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Cx_1Mab-1: A Novel Anti-mouse CXCR1 Monoclonal Antibody for Flow Cytometry

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The C-X-C motif chemokine receptor-1 (CXCR1) is a rhodopsin-like G-protein-coupled receptor, expressed on the cell surface of immune cells and tumors. CXCR1 interacts with some C-X-C chemokines, such as CXCL6, CXCL7, and CXCL8/interleukin-8, which are produced by various cells. Since CXCR1 is involved in several diseases including tumors and diabetes mellitus, drugs targeting CXCR1 have been developed. Therefore, the development of sensitive monoclonal antibodies (mAbs) for CXCR1 has been desired for the diagnosis and treatment. This study established a novel anti-mouse CXCR1 (mCXCR1) mAb, Cx_1Mab-1 (rat IgG_1, kappa), using the Cell-Based Immunization and Screening method. Cx_1Mab-1 reacted with mCXCR1-overexpressed Chinese hamster ovary-K1 (CHO/mCXCR1) and mCXCR1-overexpressed LN229 glioblastoma (LN229/mCXCR1) in flow cytometry. Cx_1Mab-1 demonstrated a high binding affinity for CHO/mCXCR1 and LN229/mCXCR1 with a dissociation constant of 2.6×10^{-9} M and 2.1×10^{-8} M, respectively. Furthermore, Cx_1Mab-1 could detect mCXCR1 by Western blot analysis. These results indicated that Cx_1Mab-1 is useful for detecting mCXCR1, and provides a possibility for targeting mCXCR1-expressing cells *in vivo* experiments.

Keywords: mouse CXCR1, CBIS method, monoclonal antibody, flow cytometry

Introduction

Chemokines can be classified into four different subfamilies: C-C, C-X-C, C-X3-C, and X-C, depending on the number and position of cysteine residues in their N-terminus. C-X-C means the first two cysteine amino acids are divided by other amino acids. Chemokines play pivotal roles in immune responses, such as infiltration and migration of immune cells by binding to its cognate receptors.[1–3]

The C-X-C motif chemokine receptor 1 (CXCR1), also referred to as C-X-C motif chemokine ligand 8 (CXCL8) receptor/interleukin-8 (IL-8) receptor alpha, is one of the G-protein coupled receptors (GPCRs) composed of 350 amino acids with structural 7 transmembrane domains.[4] CXCL6 and CXCL7 are also the chemokine ligands of CXCR1.[5] At the nearly same time that CXCR1 was discovered, the C-X-C motif chemokine receptor 2 (CXCR2), a receptor for CXCL6, CXCL7, and IL-8, was also identified.[6–8]

CXCR2 is also a receptor for CXCL2 and CXCL3 and has 77% amino acids homology with CXCR1.[9] Various cells express CXCR1/CXCR2 on the cell surface, including macrophages, neutrophils, basophils, natural killer cells, mast cells, and other leukocytes.[10,11] Furthermore, CXCR1/CXCR2 expression has also been confirmed in nonimmune cells, such as endothelial cells, fibroblasts, and tumor cells.[5,12] Owing to their homology and ligand overlapping, the functions of CXCR1/CXCR2 have been clarified in many diseases.[13]

IL-8 plays a pivotal role in the tumor microenvironment (TME) as a factor of immunosuppressive cells' recruitment, angiogenesis, and induction of epithelial-to-mesenchymal transition.[4] Functional heterogeneity of cancer-associated fibroblasts (CAFs) is regulated by IL-8 and other CXCR1/2 ligands.[14] The normal ovarian fibroblast is converted to CAFs

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by IL-8 and triggers tumor growth in mice. Aberrant CXCR1/2 ligands expression in CAFs is closely related to gastric cancer progression and poor prognosis in patients.[15] In TME of glioma, IL-8^{+}CD4^{+} T cells function in a complex manner with other IL-8-producing cells to govern the immunosuppressive properties.[16]

IL-8 expression is positively correlated with CXCR1/2 and might be a predictive marker of poor prognosis in glioma, metastatic urothelial carcinoma, and metastatic renal cell carcinoma patients.[17,18] Furthermore, the blockade of the IL-8-CXCR1/2 axis enhances the anti-programmed cell death-1 (PD-1) therapeutic effects on glioma.[19] Thus, CXCR1/2 molecules are promising novel cancer therapeutic targets for tumors.[20] Reparixin, a noncompetitive allosteric inhibitor of CXCR1/2, could prevent polymorphonuclear cell recruitment.[21]

