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Establishment of Novel Anti-Mouse CCR3 Monoclonal Antibodies (C_3Mab-6 and C_3Mab-7) by N-terminal Peptide Immunization

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The CC chemokine receptor 3 (CCR3) is a member of the G protein-coupled receptor family that is highly expressed in eosinophils and basophils. CCR3 has been proposed as a therapeutic target for human immunodeficiency virus and allergy diagnosis. Therefore, in this study, we developed specific and sensitive monoclonal antibodies (mAbs) for mouse CCR3 (mCCR3), which are useful for flow cytometry by peptide immunization. The established anti-mCCR3 mAbs, C_3Mab-6 (rat IgG_1, kappa) and C_3Mab-7 (rat IgG_1, kappa), reacted with mCCR3-overexpressed Chinese hamster ovary-K1 (CHO/mCCR3), in addition to mCCR3-endogenously expressed cell lines, such as P388 (mouse lymphoid neoplasma) and J774-1 (mouse macrophage-like) through flow cytometry. Kinetic analyses using flow cytometry indicated that the dissociation constants (K_Ds) of C_3Mab-6 for CHO/mCCR3, P388, and J774-1 cells were 8.7×10^{-9} M, 1.4×10^{-7} M, and 1.7×10^{-7} M, respectively, whereas the K_Ds of C_3Mab-7 for these cell lines were 3.7×10^{-9} M, 5.1×10^{-7} M, and 3.1×10^{-7} M, respectively. Results also indicated that C_3Mab-6 and C_3Mab-7 are useful for detecting cells expressing CCR3 through flow cytometry, thereby making them potentially beneficial for treating CCR3-expressing cells.

Keywords: mouse CCR3, monoclonal antibody, peptide immunization

Introduction

G protein-coupled receptors (GPCRs) are cell-surface signal transmission receptor proteins that transfer extracellular signals, such as light, hormones, and chemokines to intracellular molecules.[1] They play crucial roles in the pathogenesis of diseases, including cancer, inflammation, and infection.[2–4] Therefore, GPCRs are important targets for drug development.[5] Alternatively, although anti-GPCR monoclonal antibodies (mAbs) are valuable tools for therapy and diagnosis, the development of anti-GPCR mAbs is challenging. GPCRs are seven-transmembrane protein that possesses the small extracellular loop and N-terminal regions, which are antibody accessible regions.

Moreover, GPCRs have complicated three-dimensional structures, which makes their purification difficult.[6] Therefore, selecting the starting material to be used for an immunogen is important to generate anti-GPCR mAbs. Several studies have also generated mAbs applicable to flow cytometry successfully, using the N-terminal peptides of GPCR as an immunogen.[7,8] In contrast, immunization with cells expressing target GPCRs is a suitable approach against other GPCRs, such as chemokine receptors.[9–12] Besides, GPCRs expressed on plasma membranes have native conformations. Therefore, developed mAbs can react with native GPCRs on plasma membrane, but not with the denatured one.

The CC chemokine receptor 3 (CCR3), also known as CD193, is a member of the GPCR, which is highly expressed in eosinophils, basophils, and airway epithelial cells.[13,14] Since CCR3 has been proposed as a therapeutic target for human immunodeficiency virus[15–17] and allergic diseases,[18–21] the development of sensitive mAbs for CCR3 has been desired. Many anti-chemokine receptor mAbs available for flow cytometry are commercially available. Most of them are developed through the immunization of whole cells or purified proteins.

However, only a few mAbs available for flow cytometry have been developed, using immunizing peptides, for

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instance, anti-CCR2 mAb (clone SN707; Thermo Fisher Scientific, Inc., Waltham, MA) and anti-CCR5 mAb (clone T21/8; Boster Bio, Pleasanton, CA). The synthesized peptide immunization is a simple strategy and possesses several advantages to generate anti-CCR mAbs since the N-terminal regions are not conserved among the families, linearly exposed on the cell surface.[22]

Furthermore, the neutralizing activity could be expected when the N-terminal region is a ligand-binding domain. The N-terminal region of CCR3 is a binding site for eotaxin, which is a ligand for CCR3.[23,24] Therefore, anti-CCR3 mAbs targeting the N-terminal region of CCR3 will be useful as an antagonistic antibody. In this study, we developed novel anti-mouse CCR3 (mCCR3) mAbs, using N-terminal peptide immunization methods, after which we investigated its usefulness for flow cytometry, and then determined the dissociation constant to mCCR3-expressed cell lines.

