MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 41, Number 4, 2022

DOI: 10.1089/mab.2022.0001

Development of an Anti-human CCR2 Monoclonal Antibody (C_2Mab-9) by N-Terminal Peptide Immunization

Tomohiro Tanaka,[1] Guanjie Li,[2] Masaki Saito,[2] Hiroyuki Suzuki,[2] Teizo Asano,[1] Mika K. Kaneko,[1] and Yukinari Kato[1,2]

The CC chemokine receptor type-2 (CCR2) is one of the members of the G protein-coupled receptor superfamily, which are expressed on the cell surface of immune and tumor cells. CCR2 binds to the C-C motif chemokine ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1), which is produced by various cells, including tumor and immune-related cells. Therefore, the development of sensitive monoclonal antibodies (mAbs) for CCR2 has been desired for treatment and diagnosis. In this study, we established a specific anti-human CCR2 (hCCR2) mAb, C_2Mab-9 (mouse IgG_1, kappa), using the synthetic peptide immunization method. Flow cytometric and immunocytochemical results showed that C_2Mab-9 reacted with hCCR2-expressing U937 (human histiocytic lymphoma) and natural killer cells. Furthermore, C_2Mab-9 showed the moderate binding affinity for both cells. Conclusively, C_2Mab-9 can be a useful tool for analyzing hCCR2-related biological responses.

Keywords: CCR2, monoclonal antibody, flow cytometry

Introduction

G protein-coupled receptor (GPCR) is a seven-transmembrane chemokine receptor, which can trigger intracellular signals by binding its cognate chemokine ligand to influence various cellular functions.[1–3] Chemokines play important roles in immune responses, such as infiltration and migration of immune-related cells.[3,4] Depending on the number and position of the N-terminal cysteine residues, chemokines are further divided into four different subfamilies, namely CC, CXC, CX3C, and XC.[1,5]

The C-C motif chemokine ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1) is one of the C-C motif chemokine ligands. CCL2 is produced by fibroblasts, endothelial, epithelial, myeloid, and tumor cells.[5,6] In addition, CCL2 plays a crucial role in attracting monocytes, T lymphocytes, and natural killer (NK) cells.[7,8] Furthermore, CCL2 can activate several GPCRs, including the CC chemokine receptor type-2 (CCR2), CCR4, and CCR5.[9] Notably, the primary receptor of CCL2 is CCR2, whose expression has been observed in multiple cells, including monocytes, macrophages, dendritic cells, and epithelial cells.[10] The CCL2-attracted monocytes to the lung control the SARS-CoV-2 burden and the inflammatory response during infection.[11]

The CCL2–CCR2 axis has been reported in many diseases, such as immune disorders and cancers.[12,13] In cancers, high CCL2 expression influences cancer progression, angiogenesis, and metastasis.[7,14,15] CCL2 has been reported to be highly upregulated in bone tumors[15] and inflammatory breast cancer.[16] Furthermore, the invasive lesions of breast ductal carcinoma show high CCR2 and CCL2 expression.[17] Therefore, various CCR2-expressing cells are involved in disease pathogenesis by interacting with CCL2.

We have previously developed various monoclonal antibodies (mAbs) against membrane proteins, such as HER3,[18] EpCAM,[19] KLRG1,[20] TIGIT,[21] TROP2,[22,23] programmed cell death ligand 1 (PD-L1),[24] CD19,[25] CD20,[26,27] CD44,[28] CD133,[29] and podoplanin[30–37] by using the Cell-Based Immunization and Screening (CBIS) method. Moreover, we have also developed anti-GPCR mAbs against mouse CCR3,[38] mouse CCR8,[39] and human CCR9[40] using the CBIS method. In contrast, we have not established sensitive anti-GPCR mAbs, using synthetic peptide

Departments of [1]Antibody Drug Development and [2]Molecular Pharmacology, Tohoku University Graduate School of Medicine, Sendai, Japan.

immunization. In this study, we attempted to develop anti-human CCR2 (hCCR2) mAbs using synthetic peptide immunization.

