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

DOI: 10.1089/mab.2021.0063



# Development of a Novel Anti-Mouse CCR2 Monoclonal Antibody ($C_2$Mab-6) by N-Terminal Peptide Immunization

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The CC chemokine receptor type-2 (CCR2) belongs to the G-protein-coupled receptor superfamily, expressed on the cell surface of immune cells and tumors. CCR2 binds to the CC motif chemokine 2/monocyte chemoattractant protein-1, a CC chemokine, which is produced by various cells, including immune-related cells and tumors. Therefore, the development of sensitive monoclonal antibodies (mAbs) for CCR2 has been desired for treatment and diagnosis. This study established a novel, specific, and sensitive anti-mouse CCR2 (mCCR2) mAb; $C_2$Mab-6 (rat $IgG_1$, kappa), using the mCCR2 synthetic peptide immunization method. $C_2$Mab-6 reacted with mCCR2-overexpressed Chinese hamster ovary-K1 cells and L1210 (murine leukemia) cells, which express endogenous mCCR2 in flow cytometry. Furthermore, $C_2$Mab-6 showed a high binding affinity for both cells. Hence, $C_2$Mab-6 can be a useful tool for analyzing mCCR2-related biological responses, using flow cytometry.

**Keywords:** CCR2, monoclonal antibody, flow cytometry

## Introduction

Chemokines play important roles in immune responses, such as infiltration and migration,[1–4] are small-molecule proteins, which belong to the largest subfamily of cytokines.[3] They can be classified into four different subfamilies: CC, CXC, CX3C, and XC, depending on the number and position of cysteine residues in their N-terminus.[5] By binding to thier receptors, which are seven transmembrane G-protein-coupled receptors (GPCRs), chemokines can trigger an intracellular signal, which influences various cellular functions.[6]

The CC motif chemokine 2 (CCL2)/monocyte chemoattractant protein-1 is one of the CC motifs of chemokine ligands, with a molecular weight of 13,000. Various cells produce CCL2, including tumor cells, endothelial cells, epithelial cells, myeloid cells, and fibroblasts.[7,8] CCL2 also plays a crucial role in attracting monocytes, T lymphocytes, and natural killer cells.[9] Moreover, several GPCRs bind CCL2, including the CC chemokine receptor type-2 (CCR2), CCR4, and CCR5.[10] Notably, CCR2 is a primary receptor of CCL2,[11] whose expression has been observed in multiple cells, including monocytes, dendritic cells, macrophages, and epithelial cells. It has also been reported that the CCL2-CCR2 axis correlates with many diseases, such as immune disorders and cancers.[12–14]

In viral and bacterial infections, CCL2 and CCR2-expressing immune cells, such as macrophages and neutrophils, contribute to innate immune responses in respiratory organs.[15] Alternatively, in tumors, CCL2 influences cancer growth, angiogenesis, and metastasis.[9,10,16] It has also been reported as a highly upregulated chemokine in bone tumors.[17] Therefore, various cells expressing CCR2 are involved in disease pathogenesis by interacting with CCL2.

Using the Cell-Based Immunization and Screening (CBIS) method, we have developed many monoclonal antibodies (mAbs) against membrane proteins, such as anti-CD19,[18] anti-CD20,[19,20] anti-CD44,[21] anti-CD133,[22] anti-EpCAM,[23,24] anti-HER3,[25] anti-KLRG1,[26] anti-TIGIT,[27] anti-TROP2,[28,29] anti-programmed cell death ligand 1 (PD-L1),[30] and anti-podoplanin[31–41] mAbs. We have also successfully developed anti-GPCR mAbs, including anti-mouse CCR3,[42] anti-mouse CCR8,[43] and anti-human CCR9[44] mAbs, using the CBIS method. However,

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we have not established anti-GPCR mAbs, using the synthetic peptide immunization method yet. In this study, we developed anti-mouse CCR2 (mCCR2) mAbs using the synthetic peptide immunization method.

## Materials and Methods

### *Cell lines*

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). The L1210 (murine leukemia) cell line was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding mCCR2 (Accession No. NM_009915) was subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), after which the mCCR2 plasmid was transfected using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). Subsequently, CHO/mCCR2 was established using a cell sorter (SH800; Sony Corp., Tokyo, Japan), following cultivation in a medium containing 0.5 mg/mL Zeocin (InvivoGen, San Diego, CA).

CHO-K1, P3U1, CHO/mCCR2, and L1210 were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) that was supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 units/mL penicillin, 100 $\mu$g/mL streptomycin, and 0.25 $\mu$g/mL 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.