Materials and Methods

Peptides

Eurofins Genomics KK (Tokyo, Japan) synthesized a partial sequence of the N-terminal extracellular region of mCCR3 (Accession No. NP_034044.3), using cysteine as its C-terminus (mCCR3p1-19C; sequence: MAFNTDEIKTVVESFETTPC). Subsequently, the keyhole limpet hemocyanin (KLH) was conjugated at the C-terminus of the peptide (mCCR3p1-19C-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). P388 (mouse lymphoid neoplasma) and J774-1 (mouse macrophage-like) cells were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Miyagi, Japan). The synthesized DNA (Eurofins Genomics KK) encoding mCCR3 (Accession No. NM_009914.4) was subsequently subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Then, the mCCR3 plasmid was transfected into CHO-K1 and LN229 cells, using a Neon transfection system (Thermo Fisher Scientific, Inc.).

Afterward, 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 conducted. CHO-K1, P3U1, mCCR3-overexpressed CHO-K1 (CHO/mCCR3), P388, and J774-1 were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) 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, at an atmosphere of 5% CO_2 and 95% air.

Antibodies

An anti-mCCR3 mAb (clone J073E5) was purchased from BioLegend (San Diego, CA). A secondary Alexa Fluor 488-conjugated anti-rat IgG was purchased from Cell Signaling Technology, Inc., (Danvers, MA) as well.

Production of hybridomas

A 5-week-old Sprague–Dawley (SD) 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 >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 mCCR3, one SD rat was intraperitoneally immunized, using 100 μg of mCCR3p1-19C-KLH peptide with Imject Alum (Thermo Fisher Scientific, Inc.). The procedure included three additional immunization, 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 p1-19C peptide of mCCR3, using enzyme-linked immunosorbent assay (ELISA), after flow cytometry, using CHO/mCCR3, CHO-K1, P388, and J774–1 cells.

ELISA

The synthesized peptide, mCCR3p1-19C, 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 (BSA)-containing PBST for 30 minutes at 37°C. Plates were then incubated with supernatants of hybridomas, followed by a peroxidase-conjugated anti-rat immunoglobulins (1:2000 diluted; Sigma-Aldrich Corp., St. Louis, MO). Next, enzymatic reactions were conducted, using an ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.), after 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_3Mab-6 or C_3Mab-7 producing-hybridomas were collected through centrifugation at 2330 x *g* for 5 minutes, followed by filtration using Steritop (0.22 μm, Merck KGaA, Darmstadt, Germany). Filtered supernatants were subsequently applied to 1 mL Ab-Capcher ExTra (ProteNova Co., Ltd, Kagawa, Japan). 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 1M 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, CHO/mCCR3, P388, and J774-1 were harvested after an exposure to 1 mM ethylenediaminetetraacetic acid (EDTA, Nacalai Tesque, Inc.). Cells were subsequently washed with 0.1% BSA in PBS and treated with 0.01, 0.1, 1, and 10 μg/mL of primary mAbs for 30 minutes at 4°C. Then, cells were treated with Alexa Fluor 488-conjugated anti-rat IgG (1:2000; Cell Signaling Technology, Inc.), after which fluorescence data were collected, using the BD FACSLyric (BD Biosciences, Franklin Lakes, NJ).

Determination of dissociation constants (K_Ds) through flow cytometry

CHO/mCCR3, P388, and J774-1 were suspended in 100 μL of serially diluted anti-mCCR3 mAbs, after which 50 μL of Alexa Fluor 488-conjugated anti-rat IgG (1:200; Cell Signaling Technology, Inc.) was added. Afterward, fluorescence data were collected, using BD FACSLyric. The K_Ds 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-mCCR3 mAbs by peptide immunization

To develop anti-mCCR3 mAbs, one SD rat was immunized with an mCCR3p1-19C-KLH peptide (Fig. 1A). Spleen was then excised from the rat, after which splenocytes were fused with myeloma P3U1 cells (Fig. 1B). Developed hybridomas were subsequently seeded into 20 of 96-well plates and cultivated for 6 days. Afterward, wells in which cultured supernatants were positive for the mCCR3p1-19C peptide through ELISA were selected, followed by the selection of mCCR3-expressing cell-reactive and CHO-K1-nonreactive supernatants, using flow cytometry (Fig. 1C).

