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Cx_4Mab-1: A Novel Anti-Mouse CXCR4 Monoclonal Antibody for Flow Cytometry

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The CXC chemokine receptor 4 (CXCR4, CD184) is a member of the G protein-coupled receptor family that is expressed in most leukocytes. Overexpression of CXCR4 is associated with poor prognosis in not only hematopoietic malignancy but also solid tumors. Because CXCR4 is an attractive target for tumor therapy, reliable preclinical murine models using anti-CXCR4 monoclonal antibodies (mAbs) have been warranted. This study established a novel anti-mouse CXCR4 (mCXCR4) mAb using the Cell-Based Immunization and Screening method. Flow cytometric analysis showed that an anti-mCXCR4 mAb, Cx_4Mab-1 (rat IgG_{2a}, kappa), recognized mCXCR4-overexpressed Chinese hamster ovary-K1 (CHO/mCXCR4) cells and endogenously mCXCR4-expressing mouse myeloma P3X63Ag8U.1 (P3U1) cells. Furthermore, Cx_4Mab-1 did not recognize mCXCR4-knockout P3U1 cells. The dissociation constants of Cx_4Mab-1 for CHO/mCXCR4 and P3U1 were determined as 6.4×10^{-9} M and 2.3×10^{-9} M, respectively, indicating that Cx_4Mab-1 possesses a high affinity to both endogenous and exogenous mCXCR4-expressing cells. These results indicate that Cx_4Mab-1 could be a useful tool for preclinical mouse models.

Keywords: mouse CXCR4, monoclonal antibody, CBIS method

Introduction

CXC chemokine receptor 4 (CXCR4, CD184) is a member of G-protein-coupled receptors for CXCL12 (SDF-1).[1,2] CXCR4 is expressed on most leukocytes.[3–5] CXCR4 activation by CXCL12 induces cell migration, proliferation, and survival through G-proteins-dependent or -independent (JAK/STAT) pathway.[6,7] Furthermore, the CXCL12/CXCR4 axis plays a critical role in embryonic development.[8] The mice lacking CXCR4 or CXCL12 die on embryonic day 18.5. Both mice exhibit abnormal development of the cerebellum, heart, and blood vessels in the gastrointestinal tract.[9,10]

Overexpression of CXCR4 is associated with poor prognosis in various types of cancers through promoting the proliferation and metastasis.[6] CXCR4 is overexpressed in about 60% of colorectal cancers,[11] one of the most common malignancies in the world. Many studies have reported that patients with high CXCR4-expressing colorectal cancers showed frequent lymph node metastasis and distant metastasis.[12–14] The metastasized colorectal cancer in the liver, which highly expresses CXCL12, exhibited elevated expression of CXCR4 compared to the primary site.[15,16] An animal experiment using a mouse colorectal cancer CT-26 cell line revealed that CXCR4 is important for metastasis to the liver.[17] Moreover, CXCR4 supports tumor growth by inducing angiogenesis.[18,19] These findings showed that CXCR4 is an attractive target for tumor therapy.

Mouse is a commonly used animal for preclinical studies to predict the efficiency and the safety of cancer therapies.[20] For assessment of cancer therapies, two models are established. The first one is the transplantation of tumors into mice. The other is the induction of tumors in mice by genetic modification and carcinogens. Both models with immunocompetent mice are used for the evaluation of cancer treatments in the presence of host immunity, an important factor affecting the efficiency and the safety of cancer therapies.[21] However, a preclinical model using anti-mouse CXCR4 (mCXCR4) monoclonal antibodies (mAbs) has not been reported.

We have developed mAbs to mouse CCR3[22] and CCR8,[23] members of chemokine receptors, by using the Cell-Based Immunization and Screening (CBIS) method, which includes immunization of antigen-overexpressing cell and high-throughput screening of hybridoma supernatants using flow cytometry. In this report, we established a novel anti-mCXCR4 mAb using the CBIS method and evaluated its applications.

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Materials and Methods

Antibodies

Anti-mCXCR4 (CD184) mAbs (clone L276F12 and clone 2B11/CXCR4) were purchased from BioLegend (San Diego, CA) and BD Biosciences (Franklin Lakes, NJ), respectively. Alexa Fluor 488-conjugated anti-rat immunoglobulin G (IgG) was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Preparation of cell lines

LN229, Chinese hamster ovary (CHO)-K1 and P3X63Ag 8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA).

