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Development of a Sensitive Anti-Mouse CCR5 Monoclonal Antibody for Flow Cytometry

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C-C chemokine receptor 5 (CCR5), a member of the G protein-coupled receptor family, is the most common coreceptor for the human immunodeficiency virus type 1. CCR5 is also involved in the pathogenesis of tumors and inflammatory diseases. The CCR5 antagonists including monoclonal antibodies (mAbs) have been developed and evaluated in clinical trials. In this study, we developed novel mAbs for mouse CCR5 (mCCR5) using the Cell-Based Immunization and Screening (CBIS) method. One of the established anti-mCCR5 mAbs, C_5Mab-2 (rat IgG_{2b}, kappa), reacted with mCCR5-overexpressed Chinese hamster ovary-K1 (CHO/mCCR5) and an endogenously mCCR5-expressing cell line (L1210) by flow cytometry. Using flow cytometry, the dissociation constant (K_D) of C_5Mab-2 for CHO/mCCR5 was determined as 4.3×10^{-8} M. These results indicated that C_5Mab-2 is useful for the detection of mCCR5 in flow cytometry and may be applicable to obtain the proof of concept in preclinical studies.

Keywords: mouse CCR5, monoclonal antibody, Cell-Based Immunization and Screening, CBIS

Introduction

C-C chemokine receptor 5 (CCR5) is a cell membrane protein of G protein-coupled receptor (GPCR) family, which is an important regulator for lymphocyte migration and activation.[1] In the 1980s, studies showed that CCR5 is the most common coreceptor for the human immunodeficiency virus type 1 (HIV-1).[2] The lack of CCR5 offers protection against HIV infection.[3] Therefore, CCR5 antagonists were developed as anti-HIV drugs.[4] A CCR5 antagonist, maraviroc, was developed for acquired immunodeficiency syndrome (AIDS) therapy and is the only U.S. Food and Drug Administration (FDA)-approved drug for marketing.[5]

CCR5 possesses the typical structure of seven transmembrane GPCR.[1] CCR5 is expressed on macrophages, dendritic cells, and natural killer cells, which are cells of the innate immune response, as well as on T and B cells of the adaptive immune response.[4] CCR5 serves as a receptor for inflammatory β-chemokines including regulated on activation, normal T-expressed and secreted [RANTES; C-C chemokine ligand 5 (CCL5)], macrophage-inflammatory protein-1α (MIP-1α; CCL3), and MIP-1β (CCL4).[4] Upon binding to these ligands, CCR5 activates downstream signaling, which plays critical roles in the innate and adaptive immune response through the promotion of lymphocyte migration and the secretion of proinflammatory cytokines.[6] These functions mediate the progression of tumors[7,8] and inflammatory diseases.[6]

Unique functions of CCR5 in the brain have been reported. CCR5 is expressed in cortical neurons after stroke.[9] In a mouse model of traumatic brain injury, CCR5 knockdown reduces learning deficits and improves cognitive function. Furthermore, the FDA-approved CCR5 antagonist maraviroc promotes recovery of function in stroke and traumatic brain injury.[9] These results suggest that CCR5 is a promising molecular target for the recovery from stroke and traumatic brain injury.

In recent years, the CCR5 antagonists including monoclonal antibodies (mAbs) have been developed and evaluated for the treatment of tumors and inflammatory diseases in clinical trials.[6,10] We have developed anti-mouse GPCR mAbs against CCR1 (clone C_1Mab-6),[11] CCR3 (clones C_3Mab-2, C_3Mab-3, and C_3Mab-4),[12–14] CCR8 (clones C_8Mab-1, C_8Mab-2, and C_8Mab-3),[15–17] CXCR1 (clone Cx_1Mab-1),[18] CXCR3 (clone Cx_3Mab-4),[19] and CXCR4 (clone Cx_4Mab-1)[20] using the Cell-Based Immunization and Screening (CBIS) method. The CBIS method includes the immunization of antigen-overexpressed cells and high-throughput hybridoma screening using flow cytometry. In this study, a novel anti-mCCR5 mAb was developed by the CBIS method.

