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

DOI: 10.1089/mab.2022.0035

Epitope Mapping of the Novel Anti-Human CCR9 Monoclonal Antibody (C_9Mab-11) by 2×Alanine Scanning

Yu Isoda,[1,*] Tomohiro Tanaka,[1,*] Hiroyuki Suzuki,[2] Teizo Asano,[1] Kaishi Kitamura,[2] Yuma Kudo,[2] Ryo Ejima,[2] Kazuki Ozawa,[2] Takeo Yoshikawa,[3] Mika K. Kaneko,[1] and Yukinari Kato[1–3]

We recently developed a novel anti-human C-C chemokine receptor 9 (hCCR9) monoclonal antibody (mAb), C_9Mab-11, which is applicable to flow cytometry, western blotting, and enzyme-linked immunosorbent assay (ELISA). This study aims to identify the binding epitope of C_9Mab-11 by using 1×and 2×alanine (or glycine) substituted-hCCR9 peptides (1×and 2×Ala-scan) by ELISA. According to the 1×Ala-scan analysis, the response of C_9Mab-11 was diminished against M13A of the hCCR9 peptide, but was not eliminated. In the 2×Ala-scan analysis, the reactions were abolished in the substitution of P11A-N12A, N12A-M13A, and M13A-A14G of hCCR9 N-terminal peptides. The results indicate that the binding epitope of C_9Mab-11 includes Pro11, Asn12, Met13, and Ala14 of hCCR9, with the region around Met13 being particularly important. The successful identification of the C_9Mab-11 epitope might be useful for the future pathophysiological analysis of hCCR9.

Keywords: human CCR9, monoclonal antibody, epitope, ELISA, 2×Ala-scan

Introduction

The C-C chemokine receptor 9 (CCR9) is a member of the G protein-coupled receptors (GPCRs), which are highly expressed in T cells, thymocytes, B cells, plasmacytoid dendritic cells (pDCs), and intestinal cells.[1] Chemokine ligand 25 (CCL25), formerly thymus-expressed chemokine (TECK), regulates the activation and infiltration of immature T cells by binding to CCR9.[2,3] CCL25 is the only known ligand for CCR9 and is mainly secreted from the epithelial cells of the thymus and small intestine. In addition, CCR9+ T cells are gut-homing T cells because the CCR9 and CCL25 interactions potently recruit T cells to intestinal tissues.[4–6] CCR9 on immune cells guides the spatial organization and cellular communications of immune cells within tissues.

The CCR9/CCL25 axis is involved in the development of various inflammatory diseases. The increase in CCR9+ monocytes was confirmed in the synovium of patients with rheumatoid arthritis.[7] In the ovalbumin-induced allergic inflammation model, the recruitment of eosinophils and T cells was impaired at the peribronchial and perivenular levels in CCR9-deficient mice.[8] In hepatitis, CCR9+ macrophages trigger acute liver damage by collaborating with helper T1 (Th1) cells.[9] However, CCR9 axis inhibition avoided pDCs capture in the small intestine, enhanced pDCs infiltration into the liver, and protected against liver injury.[10] Therefore, managing the CCR9/CCL25 axis could be a target for immunotherapy in numerous inflammation-associated disorders.

CCR9 is highly expressed in T cell acute lymphoblastic leukemia (T-ALL), contributing to the progression of T-ALL.[11] Furthermore, CCR9 expression has been confirmed with high probability in melanomas that have metastasized to the intestine.[12,13] Thus, CCR9 is considered a therapeutic target for cancer. In the T-ALL-bearing mouse model, an anti-human CCR9 monoclonal antibody (hCCR9 mAb) (92R) and humanized 92R (Srb1) inhibited tumor growth and prolonged the survival of mice.[14,15] A clinical study of the CCR9 antagonist CCX282-B against intestinal bowel disease was conducted.[16,17] However, there are still no approved drugs despite CCR9 being an attractive target.

