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Establishment of a Sensitive Monoclonal Antibody Against Mouse CCR9 (C_9Mab-24) for Flow Cytometry

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The CC chemokine receptor 9 (CCR9), also known as CD199, is one of chemokine receptors. The CC chemokine ligand 25 (CCL25) is known to be the only ligand for CCR9. The CCR9-CCL25 interaction plays important roles in chemotaxis of lymphocytes and tumor cell migration. Therefore, CCR9-CCL25 axis is a promising target for tumor therapy and diagnosis. In this study, we established a sensitive and specific monoclonal antibody (mAb) against mouse CCR9 (mCCR9) using N-terminal peptide immunization method. The established anti-mCCR9 mAb, C_9Mab-24 (rat immunoglobulin $[IgG]_{2a}$, kappa), reacted with mCCR9-overexpressed Chinese hamster ovary-K1 (CHO/mCCR9) and mCCR9-endogenously expressed cell line, RL2, through flow cytometry. Kinetic analyses using flow cytometry showed that the dissociation constants (K_D) of C_9Mab-24 for CHO/mCCR9 and RL2 cell lines were 6.0×10^{-9} M and 4.7×10^{-10} M, respectively. Results indicated that C_9Mab-24 is useful for detecting mCCR9 through flow cytometry, thereby providing a possibility for targeting mCCR9-expressing cells *in vivo* experiments.

Keywords: mouse CCR9, monoclonal antibody, flow cytometry, dissociation constant

Introduction

Chemokines are 8–14 kDa proteins, which are known to be ligand for G protein-coupled receptors (GPCRs). Chemokine-GPCR pathway regulates cellular trafficking of lymphocytes and plays an important role in the immune system.[1,2] Chemokines are divided into four subfamilies, CC, CXC, XC, and CX3C chemokines, based on the differences of cysteine positions at their N-terminus.[3,4] Chemokine receptors are also classified into four families that correspond to their ligand subfamilies: CC chemokine receptor (CCR), CXC chemokine receptor (CXCR), CX3C chemokine receptor (CX3CR), and XC chemokine receptor (XCR).[5] Chemokines involve in the pathogenesis of several diseases, including allergic inflammatory disease, human immunodeficiency virus-associated diseases, and cancer.[6–8]

CCR9, also known as CD199, is a member of GPCR family consisting of an extracellular N-terminus, seven membrane-spanning regions, and a cytoplasmic C-terminus.[9–11] CC chemokine ligand 25 (CCL25) is known to be the only ligand for the CCR9. The CCR9-CCL25 mediates chemotaxis of lymphocytes.[9–11] Furthermore, the CCR9-CCL25 axis plays important roles in the inhibition of apoptosis,[12,13] proliferation,[14–16] invasion,[17] and metastasis[18–20] during tumor progression.[21–24] CCR9 is highly expressed in T cell lineage acute lymphoblastic leukemia (T-ALL) cells,[25] lung adenocarcinoma tissues,[26] breast cancer cell line (MDA-MB-231),[27] and ovarian cancer cell lines (OVCAR-3 and CAOV-3).[28]

The elevated expression of CCL25 is observed in T-ALL,[29] prostate,[30] breast,[28] and ovarian cancers.[28,31] The CCR9–CCL25 interaction mediates PI3K/AKT-dependent antiapoptotic signals, which results in low apoptosis and modest chemotherapeutic response.[30] Therefore, the CCR9-CCL25 interaction will be a promising target for cancer treatment and diagnosis.

In this study, we established a sensitive and specific monoclonal antibody (mAb) against mouse CCR9 (mCCR9) using N-terminal peptide immunization method and determined the dissociation constants (K_D) using mCCR9-expressed cell lines by flow cytometry.

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Materials and Methods

Peptides

Eurofins Genomics KK (Tokyo, Japan) synthesized a partial sequence of the N-terminal extracellular region of mCCR9 (accession no. NP_001160097) with cysteine at its C-terminus (mCCR9p1-19C; sequence: MMPTELTSLI PGMFDDFSYC). Subsequently, the keyhole limpet hemocyanin (KLH) was conjugated at the C-terminus of the peptide (mCCR9p1-19C-KLH).