The synthesized DNA (Eurofins Genomics KK) encoding mCXCR4 (Accession No.: NM_009911.3) was subsequently subcloned into a pCAGzeo PAcH vector (PA tag added to C-terminus of mCXCR4). The PA tag consists of 12 amino acids (GVAMPGAEDDVV).[24] The mCXCR4-PA plasmid was transfected into LN229 and CHO-K1 cells, using a Neon transfection system (Thermo Fisher Scientific Inc., Waltham, MA). Stable transfectants were established by staining with anti-mCXCR4 mAb (clone L276F12) and sorted using a cell sorter (SH800; Sony Corp., Tokyo, Japan), after which cultivation in a medium containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA) was conducted. GeneArt™ CRISPR nuclease vectors with orange fluorescent protein plasmid, which target mCXCR4 (CGGCAATGGATTGGTGATCC), were purchased from Thermo Fisher Scientific Inc., The knockout plasmid was transfected into P3U1 cells, using a Neon transfection system. mCXCR4 knockout P3U1 (BINDS-56) was established by staining with anti-mCXCR4 mAb and sorted using a cell sorter (SH800).

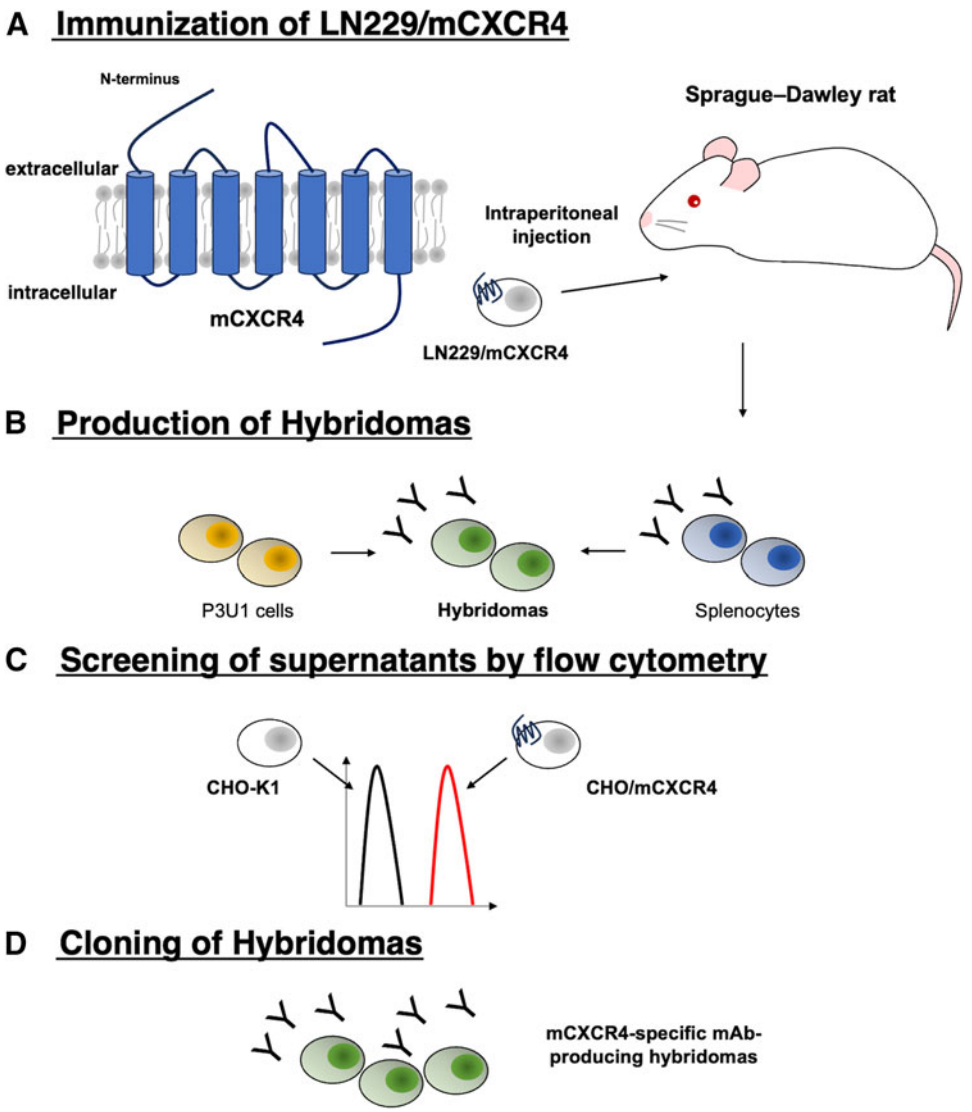

FIG. 1. The scheme of establishment of Cx_4Mab-1 by the CBIS method. **(A)** LN229/mCXCR4 cells were immunized into a Sprague–Dawley rat by intraperitoneal injections. **(B)** The splenocytes from the rat were fused with P3U1 cells. **(C)** The culture supernatants were screened through flow cytometry to select anti-mCXCR4 mAb-producing hybridomas. **(D)** Finally, Cx_4Mab-1 was established by limiting dilution and some additional screenings. CBIS, Cell-Based Immunization and Screening; mAb, monoclonal antibody.

LN229 and mCXCR4-PA-overexpressed LN229 (LN229/mCXCR4) cells were cultured in Dulbecco's modified Eagle medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc.), 100 U/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). CHO-K1, mCXCR4-PA-overexpressed CHO-K1 (CHO/mCXCR4), P3U1, and BINDS-56 cells were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc.), supplemented with 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.).

