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Development of Anti-Mouse CC Chemokine Receptor 8 Monoclonal Antibodies for Flow Cytometry

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CC chemokine receptor 8 (CCR8) belongs to the class A of G protein-coupled receptor. It is highly expressed on Treg and T helper 2 (T_H2) cells recruited to the inflammation site and is implicated in allergy and asthma. Recently, CCR8+Treg cells have been suggested to be a master regulator in the immunosuppressive tumor microenvironment; therefore, developing sensitive monoclonal antibodies (mAbs) for CCR8 has been desired. This study established a specific and sensitive mAb for mouse CCR8 (mCCR8), which is useful for flow cytometry by using the Cell-Based Immunization and Screening (CBIS) method. The established anti-mCCR8 mAb, C_8Mab-2 (rat IgG_{2b}, kappa), reacted with mCCR8-overexpressed Chinese hamster ovary-K1 (CHO/mCCR8) cells and P388 (mouse lymphoid neoplasma) or J774-1 (mouse macrophage-like) cells, which express endogenous mCCR8 by flow cytometry. C_8Mab-2, which was established by the CBIS method, could be useful for elucidating the mCCR8-related biological response by flow cytometry.

Keywords: CCR8, monoclonal antibody, CBIS, flow cytometry

Introduction

The tumor microenvironment (TME) consists of various elements, including immune and stromal cells, which affect antitumor treatment effectiveness.[1] Immunosuppressive cells, such as regulatory T cells (Treg), myeloid-derived suppressor cells, and tumor-associated macrophages (TAMs), play a pivotal role in tumor progression.[1–4] Recently, the development of cancer immunotherapy, including immune checkpoint inhibitor and chimeric antigen receptor (CAR)-T therapy, has been accelerated, and much attention has been focused on lymphocytes to develop an effective treatment against tumors.[5]

Treg cells were found as a CD4+ T cell member, which maintains self-tolerance to suppress abnormal allergic reaction and autoimmune diseases.[2,6] Treg cells are known as CD4+CD25+Foxp3+ T cells, which suppress immune response by inhibiting the proliferation of effector T cells, or secreting immunosuppressive cytokines, such as transforming growth factor-β (TGF-β), IL-10, and granzyme/perforin, which give cytotoxicity to cytotoxic T lymphocyte.[7] Treg cells are also involved in developing an immunosuppressive TME by inhibiting antitumor immunity.[8] Treg cells express cytotoxic T lymphocyte-associated-4 (CTLA-4), which block dendritic cell maturation.[2]

CC chemokine receptor 8 (CCR8) is a seven-transmembrane protein belonging to the CC chemokine receptor family. CCR8 is principally expressed on Treg and T helper 2 (T_H2) cells recruited to the inflammation site and is implicated in allergy and asthma.[9–13] Recently, CCR8+Treg cells have been suggested to be a master regulator in the immunosuppressive TME.[3,9,14] Some reports insist that the higher proportion of CCR8+Treg cells is often associated with poor prognosis in various cancer patients, including breast cancer.[8,15,16] The frequency of peripheral blood Treg cells is elevated in breast cancer patients and correlates with a better anti-CTLA-4 antibody treatment.[17,18]

Furthermore, intratumoral Treg cells have high CCR8 expression, and this type of cells is endowed with higher expressions of CD25, Foxp3, and CTLA-4 than CCR8-Treg cells.[17] CCL1/I-309, the specific ligand for CCR8 in humans, is also involved in antitumor immunosuppression via CCR8.[19–21] CCL1 is secreted by some cancer-related cells, such as TAMs, cancer-associated fibroblasts, and Treg cells, causing other Treg cells' recruitment to the tumor site, leading to angiogenesis.[22,23] CCL1 accelerates the immunosuppressive capacity of CCR8+Treg cells. CCL1-CCR8 axis also functions in melanoma metastasis to the lymph node.[24] Therefore, targeting CCR8 could show dramatic antitumor effects.[2,3,6,25]

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In our studies, we have developed many useful monoclonal antibodies (mAbs) against membrane proteins using the Cell-Based Immunization and Screening (CBIS) method.[26–28] Here, we developed a novel anti-mouse CCR8 (mCCR8) mAb, which can be used for flow cytometry.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). P388 and J774-1 were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding mCCR8 (Accession No.: NM_007720.2) was subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The mCCR8 plasmid was transfected using the Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were established by cell sorting using a cell sorter (SH800; Sony Corp., Tokyo, Japan) and cultivation in a medium containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA).

