MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 42, Number 2, 2023

DOI: 10.1089/mab.2022.0034

Open camera or QR reader and scan code to access this article and other resources online.

Epitope Mapping of Anti-Mouse CCR3 Monoclonal Antibodies (C_3Mab-6 and C_3Mab-7)

Nami Tateyama,[1,*] Teizo Asano,[1,*] Tomohiro Tanaka,[2] Yu Isoda,[1] Yuki Okada,[2] Hiyori Kobayashi,[2] Guanjie Li,[2] Ren Nanamiya,[1] Takeo Yoshikawa,[3] Mika K. Kaneko,[1,2] Hiroyuki Suzuki,[1,2] and Yukinari Kato[1–3]

One of G protein-coupled receptors, CC chemokine receptor 3 (CCR3), is expressed in eosinophils, basophils, a subset of Th2 lymphocytes, mast cells, and airway epithelial cells. CCR3 levels in the serum of colorectal cancer patients are significantly higher than in control groups. Moreover, CCR3 is essential for recruiting eosinophils into the lung. Therefore, CCR3 is considered both a therapeutic target for colorectal cancer and allergic diseases. Previously, we established anti-mouse CCR3 (mCCR3) monoclonal antibodies (mAbs), C_3Mab-6 (rat IgG_1, kappa) and C_3Mab-7 (rat IgG_1, kappa), by immunizing a rat with an N-terminal peptide of mCCR3. These mAbs can be used in flow cytometry and enzyme-linked immunosorbent assays. In this study, we performed the epitope mapping of C_3Mab-6 and C_3Mab-7 using alanine scanning. The reactivity between these mAbs and point mutants of mCCR3 were analyzed using flow cytometry. The results indicated that Phe3, Asn4, Thr5, Asp6, Glu7, Lys9, Thr10, and Glu13 of mCCR3 are essential for C_3Mab-6 binding, whereas Phe15 and Glu16 are essential for C_3Mab-7 binding.

Keywords: mouse CCR3, monoclonal antibody, epitope, flow cytometry, alanine scanning

Introduction

The CC chemokine receptor 3 (CCR3), also known as CD193, is a member of the G protein-coupled receptor.[1] CCR3 is a seven-transmembrane domain receptor with four extracellular domains, and the N-terminal region of CCR3 is located in extracellular, whereas the C-terminal region is located in intracellular. CCR3 is expressed on the surface of eosinophils, basophils, a subset of Th2 lymphocytes, mast cells, and airway epithelial cells.[2–6] Some CC chemokines, such as eotaxin-1, eotaxin-2, eotaxin-3, MCP-3, and RANTES, are known to be ligands for CCR3.[7–9]

CCR3 and its ligands play an important role in recruiting eosinophils into the lung, inducing airway hyperresponsiveness in a murine model of allergic asthma.[10–13] CCR3 also contributes to ocular allergy.[14] Moreover, CCR3 levels in the serum of colorectal cancer patients are significantly higher than in control groups.[15,16] High expression levels of eotaxins occur in some tumors such as colorectal cancer,[17] breast cancer,[18] and oral squamous cell carcinomas.[19] Therefore, CCR3 is regarded as the therapeutic target for both allergic diseases and cancers.[9,15,20–22]

Monoclonal antibodies (mAbs) are used for the treatment of inflammatory diseases and cancers.[23–26] Many therapeutic mAbs possess neutralizing activity through the blockade between the targets and their ligands.[27] The epitope identification of mAbs is important to elucidate the pharmacological function of mAbs, and is essential to avoid unexpected cross-reactivity. Previously, we produced novel anti-mouse CCR3 (mCCR3) mAbs, C_3Mab-6 (rat IgG_1, kappa), and C_3Mab-7 (rat IgG_1, kappa) by immunizing a rat with the mCCR3 N-terminal peptide.[28] C_3Mab-6 and C_3Mab-7 reacted not only with mCCR3-overexpressed Chinese hamster ovary-K1 (CHO/mCCR3) cells, but also P388 (mouse lymphoid neoplasma) and J774-1 (mouse macrophage-like) cells, which express mCCR3 endogenously in flow cytometry. However, the critical amino acids of mCCR3 for the binding of C_3Mab-6 and C_3Mab-7 have not been determined.