All cells were grown in a humidified incubator at 37°C, in an atmosphere of 5% carbon dioxide and 95% air.

Production of hybridomas

A 5-week-old Sprague–Dawley rat was purchased from CLEA Japan (Tokyo, Japan). The animal was housed under specific pathogen-free conditions. All animal experiments were performed according to the relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. The Animal Care and Use Committee of Tohoku University (Permit No.: 2022MdA-001) approved animal experiments.

To develop mAbs against mCXCR4, we intraperitoneally immunized a rat with LN229/mCXCR4 cells (1×10^9 cells) plus Alhydrogel adjuvant 2% (InvivoGen, San Diego, CA). The procedure included three additional injections every week (1×10^9 cells), which were followed by a final booster intraperitoneal injection (1×10^9 cells), 2 days before harvesting splenocytes. The harvested splenocytes were subsequently fused with P3U1 cells, using PEG1500 (Roche Diagnostics, Indianapolis, IN). For the hybridoma selection, cells were cultured in the RPMI-1640 medium with 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B, 5 μg/mL of Plasmocin, 5% Briclone (NICB, Dublin, Ireland), and hypoxanthine, aminopterin and thymidine (Thermo Fisher Scientific, Inc.). The supernatants were subsequently screened by flow cytometry using CHO/mCXCR4.

Purification of Cx_4Mab-1

The cultured supernatants of Cx_4Mab-1-producing hybridomas were filtrated with Steritop (0.22 μm; Merck KGaA, Darmstadt, Germany). The filtered supernatants were subsequently applied to 1 mL of Capto L (Cytiva, Marlborough, MA). After washing with phosphate-buffered saline (PBS), bound antibodies were eluted with an IgG elution buffer (Thermo Fisher Scientific, Inc.), followed by immediate neutralization of eluates, using 1 M Tris-HCl (pH 8.0). Finally, the eluates were concentrated, after which PBS was replaced with the elution buffer using Amicon Ultra (Merck KGaA).

