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The chemotactic receptor EBI2 regulates the homeostasis, localization and immunological function of splenic dendritic cells

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Spleen-resident dendritic cell (DC) populations occupy sentinel positions for the capture and presentation of blood-borne antigens. Here we found a difference in expression of the chemotactic receptor EBI2 (GPR183) on splenic DC subsets and that EBI2 regulated the positioning and homeostasis of DCs in the spleen. EBI2 and its main ligand, 7α,25-OHC, were required for the generation of the splenic CD4$^+$ DC subset and the localization of DCs in bridging channels. Absence of EBI2 from DCs resulted in defects in both the activation of CD4$^+$ T cells and the induction of antibody responses. Regulated expression of EBI2 on DC populations is therefore critical for the generation and correct positioning of splenic DCs and the initiation of immune responses.

Dendritic cells (DCs) are widely distributed throughout the body and can be grouped into several distinct subsets distinguishable by phenotypic markers, anatomic distribution and immunological function[1–3]. Conventional DCs that reside constitutively in secondary lymphoid organs are responsible for capturing and presenting antigens that enter directly into the organ via blood or lymph. Many such lymphoid tissue–resident DCs localize near the sites of antigen entry, such as the splenic marginal zone bridging channels and the lymph node subcapsular sinus[4], where they are poised to initiate responses to pathogens that disseminate through the blood and lymph. However, the factors that control the positioning of DCs at these ports of antigen entry remain uncharacterized.

In the spleen, resident DCs can be categorized into three subtypes on the basis of their expression of the coreceptors CD4 and CD8 (ref. 5). CD4$^+$CD8$^-$ DCs, which are further characterized by expression of the integrin CD11b (α_M) and the lectin DCIR2, predominate in this organ and represent the main population located in the marginal zone and in bridging channels[6]. Splenic CD4$^+$ DCs are involved in the capture of blood-borne antigen and its presentation to CD4$^+$ T cells, and key virus-recognition functions have been assigned to this subset[7–10]. DCIR2$^+$ DCs located in marginal zone bridging channels are important for the initiation of extrafollicular B cell responses to T cell–dependent antigens[11]. However, CD4$^+$ DCs are inefficient at cross-presenting antigen and priming CD8$^+$ T cell responses[10,12,13]. That activity has been attributed mainly to a second population of splenic DCs that reside in T cell areas and can be distinguished by expression of CD8 and the lectins CD205 and Clec9A[1,2,10,14,15]. DCs belonging to the third subset are CD4$^-$CD8$^-$ and are phenotypically and functionally similar to CD4$^+$ DCs[1,2,4]. All three DC subtypes derive from a common intrasplenic progenitor cell that is continually replenished from the blood and is maintained by local homeostatic proliferation[16–19]. How the transcriptional programs that lead to the development of a particular DC subset are activated remains elusive. Environmental cues may drive the development of the distinct DC subsets[20], which places importance on the extracellular milieu in shaping the outcome of terminal DC differentiation.

The positioning and migratory properties of each DC population are regulated by their responsiveness to chemotactic signals. An important role in the localization and migration of DCs in the spleen is served by the chemokine receptor CCR7, which mediates access of DCs to T cell zones[21–23]. The intrasplenic distribution of DCs is also influenced by the $S1P_1$ G protein–coupled receptor for sphingosine 1-phosphate, as disruption of sphingosine 1-phosphate signaling causes the accumulation of immature DCs in the marginal zone[24]. However, it remains unclear whether additional, unidentified signals mediate the segregation of DCs in the spleen. There is evidence that the G protein–coupled receptor EBI2 (GPR183) has a role in DC migration. EBI2 is reported to be expressed on DCs of both mouse and human origin, and its main ligand, 7α,25-dihydroxycholesterol (7α,25-OHC), has been shown to induce the chemotaxis of mouse splenic and bone marrow–derived DCs[25,26]. In lymphoid organs, a gradient of 7α,25-OHC is generated at the follicle perimeter by lymphoid stromal cells and guides B cells during antibody responses[27]. EBI2 and 7α,25-OHC act together to mediate the localization of B cells to the outer follicle and to the bridging channels of the spleen, and mice deficient in either EBI2 or 7α,25-OHC have severe defects in

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the generation of antibody responses[25–31]. Whether EBI2 has a related role in the localization and function of DCs during immune responses has not been established. In this study we found that EBI2 expression by DCs was required for their homeostasis, localization and ability to induce B cell and T cell responses, which emphasizes the many functions of EBI2 and oxysterols in the generation of immunity.

RESULTS

EBI2-deficient mice have fewer splenic CD4+ DCs

To provide insight into the role of EBI2 expression on DCs, we first characterized DC populations in EBI2-deficient (*Gpr183*$^{-/-}$; called '*Ebi2*$^{-/-}$' here) mice. Spleens from *Ebi2*$^{-/-}$ mice had fewer total DCs than did their wild-type counterparts (**Fig. 1a**). This was due to a specifically lower abundance of DCs of the CD4$^+$ subset, as the number of CD8$^+$ DCs and CD4$^-$CD8$^-$ DCs, as well as plasmacytoid DCs, was unaffected (**Fig. 1a**,**b**). We delineated the phenotype and maturation state of CD11c$^+$B220$^-$ DCs in *Ebi2*$^{-/-}$ mice further with a panel of established surface markers[2,32]. This analysis indicated that the number of splenic DCs with a CD11b$^+$DCIR2$^+$CD-205$^-$ phenotype was specifically affected by the absence of EBI2 (**Fig. 1a**,**b**). Thus, CD4$^+$ DCs did not simply downregulate CD4 expression in the absence of EBI2 expression; instead, the spleen was specifically depleted of CD4$^+$ DCs. In contrast to splenic DCs, resident and migratory DC populations in lymph nodes of EBI2-deficient mice were present in normal numbers (**Supplementary Fig. 1a**), as were non–lymphoid tissue DCs (**Supplementary Fig. 1b**,**c**).

As CD4$^+$ DCs downregulate their expression of CD4 after activation in culture[33], we next determined the maturation state of DCs in the spleens of EBI2-deficient mice. Expression of the surface activation markers major histocompatibility complex (MHC) class II, CD40, CD80 and CD86 was similar on *Ebi2*$^{-/-}$ and wild-type DCs of each subset (**Fig. 1c**), which indicated that the absence of EBI2 did not affect the activation or maturation state of the DCs. In addition, EBI2-deficient DCs responded normally to *in vivo* stimulation with lipopolysaccharide (LPS), as indicated by normal upregulation of the expression of surface activation markers and migration to splenic T cell areas (**Supplementary Fig. 2a**,**b**). Consistent with those results, the EBI2 ligand 7α,25-OHC had no effect on the *in vitro* maturation of DCs purified from the spleens of wild-type mice (**Supplementary Fig. 2c**). We therefore concluded that EBI2 had a major and apparently specific role in regulating the number of splenic CD4$^+$ DCs.

Expression of EBI2 on CD4+ DCs but not on CD8+ DCs

To determine whether the specific depletion of CD4$^+$ DCs in EBI2-deficient mice correlated with the expression pattern of EBI2, we analyzed the expression of EBI2 by the various DC subsets. First, we purified DCs by sorting from the spleens of wild-type mice and measured the abundance of *Ebi2* mRNA by quantitative PCR. We detected approximately tenfold higher expression of *Ebi2* in CD4$^+$ DCs than in CD8$^+$ DCs (**Fig. 2a**). CD4$^-$CD8$^-$ DCs also expressed *Ebi2,* albeit lower than the expression by CD4$^+$ DCs (**Fig. 2a**). To detect cell-surface expression of EBI2, we generated a polyclonal antibody to EBI2 and confirmed its specificity against recombinant EBI2–expressing transfectants and wild-type naive and germinal center (GC) B cells (**Supplementary Fig. 3a**,**b**). Surface EBI2 expression on splenic DCs was similar to that on splenic B cells, whereas we observed no specific binding of the EBI2-specific antibody to *Ebi2*$^{-/-}$ DCs or B cells (**Fig. 2b**). Analysis of the surface expression of EBI2 on splenic DC subsets confirmed that CD4$^+$ DCs and CD4$^-$CD8$^-$ DCs expressed EBI2, whereas we found only low EBI2 expression on CD8$^+$ DCs (**Fig. 2c**). We also detected surface expression of EBI2 on lymph node–resident CD11b$^+$ DCs and a fraction of CD8$^+$ DCs, as well as on migratory DCs (**Supplementary Fig. 3c**).