Materials and Methods

Antibodies

The anti-hCCR2 mAb (clone K036C2) was purchased from BioLegend (San Diego, CA). The peroxidase-conjugated anti-mouse immunoglobulins was purchased from Agilent Technologies, Inc. (Santa Clara, CA). The Alexa Fluor 488-conjugated anti-mouse IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Cell lines

P3X63Ag8U.1 (P3U1) was obtained from the American Type Culture Collection (Manassas, VA). U937 (The human histiocytic lymphoma) was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The human NK cells (donor lot. 4022602, purity >70%) were purchased from Takara Bio (Shiga, Japan).

P3U1 and U937 cells 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., Waltham, MA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). Subsequently, cells were grown in a humidified incubator, which was supplied with 5% CO_2 and 95% air at 37°C.

Animals

Two 6-week-old female BALB/c mice were purchased from CLEA (Tokyo, Japan). Mice were housed under specific pathogen-free conditions. Afterward, 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 >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 was used to verify death.

Hybridoma production

Two BALB/c mice were immunized using three keyhole limpet hemocyanin (KLH)-conjugated hCCR2 peptides (100 μg of each peptide), including $_{1}$-MLSTSRSRFIRNTNESGEE-$_{19}$, $_{11}$-RNTNESGEEVTTFFDYDYG-$_{29}$, and $_{21}$-TTFFDYDYGAPSHKFDVKQ-$_{39}$ (the 32nd Cys was changed to Ser in the 3rd peptide) + C-terminal cysteine. The administration was conducted through the intraperitoneal route with an Imject Alum Adjuvant (Thermo Fisher Scientific, Inc.). The procedure included three additional immunization procedures (100 μg of each peptide), followed by a final booster intraperitoneal injection (100 μg of each peptide) 2 days before their spleen cells were harvested.

The harvested spleen cells were subsequently fused with P3U1 cells, using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN). Thereafter, hybridomas were grown in an RPMI 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.

ELISA

A mixture of synthesized hCCR2 peptides, including $_{1}$-MLSTSRSRFIRNTNESGEE-$_{19}$, $_{11}$-RNTNESGEEVTTFFDYDYG-$_{29}$, and $_{21}$-TTFFDYDYGAPSHKFDVKQ-$_{39}$ + C-terminal cysteine, was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at a concentration of 1 μg/mL for each peptide and a temperature of 37°C for 30 minutes. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween20 (PBST;

