EBI2 augments Tfh cell fate by promoting interaction with IL-2–quenching dendritic cells
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T follicular helper (Tfh) cells are a subset of T cells carrying the CD4 T-cell receptor (TCR) that play a critical role in the generation of antibody-secreting plasma cells. They are defined by expression of the follicle-homing receptor CXCR5 and the transcriptional regulator BCL6. EBI2 is a G-protein-coupled receptor (GPCR) that is expressed by CD4+ T cells and is known to be upregulated in Tfh cells. However, the role of EBI2 in Tfh cell differentiation is not fully understood.

EBI2 KO Tfh cells failed to accumulate in the outer T zone at either time point and instead remained dispersed throughout the T zone. Thus, EBI2 augments Tfh cell fate by promoting positioning in the outer T zone.

WT OTII T cells also moved to the B–T zone interface in lymph nodes after immunization with alum-OVA, but EBI2-deficient T cells failed to re-localize. Activated T-cell positioning in the outer T zone was directed by 7α,25-OHC as it was dependent on the enzymes needed for its synthesis (Cyp7b1 and Ch25h) and catabolism (Hsd3b7).

Flow cytometric analysis for the early activation marker CD69 showed that co-transferred EBI2 KO and WT T cells were comparably activated at day 2 of the SRBC-OVA response (Fig. 2a), indicating similar initial exposure to cognate MHC class II–peptide complexes.

Figure 1 | EBI2 promotes positioning of newly activated CD4 T cells in the outer T zone. a, Immunohistochemical analysis of spleens for transferred WT or EBI2 KO OTII CD45.1+ T cells (blue) and endogenous B cells (IgD, brown) at 12 h, 1 day and 2 days after SRBC-OVA immunization. b, Fraction of WT and EBI2 KO OTII T cells in the outer quarter of the splenic T zone at 12 h and 1 day after SRBC-OVA immunization. Sections were stained as in Extended Data Fig. 1g. See Methods for details. c, d, As for a except mice were immunized with Listeria-OVA (c) or alum-OVA and inguinal lymph nodes were analysed (d). **P < 0.01 by Student’s t-test. Data are representative of three (a, b) or two (c–e) experiments with at least three (a) or two (b–e) mice per group.

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Figure 2 | Defective differentiation of EBI2-deficient T cells to follicular helpers. a, CD69 expression on WT OTII, EBI2 KO OTII and endogenous CD4 T cells in transfer recipient spleens 2 days after SRBC-OVA immunization. Histograms show representative FACS and graphs show summary geoMFI data for three mice of each type. b, Proliferation of co-transferred WT and EBI2 KO OTII T cells monitored by violet tracer dye dilution at days 2 and 3 after immunization. Numbers indicate mean percentage (± s.d.) of cells in the indicated gate (n = 6). c, Summary of data from b shown as a ratio of KO/WT OTII T-cell number at the indicated days. d, Flow cytometric analysis of co-transferred WT and EBI2 KO OTII T cells for PD-1 and CXCR5 at days 2 and 3 after immunization. Numbers indicate frequency of cells in gated region. e, Summary of data of the type in d: frequency (top) and number (bottom) of CXCR5+ PD-1+ CD4+ OTII T cells. f, Frequency and number of CXCR5+ PD-1+ CD4+ OTII T cells in peripheral lymph nodes of mice of the type in d, immunized with alun-OVA. ∗P < 0.05 and ∗∗P < 0.01 by analysis of variance (ANOVA) (d, f) or Student’s t-test (g). Data are representative of three (a–f) or two (g) experiments with at least three mice per group. Upregulation of the co-stimulatory molecules ICOS and OX40 also occurred to an equivalent extent (Extended Data Fig. 2a). Proliferation began by day 2 and at this point the WT and EBI2 KO cells responded similarly (Fig. 2b, c). However, by day 3, the EBI2-deficient cells were undergoing less proliferation and their numbers increased more slowly (Fig. 2b, c). This was not due to a direct effect of TGF-β, SHC or other immunomodulatory molecules on T-cell proliferation (Extended Data Fig. 2b, c). Tracking of differentiation markers on the in vivo activated T cells revealed that EBI2 KO cells were compromised in their induction of a Th cell phenotype, as assessed by CXCR5, PD-1 (Fig. 2d, e), Bcl6, and Il21 expression (Extended Data Fig. 2d–f). EBI2-deficient OTII T cells also differentiated less efficiently into Th cells in lymph nodes (Fig. 2f). We also observed reduced Th cell responses to Listeria-OVA, reduced polyclonal EBI2 KO Th cell responses to SRBCs and reduced germinal centre and plasma cell responses to these antigens (Supplementary Information and Extended Data Fig. 3a–j).

Th cell differentiation is promoted by interaction both with dendritic cells (DCs) and with B cells, and time-course studies indicate that DCs are critical early while B cells play a later role.1,2 Consistent with these requirements, WT OTII T cells showed a partial reduction in Th cell differentiation at day 3 of the response in MD4 immunoglobulin (Ig)-transgenic mice lacking cognate B cells capable of OVA antigen presentation to OTII T cells (Fig. 3a). Importantly, however, EBI2 KO OTII T cells formed fewer Th cells than WT OTII T cells in the MD4 recipients, indicating that T-cell EBI2 expression augmented Th cell development at early time points in a B-cell-independent manner.

