MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 41, Number 2, 2022

DOI: 10.1089/mab.2021.0045

Short Communications

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C_8Mab-2: An Anti-Mouse C–C Motif Chemokine Receptor 8 Monoclonal Antibody for Immunocytochemistry

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C–C motif chemokine receptor 8 (CCR8) is a G protein-coupled receptor predominantly expressed in regulatory T (Treg) and T helper 2 cells. The evidence that CCR8 expression in Treg is increased in cancers, CCR8 increases migration activity of Treg, and CCR8 induces the anti-apoptotic activity in T cell leukemia and lymphoma suggests that CCR8 is associated with cancer development. Thus, developing a specific monoclonal antibody (mAb) for CCR8 is useful for diagnostic and therapeutic purposes and the anti-CCR8 mAb becomes a remarkable experimental tool for basic research. We previously developed an anti-mouse CCR8 (mCCR8) mAb called C_8Mab-2 (rat IgG_{2b}, kappa) that was applicable to flow cytometric analysis for both endogenous and exogenous mCCR8. This study showed that C_8Mab-2 and recombinant C_8Mab-2 (recC_8Mab-2) were specifically bound to exogenously expressed mCCR8 in mCCR8-overexpressed Chinese hamster ovary-K1 cells. In addition, we found that C_8Mab-2 and recC_8Mab-2 recognized endogenous mCCR8 in P388 (a mouse lymphocyte-like cell line) and J774-1 cells (a mouse macrophage-like cell line). These data demonstrate that C_8Mab-2 and recC_8Mab-2 are useful for immunocytochemical analysis.

Keywords: mouse CCR8, C_8Mab-2, monoclonal antibody, immunocytochemical analysis

Introduction

C–C motif chemokine receptor 8 (CCR8), a member of the C–C motif chemokine receptor family, is a G protein-coupled receptor (GPCR) and is activated using C–C motif chemokine ligands CCL1/I-309, CCL16, and CCL18.[1,2] CCR8 is primarily expressed in regulatory T (Treg) and T helper 2 (T_H2) cells, but little in T helper 1 cells.[3–5] Accordingly, the receptor plays a role in allergy and asthma by recruiting $CCR8^+$ Treg and T_H2 cells to the inflammation site.[4–6]

CCR8 is also associated with cancer development. Transcriptome analyses of tumor-infiltrating Tregs revealed that the *CCR8* gene was upregulated in breast cancer, nonsmall cell lung cancer, colorectal cancer, and oral squamous cell carcinoma.[7–9] CCL1 increases the migration activity of $CCR8^+$ Treg and induces the anti-apoptotic activity of T cell leukemia and lymphoma through CCR8.[10–13] The activation of CCR8 through CCL1 promotes suppression of the $CCR8^+$ Treg function through an increase in the expression of CCR8 level in Treg.[5]

In contrast, CCL18 induces invasion, migration, and epithelial–mesenchymal transition in bladder cancer cells.[14] These reports collectively imply that CCR8 can be a diagnostic and therapeutic target molecule of cancers. Moreover, developing specific antibodies against CCR8 can be the powerful driving force for basic research to reveal unresolved CCR8 functions.

We developed monoclonal antibodies (mAbs) for GPCRs, including anti-mouse CCR3 (clone C_3Mab-2) and anti-human CCR9 (clone C_9Mab-1) using the Cell-Based Immunization and Screening (CBIS) method.[15,16] We also developed an anti-mouse CCR8 (mCCR8) clone, C_8Mab-2, and showed that C_8Mab-2 was applicable to flow cytometric

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analysis.[17] In this study, we demonstrated that C_8Mab-2 was available for immunocytochemical analysis against mCCR8, which is both endogenously and exogenously expressed in cultured mammalian cells.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 cells were provided from the American Type Culture Collection (Manassas, VA). The establishment of a mCCR8-overexpressed CHO-K1 cell line (CHO/mCCR8) was described in our previous report.[17] Mouse lymphocyte-like cell line (P388) and mouse macrophage-like cell line (J774-1) were provided from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan).

All cells were cultured in Roswell Park Memorial Institute 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% 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.), and were maintained in a humidified atmosphere at 37°C under 5% CO_2.

Antibodies

An anti-mCCR8 mAb C_8Mab-2 (rat IgG_{2b}, kappa) was developed as previously described.[17] To generate recombinant C_8Mab-2 (recC_8Mab-2), we subcloned V_H and C_H of cDNAs of C_8Mab-2 into the pCAG-Neo vector, along with V_L and C_L cDNAs of C_8Mab-2 into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. An anti-mCCR8 mAb (clone SA214G2) was purchased from BioLegend (San Diego, CA).

