

Communication

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Communication

Cx4Mab-1: A Novel Anti-Mouse CXCR4 Monoclonal Antibody for Flow Cytometry

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Abstract: 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 (CBIS) method. Flow cytometric analysis showed that an anti-mCXCR4 mAb, Cx4Mab-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, Cx4Mab-1 did not recognize mCXCR4-knockout P3U1 cells. The dissociation constants of Cx4Mab-1 for CHO/mCXCR4 and P3U1 were determined as 6.4×10^{-9} M and 2.3×10^{-9} M, respectively, indicating that Cx4Mab-1 possesses a high affinity to both endogenous and exogenous mCXCR4-expressing cells. These results indicate that Cx4Mab-1 could be a useful tool for preclinical mouse models.

Keywords: mouse CXCR4; monoclonal antibody; CBIS method

1. Introduction

C-X-C motif 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 the 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 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. In this report, we established a novel anti-mCXCR4 mAb using the CBIS method and evaluated its applications.

2. Materials and Methods

2.1. Antibodies and animals

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 IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

We purchased one five-week-old Sprague-Dawley rat from CLEA Japan (Tokyo, Japan). Animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Approved number: 2022MdA-001).

2.2. Cell lines

We obtained LN229, P3X63Ag8U.1 (P3U1), and Chinese hamster ovary (CHO)-K1 cells from the American Type Culture Collection (Manassas, VA). We synthesized DNA (Eurofins Genomics KK) encoding mCXCR4 (Accession No.: NM_009911.3) and subcloned it into a pCAGzeo PAcH vector. PA tag, twelve amino acids (GVAMPGAEDDVV).[24], was added to the C-terminus of mCXCR4. The mCXCR4-PA plasmid was transfected into LN229 and CHO-K1 cells using a Neon transfection system (Thermo Fisher Scientific Inc., Waltham, MA). We established stable transfectants using a cell sorter (SH800; Sony Corp., Tokyo, Japan) after staining them by an anti-mCXCR4 mAb (clone L276F12). We cultivated them in a medium, containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA). We purchased GeneArt™ CRISPR nuclease vectors with OFP plasmid which target mCXCR4 (CGGCAATGGATTGGTGATCC) from Thermo Fisher Scientific Inc. The knockout plasmid was transfected into P3U1 cells. mCXCR4 knockout P3U1 was established by a cell sorter (SH800), and named as BINDS-56 (http://www.med-tohoku-antibody.com/topics/001_paper_cell.htm).

All cells were grown in a humidified incubator at 37°C, in an atmosphere of 5% CO₂ and 95%

2.3. Production of hybridomas

For developing anti-mCXCR4 mAbs, a rat was immunized intraperitoneally with 1×10^9 cells of LN229/mCXCR4. We added Alhydrogel adjuvant 2% (InvivoGen, San Diego, CA) as an adjuvant in the first immunization. Three additional injections of 1×10^9 cells were performed without an adjuvant every week. We performed a final booster immunization of 1×10^9 cells intraperitoneally two days before harvesting splenocytes. We fused the harvested splenocytes with P3U1 cells using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN). Hybridoma cells were cultured in the RPMI-1640 medium, supplemented as shown above. We further added 5 µg/mL of Plasmocin, 5% Briclone (NICB, Dublin, Ireland), and hypoxanthine, aminopterin and thymidine (HAT; Thermo Fisher Scientific, Inc.) into the medium. The supernatants were screened by flow cytometry using CHO/mCXCR4. The cultured supernatants of hybridomas were filtrated and purified using *Capto L* (Cytiva, Marlborough, MA).

2.4. Flow cytometry

We harvested CHO-K1 and CHO/mCXCR4 cells using 1 mM EDTA. The cells and P3U1 and BINDS-56 were washed with 0.1% bovine serum albumin (BSA) in PBS and reacted with 10 to 0.01 µg/mL of anti-mCXCR4 mAbs or blocking buffer (control) for 30 min at 4°C. The cells were treated with 2 µg/mL of anti-rat IgG conjugated with Alexa Fluor 488. The fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corporation, Tokyo, Japan).

