MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 43, Number 2, 2024

DOI: 10.1089/mab.2023.0032

Establishment of a Novel Anti-Mouse CCR1 Monoclonal Antibody C_1Mab-6

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C-C motif chemokine receptor 1 (CCR1/CD191) is a member of G-protein-coupled receptors and is expressed on myeloid cells, such as neutrophils and macrophages. Because the CCR1 signaling promotes tumor expansion in the tumor microenvironment (TME), the modification of TME is an effective strategy for cancer therapy. Although CCR1 is an attractive target for solid tumors and hematological malignancies, therapeutic agents for CCR1 have not been approved. Here, we established a novel anti-mouse CCR1 (mCCR1) monoclonal antibody (mAb), C_1Mab-6 (rat IgG_{2b}, kappa) using the Cell-Based Immunization and Screening method. Flow cytometry and Western blot analyses showed that C_1Mab-6 recognizes mCCR1 specifically. The dissociation constant of C_1Mab-6 for mCCR1-overexpressed Chinese hamster ovary-K1 was determined as 3.9×10^{-9} M, indicating that C_1Mab-6 possesses a high affinity to mCCR1. These results suggest that C_1Mab-6 could be a useful tool for targeting mCCR1 in preclinical mouse models.

Keywords: mouse CCR1, CBIS method, monoclonal antibody, flow cytometry, Western blot

Introduction

C-C MOTIF CHEMOKINE RECEPTOR 1 (CCR1), also known as CD191, is a member of G-protein-coupled receptors for many chemokines, such as CCL3, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, and CCL23 in humans.[1] CCR1, which is expressed on neutrophils or macrophages, is important for the infiltration of these cells. The leukocyte infiltration process consists of three steps: (i) tethering, rolling, and arrest on the vessel wall, (ii) crawling on the endothelium, and (iii) transendothelial migration.[2] CCR1 contributes to stable adhesion of crawling neutrophils on the endothelium in the joints in the K/BxN serum transferred mice (a murine model for rheumatoid arthritis [RA]).[3,4]

CCR1 signaling promotes tumor invasion and metastasis. The cis-Apc(+/Delta716) Smad4(+/−) mutant mice develop spontaneous invasive colorectal cancers.[5] However, CCR1-knockout in the background decreases the invasiveness of these tumors *in vivo*.[6] CCR1-positive myeloid cells accumulate at the tumor invasive front and drive tumor invasion by producing the matrix metalloproteinases, such as MMP2 and MMP9.[7,8] Liver metastasis is observed in 25% of patients with colorectal cancers and is the main cause of death.[9,10] Analysis of disease-free survival (DFS) after curative liver resection showed that the DFS of patients with CCL15-positive liver metastases is shorter than that of CCL15-negative.[8] More CCR1-positive myeloid cells accumulated around the CCL15-positive metastases than CCL15-negative ones.[8] CCR1 signaling also plays a critical role in the development of a carcinogen-induced hepatocellular carcinoma model. The CCR1 deficiency in mice reduced the tumor development through suppression of neovascularization and intratumoral Kupffer cell accumulation.[11]

Tumor microenvironment (TME) influences the tumor progression and responsiveness to therapies.[12] Modification of TME is a strategy for improving the effect of antitumor treatment.[13] Although CCR1 is an attractive target for modifying TME and suppressing tumor progression, few preclinical studies using anti-mouse CCR1 (mCCR1) monoclonal antibodies (mAbs) have been reported.[14]

We previously developed mAbs for mouse CCR3,[15] CCR8,[16] and CXCR4[17] using the Cell-Based Immunization

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and Screening (CBIS) method.[18–25] In this study, we established a novel anti-mCCR1 mAb using the CBIS method and evaluated its applications.

