Communication

Identification of the Binding Epitope of an Anti-Mouse CCR6 Monoclonal Antibody (C6Mab-13) Using 1×Alanine Scanning

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Abstract: CC chemokine receptor 6 (CCR6) is one of the members of G protein-coupled receptor (GPCR) family that is upregulated in many immune-related cells, including B lymphocytes, effector and memory T cells, regulatory T cells, and immature dendritic cells. Coordination between CCR6 and its ligand CC motif chemokine ligand 20 (CCL20) is deeply involved in the pathogenesis of various diseases, such as cancer, autoimmune diseases, and psoriasis. Therefore, CCR6 is an attractive target for therapy and is being investigated as a diagnostic marker for patients. In a previous study, we developed an anti-mouse CCR6 (mCCR6) monoclonal antibody (mAb), C6Mab-13 (rat IgG1, kappa), applicable for flow cytometry by immunizing a rat with N-terminal peptide of mCCR6. This study investigated the binding epitope of C6Mab-13 using enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) methods with the synthesized point mutated-peptides within 1-20 amino acids region of mCCR6. In ELISA, C6Mab-13 lost the reaction to the alanine-substituted peptide of D11A. The epitope of C6Mab-13 was identified to be Asp11 in ELISA. Furthermore, in SPR analysis, the dissociation constants (K_D) could not be calculated for G9A and D11A mutants due to lack of binding. The SPR analysis demonstrated that the C6Mab-13 epitope comprises Gly9 and Asp11. Taken together, the key binding epitope of C6Mab-13 was determined to be around Asp11 on mCCR6. Based on the epitope information, C6Mab-13 could be useful for further functional analysis of mCCR6 in future studies.

Keywords: mouse CCR6; monoclonal antibody; epitope; ELISA; SPR

1. Introduction

The CC chemokine receptor 6 (CCR6) is a seven transmembrane chemokine receptor belonging to the member of G-protein-coupled receptors (GPCRs) family [1]. CCR6 is identified as a specific receptor for CC motif chemokine ligand 20 (CCL20) in 1997 [2]. CCR6 has been reported to be related to various diseases, such as cancer [3], autoimmune diseases [4], psoriasis [5], and inflammatory bowel disease (IBD) [6]. Since the expression of CCR6 is found in B cells or T cells, such as effector T cells, memory T cells, immature dendritic cells, Th17 cells, and regulatory T (Treg) cells, it affects the activity and directionality of immune cells [7-9]. In CCR6-deficient mice, the intestinal lymphoid tissue and Peyer's patch are not developed normally, and immune responses are also abnormal in contact hypersensitivity and delayed-type hypersensitivity models, indicating that CCR6 is found to be a key factor in the formation of the immune system [10].

The chemokine ligand CCL20, also known as macrophage inflammatory protein-3α (MIP-3α), liver and activation-regulated chemokine (LARC), or Exodus-1, is crucial ligand of CCR6 [1]. CCL20 also binds to scavenging receptor atypical chemokine receptor 4 (ACKR4), but G-protein canonical signaling has been occurred upon binding to CCR6 [11,12]. CCL20 is secreted by various immune-related cells, such as B cells, Th17 cells,

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dendritic cells, and natural killer cells [13]. Although there are various CC chemokine receptor-ligand pairs, the CCR6/CCL20-regulated immune response currently become a hotspot for immunological research, including disease development. The expression of CCR6 and CCL20 are dysregulated in the colonic mucosa and serum from IBD patients [14,15]. CCR6+ T cells are involved in exacerbation in an imiquimod-induced model of psoriasis [16]. Furthermore, tumor promoting effects of CCR6/CCL20 within the tumor microenvironment have been reported in many cancer types, such as renal cell carcinoma [17], gastric cancer [18], cervical cancer [19], and lung cancer [20,21]. Treg cells in peripheral blood (~60%) express CCR6 with stronger suppressive activity and higher FOXP3 expression in human oral squamous cell carcinoma patients [22]. These findings make CCL20/CCR6 axis an attractive therapeutic target for a wide variety of diseases. Development of inhibitors targeting CCR6/CCL20 axis has also been actively carried out [13].

