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Development of an Anti-Mouse CCR8 Monoclonal Antibody (C_8Mab-1) for Flow Cytometry and Immunocytochemistry

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It has been widely accepted that monoclonal antibody (mAb) is an effective tool for cancer immunotherapy. The C-C motif chemokine receptor 8 (CCR8) is highly expressed in regulatory T cells and many cancers and is associated with the progression of the cancers. However, its role in cancer progression remains unclear. Thus, the development of mAbs for CCR8 leads to cancer immunotherapy and elucidation of unknown mechanisms of CCR8-dependent cancer progression. In this study, we have developed an anti-mouse CCR8 (mCCR8) mAb (clone C_8Mab-1, rat IgG_{2a}, kappa) using the Cell-Based Immunization and Screening (CBIS) method. We showed that C_8Mab-1 and its recombinant antibody (recC_8Mab-1) bind to mCCR8-overexpressed Chinese hamster ovary (CHO)-K1 cells (CHO/mCCR8), but not to the parental CHO-K1 cells, in flow cytometry and immunofluorescence. Moreover, C_8Mab-1 and recC_8Mab-1 specifically reacted to P388 (a mouse lymphocyte-like cells) and J774-1 (a mouse macrophage-like cells), which express endogenous mCCR8, in both applications. These results suggest that C_8Mab-1, developed using the CBIS method, is useful for flow cytometry and immunocytochemistry against exogenous and endogenous mCCR8.

Keywords: CCR8, monoclonal antibody, CBIS, flow cytometry, immunofluorescence

Introduction

Immunotherapy is a powerful approach for treating many cancers. Notably, due to the effectiveness, monoclonal antibodies (mAbs) that target immune checkpoint molecules have been developed, including cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed cell death 1 (PD-1), and programmed death-ligand 1 (PD-L1).[1] CTLA-4 and PD-1 are highly expressed in regulatory T cells (Tregs) that infiltrate into the tumor microenvironment and play a significant role in the suppression of immune response in the tumor microenvironment.[2,3]

The development of various mAbs that target other molecules has been designed to find more effective strategies for cancer immunotherapy. The tumor-infiltrating Tregs highly express C-C motif chemokine receptor 8 (CCR8) on the cell surface, in addition to CTLA-4 and PD-1.[4] CCR8 belongs to the C-C motif chemokine receptor family of G protein-coupled receptor (GPCR) and is activated by CCL1, CCL8, CCL16, and CCL18.[5–8] CCR8 is upregulated in many cancers, such as bladder cancer, breast cancer, colorectal cancer, nonsmall cell lung cancer, and oral squamous cell carcinoma.[4,9–11] CCR8 is activated by CCL1 and promotes the activities of migration and antiapoptosis in Tregs and lymphoma cells.[12–14]

Moreover, CCR8 is also highly expressed in bladder cancer cells, and the receptor promotes migration, invasion, and epithelial-mesenchymal transition through activation by CCL18.[11] These studies demonstrate that CCR8 is associated with cancer progression, and CCR8 can be a target molecule for cancer immunotherapy. In contrast, regulatory mechanisms of cancer progression by CCR8 have remained unclear.

mAbs that specifically bind to CCR8 would be useful for cancer immunotherapy and elucidation of the mechanisms of cancer progression. Despite the technical difficulty in developing anti-GPCR mAbs,[15] we have developed an anti-GPCR mAbs using the Cell-Based Immunization and Screening (CBIS) method, including anti-mouse CCR3 mAb (clone C_3Mab-2),[16] anti-human CCR9 mAb (clone C_9Mab-1),[17] and anti-mouse CCR8 (mCCR8) mAb (clone C_8Mab-2).[18] In this study, we developed another

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anti-mCCR8 mAb (clone C_8Mab-1) and showed that C_8Mab-1 recognizes both exogenous and endogenous mCCR8 in flow cytometry and immunofluorescence.

Materials and Methods

Cell lines

P3X63Ag8U.1 (P3U1) and Chinese hamster ovary (CHO)-K1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). J774-1 and P388 were obtained from the Cell Resource center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). mCCR8-overexpressed CHO-K1 (CHO/mCCR8) was established in our previous study.[18] In brief, 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). mCCR8 plasmid was transfected using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). CHO/mCCR8 cells were sorted using a cell sorter (SH800; Sony Corp., Tokyo, Japan) and cultivated in a medium containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA).

P3U1, CHO-K1, P388, J774-1, and CHO/mCCR8 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 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). Cells were grown in a humidified incubator at 37°C with 5% CO_2 and 95% air.

Animals

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 conducted following the 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 No: 2019NiA-001). The rat was monitored daily for health during the four full week's duration of the experiment. A decrease of more than 25% of the 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.