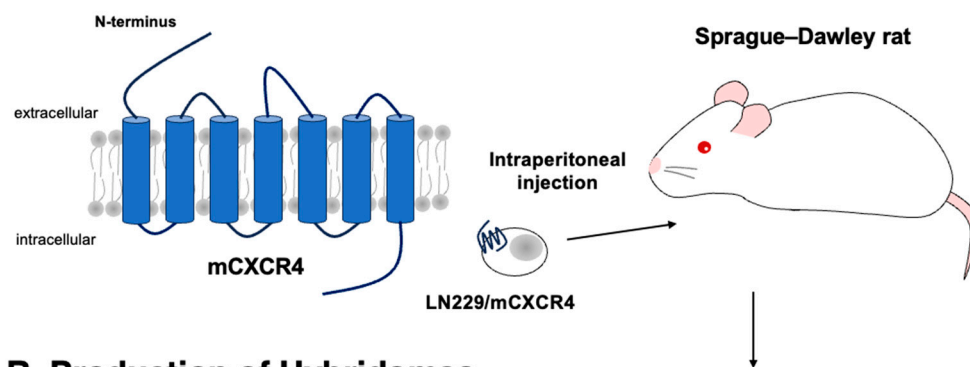
A nti-mCXCR4 mAbs were serially diluted from 10 $\mu\text{g/mL}$ to 0.61 ng/mL to determine the dissociation constant (K_D). We used FlowJo v10.8.1 (Becton, Dickinson & Company, Ashland, OR) to calculate the geometric mean of fluorescence intensities of CHO/mCXCR4 and P3U1 at each concentration. The 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).

3. Results

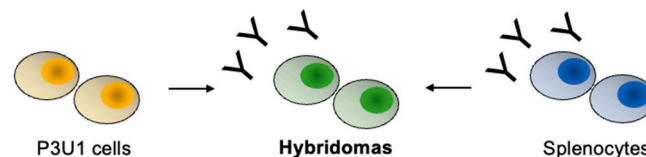
3.1. Establishment of a novel anti-mouse CXCR4 (mCXCR4) antibody

For developing anti-mCXCR4 mAbs, we immunized one rat with LN229/mCXCR4 cells as depicted in Figure 1A. The hybridomas were produced by fusion of the splenocytes with P3U1 cells using PEG1500 (Figure 1B). CHO/mCXCR4-reactive and CHO-K1-non-reactive mAbs-producing hybridomas were selected by flow cytometry (Figure 1C). After limiting dilution, a clone Cx4Mab-1 (rat IgG_{2a}, kappa) was successfully developed (Figure 1D).

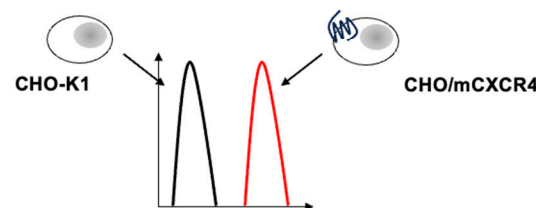
A. Immunization of LN229/mCXCR4



B. Production of Hybridomas



C. Screening of supernatants by flow cytometry



D. Cloning of Hybridomas

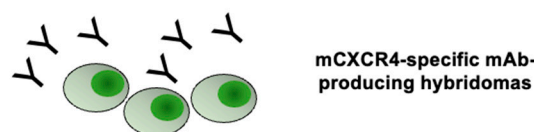


Figure 1. The scheme of establishment of Cx4Mab-1 by CBIS method. (A) We immunized LN229/mCXCR4 cells into a Sprague-Dawley rat intraperitoneally. (B) We fused the splenocytes with P3U1 cells. (C) The culture supernatants were screened by flow cytometry for selecting anti-mCXCR4 mAb-producing hybridomas. (D) Cx4Mab-1 was developed by limiting dilution.

3.2. Specificity of Cx₄Mab-1 in flow cytometry

We performed flow cytometry using Cx₄Mab-1 against CHO/mCXCR4 and P3U1 cells. We further compared its reactivity with commercially available anti-CXCR4 mAbs (clone L276F12 and clone 2B11/CXCR4). Three mAbs reacted to CHO/mCXCR4 cells in a dose-dependent manner (Figure 2). Although Cx₄Mab-1 and L276F12 did not bind to CHO-K1 cells even at 10 $\mu\text{g/mL}$, 2B11/CXCR4 reacted with CHO-K1 weakly (Supplementary Figure 1). Furthermore, three mAbs reacted to P3U1 cells dose-dependently (Figure 3A). In contrast, three mAbs did not bind to mCXCR4-knockout P3U1 (BINDS-56) cells even at 10 $\mu\text{g/mL}$ (Figure 3B). These results indicated that Cx₄Mab-1 recognizes both endogenous and exogenous mCXCR4-expressing cell lines.

