*International Immunology, Vol. 37, No. 1, pp. 53–70*
https://doi.org/10.1093/intimm/dxae045
Advance Access publication 5 August 2024



# The active form of vitamin D (calcitriol) promotes CXCR5 expression during follicular helper T cell differentiation

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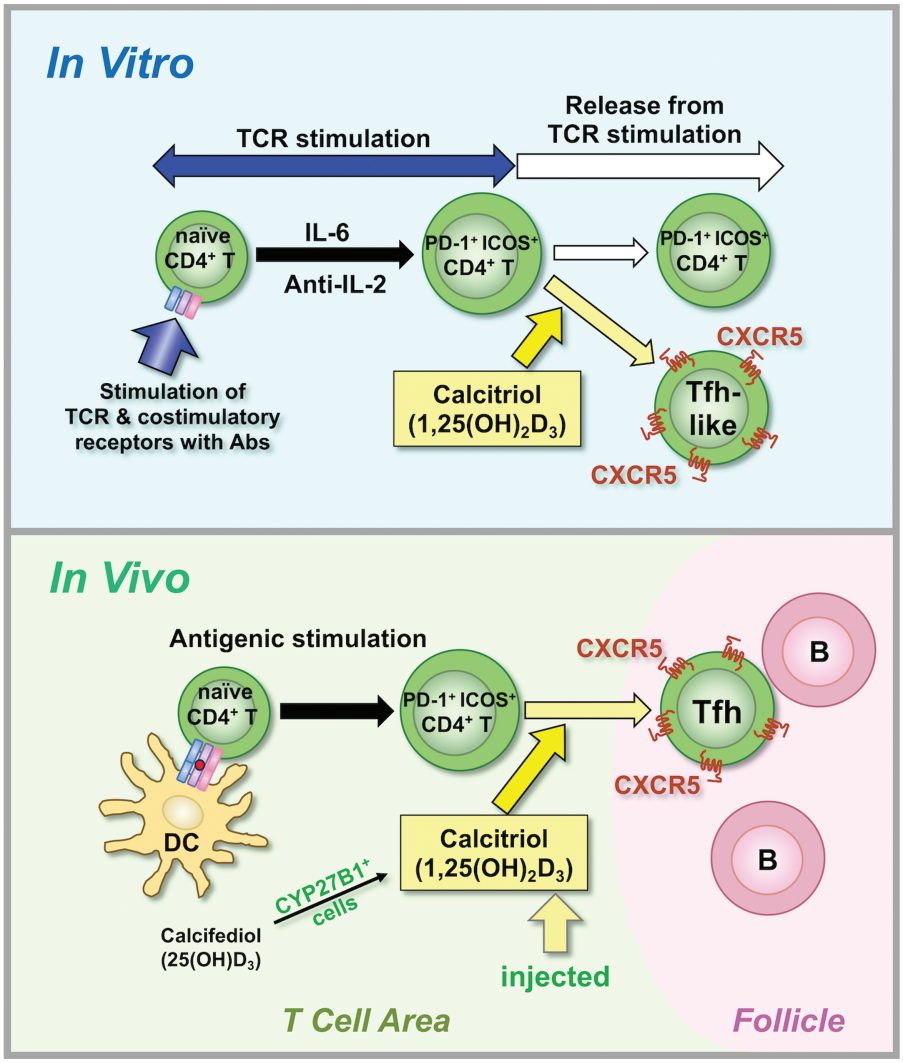
*Received* 4 November 2023, *editorial decision* 15 July 2024, *accepted* 3 August 2024

**Abstract**

**Follicular helper T (Tfh) cells promote B cell differentiation and antibody production in the B cell follicles of secondary lymphoid organs. Tfh cells express their signature transcription factor BCL6, interleukin (IL)-21, and surface molecules including inducible T cell costimulator (ICOS), programmed cell death-1 (PD-1), and C-X-C motif chemokine receptor 5 (CXCR5). Migration of Tfh cells to B cell follicles largely depends on the CXCR5 expression induced by interactions with antigen-presenting dendritic cells in the T cell area. How Tfh cells acquire sufficient levels of CXCR5 expression, however, has remained unclear. Using our *in vitro* culture system to generate CXCR5$^{low}$ Tfh-like cells from naive CD4$^+$ T cells with IL-6 in the absence of other cell types, we found that the active form of vitamin D, calcitriol, markedly enhanced CXCR5 expression after the release from persistent T cell receptor (TCR) stimulation. CH-223191, an aryl hydrocarbon receptor antagonist, further enhanced CXCR5 expression. IL-12 but not IL-4, in place of IL-6, also supported calcitriol to enhance CXCR5 expression even before the release from TCR stimulation, whereas the cell viability sharply decreased after the release. The Tfh-like cells generated with IL-6 and calcitriol exhibited chemotaxis toward C-X-C motif chemokine ligand 13 (CXCL13), expressed IL-21, and helped B cells to produce IgG antibodies *in vitro* more efficiently than Tfh-like cells generated without added calcitriol. Calcitriol injections into antigen-primed mice increased the proportion of CXCR5$^+$PD-1$^+$CD4$^+$ cells in their lymphoid organs, and enhanced T cell entry into B cell follicles. These results suggest that calcitriol promotes CXCR5 expression in developing Tfh cells and regulates their functional differentiation.**

**Graphical Abstract**




*Keywords:* antibody responses, aryl hydrocarbon receptor, IL-6, IL-12, TCR stimulation

## Introduction

Follicular helper T (Tfh) cells have pivotal roles in antibody (Ab) responses. They promote B cell proliferation and maturation to Ab-producing plasma cells in the germinal center (GC) of secondary lymphoid organs (1, 2). Interleukin (IL)-21 produced by Tfh cells plays an important role in these responses (3–5). IL-6 is a potent inducer of Tfh cell differentiation and IL-21 expression in mouse naive CD4+ T cells upon activation, while IL-12, but not IL-6, is a potent inducer of Tfh cell differentiation and IL-21 expression in human CD4+ T cells (2). On the other hand, IL-2 suppresses the differentiation of Tfh cells by limiting early commitment to the Tfh cell lineage (6). Tfh cells differentiate from naive CD4+ T cells upon interacting with antigen-presenting dendritic cells in the T cell area of lymphoid organs. Tfh cells express the transcriptional repressor BCL6, which directs Tfh cell differentiation (7–9), and surface molecules such as inducible costimulator (ICOS, CD278) and programmed cell death-1 (PD-1, CD279), which contribute to Tfh cell development and function (10–12). Tfh cells also express C-X-C motif chemokine receptor 5 (CXCR5, BLR1, CD185), which strongly contributes to their migration to B cell follicles (13–15). Indeed, mice with T cells that selectively lack CXCR5 exhibit significantly fewer and smaller GCs after immunization with a T cell-dependent antigen (15). The ligand for CXCR5 is the C-X-C motif chemokine ligand 13 (CXCL13, BLC, BCA-1) produced by follicular dendritic cells (16) and by GC Tfh cells in humans, although not in mice (17, 18), thereby attracting Tfh cells to the T–B border and finally to the follicle and GC. BCL6 is important for the Tfh cell expression of CXCR5 expression *in vivo* (8, 11, 19), but not for that in the early phase of the T cell immune response (20). Instead, BCL6 is required for maintaining the CXCR5 expression (20). ASCL2, a basic helix–loop–helix E protein, is a transcription factor expressed in Tfh cells, and ectopic expression of *Ascl2* upregulates CXCR5, but not BCL6, in T cells *in vitro* (21). Some other E protein transcription factors, including E2A, also induce CXCR5 expression, while an inhibitor of E-box protein Id3 inhibits these effects

(21, 22). Furthermore, the deletion of *Bach2* leads to the induction of CXCR5 expression even before the upregulation of *Ascl2* (23). Shaw *et al.* suggested that some E proteins, but not ASCL2, direct early Tfh cell development, and that ASCL2 is important for amplifying CXCR5 expression at later stages of Tfh differentiation into GC Tfh cells (24).

We previously found that CH-223191, an aryl hydrocarbon receptor (AhR) antagonist, moderately enhances CXCR5 expression in Tfh-like cells generated in our *in vitro* model without other cell types (25). Inducing sufficient levels of CXCR5 expression *in vitro* is difficult, however, especially in the absence of other cell types, suggesting that an unidentified physiological factor(s) is required to induce sufficient levels of CXCR5 expression in Tfh cells during their development.

In our *in vitro* model, mouse naive CD4$^+$ T cells are stimulated with immobilized Abs to CD3 and costimulatory molecules in the presence of IL-6, transforming growth factor beta (TGF-β), and blocking Abs to IL-4 and IL-2 signaling for 3 days (3d), and further cultured for 2 days without the immobilized Abs (3 + 2d). *Bcl6* expression is enhanced after the "3 + 2d" culture compared with the "3d" culture, while *Rorc(γt)* expression is downregulated after the "3 + 2d" culture compared with the "3d" culture (25). CH-223191 enhanced CXCR5 expression after the "3 + 2d," but not the "3d" culture (25). In the present study, we found that, compared with CH-223191, physiological levels of the active form of vitamin D, calcitriol (1α,25-dihydroxyvitamin $D_3$), far more dramatically enhanced CXCR5 expression after the "3 + 2d" culture, but not after the "3d" culture.

Vitamin D plays diverse roles in several physiological processes, including calcium and bone homeostasis, cell growth, glucose metabolism, and muscular and immune functions (26, 27). Vitamin D can be obtained from the diet or by UV-B-mediated synthesis. Calcifediol, also known as calcidiol, 25-hydroxycholecalciferol, and 25-hydroxyvitamin $D_3$ (25(OH)$D_3$), is the major circulating form of vitamin D and the direct precursor of calcitriol. Calcifediol is converted to calcitriol by 25-hydroxyvitamin $D_3$ 1-α-hydroxylase (CYP27B1) in the kidney and extra-renal tissues including lymphoid organs, and exerts its effect by binding to the nuclear vitamin D receptor (VDR) heterodimerized with retinoid X receptor (RXR) (28). Most immune cells express VDR. Low vitamin D levels are often associated with the development of infectious, inflammatory, and autoimmune diseases (29). Vitamin D appears to enhance and suppress immune responses, including Ab responses, depending on the situation (30). Calcitriol significantly suppresses the development of proinflammatory helper T cells, including Th17 and Th9 cells, and modestly suppresses Th1 cell development (31). The effect of calcitriol on Tfh cell development, however, remains unclear.

In the present study, we show that physiological levels of calcitriol markedly induce CXCR5 expression in Tfh-like cells generated with IL-6 or IL-12 *in vitro*. Calcitriol-treated Tfh-like cells produced more IL-21 than control-treated Tfh-like cells, and enhanced IgG production by B cells *in vitro*. Furthermore, calcitriol injections into antigen-immunized mice enhanced the proportion of PD-1$^+$CXCR5$^+$ T cells among CD4$^+$ T cells in the spleen and draining lymph nodes. We propose that calcitriol contributes to Tfh cell development, especially through enhancing CXCR5 expression.

## Methods

### *Mice and culture reagents*

Mice were obtained from Japan SLC and maintained in specific pathogen-free conditions in our animal facility. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Waseda University (#2020-A057, 2021-A003, A22-007, and A23-007). We used 7- to 12-week-old male or female C57BL/6 mice. Mouse recombinant IL-2 (rIL-2), rIL-4, rIL-6, rIL-21, rTGF-β1, rCXCL13, and anti-ICOS (clone C398.4A) monoclonal Ab (mAb) were obtained from BioLegend. Mouse rIL-12 was obtained from Fujifilm Wako Pure Chemistry. mAbs to CD28 (clone 37.51), IL-2 (clone JES6-1A12), IL-4 (clone 11B11), IL-2Rα (clone PC-61.5.3), IL-2Rβ (clone TM-β1), and interferon gamma (IFN-γ) (clone XMG1.2) were purchased from Bio X Cell. Calcitriol, EB 1089, 25(OH)$D_3$, CH-223191, and 6-formylindolo [3,2-*b*]carbazole (FICZ) were obtained from Cayman Chemical.

