

COMMUNICATION

C₇Mab-2: A novel monoclonal antibody against mouse CCR7 established by immunization of the extracellular loop domain

Haruto Yamamoto¹, Hiroyuki Suzuki*¹, Tomohiro Tanaka¹, Hiroyuki Satofuka¹, Mika K. Kaneko¹, and Yukinari Kato*¹

Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

Abstract

The chemokine receptors possess seven transmembrane helices connected by an extracellular N-terminal region, three extracellular loops (ECL1–3), three intracellular loops, and an intracellular C-terminal region. Specific monoclonal antibodies (mAbs) against chemokine receptors for flow cytometry have been developed using Cell-Based Immunization and Screening, and the N-terminal peptide immunization methods. However, there are few reports on the establishment of anti-chemokine receptor mAbs through immunization with ECL peptides. Here, an anti-mouse C–C chemokine receptor type 7 (mCCR7) mAb, C₇Mab-2 (rat immunoglobulin G_{2b} kappa), was established through immunization with the ECL3 peptide. C₇Mab-2 demonstrated reactivity to mCCR7-overexpressed Chinese hamster ovary-K1 (CHO/mCCR7) cells in flow cytometry, which was inhibited by the ECL3 peptide. C₇Mab-2 did not show cross-reactivity with other mouse CC, CXC, CX3C, and XC chemokine receptors. The dissociation constant value of C₇Mab-2 was determined to be 2.8×10^{-9} M for CHO/mCCR7 cells. Furthermore, C₇Mab-2 detected mCCR7 in immunohistochemistry. This strategy could accelerate the development of novel chemokine receptor mAbs with high affinity and specificity.

Keywords: Mouse C–C chemokine receptor type 7; Monoclonal antibody; Extracellular loop; Peptide immunization; Flow cytometry; Immunohistochemistry

*Corresponding authors:

Hiroyuki Suzuki
(hiroyuki.suzuki.b4@tohoku.ac.jp)
Yukinari Kato
(yukinari.kato.e6@tohoku.ac.jp)

Citation: Yamamoto H, Suzuki H, Tanaka T, *et al.* C₇Mab-2: A novel monoclonal antibody against mouse CCR7 established by immunization of the extracellular loop domain. *Microbes & Immunity*. 2026;3(1):172-180.
doi: 10.36922/MI025130028

Received: March 29, 2025

Revised: April 29, 2025

Accepted: June 3, 2025

Published online: July 2, 2025

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1. Introduction

Chemotactic trafficking is regulated by G protein-coupled receptors (GPCRs) on immune cells.¹ With four conserved cysteine residues forming two disulfide bonds, chemokines are cytokines that guide immune cells to the appropriate locations. They can be classified into four subfamilies: CC, CXC, XC, and CX3C, based on the number and position of cysteine residues at the N-terminus.² The biological effects of chemokines are mediated through a family of GPCRs. The chemokine receptors possess seven transmembrane helices connected by an extracellular N-terminal region, three extracellular loops (ECL1–3), three intracellular loops, and an intracellular C-terminal region.³ Several disulfide bonds connect the N-terminus to ECL3 and ECL1 to ECL2.⁴ The binding of chemokines to their specific receptors induces conformational changes and activates the

chemokine signaling pathways to regulate the migration, integration, adhesion, and proliferation of immune cells.^{5,6}

C–C chemokine receptor 7 (CCR7) is expressed on naïve T and B cells, natural killer cells, regulatory T cells, central memory T cells, dendritic cells, and tumor cells.⁷ C–C motif chemokine ligands (CCL) 19 and 21 are high-affinity CCR7 ligands that promote the migration of CCR7-positive cells to secondary lymphoid organs, including the thymus, spleen, and lymph nodes.^{8–12} Genome-wide association studies have revealed a relationship between CCL21/CCR7 and disease severity in patients with systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, or asthma.⁷ Disrupting the CCL21/CCR7 interaction with monoclonal antibodies (mAbs) or inhibitors suppresses the migration of CCR7-positive cells to inflammatory sites, thereby inhibiting disease progression.