We have previously developed various monoclonal antibodies (mAbs) against chemokine receptors, including mouse CCR2 [23], mouse CCR3 [24-27], mouse CCR4 [28], mouse CCR6 (mCCR6) [29], mouse CCR8 [30], mouse CCR9 [31], mouse CXCR6 [32], human CCR2 [33], and human CCR9 [34,35]. We have also investigated the epitope of those mAbs in previous studies [36-41]. The N-terminus of GPCRs, including CCR6, CCR9, and CXCR6, is identified as the ligand-binding domain [42-45]. Interestingly, the structure of binding between CCL20 and CCR6 has been elucidated. CCR6 and CCL20 have a shallow binding mode on the receptor surface, which induces allosteric conformational changes and is considered to trigger binding to intracellular G-proteins [46]. As well as analysis of the ligand-binding mode, the characterization of antibody epitopes is important for predicting neutralizing activity and assessment of efficacy against antigens, which regulate cellular conditions.

In this study, we performed the epitope identification of a rat anti-mCCR6 mAb (C_6Mab-13; IgG_1, kappa) using enzyme-linked immnosorbent assay (ELISA) and surface plasmon resonance (SPR) analysis against alanine-substituted N-terminal peptides of mCCR6.

2. Materials and Methods

2.1 Antibodies

A rat anti-mCCR6 mAb (clone C_6Mab-13) was previously established by immunizing a rat with a keyhole limpet hemocyanin (KLH)-conjugated N-terminal peptide of mCCR6 (1-19 amino acids [aa] + C-terminal cysteine) [29]. We purchased secondary peroxidase-conjugated anti-rat immunoglobulins from Sigma-Aldrich Corp. (St. Louis, MO)

2.2 Peptides

The mCCR6 (Accession No.: NM_001190333.1) peptide ($_1$-MNSTESYFGTD-DYDNTEYYS-$_{20}$) and 1×alanine residue-substituted peptides (Table 1) were synthesized by utilizing PEPscreen (Sigma-Aldrich Corp.).

2.3 ELISA

Synthesized mCCR6 peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc., Waltham, MA) at a concentration of 10 μg/mL for 30 min at 37℃. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc., Kyoto, Japan), wells were blocked with 1% bovine serum albumin (BSA)-containing PBST for 30 min at 37℃. The plates were incubated with 10 μg/mL of C_6Mab-13 for 30 min at 37℃, followed by a peroxidase-conjugated anti-rat immunoglobulins (1:20000 diluted; Sigma-Aldrich Corp.) for 30 min at 37℃. Enzymatic reactions were performed at room temperature 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).

2.4 Measurements of K_D via surface plasmon resonance (SPR)

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Measurements of K_D between C6Mab-13 and the epitope region peptides were performed using SPR. C6Mab-13 was immobilized on the sensor chip CM5 according to the manufacture's protocol by Cytiva (Marlborough, MA). In brief, C6Mab-13 was diluted to 10 μg/mL by the acetate buffer (pH 4.0: Cytiva) and immobilized using amine coupling reaction. The surface of the flow cell 2 of the sensor chip CM5 was treated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-hydroxysuccinimide (NHS), followed by the injection of C6Mab-13. Unreacted NHS-ester was blocked with ethanolamine after C6Mab-13 immobilization. The K_D between C6Mab-13 and alanine-substituted peptides described in Table 2 were measured using Biacore X100 (Cytiva) at 25˚C. The buffer was filtrated-PBS containing 0.05% (v/v) of Tween 20 and 0.24% (v/v) of dimethyl sulfoxide (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). A single cycle kinetics method was used to measure the binding signals. The data were analyzed by 1:1 binding kinetics to determine the association rate constant (k_a), dissociation rate constant (k_d), and dissociation constants (K_D) using BIAevaluation software (Cytiva).

3. Results

3.1. Epitope identification of C6Mab-13 by ELISA using 1×alanine-substituted mCCR6 peptides

We developed an anti-mCCR6 mAb, C6Mab-13 (rat IgG_1, kappa) by immunizing a rat with the KLH-conjugated mCCR6 N-terminal peptide [29]. To characterize the binding epitope of C6Mab-13, we synthesized 20 different 1×alanine-substituted mCCR6 peptides between Met1 to Ser20. The sequences are listed in Table 1. The results of ELISA using alanine-substituted peptides and C6Mab-13 demonstrated that C6Mab-13 bound to point mutants, such as M1A, N2A, S3A, T4A, E5A, S6A, Y7A, F8A, G9A, T10A, D12A, Y13A, D14A, N15A, T16A, E17A, Y18A, Y19A, and S20A as well as the 1-20 aa wild type (WT) sequence (positive control) (Fig. 1A). In contrast, C6Mab-13 did not react with D11A peptide (Fig. 1A). These results indicated that Asp11 was determined to be the critical aa, which is included in the C6Mab-13 epitope. The results are summarized schematically in Figure 1B.