To investigate the regulation of EBI2 on DCs after stimulation, we activated purified splenic DCs with LPS and assessed surface expression of EBI2 after overnight incubation. The already high constitutive expression of EBI2 on CD4$^+$ DCs and CD4$^-$CD8$^-$ DCs was further upregulated after activation (**Fig. 2d**), consistent with the upregulation of *EBI2* mRNA expression observed before during the maturation of human monocyte-derived DCs[34]. In addition, EBI2 was induced by LPS stimulation on most CD8$^+$ DCs (**Fig. 2d**). Therefore, EBI2 expression was modulated on distinct DC populations and during the transition from immature DC to mature DC.

EBI2 and 7α,25-OHC direct the migration of CD4+ DCs

The difference in EBI2 expression by splenic DC subsets suggested they would respond differently to the EBI2 ligand 7α,25-OHC. To test this, we purified splenic DCs and assessed the chemotactic responses of DC subpopulations to 7α,25-OHC in a Transwell assay. Gradients

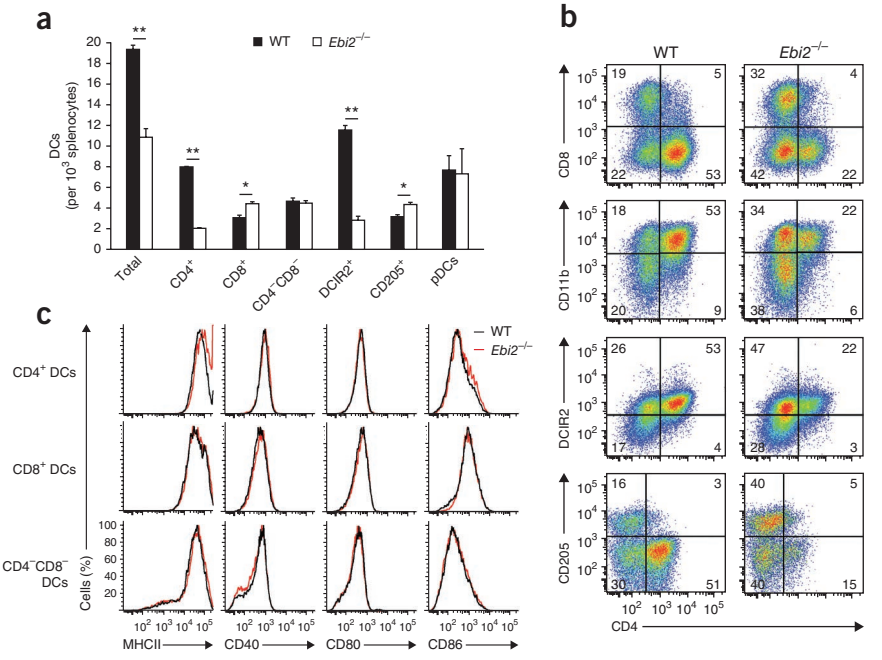

Figure 1 Lower frequency of CD4$^+$ DCs in spleens of EBI2-deficient mice. (**a**) Frequency of total DCs and DCs of various subsets (horizontal axis) in the spleens of wild-type (WT) and *Ebi2*$^{-/-}$ mice ($n = 3$ per genotype). $^*P < 0.01$ and $^{**}P < 0.001$ (Student's *t*-test). (**b**) Phenotypic comparison of wild-type and *Ebi2*$^{-/-}$ splenic DCs, assessed by flow cytometry gated on CD11c$^+$B220$^-$ cells. Numbers in quadrants indicate percent cells in each throughout. (**c**) Expression of activation markers on wild-type and *Ebi2*$^{-/-}$ splenic DCs of various subsets (left margin). Plots are gated on CD11c$^+$B220$^-$ cells. Data are representative of at least two experiments with similar results (mean and s.e.m. in **a**).

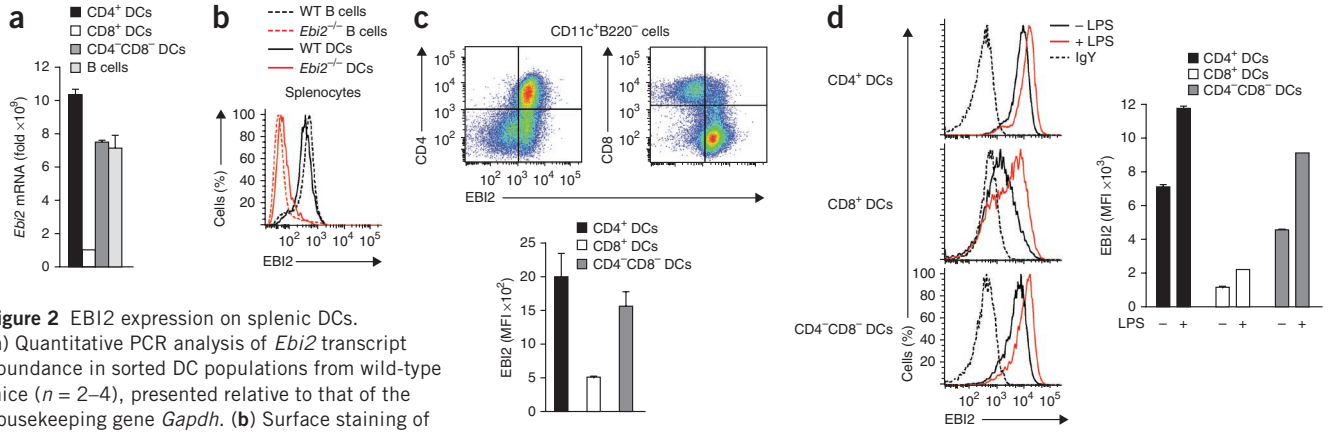

Figure 2 EBI2 expression on splenic DCs. (**a**) Quantitative PCR analysis of *Ebi2* transcript abundance in sorted DC populations from wild-type mice ($n = 2–4$), presented relative to that of the housekeeping gene *Gapdh*. (**b**) Surface staining of EBI2 on wild-type and *Ebi2*$^{-/-}$ CD11c^{+}B220^{-} DCs and B220^{+} B cells. (**c**) Surface staining of EBI2 on splenic DC populations, assessed by flow cytometry gated on CD11c^{+}B220^{-} cells (top), and geometric mean fluorescence intensity (MFI) of EBI2 staining (bottom); $n = 3$ mice per genotype. (**d**) Surface staining of EBI2 on wild-type DCs with (+ LPS) or without (– LPS) overnight stimulation with LPS (left; gated as in **c**); background fluorescence was similar for unstimulated and stimulated DCs. IgY, isotype-matched control antibody. Right, geometric mean fluorescence intensity ($n = 3$ mice). Data are representative of two experiments with similar results (mean and s.e.m. in **a**,**c**,**d**).

of 7α,25-OHC induced the migration of wild-type CD4^{+} DCs and CD4^{-}CD8^{-} DCs (**Fig. 3a**), consistent with their high expression of EBI2. In contrast, we detected only minimal migration of wild-type CD8^{+} DCs in response to 7α,25-OHC (**Fig. 3a**), and EBI2-deficient DCs did not migrate at all (**Fig. 3a**).

