 doi:10.20944/preprints202211.0071.v1

Article

Epitope Mapping of Anti-mouse CCR3 Monoclonal Antibodies using Flow Cytometry

Nami Tateyama[1], Teizo Asano[1], Hiroyuki Suzuki[2*], Guanjie Li[2], Takeo Yoshikawa[3], Tomohiro Tanaka[1], Mika K. Kaneko[1] and Yukinari Kato[1,2,3*]

[1] Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan; tateyama.nami.q6@dc.tohoku.ac.jp (N.T.); teizo.asano.a7@tohoku.ac.jp (T.A.); tomohiro.tanaka.b5@tohoku.ac.jp (T.T.); k.mika@med.tohoku.ac.jp (M.K.K.)
[2] Department of Molecular Pharmacology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan; kanketsu@med.tohoku.ac.jp (G.L.)
[3] Department of Pharmacology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan; tyoshikawa@med.tohoku.ac.jp (T.Y.)
* Correspondence: hiroyuki.suzuki.b4@tohoku.ac.jp (H.S.); yukinari.kato.e6@tohoku.ac.jp (Y.K.); Tel.: +81-22-717-8207

Abstract: The CC chemokine receptor 3 (CCR3) is a receptor for CC chemokines, including CCL5/RANTES, CCL7/MCP-3, and CCL11/eotaxin. CCR3 is expressed on the surface of eosinophils, basophils, a subset of Th2 lymphocytes, mast cells, and airway epithelial cells. CCR3 and its ligands are involved in airway hyperresponsiveness in allergic asthma, ocular allergy, and cancers. Therefore, CCR3 is an attractive target for those therapies. Previously, anti-mouse CCR3 (mCCR3) monoclonal antibodies (mAbs), C$_3$Mab-3 (rat IgG$_{2a}$, kappa), and C$_3$Mab-4 (rat IgG$_{2a}$, kappa) were developed using the Cell-Based Immunization and Screening (CBIS) method. In this study, the binding epitope of these mAbs was investigated using flow cytometry. The CCR3 extracellular domain-substituted mutant analysis showed that C$_3$Mab-3, C$_3$Mab-4, and a commercially available mAb (J073E5) recognized the N-terminal region (amino acids 1–38) of mCCR3. Next, the alanine scanning was conducted in the N-terminal region. The results revealed that Ala2, Phe3, Asn4, and Thr5 of mCCR3 are involved in C$_3$Mab-3 binding, whereas Ala2, Phe3, and Thr5 are essential to C$_3$Mab-4 binding, and Ala2 and Phe3 are crucial to J073E5 binding. These results reveal the involvement of the N-terminus of mCCR3 in the recognition of C$_3$Mab-3, C$_3$Mab-4, and J073E5.

Keywords: mouse CCR3; monoclonal antibody; epitope mapping; alanine scanning; flow cytometry

1. Introduction

Chemokines are a family of small cytokines secreted by cells and play essential roles in cell migration, inflammation, and immune responses by binding to chemokine receptors [1-4]. The CC chemokine receptor 3 (CCR3) is a receptor for CC chemokines, including CCL5/RANTES, CCL7/MCP-3, and CCL11/eotaxin [5-7]. CCR3 is expressed on the surface of eosinophils, basophils, a subset of Th2 lymphocytes, mast cells, and airway epithelial cells [8-13]. CCR3 is a family of G protein-coupled receptors (GPCRs) that transduce extracellular signals to intracellular signaling molecules [14]. The CCR3 signaling pathway is critical in eosinophil migration [15,16]. It has been reported that CCR3 and its ligands can cause airway hyperresponsiveness in a murine allergic asthma model [17-20], contributing to the ocular allergy [21]. Moreover, elevated eotaxin expression has been observed in colorectal cancer [22], breast cancer [23], and oral squamous cell carcinomas [24]. Therefore, CCR3 and its ligands are the therapeutic targets for allergic diseases and cancers [7,25].

GPCR has seven transmembrane domains, four extracellular regions, including an N-terminal region (residues 1–38), and three extracellular loops (ECL1; amino acids [aa] 96–111, ECL2; aa 176–207, and ECL3; aa 269–285). Previously, monoclonal antibodies

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 November 2022 doi:10.20944/preprints202211.0071.v1

(mAbs) have been developed against GPCRs, including an anti-mouse CCR2 mAb [26], an anti-human CCR2 mAb [27], an anti-mouse CCR3 (mCCR3) mAbs [28-30], an anti-mouse CCR4 mAb [31], an anti-mouse CCR8 mAbs [32-34], an anti-human CCR9 mAb [35], and an anti-mouse CXCR6 mAb [36]. The binding epitopes of anti-CCR2, CCR4, CCR9, and CXCR6 mAbs, which were established using the peptide immunization method, were determined by enzyme-linked immunosorbent assay (ELISA) [37-39]. However, anti-mCCR3 mAbs, C3Mab-3 (rat IgG_{2a}, kappa) [30], and C3Mab-4 (rat IgG_{2a}, kappa) were established by the Cell-Based Immunization and Screening (CBIS) method. Therefore, ELISA could not be applied to their epitope mapping.

In this study, epitope mapping of the anti-mCCR3 mAbs was conducted by flow cytometry using the extracellular region substitution and the alanine scanning methods to clarify the features of C3Mab-3 and C3Mab-4.

2. Materials and Methods

2.1. Cell lines

Chinese hamster ovary (CHO)-K1 cell was obtained from the America Type Culture Collection (ATCC, Manassas, VA, USA). The CHO/mCCR3 cells were produced in our previous study [28]. The chimera and the point mutant plasmids were transfected into CHO-K1 cells using the Neon Transfection System (Thermo Fisher Scientific Inc., Waltham, MA, USA). Stable transfectants were selected using a cell sorter (SH800; Sony Biotechnology Inc., Tokyo, Japan). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific Inc.), 100 units/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.) at 37 ℃ 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, USA).