### *Cell purification and culture*

Naive CD4$^+$ T cells were purified from spleens by negative selection using EasySep Mouse CD4$^+$ T Cell Enrichment kits (Stemcell Technologies) supplemented with biotinylated mAbs to mouse CD44 (clone IM7) and CD25 (clone PC61) (BioLegend), and subsequently by positive selection with CD62L Microbeads (Miltenyi Biotec). The purity was greater than 96%. Naive CD8$^+$ T cells were obtained similarly but by replacing the CD4$^+$ T cell enrichment kits with EasySep Mouse CD8$^+$ T Cell Enrichment kits (Stemcell Technologies). To induce Tfh-like cells, purified naive CD4$^+$ T cells were suspended (1.5–2 × $10^5$ cells/ml) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 20 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES) (pH 7.2), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS) (cDMEM), and cultured in 96- or 48-well flat-bottom plates (MS-8096R or MS-8048R, Sumitomo Bakelite) coated with mAbs to CD3ε mAb (clone 145-2C11; 3 μg/ml), ICOS (1.5 μg/ml), and lymphocyte function-associated antigen-1 (LFA-1; CD11a; clone M17/4; 1.5 μg/ml) in the presence of soluble anti-CD28 mAb (1 μg/ml), IL-6 (20 ng/ml), and TGF-β1 (1 ng/ml), and blocking mAbs to IL-4, IL-2, IL-2 receptors α (CD25) and β (CD122) (10 μg/ml each) under 10% $CO_2$ for 3 days ("3d" culture), as previously described (25). In some experiments, the cells were cultured for 2 days further in either the same well receiving an equal volume of fresh medium containing the indicated cytokines and Abs or a new culture well coated with ("5d" culture) or without ("3 + 2d" culture) mAbs to CD3, ICOS, and LFA-1 receiving an equal or the indicated volume of medium or replacing the culture supernatant with fresh medium supplemented with the indicated cytokines and blocking Abs. When the supernatant of the first culture was carried over to the second culture, however, further addition of blocking Abs had little effect

on Tfh-like cell differentiation. In some experiments, the cells were cultured for 1 day in the second culture without immobilized mAbs (3 + 1d). In some experiments, the indicated reagent, including calcitriol, the AhR antagonist CH-223191, the AhR agonist FICZ, or cycloheximide (CHX) was added to the culture. These reagents were dissolved in ethanol or dimethyl sulfoxide (DMSO), but the final solvent concentrations were less than 0.1% and did not significantly affect the results. To induce Th1 cells, naive $CD4^+$ T cells ($3 \times 10^4$ cells) were cultured in 0.2 ml of cDMEM containing IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) in 96-well plates (Sumilon) coated with 3 μg/ml of anti-CD3 and 3 μg/ml of anti-CD28. After 3 days, the cells were resuspended in 0.5 ml/well fresh cDMEM containing IL-12 (10 ng/ml) and IL-2 (50 U/ml), transferred to 48-well culture plates (Falcon), and cultured for 2 days. To induce Th0 cells, naive $CD4^+$ T cells ($3 \times 10^4$ cells) were cultured in 0.2 ml of the medium containing anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml) in 96-well plates (Sumilon) coated with 3 μg/ml of anti-CD3 and 3 μg/ml of anti-CD28. After 3 days, the cells were resuspended in 0.5 ml/well fresh medium containing anti-IFN-γ (10 μg/ml), anti-IL-4 (10 μg/ml), and IL-2 (50 U/ml), transferred to 48-well culture plates (Falcon), and cultured for 2 days. To generate inducible regulatory T (iTreg) cells, naive $CD4^+$ T cells ($3 \times 10^4$ cells) were cultured in 0.2 ml of the medium containing TGF-β1 (4 ng/ml), IL-2 (10 ng/ml), and all-*trans*-retinoic acid (10 nM; Fujifilm Wako Pure Chemistry) in 96-well plates (Sumilon) coated with 3 μg/ml of anti-CD3 and 1 μg/ml of anti-CD28 for 3 days. For intracellular staining of cytokines, the Th cells ($1 \times 10^5$ cells) were washed and cultured for 2 days in fresh medium without cytokines and blocking mAbs in 96-well plates coated with 3 μg/ml of anti-CD3 and 3 μg/ml of anti-CD28, and monensin (2 μM, Cayman) was added for the last 2 h of culture. In some experiments, RPMI 1640 medium was used in place of DMEM and the cells were cultured under 5% $CO_2$.

### *Flow cytometric analysis of cultured cells*

The cells were stained with allophycocyanin (APC)- or Alexa Fluor 647-anti-CXCR5 (CD185; clone L138D7), or together with fluorescein isothiocyanate (FITC)-anti-PD-1 (CD279; clone 29F.1A12) and phycoerythrin (PE)-anti-ICOS (CD278; clone C398.4A) or with Alexa Fluor 488-anti-CCR7 (CD197; clone 4B12) and PE-anti-CXCR4 (CD184; clone QA16A08) in the presence of anti-CD16/CD32 (clone 2.4G2) mAb (all from BioLegend). Naive $CD4^+$ T cells were incubated in the RPMI 1640 medium at 37°C for 1 h before staining with mAbs to CCR7 and CXCR4 to minimize the effect of possible ligand-dependent internalization *in vivo* (32). Stained cells were analyzed with a BD Accuri™ C6 Plus Flow Cytometer (BD). Live cells were gated based on the forward scatter (FSC) and side scatter (SSC) and the exclusion of dead cells using 7-amino-actinomycin D (7-AAD). Doublets were excluded by FSC-A/FSC-H gating. Typical gating strategies for flow cytometric analysis of cultured cells are shown in Supplementary Fig. S1. In some experiments, antigen expression levels were expressed as the change in the mean fluorescence intensity (ΔMFI), which was calculated as: (MFI of the cells stained with a fluorochrome-conjugated Ab) – (MFI of the isotype control Ab staining). Intracellular staining was performed with PE-anti-BCL6 (clone K112-91) and Alexa Fluor 647-anti-RORγt (Q31-378) (both from BD Biosciences) or with PE-anti-IL-21 (clone FFA21; eBioscience) and APC-anti-IL-17A (clone TC11-18H10.1; BioLegend) using Foxp3/Transcription Factor Staining Buffer Sets (eBioscience) according to the manufacturer's instructions.

### *Real-time polymerase chain reaction*

Total RNA was isolated from cells using a ReliaPrep RNA Cell Miniprep System (Promega) or RNeasy Mini Kit (Qiagen), and cDNA was generated using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). cDNA was used as a template for real-time polymerase chain reaction (PCR) with BrightGreen qPCR MasterMix-ROX (Applied Biosystems) and gene-specific primers. PCR and analysis were performed on a StepOnePlus Real-Time PCR system (Applied Biosystems). The relative expression of each gene was quantified with the $2^{-\Delta Ct}$ value multiplied by 1000, where ΔCt was the difference between the mean Ct value of triplicates or quadruplicates of the sample and that of the endogenous *Rplp0* control (25). The sequences of the primers for *Rplp0*, *Ccr2*, *Ccr4*, *Ccr5*, *Ccr6*, *Ccr7*, *Ccr9*, *Ccr10*, *Cxcr3*, *Cxcr4*, *Cxcr5*, *Bcl6*, *Id2*, *Prdm1*, *Rorc(γt)*, *Runx2*, *Runx3, Zfp831*, *Tbx21*, *Gata3*, and *Foxp3* are listed in Supplementary Table S1.

### *Chemotaxis assay*

To examine if the chemokine receptor CXCR5 expressed on T cells was functionally active, transwell chemotaxis assays were performed as previously described (33) with a slight modification. Briefly, 0.6 ml of cDMEM with the indicated concentration of CXCL13 was added to the lower well of transwell plates (Corning 3421), and $5 \times 10^5$ cells suspended in 0.1 ml cDMEM were added into the upper well and incubated for 2 h at 37°C. The numbers of cells that migrated into the lower wells were counted and their percentages relative to the input cell number were calculated.

### *T and B cell co-culture*

The B cells were purified from the spleens of age- and sex-matched C57BL/6 mice by negative selection using EasySep Mouse B Cell Isolation kits (Stemcell Technologies) according to the manufacturer's instructions. The indicated helper T cells (3 or $4 \times 10^4$ cells) were co-cultured with purified B cells ($1.5 \times 10^5$ cells) in the presence of soluble anti-CD3 mAb (0.1 μg/ml; BioLegend) in 0.2 ml of the medium (RPMI 1640) in 96-well round bottom plates (Falcon or Sumitomo Bakelite) for 5 or 7 days. Alternatively, T cells ($8 \times 10^4$ cells) were co-cultured with purified B cells ($1.5 \times 10^5$ cells) in the presence or absence of staphylococcal endotoxin B (SEB) from *Staphylococcus aureus* (10 μg/ml; Sigma) with soluble anti-CD28 (0.1 μg/ml) in 0.2 ml of the medium (RPMI 1640) in 96-well round bottom plates (Sumitomo Bakelite) for 5 days. Soluble anti-CD28 was added to enhance the SEB response (34). The culture supernatant was analyzed for total IgG Ab production using IgG (Total) Mouse Uncoated ELISA kits (Invitrogen)

according to the manufacturer's instructions. The change in OD450 values (ΔOD450) was calculated by subtracting OD450 readings taken from supernatants from B cells cultured alone from the OD450 values of co-cultured samples, as described previously (25, 35). In some experiments, the cultured cells were analyzed for the presence of $CD138^{high}B220^{low}$ plasma cells.

*Immunization of mice for Tfh cell induction*

To analyze Tfh cells *in vivo*, each C57BL/6 mouse was immunized by intraperitoneal (i.p.) injection with keyhole limpet hemocyanin (KLH; 100 μg) and lipopolysaccharides (10 μg) (both from MilliporeSigma) in aluminum hydroxide gel (Alum; 2 mg; Wako) as described previously with some modification (25). Two days later, the calcitriol solution (1 mM in ethanol) was diluted more than 400 times with olive oil and mice were given i.p. injections of 0.1 μg calcitriol or vehicle control. The calcitriol or vehicle injection was repeated 5 days later. Two days after the last injection, the mice were killed, and their mesenteric lymph node (MLN) cells and splenic cells were dissected out. Expression of CD4, PD-1, CXCR5, CD8α, and CD45R(B220) in the MLN and splenic cells was determined by flow cytometry with a combination of FITC-anti-CD4 (clone RM4-5), PE-anti-PD-1 (clone 29F.1A12), and APC-anti-CXCR5, or a combination of FITC-anti-CD4, PE-anti-CD8α (clone 53-6.7), biotinylated anti-CD45R (clone RA3-6B2), and APC-streptavidin (all from BioLegend).

*Immunohistochemical analysis*

Mice were immunized with KLH and treated with calcitriol as described above with modifications. Briefly, 2 days after the immunization, the calcitriol solution in ethanol was diluted more than 400 times with phosphate-buffered saline, and immediately after the dilution mice were given i.p. injections of 0.1 μg calcitriol or vehicle control. Two more days later, the injection was repeated, and 3 days after the last injection, the mice were killed and their splenic cells obtained. Frozen tissue sections were prepared as previously described with slight modifications (33). Briefly, blocks of spleens were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2). The fixed tissues were washed in 0.1 M PB, immersed in 20% sucrose in 0.1 M PB, embedded in O.C.T. compound (Sakura Finetech Japan, Tokyo, Japan), and frozen on dry ice. Frozen sections (8 μm thick) of these tissues were cut and thaw-mounted onto silane-coated glass slides. These sections were blocked in 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The sections were incubated with Alexa Fluor 488-conjugated rat anti-CD45R(B220) mAb (clone RA3-6B2; BioLegend), Armenian hamster anti-CD3ε, and biotinylated peanut agglutinin (Vector) at 4°C for at least 48 h, followed by the incubation with Cy3-conjugated goat anti-Armenian hamster IgG Ab (Jackson ImmunoResearch) and Alexa Fluor 647-conjugated streptavidin (Jackson ImmunoResearch) for 1 h at room temperature. Immunostained sections were coverslipped with glycerol and observed using a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan). Images were analyzed for the distribution of T cells in B cell follicles with ImageJ software.