To investigate whether EBI2 had a role in guiding DC localization *in vivo*, we assessed the distribution of DCs in spleens from EBI2-deficient mice. Histological staining of CD11c^{+} cells in wild-type spleen sections showed clusters of DCs in bridging channels (**Fig. 3b**), which were also DCIR2^{+}, as reported before[6] (**Fig. 3c**). DCs were absent from bridging channels in *Ebi2*$^{-/-}$ mice (**Fig. 3b**), and we detected no DCIR2^{+} cells in this location (**Fig. 3c**). In contrast, the localization of CD205^{+} DCs and lymph node DCs was not visibly affected by EBI2 deficiency (**Supplementary Fig. 4a–c**). Thus, like B cells, DCs used EBI2 to localize to the splenic bridging channels, presumably in response to the high concentrations of EBI2 ligand in these regions[25–31].

Modulating EBI2 ligand and receptor affects splenic DCs

Mice deficient in the biosynthetic enzyme cholesterol 25-hydroxylase cannot synthesize 7α,25-OHC and have abnormal localization of splenic B cells[25,27]. To investigate whether 7α,25-OHC also regulated splenic DCs *in vivo*, we analyzed the composition and distribution of DCs in spleens from mice deficient in cholesterol 25-hydroxylase (*Ch25h*$^{-/-}$ mice). Similar to EBI2-deficient mice, *Ch25h*$^{-/-}$ mice had

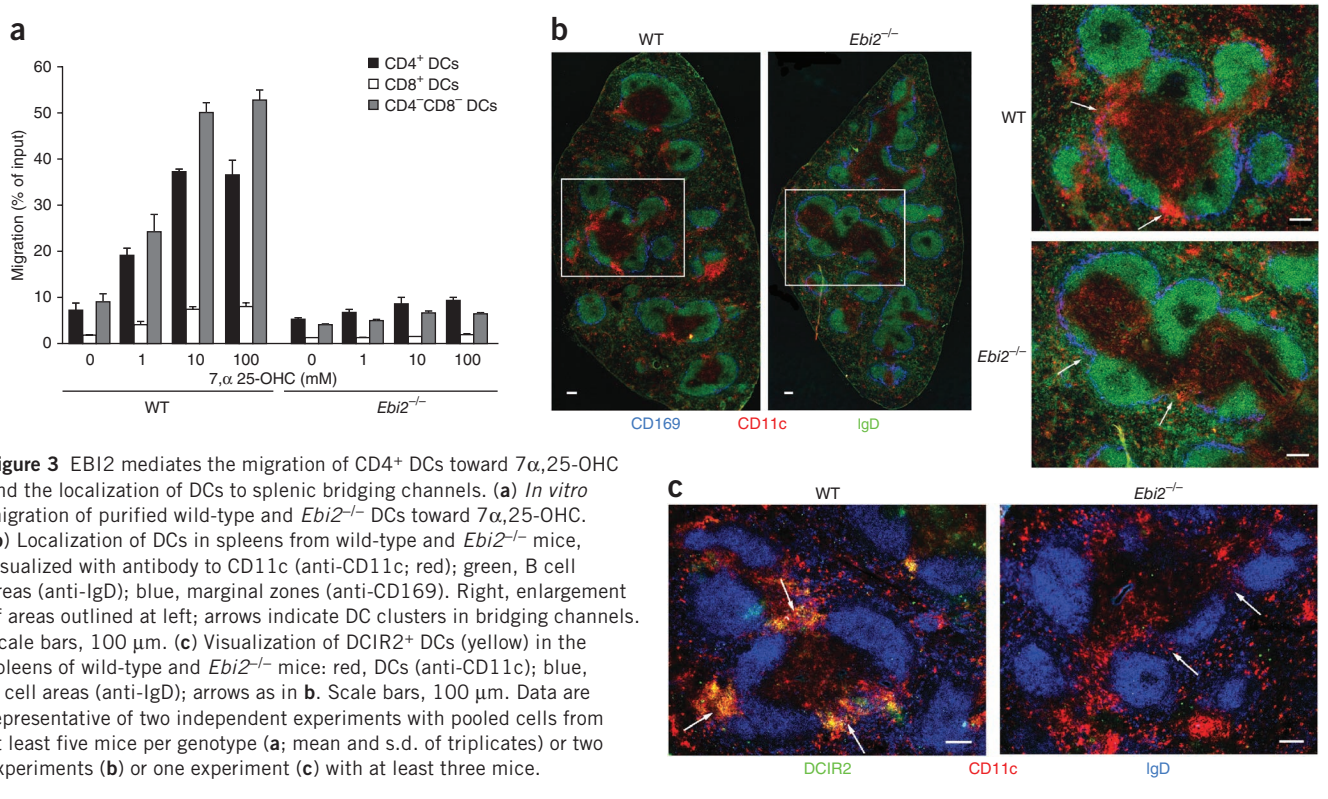

Figure 3 EBI2 mediates the migration of CD4^{+} DCs toward 7α,25-OHC and the localization of DCs to splenic bridging channels. (**a**) *In vitro* migration of purified wild-type and *Ebi2*$^{-/-}$ DCs toward 7α,25-OHC. (**b**) Localization of DCs in spleens from wild-type and *Ebi2*$^{-/-}$ mice, visualized with antibody to CD11c (anti-CD11c; red); green, B cell areas (anti-IgD); blue, marginal zones (anti-CD169). Right, enlargement of areas outlined at left; arrows indicate DC clusters in bridging channels. Scale bars, 100 μm. (**c**) Visualization of DCIR2^{+} DCs (yellow) in the spleens of wild-type and *Ebi2*$^{-/-}$ mice: red, DCs (anti-CD11c); blue, B cell areas (anti-IgD); arrows as in **b**. Scale bars, 100 μm. Data are representative of two independent experiments with pooled cells from at least five mice per genotype (**a**; mean and s.d. of triplicates) or two experiments (**b**) or one experiment (**c**) with at least three mice.

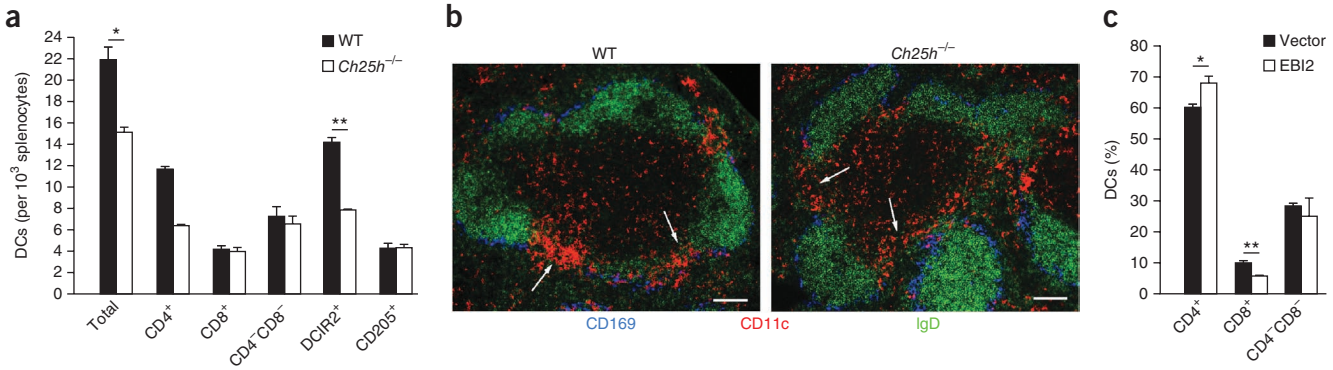

Figure 4 Production of 7α,25-OHC is required for the development of CD4+ DCs and localization of DCs in marginal zone bridging channels. (**a**) Frequency of total DCs and DCs of various subsets (horizontal axis) in wild-type and *Ch25h*$^{-/-}$ spleens. *$P < 0.05$ and **$P < 0.001$ (Student's *t*-test). (**b**) Localization of DCs in spleens from wild-type and *Ch25h*$^{-/-}$ mice (visualized as in **Fig. 3b**). Arrows indicate DCs in bridging channels. Scale bars, 100 μm. (**c**) Subset distribution of splenic DCs in lethally irradiated wild-type mice reconstituted with wild-type bone marrow cells transduced with retrovirus expressing vector alone or EBI2. Data are representative of two experiments with similar results (**a**,**c**; mean and s.e.m. of three mice (**a**) or seven to ten mice (**c**)) or two experiments with similar results with at least three mice (**b**).

selectively fewer CD4+DCIR2+ splenic DCs than did wild-type mice (**Fig. 4a**) and had no accumulation of DCs in splenic bridging channels (**Fig. 4b**). Thus, the EBI2 ligand 7α,25-OHC, which is abundant in those regions of the spleen[27], also seemed to regulate the number and localization of splenic CD4+ DCs. The less severely altered DC phenotype of *Ch25h*$^{-/-}$ mice relative to that of *Ebi2*$^{-/-}$ mice can be explained by the fact that *Ch25h*$^{-/-}$ mice produce small amounts of oxysterols related to 7α,25-OHC that can act as EBI2 ligands[25,26].