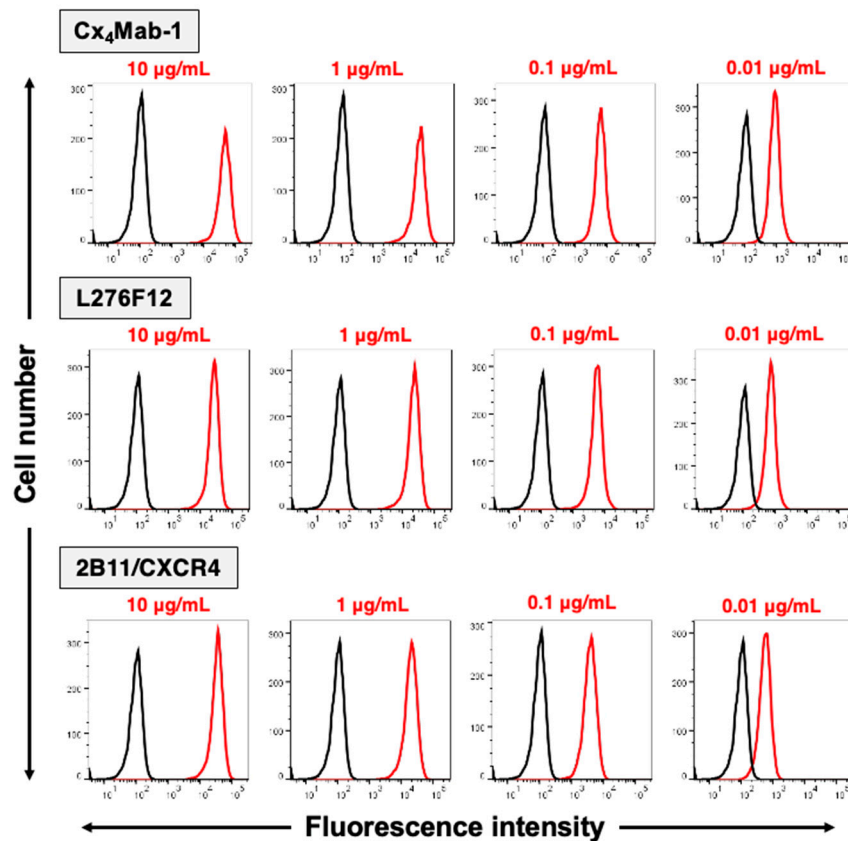


Figure 2. Flow cytometry of mCXCR4-overexpressed cells using anti-mCXCR4 mAbs. CHO/mCXCR4 cells were treated with 0.01–10 $\mu\text{g/mL}$ of Cx₄Mab-1, L276F12, or 2B11/CXCR4 followed by treatment with the secondary antibody. The red lines show the cells treated with each mAb. The black line shows the cells treated with blocking buffer and the secondary antibody (negative control).