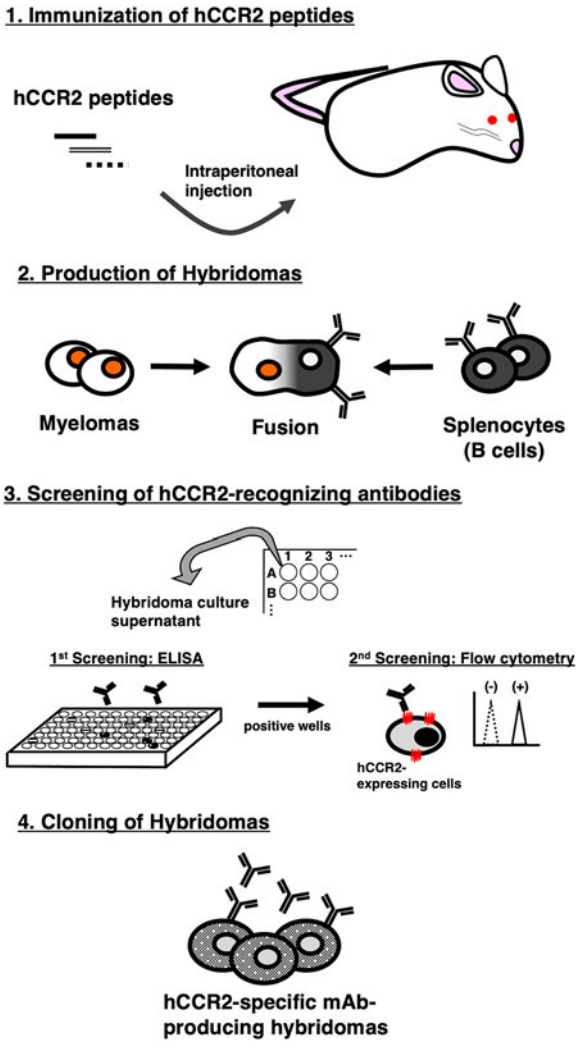

FIG. 1. A diagram showing the production of anti-hCCR2 mAbs. The mice were intraperitoneally immunized with hCCR2 peptides. Screening of hybridoma was then conducted by ELISA, followed by flow cytometry using hCCR2-expressing cells. ELISA, enzyme-linked immunosorbent assay; hCCR2, human CC chemokine receptor type-2; mAbs, monoclonal antibodies.

Nacalai Tesque, Inc.), wells were blocked with 1% bovine serum albumin (BSA)-containing PBST for 30 minutes at 37°C.

Subsequently, plates were incubated with culture supernatants, followed by peroxidase-conjugated anti-mouse immunoglobulins (1:2000 diluted). 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

U937 and human NK cells were washed with 0.1% BSA in PBS and treated with primary mAbs for 30 minutes at 4°C. Thereafter, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000), followed by the collection of fluorescence data using an SA3800 cell analyzer (Sony Corp., Tokyo, Japan).

Determination of the binding affinity by flow cytometry

U937 and human NK cells were suspended in 100 μL serially diluted anti-hCCR2 mAbs, after which Alexa Fluor 488-conjugated anti-mouse IgG (1:200) was added. Fluorescence data were subsequently collected using BD FACSLyric (BD Biosciences), followed by the calculation of the dissociation constant (K_D) through fitting binding isotherms to the built-in one-site binding models in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

Immunocytochemistry

U937 and human NK cells were centrifuged at 270×*g* for 5 minutes and the cell pellets were suspended with 4% paraformaldehyde in PBS for 10 minutes before the quenching in 50 mM NH_4Cl in PBSc/m (PBS supplemented with 0.2 mM Ca^{2+} and 2 mM Mg^{2+}) for 10 minutes. The cells were further incubated in the blocking buffer (0.5% BSA in PBSc/m) for 30 minutes, then in the primary mAbs (10 μg/mL) for 2 hours, and subsequently in an additional Alexa Fluor 488-conjugated anti-mouse IgG (1:400) and 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Inc.) for 45 minutes. Lastly, the cells were mounted using ProLong Glass Antifade Mountant. The fluorescent images were acquired using a digital microscope (BZ-X800; Keyence, Osaka, Japan) with a 40× objective.

Results

Establishment of anti-hCCR2 mAbs

We first immunized mice with the hCCR2 peptides (Fig. 1). Hybridomas were seeded into 96-well plates, after which ELISA was used to select positive wells for hCCR2 peptides, followed by the selection of U937 and NK cells-reactive supernatants using flow cytometry. We obtained reactive supernatants in 16 out of the 956 wells (1.67%) and finally established C_2Mab-9 (mouse IgG_1, kappa) after cloning by the limiting dilution.

Flow cytometry

Flow cytometry was performed using C_2Mab-9 against U937 and NK cells. Results showed that C_2Mab-9 recognized