Although the ELISA screening approach identified strong signals from mCCR3p1-19C peptide-immobilized wells, no signal was detected from control wells in 92 of 1914 wells (4.81%). The flow cytometric screening approach, therefore, identified strong signals from CHO/mCCR3 cells, and a weak or no signal from CHO-K1 cells in 31/92 (33.7%). In addition, strong signals were identified from P388 and J774-1 cells in 8 of the 31 hybridoma supernatants identified in the previous step (25.8%). After limiting dilution and several additional screenings, anti-mCCR3 mAbs, C_3Mab-6 (rat IgG_1, kappa), and C_3Mab-7 (rat IgG_1, kappa) were finally established (Fig. 1D).

Flow cytometric analysis

We conducted flow cytometry, using three anti-mCCR3 mAbs; C_3Mab-6, C_3Mab-7, and the commercially available anti-mCCR3 mAb (clone J073E5 from BioLegend: positive control) against mCCR3-expressing cell lines; CHO/mCCR3, P388, and J774-1. Although all three mAbs dose-dependently recognized CHO/mCCR3 cells at 10, 1, 0.1, and 0.01 μg/mL (Fig. 2A), parental CHO-K1 cells were not recognized (Fig. 2B). Also, although C_3Mab-7 and J073E5 did not react with P388 cells at 0.01 μg/mL (Fig. 2C, middle and right panel), C_3Mab-6 reacted with the cells even at 0.01 μg/mL

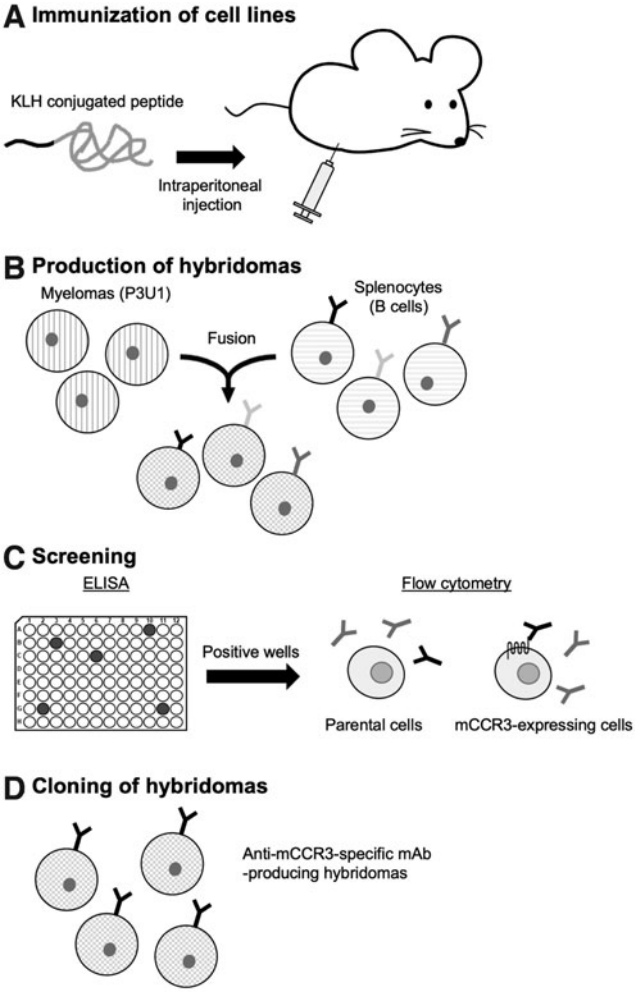

FIG. 1. A schematic illustration showing the production of anti-mCCR3 mAbs. **(A)** The mCCR3p1-19C-KLH peptide was immunized into an SD rat, using an intraperitoneal injection. **(B)** Spleen cells were then fused with P3U1 cells. **(C)** Subsequently, culture supernatants were screened through ELISA and flow cytometry to select anti-mCCR3 mAb-producing hybridomas. **(D)** After limiting dilution and some additional screenings, anti-mCCR3 mAbs were finally established. ELISA, enzyme-linked immunosorbent assay; mAbs, monoclonal antibodies.

(Fig. 2C, left panel). All three mAbs, therefore, recognized J774-1 cells at 10 and 1 μg/mL (Fig. 2C). Hence, these results suggested that C_3Mab-6 and C_3Mab-7 were specific for mCCR3, thereby making then useful for detecting endogenous mCCR3 through flow cytometry.

