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1,25-Dihydroxyvitamin D₃ Induces CCR10 Expression in Terminally Differentiating Human B Cells

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In the B cell lineage, CCR10 is known to be selectively expressed by plasma cells, especially those secreting IgA. In this study, we examined the regulation of CCR10 expression in terminally differentiating human B cells. As reported previously, IL-21 efficiently induced the differentiation of activated human CD19⁺ B cells into IgD⁻/CD38⁺ plasma cells in vitro. A minor proportion of the resulting CD19⁺ IgD⁻/CD38⁺ cells expressed CCR10 at low levels. 1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃), the active metabolite of vitamin D₃, dramatically increased the proportion of CD19⁺ IgD⁻/CD38⁺ cells expressing high levels of CCR10. The 1,25-(OH)₂D₃ also increased the number of CCR10⁺ cells expressing surface IgA, although the majority of CCR10⁺ cells remained negative for surface IgA. Thus, 1,25-(OH)₂D₃ alone may not be sufficient for the induction of IgA expression in terminally differentiating human B cells.

To further determine whether 1,25-(OH)₂D₃ directly induces CCR10 expression in terminally differentiating B cells, we next performed the analysis on the human CCR10 promoter. We identified a proximal Ets-1 site and 1,25-(OH)₂D₃-activated vitamin D receptor to the respective sites. In conclusion, 1,25-(OH)₂D₃ efficiently induces CCR10 expression in terminally differentiating human B cells in vitro. Furthermore, the human CCR10 promoter is cooperatively activated by Ets-1 and vitamin D receptor in the presence of 1,25-(OH)₂D₃. The Journal of Immunology, 2008, 180: 2786–2795.

Plasma cells represent the terminal stage of B cell differentiation and secrete large amounts of Ab. The transcription factors Blimp-1 and XBP-1 play essential roles in the plasma cell differentiation and function (1). Plasma cells exhibit preferential tissue localization in accordance with the route of immunization and the isotypes of Ig secreted. IgG-secreting plasma cells are mainly induced by a systemic route of immunization and preferentially localize in the bone marrow. In contrast, IgA-secreting plasma cells are predominantly induced by a mucosal route of immunization and preferentially localize in the mucosal tissues (2). Differential expression of tissue-specific adhesion molecules has been demonstrated in Ab-secreting cells (ASCs) depending on the route of immunization (3). Recently, chemokines and their receptors have been attracting much attention as the regulators of migration and tissue localization of lymphocytic cells (2, 4). As for plasma cells, CXCR4 has been demonstrated to guide the movement of plasma cells to splenic red pulp, lymph node medullary cords, and bone marrow, where its ligand CXCL12 is abundantly produced (5). CXCR3 mobilizes IgG-secreting plasma cells to inflammatory sites, where its ligands CXCL9, CXCL10, and/or CXCL11 are strongly up-regulated (6–8). CCR9 contributes to the localization of IgA-secreting plasma cells to the small intestine (9–11), where its ligand CCL25 is selectively produced by the intestinal epithelial cells (11–13).

CCR10 was originally identified as the receptor for CCL27 (14), which is selectively expressed by epidermal keratinocytes (15, 16). Accordingly, CCR10 has been shown to be expressed by skin-homing effector/memory T cells expressing cutaneous lymphocyte Ag (14, 17, 18). Subsequently, another ligand of CCR10 was identified and termed CCL28, which is widely expressed by the epithelial cells of various mucosal tissues (11, 19–21). Thus, certain types of CCR10-expressing cells must be present in the mucosal tissues. Previously, we have demonstrated that EBV-immortalized human B cells express CCR10, although CCR10 is not inducible by EBV-encoded latent genes (22). This was rather unexpected because the expression of CCR10 had not been described at any of the developmental or differentiation stages of B cells examined to date (23). Because EBV-immortalized B cells resemble plasma cells in terms of the continuous production of Igs (24), we hypothesized that CCR10 might be selectively expressed at the terminal differentiation stage of B cells. We have indeed demonstrated that a substantial fraction of plasma cells derived from human bone marrow expresses CCR10 and efficiently migrates to its ligands CCL27 and CCL28 (25). Furthermore, Butcher and his colleagues (26, 27) have demonstrated that IgA-ASCs present in various mucosal tissues commonly express CCR10. We have also demonstrated that CCR10 plays an important role in the homing of IgA-ASCs into the small intestine and colon (11). Consequently, it...
is now considered that the CCL28-CCR10 system comprises an important element in the common mucosal immune system by promoting the wide distribution of locally induced IgA-ASCs to various mucosal tissues in the body (2). However, the regulatory mechanism of CCR10 expression in terminally differentiating B cells has not been determined yet.