### *Antibodies*

The anti-mCCR2 mAb (clone EPR20844) was purchased from Abcam (Cambridge, United Kingdom). The secondary Alexa Fluor 488-conjugated anti-rat IgG and Alexa Fluor 488-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

### *Hybridoma production*

A 6-week-old female Sprague–Dawley (SD) rat was purchased from CLEA, Japan (Tokyo, Japan). The animal was 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 No. 2019NiA-001). The rat 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, the rat was euthanized through cervical dislocation, after which respiratory and cardiac arrest was used to verify death.

The mCCR2 peptide immunization method was used to develop mAbs against mCCR2. In brief, one SD rat was immunized, using three keyhole limpet hemocyanin-conjugated mCCR2 peptides (100 $\mu$g of each peptide), including $_{1}$-MEDNNMLPQFIHGILSTSH-$_{19}$, $_{11}$-IHGILSTSHSLFTRSI QEL-$_{29}$, and $_{21}$-LFTRSIQELDEGATTPYDY-$_{39}$+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 $\mu$g of each peptide), followed by a final booster intraperitoneal injection (100 $\mu$g of each 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.

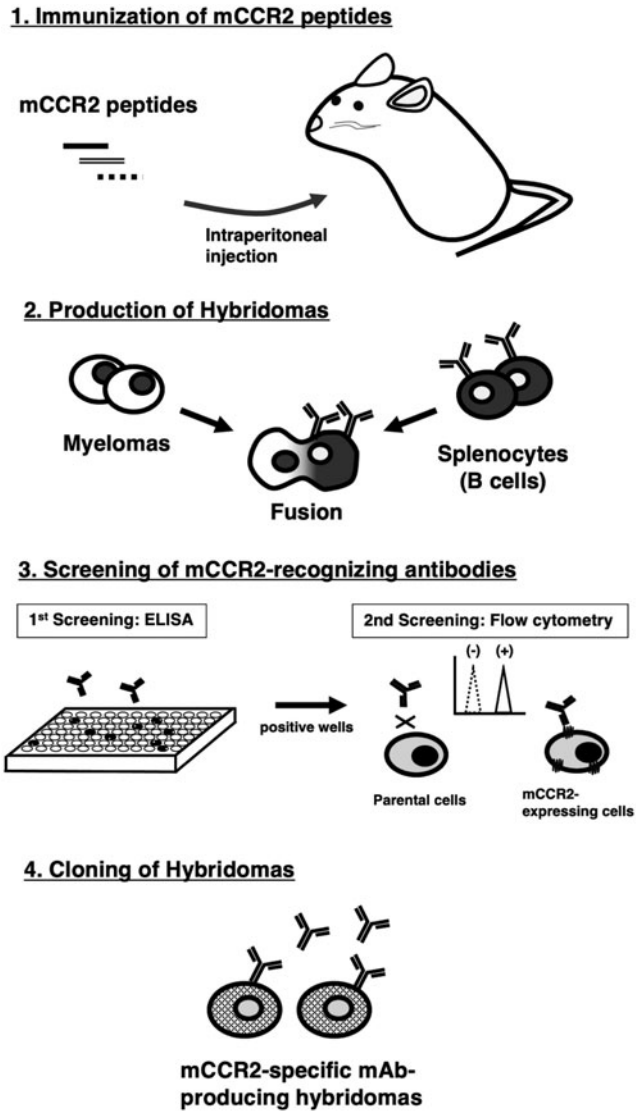


**FIG. 1.** A schematic procedure of anti-mCCR2 mAbs production. The rat was intraperitoneally immunized with mCCR2 peptides. Screening was then conducted by ELISA, using mCCR2 peptides, and flow cytometry using parental cells and mCCR2-overexpressed CHO-K1 cells. CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; mAbs, monoclonal antibodies; mCCR2, mouse CC chemokine receptor type-2.

*ELISA*

A mixture of synthesized mCCR2 peptides, including $_{1}$-MEDNNMLPQFIHGILSTSH-$_{19}$, $_{11}$-IHGILSTSHSLFTRSI QEL-$_{29}$, and $_{21}$-LFTRSIQELDEGATTPYDY-$_{39}$+C-terminal cysteine was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at a concentration of 1 $\mu$g/mL for each peptide 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 culture supernatants, followed by peroxidase-conjugated anti-rat immunoglobulins (1:2000 diluted; Agilent Technologies, Inc., Santa Clara, CA). Afterward, enzymatic reactions were conducted, using the ELISA POD substrate TMB kit (Nacalai Tesque, Inc.), following the measurement of the optical density at 655 nm, using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).