Hybridoma production

To develop mAbs against mCCR8, the CBIS method [19–25] was used. In brief, 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 1640 medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). Culture supernatants were screened using flow cytometry.

Production of the recombinant antibody

To produce recombinant C_8Mab-1 (recC_8Mab-1), we subcloned variable (V_H) and constant (C_H) regions of heavy chain cDNAs of C_8Mab-1 into the pCAG-Neo vector along with variable (V_L) and constant (C_L) regions of light chain cDNAs of C_8Mab-1 into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation). We transfected C_8Mab-1 vectors into ExpiCHO-S cells using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.). We purified the resulting mAb (recC_8Mab-1) using Protein G-Sepharose (GE Healthcare Biosciences, Pittsburgh, PA).

Flow cytometry

Cells were harvested after brief exposure to 0.25% trypsin and 1 mM of ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). They were washed with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and treated with primary mAbs (10 μg/mL) 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., Danvers, MA). Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Determination of the 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 EC800 Cell Analyzer. 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).

Immunocytochemistry

Subconfluent CHO-K1, CHO/mCCR8, P388, and J774-1 were fixed with 4% paraformaldehyde in PBS for 10 minutes and additional incubation with 50 mM of NH_4Cl in PBS supplemented with 0.2 mM of Ca^{2+} and 2 mM of Mg^{2+} (PBSc/m) for 10 minutes at room temperature. The cells were blocked with a blocking buffer (PBSc/m supplemented with 0.5% BSA) for 30 minutes followed by incubation with primary mAbs (10 μg/mL) for 1 hour and Alexa Fluor 488-conjugated anti-rat IgG (1:400; Cell Signaling Technology, Inc.) for 45 minutes at room temperature. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific, Inc.). We acquired fluorescence images on a BZ-X800 digital microscope (Keyence, Osaka, Japan) using a 40× objective.

Results

Establishment of anti-mCCR8 mAbs

To develop anti-mCCR8 mAbs, we used the CBIS method, using stable transfectants immunization and flow cytometry-mediated hybridoma screening. A SD rat was immunized with CHO/mCCR8 cells. Hybridomas were seeded into 96-well plates, and CHO/mCCR8-positive and CHO-K1-negative wells were selected. After limiting dilution, C_8Mab-1 (IgG_{2a}, kappa) was finally established. We further produced its recombinant antibody (recC_8Mab-1).

