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Development of a Novel Anti-Mouse CCR6 Monoclonal Antibody (C_6Mab-13) by N-Terminal Peptide Immunization

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The CC chemokine receptor 6 (CCR6) is a G protein-coupled receptor family member that is highly expressed in B lymphocytes, certain subsets of effector and memory T cells, and immature dendritic cells. CCR6 has only one chemokine ligand, CCL20. The CCL20–CCR6 axis has been recognized as a therapeutic target for autoimmune diseases and tumor. This study developed specific monoclonal antibodies (mAbs) against mouse CCR6 (mCCR6) using the peptide immunization method. The established anti-mCCR6 mAb, C_6Mab-13 (rat IgG_1, kappa), reacted with mCCR6-overexpressed Chinese hamster ovary-K1 (CHO/mCCR6), and mCCR6-endogenously expressed P388 (mouse lymphoid neoplasma) and J774-1 (mouse macrophage-like) cells in flow cytometry. The dissociation constant (K_D) of C_6Mab-13 for CHO/mCCR6 cells was determined to be 2.8×10^{-9} M, indicating that C_6Mab-13 binds to mCCR6 with high affinity. In summary, C_6Mab-13 is useful for detecting mCCR6-expressing cells through flow cytometry.

Keywords: mouse CCR6, monoclonal antibody, peptide immunization

Introduction

The CC chemokine receptor 6 (CCR6), also known as CD196, is a member of the G protein-coupled receptor (GPCR) family consisting of an extracellular N-terminus, seven membrane-spanning regions, and a cytoplasmic C-terminus.[1] CCR6 was first cloned in human and is a 374 amino acid-long protein.[2] CCR6 is expressed in B lymphocytes, certain subsets of effector and memory T cells, and immature dendritic cells, but not in monocytes, natural killer cells, and granulocytes.[2–5] The ligand of CCR6, CCL20/macrophage inflammatory protein-3α, is increased in inflammatory and/or autoimmune conditions.[6] CCL20 is involved in the recruitment of Th17 cells to sites of inflammation.[7] Several studies using CCR6-knockout mice or CCR6 inhibitors indicate the potential of CCR6 as a therapeutic target for autoimmune diseases.[6,8]

Furthermore, CCL20 is elevated in several tumor types, including breast, hepatocellular, pancreatic, and colorectal carcinomas.[9,10] Similarly, its expression is observed in tumor-associated macrophages, which are a significant source of the ligands in the tumors.[11,12] CCR6 expression has been shown on tumor cells. For instance, an autocrine stimulation of their proliferation and migration by the CCL20–CCR6 pathway plays a crucial role in tumor progression.[13,14] Furthermore, CCL20 in tumors recruits regulatory T (Treg) cells and Th17 in the stroma, resulting in tumor immune evasion.[15–17] In addition, it has been reported that high expression of CCR6 and CCL20 in tumors correlates with poor prognosis.[18–21]

Previously, we have developed monoclonal antibodies (mAbs) against membrane proteins, including podoplanin,[22–25] CD20,[26,27] CD44,[28,29] EpCAM,[30] TROP2,[31] EGFR,[32] HER2,[33] and HER3.[34] Furthermore, we have succeeded in the development of some anti-GPCR mAbs, including anti-mouse CCR2 mAb,[35] anti-mouse CCR3 mAbs,[36–38] anti-mouse CCR4 mAb,[39] anti-mouse CCR8 mAbs,[40–42] anti-mouse CXCR6 mAb,[43] and anti-human CCR9 mAb.[44] In this study, we established a novel anti-mouse CCR6 (mCCR6) mAb using the N-terminal peptide immunization method and determined the dissociation constant (K_D) using mCCR6-expressed cell lines by flow cytometry.

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Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). Mouse lymphoid neoplasma (P388) and mouse macrophage-like (J774-1) cells 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 mCCR6 (Accession No. NM_001190333.1) was subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). CHO-K1 cells were transfected with plasmids bearing mCCR6 with tags (PA tag at N-terminus, and RAP and MAP tags at C-terminus) using the 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) and cultivation in a medium containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA).

CHO-K1, P3U1, mCCR6-overexpressed CHO-K1 (CHO/mCCR6), P388, and J774-1 cells were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), which was supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc.), 100 U/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 an atmosphere of 5% carbon dioxide and 95% air.

