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Article

Guidance of B Cells by the Orphan G Protein-Coupled Receptor EBI2 Shapes Humoral Immune Responses

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SUMMARY

Humoral immunity depends on both rapid and long-term antibody production against invading pathogens. This is achieved by the generation of spatially distinct extrafollicular plasmablast and follicular germinal center (GC) B cell populations, but the signals that guide responding B cells to these alternative compartments have not been fully elucidated. Here, we show that expression of the orphan G protein-coupled receptor Epstein-Barr virus-induced gene 2 (EBI2, also known as GPR183) by activated B cells was essential for their movement to extrafollicular sites and induction of early plasmablast responses. Conversely, downregulation of EBI2 enabled B cells to access the center of follicles and promoted efficient GC formation. EBI2 therefore provides a previously uncharacterized dimension to B cell migration that is crucial for coordinating rapid versus long-term antibody responses.

INTRODUCTION

Dynamic changes in lymphocyte localization are fundamental to the rapid and efficient production of protective antibodies. Antibody responses are initiated by the relocalization of antigen-engaged B cells to the B zone-T zone (B-T) boundary where cognate interactions with T cells drive initial B cell proliferation (Kelsoe and Zheng, 1993; Okada and Cyster, 2006). Proliferating B cell blasts subsequently proceed down one of two independent pathways of migration and differentiation (Jacob et al., 1991; Liu et al., 1991). Responding B cells can migrate from the B-T boundary to extrafollicular areas where they are induced to rapidly expand and differentiate into plasmablasts and plasma cells (MacLennan et al., 2003). These transient antibody-secreting cells provide the most immediate source of antigen-specific antibodies. Alternatively, antigen-engaged B cells can localize in the central, follicular dendritic cell (FDC)-rich region of the follicle to form germinal centers (GCs) (MacLennan, 1994). B cells proliferating in GCs give rise to high-affinity clones and exit the GC as long-lived plasma cells and memory B cells (Manz et al., 2005; O'Connor et al., 2003). This second GC-dependent pathway of B cell differentiation provides a sustained source of antibodies with enhanced antigen neutralization potential and mediates long-term immunity against reinfection. The early changes in positioning that recruit responding B cells to either the extrafollicular or the GC pathway of antibody production are therefore crucial for coordinating rapid versus long-term humoral responses.

Lymphocyte mobility and homing is modulated by the chemoattractant receptor subfamily of G protein-coupled receptors (GPCRs) (Campbell et al., 2003; Rot and von Andrian, 2004). B cell migration and position are controlled to a large extent by the lymphoid chemokines CXCL13, CXCL12, CCL19, and CCL21 and the regulated expression of their receptors CXCR5, CXCR4, and CCR7 (Allen et al., 2004; Forster et al., 1996; Forster et al., 1999; Hargreaves et al., 2001; Nie et al., 2004; Reif et al., 2002). Homing of B cells to B cell follicles is dependent on their expression of CXCR5 (Forster et al., 1996), whereas their movement to the B-T boundary after antigen encounter is directed by the rapid upregulation of CCR7 (Reif et al., 2002). As activated B cells differentiate into plasma cells, they downregulate CXCR5 and CCR7 and upregulate CXCR4, which is critical for their localization in the splenic red pulp and subsequent accumulation in the bone marrow (Hargreaves et al., 2001). Expression of CXCR5 is retained on B cells seeding GCs and, together with CXCR4, mediates the organization of GCs (Allen et al., 2004). Although differential expression of these chemokine receptors on plasma cells and GC B cell plays an important role in the localization of these populations to their distinct microenviroments, it is unclear whether additional factors contribute to the migration of activated B cells to extrafollicular sites versus follicular GCs.

In an effort to discover additional GPCRs directing the migratory events of responding B cells, we identified Epstein-Barr virus (EBV)-induced gene 2 (EBI2) as a promising candidate. The gene encoding EBI2 (*Ebi2*, also known as *Gpr183*) was originally identified together with *Ebi1* (*Ccr7*) as the most highly upregulated gene in EBV-infected Burkitt's lymphoma cells (Birkenbach et al., 1993). EBI2 was subsequently reported to be most homologous to members of the lipid and purine GPCR family (Rosenkilde et al., 2006; Surgand et al., 2006). Although lacking any close homology partner in the chemokine receptor family, EBI2 signals through the pertussis-sensitive $G\alpha_i$ protein, similarly to many chemokine receptors (Rosenkilde et al., 2006). This orphan GPCR is predominantly expressed in lymphoid tissues and high

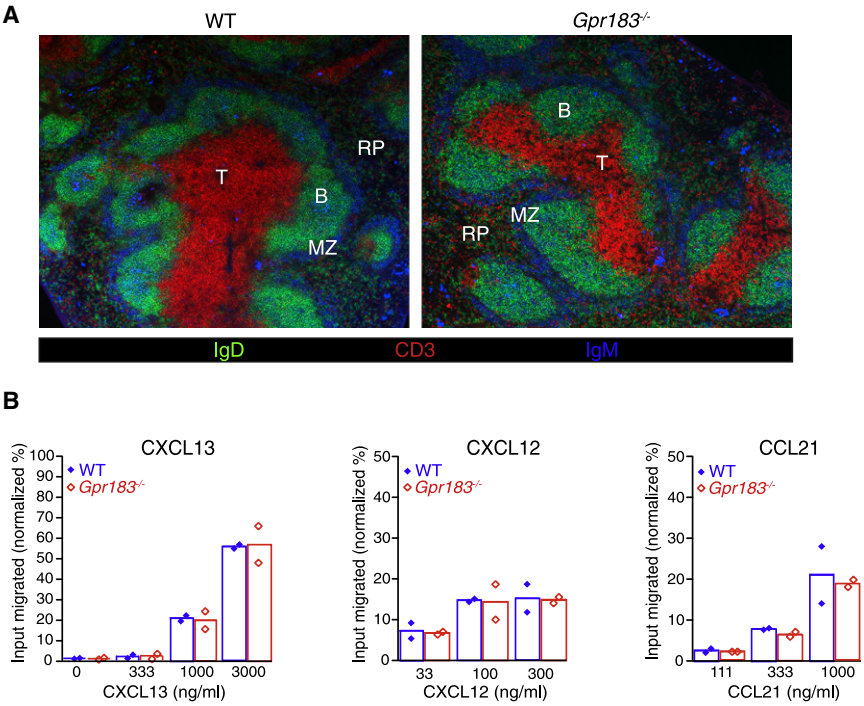

Figure 1. EBI2 Is Not Required for Normal Lymphocyte Organization and B Cell Responsiveness to Chemokines

(A) Normal splenic architecture of *Gpr183*$^{-/-}$ mice. Spleen sections were stained to detect B cell follicles ("B," stained with anti-IgD, green), T cell areas ("T," stained with anti-CD3, red), and marginal zone B cells ("MZ," stained with anti-IgM, blue). "RP" stands for red pulp. Images were taken at 10× objective magnification.

(B) Normal chemotactic response of naive polyclonal *Gpr183*$^{-/-}$ B cells to lymphoid chemokines CXCL13, CXCL12, and CCL21. Data are representative of at least three independent experiments, using cells from a minimum of five different animals of each type. The number of migrated cells was normalized to the migration of control CD45.1^{+} WT splenocytes added to all wells.

mRNA expression has been observed in naive B cells (Birkenbach et al., 1993; Rosenkilde et al., 2006). The already high constitutive expression of *Gpr183* present in naive B cells is further increased by B cell receptor (BCR)-triggered NF-κB activation, although this upregulation is only transient (Glynne et al., 2000). In contrast, GC B cell differentiation is associated with the shut down of *Gpr183* expression, which is controlled by the transcriptional repressor Bcl-6 (Shaffer et al., 2000). Despite this notable pattern of expression linked to B cell differentiation, the biological function of EBI2 remains undefined.

