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CXCL12–CXCR4 Axis Is Required for Contact-Mediated Human B Lymphoid and Plasmacytoid Dendritic Cell Differentiation but Not T Lymphoid Generation

Hirohito Minami,*,[1] Keiki Nagaharu,*,[1] Yoshiki Nakamori,*,[1] Kohshi Ohishi,† Naoshi Shimojo,‡ Yuki Kageyama,* Takeshi Matsumoto,† Yuka Sugimoto,* Isao Tawara,* Masahiro Masuya,* Hiroshi Miwa,* and Naoyuki Katayama*

We investigated the involvement of CXCL12–CXCR4 interactions in human lymphohematopoiesis by coculture with telomerized human stromal cells. CXCR4 expression was low in $CD34^+CD38^-CD45RA^-CD10^-CD7^-CD19^-$ immature hematopoietic stem/precursor cells (HSPCs) but higher in $CD34^+CD38^-CD45RA^+CD10^+CD7^{+/-}CD19^-$ early lymphoid precursors and even higher in $CD34^+CD38^+CD45RA^+CD10^+CD7^-CD19^+$ pro-B cells. Inhibition of the effect of stromal cell–produced CXCL12 by an anti-CXCR4–blocking Ab suppressed the generation of $CD45RA^+CD10^-CD7^+CD19^-$ early T lymphoid precursors (ETPs) and $CD45RA^+CD10^+CD7^-CD19^{+/-}$ B lymphoid precursors on stromal cells, but it did not affect the generation of ETPs in conditioned medium of stromal cell cultures. Replating assays showed that contact with stromal cells was critical for HSPC-derived $CD45RA^+CD10^+CD7^-CD19^-$ B lineage–biased precursors to differentiate into $CD19^+$ pro-B cells, which was suppressed by the anti-CXCR4 Ab. Conversely, HSPC-derived ETPs possessed T and B lymphoid and monocytic differentiation potential; stromal cell contact was not required for their growth but rather promoted B lymphoid differentiation. The anti-CXCR4 Ab did not affect the growth of ETPs in conditioned medium, but it suppressed their B lymphoid differentiation on stromal cells. $CD14^-CD11c^-HLA\text{-}DR^+CD123^{high}CD303^+$ plasmacytoid dendritic cells developed from HSPCs and ETPs exclusively in contact with stromal cells, which was suppressed by the anti-CXCR4 Ab. These data indicate that CXCL12 plays an essential role in stromal cell contact–mediated B lymphoid and plasmacytoid dendritic cell differentiation from immature hematopoietic and early T lymphoid precursors with a multilineage differentiation potential, but it does not participate in contact-independent generation of early T lymphoid precursors. ***The Journal of Immunology*****, 2017, 199: 2343–2355.**

The precise location and function of bone marrow stem cell niches have been intensively studied in mice (1–5). Recent studies propose that B lymphopoiesis is supported by specific cellular niches (3, 6, 7), such as CXCL12-abundant reticular cells (8) and osteoblastic cells (9). These studies also suggest that CXCL12 expressed by these niche cells plays a key role in supporting B lymphopoiesis (3, 6, 10).

CXCL12 was cloned from a stromal cell line, the conditioned medium (CM) of which supports B lymphopoiesis, and initially designated as a pre-B cell growth–stimulating factor (11). CXCL12 is now recognized as a pleomorphic chemokine that is critical for various developmental and biological processes, including homing and maintenance of hematopoietic stem cells (3) and retention of B lineage precursors in bone marrow (12). Genetically engineered mice lacking CXCL12 or its receptor CXCR4 revealed that the CXCL12–CXCR4 interaction is essential for the generation of B and NK lineage lymphoid cells, as well as plasmacytoid dendritic cells (pDCs) (10, 13–19), and it is implicated in intrathymic T lymphoid differentiation (16, 20, 21). In contrast, development of B cells was normal in genetically modified mice deficient in the second CXCL12 receptor CXCR7 (22). In humans, high levels of CXCR4 are expressed in early T and B lymphoid precursors (23, 24), and CXCL12–CXCR4 signaling is critical for intrathymic T cell differentiation (25). However, the involvement of the CXCL12–CXCR4 axis in the differentiation of human early T and B lymphoid cells and pDCs in bone marrow remains unknown.

We previously reported that telomerized human bone marrow stromal cells support the differentiation of early T and B lymphoid precursors and pDCs from human immature hematopoietic precursors (26, 27). In the current study, we investigated the role of stromal cell–derived CXCL12 in the differentiation of human T and B lymphoid cells and pDCs using this novel coculture system. Our study revealed that the CXCL12–CXCR4 interaction is crucial for stromal cell contact–mediated early B lymphoid and pDC

*Department of Hematology and Oncology, Mie University Graduate School of Medicine, Tsu, Mie 514-8507, Japan; †Blood Transfusion Service, Mie University Hospital, Tsu, Mie 514-8507, Japan; and ‡Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, Tsu, Mie 514-8507, Japan

[1]H. Minami, K.N., and Y.N. contributed equally to this work.

ORCIDs: 0000-0002-6139-4256 (K.N.); 0000-0001-8136-9907 (K.O.); 0000-0002-5366-6899 (N.S.); 0000-0003-3389-0081 (T.M.).

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Address correspondence and reprint requests to Dr. Kohshi Ohishi, Blood Transfusion Service, Mie University Hospital, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. E-mail address: koishi@clin.medic.mie-u.ac.jp

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Abbreviations used in this article: $CD7^+$ MLP, $CD34^+CD38^-CD45RA^+CD10^+CD7^+CD19^-$ multilymphoid precursor; $CD7^-$ MLP, $CD34^+CD38^-CD45RA^+CD10^+CD7^-CD19^-$ multilymphoid precursor; CM, conditioned medium; DPLP, double-positive lymphoid precursor; EBP, early B lymphoid precursor; ETP, early T lymphoid precursor; Flt3L, Flt3 ligand; 3GF, three growth factor (SCF+Flt3L+TPO); HSPC, hematopoietic stem/precursor cell; pDC, plasmacytoid dendritic cell; SCF, stem cell factor; TPO, thrombopoietin.

differentiation from immature hematopoietic and early T lymphoid precursors with a multilineage differentiation potential but not for stromal cell contact–independent generation of early T lymphoid precursors.

Materials and Methods

Isolation of hematopoietic precursors

Human umbilical cord blood samples were collected from full-term deliveries after obtaining informed consent, according to a protocol approved by the Ethics Committee of Mie University Hospital. Hematopoietic precursors were purified as reported previously with some modifications (26). Briefly, $CD34^+$ cells were separated from mononuclear cells using CD34 immunomagnetic beads (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. $CD34^+$ cells were stained with anti-CD3–FITC, anti-CD14–FITC, anti-CD15–FITC (BioLegend, San Diego, CA), anti-CD19–FITC (BD Biosciences, San Jose, CA), anti-CD10–PE (BioLegend), anti-CD34–PerCP Cy5.5 (BD Biosciences), anti-CD38–PE–Cy7 (BioLegend), anti-CD7–Brilliant Violet 421 (BD Biosciences), anti-CD45RA–Brilliant Violet 510 (BioLegend) Abs, and Zombie NIR (BioLegend), and $CD34^+CD38^-CD45RA^-CD10^-CD7^-CD19^-CD3^-CD14^-CD15^-$ hematopoietic stem/precursor cells (HSPCs) were isolated. Cell sorting was performed with a FACSAria II cell sorter (BD Biosciences), excluding Zombie NIR+ dead cells.

Flow cytometric analysis

Surface immunofluorescence staining was performed as described previously, with some modifications, using the following murine mAbs (26): anti-CD14–FITC (BioLegend), anti-CD45–FITC (BioLegend), anti-HLA-DR–FITC (BD Biosciences), anti-CD10–PE (BioLegend), anti-CD7–PE, anti-CD123 (IL-3Rα-chain)–PE (BD Pharmingen, San Diego, CA), anti-CD19–allophycocyanin (BioLegend), anti-CD303 (BDCA2)-allophycocyanin (Miltenyi Biotec), anti-CXCR7–PE (R&D Systems, Minneapolis, MN), CD14–Alexa Fluor 700 (BioLegend), anti-CD7 Brilliant Violet 421, anti-CD11c Brilliant Violet 421, and anti-CD45RA Brilliant Violet 510 (BioLegend). IgG_1-FITC, IgG_{2a}-FITC, IgG_1-PE (all from BioLegend), IgG_{2a}-PE (BD Pharmingen), IgG_{2b}-PE, IgG1–PerCP–Cy5.5, IgG_1-allophycocyanin, IgG_1–allophycocyanin–Cy7, IgG_1-PECy7, IgG_1–Alexa Fluor 700, IgG_1–Brilliant Violet 421 (all from BioLegend), and $IgG2_b$–Brilliant Violet 510 (BD Biosciences) served as isotype controls. Cells were blocked with FcR Blocking Reagent (Miltenyi Biotec) and then incubated with Abs for 30 min at 4˚C. Dead cells were excluded by staining with 7-aminoactinomycin D (BD Biosciences) or Zombie NIR.