*Statistical analysis*

The Student's unpaired two-tailed *t* test was used to analyze differences between the two conditions. One-way analysis of variance with a Tukey–Kramer multiple comparison test was used to analyze differences among more than two conditions. Values of $P < .05$ were considered significant.

## Results

*Calcitriol markedly promotes CXCR5 expression in Tfh-like cells generated with IL-6* in vitro

To search for physiological factors that induce high levels of CXCR5 expression in Tfh cells, we used our *in vitro* culture system to generate mouse Tfh-like cells from purified naive $CD4^+$ T cells without the addition of other cell types (25). Naive $CD4^+$ T cells were stimulated with immobilized mAbs to CD3, ICOS, and LFA-1 in the presence of soluble anti-CD28, IL-6, TGF-β, and blocking mAbs to IL-4, IL-2, and IL-2 receptors for 3 days, and cultured further for 2 days without immobilized mAbs ("3 + 2d culture," illustrated in Supplementary Fig. S2A). We found that calcitriol markedly induced CXCR5 expression and *Cxcr5* mRNA expression in a dose-dependent manner in Tfh-like cells (Fig. 1A and B and Supplementary Fig. S2B). Calcitriol, however, failed to induce CXCR5 expression in naive $CD8^+$ T cells under these culture conditions (Supplementary Fig. S3A). Calcitriol in amounts of 10–100 nM appeared to be optimal for inducing CXCR5 expression in $CD4^+$ T cells. The recovered cell number after the culture was moderately suppressed by calcitriol (Supplementary Fig. S3B). EB 1089, a VDR agonist, also induced CXCR5 expression (Fig. 1C), whereas the direct precursor of calcitriol, $25(OH)D_3$ (calcifediol), failed to induce the expression (Fig. 1D). Calcitriol did not significantly affect *Bcl6* expression (Fig. 1E) and moderately downregulated *Rorc(γt)* expression (Fig. 1F), consistent with previous studies suggesting that calcitriol inhibits the Th17 phenotype by inhibiting the transcription of RORγt, IL-17, IL-23R, and IL-22 (31). Expression of BCL6 protein was significantly but only moderately downregulated with 10 nM calcitriol, and RORγt protein was more markedly downregulated (Fig. 1G). We also examined the effect of calcitriol on other Tfh markers. Calcitriol and EB 1089 significantly, but only moderately, suppressed PD-1 expression (Fig. 1H) and significantly, but moderately, enhanced ICOS expression (Fig. 1I; Supplementary Fig. S3C). On the other hand, we did not detect remarkable expression of *Foxp3*, a master gene for the development of regulatory T (Treg) cells, including follicular regulatory T (Tfr) cells, in the presence or absence of calcitriol (Supplementary Fig. S3D). We also examined the effect of calcitriol on the expression of various chemokine receptor genes. The expression of *Ccr4* and *Cxcr3* was moderately enhanced and that of *Ccr6* was markedly suppressed. Among those we examined, the *Cxcr5* expression was most strongly enhanced (Fig. 2). These results suggest that calcitriol preferentially enhances CXCR5 expression and Tfh cell development.

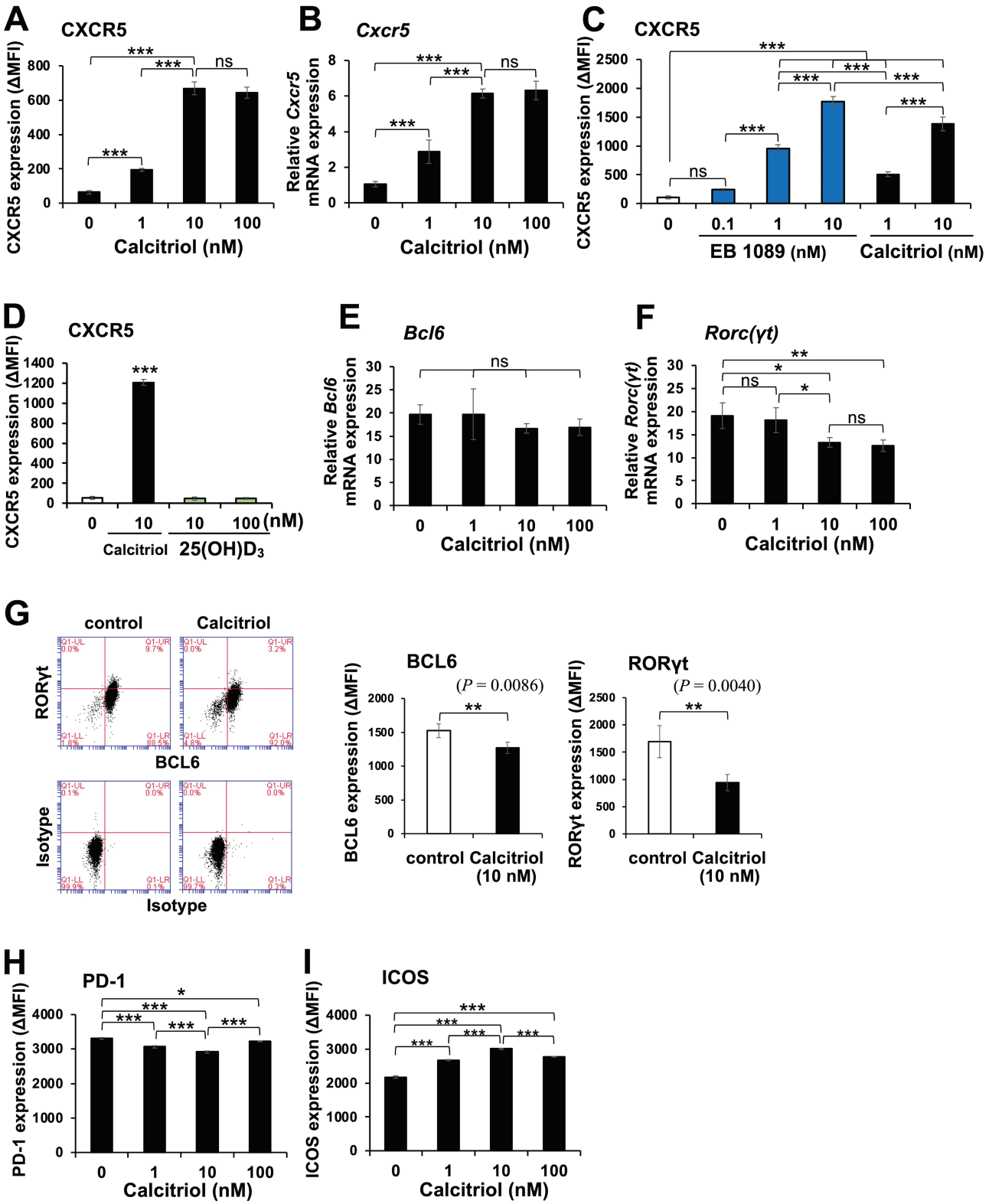



**Figure 1.** Calcitriol and the VDR agonist EB 1089 but not the direct precursor of calcitriol, 25(OH)$D_3$, enhance CXCR5 expression. Naive $CD4^+$ T cells were stimulated for 3 days with immobilized mAbs to CD3, ICOS, and LFA-1 in the presence of soluble anti-CD28, IL-6, TGF-β, and blocking mAbs to IL-4, IL-2, and IL-2 receptors in cDMEM with the indicated concentrations of calcitriol, EB 1089, or 25(OH)$D_3$. Each culture was transferred to a new well with an equal volume of fresh medium containing IL-6, TGF-β1, and the corresponding concentration of calcitriol, EB 1089, or 25(OH)$D_3$, and cultured further for 2 days. After the culture, the cells were analyzed for the expression of CXCR5 (A, C, and D), PD-1 (H), and ICOS (I) by flow cytometry (FCM) and for the expression of *Cxcr5* (B), *Bcl6* (E), and *Rorc(γt)* (F) by real-time PCR. (G) The cultured cells were fixed and permeabilized, and intracellular staining was performed to assess the expression of BCL6 and RORγt by FCM, and representative FCM profiles are shown. Results of the FCM analysis are expressed as ΔMFI ± SD in triplicate or quadruplicate cultures, and those of the real-time PCR analysis are expressed as the $2^{-\Delta Ct}$ value multiplied by 1000 (mean ± SD) in triplicate or quadruplicate cultures. Data are representative of three (B–D and G) or five (A, E, F, H, and I) independent experiments. (ANOVA for A–F, H, and I; Student's *t* test for G) $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. ANOVA, analysis of variance; ns, not significant.

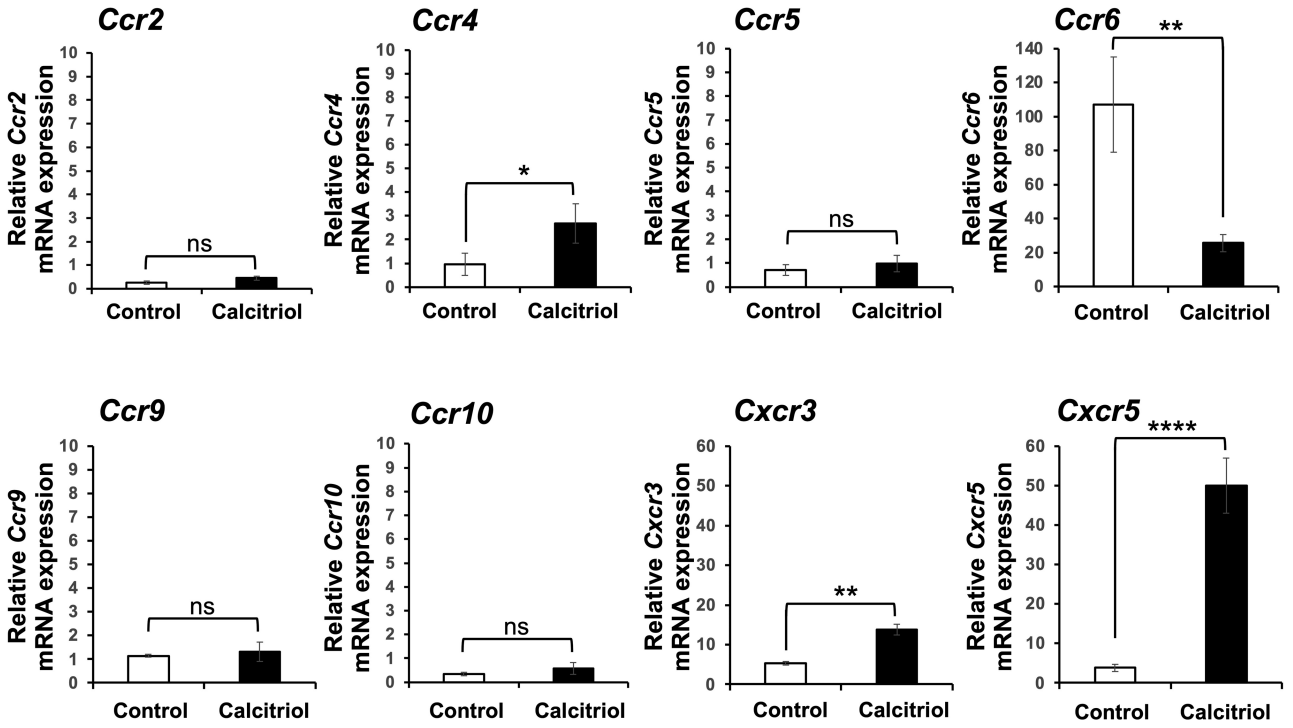



**Figure 2.** Calcitriol enhances the expression of *Cxcr5* far more markedly than that of other chemokine receptor genes. Naive CD4⁺ T cells were stimulated for 3 days and cultured without immobilized mAbs for 2 days as described in the legend of Fig. 1 in the presence or absence of calcitriol (10 nM). The expression of *Ccr2*, *Ccr4*, *Ccr5*, *Ccr6*, *Ccr9*, *Ccr10*, *Cxcr3*, and *Cxcr5* was assessed by real-time PCR. The results are expressed as the $2^{-\Delta Ct}$ value multiplied by 1000 (mean ± SD) in quadruplicate cultures. Data are representative of two independent experiments. (Student's *t* test) $^{*}P < .05$, $^{**}P < .01$, $^{****}P < .0001$. ns, not significant.