Materials and Methods

Antibodies

The anti-mouse CCR1 mAb (clone S15040E)[26] was purchased from BioLegend (San Diego, CA). We previously developed RcMab-1 against isocitrate dehydrogenase 1.[27] An anti-DYKDDDDK mAb (clone 1E6) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Alexa Fluor 488-conjugated anti-rat IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Horseradish peroxidase (HRP)-conjugated anti-rat IgG (A9542) and HRP-conjugated anti-mouse IgG (P0260) were purchased from Sigma-Aldrich (St. Louis, MO) and Agilent Technologies, Inc. (Santa Clara, CA), respectively.

Animals

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 approved by the Animal Care and Use Committee of Tohoku University (Permit No. 2022MdA-001).

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 mCCR1 (Accession No. NM_009912) 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 mCCR1 with C-terminal myc-DDK tags (hereinafter described as LN229/mCCR1 and CHO/mCCR1, respectively) were established using a cell sorter (SH800; Sony Corp., Tokyo, Japan), following cultivation in a medium containing 0.5 mg/mL G418 (Nacalai Tesque, Inc., Kyoto, Japan).

CHO-K1, P3U1, and CHO/mCCR1 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 (Thermo Fisher Scientific, Inc.), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). LN229 and LN229/mCCR1 were cultured in a Dulbecco's modified Eagle medium (Nacalai Tesque, Inc.) that was supplemented as shown above. Cells were grown in a humidified incubator, which was supplied with 5% CO_2 and 95% air at 37°C.

Hybridoma production

For developing anti-mCCR1 mAbs, a rat was immunized intraperitoneally with 1×10^9 cells of CHO/mCCR1. 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 CHO/mCCR1 were performed without an adjuvant every week. We performed a final booster immunization of 1×10^9 cells of CHO/mCCR1 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 (InvivoGen) into the medium. The hybridoma supernatants were screened by flow cytometry using LN229/mCCR1 and parental LN229. The culture supernatants of hybridomas were filtrated and purified using Ab-Capcher Extra (ProteNova, Kagawa, Japan).

Flow cytometry

CHO-K1, CHO/mCCR1, LN229, and LN229/mCCR1 cells were harvested by exposure to 1 mM EDTA (Nacalai Tesque, Inc.). The cells were washed with 0.1% bovine serum albumin in phosphate-buffered saline (PBS) and treated with anti-mCCR1 mAbs for 30 minutes at 4°C. After washing, the cells were treated with Alexa Fluor 488-conjugated anti-rat IgG. Flow cytometric analysis was performed using the SA3800 Cell Analyzer (Sony Corp.).

Determination of the binding affinity by flow cytometry

CHO/mCCR1 and LN229/mCCR1 were suspended in 100 μL serially diluted anti-mCCR1 mAbs (100 μg/mL to 6 ng/mL), after which Alexa Fluor 488-conjugated anti-rat IgG (1:200) were added. Fluorescence data were subsequently collected, using the SA3800 Cell Analyzer (Sony Corp.), following the calculation of the K_D by fitting the binding isotherms into the built-in; one-site binding model in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Western blot analysis

Cell lysates (10 μg) were boiled in sodium dodecyl sulfate (SDS) 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, they were incubated with 0.1 μg/mL of anti-DYKDDDDK or 1 μg/mL of the other primary mAbs. Then, they were incubated with HRP-conjugated anti-rat immunoglobulins (for anti-mCCR1 mAbs and RcMab-1; diluted 1:10,000) or anti-mouse immunoglobulins (for an anti-DYKDDDDK mAb; diluted 1:1000). Finally, protein bands were detected using ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) with a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Results

Establishment of anti-mCCR1 antibodies

We used the CBIS method to establish novel anti-mCCR1 antibodies (Fig. 1). The CBIS method is a high-throughput method for the establishment of antibodies against membrane proteins. CBIS method consists of two main steps:

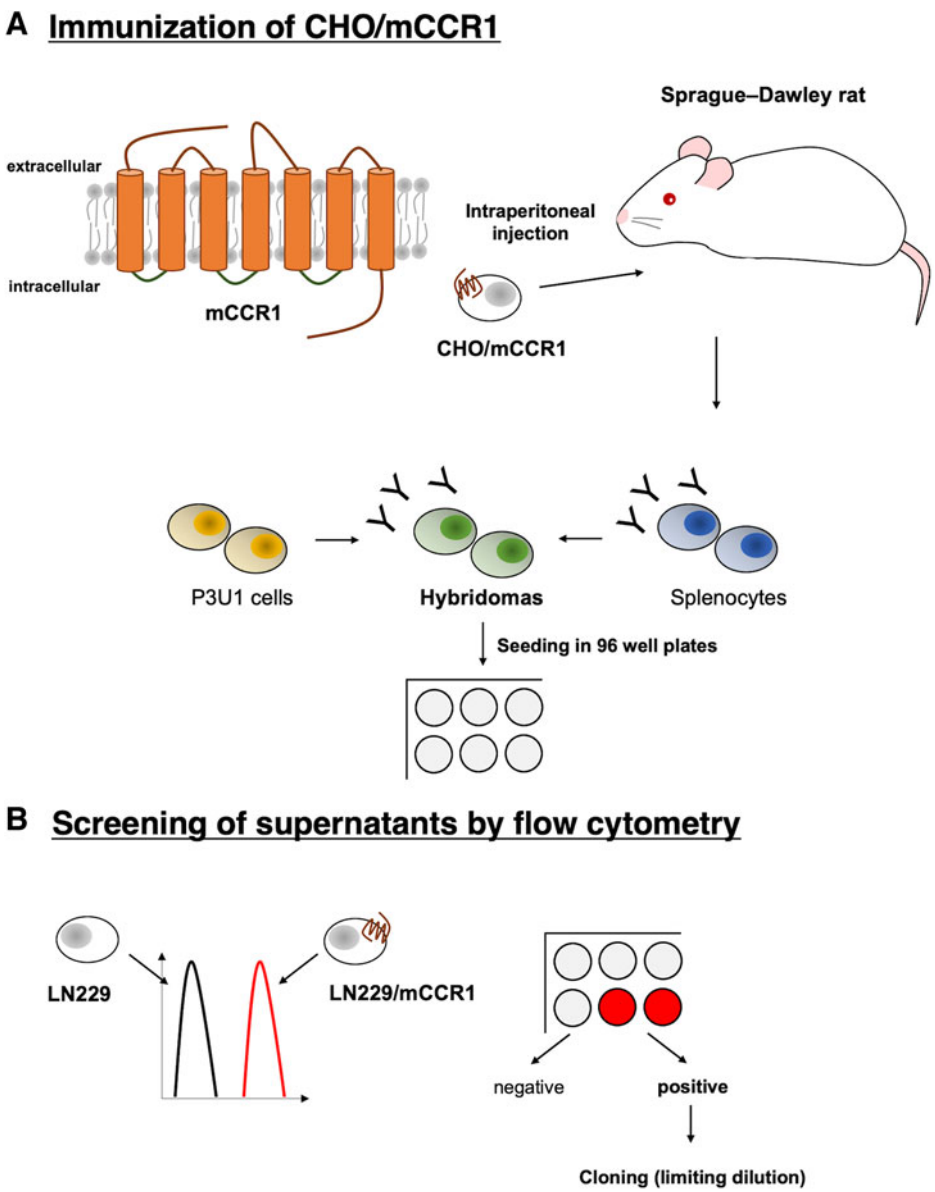

FIG. 1. The scheme of establishment of C_1Mab-6 by CBIS method. **(A)** CHO/mCCR1 cells were injected intraperitoneally into a Sprague–Dawley rat. The splenocytes of the rat were fused with P3U1 cells and seeded in 96 well plates. **(B)** The culture supernatants of each well were screened through flow cytometry to distinguish anti-mCCR1 mAb-producing hybridomas. C_1Mab-6 was established by limiting dilution and some additional screenings. CBIS, Cell-Based Immunization and Screening; CHO, Chinese hamster ovary; mAb, monoclonal antibody; mCCR1, mouse C-C motif chemokine receptor 1.