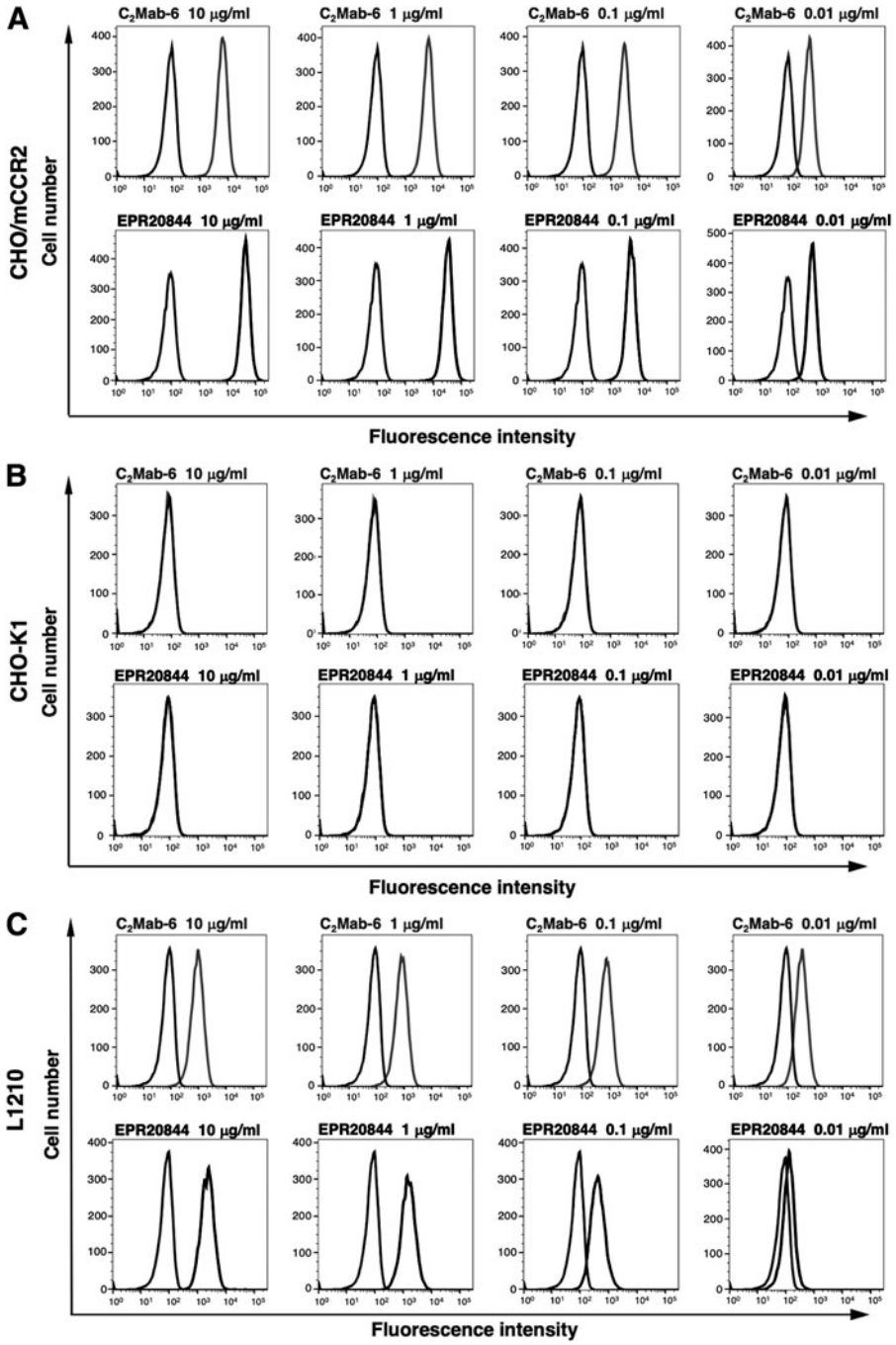


**FIG. 2.** Flow cytometry using anti-mCCR2 mAbs. CHO/mCCR2 cells **(A)**, CHO-K1 cells **(B)**, and L1210 cells **(C)** were treated with 0.01–10 μg/mL of $C_2$Mab-6 and EPR20844, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG and Alexa Fluor 488-conjugated anti-rabbit IgG, respectively. Black line represents the negative control.

*Flow cytometry*

CHO-K1 and CHO/mCCR2 cells were harvested after brief exposure to 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). CHO-K1, CHO/mCCR2, and L1210 cells were washed with 0.1% BSA in PBS and treated with primary mAbs for 30 minutes at 4°C. Afterward, cells were treated with Alexa Fluor 488-conjugated anti-rat IgG (1:1000) or Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000) as well, following the collection of fluorescence data, using a SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

*Determination of the binding affinity by flow cytometry*

CHO/mCCR2 and L1210 were suspended in 100 $\mu$L serially diluted anti-mCCR2 mAbs, after which Alexa Fluor 488-conjugated anti-rat IgG (1:200) or Alexa Fluor 488-conjugated anti-rabbit IgG (1:200) were added. Fluorescence data were subsequently collected, using BD FACSLyric (BD Biosciences), following calculation of the dissociation constant ($K_D$) by fitting the binding isotherms into the built-in; one-site binding model in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