Immunocytochemical analysis

Immunocytochemical analysis was carried out by a modification of a previously described procedure.[18] In brief, CHO-K1, CHO/mCCR8, P388, and J774-1 cells were cultured on acid-wash coverslips for 3 days. The cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 10 minutes at room temperature and quenched with PBSc/m (PBS supplemented with 0.2 mM Ca^{2+} and 2 mM Mg^{2+}) containing 50 mM NH_4Cl for 10 minutes.

Then, the cells were blocked with PBSc/m supplemented with 0.5% bovine serum albumin for 30 minutes, and they were incubated with 1 or 10 μg/mL of primary antibodies for 1 hour followed by Alexa Fluor 488-conjugated anti-rat IgG

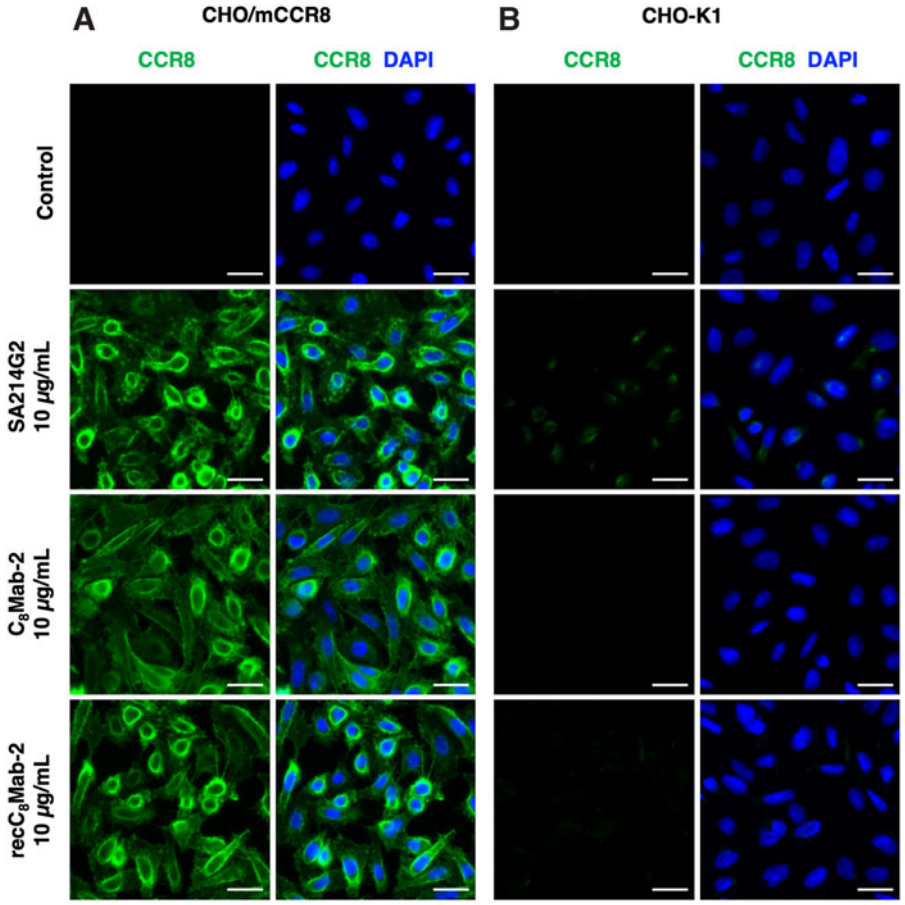

FIG. 1. Immunocytochemical analysis of exogenous mCCR8 using 10 μg/mL C_8Mab-2 and recC_8Mab-2. CHO/mCCR8 **(A)** and CHO-K1 cells **(B)** were incubated with buffer control, 10 μg/mL SA214G2, 10 μg/mL C_8Mab-2, or 10 μg/mL recC_8Mab-2 for 1 hour. They were subsequently incubated with Alexa Fluor 488-conjugated anti-rat IgG and DAPI for 45 minutes. Scale bars, 20 μm. CCR8, C–C motif chemokine receptor 8; CHO, Chinese hamster ovary; DAPI, 4′,6-diamidino-2-phenylindole; mCCR8, mouse CCR8; recC_8Mab-2, recombinant C_8Mab-2.

(1:400; Cell Signaling Technology, Inc.) for 45 minutes. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific Inc.). Fluorescence images were obtained using a 40× objective on a fluorescence microscope BZ-X800 (Keyence, Osaka, Japan).

