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Oxysterols direct immune cell migration through EBI2

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Abstract

Epstein-Barr virus (EBV)-induced gene 2 (EBI2, aka GPR183) is a G protein-coupled receptor that is required for humoral immune responses and polymorphisms in the receptor have been associated with inflammatory autoimmune diseases[1-3]. The natural ligand for EBI2 has been unknown. Here we describe identification of 7α, 25-dihydroxycholesterol (5-cholesten-3β, 7α, 25-triol; 7α, 25-OHC) as a potent and selective agonist of EBI2. Functional activation of EBI2 by 7α, 25-OHC and closely related oxysterols was verified by monitoring second messenger readouts and

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Author Contributions

S.H., S.N., M.O.R., M.D., and K.S. initiated the project and developed strategy. S.H., S.N., and M.O.R. purified activity from sheep tissue extracts. W.S., D.I.K., C.Y.C., and P.G.S. carried out corresponding work on the pig tissue. J.Z., R.K., I.C, Ra.K., R.F., and E.C.P., conducted mass-spectrometry and NMR experiments. S.L., M.V., I.P., D.G., D.N., W.L., A.K., Y.A.C., B.U.B., K.S., and A.W.S. designed and conducted pharmacological characterization of the receptor and bioactivity assays. S.R., C.M., F.G., C.S., W.S., C.Y.C., S.H., and A.W.S. proposed and/or synthesized various oxysterol ligands. T.S., C.S., and B.W.supported *in vivo* experiments with mouse models. T.Y., J.P.P., L.M.K. and J.G.C. developed the bioassay, conducted the experiments in Ch25h-deficient and transduced mice and performed the B cell chemotaxis and immunization studies. A.W.S. wrote the first draft of the manuscript which was completed by S.H., J.Z., D.N., B. W., S.R., J.G.C., C.Y.C., K.S. and commented on by all authors.

Competing financial interest statement

All authors, except for T.Y., J.P.P., L.M.K., D.I.K, and J.G.C., are current or previous employees of either Euroscreen or Novartis and some of them do hold stock or stock options in their respective companies.

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saturable, high affinity radioligand binding. Furthermore we find that 7α, 25-OHC and closely related oxysterols act as chemoattractants for immune cells expressing EBI2 by directing cell migration *in vitro* and *in vivo*. A key enzyme required for the generation of 7α, 25-OHC is cholesterol 25-hydroxylase (Ch25h)[4]. Similar to EBI2 receptor knockout mice, mice deficient in Ch25h fail to position activated B cells within the spleen to the outer follicle and mount a reduced plasma cell response after an immune challenge. This demonstrates that Ch25h generates EBI2 bioactivity *in vivo* and suggests that the EBI2 − oxysterol signaling pathway plays an important role in the adaptive immune response.

Screening of a liver extract from a peritonitis sheep sepsis model in a functional assay format detecting release of intracellular calcium identified a bioactivity specific for human EBI2 (Fig. 1a). After characterization of biophysical properties (e.g. protease resistance), two kilograms of sheep liver were extracted and fractionated over six steps of liquid chromatography (supplemental Fig. S1), which yielded pure fractions potently activating the human EBI2 receptor.

Mass spectrometry (MS) analysis of the active fractions using a NanoESI-FTMS showed four peaks with significant intensities in both step 5 (supplemental Fig. S1e; Active 1) and step 6 (supplemental Fig. S1f; Active 2), which were negligible in control samples (supplemental Fig. S2). Ions with masses m/z 383.3303, 401.3408 and 441.3332 were observed in positive ion mode, while m/z 477.3584 in negative ion mode. The ions corresponding to masses m/z 401.3408 and 383.3303 could be assigned to a protonated molecule with loss of one and two water molecules: $[M+H-2H_2O]^+$ and $[M+H-H_2O]^+$, while masses m/z 441.3332 and 477.3582 could be assigned to its sodium and acetate adducts, respectively. Accurate mass measurements allowed assigning a $C_{27}H_{46}O_3$ molecular formula to the identified 418.3447 molecular weight.

