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Development of Anti-Mouse CC Chemokine Receptor 3 Monoclonal Antibodies for Flow Cytometry

Teizo Asano,[1] Ren Nanamiya,[1] Junko Takei,[1] Takuro Nakamura,[1] Miyuki Yanaka,[1] Hideki Hosono,[1] Tomohiro Tanaka,[1] Masato Sano,[1] Mika K. Kaneko,[1] and Yukinari Kato[1,2]

CC chemokine receptor 3 (CCR3), also known as CD193, belongs to class A of G protein-coupled receptors and is present in high levels in eosinophils, basophils, and airway epithelial cells. CCR3 is considered the therapeutic target for human immunodeficiency virus (HIV) infections and allergic diseases; therefore, the development of sensitive monoclonal antibodies (mAbs) for CCR3 has been desired. This study aimed to establish a specific and sensitive mAb against mouse CCR3 (mCCR3) useful for flow cytometry analysis by employing the Cell-Based Immunization and Screening (CBIS) method. The generated anti-mCCR3 mAb, C_3Mab-2 (rat IgG_{2b}, kappa), was found to react with mCCR3-overexpressed Chinese hamster ovary-K1 (CHO/mCCR3) cells, according to flow cytometric analysis. Also, it reacted with P388 (mouse lymphoid neoplasm) or J774-1 (mouse macrophage-like) cells, which express endogenous mCCR3. Taken together, C_3Mab-2, generated by the CBIS method, can be a valuable tool for detecting mCCR3 on the surface of mouse cells.

Keywords: CCR3, monoclonal antibody, CBIS, flow cytometry, allergic diseases

Introduction

Chemokines are small cytokines that induce cell movement in response to a chemokine gradient; they play an essential role in cell migration.[1] They also have a role in allergic inflammatory diseases, human immunodeficiency virus (HIV)-associated diseases, and cancers.[2,3] Chemokines are classified into four subfamilies: CXC, CC, CX3C, and XC. All chemokines bind to and activate G protein-coupled receptors (GPCRs).[1,4] Chemokine receptors are divided into four families that correspond to their ligand subfamilies: CXC chemokine receptor (CXCR), CC chemokine receptor (CCR), CX3C chemokine receptor, and XC chemokine receptor.

CC chemokine receptor 3 (CCR3), also known as CD193, is a receptor for the CC family of chemokines, such as eotaxin, MCP-3, and RANTES. CCR3, first cloned and identified as a murine[5] or human[6] eosinophil CCR, belongs to the class A of GPCR. CCR3 is present at high levels in eosinophils, basophils, and microglial cells, as well as in subsets of mast, Th2, and airway epithelial cells. It is also present in CD31-positive endothelial cells in the choroidal neovascularization membranes of eyes with wet age-related macular degeneration.[7–10] CCR3 supports HIV Env-mediated syncytia formation, and eotaxin as its ligand can reduce the efficiency of HIV-1 infection.[9,11,12] Moreover, CCR3 is important for recruiting eosinophils into the lung; thus, it can be used to induce airway hyper-responsiveness in a murine model of allergic asthma. CCR3 also contributes to ocular allergy and, therefore, is regarded as the therapeutic target for HIV and allergic diseases.[13–16]

The development of anti-CCR3-specific monoclonal antibodies (mAbs) is necessary for allergic disease, HIV therapy, and pathological diagnosis. In this study, we have successfully developed a novel anti-CCR3 mAb using the Cell-Based Immunization and Screening (CBIS) method.[17,18] We have also investigated the usefulness of anti-CCR3 mAb for flow cytometry analysis.

Materials and Methods

Cell lines

Chinese hamster ovary K1 (CHO-K1) and P3X63Ag8U.1 (P3U1) were supplied by the American Type Culture Collection (Manassas, VA). P388 (mouse lymphoid neoplasm) and J774-1 (mouse macrophage-like) cells were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). The synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding mouse CCR3 (mCCR3) (accession no. NM_009914.4) was subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka,

[1]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.
[2]New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.

Japan). The mCCR3 plasmid was transfected using the Neon Transfection System (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were identified using a cell sorter (SH800; Sony Corp., Tokyo, Japan) and cultured in a medium containing 0.5 mg/mL of zeocin (InvivoGen, San Diego, CA).

CHO-K1, P3U1, CHO/mCCR3, P388, and J774-1 were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated 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.) in a humidified incubator at 37°C with 5% CO_2.