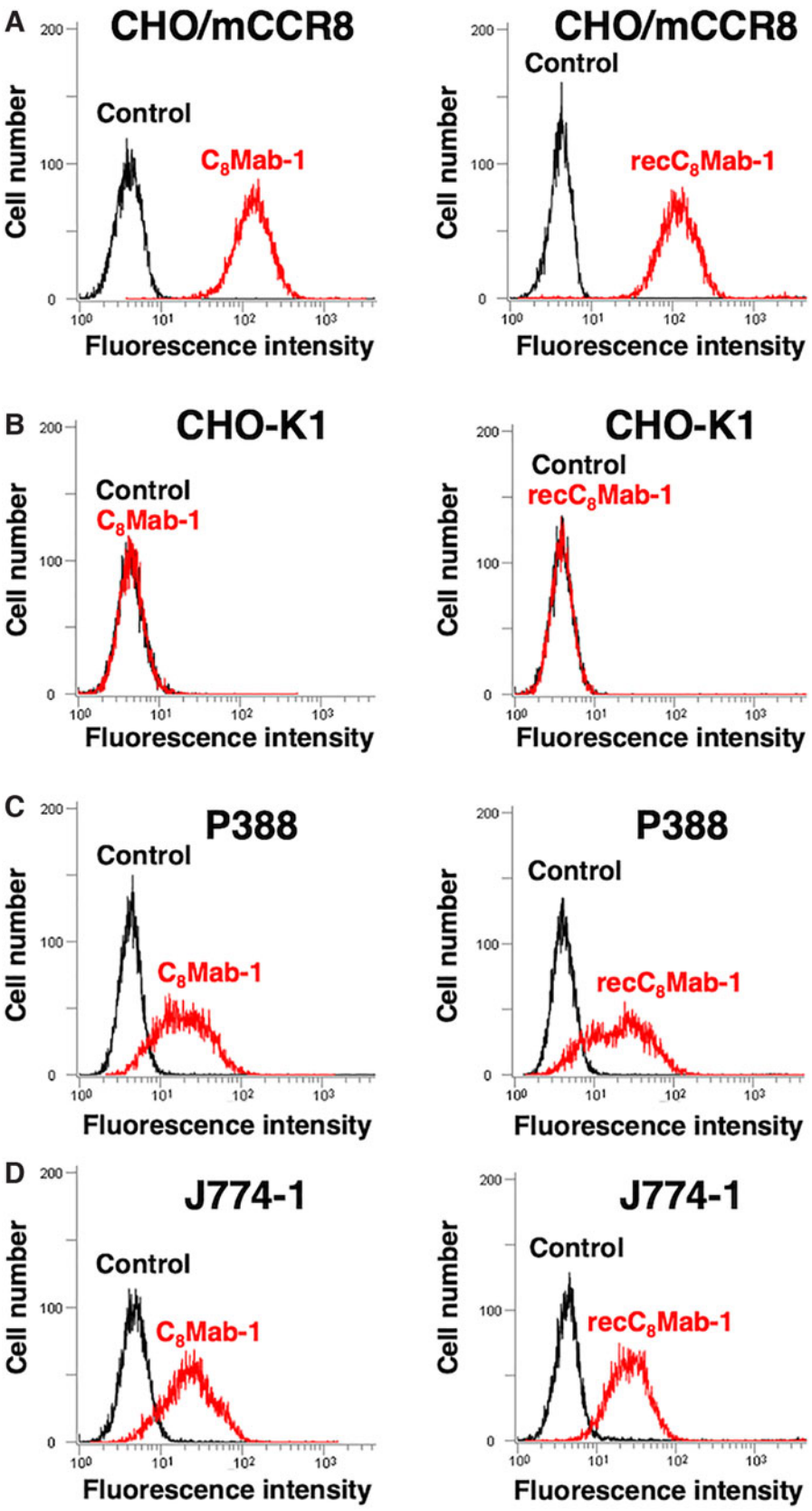

FIG. 1. Flow cytometry using anti-mCCR8 mAbs, C_8Mab-1, and recC_8Mab-1. CHO/mCCR8 **(A)**, CHO-K1 **(B)**, P388 **(C)**, and J774-1 **(D)** were treated with 10 μg/mL C_8Mab-1 or recC_8Mab-1, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG; red line, C8Mab-1 or recC8Mab-1 treated cells; black line, negative control. CHO, Chinese hamster ovary; mAb, monoclonal antibody; mCCR8, mouse C-C motif chemokine receptor 8.

Flow cytometry

We performed flow cytometry using C_8Mab-1 and recC_8Mab-1 against CHO/mCCR8 and CHO-K1. Both C_8Mab-1 and recC_8Mab-1 recognized CHO/mCCR8 (Fig. 1A), but not CHO-K1 (Fig. 1B). Both C_8Mab-1 and recC_8Mab-1 also reacted with P388 (Fig. 1C) and J774-1 (Fig. 1D), expressing endogenous mCCR8.

Determination of the binding affinity of C_8Mab-1

C_8Mab-1 might detect the conformational epitope of CCR8; therefore, we could not determine the binding affinity using Surface Plasmon Resonance. In this study, we assessed the binding affinity of C_8Mab-1 and recC_8Mab-1 with CHO/mCCR8 by flow cytometry. The K_D of C_8Mab-1 and recC_8Mab-1 for CHO/mCCR8 was 6.7×10^{-7} M and 1.1×10^{-7} M, respectively (Supplementary Fig. S1), indicating that C_8Mab-1 possesses a low affinity for CHO/mCCR8 cells.

Immunocytochemistry using C_8Mab-1 and recC_8Mab-1

We applied C_8Mab-1 and recC_8Mab-1 to immunocytochemistry against exogenously expressing mCCR8. We found that C_8Mab-1 and recC_8Mab-1 bound to CHO/mCCR8 (Fig. 2A), but not CHO-K1 (Fig. 2B), indicating that both antibodies recognize exogenous mCCR8. We also used C_8Mab-1 and recC_8Mab-1 for immunocytochemistry against endogenously expressing mCCR8 in P388 and J774-1 cells. We found that C_8Mab-1 and recC_8Mab-1 specifically recognize endogenous mCCR8 in both cells (Fig. 2C, D) Note that the intensities of the fluorescent signals were weak because the expression level of endogenous mCCR8 is expected to be low in both cells.