Cytoplasmic staining was performed as described previously (28) with some modifications. Briefly, cells were incubated with various Abs against surface Ags and Zombie NIR for 30 min at 4˚C. After washing, the cells were permeabilized and fixed with PermeaFix (Ortho, Raritan, NJ) for 20 min at room temperature, washed again, and incubated with anti-CXCL12–allophycocyanin (R&D Systems) for 30 min at 4˚C. Dead cells were distinguished by positive staining for Zombie NIR. Flow cytometric analysis was performed using FACSCanto II and BD LSRFortessa flow cytometers (BD Biosciences). All data were analyzed using BD FACSDiva software (BD Biosciences) or Fortessa flow cytometers (BD Biosciences), and processed by FlowJo software (TreeStar, San Carlos, CA).

Immunofluorescence staining

Immunofluorescence staining of osteocalcin and CXCL12 was performed as described elsewhere (29), with some modification. Stromal cells were cultured on uncoated Lab-Tek II Chamber Slides (Nalge Nunc International, Naperville, IL) at 37˚C in a humidified atmosphere with 5% CO_2 for 4 d. The cultured stromal cells were fixed with 4% phosphate-buffered paraformaldehyde (Nacalai Tesque, Kyoto, Japan) for 10 min at room temperature and washed three times with PBS for 5 min. They were treated with 0.5% Triton X-100 (Wako Pure Chemical Industries, Osaka, Japan) in PBS for 1 h, washed three times with PBS for 5 min, and stained with mouse anti-human osteocalcin–Alexa Fluor 488 (AF488; 20 μg/ml; R&D Systems), mouse anti-human CXCL12-PE (50 μg/ml; R&D Systems), mouse IgG_1 (BioLegend), and TO-PRO-3 (2 μM; Molecular Probes, Eugene, OR) at room temperature for 6 h. Images were obtained using an Olympus IX81 FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan) equipped with a 40×/1.30 NA oil-immersion objective lens and processed using Adobe Photoshop CS (Adobe Systems, Mountain View, CA).

Recombinant factors

Recombinant human thrombopoietin (TPO), human stem cell factor (SCF), human Flt3 ligand (Flt3L), and human IL-7 were purchased from PeproTech (Rocky Hill, NJ). All cytokines were used at the following concentrations unless stated otherwise: 10 ng/ml SCF, 10 ng/ml TPO, 5 or 10 ng/ml Flt3L, and 5 ng/ml IL-7.

Cocultures

Coculture was performed as described previously (26). Briefly, human telomerase reverse transcriptase–transduced telomerized stromal cells were purchased from RIKEN BioResource Center (Tsukuba, Japan). Before coculture, stromal cells were plated in a 25-cm^2 cell culture flask (Corning, Corning, NY) or a 48-well tissue culture plate (Nunc, Roskilde, Denmark) with αMEM (Life Technologies-Invitrogen, Grand Island, NY) containing 12.5% horse serum, 12.5% FCS (both from Invitrogen, Carlsbad, CA), and 1×10^{-6} M hydrocortisone (Sigma-Aldrich, St Louis, MO), as described elsewhere (26, 30). On the first day of coculture, confluent stromal cells were washed with αMEM, and isolated HSPCs, $CD45RA^+CD10^-CD7^+$ early T lymphoid precursors (ETPs), or $CD45RA^+CD10^+CD7^-$ early B lymphoid precursors (EBPs) (purity > 95%) (31) were seeded onto the confluent monolayer of stromal cells in αMEM supplemented with 20% FCS (HyClone Laboratories, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin in the presence of three growth factor (SCF+Flt3L+TPO) (3GF), based on our previous study (26). In some experiments, the culture medium of stromal cell monolayers was replaced with αMEM containing 20% FCS, and the culture medium was harvested after 7–10 d, filtered through a 0.22-μm Millex-GV Filter (Millipore, Bedford, MA), and used as CM of stromal cells. An anti-CXCR4–blocking Ab (MAB170; R&D Systems) or isotype mouse IgG2a (BioLegend) was added to the cultures at 8 μg/ml. Half of the medium was exchanged for fresh medium containing the same concentrations of cytokines every 4–5 d. Culture with OP9 cells expressing delta-1 in the presence of Flt3L and IL-7 was performed as reported previously (26, 27). In some experiments, cell culture inserts with pore sizes of 0.4 μm (BD Falcon; BD Biosciences, Franklin Lakes, NJ) were used for contact or noncontact coculture with stromal cells.

Cell division assay

The cell division assay was performed as follows. HSPCs were stained with CytoTell Green (1:250 dilution; AAT Bioquest, Sunnyvale, CA) for 30 min at 37˚C, washed twice, and stained with CD10-PE, CD34-allophycocyanin, CD38–allophycocyanin–Cy7 (BioLegend), CD7–BV-421, and CD45RA-BC510. HSPCs were sorted, cultured on stromal cells or with CM for 3 d, and analyzed for cell division using a FACSCanto II (BD Biosciences).

Cell adhesion assay

$CD34^+$ cells (2×10^5 cells per well) were preincubated with an anti-CXCR4 Ab or isotype control and then cultured on stromal cells with the anti-CXCR4 Ab (8 μg/ml) or isotype control (8 μg/ml), respectively. After 4 h, the cultures were gently pipetted, and the number of attached cells was analyzed by counting the number of detached cells.

Cytokine concentrations

Stromal cells were cultured in 24-well plates. After the stromal cells became confluent, the culture medium was replaced with 0.5 ml of αMEM with 20% FCS, and a portion of the CM was harvested at days 0, 7, and 14. In addition, $CD34^+$ cells (2×10^4 cells per well) were cultured with or without stromal cells, and a portion of CM was similarly harvested. The concentrations of CXCL12 in CM were analyzed using a LEGENDplex Multi-Analyte Flow Assay (BioLegend), according to the manufacturer's recommended method. Data were obtained using an LSRFortessa X-20 (BD Biosciences) and processed by LEGENDplex Data Analysis Software 8.0 (BioLegend). CXCL12 concentrations were also analyzed using a Human CXCL12/CXCL12 alpha Quantikine ELISA Kit (R&D Systems), as per the manufacturer's recommended method. Plates were analyzed using a plate reader (Multiskan JX; Thermo Labsystems, Helsinki, Finland).

Statistics

The Student *t* test was used to assess statistical significance.

Results

Expression of CXCR4 and production of CXCL12

To examine the potential role of CXCL12–CXCR4 interactions in human early lymphopoiesis, we examined the expression of CXCR4 in the early stage of hematopoietic and lymphoid precursors in cord

blood (32). Expression levels of CXCR4 were low in $CD34^+CD38^-CD45RA^-CD10^-CD7^-CD19^-$ HSPCs but were higher in $CD34^+CD38^-CD45RA^+CD10^+CD7^+CD19^-$ multilymphoid precursors ($CD7^+$ MLPs) and $CD34^+CD38^-CD45RA^+CD10^+CD7^-CD19^-$ multilymphoid precursors ($CD7^-$ MLPs) (32) and were further elevated on $CD34^+CD38^+CD45RA^+CD10^+CD7^-CD19^+$ pro-B cells (Fig. 1A). High levels of CXCR4 expression in early lymphoid precursors (24) and pro-B cells (23) in fetal bone marrow have also been observed in other studies. In contrast, little or no CXCR7, a second CXCL12 receptor, was detected in HSPCs and MLPs of cord blood (data not shown), consistent with data from another study (33). Conversely, flow cytometry showed that CXCL12 was expressed on the cell surface at low levels and in cytoplasmic fractions of telomerized stromal cells (Fig. 1B). Immunofluorescence showed that the cytoplasm of stromal cells was stained positively for CXCL12 and osteocalcin, a marker of osteoblast lineage cells (Fig. 1C). The expression of osteocalcin was consistent with prior studies showing that telomerized bone marrow stromal cells consist predominantly of osteogenic cells (34). To examine the production of CXCL12 from stromal cells and hematopoietic precursors, we assessed the concentrations of CXCL12 in cultures containing confluent stromal cells, $CD34^+$ hematopoietic precursors, or $CD34^+$ cells on stromal cells. The concentration of CXCL12 was increased and reached a plateau at around day 7. Little or no CXCL12 was detected by culture of $CD34^+$ hematopoietic precursors alone, and the concentration of CXCL12 was not increased by coculture of hematopoietic precursors on stromal cells (Supplemental Fig. 1). The concentrations of CXCL12 (3.5–32.5 ng/ml) in CM of the cultures containing confluent stromal cells were higher than the physiological concentration of CXCL12 in human plasma (~0.6 ng/ml) (35, 36). These data suggest that the CXCL12–CXCR4 axis might play a role in human early lymphoid differentiation supported by stromal cells.

Effect of an anti-CXCR4 Ab on the generation of lymphoid precursors from immature hematopoietic and early lymphoid precursors

To elucidate the potential role of the CXCL12–CXCR4 interaction in early lymphoid differentiation on stromal cells, HSPCs, $CD7^+$ MLPs, or $CD7^-$ MLPs were isolated from cord blood and cultured on stromal cells with 3GF in the presence of an anti-CXCR4–blocking Ab or isotype control. In the control cultures, $CD45RA^+CD14^-$ cells, including $CD45RA^+CD10^-CD7^+$ ETPs (31), $CD45RA^+CD10^+CD7^+$ early double-positive lymphoid precursors (DPLPs), and $CD45RA^+CD10^+CD7^-$ early B lymphoid precursors (EBPs), as well as $CD14^+$ monocytic cells, were generated from HSPCs, $CD7^+$ MLPs, and $CD7^-$ MLPs. Compared with HSPCs, $CD7^+$ and $CD7^-$ MLPs tended to differentiate further into $CD45RA^+CD10^+CD19^+$ pro-B cells (Fig. 2Ai, 2Aiii, 2Av). Addition of the anti-CXCR4 Ab to the cultures inhibited the generation of $CD45RA^+CD14^-$ cells, ETPs, DPLPs, EBPs, and pro-B cells from HSPCs, $CD7^+$ MLPs, and $CD7^-$ MLPs. The suppressive effect exerted by the anti-CXCR4 Ab was more apparent in cultures with $CD7^+$ MLPs or $CD7^-$ MLPs than with HSPCs, because few or no $CD45RA^+CD14^-$ cells were detected in cultures of MLPs with the anti-CXCR4 Ab. The generation of pro-B cells was almost completely suppressed by the anti-CXCR4 Ab, whereas the development of $CD14^+$ monocytic cells was not significantly affected (Fig. 2A).