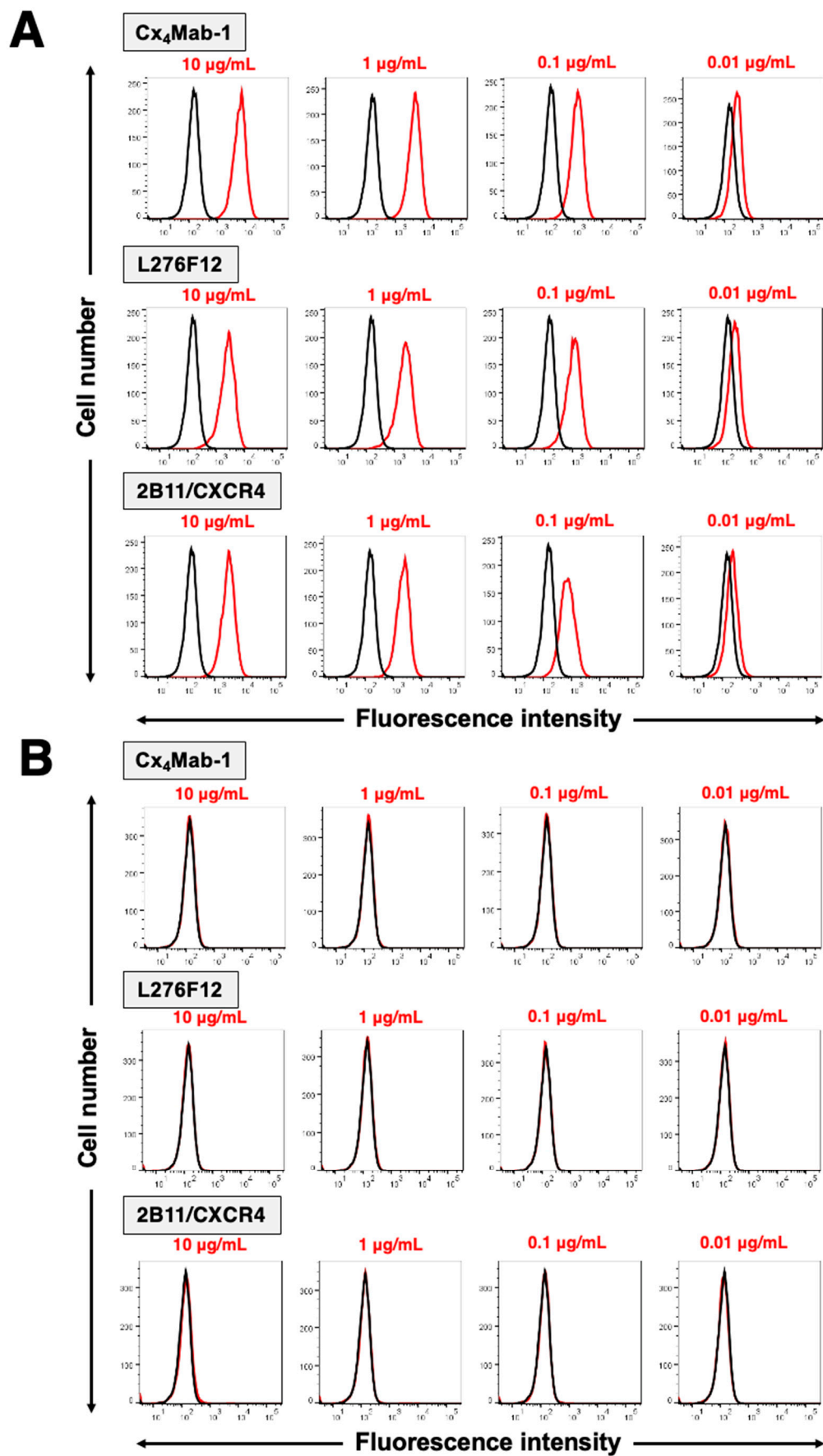


Figure 3. Flow cytometry of endogenously mCXCR4-expressed cells using anti-mCXCR4 mAbs. P3U1 (A) and mCXCR4-knockout P3U1 (BINDS-56) (B) cells were treated with 0.01–10 µg/mL of Cx₄Mab-1, L276F12, or 2B11/CXCR4 followed by treatment with the secondary antibody. The red line

shows the cells treated with each mAb. The black line shows the cells treated with blocking buffer and the secondary antibody (negative control).

3.3. Affinity of Cx₄Mab-1 against mCXCR4-expressing cells

For determining the K_D of Cx₄Mab-1 to mCXCR4, the kinetic analysis was performed using flow cytometry against CHO/mCXCR4 and P3U1 cells. The geometric mean of Cx₄Mab-1-treated cells was plotted. The K_D values of Cx₄Mab-1 for CHO/mCXCR4 and P3U1 were determined as 6.4×10^{-9} M and 2.3×10^{-9} M, respectively (Figure 4A). The K_D of L276F12 for CHO/mCXCR4 and P3U1 were determined as 1.2×10^{-8} M and 1.5×10^{-9} M, respectively (Figure 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 (Figure 4C). These results indicated that Cx₄Mab-1 possesses a high affinity to both endogenous and exogenous mCXCR4-expressing cells.