Metastasis to the lymph node is an important predictive factor for patients with cancer.¹³ The elevated expression of CCR7 is associated with lymph node metastasis in many solid tumors, including esophageal,¹⁴ gastric,¹⁵ colorectal,¹⁶ pancreatic,¹⁷ thyroid,¹⁸ oral,¹⁹ and non-melanoma skin cancers.²⁰ CCR7, but not others, specifically drives cancer cell homing to lymph nodes and other secondary lymphoid organs, where the ligands CCL19 and CCL21 are constitutively expressed by stroma cells.²¹ Therefore, developing specific mAbs against mouse CCR7 (mCCR7) is essential to targeting the CCR7-expressing cells in preclinical mouse disease models.

Specific mAbs against various chemokine receptors have been developed, including mouse CXCR1 (mCXCR1; clone C_{x1}Mab-1),²² mouse CXCR3 (mCXCR3; clone C_{x3}Mab-4),²³ mouse CXCR4 (mCXCR4; clone C_{x4}Mab-1),²⁴ mouse CCR1 (mCCR1; clone C₁Mab-6),²⁵ mouse CCR3 (mCCR3; clones C₃Mab-2, C₃Mab-3, and C₃Mab-4),²⁶ mouse CCR5 (mCCR5; clone C₅Mab-2),²⁷ mCCR7 (clone C₇Mab-7),²⁸ and mouse CCR8 (mCCR8; clones C₈Mab-1, C₈Mab-2, and C₈Mab-3)²⁹ using the Cell-Based Immunization and Screening (CBIS) method. This method involves immunization with antigen-overexpressed cells and flow cytometry-based high-throughput screening. Furthermore, specific mAbs against mouse CCR2 (mCCR2; clone C₂Mab-6),³⁰ mCCR3 (clones C₃Mab-6 and C₃Mab-7),³¹ mouse CCR4 (mCCR4; clone C₄Mab-1),³² mouse CCR6 (mCCR6; clone C₆Mab-13),³³ mouse CCR9 (mCCR9; clone C₉Mab-24),³⁴ mouse CXCR5 (mCXCR5; clone C_{x5}Mab-3),³⁵ and mouse CXCR6 (mCXCR6; clone C_{x6}Mab-1)³⁶ have also been established using the N-terminal peptide immunization. In contrast, there are few reports on the establishment of anti-chemokine receptor mAbs by immunization with ECL peptides.

2. Materials and methods

2.1. Cell lines

Mouse myeloma cell line P3X63Ag8.U1 (P3U1) and Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection (USA). The mCCR7-overexpressed CHO-K1 (CHO/mCCR7) cell line was previously established.²⁸ Stable transfectants of the following chemokine receptors were previously established:³⁵ CHO/mCCR1, CHO/mCCR2, CHO/mCCR3, CHO/PA-mCCR4, CHO/mCCR5, CHO/PA-mCCR6, CHO/mCCR8, CHO/mCCR9, CHO/PA-mCCR10, CHO/mCXCR1, CHO/mCXCR2, CHO/mCXCR3, CHO/mCXCR4, CHO/mCXCR5, CHO/mCXCR6, CHO/mCX3CR1, and CHO/mXCR1. These cells were maintained as described previously.³⁵

2.2. Peptides

Eurofins Genomics KK (Japan) synthesized partial sequences of the ECLs of mCCR7 as follows: mCCR7-1 (SEAKSWIFGVYLC), mCCR7-2 (ELLYSGLQKNSGEDTLRC), and mCCR7-3 (CETSKQLNIAVDVTYS). Subsequently, the keyhole limpet hemocyanin (KLH) was conjugated to the N-terminus of mCCR7-3 or the C-terminus of mCCR7-1 and mCCR7-2.

2.3. Hybridoma production

The Animal Care and Use Committee of Tohoku University approved the animal study (Permit number: 2022MdA-001). Three 6-week-old female Sprague–Dawley (SD) rats (CLEA Japan, Japan) were intraperitoneally immunized with 100 µg of the KLH-conjugated mCCR7 peptides mixed with 2% Alhydrogel adjuvant (InvivoGen, USA). Hybridomas were generated as described previously.³⁶

2.4. Enzyme-linked immunosorbent assay

The synthesized mCCR7 peptides (1 µg/mL) were immobilized on immunoplates. After blocking with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (Nacalai Tesque, Inc., Japan) and 1% bovine serum albumin (BSA), the plates were treated with hybridoma supernatants. Enzymatic reactions were performed using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc., Japan). Optical density was detected at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).³⁶