Similar observations were made in B-cell-deficient μMT mice (Extended Data Fig. 4a). These findings led us to test whether EBI2 was required in T cells for some type of interaction with DCs. Ablation of DCs using Zbtb46-diphtheria toxin receptor (DTR) mice caused a complete block in Th cell generation (Extended Data Fig. 4b, c). In the case of conventional DCs (CD11c+), this effect was abrogated by CD11c-DCs (Fig. 4b, c). In contrast, ablation of EBI2 KO T cells was broadly distributed and only partly overlapped with the DCIR2+ DCs (Fig. 4b, c).
with deficiencies in DCs, we established that CD4+ but not CD8+ DCs were important for Tfh cell induction by SRBC-OVA (Supplementary Information and Extended Data Fig. 4f–k).

ICOS signalling is important for Tfh cell differentiation,1,2 and splenic DCs upregulated ICOS ligand (ICOSL) mRNA after activation by SRBCs, with the extent of upregulation being greater in CD4+ than CD8+ DCs (Fig. 3d). However, flow cytometric analysis showed CD4+ DCs activated for 12 h had lower surface ICOSL staining (Fig. 3e and Extended Data Fig. 4l). Since ICOSL undergoes rapid ectodomain shedding after ICOS engagement,19,20 we considered the possibility that surface levels were reduced because of interactions with ICOS-high activated T cells. Consistent with this idea, when SRBC-immunized mice were also treated with an ICOS blocking antibody to prevent ICOS-induced ICOSL shedding,19,20 CD4+ but not CD8+ DCs showed increased ICOSL surface abundance compared with unimmunized mice (Fig. 3e and Extended Data Fig. 4l). We took advantage of the sensitivity of ICOSL to ICOS-induced shedding as a method to measure the amount of interaction between DCs and cognate T cells. Twelve hours after SRBC-OVA immunization, ICOSL levels were higher on CD4+ DCs in mice harbouring EB2-deficient OTII T cells than WT OTII T cells (Fig. 3f). These data suggest that the reduced Tfh differentiation of EB2 KO OTII T cells occurs at least in part because of lower ICOS engagement with CD4+ DCs. However, in mice treated with an ICOS blocking antibody, although Tfh differentiation of control OTII T cells was reduced, EB2-deficient OTII T cells were still more defective (Fig. 3g), indicating an ICOS-independent influence of EB2 in augmenting Tfh cell fate. An assessment of mRNA levels of other factors established to have an effect on Tfh differentiation (IL-6, transforming growth factor-β (TGF-β)) showed that they were similarly expressed in CD4+ and CD8+ DCs and they were therefore not considered likely factors accounting for the EB2-dependence of Tfh cell differentiation (Extended Data Fig. 4n).
To search for surface or secreted DC-derived factors that might augment Tfh cell differentiation, we performed RNA sequencing (RNA-seq) analysis on CD4+ DCs from the spleens of saline or SRBC-immunized mice. This analysis revealed CD25, the high affinity IL-2 receptor α-chain, as one of the most strongly induced genes in SRBC-activated DCs (Extended Data Fig. 5a). CD25 mRNA induction occurred rapidly after immunization and remained elevated for at least 2 days (Fig. 4a), and many CD4+ DCs were surface positive for CD25 over this time frame (Fig. 4b). CD8+ DCs showed little induction of CD25 mRNA or protein under these immunization conditions (Fig. 4a, b). Analysis of lymph node DCs after OVA plus alum immunization revealed upregulation of CD25 on migratory CD11b+ DCs at day 1 and 2 (Extended Data Fig. 5b). Staining of spleen sections identified CD25+ cells in the unstimulated T zone that are likely CD25hi regulatory T cells, but also showed broad induction of CD25 in the outer T zone within 12 h of SRBC immunization in a pattern resembling the DCIR2+ DC distribution (Fig. 4c). Similar appearance of CD25 staining in the T zone was seen in T-cell-deficient mice, providing evidence that expression by activated DCs was being detected (Extended Data Fig. 5c). CD25 needs to associate with CD122 (IL-2Rγ) to transmit signals in response to IL-2 (ref. 10). Despite expression of CD25, activated CD4+ T cells showed minimal CD222 mRNA and protein expression and they did not respond to IL-2 as assessed by intracellular pSTAT5 staining (Extended Data Fig. 5d–f). These data led us to consider the possibility that activated DCs express CD25 to alter IL-2 availability in the outer T zone.

IL-2 has pleiotropic roles in directing T-cell fate, including a negative influence on Tfh cell differentiation5–10. Despite equivalent IL-2 production and receptor expression (Supplementary Information and Extended Data Fig. 5g–i), EBI2 KO T cells showed more IL-2Rγ γ- to transmit signals in response to IL-2 (ref. 10). Despite expression of CD25, activated CD4+ T cells showed minimal CD222 mRNA and protein expression and they did not respond to IL-2 as assessed by intracellular pSTAT5 staining (Extended Data Fig. 5d–f). These data led us to consider the possibility that activated DCs express CD25 to alter IL-2 availability in the outer T zone. Consistent with this possibility, when in vivo activated DCs were incubated briefly in vitro they were found to release soluble CD25 (sCD25) into the culture supernatant (Fig. 4f). DC production of sCD25 was not dependent on interaction with T cells (Fig. 4f). Analysis of spleen tissue extracts showed elevated sCD25 production at 6 h after SRBC immunization and this was maintained at 24 h (Fig. 4g) and occurred in a T-cell-independent and DC-dependent manner (Fig. 4h). To determine whether the sCD25 functioned as an IL-2 antagonist, supernatants from cultures of activated CD4+ DCs were tested in a bioassay for their ability to inhibit IL-2R signalling. Regulatory T cells were used as the reporter cells in this bioassay since they were more sensitive to low-dose IL-2 than Tfh cells (J.L. and J.G.C., unpublished observations). Supernatants from cultured SRBC-activated WT but not CD25 KO CD4+ DCs were able to inhibit IL-2R signalling in T cells (Extended Data Fig. 5i).