The 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) is the biologically active metabolite of vitamin D₃, which binds and activates the nuclear vitamin D receptor (VDR). The activated VDR dimerizes with another nuclear receptor, one of the retinoid X receptors, and the heterodimer binds with high affinity to vitamin D response elements (VDREs) in the promoter region of target genes (28). Besides the role in calcium homeostasis, 1,25-(OH)₂D₃ is also known to exert potent immunomodulatory activities (29–31). For example, 1,25-(OH)₂D₃ suppresses the production of Th1 cytokines such as IFN-γ and IL-2, and consequently leads to the enhanced production of Th2 cytokines such as IL-4 and IL-5, thus potentially promoting humoral immune responses (29, 30). The 1,25-(OH)₂D₃ also promotes innate immunity by directly inducing the gene expression of antimicrobial peptides cathelicidin and β-defensin 2 in various human cell types (32, 33). For example, 1,25-(OH)₂D₃ is involved in the enhanced killing of intracellular Mycobacterium tuberculosis by TLR-triggered human macrophages via the induction of cathelicidin (33). Moreover, if supplied as an adjuvant supplement in systemic immunization, 1,25-(OH)₂D₃ has been shown to promote common mucosal immune responses, as evidenced by the increased mucosal secretion of IgA and IgG Abs (29, 34). Recently, Sigmundsdottir et al. (35) have demonstrated that 1,25-(OH)₂D₃ efficiently induces CCR10 in terminally differentiating B cells and thus enable them to migrate to various mucosal tissues via CCL27, which is widely produced by the mucosal epithelial cells (11, 19–21). In the present study, we have demonstrated that 1,25-(OH)₂D₃ efficiently induces CCR10 in terminally differentiating human B cells in vitro. Furthermore, we have revealed that the human CCR10 promoter is directly activated by Ets-1 and VDR in the presence of 1,25-(OH)₂D₃.

Materials and Methods

Cell lines

BJAB and Ramos (EBV-negative Burkitt’s lymphoma cell lines), Daudi and Raji (EBV-positive Burkitt’s lymphoma cell lines), and BCL-5M and BCL-TOS (EBV-immortalized B cell lymphoblastic cell lines (LCLs)) were described previously (22). BALL-1 (a human B acute lymphoblastic leukemia cell line) was obtained from the Health Science Research Resources Bank. All of these cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acid, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-ME. Human myeloma cell lines KMS-12BM and KMS-12PE were obtained from the Health Science Research Resources Bank. Human myeloma cell lines L-363, OPM-2, AMO-1, SK-MM-2, and KARPAS-620 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen. All of the myeloma cell lines were cultured in RPMI 1640 supplemented with 15% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Human embryonic kidney (HEK) 293T cells were cultured in DMEM supplemented with 10% FCS, 1% nonessential amino acids, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Induction of human B cell differentiation

Human rIL-21 was purchased from Invitrogen Life Technologies. Anti-CD40/TNF-F5/5 (82111) and anti-IgM F(ab)² were purchased from R&D Systems and Jackson ImmunoResearch Laboratories, respectively. The 1,25-(OH)₂D₃ was purchased from Cayman Chemical. All-trans retinoic acid (RA) was purchased from Sigma-Aldrich. 22-Oxa-1,25-(OH)₂D₃ (OCT), an analog of 1,25-(OH)₂D₃ and a VDR agonist, was provided by Chugai Pharmaceutical. TEI-9647, a VDR antagonist, was provided by Teijin Institute for Bio-Medical Research. Unusual transfusion blood samples were provided by Osaka Red Cross Hospital. The use of the blood samples was approved by the ethical committee of Kinki University School of Medicine. PBMC were prepared using Ficoll-Paque. CD19⁺ B cell lines were purified from PBMC by negative selection using BD IMag Human B Lymphocyte Enrichment Set-DM and BD IMagnet (BD Biosciences). The preparations were typically >90% CD19⁺ B cells. Induction of B cell differentiation into plasma cells was performed essentially as described previously (36). Briefly, purified CD19⁺ B cells were cultured in the presence of anti-IgM F(ab)², at 5 µg/ml, anti-CD40 at 1 µg/ml, and IL-2 at 100 ng/ml for 6 days. On day 3, 1,25-(OH)₂D₃, OCT, TEI-9647, and RA were added to the cultures, as indicated.

Flow cytometry

FITC-labeled anti-CD38 (HT2Z), PE-labeled anti-IgD (I6-A2–6), and PerCP-Cy5.5-labeled anti-CD19 (SI25C1) were purchased from BD Biosciences. PE-labeled anti-IgA was purchased from Southern Biotechnology Associates. PerCP-Cy5.5-labeled control mouse IgG1 was purchased from BD Biosciences. Other labeled isotype-matched control mouse Abs were purchased from Beckman Coulter. Allopolyphycocyanin-conjugated anti-CCR10 (314305) and allopolyphycocyanin-conjugated rat IgG2a isotype control were purchased from R&D Systems. Cells were suspended in ice-cold PBS containing 2% FCS and 0.05% sodium azide (staining buffer) and treated with normal human serum for 20 min to block FcRs. Cells were incubated for 30 min with a mixture of FITC-CD38, PE-IgD, PerCP-Cy5.5-CD19, and allopolyphycocyanin-CCR10, at a minimum of appropriate isotype-matched control IgGs. After washing, the cells were immediately analyzed on FACS caliber (BD Biosciences) with appropriate gates and quantified with respect to the isotype-matched control IgGs.