Antibodies

An anti-mCCR3 mAb (clone J073E5) was purchased from BioLegend (San Diego, CA). The Alexa Fluor 488-conjugated anti-rat secondary IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Hybridoma production

One 6-week-old Sprague Dawley (SD) rat (CLEA Japan, Tokyo, Japan) was housed under specific pathogen-free conditions. All animal experiments were performed according to relevant guidelines and regulations to minimize animal suffering and distress. The animal experiments were approved by the Animal Care and Use Committee of Tohoku University (permit number: 2019NiA-001). The rat was monitored daily for health during the 4-week experiment. A reduction of >25% of the total body weight was defined as a humane end point. The rat was euthanized by cervical dislocation, and its death was verified by respiratory and cardiac arrest. We employed the absorption step-added CBIS (abCBIS) method to develop mAbs against mCCR3. In brief, an SD rat was immunized with 1×10^9 CHO/mCCR3 cells with the Imject Alum (Thermo Fisher Scientific, Inc.) by intraperitoneal injection. The procedure included three additional immunizations, followed by a final booster intraperitoneal injection administered 2 days before the harvest of spleen cells. The harvested spleen cells were subsequently fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The resultant hybridomas were grown in an RPMI medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). Culture supernatants were mixed with CHO-K1 cells for 30 minutes to absorb the antibodies that react to CHO-K1 cells. The supernatants were then collected for centrifugation and screened with CHO/mCCR3 cells by flow cytometry to identify the hybridomas producing mCCR3-specific mAbs.

Flow cytometry

The CHO/mCCR3 cells were harvested after a brief incubation with 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). They were then washed with 0.1% bovine serum albumin in phosphate-buffered saline, treated with primary mAbs for 30 minutes at 4°C, and incubated with Alexa Fluor 488-conjugated anti-rat IgG (1:2000; Cell Signaling Technology, Inc.). The fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp.).

Determination of antibody-binding affinity by flow cytometry

P388 and J774-1 were suspended in 100 μL of serially diluted anti-mCCR3 mAbs, and Alexa Fluor 488-conjugated anti-rat IgG (1:200; Cell Signaling Technology, Inc.) was added. The fluorescence data were collected using the BD FACSLyric (BD Biosciences, Franklin Lakes, NJ). The dissociation constant (K_D) was calculated by fitting binding isotherms to built-in one-site binding models in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

Results

Creation of anti-mCCR3 mAbs using the abCBIS method

We employed the abCBIS method to develop anti-mCCR3 mAbs and using the stable mCCR3 transfectants for both the immunization and flow cytometric screening (Fig. 1). In this study, one rat was immunized with CHO/mCCR3 cells overexpressing mCCR3 to develop anti-mCCR3 mAbs. The developed hybridomas were seeded into 96-well plates and cultivated for 6 days. The culture supernatants from the wells were mixed with CHO-K1 cells to absorb the CHO-K1-reacting antibodies. The supernatants were again collected after centrifugation.

Wells positive for CHO/mCCR3 were selected using flow cytometry. The first screening method observed the supernatant from 48 of the 956 wells with hybridomas (5.02%) displayed strong antibody-binding signals with the CHO/mCCR3 cells and weak or no signals with the CHO–K1 cells. Then, the 48 hybridoma clones were tested with P388 (lymphoid neoplasms) and J774-1 (macrophage-like cell lines) cells in the second screening. The supernatants from 5 of the 48 hybridoma culture (10.4%) exhibited robust antibody-binding signals with P388 and J774-1 cells. After limiting dilution and several additional screenings, an anti-mCCR3 mAb, C_3Mab-2 (rat IgG_{2b}, kappa), was finally established.

Flow cytometry analysis

We performed flow cytometry using C_3Mab-2 against CHO/mCCR3, P388, J774-1, and CHO-K1 cells. C_3Mab-2 recognized the CHO/mCCR3 cells in a dose-dependent manner (Fig. 2A), but not the parental CHO-K1 cells (Fig. 2B). Another anti-mCCR3 mAb (clone J073E5 from BioLegend), which acted as the positive control, also recognized CHO/mCCR3 in a dose-dependent manner (Fig. 2A), but not CHO-K1 (Fig. 2B). Although C_3Mab-2 reacted with CHO/mCCR3 cells at >0.1 μg/mL, J073E5 reacted with CHO/mCCR3 cells even at 0.01 μg/mL (Fig. 2A). C_3Mab-2 also detected the endogenous mCCR3 on P388 (Fig. 2C) and J774-1 cells (Fig. 2D) in a dose-dependent manner. These results suggest that C_3Mab-2 is specific for mCCR3 and is useful for detecting endogenous mCCR3 in flow cytometric analysis.