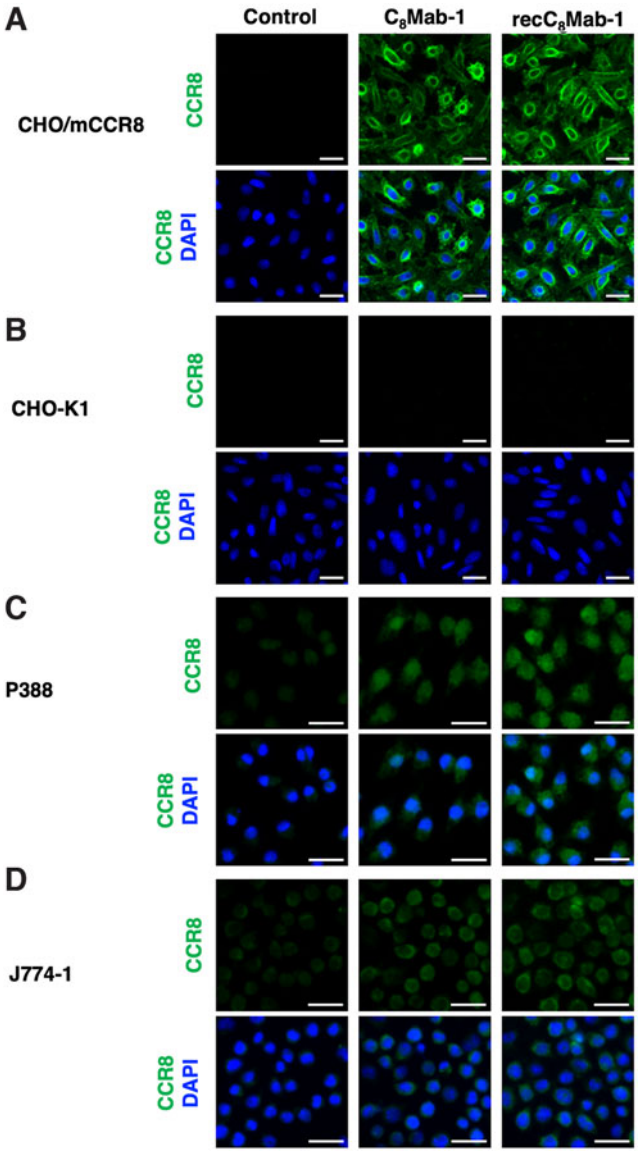

FIG. 2. Immunocytochemistry using C_8Mab-1 and recC_8Mab-1. CHO/mCCR8 **(A)**, CHO-K1 cells **(B)**, P388 **(C)**, and J774-1 **(D)** were treated with buffer control, 10 μg/mL C_8Mab-1, or recC_8Mab-1, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. DAPI was used for nuclear staining. Scale bars, 20 μm. DAPI, 4′,6-diamidino-2-phenylindole.

Discussion

In this study, we developed a novel mAb for mCCR8, C_8Mab-1, using the CBIS method. Both C_8Mab-1 and recC_8Mab-1 bound to mCCR8 with low affinity (Supplementary Fig. S1), but they specifically recognized exogenous and endogenous mCCR8 in both flow cytometry (Fig. 1) and immunocytochemistry (Fig. 2).

These mAbs would be useful for elucidation of the mechanism of mCCR8 in cancer progression, such as analysis of the expression level and intracellular distribution of mCCR8 in target cells, and isolation of $CCR8^+$ cells by combining with a cellular sorting technique. Confirming other applications of C_8Mab-1 and recC_8Mab-1, including western blotting, immunohistochemistry, and immunoprecipitation, is required to promote basic research in the future. In contrast, flow cytometric analysis revealed that C_8Mab-1 and recC_8Mab-1 bind to mCCR8 with low affinity. We previously developed another mCCR8 mAb (C_8Mab-2), which binds to mCCR8 with moderate affinity in flow cytometry.[18] To uncover amino acid sequences of the epitopes of C_8Mab-1 and C_8Mab-2 would be advantageous for developing novel mAbs that possess higher affinity.

Recent studies have revealed that anti-CCR8 mAbs and a CCR8-specific nanobody display antitumor activity in bladder cancer, colon cancer, and nonsmall cell lung cancer.[26–28] A mechanism of the antitumor activity is the depletion of $CCR8^+$ Tregs at the tumor microenvironment.[27,29] In contrast, we have not evaluated the antitumor activity of C_8Mab-1 and recC_8Mab-1. It is important to investigate their antitumor activity by Tregs depletion using *in vivo* mice tumor models. Furthermore, since CCR8 is highly expressed in cancer cells, the evaluation of their antitumor activity via antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity is also thought to be important.

CCR8 plays an important role in other physiological functions. The receptor is involved in the promotion of the onset of allergy and inflammatory diseases by recruiting Tregs, T helper 2 (T_H2) cells, and eosinophils into the inflammation site.[30–33] CCR8 expressed in neurons causes

diabetic neuropathy via stimulation by CCL1.[34] Thus, it is expected that C_8Mab-1 and recC_8Mab-1 are also effective for treating these diseases.

In summary, we developed a novel mAb for mCCR8, C_8Mab-1, in this study. C_8Mab-1 and recC_8Mab-1 are applicable to flow cytometry and immunocytochemistry. These mAbs would be valuable for elucidating the roles of CCR8 in cancer.

Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1

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