Departments of [1]Antibody Drug Development, [2]Molecular Pharmacology, and [3]Pharmacology, Tohoku University Graduate School of Medicine, Sendai, Japan.
*These authors contributed equally to this study.

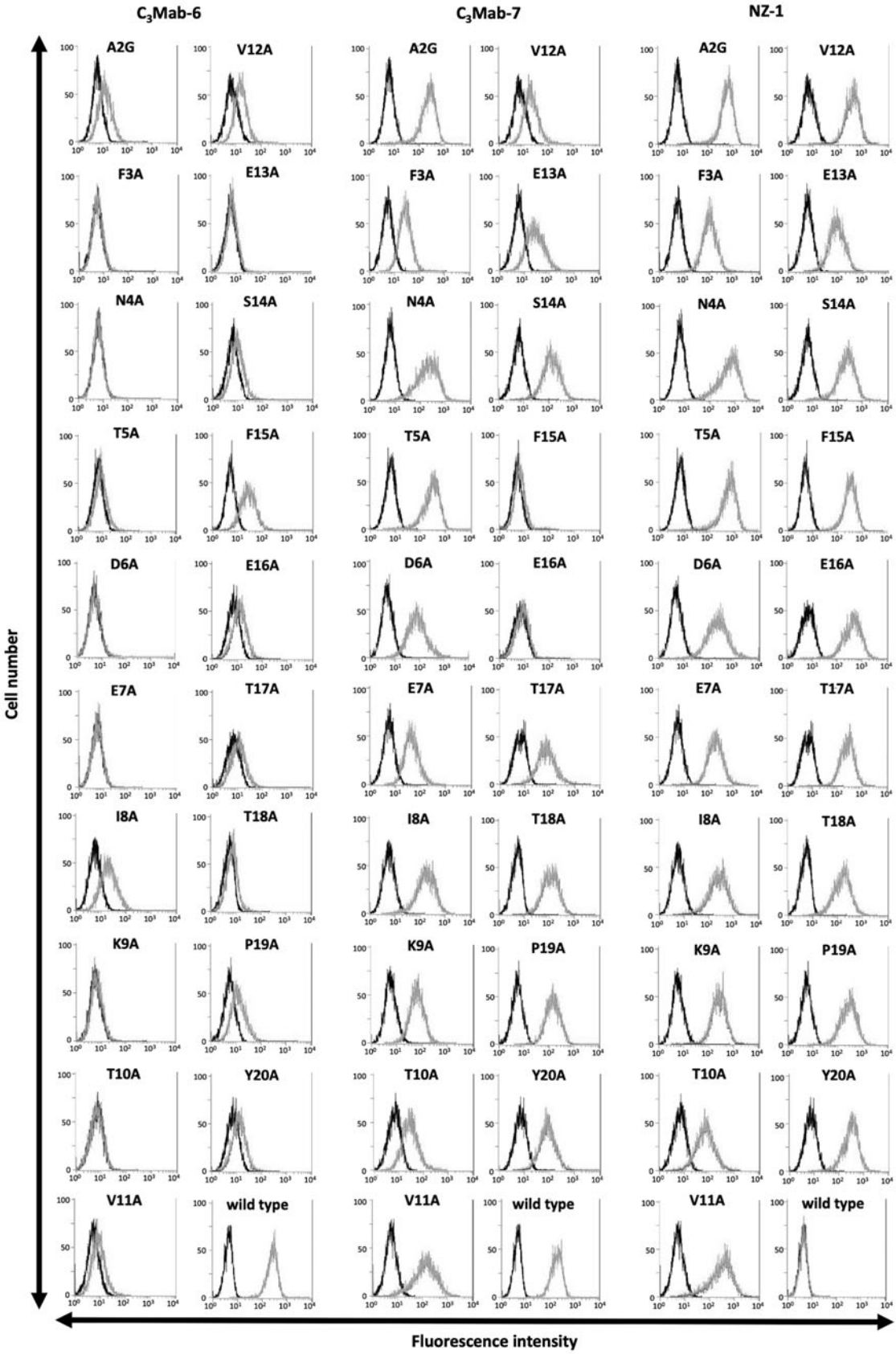

FIG. 1. Determination of the C_3Mab-6 and C_3Mab-7 epitope for mCCR3 by flow cytometry. C_3Mab-6 (1 μg/mL; gray line), C_3Mab-7 (1 μg/mL; gray line), or NZ-1 (1 μg/mL; gray line) were treated with mCCR3 point mutants-overexpressed CHO-K1 cells for 30 minutes at 4°C, followed by the addition of the secondary antibody. The black line represents the negative control. CHO-K1, Chinese hamster ovary-K1; mCCR3, mouse CC chemokine receptor 3.

In this study, we determined the binding epitope of C_3Mab-6 and C_3Mab-7 to mCCR3 using alanine scanning by flow cytometry.[29–34]

Materials and Methods

Plasmids of mCCR3 point mutants

The pEX-A2J2 plasmid subcloned mCCR3 (accession No. NM_009914.4) was purchased from Eurofins Genomics KK (Tokyo, Japan).[28,35–37] Substitutions of amino acids to alanine in the mCCR3 sequence were conducted by QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies, Inc., Santa Clara, CA). PCR fragments bearing the desired mutations were inserted into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and a PA16 tag[38–41] was added at the N-terminus using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan). The alanine (glycine) substitution mutants (A2G, F3A, N4A, T5A, D6A, E7A, I8A, K9A, T10A, V11A, V12A, E13A, S14A, F15A, E16A, T17A, T18A, P19A, and Y20A) were generated using QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies Inc., Santa Clara, CA, USA).

Cell lines

CHO-K1 cells were obtained from the America Type Culture Collection (ATCC, Manassas, VA). The CHO/mCCR3 cells were produced in our previous study.[37] mCCR3 point mutant plasmids were transfected into CHO-K1 cells using the Neon Transfection System (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were selected using a cell sorter (SH800; Sony Corp., Tokyo, Japan). CHO/mCCR3 cell and stable transfectants were cultured in 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 streptomycin, and