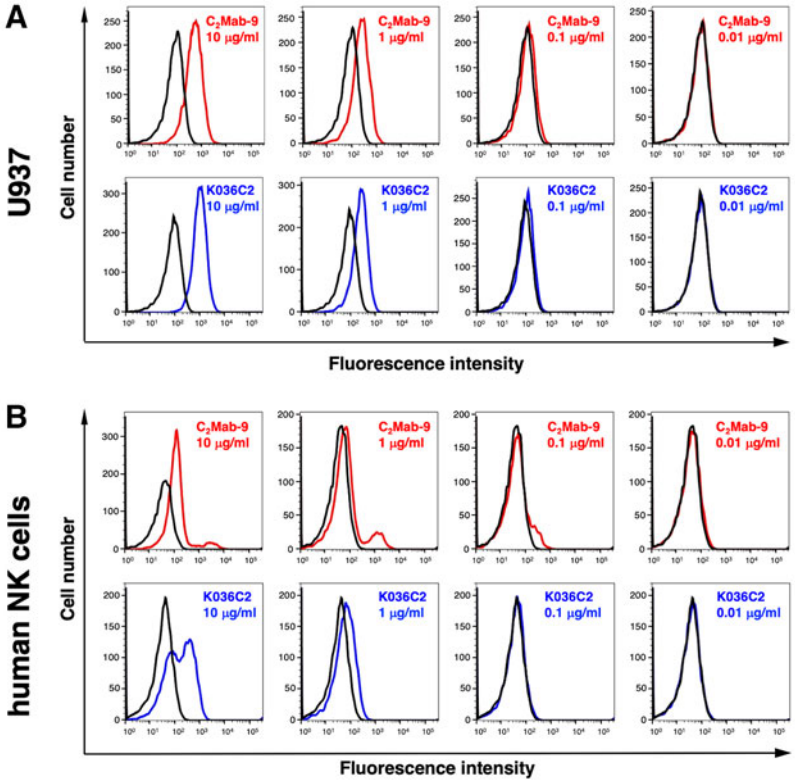

FIG. 2. Flow cytometry results using anti-hCCR2 mAbs. U937 (human histiocytic lymphoma) cells **(A)** and human NK cells **(B)** were treated with 0.01–10 μg/mL of C_2Mab-9 and K036C2, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. The black line represents the negative control. NK, natural killer.

U937 (Fig. 2A) and NK cells (Fig. 2B) in a dose-dependent manner. Another anti-hCCR2 mAb (clone K036C2) also recognized U937 (Fig. 2A) and NK cells (Fig. 2B) dose-dependently.

Determination of the binding affinity of C_2Mab-9

The binding affinity of C_2Mab-9 was assessed with U937 and NK cells based on flow cytometry. Results showed that the K_D of C_2Mab-9 for U937 and NK cells was 2.9×10^{-8} M and 7.9×10^{-8} M, respectively (Supplementary Fig. S1). These results indicated that C_2Mab-9 possesses moderate affinities for U937 and NK cells.

Immunocytochemistry

Immunocytochemistry was performed using C_2Mab-9 against U937 and NK cells. Results showed that C_2Mab-9 and anti-hCCR2 mAb (clone K036C2), but not buffer control, were bound to U937 and NK cells (Fig. 3), indicating that C_2Mab-9 specifically recognizes endogenous hCCR2 in immunocytochemistry.

Discussion

The GPCRs possess various functions in normal and pathological conditions and they have been focused on as pivotal drug targets.[41] Although it is scientifically well known to be difficult to develop anti-GPCR mAbs,[42] we have successfully established various anti-GPCR mAbs, including anti-mouse CCR3 mAbs,[38] mouse CCR8 mAbs,[39] and human CCR9 mAbs[40] using the CBIS method. In this study, we developed a novel anti-hCCR2 mAb (C_2Mab-9) through peptide immunization. The K_D of C_2Mab-9 for U937 and NK cells was 2.9×10^{-8} M and 7.9×10^{-8} M, respectively, indicating that C_2Mab-9 possesses a moderate affinity against endogenous hCCR2-expressing cells.