Chemotaxis assay

Assays were performed using a 96-well ChemoTx chamber (NeuroProbe) with 8-µm pores, as described previously (25). Cells were suspended in RPMI 1640 containing 10% FCS and 10 mM HEPES (chemotaxis medium), and placed over the lower wells containing chemotaxis medium only or CCL27 (R&D Systems). After 4 h at 37°C, input cells and cells in the lower wells were stained with FITC-labeled anti-CD38 and PC5-labeled control mouse IgG1. After washing, the cells were immediately analyzed on FACS caliber with appropriate gates and quantified with respect to the isotype-matched control IgGs.

RT-PCR

Template cDNAs were generated from total RNAs from various human B cell lines using TRIzol reagent, as described previously (22). From the cultured B cells, the dead cells were first removed by using MACS Dead Cell Removal Kit (Miltenyi Biotec), and template cDNAs were generated using Cells-to-DNA Kit (Applied Biosystems). For semiquantitative RT-PCR, template cDNAs equivalent to 20 ng of total RNAs were amplified in a solution of 20 µl containing 10 pmol each primer, 0.2 µM dNTP, and 1 U of Ex-Taq polymerase (Takara Bio). The amplification conditions consisted of denaturation at 94°C for 30 s (5 min for the first cycle), annealing at 60°C for 30 s, and extension at 72°C for 30 s (5 min for the last cycle) for 36 cycles for CCR10, VDR, Blimp-1, and BCL-6; for 33 cycles for XBP-1; 32 cycles (B cell lines) or 36 cycles (myeloma cell lines) for Ets-1; 33 cycles for CCR10, VDR, Blimp-1, and BCL-6; 32 cycles for XBP-1; 31 cycles for OCT; 27 cycles for OCT; 26 cycles for CCR10, VDR, OCT; 24 cycles for OCT; 23 cycles for CCR10, VDR, OCT; 22 cycles for OCT; 21 cycles for CCR10, VDR, OCT; 20 cycles for OCT; 19 cycles for CCR10, VDR, OCT; 18 cycles for OCT; 17 cycles for CCR10, VDR, OCT; 16 cycles for OCT; 15 cycles for CCR10, VDR, OCT; 14 cycles for OCT; 13 cycles for CCR10, VDR, OCT; 12 cycles for OCT; 11 cycles for CCR10, VDR, OCT; 10 cycles for OCT; 9 cycles for CCR10, VDR, OCT; 8 cycles for OCT; 7 cycles for CCR10, VDR, OCT; 6 cycles for OCT; 5 cycles for CCR10, VDR, OCT; 4 cycles for OCT; 3 cycles for CCR10, VDR, OCT; 2 cycles for CCR10, VDR, OCT; 1 cycle for CCR10, VDR, OCT; 0 cycles for CCR10, VDR, OCT. The primers used for RT-PCR were as follows: +5'-GGTTAATGAGATGGTACCA CCCAGC for Ets-1; +5'-AGTGGCCATCTGCTGATGCCTC-3' and -5'-GC ACCGCACAGGCTGTCTCA-3' for VDR; +5'-TGCTGATATCGCC GATCTACTG-3' and -5'-TCTAGTGGCGAAGCTCTGTTGCT-3' for CCR10; +5'-GAATGGAATACATACAAAGGG-3' and -5'-CATT TTGCTTCGCTGGTGCT-3' for Blimp-1; +5'-CAAGAAAGTTCT AGGAAGCGCCCG-3' and -5'-GATTGTCACACTAAGTTGAT TT-3' for BCL-6; +5'-CCTTTGTAGTTGGAACCCAG-3' and -5'-GG CCTGTTTATATGTTGG-3' for XBP-1; +5'-GCAAGGTTCTCCG TGCACATTGG-3' and -5'-GCTGGTCCACCCCTCTTTTGG TC-3' for GAPDH. Real-time PCR was performed on 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Gene Expression Assays for CCR10, VDR, and GAPDH. The PCR conditions were essentially as described previously (11). Gene expression was quantified by using Sequence Detection System Software (Applied Biosystems).
Promoter-reporter plasmids and transcription factor expression vectors