Database query with $C_{27}H_{46}O_3$ or mass 418.3447 provided mainly oxidized cholesterol or vitamin D derivatives. We found around 50 candidates when querying public databases (CHEBI, HMDB, Kegg, LipidMap, Metline, BioCyc, and LipidBank) but very few were commercially available. Among the tested compounds two related oxysterols, cholest-5-ene-3β, 7β, 25-triol (7β, 25-OHC) and cholest-5-ene-3β, 7β-diol (7β-OHC) induced specific activation of the human EBI2 receptor with EC_{50} values close to 50 nM and 15 μM respectively, while the 7-methoxy derivate of 7β-OHC did not activate EBI2, indicating some structure activity relationship (supplemental material Fig. S3).

In contrast to 7α, 25-OHC for which a clear metabolic pathway has been described[4], the generation of 7β, 25-OHC *in vivo* is still enigmatic[5]. This information prompted us to synthesize 7α, 25-OHC (Fig. 1b) which proved to be more potent than 7β, 25-OHC (Fig. 1c). While the purification from septic sheep liver was in progress, we also initiated a parallel approach using pig liver tissue (starting material 10 kg). After five liquid chromatography separation steps, ions with masses m/z 365.3202, 383.3311, 401.3411 and 441.333 were observed in positive ion mode, rationalized as $M+H-3H_2O$, $M+H-2H_2O$, $M+H-H_2O$, and M+Na, respectively. In order to enable ^{1}H-NMR analysis of the active fraction a scale-up of the purification using 35 kg pig liver tissue yielded about 100 micrograms of substantially pure EBI2 ligand. ^{1}H-NMR analysis comparing EBI2 ligand purified from pig

liver with synthetic 7α, 25-OHC confirmed the molecular identity of the two compounds (supplemental material Fig. S4), suggesting that 7α, 25-OHC is the naturally occurring receptor ligand.

After identification of 7α, 25-OHC as an EBI2 agonist, we conducted a pharmacological characterization of 7α, 25-OHC and closely related oxysterols using two functional second messenger readouts (release of intracellular calcium (Fig. 1c) and binding of GTPγS; see Table 1). Other than the hydroxylation at the 3 position, hydroxylations at the 7 and at the 25 positions were necessary for potent activation of EBI2. At the 7 position the axial alpha position was much preferred over the equatorial beta position. Hydroxylation of the side chain at the 25 position was preferred but hydroxylation at the 27 position also led to a strong activation. Parental cells lacking the EBI2 receptor did not respond to the tested oxysterols (data for 7α, 25-OHC shown in Fig 1c; data for other oxysterols omitted for clarity of the figure). In order to characterize binding of oxysterols to the EBI2 receptor we developed a suitable radioligand. Reduction of 7- keto, 25-OHC using tritiated L-selectride led to the formation of [^{3}H]-7, 25-OHC. Separation of the two stereoisomers yielded radio-labeled [^{3}H]-7α, 25-OHC as well as [^{3}H]-7β, 25-OHC (supplemental material Fig. S5a). Using membranes from a cell line stably expressing human EBI2 we found saturable, high affinity binding of [^{3}H]-7α, 25-OHC to EBI2 (supplemental Fig. S5b) with a dissociation constant (K_d) of 25 ±10 nM (n=3). This binding was displaced by increasing concentrations of 7α, 25-OHC or closely related oxysterols (Fig. 1d, Table 1). The rank order of the different oxysterol compounds was identical to the functional assays. Using the other stereoisomer [^{3}H]-7β, 25-OHC no specific binding to EBI2 membranes was detected. Membranes from parental cells lacking EBI2 receptor did not bind [^{3}H]-7α, 25-OHC (data not shown).

Oxysterols have pleiotropic physiological activities: they regulate the expression of genes that participate in both sterol and fat metabolism, serve as substrates for the synthesis of bile acids, and are intermediates in the transfer of sterols from the periphery to the liver[6]. In addition, oxysterol-dependent activation of the nuclear hormone receptor LXR has recently been implicated in the acquired immune response[7]. In order to assess possible interaction of the most potent EBI2 ligands with other receptors we have tested 7α, 25-OHC and 7α, 27-OHC in a panel of reporter gene assays against 8 different nuclear hormone receptors including LXR and 31 different GPCRs. In none of these assays did we see a significant interaction between 7α, 25-OHC or 7α, 27-OHC and the tested partner (see supplemental material).