To determine whether DCs were regulating T-cell differentiation in vivo by production of CD25, we generated bone marrow (BM) chimaeric mice that lacked CD25 in DCs (Supplementary Information and Extended Data Fig. 6a–e). In these recipients, control (EBI2 Het) OTII T cells were compromised in their ability to take on a Tfh cell fate whereas EBI2 KO T cells were only mildly affected (Fig. 4i). Staining of tissue sections from the DT-treated BM chimaeras established that the high CD25 abundance in the outer T zone was dependent on CD25 expression by DCs (Extended Data Fig. 6f). Splenic Tfh cell responses to Listeria-OVA and lymph node Tfh cell responses to alum-OVA were also diminished in mice lacking CD25 on DCs (Extended Data Fig. 6g, h). Moreover, mice lacking CD25 in DCs and harbouring transgenic B cells specific for hen egg lysozyme (HEL) mounted reduced plasma cell and germinal centre responses to HEL-conjugated SRBCs (Fig. 4j and Extended Data Fig. 6i, j), which was associated with reduced serum anti-HEL IgG1 and IgG2b antibody levels (Fig. 4k). To further test whether reduced exposure of EB2 KO T cells to sCD25 could account for the defective Tfh cell induction, we treated mice with recombinant sCD25 at day 0 and 1 of the OTII T-cell response to SRBC-OVA. This treatment was sufficient to elevate sCD25 levels in tissue extracts (Extended Data Fig. 6k), to antagonize pSTAT5 over-induction in the EB2 KO T cells (Fig. 4l) and to partly rescue Tfh cell differentiation (Fig. 4m). A full restoration of the Tfh response was not expected given that EB2-deficient cells are also compromised in accessing ICOSL and possibly other Tfh-cell-promoting signals from cells in the outer T zone.

This work establishes a role for EBI2 and 70α, 25-OHC in positioning activated T cells at the follicle–T-zone interface, promoting contact with Tfh cell-promising ICOSL1–25+ DCs (Supplementary Information and Extended Data Fig. 7). Given that interactions both with DCs and with B cells are important for full Tfh cell differentiation12–13, we suggest that T-cell EB2U pigregation initially acts to favour interaction with Tfh-cell-promoting DCs and subsequently with activated B cells. While EB2U pigregation is important for Tfh cell induction, the receptor is downregulated at week 2 of the response and this may facilitate Tfh cell retention in germinal centres14. Soluble CD25 was first detected in human serum ~30 years ago and it has since been reported in human and mouse serum in many studies and correlated with various disease conditions21–23. Although there has been evidence that sCD25 can antagonize certain IL-2 functions20,22,24, the significance of sCD25 in vivo has been unclear. Moreover, the function of CD25 in myeloid cells has been mysterious20,25,26, with some in vitro studies suggesting it suppresses27 and others that it augments28,29 T-cell responses. We show that DC production of CD25 plays an important role in quenching IL-2 in the outer T zone and it thereby cooperates with other factors, including ICOSL, to facilitate Tfh cell differentiation (Extended Data Fig. 7). While our findings show DCs produce sCD25, we do not exclude the possibility that membrane-associated CD25 on DCs also has a regulatory role. Strengths of IL-2 signalling influence Treg cell activity, Th17, Th1 and Th2 cell development, and CD8+ T-cell proliferation and differentiation10,30. We suggest that CD25-mediated IL-2-quenching by DCs will be a general mechanism acting to guide a range of IL-2-sensitive cell activation and differentiation processes.

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1. Ramiscal, R. R. & Vinuesa, C. G. T-cell subsets in the germinal center. Immunol. Rev. 252, 146–155 (2013).
2. Crotty, S. T follicular helper cell differentiation, function, and roles in disease. Immuinology 41, 529–542 (2014).
3. Garside, P. et al. Visualization of specific B and T lymphocyte interactions in the lymph node. Science 281, 96–99 (1998).
4. Haynes, N. M. et al. Role of Cxcr5 and Ccr7 in follicular Th cell positioning and appearance of a programmed cell death gene high germinal center-associated subpopulation. J. Immunol. 179, 5099–5108 (2007).
5. Lee, S. K. et al. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. J. Exp. Med. 208, 1377–1388 (2011).
6. Johnston, R. J., Choi, Y. S., Diamond, J. A., Yang, J. A. & Crotty, S. STAT5 is a potent negative regulator of Tfh cell differentiation. J. Exp. Med. 209, 243–250 (2012).
7. Ballesteros-Tato, A. et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. Immunity 36, 847–856 (2012).
8. Nurieva, R. I. et al. STAT5 protein negatively regulates T follicular helper (Th) cell generation and function. J. Biol. Chem. 287, 11234–11239 (2012).
9. Ostreich, K. J., Mohn, S. E. & Weinmann, A. S. Molecular mechanisms that control the expression and activity of Bcl-6 in Th1 cells to regulate flexibility with a Th1-like gene profile. Nature Immunol. 13, 405–411 (2012).
10. Liao, W., Lin, J. X. & Leonard, W. J. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity 38, 13–25 (2013).