We further investigated how lymphoid differentiation changes depend on the state of cell attachment to stromal cells and how the anti-CXCR4 Ab influences lymphoid differentiation. To examine the effect of the anti-CXCR4 Ab on lymphoid precursors without direct contact with stromal cells, HSPCs were cultured with CM collected from cultures of confluent stromal cells instead of stromal cells. Compared with the cultures with CM, the numbers of total cells and $CD45RA^+CD14^-$ cells were significantly lower, but the proportion of B lineage cells, including DPLPs, EBPs, and pro-B cells, among $CD45RA^+CD14^-$ cells was higher in the cultures on stromal cells (Fig. 2B). Because the proportion of ETPs among $CD45RA^+CD14^-$ cells was similar between the cultures, a higher number of ETPs was consistently produced in the cultures with CM than in those with stromal cells. The anti-CXCR4 Ab strongly inhibited the generation of $CD45RA^+CD14^-$ cells, including ETPs, DPLPs, EBPs, and pro-B cells, on stromal cells. However, in the cultures with CM, the anti-CXCR4 Ab did not significantly affect the generation of ETPs, DPLPs, or EBPs, whereas the development of $CD19^+$ pro-B cells was suppressed. These data suggest that adhesion to stromal cells suppresses the growth of immature hematopoietic precursors but promotes their differentiation toward B lymphoid cells and that anti-CXCR4 Ab inhibits the generation of early T and B lymphoid precursors in contact with stromal cells but not the generation of early T lymphoid precursors without direct contact with stromal cells.

To further elucidate the effect of stromal cell adhesion on the growth of HSPCs, we analyzed the cell division history by labeling HSPCs with CytoTell Green and culturing them on stromal cells or with CM. The cell division assay showed that HSPCs proliferated more slowly on stromal cells than with CM (Supplemental Fig. 2). The importance of direct interactions between hematopoietic precursors and stromal cells for B lymphoid differentiation was confirmed by physically separating hematopoietic precursors from stromal cells with culture inserts. As expected, higher percentages of $CD10^+CD19^-$ EBPs and $CD10^+CD19^+$ pro-B cells were generated in the cultures under the culture inserts in contact with stromal cells than in the cultures above the culture inserts (Supplemental Fig. 3).

Taken together, these data indicate that the CXCL12–CXCR4 interaction is primarily important for stromal cell contact–dependent lymphoid development.

Differentiation potential of ETPs and EBPs generated in cultures

We previously reported that telomerized stromal cells support the generation of ETPs and pro-B cells (26), but the differentiation pathway of these lymphoid precursors has not been defined. To determine the effect of the anti-CXCR4 Ab on generating relatively more mature lymphoid precursors, we examined the lymphoid differentiation pathway and the role of stromal cell contact in the differentiation process from immature hematopoietic precursors. To this end, HSPCs were cultured with 3GF on stromal cells for 14 d and then ETPs and EBPs were isolated from the $CD45RA^+CD14^-$ fraction of cells and recultured on stromal cells or with CM for another 14 d. After reculture on stromal cells, the ETPs gave rise to $CD10^-CD7^+$, $CD10^+CD7^+$, and $CD10^+CD7^-CD19^{+/-}$ lymphoid cells, whereas the EBPs mainly differentiated to pro-B cells (Fig. 3). In cultures containing CM, differentiation toward pro-B cells from ETPs and EBPs was reduced considerably compared with cultures on stromal cells (Fig. 3). A significant number of $CD14^+$ monocytic cells had developed in all cultures (data not shown). To assess the differentiation potential for T lineage cells, ETPs and EBPs were recultured further on Notch ligand delta-1–expressing OP9 stromal cells in the presence of Flt3L and IL-7 (37). After 21 d of culture, both types of lymphoid precursors were induced to differentiate into $CD1a^+CD5^+$ pre-T cells (Fig. 3). Similar data were obtained by culture of ETPs and EBPs derived from HSPCs cultured in the presence of CM (data not shown). These data

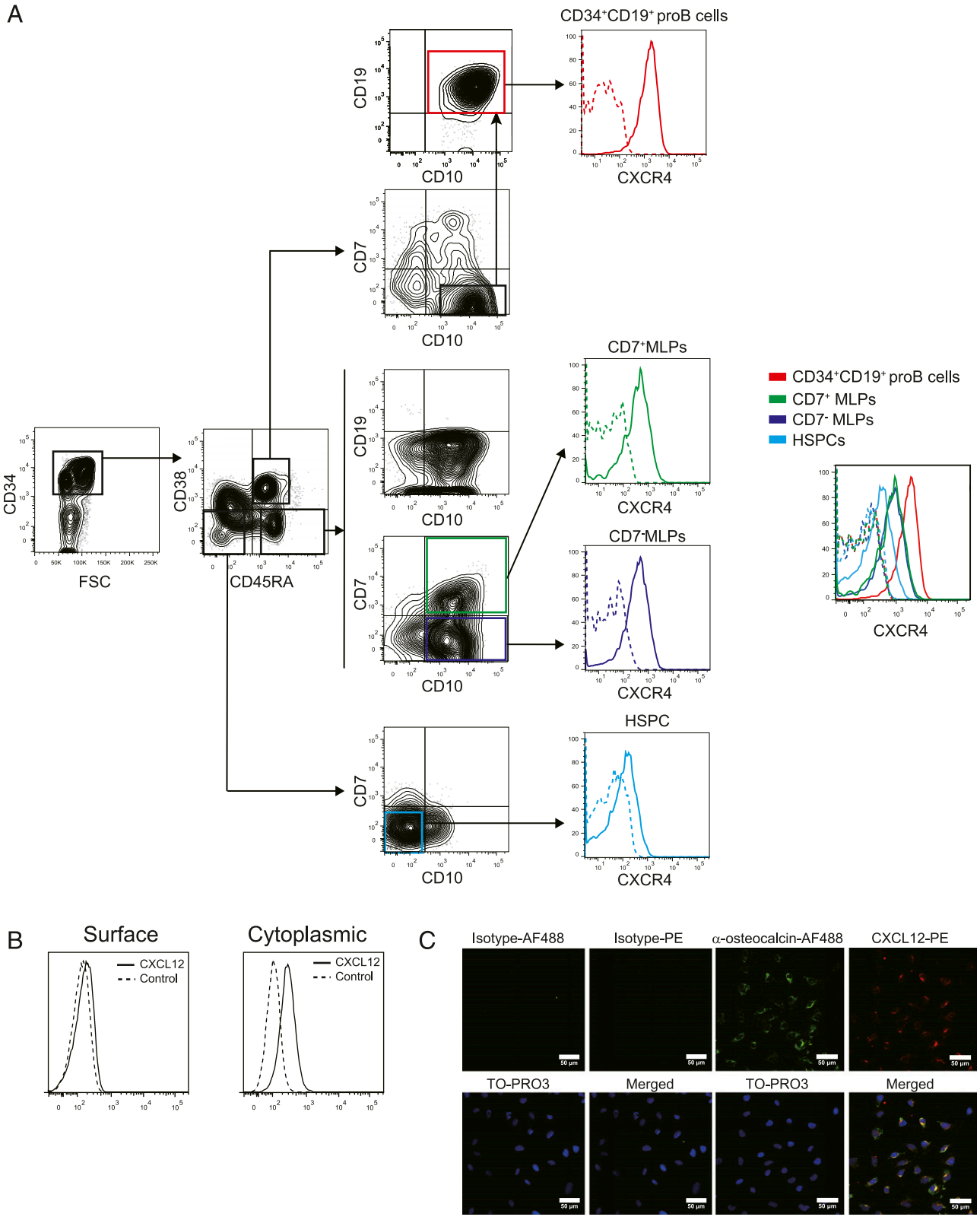

FIGURE 1. Expression of CXCR4 in immature hematopoietic and early lymphoid precursors and CXCL12 expression in stromal cells. (**A**) Expression of CXCR4 on HSPCs, $CD7^+$ MLPs, $CD7^-$ MLPs, and $CD34^+CD38^+CD45RA^+CD10^+CD7^-CD19^+$ pro-B cells. Data are representative of three independent experiments. (**B**) Surface and intracellular CXCL12 expression in stromal cells was analyzed by flow cytometry. (**C**) Expression of CXCL12 in stromal cells was examined immunocytochemically. Osteocalcin is green, CXCL12 is red, and TO-PRO-3 nuclear staining is blue (original magnification ×400).

show that ETPs have differentiation potentials for T lymphoid, B lymphoid, and monocytic cells, whereas EBPs are B lineage–biased lymphoid precursors retaining the differentiation potential for T lymphoid and monocytic cells. The results also indicated that direct contact with stromal cells induces ETPs and EBPs to differentiate toward pro-B cells.