In this study, we demonstrate a critical role for EBI2 in the regulation of a T cell-dependent antibody response. We show that expression of EBI2 by antigen-specific B cells during the early stages of the response was essential for their normal migration to extrafollicular areas of the spleen and differentiation into plasmablasts. On the other hand, downregulation of EBI2 was found to mediate migration of B cells to the central regions of the B cell follicle and enabled the efficient generation of antigen-specific GC B cells. B cells are therefore guided through secondary lymphoid microenvironments by the modulation of EBI2 expression during T cell-dependent B cell responses. This differential migration ultimately directs plasmablast versus GC B cell differentiation and thus provides a mechanism for balancing rapid versus long-term antibody responses.

RESULTS

EBI2 Deficiency Does Not Affect Lymphoid Architecture nor Chemotaxis to Lymphoid Chemokines

To provide insights into the role of EBI2 in B cell responses, we generated EBI2-deficient mice. Mice lacking the entire EBI2 coding region in all cells (*Gpr183*$^{-/-}$ mice) were generated on a C57BL/6 genetic background with a standard gene targeting approach (Figure S1 available online). EBI2-deficient mice exhibited normal viability and gross phenotype as well as normal numbers of immature, follicular, marginal zone and peritoneal B1 B cells and CD4^{+} and CD8^{+} T cells (data not shown). Histological analysis revealed typical partitioning of B and T cells within the splenic white pulp and resolution of follicular and marginal zone B cell populations (Figure 1A). The normal splenic architecture of EBI2-deficient mice contrasts with the disorganization of splenic lymphocytes observed in mice lacking either CXCR5 or CCR7 (Forster et al., 1996; Forster et al., 1999). Thus, not only was EBI2 not required for the gross organization of lymphocytes in the spleen but its expression also did not substantially influence the in vivo chemotactic activities of the primary lymphocyte chemokine receptors. Accordingly, EBI2-deficient B cells exhibited normal cell-surface expression of CXCR5, CXCR4, and CCR7 (data not shown) and normal in vitro migration to their ligands (CXCL13, CXCL12, and CCL21 respectively) (Figure 1B).

Differential Expression of EBI2 Regulates B Cell Localization within the Follicle

In the absence of a gross defect in the localization of B cells within *Gpr183*$^{-/-}$ mice, we postulated that a potential role for EBI2 in regulating B cell migration might depend on its differential expression between B cells present within the same physiological compartment. To directly test this proposition, we transferred purified naive B cells from wild-type (WT) and EBI2-deficient mice into CD45.1 congenic WT mice and assessed their positioning within recipient spleens after 18–20 hr by immunohistology. In contrast to the even distribution of donor WT B cells within splenic B cell areas, EBI2-deficient B cells accumulated in the center of B cell follicles, colocalizing with CD21^{+}CD35^{+} follicular dendritic cells (FDCs) (Figure 2A). Transferred EBI2-deficient B cells exhibited a similar pattern of localization within the follicles of recipient lymph nodes (Figure S2). When GCs were present within recipient follicles, transferred EBI2-deficient B cells were also attracted toward the FDC network but did not enter GCs and instead congregated around them (Figure 2B). These data

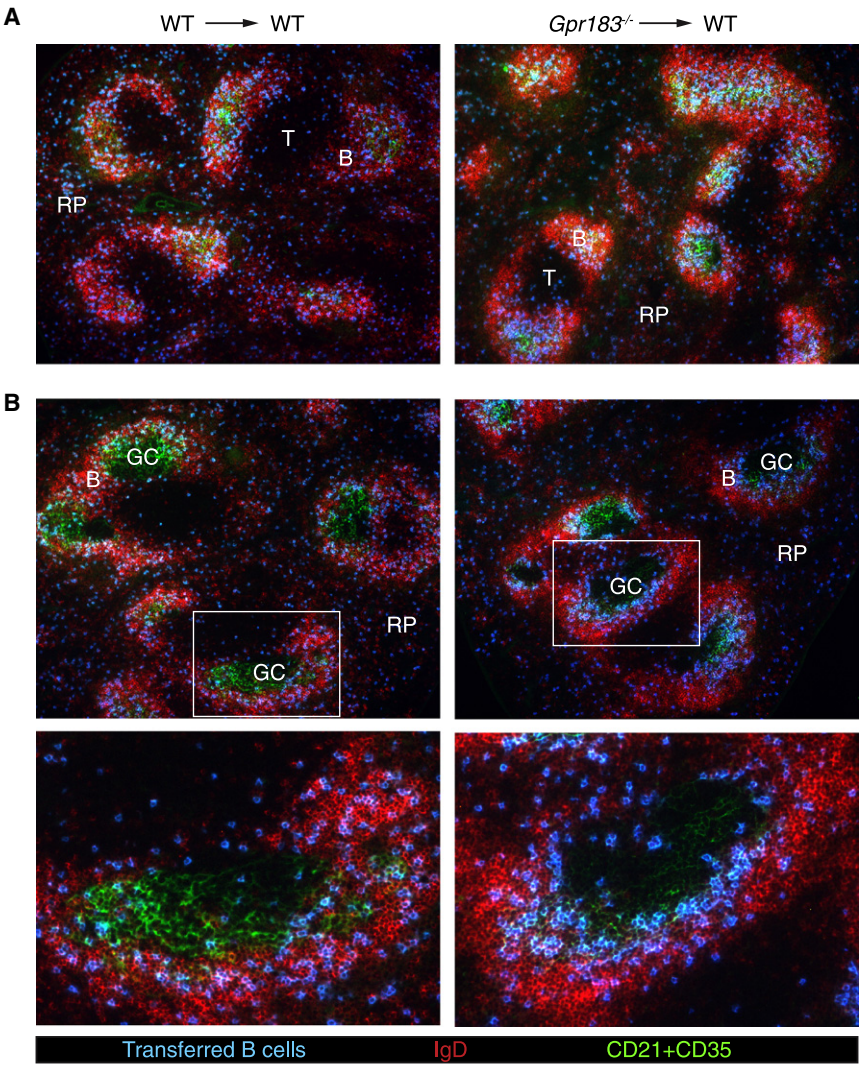

Figure 2. EBI2 Controls the Positioning of Naive B Cells in Follicles

Adoptively transferred naive EBI2-deficient B cells accumulate in the center of B cell follicles in FDC-dense areas and around GCs. Purified polyclonal *Gpr183*$^{-/-}$ or WT B cells were allowed to home in WT recipient mice for 18–20 hr and were distinguished from host B cells with the congenic marker CD45.2 (cyan). FDCs were detected with anti-CD21+CD35 (green) and B cell follicles visualized with anti-IgD (blue). Recipient mice were either naive (A) or had been immunized 6 days prior to B cell transfer with SRBCs (B). The inset shows accumulation of *Gpr183*$^{-/-}$ B cells around a GC. T, T cell areas; RP, red pulp. Images were taken at 10× objective magnification. Representative sections from one of three similar experiments are shown.

demonstrate that differential expression of EBI2 among B cells had a dramatic effect on their migration, with B cells expressing relatively high amounts of EBI2 localizing to the outer follicle and those with low EBI2 expression moving toward the central FDC-rich region. Modulation of EBI2 expression is therefore a potentially powerful mechanism for relocating B cells to different microenvironments within secondary lymphoid tissues.

