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EBI2 Guides Serial Movements of Activated B Cells and Ligand Activity Is Detectable in Lymphoid and Nonlymphoid Tissues

Lisa M. Kelly, João P. Pereira,1 Tangsheng Yi, Ying Xu, and Jason G. Cyster

EBV-induced gene 2 (EBI2) was recently shown to direct the delayed movement of activated B cells to interfollicular and outer follicular regions of secondary lymphoid organs and to be required for mounting a normal T-dependent Ab response. In this study, we show that EBI2 promotes an early wave of Ag-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 zone–T zone 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 nonlymphoid tissues. Production of EBI2 ligand activity by a cell line is sensitive to statins, suggesting production in a 3-hydroxy-3-methyl-glutaryl-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 Ab response and they suggest that EBI2 responds to a broadly distributed lipid ligand. The Journal of Immunology, 2011, 187: 3026–3032.

B cells migrate into lymphoid follicles in a CXCR5-dependant manner, responding to the CXCR5 ligand CXCL13, which is abundantly displayed on follicular stromal cells (1–3). Within the follicle B cells migrate at an average velocity of 6 μm/ min in a “random walk,” surveying for Ags displayed by sinus-associated macrophages, follicular dendritic cells, or conduits or that have diffused into the follicle (3). Within 6 h after Ag encounter, Ag-engaged B cells move to the B zone–T zone (B–T) boundary in a CCR7-dependent manner, responding to CCL21 and CCL19 made by T zone stromal cells, to interact with Th 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 follicular and interfollicular 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, 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 was also found in some T cells and myeloid cells (9–14). Studies in two EBI2-knockout (KO) mouse lines established that EBI2 was required for B cells to correctly localize to interfollicular and outer follicular niches at days 2–3 of the T-dependent Ab 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 Ab response, establishing a role for this receptor in humoral immunity (12, 13).

Based on sequence alignments, EBI2 has been clustered with a number of GPCR 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).

In this study 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 h, 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 interfollicular regions is promoted by CD40 engagement. Finally, we employ a bioassay to provide evidence for EBI2 ligand activity in lymphoid tissues, and also multiple nonlymphoid tissues, and we suggest that 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-Igh6 Thy1.1Gpi1/J (IgMa) and bm12 mice were from The Jackson Laboratory. CXCR5−/− (MGI 2158677) (18), pT (MGI 1857881) (19), EBI2−/− (MGI 4399081) (12), CD40-deficient (MGI 2182733), MD4 (MGI 2384162), and OTHI (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...
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; Bio-Legend) 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 (BD Biosciences), and data were analyzed with FlowJo software (Tree Star). For cell sorting for in vivo analysis of Ebi2 expression, cell suspensions were first prepared from spleens in HBSS (University of California, San Francisco, Cell Culture Facility) containing 0.5% FBS and 0.5% fatty acid-free BSA (Calbiochem). Cells at a density of 4 × 10^5 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 i.p. immunized with 50 µg hen egg lysozyme (HEL)-OVA in RIBI adjuvant system (Sigma-Aldrich) 1 d after receiving splenocytes containing 5–10^8 wild-type (WT), EBI2 KO, or CD40 KO MD4 B cells and 2.5–5 × 10^7 OTII T cells transferred i.v. For CD40L-blocking experiments, mice were injected i.v. with 1 mg anti-mouse CD40L (clone MR1; BioExpress) 24 h after immunization. Spleens were harvested and digested with 2 mg/ml collagenase type II (Worthington Biochemical) or frozen for sectioning. For positioning studies, splenocytes containing 10–40 × 10^3 MD4 cells were transferred i.v. and the following day mice were injected with 1 mg HEL (Sigma-Aldrich) i.v. Splenocytes containing 30 × 10^3 MD4 B cells were transferred into CD40 KO recipients or lethally irradiated recipients reconstituted for at least 6 wk with CD40-deficient bone marrow. The next day, 1 mg HEL was injected i.v., followed 6 h subsequently by anti-CD40-α and anti-CD40-β (clone 45-4.5, University of California, San Francisco, Hybridoma Core). Mice were analyzed 36 h later. Bmi12 experiments were performed as in Pereira et al. (12). Purified and labeled WT and CD40-deficient B6 B cells (20 × 10^6 each) were mixed and stimulated with anti-IgM for 6 h and then transferred to bm12 recipients for 2 d. B cells were isolated by negative selection using mouse CD43 Dynabeads (Invitrogen), following the manufacturer’s protocol. In vitro analysis of Ebi2 and Ccr7 expression was performed by culturing 10^5 purified B cells with 13 µg/ml anti-IgM F(ab')2, goat anti-mouse IgM; Jackson ImmunoResearch Laboratories). For homeostatic positioning, 30 × 10^3 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 7-µm cryosections were fixed and stained immunochemically as described (20) with combinations of goat anti-mouse IgD (Accurate Chemical and Scientific), biotin anti-IgM(D5-1; BD Biosciences), B220 FITC (RA3-6B2; BioLegend), and/or biotin anti-IgD (AMS9.1; BD Biosciences) followed by HRP-conjugated donkey anti-goat IgD (H+L), HRP-conjugated anti-FITC, alkaline phosphatase-conjugated anti-FITC, and/or alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories). For immunofluorescence, staining was with FITC-conjugated anti-IgD (AMS9.1; BD Biosciences) and PE-conjugated anti-IgD (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 intestinal fluid-enriched extracts were prepared as previously described (21). Briefly, organs were weighed and mashed in 10 vol (assuming a density of 1 mg/ml) of sterile chemotaxis media (RPMI plus 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 (12) of a M12 B cell line transduced with an EBI2-deficient bone marrow. The next day, 1 mg HEL was injected i.v., followed 6 h subsequently by anti-CD40-α and anti-CD40-β (clone 45-4.5, University of California, San Francisco, Hybridoma Core). Mice were analyzed 36 h later. Bmi12 experiments were performed as in Pereira et al. (12). Purified and labeled WT and CD40-deficient B6 B cells (20 × 10^6 each) were mixed and stimulated with anti-IgM for 6 h and then transferred to bm12 recipients for 2 d. B cells were isolated by negative selection using mouse CD43 Dynabeads (Invitrogen), following the manufacturer’s protocol. In vitro analysis of Ebi2 and Ccr7 expression was performed by culturing 10^5 purified B cells with 13 µg/ml anti-IgM F(ab')2, goat anti-mouse IgM; Jackson ImmunoResearch Laboratories). For homeostatic positioning, 30 × 10^3 B cells were isolated by negative selection as described and labeled with 2.5 µM CFSE (Molecular Probes).