Preparation of cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). RL2 cells (mouse leukemia cells) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Miyagi, Japan). The expression plasmid of mCCR9 (pCMV6neo-mCCR9-Myc-DDK) was purchased from OriGene Technologies, Inc. (Rockville, MD). The mCCR9 plasmid was transfected into CHO-K1 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) using Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), containing 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.), and 0.5 mg/mL of G418 (Nacalai Tesque, Inc.).

CHO-K1, P3U1, mCCR9-overexpressed CHO-K1 (CHO/mCCR9), and RL2 were cultured in RPMI 1640 medium with 10% heat-inactivated FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B. Cells were grown in a humidified incubator at 37°C, at an atmosphere of 5% CO_2 and 95% air.

Antibodies

Anti-mCCR9 mAbs (clone 9B1 and CW-1.2) were purchased from BioLegend (San Diego, CA). A secondary Alexa Fluor 488-conjugated anti-rat immunoglobulin (IgG) was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Production of hybridomas

A 5-week-old Sprague–Dawley rat was purchased from CLEA Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions. All animal experiments were also conducted according to relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. The Animal Care and Use Committee of Tohoku University (permit no. 2019NiA-001) approved animal experiments. The rat was monitored daily for health during the full 4-week duration of the experiment. A reduction of more than 25% of the total body weight was defined as a humane endpoint. During sacrifice, the rat was euthanized through cervical dislocation, after which death was verified through respiratory and cardiac arrest.

To develop mAbs against mCCR9, one rat was immunized intraperitoneally, using 100 μg mCCR9p1-19C-KLH peptide with Imject Alum (Thermo Fisher Scientific, Inc.). The procedure included three additional immunizations, which were followed by a final booster intraperitoneal injection, 2 days before the harvest of spleen cells. Harvested spleen cells were subsequently fused with P3U1 cells, using PEG1500 (Roche Diagnostics, Indianapolis, IN), after which hybridomas were grown in an RPMI1640 medium supplemented with hypoxanthine, aminopterin, and thymidine for the selection (Thermo Fisher Scientific, Inc.). Supernatants were subsequently screened with the mCCR9p1-19C peptide, using enzyme-linked immunosorbent assay (ELISA), after flow cytometry, using CHO/mCCR9 and CHO-K1 cells.

Enzyme-linked immunosorbent assay

The synthesized peptide, mCCR9p1-19C, was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at a concentration of 1 μg/mL for 30 minutes at 37°C. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc.), wells were blocked with 1% bovine serum albumin-containing PBST for 30 minutes at 37°C. Plates were then incubated with supernatants of hybridomas, followed by a peroxidase-conjugated anti-rat IgG (1:20000 diluted; Sigma-Aldrich Corp., St. Louis, MO). Next, enzymatic reactions were conducted, using an ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.), followed by the measurement of the optical density at 655 nm, using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Purification of mAbs

The cultured supernatants of C_9Mab-24-expressing hybridomas were collected through centrifugation at 2330 × g for 5 minutes, followed by filtration using Steritop (0.22 μm; Merck KGaA, Darmstadt, Germany). Filtered supernatants were subsequently applied to 1 mL Protein G-Sepharose (Cytiva, Marlborough, MA). After washing with PBS, bound antibodies were eluted with an IgG elution buffer (Thermo Fisher Scientific, Inc.), followed by an immediate neutralization of eluates, using 1 M Tris-HCl. Finally, eluates were concentrated, after which PBS was used to replace the elution buffer, using Amicon Ultra (Merck KGaA).

Flow cytometric analyses

CHO-K1 and CHO/mCCR9 cells were harvested after an exposure to 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). CHO-K1, CHO/mCCR9, and RL2 cells were washed with 0.1% bovine serum albumin in PBS and treated with 0.01, 0.1, 1, and 10 μg/mL primary mAbs for 30 minutes at 4°C. Then, cells were treated with Alexa Fluor 488-conjugated anti-rat IgG (1:2000 diluted), after which fluorescence data were collected, using the BD FACSLyric (BD Biosciences, Franklin Lakes, NJ).

Determination of dissociation constants (K_D) through flow cytometry

CHO/mCCR9 and RL2 cells were suspended in 100 μL serially diluted anti-mCCR9 mAbs, after which 50 μL Alexa Fluor 488-conjugated anti-rat IgG (1:200 diluted) was added.