EBI2 Regulates in B Cell Responses to T Cell-Dependent Antigen

The best characterized example of modulated EBI2 expression is over the course of T cell-dependent B cell responses, during which antigen-engagement results in a transient increase in *Gpr183* expression and subsequent GC B cell differentiation is associated with *Gpr183* downregulation (Glynne et al., 2000; Shaffer et al., 2000). To determine whether EBI2 may play a role in such responses, we initially examined the response of *Gpr183*$^{-/-}$ mice to the classical T cell-dependent antigen sheep red blood cells (SRBCs). Histological and flow cytometric analysis revealed that EBI2-deficient mice produced reduced numbers of IgG1^{+}B220^{lo} extrafollicular plasma cells in response to SRBC immunization (Figures 3A and 3B). In contrast, EBI2 deficiency did not affect the corresponding GC response (Figure 3A) nor did it alter in vitro B cell activation in response to a variety of BCR-dependent and -independent stimuli (Figure 3C). Expression of EBI2 was also not required for normal plasma cell differentiation in vitro (Figure 3D). EBI2 therefore appears to play a specific and nonredundant role in the in vivo generation of the extrafollicular plasma cells in response to T cell-dependent antigen.

Expression of EBI2 by B Cells Is Required for Generation of Early T Cell-Dependent Antibody Responses

To analyze in more detail the EBI2-mediated regulation of early B cell responses, we bred anti-hen egg lysozyme (HEL) SW_{HEL} immunoglobulin heavy chain gene-targeted mice onto the *Gpr183*$^{-/-}$ background (Phan et al., 2003). Adoptive transfer of EBI2-deficient SW_{HEL} B cells into WT recipients and challenge with a reduced HEL affinity mutant ($HEL^{2\times}$) conjugated to SRBCs (Paus et al., 2006) allowed direct assessment of the B cell-intrinsic function of EBI2 in a T cell-dependent antibody response. Analysis of *Gpr183* expression in WT SW_{HEL} B cells confirmed the rapid upregulation of *Gpr183* after antigen binding and revealed a strong modulation of the expression of this receptor in differentiating SW_{HEL} B cells in vivo, including marked downregulation in GC B cells (Figure 4A) as previously described (Shaffer et al., 2000).

Defects were observed in the response of EBI2-deficient SW_{HEL} B cells from as early as 2 days after immunization. At this point a slight delay in the proliferative expansion of EBI2-deficient relative to WT SW_{HEL} B cells was apparent by comparison of their respective CFSE dilution profiles (Figure 4B). Partly as a consequence of this, responding EBI2-deficient SW_{HEL} B cells were detected at reduced frequencies in the spleen of recipient animals throughout the first 5 days after immunization (Figure 4C).

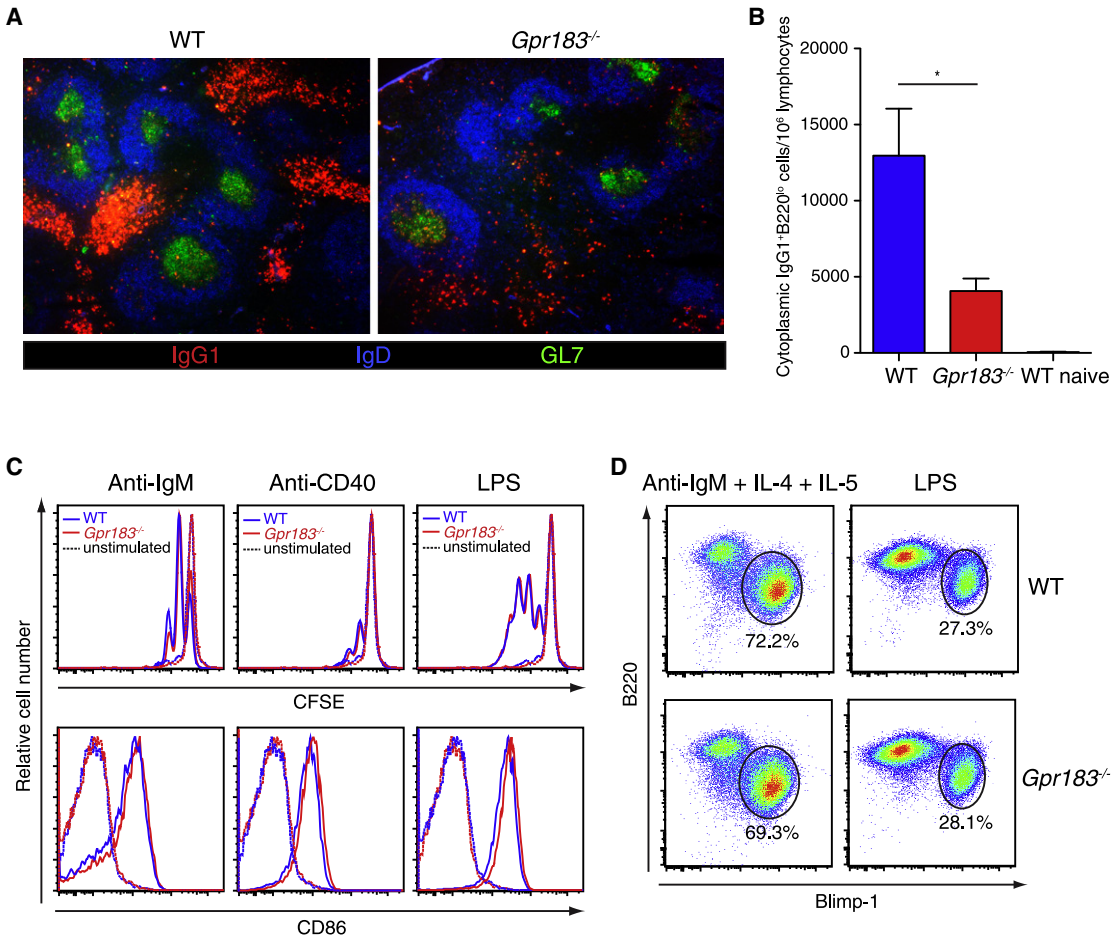

Figure 3. EBI2 Plays a Role in B Cell Responses to T Cell-Dependent Antigen In Vivo but Is Not Required for B Cell Activation and Differentiation In Vitro

(A and B) Reduced generation of IgG1$^+$ plasma cells in the spleen of *Gpr183*$^{-/-}$ mice 6 days after immunization with SRBCs.

(A) Staining of spleen sections for detecting IgG1$^+$ cells (red), GCs (anti-GL7, green), and B cell follicles (anti-IgD, blue).

(B) Quantification of IgG1$^+$ plasma cells by flow cytometry. Plasma cells were identified by high cytoplasmic staining for IgG1 and low B220 expression. The frequency of cells with this phenotype was increased by a factor of >10^4 in immunized WT mice compared to naive mice, indicating that they had been specifically induced by immunization. Means ± SEM are shown (n = 3); $p < 0.05$ (unpaired two-tailed Student's t test). Data are representative of two similar experiments.

(C and D) Normal in vitro activation, proliferation, and differentiation of EBI2-deficient B cells.

(C) Stimulation of *Gpr183*$^{-/-}$ and WT B cells with BCR-dependent (anti-IgM) and BCR-independent T cell-derived (anti-CD40) and Toll-like receptor-mediated signals. Overlays of CFSE dilution and CD86 expression profiles of *Gpr183*$^{-/-}$ and WT B cells, gated on live B220$^+$ cells, are shown.

(D) Stimulation of B cells from *Gpr183*$^{-/-}$ *Prdm1*$^{+/gfp}$ and *Prdm1*$^{+/gfp}$ reporter mice for detecting the generation of plasma cells. Mean percentages of Blimp-1-expressing (GFP$^+$) B220^{lo} cells in duplicate B cell cultures with anti-IgM plus IL-4 and IL-5 or LPS are indicated.