The reaction of C_3Mab-2 to CHO/mCCR3 was lost when trypsin was used for harvesting CHO/mCCR3 cells (data not shown), indicating that the epitope of C_3Mab-2 is trypsin-sensitive. In contrast, the reaction of J073E5 to CHO/mCCR3 was observed even when trypsin was used for harvesting CHO/mCCR3 cells (data not shown). These results indicate that the epitope of C_3Mab-2 is different from that of J073E5.

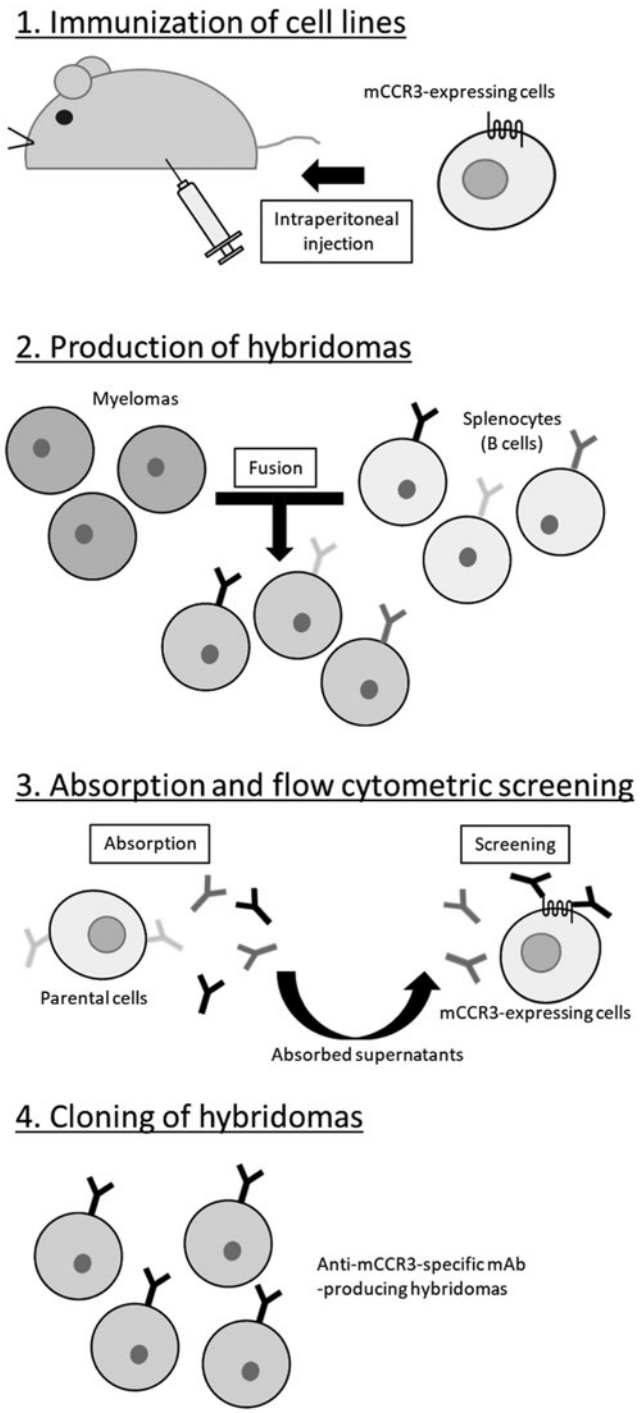

FIG. 1. Production of anti-mCCR3 mAbs. Procedure of abCBIS method. CHO/mCCR3 cells were immunized into SD rat using intraperitoneal injection. Screening was performed using flow cytometry. CBIS, Cell-Based Immunization and Screening; mAbs, monoclonal antibodies; CHO/mCCR3, Chinese hamster ovary/mouse CC chemokine receptor 3; SD, Sprague Dawley.

Determination of the binding affinity using C_3Mab-2 against P388 and J774-1 cells by flow cytometry

We conducted a kinetic analysis to assess the binding affinity of C_3Mab-2 to P388 and J774-1 cells using flow cytometry. The geometric mean of fluorescence intensity was plotted versus the concentrations of C_3Mab-2 and fitted by one-site binding models. The K_D of C_3Mab-2 for P388 and J774-1 was determined to be 2.1×10^{-7} M and 3.7×10^{-7} M, respectively, indicating the moderate affinity of C_3Mab-2 for mCCR3-expressing cells.