2.2. Plasmid construction

Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding mCCR3 (Accession No.: NM_009914.4) [28-30] and mouse CCR8 (mCCR8; Accession No.: NM_007720.2) [32-34] were subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. Chimeric mutants mCCR8 (mCCR3p1-38), mCCR8 (mCCR3p96-111), mCCR8 (mCCR3p176-207), and mCCR8 (mCCR3p269-285) were produced with a RAP [40,41] and a MAP tag [42,43] at their C-terminus using the HotStar HiFidelity polymerase kit (Qiagen Inc., Hilden, Germany). Alanine (glycin) substitutions in the mCCR3 N-terminal region were conducted using QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies Inc., Santa Clara, CA, USA). PCR fragments bearing the desired mutations were inserted into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation) using the In-Fusion HD Cloning Kit (TaKaRa Bio, Inc., Shiga, Japan).

2.3. Antibodies

C3Mab-3 [30] and C3Mab-7 [29] were described previously. C3Mab-4 was also established together with C3Mab-3. An anti-mCCR3 mAb (clone J073E5) was purchased from BioLegend (San Diego, CA, USA). A secondary Alexa Fluor 488-conjugated anti-rat IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) as well.

2.4. 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 primary mAbs (1 μg/ml) for 30 min at 4 ℃ and subsequently with Alexa Fluor 488-conjugated anti-rat IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA). Fluorescence data were obtained using the EC800 Cell Analyzer (Sony Biotechnology Inc.).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 November 2022 doi:10.20944/preprints202211.0071.v1

3. Results

3.1. Determination of the epitope of anti-mCCR3 mAbs by flow cytometry using chimeric proteins.

C3Mab-3 and C3Mab-4 were established using the CBIS method, and are applicable to flow cytometry, but not to ELISA. To investigate the binding epitope of C3Mab-3 and C3Mab-4, we focused on four extracellular regions of mCCR3 including the N-terminal region (aa 1-38), ECL1 (aa 96-111), ECL2 (aa 176-207), and ECL3 (aa 269-285). The four extracellular regions of mCCR3 were substituted into the corresponding regions of mCCR8, which possesses a similar amino acid structure to mCCR3. As shown in Fig. 1, mCCR8 (mCCR3p1-38), mCCR8 (mCCR3p96-111), mCCR8 (mCCR3p176-207), and mCCR8 (mCCR3p269-285) were generated. The chimeric proteins were transiently expressed on CHO-K1 cells, and their reactivities to C3Mab-3, C3Mab-4, and commercially available J073E5 were analyzed using flow cytometry. As shown in Fig. 2, C3Mab-3, C3Mab-4 and J073E5 reacted with mCCR8 (mCCR3p1-38) and mCCR3. In contrast, they did not react with mCCR8 (mCCR3p96-111), mCCR8 (mCCR3p176-207), and mCCR8 (mCCR3p269-285). These results show that the N-terminal region of mCCR3 is recognized by C3Mab-3, C3Mab-4, and J073E5.

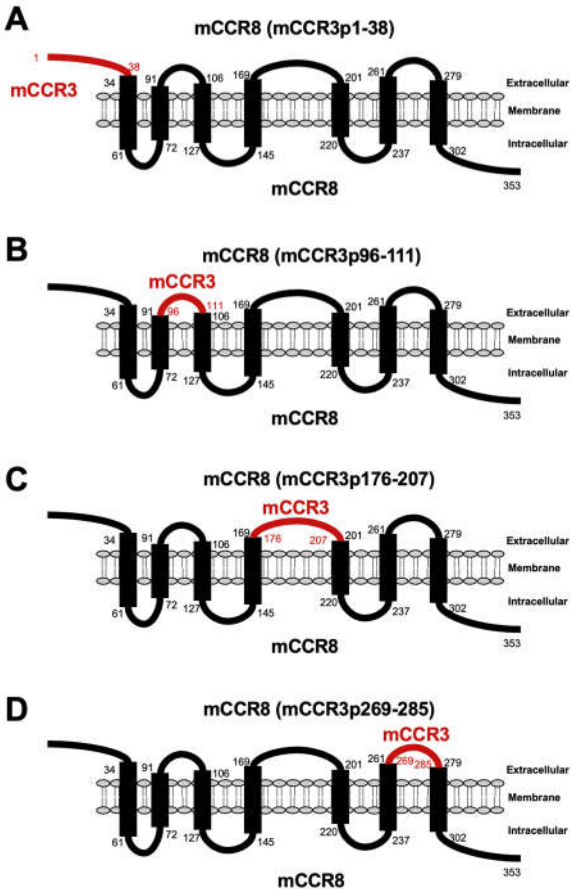

Figure 1. Schematic illustration of mCCR8 and mCCR3 chimeric proteins. The four extracellular regions of mCCR3, including the N-terminal region (residues 1–38), ECL1 (residues 96–111), ECL2 (residues 176–207), and ECL4 (residues 269–285) were substituted into the corresponding regions of mCCR8. ECL, extracellular loop.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 November 2022 doi:10.20944/preprints202211.0071.v1