Kinetic analysis of the interaction of C_3Mab-6 and C_3Mab-7 with mCCR3-expressed cells through flow cytometry

To assess the apparent K_Ds of C_3Mab-6 and C_3Mab-7 with mCCR3-expressing cells, we conducted kinetic analysis of the interaction of C_3Mab-6 and C_3Mab-7 with CHO/mCCR3, P388, and J774-1, using flow cytometry. The geometric mean of the fluorescence intensity was then plotted versus the

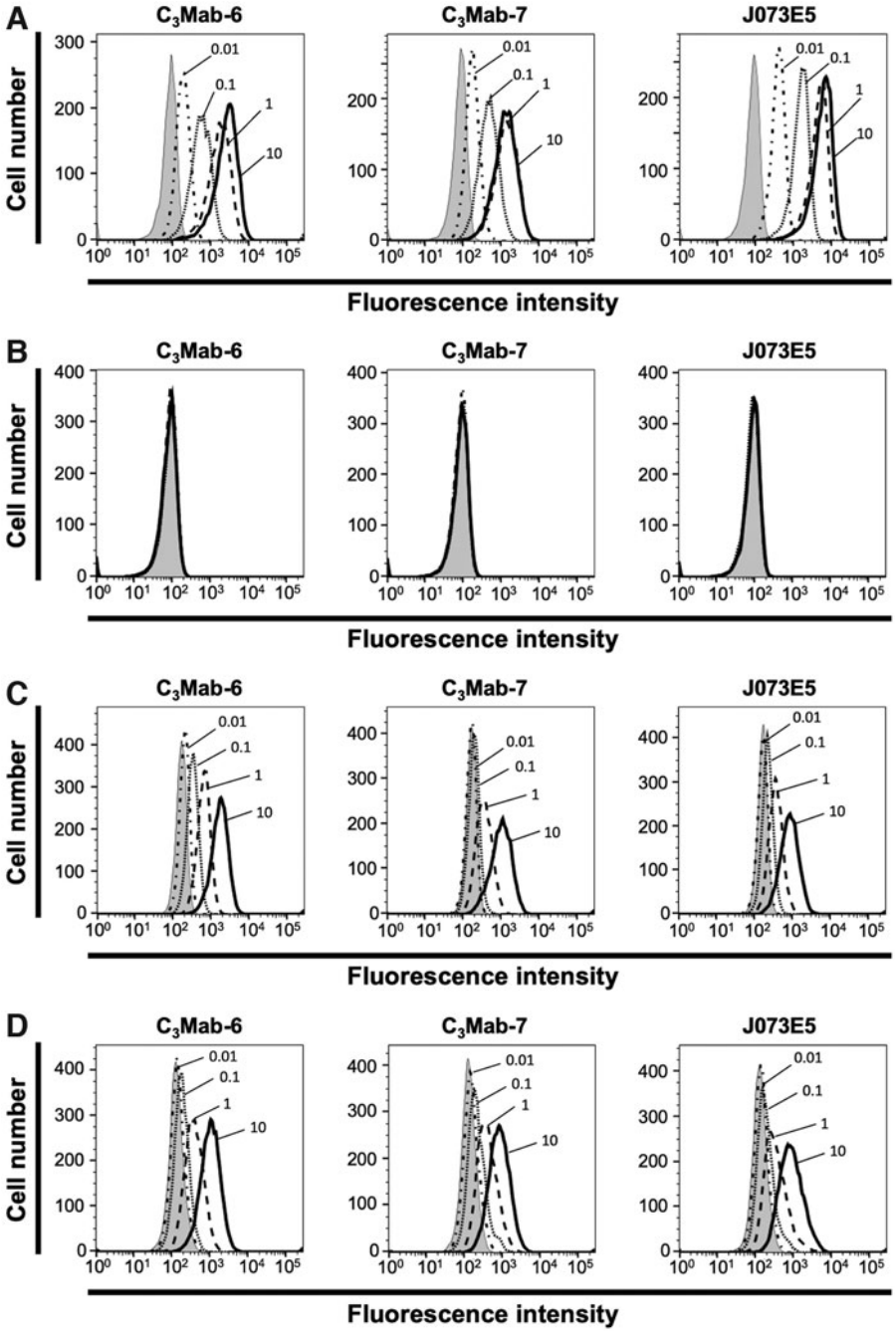

FIG. 2. Flow cytometry using anti-mCCR3. CHO/mCCR3 **(A)**, CHO-K1 **(B)**, P388 **(C)**, and J774-1 **(D)** cells were treated with 0.01 μg/mL (dashed-dotted line), 0.1 μg/mL (dotted line), 1 μg/mL (dashed line), and 10 μg/mL (solid line) of C_3Mab-6 (left panels), C_3Mab-7 (middle panels), or J073E5 (right panels), followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. Filled, without first Ab as negative controls. CHO, Chinese hamster ovary.