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Oxysterols direct immune cell migration via EBI2.

Oxysterols direct B-cell migration through EBI2.

et al.

Nature

Immunity

molecular signatures in naive and memory immune responses. Immunity 42, 704–718 (2015).

Meredith, M. M. et al. Expression of the zinc finger transcription factor ZDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. J. Exp. Med. 209, 1153–1165 (2012).

Goenka, R. et al. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. J. Immunol. 187, 1091–1095 (2011).

Yi, T. & Cyster, J. G. EBI2 mediates B cell segregation between the outer and centre follicle. Nature 460, 1122–1126 (2009).

Hannedouche, S. et al. Oxysterols direct immune cell migration via EBI2. Nature 475, 524–527 (2011).

Liu, C. et al. Oxysterols direct B-cell migration through EBI2. Nature 475, 519–523 (2011).

Suan, D. et al. T follicular helper cells have distinct modes of migration and molecular signatures in naive and memory immune responses. Immunology 12, 704–718 (2015).

Russell, S. E., Moore, A. C., Fallon, P. G. & Walsh, P. T. Soluble IL-2R α chain induced Th17 autoimmune and inflammatory diseases. Nature Rev. Immunol. 15, 283–294 (2015).

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Author Contributions J.L. designed and performed experiments, interpreted the results and prepared the manuscript. E.L. performed several experiments including staining and quantitation of cell distribution in sections and helped prepare the manuscript. T.Y. performed experiments identifying the defects in EBI2 KO T cells. J.G.C. designed experiments, supervised research and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.G.C. (jason.cyster@ucsf.edu).
METHODS

Mice and bone marrow chimaeras. Wild-type C57BL/6NCr and C57BL/6-cBrd/+ (B6-129.S7) mice of 7–9 weeks of age were purchased from the National Cancer Institute. Ebi2−/− (containing a green fluorescent protein (GFP) reporter in place of the Ebi2 coding exon), Cyp7b1−/−, Ch25h−/−, Hsd3b7−/−, C7r−/−, HEL-specific MD4 Ig-transgenic, HEL-specific H2-10 mice and OVA-specific OTII TCR-transgenic mice have been described (refs 11, 17, 31 and references therein). B-cell-deficient μMT mice were provided by T. Dranoff and C. Allen. Cd28−/− mice were provided by K. M. Ansel. Tcell−/−, Cd47−/−, Rag2−/−, Zeb2−/−DTR mice were from Jackson Laboratories. Ifng−/− CD11c−Cre− mice were from Jax and provided by S. Sanjabi. Cd25−/− mice were from Jax and provided by M. Muschen. BM chimaeras were generated as described13 and analysed after 6–12 weeks. Mixed BM chimaeras were made by mixing equal amounts of the two types of BM before transfer. The sample sizes were guided by previous studies in our laboratory. No animals were excluded from analysis, and sample size estimates were not used. The mouse genotype was not blinded from the investigator. Mice of a given genotype were randomly assigned to groups. However, littermate mice were evenly distributed into control or treatment groups and mice of both groups were co-caged whenever possible. In experiments involving transfers of OTII T cells, since the TCR transgene is on the Y chromosome, male mice were used as donors and recipients. In other experiments, similar numbers of male and female mice were used. All mice were adult and were studied between 7 and 20 weeks of age. Animals were housed in a specific-pathogen free environment in the Laboratory Animal Research Center at the University of California, San Francisco, and all experiments conformed to ethical principles and guidelines approved by the Institutional Animal Care and Use Committee.

Adoptive transfer, immunizations, DC ablation and treatments. For analysis of CD4+ T-cell position, activation or Th differentiation in spleens, ×10^6 to ×10^7 ×10^5 ×10^7 WT and/or EBI2 KO OTII cells were adoptively transferred into mice. One day after cell transfer, recipients were immunized intraperitoneally with 2 × 10^6 SRBCs (Colorado Serum Company) conjugated with OVA (Sigma-Aldrich) as described14 with minor modifications detailed below, with 2.5 μg OVA plus 2.5 μg lipopolysaccharide (Escherichia coli 0111:B4, Sigma-Aldrich), intravenously with 2 × 10^6 heat-killed Listeria-OVA as described15, or subcutaneously with 25 μg OVA in 200 μl Alum (InovioGen). For conjugation of OVA with SRBCs, 1 ml of SRBCs was washed with PBS three times, incubated with 4 ml of 30 mg ml−1 ice-cold OVA in PBS and crosslinked with 1 ml of 100 mg ml−1 EDCI (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide, Sigma-Aldrich) for 1 h on ice with occasional mixing, followed by washing four times in PBS to remove the free OVA and confirmation of the conjugation by flow cytometry. For HEL-specific antibody responses, 1 × 10^6 H2-10 cells were adoptively transferred into desired recipients. One day after cell transfer, recipients were intraperitoneally immunized with SRBCs conjugated with a low affinity mutant of HEL termed HEL2 × (ref. 35) as described17. To visualize cell proliferation, cells were labelled with CellTrace violet tracer (Molecular Probes, Invitrogen) according to the manufacturer’s instructions. For DC ablation, Zeb2−/−DTR full or mixed chimaeras were injected intraperitoneally with 20 μg DT (Sigma-Aldrich) per gram of body weight 3 days before cell transfer and received 4 μg DT per gram of body weight on the third day after the initial DT injection and in some cases again 3 days later. To block ICOS, mice were injected intra-nously with anti-mouse ICOS antibody (rat IgG2b, clone 7E.17G9, BioXcell) or rat IgG2b isotype control 1 day before and 2 days after cell transfer (0.5 mg per mouse per injection). To block IL-2, 2 doses of recombinant CD25 mouse protein (25 μg per mouse per dose, Sino Biological) were injected into mice at the same time with immunization or 1 day after immunization.