To determine whether higher expression of EBI2 could affect splenic CD4+ DC numbers, we introduced an EBI2-expressing retroviral vector into wild-type hematopoietic cells by transduction of bone marrow stem cells and reconstitution of lethally irradiated wild-type mice. We identified DCs transduced with EBI2-expressing or control vector through the use of a green fluorescent protein reporter and determined the frequency of DCs in each subset. Enforced EBI2 expression resulted in a slightly but consistently higher percentage of CD4+ DCs (**Fig. 4c**). Thus, whereas absence of expression of EBI2 or its ligand resulted in fewer CD4+ DCs, overexpression of EBI2 resulted in expansion of this DC subset.

EBI2 functions intrinsically in splenic CD4+ DCs

We next assessed whether the requirement for EBI2 expression for the accumulation and localization of CD4+ DCs in the spleen was intrinsic to DCs. For this, we generated mixed–bone marrow chimeras by reconstituting lethally irradiated C57BL/6 (B6) congenic B6.SJL-*Ptprc*^{a} (CD45.1+) mice with a 50:50 mixture of B6.SJL-*Ptprc*^{a} bone marrow (CD45.1+) plus either *Ebi2*$^{-/-}$ or C57BL/6 (B6) control bone marrow (each C57BL/6 (CD45.2+)). At 8 weeks after reconstitution, we assessed the subset compositions of DCs originating from *Ebi2*$^{-/-}$ (CD45.2+) and B6.SJL-*Ptprc*^{a} (CD45.1+) bone marrow. *Ebi2*$^{-/-}$ DC populations had a lower proportion of DCs with a CD4+DCIR2+ phenotype than did B6.SJL-*Ptprc*^{a} DCs (**Fig. 5a**). Accordingly, although *Ebi2*$^{-/-}$ and B6.SJL-*Ptprc*^{a} DCs developed in the same environment, *Ebi2*$^{-/-}$ cells were greatly under-represented in the CD4+ and DCIR2+ DC populations (**Fig. 5b**), which indicated that EBI2-deficient CD4+DCIR2+ DCs were not generated in normal numbers. In contrast, the ratio of B6.SJL-*Ptprc*^{a} DCs to *Ebi2*$^{-/-}$ DCs was ~1 for CD8+, CD4−CD8− and CD205+ DCs and the ratio of B6.SJL-*Ptprc*^{a} DCs to B6 wild-type DCs was ~1 in all splenic DC compartments (**Fig. 5b**). Thus, as for intact mice, DC-intrinsic EBI2 deficiency resulted in selective loss of CD4+ DCIR2+ DCs in these chimeras.

To confirm the DC-intrinsic requirement for EBI2 expression, we analyzed the generation of CD4+ DCs in mice with DC-specific ablation of EBI2, generated by crossing mice with *lox*P-flanked alleles (*Ebi2*$^{fl/fl}$ mice) with mice with transgenic expression of Cre recombinase from the promoter of the gene encoding the common DC marker CD11c, which results in deletion of *lox*P-flanked genes specifically in DCs (*Cd11c*-Cre+ mice). The *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ progeny had a lower frequency of DCs of the CD4+DCIR2+ subset than did *Ebi2*$^{fl/fl}$ or *Cd11c*-Cre+ control mice (**Fig. 5c** and data not shown). As a result of this defect in the generation of CD4+DCIR2+ DCs, *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice had fewer total splenic DCs than did *Ebi2*$^{fl/fl}$ mice, but had an abundance of CD8+CD205+ and CD4−CD8− DCs once again similar to that of *Ebi2*$^{fl/fl}$ mice (**Fig. 5d**). Histological analysis of spleens from *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice also showed that localization of DCs in bridging channels required DC-intrinsic EBI2 expression (**Fig. 5e**).

EBI2 does not control DC precursors or DC survival

To investigate the role of EBI2 in DC development in more detail, we next sought to determine whether EBI2 delivered signals that either directly promoted the development of CD4+ DCs or their immediate precursors or regulated the turnover or survival of CD4+ DCs. Culture of *Ebi2*$^{-/-}$bone marrow cells with the cytokine Flt3L generated DC populations with a phenotype similar to that of wild-type DCs (**Fig. 6a** and data not shown), which indicated that the equivalents of CD8+ and CD8− DCs (CD24^{hi} and CD11b^{hi} cells, respectively) developed normally *in vitro*. EBI2 was also not required for early DC development, as the number of macrophage and DC progenitors, common DC progenitors and precursors to conventional DCs in the bone marrow, as well as splenic precursors to conventional DCs, were unaffected in *Ebi2*$^{-/-}$ mice (**Fig. 6b**).

To determine if EBI2 deficiency had an effect on the turnover of CD4+ DCs, we compared the rate of *in vivo* incorporation of the thymidine analog BrdU into splenic *Ebi2*$^{-/-}$ and wild-type DCs. To exclude the possibility of a contribution of DC-extrinsic factors that affect turnover rates, we used *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice in these experiments. Over a 48-hour period, the percentage of DCs that incorporated BrdU was similar for all of the DC subsets in *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice (**Fig. 6c**). Accordingly, a similar percentage of DCs in each subset expressed the nuclear proliferation antigen Ki67 (**Fig. 6d**).

To establish whether EBI2 had a role in promoting DC survival, we compared the frequency of DCs undergoing apoptosis in spleens from *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice. The spleens of

