Published in final edited form as:

J Immunol. 2011 September 15; 187(6): 3026–3032. doi:10.4049/jimmunol.1101262.

EBI2 guides serial movements of activated B cells and ligand activity is detectable in lymphoid and non-lymphoid tissues¹

Lisa M. Kelly, João P. Pereira², Tangsheng Yi, Ying Xu, and Jason G. Cyster^{*}

Howard Hughes Medical Institute and Department of Microbiology and Immunology, University of California San Francisco, 513 Parnassus Ave., San Francisco, CA 94143

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 (1-3). Within the follicle B cells migrate at an average velocity of 6 $\mu\text{m}/\text{min}$ in a 'random' walk, surveying for antigens displayed by sinus-associated macrophages, follicular dendritic cells (FDCs), conduits, or that have diffused into the follicle (3). 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 (4). 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 (5, 6). Plasmablasts then emerge, particularly in interfollicular regions, and germinal center (GC) B cells soon accumulate at the follicle center (6, 7).

EBI2 is an orphan GPCR that was identified during a screen for EBV-induced genes (8). 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

¹JGC is an Investigator of the Howard Hughes Medical Institute. This work was supported in part by NIH grant AI40098.

^{*}Address correspondence to: Jason G. Cyster, Howard Hughes Medical Institute and Department of Microbiology and Immunology, University of California San Francisco, 513 Parnassus Ave., San Francisco, CA 94143-0414; phone (415) 502-6427; Fax (415) 502-8424; Jason.Cyster@ucsf.edu.

²Current address: Department of Immunobiology, Yale University School of Medicine, The Anlyan Center, 300 Cedar Street, S625B, New Haven, CT 06519

was also found in some T cells and myeloid cells (9-14). 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 (12, 13). As B cells differentiate into GC cells they downregulate EBI2 and this is important for the cells to participate in the GC response (12, 13). 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 (12, 13).

Based on sequence alignments, EBI2 has been clustered with a number of G-protein coupled receptor subgroups, most commonly with subsets of lipid receptors (15-17). Although one study suggested EBI2 may be a constitutively active receptor (16), the *in vivo* studies provided strong evidence that EBI2 is responsive to an extrinsic ligand (12, 13).

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^aThy1^aGPi1^a/J (IgMa) and bm12 mice were from The Jackson Laboratory. CXCR5^{-/-} (MGI 2158677; (18)), *plt* (MGI 1857881; (19)), EBI2^{-/-} (MGI 4399081; (12)), 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 (12). 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 (20), 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 4×10^7 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 NH₄Cl. 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 μ g HEL-OVA in RIBI adjuvant system (Sigma) one day after receiving splenocytes containing $5\text{--}10\times 10^6$ WT, EBI2 KO or CD40 KO MD4 B cells and $2.5\text{--}5\times 10^6$ 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 $10\text{--}40\times 10^6$ MD4 cells were transferred intravenously and the following day mice were injected with 1 mg HEL (Sigma Aldrich) intravenously. Splenocytes containing 30×10^6 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 μ g anti-CD40 (clone FGK4.5, UCSF Hybridoma Core). Mice were analyzed 36 h later. Bm12 experiments were performed as in (12). Purified and labeled wild-type and CD40-deficient B6 B cells (20×10^6 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 μ g ml⁻¹ anti-IgM (F(ab')₂ goat anti-mouse IgM, Jackson Immunoresearch). For homeostatic positioning, 30×10^6 B cells were isolated by negative selection as described and labeled with 2.5 μ M CFSE (Molecular Probes).

Immunohistochemistry and immunofluorescent microscopy

Tissue was prepared and 7 μ m cryosections were fixed and stained immunohistochemically as described (20) 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 μ m filter. Clean supernatants were collected after centrifugation and tested for bioactivity by transwell chemotaxis assays (12) of M12 B cell line transduced with an EBI2-IRES-GFP retroviral construct (12) 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% CO₂. In some cases, HEK293 cells were cultured in chemotaxis media containing the indicated concentrations of cyclooxygenase inhibitor (Ibuprofen, Sigma), cytosolic phospholipase A1 and A2 inhibitor (AACOCF₃, 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% CO₂.

EBI2 ligand fractionations

Mouse tissue extracts and HEK293 culture supernatants were prepared for reverse-phase high pressure liquid chromatography (RP-HPLC) by adjusting trifluoroacetic 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 (12, 13) (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 (22). 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) (13). 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. 2a) in agreement with other studies (9-11, 13). 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 (4), 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 (12, 13), 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-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 (13). 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 (19, 23). 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 (4)). 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.

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 (24). CXCR5-deficiency led to a less efficient distribution of cells along the boundary but did not allow their spread through the T zone (4). 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 (18, 25). 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). 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. 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. S2). 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.

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 (12, 13)). 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 (5, 26) and this was observed in our experiments (Fig. 4c). In the absence of T cell help, antigen-engaged B cells fail to relocate from the B-T boundary at

day 2 and many of the cells die in this location (27). 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 (27) 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 relocate to outer and inter follicular regions, while the EBI2 KO MD4 B cells did not relocate to these areas (Fig. 4d). In the absence of CD40 signaling, many of the antigen-engaged B cells were eliminated by 2 days of antigen exposure (Fig. 4d) as expected (27). 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-Ab responsive T cells (27). 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. 2e). 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 relocated to the outer follicle (Fig. 4a), 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.

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 (21). 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 (16). 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. 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 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 (14) 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 (3), 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 entry (3). 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 (3). It seems possible that B cells that have encountered low amounts of antigen in the follicle (or while entering the tissue from circulation) initially relocate 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 IFN α/β 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 (4) – 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 (12)). The propensity of EBI2 over-expressing cells to travel selectively to the outer follicle (12) 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 (16); 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 7 α ,25-dihydroxycholesterol and 7 α ,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 (14). Polymorphisms in human EBI2 were also associated with type I diabetes and other inflammatory diseases (14). EBI2 is expressed in a range of myeloid cells as well as some T cells (12, 14) and the rat studies suggested EBI2 may regulate IRF7-mediated gene expression in macrophages (14). We can therefore anticipate a broad role for EBI2 in influencing cell migration and immune function during innate as well as adaptive immune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Kevan Shokat and Morris Feldman for advice and use of HPLC, David Julius and David King for helpful suggestions regarding fractionation, Jinping An for excellent technical assistance and Jesse Green and Jagan Muppidi for comments on the manuscript.

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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 WT or EBI2^{-/-} 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.

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.

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.

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 (IgMaDa) 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).

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.

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).