T-independent responses and upon initial Ag exposure to naive B cells (Qi et al., 2006). It is also possible that DCs in these areas can interact with helper T cells that relocalize to the IF/OF and deliver signals to them, thus indirectly affecting the B cell response.

Activated B cells in the OF and IF may also interact with macrophages. During infection, SCS macrophages and other cell types may become infected. These cells can block further infection of other cell types and also produce effector molecules such as type I IFN which can subsequently influence the immune responses directly and by recruiting other cell types (Iannacone et al.,

2010). It is interesting to consider the role of infected macrophages both in producing effector molecules that may skew the outer follicle milieu, and as a continuous source of Ag. Subcutaneously administered IC are rapidly cleared from SCS macrophages, resulting in complete transfer to FDC by 8 hr (Phan et al., 2007). However, during an infection, Ag may continuously be generated in the periphery to drain to the LN, or may be continuously produced locally. As these macrophages are not proficient at degrading Ag, it has been suggested that this site may be enriched for infection and Ag retention (Cyster, 2010). This may generate a more constant exposure to Ag at inter and outer follicular regions, and EBI2 may allow cells to efficiently access these areas.

EBI2 is required for B cells to localize to outer and inter follicular areas at d2 of a T-dependent immune response (Gatto et al., 2009; Chapter 2). However, by d3-4, EBI2-deficient B cells are able to localize toward the outer follicle, and PB and GC cells appear normally positioned. It is possible that chemokine receptors known to have roles in these processes are sufficient to direct activated B cells to the appropriate positions even in the absence of EBI2, ie, continued downregulation of CCR7 to allow activated cells to migrate away from the T zone and T zone-proximal follicle area, and downregulation of CXCR5 and upregulation of CXCR4 to position PB. However, it is also possible that other receptors may direct activated B cells to the outer follicle, with delayed action compared to EBI2. CXCR3 recognizes the inflammatory chemokine CXCL9, which is induced in the outer follicle in some infections. CXCR3 has recently been shown to be upregulated on CD8+ T cells by d3 of a viral infection and is

required for responding T cells to migrate out of the T zone to infected MZM in the OF (Hu et al., 2011). It will be interesting to determine if CXCR3 has a role in positioning activated B cells to this area with delayed kinetics relative to EBI2.

EBI2 and early PB generation

EBI2 deficiency leads to decreased generation of PB and serum Ab, but this effect has been variable depending on the system examined. In the T-dependent immune response to NP-KLH, we observed significantly decreased Ag-specific serum IgG1, while Ag-specific IgM production was not decreased (Chapter 2). Another group has shown that in response to the T-dependent response to SRBC, production of IgG1+ PB is significantly decreased, and we have confirmed this observation, and noted that IgM+ PB are increased in a B cell intrinsic manner (Gatto et al., 2009). In response to HEL-SRBC immunization, total PB generation is decreased in EBI2 deficient B cells, and both antigen-specific IgG1 and IgM is decreased; conversely, activated B cells constitutively expressing EBI2 were shown to have both increased IgM and IgG1 in serum (Gatto et al., 2009). In some circumstances, it seems that EBI2 is required for total PB generation, while in others, isotype switching is also affected. The differences among these systems have not yet been reconciled. It is possible that Ag affinity and density is different in these systems, although Gatto et al report similar observations in unpublished data with Ag over a 10,000-fold range (Gatto et al., 2009). Furthermore, effects on isotype switching have so far only been

observed in switching to IgG1, so it will be important to determine if this effect extends to other isotypes.

EBI2 may also be more relevant in response to certain Ags and immunizations. As discussed above, Ag distribution varies depending on Ag size and solubility. EBI2 may be most important during immune responses in which Ag is preferentially retained near the OF, which may involve large Ag, chronic infection in which Ag is continually draining to the OF, or locally produced Ag in the case of infected CD169+ macrophages, for example. Most of the investigations into the role of EBI2 in the immune response have focused on the spleen, although similar homing patterns are observed in pLN (Pereira et al., 2010). It may be interesting to examine responses in the LN induced by subcutaneous immunization.

