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C_9Mab-1: An Anti-Human CCR9 Monoclonal Antibody for Immunocytochemistry

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C–C motif chemokine receptor 9 (CCR9) is a G protein-coupled receptor, which is highly expressed in T-lymphocytes and different cancer cells. CCR9 aggravates immune diseases and cancer progression and is considered a biomarker and a therapeutic target of diseases. The development of specific monoclonal antibody (mAbs) for human CCR9 (hCCR9) is required to diagnose and treat immune diseases and cancers. Previously, we established the cell-based immunization and screening (CBIS) method, which does not need purified target proteins. Anti-hCCR9 mAb (clone C_9Mab-1; mouse IgG_1, kappa) was also developed using the CBIS method. C_9Mab-1 is usable for flow cytometry against exogenously and endogenously expressing hCCR9. This study showed that C_9Mab-1 and its recombinant antibody (recC_9Mab-1) specifically detected exogenous hCCR9 stably overexpressed in Chinese hamster ovary (CHO)-K1 cells and endogenous hCCR9 expressed in a human T-lymphoblastic leukemia cell line MOLT-4 cells through immunocytochemistry. This study provides a new application of C_9Mab-1 and recC_9Mab-1 in immunocytochemistry.

Keywords: human CCR9, C_9Mab-1, monoclonal antibody, immunocytochemistry

Introduction

C–C MOTIF CHEMOKINE receptor 9 (CCR9) is a G protein-coupled receptor (GPCR) that is highly expressed in the thymus and is distributed in mature and immature T-lymphocytes.[1,2] C–C motif chemokine ligand 25 (CCL25)/thymus-expressed chemokine (TECK) is a selective agonist of CCR9 and is expressed in the thymus and small intestine.[3] Activation of CCR9 by CCL25/TECK induces localization of mucosal T-lymphocytes to the microvessels of the small intestine, gut walls, and the gut-associated lymphoid tissue.[4,5] In patients with small bowel Crohn's disease, the frequency of $CCR9^+$ lymphocytes was elevated in peripheral blood. The homing of $CCR9^+$ T-lymphocytes to the small intestine mucosa advances the local inflammation.[6,7] Moreover, upregulation of CCR9 in the heart of myocardial infarction potentiates inflammatory response and apoptosis in cardiomyocytes.[8] An increase in the expression of CCR9 in peripheral blood monocytes of rheumatoid arthritis augments chemotaxis of the monocytes and differentiation of the monocytes to macrophages. These events would promote infiltration of the monocytes/macrophages in the synovium.[9]

CCR9 is overexpressed in different cancers, including acute lymphoblastic leukemia,[10] lymphoma,[11,12] melanoma,[13,14] ovarian cancer,[15] prostate cancer,[16] non-small cell lung cancer,[17] pancreatic cancer,[18] hepatocellular carcinoma,[19] and breast cancer.[20,21] Moreover, CCR9 expression is high in circulating tumor cells of patients with solid tumors.[22] As a result, CCR9 promotes proliferation, migration, invasion, metastasis, tumorigenesis, and chemoresistance of the cancer cells and thus has been considered a biomarker and a therapeutic target. However, molecular mechanisms of cancer development by CCR9 have remained unresolved.

Monoclonal antibodies (mAbs) that specifically detect human CCR9 (hCCR9) in many applications would be beneficial for the diagnosis of the CCR9-dependent immune and inflammatory diseases and cancers. Additionally, the mAbs would also be useful for elucidation of CCR9 functions.

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Despite the technical challenge in the development of anti-GPCR antibodies,[23] mAbs of GPCRs, including anti-mouse CCR3 mAb (clone C_3Mab-2)[24] and anti-mouse CCR8 mAb (clone C_8Mab-1),[25] has been developed using the cell-based immunization and screening (CBIS) method. Anti-hCCR9 mAb (clone C_9Mab-1; mouse IgG_1, kappa) was also developed using the CBIS method.[26] C_9Mab-1 recognizes hCCR9 with high binding affinity and is usable for flow cytometry against exogenous and endogenous hCCR9. In this study, it was shown that C_9Mab-1 and its recombinant antibody (recC_9Mab-1) are applicable for immunocytochemistry against exogenous and endogenous hCCR9.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 cells were purchased from the American Type Culture Collection (Manassas, VA). CHO-K1 cell line stably expressing hCCR9 (CHO/hCCR9) was established in a previous report.[26] A human T-lymphoblastic leukemia cell line (MOLT-4 cells) was provided from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). CHO-K1, CHO/hCCR9, and MOLT-4 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% of heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). The cells were grown in a humidified atmosphere at 37°C with 5% CO_2.

