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Development of a Sensitive Anti-Human CCR9 Monoclonal Antibody (C_9Mab-11) by N-Terminal Peptide Immunization

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The C-C chemokine receptor 9 (CCR9) belongs to the G-protein-coupled receptor superfamily, and is highly expressed on the T cells and intestinal cells. CCR9 regulates various immune responses by binding to the C-C chemokine ligand, CCL25, and is involved in inflammatory diseases and tumors. Therefore, the development of sensitive monoclonal antibodies (mAbs) for CCR9 is necessary for treatment and diagnosis. In this study, we established a specific anti-human CCR9 (hCCR9) mAb; C_9Mab-11 (mouse IgG_{2a}, kappa), using the synthetic peptide immunization method. C_9Mab-11 reacted with hCCR9-overexpressed Chinese hamster ovary-K1 (CHO/hCCR9) and hCCR9-endogenously expressed MOLT-4 (human T-lymphoblastic leukemia) cells in flow cytometry. The dissociation constant (K_D) of C_9Mab-11 for CHO/hCCR9 and MOLT-4 cells were determined to be 1.2×10^{-9} M and 4.9×10^{-10} M, respectively, indicating that C_9Mab-11 possesses a high affinity for both exogenously and endogenously hCCR9-expressing cells. Furthermore, C_9Mab-11 clearly detected hCCR9 protein in CHO/hCCR9 cells using western blot analysis. In summary, C_9Mab-11 can be a useful tool for analyzing hCCR9-related biological responses.

Keywords: human CCR9, monoclonal antibody, flow cytometry, western blot

Introduction

Chemokines are 8- to 12-kDa chemotactic cytokines that regulate cell migration by binding to seven-transmembrane G-protein-coupled receptors (GPCRs). Chemokines play pivotal roles during development, inflammation, and pathological processes, including cancer progression.[1] They can be divided into four categories: CC, CXC, CX3C, and XC subfamilies, depending on the number and position of N-terminal cysteine residue.

The C-C chemokine receptor 9 (CCR9), a member of GPCRs, is highly expressed in gut-homing T cells, thymocytes, B cells, dendritic cells, and intestinal cells.[2] CCR9 on immature T cells contributes to T cell activation and infiltration by binding to its dedicated ligand, CCL25/thymus-expressed chemokine.[3] CCL25 is mainly secreted from epithelial cells of the thymus and small intestine, and inducts the T cells into intestinal tissues.[4–6] CCR9/CCL25 has been reported to be involved in various inflammatory diseases.[7] CCR9 expression is increased in mice myocardial infarction model, and abrogation of CCR9 improved the mice survival.[8]

In hepatitis, $CCR9^+$ macrophages induced acute liver damage by interacting with helper T1 (Th1) cells.[9] In dextran sulfate sodium-induced mice colitis, a model of inflammatory bowel disease (IBD), both CCR9 and CCL25 expression were elevated. In the colitis model, CCR9-knockout mice exhibited higher IBD score and mortality.[10] Therefore, the targeting CCR9/CCL25 could be an attractive therapeutic strategy because of its involvement with inflammation-associated diseases. A clinical trial of the CCR9 antagonist CCX282-B against IBD had been conducted.[11,12] CCR9 was also found to be expressed in T-acute lymphoblastic leukemia (T-ALL), which contributes to the progression of T-ALL.[13] $CD4^+CCR9^+$T cells have been found to express large amounts of interleukin 21, inducible T cell co-stimulators, and the transcription factors, including Bcl-6. Furthermore, the $CD4^+CCR9^+$T cells support antibody production from B cells.[14]

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Previously, we have developed various anti-GPCR monoclonal antibodies (mAbs) against mouse CCR3[15–17] and mouse CCR8[18–20] by using the Cell-Based Immunization and Screening (CBIS) method. Furthermore, anti-human CCR9 (hCCR9) mAb (clone C_9Mab-1) was also established using the CBIS method.[21] We also identified the epitope of C_9Mab-1 on the N-terminal region of the hCCR9 protein.[22] The aim of this study is to obtain more sensitive anti-hCCR9 mAbs by the synthetic peptide immunization method using the epitope region of C_9Mab-1.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). MOLT-4 (a human T-lymphoblastic leukemia cell line) was provided from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). The expression plasmid of hCCR9 (pCMV6neoCCR9-Myc-DDK) was purchased from OriGene Technologies, Inc. (Rockvile, MD) and was transfected into CHO-K1 cells using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were selected by limiting dilution and cultivation in a medium containing 0.5 mg/mL of G418 (Nacalai Tesque, Inc., Kyoto, Japan).