The proximal CCR10 promoter region (from −1 to −1500 bp upstream from the start codon) was amplified by nested PCR from human genomic DNA using Pyrobest DNA polymerase (Takara Bio) and the following primers: 5′-ACCACCGAGGTTGACATCATC-3′ and 5′-ACCTGTGGCTGGCAGCG-3′ for the first PCR, and 5′-TTCGAGCTCTACAGGTTGTTGAC-3′ for the second PCR. The amplified fragments were digested at the SalI/HindIII sites of pGL3-basic luciferase reporter plasmid (Promega) to generate pGL3-CCR10 (−1500/+1). Serially 5′-truncated promoter fragments (−976/+1, −694/+1, −514/+1, −348/+1, −212/+1, −171/+1, −137/+1, −126/+1, −110/+1, −56/+1) were also PCR amplified and inserted into the XhoI/HindIII sites of pGL3-basic. Site-directed mutagenesis was performed by using the overlap PCR method (37). The plasmids with mutated sequences were as follows: pGL3-CCR10 (−171/Ets-1/−1), from ACAGGAGGA to ACACGGAGGA; pGL3-CCR10 (−171/Karos/−1), from GAGGGCGGAG to GAAGTGGGAAAGT in GAAT GCCTAAGT; pGL3-CCR10 (−171/VDR/−1), from GGGCTCATCAGG to GTGATCTAAGGGCTsql. An Ets-1 expression vector (pcDNA-Ets-1) was provided by M. Ouchida (Okayama University, Graduate School of Medicine, Okayama, Japan). VDR was amplified from the cDNA of 1,25-(OH)2D3-treated KARPAS-620 cells using the 5′-TTT GATATCCCTTCAGGAGTTGGAAGCT-3′ and 5′-TTTTCCTCCAGGACGGTGTCCTGCTAGGAG-3′ primers: 5′-TTTTCCTCCAGGACGGTGTCCTGCTAGGAG-3′, digested at the EcoRV/XhoI sites (underlined sequences), and cloned into pcDNA3 to generate pcDNA3-VDR.

Luciferase reporter assay

Transient transfection was performed using DMRIE-C transfection reagent (Invitrogen Life Technologies), following the manufacturer’s protocol. KARPAS-620 cells (3–4×104) and HEK293T cells (1–2×104) were cotransfected with 1.5 μg of pSV-IgA and/or 1.5 μg of pGL3-CCR10 (−171/VDR/−1), from GGGCTCATCAGG to GTGATCTAAGGGCTsql. The cells were treated with either medium only or with 100 nM 1,25-(OH)2D3 for 24 h by using NucBuster Protein Extraction Kit (EMD Biosciences), which is an ELISA-like colorimetric assay alternative to the bioluminescence assay. In brief, nuclear extracts were prepared from cells treated with 1,25-(OH)2D3, however, the frequencies of CCR10 expression in IL-21-induced CD19+ IgD−CD38+ cells was observed after 6 days of culturing (25–40% from 6 donors) (Fig. 1A). We confirmed that IL-21 was essential for the efficient induction of these cells (data not shown). When added to the cultures on day 3, 1,25-(OH)2D3 further increased the percentage of IgD−CD38+ cells (40–65% from 6 donors) and also, as reported previously (38), up-regulated the levels of CD38 expression (mean ± SD of mean fluorescence intensity from 6 donors: 1463 ± 183 with 1,25-(OH)2D3 vs 768 ± 102 without 1,25-(OH)2D3, p < 0.001) (Fig. 1A). We then examined CCR10 expression in the resulting CD19+ IgD−CD38+ cells. As shown in Fig. 1B, a minor proportion of cells in the CD19+IgD−CD38+ cells spontaneously expressed CCR10 (8–18% from 6 donors), although their surface CCR10 levels were relatively low (see below). 1,25-(OH)2D3 dramatically increased the proportion of CCR10+ cells in the CD19+IgD−CD38+ cells in a dose-dependent manner (34–52% from 6 donors at 100 nM). The surface levels of CCR10 expression were also greatly enhanced by 1,25-(OH)2D3 (mean ± SD of mean fluorescence intensity from 6 donors: 524 ± 158 at 100 nM vs 86 ± 30 at 0 nM, p < 0.001). At 1000 nM 1,25-(OH)2D3, however, the frequencies of CCR10+ cells were similarly modest to those obtained at 100 nM (data not shown). To further confirm that the observed up-regulation in CCR10 expression was truly mediated by 1,25-(OH)2D3, we also examined the effects of OCT (1,25-(OH)2D3 analog) and TGF-β (a VDR antagonist) on CCR10 expression in IL-21-induced CD19+ IgD−CD38+ cells. As shown in Fig. 1C, OCT increased the number of CCR10+ cells as efficiently as 1,25-(OH)2D3 did, whereas TGF-β effectively suppressed 1,25-(OH)2D3- and OCT-induced increases in CCR10+ cells. By real-time PCR, we further verified the strong increases in CCR10 mRNA in the B cell cultures treated with 1,25-(OH)2D3 (Fig. 1D). Furthermore, as shown in Fig. 1E, 1,25-(OH)2D3-treated CD19+IgD−CD38+ cells exhibited much enhanced chemotactic responses toward CCL27, the CCR10-specific chemokine ligand (14). Collectively, these results clearly indicate that 1,25-(OH)2D3 is a potent inducer of CCR10 expression in IL-21-induced terminally differentiating human B cells.