CHO-K1, P3U1, CHO/mCCR8, P388, and J774-1 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). Cells were grown in a humidified incubator at 37°C with 5% CO_2 and 95% air.

Antibodies

An anti-mCCR8 mAb (clone SA214G2) was purchased from BioLegend (San Diego, CA). Secondary Alexa Fluor 488-conjugated anti-rat IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Hybridoma production

A female Sprague-Dawley (SD) rat (6-week-old) was purchased from CLEA Japan (Tokyo, Japan). The animal was housed under specific pathogen-free conditions. All animal experiments were performed according to relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2019NiA-001). The rat was monitored daily for health during the full 4-week duration of the experiment.

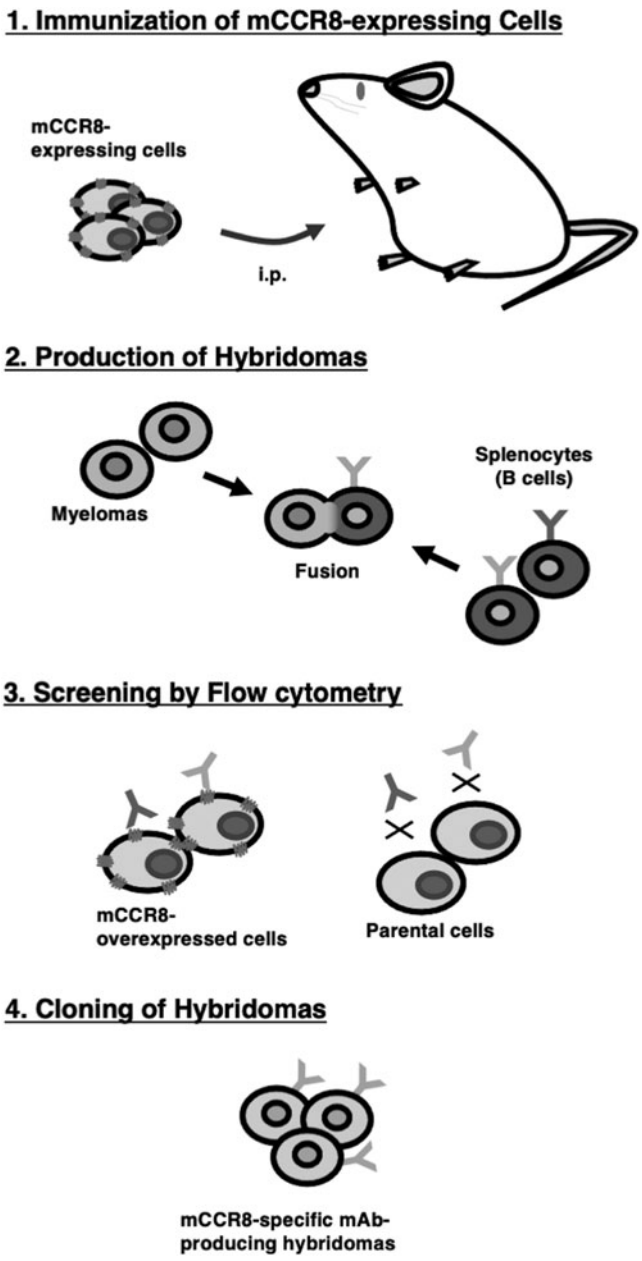
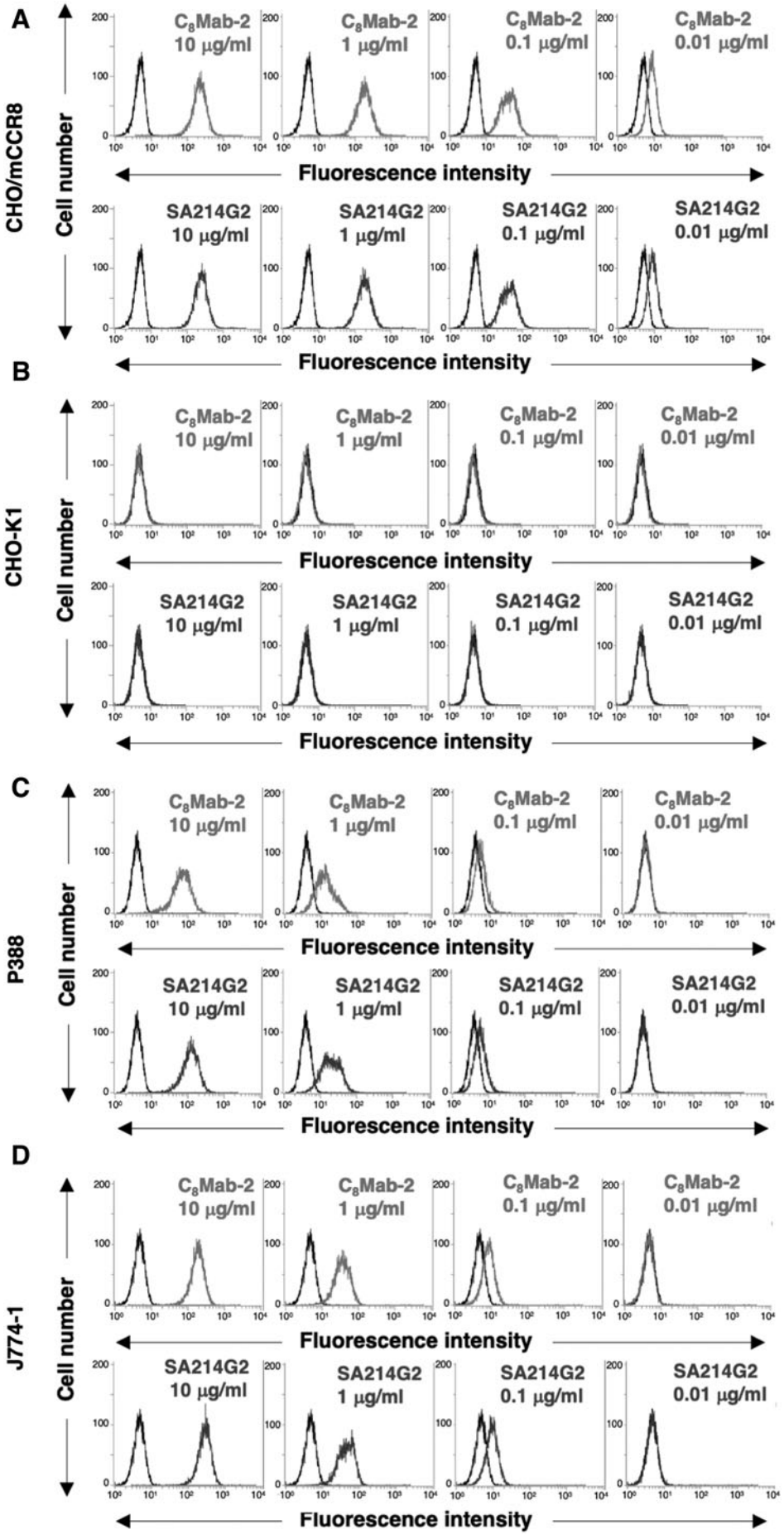


FIG. 1. Production of anti-mCCR8 mAbs. The procedure of CBIS. CHO/mCCR8 cells were immunized into an SD rat using intraperitoneal injection. Screening was performed by flow cytometry. CBIS, Cell-Based Immunization and Screening; CHO, Chinese hamster ovary; mAbs, monoclonal antibodies; mCCR8, mouse CC chemokine receptor 8; SD, Sprague-Dawley.