Results

C_8Mab-2 and recC_8Mab-2 recognize exogenously expressed mCCR8 in CHO-K1 cells

To investigate whether C_8Mab-2 and recC_8Mab-2 recognize mCCR8 in immunocytochemistry, we used stable CHO/mCCR8 cells, which highly express mCCR8 protein.[17] It was revealed that both C_8Mab-2 (10 μg/mL) and recC_8Mab-2 (10 μg/mL), but not the buffer control, detected mCCR8 in CHO/mCCR8 cells (Fig. 1A). In contrast, both antibodies showed no signal from parental CHO-K1 cells during image acquisition (Fig. 1B). A commercially available anti-mCCR8 antibody (SA214G2) was used as a positive control and bound to CHO/mCCR8 cells, but background signals were also obtained from CHO-K1 cells (Fig. 1A, B). These data suggest that C_8Mab-2 and recC_8Mab-2 specifically recognize mCCR8.

The result of flow cytometry demonstrated that the fluorescence intensity obtained from 1 and 10 μg/mL of C_8Mab-2 labeling was comparable with those of CHO/mCCR8 cells.[17] Thus, in this study, we also used 1 μg/mL C_8Mab-2, recC_8Mab-2, and SA214G2 for immunocytochemistry. As a result, 1 μg/mL C_8Mab-2 and recC_8Mab-2 detected mCCR8 with a similar fluorescent intensity to 10 μg/mL in CHO/mCCR8 cells (Supplementary Fig. S1).

C_8Mab-2 and recC_8Mab-2 recognize endogenously expressed mCCR8 in P388 and J774-1 cells

Using immunocytochemistry, we examined whether C_8Mab-2 and recC_8Mab-2 recognize endogenous mCCR8 in P388 and J774-1 cells. As the expression levels of endogenous mCCR8 in both cells are expected to be lower than the level of exogenous mCCR8 in CHO/mCCR8 cells, we obtained fluorescence images of endogenous mCCR8 using longer exposure times. Thus, C_8Mab-2, recC_8Mab-2, and SA214G2 specifically detected endogenous mCCR8 in P388 and J774-1 cells (Fig. 2A, B). Moreover, the fluorescence intensity from recC_8Mab-2 was slightly stronger than the intensity from C_8Mab-2, indicating that the activity of recC_8Mab-2 was stronger than that of C_8Mab-2.

Discussion

Using flow cytometry, we previously evaluated that C_8Mab-2 selectively binds to exogenously expressed mCCR8 in CHO/mCCR8 cells and endogenous mCCR8 in