### *Calcitriol induces high levels of CXCR5 expression after the release from persistent T cell receptor stimulation*

The combination of IL-6 and TGF-β can induce Th17 differentiation (36). As we previously demonstrated, however, 3 days of Ab-dependent stimulation in the presence of IL-6 and TGF-β (first "3d" culture) followed by 2 days of culture without immobilized Abs ("3 + 2d" culture, illustrated in Fig. 3A and Supplementary Fig. S2A) upregulates *Bcl6* expression and downregulates *Rorc(γt)* expression, compared with the first "3d" culture (25). Calcitriol-induced CXCR5 and *Cxcr5* expression after the "3 + 2d" culture but not after the "3d" culture or the "5d" culture in which the cells were continuously stimulated with immobilized mAbs (illustrated in Fig. 3A and Supplementary Fig. S2A; Fig. 3B and C). Calcitriol did not significantly affect *Bcl6* or *Zfp831* expression (Fig. 3D and E). *Zfp831* encodes the transcription factor ZFP831, which directly contributes to the upregulation of *Bcl6* expression (37). On the other hand, calcitriol suppressed *Rorc(γt)* expression after the "3 + 2d" or "5d" culture but not after the "3d" culture (Fig. 3F).

We also examined if calcitriol is required during the first "3d" culture or the second "2d" culture for potentiating CXCR5 and *Cxcr5* expression. Calcitriol added in the second culture more efficiently induced CXCR5 and *Cxcr5* expression than that added only in the first culture (Fig. 3G and H), suggesting that calcitriol induces CXCR5 expression mainly after the release from T cell receptor (TCR) stimulation. To obtain some insight into the mechanism of the calcitriol-induced *Cxcr5* expression, we added CHX, a protein synthesis inhibitor, into the second culture for 1 day. Unexpectedly, CHX significantly enhanced *Cxcr5* expression even without added calcitriol (Fig. 4A). The results suggest a possibility that synthesis of transcriptional repressors may participate in the regulation of *Cxcr5* expression and that calcitriol may repress the repressor expression. Indeed, we found that calcitriol significantly repressed the expression of *Id2* that encodes a repressor of *Cxcr5* expression (38) in the "3 + 2d" culture but not "3d" or "5d" cultures (Fig. 4B and C). Concerning other repressors of *Cxcr5*, calcitriol moderately suppressed *Runx3* expression, but failed to suppress the expression of *Prdm1* and *Runx2* (Fig. 4B).

### *IL-6, IL-21, and IL-12, but not IL-4, support the calcitriol-induced CXCR5 expression*

IL-6 and IL-21 redundantly induce mouse Tfh cell differentiation (39), and IL-6 induces IL-21 production (2, 40). IL-12 is the most potent initiator of the human Tfh cell program (41, 42) and induces the Tfh-like cell phenotype in mouse cells in the early stage (43). As with IL-6, we found that IL-21 and IL-12, but not IL-4, could support calcitriol to enhance CXCR5 expression in mouse CD4⁺ T cells after the "3 + 2d" culture (Fig. 5A). Unlike IL-6, IL-12 supported to enhance CXCR5 expression immediately after the first "3d" culture (Fig. 5B). The "3d" culture with IL-12 induced the expression of BCL6 protein and mRNA at lower levels than that with IL-6, and calcitriol did not affect the expression (Supplementary Fig. S4A). After the "3d" culture with IL-12, the expression of RORγt, *Gata3*, and

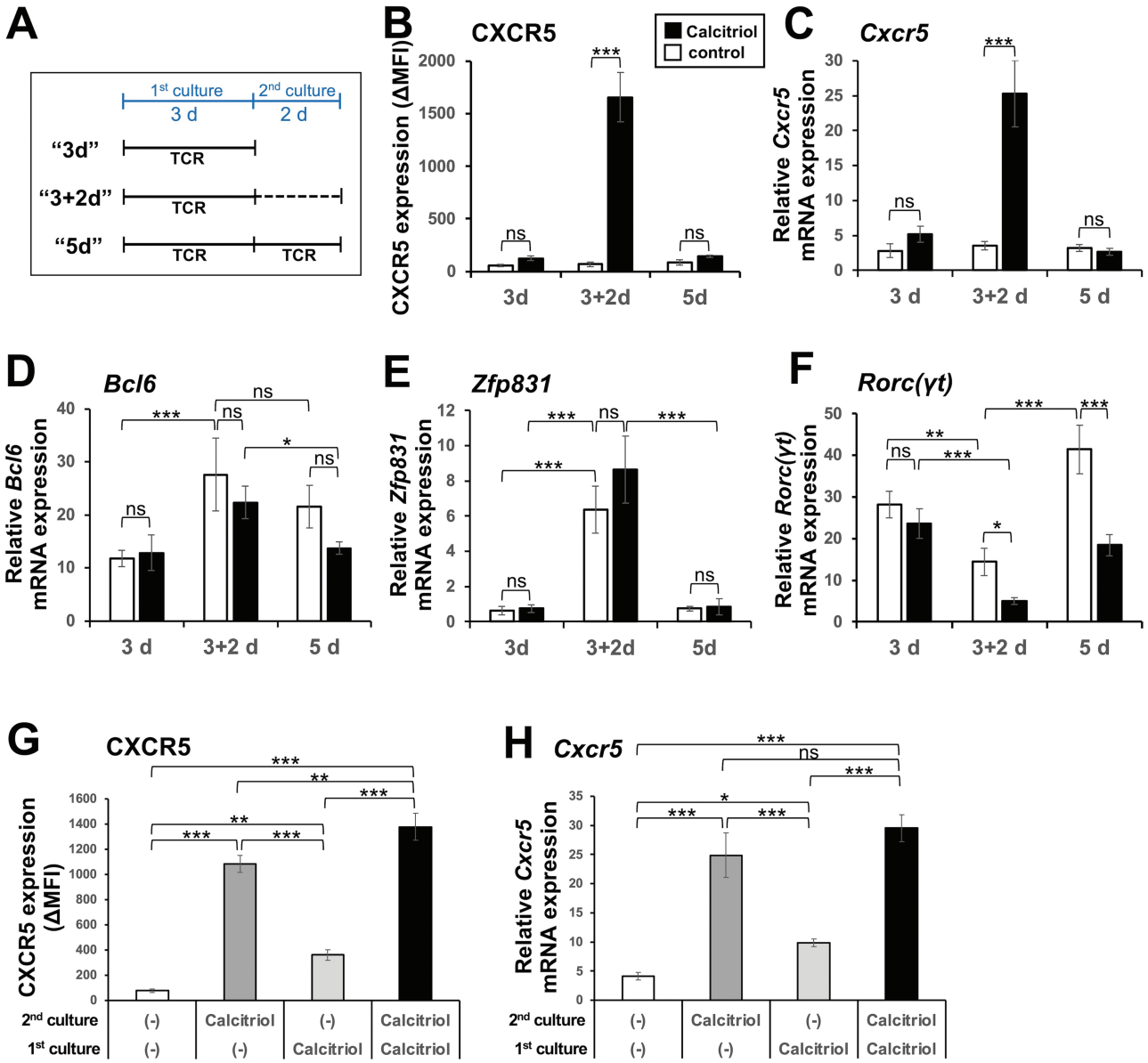



**Figure 3.** Calcitriol induces high levels of CXCR5 expression after the release from persistent TCR stimulation. Naive CD4+ T cells were cultured as described in the legend of Fig. 1 for 3 days ("3d" culture) with or without 10 nM calcitriol. Aliquots of the cultures were transferred and cultured in new mAb-coated plates as for the "3d" culture ("5d" culture) or uncoated plates ("3 + 2d" culture) with the addition of an equal volume of fresh medium containing IL-6 and TGF-β1 with or without 10 nM calcitriol for 2 days (A–F). Other aliquots of the cells were resuspended and cultured in new uncoated plates with twice the volume of fresh medium containing IL-6, TGF-β1, and mAbs to IL-2, CD25, and CD122 with or without 10 nM calcitriol for 2 days (G and H). (A) Schematic illustration for the "3d," "3 + 2d," and "5d" culture. The cultured cells were analyzed for the expression of CXCR5 (B and G) by FCM or for the expression of *Cxcr5* (C and H), *Bcl6* (D), *Zfp831* (E), and *Rorc(γt)* (F) by real-time PCR. Results of the FCM analysis are expressed as ΔMFI ± SD in triplicate cultures, and those of the real-time PCR analysis are expressed as the $2^{-\Delta Ct}$ value multiplied by 1000 (mean ± SD) in triplicate or quadruplicate cultures. Data are representative of three independent experiments. (ANOVA) $*P < .05$, $**P < .01$, $***P < .001$. ANOVA, analysis of variance; ns, not significant.

even *Tbx21*, was low (Supplemental Fig. S4A and B). After the "3 + 2d" culture with IL-12, calcitriol did not significantly suppress PD-1 expression but enhanced ICOS expression, as observed in the culture with IL-6 (Fig. 5B). The number of viable cells, however, was very low after the "3 + 2d" culture with IL-12 in the presence or absence of added calcitriol compared with that with IL-6 (Fig. 5B, Supplementary Fig. S4C). Even an overnight culture without TCR stimulation after the "3d" culture reduced the viable cell number to one-third (approximately $1.5 \times 10^5$ cells/ml with or without calcitriol) of that observed immediately after the "3d" culture. These results suggest that IL-12 can also support calcitriol to enhance CXCR5 expression and induce the development of Tfh-like cells to some extent, but that an additional factor(s) may be required for IL-12 to support the survival of mouse Tfh-like cells.