## Results

*Establishment of anti-mCCR2 mAbs*

To develop anti-mCCR2 mAbs, we used the mCCR2 peptide immunization method. Hybridoma screening was conducted using ELISA and flow cytometry (Fig. 1). A rat was immunized with three kinds of mCCR2 synthetic peptides. Subsequently, hybridomas were seeded into 96-well plates, after which ELISA was used to extract positive wells for mCCR2 peptides, followed by the selection of CHO/mCCR2-reactive and CHO-K1-nonreactive supernatants using flow cytometry. Afterward, we obtained CHO/mCCR2-reactive supernatants in 3 of 1916 wells (0.16%), following the establishment of $C_2$Mab-6 (rat $IgG_1$, kappa) after cloning by limiting dilution.

*Flow cytometry*

Flow cytometry was conducted using $C_2$Mab-6 against CHO/mCCR2, CHO-K1, and L1210. Results showed that $C_2$Mab-6 dose-dependently recognized CHO/mCCR2 (Fig. 2A), but not CHO-K1 (Fig. 2B). Another anti-mCCR2 mAb (clone EPR20844 from Abcam) also dose-dependently recognized CHO/mCCR2 (Fig. 2A), but not CHO-K1 (Fig. 2B). However, both $C_2$Mab-6 and EPR20844 reacted with CHO/mCCR2, even at 0.01 $\mu$g/mL concentration (Fig. 2A). Both $C_2$Mab-6 and EPR20844 also reacted dose-dependently with endogenous mCCR2-expressing L1210 (Fig. 2C).

*Determination of the binding affinity of $C_2$Mab-6*

The binding affinity of $C_2$Mab-6 was assessed with CHO/mCCR2 and L1210, using flow cytometry. Results showed that the $K_D$ of $C_2$Mab-6 for CHO/mCCR2 and L1210 were $1.6\times10^{-9}$ and $1.8\times10^{-10}$ M, respectively (Fig. 3). In contrast, the $K_D$ of the commercially available anti-mCCR2 mAb (clone EPR20844) for CHO/mCCR2 and L1210 were $7.6\times10^{-9}$ and $3.3\times10^{-9}$ M, respectively (Fig. 4). These results therefore indicate that $C_2$Mab-6 possesses a high affinity for CHO/mCCR2 and L1210 cells.

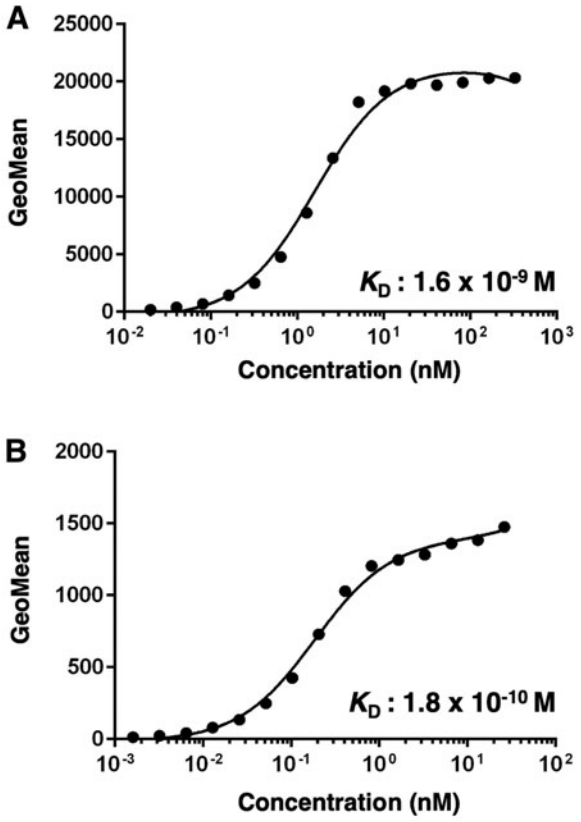


**FIG. 3.** The determination of the binding affinity of $C_2$Mab-6. CHO/mCCR2 **(A)** or L1210 **(B)** cells were suspended in 100 µL serially diluted $C_2$Mab-6 (25–0.0015 µg/mL for CHO/mCCR2 and 2–0.0001 µg/mL for L1210). Then, cells were treated with Alexa Fluor 488-conjugated anti-rat IgG. Fluorescence data were subsequently collected using a BD FACSLyric, following the calculation of the dissociation constant ($K_D$) by GraphPad PRISM 8.