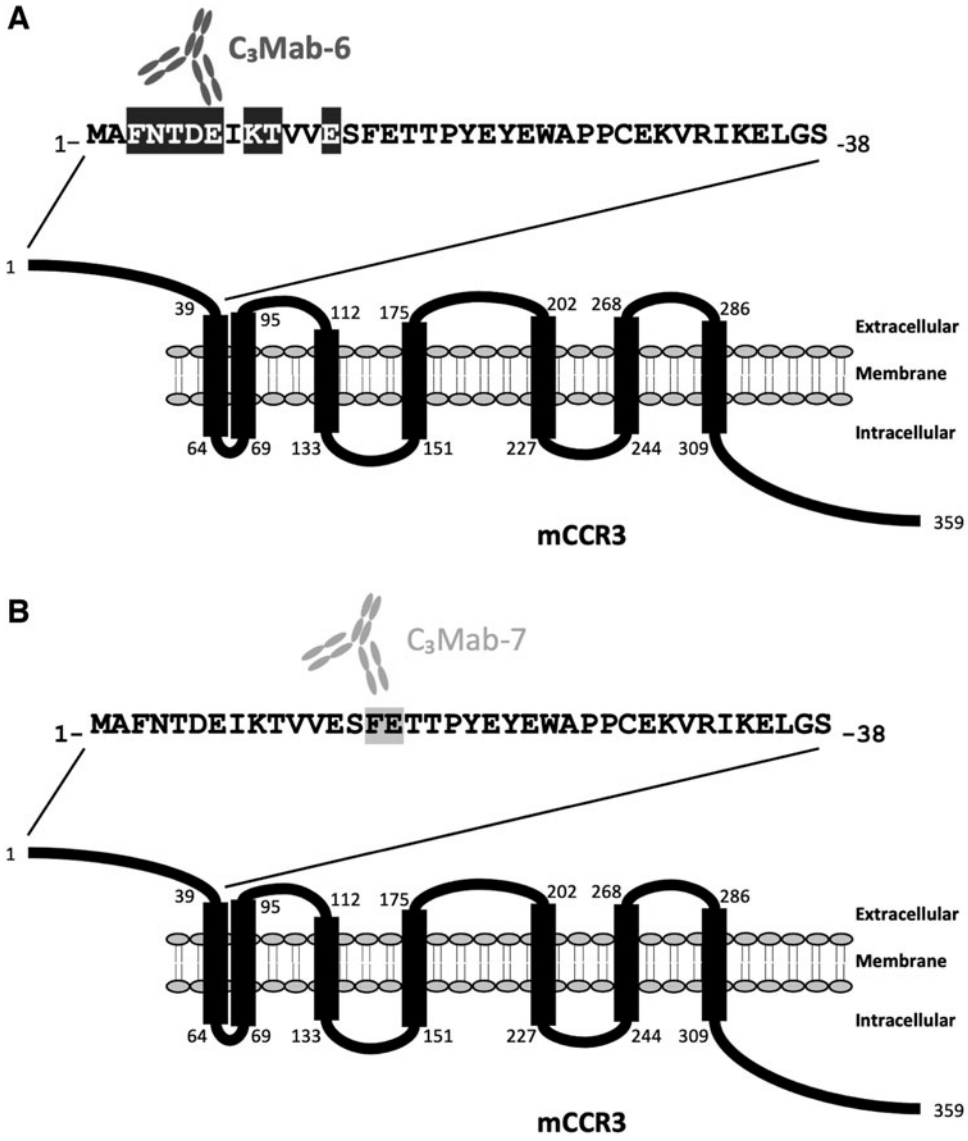

FIG. 2. Schematic illustration of C_3Mab-6 and C_3Mab-7 epitope for mCCR3. **(A)** C_3Mab-6 epitope for mCCR3 involves Phe3, Asn4, Thr5, Asp6, Glu7, Lys9, Thr10, and Glu13 of mCCR3. **(B)** C_3Mab-7 epitope for mCCR3 involves Phe15 and Glu16 of mCCR3.

0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere containing 5% CO_2. The stable transfectants were cultivated in a medium containing 0.5 mg/mL Zeocin (InvivoGen, San Diego, CA).

Flow cytometry

Cells were harvested after brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin in phosphate-buffered saline, cells were treated with C_3Mab-6, C_3Mab-7, or NZ-1 (1 μg/mL) for 30 minutes at 4°C and subsequently with Alexa Fluor 488-conjugated anti-rat IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using EC800 Cell Analyzer (Sony Corp.).

Results

To investigate the epitope of C_3Mab-6 and C_3Mab-7 for mCCR3, we conducted alanine scanning. We constructed 19 alanine substitution mutants of mCCR3. Mutant proteins were stably expressed on CHO-K1 cells, and the reactivities against C_3Mab-6, C_3Mab-7, or anti-PA16 tag mAb (NZ-1) were analyzed using flow cytometry.

First, we investigated the cell surface expression of mCCR3 mutants on CHO-K1 cells using NZ-1. The reactivities of NZ-1 against each mCCR3 mutant were confirmed in all the mutants (Fig. 1, right). Since PA16 tag was not conjugated in the wild-type mCCR3, NZ-1 did not react with it (Fig. 1, right). Next, we examined the reactivities of C_3Mab-6 and C_3Mab-7 against mCCR3 mutants. C_3Mab-6 reacted with 11 mutants (A2G, I8A, V11A, V12A, S14A, F15A, E16A, T17A, T18A, P19A, and Y20A) and wild-type mCCR3 (Fig. 1, left).