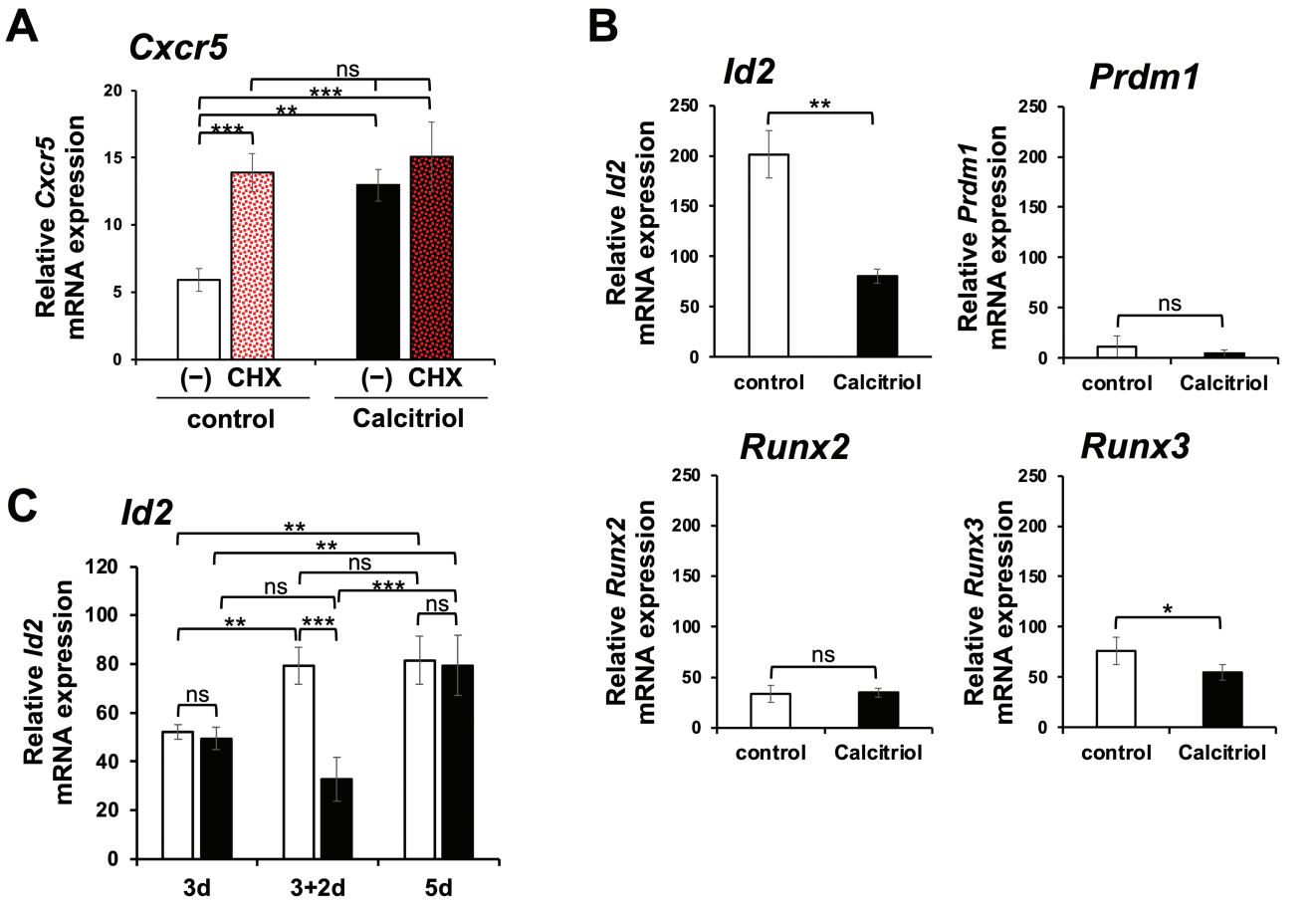



**Figure 4.** The effect of CHX added in the second culture on the expression of *Cxcr5* and the effect of calcitriol on the expression of *Id2* and other repressors of *Cxcr5*. (A) Naive CD4+ T cells were stimulated for 3 days with immobilized mAbs to CD3, ICOS, and LFA-1 in the presence of soluble anti-CD28, IL-6, TGF-β1, and blocking mAbs to IL-4, IL-2, and IL-2 receptors with or without calcitriol (10 nM). Each culture was transferred to a new well with an equal volume of fresh medium containing IL-6 and TGF-β1 with or without calcitriol and CHX (10 μg/ml), and cultured further for 1 day. The expression of *Cxcr5* was assessed by real-time PCR. The "3d" (C), "3 + 2d" (B and C), and "5d" (C) cultures were performed with or without calcitriol (10 nM). The cultured cells were assessed for the expression of *Id2* (B and C), *Prdm1* (B), *Runx2* (B), and *Runx3* (B) by real-time PCR. The results are expressed as the $2^{-\Delta Ct}$ value multiplied by 1000 (mean ± SD) in triplicate or quadruplicate cultures. Data are representative of two (A and C) or three (B) independent experiments. (ANOVA for A and C; Student's *t* test for B) **P* < .05, ***P* < .01, ****P* < .001. ANOVA, analysis of variance; ns, not significant.

### *Calcitriol induces chemotactic activity toward the CXCR5 ligand CXCL13 in IL-6-induced Tfh-like cells*

We used transwell migration assays to examine if Tfh-like cells generated by the "3 + 2d" culture in the presence of calcitriol and IL-6 could migrate toward the CXCR5 ligand CXCL13. The calcitriol-treated cells exhibited significant chemotaxis toward CXCL13 (Fig. 6), suggesting that the surface CXCR5 protein expressed on calcitriol-treated Tfh-like cells is functionally active.

In addition to the CXCR5 expression, reduced expression of CCR7 is required for the migration of Tfh cells into B cell follicles (15). CCR7 is a receptor for the T zone chemokines CCL19 (ELC) and CCL21 (SLC), and provides a counteracting signal to retain activated T cells in the T cell area. CCR7 and CXCR4 are expressed on naive T cells and downregulated during differentiation to effector T cells (44). We confirmed that Tfh-like cells generated *in vitro* with or without calcitriol did not significantly express CCR7 or CXCR4 (Supplementary Fig. S5A).

### *The AhR antagonist CH-223191 enhances calcitriol-induced CXCR5 expression*

We previously reported that the AhR antagonist CH-223191 enhances CXCR5 expression in Tfh-like cells, while the AhR agonist FICZ suppresses CXCR5 expression (25). Calcitriol far more efficiently enhanced CXCR5 and *Cxcr5* expression in Tfh-like cells than CH-223191, but CH-223191 further enhanced the calcitriol-induced CXCR5 and *Cxcr5* expression (Fig. 7). Under the present condition, however, CH-223191 alone did not significantly enhance CXCR5 or *Cxcr5* expression (Fig. 7), although direct comparison in the absence of calcitriol confirmed that CH-223191 significantly enhanced the expression (Supplementary Fig. S5B). On the other hand, FICZ suppressed the calcitriol-induced CXCR5 and *Cxcr5* expression. The combination of calcitriol and CH-223191 moderately but further suppressed PD-1 expression and enhanced ICOS expression compared with calcitriol alone (Fig. 7A). The combination had no significant effect on *Bcl6* expression, but significantly suppressed *Rorc(γt)* expression

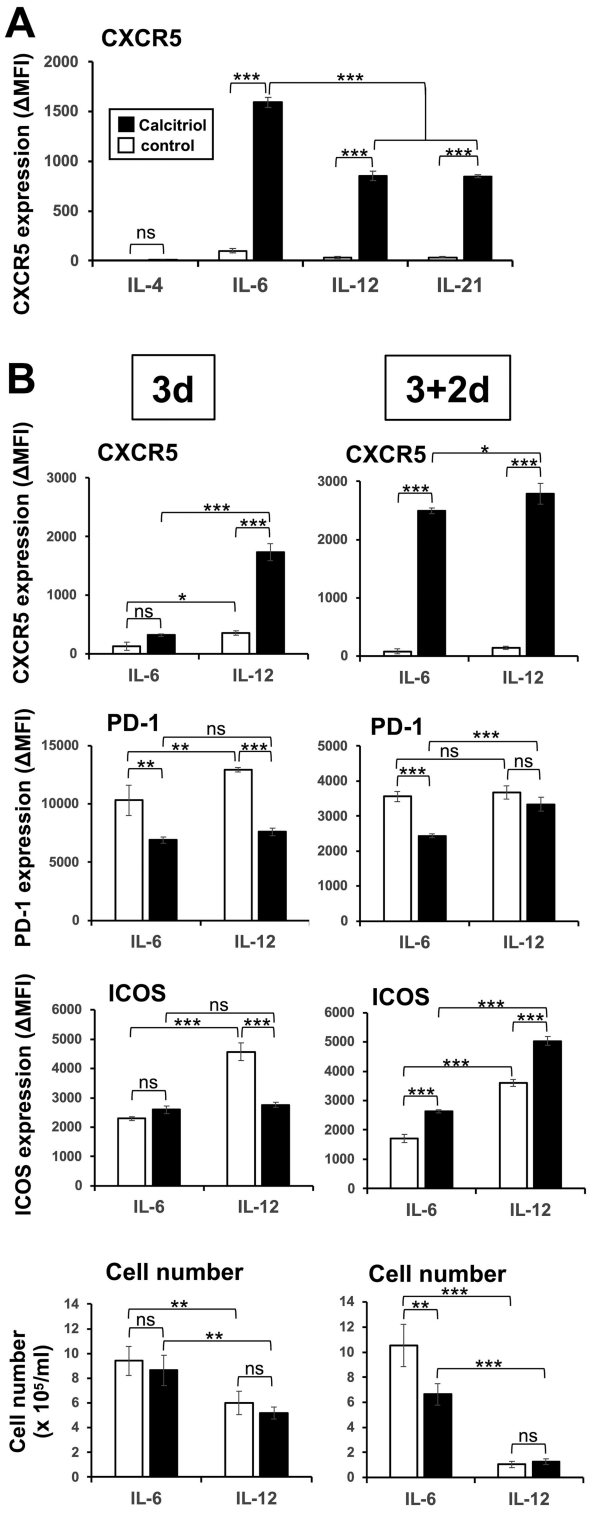



**Figure 5.** IL-12 and IL-21 also support calcitriol-induced CXCR5 expression. The "3d" or "3 + 2d" culture was performed in the presence of IL-4, IL-12, or IL-21 in place of IL-6 with or without added calcitriol (10 nM). (A) CXCR5 expression after the "3 + 2d" culture was assessed by FCM. (B) After the "3d" or "3 + 2d" culture in the presence of IL-6 or IL-12, expression of CXCR5, PD-1, and ICOS was assessed by FCM, and the recovered cell number was determined. Results of the FCM analysis are expressed as ΔMFI ± SD in triplicate cultures. Data are representative of three independent experiments. (ANOVA) $*P < .05$, $**P < .01$, $***P < .001$. ANOVA, analysis of variance; ns, not significant.

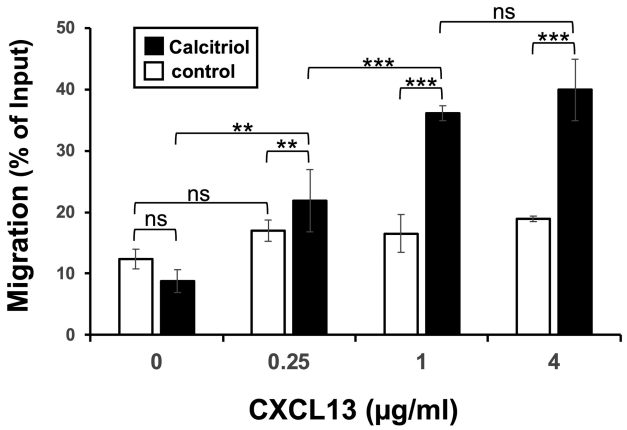



**Figure 6.** Calcitriol induces significant chemotactic activity toward CXCL13 in Tfh-like cells. Tfh-like cells were generated in DMEM with IL-6 from naive CD4⁺ T cells by the "3 + 2d" culture with or without added calcitriol (10 nM). Cells were assessed for their ability to migrate toward graded concentrations of CXCL13 during 2 h of incubation by transwell chemotaxis assay. The percentage of cells migrating from the upper well to the lower well with CXCL13 was determined. Results are expressed as mean percentage ± SD of triplicate samples. Data are representative of two independent experiments. (ANOVA) $**P < .01$, $***P < .001$. ANOVA, analysis of variance; ns, not significant.

(Fig. 7B). These results suggest that AhR activity modulates calcitriol-induced CXCR5 expression in Tfh-like cells.

### *Calcitriol suppresses Tfh-like cell production of IL-17A and enhances their production of IL-21, and induces IgG production in B cells*

Tfh-like cells generated with calcitriol *in vitro* produced more IL-21 and much less IL-17A than those generated without added calcitriol (Fig. 8A). In co-cultures with B cells, Tfh-like cells generated with calcitriol more efficiently promoted IgG production than those generated without added calcitriol or Th0 or Th1 cells in the presence of soluble anti-CD3 mAb (Fig. 8B). Tfh-like cells generated with or without calcitriol-induced $CD138^{high}B220^{low}$ cells (Supplementary Fig. S6), suggesting that they induced plasma cells. These results suggest that calcitriol upregulates the B cell helper activity of Tfh cells. As the stimulation with anti-CD3 might not involve direct T–B cell interactions, we also examined if similar upregulation was observed with SEB, a superantigen, instead of anti-CD3. Tfh-like cells generated with calcitriol more efficiently promoted IgG production in the presence of SEB than those generated without added calcitriol, and induced $CD138^{high}B220^{low}$ cells (Fig. 9). In the absence of SEB or T cells, these responses were limited. The results suggest that calcitriol upregulates the B cell helper activity of Tfh-like cells also upon direct interactions with B cells.