2.5. Flow cytometry

Cells were incubated with C₇Mab-2 in a blocking buffer (0.1% BSA in PBS) at 4°C for 30 min. For the peptide inhibition assay, C₇Mab-2 (2 µg/mL) was pre-incubated with dimethyl sulfoxide (DMSO) or 1 µg/mL mCCR7-3

peptide for 15 min, and then incubated with the cells for 30 min at 4°C. Data were collected using the SA3800 Cell Analyzer (Sony Biotechnology, Japan) and analyzed as described previously.³⁶

2.6. Determination of dissociation constant

Cells were treated with C₇Mab-2 (0.006 to 100 µg/mL). The cells were then incubated with Alexa Fluor 488-conjugated anti-rat immunoglobulin G (IgG) at 4°C for 30 min. The dissociation constant (K_D) of C₇Mab-2 for CHO/mCCR7 was determined using GraphPad PRISM 6 (USA), as described previously.³⁶

2.7. Immunohistochemical analysis

The preparation of cell sections and antigen retrieval were performed as described previously.²⁸ After blocking, the sections were treated with C₇Mab-2 (20 µg/mL) for 1 h. For the peptide inhibition assay, C₇Mab-2 (20 µg/mL) was pre-incubated with 2 µg/mL of the mCCR7-3 peptide or DMSO for 15 min and then incubated with the cell blocks for 1. Color development was achieved as described previously.²⁸

3. Results

3.1. Development of an anti-mouse CCR7 monoclonal antibody, C₇Mab-2, by immunization with three extracellular loop peptides

Three SD rats were immunized with the KLH-conjugated mCCR7 peptides, respectively (Figure 1A). Hybridomas were produced by fusion with P3U1 cells (Figure 1B). Then, positive wells for each unconjugated mCCR7 peptide were selected using enzyme-linked immunosorbent assay (ELISA) (Figure 1C). The ELISA screening identified 11 of 1,534 wells for mCCR7-1 (0.7%), 78 of 1,534 wells for mCCR7-2 (5.1%), and 93 of 1,438 wells for mCCR7-3 (6.5%) that strongly reacted with the respective mCCR7 peptide. Second screenings were subsequently performed using flow cytometry (Figure 1C). Among the 93 ELISA-positive wells for mCCR7-3, 11 wells showed reactivity with CHO/mCCR7 cells but not with CHO-K1 cells. No flow cytometry-positive wells were obtained from hybridomas derived from mCCR7-1-KLH- and mCCR7-2-KLH-immunized rat. The anti-mCCR7 mAb-producing hybridomas derived from KLH-mCCR7-3-immunized rat were further cloned by limiting dilution, and C₇Mab-2 (rat IgG_{2b}, kappa) was finally established (Figure 1D).

3.2. Flow cytometry using C₇Mab-2

Flow cytometry was performed using C₇Mab-2 against CHO/mCCR7 cells and CHO-K1 cells. C₇Mab-2 recognized CHO/mCCR7 cells at concentrations ranging from 0.01 to 10 µg/mL, whereas CHO-K1 cells were not

recognized even at 10 µg/mL (Figure 2A). A peptide-blocking assay demonstrated that C₇Mab-2 reacted with CHO/mCCR7 cells, and this reactivity was completely neutralized by the mCCR7-3 peptide (Figure 2B).

3.3. Reactivity of C₇Mab-2 to various chemokine receptor-expressing CHO-K1 cells

Anti-mouse CC, CXC, CX3C, and XC chemokine receptor mAbs have previously been established and evaluated using CHO-K1 cells expressing these receptors.³⁵ Among 18 CHO-K1 cells expressing mouse CC, CXC, CX3C, and XC chemokine receptors, C₇Mab-2 recognized only CHO/mCCR7 cells, but not others (Figure 3).

