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Identification of the Binding Epitope of an Anti-mouse CCR4 Monoclonal Antibody, C_4Mab-1

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C-C chemokine receptor 4 (CCR4) is one of G protein-coupled receptors, and interacts with chemokines, CCL17 and CCL22. CCR4 is expressed on T cells such as helper T type 2 cells, regulatory T cells, and interleukin 17-producing T helper cells. CCR4 is associated with T cells trafficking into the tumor microenvironment, and is associated with tumor progression or metastasis. Therefore, CCR4 may be a potential thera-peutic option for T cell malignancies. C_4Mab-1 is a novel anti-mouse CCR4 (mCCR4) monoclonal antibody produced by mCCR4 N-terminal peptide immunization. C_4Mab-1 is useful for flow cytometric analysis. In this study, we conducted the epitope mapping of C_4Mab-1 using enzyme-linked immunosorbent assay (ELISA) and peptide blocking assay. The result of ELISA indicated that Thr7, Asp8, and Gln11 of mCCR4 are the critical amino acids for the C_4Mab-1 binding. Furthermore, peptide blocking assay by flow cytometry showed that Thr7, Asp8, and Gln11 of mCCR4 are essential for C_4Mab-1 binding to mCCR4-overexpressed Chinese hamster ovary-K1 (CHO/mCCR4) cells, and Val6, Thr9, and Thr10 are involved in the C_4Mab-1 binding to CHO/mCCR4 cells. These results indicate that the critical binding epitope of C_4Mab-1 includes Thr7, Asp8, and Gln11 of mCCR4.

Keywords: human CCR4, C_4Mab-1, epitope, monoclonal antibody, enzyme-linked immunosorbent assay, flow cytometry

Introduction

Chemokines are a family of small cytokines secreted by cells, and act as a chemoattractant to guide the migration of cells that induce directional movement of leukocytes to sites of inflammation or injury.[1,2] The chemokines also play fundamental roles in function of the immune system.[3] Chemokines interact with chemokine receptor, and this interaction transduces extracellular signals to intracellular signals. Chemokine receptors belong to G protein-coupled receptors, which is characterized by a seven-transmembrane receptor. Because of their crucial role in cell migration, chemokine receptors are regarded as important therapeutic targets for inflammatory diseases and cancer.[4]

C-C chemokine receptor 4 (CCR4) is one of a chemokine receptor family. CCR4 is a receptor for CC chemokine ligand 17 (CCL17; thymus and activation-regulated chemokine) and CC chemokine ligand 22 (CCL22; macrophage-derived chemokine).[5] CCR4 is expressed on helper T type 2 (Th2) cells and is upregulated by T cell receptor activation.[6,7] CCR4 is also expressed on other T cell subsets such as regulatory T (Treg) cells and interleukin 17-producing helper T (Th17) cells.[7–10] It has been reported that CCR4 is associated with T cells trafficking into the tumor microenvironment,[11] and is overexpressed on malignant T cells.[12,13]

Moreover, high CCR4+ Treg levels are found in murine and human solid tumors, and/or are associated with tumor progression or metastasis.[14–22] Therefore, CCR4 could be a diagnostic and prognostic marker, and targeting CCR4 has become a promising therapeutic option for T cell malignancies such as adult T cell leukemia/lymphoma (ATLL), and cutaneous T cell lymphomas (CTCLs).[23,24]

Mogamulizumab is a humanized anti-CCR4 monoclonal antibody (mAb) with a defucosylated Fc region.[25,26] Mogamulizumab has an antibody-dependent cellular cytotoxicity (ADCC) activity, and is an effective antibody drug

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for patients with CCR4-positive ATLL and CTCLs.[27,28] Moreover, mogamulizumab binds to the N-terminal residues from 12th to 29th of human CCR4.[28] Furthermore, it has been reported that the N-terminus of the chemokine receptor, such as CCR2, CCR3, CCR5, and CXCR1, is important for the ligand binding.[2,29] Therefore, N-terminus of CCR4 is an attractive target region for producing anti-CCR4 mAbs.