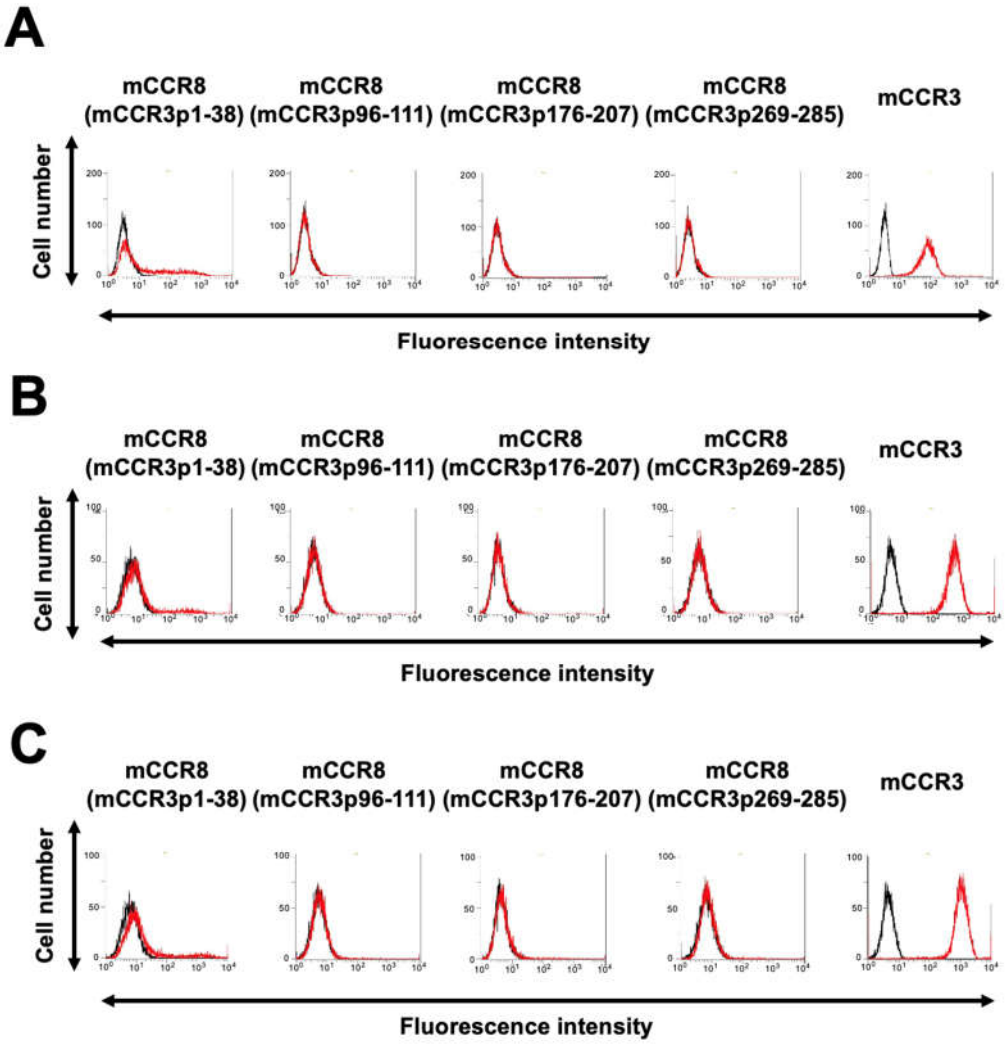

Figure 2. Determination of the epitope of anti-mCCR3 mAbs by flow cytometry using chimeric proteins. C3Mab-3 (1 μg/mL) (A), C3Mab-4 (1 μg/mL) (B), and J073E5 (1 μg/mL) (C) were treated with CHO-K1 cells which were transiently expressed chimeric proteins for 30 min at 4°C, followed by the addition of Alexa488-conjugated anti-rat IgG. Red lines show the cells with anti-mCCR3 mAb treatment, and black lines show cells without anti-mCCR3 mAbs treatment as a negative control.

3.2. Determination of the C3Mab-3 epitope by flow cytometry using alanine scanning.

Next, alanine scanning was conducted in the N-terminal region except for Cys28. Thirty-six alanine substitution mutants of mCCR3 were constructed, and the mutant proteins were transiently expressed on CHO-K1 cells. The reactivity against C3Mab-3, C3Mab-4, and J073E5 was assessed using flow cytometry. As shown in Fig. 3A, C3Mab-3 did not react with the four mutants (A2G, F3A, N4A, and T5A). In contrast, C3Mab-3 reacted with the other 32 mutants. These results showed that four residues (Ala2, Phe3, Asn4, and Thr5) of mCCR3 are important for C3Mab-3 binding (Fig. 3B). C3Mab-4 did not react with three mutants (A2G, F3A, and T5A) but reacted with others (Fig. 4A), indicating that three residues (Ala2, Phe3, and Thr5) of mCCR3 are important for C3Mab-4 binding (Fig. 4B). J073E5 did not react with two mutants (A2G and F3A) but reacted with others (Fig. 5A), indicating that two residues (Ala2 and Phe3) of mCCR3 are important for J073E5 binding (Fig. 5B). The cell surface expression of mCCR3 mutants on CHO-K1 cells was confirmed using anti-mCCR3 mAbs, C3Mab-7. It has already been confirmed that Phe15 and Glu16 are essential for C3Mab-7 binding (manuscript submitted). We could confirm the cell surface expression of four mutants (A2G, F3A, N4A, and T5A) of mCCR3 using C3Mab-7 (Fig. 6).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 November 2022 doi:10.20944/preprints202211.0071.v1