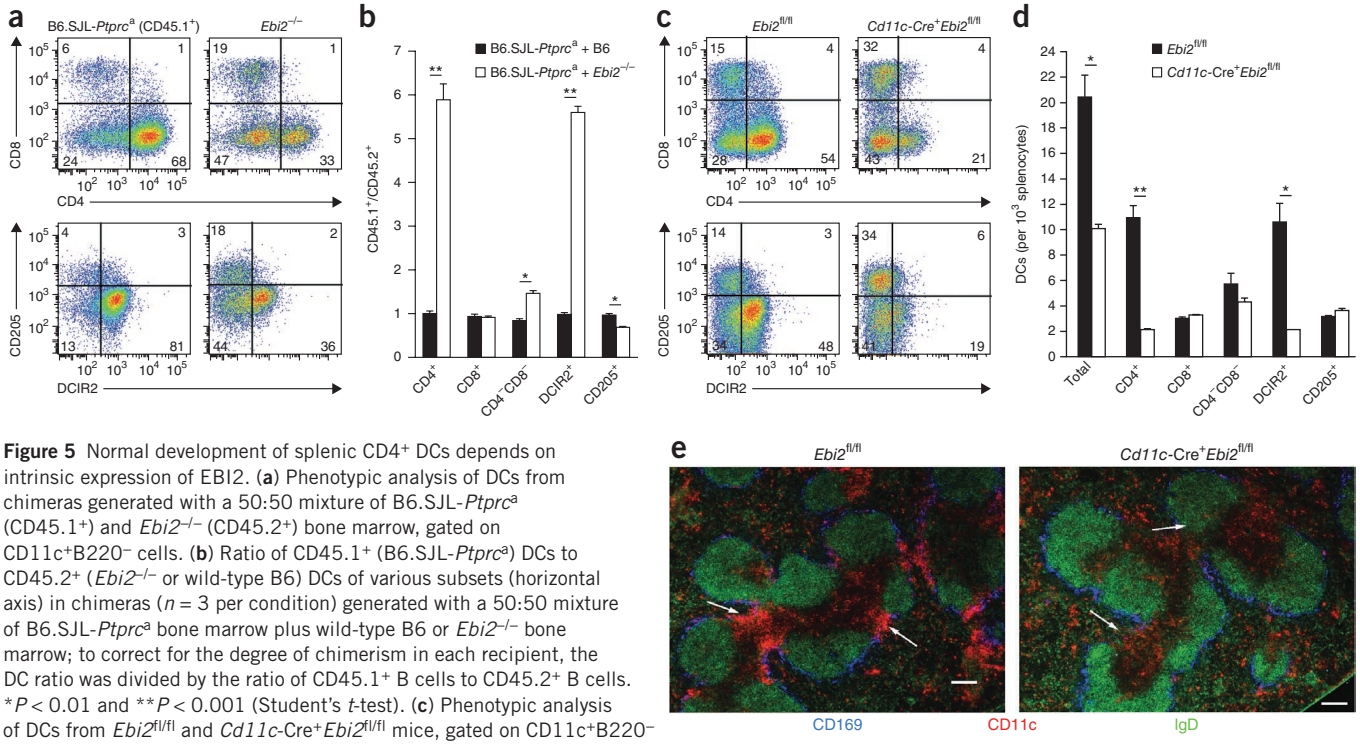

Figure 5 Normal development of splenic CD4+ DCs depends on intrinsic expression of EBI2. (**a**) Phenotypic analysis of DCs from chimeras generated with a 50:50 mixture of B6.SJL-*Ptprc*^{a} (CD45.1+) and *Ebi2*$^{-/-}$ (CD45.2+) bone marrow, gated on CD11c+B220− cells. (**b**) Ratio of CD45.1+ (B6.SJL-*Ptprc*^{a}) DCs to CD45.2+ (*Ebi2*$^{-/-}$ or wild-type B6) DCs of various subsets (horizontal axis) in chimeras ($n = 3$ per condition) generated with a 50:50 mixture of B6.SJL-*Ptprc*^{a} bone marrow plus wild-type B6 or *Ebi2*$^{-/-}$ bone marrow; to correct for the degree of chimerism in each recipient, the DC ratio was divided by the ratio of CD45.1+ B cells to CD45.2+ B cells. $^*P < 0.01$ and $^{**}P < 0.001$ (Student's *t*-test). (**c**) Phenotypic analysis of DCs from *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice, gated on CD11c+B220− cells. (**d**) Frequency of total DCs and DCs of various subsets (horizontal axis) in spleens from *Ebi2*$^{fl/fl}$ and *CD11c*-Cre+*Ebi2*$^{fl/fl}$ mice ($n = 3$ per genotype). $^*P < 0.01$ and $^{**}P < 0.001$ (Student's *t*-test). (**e**) Localization of DCs in the spleen of *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice (visualized as in **Fig. 3b**). Arrows indicate DC clusters in bridging channels. Scale bars, 100 μm. Data are representative of two experiments (**a**), two experiments with similar results (**b**; mean and s.e.m.), three experiments with similar results (**c**), three experiments (**d**; mean and s.e.m.) or two experiments with at least three mice (**e**).

both groups of mice had a similar percentage of DCs that expressed active caspase-3 or bound annexin V for all three DC subsets (**Fig. 6e**,**f**). Consistent with those results, 7α,25-OHC did not improve DC survival in culture (**Supplementary Fig. 5**). Together these findings indicated that EBI2 did not function by promoting the proliferation or survival of CD4+ DCs or by promoting the development of their direct precursors. As we found no evidence that CD4+ DCs localized elsewhere in the body of EBI2-deficient mice (**Supplementary Fig. 1** and data not shown), it seemed likely that EBI2 controlled splenic CD4+ DCs by specifically regulating their differentiation in the spleen.

Responses to particulate antigen require DC EBI2

Although it is established that EBI2 expression by B cells is critical for the induction of antibody responses, the importance of EBI2 expression by DCs in immune responses remains unclear. To test the ability of EBI2-deficient DCs to prime antibody responses, we adoptively transferred CD45.1+ SW_{HEL} B cells (which are specific for hen egg lysozyme (HEL)) into *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ or *Ebi2*$^{fl/fl}$ recipient mice and challenged the recipients intravenously with HEL^{2X} protein (an HEL mutant with lower affinity) conjugated to sheep red blood cells (SRBCs)[28,35,36]. We then assessed the population expansion and differentiation of SW_{HEL} B cells 5 d after immunization. The frequency of SW_{HEL} B cells that responded to HEL^{2X}-conjugated SRBCs in *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice was only 10–20% that in immunized control *Ebi2*$^{fl/fl}$ recipients, and neither GC B cells nor plasma cells were generated in normal numbers (**Fig. 7a**). There was a lower frequency of CD38^{lo}B220^{hi} SW_{HEL} GC B cells in particular in *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ recipients than in *Ebi2*$^{fl/fl}$ recipients, as well as a lower proportion of SW_{HEL} B cells that had undergone isotype switching (**Fig. 7b**). As anticipated from the diminished response of SW_{HEL} B cells, *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice had 80–90% lower serum concentrations of immunoglobulin M (IgM) and IgG1 antibodies to HEL than did *Ebi2*$^{fl/fl}$ mice (**Fig. 7c**). The defect in the B cell response induced in *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice was not apparent in the early stages of the response, as SW_{HEL} B cells showed normal proliferation and upregulation of the activation marker CD86 on day 3 after immunization (**Supplementary Fig. 6**). This finding suggested that the B cell response was affected, as it became more dependent on T cell help, and therefore that DCs in *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice were inefficient in priming CD4+ T cell responses.

To assess the requirement for EBI2 expression by DCs in the induction of CD4+ T cell responses, we adoptively transferred OT-II T cells (which have transgenic expression of an ovalbumin-specific T cell antigen receptor) into *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ or *Ebi2*$^{fl/fl}$ recipient mice, which we immunized with ovalbumin-conjugated SRBCs. We identified OT-II T cells by their expression of Thy-1.1 and T cell antigen receptor α-chain variable region 2. Analysis of the frequency of OT-II T cells at various time points after immunization indicated that their population expansion was lower in *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice than in *Ebi2*$^{fl/fl}$ mice (**Fig. 7d**). The generation of follicular helper T cells (identified by their expression of the chemokine receptor CXCR5 and the costimulatory molecule PD-1) was also impaired in *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice (**Fig. 7e**), as was the GC B cell response (**Fig. 7f**).

The results reported above demonstrated that the induction of B cell and T cell responses to intravenous particulate antigen required EBI2 expression on DCs. To determine whether EBI2-deficient DCs had an intrinsic defect in supporting T cell–dependent antibody responses, we assessed the ability of DCs in *Cd11c*-Cre+*Ebi2*$^{fl/fl}$ mice to prime antibody responses to a soluble antigen delivered to