A phase 2 clinical trial using navarixin (SCH-527123), a selective CXCR1/2 antagonist, has been conducted in combination with anti-PD-1 mAb, pembrolizumab, in advanced solid tumors.[22,23] Navarixin blocks neutrophil recruitment and suppresses the tumor cell growth in a murine melanoma model. CXCR1/2 are attractive targets for treatment against multiple cancers.

CXCR1/2 and their chemokine ligands regulate the immune system, which mediates the progression of diseases such as cancer. Therefore, CXCR1/2-targeting antibodies will contribute to the diagnosis and therapy. Using the Cell-Based Immunization and Screening (CBIS) method, we have developed many monoclonal antibodies (mAbs) against C-C chemokine receptors, including mouse CCR3,[24] mouse CCR8,[25–27] and human CCR9[28] mAbs. Next, we have been trying to establish the anti-C-X-C motif chemokine receptor mAbs.

We recently obtained an anti-mouse CXCR3 mAb,[29] an anti-mouse CXCR4 mAb,[30] and an anti-mouse CXCR6 mAb.[31] In this study, we have successfully developed anti-mouse CXCR1 (mCXCR1) mAbs using the CBIS method that is applicable to flow cytometry and Western blot analysis.

Materials and Methods

Cell lines

LN229, Chinese hamster ovary (CHO)-K1, and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). pCMV6neo-myc-DDK vector with mCXCR1 (Accession No.: NM_178241) was purchased from OriGene Technologies, Inc., (Rockville, MD). The plasmid was transfected into the cell lines using a Neon Transfection System (Thermo Fisher Scientific, Inc., Waltham, MA). Subsequently, LN229 and CHO-K1, which stably overexpressed mCXCR1 with C-terminal myc-DDK tags (hereinafter described as LN229/mCXCR1 and CHO/mCXCR1, respectively) were established using a cell sorter (SH800; Sony Corp., Tokyo, Japan), after cultivation in a medium containing 0.5 mg/mL G418 (Nacalai Tesque, Inc., Kyoto, Japan).

CHO-K1, P3U1, and CHO/mCXCR1 were also cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc.) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). LN229 and LN229/mCXCR1 were cultured in a Dulbecco's modified Eagle's medium (Nacalai Tesque, Inc.) that was supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). Then, cells were grown in a humidified incubator, which was supplied with 5% CO_2 and 95% air at 37°C.

Antibodies

The anti-mCXCR1/IL-8 RA mAb (clone 1122A) was purchased from R&D Systems, Inc., (Minneapolis, MN). An anti-DYKDDDDK mAb (clone 1E6) and an anti-β-actin mAb (clone AC-15) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and Sigma-Aldrich Corp. (St. Louis, MO), respectively. Alexa Fluor 488-conjugated anti-rat IgG and Alexa Fluor 488-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology, Inc., (Danvers, MA).

Hybridoma production

The animal was housed under specific pathogen-free conditions. All animal experiments were approved by the