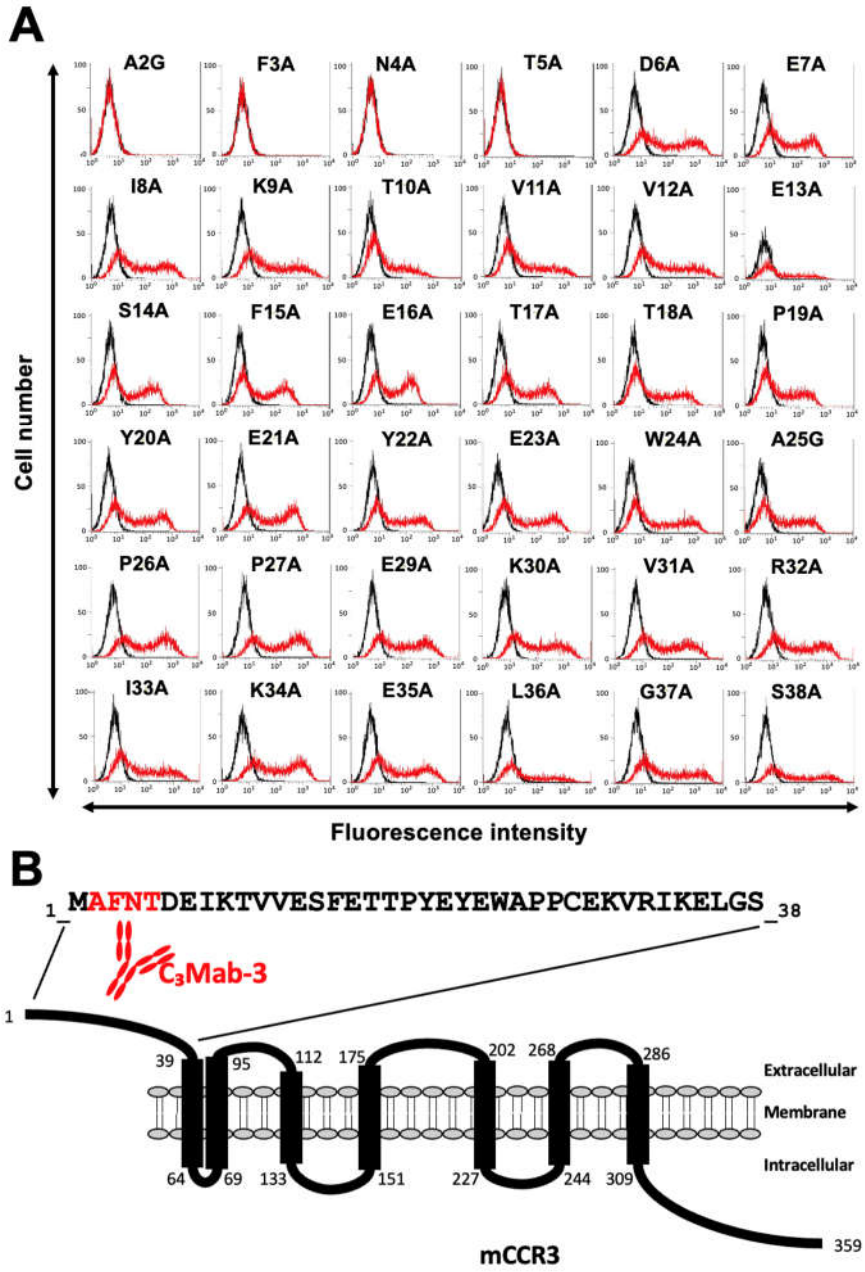

Figure 3. Determination of the C3Mab-3 epitope by flow cytometry using alanine scanning. (A) C3Mab-3 (1 μg/mL) was treated with CHO-K1 cells which were transiently expressed mutant proteins for 30 min at 4°C, followed by the addition of Alexa488-conjugated anti-rat IgG. Red lines show the cells with C3Mab-3 treatment, and black lines show cells without Ab treatment as a negative control. (B) The C3Mab-3 epitope for mCCR3 involves Ala2, Phe3, Asn4, and Thr5 of mCCR3.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 November 2022 doi:10.20944/preprints202211.0071.v1

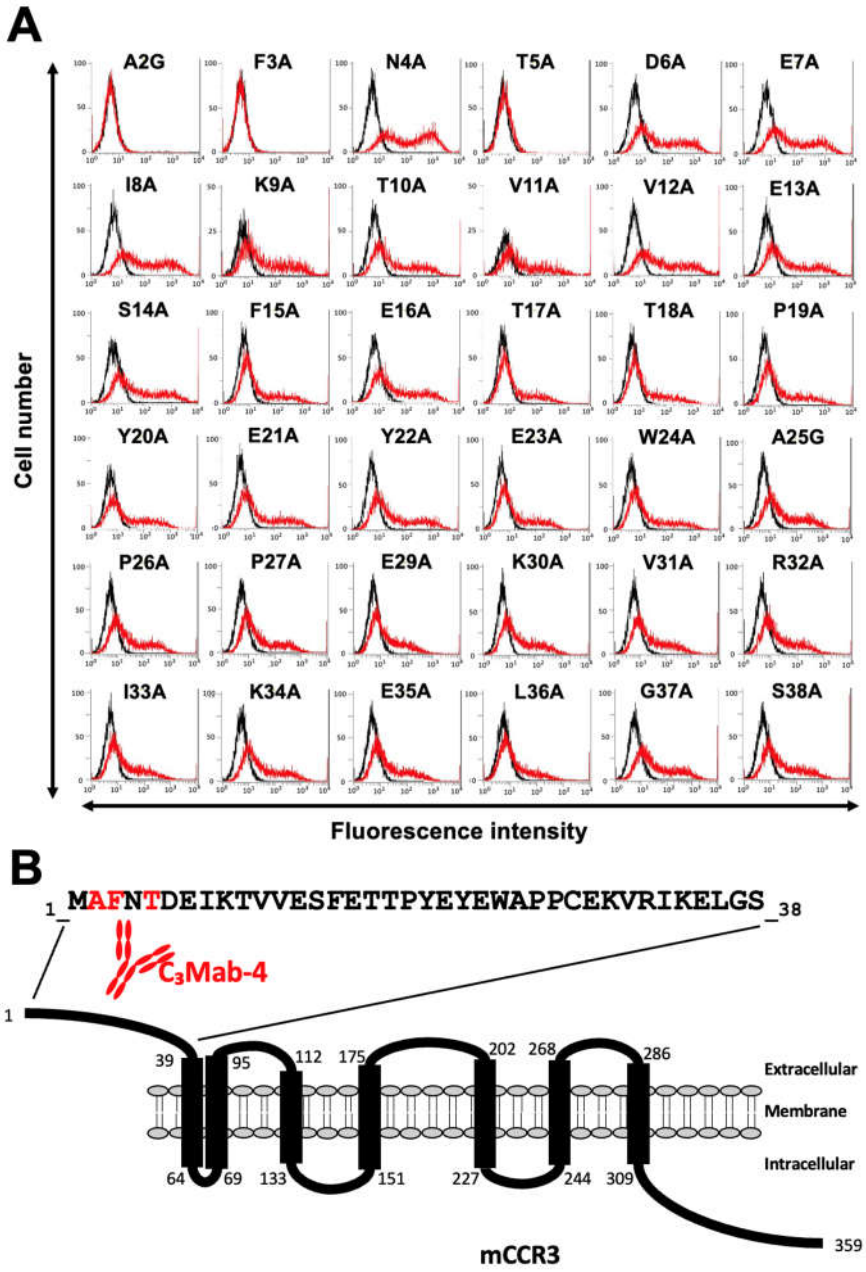

Figure 4. Determination of the C3Mab-4 epitope by flow cytometry using alanine scanning. (A) C3Mab-4 (1 μg/mL) was treated with CHO-K1 cells which were transiently expressed mutant proteins for 30 min at 4°C, followed by the addition of Alexa488-conjugated anti-rat IgG. Red lines show the cells with C3Mab-4 treatment, and black lines show cells without Ab treatment as a negative control. (B) The C3Mab-4 epitope for mCCR3 involves Ala2, Phe3, and Thr5 of mCCR3.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 November 2022 doi:10.20944/preprints202211.0071.v1