In contrast, C_3Mab-6 did not react with 8 mutants (F3A, N4A, T5A, D6A, E7A, K9A, T10A, and E13A) (Fig. 1, left). C_3Mab-7 reacted with 17 mutants (A2G, F3A, N4A, T5A, D6A, E7A, I8A, K9A, T10A, V11A, V12A, E13A, S14A, T17A, T18A, P19A, and Y20A) and wild-type mCCR3, whereas 2 mutants (F15A and E16A) lost their reactivity to C_3Mab-7 (Fig. 1, middle). These results indicated that eight amino acids of mCCR3 (Phe3, Asn4, Thr5, Asp6, Glu7, Lys9, Thr10, and Glu13) are critical for C_3Mab-6 binding (Fig. 2A), whereas two amino acids (Phe15 and Glu16) are critical for that on C_3Mab-7 (Fig. 2B).

Discussion

Therapeutic mAbs possesses biological activities, including neutralizing activity against the physiological ligands.[27] The epitope determination in the target protein is necessary to understand the properties of mAbs. Previously, we established two anti-mCCR3 mAbs, C_3Mab-6 and C_3Mab-7, which were established by immunizing the N-terminal peptide of mCCR3 ($_1$-MAFNTDEIKTVVESFETTP-$_{19}$).[28] Therefore, the binding epitope of C_3Mab-6 and C_3Mab-7 are included within the residues.

In this study, we performed the epitope mapping of C_3Mab-6 and C_3Mab-7 using alanine scanning of the mCCR3 N-terminal domain. The results showed that C_3Mab-6 and C_3Mab-7 have different binding regions of mCCR3 (Fig. 1). The C_3Mab-6 epitope is mainly located in the first half of the N-terminal region, whereas that of C_3Mab-7 is located in another half of the N-terminal region (Fig. 2). Structural analyses showed that eotaxin binds to the N-terminus of human CCR3 (residues 8–23).[42,43] Therefore, it is expected that both C_3Mab-6 and C_3Mab-7 may compete with eotaxin for binding to mCCR3.

Zhu *et al.* showed that the dissociation constants (K_D) of CCR3's binding to eotaxin-1, -2, and -3 are 2.1, 9.7, and 1.2×10^{-9} M, respectively.[44] We previously showed that C_3Mab-6 and C_3Mab-7 bound to CHO/mCCR3 with K_D of 8.7×10^{-9} M and 3.7×10^{-9} M, respectively.[28] The K_D values of C_3Mab-6 and C_3Mab-7 to CHO/mCCR3 were comparable with that of eotaxin. Therefore, we expect that C_3Mab-6 and C_3Mab-7 treatments would block eotaxin's binding to CHO/mCCR3.

In future studies, the neutralizing activities of C_3Mab-6 and C_3Mab-7 should be further assessed. Moreover, these mAbs will be useful for depleting mCCR3-expressing eosinophils and basophils, and targeting mCCR3-positive 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 nos. JP22ama121008 (to Y.K.), JP22am0401013 (to Y.K.), JP22bm1004001 (to Y.K.), JP22ck0106730 (to Y.K.), and JP21am0101078 (to Y.K.), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) grant nos. 21K15523 (to T.A.), 21K20789 (to T.T.), 22K06995 (to H.S.), 21K07168 (to M.K.K.), and 22K07224 (to Y.K.).