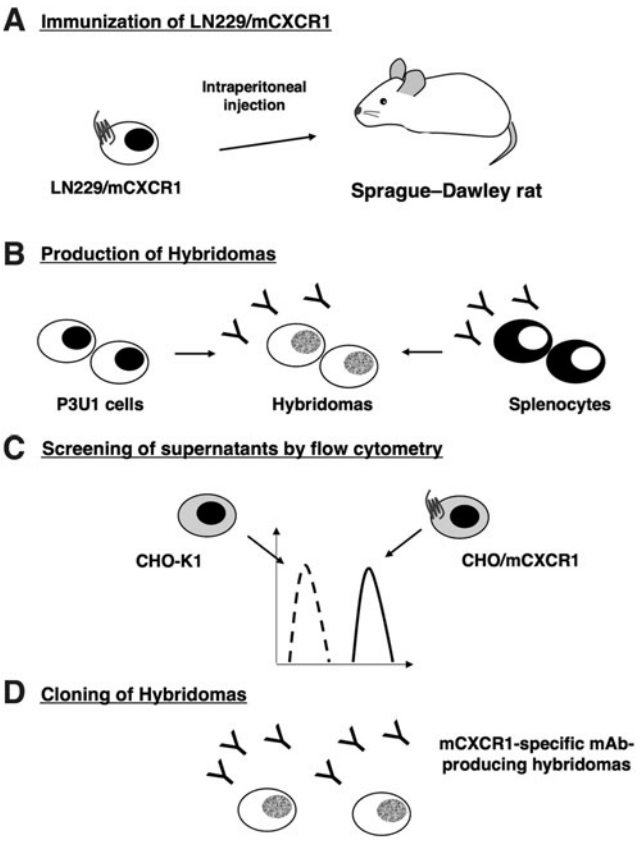

FIG. 1. A schematic procedure of anti-mCXCR1 mAbs production. The procedure of CBIS. **(A)** LN229/mCXCR1 cells were immunized into a Sprague-Dawley rat using intraperitoneal injection. **(B)** The harvested splenocytes were fused with P3U1 cells using polyethylene glycol 1500. **(C)** The culture supernatants of hybridoma were screened by flow cytometry. **(D)** Cx_1Mab-1 was established by limiting dilution. CBIS, Cell-Based Immunization and Screening; mCXCR, mouse C-X-C motif chemokine receptor.

Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

For developing anti-mCXCR1 mAbs, a 6-week-old female Sprague-Dawley rat (CLEA Japan, Tokyo, Japan) was immunized intraperitoneally with 1×10^9 cells of LN229/mCXCR1. The immunogen was harvested after brief exposure to 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). We added Alhydrogel adjuvant 2% (InvivoGen, San Diego, CA) as an adjuvant in the first immunization. Three additional injections of 1×10^9 cells of LN229/mCXCR1 were performed without an adjuvant every week. We performed a final booster immunization of 1×10^9 cells of LN229/mCXCR1 intraperitoneally 2 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 hypoxanthine, aminopterin, and thymidine (Thermo Fisher Scientific, Inc.), 5% Briclone (NICB, Dublin, Ireland), and 5 μg/mL of plasmocin into the medium. The hybridoma supernatants were screened by flow cytometry using CHO/mCXCR1 and parental CHO-K1. The culture supernatants of hybridomas were filtrated and purified using Ab-Capcher Extra (ProteNova, Kagawa, Japan).

Flow cytometry

CHO-K1 and CHO/mCXCR1 cells were harvested after brief exposure to 2.5 g/L-Trypsin/1 mmol/L-EDTA solution with phenol red (Nacalai Tesque, Inc.). CHO-K1, CHO/mCXCR1, LN229, and LN229/mCXCR1 cells were washed with 0.1% bovine serum albumin in phosphate-buffered saline (PBS) and treated with primary mAbs for 30 min at 4°C. Afterward, cells were treated with Alexa Fluor 488-conjugated