immunization of antigen-overexpressing cells (Fig. 1A) and screening of hybridoma supernatants using flow cytometry (Fig. 1B). After immunization of CHO/mCCR1 and screening of hybridoma supernatants with LN229/mCCR1, C_1Mab-6 (rat IgG_{2b}, kappa) was finally developed.

Flow cytometry using anti-mCCR1 mAbs

To check the specificity and reactivity of C_1Mab-6, we performed flow cytometry against LN229/mCCR1, LN229, CHO/mCCR1, and CHO-K1. S15040E is a commercially available anti-mCCR1 mAb.[26] C_1Mab-6 bound to LN229/mCCR1 and CHO/mCCR1 cells in a dose-dependent manner (Figs. 2A and 3A). In contrast, S15040E exhibited lower reactivity against both cells compared to C_1Mab-6 at a concentration of 0.01 μg/mL. C_1Mab-6 and S15040E did not react with LN229 and CHO-K1 even at 10 μg/mL (Figs. 2B and 3B), indicating that both mAbs are specific to mCCR1.

Determination of dissociation constants of anti-mCCR1 mAbs

Next, we determined the dissociation constants (K_D) of anti-mCCR1 mAbs, which is expressed on the cell surface using flow cytometry. The geometric mean of the fluorescence intensity at each concentration of C_1Mab-6 and S15040E was plotted. By fitting one-site binding models, the K_D values of C_1Mab-6 and S15040E for LN299/mCCR1 were determined as 1.0×10^{-8} and 1.7×10^{-7} M, respectively