CHO-K1, P3U1, CHO/hCCR9, and MOLT-4 were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc.) that was 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.). Then, cells were grown in a humidified incubator, which was supplied with 5% CO_2 and 95% air at 37°C.

Hybridoma production

Two 6-week-old female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Mice were housed under specific pathogen-free conditions. Then, animal experiments were conducted, following relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. The Animal Care and Use Committee of Tohoku University approved animal experiments (permit number: 2019NiA-001). The mouse health was monitored daily during the entire 4-week duration of the experiment, and a reduction of more than 25% of the total body weight was denoted as a humane endpoint. Subsequently, mice were euthanized through cervical dislocation, after which respiratory and cardiac arrest were used to verify death.

We immunized two BALB/c mice with keyhole limpet hemocyanin-conjugated hCCR9 peptide (100 μg): $_4$-TDFTSPIPNMADDYGSEST-$_{22}$ + C-terminal cysteine. The administration was conducted through the intraperitoneal route with an Imject Alum (Thermo Fisher Scientific, Inc.). The procedure included three additional immunization procedures (100 μg of peptide), followed by a final booster intraperitoneal injection (100 μg of peptide) 2 days before its spleen cells were harvested.

The harvested spleen cells were subsequently fused with P3U1 cells, using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN). Then, hybridomas were grown in an RPMI 1640 medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). Cultured supernatants were finally screened using enzyme-linked immunosorbent assay (ELISA) and flow cytometric analysis.

Enzyme-linked immunosorbent assay

A synthesized hCCR9 peptide ($_4$-TDFTSPIPNMA DDYGSEST-$_{22}$ + C-terminal cysteine) was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at a concentration of 1 μg/mL peptide at 37°C for 30 minutes. 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 culture supernatants, followed by peroxidase-conjugated anti-mouse immunoglobulins (1:2000 diluted; Agilent Technologies, Inc., Santa Clara, CA). Enzymatic reactions were conducted using the 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).

Flow cytometry

Cells were washed with 0.1% BSA in PBS and treated with C_9Mab-11 or recombinant C_9Mab-1 (recC_9Mab-1)[23] for 30 minutes at 4°C. Then, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA), followed by the collection of fluorescence data, using SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Determination of the binding affinity by flow cytometry

CHO/hCCR9 and MOLT-4 cells were suspended in 100 μL serially diluted anti-hCCR9 mAbs, after which Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.) was added. Fluorescence data were subsequently collected, using BD FACSLyric (BD Biosciences), followed by the calculation of the dissociation constant (K_D) by fitting the binding isotherms into the built-in one-site binding model in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Western blot analysis

Cell lysates (10 μg) were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.). Proteins were electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% nonfat milk (Nacalai Tesque, Inc.), PVDF membranes were incubated with 1 μg/mL of C_9Mab-11 and 1 μg/mL of an anti-DYKDDDDK mAb (clone 1E6; FUJIFILM Wako Pure Chemical Corporation), followed by incubation with peroxidase-conjugated anti-mouse IgG (1:2000; Agilent Technologies, Inc.); or 1 μg/mL of an anti-isocitrate dehydrogenase 1 (IDH1) mAb (clone RcMab-1),[24,25] followed by incubation with peroxidase-conjugated anti-rat IgG (1:10,000; Sigma-Aldrich Corp., St. Louis, MO). Blots were developed using Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and imaged with a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Results

Establishment of anti-hCCR9 mAbs

We first immunized mice with the hCCR9 peptide (Fig. 1). Hybridomas were seeded into 96-well plates, after which ELISA was used to select reactive wells for hCCR9 peptide, followed by the selection of CHO/hCCR9 and MOLT-4 cells-reactive supernatants of hybridoma using flow cytometry. We obtained reactive supernatants in 63 of 958 wells (6.58%), and finally established C_9Mab-11 (mouse IgG_{2a}, kappa) after cloning by the limiting dilution.

