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Original Articles

Epitope Mapping of an Anti-Human CCR9 Monoclonal Antibody (C_9Mab-1) Using Enzyme-Linked Immunosorbent Assay

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

One of G protein-coupled receptors, CCR9, is mainly expressed in the thymocytes and the small bowel. The ligand of CCR9 is CCL25 (TECK), and the CCR9–CCL25 axis controls T cell maturation and intestinal immune response. CCR9 is related to graft-versus-host disease and autoimmune diseases. Recent studies have been reported that CCR9 is also associated with tumor proliferation, apoptosis, migration, and drug resistance. Therefore, CCR9-targeting therapy is receiving a lot of attention. Previously, we developed an anti-human CCR9 (hCCR9) monoclonal antibody, C_9Mab-1 (IgG_1, kappa), which can be used for flow cytometry, by immunizing mice with hCCR9-overexpressed Chinese hamster ovary-K1 cells. In this study, we examined the critical epitope of C_9Mab-1, using enzyme-linked immunosorbent assay (ELISA) with synthesized peptides. First, we performed ELISA with deletion mutants, and C_9Mab-1 reacted to the 1–20 amino acids sequence of hCCR9. Next, we analyzed the reaction to 20 point mutants, and C_9Mab-1 did not recognize the alanine-substituted peptides of I10A, P11A, N12A, M13A, A14G, D16A, and Y17A. The results indicate that the binding epitope of C_9Mab-1 includes Ile10, Pro11, Asn12, Met13, Ala14, Asp16, and Tyr17 of hCCR9.

Keywords: human CCR9, C_9Mab-1, epitope mapping, monoclonal antibody, enzyme-linked immunosorbent assay

Introduction

Chemokines are 8–14 kDa protein, which regulate the development, homeostasis, and function of the immune system. They are divided into four subfamilies of C, CC, CXC, and CX3C based on the differences of two cysteine positions at the N-terminus.[1,2] More than 50 chemokines and 20 chemokine receptors have been found so far.[3] Chemokine receptors are G protein-coupled receptors with seven helical transmembrane regions. CCR9 is one of the chemokine receptors with a molecular weight of ~40,000.[4]

CCR9 is mainly expressed on developing thymocytes and the small intestine.[5,6] Although many chemokines recognize multiple receptors, and a single receptor can bind multiple chemokines, CCL25 (TECK) is the only ligand for CCR9.[7,8] CCR9–CCL25 interaction plays important roles in proper T cell development and intestinal immune response and inflammation.[7,9,10]

It has been reported that CCR9 expression is associated with various diseases. $CCR9^+$ plasmacytoid dendritic cells induce regulatory T cell function, resulting in suppression of antigen-specific immune responses, including acute graft-versus-host disease.[11] In patients with small bowel Crohn's or celiac disease, but not in patients with purely colonic Crohn's disease, $CCR9^+$ lymphocytes were elevated remarkably in peripheral blood lymphocytes. In contrast, $CCR9^+$ T cells in inflamed small intestine were significantly lower than in normal small intestinal mucosa.[12] Moreover, in the past few years, it has been discovered that CCR9–CCL25 interaction is related to tumor proliferation, metastasis, and drug resistance.

Departments of [1]Antibody Drug Development and [2]Molecular Pharmacology, Tohoku University Graduate School of Medicine, Sendai, Japan.
[i]ORCID ID (https://orcid.org/0000-0001-5385-8201).

CCR9 is highly expressed in a variety of cancers, such as T-lineage acute lymphoblastic leukemia, large B cell lymphoma, melanoma, ovarian cancer, and breast cancer.[4] Shen et al. reported that pancreatic cancer cells expressed CCR9, and its activation enhances proliferation of these cells.[13] Cutaneous melanoma has a clear pattern of metastasis to the small intestine, which is caused by activation of CCR9.[14] CCR9–CCL25 interaction also mediates PI3K/AKT-dependent anti-apoptotic signals in prostate cancer cells, resulting in low apoptosis and modest chemotherapeutic response.[15] Therefore, CCR9 has been expected to be a new target for cancer treatment.

In a previous study, we established an anti-human CCR9 (hCCR9) monoclonal antibody (mAb), C_9Mab-1 (mouse IgG_1, kappa), which is useful for flow cytometry by using a Cell-Based Immunization and Screening (CBIS) method.[16] To clarify further characteristics of C_9Mab-1, we herein performed epitope mapping using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

hCCR9 peptides

hCCR9 peptides (accession No.: NM_031200), including 4 deletion mutants (Table 1) and 20 point mutants (Table 2), were synthesized by utilizing PEPScreen (Sigma-Aldrich Corp., St. Louis, MO). One cysteine residue was replaced by serine: C38S.