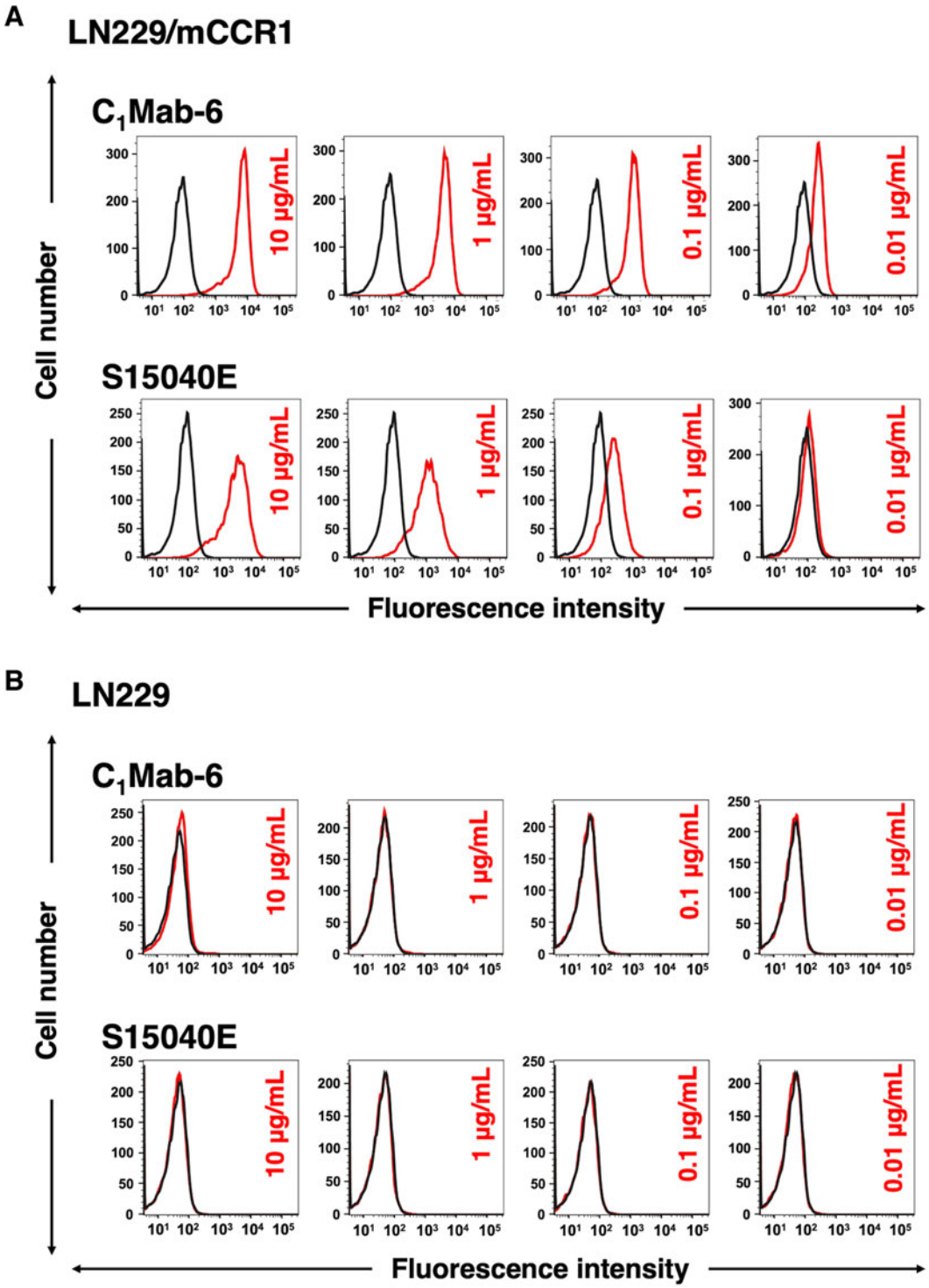

FIG. 2. Flow cytometry of anti-mCCR1 mAbs against LN229/mCCR1. LN229/mCCR1 **(A)** and LN229 **(B)** cells were treated with 0.01–10 μg/mL of C_1Mab-6 or S15040E, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG antibodies. The red lines show the cells treated with each mAb. The black lines show the cells treated with blocking buffer and Alexa Fluor 488-conjugated anti-rat IgG antibodies (negative control).

(Fig. 4A, B). Furthermore, the K_D values of C_1Mab-6 and S15040E for CHO/mCCR1 were calculated as 3.9×10^{-9} and 3.5×10^{-8} M, respectively (Fig. 4C, D). These results indicate that C_1Mab-6 possesses much higher affinity than S15040E.

Western blot using anti-mCCR1 mAbs

Finally, we performed Western blot analysis using anti-mCCR1 mAbs. Lysates of CHO-K1 and CHO/mCCR1 were probed. The molecular weight of mCCR1-mycDDK is about