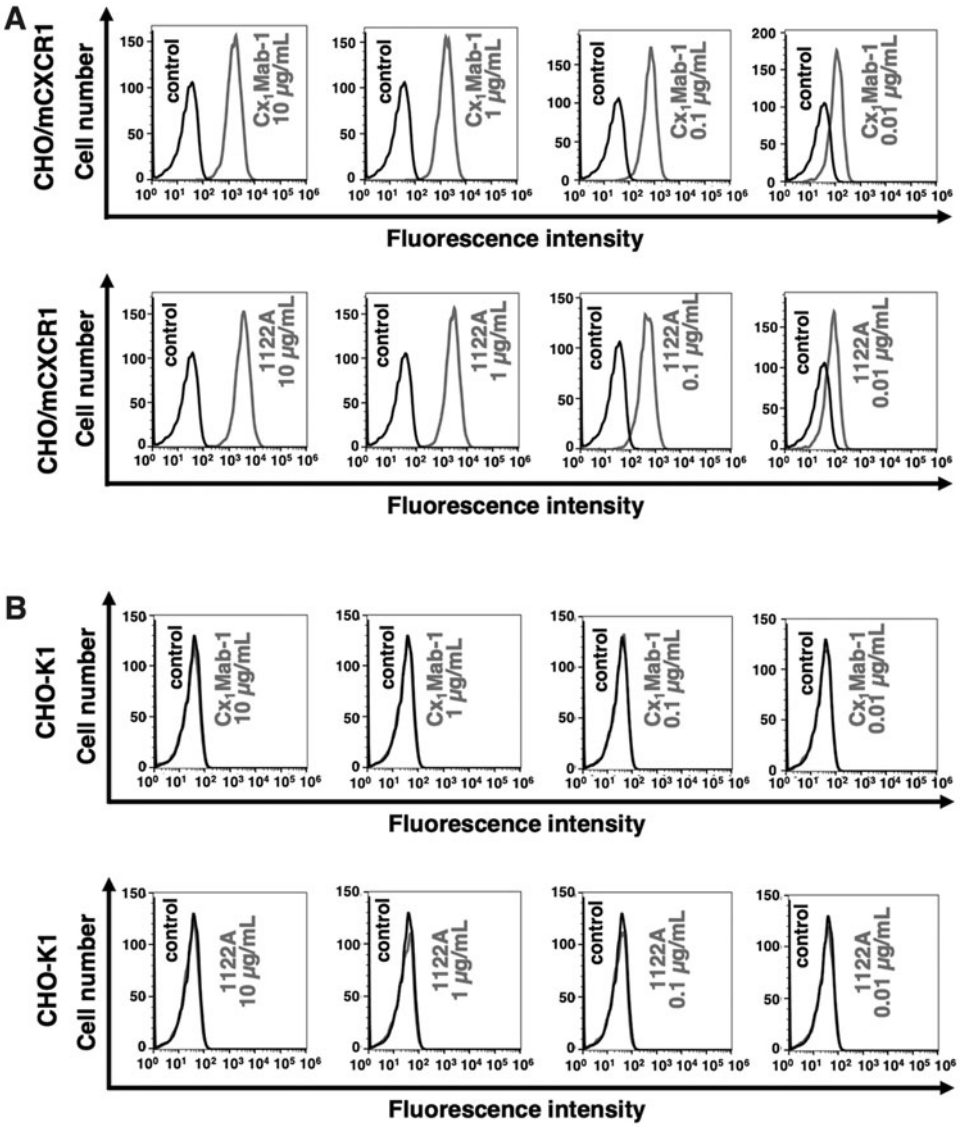

FIG. 2. Flow cytometric analysis of anti-mCXCR1 mAbs against CHO/mCXCR1 and CHO-K1. CHO/mCXCR1 **(A)** and CHO-K1 cells **(B)** were treated with 0.01–10 μg/mL of Cx_1Mab-1 and 1122A, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG and Alexa Fluor 488-conjugated anti-rabbit IgG, respectively. CHO, Chinese hamster ovary.

anti-rat IgG (1:1000) or Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000). The fluorescence data was collected using an SA3800 Cell Analyzer (Sony Corp.).

Determination of the binding affinity by flow cytometry

CHO/mCXCR1 and LN229/mCXCR1 were suspended in 100 μL serially diluted anti-mCXCR1 mAbs (25 to 0.006 μg/mL), after which Alexa Fluor 488-conjugated anti-rat IgG (1:200) or Alexa Fluor 488-conjugated anti-rabbit IgG (1:200) was added. Fluorescence data were subsequently collected, using the SA3800 Cell Analyzer (Sony Corp.). The dissociation constant (K_D) was calculated by fitting the binding isotherms into the built-in one-site binding model in GraphPad PRISM 10 (GraphPad Software, Inc., La Jolla, CA).

Western blot analysis

Cell lysates were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.), after which proteins were separated on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred to polyvinylidene difluoride membranes (Merck KGaA). After blocking with 4% skim milk (Nacalai Tesque, Inc.) in 0.05% Tween 20-containing PBS, membranes were incubated with 1 μg/mL of Cx_1Mab-1, 1 μg/mL of an anti-mCXCR1/IL-8 RA mAb (clone 1122A), 1 μg/mL of an anti-DYKDDDDK mAb (clone 1E6), or 1 μg/mL of an anti-β-actin mAb (clone AC-15).