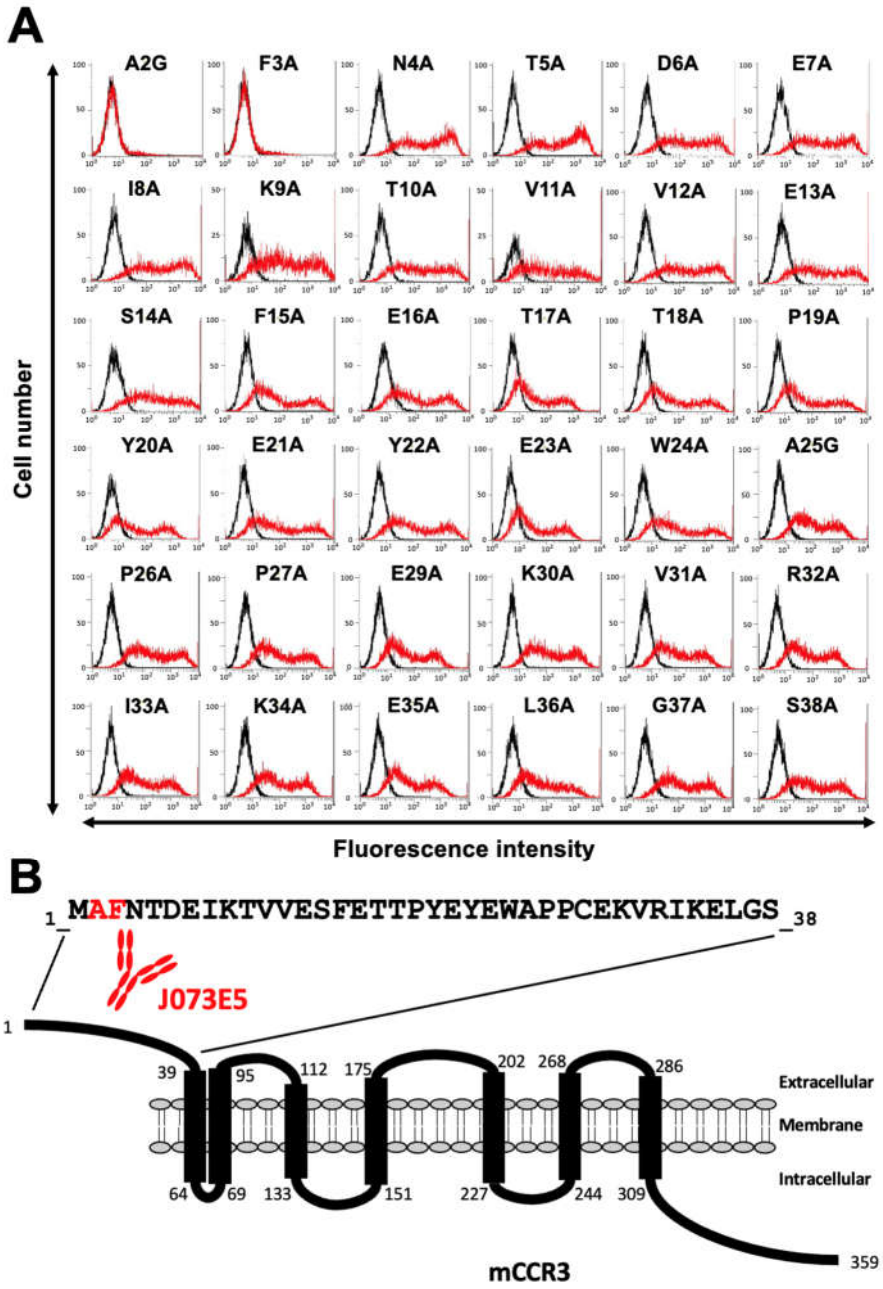

Figure 5. Determination of the J073E5 epitope by flow cytometry using alanine scanning. (A) J073E5 (1 μg/mL) was treated with CHO-K1 cells which were transiently expressed mutant proteins for 30 min at 4°C, followed by the addition of Alexa488-conjugated anti-rat IgG. Red lines show the cells with J073E5 treatment, and black lines show cells without Ab treatment as a negative control. (B) The J073E5 epitope for mCCR3 involves Ala2, and Phe3 of mCCR3.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 November 2022 doi:10.20944/preprints202211.0071.v1