B cells in different stages of maturation show a distinct profile of EBI2 expression. It is abundant in naive B cells and further upregulated early after their activation by antigen, while B cells from germinal centers sharply down-regulate EBI2 expression. Once affinity maturation has been completed and the B cells differentiate into plasma or memory cells, EBI2 is again expressed. This expression pattern combined with the recent finding that EBI2 is a key regulator positioning B cells in lymphoid organs[1, 2] prompted us to investigate oxysterols as chemoattractants in a transwell assay system. For this experiment we used either an EBV-infected human B cell line or a Burkitt's lymphoma pre B cell line called RS11846[8, 9]. Both cell lines displayed high endogenous EBI2 expression (supplemental Fig.

6). We found that 7α, 25-OHC can potently attract EBV-infected B cells or RS11846 cells (Fig. 2a, 2b). Similar to classical chemokines we found a bell shaped activity curve in the transwell assay that indicates reduced attraction at high compound concentrations. Oxysterols closely related to 7α, 25-OHC can also attract RS11846 cells albeit with lower potency. The rank order of different oxysterols in the migration assay was identical to the rank order in the functional and binding assays. The attraction of EBI2 expressing RS11846 cells could be blocked by pertussis toxin, a reagent which blocks signaling of Gαi- coupled receptors (Fig. 2c). Oxysterol-mediated chemoattraction is not limited to B-lineage cells but can also be observed with other immune cells (e.g. dendritic cells) expressing EBI2. Messenger RNA expression analysis of different human immune cells indicated that various myeloid cells highly express EBI2([10] and supplemental Fig. 7). Testing of bone marrow-derived dendritic cells[11] from wild type and Ebi2(−/−) animals in the transwell migration assay showed that only wild type cells migrate towards 7α, 25-OHC (Fig. 2d).

Identification of the chemoattractant properties of oxysterols on B cells and the previous findings regarding EBI2-deficient B cells[1, 2] suggested that ablation of enzymes necessary for ligand production will have an impact on the B cell trafficking after an immune challenge. The defined biosynthetic pathway of the more potent 7α, 25-OHC requires the activity of cholesterol 25-hydroxylase (Ch25h)[4] and oxysterol 7α-hydroxylase (Cyp7b1)[4]. Both of these enzymes are abundantly expressed in spleen and lymph nodes (Fig. 3a). To determine whether Ch25h deficiency affected EBI2 ligand production, tissue extracts were prepared from wild type and Ch25h(−/−)[12] spleen and lymph nodes and tested for their ability to attract an EBI2 expressing cell line in a bioassay that has subnanomolar sensitivity for 7α, 25-OHC (supplemental. Fig. S8c). While wild type tissue extracts showed readily identifiable activity, extracts from Ch25h(−/−) mice lacked activity in this bioassay (Fig. 3b). In addition, spleen extracts from mice reconstituted with bone marrow transduced with a Ch25h expressing retroviral vector showed a greatly increased tendency to attract EBI2 expressing cells (Fig. 3c). Consistent with evidence that Ch25h expression is increased by LPS exposure[13], the bioactivity in spleen extracts from 6 and 16 h treated mice was increased several fold (supplemental Fig. S8a,b). Using mass spectrometry we assessed production of 7α, 25-OHC in the spleen of unchallenged and LPS-challenged mice (supplemental Fig. S8d). A peak corresponding to 7α, 25-OHC was clearly detectable in tissue prepared from LPS-challenged wild type mice, but was close to the detection limit or undetectable in unchallenged wild type or Ch25h(−/−) mice. The origin of the weak signal detected in some Ch25h(−/−) mice derived samples is not yet clear and could be due to the production of low amounts of 7α, 25-OHC via enzymes other than Ch25h[14-16]. In summary these results indicate that in an LPS immune challenge 7α, 25-OHC is generated in the spleen in a Ch25h-dependent manner.