Afterward, fluorescence data were collected, using BD FACSLyric. The K_D were subsequently calculated by fitting saturation binding curves to the built-in one-site binding models in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

Results

Development of anti-mCCR9 mAbs by peptide immunization

To develop anti-mCCR9 mAbs, one rat was immunized with an mCCR9p1-19C-KLH peptide (Fig. 1A). Spleen was then excised from the rat, after which splenocytes were fused with myeloma P3U1 cells using PEG1500 (Fig. 1B). Developed hybridomas were subsequently seeded into 20 of 96-well plates and cultivated for 7 days. Then, wells in which cultured supernatants were positive for the mCCR9p1-19C peptide through ELISA were selected (Fig. 1C).

The ELISA screening approach identified strong signals from mCCR9p1-19C peptide-immunized wells, no signal was detected from control wells in 152 of 1916 wells (7.93%). Afterward, ELISA-positive wells were screened using flow cytometry for the selection of mCCR9-expressing cell-reactive and CHO-K1-nonreactive supernatants (Fig. 1C). The flow cytometric screening approach identified strong signals from CHO/mCCR9 cells, and a weak or no signal from CHO-K1 cells in 37/152 (24.3%). After limiting dilution and several additional screenings, anti-mCCR9 mAbs, C_9Mab-24 (rat IgG_{2a}, kappa) was finally established (Fig. 1D).

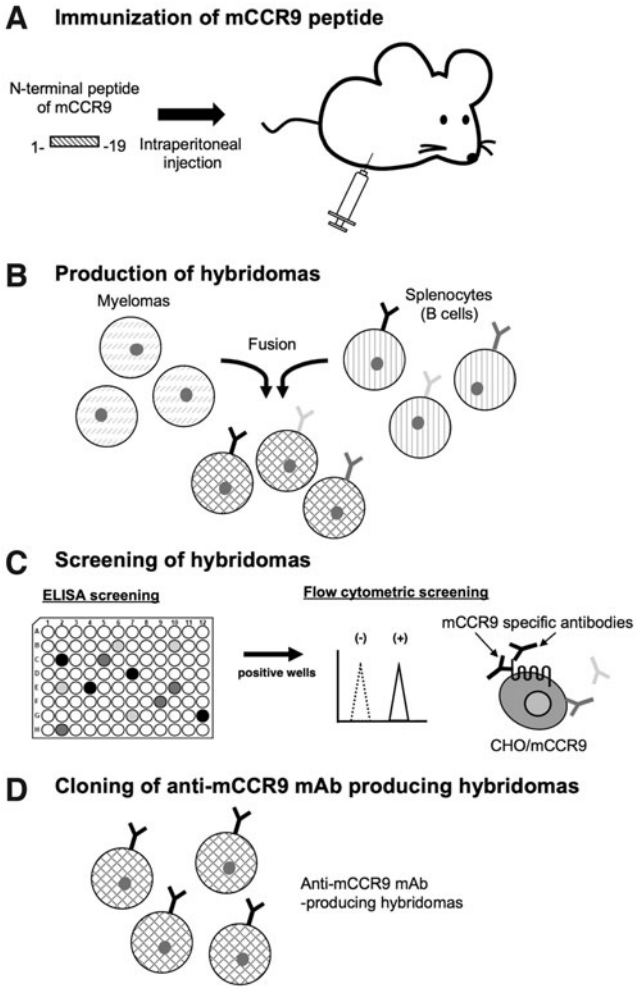

FIG. 1. Schematic illustration of the production of anti-mCCR9 mAbs. **(A)** mCCR9p1-19C-KLH peptide was immunized into one SD rat using an intraperitoneal injection. **(B)** Spleen was excised and spleen cells were fused with P3U1 cells. **(C)** Culture supernatants were screened by ELISA and then flow cytometry to select hybridomas that are producing anti-mCCR9 mAb. **(D)** After limiting dilution and some additional screenings, mCCR9-specific mAb was finally established. ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; P3U1, P3X63Ag8U.1; mAb, monoclonal antibody; mCCR9, mouse CCR9; SD, Sprague–Dawley.