Previously, we developed various anti-mouse and anti-human GPCR mAbs,[18–25] including an anti-hCCR9 mAb (clone C_9Mab-11).[26] We further characterized the binding

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.

epitopes of these mAbs.[27–29] The binding epitope of Cx_6Mab-1, an anti-mouse CXCR6 mAb, was identified by enzyme-linked immunosorbent assay (ELISA) using the two alanine scanning method (2×Ala-scan).[29] We demonstrated that the 2×Ala-scan was an effective method for epitope mapping of mAbs.

In this study, we determined the epitope of C_9Mab-11 using the 1×and 2×Ala-scan strategies.

Materials and Methods

Peptides

The hCCR9 (Accession No. NM_031200) peptide ($_4$-TDFTSPIPNMADDYGSEST-$_{22}$), one-alanine (or glycine) residue-substituted peptide (Supplementary Table S1), and two alanine (or glycine) residue-substituted peptides (Table 1) were synthesized using PEPScreen (Sigma-Aldrich Corp., St. Louis, MO).

Enzyme-linked immunosorbent assay

Synthesized hCCR9 peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham, MA) at a 1 μg/mL concentration for 30 minutes at 37°C. After washing with phosphate-buffered saline containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc., Kyoto, Japan), the wells were blocked with 1% bovine serum albumin-containing PBST for 30 minutes at 37°C. The plates were incubated with 1 μg/mL of C_9Mab-11, followed by peroxidase-conjugated anti-mouse immunoglobulin (1:2000 diluted; Agilent Technologies Inc., Santa Clara, CA). Enzymatic reactions were performed using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.). The optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Table 1. Identification of the C_9Mab-11 Epitope Using 2×Alanine-Substituted hCCR9 Peptides

Peptides	*Sequences*	*C_9Mab-11*
WT	TDFTSPIPNMADDYGSEST	+++
T4A–D5A	AAFTSPIPNMADDYGSEST	+++
D5A–F6A	TAATSPIPNMADDYGSEST	+++
F6A–T7A	TDAASPIPNMADDYGSEST	+++
T7A–S8A	TDFAAPIPNMADDYGSEST	+++
S8A–P9A	TDFTAAIPNMADDYGSEST	+++
P9A–I10A	TDFTSAAPNMADDYGSEST	+++
I10A–P11A	TDFTSPAANMADDYGSEST	+++
P11A–N12A	TDFTSPIAAMADDYGSEST	-
N12A–M13A	TDFTSPIPAAADDYGSEST	-
M13A–A14G	TDFTSPIPNAGDDYGSEST	-
A14G–D15A	TDFTSPIPNMGADYGSEST	+++
D15A–D16A	TDFTSPIPNMAAAYGSEST	+++
D16A–Y17A	TDFTSPIPNMADAAGSEST	+++
Y17A–G18A	TDFTSPIPNMADDAASEST	+++
G18A–S19A	TDFTSPIPNMADDYAAEST	+++
S19A–E20A	TDFTSPIPNMADDYGAAST	+++
E20A–S21A	TDFTSPIPNMADDYGSAAT	+++
S21A–T22A	TDFTSPIPNMADDYGSEAA	+++

+++, OD655≧0.3; -, OD655<0.1.
hCCR9, human C-C chemokine receptor 9; WT, wild-type.

Results and Discussion

Epitope mapping of C_9Mab-11 with alanine-substituted hCCR9 peptides

We previously established an anti-hCCR9 mAb (clone C_9Mab-11) by immunizing the hCCR9 N-terminal peptide ($_4$-TDFTSPIPNMADDYGSEST-$_{22}$) + C-terminal cysteine residue with keyhole limpet hemocyanin.[26] To investigate the binding epitope of C_9Mab-11, we first synthesized one-alanine- (or glycine-) substituted peptides of hCCR9, referred to as the 1×Ala-scan method (Supplementary Table S1). C_9Mab-11 reacted with all one-alanine- (or glycine-) substituted and wild-type (WT) hCCR9 peptides. However, compared with other substituted peptides, the reaction of C_9Mab-11 against M13A was attenuated (Supplementary Fig. S1).