Antibodies

C_9Mab-1 was developed in a previous report.[26] To produce recC_9Mab-1, we subcloned variable region of heavy chain (V_H) and constant region of heavy chain (C_H) of C_9Mab-1 cDNAs into the pCAG-Neo vector, and variable region of light chain (V_L) and constant region of light chain (C_L) of C_9Mab-1 cDNA into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. An anti-hCCR9 mAb (clone 112509) was obtained from R&D Systems (Minneapolis, MN). Alexa Fluor 488-conjugated anti-mouse IgG was obtained from Cell Signaling Technology, Inc., (Danvers, MA).

Immunocytochemistry

The whole procedure of immunocytochemistry was conducted at room temperature. First, CHO-K1 and CHO/hCCR9 cells were attached on sterile acid-wash coverslips and were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA) for 10 minutes. The residual PFA was washed out once in PBS supplemented with 0.2 mM Ca^{2+} and 2 mM Mg^{2+} (PBSc/m) and quenched with PBSc/m containing 50 mM NH_4Cl for 10 minutes. Then, the cells were incubated in the blocking buffer (PBSc/m containing 0.5% bovine serum albumin) for 30 minutes, incubated with primary antibodies (10 μg/mL in the blocking buffer) for 1 hour. Subsequently, the cells were incubated with the mixture of Alexa Fluor 488-conjugated anti-mouse IgG (1:400) and 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Inc.) (in the blocking buffer) for 45 minutes. Finally, the cells were mounted using ProLong Glass Antifade Mountant (Thermo Fisher Scientific, Inc.). In contrast, the suspension of MOLT-4 cells was centrifuged at 270 × *g* for 2 minutes, and the obtained cell pellet was fixed with 4% PFA in PBS for 10 minutes.

After centrifugation, the cells were rinsed once in PBSc/m and quenched using 50 mM NH_4Cl in PBSc/m for 10 minutes. The cells were subsequently blocked with the blocking buffer for 30 minutes, incubated with primary antibodies (10 μg/mL in the blocking buffer) for 1 hour, and further incubated with Alexa Fluor 488-conjugated anti-mouse IgG (1:400) and DAPI (in the blocking buffer) for 45 minutes. Finally, the cells were suspended in ProLong Glass Antifade Mountant. Fluorescence images were obtained using a 40 × objective on a BZ-X800 digital microscope (Keyence, Osaka, Japan).

Results and Discussion

To investigate whether C_9Mab-1 and recC_9Mab-1 were usable for immunocytochemistry, CHO/hCCR9 cells were immunolabeled with the antibodies. It was found that C_9Mab-1 and recC_9Mab-1 were primarily bound to hCCR9 at the plasma membrane, whereas buffer control failed to detect it (Fig. 1A). Additionally, a commercially available anti-hCCR9 antibody (clone 112509) also recognized hCCR9 at the plasma membrane (Fig. 1A, arrowheads). In contrast, C_9Mab-1, recC_9Mab-1, and 112509 did not detect hCCR9 in parental CHO-K1 cells (Fig. 1B). The result suggests that C_9Mab-1 and recC_9Mab-1 as well as 112509 specifically recognize exogenously expressing hCCR9 in CHO/hCCR9 cells.

Immunolabeling of endogenous hCCR9 expressed in MOLT-4 cells was further examined. As a result, C_9Mab-1, recC_9Mab-1, and 112509, but not buffer control, visualized endogenous hCCR9 (Fig. 2), proposing that C_9Mab-1 and recC_9Mab-1 specifically recognize endogenous CCR9 in MOLT-4 cells. Moreover, the fluorescent signals of CCR9 were mainly observed at the cell periphery. They were separated from the nucleus, showing that C_9Mab-1, recC_9Mab-1, and 112509 detected hCCR9 at the plasma membrane in MOLT-4 cells (Fig. 2, arrowheads in the enlarged images).

These data demonstrate that C_9Mab-1 and recC_9Mab-1 specifically bind to exogenous and endogenous hCCR9 in immunocytochemistry. Thus, those antibodies would provide valuable information on the diagnosis of CCR9-related immune and inflammatory diseases (e.g., asthma, cardiovascular disease, hepatitis, inflammatory bowel disease, and rheumatoid arthritis)[8] and cancers. Moreover, identifying the cellular distribution of hCCR9 would facilitate research to elucidate the unresolved physiological roles of CCR9.