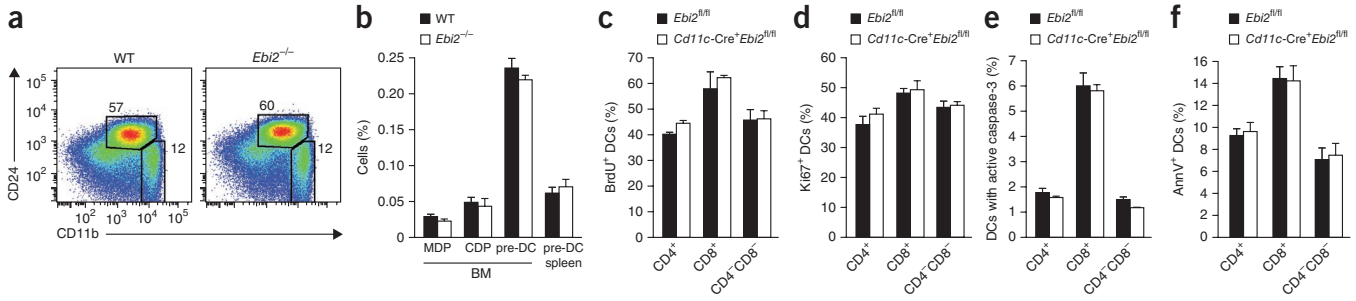

Figure 6 EBI2 is not required for normal development of DC precursors or survival of splenic DCs. (**a**) Phenotype of DCs generated *in vitro* from wild-type or *Ebi2*$^{-/-}$ bone marrow cultured with Flt3L (plots gated on CD11c$^+$B220$^-$ cells). Numbers adjacent to outlined areas indicate percent cells that are equivalents of CD8$^+$ DCs (top left) or of CD8$^-$ DCs (bottom right). (**b**) Frequency of CD3$^-$CD19$^-$B220$^-$NK1.1$^-$Gr-1$^-$CD11b$^-$CD11c$^-$(Lin$^-$)Sca-1$^-$Flt3$^+$CD115$^+$CD117^{hi} macrophage and DC progenitors (MDP), Lin$^-$Sca-1$^-$ Flt3$^+$CD115$^+$CD117^{lo} common DC progenitors (CDP) and CD3$^-$B220$^-$NK1.1$^-$M HCII$^-$CD11c$^+$Flt3$^+$CD172α^{lo} precursors of conventional DCs (pre-DCs) in bone marrow (BM) and spleen from wild-type and *Ebi2*$^{-/-}$ mice. (**c**) Incorporation of BrdU by various subsets (horizontal axis) of splenic DCs from *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice given intraperitoneal injection of BrdU and then given BrdU in drinking water for 48 h before analysis. (**d**–**f**) Frequency of Ki67-expressing DCs (**d**), DCs expressing active caspase-3 (**e**) or DCs that bind annexin V (AnnV$^+$; **f**) in various subsets (horizontal axes) of splenic DCs from *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice. Data are representative of two experiments (mean and s.e.m. of three mice per genotype in **b**–**f**).

the EBI2-expressing DC populations. We exploited the restricted expression of the signal-regulatory protein Sirpβ1 on CD4$^+$ DCs and some CD4$^-$CD8$^-$ DCs, but not on CD8$^+$ DCs[37] (**Fig. 7g**), to deliver antigen directly to CD4$^+$ DCs, and used rat monoclonal antibody to Sirpβ1 as an antigen. We injected *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice intravenously with rat antibody to Sirpβ1 and then measured their titers of IgG antibodies to rat. The antibody responses elicited this way were not impaired by the absence of EBI2 on DCs but were instead higher in *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice than in their *Ebi2*$^{fl/fl}$ counterparts (**Fig. 7h**), possibly due to the slightly higher expression of Sirpβ1 on DCs in *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice (**Fig. 7g**). Thus, delivery of antigen to CD4$^+$ DCs bypassed the requirement for EBI2 for the induction of antibody responses, which indicated that EBI2-deficient DCs were functionally able to prime antibody responses but did not support responses to particulate antigen because of their aberrant localization in the spleen.

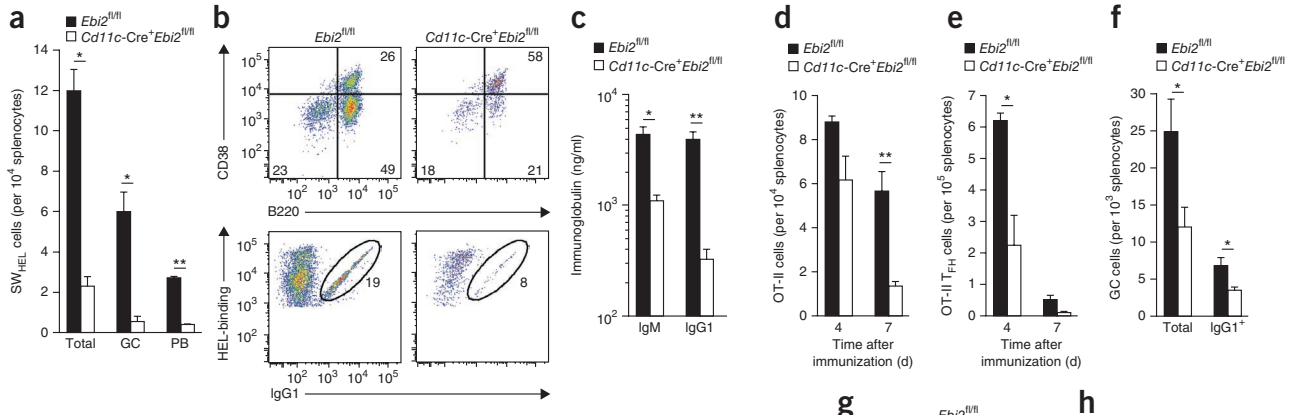

Figure 7 EBI2 expression on DCs is required for normal induction of B cell and T cell responses. (**a**) Frequency of total SW$_{HEL}$ B cells and cells with a B220^{hi}CD38^{lo} GC phenotype or B220^{lo}CD38^{lo} plasmablast (PB) phenotype after adoptive transfer of SW$_{HEL}$ B cells into *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice, assessed on day 5 after immunization of recipients with HEL^{2X}-conjugated SRBCs. (**b**) Phenotype of responding SW$_{HEL}$ B cells in recipients 5 d after transfer and immunization as in **a** (gated on donor-derived (CD45.1$^+$CD45.2$^-$) HEL-binding B cells). Numbers adjacent to outlined areas (bottom row) indicate percent HEL-binding IgG1$^+$ cells. (**c**) Concentration of anti-HEL IgM and IgG1 in serum of recipients as in **a**. (**d**,**e**) Frequency of total OT-II cells (**d**) and OT-II follicular helper T (T$_{FH}$) cells (**e**) after adoptive transfer of OT-II T cells into *Ebi2*$^{fl/fl}$ and *CD11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice, assessed 4 or 7 d after immunization of recipients with ovalbumin-conjugated SRBCs. (**f**) Frequency of endogenous B220^{hi}CD38^{lo} GC B cells in mice as in **d**,**e**, 7 d after transfer and immunization. (**g**) Expression of Sirpβ1 on splenic DC populations in *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice (gated on CD11c$^+$B220$^-$ cells). (**h**) Anti-rat immunoglobulin (Ig) titers in serum from *Ebi2*$^{fl/fl}$ and *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice 2 and 4 weeks after immunization with rat monoclonal antibody to Sirpβ1. Background titers in mice immunized with isotype-matched control monoclonal antibody (GL117) were <80. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*-test). Data are representative of three experiments (**a**–**c**), two experiments (**d**–**g**) or one experiment (**h**; mean and s.e.m. of three to four mice per genotype in **a**, **c**–**f**, **h**).

DISCUSSION

Although a variety of transcription factors have been shown to facilitate the development of either $CD8^+$ or $CD8^-$ conventional DCs[20], EBI2 is the first cell surface receptor to our knowledge shown to be required for the generation of a specific DC subset. Furthermore, EBI2 is the only known factor to our knowledge found to control the production of $CD4^+$ DCs but not the closely related $CD4^-CD8^-$ subset. Our study has shown that EBI2 expression on DCs regulated their access to splenic bridging channels and was crucial for the induction of B cell and T cell responses to blood-borne particulate antigen.

A question raised by our investigations is whether the signals delivered to DCs via EBI2 are directly responsible for promoting the development of $CD4^+$ DCs. This question is particularly salient given the fact that the only known function of EBI2 is to mediate cellular chemotaxis in response to 7α,25-OHC. As DC populations were generated normally from EBI2-deficient bone marrow *in vitro,* EBI2 does not seem to directly mediate the development of $CD4^+$ DCs. Furthermore, as EBI2 deficiency selectively resulted in a smaller $CD4^+$ DC subset, the development and migration to the spleen of the common immediate DC precursor[16,17] seems to occur normally in absence of EBI2. Accordingly, we found normal frequencies of precursors of conventional DCs in the spleens of EBI2-deficient mice.