Table 1. Identification of C6Mab-13 epitope using point mutant peptides of mCCR6 by enzyme-linked immunosorbent assay.

Peptides	*Sequences*	*C6Mab-13 reactivity*
p1_20 (WT)	MNSTESYFGTDDYDNTEYYS	+++
M1A	ANSTESYFGTDDYDNTEYYS	+++
N2A	MASTESYFGTDDYDNTEYYS	+++
S3A	MNATESYFGTDDYDNTEYYS	+++
T4A	MNSAESYFGTDDYDNTEYYS	+++
E5A	MNSTASYFGTDDYDNTEYYS	+++
S6A	MNSTEAYFGTDDYDNTEYYS	+++
Y7A	MNSTESAFGTDDYDNTEYYS	+++
F8A	MNSTESYAGTDDYDNTEYYS	+++
G9A	MNSTESYFATDDYDNTEYYS	+++
T10A	MNSTESYFGADDYDNTEYYS	+++
D11A	MNSTESYFGTADYDNTEYYS	-
D12A	MNSTESYFGTDAYDNTEYYS	+++
Y13A	MNSTESYFGTDDADNTEYYS	+++
D14A	MNSTESYFGTDDYANTEYYS	+++

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N15A	MNSTESYFGTDDYDATEYYS	+++
T16A	MNSTESYFGTDDYDNAEYYS	+++
E17A	MNSTESYFGTDDYDNTAYYS	+++
Y18A	MNSTESYFGTDDYDNTEAYS	+++
Y19A	MNSTESYFGTDDYDNTEYAS	+++
S20A	MNSTESYFGTDDYDNTEYYA	+++

+++, OD655≧0.3; —, OD655<0.1

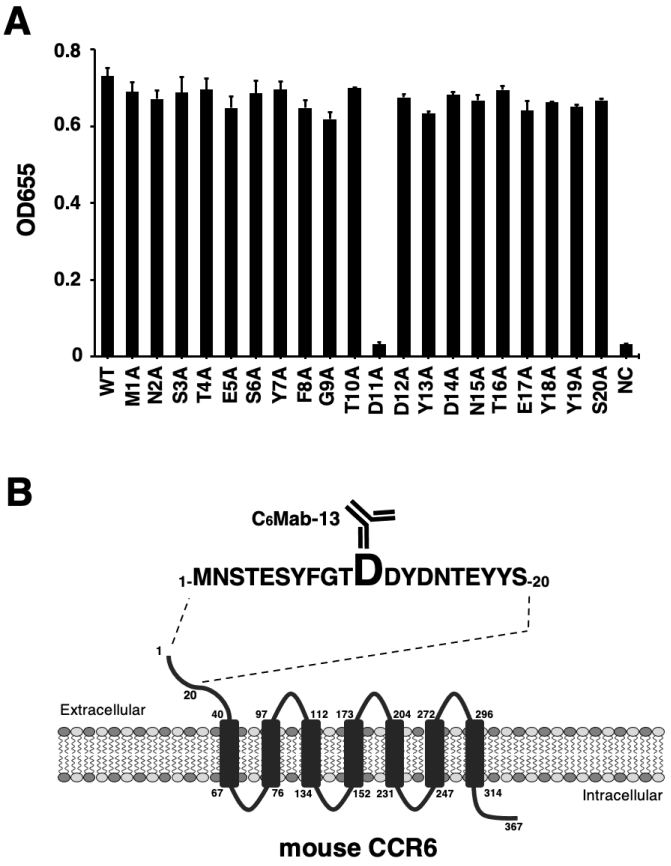

Figure 1. Determination of C6Mab-13 epitope by ELISA using alanine-substituted peptides of mCCR6. (**A**) Synthesized peptides of mCCR6 (10 μg/mL respectively) were immobilized on immunoplates for 30 min at 37°C. The plates were incubated with 10 μg/mL of C6Mab-13, followed by the treatment of peroxidase-conjugated anti-rat immunoglobulins. Optical density was measured at 655 nm (OD655) using a microplate reader. (**B**) mCCR6 and the epitope of C6Mab-13 were schematically illustrated. The C6Mab-13 epitope of mCCR6 comprises Asp11 from ELISA experiments.