Recently, Mora et al. (39) have reported that dendritic cells (DCs) derived from mucosal tissues, but not those derived from nonmucosal tissues, strongly induce IgA secretion and CCR9 expression in activated B cells. All-trans retinoic acid (RA) is partly responsible for the specific function of mucosal tissue DCs. Furthermore, B cells treated with RA and cocultured with DCs migrated to CCL25, the CCR9 ligand (4), but not to CCL28, the CCR10 ligand (19, 20). The latter observation led the authors to conclude that CCR10 expression was not induced by RA (39). However, this does not formally exclude the induction, to a certain extent, of CCR10 expression in terminally differentiating B cells by RA. We therefore examined the effect of RA on the surface expression of CCR10 in IL-21-induced CD19+CD38+ cells. As shown in Fig. 1F, RA at 100 nM, but not at 10 nM (data not shown), slightly increased the number of CCR10+ cells in the resulting CD19+IgD−CD38+ cells; however, its effect was much weaker than that of 1,25-(OH)2D3. In combination, RA at 100 nM, but not at 10 nM (data not shown), also enhanced the 1,25-(OH)2D3-induced increases in the number of CCR10+ cells in the CD19+IgD−CD38+ cells. This is probably because RA inhibits
FIGURE 1. Effects of 1,25-(OH)2D3 on CCR10 expression in terminally differentiating human B cells. A, IL-21-induced terminal differentiation of activated B cells. CD19+ B cells were prepared from PBMCs obtained from healthy donors (n = 6) by negative selection (purity, >90%). An aliquot of cells was analyzed for the surface expression of IgD and CD38 (left). The remaining cells were cultured in the presence of anti-IgM (5 μg/ml), anti-CD40 (1 μg/ml), and IL-21 (100 ng/ml) for 6 days. 1,25-(OH)2D3 was added at a concentration of 100 nM on day 3. The cells were stained for CD19, IgD, and CD38 (right). CD19+ IgD− CD38+ cells represent plasma cells. The representative results obtained from six donors are shown.

B, Induction of CCR10 by 1,25-(OH)2D3. CD19+ B cells were cultured as described in A. 1,25-(OH)2D3 was added on day 3, as indicated. The cells were stained for CD19, IgD, CD38, and CCR10. The representative results obtained from three donors are shown.

C, Effects of OCT (an analog of 1,25-(OH)2D3) and TEI-9647 (a VDR antagonist) on surface expression of CCR10. CD19+ B cells were cultured as described in A. 1,25-(OH)2D3 (10 nM), OCT (10 nM), and TEI-9647 (1 μM) were added on day 3, as indicated. Data represent mean ± SEM from three cultures. The representative results from three donors are shown.

D, Real-time PCR for CCR10. CD19+ B cells were cultured as described in A. 1,25-(OH)2D3 was added at a concentration of 100 nM on day 3. Data represent mean ± SEM from three cultures. The representative results from three donors are shown.

E, Chemotactic response of CD19+CD38+ cells to CCL27. CD19+ B cells were cultured as described in A. 1,25-(OH)2D3 was added at a concentration of 100 nM on day 3. Chemotaxis assays
the 1,25-(OH)\textsubscript{2}D\textsubscript{3}-mediated feedback induction of CYP24A1, which encodes 1,25-(OH)\textsubscript{2}D\textsubscript{3}-24-hydroxylase, the enzyme that inactivates 1,25-(OH)\textsubscript{2}D\textsubscript{3} (data not shown) (40). In contrast, we observed no significant increase in the number of CCR9\textsuperscript{+} cells in IL-21-induced CD19\textsuperscript{+} IgD\textsuperscript{+} CD38\textsuperscript{+} cells upon treatment with RA or 1,25-(OH)\textsubscript{2}D\textsubscript{3}, alone or in combination (data not shown). As reported by Mora et al. (39), RA may require the presence of DCs for its efficient induction of CCR9 in activated B cells.