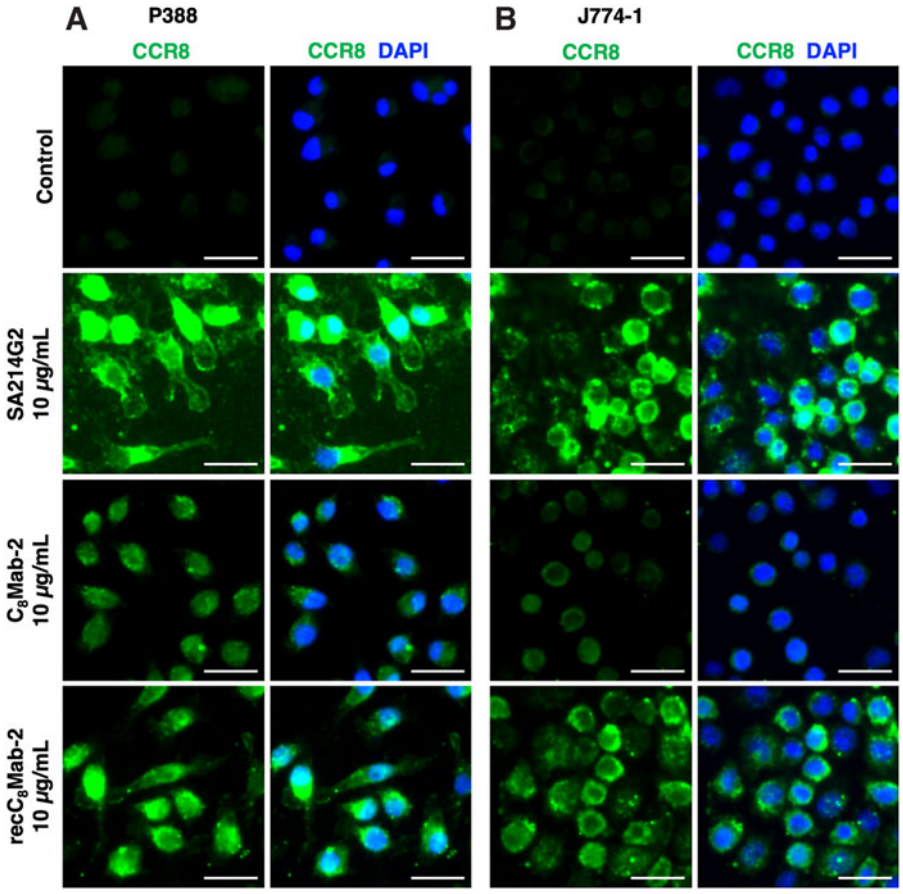

FIG. 2. Immunocytochemical analysis of endogenous mCCR8 using 10 μg/mL C_8Mab-2 and recC_8Mab-2. P388 **(A)** and J774-1 cells **(B)** were incubated with buffer control, 10 μg/mL SA214G2, 10 μg/mL C_8Mab-2, or 10 μg/mL recC_8Mab-2 for 1 hour. They were subsequently incubated with Alexa Fluor 488-conjugated anti-rat IgG and DAPI for 45 minutes. Scale bars, 20 μm.

P388 and J774-1 cells.[17] This study demonstrates that using immunocytochemical analysis, C_8Mab-2 recognizes both exogenous and endogenous mCCR8. In immunocytochemistry, the specificity of C_8Mab-2 and recC_8Mab-2 was demonstrated by two pieces of evidence: (1) no visible (or very weak) signal was obtained from control samples (e.g., CHO-K1 cells treated with C_8Mab-2/recC_8Mab-2; CHO/mCCR8, P388, or J774-1 cells treated with buffer control) and (2) mCCR8 was distributed in the cell membrane in CHO/mCCR8, P388, and J774-1 cells.

Immunocytochemical analysis of mCCR8 using C_8Mab-2 and recC_8Mab-2 would provide us valuable information for basic research. The intracellular distribution of mCCR8 would be identified in detail when the cells are colabeled with any organelle markers, such as plasma membranes. The expression level of the mCCR8 protein in cancer cells would be detected from fluorescence intensity data. Furthermore, using image cytometry, T cell types that highly express mCCR8 would be revealed when they are isolated from mice and the cell population is colabeled with surface antigen markers (e.g., CD4, CD25, and CD127).

Some anti-GPCR antibodies have been approved for clinical use. Mogamulizumab, an anti-CCR4 mAb, is used for human T cell lymphoma.[18] Erenumab, an anti-calcitonin gene-related peptide receptor mAb, is used for migraines.[19] Contrastingly, the availability of anti-mCCR8 antibodies for cancer therapy has been investigated *in vivo* and *in vitro*. For instance, treatment of colon cancer with a commercially available anti-mCCR8 mAb suppresses tumor growth.[20] Blockage of human CCR8 with a commercially available anti-human CCR8 mAb destabilizes $CCR8^+$ Tregs, resulting in reactivation of antitumor immunity in muscle-invasive bladder cancer.[21]

Van Damme *et al.*[22] developed a fusion antibody of a CCR8-specific nanobody and an antibody-dependent cell-mediated cytotoxicity (ADCC)-prone Fc region. The anti-CCR8 nanobody–Fc fusion significantly reduced the growth of nonsmall cell lung carcinoma.

In general, the development of anti-GPCR antibodies is technically difficult because of the variable three-dimensional conformation of GPCR, small extracellular epitopes of GPCR, and difficult preparation of homogeneous functional antigens.[23] In the CBIS method, mammalian cell lines stably expressing a target molecule were used as an immunogen, meaning that GPCR-harboring conformational epitopes and physiological post-translational modification are used as antigens. MAbs developed using the CBIS method are expected to be highly suitable for detecting GPCR expressed in cells. Thus, in the future, it is necessary to investigate whether C_8Mab-2 and recC_8Mab-2 possess ADCC activity, complement-dependent cytotoxicity, and antitumor activity.

In conclusion, we clarified that C_8Mab-2 and recC_8Mab-2 could be useful tools for immunocytochemistry in detecting the expression and intracellular distribution of mCCR8 in Tregs and cancer cells including T cell lymphoma.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported, in part, by Japan Agency for Medical Research and Development (AMED) under Grant Nos. JP22ama121008 (to Y.K.), JP21am0401013 (to Y.K.), and JP21am0101078 (to Y.K.), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) Grant Nos. 21K06059 (to M.S.), 21K20789 (to T.T.), 21K15523 (to T.A.), 20K16322 (to M.S.), 21K07168 (to M.K.K.), and 19K07705 (to Y.K.).

Supplementary Material

Supplementary Figure S1

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Received: September 15, 2021
Accepted: February 14, 2022