FIG. 2. Flow cytometry using anti-mCCR8. **(A)** CHO/mCCR8 cells were treated with 0.01–10 μg/mL of C_8Mab-2 or SA214G2, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG; black line, negative control. **(B)** CHO-K1 cells were treated with 0.01–10 μg/mL of C_8Mab-2 or SA214G2, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG; black line, negative control. **(C)** P388 cells were treated with 0.01–10 μg/mL of C_8Mab-2 or SA214G2, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG; black line, negative control. **(D)** J774-1 cells were treated with 0.01–10 μg/mL of C_8Mab-2 or SA214G2, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG; black line, negative control.



A reduction of >25% of total body weight was defined as a humane endpoint. The rat was euthanized by cervical dislocation, and death was verified by respiratory and cardiac arrest.

To develop mAbs against mCCR8, the CBIS method[26] was used. Briefly, one SD rat was immunized with CHO/mCCR8 cells (1×10^9) by the intraperitoneal route together with the Imject Alum (Thermo Fisher Scientific, Inc.). The procedure included three additional immunizations followed by a final booster intraperitoneal injection administered 2 days before spleen cells were harvested. Harvested spleen cells were subsequently fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The hybridomas were grown in an RPMI medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). Culture supernatants were screened by flow cytometry.

Flow cytometry analysis

Cells were harvested after brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). They were washed with 0.1% bovine serum albumin in phosphate-buffered saline and treated with primary mAbs for 30 minutes at 4°C. Then, the cells were treated with Alexa Fluor 488-conjugated anti-rat IgG (1:2000; Cell Signaling Technology, Inc.). Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Determination of binding affinity by flow cytometry

CHO/mCCR8 was suspended in 100 μL of serially diluted anti-mCCR8 mAbs, and Alexa Fluor 488-conjugated anti-rat IgG (1:200; Cell Signaling Technology, Inc.) was added. Fluorescence data were collected using the BD FACSLyric (BD Biosciences, Franklin Lakes, NJ). The dissociation constant (K_D) was calculated by fitting binding isotherms to built-in, one-site binding models in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

Results

Establishment of anti-mCCR8 mAbs

To develop anti-mCCR8 mAbs, we employed the CBIS method, using stable transfectants for immunization and flow cytometry (Fig. 1). A rat was immunized with CHO/mCCR8 cells, which overexpress mCCR8. Hybridomas were seeded into 96-well plates, and CHO/mCCR8-positive and CHO-K1-negative wells were selected. The first screening approach identified strong signals from CHO/mCCR8 cells and weak or no signals from CHO-K1 cells in 73 of 1916 wells (3.8%). After limiting dilution, C_8Mab-2 (IgG_{2b}, kappa) was finally established.

Flow cytometry

We performed flow cytometry using C_8Mab-2 against CHO/mCCR8 and CHO-K1. C_8Mab-2 recognized CHO/mCCR8 dose dependently (Fig. 2A), but not CHO-K1 (Fig. 2B). Another anti-mCCR8 mAb (clone SA214G2 from BioLegend: positive control) also recognized CHO/mCCR8 dose dependently (Fig. 2A), but not CHO-K1 (Fig. 2B). Both C_8Mab-2 and SA214G2 reacted with CHO/mCCR8 even in 0.01 μg/mL (Fig. 2A). Both C_8Mab-2 and SA214G2 also reacted with P388 (Fig. 2C) and J774-1 (Fig. 2D), expressing endogenous mCCR8 dose dependently.