Next, we investigated whether CCR10 expression was coregulated with IgA expression in terminally differentiating B cells. As shown in Fig. 1G, 1,25-(OH)\textsubscript{2}D\textsubscript{3} did not significantly increase the IgA content in the culture supernatants of IL-21-induced CD19\textsuperscript{+} IgD\textsuperscript{+} CD38\textsuperscript{+} cells. However, the potent growth-inhibitory effect of 1,25-(OH)\textsubscript{2}D\textsubscript{3} might paradoxically reduce the total amount of IgA produced by IL-21-induced CD19\textsuperscript{+} IgD\textsuperscript{+} CD38\textsuperscript{+} cells as a whole (41, 42). We therefore also examined surface IgA expression in the resulting CD19\textsuperscript{+} CD38\textsuperscript{+} cells. Although surface IgA\textsuperscript{+} cells were detected only at low levels in IL-21-induced CD19\textsuperscript{+} CD38\textsuperscript{+} cells, their levels were significantly increased upon treatment with 1,25-(OH)\textsubscript{2}D\textsubscript{3} (Fig. 1H). Furthermore, the increase in the number of surface IgA\textsuperscript{+} cells by 1,25-(OH)\textsubscript{2}D\textsubscript{3} was more pronounced in the CCR10\textsuperscript{+} fraction than in the CCR10\textsuperscript{−} fraction (mean percentage ± SD from 3 donors: IgA CCR10\textsuperscript{+} cells, from 2.0 ± 1.1 to 3.8 ± 1.9; IgA CCR10\textsuperscript{−}, from 0.7 ± 0.6 to 6.5 ± 0.3). Nevertheless, the majority of CCR10\textsuperscript{+} cells remained negative for surface IgA. Thus, 1,25-(OH)\textsubscript{2}D\textsubscript{3} alone may not be sufficient in inducing IgA expression in terminally differentiating human B cells. In contrast, RA had no significant effect on surface IgA expression in IL-21-induced CD19\textsuperscript{+} CD38\textsuperscript{+} cells, including the CCR10\textsuperscript{+} fraction (data not shown). This may again be due to its requirement of DCs for the efficient induction of IgA expression in activated B cells (39).

Identification of the major regulatory elements in the human CCR10 promoter

Next, we examined the effect of 1,25-(OH)\textsubscript{2}D\textsubscript{3} on CCR10 expression in a human myeloma cell line KARPAS-620. As shown in Fig. 2A, 1,25-(OH)\textsubscript{2}D\textsubscript{3} up-regulated CCR10 expression in KARPAS-620 cells. 1,25-(OH)\textsubscript{2}D\textsubscript{3} also up-regulated VDR expression, as reported previously (43). By real-time PCR, it was determined that 1,25-(OH)\textsubscript{2}D\textsubscript{3} up-regulated the levels of CCR10 mRNA and VDR mRNA in KARPAS-620 cells several folds (Fig. 2B). 1,25-(OH)\textsubscript{2}D\textsubscript{3} also up-regulated the surface expression of CCR10 in KARPAS-620 cells (Fig. 2C). We also confirmed that OCT (an analog of 1,25-(OH)\textsubscript{2}D\textsubscript{3}) increased the surface expression of CCR10, whereas TEI-9647 (a VDR antagonist) suppressed the up-regulation of surface CCR10 induced by 1,25-(OH)\textsubscript{2}D\textsubscript{3} or OCT. These results were highly concomitant with those obtained from IL-21-induced normal CD19\textsuperscript{+} CD38\textsuperscript{+} cells (Fig. 1). Thus, KARPAS-620 cell line can be used as a model for the analysis of the transcriptional regulation of CCR10 expression in terminally differentiating human B cells.

We transfected a luciferase reporter plasmid pGL3 or pGL3-CCR10 (−1500/−1), which was inserted with the human CCR10 promoter fragment from −1500 to −1 bp, into KARPAS-620 cells and a human embryonic kidney cell line HEK293T. As shown in Fig. 3A, compared with the control pGL3, pGL3-CCR10 (−1500/−1) showed a high basal promoter activity only in KARPAS-620 cells.
cells. Furthermore, 1,25-(OH)2D3 strongly up-regulated the promoter activity of pGL3-CCR10 (−1500/−1) in KARPAS-620 cells, but not in HEK293T cells. Thus, the CCR10 promoter fragment from −1500 to −1 bp was proven to be specifically active and responsive to 1,25-(OH)2D3 only in KARPAS-620 cells.

As shown in Fig. 3B (left), the on-line program TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html) revealed potential binding sites for various transcription factors in the CCR10 promoter region from −1500 to −1 bp. To identify the actual regulatory elements in the CCR10 promoter, we next generated a series of pGL3 reporter plasmids carrying progressively 5′-truncated promoter fragments and transfected them to KARPAS-620 cells. As shown in Fig. 3C, we observed dramatic increases in basal promoter activity and induction by 1,25-(OH)2D3 with the promoter fragment from −126 to −1 bp in comparison with that from −137 to −1 bp, whereas the induction by 1,25-(OH)2D3 was much reduced with the promoter fragment from −110 to −1 bp, and there was essentially no promoter activity with the promoter fragment from −56 to −1 bp. These results suggested the presence of the major regulatory elements to lie within the promoter region between −171 and −56 bp; a possible negative regulatory element within −137 to −126 bp; a major VDRE within −126 to −110 bp; and a critical promoter element within −110 to −56 bp. As shown in Fig. 3B (left), TFSEARCH revealed an Ikaros site to lie within −136 to −125 bp and an Ets-1 site within −71 to −62 bp; however, no potential VDRE was found to lie within −126 to
To prove the specific binding of Ets-1 and 1,25-(OH)\(_2\)D\(_3\)-activated VDR to the respective elements in the human CCR10 promoter,

we used the NoShift transcription factor assay, which is an ELISA-like colorimetric assay alternative to the EMSA. As shown in Fig. 4A, the specific binding of Ets-1 to the Ets-1 element was demonstrated by using the nuclear extracts from CCR10-expressing human myeloma cell lines KARPAS-620 and KMS-12BM. Furthermore, as shown in Fig. 4B, the specific binding of VDR to the putative VDRE was also confirmed by using the nuclear extracts from these myeloma cell lines treated with 1,25-(OH)\(_2\)D\(_3\).