EBI2 ligand bioactivity in mouse cell line supernatants, as well as 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 at 5% CO2. In some cases, HEK293 cells were cultured in chemotaxis media containing the indicated concentrations of cyclooxygenase inhibitor (ibuprofen; Sigma-Aldrich), cystolic phospholipase A1, and A2 inhibitor (arachidonyl trifluoromethyl ketone; BIOMOL), cyclooxygenases and lipooxygenase inhibitor (5,8,11,14-eicosatetraenoy acid; BIOMOL), or 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor (atorvastatin and mevastatin; Sigma-Aldrich) for 12 h at 37˚C at 5% CO2.

EBI2 ligand fractionations
Mouse tissue extracts and HEK293 culture supernatants were prepared for reversed phase HPLC by adjusting trifluoroacetic acid (TFA) to 0.1% and acetonitrile to 10%. Small precipitates were removed by centrifugation. Supernatants were fractionated with reversed phase C18 Sep-Pak columns (Waters) by serial washes with increasing concentrations of acetonitrile plus 0.1% TFA. Semi-preparative reversed phase HPLC was performed on a Varian ProStar solvent delivery system equipped with a semi-preparative C18 ZORBAX StableBond column (300 A˚ pore size; Agilent Technologies/WVR International) and an analytical C18 ZORBAX StableBond column (80 A˚ pore size; Agilent Technologies/WVR International) using an acetonitrile (0.1% TFA/H_2O (0.1% TFA) gradient (10–100%) as the mobile phase and monitored by ultraviolet scan between wavelengths of 180 and 360 nm. One-minute fractions were collected, lyophilized, and tested for bioactivity by chemotaxis assay.