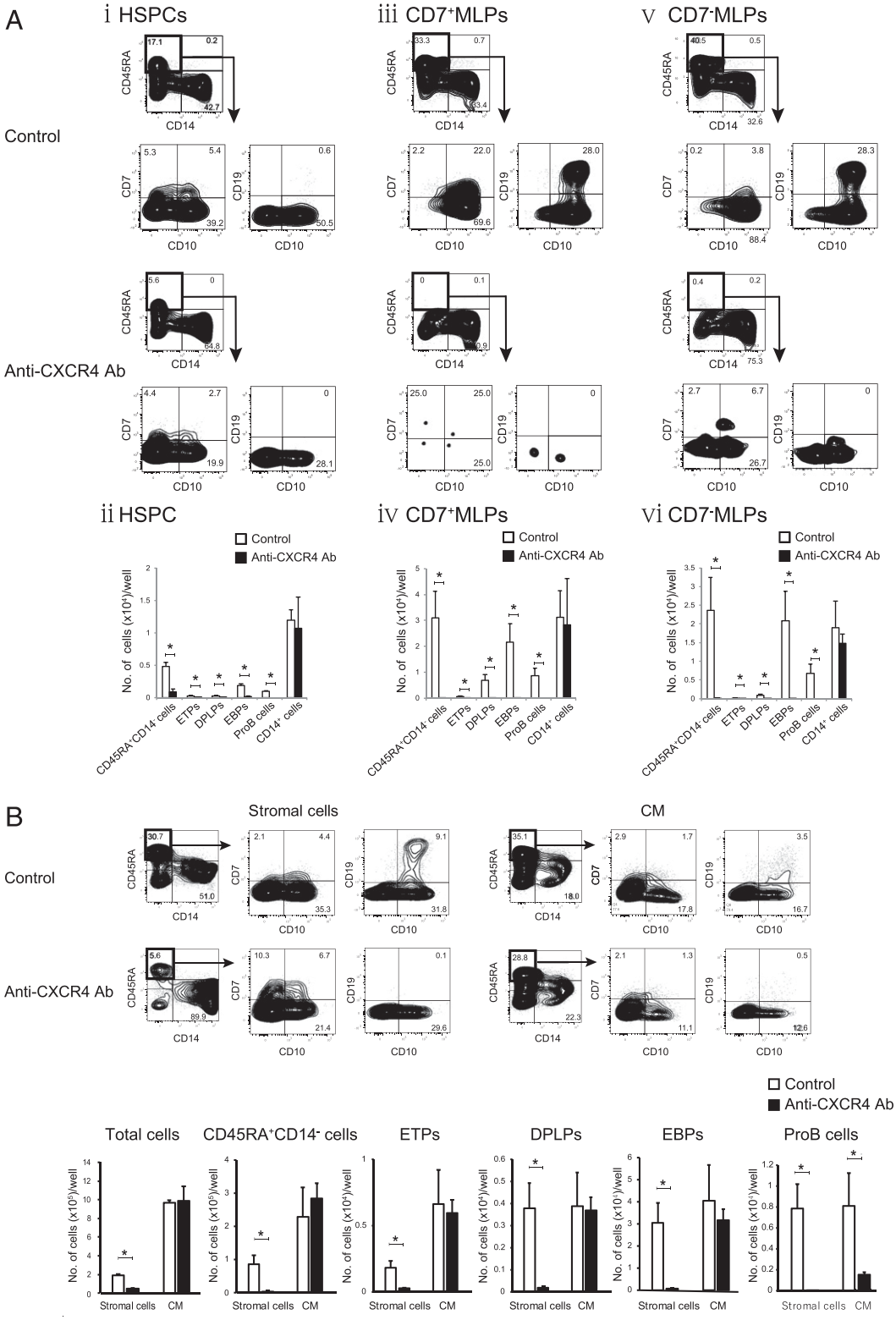

FIGURE 2. Effect of the anti-CXCR4 Ab on immature hematopoietic and early lymphoid precursors. (**A**) HSPCs, $CD7^+$ MLPs, or $CD7^-$ MLPs (1000 cells per well) were cultured on stromal cells with an anti-CXCR4 Ab or isotype control for 21 d, and the expression of CD10, CD7, and CD19 in $CD45RA^+CD14^-$ cells (**i**, **iii**, and **v**) and the numbers of $CD45RA^+CD14^-$ cells, ETPs, DPLPs, EBPs, pro-B cells, and $CD14^+$ cells (**ii**, **iv**, and **vi**) were analyzed. (**B**) HSPCs (2000 cells per well) were cultured on stromal cells or with CM in the presence of the anti-CXCR4 Ab or isotype control and analyzed at 21 d. Representative data of five experiments are shown. $*p < 0.05$.

Anti-CXCR4 Ab suppresses stromal cell contact–mediated differentiation of EBPs

Based on the above results, we first examined the effect of the anti-CXCR4 Ab on EBPs, which were similarly generated from HSPCs cultured with 3GF for 14 d, in cultures on stromal cells or with CM for another 14 d. In the control culture on stromal cells, pro-B cells appeared at day 7 (Fig. 4A) and became the major population among $CD45RA^+CD14^-$ cells at day 14 (Fig. 4B). However, the

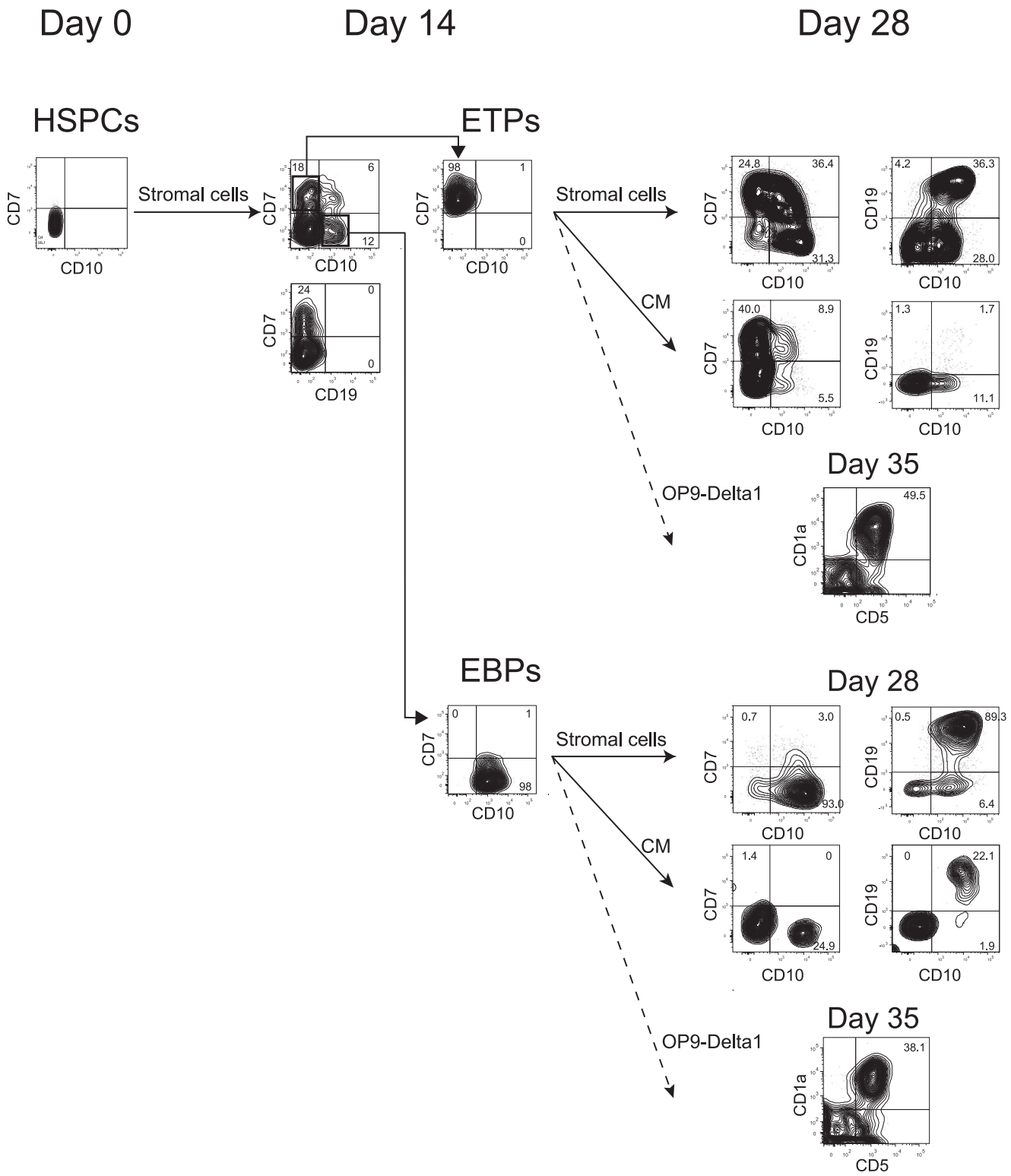

FIGURE 3. Differentiation potential of lymphoid precursors and the effect of stromal cell contact. HSPCs were cultured on stromal cells for 14 d and then ETPs and EBPs were isolated. Both precursors were recultured on stromal cells or with CM for another 14 d. The expression of CD10, CD7, and CD19 in CD45RA$^+$CD14$^-$ cells at days 0, 14, and 28 is shown. Both types of precursors were also recultured on delta-1–expressing OP9 stromal cells with Flt3L and IL-7 for 21 d and then assessed for expression of CD1a and CD5. Representative data of four experiments are shown.

anti-CXCR4 Ab suppressed differentiation into pro-B cells from days 7 to 14, and only a small number of CD45RA$^+$CD14$^-$ cells, including CD10$^+$ B lineage cells, was detected in cultures with the anti-CXCR4 Ab at day 14 (Fig. 4A–C). In control cultures with CM, the differentiation toward pro-B cells was diminished considerably compared with control cultures on stromal cells, as described previously. The presence of the anti-CXCR4 Ab in cultures with CM further suppressed their B lymphoid differentiation, and few or no CD45RA$^+$CD14$^-$ cells remained at day 14 (Fig. 4D, 4E). These findings imply that the CXCL12–CXCR4 axis is crucial for stromal cell contact–induced B lymphoid differentiation at a later stage from CD10$^+$CD19$^-$ EBPs to CD10$^+$CD19$^+$ pro-B cells.