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Materials and Methods

Preparation of cell lines

L1210 (mouse lymphocytic leukemia) was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). LN229, Chinese hamster ovary (CHO)-K1, and P3X63Ag8U.1 (P3U1) were obtained from the American Type Culture Collection (Manassas, VA).

The synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding mCCR5 (Accession No.: NM_009917) was subsequently subcloned into pCAGzeo and pCAGzeo-cPA vectors (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The PA tag can be detected by an anti-human podoplanin mAb (clone NZ-1).[21] The mCCR5 plasmids were transfected into CHO-K1 and LN229 cells, using a Neon transfection system (Thermo Fisher Scientific Inc., Waltham, MA). Stable transfectants were established through cell sorting 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 performed.

L1210, CHO-K1, mCCR5-overexpressed CHO-K1 (CHO/mCCR5), and P3U1 were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc.), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). LN229 and mCCR5-overexpressed LN229 (LN229/mCCR5 or LN229/mCCR5-PA) were cultured in Dulbecco's modified Eagle medium (DMEM; Nacalai Tesque, Inc.), supplemented with 10% FBS, 100 units/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. All cells were grown in a humidified incubator at 37°C, in an atmosphere of 5% CO_2 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 number: 2022MdA-001) approved animal experiments. The rat was monitored daily for health during the complete 4-week duration of the experiment. A reduction of >25% of the total body weight was defined as a humane endpoint. During the sacrifice, the rat was euthanized through cervical dislocation, after which death was verified through respiratory and cardiac arrest.

To develop mAbs against mCCR5, we intraperitoneally immunized one rat with CHO/mCCR5 (1×10^9 cells) plus Alhydrogel adjuvant 2% (InvivoGen). The procedure included three additional injections every week (1×10^9 cells/rat), which were followed by a final booster intraperitoneal injection (1×10^9 cells/rat), 2 days before harvesting spleen cells. The harvested spleen cells were subsequently fused with P3U1 cells, using PEG1500 (Roche Diagnostics, Indianapolis, IN), after which hybridomas were grown in the RPMI-1640 medium with 10% FBS, 5% Briclone (NICB, Dublin, Ireland), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B. For the hybridoma selection, hypoxanthine, aminopterin, and thymidine (HAT; Thermo Fisher Scientific Inc.) were added into the medium. The supernatants were subsequently screened using flow cytometry using LN229/mCCR5-PA and LN229.

Antibodies

An anti-mCCR5 mAb (clone HM-CCR5, Armenian hamster IgG) conjugated with Alexa Fluor 488 was purchased from BioLegend (San Diego, CA). Alexa Fluor 488-conjugated anti-rat IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

The cultured supernatant of C_5Mab-2 hybridomas was applied to 1 mL of Ab-Capcher (ProteNova, Kagawa, Japan). After washing with phosphate-buffered saline (PBS), the antibodies were eluted with an IgG elution buffer (Thermo Fisher Scientific Inc.). Finally, the eluates were concentrated, and the elution buffer was replaced with PBS using Amicon Ultra (Merck KGaA, Darmstadt, Germany).

Flow cytometric analysis

Cells were harvested after a brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA, Nacalai Tesque, Inc.). The cells were subsequently washed with 0.1% bovine serum albumin (BSA) in PBS (blocking buffer) and treated with 0.01, 0.1, 1, and 10 μg/mL of primary mAbs for 30 minutes at 4°C. The C_5Mab-2-treated cells were further incubated with Alexa Fluor 488-conjugated anti-rat IgG (1:2,000). The fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp.).