To test whether 7α, 25-OHC regulates B cell migration *in vivo*, we tested Ch25h(−/−) mice[12] in an adoptive transfer experiment. Ch25h(−/−) or wild type mice received transfers of hen egg lysozyme (HEL)-specific IgM^a MD4 B cells and ovalbumin (OVA)-specific OTII T cells at day -1 and were immunized with HEL-OVA at day 0 and analyzed at day 2 for their immune response. Visualization of activated B cells (IgM^a) in the spleen relative to the position of marginal metallophilic macrophages (Moma1) (Fig. 3d) or endogenous

follicular B cells (Supplemental Fig. S9) showed that at day 2 after immune challenge in wild type recipients, many of the activated B cells had moved to the back of the follicle and interfollicular regions while in Ch25h(−/−) mice activated B cells largely failed to move to these regions and instead remained near the follicle/T zone interface or moved into the follicle (Fig. 3d, e and supplemental Fig. S9). The defective distribution of activated B cells in Ch25h-deficient mice was similar to that reported for day 2 activated EBI2-deficient B cells[1]. Migration assays with spleen cells prepared at the same time point showed that the day 2 activated wild-type MD4 B cells responded more vigorously than naïve endogenous B cells to 7α, 25-OHC (Fig. 3f). Both the baseline naïve B cell response and the increased response of activated B cells required EBI2 expression (supplemental Fig. S10) and MD4 B cells that had not been exposed to antigen did not show an augmented response (supplemental Fig. S10). EBI2 bioactivity was similar in spleen extracts from day 2 immunized and unimmunized mice (supplemental Fig. S10). Ch25h and Cyp7b1 transcript abundance was low in B and T lymphocytes but enriched in stromal cells (Fig. 3a) suggesting that the enzymes are expressed in adhesive myeloid or mesenchymal cells, consistent with studies on other tissues[13, 17, 18]. Although it has not been possible to determine enzyme distribution in tissue sections with existing antibody reagents and no tools are available to visualize ligand distribution, based on the EBI2-dependent homing properties of activated B cells, we speculate that ligand abundance is higher in outer and inter follicular regions compared to the follicle center. Finally, we tested whether ligand deficiency led to a defect in the plasma cell response. Five days following immunization with a T-dependent antigen (sheep red blood cells, SRBC) the Ch25h-deficient mice showed a ~3-fold defect in the magnitude of their IgG1 plasma cell response (Fig. 3g), an impairment very similar to that observed in EBI2-deficient mice[1,2].

We have identified the natural ligand for EBI2 and delineate its physiological function as a chemoattractant in the immune system[1, 2]. Our results provide an unanticipated link between recent results showing a crucial role for EBI2 in promoting B cell localization within lymphoid organs, of EBI2 connecting through IRF7 to regulate a macrophage-associated autoimmune disease[3] and studies highlighting how oxysterols shape the innate and adaptive immune responses[12, 13, 19]. Together with these published reports, our results establish a new physiological dimension for oxysterols as bioactive signaling molecules.

Materials and Methods Summary

Compounds

: 7α, 25-OHC (CAS No. 64907-22-8) and 7β, 25-OHC (CAS No. 64907-21-7) were synthesized either in a one step reaction from the 7-keto precursor (see the supplemental material), or in a four step synthesis according to published procedures[20]. 7α, 27-OHC (CAS No. 144300-24-3), 7β, 27-OHC (CAS No. 240129-43-5) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 7α-OHC (CAS No. 566-26-7) was batch synthesized at Novartis. 7β-OHC (CAS No. 566-27-8), 25-OHC (CAS No. 2140-46-7), and cholesterol (CAS No. 57-88-5) were purchased from Sigma-Aldrich (Buchs, Switzerland).

EBI2 recombinant cell line

The human EBI2 gene was cloned using RT-PCR from HL-60 cDNA and corresponded to the Genbank accession number NP_004942. The human EBI2 was inserted into the pEFIN3 vector at the EcoR1 and Xbal sites in the multiple cloning site. Plasmids expressing human EBI2 were stably transfected in CHO-K1 expressing the apo-aequorin and Gqi5 to yield CHO/Aeq/Gqi5/hEBI2.