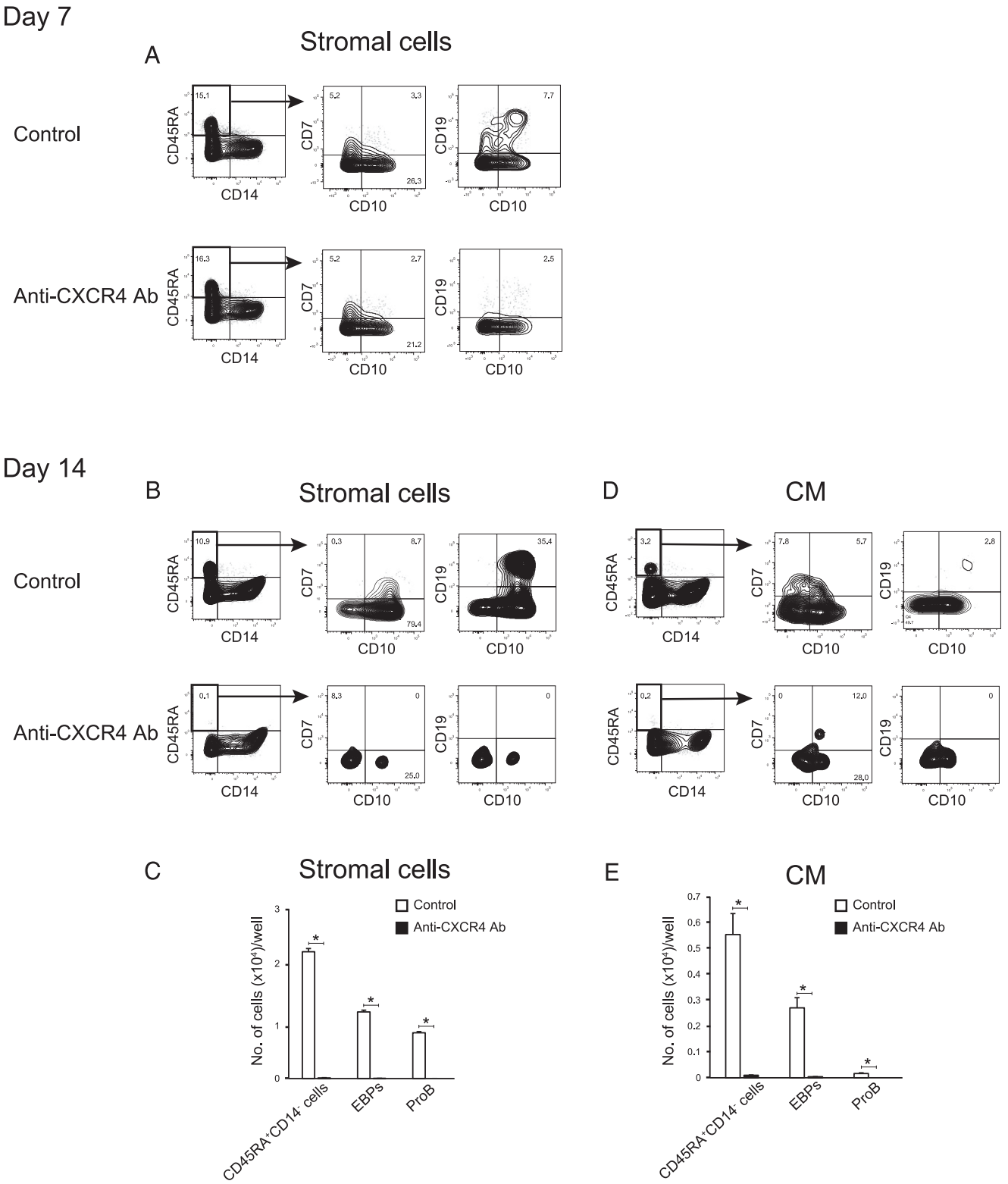

FIGURE 4. Effect of the anti-CXCR4 Ab on EBPs. HSPCs were cultured on stromal cells for 14 d, and EBPs (purity > 95%) were isolated and cultured (3000 cells per well) on stromal cells or with CM in the presence of the anti-CXCR4 Ab or isotype control for 7 or 14 d. Expression of CD10, CD7, and CD19 in $CD45RA^+CD14^-$ cells at day 7 (**A**) and day 14 (**B** and **D**) and numbers of $CD45RA^+CD14^-$, EBPs, and pro-B cells at day 14 (**C** and **E**) in cultures. Similar data were observed in two other separate experiments. $*p < 0.05$.

Differential action of the anti-CXCR4 Ab on ETPs with or without contact with stromal cells

We next examined the effect of the anti-CXCR4 Ab on ETPs, which were obtained by culture of HSPCs with 3GF for 14 d with CM, in cultures on stromal cells or with CM. In control cultures on stromal cells, ETPs were induced to differentiate toward $CD10^+$ B lineage cells (Fig. 5Ai). The anti-CXCR4 Ab inhibited contact-mediated lymphoid differentiation of ETPs. Notably, few