Previously, we produced a novel anti-mouse CCR4 (mCCR4) mAb, C_4Mab-1 (rat IgG_1, kappa), using the mCCR4 N-terminal peptide immunization.[30] C_4Mab-1 reacted with mCCR4-overexpressed Chinese hamster ovary-K1 (CHO/mCCR4) cells, P388 (mouse lymphoid neoplasma) cells and J774-1 (mouse macrophage-like) cells in flow cytometry. However, critical amino acids of mCCR4 for C_4Mab-1 binding has not been determined. Epitope identification of mAbs is important to elucidate the pharmacological function of mAbs and is essential to avoid unexpected cross-reactivity. In this study, we conducted the determination of the binding epitope of C_4Mab-1 on mCCR4 using enzyme-linked immunosorbent assay (ELISA) and peptide blocking assay using flow cytometry.

Materials and Methods

Cell lines

CHO-K1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). CHO/mCCR4 was previously established by transfecting plasmid that encodes mCCR4 with PA tag[31–34] at N-terminus and RAP tag[35,36] and MAP tag[37,38] at C-terminus into CHO-K1 cells using the Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). CHO/mCCR4 cells were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) that was supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). Cells were grown in a humidified incubator, which was supplied with 5% CO_2 and 95% air at 37°C.

Enzyme-linked immunosorbent assay

The mCCR4 peptides (Accession No.: NM_009916), including 20 point mutants (Table 1), were synthesized by utilizing PEPScreen (Sigma-Aldrich Corp., St. Louis, MO).[39–61] Each peptide was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at a concentration of 10 μg/mL for 30 minutes at 37°C. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), wells were blocked with 1% bovine serum albumin (BSA)-containing PBST for 30 minutes at 37°C.

The plates were then incubated with C_4Mab-1 (1 μg/mL), followed by a 1:10,000 dilution of peroxidase-conjugated anti-rat immunoglobulins (Sigma-Aldrich Corp.). 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).

Peptide blocking assay

CHO/mCCR4 cells were washed with 0.1% BSA in PBS. C_4Mab-1 (1 μg/mL) was incubated with each peptide (10 μg/mL) for 30 minutes at 4°C. CHO/mCCR4 cells were treated with peptide mixed C_4Mab-1, and further with Alexa Fluor 488-conjugated anti-rat IgG (1:1000). The fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Table 1. Identification of the C_4Mab-1 Epitope Using Alanine-Substituted Mouse C-C Chemokine Receptor 4 Peptides

Peptides	*Sequence*	*Reactivity with C_4Mab-1*
WT	MNATEVTDTTQDETVYNSYY	+++
M1A	ANATEVTDTTQDETVYNSYY	+++
N2A	MAATEVTDTTQDETVYNSYY	+++
A3G	MNGTEVTDTTQDETVYNSYY	+++
T4A	MNAAEVTDTTQDETVYNSYY	+++
E5A	MNATAVTDTTQDETVYNSYY	+++
V6A	MNATEATDTTQDETVYNSYY	++
T7A	MNATEVADTTQDETVYNSYY	–
D8A	MNATEVTATTQDETVYNSYY	–
T9A	MNATEVTDATQDETVYNSYY	+++
T10A	MNATEVTDTAQDETVYNSYY	+++
Q11A	MNATEVTDTTADETVYNSYY	–
D12A	MNATEVTDTTQAETVYNSYY	+++
E13A	MNATEVTDTTQDATVYNSYY	+++
T14A	MNATEVTDTTQDEAVYNSYY	+++
V15A	MNATEVTDTTQDETAYNSYY	+++
Y16A	MNATEVTDTTQDETVANSYY	+++
N17A	MNATEVTDTTQDETVYASYY	+++
S18A	MNATEVTDTTQDETVYNAYY	+++
Y19A	MNATEVTDTTQDETVYNSAY	+++
Y20A	MNATEVTDTTQDETVYNSYA	+++