Flow cytometry

Flow cytometry was performed using C_9Mab-11 and recC_9Mab-1 against exogenously hCCR9-overexpressed CHO/hCCR9 and endogenously hCCR9-expressed MOLT-4 cells. Results showed that C_9Mab-11 recognized

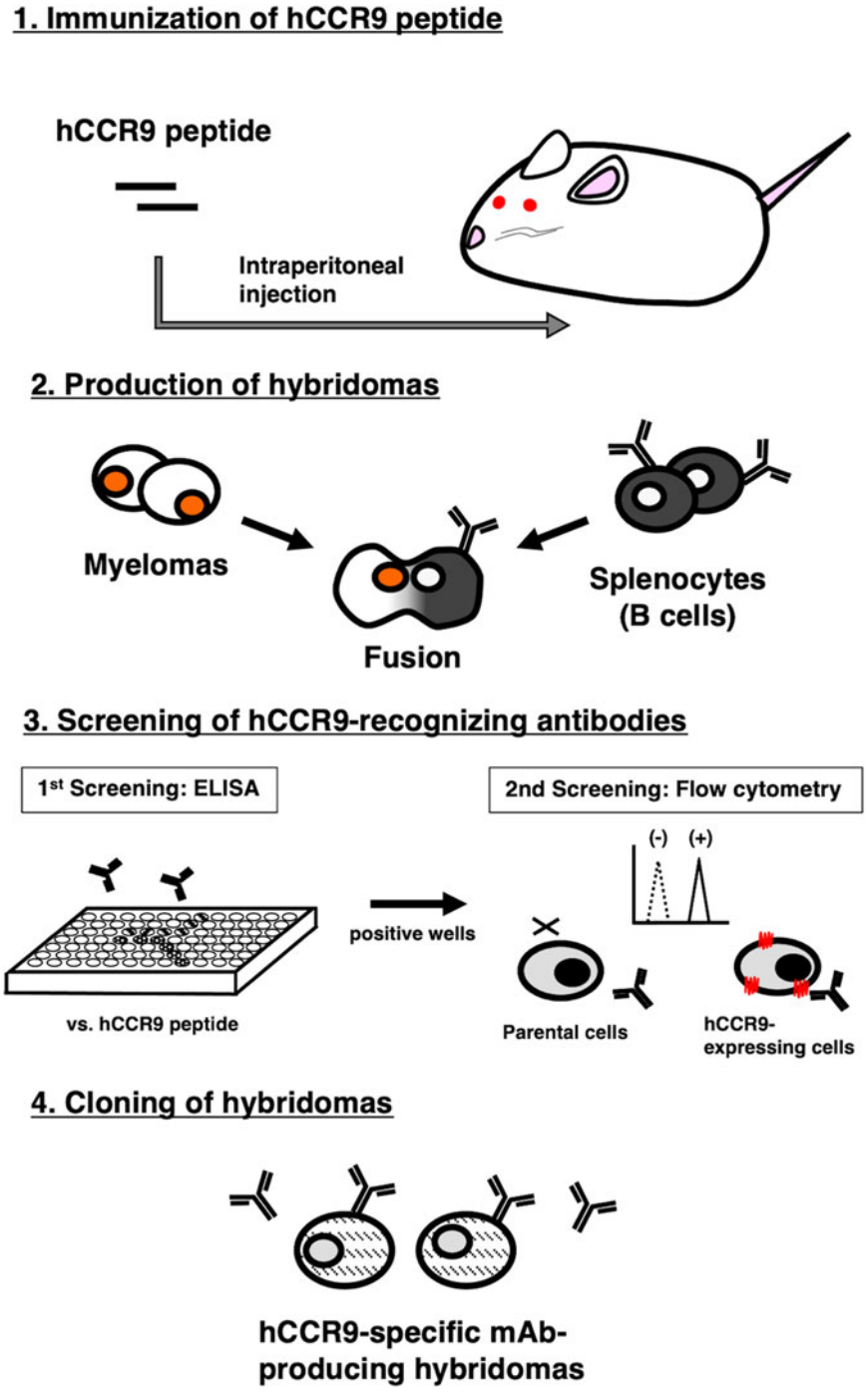

FIG. 1. A schematic illustration about the production of anti-hCCR9 mAbs. The mice were intraperitoneally immunized with the hCCR9 peptide. Screening of hybridoma was then conducted by ELISA, followed by flow cytometry using hCCR9-expressing cells. ELISA, enzyme-linked immunosorbent assay; mAbs, monoclonal antibodies; hCCR9, human CCR9.