Determination of dissociation constant (K_D) by flow cytometry

CHO/mCCR5 cells were suspended in serially diluted C_5Mab-2 for 30 minutes at 4°C. The cells were treated with Alexa Fluor 488-conjugated anti-rat IgG (1:200). CHO/mCCR5 cells were suspended in serially diluted HM-CCR5 conjugated with Alexa Fluor 488. The fluorescence data were collected, using the SA3800 Cell Analyzer. The K_D was subsequently calculated by fitting saturation binding curves to the built-in, one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Results

Development of anti-mCCR5 mAbs using the CBIS method

To develop anti-mCCR5 mAb, one rat was immunized with CHO/mCCR5 cells (Fig. 1A). The spleen was then excised from the rat, and splenocytes were fused with myeloma P3U1 cells (Fig. 1B). The developed hybridomas were subsequently seeded into 96-well plates and cultivated for 6 days. The positive wells were screened by the selection of LN229/mCCR5-PA-reactive and LN229-nonreactive supernatants using flow cytometry (Fig. 1C). After the limiting dilution and several additional screenings, an anti-mCCR5 mAb, C_5Mab-2 (rat IgG_{2b}, kappa), was finally established (Fig. 1D).

Flow cytometric analysis using C_5Mab-2

We conducted flow cytometry using two anti-mCCR5 mAbs: C_5Mab-2 and HM-CCR5 (from BioLegend) against

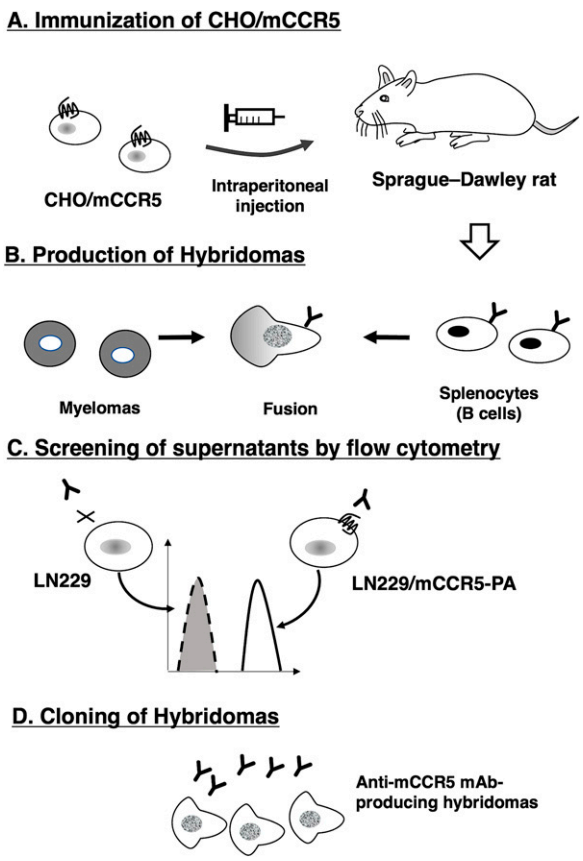

FIG. 1. The production of an anti-mCCR5 mAb, C_5Mab-2. **(A)** CHO/mCCR5 cells were immunized into a Sprague–Dawley rat. **(B)** The spleen cells were fused with P3U1 cells. **(C)** To select anti-mCCR5 mAb-producing hybridomas, the supernatants were screened by flow cytometry using LN229 and LN229/mCCR5-PA cells. **(D)** After limiting dilution, an anti-mCCR5 mAb, C_5Mab-2 was finally established.

CHO/mCCR5, CHO-K1, and L1210 cells. C_5Mab-2 recognized CHO/mCCR5 cells dose-dependently at 10, 1, 0.1, and 0.01 μg/mL (Fig. 2A). In contrast, HM-CCR5 needed >0.1 μg/mL for the detection of CHO/mCCR5 clearly (Fig. 2A). Parental CHO-K1 cells were not recognized even at 10 μg/mL of all mAbs (Fig. 2B). The superior reactivity of C_5Mab-2 compared with HM-CCR5 was also observed in LN229/mCCR5 cells (Supplementary Fig. S1).