Antibodies

An anti-mCCR6 mAb (clone 29-2L17) was bought from BioLegend (San Diego, CA). Secondary Alexa Fluor 488-conjugated anti-rat IgG and Alexa Fluor 488-conjugated anti-Armenian hamster IgG were bought from Cell Signaling Technology, Inc. (Danvers, MA).

Peptides

Eurofins Genomics KK (Tokyo, Japan) synthesized a partial sequence of the N-terminal extracellular region of mCCR6 ($_1$-MNSTESYFGTDDYDNTEYY-$_{19}$) plus C-terminal cysteine (mCCR6p1-19C). Subsequently, the keyhole limpet hemocyanin (KLH) was conjugated at the C-terminus of the peptide (mCCR6p1-19C-KLH).

Production of hybridomas

A 7-week-old Sprague–Dawley (SD) rat was purchased from CLEA Japan (Tokyo, Japan), and was housed under specific pathogen-free conditions. All animal experiments were conducted 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 No. 2019NiA-001). The rat was monitored daily for their health during the full 4-week duration of the experiment. A reduction of more than 25% of total body weight was defined as a humane endpoint. The rat was euthanized through cervical dislocation and death was verified through respiratory and cardiac arrest.

To develop mAbs against mCCR6, one rat was intraperitoneally immunized, using 300 μg mCCR6p1-19C-KLH peptide with Imject Alum (Thermo Fisher Scientific Inc.). The procedure included three additional immunization sessions, which was followed by a final booster intraperitoneal injection, administered 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 RPMI 1640 medium supplemented with hypoxanthine, aminopterin, and thymidine for the selection (Thermo Fisher Scientific Inc.). Supernatants were subsequently screened with the mCCR6p1-19C peptide, using enzyme-linked immunosorbent assay (ELISA), after flow cytometry, using CHO/mCCR6, CHO-K1, P388, and J774-1 cells.

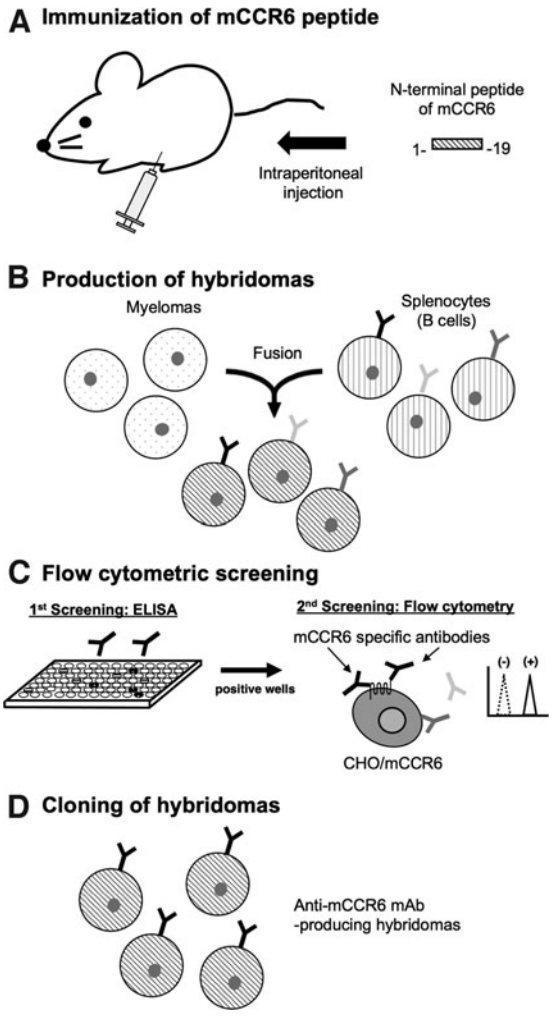

FIG. 1. Schematic illustration of the production of anti-mCCR6 mAbs. **(A)** An SD rat was immunized with N-terminal mCCR6 peptide using an intraperitoneal injection. **(B)** Spleen cells were fused with P3U1 cells. **(C)** Culture supernatants were screened with ELISA and flow cytometry to select hybridomas that are producing anti-mCCR6 mAbs. **(D)** After limiting dilution and some additional screenings, anti-mCCR6 mAbs were finally established. CCR6, CC chemokine receptor 6; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; mAbs, monoclonal antibodies; mCCR6, mouse CCR6; SD, Sprague–Dawley.