Enzyme-linked immunosorbent assay

Synthesized hCCR9 peptides (Tables 1 and 2) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham, MA) at a concentration of 10 μg/mL for 30 minutes at 37°C. After washing with phosphate-buffered saline containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc., Kyoto, Japan), 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-1, followed by a peroxidase-conjugated anti-mouse immunoglobulins (1:2000 diluted; Agilent Technologies, Inc., Santa Clara, CA). Enzymatic reactions were performed using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Results

Epitope mapping of C_9Mab-1 using deletion mutants

We previously established an anti-hCCR9 mAb (C_9Mab-1), which is useful for flow cytometry by the CBIS method.[16] To reveal the binding epitope of C_9Mab-1, we synthesized 4 peptides (Table 1), which consist of 1–20 amino acids (aa), 11–30 aa, 21–40 aa, and 31–48 aa of hCCR9, and performed ELISA. The results showed that C_9Mab-1 recognized the 1–20 aa ($_1$-MTPTDFTSPIPN MADDYGSE-$_{20}$) sequence of hCCR9 (Fig. 1A). The results are summarized in Figure 1B.

Table 1. Identification of C_9Mab-1 Epitope Using Deletion Mutants by Enzyme-Linked Immunosorbent Assay

Peptides	*Sequence*	*C_9Mab-1*
1–20	MTPTDFTSPIPNMADDYGSE	+++
11–30	PNMADDYGSESTSSMEDYVN	–
21–40	STSSMEDYVNFNFTDFYSEK	–
31–48	FNFTDFYSEKNNVRQFAS	–

+++, OD655 $\geqq$0.6; –, OD655 < 0.1.

Table 2. Identification of C_9Mab-1 Epitope Using Point Mutants by Enzyme-Linked Immunosorbent Assay

Mutant peptides	*Sequence*	*C_9Mab-1*
M01A	ATPTDFTSPIPNMADDYGSE	+++
T02A	MAPTDFTSPIPNMADDYGSE	+++
P03A	MTATDFTSPIPNMADDYGSE	+++
T04A	MTPADFTSPIPNMADDYGSE	+++
D05A	MTPTAFTSPIPNMADDYGSE	+++
F06A	MTPTDATSPIPNMADDYGSE	+++
T07A	MTPTDFASPIPNMADDYGSE	+++
S08A	MTPTDFTAPIPNMADDYGSE	+++
P09A	MTPTDFTSAIPNMADDYGSE	+++
I10A	MTPTDFTSPAPNMADDYGSE	–
P11A	MTPTDFTSPIANMADDYGSE	–
N12A	MTPTDFTSPIPAMADDYGSE	–
M13A	MTPTDFTSPIPNAADDYGSE	–
A14G	MTPTDFTSPIPNMGDDYGSE	–
D15A	MTPTDFTSPIPNMAADYGSE	+
D16A	MTPTDFTSPIPNMADAYGSE	–
Y17A	MTPTDFTSPIPNMADDAGSE	–
G18A	MTPTDFTSPIPNMADDYASE	+++
S19A	MTPTDFTSPIPNMADDYGAE	+++
E20A	MTPTDFTSPIPNMADDYGSA	+++

+++, OD655 $\geqq$0.6; +, 0.1$\leqq$ OD655 < 0.3; –, OD655 < 0.1.

Epitope mapping of C_9Mab-1 using point mutants

Then, we synthesized 20 point mutant peptides of 1–20 aa of hCCR9 (Table 2). C_9Mab-1 exhibited reaction with M01A, T02A, P03A, T04A, D05A, F06A, T07A, S08A, P09A, D15A, G18A, S19A, E20A, and wild-type 1–20 aa (Fig. 2A). In contrast, C_9Mab-1 did not react with I10A, P11A, N12A, M13A, A14G, D16A, and Y17A (Fig. 2A), indicating that Ile10, Pro11, Asn12, Met13, Ala14, Asp16, and Tyr17 are included in the critical epitope of C_9Mab-1. The results are summarized in Figure 2B.

Discussion

CCR9 is a seven-transmembrane receptor with four extracellular domains. Among the four extracellular regions, the first one at N-terminus is the largest with 48 aa, and the others are relatively small. So, we hypothesized that the epitope of C_9Mab-1 might be located at the extracellular region of the N-terminus. ELISA revealed that Ile10, Pro11, Asn12, Met13, Ala14, Asp16, and Tyr17 were critical aa, which were included in the epitope of C_9Mab-1.

Information about the binding epitope is important to elucidate the functional effect of mAbs. The ligand binding sites of several chemokine receptors have been defined, and the studies showed that the N-terminus of some receptors, such as CCR2, CCR3, CCR5, and CXCR1, is important for