concentration of C_3Mab-6 or C_3Mab-7, after fitting through one-site binding models in GraphPad PRISM 8. The K_Ds of C_3Mab-6 for CHO/mCCR3, P388, and J774-1 cells were determined as 8.7×10^{-9} M, 1.4×10^{-7} M, and 1.7×10^{-7} M, respectively. The K_Ds of C_3Mab-7 for these cell lines were 3.7×10^{-9} M, 5.1×10^{-7} M, and 3.1×10^{-7} M, respectively (Fig. 3). These results, therefore, indicate that C_3Mab-6 or C_3Mab-7 possesses a high affinity for CHO/mCCR3, with a moderate affinity for P388 and J774-1 cells.

Discussion

GPCRs expressed on the plasma membrane have native conformations. Therefore, immunization with cells, which express target GPCRs, is a suitable approach against GPCRs, such as chemokine receptors.[9–12] By using the Cell-Based Immunization and Screening (CBIS) method, in which antigen-expressing cell lines are used for immunization and screening, we have established useful mAbs against many

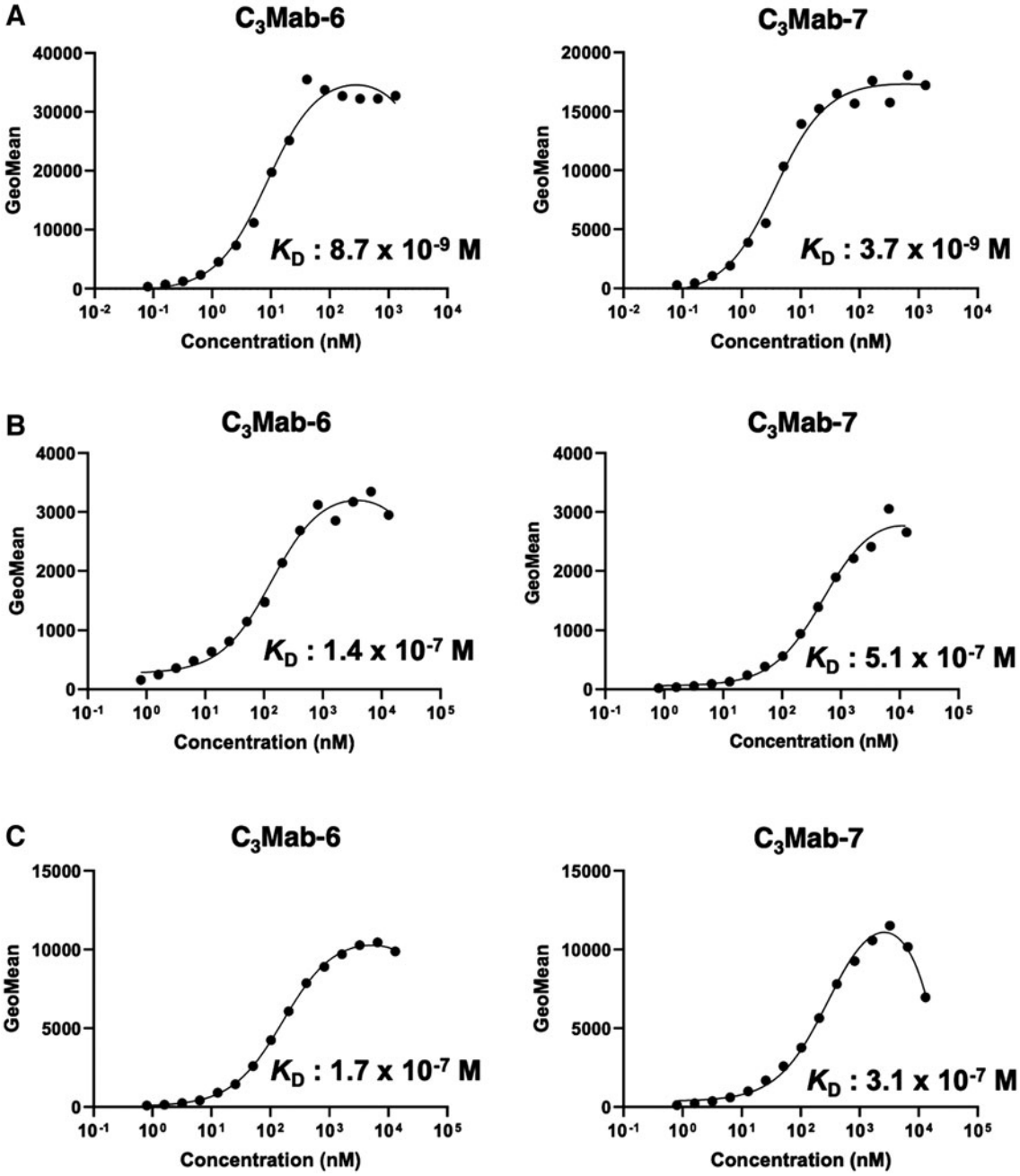

FIG. 3. Determination of the K_Ds of C_3Mab-6 and C_3Mab-7 against mCCR3-expressing cells. CHO/mCCR3 **(A)** was suspended in 100 μL serially diluted C_3Mab-6 or C_3Mab-7 (0.006–100 μg/mL). Then, P388 **(B)** and J774-1 **(C)** were suspended in 100 μL serially diluted C_3Mab-6 or C_3Mab-7 (0.06–1000 μg/mL). The Alexa Fluor 488-conjugated anti-rat IgG was subsequently added, after which fluorescence data were obtained, using BD FACSLyric.

membrane proteins, including GPCRs, such as CCR3,[11] CCR8,[12] CCR9,[10] and other membrane proteins, such as podoplanin,[25–28] CD20,[29,30] CD44,[31] EpCAM,[32] TROP2,[33] EGFR,[34] and HER3[35] in previous studies.

Although developed mAbs through the CBIS method reacts with native GPCRs on plasma membranes, solubilized and denatured proteins have remained unreactive. Therefore, they are useful for flow cytometry. In contrast, many of them are not useful for ELISA and Western blot. In this study, we developed novel anti-CCR3 mAbs, C_3Mab-6 and C_3Mab-7, through immunization with the N-terminal peptide of mCCR3. As observed, C_3Mab-6 and C_3Mab-7 were useful not only for ELISA but also for flow cytometry. This result, therefore, proposes that the N-terminal region of mCCR3 forms a linear structure.