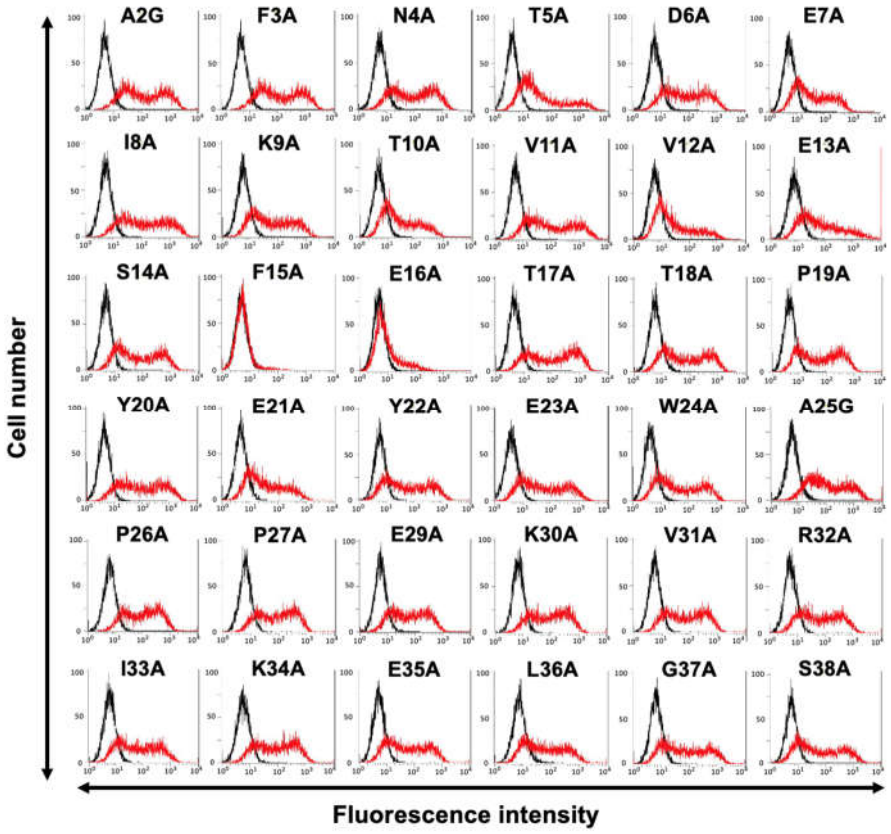

Figure 6. Cell surface expression of mCCR3 mutants on CHO-K1 cells by flow cytometry. C3Mab-7 (1 μg/mL) was treated with CHO-K1 cells which were transiently expressed mutant proteins for 30 min at 4°C, followed by the addition of Alexa488-conjugated anti-rat IgG. Red lines show the cells with C3Mab-7 treatment, and black lines show cells without Ab treatment as a negative control.

4. Discussion

We have established various mAbs against membrane proteins using the CBIS method. Because the mAbs sometimes recognize the conformational epitope, they can be applied to flow cytometry but not to Western blotting and ELISA. Two anti-mCCR3 mAbs examined in this study, C3Mab-3 and C3Mab-4, were established using the CBIS method [30]. We first attempted to identify their epitope using synthetic peptides using ELISA. However, they did not recognize the synthetic peptides, including the mCCR3 N-terminal region (p1–19), which contains the epitope determined using flow cytometry (Fig. 3 and 4). These results suggest that the residues participate in the formation of conformational epitope and/or undergo the post-translational modification on the cell surface. Furthermore, we could not exclude the possibility of the first Met as their epitopes. In the case of C3Mab-3 and C3Mab-4 epitopes, Asn4 and Thr5 are involved in the recognition. Although Asn and Thr are known to be *N*- and *O*- glycosylated respectively, there is no report on the glycosylation of Asn4 and Thr5 of mCCR3. Further studies were required to analyze the involvement of the posttranslational modification of these residues in the recognition by C3Mab-3 and C3Mab-4.

Other anti-mCCR3 mAbs, C3Mab-6, and C3Mab-7, were also developed by mCCR3 N-terminal peptide immunization [29]. It was found that they could recognize the synthetic peptide of the mCCR3 N-terminal region (p1-19) using ELISA and cell surface expressed mCCR3 by flow cytometry. Furthermore, Phe3, Asn4, Thr5, Asp6, Glu7, Lys9, Thr10, and Glu13 of mCCR3 were determined as C3Mab-6 epitope, whereas Phe15 and Glu16 as C3Mab-7 epitope (manuscript submitted). These results indicate that C3Mab-6 and C3Mab-7 recognize both the naked N-terminal peptide and cell surface expressed mCCR3. Further studies are essential to understand the difference of mCCR3 recognitions between C3Mab-3/C3Mab-4 and C3Mab-6/C3Mab-7.

It has been reported that a CCR3 ligand, CCL11/eotaxin binds to the N-terminal region of CCR3 [44,45]. Therefore, our established anti-mCCR3 mAbs could compete with the ligand binding to mCCR3 and have neutralizing activity. Shen *et al.* reported that anti-CCR3 mAb could significantly suppress airway eosinophilia and mucus overproduction in asthmatic mice; therefore, the blockage of the CCR3 axis may be an attractive strategy for asthma therapy [46]. In future studies, we would like to examine the neutralizing activities of these anti-mCCR3 mAbs.

Author Contributions: NT, GL, TT, TY, and TA performed the experiments. MKK and YK designed the experiments. NT, TA, HS, and YK analyzed the data. TA, HS, and YK wrote the manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

Funding: The present study was supported in part by the Japan Agency for Medical Research and Development (AMED; grant nos: JP22ama121008, JP21am0401013, JP22bm1004001, JP22ck0106730 and JP21am0101078).

Acknowledgments: The authors would like to thank Ms. Ms. Saori Okuno and Ms. Saori Handa (Department of Antibody Drug Development, Tohoku University Graduate School of Medicine) for technical assistance of the experiments.

Conflicts of Interest: The authors declare no conflict of interest involving this article.