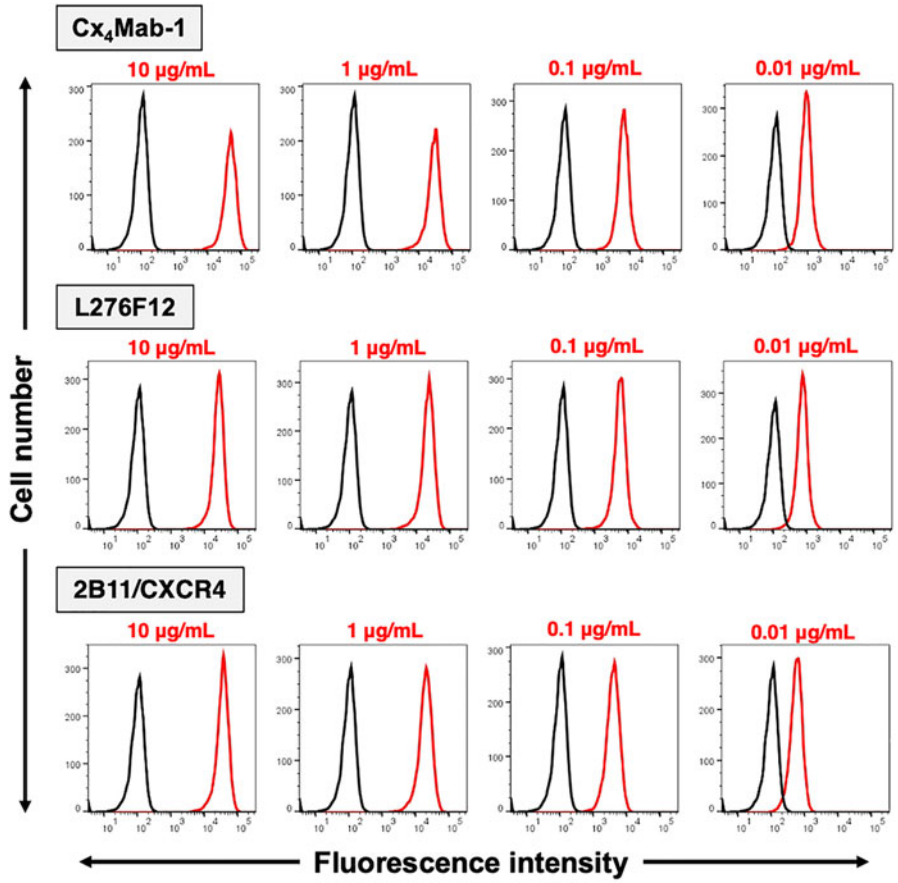

FIG. 2. Flow cytometry of mCXCR4-overexpressed cells using anti-mCXCR4 mAbs. CHO/mCXCR4 cells were treated with 0.01–10 μg/mL of Cx_4Mab-1, L276F12, or 2B11/CXCR4 followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. The red lines show the cells treated with each mAb. The black line shows the cells treated with blocking buffer and Alexa Fluor488-conjugated anti-rat IgG (negative control). CHO, Chinese hamster ovary; IgG, immunoglobulin G.