Tissue extracts preparation

Extraction of sheep tissue was done by methanol / water/ acetic acid (90:9:1) while pig tissue was extracted using hexane/isopropanol (1:1). Methods for separation of crude extracts as well as details on the pharmacological characterization and spectroscopic methods used can be found in the supplemental material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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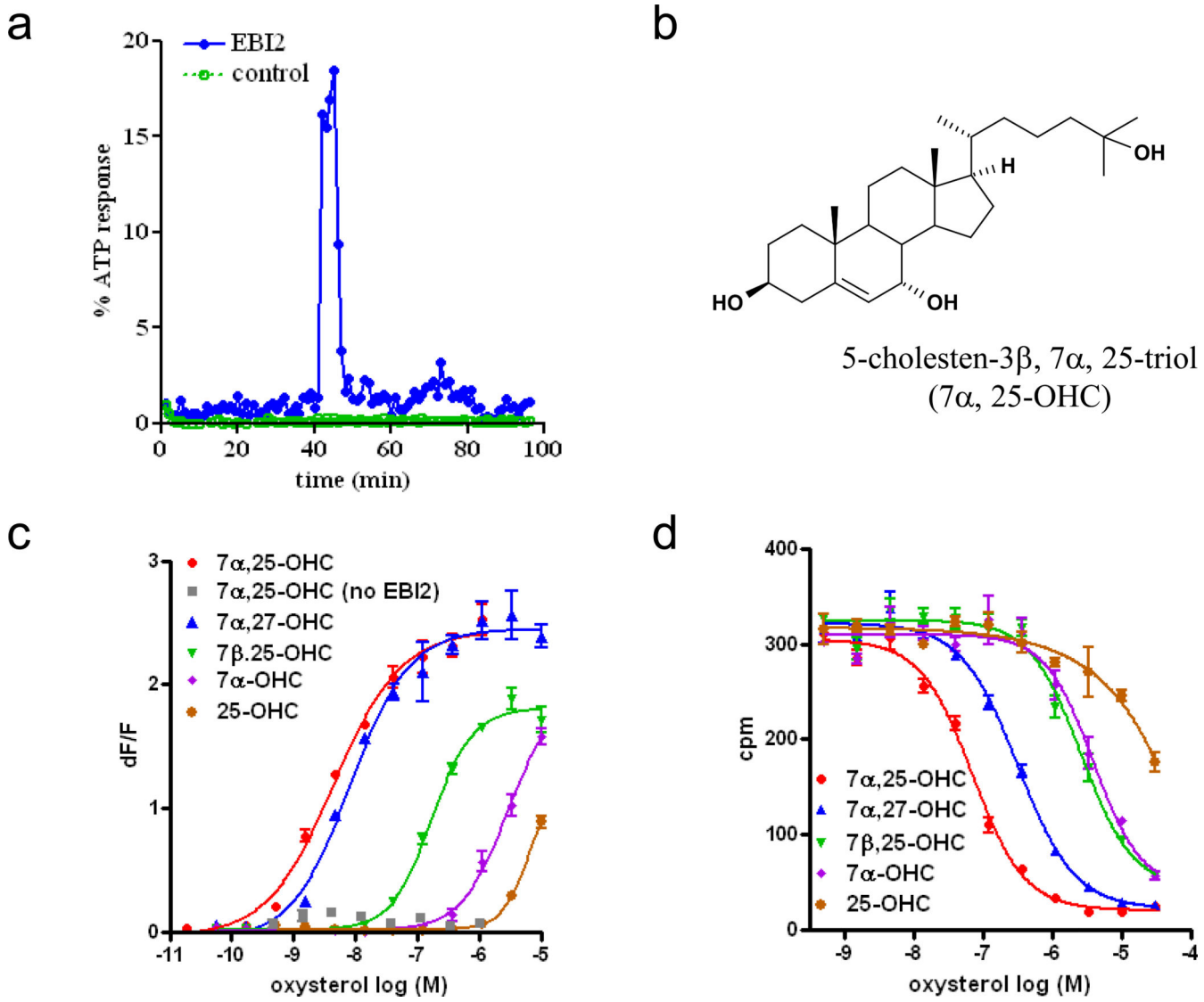