Phenotypic analysis of responding EBI2-deficient SW_{HEL} B cells confirmed that the specific defect in T cell-dependent plasmablast generation identified previously in *Gpr183*$^{-/-}$ mice (Figures 3A and 3B) could be attributed to the absence of EBI2 from responding B cells. On day 4.5 after activation, 50%–60% of WT SW_{HEL} B cells had acquired a phenotype characteristic of plasmablasts, distinguished by downregulation of B220, upregulation of CD138, and the concomitant switch from surface to cytoplasmic immunoglobulin expression (Figure 4D). Among EBI2-deficient SW_{HEL} B cells, however, the proportion of cells with this phenotype was typically <20% (Figure 4D) with total plasmablast frequencies being reduced to 10%–20% of WT (Figure 4E). In contrast, total frequencies of GC B cells (Figure 4F) and early memory B cells (data not shown) generated from EBI2-deficient SW_{HEL} donor B cells did not differ from the WT control. As anticipated from the impaired generation of early plasmablasts, the serum anti-HEL IgM and IgG1 concentrations in recipients of EBI2-deficient SW_{HEL} B cells were only 10%–20% of those in recipients of WT SW_{HEL} B cells (Figure 4G), particularly during the initial phase of the response prior to the emergence of plasma cells from GCs. Expression of EBI2 by responding B cells was therefore specifically required to ensure efficient generation of T cell-dependent extrafollicular plasmablasts and the associated early antibody response.

EBI2 Regulates B Cell Migration during Early T Cell-Dependent Responses

The results obtained thus far indicated that EBI2 regulates both B cell migration and the generation of the early T cell-dependent

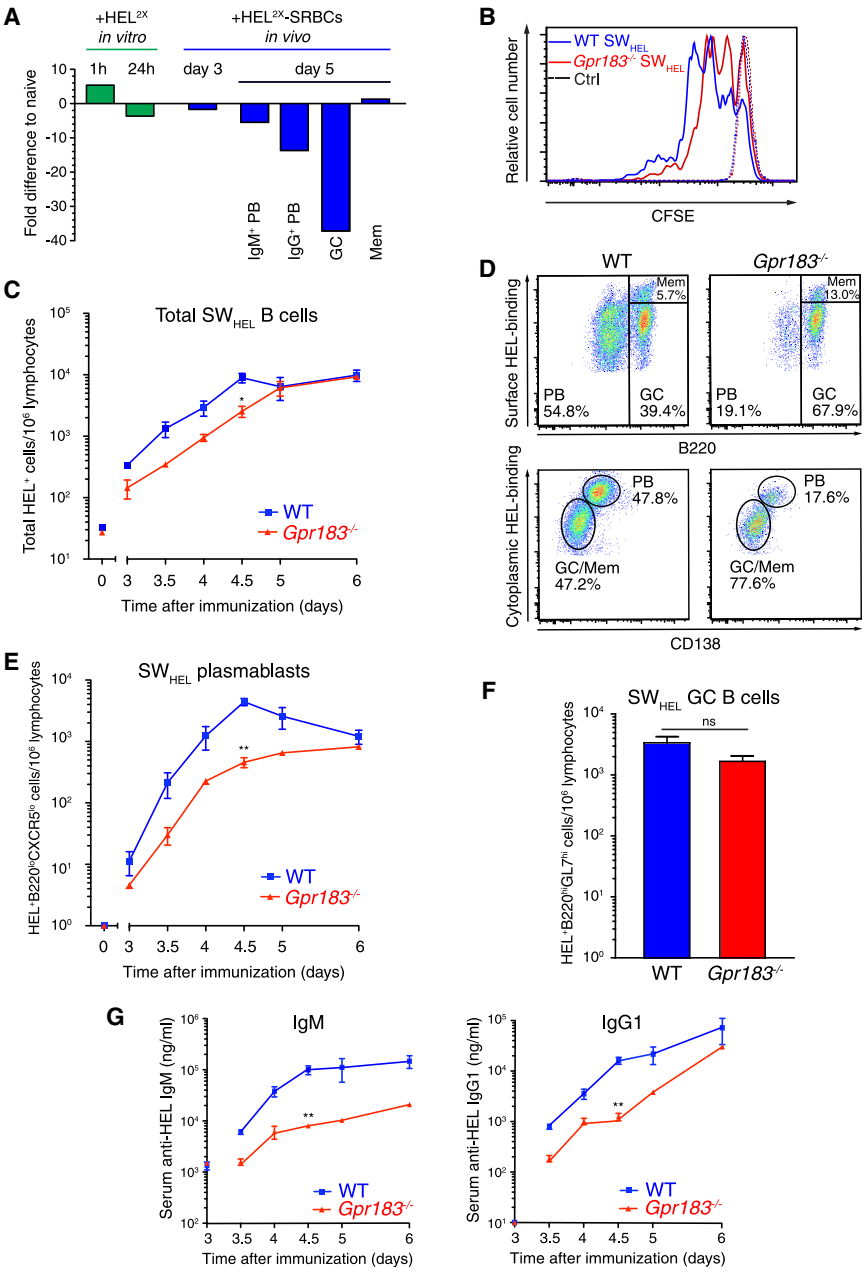

Figure 4. Expression of EBI2 by B Cells in the Initial Stages after Activation Is Required for Generation of Early Antibody Responses

(A) Quantitative PCR analysis of modulation of *Gpr183* expression in sorted WT SW_{HEL} B cells after in vitro stimulation with $HEL^{2\times}$ or in vivo activation with $HEL^{2\times}$ coupled to SRBCs. Fold differences to naive SW_{HEL} B cells are shown.

(B–F) Defective response of *Gpr183*$^{-/-}$ SW_{HEL} B cells to immunization with $HEL^{2\times}$-SRBCs.

(B) Overlay of CFSE dilution profiles of *Gpr183*$^{-/-}$ and WT SW_{HEL} B cells 2 days after immunization. Plots are gated on donor-derived ($CD45.2^{+}CD45.1^{-}$) HEL-binding B cells.

(C) Total frequencies of donor-derived HEL-binding B cells determined by flow cytometry.

(D) Phenotypic analysis of SW_{HEL} B cells on day 4.5 shows reduced plasmablast (PB) and increased GC populations. Plots are gated on donor-derived HEL-binding B cells. "Mem" represents memory.

(E) Total frequencies of SW_{HEL} plasmablasts identified by flow cytometry on the basis of their $B220^{lo}CXCR5^{lo}$ phenotype.

(F) Total frequency of SW_{HEL} GC B cells on day 4.5 after immunization. Donor derived HEL-binding GC B cells were determined by flow cytometry on the basis of their $B220^{hi}GL7^{hi}$ phenotype.

(G) Anti-HEL IgM and IgG1 concentrations in serum of recipient mice quantified by ELISA. Means ± SEM are shown (n = 3); levels of statistical significance are indicated for day 4.5 (*$p < 0.05$, **$p < 0.01$; ns, not significant, unpaired two-tailed Student's t test). Data are representative of three similar experiments.

plasmablast response. To explore whether these two activities of EBI2 may be linked, we next assessed by histological analysis the movement of EBI2-deficient SW_{HEL} B cells within the spleen during the early stages of their response to $HEL^{2\times}$-SRBC. Within 1 day of initial challenge, antigen-stimulated WT SW_{HEL} B cells were localized along the B-T boundary (Figure 5, day 1) as a result of CCR7-induced migration (Reif et al., 2002). Despite normal upregulation of CCR7 (Figure 6A), most EBI2-deficient SW_{HEL} B cells did not distribute uniformly along the B-T boundary, with many migrating deep into T cell areas (Figure 5, day 1).