Then, they were incubated again with horseradish peroxidase-conjugated anti-rat immunoglobulins (for Cx_1Mab-1; diluted 1:10,000; Sigma-Aldrich Corp.), anti-mouse immunoglobulins (for anti-DYKDDDDK and anti-β-actin; diluted 1:1000; Agilent Technologies, Inc., Santa Clara, CA), or anti-rabbit immunoglobulins (for 1122A; diluted 1:1000; Agilent Technologies, Inc.). Finally, protein bands were detected using ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc.) or ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) with a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

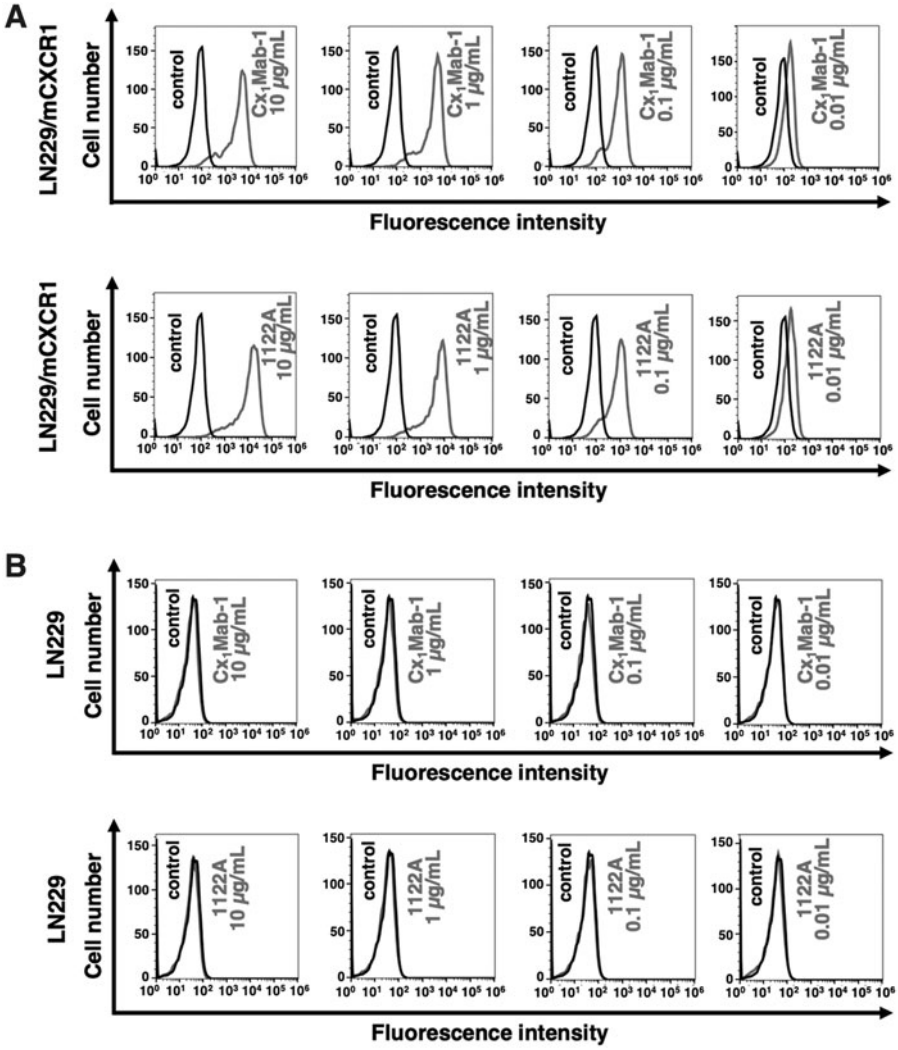

FIG. 3. Flow cytometric analysis of anti-mCXCR1 mAbs against LN229/mCXCR1 and LN229. LN229/mCXCR1 cells **(A)** and LN229 cells **(B)** were treated with 0.01–10 μg/mL of Cx_1Mab-1 and 1122A, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG and Alexa Fluor 488-conjugated anti-rabbit IgG, respectively.