Figure 1. Identification, structure, and pharmacological characterization of 7α, 25-OHC

a: Bioactivity profile measured by mobilization of intracellular calcium of fractions from septic sheep liver extracts tested on EBI2 expressing cells (blue) or tested on a control cell line (green) expressing an unrelated GPCR called CCRL2. Activity is plotted relative to an ATP response of an endogenous ATP receptor.

b: Chemical structure of 5-cholesten-3β, 7α, 25-triol (7α, 25-OHC).

c: Calcium mobilization: Dose response curve induced by several related oxysterols in a CHO cell line stably expressing EBI2. Data for 7α, 25-OHC on the parental cell line lacking expression of EBI2 is given as (7α, 25-OHC, no EBI2). Data shown are from a duplicate experiment.

d: Radioligand binding assay: Displacement of 10 nM ^{3}H-7α, 25-OHC bound to membranes from EBI2 expressing CHO cells by increasing concentrations of unlabeled oxysterols. Data from quadruplicate experiments are shown.

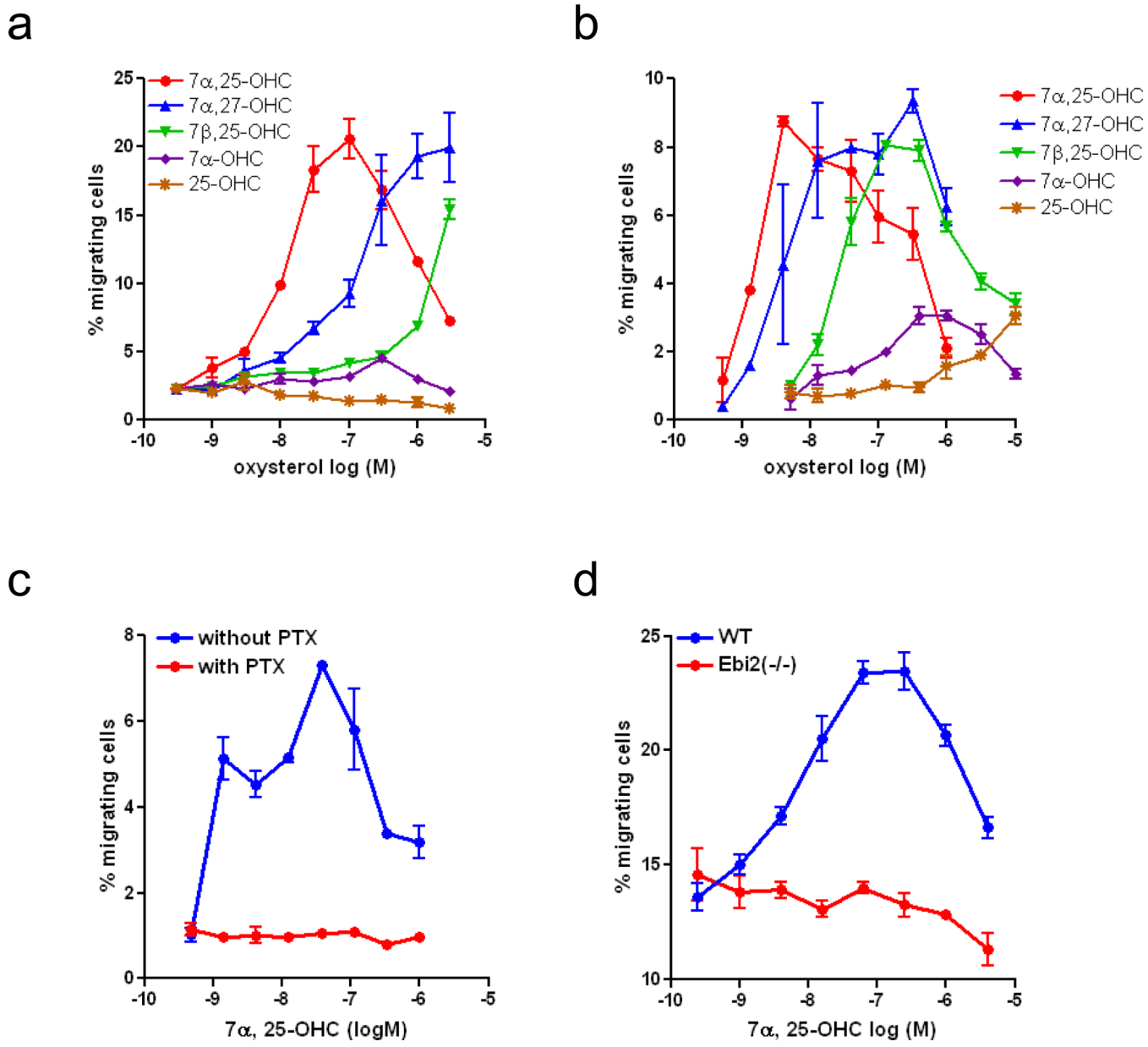

Figure 2. Oxysterol-mediated immune cell migration

a: Migration of EBV-infected B cells towards 7α, 25-OHC and closely related oxysterols.

b: Migration of RS11846 cells towards 7α, 25-OHC and closely related oxysterols.

c: Oxysterol-mediated RS11846 cell migration in the presence (red) or absence (blue) of pertussis toxin (PTX).

d: Migration of bone marrow-derived dendritic cells (BMDC) from wild type (blue) or Ebi2(−/−) mice (red).

Data for all four experiments were conducted in a transwell assay system with duplicate (a,b,c) or quadruplicate (d) samples.