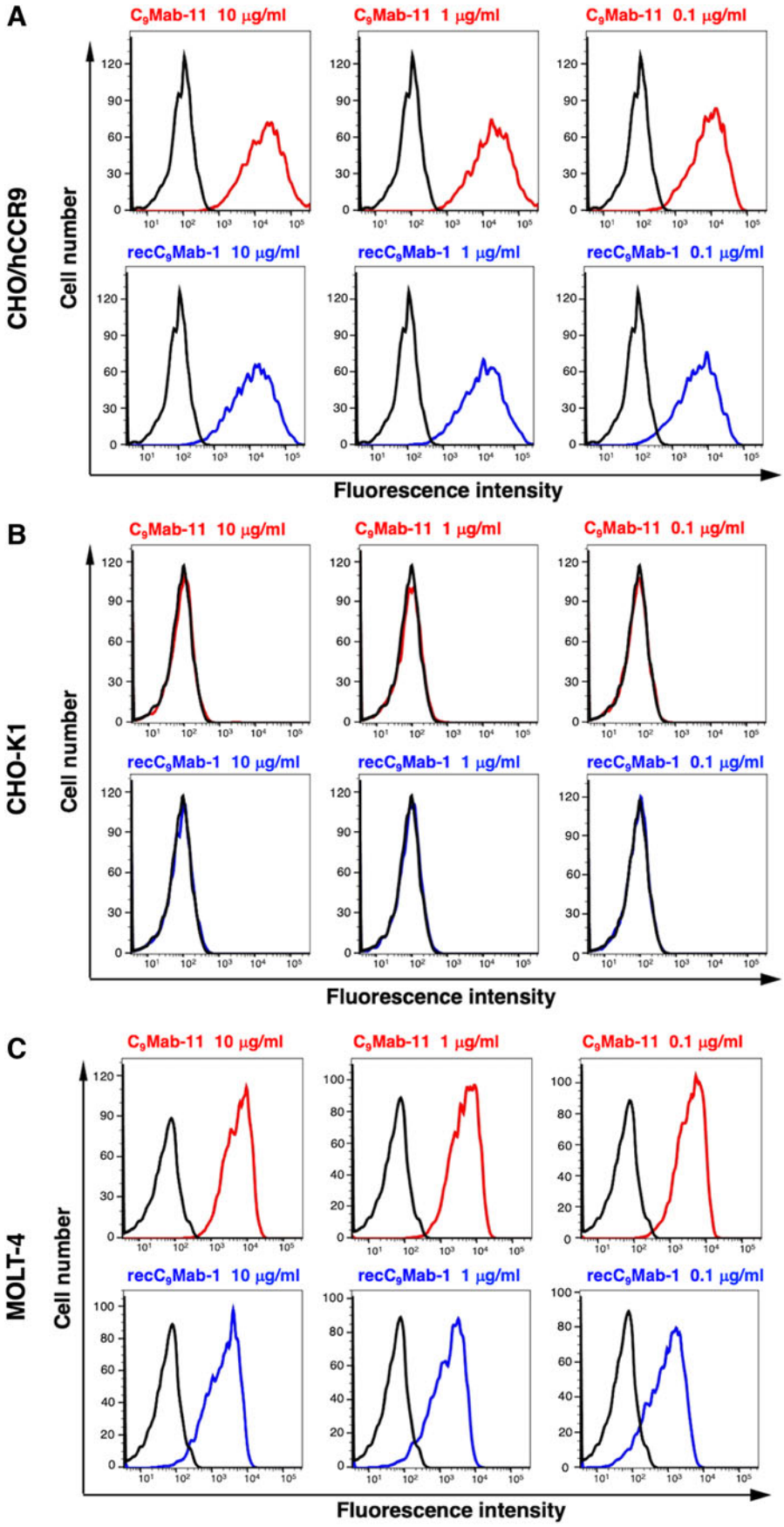

FIG. 2. Flow cytometry using anti-hCCR9 mAbs. CHO/hCCR9 cells **(A)**, CHO-K1 cells **(B)**, and MOLT-4 cells **(C)** were treated with 0.1, 1, 10 μg/mL of C_9Mab-11 and recC_9Mab-1, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Black line represents the negative control. CHO, Chinese hamster ovary; recC_9Mab-1, recombinant C_9Mab-1.

CHO/hCCR9 in a dose-dependent manner (Fig. 2A) but did not react with parental CHO-K1 cells (Fig. 2B). C_9Mab-11 reacted with MOLT-4 cells in a dose-dependent manner (Fig. 2C). Although recC_9Mab-1 reacted with both CHO/hCCR9 (Fig. 2A) and MOLT-4 (Fig. 2C) in the similar way with C_9Mab-11, the reactivity of C_9Mab-11 is more sensitive than recC_9Mab-1 at a concentration of 1 μg/mL.