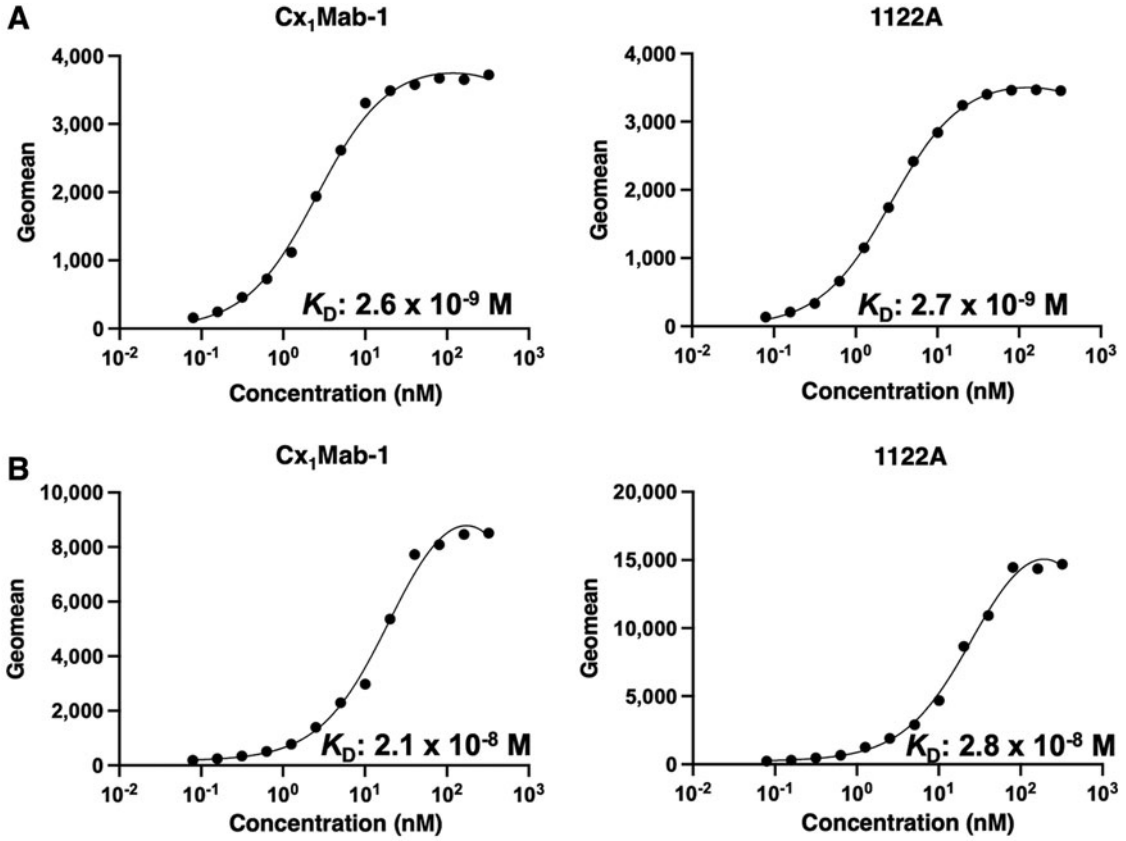

FIG. 4. The determination of the binding affinity of anti-mCXCR1 mAbs. CHO/mCXCR1 **(A)** or LN229/mCXCR1 **(B)** cells were suspended in 100 μL serially diluted Cx_1Mab-1 or 1122A (25 to 0.006 μg/mL). Then, cells were treated with Alexa Fluor 488-conjugated anti-rat IgG or Alexa Fluor 488-conjugated anti-rabbit IgG. Fluorescence data were subsequently collected using the SA3800 Cell Analyzer. The dissociation constant (K_D) was calculated by GraphPad PRISM 10.

Results

Establishment of anti-mCXCR1 mAbs

To develop anti-mCXCR1 mAbs, we employed the CBIS method using mCXCR1-overexpressed cells. Hybridoma screening was conducted using flow cytometry (Fig. 1). A rat was intraperitoneally immunized with LN229/mCXCR1. Subsequently, hybridomas were seeded into 96-well plates, after which flow cytometric analysis was used to extract CHO/mCXCR1-reactive and CHO-K1-nonreactive supernatants. Afterward, we obtained CHO/mCXCR1-reactive supernatants in 78 of 958 wells (8.1%). We finally established clone Cx_1Mab-1 (rat IgG_1, kappa) by limiting dilution and additional screening.