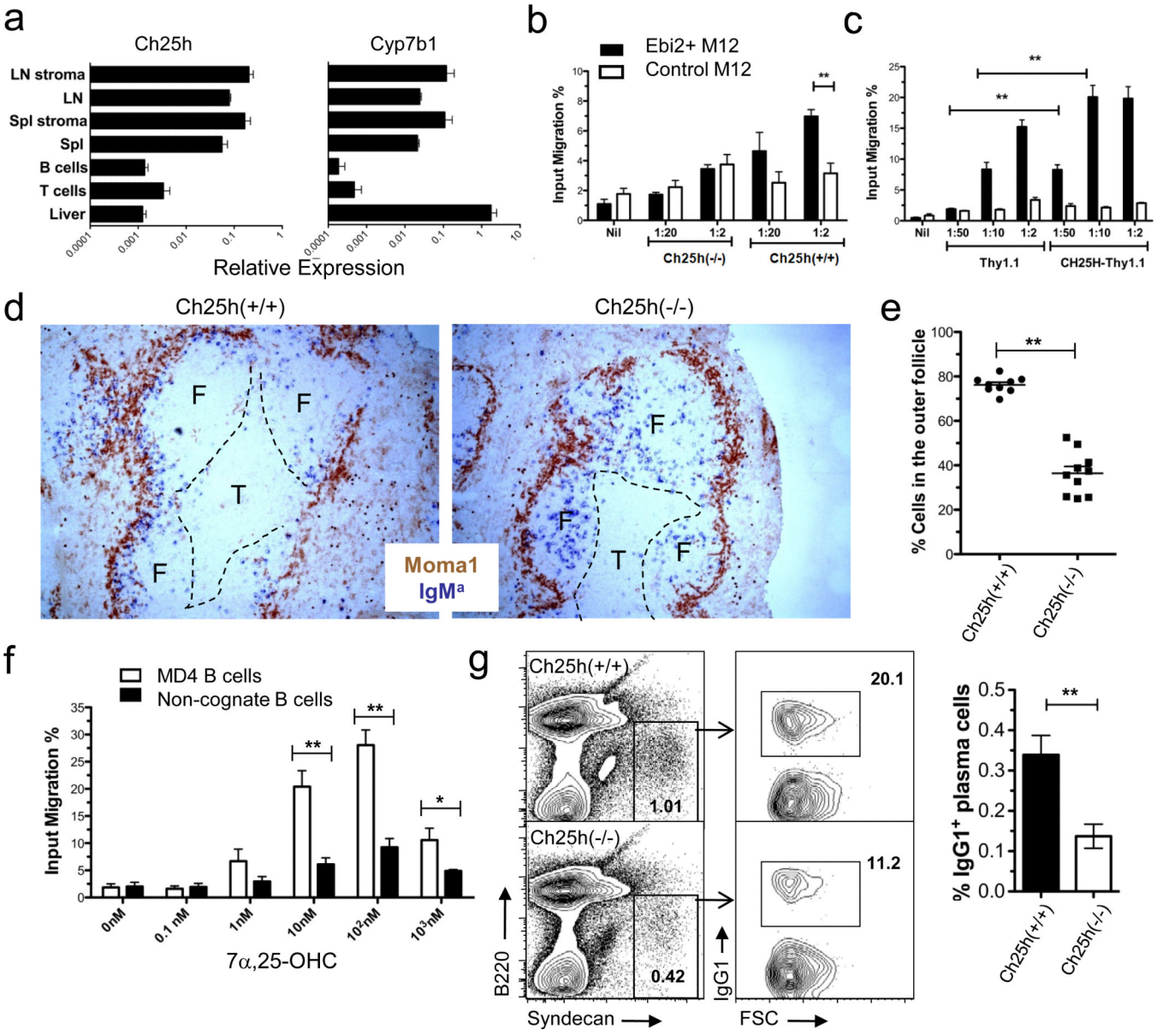

Figure 3. CH25H expression regulates EBI2 bioactivity *in vivo* and is required for mounting a T-dependent antibody response

a: Quantitative PCR analysis of Ch25h and Cyp7b1 transcript abundance in the indicated tissues or cell preparations, relative to HPRT.

b: Bioassay of spleen extracts from Ch25h(−/−) or wild type animals using a reporter cell line (M12) with or without EBI2.

c: Bioassay of spleen extracts from mice reconstituted with bone marrow transduced with a CH25H expressing retroviral vector (CH25H-Thy1.1) or a control vector (Thy1.1).

d: Spleen sections from the indicated mice after immune challenge (day 2). Visualization of antigen-sepcific transferred (IgM^a, blue) B cells in Ch25h(+/+) mice indicates that many of the activated B cells have moved to the back of the follicle and interfollicular regions, near Moma1+ marginal metallophilic macrophages (brown). In the Ch25h(−/−), the activated B

cells largely fail to move to these regions and instead remain near the follicle/T cell zone interface or move into the follicle. Dashed lines indicate the follicle (F) T zone (T) boundary, identified in serial sections using IgD staining. These images are representative of multiple sections from three mice of each genotype.