We propose that rather than directly promoting the generation of $CD4^+$ DCs via as-yet-undetermined signaling pathways, EBI2 may instead guide $CD4^+$ DCs or their precursors to the appropriate microanatomical niche in which spatially restricted cues are ultimately responsible for driving $CD4^+$ DC differentiation. We found here that EBI2 and its ligand were absolutely required for the positioning of splenic DCs in the marginal zone bridging channels. Although a residual $CD4^+$ DC population was present in both *Ebi2*$^{-/-}$ mice and *Ch25h*$^{-/-}$ mice, these DCs did not cluster in the bridging channels, which indicated that EBI2 expression on $CD4^+$ DCs was required for their localization to these areas.

If spatially restricted tropic factors present in the marginal zone and bridging channels are indeed required for the establishment of $CD4^+$ DCs, one possible source of these factors is cells of the B lineage. Both the splenic marginal zone and bridging channels are rich in B cells and plasmablasts, which have been shown to associate closely with DCs[11,16,38]. A candidate tropic factor in this case would be lymphotoxin-$\alpha_1\beta_2$, a membrane cytokine known to be required for the homeostasis of $CD4^+$ DCs[16] and to be expressed mainly by B cells in the spleen[39]. It remains possible that in the absence of EBI2 expression, a large number of the $CD4^+$ DCs leave the spleen and relocate elsewhere in the body. However, we have not detected $CD4^+$ DCs in the blood (data not shown) or nonlymphoid organs. Thus, EBI2 most probably acts by potentiating the differentiation of $CD4^+$ DCs through its ability to regulate their localization in the spleen.

Although $CD4^+$ DCs are generally most efficient at presenting antigen on MHC class II, $CD8^+$ and $CD4^-CD8^-$ DCs are able to present antigen to $CD4^+$ T cells via MHC class II[8,10,12,13], and antigen targeted to $CD8^+$ DCs has been shown to elicit good antibody responses[14,40]. Nevertheless, our findings have demonstrated that $CD8^+$ DCs and $CD4^-CD8^-$ DCs were not able to compensate for a grossly smaller $CD4^+$ DC subset and that an intact population of $CD4^+$ DCs in marginal zone bridging channels was crucial for the induction of B cell and T cell responses to blood-borne antigen. As $CD4^-CD8^-$ DCs expressed EBI2 and migrated toward 7α,25-OHC, it is possible that although they were present in normal numbers, they were displaced from the marginal zone bridging channels. This may have contributed to the lower responsiveness of *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice to particulate antigen. $CD4^+$ DCs in the bridging channel are strategically located to capture intravenously administered particulate antigens (such as the HEL-conjugated SRBCs used in our study here) that are trapped in the splenic marginal zone. The response to the same antigen injected intraperitoneally was not impaired (data not shown), and when we delivered antigen to $CD4^+$ DCs through the use of antibody to Sirpβ1, EBI2 expression on DCs was no longer required for the induction of antibody responses. We conclude that EBI2-deficient splenic DCs were functionally able to present antigen and interact with T cells but were unable to access blood-borne antigen trapped in the marginal zone because of their inability to localize near this area. A further explanation for the superior ability of $CD4^+$ DCs to elicit antibody responses may be that they are localized in the migration pathway of lymphocytes homing to the spleen and therefore are ideally positioned for the presentation of captured blood-borne antigen to recirculating naive B cells and T cells. Published findings have shown that B cells accumulate rapidly in bridging channels after antigen delivery to $DCIR2^+$ DCs and are activated by antigen-bearing DCs[11]. Similarly, DCs residing in the marginal zone may capture and present HEL-conjugated SRBCs to incoming B cells and may be directly involved in their activation. However, as the antibody response to SRBCs is heavily dependent on the efficient delivery of T cell help to responding B cells, it is likely that impaired priming of $CD4^+$ T cells contributed to the defects in the B cell responses of *Cd11c*-Cre$^+$*Ebi2*$^{fl/fl}$ mice.

Although several cell types of the immune system are reported to express EBI2 (refs. 25,26,41), the only previously characterized function of this receptor to our knowledge has been in the context of B cell migration and differentiation. Our study has extended knowledge of the roles of EBI2 in the immune response. We have now identified EBI2 as the chemotactic receptor responsible for the accumulation of DCs in bridging channels and as another factor required for the homeostasis of splenic $CD4^+$ DCs. This information may prove valuable in designing DC-based vaccines and in evaluating the therapeutic benefits of oxysterols.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

D.G. and R.B. conceived of the project and wrote the manuscript; D.G. and K.W. did most of the experiments; I.C. provided antibody to Sirpβ1 and analyzed anti-rat IgG responses; D.M.-D. produced and analyzed cells transfected to express EBI2; P.S. and D.C. organized the production and purification of antibody to EBI2; and G.K. helped analyze responses in EBI2-deficient mice.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. *Ebi2*$^{-/-}$, SW_{HEL} and OT-II mice have been described[28,42,43]. *Cd11c*-Cre mice[44] and *Ch25h*$^{-/-}$ mice[45] were from The Jackson Laboratory. SW_{HEL} mice were bred onto a CD45.1^{+} congenic C57BL/6 background and OT-II mice were bred onto a Thy-1.1^{+} congenic C57BL/6 background; all other mice were maintained on a C57BL/6 background. Mice were bred and housed in a specific pathogen–free environment at Australian Bioresources and the Garvan Institute. Experiments were carried according to protocols approved by the Garvan/St Vincent's Animal Ethics Committee.

Isolation and culture of DCs. Spleens and peripheral lymph nodes (inguinal, axillary, brachial and cervical) were digested with 2 mg/ml collagenase D (Roche) and 20 μg/ml DNase I (Roche) as described[5]. Kidneys and lungs were isolated from perfused mice, cut into small pieces and digested for 1 h with 2 mg/ml collagenase D and 20 μg/ml DNase I. Cell suspensions were purified by Ficoll gradient centrifugation according to the manufacturer's instructions (GE Healthcare). DCs were purified from spleen cell suspensions by negative depletion with magnetic cell sorting. DC-enriched cell preparations were cultured overnight with 10 μg/ml LPS (Sigma-Aldrich) and/or 'titrated' amounts of 7α, 25-OHC (Avanti Polar Lipids) in RPMI-1640 medium supplemented with 10% FCS. For generation of DCs from bone marrow cultures, bone marrow cells were cultured for 10 d at a density of 1.5×10^6 cells/ml in RPMI-1640 medium supplemented with 10% FCS containing 300 ng/ml mouse Flt3L (R&D Systems).

Generation of anti-EBI2. Polyclonal EBI2-specific antibodies were generated in chickens with the synthetic peptide MANNFTTPLATSHGNNC for immunization (amino-terminal 17 amino acids of mouse EBI2). Peptide was conjugated to keyhole limpet hemocyanin via the carboxy-terminal cysteine residue. Hens were injected with the keyhole limpet hemocyanin–conjugated peptide (four injections over a 7-week period). Antibodies (IgY) were collected from both preimmune and immune eggs. Peptide-specific titers were determined by enzyme-linked immunosorbent assay against an ovalbumin (OVA) conjugate of the peptide. Antibodies from immune eggs were purified on an affinity matrix consisting of EBI2 peptide conjugated to agarose. Flow-through of the affinity purification was used as a negative control in flow cytometry. Antibodies were biotinylated with 20-fold molar excess of EZ-link NHS-PEG4-Biotin (Pierce), and unbound biotinylation reagent was removed by dialysis. Nonspecific binding activity of the EBI2 antibodies was removed by incubation for 1 h at 4 °C with EBI2-deficient splenocytes.