Results
EBI2 promotes movement of naive B cells through the outer follicle
EBI2 is abundantly expressed in naive B cells, and when naive cells lack this receptor they have a propensity to be enriched at the follicle center and underrepresented at the follicle periphery in spleen, lymph nodes (LNs), and Peyer’s patches (12, 13) (Supplemental Fig. 1A). Isolated lymphoid follicles in the intestine are rudimentary B cell-rich aggregates that do not have all the features of secondary lymphoid organs (22). However, in isolated lymphoid follicles we also found that EBI2 favored access of naive B cells to the outer follicle (Fig. 1A). In short-term transfer experiments, a bias in the distribution of EBI2-deficient naive B cells between the outer and center follicle could also be detected, although it appeared less marked than in mixed bone marrow chimeras (Supplemental Fig. 1B) (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 1 h, intermediate expression at 2 h, and a return to levels similar to naive cells at 6 h (Fig. 2A), in agreement with other studies (9–11, 13). In 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 h but being significantly upregulated over control levels at 6 h (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, WT or EBI2-deficient HEL-specific MD4 Ig-transgenic B cells were transferred to WT hosts and then the
mice were systemically immunized with soluble HEL. Prior to Ag injection, EBI2-deficient B cells were distributed in follicles with a bias for the follicle center (Fig. 2C). Three hours after Ag 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 h 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), although they tended to distribute more extensively into the T zone (Fig. 2C). Transcript abundance in 6 h activated B cells was close to the levels in naive cells (relative to hypoxanthine phosphoribosyltransferase [HPRT]), amounts that generate sufficient EBI2 to influence B cell behavior (Fig. 1); direct assessment of EBI2 protein levels awaits generation of an Ab reagent. At 10 h 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 Ag-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 Ag 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 h after B cell activation, we examined the distribution of Ag-engaged B cells in plt/plt mice that are deficient in CCR7 ligand expression in lymphoid tissues (19, 23). In these mice, 6 h activated B cells failed to move to a location corresponding to the B–T boundary and instead accumulated in the outer follicle (Fig. 3A and Ref. 4). Strikingly, 6 h 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 h Ag 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\[^{\text{a}}\] 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 h 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 (Supplemental Fig. 2A), consistent with earlier studies (18, 25). CXCR5 EBI2 double KO (DKO) B cells also failed to access follicles, remaining mostly in the red pulp, although with small numbers of cells reaching the T zone (Supplemental Fig. 2A). At 6 h after activation, CXCR5 KO

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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) bone marrow chimeras, stained as indicated. Original magnification \(\times 20\). B, Spleen and peripheral LN sections from WT or EBI2-deficient mice that had received 1 d transfer of WT (Igh\(^{a}\)) B cells. Stained to detect the transferred B cells (IgM\(^{a}\)D\(^{a}\), blue) and endogenous B cells with Abs to total IgD (1, 3, and 6 h) or B220 (10 h) (brown). Original magnification \(\times 10\). F, follicle (a single follicle is labeled); IF, interfollicular region; MZ, marginal zone; OF, outer follicle; T, T zone.

**FIGURE 2.** EBI2 is rapidly upregulated after B cell activation and promotes early movement to the outer follicle. A and 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. Quantitative PCR data were standardized against HPRT and data were 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 Ag injection. Transferred MD4 B cells were detected by staining for IgM\(^{a}\) and IgD\(^{a}\) (blue) and endogenous B cells with Abs to total IgD (1, 3, and 6 h) or B220 (10 h) (brown). Original magnification \(\times 10\). Black arrows highlight interfollicular regions. Views are representative of at least two mice of each type. F, follicle; T, T zone.
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, Supplemental Fig. 2B). CXCR5 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 (Supplemental Fig. 2C). These data provide further evidence that the EBI2 ligand is present in interfollicular regions, and they 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 Ag, many activated B cells are redistributed to interfollicular and outer follicular regions in a strictly EBI2-dependent manner (Fig. 4A and Refs. 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, Ag-engaged B cells fail to relocalize 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 transfected WT or EBI2 KO MD4 B cells into CD40-deficient hosts, immunized them with soluble HEL in the absence of adjuvant to activate the B cells but avoid recruiting helper T cells (27), and then treated cells 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 follicular and interfollicular regions, whereas the EB12 KO MD4 B cells did not relocalize to these areas (Fig. 4D). In the absence of CD40 signaling, many of the Ag-engaged B cells were eliminated by 2 d Ag exposure (Fig. 4D) as expected (27). These findings suggest that CD40 engagement may be sufficient to augment EB12 function in Ag-activated B cells, helping facilitate their movement to interfollicular 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 d, as expected, WT B cells became concentrated in the outer follicle. In 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. Whereas most WT B cells had relocalized 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 (Supplemental Fig. 3). 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. Taken 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 EB12 ligand bioactivity

To test for the presence of EB12 ligand within lymphoid tissues, we generated tissue extracts using a procedure we had previously employed in our analysis of interstitial sphingosine 1-phosphate concentrations (21). To our surprise, extracts prepared from spleen, LNs, and thymus showed a readily detectable attractant activity for EB12-transduced but not control cells (Fig. 5A). Bioactivity was also detected in a number of nonlymphoid tissues, including brain, kidney, liver, and lung, but not plasma (Fig. 5A). Chemotaxis data by this bioactivity was sensitive to PTX pretreatment of the EB12-expressing cells (Fig. 5B), providing evidence that EB12 is a Gα-coupled receptor, in agreement with a previous report (16). We next tested whether the bioactivity was proteinaceous in nature by treatment with proteinase K. Whereas this treatment readily destroyed SDF1 (CXCL12) activity, it had no effect on the EB12 ligand activity (Fig. 5C). The resistance of EB12 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 (Supplemental Fig. 4).