## Discussion

It has been reported that the development of therapeutic agents targeting the CCL2-CCR2 axis can be effective for treating or managing various diseases.[45] Nonetheless, anti-GPCR mAbs are difficult to develop, due to problems with folded-protein structures, small epitope regions, and purification of functional antigens.[45] Therefore, we have tried developing various anti-GPCR mAbs, including anti-mouse CCR3,[42] anti-mouse CCR8,[43] and anti-human CCR9[44] mAbs, using the CBIS method. However, we have not established the production of anti-GPCR mAbs, using synthetic peptide immunization. Thus, in this study, we developed a novel anti-mCCR2 mAb ($C_2$Mab-6) through peptide immunization. The $K_D$ of $C_2$Mab-6 for CHO/mCCR2 and L1210 were $1.6\times10^{-9}$ and $1.8\times10^{-10}$ M, respectively, indicating that $C_2$Mab-6 possesses a high affinity against mCCR2-expressing cells. We demonstrated peptide immunization of the GPCR N-terminal region as the powerful method for establishing high-affinity mAbs.

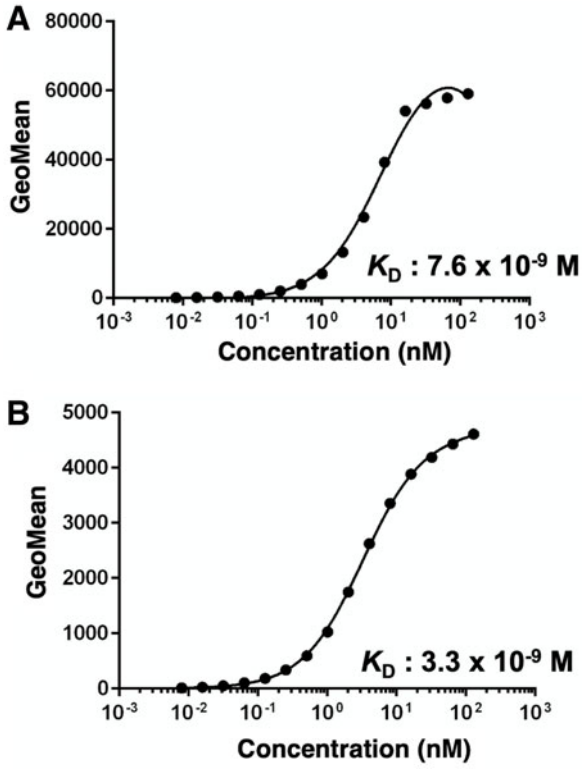


**FIG. 4.** The determination of the binding affinity of EPR20844. CHO/mCCR2 **(A)** or L1210 **(B)** cells were suspended in 100 μL serially diluted EPR20844 (10–0.0006 μg/mL). Cells were then treated with Alexa Fluor 488-conjugated anti-rabbit IgG. Fluorescence data were subsequently collected using a BD FACSLyric, following the calculation of the dissociation constant ($K_D$) by GraphPad PRISM 8.

In the tumor microenvironment (TME), CCR2 has been focused on as an immunoregulatory molecule. Tumor-secreted CCL2 recruits CCR2-expressing monocytes into the tumor, where it differentiates into M2 macrophages, known as tumor-associated macrophages (TAM).[10,13] Previously, in an esophageal squamous cell carcinoma (ESCC) cohort study, CCL2 expression and TAM accumulation became poor prognostic factors.[46] In breast and pancreatic cancers, high serum CCL2 levels were associated with malignancy.[47,48] In addition, studies have shown that the CCL2-CCR2 axis contributes to the construction of TMEs for immune evasion by recruiting immunosuppressive cells, such as $CCR2^+$ myeloid-derived suppressor cells, Th2 cells, and regulatory T cells. Besides, deleting CCR2 suppresses PD-1 expression in cytotoxic T lymphocytes.

CCL2 expression was associated with PD-1-related gene signatures in patients with ESCC.[46] Moreover, as a therapeutic approach, the CCR2 antagonist enhanced the effect of anti-PD-1 therapy on bladder cancer[49] and glioma.[50] Thus, it is of interest to evaluate the neutralizing activity of $C_2$Mab-6 for clinical applications in immune therapy. Recently, evaluating the tumor and tumor immune microenvironment was proposed to be essential for cancer diagnosis.[51] Nevertheless, further evaluation should apply $C_2$Mab-6 to immunohistochemistry for cancer diagnosis.

In our previous studies, we changed the isotype of mAbs into mouse $IgG_{2a}$ to retain the antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which led to high antitumor activities in mouse xenograft models.[52–54] However, since the subclass of $C_2$Mab-6 was rat $IgG_1$, it did not possess ADCC and CDC. Therefore, in further studies, we will convert the subclass of $C_2$Mab-6 into mouse $IgG_{2a}$ to evaluate whether the depletion of CCR2-expressing immunosuppressive cells can enhance antitumor activities in mouse syngeneic and xenograft models of human cancers.

### 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 numbers: JP21am0401013 (to Yukinari Kato) and JP21am0101078 (to Yukinari Kato).