Flow cytometric analysis

We conducted flow cytometric analysis using three anti-mCCR9 mAbs, C_9Mab-24 and the commercially available anti-mCCR9 mAbs (clone 9B1 and CW-1.2 from BioLegend), against mCCR9-expressed cell lines CHO/mCCR9 and RL2. All three mAbs recognized CHO/mCCR9 cells dose-dependently (Fig. 2A), whereas parental CHO-K1 cells were not recognized by them except for 10 μg/mL of CW-1.2 (Fig. 2B). Although 9B1 did not react with CHO/mCCR9 cells at 0.01 μg/mL, C_9Mab-24 and CW-1.2 reacted with the cells even at 0.01 μg/mL (Fig. 2A). All three mAbs also reacted with RL2 cells dose-dependently, but 9B1 did not react with RL2 cells at 0.1 and 0.01 μg/mL (Fig. 2C). These results suggested that C_9Mab-24 is specific for mCCR9, and is useful for detecting exogenous and endogenous mCCR9 through flow cytometry.

Kinetic analysis of the interaction of anti-mCCR9 mAbs with mCCR9-expressed cells through flow cytometry

To determine the apparent K_D of anti-mCCR9 mAbs with mCCR9-expressed cells, we conducted kinetic analysis of the interaction of C_9Mab-24, 9B1, and CW-1.2 with CHO/mCCR9 and RL2 cells using flow cytometry. The geometric means of the fluorescence intensity were then plotted versus the concentration of anti-mCCR9 mAbs, following fitting through one-site binding models in GraphPad PRISM 8. The K_D of C_9Mab-24 for CHO/mCCR9 and RL2 cells were determined as 6.0×10^{-9} M (Fig. 3A) and 4.7×10^{-10} M (Fig. 3B), respectively. The K_D of 9B1 for CHO/mCCR9 and RL2 cells were determined as 2.7×10^{-8} M (Fig. 3C) and 8.7×10^{-8} M (Fig. 3D), respectively. The K_D of CW-1.2 for CHO/mCCR9 and RL2 cells were determined as 4.3×10^{-9} M (Fig. 3E) and 2.9×10^{-10} M (Fig. 3F), respectively. These results indicate that C_9Mab-24 and CW-1.2 possess a high affinity for both CHO/mCCR9 cells and RL2 cells.

Discussion

Previously, we have succeeded in development of mAbs against chemokine receptors, including mouse CCR2,[32] human CCR2,[33] mouse CCR3,[34–36] mouse CCR4,[37] mouse CCR8,[38–40] human CCR9,[41] and mouse CXCR6.[42] In this study, we developed a novel anti-mCCR9