EBI2 and GC B cells

The activity of EBI2 in B cells later in the immune response will also be interesting to analyze. EBI2 transcripts are downregulated in GC B cells and the GFP reporter is strongly downregulated as well, leading GCs to appear as GFP negative “islands” in the center of follicles, surrounded by GFP-positive naive B cells in sections of EBI2-GFP/+ mouse lymphoid tissues (Chapter 2). Bcl6 is highly expressed by GC B cells and targets EBI2, leading to repression of EBI2 (Basso et al., 2010; Shaffer et al., 2001); a bcl6 hypomorph has recently been reported to be defective in EBI2 downregulation in preGC B cells (Kitano et al., 2011). We have not observed perturbed organization of the GC in the absence of

EBI2, as expected since EBI2 is normally downregulated in these cells. However, we have not analyzed GC organization of EBI2 KO B cells in competition with WT. Centrocytes express higher amounts of EBI2 than centroblasts, although whether this effect is from late centrocytes which may be exiting the GC and re-expressing EBI2 or from contamination of the centrocyte population with EBI2-expressing memory cells, is unknown (Luckey et al., 2006).

Memory B cell localization is still under investigation, and it is difficult to stain for these cells in sections as they express some markers common to naive or GC B cells. However, recent attempts have suggested that IgM+ memory cells are found in the B cell follicle, while IgG+ memory cells exist adjacent to residual germinal centers (Aiba et al., 2010). Re-expression of EBI2 could help memory B cells leave the GC, and analysis of the localization of EBI2 KO memory B cells could help test this hypothesis. We have not seen a difference in the number of GFP-expressing B cells still residing in GC of EBI2GFP/GFP (KO) versus EBI2GFP/+ mice, implying that EBI2 is not required for exiting the GC. However, without careful analysis of memory B cell numbers and survival, it is not clear if memory B cells have decreased survival without EBI2.

EBI2 and ASC

EBI2 transcripts in ASC appear to be decreased compared to naïve B cells, although to a lesser extent than in GC (Gatto and Brink, 2010; Luckey et al., 2006), but EBI2 reporter GFP is still expressed in these cells. It remains to be determined if EBI2 plays a role in ASC localization. ASC localize to areas that we

predict have high EBI2L concentrations, such as bridging channels of the spleen, which may reflect ongoing contributions of EBI2 to ASC localization. However, decreased CXCR5 and increased CXCR4 expression may be sufficient to account for ASC localization to the red pulp. Thus far, EBI2-deficient ASC have not been observed to be mislocalized, although subtle effects in positioning may be difficult to discern in the red pulp. CXCR4-deficient ASCs seem to reside closer to the follicles than WT ASCs, which may reflect ongoing activity of EBI2 in these cells (Hargreaves et al., 2001).

EBI2 regulation

So far, EBI2 expression has been tracked at the transcriptional and functional level. Use of an EBI2-GFP reporter line has also been informative for predicting EBI2 protein expression, although the long half-life of GFP may confound these results (Chapter 2). EBI2 transcript analysis does not always seem to faithfully report the amount of functional EBI2 expressed by a cell. While the initial increase of EBI2 within an hour following BCR stimulation is reflected in EBI2 activity in positioning B cells at this timepoint, at later points, such as d2 of the immune response, EBI2 is functionally upregulated despite being transcriptionally decreased compared to naive B cells (Chapter 3). Activated cells may still have a high amount of EBI2 while having less than naive B cells, since naïve B cells highly express EBI2. However, decreased transcription and increased function may suggest that EBI2 can be regulated post-transcriptionally. The half-life of EBI2 protein is unknown. It will be useful to develop tools to track

EBI2 protein, for example to monitor surface levels of EBI2 throughout the immune response.