References

1. New DC, Wong YH. CC chemokine receptor-coupled signalling pathways. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai) 2003;35:779–788.
2. Willems LI, Ijzerman AP. Small molecule antagonists for chemokine CCR3 receptors. Med Res Rev 2010;30:778–817.
3. Stellato C, Brummet ME, Plitt JR, et al. Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells. J Immunol 2001;166:1457–1461.
4. He J, Chen Y, Farzan M, et al. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature 1997; 385:645–649.
5. Heath H, Qin S, Rao P, et al. Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J Clin Invest 1997;99:178–184.
6. Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. Science 1997;277:2005–2007.
7. Wise EL, Duchesnes C, da Fonseca PC, et al. Small molecule receptor agonists and antagonists of CCR3 provide insight into mechanisms of chemokine receptor activation. J Biol Chem 2007;282:27935–27943.
8. Bisset LR, Schmid-Grendelmeier P. Chemokines and their receptors in the pathogenesis of allergic asthma: Progress and perspective. Curr Opin Pulm Med 2005;11:35–42.
9. Mackay CR. Moving targets: Cell migration inhibitors as new anti-inflammatory therapies. Nat Immunol 2008;9:988–998.
10. Fulkerson PC, Fischetti CA, McBride ML, et al. A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. Proc Natl Acad Sci U S A 2006;103:16418–16423.