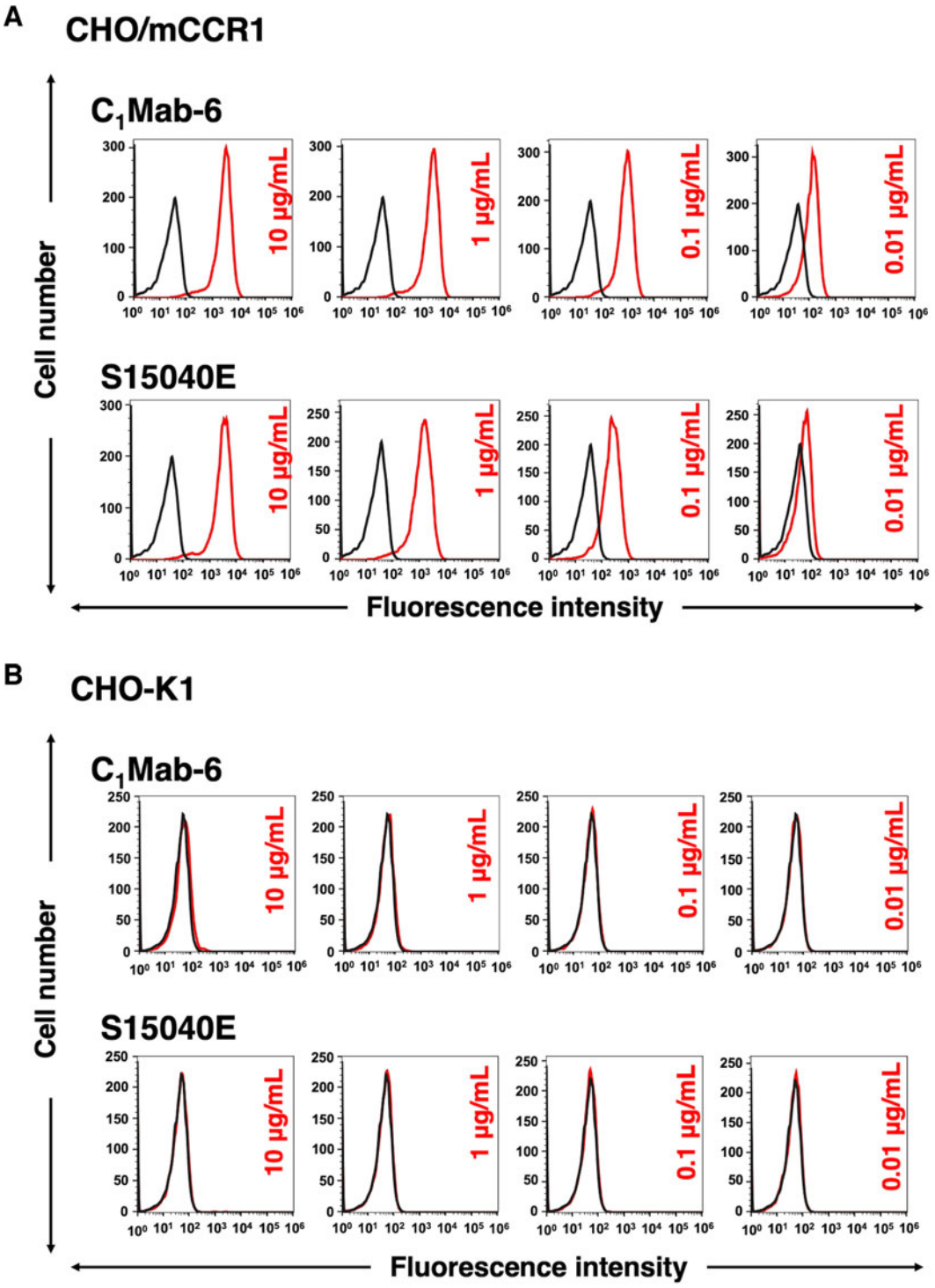

FIG. 3. Flow cytometry of anti-mCCR1 mAbs against CHO/mCCR1. CHO/mCCR1 **(A)** and CHO-K1 **(B)** cells were treated with 0.01–10 μg/mL of C_1Mab-6 or S15040E, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG antibodies. The red lines show the cells treated with each mAb. The black lines show the cells treated with blocking buffer and Alexa Fluor 488-conjugated anti-rat IgG antibodies (negative control).

44,000. C_1Mab-6 and an anti-DYKDDDDK mAb (clone 1E6) detected mCCR1 as about a 44-kDa band from lysates of CHO/mCCR1 (Fig. 5), although S15040E could not detect mCCR1 in the lysates of CHO/mCCR1. C_1Mab-6 did not detect any bands from the lysates of CHO-K1, indicating that C_1Mab-6 can recognize mCCR1 specifically in Western blot analysis.