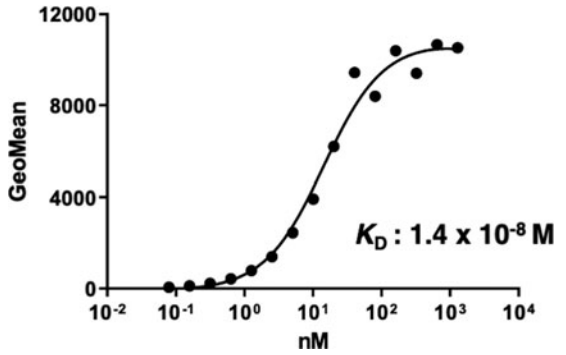


FIG. 3. Determination of the binding affinity of C_8Mab-2. CHO/mCCR8 was suspended in 100 µL of serially diluted C_8Mab-2 (0.006–100 µg/mL). Then, Alexa Fluor 488-conjugated anti-rat IgG was added. Fluorescence data were collected using the BD FACSLyric.

Determination of the binding affinity of C_8Mab-2

We then assessed the apparent binding affinity of C_8Mab-2 with CHO/mCCR8 by flow cytometry. The K_D of C_8Mab-2 for CHO/mCCR8 was 1.4×10^{-8} M (Fig. 3), indicating that C_8Mab-2 possesses moderate affinity for CHO/mCCR8 cells.

Discussion

G protein-coupled receptors (GPCRs), including CCR8, are expressed on the cell surface, transmit signals to intracellular molecules about various extracellular conditions, and govern broad cell dynamics, such as homeostasis, proliferation, and migration. GPCRs are also deeply involved in cancer and inflammatory diseases.[29] Therefore, GPCRs are focused as targets for many diseases, and the development of therapeutic agents, such as anti-GPCR antibodies, has been investigated. The development of anti-GPCR antibodies has been reported to be difficult due to the complexity of its folded three-dimensional structure, the small exposed area of extracellular epitopes, and the difficulty of purifying a functional protein as an antigen.[30] The preparation of immunogens that maintain a biological and functional structure is vital for obtaining competent antibodies.

CC chemokine receptor belongs to the GPCR family and regulates various cellular dynamics. CCR8 is a 41-kDa seven-transmembrane CC chemokine receptor in humans, which possesses 355 amino acids. mCCR8 shares over 70% amino acid sequence identity with the human protein. It has been reported that CCR8+Treg cells function to suppress the immune response in the TME.[2] The presence of CCR8+Treg cells in a tumor is often associated with poor prognosis in various cancer patients, including breast cancer.[15] Several mAbs for CCR8 were initially developed in the research for the detection or neutralization of CCR8[31–33] and are also commercially available (Supplementary Table S1). Half of them were developed by immunizing rabbits with synthetic peptides, and applications are limited to Western blotting. Maintaining the structure of the GPCR, such as CC chemokine

receptors, is essential, primarily when it is used for flow cytometry, in which mAbs recognize the extracellular epitopes of the GPCR protein in immunology.[30]

Using the CBIS method, unlike immunizing synthetic peptides, the biological structure and modification of proteins, such as glycosylation and folding, could be retained. In commercial products, most of the mAbs applicable for flow cytometry have also been developed by cell-based immunization (Supplementary Table S1). The cell-based method might be presenting a more natural and functional antigen than synthetic peptides as an immunogen. Furthermore, it is easier to acquire mAbs against multipass-transmembrane proteins because no purified proteins are required for immunogens and screening.

Previously, we developed mAbs, such as programmed cell-death ligand 1 (PD-L1),[27] EpCAM,[34] TROP2,[35] CD20,[36] CD44,[28] and CD133,[26] using the CBIS method. In this study, we successfully developed a sensitive anti-mCCR8 mAb, C_8Mab-2, that can be used for flow cytometry using the CBIS method. C_8Mab-2 recognized overexpressed and endogenously expressed mCCR8 in each cell line (Fig. 2). Because CCR8 is considered the next pivotal immune checkpoint molecule,[3] C_8Mab-2 might be useful for investigating the molecular mechanism in various CCR8-related diseases using mouse *in vivo* models.

Authors' Contributions

T.T., R.N., J.T., T.N., M.Y., H.H., M.S., and T.A. performed experiments; M.K.K. designed the experiments; and T.T. and Y.K. wrote the article.

Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Table S1

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