### References

1. Luther SA, and Cyster JG: Chemokines as regulators of T cell differentiation. Nat Immunol 2001;2:102–107.
2. Sokol CL, and Luster AD: The chemokine system in innate immunity. Cold Spring Harb Perspect Biol 2015;7:a016303.
3. Nagarsheth N, Wicha MS, and Zou W: Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. Nat Rev Immunol 2017;17:559–572.
4. Balkwill F: Cancer and the chemokine network. Nat Rev Cancer 2004;4:540–550.
5. Hughes CE, and Nibbs RJB: A guide to chemokines and their receptors. FEBS J 2018;285:2944–2971.
6. Schöneberg T, and Liebscher I: Mutations in G protein-coupled receptors: Mechanisms, pathophysiology and potential therapeutic approaches. Pharmacol Rev 2021;73:89–119.
7. Bianconi V, Sahebkar A, Atkin SL, and Pirro M: The regulation and importance of monocyte chemoattractant protein-1. Curr Opin Hematol 2018;25:44–51.
8. Do HTT, Lee CH, and Cho J: Chemokines and their receptors: Multifaceted roles in cancer progression and potential value as cancer prognostic markers. Cancers (Basel) 2020;12:287.
9. Lim SY, Yuzhalin AE, Gordon-Weeks AN, and Muschel RJ: Targeting the CCL2-CCR2 signaling axis in cancer metastasis. Oncotarget 2016;7:28697–28710.
10. Xu M, Wang Y, Xia R, Wei Y, and Wei X: Role of the CCL2-CCR2 signalling axis in cancer: Mechanisms and therapeutic targeting. Cell Prolif 2021;54:e13115.
11. Kadomoto S, Izumi K, and Mizokami A: Roles of CCL2-CCR2 axis in the tumor microenvironment. Int J Mol Sci 2021;22:8530.
12. Hao Q, Vadgama JV, and Wang P: CCL2/CCR2 signaling in cancer pathogenesis. Cell Commun Signal 2020;18:82.
13. Urbantat RM, Vajkoczy P, and Brandenburg S: Advances in chemokine signaling pathways as therapeutic targets in glioblastoma. Cancers (Basel) 2021;13:2983.
14. Moadab F, Khorramdelazad H, and Abbasifard M: Role of CCL2/CCR2 axis in the immunopathogenesis of rheumatoid arthritis: Latest evidence and therapeutic approaches. Life Sci 2021;269:119034.