Flow cytometry and cell sorting. All antibody conjugates were from Biologend or BD Biosciences. EB2 surface staining, T-cell staining, germinal centre B cell staining and intracellular Ig staining for plasma cells were performed as described18,19,20,21,22,23,24. To stain CD11c, cells were performed with PE-Cy7-conjugated anti-CD11c (clone A20, Biolegend). Anti-CD45.1 (clone A20, Biolegend), biotin-conjugated anti-DCIR2 (clone 33D1, Biologend) or biotin-conjugated anti-CD25 (clone PC61.5, BD Biosciences) followed by HRP-conjugated anti-FITC, AP-conjugated anti-FITC, and/or AP-conjugated SA (Jackson ImmunoResearch). For staining of DCIR2 and CD25, a tyramide amplification kit was used (TSA Biotin System; Perkin Elmer). For immunohistochemistry, staining was performed with biotin conjugated anti-CD45.1, rabbit anti-GFP (Molecular Probes), and goat anti-mouse IgM (GAM/GD/Fcy7/8, Cedarlane Laboratories), followed by AMCA-conjugated donkey anti-goat IgG (Jackson ImmunoResearch), Alexa 488-conjugated donkey anti-rabbit IgG and Alexa 647-conjugated streptavidin (Invitrogen). Images were captured with a Zeiss AxioObserver Z1 inverted microscope.

Image quantification. Immunofluorescence images were analysed using IMARIS (version 7.3.0). White-pulp cords containing circular T zones were used to quantify outer T zone positioning of co-transferred WT (red) and EB2 HET or KO cells (green). OTII cells were defined using the Spots function in IMARIS and coordinates for each OTII cell were exported into R. The centre and average radius of the T zone was measured using the Measurement Points function in IMARIS. The ‘outer T zone’ was defined as the area further than three-quarters of the average radius from the centre of the T zone. The distance of each OTII cell from the centre of the T zone and the proportion of cells in the outer T zone was calculated using R. An average of 70 cells were present for each co-transferred group per T zone (average of 140 total). IHC images were analysed manually using the Cell Counter Plugin in ImageJ (version 1.49). OTII cells that were in contact with DCIR2+ DCs were distinguished from alone OTII cells using separate counters.

Soluble CD25 ELISA and bioassay. To test the production of sCD25 by DCs in vitro, mice were first intraperitoneally immunized with SRBCs. At the time of analysis, spleen CD4+ DCs were enriched by depletion of T, B and natural killer (NK) cells with a cocktail of anti-conjugated antibodies and isolated by positive selection using biotin-conjugated anti-DCIR2 to purities of over 90% (Milenyi Biotech). The purified DCs were cultured in vitro for 8 h and the presence of soluble CD25 in culture supernatants was detected using a CD25 ELISA kit (R&D Systems). To detect sCD25 in spleen tissue, each spleen was mashed into 1 ml of medium through a 70 μm cell strainer and centrifuged at 300g for 10 min and 3,000g for 15 min at 4 °C. The cell-free supernatant was subjected to the ELISA assay. To test for antigen of IL-2 mediated signalling, DC culture supernatants from 24 h stimulation were added to 1 h stimulation of recombinant mouse IL-2 (2 μg/ml) for 2 h and added to splenocytes at 37 °C for 30 min. sCD25 levels in DC25+ CD4+ T cells were analysed as described above.

Detection of SRBC- or HEL-specific antibody responses. To assay for anti-SRBC or anti-HEL IgM and IgG from mouse serum, 50 μl of SRBCs or HEL-conjugated mouse RBCs (5 × 10^8 cells per millilitre in PBS) were incubated with 2 μl of serum for 1 h at room temperature. After washing, the RBCs were incubated with fluorescent antibodies against mouse IgM, IgG1 and IgG2b for flow cytometric analysis.