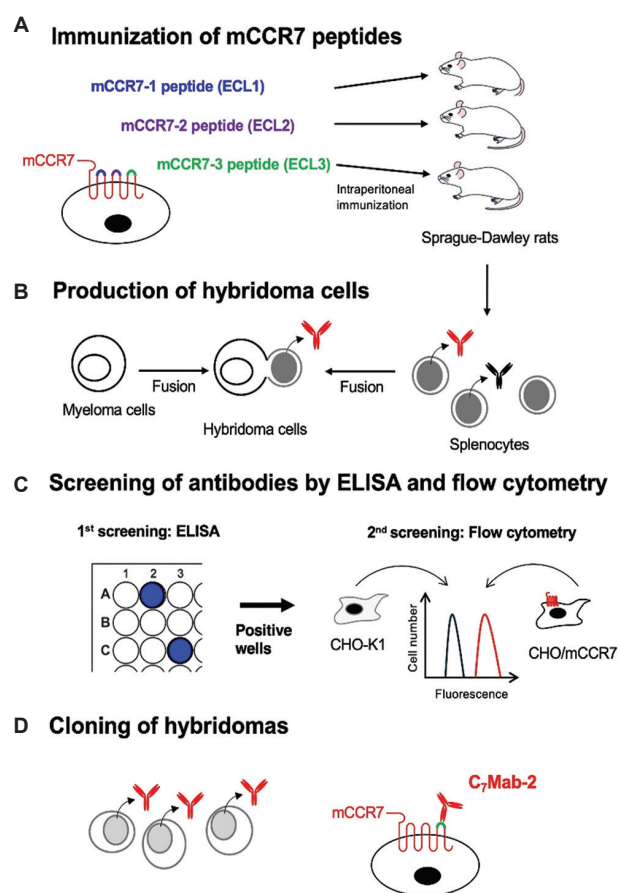


Figure 1. Schematic representation of anti-mCCR7 mAb production. (A) The KLH-conjugated mCCR7 ECL peptides (mCCR7-1, mCCR7-2, and mCCR7-3) were immunized into Sprague-Dawley rats. (B) The spleen cells were fused with P3U1 cells. (C) To select anti-mCCR7 mAb-producing hybridomas, the supernatants were screened by ELISA and flow cytometry using CHO-K1 cells and CHO/mCCR7 cells. (D) The anti-mCCR7 mAb-producing hybridomas from KLH-mCCR7-3-immunized rat were further cloned by limiting dilution, and C₇Mab-2 (rat IgG_{2b}, kappa) was finally established. Abbreviations: CHO-K1: Chinese hamster ovary-K1; ECL: Extracellular loop; ELISA: Enzyme-linked immunosorbent assay; KLH: Keyhole limpet hemocyanin; mAb: Monoclonal antibody; mCCR7: Mouse C-C chemokine receptor type 7.