### *Calcitriol injections in antigen-primed mice upregulate the proportion of $CXCR5^{+}PD\text{-}1^{+}$ cells among $CD4^{+}$ cells in their lymphoid organs*

We examined if calcitriol injections in antigen-primed mice affect the proportion of Tfh cells in lymphoid organs. A schematic diagram of the experimental protocol is shown in Fig. 10A. $CD4^{+}$ cells were gated from the spleen and MLN cells

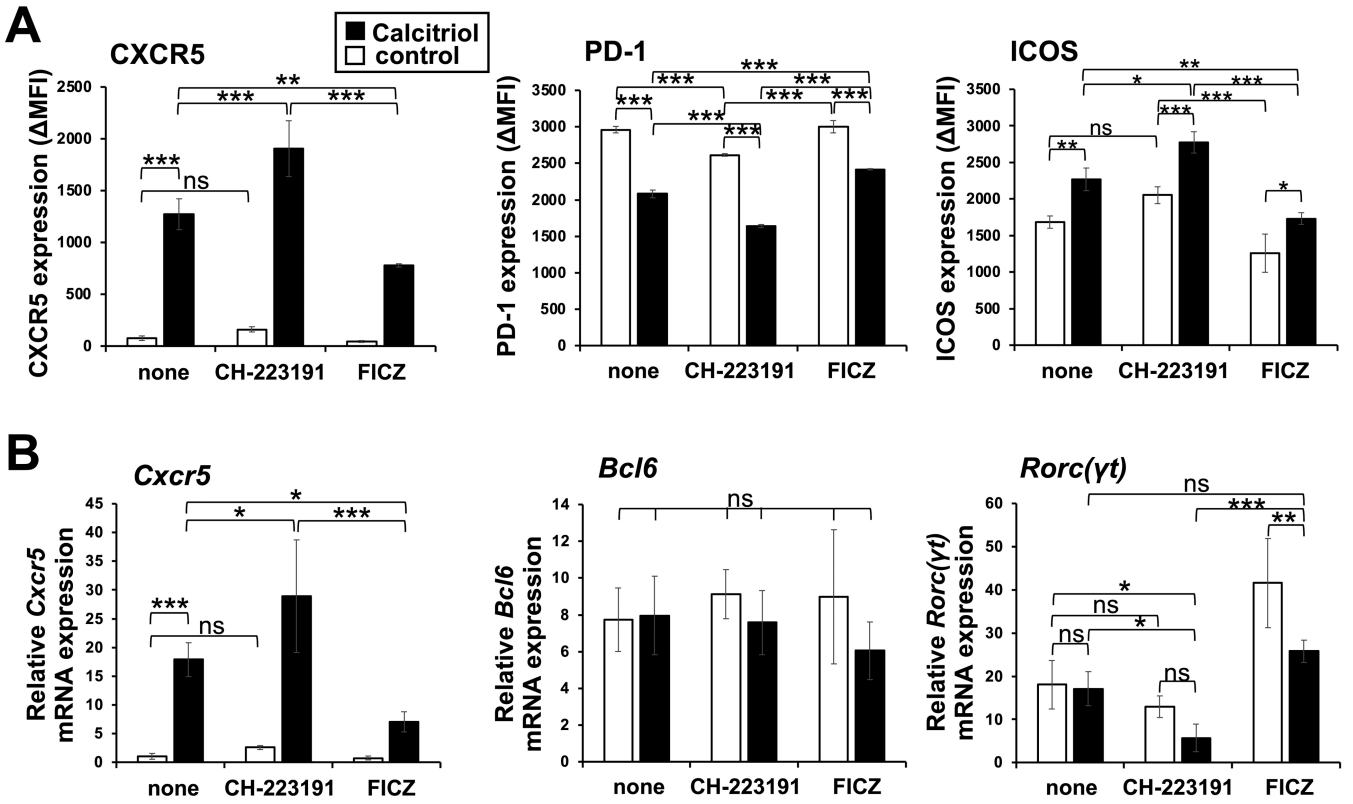



**Figure 7.** Calcitriol-induced CXCR5 expression is enhanced by the AhR antagonist CH-223191 and suppressed by the AhR agonist FICZ. Tfh-like cells were generated in DMEM with IL-6 from naive CD4+ T cells by the "3 + 2d" culture with or without CH-223191 (2.5 μM) or FICZ (100 nM) in the presence or absence of calcitriol (10 nM). (A) Expression of CXCR5, PD-1, and ICOS was assessed by FCM assay. Results are expressed as ΔMFI ± SD ($n = 3$). (B) Expression of *Cxcr5*, *Bcl6*, and *Rorc(γt)* was assessed by real-time PCR. Results are expressed as the $2^{-\Delta Ct}$ value multiplied by 1000 (mean ± SD) in triplicate or quadruplicate cultures. Data are representative of three independent experiments. (ANOVA) $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. ANOVA, analysis of variance; ns, not significant.

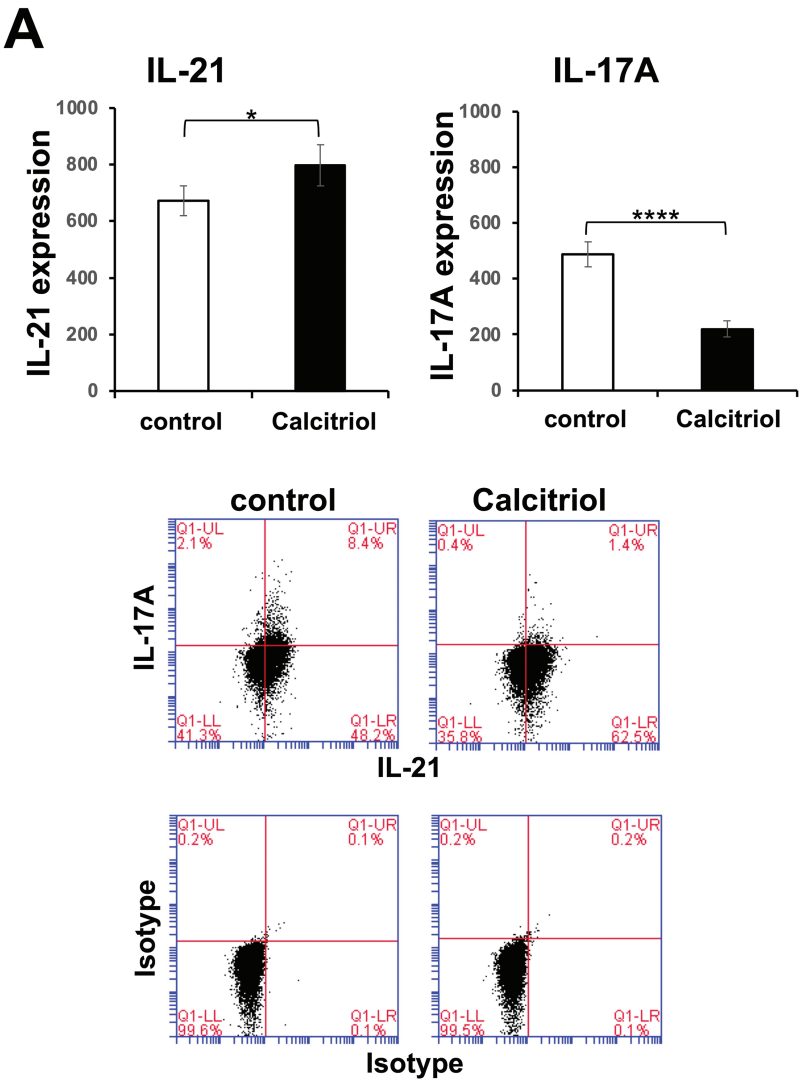
of treated mice, and assessed for their expression of CXCR5 and PD-1, as shown in Supplementary Fig. S7A. Calcitriol injections enhanced the proportion of CXCR5+PD-1+ cells among CD4+ cells in the spleens and MLNs of mice immunized intraperitoneally (Fig. 10B). The weight of the MLNs was higher in calcitriol-treated mice than vehicle-treated mice, although the MLN cell number varied widely and was not significantly increased by calcitriol injections (Supplementary Fig. S7B). As for the spleen, however, calcitriol injections did not exert significant effects on spleen weight or cell number after red blood lysis. The proportions of CD4+ cells, CD8+ cells, and B cells were also determined by gating as shown in Supplementary Fig. S7C. Calcitriol injections did not significantly affect these proportions in the spleen or MLNs (Supplementary Fig. S7B). These results suggest that calcitriol is also likely to enhance CXCR5 expression and differentiation of follicular T cells *in vivo*.

### *Calcitriol injections in antigen-primed mice enhance the entry of T cells into B cell follicles*

We directly examined if calcitriol injections affect the T cell distribution in B cell follicles in the spleens of immunized mice. T cells, B cells, and GCs in their tissue sections were visualized (Fig. 11A and Supplementary Fig. S8), and B cell follicle areas were selected using ImageJ (Fig. 11B). Immunohistochemical analyses indicated that the ratio of T cells (red) to B cells (green) in B cell follicles of the spleen was significantly higher in calcitriol-injected mice than that in control mice (Fig. 11C). These results suggest that calcitriol enhances the entry of T cells into B cell follicles.

## Discussion

Our findings demonstrated that physiological levels of calcitriol markedly enhanced CXCR5 expression after the release from the sustained TCR stimulation during the Tfh-like cell development *in vitro*. Expression of other Tfh cell markers, including BCL6, PD-1, ICOS, and *Zfp831*, was affected only moderately, if at all. Tfh-like cells generated with calcitriol *in vitro* migrated toward the CXCR5 ligand CXCL13, produced IL-21, and promoted B cell production of IgG antibodies *in vitro* more efficiently than those generated without calcitriol, suggesting that calcitriol promotes CXCR5 expression in developing Tfh cells and regulates their functional differentiation. In CD8+ T cells, however, calcitriol did not significantly induce CXCR5 expression under the present culture condition. On the other hand, CXCR5 expression is also important for B cells to migrate into the follicles. Calcitriol did not significantly induce or enhance CXCR5 expression



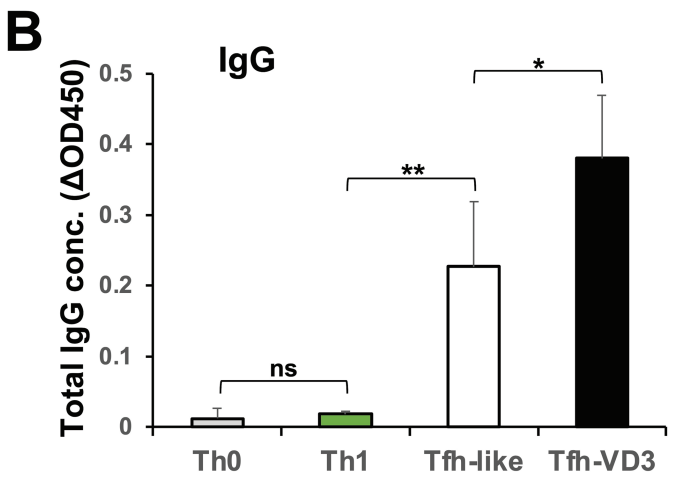




**Figure 8.** Calcitriol suppresses the production of IL-17A by Tfh-like cells and enhances their production of IL-21, and induces IgG production in B cells. Tfh-like cells were generated in RPMI 1640 with IL-6 from naive $CD4^+$ T cells by the "3 + 2d" culture with or without 10 nM calcitriol. (A) Tfh-like cells ($1 \times 10^5$ cells) were resuspended and cultured in fresh RPMI 1640 medium in 96-well plates coated with 3 μg/ml of anti-CD3 and 3 μg/ml of anti-CD28 for 2 days, and monensin (2 μM) was added for the last 2 h of the culture. Expression of IL-21 and IL-17A was assessed by FCM assay. Results are expressed as ΔMFI ± SD in quadruplicate cultures. Representative FCM profiles are shown. (B) Th0 and Th1 cells were obtained as described in the Methods section. Th0, Th1, or Tfh-like cells ($3 \times 10^4$ cells) were co-cultured with purified B cells ($1.5 \times 10^5$ cells) in the presence of soluble anti-CD3 mAb (0.1 μg/ml) in 0.2 ml of RPMI 1640 medium in 96-well round bottom plates for 7 days. The culture supernatant was analyzed for total IgG Ab production by ELISA. Results are expressed as ΔOD450 ± SD in triplicate or quadruplicate cultures. ΔOD450 values were calculated by subtracting OD450 readings taken from supernatants from B cells cultured alone from the OD450 values of co-cultured samples. Data are representative of four independent experiments. (Student's *t* test for A; ANOVA for B) $^*P < .05$, $^{**}P < .01$, $^{****}P < .0001$. ANOVA, analysis of variance; ns, not significant.