ELISA

The synthesized peptide (MNSTESYFGTDDYDNTE YYC), was immobilized on Nunc Maxisorp 96 well immunoplates (Thermo Fisher Scientific Inc.) at a concentration of 1 μg/mL for 30 min 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 (BSA)-containing PBST for 30 min at 37°C. Plates were then incubated with supernatants of

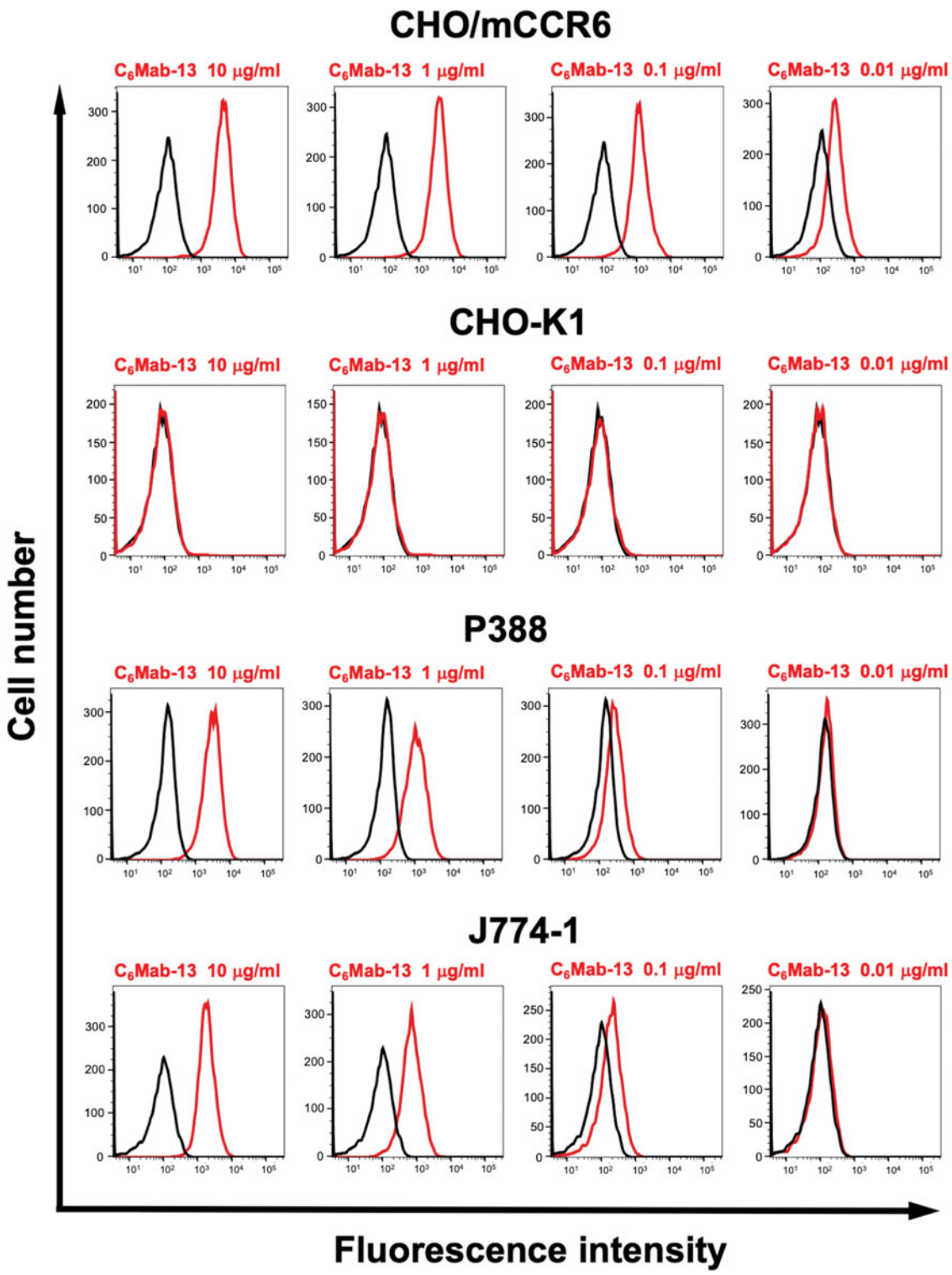

FIG. 2. Flow cytometric analyses using C_6Mab-13. CHO/mCCR6, CHO-K1, P388, and J774-1 cells were treated with 10, 1, 0.1, or 0.01 μg/mL of C_6Mab-13, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. Fluorescence data were obtained using SA3800 Cell Analyzer. Black line, negative control.