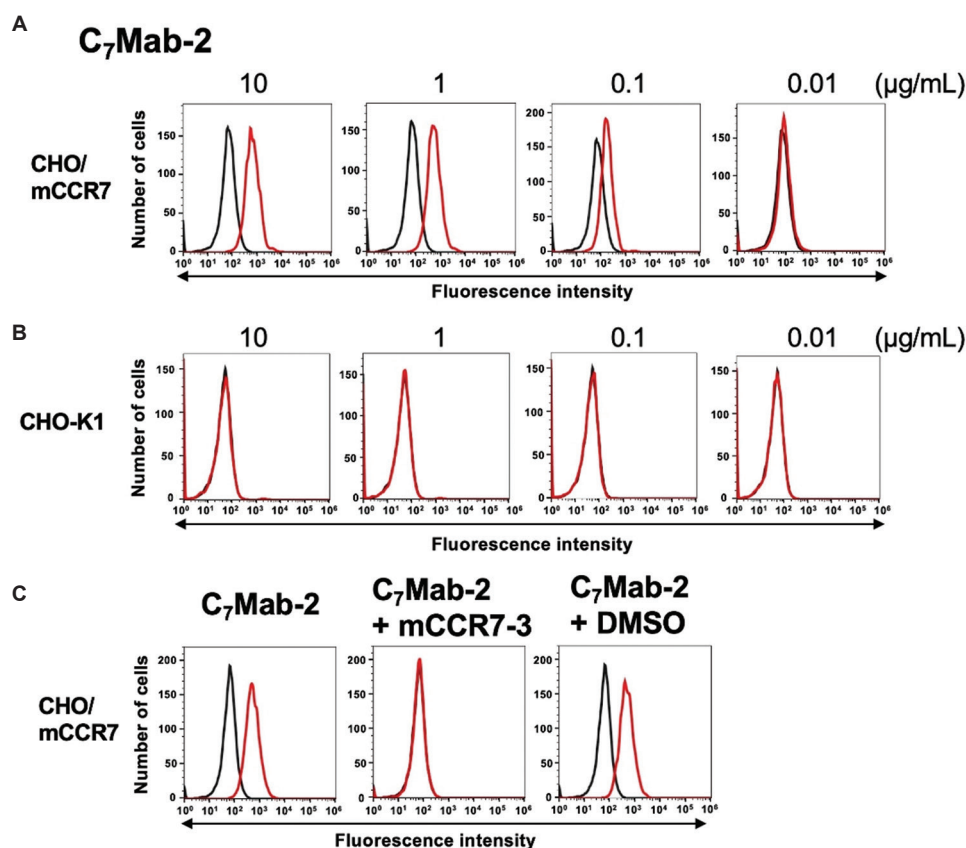


Figure 2. Flow cytometry analysis of C_7 Mab-2 against CHO/mCCR7 cells and CHO-K1 cells. (A and B) CHO/mCCR7 cells (A) and CHO-K1 cells (B) were treated with 0.01, 0.1, 1, and 10 $\mu\text{g}/\text{mL}$ of C_7 Mab-2 (red line). The mAb-treated cells were further incubated with Alexa Fluor 488-conjugated anti-rat IgG. The black line represents the negative control (blocking buffer). The dose-dependent reactivities of C_7 Mab-2 to CHO/mCCR7 cells were investigated in at least three independent experiments. (C) Peptide-blocking assay using C_7 Mab-2 with mCCR7-3 peptide. C_7 Mab-2 (2 $\mu\text{g}/\text{mL}$) with mCCR7-3 (1 $\mu\text{g}/\text{mL}$, red line) or control (1% DMSO in blocking buffer, red line) was reacted with CHO/mCCR7 for 30 min at 4°C, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. The black line represents the negative control (blocking buffer).

Abbreviations: CHO-K1: Chinese hamster ovary-K1; DMSO: Dimethyl sulfoxide; IgG: Immunoglobulin G; mCCR7: Mouse C-C chemokine receptor type 7.

3.4. Dissociation constant of C_7 Mab-2

The binding affinity of C_7 Mab-2 was evaluated using flow cytometry. The K_D of C_7 Mab-2 for CHO/mCCR7 cells was $2.8 \pm 0.3 \times 10^{-9}$ M (Figure 4).

3.5. Immunohistochemistry using C_7 Mab-2

To examine the suitability of C_7 Mab-2 for immunohistochemistry, the sections of CHO-K1 cells and CHO/mCCR7 cells were stained with C_7 Mab-2. The membranous and cytoplasmic staining were observed in CHO/mCCR7 cells (Figure 5A), but not in CHO-K1 cells (Figure 5B). Furthermore, this reactivity was completely neutralized by the mCCR7-3 peptide (Figure 5C and D).

4. Discussion

This study established an anti-mCCR7 mAb, C_7 Mab-2, by immunizing with the ECL3 peptide. C_7 Mab-2 can be applied to flow cytometry (Figure 2) and immunohistochemistry

(Figure 5) to detect mCCR7-positive cells. The reactivity (Figure 2) and affinity (Figure 4) of C_7 Mab-2 are similar to another anti-mCCR7 mAb, C_7 Mab-7, which was established through the CBIS method.²⁸ It is critical to investigate whether C_7 Mab-2 can detect endogenous mCCR7 using cells from secondary lymphoid organs, including the lymph node, thymus, and spleen. Furthermore, this study also confirmed that C_7 Mab-2 recognizes mCCR7, but not other CC, CXC, CX3C, and XC chemokine receptors (Figure 3). The ECL1–3 peptides were immunized, and the ELISA-positive wells in hybridomas were obtained from each peptide-immunized rat. However, it was unable to obtain flow cytometry-positive wells in hybridomas derived from ECL1 and ECL2 peptide-immunized rats. Among hybridomas from the ECL3 peptide-immunized rat, only 10% of ELISA-positive supernatants recognized CHO/mCCR7 in flow cytometry, indicating that conformational changes and modifications, including glycosylation³⁷ or disulfide bond formation,⁴ would restrict the recognition