We next investigated the reactivity of C_5Mab-2 against an endogenously mCCR5-expressing cell line, L1210. C_5Mab-2 reacted with L1210 at >0.1 μg/mL (Fig. 2C). In contrast, HM-CCR5 could react with L1210 at >1 μg/mL, but the reaction was weak at 0.1 μg/mL. These results suggested that C_5Mab-2 specifically recognizes mCCR5 and is also useful for detecting endogenous mCCR5 by flow cytometry.

Determination of the binding affinity of C_5Mab-2 using flow cytometry

To determine the K_D of anti-mCCR5 mAbs against CHO/mCCR5, we conducted flow cytometry, and the geometric mean of the fluorescence intensity was plotted versus the concentration of C_5Mab-2. The K_D values of C_5Mab-2 and HM-CCR5 for CHO/mCCR5 were determined as 4.3×10^{-8} M and 1.6×10^{-7} M, respectively (Fig. 3). These results indicate that C_5Mab-2 possesses the superior affinity to CHO/mCCR5 compared with HM-CCR5.

Discussion

In this study, we developed a novel anti-mCCR5 mAb, C_5Mab-2 using the CBIS method and showed the usefulness for flow cytometry to detect the exogenous and endogenous mCCR5 (Fig. 2). The understanding of the structural-based CCR activation is important for the development of therapeutic agents. Among the CCR family members, CCR5 has been structurally solved in both inactive and active states.[22,23] Recently, the structures of CCR8 in complex with either the antagonistic mAb or the endogenous ligand CCL1 were determined, which provides the specific activation mechanism by CCL1 and inhibition by mAb.[24] We previously determined the epitope of C_3Mab-3 and C_3Mab-4 (anti-mouse CCR3 mAbs)[12] and C_8Mab-2 (an anti-mouse CCR8 mAb)[25] using the chimeric proteins and the alanine scanning in flow cytometry. We should determine the epitope and antagonistic activity of C_5Mab-2 in future studies.

CCR5 is the principal HIV coreceptor[2] and is involved in the disease progression in tumors[7] and inflammatory diseases.[26] CCR5 has been implicated in the inflammatory complications

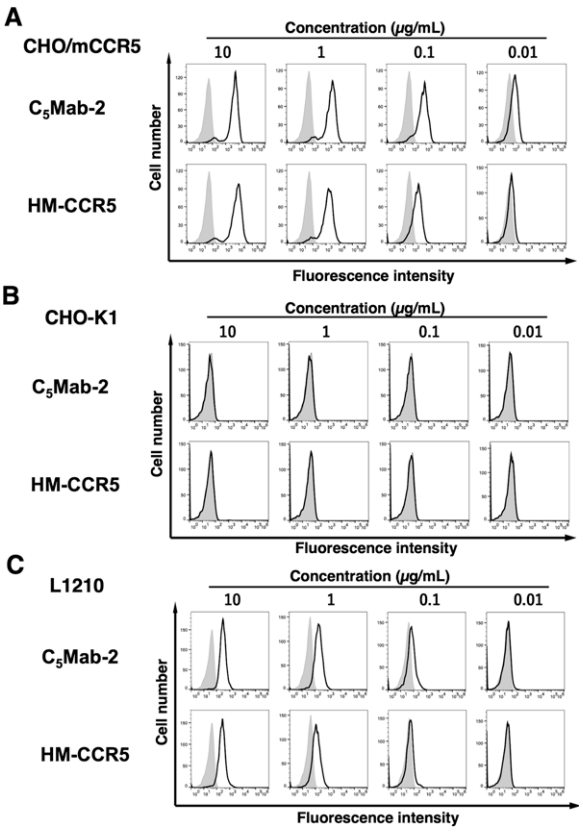

FIG. 2. Flow cytometry of mCCR5-expressed cells using C_5Mab-2 and HM-CCR5. CHO/mCCR5 **(A)**, CHO-K1 **(B)**, and L1210 **(C)** cells were treated with 0.01–10 μg/mL of C_5Mab-2 or HM-CCR5 conjugated with Alexa Fluor 488 (black line). The C_5Mab-2-treated cells were further incubated with anti-rat IgG conjugated with Alexa Fluor 488. The *filled gray* represents the negative control (blocking buffer).