Quantitative RT–PCR. Total RNA from sorted cells was isolated and reverse-transcribed, and quantitative PCR was performed as described18. Data were analysed using the ΔΔCt method and are expressed as fold changes relative to controls. RNA-seq analysis. Spleens were taken 1 h after saline or SRBC immunization. CD4+ DCs were pre-enriched using MACS manual cell separation columns with anti-CD11c microbeads (Miltenyi Biotech) and further sorted on the basis of surface markers of CD11c+1−Ab +CD4+CD8−. Cells were sorted twice on a FACSAria III to purities of over 99%. Sorted DCs (10^6) were snap frozen and then RNA was extracted with the QIAGEN RNeasy Kit. RNA quality was checked with an Agilent 2100 Bioanalyzer (RNA integrity number > 9 for all samples). Barcoded sequencing libraries were generated with 100 ng of RNA with an Ovation RNA-Seq System V2 and Encore Rapid Library System. Sequencing was performed on an Illumina HiSeq 2500 with 100-bp pair-end reads. Sequence reads were reported as FASTQ files, which were aligned to the mm9 mouse genome with STAR (Spliced Transcript Alignment to a Reference). Generation of log2FC values and further analyses were performed with a Bioconductor package on RStudio. The RNA-seq data have been deposited in the Gene Expression Omnibus (NCBI) data repository under accession code GEO: GSE71165.
31. Yi, T. et al. Oxysterol gradient generation by lymphoid stromal cells guides activated B cell movement during humoral responses. *Immunity* **37**, 535–548 (2012).

32. Carlsson, F., Getahun, A., Rutemark, C. & Heyman, B. Impaired antibody responses but normal proliferation of specific CD4+ T cells in mice lacking complement receptors 1 and 2. *Scand. J. Immunol.* **70**, 77–84 (2009).

33. Muraille, E. et al. Distinct in vivo dendritic cell activation by live versus killed *Listeria monocytogenes*. *Eur. J. Immunol.* **35**, 1463–1471 (2005).

34. Allen, C. D., Okada, T., Tang, H. L. & Oyster, J. G. Imaging of germinal center selection events during affinity maturation. *Science* **315**, 528–531 (2007).