Flow cytometric analysis

Flow cytometric analysis was conducted using Cx_1Mab-1 and another anti-mCXCR1/IL-8 RA mAb (clone 1122A from R&D Systems, Inc.) against CHO-K1, CHO/mCXCR1, LN229, and LN229/mCXCR1. Results showed that Cx_1Mab-1 and 1122A recognized CHO/mCXCR1 dose dependently, but not parental CHO-K1 cells (Fig. 2). Both mAbs also recognized LN229/mCXCR1 dose dependently, but not parental LN229 cells (Fig. 3). Both Cx_1Mab-1 and 1122A reacted with CHO/mCXCR1 and LN229/mCXCR1 even at 0.01 μg/mL concentration (Figs. 2, 3), indicating that both mAbs are highly sensitive.

Determination of the binding affinity of Cx1Mab-1

The binding affinity of Cx_1Mab-1 and 1122A was assessed with CHO/mCXCR1 and LN229/mCXCR1 using flow cytometry. Results showed that the K_D of Cx_1Mab-1 for CHO/mCXCR1 and LN229/mCXCR1 was 2.6×10^{-9} M and 2.1×10^{-8} M, respectively (Fig. 4). In contrast, the K_D of 1122A for CHO/mCXCR1 and LN229/mCXCR1 was 2.7×10^{-9} M and 2.8×10^{-8} M, respectively (Fig. 4). These results indicate that Cx_1Mab-1 possesses a moderate affinity for exogenously overexpressed mCXCR1 in CHO-K1 and LN229 cells.

Western blot analysis

Western blot analysis was performed to further assess the availability of Cx_1Mab-1. Lysates of CHO-K1, CHO/mCXCR1, LN229, and LN229/mCXCR1 cells were probed. As demonstrated in Figure 5, Cx_1Mab-1 detected the ~48-kDa band of mCXCR1 in lysates from CHO/mCXCR1 and LN229/mCXCR1 cells, whereas this band was not present in lysates from CHO-K1 and LN229 cells, indicating that