Structural analyses showed that eotaxin binds to the N-terminus of human CCR3 (residues 8–23).[23,24] The K_Ds of CCR3's binding to eotaxin-1, -2, and -3 are 2.1, 9.7, 1.2×10^{-9} M, obtained from competitive radioligand binding assays using CCR3-expressing CHO cells, respectively.[36] Because eotaxin, C3Mab-6, and C3Mab-7 recognize N-terminal region of mCCR3, we need further investigation

to reveal the essential amino acids recognized by C_3Mab-6 and C_3Mab-7.[24] In the future study, it is interesting to evaluate the neutralizing activity of C_3Mab-6 and C_3Mab-7 to eotaxin.

In the future study, we will investigate activities of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Since rat IgG_1 does not possess ADCC and CDC activities, we will also convert the subclass of C_3Mab-6 and C_3Mab-7 into mouse IgG_{2a}, then evaluate their ADCC and CDC activities. This antibody is useful for treating mCCR3-expressing cancers and depleting mCCR3-expressing eosinophils and basophils.

In a previous study, we developed other anti-mCCR3 mAbs; C_3Mab-2 and C_3Mab-3, using the CBIS method, and showed their usefulness in flow cytometry.[11] As observed, C_3Mab-3, C_3Mab-6, and C_3Mab-7 recognized both EDTA-treated and trypsin-treated CHO/mCCR3 cells. In contrast, C_3Mab-2 recognized only EDTA-treated cells, but not trypsin-treated cells. These results suggest that the epitope of C_3Mab-2 will be different from that of other three mAbs, and affected by trypsin. In contrast, at least the N-terminal 19 amino acids, recognized by C_3Mab-6 and C_3Mab-7, were not affected by trypsin. Future studies are required to identify the epitope of C_3Mab-2 and C_3Mab-3.

Author Disclosure Statement

No competing financial interests exist.

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