Specific binding of Ets-1 and 1,25-(OH)\(_2\)D\(_3\)-activated VDR to the respective elements in the CCR10 promoter

To prove the specific binding of Ets-1 and 1,25-(OH)\(_2\)D\(_3\)-activated VDR to the respective elements in the human CCR10 promoter,
differentiation of human B cells in vitro (36), we have demonstrated that 1,25-(OH)2D3 robustly induces CCR10 expression in terminally differentiating human B cells (Fig. 1). Furthermore, we have demonstrated that Ets-1 and 1,25-(OH)2D3-activated VDR cooperatively activate the CCR10 promoter via an Ets-1 site at −71 to −62 bp and a major VDRE at −124 to −110 bp (Fig. 3). We have also demonstrated that CCR10-expressing human myeloma cell lines and EBV-LCLs consistently express both Ets-1 and VDR at high levels (Fig. 5). Previous studies have demonstrated that 1,25-(OH)2D3, if supplied as an adjuvant supplement, promotes common mucosal immune responses even upon systemic immunization (29, 34). Based on the present results, 1,25-(OH)2D3 may promote mucosal immunity partly by inducing CCR10 expression in terminally differentiating B cells. However, we observed only a marginal increase in IgA expression in terminally differentiating human B cells by 1,25-(OH)2D3 (Fig. 1). Therefore, 1,25-(OH)2D3 alone may not be sufficient to promote mucosal IgA responses in vivo. Furthermore, Chen et al. (42) have recently reported that 1,25-(OH)2D3 inhibits the ongoing proliferation of activated B cells by inducing their apoptosis, and thereby significantly inhibits the generation of plasma cells and memory B cells in vitro, although the up-regulation of the genetic programs involved in B cell differentiation was not strongly affected. Therefore, the effect of 1,25-(OH)2D3 on the terminal differentiation of B cells may be critically dependent on the timing of their exposure to 1,25-(OH)2D3.

Vitamin D3 (cholecalciferol) is generated in the skin from 7-dehydrocholesterol in response to sun exposure (48). Vitamin D3 is then transported to the liver and converted to 25-hydroxyvitamin D3, which is the main circulating vitamin D3 metabolite (48). The key enzyme 25-hydroxyvitamin D3 1α-hydroxylase, which is encoded by CYP27B1, catalyzes the synthesis of the active form 1,25-(OH)2D3 from 25-hydroxyvitamin D3 mainly in the kidney (48). Among the hematopoietic cells, macrophages and mature DCs express CYP27B1 at high levels, whereas B cells express CYP27B1 only at low levels (31, 49). Thus, macrophages and mature DCs, especially those in mucosal tissues, may produce 1,25-(OH)2D3 to induce CCR10 expression in terminally differentiating B cells in vivo.

Upon activation, B cells are known to up-regulate VDR (47). However, only after a strong activation, such as the concomitant cross-linking of Ig receptor and ligation of CD40, do activated B cells become responsive to 1,25-(OH)2D3 (50). Thus, the elevated expression and function of VDR in terminally differentiating B cells may in part explain the plasma cell stage-specific expression of CCR10. The stage-specific expression of CCR10 may also be regulated by a negative regulatory element(s) in the CCR10 promoter. We have indeed mapped a negative regulatory element in close vicinity to the VDRE (Fig. 3). Sequence analysis of this region has revealed a putative Ikaros site immediately upstream of the VDRE (Fig. 3). The Ikaros family of zinc finger transcription factors (Ikaros, Aiolos, and Helios) is an important regulator of lymphoid development and differentiation (51). Ikaros and Aiolos have been shown to function as strong transcriptional repressors (52). The Ikaros family genes are transcribed as large numbers of isoforms through extensive alternative splicing. Shorter isoforms tend to behave as dominant-negative isoforms upon heterodimerization with longer isoforms and even with the isoforms of other family members (51). Thus, the mechanisms of transcriptional regulation involving the Ikaros family are highly complex. Future studies are necessary to identify the actual transcription factor(s) binding to the putative Ikaros site in the CCR10 promoter. Such studies may provide further insight into the transcriptional regulation of the plasma cell stage-specific gene expression.