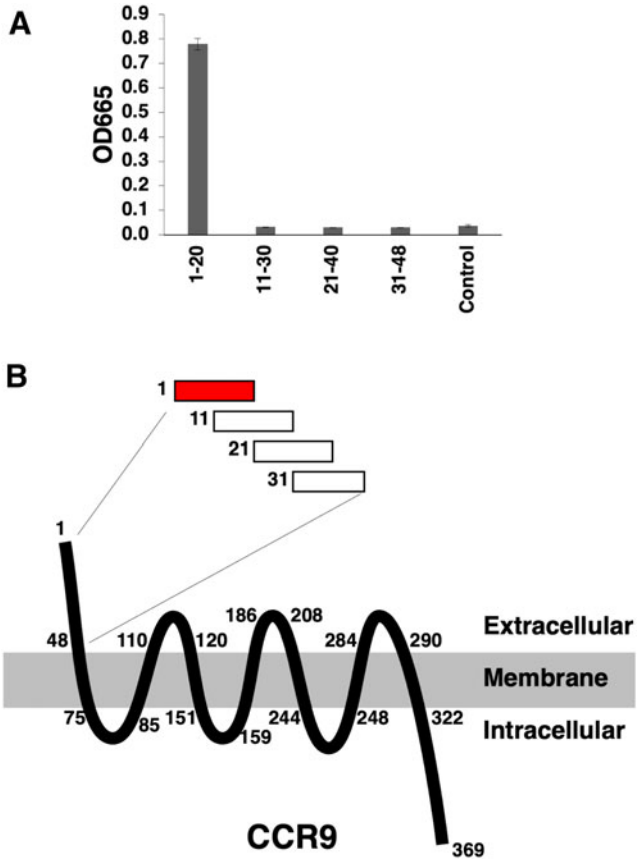

FIG. 1. Determination of the C_9Mab-1 epitope for hCCR9 by ELISA using deletion mutants. **(A)** Synthesized peptides of hCCR9 were immobilized on immunoplates. The plates were incubated with C_9Mab-1 (1 μg/mL), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Schematic illustration of hCCR9 and the C_9Mab-1 epitope. ELISA, enzyme-linked immunosorbent assay; hCCR9, human CCR9.

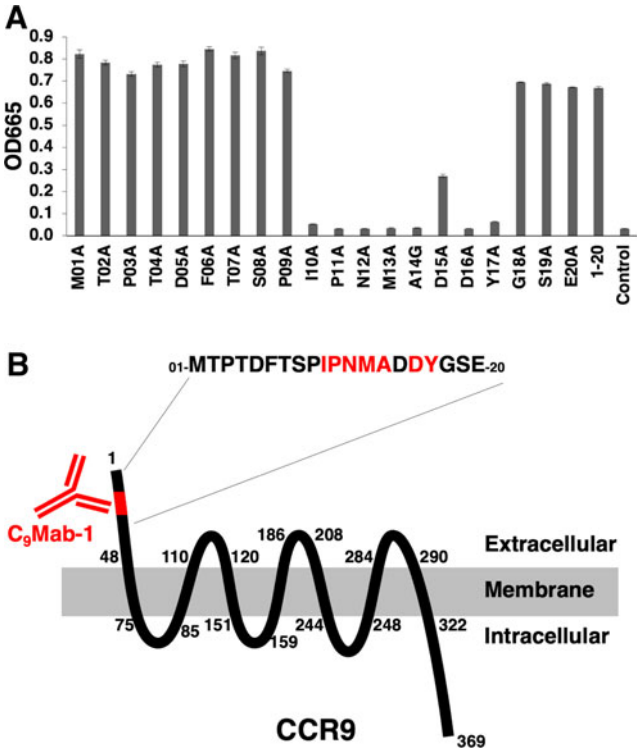

FIG. 2. Determination of the C_9Mab-1 epitope of hCCR9 by ELISA using point mutants. **(A)** Synthesized peptides of hCCR9 were immobilized on immunoplates. The plates were incubated with C_9Mab-1 (1 μg/mL), followed by peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Schematic illustration of hCCR9 and the C_9Mab-1 epitope. The C_9Mab-1 epitope of hCCR9 involves Ile10, Pro11, Asn12, Met13, Ala14, Asp16, and Tyr17.

ligand binding.[8] Some studies showed that the mAbs against the N-terminus of CCR9 inhibited the interaction between CCR9 and CCL25,[7,17] therefore, the N-terminus of CCR9 also appears to be important for ligand binding. Chamorro et al. established an anti-hCCR9 mAb, clone 91R (mouse IgG_{2b}), which recognized the N-terminal region of CCR9 by gene gun immunization with full-length hCCR9 coding sequence inserted in a eukaryotic expression vector.

The clone 91R showed antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity against acute T lymphoblastic leukemia MOLT-4 cells and significantly inhibited the tumor growth of xenografts.[17] From these studies, it is expected that C_9Mab-1 has an ability to block CCR9–CCL25 binding and antitumor activities against CCR9-expressing cells. We will examine these activities of C_9Mab-1 in a future study.

In this study, we investigated the binding epitope of C_9Mab-1 using synthesized peptides. Peptide scanning is simple, relatively inexpensive, and useful for determining a linear epitope. However, peptide structure is unfolded and different from native proteins, so, it is better to examine using other ways together. X-ray crystallography is the most accurate method for epitope mapping; however, this method requires difficult techniques and costs money and time.[18]

In a previous study, we developed a novel epitope mapping method named RIEDL insertion for epitope mapping (REMAP)[19,20] using a RIEDL tag system.[21] This method is useful for determining both linear and conformational epitopes. In a future study, we will also investigate the critical epitope of C_9Mab-1 using the REMAP method and compare those results.

Author Disclosure Statement

No competing financial interests exist.

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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: yukinarikato@med.tohoku.ac.jp

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