11. Fukuda K, Kuo CH, Morohoshi K, et al. The murine CCR3 receptor regulates both eosinophilia and hyperresponsiveness in IgE-mediated allergic conjunctivitis. Br J Ophthalmol 2012;96:1132–1136.
12. Chantry D, Burgess LE. Chemokines in allergy. Curr Drug Targets Inflamm Allergy 2002;1:109–116.
13. Pease JE. Asthma, allergy and chemokines. Curr Drug Targets 2006;7:3–12.
14. Miyazaki D, Nakamura T, Komatsu N, et al. Roles of chemokines in ocular allergy and possible therapeutic strategies. Cornea 2004;23:S48–S54.
15. Gerard C, Rollins BJ. Chemokines and disease. Nat Immunol 2001;2:108–115.
16. Ben-Baruch A. Organ selectivity in metastasis: Regulation by chemokines and their receptors. Clin Exp Metastasis 2008;25:345–356.
17. Cho H, Lim SJ, Won KY, et al. Eosinophils in colorectal neoplasms associated with expression of CCL11 and CCL24. J Pathol Transl Med 2016;50:45–51.
18. Thomas JK, Mir H, Kapur N, et al. CC chemokines are differentially expressed in Breast Cancer and are associated with disparity in overall survival. Sci Rep 2019;9:4014.
19. Lorena SC, Oliveira DT, Dorta RG, et al. Eotaxin expression in oral squamous cell carcinomas with and without tumour associated tissue eosinophilia. Oral Dis 2003;9:279–283.
20. Grozdanovic M, Laffey KG, Abdelkarim H, et al. Novel peptide nanoparticle-biased antagonist of CCR3 blocks eosinophil recruitment and airway hyperresponsiveness. J Allergy Clin Immunol 2019;143:669–680.e612.
21. Elsner J, Escher SE, Forssmann U. Chemokine receptor antagonists: A novel therapeutic approach in allergic diseases. Allergy 2004;59:1243–1258.
22. Gangur V, Birmingham NP, Thanesvorakul S, et al. CCR3 and CXCR3 as drug targets for allergy: Principles and potential. Curr Drug Targets Inflamm Allergy 2003;2:53–62.
23. Holgate ST, Polosa R. Treatment strategies for allergy and asthma. Nat Rev Immunol 2008;8:218–230.
24. Lee YT, Tan YJ, Oon CE. Molecular targeted therapy: Treating cancer with specificity. Eur J Pharmacol 2018; 834:188–196.
25. Boushey HA, Jr. Experiences with monoclonal antibody therapy for allergic asthma. J Allergy Clin Immunol 2001; 108:S77–S83.
26. Sarinho E, Cruz AA. Anti-IgE monoclonal antibody for the treatment of the asthma and other manifestations related to allergic diseases. J Pediatr (Rio J) 2006;82:S127–S132.
27. Jin S, Sun Y, Liang X, et al. Emerging new therapeutic antibody derivatives for cancer treatment. Signal Transduct Target Ther 2022;7:39.
28. Asano T, Suzuki H, Goto N, et al. Establishment of novel anti-mouse CCR3 monoclonal antibodies (C(3)Mab-6 and C(3)Mab-7) by N-terminal peptide immunization. Monoclon Antib Immunodiagn Immunother 2022;41:94–100.
29. Sayama Y, Sano M, Kaneko MK, et al. Epitope analysis of an anti-whale podoplanin monoclonal antibody, PMab-237, using flow cytometry. Monoclon Antib Immunodiagn Immunother 2020;39:17–22.