Determination of the binding affinity of C_9Mab-11

The binding affinity of C_9Mab-11 was assessed with CHO/hCCR9 and MOLT-4 cells using flow cytometry. Results showed that the K_D values of C_9Mab-11 for CHO/hCCR9 and MOLT-4 cells were 1.2×10^{-9} M and 4.9×10^{-10} M, respectively (Fig. 3A, B). These results indicated that C_9Mab-11 possesses high affinity for CHO/hCCR9 and MOLT-4 cells.

Western blot analyses

We investigated whether C_9Mab-11 can be used for western blot analysis by analyzing CHO-K1 and CHO/hCCR9 cell lysates. As shown in Figure 4, C_9Mab-11 could clearly detect hCCR9 as around 48-kDa band (hCCR9 + Myc-DDK tag) in CHO/hCCR9 cell lysates, whereas no band was detected in parental CHO-K1 cells. An anti-DYKDDDDK mAb (clone 1E6) was used as a positive control and could also detect a band of the same position in CHO/hCCR9 cell lysates. An anti-IDH1 mAb (clone RcMab-1) was used for internal control. These results indicate that C_9Mab-11 can detect hCCR9 in western blot analyses.

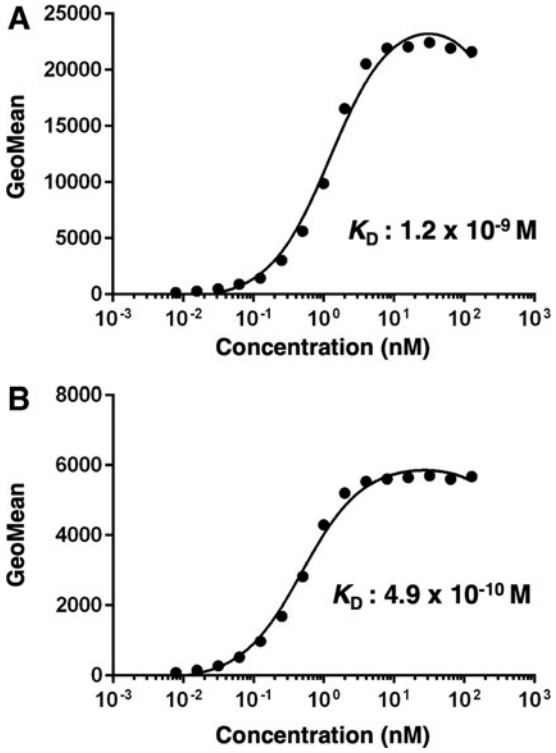

FIG. 3. The determination of the binding affinity of C_9Mab-11. CHO/hCCR9 **(A)** or MOLT-4 **(B)** cells were suspended in 100 µL serially diluted C_9Mab-11 (10–0.0006 µg/mL for both cells). Then, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were subsequently collected using BD FACSLyric, after the calculation of the dissociation constant (K_D) by GraphPad PRISM 6.

Discussion

Using the CBIS method, we have developed many mAbs, which could recognize the biological structure and modification, including folding and glycosylation in the membrane proteins.[16,18,20,21,23,26–32] We also developed an anti-hCCR9 mAb (clone C_9Mab-1) by using CBIS method.[21] By evaluating the mAb epitope, we could find a better peptide immunogen for the same target protein. We, therefore, identified the epitope of C_9Mab-1 as Ile10, Pro11, Asn12, Met13, Ala14, Asp16, and Tyr17 of hCCR9 by ELISA.[22] In this study, we immunized mice with the N-terminal peptide of hCCR9, including these amino acids, and obtained a highly sensitive mAb (clone C_9Mab-11).

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.[33] The design of antigenic epitope is important for antibody production by peptide immunization method. Thus, the combination with CBIS and peptide immunization method considered to be an effective method for efficiently obtaining highly sensitive mAbs. We successfully established anti-GPCRs mAbs against the mCCR3[15–17] and mCCR8[18,19] by CBIS method.

In the future study, we will try to obtain more highly sensitive anti-GPCR mAbs by using the same strategy of C_9Mab-11 development. In our previous study, numerous mAbs against membrane proteins, such as EGFR,[34] HER2,[35] HER3,[36] CD20,[26] CD44,[32] CD133,[37] EpCAM,[28] TROP2,[29,30] and podoplanin[27,31,38–40] have been produced by using CBIS method and the epitopes of those mAbs have been already identified.[41–44] It is expected that more sensitive and useful mAbs will be obtained against these proteins by immunizing the identified epitopes.