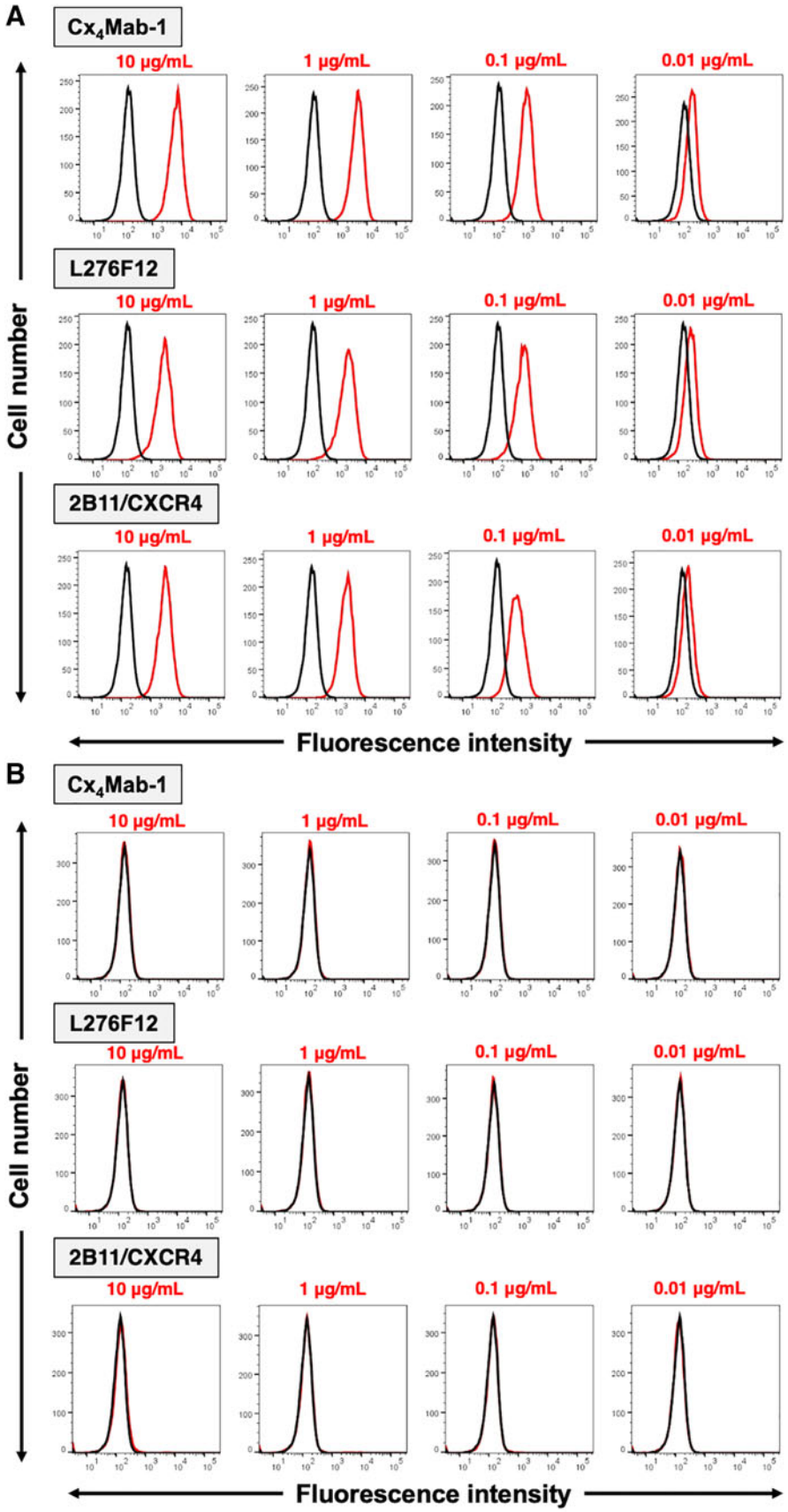

FIG. 3. Flow cytometry of endogenously mCXCR4-expressed cells using anti-mCXCR4 mAbs. P3U1 **(A)** and mCXCR4-knockout P3U1 (BINDS-56) **(B)** cells were treated with 0.01–10 μg/mL of Cx_4Mab-1, L276F12, or 2B11/CXCR4 followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. The red line shows the cells treated with each mAb. The black line shows the cells treated with blocking buffer and Alexa Fluor 488-conjugated anti-rat IgG (negative control).

Flow cytometry

CHO-K1 and CHO/mCXCR4 cells were harvested after a brief exposure to 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). The cells were washed with 0.1% bovine serum albumin in PBS and treated with 10 to 0.01 μg/mL anti-mCXCR4 mAbs (clone L276F12 and clone 2B11/CXCR4) and Cx_4Mab-1 for 30 min at 4°C. The cells were treated with 2 μg/mL Alexa Fluor 488-conjugated anti-rat IgG. The fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corporation, Tokyo, Japan).

To determine the dissociation constant (K_D), anti-mCXCR4 mAbs were serially diluted from 10 μg/mL to 0.61 ng/mL. The geometric mean of fluorescence intensities of CHO/mCXCR4 and P3U1 at each concentration was calculated by FlowJo v10.8.1 (Becton, Dickinson & Company, Ashland, OR). The K_D was estimated by fitting saturation binding curves to the built-in; one-site binding models in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