Flow cytometry. Cell suspensions were stained and analyzed on a FACSCanto II (BD Biosciences) or SORP LSRII (BD Biosciences) as described[28,46]. The following antibodies and reagents were used for cell staining: allophycocyanin–anti-CD11c (N418; eBiosciences), fluorescein isothiocyanate–anti-CD11c (HL3; BD Biosciences), phycoerythrin–anti-CD4 (RM4-5; BD Biosciences), allophycocyanin–anti-CD4 (RM4-5; BD Biosciences), fluorescein isothiocyanate–anti-CD4 (RM4-5; BD Biosciences), peridinin chlorophyll protein–anti-CD4 peridinin chlorophyll protein (RM4-5; BD Biosciences), Pacific Blue–anti-CD8 (53-6.7; BD Biosciences), peridinin chlorophyll protein–cynanine 5.5–anti-CD8 (53-6.7; Biolegend), phycoerythrin-indotricarbocyanine–anti-CD45R (anti-B220; RA3-6B2; BD Biosciences), peridinin chlorophyll protein–cyanine 5.5–anti-CD11b (M1/70; BD Biosciences), biotin–anti-DCIR2 (33D1; eBiosciences), phycoerythrin–anti-CD205 (205yekta; eBiosiences), phycoerythrin–anti-CD86 (GL1; BD Biosciences), fluorescein isothiocyanate–conjugated antibody to MHC class II (M5/114.15.2; eBiosciences), biotin–anti-CD40 (HM40.3; eBiosciences), biotin–anti-CD80 (16-10A1; BD Biosciences), peridinin chlorophyll protein–cyanine 5.5–anti-CD45.1 (A20; eBiosciences), fluorescein isothiocyanate–anti-CD45.2 (104, BD Biosciences), phycoerythrin-indotricarbocyanine–anti-CD45.2 (104; eBiosciences), Alexa Fluor 647–anti-Ki67 (B56; BD Biosciences), fluorescein isothiocyanate–anti-IgG1 (A85-1; BD Biosciences), phycoerythrin–anti-CD38 (90; BD Biosciences), eFluor450–anti-Thy-1.1 (HIS51; eBiosciences), allophycocyanin-conjugated antibody to T cell antigen receptor α-chain variable region 2 (B20.1; eBiosciences), biotin–anti-CXCR5 (2G8; BD Biosciences), phycoerythrin–anti-PD1 (J43; BD Biosciences), phycoerythrin–anti-CD207 (10E2; Biolegend), allophycocyanin–anti-CD103 (2E7; Biolegend), biotin–anti-CD3 (145-2C11; eBioscience), biotin–anti-NK1.1 (PK136; Biolegend), biotin–anti CD11b (M1/70; BD Biosciences), allophycocyanin–anti-CD19 (1D3; BD Biosciences), allophycocyanin–anti-Sca-1 (D7; Biolegend), phycoerythrin–anti-CD135 (A2F10; Biolegend), Alexa Fluor 488–anti-CD115 (AFS98; Biolegend), Brilliant Violet 421–anti-CD117 (2B8; Biolegend), Pacific Blue–conjugated antibody to MHC class II (M5/114.15.2; Biolegend), fluorescein isothiocyanate–anti-CD172α (P84; Biolegend), streptavidin-phycoerythrin (BD Biosciences), streptavidin–peridinin chlorophyll protein–cyanine 5.5 (BD Biosciences), streptavidin-phycoerythrin-indotricarbocyanine (eBiosciences), streptavidin-allophycocyanin (BD Biosciences), streptavidin–Qdot 605 (Invitrogen). For staining of apoptotic cells phycoerythrin-conjugated antibody to caspase 3 (C92-605; BD Biosciences) and fluorescein isothiocyanate–conjugated antibody to annexin V (556420; BD Biosciences) were used according to the manufacturer's instructions.

Quantitative RT-PCR. DC populations were sorted into TRIzol reagent (Invitrogen) and *Ebi2* expression was quantified as described[28]. Expression of GAPDH (glyceraldehyde phosphate dehydrogenase) was used for copy-number normalization.

Transwell migration assay. DCs were purified from spleens of *Ebi2*$^{-/-}$ and wild-type mice by magnetic cell sorting and were allowed to transmigrate for 3 h across 5-μm Transwell filters (Corning Costar) as described[47]. Cells that had migrated toward7α,25-OHC (Avanti Polar Lipids) were counted by flow cytometry.

Immunofluorescence microscopy. Spleen and inguinal lymph node sections were cut, prepared and analyzed as described[28]. DCs were stained with biotinylated anti-CD11c (HL3; BD Biosciences) and Alexa Fluor 555–conjugated streptavidin (Invitrogen) or allophycocyanin–anti-CD11c (N418; eBioscience), and B cell follicles were stained with fluorescein isothiocyanate–anti-IgD (11-26c.2a; BD Biosciences) or Alexa Fluor 647–anti-IgD (11-26c.2a; BioLegend). Metallophilic macrophages were visualized with Alexa Fluor 647–anti-CD169 (Ser-4) with an antibody labeling kit from Molecular Probes. DCIR2^{+} and CD205^{+} cells were stained with biotinylated anti-DCIR2 (33D1; eBiosciences) and anti-CD205 (NLDC-145; Biolegend), respectively. Fluorescein isothiocyanate–conjugated antibody to MHC class II (M5/114.15.2; eBiosciences) and biotinylated anti-CD11b (M1/70; BD Biosciences) were used for staining of lymph nodes.

Generation of bone marrow chimeras. B6.SJL-*Ptprc*^{a} mice were lethally irradiated and reconstituted by intravenous injection of a 1:1 mixture of 2×10^7 *Ebi2*$^{-/-}$ or C57BL/6 and B6.SJL-*Ptprc*^{a} bone marrow cells. Mice were analyzed 2–3 months after reconstitution. Retroviral transduction of hematopoietic stem cells was done as described with an MSCV2.2-based retroviral vector expressing EBI2 and enhanced green fluorescent protein as an expression marker[28]. Bone marrow cells from C57BL/6 mice were transduced with retroviral supernatants and were used to reconstitute lethally irradiated C57BL/6 recipient mice. Chimeric mice were analyzed 8–10 weeks after reconstitution and had normal frequencies of DCs, with 2–20% of DCs being transduced, as assessed by expression of green fluorescent protein.

BrdU labeling. Mice were injected intraperitoneally with 2 mg BrdU (5-bromodeoxyuridine) and were maintained for 48 h with water containing 0.8 mg/ml of BrdU. Splenocytes were prepared and stained with an FITC BrdU flow kit according to the manufacturer's instructions (BD Biosciences).

Adoptive transfer and immunization. The adoptive-transfer system used for analysis of the responses of SW_{HEL} B cells, mutant HEL^{2X} protein and its conjugation to SRBCs have been described[28,35,36]. For conjugation of SRBCs to OVA, SRBCs were incubated for 1 h with 10 mg/ml OVA (Sigma-Aldrich) in 0.35 M mannitol and 0.01 NaCl in the presence of 10 mg/ml N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (Sigma-Aldrich). The conjugation efficiency was checked by flow cytometry with fluorescein isothiocyanate–conjugated polyclonal rabbit anti-OVA (ab85584; Abcam). OVA-conjugated SRBCs (2×10^8) were injected intravenously together with OT-II T cells (5×10^4) into *Cd11c*-Cre^{+}*Ebi2*$^{fl/fl}$ or *Ebi2*$^{fl/fl}$ recipient mie. Immunization with rat monoclonal antibody to Sirpβ1 (7E11; purified in-house from hybridoma supernatant) was done intravenously with 2 μg antigen.

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Enzyme-linked immunosorbent assay. Serum concentrations of anti-HEL IgM and IgG1 analyzed by enzyme-linked immunosorbent assay as described[42].

Anti-rat immunoglobulin titers were determined with horseradish peroxidase–conjugated donkey antibody to mouse IgG (Chemicon International) as described[14,40].

Statistical analysis. Statistical differences between means were assessed by an unpaired two-tailed Student's *t*-test.

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