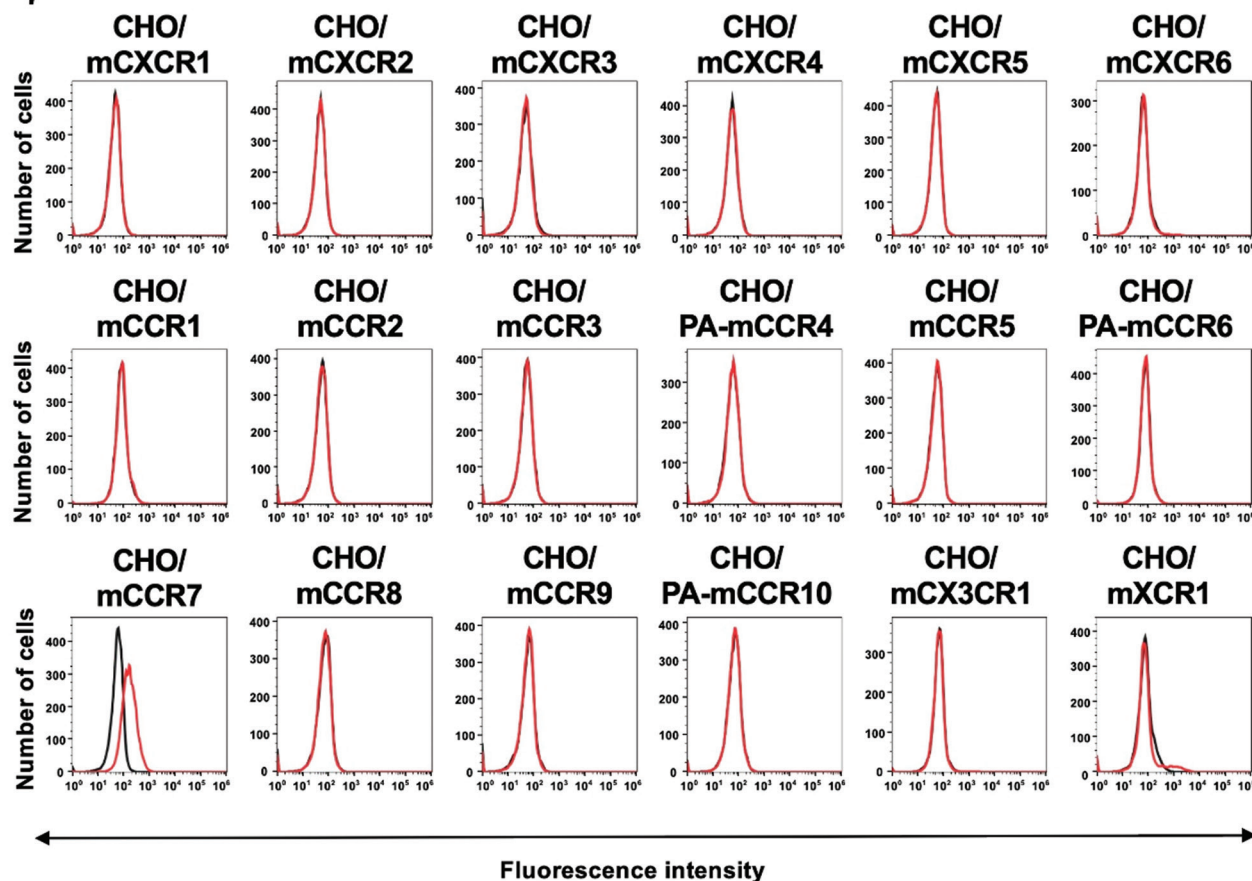
C₇Mab-2

Figure 3. Flow cytometry analysis of C₇Mab-2 in CC, CXC, CX3C, and XC chemokine receptor-expressing CHO-K1 cells. Eighteen mouse CC, CXC, CX3C, and XC chemokine receptor-expressing CHO-K1 cells were treated with 1 µg/mL of C₇Mab-2 (red line) or control blocking buffer (black line), followed by treatment with Alexa fluor 488-conjugated anti-rat IgG. Fluorescence data were collected using the SA3800 cell analyzer. Each receptor expression was previously confirmed by flow cytometry.