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

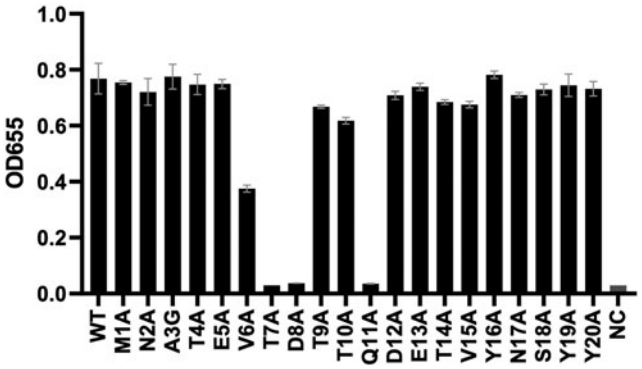

FIG. 1. Determination of the C_4Mab-1 epitope for mCCR4 by ELISA using point mutant peptides. Synthesized peptides of mCCR4 were immobilized on immunoplates. The plates were incubated with C_4Mab-1 (1 μg/mL), followed by incubation with peroxidase-conjugated anti-rat immunoglobulins. ELISA, enzyme-linked immunosorbent assay; NC, negative control; mCCR4, mouse C-C chemokine receptor 4.

Results

Epitope mapping by ELISA

C_4Mab-1 was established using the mCCR4 N-terminal peptide immunization. Therefore, we synthesized 20 alanine-substituted mCCR4 N-terminal peptides to investigate the critical epitope of C_4Mab-1 on mCCR4 (Table 1). Each alanine-substituted mutant peptide was immobilized. None of peptide was immobilized as a negative control (NC). The results of ELISA showed that C_4Mab-1 reacted with 16 alanine-substituted mutant peptides (M1A, N2A, A3G, T4A, E5A, T9A, T10A, D12A, E13A, T14A, V15A, Y16A, N17A, S18A, Y19A, Y20A) as well as the 1–20 amino acids wild-type sequence (WT; positive control) (Fig. 1).

In contrast, C_4Mab-1 did not react with three mutant peptides, T7A, D8A, and Q11A, as well as the NC (Fig. 1), These results indicated that Thr7, Asp8, and Gln11 of mCCR4 are the critical amino acids for the C_4Mab-1 binding. The results are summarized in Table 1.

Peptide blocking assay by flow cytometry

We then performed a peptide blocking assay by flow cytometry. C_4Mab-1 was mixed with each peptide or blocking buffer (–peptide). The reactivity between CHO/mCCR4 and peptide mixed C_4Mab-1 was measured by flow cytometry. C_4Mab-1 reacted with CHO/mCCR4 (Fig. 2A, –peptide). This reaction was completely neutralized by mixing WT, M1A, N2A, A3G, T4A, E5A, D12A, E13A, T14A, V15A, Y16A, N17A, S18A, Y19A, and Y20A peptide with C_4Mab-1, and almost neutralized by mixing V6A, T9A, and T10A peptide with C_4Mab-1. In contrast, T7A, D8A, and Q11A peptides did not block the reaction of C_4Mab-1 with CHO/mCCR4 (Fig. 2A). These results