Discussion

Purified proteins and peptides are usually used as immunogens to generate antibodies. It takes much time and effort to collect purified proteins, especially membrane proteins. Although peptides are commercially available and easy to obtain, the mAbs generated by immunizing with peptides often do not recognize the native proteins.[19] Although many commercially available anti-mCCR3 polyclonal antibodies are useful in Western blot, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA), they may not be effective in flow cytometry. Of the seven anti-mCCR3 mAbs that we commercially obtained, only five are useful in flow cytometry (Supplementary Table S1). The CBIS method, which uses antigen-expressing cell lines for both immunization and screening, can help us effectively develop mAbs that are useful in flow cytometry. We recently have succeeded in developing numerous useful mAbs that target membrane proteins, including podoplanin,[20–23] CD20,[24] CD44,[25] and TROP2.[26]

In this study, we have used the modified CBIS method, abCBIS, and successfully generated a novel anti-mCCR3 mAb, C_3Mab-2, which is useful for flow cytometry. Grimaldi *et al*. previously reported that anti-mCCR3 mAbs were generated by immunizing rats with Y3/mCCR3 cells and screening with NIH3T3/mCCR3 and NIH3T3 cells.[27] Likewise, different kinds of cells, such as Y3 and NIH3T3, should be used for immunization and screening, respectively, because using parental cells for immunization may produce nonspecific antibodies that may hinder the screening for specific anti-mCCR3 antibodies. In this study, the same parental cells, such as CHO-K1 cells, were used for immunization and screening in the abCBIS method. We could obtain specific mAbs against targets because almost all the nonspecific antibodies were removed during the absorption step.

In this study, we successfully established a novel anti-mCCR3 mAb, C_3Mab-2, which is useful for flow cytometry using the abCBIS method. Notably, C_3Mab-2 reacted with endogenous mCCR3 in P388 and J774-1 cells in the same way as the J073E5 mAb (BioLegend). In the future study, we will determine whether C_3Mab-2 is useful for Western blot, immunohistochemistry, and ELISA because there is no anti-mCCR3 mAb that is useful in all the applications (Supplementary Table S1).

Chemokines and their receptors are attractive therapeutic targets; some drugs that target chemokine receptors have been launched. For example, Maraviroc is a CCR5 antagonist. CCR5 binds to gp120, which is on the surface of the HIV envelope and essential for virus entry into cells.[28–30] Therefore, maraviroc is used as an antiretroviral drug to block HIV entry. Plerixafor is a CXCR4 antagonist and an immunostimulant used in patients with lymphoma and multiple myeloma. Mogamulizumab, a mAb against CCR4, is used as an anticancer drug.[31,32] The anti-mCCR3 mAbs effective in multipurpose analyses will also allow us to explore mCCR3-mediated allergic diseases in a mouse model, and might lead to the development of CCR3-targeting drugs.