30. Kaneko MK, Sayama Y, Sano M, et al. The epitope of PMab-210 is located in platelet aggregation-stimulating domain-3 of pig podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38:271–276.
31. Kato Y, Sayama Y, Sano M, et al. Epitope analysis of an antihorse podoplanin monoclonal antibody PMab-219. Monoclon Antib Immunodiagn Immunother 2019;38:266–270.
32. Sano M, Kaneko MK, Kato Y. Epitope mapping of monoclonal antibody PMab-233 against tasmanian devil podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38:261–265.
33. Sayama Y, Sano M, Furusawa Y, et al. Epitope mapping of PMab-225 an anti-alpaca podoplanin monoclonal antibody using flow cytometry. Monoclon Antib Immunodiagn Immunother 2019;38:255–260.
34. Chang YW, Yamada S, Kaneko MK, et al. Epitope mapping of monoclonal antibody PMab-38 against dog podoplanin. Monoclon Antib Immunodiagn Immunother 2017; 36:291–295.
35. Asano T, Suzuki H, Tanaka T, et al. C3Mab-3: A monoclonal antibody for mouse CC chemokine receptor 3 for flow cytometry. Monoclon Antib Immunodiagn Immunother 2022;41:74–79.
36. Saito M, Harigae Y, Li G, et al. C3Mab-2: An anti-mouse CCR3 monoclonal antibody for immunocytochemistry. Monoclon Antib Immunodiagn Immunother 2022;41:45–49.
37. Asano T, Nanamiya R, Takei J, et al. Development of anti-mouse CC chemokine receptor 3 monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:107–112.
38. Tamura R, Oi R, Akashi S, et al. Application of the NZ-1 Fab as a crystallization chaperone for PA tag-inserted target proteins. Protein Sci 2019;28:823–836.
39. Tabata S, Kitago Y, Fujii Y, et al. An anti-peptide monoclonal antibody recognizing the tobacco etch virus protease-cleavage sequence and its application to a tandem tagging system. Protein Expr Purif 2018;147:94–99.
40. Fujii Y, Kaneko M, Neyazaki M, et al. PA tag: A versatile protein tagging system using a super high affinity antibody against a dodecapeptide derived from human podoplanin. Protein Expr Purif 2014;95:240–247.
41. Fujii Y, Matsunaga Y, Arimori T, et al. Tailored placement of a turn-forming PA tag into the structured domain of a protein to probe its conformational state. J Cell Sci 2016; 129:1512–1522.
42. Ye J, Kohli LL, Stone MJ. Characterization of binding between the chemokine eotaxin and peptides derived from the chemokine receptor CCR3. J Biol Chem 2000;275: 27250–27257.
43. Millard CJ, Ludeman JP, Canals M, et al. Structural basis of receptor sulfotyrosine recognition by a CC chemokine: The N-terminal region of CCR3 bound to CCL11/eotaxin-1. Structure 2014;22:1571–1581.
44. Zhu JZ, Millard CJ, Ludeman JP, et al. Tyrosine sulfation influences the chemokine binding selectivity of peptides derived from chemokine receptor CCR3. Biochemistry 2011;50:1524–1534.

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

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

Received: September 26, 2022
Accepted: March 21, 2023