Defective migration of EBI2-deficient SW_{HEL} B cells became increasingly evident as the response progressed. By day 3, many WT SW_{HEL} B cells had moved to lateral poles of B cell follicles proximal to bridging and interfollicular regions. This was not evident, however, in the response of EBI2-deficient SW_{HEL} B cells, which remained localized in central follicular areas proximal to the T cell zone (Figure 5, day 3). Responding EBI2-deficient SW_{HEL} B cells still persisted in this location 12 hr later, whereas a large fraction of WT SW_{HEL} B cells had by this time migrated into the bridging channels and red pulp (Figure 5, day 3.5). The relative absence of extrafollicular EBI2-deficient SW_{HEL} B cells continued until the peak of the WT extrafollicular plasmablast response on day 4.5, with EBI2-deficient B cells instead predominantly moving into GCs (Figure 5, day 4.5). The failure of EBI2-deficient B cells to form a robust extrafollicular plasmablast response therefore correlated directly with greatly reduced migration of responding B cells to the bridging channel and red pulp areas in which these responses occur (MacLennan et al., 2003). Aberrant migration of responding EBI2-deficient B cells was not the result of a defect in regulation of chemokine receptor expression, given that EBI2-deficient SW_{HEL} B cell subpopulations expressed normal amounts of CXCR5 and CXCR4 (Figure 6B). As was observed for naive B cells (Figure 2), responding B cells therefore required EBI2 in order to move away from the central area

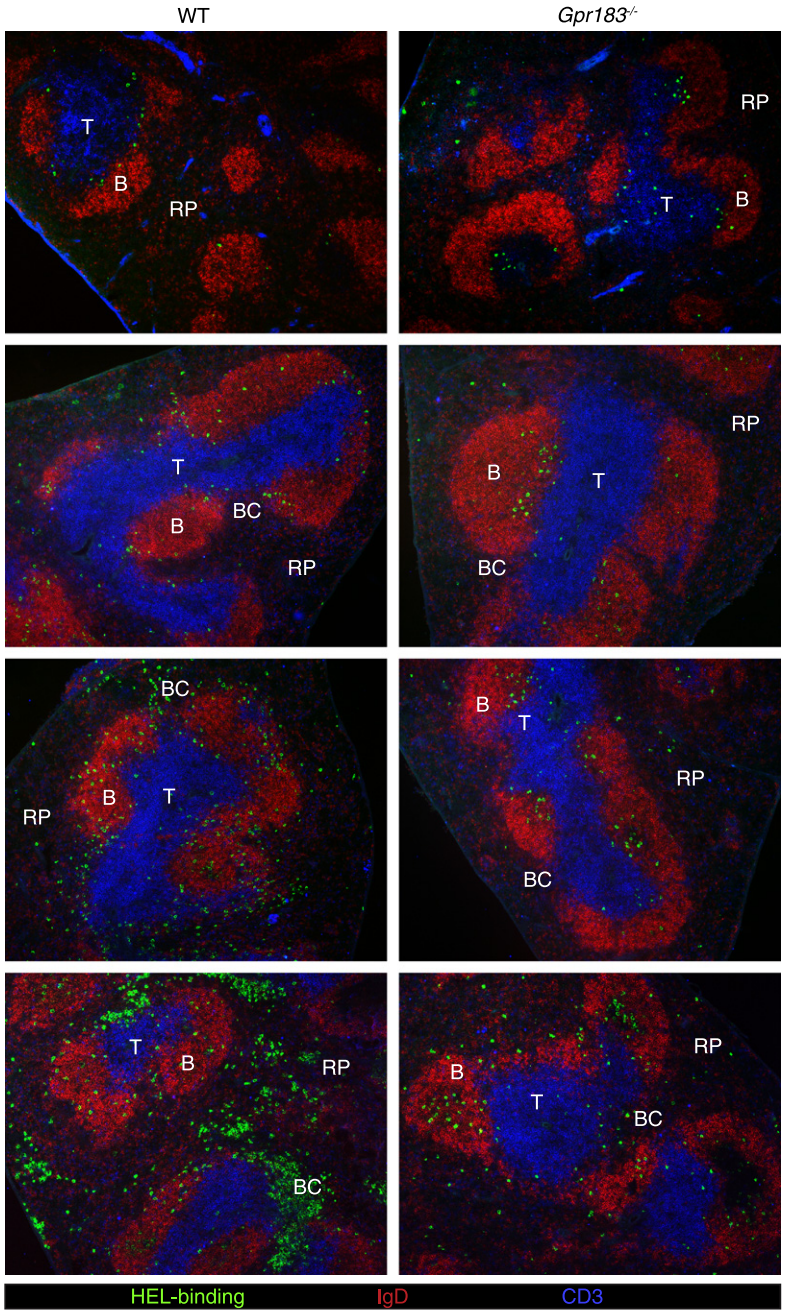

Figure 5. EBI2 Fine-Tunes the Position of Responding B Cells

Aberrant migration pattern of *Gpr183*$^{-/-}$ SW_{HEL} B cells at the indicated time points after challenge $HEL^{2\times}$-SRBCs. Immunofluorescent staining of spleen sections for detecting the localization of HEL-binding B cells (green) is shown. The following abbreviations are used: B, B cell follicles (stained with anti-IgD, blue); T, T cell areas (stained with anti-CD3, red); BC, bridging channel; and RP, red pulp. Images were taken at 10× objective magnification. Representative sections from one of three similar experiments are shown.

of the follicle. On the basis of this observation, it is likely that EBI2 controls plasmablast differentiation primarily by facilitating the migration of responding B cells to the extrafollicular microenvironments where this differentiation process is sustained.

Downregulation of EBI2 during B Cell Differentiation Promotes Efficient GC Responses

The migration of EBI2-deficient naive B cells to the FDC-rich areas of the central follicle (Figure 2) coupled with the extensive downregulation of *Gpr183* expression associated with GC B cell differentiation (Shaffer et al., 2000) (Figure 4A) suggested that the impetus for central follicular localization provided by downregulation of EBI2 on responding B cells is important for establishing a GC response. To test this proposition, SW_{HEL} B cells were engineered to constitutively express EBI2 by retroviral-mediated gene transfer into SW_{HEL} hematopoietic stem cells. Mature, naive SW_{HEL} B cells transduced with empty or *Gpr183*-containing vector (GFP^+) were purified by FACS sorting and challenged with $HEL^{2\times}$-SRBC in adoptive transfer. Whereas the usual proportion of control SW_{HEL} B cells acquired a $B220^{hi}PNA^{hi}$ GC phenotype on day 5, the frequency of GC B cells in responses from SW_{HEL} B cells that were unable to downregulate EBI2 was reduced to 25% of empty vector controls (Figure 7A). Histological analysis confirmed the smaller size of GCs forming from SW_{HEL} B cells with constitutive EBI2 expression (Figure 7B). Instead, these B cells differentiated en masse into extrafollicular plasmablasts (Figures 7A and 7B) and thus produced increased early anti-HEL IgM and IgG1 concentrations in the sera of recipient mice (Figure 7C). Despite the dramatic shift in B cell differentiation evident from constitutive EBI2 expression, the overall numbers of responding B cells were not affected (Figure 7D). It is evident, therefore, that enforced expression of EBI2 drives extrafollicular plasmablast differentiation, whereas its downregulation facilitates GC B cell differentiation.

DISCUSSION

The early control of extrafollicular versus GC localization of responding B cells is controlled to a large extent by the activity of the chemokine receptors CXCR4, CXCR5, and CCR7. However, CXCR4-deficient plasma cells are not impacted in their access to extrafollicular sites, and GC clusters form, albeit with disorganized structure or orientation, in absence of CXCR4- or CXR5-mediated chemotaxis (Allen et al., 2004; Hargreaves