35. Paus, D. et al. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J. Exp. Med.* **203**, 1081–1091 (2006).

36. Meli, A. P. & King, I. L. Identification of mouse T follicular helper cells by flow cytometry. *Methods Mol. Biol.* **1291**, 3–11 (2015).
Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | EBI2 and 7α,25-OHC promote positioning of newly activated CD4 T cells in the outer T zone. a, Flow cytometric analysis of EBI2 expression on splenic OTII T cells and endogenous B cells in transfer recipients at 0 and 12 h after SRBC-OVA immunization. EBI2 KO cells were used as a staining control. Left histograms show example FACS data and right panel shows summary data across the indicated time points as geometric mean fluorescence intensity (geoMFI). b, EBI2 expression on OTII and endogenous T cells in transfer recipients 2 days after saline or lipopolysaccharide-OVA immunization. Left histograms show example flow cytometric data and right panel shows summary geoMFI data for four mice. c, Summary geoMFI time course data of EBI2 expression on lymph node OTII T cells in transfer recipients at the indicated times after alum-OVA immunization. d, GFP expression in EBI2GFP/CD4 T cells that were unstimulated (naive) or treated with anti-CD3 plus anti-CD28 for 2 days. Left histogram shows example flow cytometric data and right panel shows summary geoMFI data for three mice. e, Ebi2 mRNA abundance in cells of the type in d, determined by RT–qPCR and shown relative to the naive cells. f, Migration of OTII T cells and endogenous cells to the indicated amounts of 7α,25-OHC in transwell assays. Cells were from unimmunized (0 h) or immunized (day 1, 2) transfer recipient mice in one experiment (left) or from 12 h immunized transfer recipients in a second experiment (right). Data are shown as percentage of input cells of each type that migrated. g, Immunofluorescence analysis of spleen showing the distribution of co-transferred WT CD45.1+ (red) and EBI2 het or KO (GFP+, green) OTII T cells and endogenous B cells (IgD, blue) at 12 h and 1 day after immunization. h, i, Immunohistochemical analysis of WT spleens (h) and inguinal lymph nodes (i) showing the distribution of transferred control (WT) or EBI2 deficient (KO) OTII CD45.1+ T cells (blue) and endogenous B cells (IgD, brown) at day 2 after lipopolysaccharide-OVA immunization (h) or day 1 after alum-OVA immunization (i). j, Immunohistochemical analysis of Cyp7b1, Ch25h or Hsd3b7 control (het, upper panels), or KO (lower panels) spleens showing the distribution of transferred WT OTII T cells (CD45.1, blue) and endogenous B cells (IgD, brown) at day 2 after SRBC-OVA immunization. k, CCR7 expression on WT and EBI2 KO OTII T cells in transfer recipient spleens at the indicated days after SRBC-OVA immunization. **P < 0.01 by ANOVA (a, c) or Student’s t-test (b, d, f). Data are representative of two (a–i, k) or three (j) independent experiments with at least three (a–c, k) or two (d–j) mice per group (error bars (e), s.e.m.).
Extended Data Figure 2 | Defective differentiation of EBI2-deficient T cells to follicular helpers. a, ICOS and OX40 expression on WT, EBI2-deficient (KO) OTII and endogenous CD4 T cells in transfer recipient spleens two days after SRBC-OVA immunization. b, c, In vitro proliferation of WT and EBI2 KO T cells in response to anti-CD3 plus anti-CD28 in the presence of the indicated amounts of 7α,25-OHC, shown as violet tracer dye dilution profiles (b) and total CD4 T-cell numbers (c) at day 3 of culture. Numbers in b indicate frequency of cells that have undergone two or more divisions. d, Flow cytometric analysis of co-transferred WT and EBI2 KO OTII T cells for CXCR5 and intracellular Bcl6 expression at the indicated days after SRBC-OVA immunization. Numbers indicate frequency of cells in gated region. e, Summary of data of the type in d. Upper plot shows frequency and lower plot number of CXCR5⁺ Bcl6hi OTII T cells. f, Il21 mRNA abundance in CXCR5⁺ PD-1hi control (Het) or EBI2 KO OTII T cells sorted from recipient spleens at day 3 after immunization with SRBC-OVA, determined by RT–qPCR and shown relative to the Het control. g, Frequency and number of CXCR5⁺ PD-1hi (left) or CXCR5⁺ Bcl6hi (right) WT and EBI2 KO OTII T cells in mice that received the cells as separate transfers, at day 3 after SRBC-OVA immunization. **P < 0.01 by ANOVA (e) or Student’s t-test (f, g). Data are representative of three (a, d, e, g) or two (b, c, f) independent experiments with at least three mice per group (error bars (f), s.e.m.).
Extended Data Figure 3 | EBI2-deficient T cells support reduced plasma cell and germinal centre response. a, Frequency and number of CXCR5+ PD-1hi CD4+ control (WT) and EBI2 KO OTII T cells in spleens of day 3 *Listeria*-OVA immunized transfer recipients. b, PD-1 and CXCR5 flow cytometric analysis of control (Het) and EBI2 KO polyclonal CD4 T cells co-transferred to OTII recipients, 8 days after immunization with unconjugated SRBCs. c, Summary of data of the type in b shown as CXCR5+ PD-1hi cell frequency and number. d, Flow cytometric analysis for the germinal centre markers FAS and GL7 on endogenous B cells in OTII TCR transgenic mice that received no cells, control (HET) CD4 T cells or EBI2 KO CD4 T cells, or in WT B6 control mice, 12 days after immunization with unconjugated SRBCs. e, Summary of data from d shown as number of FAS+ GL7+ germinal centre B cells. f, Flow cytometric analysis for CD138hi B220lo plasma cells (top) and intracellular IgM and IgG1 staining of these cells (bottom) in mice of the type in d. g, Summary of data from f shown as number of cells. h, Serum anti-SRBC antibody levels in mice of the type in d, determined by flow cytometric analysis of SRBCs stained with immune sera, plotted as geoMFI. i, j, Number of Fas+ GL7+ germinal centre cells and CD138hi B220int plasma cells in *Listeria*-OVA immunized CD28 KO mice that had received control (het) or EBI2 KO OTII cells, analysed at day 5. **P < 0.01 by ANOVA (g, h) or Student’s t-test (a, e, c, i, j). Data are representative of two independent experiments with at least three mice per group.
Extended Data Figure 4 | See next page for caption.