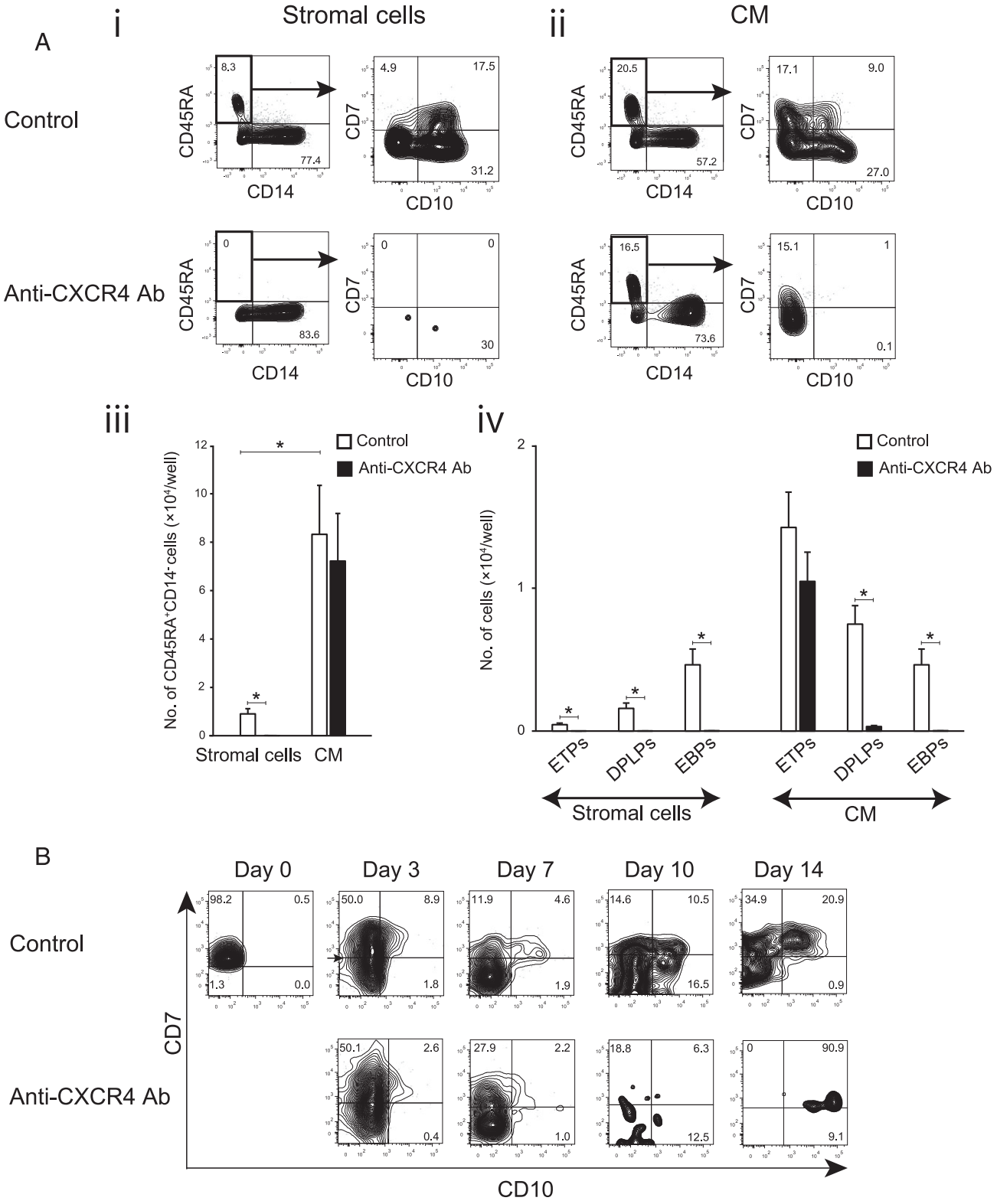

FIGURE 5. Effect of the anti-CXCR4 Ab on ETPs cultured on stromal cells or with CM. (**A**) HSPCs were cultured for 14 d with CM, and ETPs (2500 cells per well) (purity > 95%) were cultured on stromal cells or with CM, in the presence or absence of anti-CXCR4 Ab for 14 d. Expression of CD10 and CD7 in $CD45RA^+CD14^-$ cells (**i** and **ii**) and numbers of $CD45RA^+CD14^-$ cells (**iii**) and ETPs, DPLPs, and EBPs (**iv**) are shown. Representative data of four separate experiments are shown. (**B**) ETPs were cultured on stromal cells with or without anti-CXCR4 Ab, and the expression of CD10 and CD7 in $CD45RA^+CD14^-$ cells was analyzed at days 3, 7, 10, and 14. Data are representative of three independent experiments. $*p < 0.05$.

or no $CD45RA^+$ cells, including $CD7^+$ or $CD10^+$ lymphoid cells, were detected (Fig. 5Ai, 5Aiii, 5Aiv). Compared with control cultures on stromal cells, the numbers of $CD45RA^+CD14^-$ cells and ETPs were remarkably higher in control cultures with CM, although the proportion of $CD10^+$ B lineage cells among $CD45RA^+CD14^-$ cells was relatively lower (Fig. 5Ai, 5Aii). The anti-CXCR4 Ab did not affect the growth of ETPs in CM, whereas the generation of $CD10^+$ B lineage cells was also

suppressed (Fig. 5Aii, 5Aiv). Thus, the anti-CXCR4 Ab acted on ETPs differently, depending on whether the T lymphoid precursors were in direct contact with stromal cells. These data reveal that CXCL12–CXCR4 engagement is conditionally required for differentiation of ETPs in contact with stromal cells but is dispensable for their growth without contact with stromal cells.

Few or no CD45RA$^+$ cells were detected at day 14 after replating of ETPs on stromal cells with the anti-CXCR4 Ab (Fig. 5Ai). We elucidated the action of the anti-CXCR4 Ab in ETPs in contact with stromal cells more precisely by incubating the ETPs on stromal cells, with or without the anti-CXCR4 Ab, and sequentially assessing the proportion of CD10$^+$ and/or CD7$^+$ cells in the CD45RA$^+$CD14$^-$ fraction of cells. In control cultures, ETPs differentiated gradually toward CD10$^+$ B lineage cells from day 3 to 14. The anti-CXCR4 Ab suppressed the differentiation to CD10$^+$ B lineage cells during the initial 7 d and then few CD7$^+$ or CD10$^+$ lymphoid cells were detected in the CD45RA$^+$CD14$^-$ fraction of cells at day 10 (Fig. 5B). These findings imply that ETPs, the differentiation of which to B-lineage cells was inhibited by blocking of CXCL12–CXCR4 axis, became dependent on CXCL12 for their survival after culture on stromal cells. To clarify how CXCL12 dependency changed during culture, ETPs were cultured for 14 d on stromal cells, and the anti-CXCR4 Ab was added during the 14 d of culture or during the initial or latter 7 d of culture, and the numbers of CD45RA$^+$CD14$^-$ cells, ETPs, and EBPs were analyzed after 14 d. CD45RA$^+$CD14$^-$ cells were not detected when the anti-CXCR4 Ab was present during the entire 14 d or during the latter 7 d of culture. However, a significant number of CD45RA$^+$CD14$^-$ cells, including ETPs and EBPs, was observed when the anti-CXCR4 Ab was present during the initial 7 d of culture (Fig. 6A). These data suggest that CXCL12 dependency increased in the later period following culture on stromal cells. To understand the mechanism underlying the change in CXCR12 dependency, expression of CXCR4 in the CD45RA$^+$CD14$^-$ fraction of cells was assessed before and after culture of ETPs on stromal cells or with CM. After 7 d of culture, a proportion of CD7$^+$ cells had differentiated toward CD10$^+$ B lineage cells, and the generated CD10$^+$CD7$^+$ population expressed higher levels of CXCR4. The CXCR4 expression levels were even higher in the CD10$^-$CD7$^+$ population after culture on stromal cells compared with the CD10$^-$CD7$^+$ population before culture (Fig. 6B). However, in cultures with

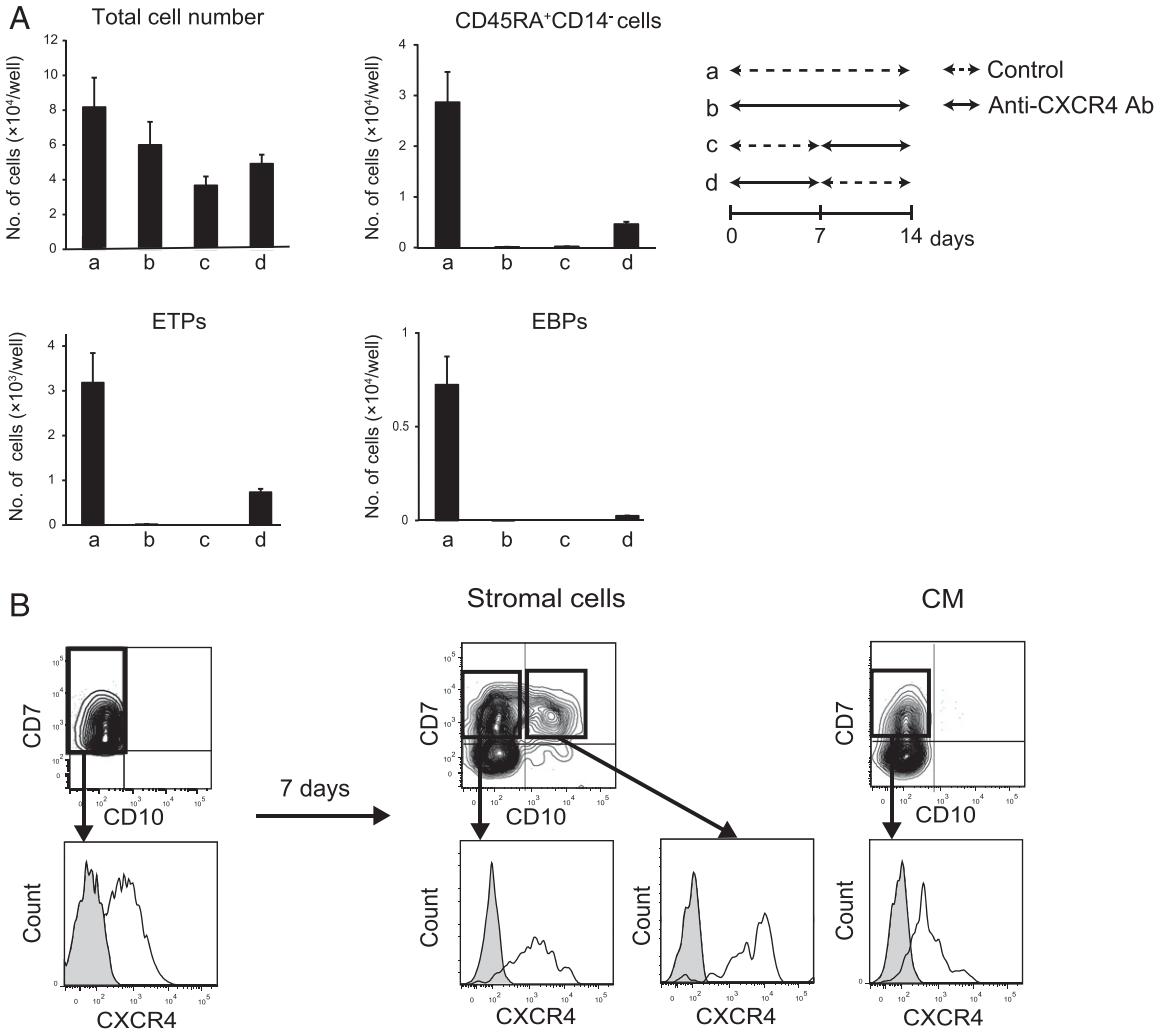

FIGURE 6. Effect of the anti-CXCR4 Ab on ETPs during initial and latter culture periods. (**A**) ETPs (3000 cells per well) (purity > 95%) were cultured on stromal cells in the presence of the (a) isotype control or (b) anti-CXCR4 Ab for 14 d, (c) during the last 7 d, or (d) during the initial 7 d. Numbers of total cells, CD45RA$^+$CD14$^-$ cells, ETPs, and EBPs were analyzed. (**B**) HSPCs were cultured for 14 d with CM and then ETPs were isolated and cultured with CM or on stromal cells for 7 d. CXCR4 expression levels in the CD10$^-$CD7$^+$ cell fraction before and after culture and in the CD10$^+$CD7$^-$ cell fraction after culture are shown. Representative data of three independent experiments are shown.