Furthermore, we synthesized 18 peptides, substituting two amino acid sequences with two alanine (or glycine) residues (2×Ala-scan method). For instance, a peptide (T4A–D5A) indicates the alanine substitution of the fourth Thr and the fifth Asp of the hCCR9 peptide (Table 1). As shown in Figure 1A, C_9Mab-11 exhibited reactions with T4A–D5A, D5A–F6A, F6A–T7A, T7A–S8A, S8A–P9A, P9A–I10A, I10A–P11A, A14G–D15A, D15A–D16A, D16A–Y17A, Y17A–G18A, G18A–S19A, S19A–E20A, E20A–S21A, S21A–T22A, and WT of hCCR9 peptides. However, C_9Mab-11 did not react with P11A–N12A, N12A–M13A, and M13A–A14G (Fig. 1A), indicating that Pro11, Asn12, Met13, and Ala14 are included in the critical epitope of C_9Mab-11. The results are summarized in Table 1. Figure 1B describes a schematic illustration of hCCR9 and the estimated binding epitope of C_9Mab-11.

The 2×Ala-scan is a useful method for identifying the epitope of mAbs. Considering the results of the 1×Ala-scan (Supplementary Fig. S1), we speculate that amino acids in the region centered on Met13 of hCCR9 are pivotal epitope for C_9Mab-11 binding. Anti-hCCR9 mAbs, 91R (mouse IgG_{2b}), and 92R (mouse IgG_{2a}) generated by immunization of plasmid bearing-hCCR9 cDNA using the gene gun method have been reported to possess antitumor effects in mouse models.[14,15,30] The hotspot of epitopes of 91R and 92R are $_{11}$-PNMADD-$_{16}$ of hCCR9,[14] consistent with the epitope of C_9Mab-11 in this study (Fig. 1). Since the subclass of C_9Mab-11 is also mouse IgG_{2a},[26] C_9Mab-11 is expected to have an antitumor effect on CCR9-expressing cancers. In addition to antitumor activity, chimeric antigen receptor-T cells that target hCCR9 have high antitumor activity against T-ALL.[31] C_9Mab-11 could contribute to the development of novel immunotherapy modalities.