EBI2L

EBI2 was originally identified as an orphan GPCR, and its ligand has been very recently discovered to be 7α,25-dihydroxycholesterol (7α,25-OHC) (Hannedouche et al., 2011; Liu et al., 2011). Cells migrate to 7α,25-OHC and related oxysterols in an EBI2-dependent manner. The details of the production, regulation, and degradation of EBI2L are still under intense investigation. Since EBI2 is required for B cells to access the OF, and since EBI2 deficient B cells cluster toward the center of the follicle, we have hypothesized the EBI2L concentration is highest at the edges of the follicle and decreases toward the center. It is not known whether this gradient is established due to production of ligand at the OF with a passive decrease in concentration toward the center, or if ligand concentration is actively reduced toward the center due to degradation there. We observed the lymphotoxin blockade perturbs EBI2-mediated segregation, implying that the EBI2L gradient is disrupted in this process. A variety of cells that may establish or maintain the gradient are lymphotoxin dependent, including FDC, MRC, and CD169+ macrophages, so it will be interesting to investigate which of these cells are important in this process (Pereira et al., 2010). The generation of 7α,25-OHC requires cellular cholesterol 25-hydroxylase and oxysterol 7α-hydroxylase (cyp7b1), so the localization and regulation of these enzymes is also being analyzed.

Chemokine production is known to change during infection and immunization. While inflammatory chemokines are induced, homeostatic chemokine production also changes. CCL21 and CXCL13 transcript and protein abundance is transiently decreased in the spleen following viral or bacterial infection, as well as immunizations (Mueller et al., 2007). While CXCL13 protein is normally found throughout the follicle, especially toward the center on FDC but also more broadly, during infection, central follicular CXCL13 is decreased. At least in some circumstances, CXCL13 can be seen on ER-TR7+ stromal cells in the outer follicle, which may help activated cells move to the edge of the follicle during infection or immunization, or decrease their attraction toward the center (Mueller and Ahmed, 2008). It will be interesting to examine EBI2L production throughout infection and immunization; however, this effect required IFNgamma and thus may be most relevant in Th1 immune responses, while EBI2 may play a more dominant role in Th2 responses (Mueller et al., 2007). LPS may increase EBI2L production, showing that this chemoattractant may be modulated during infection (Hannedouche et al., 2011).

EBI2 and other B cell activation states

The role of EBI2 in T-independent responses remains unknown. Following T-independent immunizations, such as with NP-Ficoll, responding B cells have been shown to localize to the outer follicle and interfollicular areas of the spleen early after activation (Garcia de Vinuesa et al., 1999). However, the receptor that mediates this localization is not known. This may be particularly physiologically

relevant in response to blood-borne bacteria, in which cognate B cells, which may be enriched in the MZ, migrate to both the B:T boundary and IF or bridging zones in the spleen (Balazs et al., 2002). T-independent responses are divided into type I, induced by TLR activation, and type II, induced by BCR activation by repeating polysaccharides. TLR stimulation has thus far not shown increases in EBI2 transcript, although we have shown that EBI2 transcription does not always correlate with activity. We have not compared the ability of highly repetitive polysaccharides to modulate EBI2 activity through BCR activation.

The role of EBI2 in anergy remains unknown. Anergic B cells are excluded from the B cell follicle and reside at the B:T boundary. Unlike naive B cells that migrate to the B:T boundary upon recognition of cognate Ag, anergic B cells do not have increased CCR7 levels but instead depend on decreased expression of CXCR5 for this localization. CCR7-deficient anergic B cells localize to the outer follicle, suggesting that EBI2 may be active in anergic cells (Ekland et al., 2004). If so, EBI2 may help keep anergic B cells near the follicle, as we have shown that without EBI2, activated B cells may enter deeper into the T zone. We have shown this to be especially evident in the absence of CXCR5, and thus may be relevant for anergic cells which express decreased CXCR5 compared to naive B cells.

Summary

Here we have shown that the GPCR EBI2 plays a role in positioning naïve and activated B cells. EBI2 is highly expressed on naïve B cells, and this

expression is further increased immediately following B cell activation. EBI2 positions B cells to the OF and IF both before and after B cells receive T cell help. Downregulation of EBI2 is necessary for GC formation, and EBI2 is required for efficient generation of a T-dependent antibody response. The mechanism of this requirement is still under investigation, as are still broader roles of EBI2 and its ligand in many aspects of the immune response.

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