Several studies have investigated the availability of the CCL25–CCR9 axis as a therapeutic target of immune and inflammatory diseases. For example, administrations of mAbs against mouse CCR9 (mCCR9) and mouse CCL25 (mCCL25) inhibit CCR9-mediated calcium mobilization and attenuate inflammation during the early stage of chronic murine ileitis.[27] Anti-mCCL25 mAb reduced skin allograft rejection in mice through the neutralization of CCL25 and the subsequent suppressing migration of immune cells, possibly mCCR9-positive T cells, at the skin allograft.[28]

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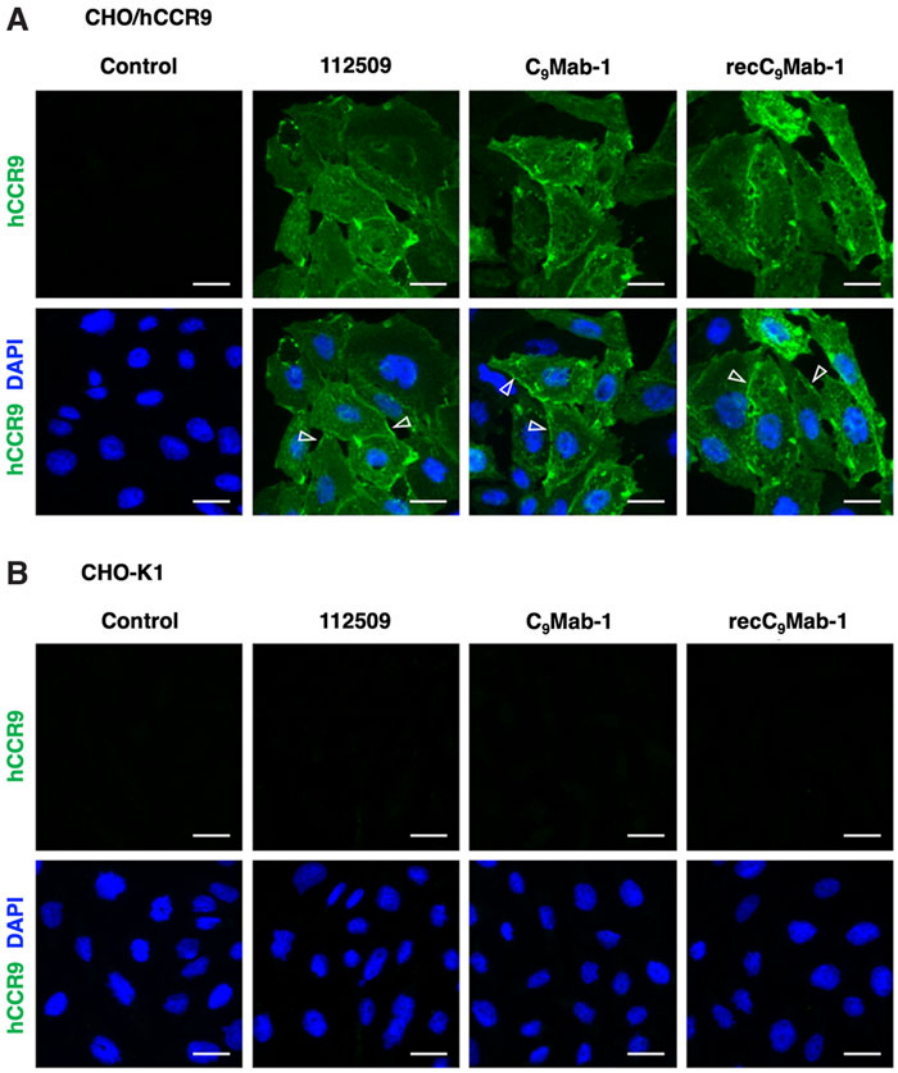

FIG. 1. Immunocytochemistry against exogenous hCCR9 using C_9Mab-1 and recC_9Mab-1. CHO/hCCR9 cells **(A)** and CHO-K1 cells **(B)** were treated with buffer control, 112509 (10 μg/mL), C_9Mab-1 (10 μg/mL), or recC_9Mab-1 (10 μg/mL) for 1 hour. Subsequently, the cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG and DAPI for 45 minutes. Arrowheads indicate the CCR9 signals distributed at the plasma membrane: Scale bars, 20 μm. CHO, Chinese hamster ovary; CCR9, C–C motif chemokine receptor 9; DAPI, 4′,6-diamidino-2-phenylindole; hCCR9, human CCR9.