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

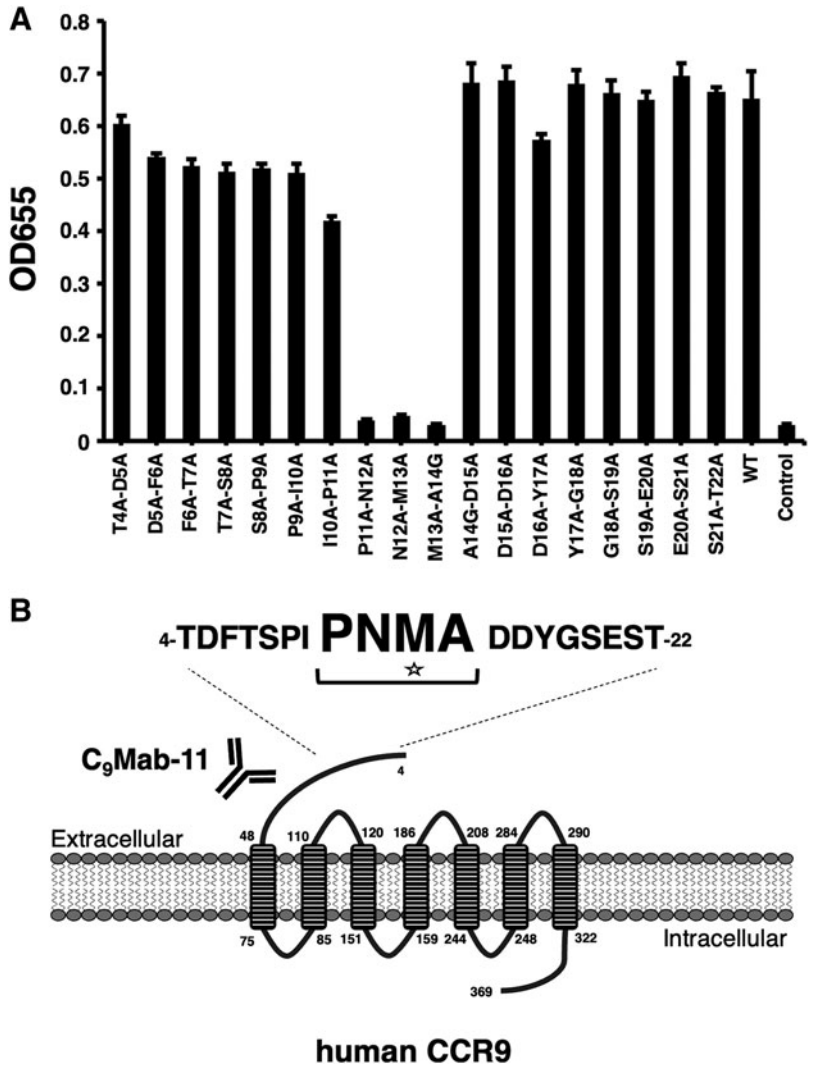

FIG. 1. Epitope identification of the C_9Mab-11 by ELISA using the 2×Ala-scan method. **(A)** The 2×alanine-substituted and WT of hCCR9 peptides were immobilized on immunoplates. The plates were incubated with C_9Mab-11 (1 μg/mL), followed by peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Schematic illustration of hCCR9 and the critical epitope of C_9Mab-11. The C_9Mab-11 epitope involves Pro11, Asn12, Met13, and Ala14 of hCCR9. Star describes the estimated epitope from Supplementary Figure S1. 2×Ala-scan, 2×alanine scanning; ELISA, enzyme-linked immunosorbent assay; hCCR9, human C-C chemokine receptor 9; WT, wild-type.

Research (KAKENHI) grant nos. 21K20789 (to T.T.), 22K06995 (to H.S.), 21K07168 (to M.K.K.), and 22K07224 (to Y.K.).

Supplementary Material

Supplementary Table S1
Supplementary Figure S1

References

1. Ozga AJ, Chow MT, Luster AD. Chemokines and the immune response to cancer. Immunity 2021;54:859–874.
2. Tu Z, Xiao R, Xiong J, et al. CCR9 in cancer: Oncogenic role and therapeutic targeting. J Hematol Oncol 2016;9:10.
3. Wurbel MA, Malissen M, Guy-Grand D, et al. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes. Blood 2001;98:2626–2632.
4. Zaballos A, Gutiérrez J, Varona R, et al. Cutting edge: Identification of the orphan chemokine receptor GPR-9-6 as CCR9, the receptor for the chemokine TECK. J Immunol 1999;162:5671–5675.
5. Wurbel MA, Philippe JM, Nguyen C, et al. The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9. Eur J Immunol 2000; 30:262–271.
6. Campbell DJ, Butcher EC: Intestinal attraction: CCL25 functions in effector lymphocyte recruitment to the small intestine. J Clin Invest 2002;110:1079–1081.
7. Schmutz C, Cartwright A, Williams H, et al. Monocytes/macrophages express chemokine receptor CCR9 in rheumatoid arthritis and CCL25 stimulates their differentiation. Arthritis Res Ther 2010;12:R161.