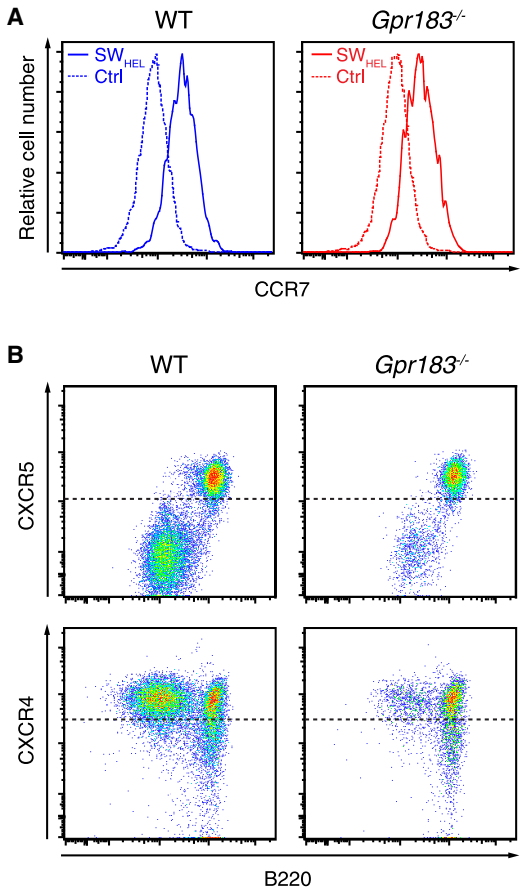

Figure 6. EBI2 Deficiency Does Not Affect the Regulation of Chemokine Receptor Expression of Responding B Cells

(A) Analysis of CCR7 upregulation on *Gpr183*$^{-/-}$ and WT SW_{HEL} B cells on day 1. Resting levels on non-HEL-binding CD45.2^{+} B cells are shown as controls. (B) Unchanged levels of CXCR5 and CXCR4 expression on responding *Gpr183*$^{-/-}$ and WT SW_{HEL} B cells on day 4.5. Plots are gated on donor-derived HEL-binding B cells and are representative of at least three mice.

et al., 2001). Thus, the existing experimental evidence points toward the involvement of an additional signal in the organization of early migratory events in T cell-dependent B cell responses. Our findings demonstrate that EBI2-driven positioning of B cells is a critical component of the mechanism that segregates activated B cells into the extrafollicular versus GC compartments. First, EBI2-dependent migration of responding B cells toward the bridging channels was required for the generation of the early extrafollicular plasmablast response, a process that was greatly enhanced by enforced expression of EBI2. Furthermore, the downregulation of EBI2 expression associated with GC B cell differentiation was sufficient to drive the accumulation of B cells in the deep FDC-rich regions of the follicle where GCs originate and enhanced the formation of GC responses. Although it is clear from the migration pattern of naive EBI2-deficient B cells that other activation-induced changes are typically required for B cells to enter GCs, our data indicate that the downregulation of *Gpr183* by Bcl-6 is one of the critical activities of this transcription factor in mediating GC B cell differentiation (Shaffer et al., 2000).

EBI2 remains an orphan GPCR and the identity and source of its ligand are yet to be described. Molecular studies of EBI2 have suggested that this receptor has constitutive activity, similar to that observed for many herpesvirus-encoded 7TM receptors (Benned-Jensen and Rosenkilde, 2008; Rosenkilde et al., 2006). It is conceivable that heterodimerization of EBI2 with other chemokine receptors could positively or negatively regulate their activity (Levoye et al., 2006). Accordingly, EBI2 deficiency would be expected to affect the in vitro chemotactic responses of B cells to chemokines. Such an outcome was not the case as far as tested. Thus, although our results do not exclude a constitutive receptor activity, they indicate that the potential agonist-independent signaling of EBI2 has no detectable impact on the migration of B cells to chemokines. Nevertheless, it remains possible that EBI2 modulates B cell responsiveness to chemokines when bound to its ligand. This orphan GPCR therefore is most likely to regulate B cell localization through a ligand that exhibits a spatially defined pattern of production. The migratory behavior of both naive and activated EBI2-deficient B cells indicated that these B cells failed to be attracted to the periphery of B cell follicles and extrafollicular regions, suggesting the presence of an agonist in these areas. Accumulation of B cells and plasma cells in the marginal-zone-bridging channels has been observed as a result of unbalanced chemokine responsiveness (Hargreaves et al., 2001; Reif et al., 2002). Similarly, autoreactive B cell blasts tend to localize to the red pulp-T zone border (Phan et al., 2003; Seo et al., 2002; William et al., 2002). The reasons for this homing pattern are unclear and suggest the existence of an unknown factor driving the lodgment of cells to this splenic subcompartment. Although our findings reveal EBI2 as a good candidate for mediating such localization, the extent of its contribution remains to be defined. Nevertheless, EBV-infected B cells, which are induced to express high amounts of EBI2 (Birkenbach et al., 1993), have been reported to migrate to extrafollicular regions and to avoid GCs during infectious mononucleosis (Niedobitek et al., 1992), which is in accordance with our results on EBI2 function.

For this study we have used the intermediate affinity mutant antigen $HEL^{2\times}$ for immunization. However, we have observed a similar defect in the plasmablast differentiation of EBI2-deficient SW_{HEL} B cells over a 10,000-fold affinity range by using WT HEL or the low-affinity mutant $HEL^{3\times}$ (data not shown). This indicates that increasing or decreasing BCR signal strength, which is known to regulate the plasmablast response (Benson et al., 2007; Paus et al., 2006), does not correct or exacerbate the defective response of EBI2-deficient B cells. Consistent with this result, the in vitro activation and proliferation of B cells was not affected by absence of EBI2, and therefore a contribution of EBI2 to the signals required for efficient B cell stimulation is unlikely. Notably, expression of EBI2 was also not required for normal in vitro plasmablast differentiation. It is still possible, however, that signals delivered by EBI2 can directly trigger or regulate gene expression programs that drive plasmablast differentiation. On the other hand, the guidance of responding B cells to distinct microenvironments mediated by modulation of EBI2 expression may subject them to alternative extracellular milieus that could direct their subsequent lineage commitment. Thus, EBI2-deficient B cells may predominantly form GC B cells in vivo because of the fact that they remain in a microenvironment proximal to the FDC network, in which antigen deposits and