hybridomas, followed by peroxidase-conjugated anti-rat immunoglobulins (1:20000 diluted; Sigma-Aldrich Corp., St. Louis, MO). Next, enzymatic reactions were conducted, using ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.), followed by measurement of the optical density at 655 nm, using iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Flow cytometry

Cells were harvested after a brief exposure to 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). They were washed with 0.1% BSA in PBS and treated with 10, 1, 0.1, or 0.01 μg/mL primary mAbs for 30 minutes at 4°C. The cells were then treated with Alexa Fluor 488-conjugated

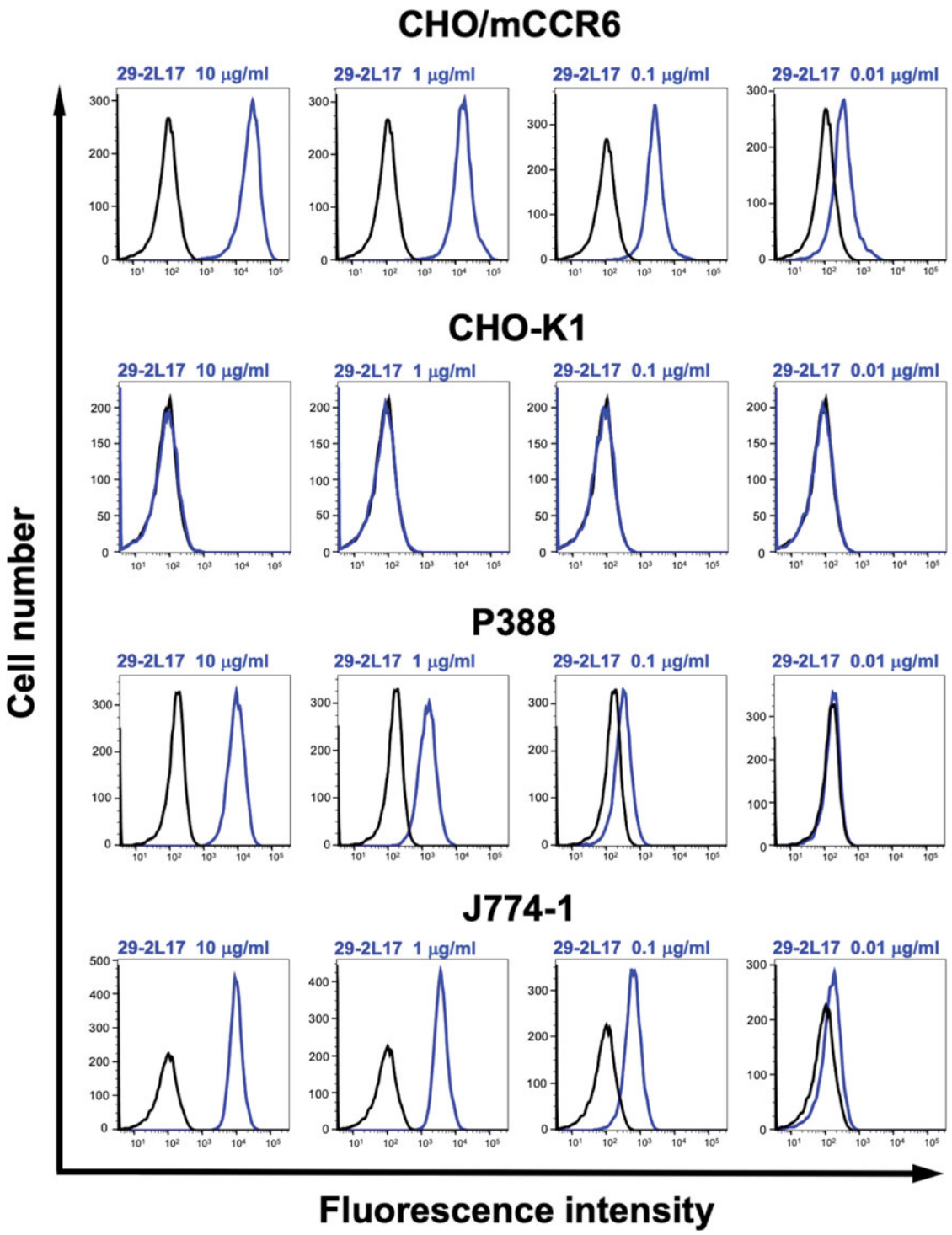

FIG. 3. Flow cytometric analyses using 29-2L17. CHO/mCCR6, CHO-K1, P388, and J774-1 cells were treated with 10, 1, 0.1, or 0.01 μg/mL of 29-2L17, followed by treatment with Alexa Fluor 488-conjugated anti-Armenian hamster IgG. Fluorescence data were obtained using SA3800 Cell Analyzer. Black line, negative control.

anti-rat IgG (1:1000) or Alexa Fluor 488-conjugated anti-Armenian hamster IgG (1:1000). Fluorescence data were obtained using SA3800 Cell Analyzer (Sony Corp.).