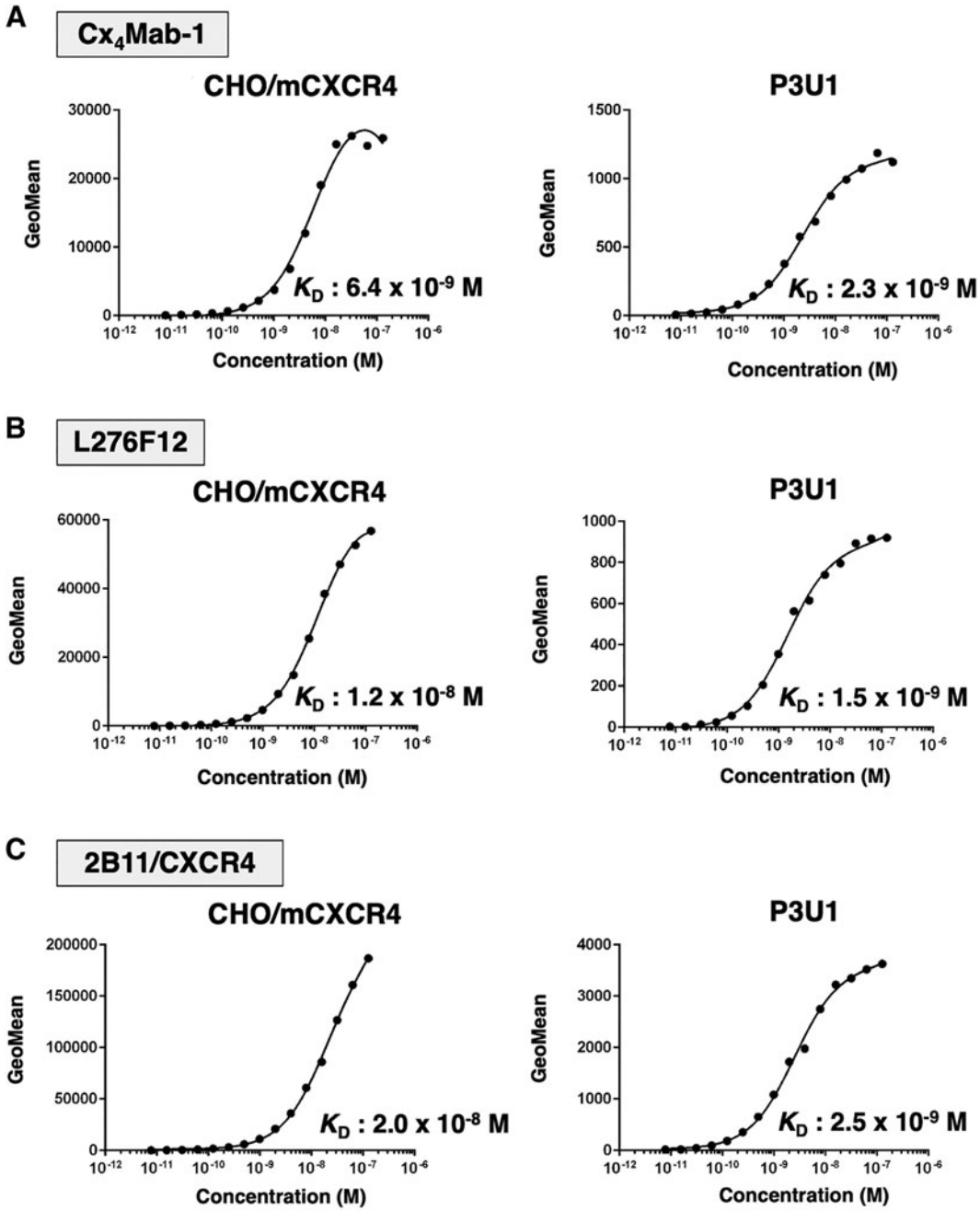

FIG. 4. Kinetic analyses of anti-mCXCR4 mAbs against mCXCR4-expressed cells through flow cytometry. The determination of the binding affinity of Cx_4Mab-1 **(A)**, L276F12 **(B)**, and 2B11/CXCR4 **(C)** against CHO/mCXCR4 or P3U1 cells by flow cytometry. The dots show the geometric mean of fluorescence intensity of CHO/mCXCR4 and P3U1 at each concentration. The solid lines are the fitting curve calculated by GraphPad PRISM 8.

Results

Establishment of a novel anti-mCXCR4 antibody

To establish anti-mCXCR4 mAbs, a rat was immunized with LN229/mCXCR4 cells (Fig. 1A). The hybridomas were produced by the fusion of the splenocytes with P3U1 cells using polyethylene glycol (Fig. 1B). The hybridomas, which are producing CHO/mCXCR4-reactive and CHO-K1-nonreactive mAbs, were selected by flow cytometry (Fig. 1C). After limiting dilution, a clone Cx_4Mab-1 (rat IgG_{2a}, kappa) was established (Fig. 1D).