In the tumor microenvironment (TME), CCR2 has an important function as an immunoregulatory molecule.[9,13,43] CCL2 expression was associated with PD-1–related gene signatures in patients with esophageal squamous cell carcinoma.[43] The tumor-secreted CCL2 recruits CCR2-expressing monocytes into the tumor, where they differentiate into M2 macrophages, known as tumor-associated macrophages

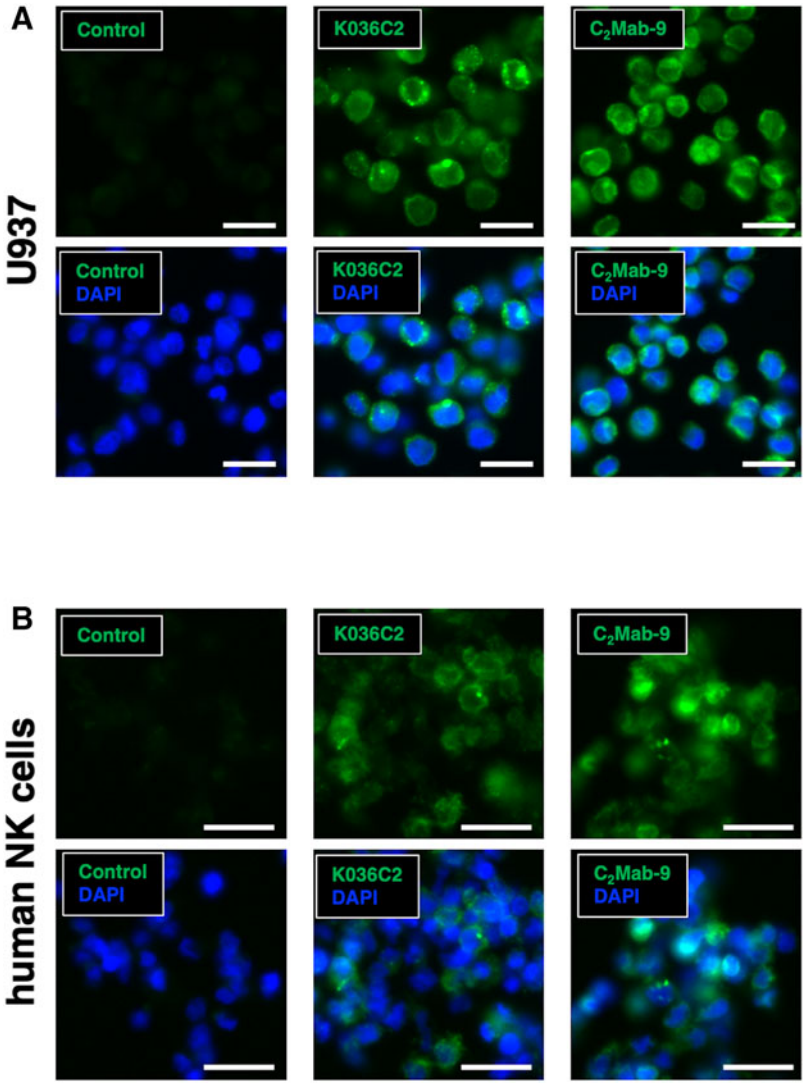

FIG. 3. Immunocytochemistry using anti-hCCR2 mAbs. U937 cells **(A)** and NK cells **(B)** were treated with control, K036C2 (10 μg/mL), and C_2Mab-9 (10 μg/mL), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG and DAPI. Scale bars: 20 μm. DAPI, 4′,6-diamidino-2-phenylindole.

(TAMs). TAMs express cytokines and chemokines that can suppress antitumor immunity and promote tumor progression.[5] In brain tumors, CCL2–CCR2 signaling triggers the infiltration of TAM and regulatory T cells, which contributes the formation of immunosuppressive TME.[44–46]

In addition, interactions between CCL2–CCR2 have been shown to recruit immunosuppressive cells such as $CCR2^+$ myeloid-derived suppressor cells[47] and metastasis-promoting monocytes.[48] In anti-PD-1–resistant glioma-bearing mice model, CCR2 deficiency unmasked an anti-PD-1 effect and enhanced the survival. Furthermore, the CCR2 antagonist (CCX872) enhanced the effect of anti-PD-1 therapy in the glioma.[49] Therefore, it is important to evaluate the neutralizing activity of C_2Mab-9 in the future.