Determination of K_D by flow cytometry

The CHO/mCCR6 cells were suspended in 100 μL of serially diluted anti-mCCR6 mAbs and then 50 μL of Alexa Fluor 488-conjugated anti-rat IgG (1:200) or Alexa Fluor 488-conjugated anti-Armenian hamster IgG (1:200) was added. Fluorescence data were obtained using the BD FACSLyric (BD Biosciences, Franklin Lakes). The K_D was calculated by fitting saturation binding curves to built-in one-site binding models in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla).

Results

Establishment of anti-mCCR6 mAbs using the peptide immunization method

To develop anti-mCCR6 mAbs, we employed the peptide immunization method (Fig. 1). One SD rat was immunized with mCCR6p1-19C-KLH (Fig. 1A). The spleen of the rat was excised, and splenocytes were subsequently fused with myeloma cells using PEG1500 (Fig. 1B). The developed hybridomas were seeded into 96-well plates and cultivated for 7 days.

Culture supernatants that were positive for mCCR6p1-19C peptide were screened using ELISA (Fig. 1C). The first screening approach identified signals from mCCR6p1-19C in 477 out of 1916 wells (24.9%). We selected the top 82 wells for second screening. The second screening procedure identified signals from CHO/mCCR6 cells in 20 out of the 82 hybridoma supernatants identified in the previous step (24.4%). After limiting dilution and several additional flow cytometric screenings, an anti-mCCR6 mAb, C_6Mab-13 (rat IgG_1, kappa) was eventually established (Fig. 1D).

Flow cytometry analysis

We performed flow cytometry using C_6Mab-13, and commercially available anti-mCCR6 mAb (clone 29-2L17) against CHO/mCCR6, CHO-K1, P388, and J774-1 cells. C_6Mab-13 dose-dependently recognized CHO/mCCR6 cells at 10, 1, 0.1, and 0.01 μg/mL, whereas C_6Mab-13 did not react to CHO-K1 cells even at 10 μg/mL (Fig. 2). C_6Mab-13 also reacted to P388 and J774-1 cells at 10, 1, and 0.1 μg/mL, and did not react at 0.01 μg/mL (Fig. 2). Another anti-mCCR6 mAb (clone 29-2L17) also recognized CHO/mCCR6 and J774-1 cells in a dose-dependent manner at 10, 1, 0.1, and 0.01 μg/mL, and P388 cells at 10, 1, and 0.1 μg/mL, but not CHO-K1 cells (Fig. 3). These results suggest that C_6Mab-13 is useful for detecting exogenously and endogenously expressed mCCR6 using flow cytometry.

Determination of the K_D between anti-mCCR6 mAbs and CHO/mCCR6 cells by flow cytometry

To assess the K_D of C_6Mab-13 and 29-2L17 with mCCR6-expressing cells, we performed the kinetic analysis of the interactions of C_6Mab-13 and 29-2L17 with CHO/mCCR6 cells using flow cytometry. The geometric mean of fluorescence intensity was plotted versus the concentrations of C_6Mab-13 or 29-2L17, and fitted by one-site binding models in GraphPad Prism 6. The K_D of C_6Mab-13 for CHO/mCCR6 cells was determined to be 2.8×10^{-9} M (Fig. 4A). In addition, the K_D of 29-2L17 for CHO/mCCR6 cells was determined to be 8.2×10^{-9} M (Fig. 4B). These results show that C_6Mab-13 and 29-2L17 possesses a high affinity for CHO/mCCR6 cells.