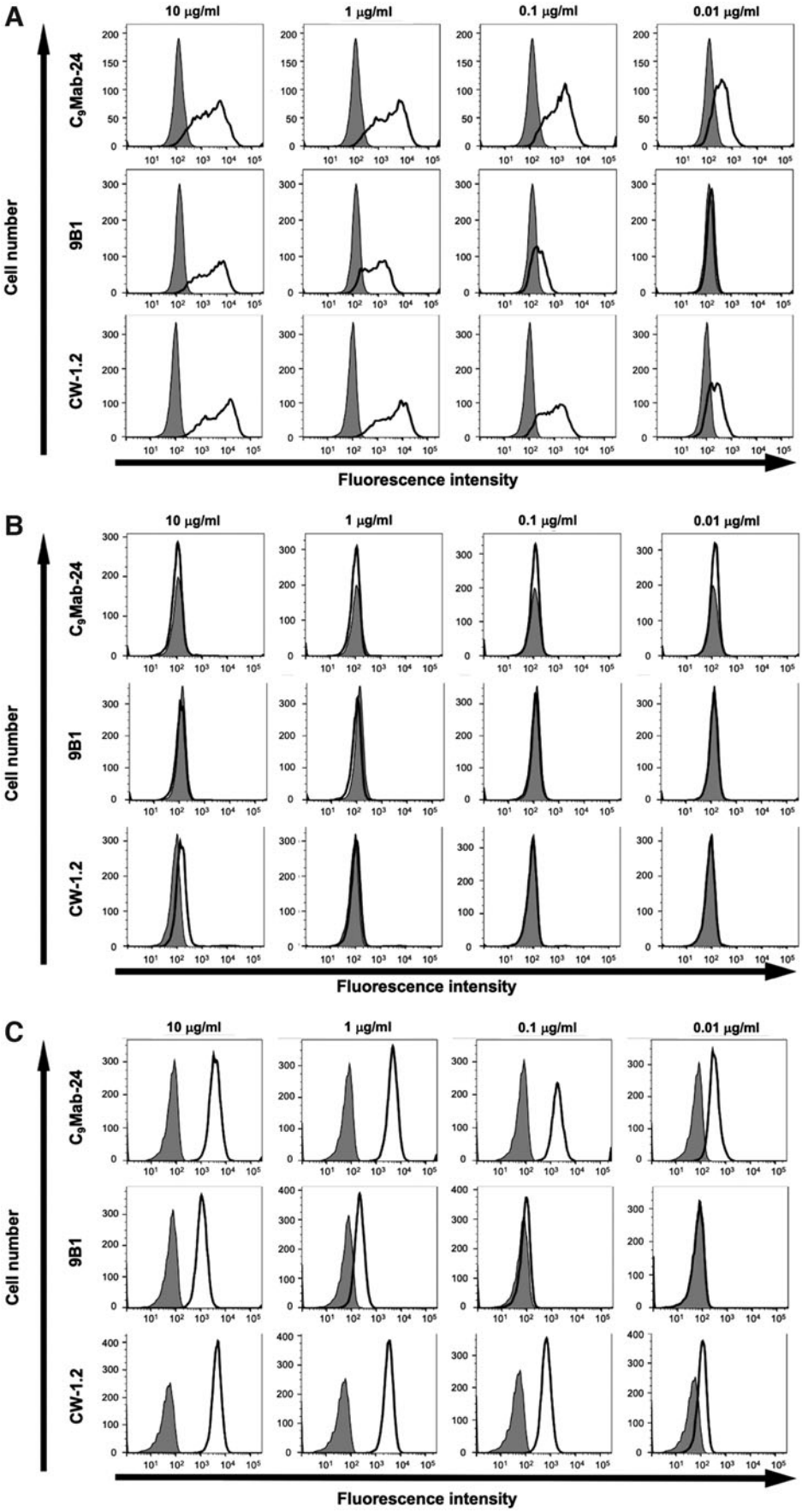

FIG. 2. Flow cytometry using anti-mCCR9 mAbs. CHO/mCCR9 **(A)**, CHO-K1 **(B)**, and RL2 **(C)** cells were treated with 0.01, 0.1, 1, and 10 μg/mL of C_9Mab-24 (upper panels), 9B1 (middle panels), or CW-1.2 (lower panels), followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. Filled, without primary Ab as negative controls. CHO, Chinese hamster ovary; IgG, immunoglobulin.