Abbreviations: CHO-K1: Chinese hamster ovary-K1; IgG: Immunoglobulin G; mCCR: Mouse C-C chemokine receptor; mCXCR: Mouse C-X-C motif chemokine receptor; mXCR: Mouse X-C motif chemokine receptor.

of mAbs. Future investigation should involve the determination of the critical epitope of C₇Mab-2, which may help the understanding of the recognition mechanism of mCCR7. Notably, the Cx₆Mab-1 epitope was previously identified using 1× and 2× alanine scanning methods.³⁸

Structural information on chemokine receptors is required to develop drugs that fulfill the requirements. Much effort has been made to determine the structures in complex with either synthetic ligands³⁹⁻⁴¹ or native chemokines^{42,43} by X-ray crystallography. The cryo-electron microscopy (cryo-EM) has reportedly determined the structures of several chemokine receptor-ligand complexes.⁴⁴⁻⁴⁷ Although the structure of the CCR7-ligand complex has not been determined, the crystal structure of CCR7 with Cmp2105, an intracellular allosteric CCR7 receptor antagonist, was previously determined.⁴⁸ Recently,

the structures of chemokine receptor-mAb complexes have been determined by means of the cryo-EM, providing a detailed structural and mechanistic framework of chemokine receptor activation and inhibition.⁴⁹ Since C₇Mab-2 is known to recognize ECL3 of mCCR7, it could help the structural analysis of mCCR7 in future studies.

Several *in vitro* and *in vivo* preclinical tumor models have demonstrated that increased CCR7 expression promotes tumor growth and metastasis, whereas reduced CCR7 expression suppresses these processes.⁵⁰ For example, in an orthotopic model, mCCR7-overexpressing mouse mammary tumor cells (PyVmT) demonstrated enhanced metastasis to the lymph nodes. In contrast, the control cells did not migrate to the lymph nodes but metastasized to the lungs. Additionally, mCCR7 overexpression significantly increased tumor growth in

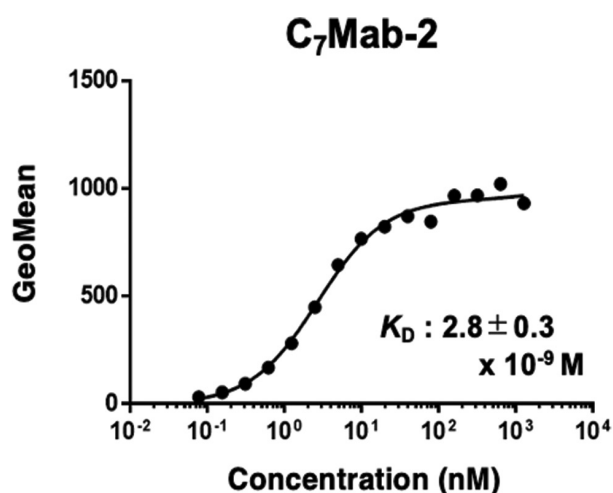


Figure 4. Binding affinity of C_7 Mab-2. CHO/mCCR7 cells were incubated with serially diluted C_7 Mab-2, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. Fluorescence data were collected using the SA3800 cell analyzer, and the K_D was calculated using GraphPad PRISM 6. The representative results were shown. Three independent experiments were conducted to determine the average K_D (mean \pm standard deviation). Abbreviations: CHO: Chinese hamster ovary; IgG: Immunoglobulin G; K_D : Dissociation constant; mCCR7: Mouse C–C chemokine receptor type 7.

PyVmT cells both *in vitro* and *in vivo* compared to the control.⁵¹ Furthermore, in a mouse melanoma model, mCCR7-overexpressed B16 melanoma cells exhibited a significantly higher rate of lymph node metastasis than control cells, although the primary tumor size remained unchanged.⁵² To target the mCCR7-positive tumors *in vivo*, C_7 Mab-2 (rat IgG_{2b}) should be converted to mouse IgG_{2a} mAb. Through the determination of heavy-chain variable domain and light-chain variable domain sequences of C_7 Mab-2, a large amount of recombinant mAbs can be generated for use in preclinical studies.

In a syngeneic mouse model of oral cancers, the growth of tumors was significantly decreased in mCCR7-knockout (KO) mice.⁵³ Single-cell RNA sequencing analysis showed that the M2 macrophage proportion in the KO group was lower compared to the control.⁵³ mCCR7 stimulates the polarization of M2 macrophages, which promotes the migration, invasion, and proliferation of tumor cells.⁵³ Therefore, the depletion of mCCR7-expressing cells by anti-mCCR7 mAbs, such as class-switched and defucosylated mouse IgG_{2a}-type C_7 Mab-2, could help investigate the effect of mCCR7-expressing cell depletion on tumor growth.