e: Fraction of day 2 activated B cells located in the outer follicle. Sections were stained as in d and IgMa cells within ~70um of the Moma1+ cells were enumerated and divided by the total number of IgMa cells in the follicle. Each point corresponds to an individual follicle. Enumeration was performed on sections from three mice.

f: Migration of antigen-specific (MD4) B cells and non-cognate B cells from spleens of day 2 immunized wild type mice in response to 7α, 25-OHC.

g: Plasma cell response in Ch25h(+/+) and Ch25h(−/−) mice at day 5 following immunization with sheep red blood cells. Left profiles show spleen cells stained for the indicated markers with plasma cells identified as $B220^{lo}Syndecan^{hi}$ and further stained to detect intracellular IgG1. Numbers indicate percent of total cells in the indicated gate. Right bar graph shows a summary of the data for 9 mice of each type (mean±SE).

*p<0.05, **p<0.01 (unpaired student's T-test)

Table 1

Pharmacological characterization of different oxysterols in calcium fluorescence (FLIPR™), GTPγ^{35}S binding, and radioligand binding assay using [^{3}H]-7α, 25-OHC.

compound	Ca^{2+} Fluorescence EC_{50} (nM)	GTPγ^{35}S Binding EC_{50} (nM)	[^{3}H]-7α, 25-OHC Binding IC_{50} (nM)
7α, 25-OHC	2 ± 2	8 ± 4	70 ± 4
7α, 27-OHC	5 ± 4	5 ± 2	362 ± 32
7β, 25-OHC	121 ± 54	221 ± 105	2632 ± 110
7β, 27-OHC	310 ± 261	125 ± 66	>10000
7α-OHC	1453 ± 1163	498 ± 267	3536 ± 431
25-OHC	3032 ± 2645	727 ± 232	>10000
7β-OHC	>10000	>10000	>10000
cholesterol	>10000	>10000	>10000

n = 3-4, values are given with standard deviation of the mean