The N-terminus of several GPCRs, including CCR2, CCR3, CCR5, and CXCR1 was defined as the ligand-binding region.[45] The N-terminus of CCR9 is also thought to be important for the ligand binding, because the mAb, which binds to N-terminus of CCR9, was reported to block the interaction between CCR9 and CCL25.[46,47] The CCR9/CCL25 expression is increased in various inflammatory diseases, such as myocardial infarction, rheumatoid arthritis, IBD, asthma, and tumors[8–10,13]; therefore, the CCR9/CCL25 interaction is expected as an ideal therapeutic target.

The C_9Mab-11 may have therapeutic effects for the aforementioned diseases. In the immune system, CCR9 plays a vital role in the regulation of T cells. T cells recruitment to the intestine and colon is triggered by CCR9 stimulation.[48,49] A tumor acidity-responsive nanoparticle delivery system (NP-siCD47/CCL25) can release CCL25 protein and CD47 small interfering RNA in tumor. The released CCL25 promoted the infiltration of $CCR9^+CD8^+$ T cells in tumor, which resulted in inhibition of tumor growth and metastasis through a T cell-dependent immunity.[50] Therefore, CCR9+ T cells have positive effect for cancer treatment, through activation

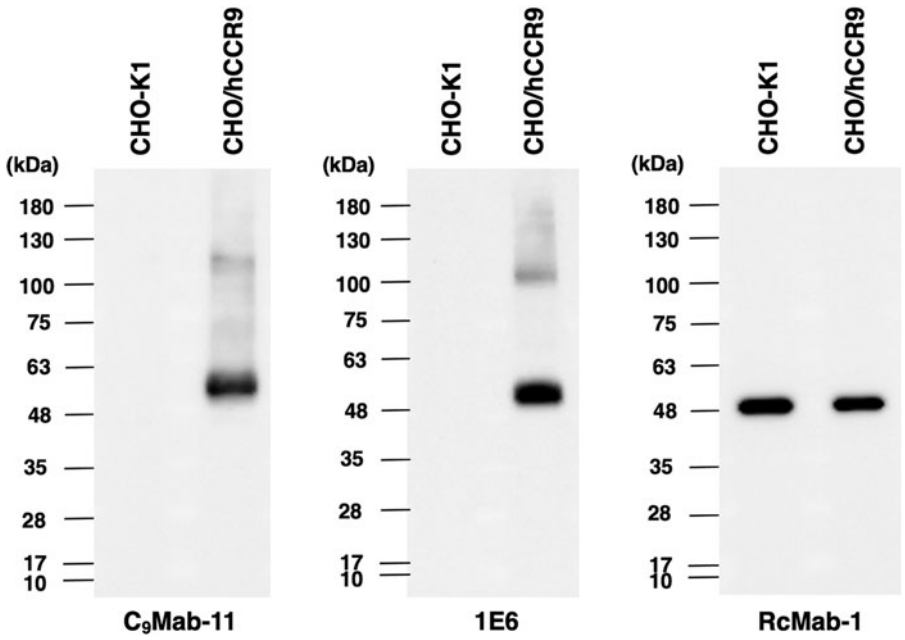

FIG. 4. The detection of hCCR9 by western blot analysis. Cell lysates of CHO-K1 and CHO/hCCR9 (10 μg) were electrophoresed and transferred onto PVDF membranes. The membranes were incubated with 1 μg/mL of C_9Mab-11, 1 μg/mL of an anti-DYKDDDDK mAb (clone 1E6), and 1 μg/mL of an anti-IDH1 mAb (clone RcMab-1) and subsequently with peroxidase-conjugated anti-mouse or anti-rat immunoglobulins. IDH1, isocitrate dehydrogenase 1; PVDF, polyvinylidene difluoride.

of tumor immunity. Recently, it has shown that chimeric antigen receptor T cells targeting CCR9 possess antileukemic activity against T-ALL *in vitro* and *in vivo*.[51] C_9Mab-11 is expected to be effective in cancer immunotherapy.

C_9Mab-11 is applicable to various experiments, such as flow cytometry, ELISA, and western blot analysis. Therefore, C_9Mab-11 will also contribute to further research related to CCR9 functions.

Author Disclosure Statement

No competing financial interests exist.

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