Discussion

Anti-CCR1 therapies are potential treatments to improve the quality of life of multiple myeloma (MM) patients. MM is an incurable plasma B cell malignancy.[28] Systemic osteopenia is observed in most MM patients.[29] Osteopenia

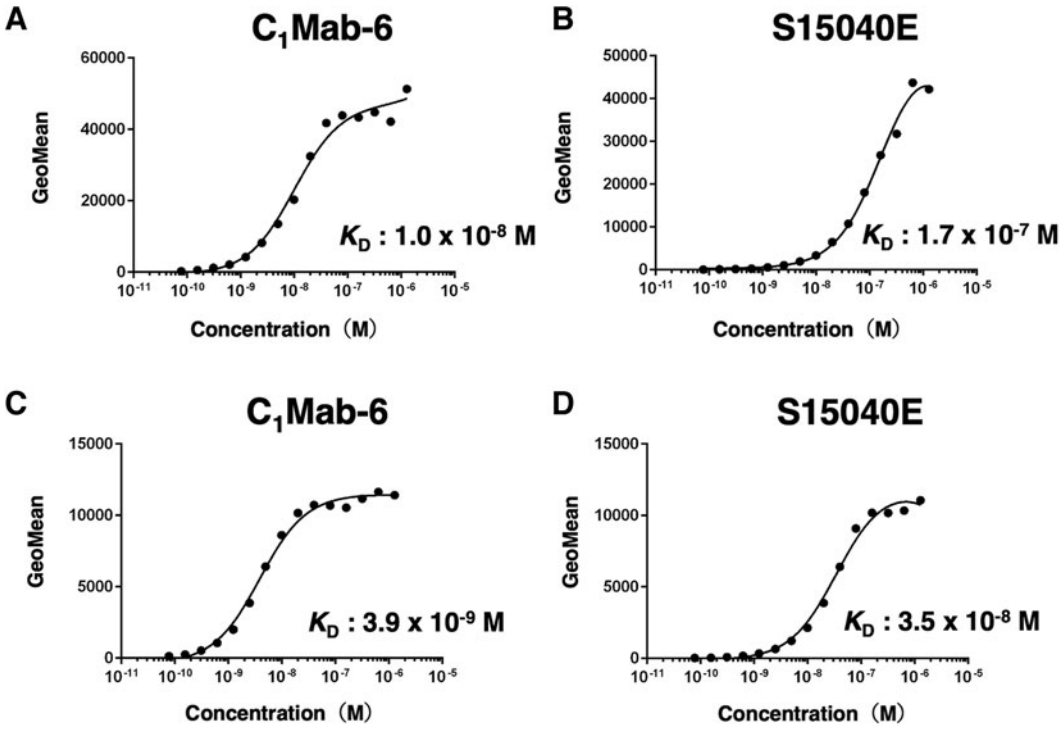

FIG. 4. Determination of dissociation constants of anti-mCCR1 mAbs against mCCR1-overexpressed cells. The binding affinity of C_1Mab-6 and S15040E against LN229/mCCR1 cells **(A, B)** and CHO/mCCR1 cells **(C, D)** were determined by flow cytometry. The dots show the geometric mean of fluorescence intensity at each concentration. The solid lines are the fitting curve calculated by GraphPad PRISM 6.

causes bone fractures and impacts their quality of life. Serum levels of CCL3 are elevated in MM patients and correlate with the extent of bone disease.[30] MM cells produce CCL3, which activates osteoclast.[31,32] Neutralization of CCL3 with anti-CCL3 antibodies blocks osteolysis in mice.[32] CCL3 is a ligand activating CCR1 and CCR5.[1] CCX721, a small molecule antagonist against CCR1 not CCR5, dramatically reduced the osteolysis induced by 5TGM1, a murine MM cell line.[33] Additionally, CCX721 also reduces tumor burden of 5TGM1 cells inoculated intravenously.[33] CCX721 does not block the proliferation of 5TGM1 cells *in vitro* or inoculated subcutaneously.[33] These