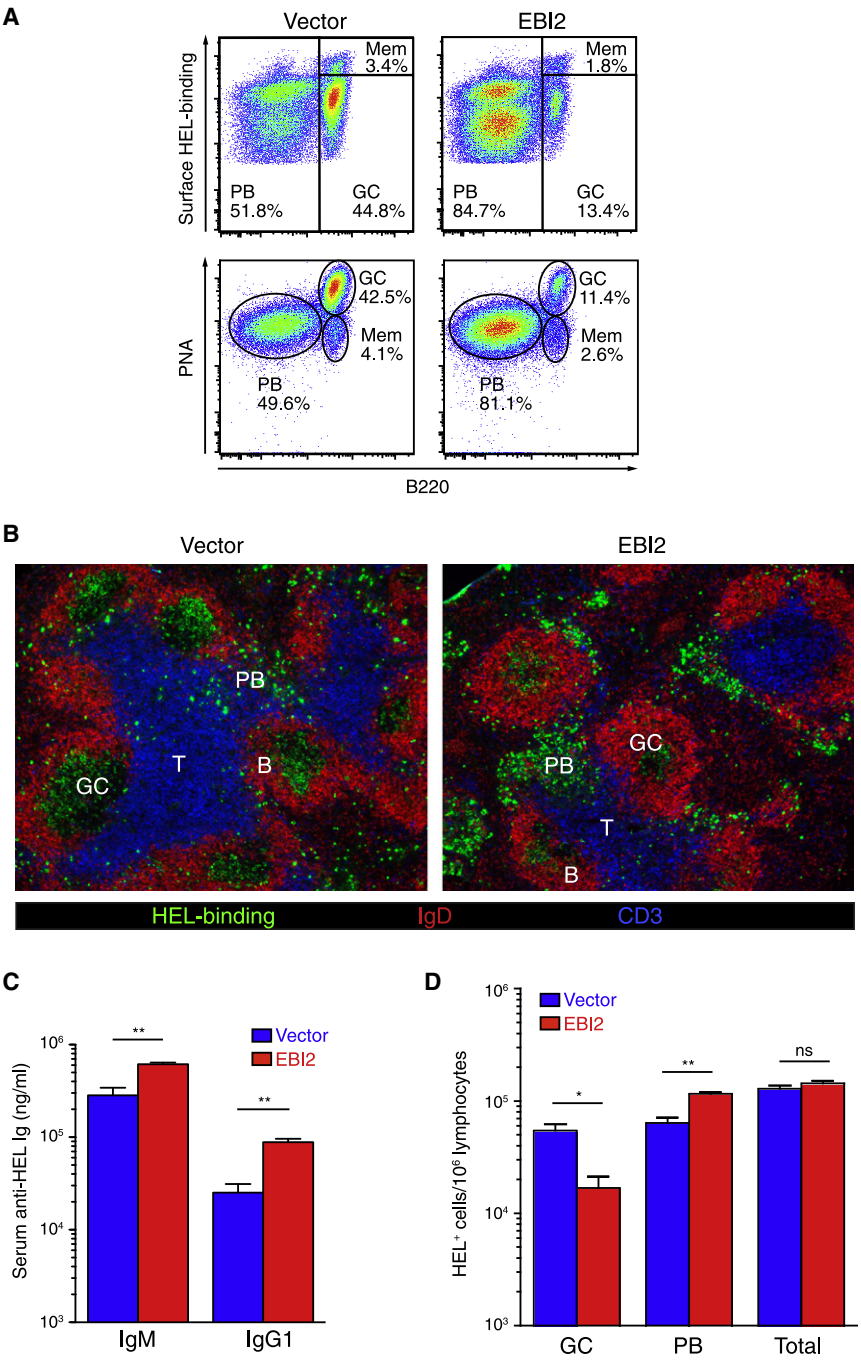

Figure 7. Downregulation of EBI2 during B Cell Differentiation Promotes Efficient GC Responses

Abnormal response of SW_{HEL} B cells with enforced EBI2 expression 5 days after immunization with HEL^{2X}-SRBCs. Constitutive expression of EBI2 was achieved through retroviral-mediated gene transfer of bone marrow cells.

(A) Phenotypic analysis of SW_{HEL} B cells to shows reduced GC and increased plasmablast (PB) populations. Plots are gated on donor-derived HEL-binding B cells. "Mem" represents memory. Mean percentages are indicated; $p < 0.01$.

(B) Immunofluorescent staining of spleen sections to detect HEL-binding B cells (green) in GC structures (indicated with GC). HEL-specific plasmablasts can be distinguished by the bright cytoplasmic staining (indicated with PB). The following abbreviations are used: B, B cell follicles (stained with anti-IgD, blue); T, T cell areas (stained with anti-CD3, red). Images were taken at 10× objective magnification.

(C) Anti-HEL IgM and IgG1 concentrations in serum of recipient mice quantified by ELISA.

(D) Total frequencies of donor-derived HEL-binding B cells and SW_{HEL} B cells with a GC or plasmablasts phenotype as distinguished in (A). Means ± SEM are shown (n = 3); *$p < 0.05$, **$p < 0.01$; ns, not significant (unpaired two-tailed Student's t test). Data are representative of two similar experiments.

follicular T helper cells drive their proliferation and selection into the long-term effectors of humoral immunity. At the same time, because the factors and myeloid cell populations supporting maturation and survival of plasmablasts are concentrated outside of B cell follicles, they are not encountered by most responding EBI2-deficient B cells (Garcia De Vinuesa et al., 1999; Mohr et al., 2009). The control of EBI2 expression, via NF-κB, Bcl-6, or other pathways, therefore plays an important role in determining the balance between plasmablast and GC differentiation. In vitro, cytokines such as interleukin (IL)-4, IL-6, and IL-10 have been shown to modulate *Gpr183* expression (Lam et al., 2008; Shaffer et al., 2000). In contrast, we have only seen minimal differences in the expression of *Gpr183* mRNA in SW_{HEL} B cells stimulated with HEL antigen of different affinities (data not shown). Although regulation of EBI2 is an important component of the early commitment of responding B cells to the extrafollicular versus GC pathway of differentiation, it is clear that this fundamental decision in B cell fate is a complex interplay of many signals in vivo. However, it remains possible that abnormal regulation of EBI2 expression leads to autoimmune and inflammatory diseases in which the balance between plasmablast and GC B cell differentiation is lost.

The importance of EBI2 for B cell function was first suggested by the dramatic upregulation of this receptor in EBV-transformed B cells and further inferred from its regulation in activated and GC B cells (Birkenbach et al., 1993; Glynne et al., 2000; Shaffer et al., 2000). An involvement of EBI2 in pathology has also been suggested by its dysregulated expression in B cell-associated autoimmune and neoplastic diseases (Aalto et al., 2001; Ye et al., 2003). However, the function of EBI2 and the significance and implications of its modulation have long awaited clarification. This study provides evidence for a biological function of EBI2 and indicates that this receptor provides an extra dimension to B cell migration and differentiation. Modulation of EBI2 expression is necessary for

ensuring both the rapid and long-term antibody production that are required for optimal protection against pathogens. Identification of the putative ligand for EBI2 and elucidation of the molecular mechanisms by which it controls B cell migration and differentiation may prove valuable in designing new vaccine strategies and potential therapeutics for immune disorders.

EXPERIMENTAL PROCEDURES

Mice

Generation of conditionally EBI2-deficient mice was performed by Ozgene (Perth, Australia). Gene targeting was performed in C57BL/6 Bruce 4 ES cells with a targeting construct in which the second exon of *Gpr183*, which contains the entire EBI2 coding sequence, was flanked by *loxP* sites located 208 bp upstream of the ATG start codon and 554 bp downstream of the TAA stop codon. A *FRT*-flanked PGK-Neo^r cassette was inserted immediately 5′ of the downstream *loxP* site and contiguous 5′ homology (6407 bp) and 3′ homology arms (6030 bp) were added (Figure S1A). Chimeric mice established from the injection of a homologously recombined ES cell clone into BALB/c blastocysts were bred directly with C57BL/6 *ACT:FLPe* transgenic mice (Rodriguez et al., 2000) so that germ-line deletion of the *FRT*-flanked PGK-Neo^r cassette from the targeted allele could be obtained. Germ-line deletion of exon 2 to generate a null allele of *Gpr183* was achieved by subsequent breeding to C57BL/6 *Zp3-CRE* transgenic mice (Lewandoski et al., 1997) (Figure S1A). *ACT:FLPe* and *Zp3-CRE* transgenes were removed and homozygous *Gpr183*$^{-/-}$ mice were generated through subsequent backcrossing of progeny carrying the null allele of *Gpr183.* Mice were screened by PCR analysis of tail-tip DNA with the primers 5′-TCCCTTTGAACCTGACTTTTG-3′ (P1), 5′-CTGTCAACCACCAGCAGAAC-3′ (P2), and 5′-CGTGCACATGTTTT CAGTGG-3′ (P3) as shown in Figure S1. Analysis of bone marrow, thymus, spleen, lymph nodes, and peritoneal cavity cells did not reveal any defect in the development of the immune system of *Gpr183*$^{-/-}$ mice. SW$_{HEL}$ mice and *Prdm1*$^{+/gfp}$ reporter mice have been described previously (Kallies et al., 2004; Phan et al., 2003). C57BL/6 and CD45.1 congenic C57BL/6 (B6.SJL/ptprc^a) mice were obtained from the Animal Resources Centre (Perth, Australia). All mice were maintained on a C57BL/6 genetic background and housed in a specific pathogen-free environment in the Garvan Institute Biological Testing Facility. All experimental protocols were approved by the Garvan/St. Vincent's Animal Ethics Committee.