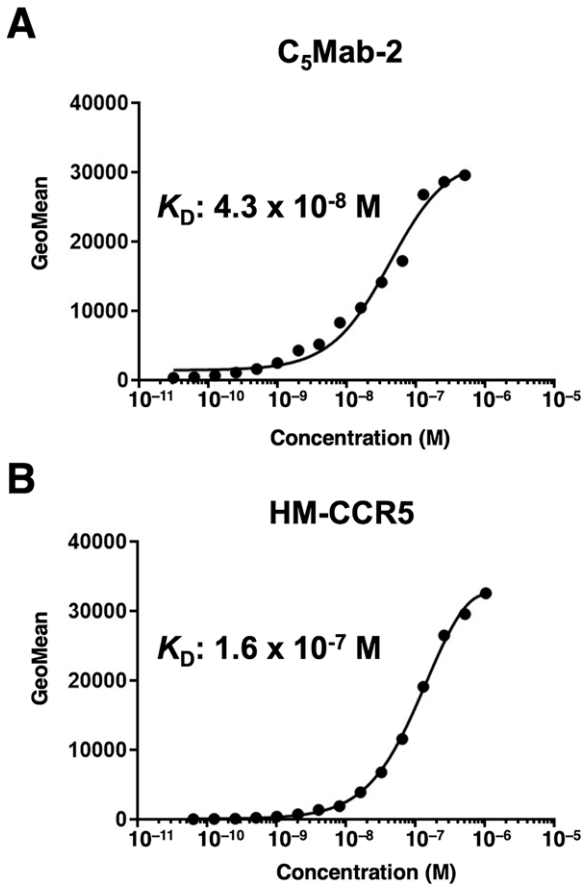

FIG. 3. The binding affinity of C_5Mab-2. **(A)** CHO/mCCR5 cells were suspended in serially diluted C_5Mab-2. The cells were treated with anti-rat IgG conjugated with Alexa Fluor 488. **(B)** CHO/mCCR5 cells were suspended in serially diluted HM-CCR5 conjugated with Alexa Fluor 488. The fluorescence data were subsequently collected using the SA3800 Cell Analyzer, followed by the calculation of the K_D using GraphPad PRISM 6.

of coronavirus disease 2019 (COVID-19).[27] Therefore, CCR5 antagonists have been tested in various clinical researches.[28] Furthermore, an anti-CCR5 mAb (PRO 140) has been developed and investigated for HIV, breast cancer, and COVID-19 in phase II or III trials.[6,10]

Using mouse models, CCR5 has been implicated to have a novel function in neural systems. CCR5 was first identified to be involved in plasticity and memory in a reverse genetic screening. In total, 148 transgenic and knockout mutant mouse strains were screened for contextual memory phenotypes. The CCR5 knockout resulted in memory enhancements for contextual conditioning when tested 24 hours after training.[29] In addition, decreasing the function of CCR5 enhances the long-term potentiation and hippocampus-dependent memory, whereas neuronal CCR5 overexpression caused memory deficits in mice.[29] These results indicated that CCR5 is a suppressor for cortical plasticity and hippocampal learning and memory. Furthermore, an age-related increase in CCL5-CCR5 expression in neurons leads to impairments in memory linking in middle-aged mice that can be reversed by the FDA-approved CCR5 inhibitor, maraviroc.[30] These results suggested that the CCL5-CCR5 axis is a potential target for memory deficits. MAb therapies have been applied to neural disorders such as Alzheimer's disease.[31] CCR5 plays an important role in modulating $A\beta_{1-42}$-induced learning and memory deficits in a mouse model, which suggests that CCR5 antagonists are a potential treatment to improve cognitive deficits associated with Alzheimer's disease.[32] Therefore, C_5Mab-2 could contribute to the treatment of memory deficits in mouse preclinical studies.

Authors' Contribution

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

Author Disclosure Statement

The authors have no conflict of interest.

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Supplementary Material

Supplementary Figure S1

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