15. Cuypers F, Schäfer A, Skorka SB, Surabhi S, Tölken LA, Paulikat AD, Kohler TP, Otto SA, Mettenleiter TC, Hammerschmidt S, Blohm U, and Siemens N: Innate immune responses at the asymptomatic stage of influenza A viral infections of *Streptococcus pneumoniae* colonized and non-colonized mice. Sci Rep 2021;11:20609.
16. Midavaine É, Côté J, and Sarret P: The multifaceted roles of the chemokines CCL2 and CXCL12 in osteophilic metastatic cancers. Cancer Metastasis Rev 2021;40:427–445.
17. Loberg RD, Day LL, Harwood J, Ying C, St John LN, Giles R, Neeley CK, and Pienta KJ: CCL2 is a potent regulator of prostate cancer cell migration and proliferation. Neoplasia 2006;8:578–586.
18. Yamada S, Kaneko MK, Sayama Y, Asano T, Sano M, Yanaka M, Nakamura T, Okamoto S, Handa S, Komatsu Y, Nakamura Y, Furusawa Y, Takei J, and Kato Y: Development of novel mouse monoclonal antibodies against human CD19. Monoclon Antib Immunodiagn Immunother 2020;39:45–50.
19. Furusawa Y, Kaneko MK, and Kato Y: Establishment of C20Mab-11, a novel anti-CD20 monoclonal antibody, for the detection of B cells. Oncol Lett 2020;20:1961–1967.
20. Furusawa Y, Kaneko MK, and Kato Y: Establishment of an anti-CD20 monoclonal antibody (C20Mab-60) for immunohistochemical analyses. Monoclon Antib Immunodiagn Immunother 2020;39:112–116.
21. Yamada S, Itai S, Nakamura T, Yanaka M, Kaneko MK, and Kato Y: Detection of high CD44 expression in oral cancers using the novel monoclonal antibody, C44Mab-5. Biochem Biophys Rep 2018;14:64–68.
22. Itai S, Fujii Y, Nakamura T, Chang YW, Yanaka M, Saidoh N, Handa S, Suzuki H, Harada H, Yamada S, Kaneko MK, and Kato Y: Establishment of CMab-43, a sensitive and specific anti-CD133 monoclonal antibody, for immunohistochemistry. Monoclon Antib Immunodiagn Immunother 2017;36:231–235.
23. Hosono H, Ohishi T, Takei J, Asano T, Sayama Y, Kawada M, Kaneko MK, and Kato Y: The anti-epithelial cell adhesion molecule (EpCAM) monoclonal antibody EpMab-16 exerts antitumor activity in a mouse model of colorectal adenocarcinoma. Oncol Lett 2020;20:383.
24. Kaneko MK, Ohishi T, Takei J, Sano M, Nakamura T, Hosono H, Yanaka M, Asano T, Sayama Y, Harada H, Kawada M, and Kato Y: AntiEpCAM monoclonal antibody exerts antitumor activity against oral squamous cell carcinomas. Oncol Rep 2020;44:2517–2526.
25. Asano T, Ohishi T, Takei J, Nakamura T, Nanamiya R, Hosono H, Tanaka T, Sano M, Harada H, Kawada M, Kaneko MK, and Kato Y: AntiHER3 monoclonal antibody exerts antitumor activity in a mouse model of colorectal adenocarcinoma. Oncol Rep 2021;46:173.
26. Asano T, Nanamiya R, Tanaka T, Kaneko MK, and Kato Y: Development of antihuman killer cell lectin-like receptor subfamily G member 1 monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:76–80.
27. Takei J, Asano T, Nanamiya R, Nakamura T, Yanaka M, Hosono H, Tanaka T, Sano M, Kaneko MK, Harada H, and Kato Y: Development of anti-human T cell immunoreceptor with Ig and ITIM domains (TIGIT) monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:71–75.
28. Sayama Y, Kaneko MK, and Kato Y: Development and characterization of TrMab6, a novel antiTROP2 monoclonal antibody for antigen detection in breast cancer. Mol Med Rep 2021;23:92.
29. Sayama Y, Kaneko MK, Takei J, Hosono H, Sano M, Asano T, and Kato Y: Establishment of a novel anti-TROP2 monoclonal antibody TrMab-29 for immunohistochemical analysis. Biochem Biophys Rep 2021;25:100902.
30. Yamada S, Itai S, Nakamura T, Yanaka M, Chang YW, Suzuki H, Kaneko MK, and Kato Y: Monoclonal antibody L1Mab-13 detected human PD-L1 in lung cancers. Monoclon Antib Immunodiagn Immunother 2018;37:110–115.
31. Hosono H, Asano T, Takei J, Sano M, Tanaka T, Kaneko MK, and Kato Y: Development of an anti-elephant podoplanin monoclonal antibody PMab-265 for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:141–145.
32. Tanaka T, Asano T, Sano M, Takei J, Hosono H, Nanamiya R, Nakamura T, Yanaka M, Harada H, Fukui M, Suzuki H, Uchida K, Nakagawa T, Kato Y, and Kaneko MK: Development of monoclonal antibody PMab-269 against California Sea Lion podoplanin. Monoclon Antib Immunodiagn Immunother 2021;40:124–133.
33. Kato Y, Furusawa Y, Sano M, Takei J, Nakamura T, Yanaka M, Okamoto S, Handa S, Komatsu Y, Asano T, Sayama Y, and Kaneko MK: Development of an anti-sheep podoplanin monoclonal antibody PMab-256 for immunohistochemical analysis of lymphatic endothelial cells. Monoclon Antib Immunodiagn Immunother 2020;39:82–90.
34. Kato Y, Furusawa Y, Itai S, Takei J, Nakamura T, Sano M, Harada H, Yamada S, and Kaneko MK: Establishment of an anticetacean podoplanin monoclonal antibody PMab-237 for immunohistochemical analysis. Monoclon Antib Immunodiagn Immunother 2019;38:108–113.
35. Furusawa Y, Takei J, Sayama Y, Yamada S, Kaneko MK, and Kato Y: Development of an anti-bear podoplanin monoclonal antibody PMab-247 for immunohistochemical analysis. Biochem Biophys Rep 2019;18:100644.
36. Furusawa Y, Yamada S, Itai S, Nakamura T, Takei J, Sano M, Harada H, Fukui M, Kaneko MK, and Kato Y: Establishment of a monoclonal antibody PMab-233 for immunohistochemical analysis against Tasmanian devil podoplanin. Biochem Biophys Rep 2019;18:100631.
37. Kato Y, Furusawa Y, Yamada S, Itai S, Takei J, Sano M, and Kaneko MK: Establishment of a monoclonal antibody PMab-225 against alpaca podoplanin for immunohistochemical analyses. Biochem Biophys Rep 2019;18:100633.
38. Furusawa Y, Yamada S, Nakamura T, Sano M, Sayama Y, Itai S, Takei J, Harada H, Fukui M, Kaneko MK, and Kato Y: PMab-235: A monoclonal antibody for immunohistochemical analysis against goat podoplanin. Heliyon 2019;5:e02063.
39. Kato Y, Yamada S, Furusawa Y, Itai S, Nakamura T, Yanaka M, Sano M, Harada H, Fukui M, and Kaneko MK: PMab-213: A monoclonal antibody for immunohistochemical analysis against pig podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38:18–24.
40. Furusawa Y, Yamada S, Itai S, Nakamura T, Yanaka M, Sano M, Harada H, Fukui M, Kaneko MK, and Kato Y: PMab-219: A monoclonal antibody for the immunohistochemical analysis of horse podoplanin. Biochem Biophys Rep 2019;18:100616.
41. Furusawa Y, Kaneko MK, Nakamura T, Itai S, Fukui M, Harada H, Yamada S, and Kato Y: Establishment of a monoclonal antibody PMab-231 for tiger podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38:89–95.