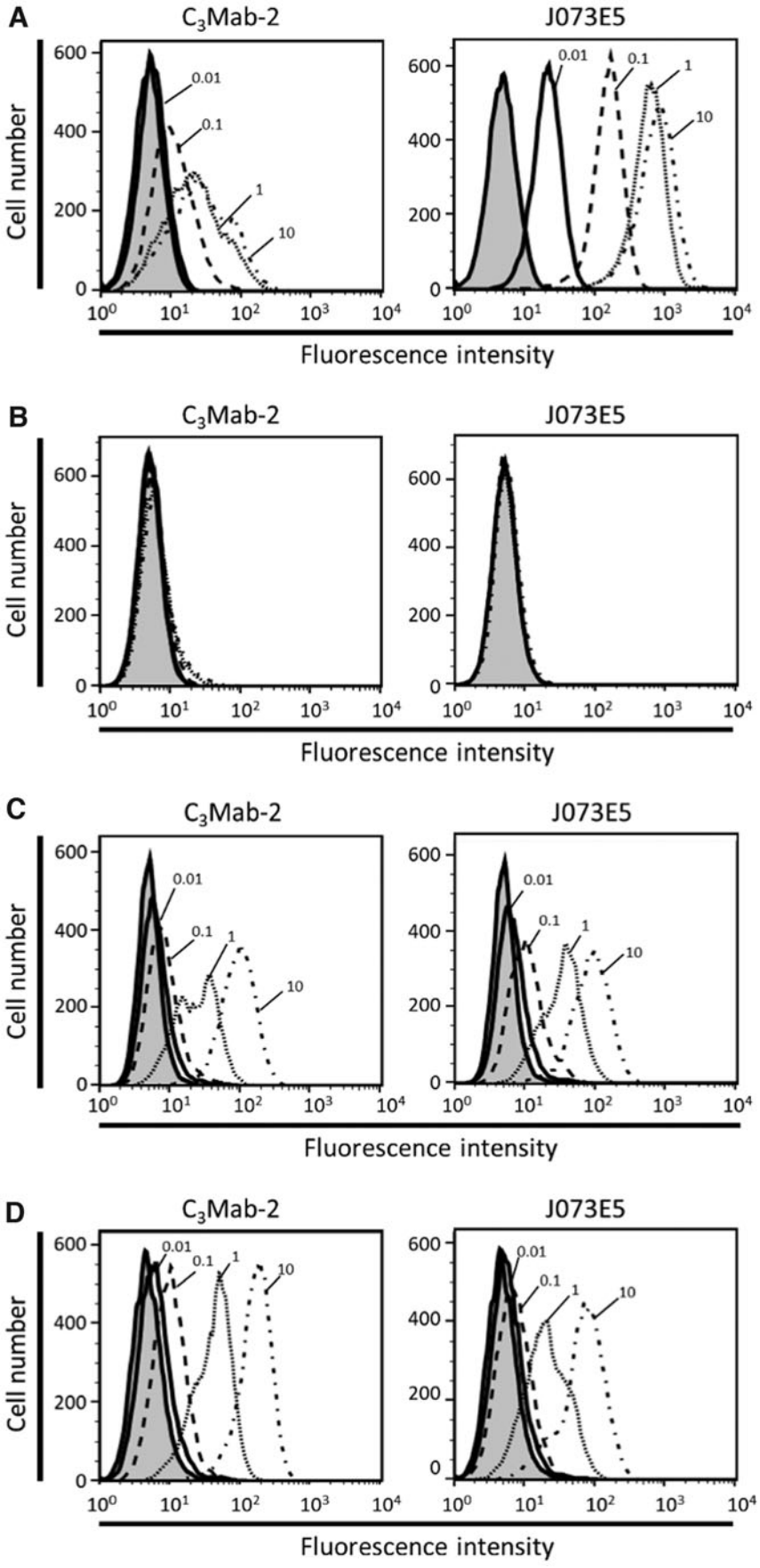

FIG. 2. Flow cytometry using anti-mCCR3. CHO/mCCR3 **(A)**, CHO-K1 **(B)**, P388 **(C)**, and J774-1 **(D)** cells were treated with 0.01 μg/mL (solid line), 0.1 μg/mL (dashed line), 1 μg/mL (dotted line), and 10 μg/mL (dashed-dotted line) of C_3Mab-2 or J073E5 (BioLegend), followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG; filled, negative control.

Author Disclosure Statement

T.A., R.N., J.T., T.N., M.Y., H.H., T.T., and M.S. performed experiments; M.K.K. designed the experiments; T.A. and Y.K. wrote the article.