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.), JP22am0401013 (to Y.K.), JP22bm1004001 (to Y.K.), JP22ck0106730 (to Y.K.), and JP21am0101078 (to Y.K.).

Supplementary Material

Supplementary Figure S1

References

1. Hughes CE, and Nibbs RJB: A guide to chemokines and their receptors. FEBS J 2018;285:2944–2971.
2. 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.
3. Sokol CL, and Luster AD: The chemokine system in innate immunity. Cold Spring Harb Perspect Biol 2015;7:a016303.
4. Luther SA, and Cyster JG: Chemokines as regulators of T cell differentiation. Nat Immunol 2001;2:102–107.
5. Urbantat RM, Vajkoczy P, and Brandenburg S: Advances in chemokine signaling pathways as therapeutic targets in glioblastoma. Cancers (Basel) 2021;13:2983.
6. 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.
7. Lim SY, Yuzhalin AE, Gordon-Weeks AN, and Muschel RJ: Targeting the CCL2-CCR2 signaling axis in cancer metastasis. Oncotarget 2016;7:28697–28710.
8. O'Connor T, and Heikenwalder M: CCL2 in the tumor microenvironment. Adv Exp Med Biol 2021;1302:1–14.
9. 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.
10. Kadomoto S, Izumi K, and Mizokami A: Roles of CCL2-CCR2 axis in the tumor microenvironment. Int J Mol Sci 2021;22:8530.
11. Vanderheiden A, Thomas J, Soung AL, Davis-Gardner ME, Floyd K, Jin F, Cowan DA, Pellegrini K, Shi PY, Grakoui A, Klein RS, Bosinger SE, Kohlmeier JE, Menachery VD, and Suthar MS: CCR2 signaling restricts SARS-CoV-2 infection. mBio 2021;12:e0274921.
12. Hao Q, Vadgama JV, and Wang P: CCL2/CCR2 signaling in cancer pathogenesis. Cell Commun Signal 2020;18:82.
13. Talbot J, Bianchini FJ, Nascimento DC, Oliveira RD, Souto FO, Pinto LG, Peres RS, Silva JR, Almeida SC, Louzada-Junior P, Cunha TM, Cunha FQ, and Alves-Filho JC: CCR2 expression in neutrophils plays a critical role in their migration into the joints in rheumatoid arthritis. Arthritis Rheumatol 2015;67:1751–1759.
14. 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.
15. 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.
16. Rogic A, Pant I, Grumolato L, Fernandez-Rodriguez R, Edwards A, Das S, Sun A, Yao S, Qiao R, Jaffer S, Sachidanandam R, Akturk G, Karlic R, Skobe M, and Aaronson SA: High endogenous CCL2 expression promotes the aggressive phenotype of human inflammatory breast cancer. Nat Commun 2021;12:6889.
17. Fang WB, Sofia Acevedo D, Smart C, Zinda B, Alissa N, Warren K, Fraga G, Huang LC, Shyr Y, Li W, Xie L, Staggs V, Hong Y, Behbod F, and Cheng N: Expression of CCL2/CCR2 signaling proteins in breast carcinoma cells is associated with invasive progression. Sci Rep 2021;11:8708.
18. 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: Anti-HER3 monoclonal antibody exerts antitumor activity in a mouse model of colorectal adenocarcinoma. Oncol Rep 2021;46:173.
19. 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.
20. 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.
21. 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.
22. Sayama Y, Kaneko MK, and Kato Y: Development and characterization of TrMab-6, a novel anti-TROP2 monoclonal antibody for antigen detection in breast cancer. Mol Med Rep 2021;23:92.
23. 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.
24. Yamada S, Itai S, Nakamura T, Yanaka M, Chang YW, Suzuki H, Kaneko MK, and Kato Y: Monoclonal antibody L(1)Mab-13 detected human PD-L1 in lung cancers. Monoclon Antib Immunodiagn Immunother 2018;37:110–115.
25. 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.