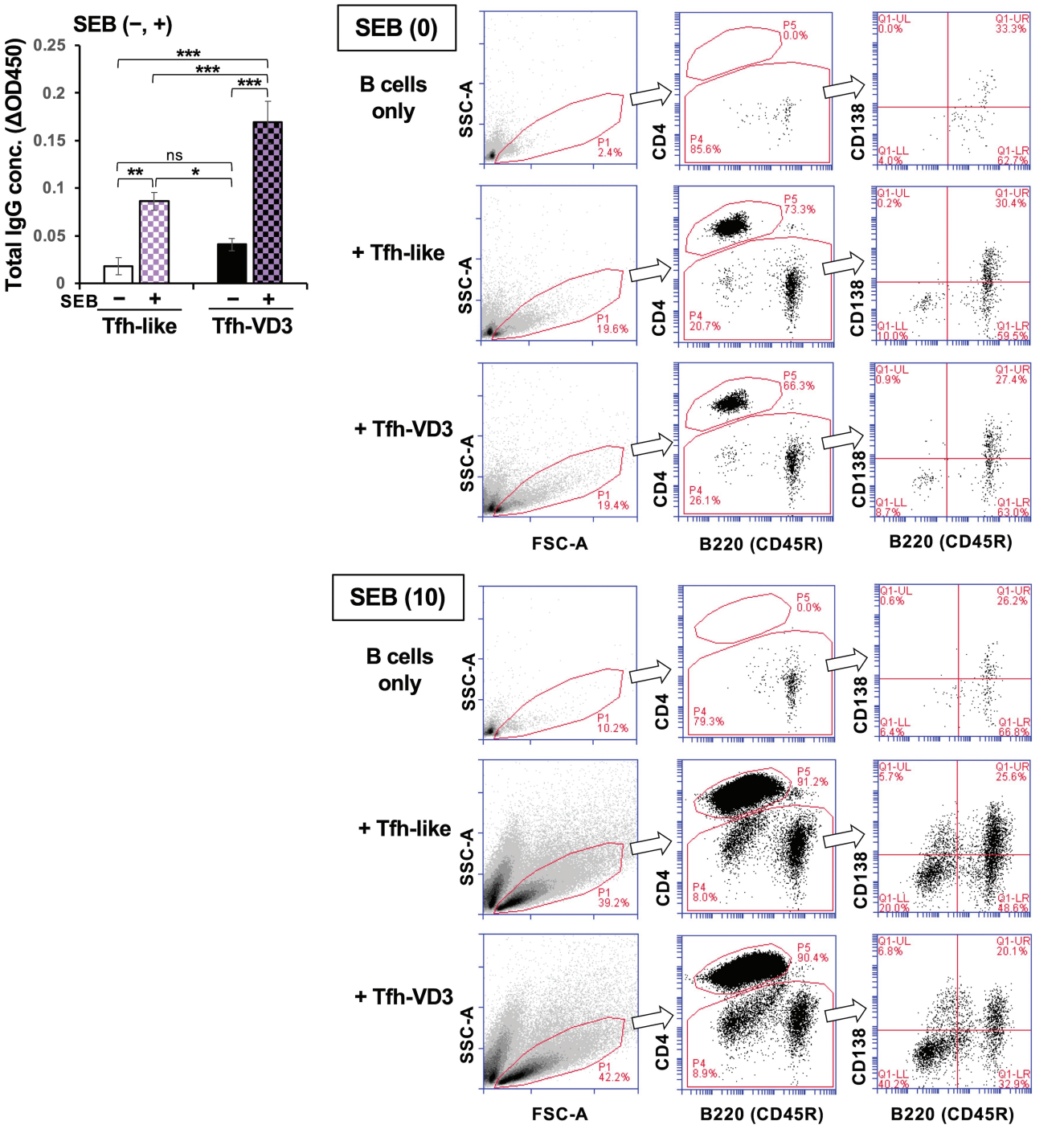



**Figure 9.** Th-like cells generated with calcitriol more efficiently promote IgG production in the presence of SEB than those generated without added calcitriol, and induce plasma cells. Tfh-like cells ($8 \times 10^4$ cells) generated with or without calcitriol in the RPMI medium were co-cultured with B cells ($1.5 \times 10^5$ cells) in the presence of soluble anti-CD28 (0.1 μg/ml) with or without SEB (10 μg/ml) for 5 days. The culture supernatant was analyzed for total IgG Ab production by ELISA. Results are expressed as ΔOD450 ± SD in triplicate cultures. ΔOD450 values were calculated by subtracting OD450 readings taken from supernatants from B cells cultured alone from the OD450 values of co-cultured samples. The mean OD450 (± SD) readings from supernatants from B cells cultured alone were 0.049 ± 0.003 and 0.043 ± 0.001 for the culture with and without SEB, respectively. Data are representative of two independent experiments. (ANOVA) $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. ANOVA, analysis of variance; ns, not significant. Representative FCM profiles after the co-culture are also shown.

in CD19$^+$ bone marrow cells or splenic B cells in our trial experiments.

The CXCR5 expression levels induced in CD4$^+$ T cells by calcitriol were far greater than those induced by any other factors we examined. The AhR agonist CH-223191 could only modestly upregulate the CXCR5 expression, but could further enhance calcitriol-induced CXCR5 expression. Therefore, some background AhR activity appears

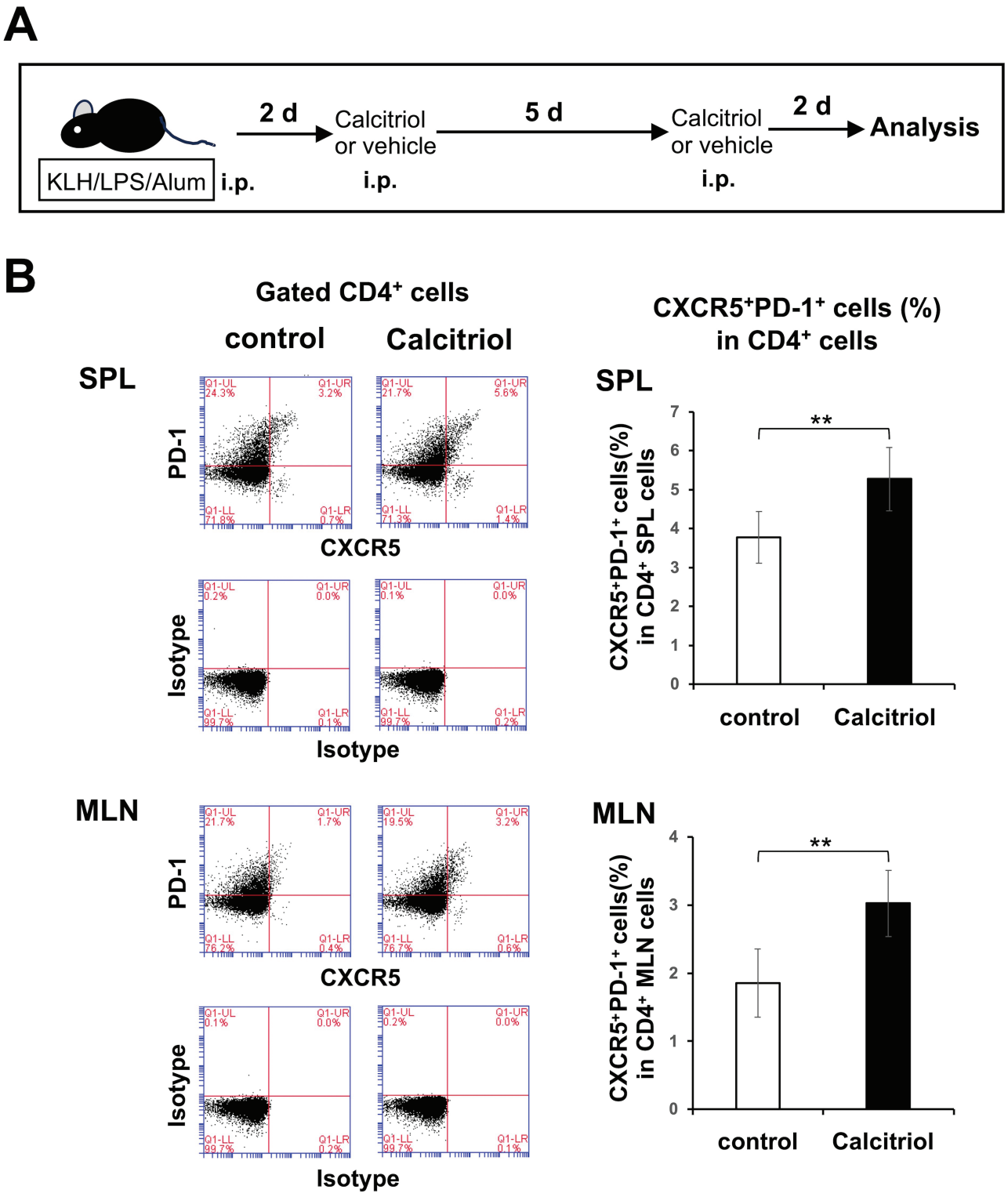



**Figure 10.** Injections of calcitriol in antigen-primed mice upregulate the proportion of CXCR5$^+$PD-1$^+$ cells among CD4$^+$ cells in the spleen and lymph nodes. (A) A schematic diagram of the experimental protocol is shown. C57BL/6 mice were given intraperitoneal (i.p.) injections of a mixture of 100 μg KLH, 10 μg LPS, and 2 mg alum. Two days later, mice were given i.p. injections of 0.1 μg calcitriol or vehicle control. Five days after that, the calcitriol or vehicle control injection was repeated. Two days after the last injection, their splenic cells and MLN cells were analyzed. (B) Gated CD4$^+$ cells from the spleen and MLNs were analyzed for the expression of CXCR5 and PD-1 by FCM, and representative FCM profiles are shown. Results are expressed as the mean percentage of CXCR5$^+$PD-1$^+$ cells ± SD in 6 mice per group. Data are representative of three independent experiments. (Student's *t* test) $^{**}P < .01$. LPS, lipopolysaccharides.