Extended Data Figure 4 | T-cell EBI2 is required for CD4+ DC-mediated augmentation of Tfh cell induction. a, Frequency and number of CXCR5+PD-1hi OTII T cells in μMT recipients determined by flow cytometric analysis. b, Flow cytometric analysis for CD11c and MHC class II on splenocytes from Zbtb46-DTR mice treated with saline or DT for 1 day. Graph shows summary data for DC number in four mice of each type. c, Frequency and number CXCR5+PD-1hi WT and EBI2 KO OTII T cells in spleens from Zbtb46-DTR BM chimaeras treated with saline or diphtheria toxin (DT), at day 3 after immunization with SRBC-OVA. d, Immunohistochemical analysis of spleen sections from WT mice without immunization (saline) or SRBC immunized for the indicated times, stained to detect IgD+ B cells (blue) and DCIR2+ DCs (brown). e, Immunohistochemical analysis of spleen sections from recipients of WT or EBI2 KO OTII T cells at 12 h and 1 day after immunization SRBC-OVA immunization, stained for OTII CD45.1+ T cells (blue) and DCIR2+ DCs (brown). f, Immunohistochemical analysis of spleen sections from WT:Zbtb46-DTR or CCR7 KO:Zbtb46-DTR mixed BM chimaeras treated with DT, at day 2 after immunization. g, Frequency and number of CXCR5+PD-1hi WT and EBI2 KO OTII T cells in spleens from WT:Zbtb46-DTR (control) or CCR7 KO:Zbtb46-DTR mixed BM chimaeras treated with DT, at day 3 after immunization. h, Frequency and number of CXCR5+PD-1hi control (het) and EBI2 KO co-transferred OTII T cells in spleens of CD47 KO recipients at day 3 after SRBC-OVA immunization. i, Number of total and CD4+ DC in spleens from Irf4−/−CD11c-Cre− or + mice. j, As for g but in Irf4−/−CD11c-Cre− or + recipient mice. k, As for g but in Batf3 KO recipient mice. l, ICOSL surface levels for DCs from mice immunized 12 h earlier with saline or SRBCs and treated with control or ICOS blocking antibody. m, ICOSL surface levels for CD4+ or CD8+ DCs from CD28 KO mice immunized 12 h earlier with saline or SRBCs. n, Il6 and Tgfβ mRNA abundance in sorted CD4+ and CD8+ splenic DCs from mice treated with saline or SRBC 6 h earlier, determined by RT–qPCR, shown relative to the control CD8+ DC. **P < 0.01 by ANOVA (g, k) or Student’s t-test (a–c, i, j, m). Data are representative of three (a–e) or two (f–n) independent experiments with at least three (a–c, g–n) or two (d–f) mice per group (error bars (n), s.e.m.).
Extended Data Figure 5 | See next page for caption.
Extended Data Figure 5 | DCs produce membrane and soluble CD25 and inhibit IL-2R signalling. a, Heat map of RNA-seq data from sorted CD4+ splenic DC showing the top 15 most induced genes at 1 h after SRBC versus saline immunization. b, CD25 surface levels in CD11b+ migratory and resident DCs from lymph nodes of mice immunized with saline or alum-OVA 2 days earlier. Graph on right shows summary data for total number of migratory CD25+ DCs. c, Immunohistochemical analysis of spleen sections from TCRβδ KO mice immunized 1 day earlier with saline or SRBC, stained to detect IgD (blue) and CD25 (brown). d, e, CD122 (Il2rb) mRNA determined by RT–qPCR (d) and surface staining (e) on the indicated cell types isolated from spleens of WT OTII T-cell recipients at day 0, 1 and 2 after SRBC-OVA immunization. Transcript data are plotted relative to the signal in CD4+ DCs at day 0. f, Intracellular flow cytometric analysis of pSTAT5 in CD4+ DCs or, as a positive control CD4+ T cells, that were untreated or incubated with IL-2 (200 pg ml−1) or, as a further positive control, GM-CSF (100 pg ml−1). g, Il2 mRNA in control (Het) and EBI2 KO OTII T cells isolated from recipient mice at the indicated times after SRBC-OVA immunization. h, Intracellular flow cytometry for IL-2 in cells of the type in f at 0, 12 and 24 h. Percentages show mean (±s.e.m.) for three mice at each time point. i, Flow cytometric analysis of CD25 expression on co-transferred WT and EBI2 KO OTII T cells in WT recipients at the indicated days after SRBC-OVA immunization. j, Prdm1 (encoding Blimp1) transcript levels in sorted CXCR5+PD-1hi control (het) and EBI2 KO OTII T cells from SRBC-OVA immunized mice at day 3, plotted relative to the mean level in the Het group. k, Summary of pSTAT5 staining data for OTII T cells from mice immunized 1 day earlier with SRBC-OVA, incubated with the indicated amounts of 7α,25-OHC plus IL-2 (200 pg ml−1) for 1 h. l, Flow cytometry of pSTAT5 in CD25+ (regulatory) T cells exposed to the indicated amounts of IL-2 that had been pre-mixed with supernatants (s/n) from 8 h cultures of splenic CD4+ DCs from WT or CD25 KO mice immunized with saline or SRBCs 1 day before. Graph on right shows summary data from one experiment. **P < 0.01 by ANOVA (b, l) or Student’s t-test (j). Data are representative of one (a) or two (b–l) independent experiments with at least two (a) or three (b–l) mice per group (error bars (g, j), s.e.m.).
Extended Data Figure 6  |  DC CD25 expression reduces IL-2 signalling in activated CD4 T cells, favouring their differentiation to follicular helpers. a, Diagram of CD25 KO:Zbtb46-DTR BM chimaera generation and time line of experiment. DTx, DT treatment. b–d, Numbers (b), surface marker expression (c) and outer T zone positioning (d) of CD4+ DCIR2+ DCs in WT:Zbtb46-DTR and CD25 KO:Zbtb46-DTR mixed BM chimaeras pre-treated with DT, at day 1 after saline or SRBC immunization. e, Number of Foxp3+ CD25+ regulatory T cells in mice of the type in b except that the mice were immunized for three days. f, Immunohistochemical analysis of spleen sections from mice of the type in b, stained to detect IgD (blue) and CD25 (brown). g, h, Frequency and number of CXCR5+PD-1hi control (EBI2 Het) and EBI2 KO OTII T cells in splens (g) or lymph nodes (h) from WT:Zbtb46-DTR or CD25 KO:Zbtb46-DTR mixed BM chimaeras pre-treated with saline or DT, at day 3 after immunization with Listeria-OVA (g) or alum-OVA (h). i, j, Flow cytometric analysis for HEL-binding CD138+ plasma cells (i) and HEL-binding GL7+ Fas+ germinal centre B cells (j) in spleens from WT:Zbtb46-DTR (control) or CD25 KO:Zbtb46-DTR mixed BM chimaeras that had received Hy10 B cells and been treated with DT, at day 5 after immunization with HEL-SRBC. k, Soluble CD25 detected by ELISA in spleen extracts taken from 12 h SRBC immunized mice, at day 1 after saline or recombinant CD25 treatment. **P < 0.01 by Student’s t-test (h, k).
Extended Data Figure 7 | Model of how EBI2-dependent positioning of activated T cells in association with CD25⁺ DCs in the outer T zone favours Tfh cell differentiation. Initially, cognate T cells throughout the T zone are activated by antigen recognition and promptly start upregulating EBI2 and making IL-2. EBI2 guides cells to the 7α,25-OHC high outer T zone and in this location they interact with activated DCs producing membrane and shed CD25 that binds and quenches IL-2. This limits IL-2R signalling on the T cell via pSTAT5 and allows induction of Bcl6 by other inputs such as ICOSL. T cells that lack EBI2 or remain in the inner T zone for other reasons are exposed to autocrine IL-2 and this induces Blimp1, a repressor of Bcl6 (ref. 2), disfavouring the Tfh cell fate.