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 (arachidonyl trifluoromethyl ketone), cyclooxygenase (ibuprofen), and lipoxygenase (5,8,11,14-eicosatetraynoic acid) pathways had variable but not convincing inhibitory effects (Supplemental Fig. 4C). However, treatment with either of two statins, inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA reductase, led to a decrease in migration of EB12-transduced cells without affecting the background migration of the control cells (Fig. 5E, Supplemental Fig. 4C). These observations provide evidence that EB12 ligand biosynthesis depends on cells having an intact cholesterol biosynthetic pathway.

CD40 engagement promotes sustained EB12-dependent responsiveness to bioactivity

We took advantage of the identification of EB12 ligand activity in tissue extracts to test whether EB12-dependent chemotactic function was detectable in day 2 activated B cells, a time point when EB12 transcript levels were slightly reduced but the in vivo positioning data showed EB12 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 EB12-dependent (Fig. 6A). Endogenous (naive) B cells did not show an EB12-dependent response, likely because the extracts contained only low amounts of ligand (Fig. 6A). Moreover, stimulation of HEL-Ag–exposed B cells for 2 d with anti-CD40 led to an EB12-dependent migratory response to spleen extracts.
exposed B cells by CD40 engagement.

CXCL13-deficient extracts likely reflects the presence of other assay (Fig. 6). Extracts prepared from CXCL13-deficient spleens and mice. **p < 0.01, unpaired two-tailed Student t test. (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 Ag plus anti-CD40 (Fig. 6B), whereas cells exposed to Ag 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 Ag-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 h, CCR7 upregulation dominantly influences cell location but EBI2 functions together with CXCRR5 to distribute the activated cells along the length of the B–T boundary. Subsequent movement of activated B cells to interfollicular 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 G_i-coupled chemoattractant receptor and provide evidence that EBI2 ligand is a lipid and is present not only in lymphoid tissues but also in many nonlymphoid tissues. The widespread distribution of ligand is consistent with our finding that EBI2 is active in intestinal isolated lymphoid follicles. 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 naive B cells. However, naive B cell migration to the outer follicle can take place in the absence of EBI2. Because 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 naive B cell migration in the outer follicle 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 Ag entry (3). Recent studies have highlighted a role for LN subcapsular sinus macrophages, located between the incoming lymph and the outer follicle, in presenting Ags to B cells (3). It seems possible that B cells that have encountered low amounts of Ag in the follicle (or while entering the tissue from circulation) initially relocalize to the outer follicle to survey for further incoming Ag on such macrophages, improving their chance of internalizing sufficient Ag to later interact productively with Th 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 in instruct appropriate differentiation of the cells.

CCR7 and CCR7 ligands are critical for movement of 6 h 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 h activated B cells. Thus, CCR7 normally comes to dominate over the EBI2-dependent outer follicle tropism by 6 h, 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 that EBI2 ligand is present at the B–T boundary as well as in interfollicular and outer follicular regions, a suggestion supported by the circumferential distribution of WT naive B cells around follicles in spleens of mixed bone marrow chimeras (Supplemental Fig. 1A and Ref. 12). The propensity of EBI2-overexpressing 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 d in the presence of Th 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, whereas 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 that 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 suggest 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 no. WO/2010/066689). It will be important in future studies to test whether these oxysterols are physiological EBI2 ligands in lymphoid and nonlymphoid 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 IFN regulatory factor 7-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.

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Disclosures
The authors have no financial conflicts of interest.

References
Suppl. Figure 1
Suppl. Figure 2

**A**

EBI2 HET CXCR5 KO MD4

EBI2 KO CXCR5 KO MD4

**B**

EBI2 HET CXCR5 KO MD4

EBI2 KO CXCR5 KO MD4

**C**

- IgM+D+
  - B220

- IgM+D+
  - IgD

- EBI2 HET CXCR5 KO
- EBI2 KO CXCR5 KO

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Suppl. Figure 2
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 WT or EBI2-/- Ighb (red) and WT Igha (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).

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 IgMa and IgDa (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.

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.

Supplemental Figure 4: Detection of hydrophobic EBI2 ligand bioactivity and statin-sensitivity of bioactivity generation by HEK293 cells: (A, B) 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. (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.