Specificity of Cx4Mab-1 in flow cytometry

We performed flow cytometry using Cx_4Mab-1 and commercially available mAbs (clone L276F12 and clone 2B11/CXCR4) against CHO/mCXCR4 and P3U1 cells. Three mAbs reacted to CHO/mCXCR4 cells in a dose-dependent manner (Fig. 2). Although Cx_4Mab-1 and L276F12 did not bind to CHO-K1 cells even at 10 μg/mL, 2B11/CXCR4 reacted with CHO-K1 weakly (Supplementary Fig. S1). Furthermore, three mAbs reacted to P3U1 cells in a dose-dependent manner (Fig. 3A). In contrast, three mAbs did not bind to mCXCR4-knockout P3U1 (BINDS-56) cells even at 10 μg/mL (Fig. 3B). These results indicated that Cx_4Mab-1 recognizes both endogenous and exogenous mCXCR4-expressing cell lines.

Affinity of Cx4Mab-1 against mCXCR4-expressing cells

To determine the K_D of Cx_4Mab-1 to mCXCR4, we performed kinetic analysis via flow cytometry using CHO/mCXCR4 and P3U1 cells. The geometric mean of the fluorescence intensity of each concentration of Cx_4Mab-1 was plotted. The K_D values of Cx_4Mab-1 for CHO/mCXCR4 and P3U1 were determined as 6.4×10^{-9} M and 2.3×10^{-9} M, respectively (Fig. 4A). The K_D values of L276F12 for CHO/mCXCR4 and P3U1 were determined as 1.2×10^{-8} M and 1.5×10^{-9} M, respectively (Fig. 4B). The K_D values of 2B11/CXCR4 for CHO/mCXCR4 and P3U1 were determined as 2.0×10^{-8} M and 2.5×10^{-9} M, respectively (Fig. 4C). These results indicated that Cx_4Mab-1 possesses a high affinity to both endogenous and exogenous mCXCR4-expressing cells.

Discussion

In this study, we compared the sensitivity, specificity, and affinity of three anti-mCXCR4 mAbs (Cx_4Mab-1, L276F12, and 2B11/CXCR4) using flow cytometry. We found no difference in the sensitivity among three mAbs (Fig. 2). In contrast, 2B11/CXCR4 reacted with CHO-K1 weakly (Supplementary Fig. S1), indicating that 2B11/CXCR4 might cross-react with other molecules. Cx_4Mab-1 possesses the best affinity among three mAbs against CHO/mCXCR4 (Fig. 4A). In contrast, L276F12 shows the best affinity among three mAbs against P3U1 (Fig. 4B), indicating that Cx_4Mab-1 and L276F12 demonstrate better affinity than 2B11/CXCR4. Overall, three mAbs possess high sensitivity, and affinity against mCXCR4.

CXCL12-CXCR4 signaling contributes to tumor growth and metastasis in various types of cancer.[6] Therefore, CXCR4-targeted therapies have been developed. Ulocuplumab is a fully human IgG_4 anti-human CXCR4 (hCXCR4) mAb.[25] Ulocuplumab induced apoptosis of leukemia cells from chronic lymphocytic leukemia patients by blocking CXCL12 binding to hCXCR4. In addition, a phase Ib/II study in patients with relapsed/refractory multiple myeloma reported that the combination of ulocuplumab with lenalidomide and dexamethasone showed a high response rate (55.2%) and a clinical benefit rate (72.4%).[26] PF-06747143, another humanized IgG_1 anti-hCXCR4 mAb, also showed antitumor activities in multiple hematologic cancer models.[27]

A potential side effect of anti-CXCR4 therapy is the toxicity to normal leukocytes and hematopoietic stem cells (HSCs). The CXCL12/CXCR4 axis is essential for hematopoiesis in fetuses and adults. Deficiencies of CXCR4 or CXCL12 exhibit hematopoietic defects in fetal mice.[9,10] Studies using CXCR4 conditional knockout mice demonstrated that CXCL12/CXCR4 axis also plays critical roles in hematopoiesis in adult.[28] In the bone marrow, CXCL12-CXCR4 signaling tethers HSCs in the niches where the quiescent HSC pool is maintained by supplying the requisite factors. AMD3100 (Plerixafor/Mozobil), an antagonist of CXCR4, induces HSC mobilization from bone marrow to peripheral blood.[29] Cx_4Mab-1 could be a useful tool for developing anti-CXCR4 therapies in preclinical murine models.

Authors' Contributions

T.O., H.S., and T.T. performed the experiments. M.K.K. and Y.K. designed the experiments. H.S. and M.K.K. analyzed the data. T.O. and Y.K. wrote the article. All authors have read and agreed to the published version of the article.

Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1

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