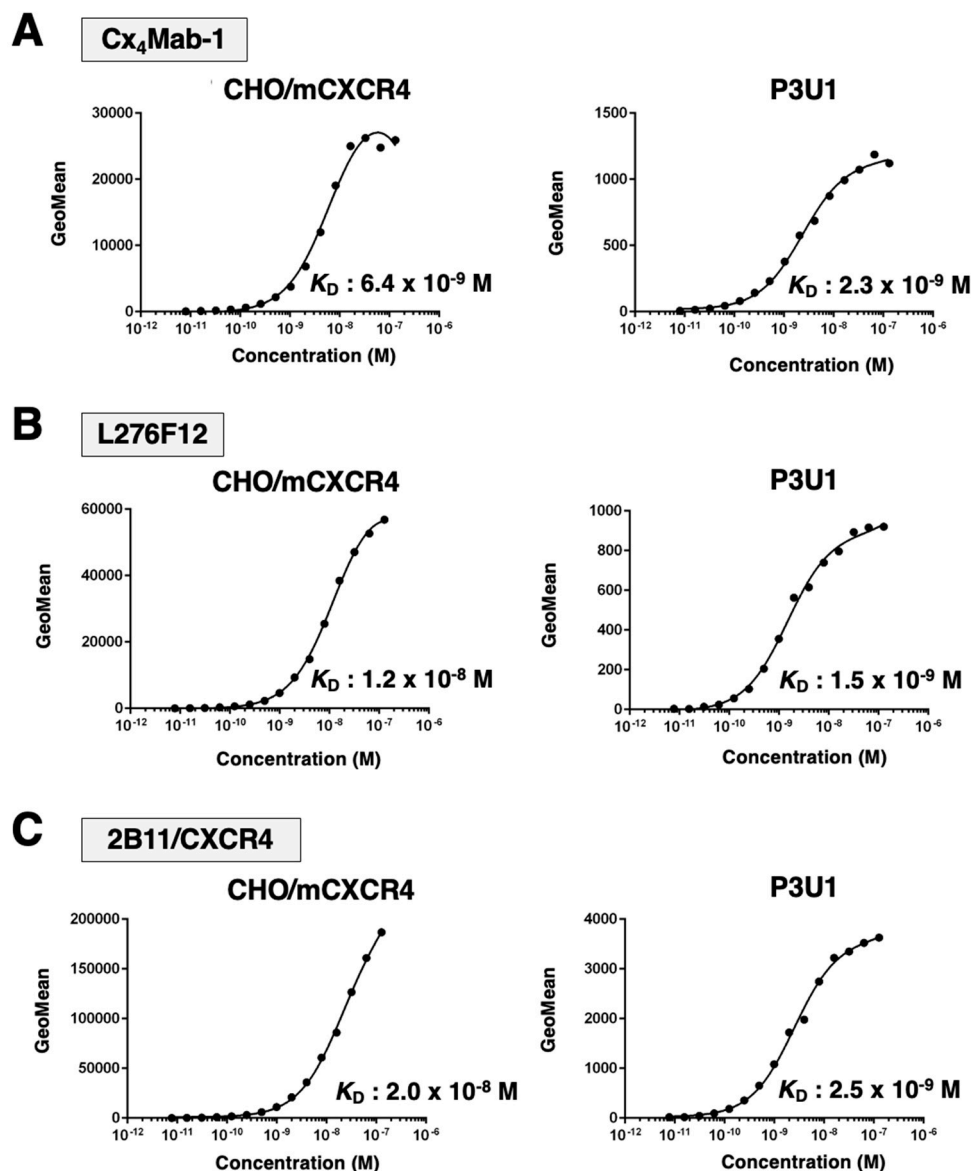


Figure 4. Kinetic analyses of anti-mCXCR4 mAbs against mCXCR4-expressed cells through flow cytometry. The determination of the binding affinity of Cx₄Mab-1 (A), L276F12 (B), and 2B11/CXCR4 (C) against CHO/mCXCR4 or P3U1 cells by flow cytometry. The dots show the geometric mean of fluorescence intensity of CHO/mCXCR4 and P3U1 at each concentration. The solid lines are the fitting curve calculated by GraphPad PRISM 8.

4. Discussion

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 IgG₄ anti-human CXCR4 (hCXCR4) mAb [25]. Ulocuplumab induced apoptosis of leukemia cells from chronic lymphocytic leukemia (CLL) patients by blocking CXCL12 binding to hCXCR4. Additionally, 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 IgG₁ 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. 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 hematopoietic stem cells (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]. Cx4Mab-1 could be a useful tool for developing anti-CXCR4 therapies in preclinical murine models.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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