References

1. Kufareva, I.; Salanga, C.L.; Handel, T.M. Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies. *Immunol Cell Biol* **2015**, *93*, 372-383, doi:10.1038/icb.2015.15.
2. Zlotnik, A.; Yoshie, O. Chemokines: a new classification system and their role in immunity. *Immunity* **2000**, *12*, 121-127, doi:10.1016/s1074-7613(00)80165-x.
3. Charo, I.F.; Ransohoff, R.M. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* **2006**, *354*, 610-621, doi:10.1056/NEJMra052723.
4. Murphy, P.M.; Baggiolini, M.; Charo, I.F.; Hébert, C.A.; Horuk, R.; Matsushima, K.; Miller, L.H.; Oppenheim, J.J.; Power, C.A. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* **2000**, *52*, 145-176.
5. Wise, E.L.; Duchesnes, C.; da Fonseca, P.C.; Allen, R.A.; Williams, T.J.; Pease, J.E. Small molecule receptor agonists and antagonists of CCR3 provide insight into mechanisms of chemokine receptor activation. *J Biol Chem* **2007**, *282*, 27935-27943, doi:10.1074/jbc.M703255200.
6. Bisset, L.R.; Schmid-Grendelmeier, P. Chemokines and their receptors in the pathogenesis of allergic asthma: progress and perspective. *Curr Opin Pulm Med* **2005**, *11*, 35-42, doi:10.1097/01.mcp.0000144502.50149.e0.
7. Mackay, C.R. Moving targets: cell migration inhibitors as new anti-inflammatory therapies. *Nat Immunol* **2008**, *9*, 988-998, doi:10.1038/ni.f.210.
8. Willems, L.I.; Ijzerman, A.P. Small molecule antagonists for chemokine CCR3 receptors. *Med Res Rev* **2010**, *30*, 778-817, doi:10.1002/med.20181.
9. Stellato, C.; Brummet, M.E.; Plitt, J.R.; Shahabuddin, S.; Baroody, F.M.; Liu, M.C.; Ponath, P.D.; Beck, L.A. Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells. *J Immunol* **2001**, *166*, 1457-1461, doi:10.4049/jimmunol.166.3.1457.
10. He, J.; Chen, Y.; Farzan, M.; Choe, H.; Ohagen, A.; Gartner, S.; Busciglio, J.; Yang, X.; Hofmann, W.; Newman, W.; et al. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* **1997**, *385*, 645-649, doi:10.1038/385645a0.
11. Heath, H.; Qin, S.; Rao, P.; Wu, L.; LaRosa, G.; Kassam, N.; Ponath, P.D.; Mackay, C.R. Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J Clin Invest* **1997**, *99*, 178-184, doi:10.1172/jci119145.
12. Sallusto, F.; Mackay, C.R.; Lanzavecchia, A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* **1997**, *277*, 2005-2007, doi:10.1126/science.277.5334.2005.
13. New, D.C.; Wong, Y.H. CC chemokine receptor-coupled signalling pathways. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* **2003**, *35*, 779-788.
14. Kroeze, W.K.; Sheffler, D.J.; Roth, B.L. G-protein-coupled receptors at a glance. *J Cell Sci* **2003**, *116*, 4867-4869, doi:10.1242/jcs.00902.
15. Kampen, G.T.; Stafford, S.; Adachi, T.; Jinquan, T.; Quan, S.; Grant, J.A.; Skov, P.S.; Poulsen, L.K.; Alam, R. Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood* **2000**, *95*, 1911-1917.
16. Zhu, Y.; Bertics, P.J. Chemoattractant-induced signaling via the Ras-ERK and PI3K-Akt networks, along with leukotriene C4 release, is dependent on the tyrosine kinase Lyn in IL-5- and IL-3-primed human blood eosinophils. *J Immunol* **2011**, *186*, 516-526, doi:10.4049/jimmunol.1000955.