Retroviral Transduction of Hematopoietic Stem Cells

The coding sequence of EBI2 was amplified by PCR from C57BL/6 genomic DNA and inserted into an MSCV2.2-based retroviral vector containing eGFP as an expression marker downstream of the internal ribosomal entry site (IRES). Retrovirus-containing supernatant was generated with the Phoenix-E packaging cell line together with pCL-Eco packaging vector as described (Swift et al., 2001). Bone marrow cells were extracted from the femur of SW$_{HEL}$ mice and cultured for 24 hr in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of 50 ng/ml mSCF, 20 ng/ml IL-3, and 50 ng/ml IL-6 (all from R&D Systems) before spin-infecting with retroviral supernatants and 4 μg/ml polybrene (Sigma). After infection, cells were cultured for additional 24 hr and then used for reconstituting lethally irradiated C57BL/6 recipient mice. Chimeric mice had frequencies of HEL-binding B cells comparable to SW$_{HEL}$ mice, with 30%–50% of B cells being transduced, as assessed by GFP expression.

Adoptive Transfers and Immunizations

The adoptive transfer system, mutant HEL$^{2\times}$ protein, and its conjugation to SRBCs have been described in detail previously (Brink et al., 2008; Paus et al., 2006). In brief, small numbers (3×10^4) of HEL-binding B cells from spleen of a donor *Gpr183*$^{-/-}$ or WT SW$_{HEL}$ mouse were transferred intravenously into nonirradiated CD45.1 congenic C57BL/6 recipient mice together with 2×10^8 HEL$^{2\times}$-SRBC. SW$_{HEL}$ donor B cells were identified by flow cytometry on the basis of their CD45.2$^+$CD45.1$^-$ phenotype as well as their ability to bind HEL. Detailed phenotypic analysis of SW$_{HEL}$ B cells on day 5 has indicated that the after subpopulations of B cells can be distinguished on the basis of the relative expression of B220 and cell-surface BCR (HEL binding): GC B cells (B220^{hi}BCR^{lo}), IgM$^+$ plasmablasts (B220^{lo}BCR^{hi}), IgG$^+$ plasmablasts (B220^{lo}BCR^{lo}), and early memory B cells (B220^{hi}BCR^{hi}) (Chan et al., 2009). For analysis of the positioning of naive B cells, *Gpr183*$^{-/-}$ or WT B cells were purified by negative depletion with magnetic cell sorting. B cell purity was 95%–99%. $3x10^7$ B cells were injected intravenously into CD45.1 congenic mice and spleens and inguinal lymph nodes of recipient mice were collected 18-20 hr later.

Quantitative RT-PCR

HEL-binding B cell populations were sorted into Trizol reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. First-strand cDNA was synthesized with Oligo(dT) primers and AMV Reverse Transcriptase (Promega). Quantification of *Gpr183* transcripts in the SW$_{HEL}$ populations was performed with the SYBR Green PCR Master Mix (Invitrogen) and the Rotor-Gene system (Corbett). Each sample was assessed in duplicates and the analysis was repeated with a second set of samples. Expression of GAPDH was used for copy number normalization. The primer pairs used were as follows: EBI2 forward 5′-CAGCTTTACCCACTCGGATA-3′, EBI2 reverse 5′-AAGAAGCGGTCTATGCTCAA-3′, GAPDH forward 5′- TGAAGCA GGCATCTGAGGG-3′, and GAPDH reverse 5′- CGAAGGTGGAAGAGTGGG AG-3′. Quantification of *Gpr183* expression relative to naive SW$_{HEL}$ B cells was determined with the comparative threshold cycle method.

Flow Cytometry

Splenocytes were prepared, stained for surface molecules, and analyzed on a FACSCanto (BD Biosciences) as previously described (Phan et al., 2005). Intracellular staining was performed after fixation with 10% formalin and permeabilization with 0.2% polyethylene sorbitan monolaurate. HEL-binding B cells were detected with 200 ng/ml HEL (Sigma-Aldrich) and purified HyHEL9 hybridoma supernatant conjugated to Alexa Fluor 647 (Molecular Probes). The following antibodies and reagents were used for cell staining: anti-CD184/CXCR4-biotin (2B11; BD Biosciences), anti-CXCR5-biotin (2G8; BD Biosciences), anti-CCR7-biotin (4B12; eBiosciences), anti-CD138-biotin (281.2; BD Biosciences), anti-IgG1-biotin (A85-1; BD Biosciences), PNA-biotin (Vector Laboratories), streptavidin-PE (BD Biosciences), anti-CD86-PE (GL1; BD Biosciences), anti-CD45.1-PE/Cy7 (A20; eBiosciences), anti-CD45.2-PerCP/Cy5.5 (104; eBiosciences), anti-GL7-FITC (GL7; BD Biosciences), and anti-CD45R/B220-Pacific blue (RA3-6B2; BD Biosciences).

ELISA

HyHEL10 anti-HEL Ig isotype standards were produced and serum anti-HEL IgM and IgG1 concentrations analyzed by ELISA as previously described (Phan et al., 2003).

Immunofluorescence Microscopy

Spleen or lymph node sections (5–7 μm) were fixed with acetone and blocked with 30% horse serum. HEL-binding B cells were detected with 100 ng/ml HEL (Sigma) and then detected with polyclonal rabbit anti-HEL serum and sheep anti-rabbit IgG-FITC (Chemicon). T cell areas were revealed with biotinylated anti-CD3 (500A2; eBiosciences) and Alexa Fluor 555-conjugated streptavidin (Invitrogen), B cell follicles were revealed with anti-IgD-Alexa Fluor 647 (11-26c.2a; BioLegend), marginal zone B cells were revealed with anti-IgM-FITC (R6-60.2; BD Biosciences), GC B cells were revealed with anti-GL7-FITC (GL7; BD Biosciences); FDCs were revealed with anti-CD21/35 (7G6; BD Biosciences), and IgG1$^+$ cells were revealed with biotinylated anti-IgG1 (A85.1; BD Biosciencies) and Alexa Fluor 555-conjugated streptavidin (Invitrogen) as indicated. Adoptively transferred naive *Gpr183*$^{-/-}$ and WT B cells were distinguished with biotinylated anti-CD45.2 (104; BD Biosciences) and then with HRP-conjugated streptavidin and TSA-direct Cy3-tyramide (PerkinElmer). All images were obtained at ×10 objective magnification.

Transwell Migration Assays

Splenocytes from *Gpr183*$^{-/-}$ and WT mice were allowed to transmigrate across 5 μm transwell filters (Corning Costar Corp) for 3 hr and were enumerated by flow cytometry as described (Allen et al., 2004). Chemokines were obtained from R&D Systems. Transwell assays were performed in duplicate for each chemokine concentration and were repeated with cells from a minimum of five different animals of each type. The number of migrated cells

was normalized to the migration of control CD45.1$^+$ WT splenocytes added to all wells.

In Vitro Cultures

Cell suspensions from spleens or lymph nodes of *Gpr183*$^{-/-}$ and WT mice were prepared in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 55 μM 2-ME and stimulated with 10 μg/ml goat anti-mouse IgM (Jackson ImmunoResearch), 5 μg/ml anti-CD40 mAb (HM40-3; BD Biosciences), or 2.5 μg/ml LPS. CD86 upregulation on B cells compared to unstimulated cells was analyzed by flow cytometry 24 hr after stimulation. For analysis of proliferation, B cells purified by magnetic cell sorting were labeled with CFSE prior to culturing (Phan et al., 2003) and cell division was assessed by flow cytometry after 3 days. For analysis of plasma cell differentiation, B cells were purified from from *Gpr183*$^{-/-}$ *Prdm1*$^{+/gfp}$ and *Prdm1*$^{+/gfp}$ reporter mice and cultured for 4 days with 10 μg/ml goat anti-mouse IgM (Jackson ImmunoResearch) plus 10 ng/ml IL-4 and 2 ng/ml IL-5 (Sigma-Aldrich) or 10 μg/ml LPS.

SW_{HEL} splenocytes were stimulated with 500 ng/ml $HEL^{2\times}$ for 1 and 24 hr before analysis of *Gpr183* expression.

Statistical Analysis

Levels of statistical significance between means were calculated by unpaired two-tailed Student's t test.

SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00313-6.

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