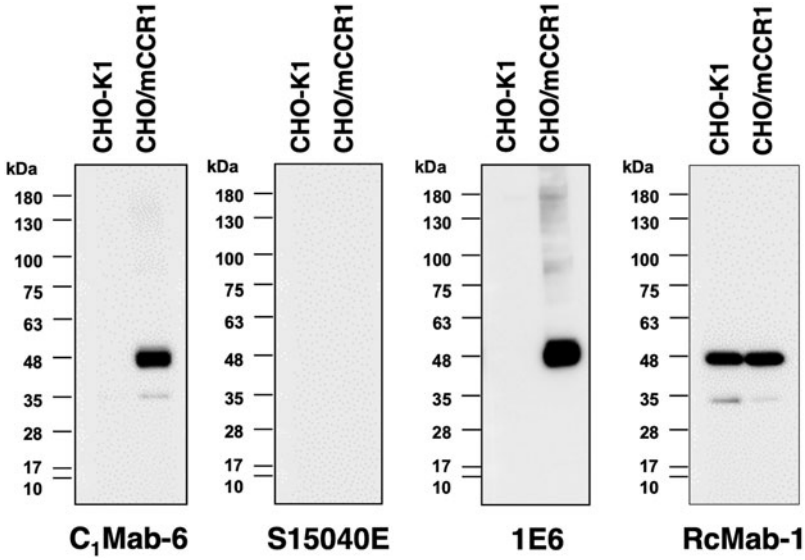

FIG. 5. Western blot analysis using anti-mCCR1 mAbs. Cell lysates of CHO-K1 and CHO/mCCR1 cells were electrophoresed, and proteins were transferred onto PVDF membranes. After blocking, membranes were incubated with C_1Mab-6, S15040E, anti-DYKDDDDK mAb (1E6), or RcMab-1, and then incubated with HRP-conjugated anti-rat IgG or anti-mouse IgG. RcMab-1 (an anti-IDH1 mAb) (an internal control). HRP, horseradish peroxidase; IDH1, isocitrate dehydrogenase 1; PVDF, polyvinylidene difluoride.

results indicated that CCR1 blockage inhibits tumor burden and osteolysis by MM through modifying the bone marrow microenvironment.

RA is a chronic inflammatory disease characterized by massive infiltration of synovial tissue and synovial fluid with immune cells in affected joints.[34] CCR1 is abundantly expressed by monocytes/macrophages of RA, which mediate the inflammation at these sites.[35] CCX354, an analog of CCX721, was developed for the treatment of RA.[36] CCX354 exhibited good safety and clinical activities to RA patients in a phase II study.[37] However, once daily oral administration is needed because the half-life of CCX354 in the plasma of humans is $\sim$6 hours.[36] Antibodies are therapeutic drugs stable in serum (the half-life of antibodies is around 3 weeks).[38] C_1Mab-6 can bind to mCCR1-expressed cells with a high affinity (Fig. 4). C_1Mab-6 could be useful tools for developing anti-mCCR1 therapies in preclinical murine models.

Acknowledgment

This study was previously published in preprint.org (doi: 10.20944/preprints202312.2059.v1).

Authors' Contributions

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

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported in part by Japan Agency for Medical Research and Development (AMED) under grant numbers: JP23ama121008 (to Yukinari Kato), JP23am0401013 (to Yukinari Kato), 23bm1123027h0001 (to Yukinari Kato), and JP23ck0106730 (to Yukinari Kato), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) grant numbers: 23K19494 (to Tsunenori Ouchida), 21K20789 (to Tomohiro Tanaka), 22K06995 (to Hiroyuki Suzuki), 21K07168 (to Mika K. Kaneko), and 22K07224 (to Yukinari Kato).

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Received: December 26, 2023
Accepted: February 5, 2024