17. Fulkerson, P.C.; Fischetti, C.A.; McBride, M.L.; Hassman, L.M.; Hogan, S.P.; Rothenberg, M.E. 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, doi:10.1073/pnas.0607863103.
18. Fukuda, K.; Kuo, C.H.; Morohoshi, K.; Liu, F.T.; Ono, S.J. The murine CCR3 receptor regulates both eosinophilia and hyperresponsiveness in IgE-mediated allergic conjunctivitis. *Br J Ophthalmol* **2012**, *96*, 1132-1136, doi:10.1136/bjophthalmol-2011-301399.
19. Chantry, D.; Burgess, L.E. Chemokines in allergy. *Curr Drug Targets Inflamm Allergy* **2002**, *1*, 109-116, doi:10.2174/1568010023344995.
20. Pease, J.E. Asthma, allergy and chemokines. *Curr Drug Targets* **2006**, *7*, 3-12, doi:10.2174/138945006775270204.
21. Miyazaki, D.; Nakamura, T.; Komatsu, N.; Nawata, N.; Ikeda, Y.; Inoue, Y.; Higashi, H.; Ono, S.J. Roles of chemokines in ocular allergy and possible therapeutic strategies. *Cornea* **2004**, *23*, S48-54, doi:10.1097/01.ico.0000136671.50777.fe.
22. Cho, H.; Lim, S.J.; Won, K.Y.; Bae, G.E.; Kim, G.Y.; Min, J.W.; Noh, B.J. Eosinophils in Colorectal Neoplasms Associated with Expression of CCL11 and CCL24. *J Pathol Transl Med* **2016**, *50*, 45-51, doi:10.4132/jptm.2015.10.16.
23. Thomas, J.K.; Mir, H.; Kapur, N.; Bae, S.; Singh, S. CC chemokines are differentially expressed in Breast Cancer and are associated with disparity in overall survival. *Sci Rep* **2019**, *9*, 4014, doi:10.1038/s41598-019-40514-9.
24. Lorena, S.C.; Oliveira, D.T.; Dorta, R.G.; Landman, G.; Kowalski, L.P. Eotaxin expression in oral squamous cell carcinomas with and without tumour associated tissue eosinophilia. *Oral Dis* **2003**, *9*, 279-283, doi:10.1034/j.1601-0825.2003.00958.x.
25. Gerard, C.; Rollins, B.J. Chemokines and disease. *Nat Immunol* **2001**, *2*, 108-115, doi:10.1038/84209.
26. Tanaka, T.; Li, G.; Asano, T.; Saito, M.; Kaneko, M.K.; Suzuki, H.; Kato, Y. Development of a Novel Anti-Mouse CCR2 Monoclonal Antibody (C(2)Mab-6) by N-Terminal Peptide Immunization. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 80-86, doi:10.1089/mab.2021.0063.
27. Tanaka, T.; Li, G.; Saito, M.; Suzuki, H.; Asano, T.; Kaneko, M.K.; Kato, Y. Development of an Anti-human CCR2 Monoclonal Antibody (C(2)Mab-9) by N-Terminal Peptide Immunization. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 188-193, doi:10.1089/mab.2022.0001.
28. Asano, T.; Nanamiya, R.; Takei, J.; Nakamura, T.; Yanaka, M.; Hosono, H.; Tanaka, T.; Sano, M.; Kaneko, M.K.; Kato, Y. Development of Anti-Mouse CC Chemokine Receptor 3 Monoclonal Antibodies for Flow Cytometry. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 107-112, doi:10.1089/mab.2021.0009.
29. Asano, T.; Suzuki, H.; Goto, N.; Tanaka, T.; Kaneko, M.K.; Kato, Y. 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, doi:10.1089/mab.2021.0065.
30. Asano, T.; Suzuki, H.; Tanaka, T.; Saito, M.; Li, G.; Goto, N.; Nanamiya, R.; Kaneko, M.K.; Kato, Y. C(3)Mab-3: A Monoclonal Antibody for Mouse CC Chemokine Receptor 3 for Flow Cytometry. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 74-79, doi:10.1089/mab.2021.0062.
31. Takei, J.; Suzuki, H.; Asano, T.; Tanaka, T.; Kaneko, M.K.; Kato, Y. Development of a Novel Anti-Mouse CCR4 Monoclonal Antibody (C(4)Mab-1) by N-Terminal Peptide Immunization. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 87-93, doi:10.1089/mab.2021.0064.
32. Saito, M.; Suzuki, H.; Tanaka, T.; Asano, T.; Kaneko, M.K.; Kato, Y. Development of an Anti-Mouse CCR8 Monoclonal Antibody (C(8)Mab-1) for Flow Cytometry and Immunocytochemistry. *Monoclon Antib Immunodiagn Immunother* **2022**, doi:10.1089/mab.2021.0069.
33. Suzuki, H.; Saito, M.; Asano, T.; Tanaka, T.; Kitamura, K.; Kudo, Y.; Kaneko, M.K.; Kato, Y. C(8)Mab-3: An Anti-Mouse CCR8 Monoclonal Antibody for Immunocytochemistry. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 110-114, doi:10.1089/mab.2022.0002.
34. Tanaka, T.; Nanamiya, R.; Takei, J.; Nakamura, T.; Yanaka, M.; Hosono, H.; Sano, M.; Asano, T.; Kaneko, M.K.; Kato, Y. Development of Anti-Mouse CC Chemokine Receptor 8 Monoclonal Antibodies for Flow Cytometry. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 65-70, doi:10.1089/mab.2021.0005.
35. Nanamiya, R.; Takei, J.; Asano, T.; Tanaka, T.; Sano, M.; Nakamura, T.; Yanaka, M.; Hosono, H.; Kaneko, M.K.; Kato, Y. Development of Anti-Human CC Chemokine Receptor 9 Monoclonal Antibodies for Flow Cytometry. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 101-106, doi:10.1089/mab.2021.0007.
36. Kitamura, K.; Suzuki, H.; Kaneko, M.K.; Kato, Y. Cx(6)Mab-1: A Novel Anti-Mouse CXCR6 Monoclonal Antibody Established by N-Terminal Peptide Immunization. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 133-141, doi:10.1089/mab.2022.0010.
37. Tanaka, T.; Li, G.; Asano, T.; Kaneko, M.K.; Suzuki, H.; Kato, Y. Epitope Mapping of the Anti-Human CCR2 Monoclonal Antibody C(2)Mab-9. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 150-156, doi:10.1089/mab.2022.0012.
38. Asano, T.; Suzuki, H.; Tanaka, T.; Kaneko, M.K.; Kato, Y. Identification of the Binding Epitope of an Anti-mouse CCR4 Monoclonal Antibody, C(4)Mab-1. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 214-220, doi:10.1089/mab.2022.0015.
39. Takei, J.; Asano, T.; Li, G.; Saito, M.; Suzuki, H.; Kaneko, M.K.; Kato, Y. Epitope Mapping of an Anti-Human CCR9 Monoclonal Antibody (C(9)Mab-1) Using Enzyme-Linked Immunosorbent Assay. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 239-242, doi:10.1089/mab.2021.0037.
40. Fujii, Y.; Kaneko, M.K.; Ogasawara, S.; Yamada, S.; Yanaka, M.; Nakamura, T.; Saidoh, N.; Yoshida, K.; Honma, R.; Kato, Y. Development of RAP Tag, a Novel Tagging System for Protein Detection and Purification. *Monoclon Antib Immunodiagn Immunother* **2017**, *36*, 68-71, doi:10.1089/mab.2016.0052.

41. Miura, K.; Yoshida, H.; Nosaki, S.; Kaneko, M.K.; Kato, Y. RAP Tag and PMab-2 Antibody: A Tagging System for Detecting and Purifying Proteins in Plant Cells. *Front Plant Sci* **2020**, *11*, 510444, doi:10.3389/fpls.2020.510444.
42. Fujii, Y.; Kaneko, M.K.; Kato, Y. MAP Tag: A Novel Tagging System for Protein Purification and Detection. *Monoclon Antib Immunodiagn Immunother* **2016**, *35*, 293-299, doi:10.1089/mab.2016.0039.
43. Wakasa, A.; Kaneko, M.K.; Kato, Y.; Takagi, J.; Arimori, T. Site-specific epitope insertion into recombinant proteins using the MAP tag system. *J Biochem* **2020**, *168*, 375-384, doi:10.1093/jb/mvaa054.
44. Ye, J.; Kohli, L.L.; Stone, M.J. Characterization of binding between the chemokine eotaxin and peptides derived from the chemokine receptor CCR3. *J Biol Chem* **2000**, *275*, 27250-27257, doi:10.1074/jbc.M003925200.
45. Millard, C.J.; Ludeman, J.P.; Canals, M.; Bridgford, J.L.; Hinds, M.G.; Clayton, D.J.; Christopoulos, A.; Payne, R.J.; Stone, M.J. 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, doi:10.1016/j.str.2014.08.023.
46. Shen, H.H.; Xu, F.; Zhang, G.S.; Wang, S.B.; Xu, W.H. CCR3 monoclonal antibody inhibits airway eosinophilic inflammation and mucus overproduction in a mouse model of asthma. *Acta Pharmacol Sin* **2006**, *27*, 1594-1599, doi:10.1111/j.1745-7254.2006.00446.x.