CM, most $CD7^+$ cells were still negative for CD10, and the expression levels of CXCR4 in the $CD10^-CD7^+$ population remained as low as before culture (Fig. 6B). Taken together, these data led us to speculate that ETPs become more dependent on CXCL12 upon attachment to stromal cells as they differentiate toward B lineage cells.

Effect of the anti-CXCR4 Ab on pDC generation

We previously reported that telomerized stromal cells support the generation of pDCs from human hematopoietic precursors (26). We further examined the role of the CXCL12–CXCR4 axis in the generation of pDCs by culture of HSPCs with 3GF on stromal cells or with CM in the presence or absence of the anti-CXCR4 Ab.

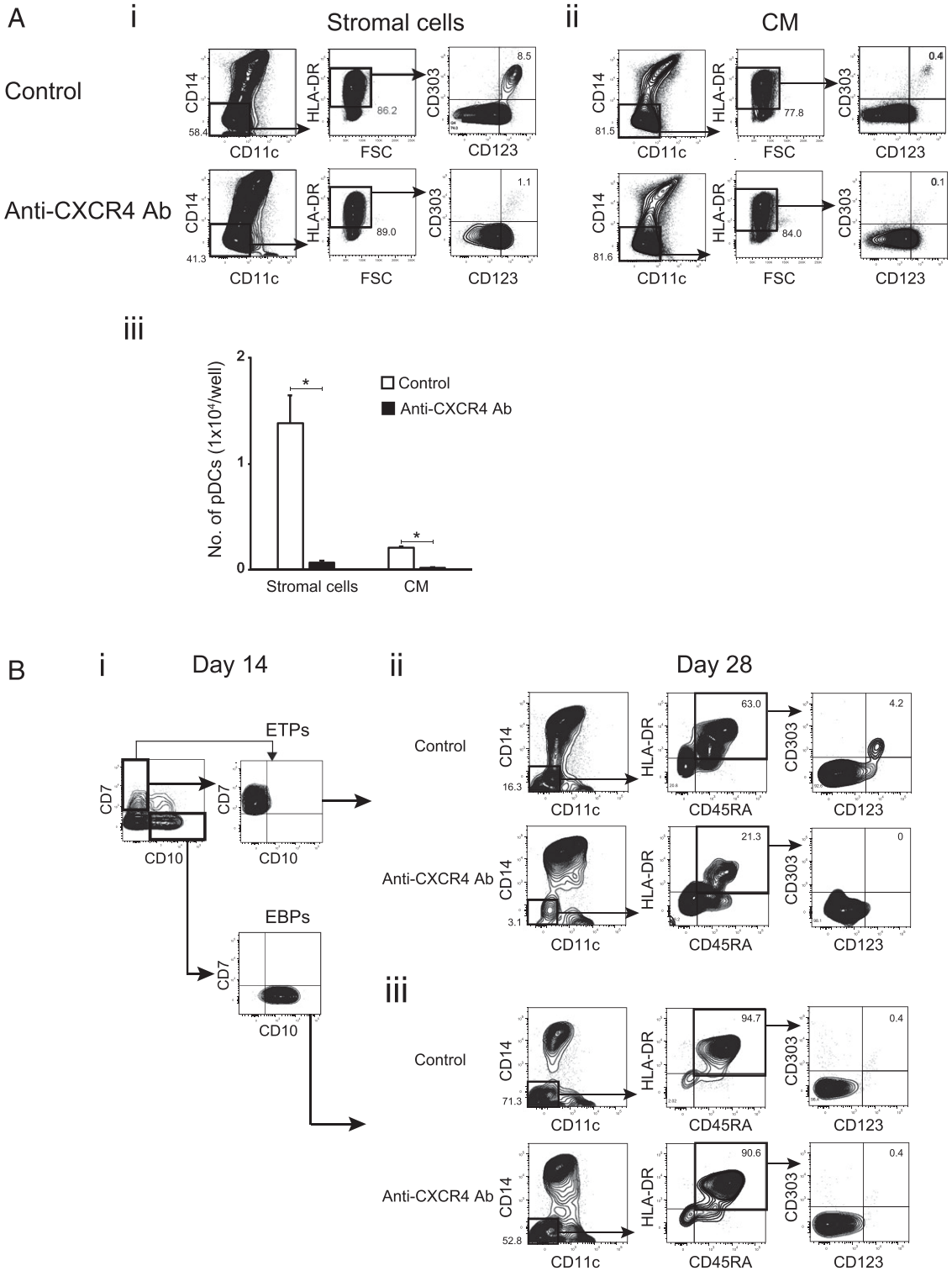

FIGURE 7. Effect of the anti-CXCR4 Ab on the generation of pDCs. (**A**) HSPCs (4000 cells per well) were cultured on stromal cells (A**i**) or with CM (A**ii**) in the presence of the anti-CXCR4 Ab or isotype control. (**B**) After 21 d, the generation of $CD14^-CD11c^-HLA\text{-}DR^+CD123^{high}CD303^+$ pDCs was analyzed. ETPs and EBPs, which were generated from HSPCs cultured for 14 d with stromal cells (B**i**), were recultured on stromal cells for another 14 d in the presence of the anti-CXCR4 Ab or isotype control and then the generation of pDCs from ETPs (B**ii**) or EBPs (B**iii**) was similarly assessed. Representative data of three separate experiments are shown. $*p < 0.05$.

In control cultures, a significantly higher number of HLA-DR$^+$ CD11c$^-$CD123^{high}CD303$^+$ pDCs had developed on stromal cells compared with CM (Fig. 7Ai, 7Aii). The anti-CXCR4 Ab suppressed the generation of pDCs on stromal cells (Fig. 7Ai–Aiii). We further examined the differentiation pathway of pDCs and the role of the CXCL12–CXCR4 interaction in the pDC-differentiation process. To this end, ETPs or EBPs, which were derived from HSPCs cultured for 14 d on stromal cells (Fig. 7Bi), were cultured on stromal cells in the presence of the anti-CXCR4 Ab or isotype control. As shown in Fig. 7B, pDCs developed from ETPs (Fig. 7Bii), but not EBPs (Fig. 7Biii), on stromal cells, which was also suppressed by the anti-CXCR4 Ab. These findings suggest that the ETPs generated in cultures possess differentiation potentials for T lymphoid, B lymphoid, and monocytic cells, as well as pDCs (Fig. 8). Furthermore, the CXCL12–CXCR4 interaction is essential for stromal cell–mediated pDC differentiation from immature hematopoietic precursors and ETPs with a multilineage differentiation capacity.

Discussion

In this study, we determined the role of the CXCL12–CXCR4 interaction in the differentiation of human HSPCs using a unique coculture system that supports the differentiation of various lineages of lymphoid cells and pDCs (26). Stromal cell contact was crucial for early B lymphoid and pDC differentiation, and the anti-CXCR4 Ab suppressed the stromal cell contact–mediated B lymphoid and pDC differentiation from HSPCs and ETPs. Conversely, direct contact with stromal cells was not needed for the generation of ETPs; rather, it promoted B lymphoid differentiation. The anti-CXCR4 Ab did not affect the stromal cell–independent ETP generation, but it inhibited stromal cell contact–mediated B lymphoid differentiation. These data demonstrate that stromal cell–derived CXCL12 plays a critical role in stromal cell contact–mediated human early B lymphoid and pDC differentiation from immature hematopoietic precursors and ETPs with a multilineage differentiation potential, but it is dispensable for stromal cell contact–independent generation of ETPs (Fig. 8).

We found that ETPs generated by culture of HSPCs possessed a multilineage differentiation capacity for T and B lymphoid cells, pDCs, and monocytic cells, whereas EBPs were B lineage–biased lymphoid precursors. A difference in the differentiation potential between CD34$^+$CD45RA^{high}CD7$^+$ and CD34$^+$CD45RA^{high}lin$^-$ CD10$^+$ lymphoid precursors has been suggested by another study (38). We found that direct contact with stromal cells was not necessary for the generation of ETPs and that the generated ETPs underwent differentiation into mature T lineage cells on delta-1–expressing OP9 stromal cells, but they differentiated toward B lineage cells and pDCs upon contact with telomerized stromal cells. It has been demonstrated in vivo in mice that ETPs undergo T lineage differentiation in the thymic environment, but they differentiate toward B lymphoid cells upon blockade of Notch signaling (39). These findings, together with our results, imply that ETPs can be generated from immature hematopoietic precursors without contact with stromal cells, but they adopt a different cell fate depending on the interaction with tissue-specific niches in the thymus or bone marrow. Conversely, stromal cell contact was crucial for differentiation of pDCs and B lymphoid cells. Prior studies have suggested the importance of direct contact with stromal cells for human early B lymphoid differentiation (40, 41). In terms of pDC differentiation, one study showed that direct contact with delta-1–expressing OP9 stromal cells is important for development of pDCs (42). Other studies have reported that pDC generation is supported by Flt3L and TPO (43, 44). We observed generation of a significant number of pDCs with 3GF by culture in the absence of stromal cells or CM (data not shown). It is possible that stromal cells may produce inhibitory factors of pDC differentiation, but the direct interaction with stromal cells promotes pDC differentiation, overcoming the effect of inhibitory factors.