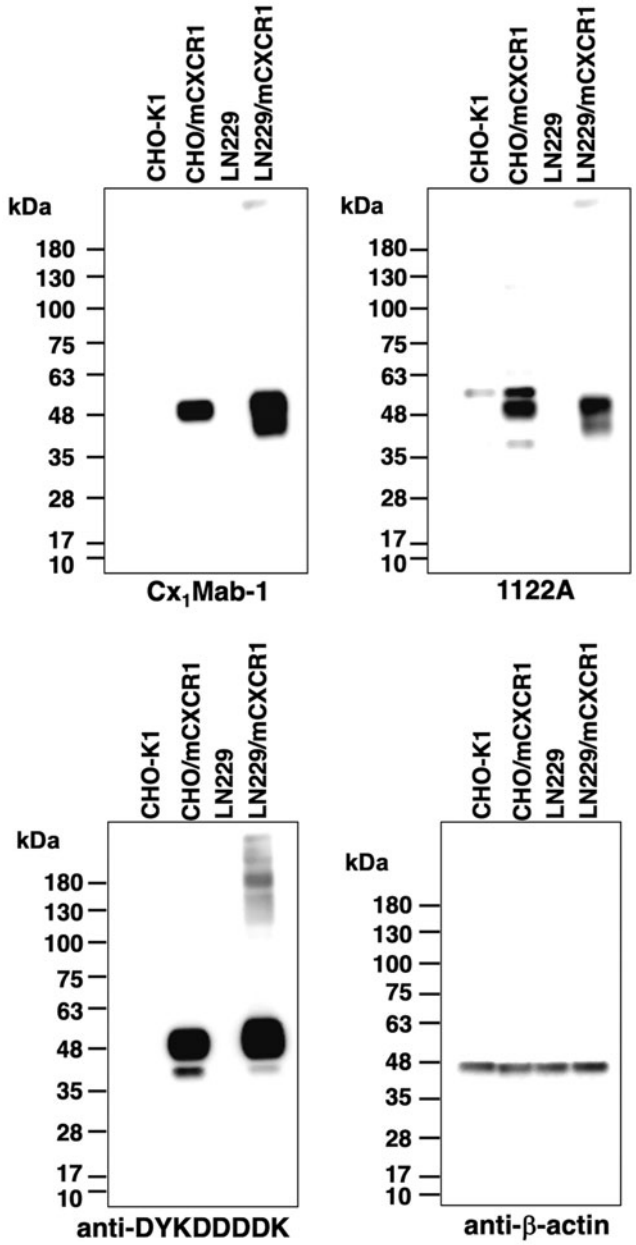

FIG. 5. Western blot analysis using anti-mCXCR1 mAbs. Cell lysates (10 μg) of CHO-K1, CHO/mCXCR1, LN229, and LN229/mCXCR1 cells were electrophoresed and proteins were transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with 1 μg/mL of Cx_1Mab-1, 1122A, an anti-DYKDDDDK mAb (1E6), or an anti-β-actin mAb (AC-15), and then incubated with peroxidase-conjugated anti-rat IgG or anti-mouse IgG.

Cx_1Mab-1 specifically detects mCXCR1. Clone 1122A showed a weak nonspecific band in CHO-K1 (Fig. 5); therefore, Cx_1Mab-1 is more specific than clone 1122A in Western blot analysis.

Discussion

The TME is composed of various cells including cancer cells, immune cells, and stromal cells.[32] Chemokines and their receptors expressed in these cells modulate a complex TME.[23,32] Neutrophil is one of the major mediators of inflammation. IL-8 secreted from $CD4^+$ T cells recruited CXCR1/2-expressed neutrophils to the inflammation site.[33] The neutrophils promote inflammation by generating reactive oxygen species.[19,34] In this study, we established Cx_1Mab-1 that recognizes mCXCR1 with high sensitivity (Figs. 2, 3). Therefore, Cx_1Mab-1 would contribute to detecting and isolating the mCXCR1-positive lymphocytes.

Tumor-associated macrophage (TAM) plays a vital role as a tumor immune regulator in TME.[35] TAM produces IL-8 that attenuates cancer immunity by suppressing the infiltration of $CD8^+$ T cells and enhances programmed cell death-1 ligand-1 (PD-L1) expression in TAM itself.[36] Blocking IL-8 in TAM by neutralizing antibody suppresses epithelial ovarian cancer migration.[37] Furthermore, CXCR1/2-positive neutrophils and myeloid-derived suppressor cells in cancer patients also play an important role in immune suppression by obstructing the contact between $CD8^+$ T cells and the target tumor cells.[19] Therefore, it is interesting to investigate whether Cx_1Mab-1 interferes with the IL-8-CXCR1 interaction. Furthermore, the identification of epitope is essential to know the property of Cx_1Mab-1.

Therapeutic drugs that target CXCR1/2 have been developed for cancers.[12] SCH527123 is an orally available allosteric inhibitor of CXCR1/2 that can inhibit the neutrophil activity.[22,38] Reparixin selectively blocks CXCR1 that is expressed in human breast cancer stem cells (CSCs) in preclinical models.[39] Furthermore, reparixin significantly decreases tumor growth in combination with paclitaxel or docetaxel compared with chemotherapy alone.[39,40] Anti-CXCR1 mAbs also have the potential to affect CSCs *in vitro*.[39] Furthermore, the blockers for CXCR1/2 potentiate the efficacy of immune checkpoint inhibitors (ICIs) through suppression of neutrophils and myeloid-derived suppressor cells.[19,41,42]

Several clinical trials demonstrate the promise of combination therapies treating CXCR1/2-targeted drugs and ICIs.[4,43] We have previously enhanced antibody-dependent cellular cytotoxicity (ADCC) activity complement-dependent cytotoxicity (CDC) by modifying mAb isotypes and defucosylation in mAbs.[44–46] Since Cx_1Mab-1 is rat IgG_1 that shows no ADCC and CDC activities, it will be converted into a mouse IgG_{2a} version to verify the effect of depletion of mCXCR1-positive immunosuppressive cells in preclinical models.

Authors' Contributions

G.L., T.T., and T.O. performed the experiments. M.K.K. and Y.K. designed the experiments. G.L., T.T., and M.K.K. analyzed the data. T.T., H.S., 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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