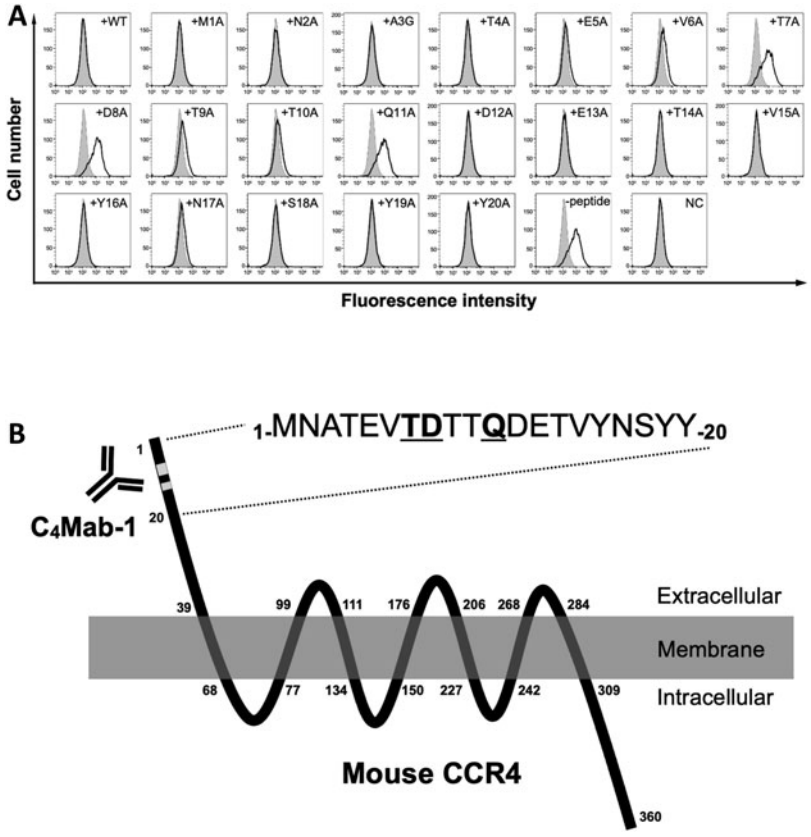

FIG. 2. Determination of the C_4Mab-1 epitope for mCCR4 by peptide blocking assay. **(A)** C_4Mab-1 (1 μg/mL) plus each point mutant peptide (10 μg/mL), control (blocking buffer; –peptide) was reacted with CHO/mCCR4 cells for 30 minutes at 4°C, followed by the addition of secondary antibodies. **(B)** Schematic illustration of mCCR4 and the C_4Mab-1 epitope. Underlines indicate the critical amino acids for C_4Mab-1 binding. CHO, Chinese hamster ovary.

indicated that Thr7, Asp8, and Gln11 of mCCR4 are essential for C_4Mab-1 binding to CHO/mCCR4. The results are summarized in Figure 2B.

Discussion

Epitope identification of mAbs is important to elucidate the pharmacological function of mAbs and is essential to avoid unexpected cross-reactivity. We previously developed an anti-mCCR4 mAb (clone C_4Mab-1) by immunizing the mCCR4 N-terminal peptide.[30] C_4Mab-1 can be applicable to flow cytometric analysis. However, its epitope has not been determined. In this study, we identified the epitope of C_4Mab-1 using two methods, ELISA and peptide blocking assay. The results of ELISA and peptide blocking assay indicated that three amino acids, Thr7, Asp8, and Gln11 of mCCR4, are critical for C_4Mab-1 binding (Figs. 1 and 2).

Molecular targeted therapy is one of the strategies for cancer treatment, and mAb is an important modality for it. The main antitumor mechanisms of mAbs are neutralization of growth factors, ADCC, and complement-dependent cytotoxicity (CDC). In addition, mogamulizumab can deplete Treg cells, which is expected for the application to solid tumors as an immunomodulator.[62,63] To confirm whether C_4Mab-1 can be available for cancer therapy, further investigations are required. For investigation of ADCC and CDC activities, C_4Mab-1 needs to be changed into mouse IgG_{2a}.[64–70]

A neutralizing mAb blocks interaction between a receptor and its ligand. The ligand-binding region of CCR4 to its ligands has not been reported; in contrast, the N-terminal regions of several chemokine receptors, such as CCR2, CCR3, CCR5, and CXCR1, have been determined to be critical for the ligand–receptor binding.[29] Thus, the N-terminus of CCR4 may also be important for the ligand binding. C_4Mab-1 binds to N-terminus region of mCCR4; therefore, C_4Mab-1 is a potential neutralizing mAb. In a future study, we will investigate neutralization activity of C_4Mab-1.

Author Disclosure Statement

No competing financial interests exist.

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