8. López-Pacheco C, Soldevila G, Du Pont G, et al. CCR9 Is a key regulator of early phases of allergic airway inflammation. Mediators Inflamm 2016;2016:3635809.
9. Nakamoto N, Ebinuma H, Kanai T, et al. CCR9+ macrophages are required for acute liver inflammation in mouse models of hepatitis. Gastroenterology 2012;142:366–376.
10. Koda Y, Nakamoto N, Chu PS, et al. CCR9 axis inhibition enhances hepatic migration of plasmacytoid DCs and protects against liver injury. JCI Insight 2022;7(17):e159910.
11. Qiuping Z, Qun L, Chunsong H, et al. Selectively increased expression and functions of chemokine receptor CCR9 on CD4+ T cells from patients with T-cell lineage acute lymphocytic leukemia. Cancer Res 2003;63:6469–6477.
12. Amersi FF, Terando AM, Goto Y, et al. Activation of CCR9/CCL25 in cutaneous melanoma mediates preferential metastasis to the small intestine. Clin Cancer Res 2008; 14:638–645.
13. Hwang ST: Chemokine receptors in melanoma: CCR9 has a potential role in metastasis to the small bowel. J Invest Dermatol 2004;122:xiv–xv.
14. Somovilla-Crespo B, Martín Monzón MT, Vela M, et al. 92R Monoclonal antibody inhibits human CCR9(+) leukemia cells growth in NSG mice xenografts. Front Immunol 2018;9:77.
15. Santamaria S, Delgado M, Botas M, et al. Therapeutic potential of an anti-CCR9 mAb evidenced in xenografts of human CCR9(+) tumors. Front Immunol 2022;13:825635.
16. Zhang J, Romero J, Chan A, et al. Biarylsulfonamide CCR9 inhibitors for inflammatory bowel disease. Bioorg Med Chem Lett 2015;25:3661–3664.
17. Walters MJ, Wang Y, Lai N, et al. Characterization of CCX282-B, an orally bioavailable antagonist of the CCR9 chemokine receptor, for treatment of inflammatory bowel disease. J Pharmacol Exp Ther 2010;335:61–69.
18. Asano T, Suzuki H, Tanaka T, et al. C(3)Mab-3: A monoclonal antibody for mouse CC chemokine receptor 3 for flow cytometry. Monoclon Antib Immunodiagn Immunother 2022;41:74–79.
19. 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.
20. Nanamiya R, Takei J, Asano T, et al. Development of anti-human CC chemokine receptor 9 monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:101–106.
21. Tanaka T, Li G, Saito M, et al. 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.
22. Takei J, Suzuki H, Asano T, et al. 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.
23. Suzuki H, Saito M, Asano T, et al. C(8)Mab-3: An anti-mouse CCR8 monoclonal antibody for immunocytochemistry. Monoclon Antib Immunodiagn Immunother 2022;41: 110–114.
24. Kitamura K, Suzuki H, Kaneko MK, et al. 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.
25. Saito M, Suzuki H, Tanaka T, et al. Development of an anti-mouse CCR8 monoclonal antibody (C(8)Mab-1) for flow cytometry and immunocytochemistry. Monoclon Antib Immunodiagn Immunother 2022;41(6):333–338; doi: 10 .1089/mab.2021.0069
26. Tanaka T, Isoda Y, Suzuki H, et al. Development of a sensitive anti-human CCR9 monoclonal antibody (C9Mab-11) by N-terminal peptide immunization. Monoclon Antib Immunodiagn Immunother 2022;41(6):303–310; doi: 10 .1089/mab.2022.0027
27. Takei J, Asano T, Li G, et al. 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.
28. Tanaka T, Li G, Asano T, et al. Epitope mapping of the anti-human CCR2 monoclonal antibody C(2)Mab-9. Monoclon Antib Immunodiagn Immunother 2022;41:150–156.
29. Isoda Y, Tomohiro T, Suzuki H, et al. Epitope mapping of an anti-mouse CXCR6 monoclonal antibody (Cx6Mab-1) using the 2×Alanine scanning method. Monoclon Antib Immunodiagn Immunother 2022;41(5):275–278; doi: 10 .1089/mab.2022.0019
30. Chamorro S, Vela M, Franco-Villanueva A, et al. Antitumor effects of a monoclonal antibody to human CCR9 in leukemia cell xenografts. MAbs 2014;6:1000–1012.
31. Maciocia PM, Wawrzyniecka PA, Maciocia NC, et al. Anti-CCR9 chimeric antigen receptor T cells for T-cell acute lymphoblastic leukemia. Blood 2022;140:25–37.

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

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

Received: October 2, 2022
Accepted: March 21, 2023