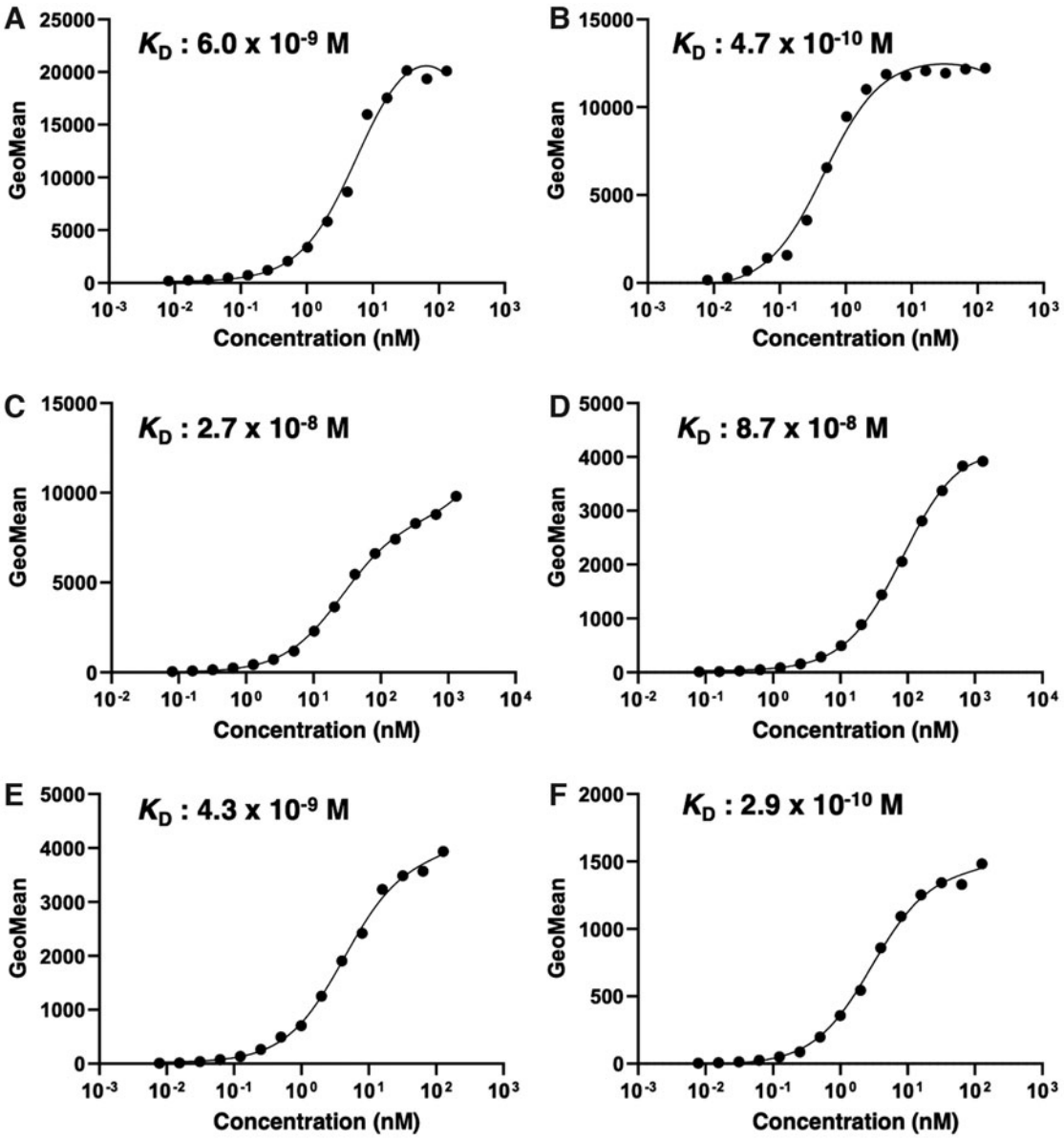

FIG. 3. Determination of the K_D of anti-mCCR9 mAbs against mCCR9-expressing cells. CHO/mCCR9 **(A, C, E)** and RL-2 **(B, D, F)** were suspended in 100 μL serially diluted C_9Mab-24 (0.0006–10 μg/mL, **A, B**), 9B1 (0.006–100 μg/mL, **C, D**) or CW-1.2 (0.0006–10 μg/mL, **E, F**). Alexa Fluor 488-conjugated anti-rat IgG was then added. Fluorescence data were obtained using BD FACSLyric. K_D were calculated by fitting saturation binding curves to the built-in one-site binding models in GraphPad PRISM 8.

mAb, C_9Mab-24 (rat IgG_{2a}, kappa), through immunization with the N-terminal peptide of mCCR9. C_9Mab-24 can detect both exogenous and endogenous mCCR9 in flow cytometric analysis (Fig. 2).

The specific and sensitive antibodies are essential to develop therapeutic antibodies. Therapeutic antibodies possess several mechanisms of action, including neutralization,[43] antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC).[44] Furthermore, therapeutic antibodies can be utilized as a drug delivery carrier,[45] Sharma et al. reported that blocking of the CCR9-CCL25 interaction by anti-CCR9 mAb abrogates the CCL25-mediated resistance to a chemotherapeutic agent, etoposide in prostate cancer.[30] It has been also reported that anti-CCR9 mAb inhibited the ovarian cancer migration and invasiveness toward chemotactic gradients of CCL25.[28] Therefore, CCR9 is an attractive therapeutic target and anti-CCR9 mAbs are useful for cancer treatment.

In this study, we showed the specificity (Fig. 2) and high affinities (Fig. 3A, B) of C_9Mab-24. It has been reported that GPCRs, including CCR9, CCR2, CCR3, CCR5, and CXCR1, interact with their ligands at the N-terminal region.[11,46–48] Since C_9Mab-24 was developed by immunizing N-terminal peptide of mCCR9, C_9Mab-24 is expected to inhibit the binding of CCL25 to CCR9. Moreover, rat IgG_{2a} possesses ADCC and CDC activities. Therefore, C_9Mab-24 could be used for the elimination of mCCR9-positive cells *in vivo*.

Author Disclosure Statement

No competing financial interests exist.

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