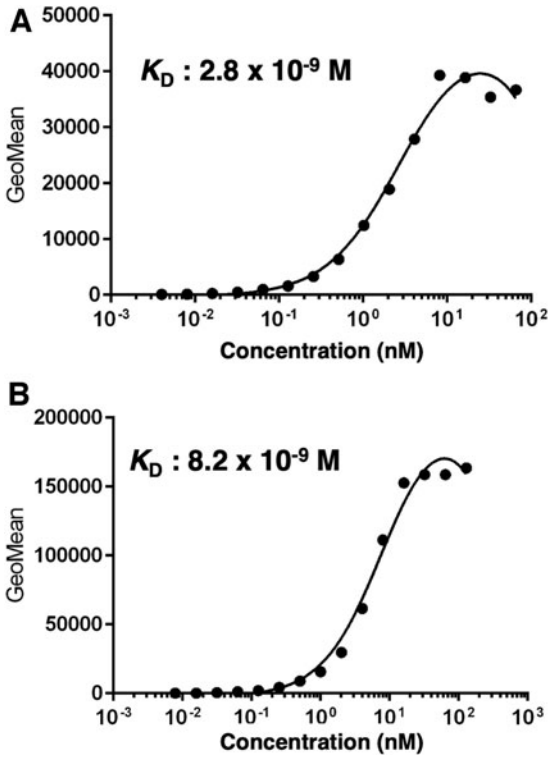

FIG. 4. Determination of the binding affinity of C_6Mab-13 and 29-2L17. CHO/mCCR6 cells were suspended in 100 µL serially diluted C6Mab-13 (0.00031–5 µg/mL) **(A)** or 29-2L17 (0.00061–10 µg/mL) **(B)**. Subsequently, Alexa Fluor 488-conjugated anti-rat IgG and anti-Armenian hamster IgG were added, respectively. Fluorescence data were collected using the FACSLyric.

Discussion

The development of the mAbs for GPCRs is difficult because of the structural complexity of GPCRs. The Cell-Based Immunization and Screening (CBIS) method is a useful technique to develop mAbs against complex structured antigens, including GPCRs (CCR3, CCR8, and CCR9)[38,42,44] and four-transmembrane protein (CD20).[26,27] We previously developed an anti-human CCR9 mAb (clone C_9Mab-1) by using CBIS method and determined that C_9Mab-1 epitope is located on the N-terminus of the human CCR9.[44,45] Therefore, we have developed mAbs against GPCRs, including mouse CCR2,[35] mouse CCR3,[37] mouse CCR4,[39] and mouse CXCR6[43] by N-terminal peptide immunization.

In this study, we developed a novel mAb against mCCR6 (C_6Mab-13) recognizes exogenous and endogenous mCCR6 by flow cytometry (Fig. 2). Although most mAbs, developed using the peptide immunization method, react with peptide, they will not react with native GPCRs on plasma membranes. Therefore, they are useful for ELISA and Western blot, but many of them are not useful for flow cytometry. In this study, we demonstrated that C_6Mab-13 can be available for flow cytometric analysis. Moreover, C_6Mab-13 binds to CHO/mCCR6 cells with high affinity (Fig. 4A).

CCL20 makes interactions with all three extracellular domains of CCR6 and N-terminal residues from Tyr27 to Leu38. In contrast, N-terminal residues before Tyr27 in CCR6 are not involved in CCL20 interaction.[46] The major sites of interaction on CCR6 are the second extracellular domain and N-terminal residues from Tyr27 to Leu38 in CCR6. The epitope of C_6Mab-13 is located from 1st to 19th amino acids of mCCR6; therefore, C_6Mab-13 might not possess neutralizing activity for CCL20.

The recruitment of Th17 cells through the CCL20–CCR6 pathway plays a critical role in the development of autoimmune diseases, including inflammatory bowel disease, psoriasis, rheumatoid arthritis, and multiple sclerosis.[6] Therefore, antibody-mediated inhibition of CCR6-positive cells migration to the sites of inflammation could control the diseases. In future study, we will try to develop the mAbs, which have neutralizing activity for CCL20 by using peptide immunization method.

The CCR6-positive Treg cells infiltrating tumors have been reported to play a critical role in the suppression of tumor immunity.[47] Therefore, the depletion of Treg cells and/or blocking of infiltration of Treg cells into tumor tissues by modulating the CCR6-CCL20 axis are promising strategies of treatment.[7,9] Recently, Robert et al reported that bispecific antibodies against CCR6 and CXCR3 effectively block cell chemotaxis and induce specific antibody-dependent cell-mediated cytotoxicity (ADCC).[48] We will convert the subclass of C_6Mab-13 into mouse IgG_{2a} to investigate whether C_6Mab-13 possesses the ADCC activity *in vitro* and evaluate the depletion of Treg cells and antitumor activities *in vivo* animal models.

Author Disclosure Statement

No competing financial interests exist.

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