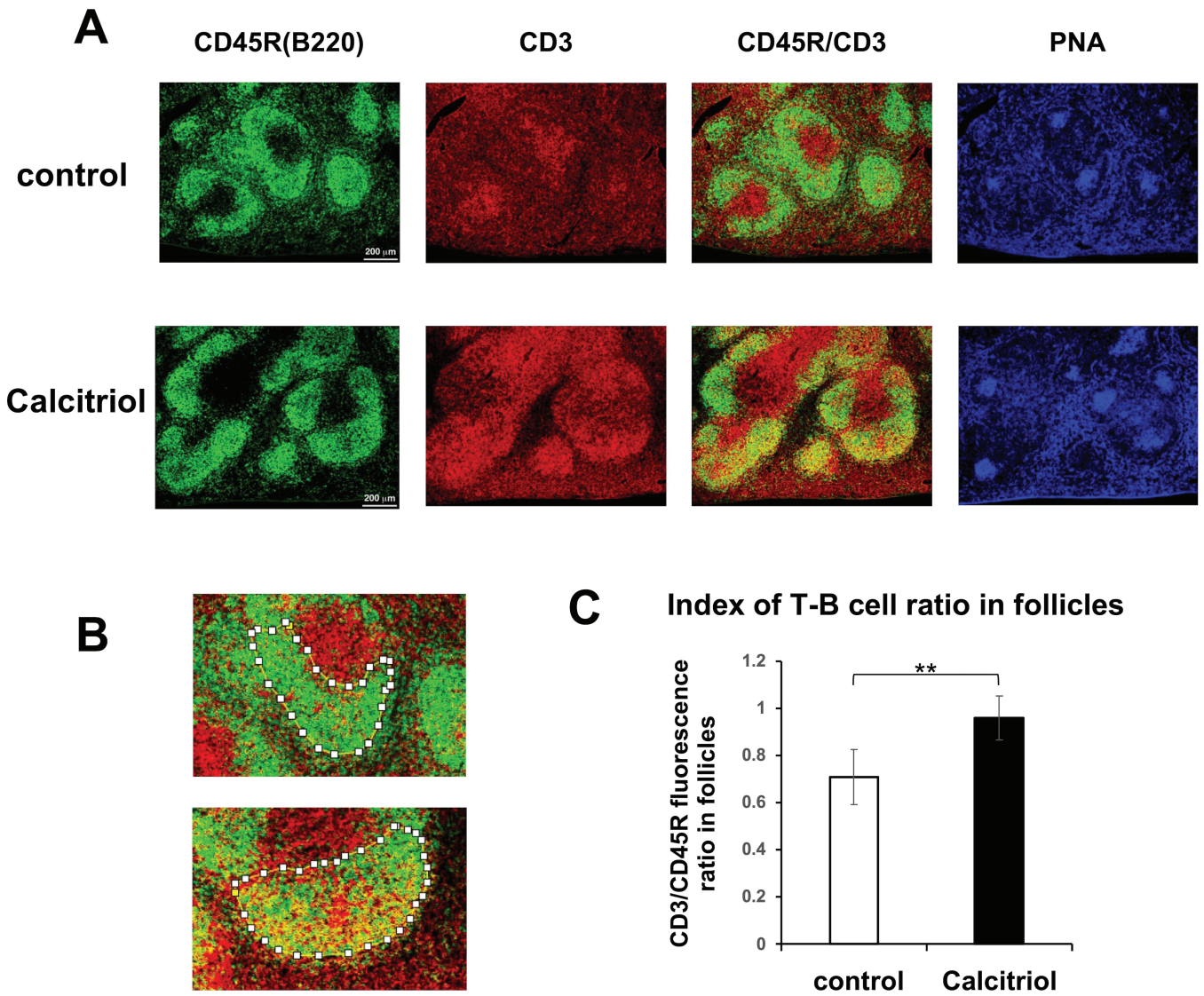



**Figure 11.** Calcitriol injections in antigen-primed mice increase T cells distributed in B cell follicles. Mice were given intraperitoneal (i.p.) injections of a mixture of KLH and LPS in alum as described in the legend for Fig. 10. Two days later, mice were given i.p. injections of 0.1 μg calcitriol or vehicle control. Two days after that, the calcitriol or vehicle control injection was repeated. Three days after the last injection, histochemical analysis was performed on their spleens for staining B cells with anti-CD45R(B220), T cells with anti-CD3, and GCs with PNA. (A) Representative images of follicles in the spleen from calcitriol- or vehicle control-treated mice. (B) Examples of the image selection of a B cell follicle with ImageJ. (C) Relative T–B cell ratios were estimated by comparing the red and green fluorescence intensities in B cell follicle images of spleens from calcitriol-treated and vehicle control-treated mice. Results are expressed as mean red/green fluorescence intensity ratio ± SD in 44 follicles from 4 mice per group. Data are representative of two independent experiments. (Student's *t* test) $^{**}P < .01$. LPS, lipopolysaccharides; PNA, peanut agglutinin.

to have suppressive effects on the CXCR5 expression induced by calcitriol with IL-6 and TGF-β. AhR agonists or their precursors, such as aromatic amino acids present in the medium and FCS, might contribute to the AhR activity (25). Our previous study indicated that CH-223191 and the AhR agonist FICZ downregulate and upregulate *Rorc(γt)* expression, respectively, in Tfh-like cells (25). In the present study, we confirmed that FICZ significantly upregulated the *Rorc(γt)* expression i.e. critical for Th17 cell differentiation, but CH-223191 did not significantly affect the expression (Fig. 7B). The difference might be partly due to the AhR agonist levels in the FCS lots used in these experiments. On the other hand, calcitriol inhibits the transcription of the Th17 markers including RORγt, IL-17, IL-23R, and IL-22 (31). Indeed, calcitriol significantly suppressed RORγt protein expression (Fig. 1G), although it moderately or not significantly suppressed *Rorc(γt)* mRNA expression (Fig. 1F and 7B). However, the combination of calcitriol and CH-223191 significantly suppressed the *Rorc(γt)* expression (Fig. 7B). Therefore, it may be possible that calcitriol and CH-223191 coordinately inhibited Th17 phenotype, resulting in the upregulation of the Tfh phenotype. Another synthetic reagent, TWS119, a glycogen synthetase kinase 3β inhibitor that can upregulate *Ascl2* expression, also showed only

modest effects on the CXCR5 expression in our preliminary experiments with our culture system.

We also found that the protein synthesis inhibitor CHX added in the second culture enhanced *Cxcr5* expression even without added calcitriol. The results let us hypothesize that calcitriol may repress the expression of transcriptional repressors of *Cxcr5* during the second culture. The *Id2*, *Prdm1*, *Runx2*, and *Runx3* genes encode transcriptional repressors of *Cxcr5* (38). We found that calcitriol markedly suppressed the expression of *Id2* but not *Prdm1* or *Runx2* after the release from TCR stimulation. Calcitriol suppressed *Runx3* expression only moderately. Therefore, *Id2* expression is most likely to participate in the repression of *Cxcr5* expression, and thus the repression may be released by calcitriol. As BCL6 is known to repress these repressors (38), calcitriol may enhance or supplement its effect. Further studies will be needed to confirm this hypothesis by examining the direct binding of ID2 protein to the regulatory region of the *Cxcr5* gene and the actual regulation of its expression.

VDR-mediated signaling, however, may not be solely responsible for the induction of sufficient levels of CXCR5 expression *in vivo*. Tfh cells are essential for the formation and maintenance of GCs (8), but spleens from VDR-deficient mice showed a normal GC appearance (45), suggesting that VDR signaling is not essential for Tfh cell development, including their CXCR5 expression. Accordingly, under some experimental settings in mice, Ab production does not require vitamin D or VDR (46). On the other hand, vitamin D insufficiency is often involved in the development of infectious, inflammatory, and autoimmune diseases (29). Because vitamin D appears to both enhance and suppress immune responses, including Ab responses depending on the situation (30), it may be that proper vitamin D levels contribute to the Tfh cell-dependent control of the quality and quantity of Abs for proper humoral responses *in vivo*. In addition, it is likely that other physiological factors or mechanisms independent of VDR redundantly contribute to the expression of CXCR5 and Tfh cell development. The oncoprotein survivin may be another candidate factor, as it can be extracellularly released, incorporated by other cells, and bind tightly to the side of the BCL6 BTB domain, a primary interaction site with its co-repressors, and upregulate CXCR5 (47).

Thus, in the present study, instead of analyzing VDR-deficient mice, we examined if injections of calcitriol in antigen-primed normal mice affect the proportion of $CXCR5^+$ T cells in lymphoid organs and the distribution of T cells in B cell follicles. We found that calcitriol injections upregulated the proportion of $CXCR5^+PD\text{-}1^+$ cells among $CD4^+$ cells and the ratio of T cells to B cells in follicles, suggesting that calcitriol enhances follicular T cell differentiation and migration to B cell follicles. In lymphoid organs, calcitriol may be provided by several types of CYP27B1-expressing cells, including dendritic cells, macrophages, B cells, and T cells, especially upon activation (48). In fact, our preliminary immunohistochemical analyses indicated that $CYP27B1^+$ cells were distributed in draining lymph nodes obtained from immunized mice. Nonetheless, the balance between CYP27B1 and the major calcitriol catabolizing enzyme CYP24A1 may contribute to the local calcitriol concentration despite the presence of 25(OH)$D_3$. It remains to be determined if calcitriol-treated follicular T cells can contribute to optimal Ab responses with proper quality and quantity.

IL-12 in place of IL-6 in our *in vitro* culture system to generate Tfh-like cells also upregulated CXCR5 expression and some other Tfh cell phenotypes even immediately after the "3d" culture without release from TCR stimulation. Incubation without TCR stimulation after the "3d" culture, however, quickly decreased the number of live cells. This might be due in part to the lack of IL-21, a Tfh cell growth factor, because IL-12 does not induce IL-21 expression in mouse $CD4^+$ T cells unlike in human $CD4^+$ T cells (41, 42, 49). Our preliminary experiments, however, indicated that adding IL-21 to the second culture of the "3 + 2d" culture with IL-12 failed to restore the cell viability. Adding low concentrations of IL-2 to the second culture may be another method of inducing Tfh1-like cells, as reported (34). Although IL-2 generally suppresses the development of both mouse and human Tfh cells (6, 50), IL-2 administration inhibits early commitment to the Tfh cell lineage rather than affecting already differentiated Tfh cells (6). Therefore, the delayed addition of IL-2 in the presence of calcitriol may support the viability and maintenance of Tfh-like cells.

Calcitriol can augment the differentiation of Treg cells, but their differentiation requires IL-2 signaling (51). Calcitriol might be able to augment the differentiation of Tfr cells as well. Tfh-like cells generated with calcitriol in our *in vitro* culture system did not express *Foxp3*. On the other hand, in our *in vivo* experiments, we did not determine if calcitriol injections affected *Foxp3* expression in the $CXCR5^+PD\text{-}1^+CD4^+$ cells in the spleen or MLNs. The majority of Tfr cells are thought to be thymus-derived natural Treg populations, while some of them can also derive from $Foxp3^-$ precursors (52). We attempted to generate $CXCR5^+Foxp3^+$ T cells from naive $CD4^+$ T cells in the presence of calcitriol by modifying the concentrations of IL-2, IL-6, TGF-β, and mAbs to IL-2 and IL-2 receptors and by adding retinoic acid to our *in vitro* culture system. Our preliminary results, however, indicated that *Foxp3* expression was reciprocal to *Cxcr5* and *Bcl6* expression, mainly depending on the IL-2 signal levels even in the presence of calcitriol. Further studies are required to determine if calcitriol contributes to the induction of Tfr-like cells.

In summary, calcitriol markedly enhanced CXCR5 expression during the development of Tfh-like cells from naive $CD4^+$ T cells *in vitro*. Tfh-like cells generated with calcitriol were capable of chemotaxis toward CXCL13, producing IL-21, and promoting B cell production of IgG Abs more efficiently than those generated without calcitriol. Injections of calcitriol into antigen-primed mice upregulated the proportion of follicular T cells. Calcitriol is likely to contribute to Tfh cell development, especially through regulating their CXCR5 expression. The precise role of calcitriol in Ab responses through regulation of Tfh cell differentiation and the possible development of Tfr cells *in vivo* requires further investigation.

## Acknowledgments

We thank Y. Totani for helpful advice regarding the ImageJ analysis.

## Supplementary data

Supplementary data are available at *International Immunology* Online.

*Conflict of interest statement*. None declared.


## Funding

This work was partially supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (19K07071 to M.I. and E.I.).


## Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Author contributions

M.I. designed the study, supervised the experiments, and wrote the article. E.I. supervised the study and provided constructive discussion and support. S.-Y.S. provided constructive advice and technical support. M.I., A.T., R.S., and S.-Y.S. performed the experiments.

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