Our results using the anti-CXCR4 Ab showed that CXCL12–CXCR4 engagement was critical for B lymphoid and pDC differentiation evoked by contact with stromal cells. B lymphoid differentiation proceeded to some extent without contact with stromal cells, which was also suppressed by the anti-CXCR4 Ab. Nonetheless, such contact-independent B lymphoid differentiation

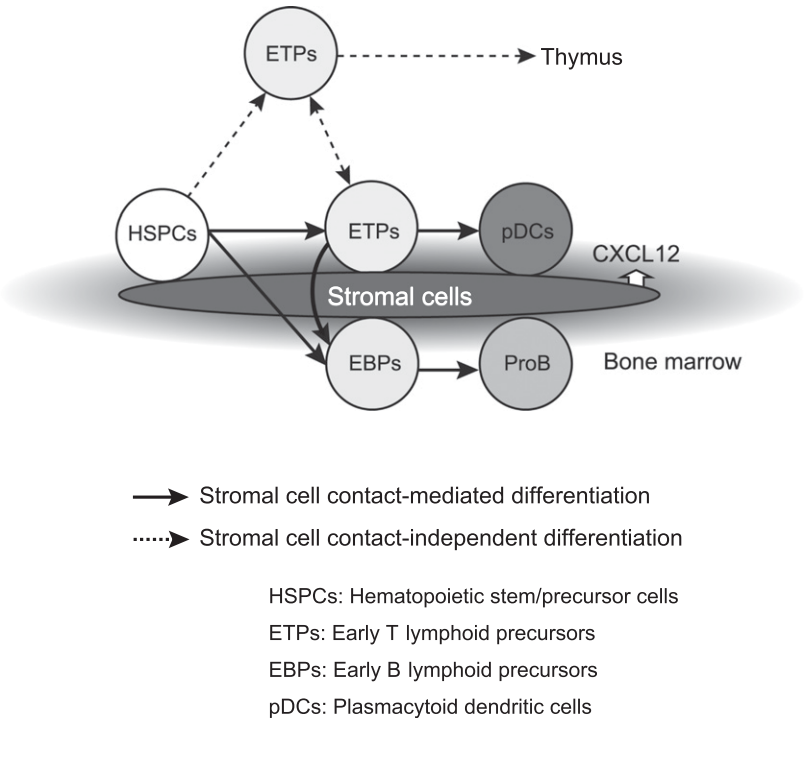

FIGURE 8. Role of the CXCL12–CXCR4 axis in human early T and B lymphoid and pDC differentiation in the context of the interaction with stromal cells.

was limited. Therefore, our findings extend previous studies of gene-engineered mice (13–15, 17) and provide a new notion that CXCL12 plays an essential role in stromal cell contact–mediated B lymphoid and pDC differentiation. CXCL12 was not needed for the generation or growth of ETPs under a condition without contact with stromal cells, but it was required for their generation on stromal cells, probably because the ETPs were induced to differentiate toward B lymphoid precursors that are dependent on CXCL12 for survival and differentiation. Glodek et al. (45) found that CXCL12 specifically stimulates sustained adhesion of pro- and pre-B cells to stromal cells through the VCAM-1–VLA-4 interaction. CXCR4-mediated activation of integrin pathways has been observed in immature hematopoietic precursors and neutrophils in the bone marrow environment (46, 47). We also observed that blocking of the CXCL12–CXCR4 interaction with the anti-CXCR4 Ab suppressed the adhesion of HSPCs to the stromal cells (Supplemental Fig. 4). Although the precise mechanisms remain undefined, such cross-talk between CXCL12–CXCR4 and adhesion signaling might play a crucial role in stromal cell contact–mediated lymphoid and pDC differentiation.

Our data indicate that the CXCL12–CXCR4 axis may play a central role in the regulation of stromal cell contact–mediated lymphohematopoiesis in bone marrow. The CXCL12–CXCR4 axis has been revealed to be involved in the pathogenesis of various hematologic diseases, including pDC neoplasms (48–51). Activation mutations of the CXCR4 gene have been identified in warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis syndrome (50, 52–54) and Waldenström's macroglobulinemia (55, 56), suggesting that dysregulation of CXCR4 signaling may cause immunological disorders or hematological malignancies. Further studies are warranted to understand the precise mechanism underlying the CXCL12-CXCR4–mediated regulation of stromal cell contact in physiological and pathological states.

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Disclosures

The authors have no financial conflicts of interest.

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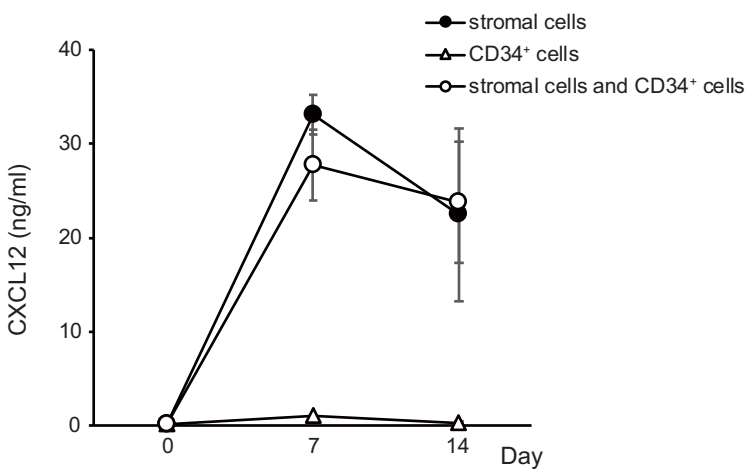
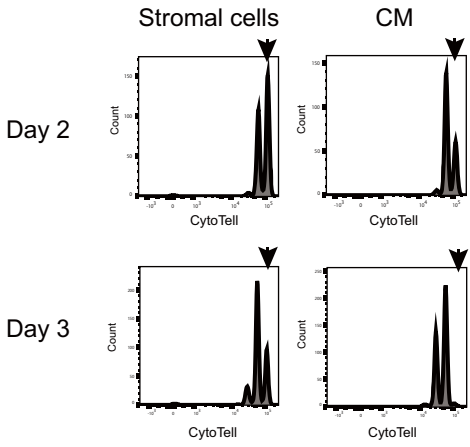
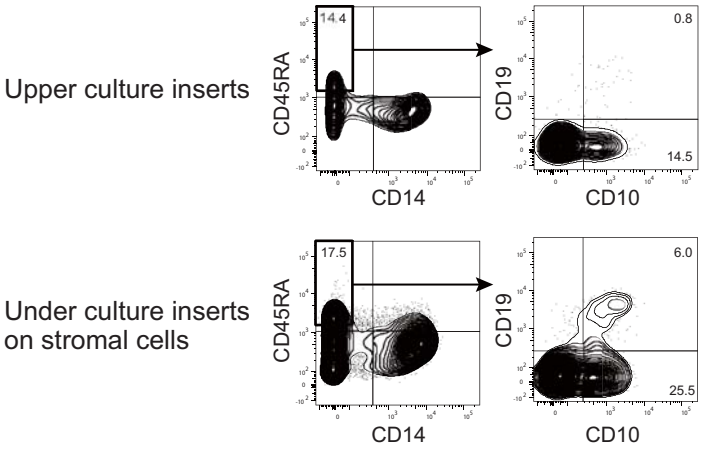
Supplemental Figure 1



Supplemental Figure 1. Production of CXCL12 from stromal cells and hematopoietic precursors. Concentrations of CXCL12 in CM were assessed at day 0, 7, and 14 after culture of confluent stromal cells, 2×10^4 $CD34^+$ cells, or 2×10^4 $CD34^+$ cells on stromal cells.

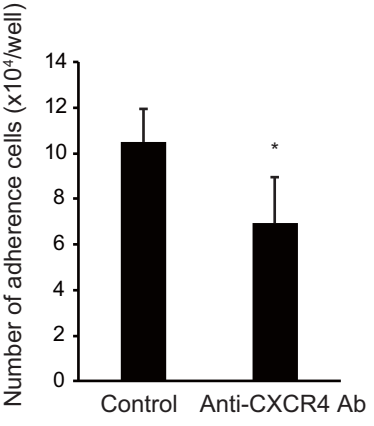


Supplemental Figure 2. Cell division analysis of HSPCs in cultures on stromal cells or with CM. HSPCs labeled with CytoTell™ Green were cultured on stromal cells or with CM for 3 days and then analyzed for cell division.



Supplemental Figure 3. Inhibition of direct contact between HSPCs and stromal cells with culture inserts impairs B-lymphoid differentiation. HSPCs (200 cells/well) were cultured above and under culture inserts on stromal cells for 3 weeks and then analyzed for the expression of CD10 and CD19 in the $CD45RA^{+}CD14^{-}$ cell fraction of cultured cells. Representative data of three separate experiments are shown.

Supplemental Figure 4



Supplemental Figure 4. Effect of the CXCL12-CXCR4 interaction on the adhesion of hematopoietic precursors to stromal cells. $CD34^+$ cells (2×10^5 cells/well) were pre-incubated with the anti-CXCR4 Ab or isotype control for 30 minutes and then cultured on stromal cells. After 4 hours, these cultures were gently pipetted and analyzed for the number of adhered cells by counting detached cell numbers. *P<0.05. Representative data of three separate experiments are shown.