42. Asano T, Nanamiya R, Takei J, Nakamura T, Yanaka M, Hosono H, Tanaka T, Sano M, Kaneko MK, and Kato Y: Development of anti-mouse CC chemokine receptor 3 monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:107–112.
43. Tanaka T, Nanamiya R, Takei J, Nakamura T, Yanaka M, Hosono H, Sano M, Asano T, Kaneko MK, and Kato Y: Development of anti-mouse CC chemokine receptor 8 monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:65–70.
44. Nanamiya R, Takei J, Asano T, Tanaka T, Sano M, Nakamura T, Yanaka M, Hosono H, Kaneko MK, and Kato Y: Development of anti-human CC chemokine receptor 9 monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:101–106.
45. Jo M, and Jung ST: Engineering therapeutic antibodies targeting G-protein-coupled receptors. Exp Mol Med 2016; 48:e207.
46. Yang H, Zhang Q, Xu M, Wang L, Chen X, Feng Y, Li Y, Zhang X, Cui W, and Jia X: CCL2-CCR2 axis recruits tumor associated macrophages to induce immune evasion through PD-1 signaling in esophageal carcinogenesis. Mol Cancer 2020;19:41.
47. Sanford DE, Belt BA, Panni RZ, Mayer A, Deshpande AD, Carpenter D, Mitchem JB, Plambeck-Suess SM, Worley LA, Goetz BD, Wang-Gillam A, Eberlein TJ, Denardo DG, Goedegebuure SP, and Linehan DC: Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: A role for targeting the CCL2/CCR2 axis. Clin Cancer Res 2013;19:3404–3415.
48. Izumi K, Fang LY, Mizokami A, Namiki M, Li L, Lin WJ, and Chang C: Targeting the androgen receptor with siRNA promotes prostate cancer metastasis through enhanced macrophage recruitment via CCL2/CCR2-induced STAT3 activation. EMBO Mol Med 2013;5:1383–1401.
49. Tu MM, Abdel-Hafiz HA, Jones RT, Jean A, Hoff KJ, Duex JE, Chauca-Diaz A, Costello JC, Dancik GM, Tamburini BAJ, Czerniak B, Kaye J, and Theodorescu D: Inhibition of the CCL2 receptor, CCR2, enhances tumor response to immune checkpoint therapy. Commun Biol 2020;3:720.
50. Flores-Toro JA, Luo D, Gopinath A, Sarkisian MR, Campbell JJ, Charo IF, Singh R, Schall TJ, Datta M, Jain RK, Mitchell DA, and Harrison JK: CCR2 inhibition reduces tumor myeloid cells and unmasks a checkpoint inhibitor effect to slow progression of resistant murine gliomas. Proc Natl Acad Sci U S A 2020;117:1129–1138.
51. Wellenstein MD, and de Visser KE: Cancer-cell-intrinsic mechanisms shaping the tumor immune landscape. Immunity 2018;48:399–416.
52. Takei J, Kaneko MK, Ohishi T, Hosono H, Nakamura T, Yanaka M, Sano M, Asano T, Sayama Y, Kawada M, Harada H, and Kato Y: A defucosylated antiCD44 monoclonal antibody 5mG2af exerts antitumor effects in mouse xenograft models of oral squamous cell carcinoma. Oncol Rep 2020;44:1949–1960.
53. Itai S, Ohishi T, Kaneko MK, Yamada S, Abe S, Nakamura T, Yanaka M, Chang YW, Ohba SI, Nishioka Y, Kawada M, Harada H, and Kato Y: Anti-podocalyxin antibody exerts antitumor effects via antibody-dependent cellular cytotoxicity in mouse xenograft models of oral squamous cell carcinoma. Oncotarget 2018;9:22480–22497.
54. Hosono H, Takei J, Ohishi T, Sano M, Asano T, Sayama Y, Nakamura T, Yanaka M, Kawada M, Harada H, Kaneko MK, and Kato Y: AntiEGFR monoclonal antibody 134mG2a exerts antitumor effects in mouse xenograft models of oral squamous cell carcinoma. Int J Mol Med 2020;46:1443–1452.

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*Received: December 9, 2021*
*Accepted: January 25, 2022*