It is known that IgA-ASCs in the intestinal tissues frequently express CCR9 and/or CCR10 (26, 27). Recently, Mora et al. (39) have demonstrated that DCs in the gut-associated lymphoid tissues (GALT), but not those in the nonmucosal lymphoid tissues, induce the expression of αββ2 and CCR9 in activated B cells. Moreover, DCs in GALT efficiently induce IgA secretion in activated B cells (39). In this context, DCs in GALT produce RA, the active metabolite of vitamin A, which plays an essential role in the induction of gut tropism and IgA secretion in activated B cells by mucosal DCs (39). However, RA alone is not sufficient and requires the presence of DCs for the observed effects (39). Thus, additional signals via cytokines and/or surface molecules provided by DCs may be required for the induction of gut tropism and IgA secretion by RA (39). In the present study, we have demonstrated that, at high concentrations, RA moderately induces CCR10 expression in terminally differentiating human B cells (Fig. 1). RA also enhances 1,25-(OH)2D3-mediated CCR10 induction in terminally differentiating B cells (Fig. 1). However, we did not observe the induction of CCR9 or β2 integrin in terminally differentiating B cells by RA or 1,25-(OH)2D3 in the present culture conditions (data not shown). This appears to be partly in agreement with the conclusion by Mora et al. (39) that RA requires the presence of DCs for the efficient induction of αββ2 and CCR9 in activated B cells.

The 1,25-(OH)2D3 has been shown to induce the expression of the antimicrobial peptides cathelicidin and β-defensin 2 in various types of human cells (32, 33). Because CCL28, the CCR10 ligand expressed by various mucosal epithelial cells (11, 19–21), is also a chemokine with a potent antimicrobial activity (21), 1,25-(OH)2D3 induces the expression of CCL28 and other antimicrobial peptides in mucosal tissue cells, thus possibly orchestrating both innate and acquired immunity in the mucosal tissues. However, we did not observe 1,25-(OH)2D3 to exert any strong effect on the constitutive expression of CCL28 in two human colon epithelial cell lines T84 and Caco-2 (data not shown), even though both of the cell lines strongly up-regulated the expression of CYP24A1 in response to 1,25-(OH)2D3 (40). Furthermore, we detected CCL28 mRNA in the intestinal tissues of VDR-deficient mice (53) at levels comparable to those of control wild-type mice (data not shown).

Recently, Sigmundsdottir et al. (35) have demonstrated that 1,25-(OH)2D3 induces CCR10 expression in activated human T cells. The authors have suggested the presence of a possible VDRE(s) within the CCR10 promoter region from −96 to −54 bp, by demonstrating the specific binding of the complex of VDR and its heterodimeric partner retinoid X receptor to this region by a gel-shift assay (35). They have also noted that the region is well conserved between humans and mice (35). However, they did not perform the promoter analysis to prove the functional importance of the suggested region for the induction by 1,25-(OH)2D3. In the present study, we have mapped a strong VDRE at −124 to −110 bp in the human CCR10 promoter, which thus locates in the upstream of the region suggested by Sigmundsdottir et al. (35). We also observed a weak up-regulation of the promoter activity by 1,25-(OH)2D3 with the reporter plasmids missing the major VDRE, but carrying the region suggested by Sigmundsdottir et al. (35) (see pGL3-CCR10 (−110/−1) in Fig. 3C and pGL3-CCR10 (−171/VDRE) in Fig. 3D). Therefore, the region suggested by Sigmundsdottir et al. (35) may also contain a weak VDRE.

Unexpectedly, the VDRE that we have identified in the human CCR10 promoter is not present in the mouse CCR10 promoter. In fact, by aligning the human and mouse CCR10 promoter regions from −1500 to −1 bp, there are only three short stretches spanning from −1258 to −996 bp, from −711 to −659 bp, and from −100 to −79 bp.
to ~1 bp that are homologous between the two species (the nuclotide numbers are from the human sequence). Therefore, the major regulatory elements that we have found in the region from -137 to -110 bp are totally missing in the mouse sequence. Furthermore, our preliminary studies demonstrated that 1,25-(OH)2D3 was mostly unable to induce CCR10 expression in B220+CD138+ terminally differentiating mouse B cells generated in vitro by culturing naive B cells with anti-IgM + anti-CD40 + IL-21 (54), anti-IgM + IL-5 + IL-6 (39), or LPS + IL-5 + TGFB (55) (data not shown). Moreover, we detected CCR10 mRNA in the intestinal tissues of VDR-deficient mice (53) at levels comparable to those in control wild-type mice (data not shown). The B220+IgA+ fraction of the mesenteric lymph node obtained from VDR-deficient mice also contained CCR10-expressing cells at levels comparable to those from wild-type mice (data not shown). Notably, Sigmundsdottir et al. (35) also mentioned in their discussion that 1,25-(OH)2D3 induced CCR10 expression in mouse T cells much less efficiently than in human T cells. Therefore, there may be a substantial species difference in the role of 1,25-(OH)2D3 in the CCR10 expression in skin-homing T cells and terminally differentiating B cells between humans and mice. However, it may not be so surprising that humans and nocturnal animals such as mice have evolved differently with regard to their requirement for the sunlight exposure and the resulting 1,25-(OH)2D3.

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Disclosures

The authors have no financial conflict of interest.

References


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