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Supplementary Material

Supplementary Table S1

References

1. Zlotnik A, and Yoshie O: Chemokines: A new classification system and their role in immunity. Immunity 2000;12: 121–127.
2. Gerard C, and Rollins BJ: Chemokines and disease. Nat Immunol 2001;2:108–115.
3. Ben-Baruch A: Organ selectivity in metastasis: Regulation by chemokines and their receptors. Clin Exp Metastasis 2008;25:345–356.
4. Murphy PM, Baggiolini M, Charo IF, Hébert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, and Power CA: International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 2000;52:145–176.
5. Post TW, Bozic CR, Rothenberg ME, Luster AD, Gerard N, and Gerard C: Molecular characterization of two murine eosinophil beta chemokine receptors. J Immunol 1995;155: 5299–5305.
6. Combadiere C, Ahuja SK, and Murphy PM: Cloning and functional expression of a human eosinophil CC chemokine receptor. J Biol Chem 1995;270:16491–16494.
7. Willems LI, and Ijzerman AP: Small molecule antagonists for chemokine CCR3 receptors. Med Res Rev 2010;30: 778–817.
8. Stellato C, Brummet ME, Plitt JR, Shahabuddin S, Baroody FM, Liu MC, Ponath PD, and Beck LA: Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells. J Immunol 2001;166:1457–1461.
9. He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, Mackay CR, Sodroski J, and Gabuzda D: CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature 1997;385:645–649.
10. Kuse Y, Tsuruma K, Kanno Y, Shimazawa M, and Hara H: CCR3 is associated with the death of a photoreceptor cell-line induced by light exposure. Front Pharmacol 2017;8:207.
11. Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, Parmentier M, Collman RG, and Doms RW: A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell 1996;85:1149–1158.
12. Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, and Sodroski J: The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 1996;85:1135–1148.
13. Grozdanovic M, Laffey KG, Abdelkarim H, Hitchinson B, Harijith A, Moon HG, Park GY, Rousslang LK, Masterson JC, Furuta GT, Tarasova NI, Gaponenko V, and Ackerman SJ: Novel peptide nanoparticle-biased antagonist of CCR3 blocks eosinophil recruitment and airway hyperresponsiveness. J Allergy Clin Immunol 2019;143:669–680.e612.
14. Fukuda K, Kuo CH, Morohoshi K, Liu FT, and Ono SJ: The murine CCR3 receptor regulates both eosinophilia and hyperresponsiveness in IgE-mediated allergic conjunctivitis. Br J Ophthalmol 2012;96:1132–1136.
15. Elsner J, Escher SE, and Forssmann U: Chemokine receptor antagonists: A novel therapeutic approach in allergic diseases. Allergy 2004;59:1243–1258.
16. Song DJ, Shim MH, Lee N, Yoo Y, and Choung JT: CCR3 monoclonal antibody inhibits eosinophilic inflammation and mucosal injury in a mouse model of eosinophilic gastroenteritis. Allergy Asthma Immunol Res 2017;9:360–367.
17. 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.
18. 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.
19. Greenbaum JA, Andersen PH, Blythe M, Bui HH, Cachau RE, Crowe J, Davies M, Kolaskar AS, Lund O, Morrison S, Mumey B, Ofran Y, Pellequer JL, Pinilla C, Ponomarenko JV, Raghava GP, van Regenmortel MH, Roggen EL, Sette A, Schlessinger A, Sollner J, Zand M, and Peters B: Towards a consensus on datasets and evaluation metrics for developing B-cell epitope prediction tools. J Mol Recognit 2007;20:75–82.
20. 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.
21. 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.
22. 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.
23. 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.
24. 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.
25. 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.
26. 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;23:1.

27. Grimaldi JC, Yu NX, Grunig G, Seymour BW, Cottrez F, Robinson DS, Hosken N, Ferlin WG, Wu X, Soto H, O'Garra A, Howard MC, and Coffman RL: Depletion of eosinophils in mice through the use of antibodies specific for C-C chemokine receptor 3 (CCR3). J Leukoc Biol 1999; 65:846–853.
28. Levy JA: HIV pathogenesis: 25 years of progress and persistent challenges. AIDS 2009;23:147–160.
29. Trkola A, Dragic T, Arthos J, Binley JM, Olson WC, Allaway GP, Cheng-Mayer C, Robinson J, Maddon PJ, and Moore JP: CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. Nature 1996; 384:184–187.
30. Mondor I, Moulard M, Ugolini S, Klasse PJ, Hoxie J, Amara A, Delaunay T, Wyatt R, Sodroski J, and Sattentau QJ: Interactions among HIV gp120, CD4, and CXCR4: Dependence on CD4 expression level, gp120 viral origin, conservation of the gp120 COOH- and NH2-termini and V1/V2 and V3 loops, and sensitivity to neutralizing antibodies. Virology 1998;248:394–405.
31. Ishii T, Ishida T, Utsunomiya A, Inagaki A, Yano H, Komatsu H, Iida S, Imada K, Uchiyama T, Akinaga S, Shitara K, and Ueda R: Defucosylated humanized anti-CCR4 monoclonal antibody KW-0761 as a novel immunotherapeutic agent for adult T-cell leukemia/lymphoma. Clin Cancer Res 2010;16:1520–1531.
32. Remer M, Al-Shamkhani A, Glennie M, and Johnson P: Mogamulizumab and the treatment of CCR4-positive T-cell lymphomas. Immunotherapy 2014;6:1187–1206.

Address correspondence to:
Yukinari Kato
New Industry Creation Hatchery Center
Tohoku University
2–1, Seiryo-machi, Aoba-ku
Sendai 980-8575
Miyagi
Japan

E-mail: yukinarikato@med.tohoku.ac.jp

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