5. Conclusion

An anti-mCCR7 mAb, C_7 Mab-2, was established by immunization with the ECL3 peptide. C_7 Mab-2 can be used in flow cytometry and immunohistochemistry

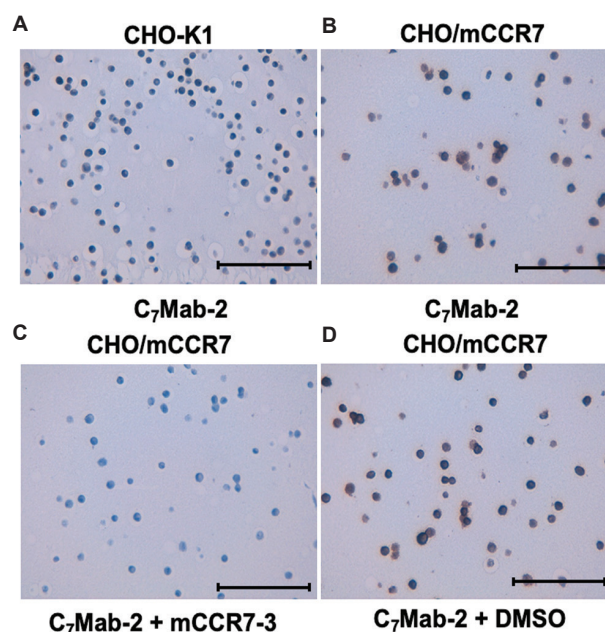


Figure 5. Immunohistochemistry of paraffin-embedded cell sections of CHO/mCCR7 cells and CHO-K1 cells using C_7 Mab-2. (A and B) Sections of CHO-K1 cells (A) and CHO/mCCR7 cells (B) were treated with 20 μ g/mL of C_7 Mab-2, followed by treatment with Histofine Simple Stain Mouse MAX PO (Rat). (C and D) Peptide-blocking assay using C_7 Mab-2 with mCCR7-3 peptide. C_7 Mab-2 (20 μ g/mL) with mCCR7-3 (2 μ g/mL, C) or control (1% DMSO in blocking buffer, D) were reacted with the sections of CHO/mCCR7 cells, followed by treatment with Histofine Simple Stain Mouse MAX PO (rat). Color was developed using DAB, and counterstaining was performed using hematoxylin. Scale bar: 100 μ m. Magnification: x200.

Abbreviations: CHO: Chinese hamster ovary; DAB: 3,3'-diaminobenzidine tetrahydrochloride; DMSO: Dimethyl sulfoxide; mCCR7: Mouse C–C chemokine receptor type 7.

experiments and is expected to provide proof of concept in preclinical studies.

Acknowledgments

None.

Funding

This research was supported in part by the Japan Agency for Medical Research and Development (AMED) under Grant Numbers: JP24am0521010 (to Y. Kato), JP24ama121008 (to Y. Kato), JP24ama221339 (to Y. Kato), JP24bm1123027 (to Y. Kato), and JP24ck0106730 (to Y. Kato), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) under Grant Numbers. 22K06995 (to H. Suzuki), 24K18268 (to T. Tanaka), 24K11652 (to H. Satofuka), and 25K10553 (to Y. Kato).

Conflict of interest

The authors declare that they have no competing interest.

Author contributions

Conceptualization: Mika K. Kaneko, Yukinari Kato

Formal analysis: Hiroyuki Suzuki

Funding acquisition: Tomohiro Tanaka, Hiroyuki Satofuka, Hiroyuki Suzuki, Yukinari Kato

Investigation: Haruto Yamamoto, Hiroyuki Suzuki, Tomohiro Tanaka, Hiroyuki Satofuka

Methodology: Mika K. Kaneko

Writing—original draft: Hiroyuki Suzuki

Writing—review & editing: Yukinari Kato

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

Consent for publication

Not applicable.

Availability of data

The data of this study are available in the article.

Further disclosure

The paper has been uploaded to a preprint server (doi: 10.20944/preprints202503.0581.v1).

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