26. Furusawa Y, Kaneko MK, and Kato Y: Establishment of C(20)Mab-11, a novel anti-CD20 monoclonal antibody, for the detection of B cells. Oncol Lett 2020;20:1961–1967.
27. Furusawa Y, Kaneko MK, and Kato Y: Establishment of an anti-CD20 monoclonal antibody (C(20)Mab-60) for immunohistochemical analyses. Monoclon Antib Immunodiagn Immunother 2020;39:112–116.
28. 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, C(44)Mab-5. Biochem Biophys Rep 2018;14:64–68.
29. 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.
30. 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.
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. 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.
33. Oki H, Honma R, Ogasawara S, Fujii Y, Liu X, Takagi M, Kaneko MK, and Kato Y: Development of sensitive monoclonal antibody PMab-2 against rat podoplanin. Monoclon Antib Immunodiagn Immunother 2015;34:396–403.
34. 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.
35. Yamada S, Ogasawara S, Kaneko MK, and Kato Y: LpMab-23: a cancer-specific monoclonal antibody against human podoplanin. Monoclon Antib Immunodiagn Immunother 2017;36:72–76.
36. Honma R, Fujii Y, Ogasawara S, Oki H, Liu X, Nakamura T, Kaneko MK, Takagi M, and Kato Y: Establishment of novel monoclonal antibody PMab-32 against rabbit podoplanin. Monoclon Antib Immunodiagn Immunother 2016;35:41–47.
37. Yamada S, Itai S, Nakamura T, Yanaka M, Saidoh N, Chang YW, Handa S, Harada H, Kagawa Y, Ichii O, Konnai S, Kaneko MK, and Kato Y: PMab-52: specific and sensitive monoclonal antibody against cat podoplanin for immunohistochemistry. Monoclon Antib Immunodiagn Immunother 2017;36:224–230.
38. 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.
39. 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.
40. 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.
41. Schöneberg T, and Liebscher I: Mutations in G protein-coupled receptors: mechanisms, pathophysiology and potential therapeutic approaches. Pharmacol Rev 2021;73:89–119.
42. Jo M, and Jung ST: Engineering therapeutic antibodies targeting G-protein-coupled receptors. Exp Mol Med 2016;48:e207.
43. 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.
44. Panek WK, Pituch KC, Miska J, Kim JW, Rashidi A, Kanojia D, Lopez-Rosas A, Han Y, Yu D, Chang CL, Kane JR, Zhang P, Cordero A, and Lesniak MS: Local application of autologous platelet-rich fibrin patch (PRF-P) suppresses regulatory T cell recruitment in a murine glioma model. Mol Neurobiol 2019;56:5032–5040.
45. Ye XZ, Xu SL, Xin YH, Yu SC, Ping YF, Chen L, Xiao HL, Wang B, Yi L, Wang QL, Jiang XF, Yang L, Zhang P, Qian C, Cui YH, Zhang X, and Bian XW: Tumor-associated microglia/macrophages enhance the invasion of glioma stem-like cells via TGF-β1 signaling pathway. J Immunol 2012;189:444–453.
46. Crane CA, Ahn BJ, Han SJ, and Parsa AT: Soluble factors secreted by glioblastoma cell lines facilitate recruitment, survival, and expansion of regulatory T cells: implications for immunotherapy. Neuro Oncol 2012;14:584–595.
47. Huang B, Lei Z, Zhao J, Gong W, Liu J, Chen Z, Liu Y, Li D, Yuan Y, Zhang GM, and Feng ZH: CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers. Cancer Lett 2007;252:86–92.
48. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA, and Pollard JW: CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 2011;475:222–225.
49. 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.

Address correspondence to:
Yukinari Kato
Department of Molecular Pharmacology
Tohoku University Graduate School of Medicine
2-1, Seiryo-machi, Aoba-ku
Sendai
Miyagi 980-8575
Japan

E-mail: yukinari.kato.e6@tohoku.ac.jp

Received: January 9, 2022
Accepted: July 11, 2022