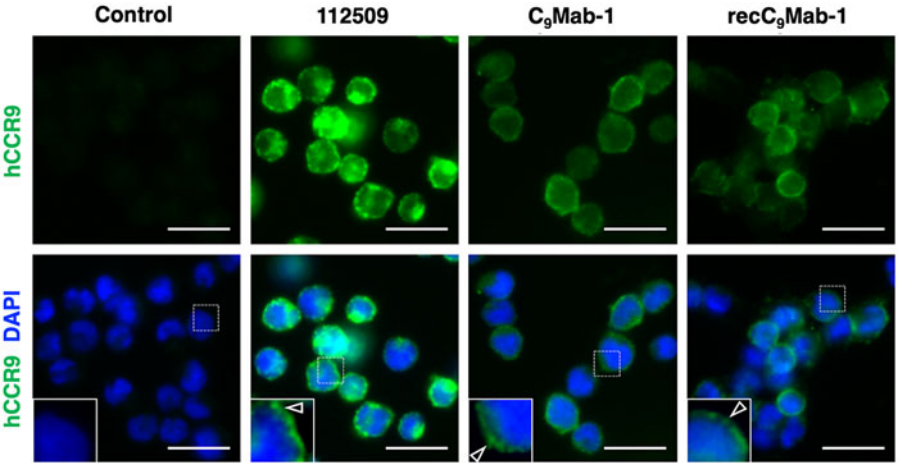

FIG. 2. Immunocytochemistry against endogenous hCCR9 using C_9Mab-1 and recC_9Mab-1. MOLT-4 cells were treated with buffer control, 112509 (10 μg/mL), C_9Mab-1 (10 μg/mL), or recC_9Mab-1 (10 μg/mL) for 1 hour. Subsequently, the cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG and DAPI for 45 minutes. Insets exhibit enlarged images of the boxed areas. Arrowheads indicate the CCR9 signals distributed at the plasma membrane. The brightness of the enlarged images was slightly modified for presentation purposes: Scale bars, 20 μm.

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It was speculated that C_9Mab-1 could attenuate symptoms of immune diseases. Previously, it was identified that seven amino acids of the N-terminus of hCCR9 (Ile10, Pro11, Asn12, Met13, Ala14, Asp16, and Tyr17) are the binding epitope of C_9Mab-1.[29] However, current studies have shown that chemokine ligands bind to the N-termini of their chemokine receptors, for example, the binding of CCL11/eotaxin-1 to CCR2,[30] CCL24/eotaxin-2 to CCR3,[31] CXCL8 to CXCR2,[32] and CXCL12 to CXCR3.[33] Additionally, anti-CCR9 mAbs that bind to the N-terminus of CCR9 decrease the interaction between CCR9 and CCL25/TECK.[34,35] Thus, the series of evidence indicates a possibility that C_9Mab-1 and recC_9Mab-1 inhibit the hCCR9 activity by inhibiting the interaction of CCL25/TECK with hCCR9 and thereby useful for immune disease therapy.

Mouse anti-CCR9 mAbs (clone 91R and 92R) exhibit antitumor effects in leukemia cell xenograft mouse models and *in vitro*.[34,36] Since the subclass of C_9Mab-1 and recC_9Mab-1 are IgG_1, the antibodies are less likely to possess antitumor activity. We previously converted the subclass of anti-epidermal growth factor receptor mAb (clone EMab-134) from IgG_1 to IgG_{2a} (named as 134-mG_{2a}), and demonstrated that the defucosylated 134-mG_{2a} (named as 134-mG_{2a}-f) possesses antitumor activities *in vitro* and *in vivo*.[37] In the future, it is of interest to investigate whether the subclass-converted C_9Mab-1 possesses antitumor activity.

In conclusion, this study demonstrates that C_9Mab-1 and recC_9Mab-1 are applicable for detecting exogenous and endogenous hCCR9 in immunocytochemistry. Thus, the mAbs would become valuable tools for the diagnosis and medical therapy of CCR9-dependent immune diseases and cancers. The mAbs would also be useful for the elucidation of the functions of CCR9.

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Author Disclosure Statement

No competing financial interests exist.

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