3.2. Epitope identification of C6Mab-13 by SPR using 1×alanine-substituted mCCR6 peptides

To confirm the epitope of C6Mab-13, we next measured the binding affinity between C6Mab-13 and synthesized peptides, including 20 point mutants and WT of mCCR6, using Biacore X100. The sequences of peptides are described in Table 1. The measured values are summarized in Table 2. The association rate constant (k_a), dissociation rate constant (k_d), and dissociation constants (K_D) of G9A and D11A were not determined. These results demonstrated that Gly9 and Asp11 were determined to be the critical aa of the C6Mab-13 epitope.

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Mutant peptides of F8A, T10A, Y13A, and D14A increased the K_D values by 15.5-, 4.4-, 16.5-, and 2.8-fold, respectively (Table 2), indicating that Phe8, Thr10, Tyr13, and Asp14 might contribute to C_6Mab-13-binding to mCCR6.

Table 2. The K_D between C_6Mab-13 and 1×Alanine-substituted peptides by surface plasmon resonance.

Peptides	k_a *(/ms)*	k_d *(/s)*	K_D *(M)*
p1_20 (WT)	6.84×10^3	3.77×10^{-3}	5.52×10^{-7}
M1A	6.94×10^3	4.15×10^{-3}	5.99×10^{-7}
N2A	7.86×10^3	4.23×10^{-3}	5.38×10^{-7}
S3A	7.62×10^3	4.53×10^{-3}	5.94×10^{-7}
T4A	7.92×10^3	4.55×10^{-3}	5.75×10^{-7}
E5A	8.20×10^3	4.64×10^{-3}	5.65×10^{-7}
S6A	9.05×10^3	5.25×10^{-3}	5.81×10^{-7}
Y7A	8.16×10^3	3.45×10^{-3}	4.23×10^{-7}
F8A	1.43×10^3	1.23×10^{-2}	8.55×10^{-6}
G9A	ND	ND	ND
T10A	1.31×10^4	3.15×10^{-2}	2.40×10^{-6}
D11A	ND	ND	ND
D12A	7.43×10^3	7.09×10^{-3}	9.55×10^{-7}
Y13A	1.43×10^3	1.30×10^{-2}	9.12×10^{-6}
D14A	6.87×10^3	1.05×10^{-2}	1.53×10^{-6}
N15A	6.19×10^3	5.61×10^{-3}	9.06×10^{-7}
T16A	6.23×10^3	5.17×10^{-3}	8.30×10^{-7}
E17A	6.38×10^3	6.67×10^{-3}	1.05×10^{-6}
Y18A	5.23×10^3	5.56×10^{-3}	1.06×10^{-6}
Y19A	5.75×10^3	6.02×10^{-3}	1.05×10^{-6}
S20A	4.68×10^3	5.96×10^{-3}	1.27×10^{-6}

ND, not determined.

4. Discussion

This study examines the binding epitope of C_6Mab-13 by 1×alanine-substituted peptide scanning method using ELISA and SPR. We concluded the pivotal epitope as Asp11 using ELISA, and as Gly9 and Asp11 using SPR. They are located outside the region of all three extracellular domains of CCR6 and N-terminal residues from Tyr27 to Leu38, to which the chemokine ligand CCL20 binds [46]. Therefore, C_6Mab-13 might not show the neutralizing activity for CCL20. In contrast, structural changes might occur when C_6Mab-13 binds to CCR6. Also, it has been reported that different affinities of antibodies dramatically alter the functional activities [47]. Therefore, we plan to investigate the neutralizing activity of C_6Mab-13 between CCL20 and CCR6 in the future study.

The epitope mapping results using ELISA (Figure 1) and SPR (Table 2) indicated the similar region of mCCR6 as the binding epitope. However, Gly9 is shown as the critical aa by only SPR analysis (Table 2). The experimental system is different between two experiments such as; i) the synthesized peptides were immobilized on immunoplates in ELISA, whereas C_6Mab-13 was immobilized on a sensor chip CM5 in SPR analysis; ii) the reaction time between the antigen and the antibody is different; iii) the secondary antibody is used only in ELISA. These different conditions may lead to the inconsistent results of two experiments in this study.

In SPR analysis, mutant peptides of F8A, T10A, Y13A, and D14A increased the K_D values by 15.5-, 4.4-, 16.5-, and 2.8-fold, respectively (Table 2). These results indicate that Phe8, Thr10, Tyr13, and Asp14 might contribute to C_6Mab-13-binding to mCCR6. In the future, we will perform the cell-based alanine-scanning or 2×alanine-scanning methods for more detailed epitope analysis for C_6Mab-13 as we previously clarified the epitopes of mAbs [36,38,40]. We further plan to apply a RIEDL insertion for epitope mapping (REMAP) method for the epitope mapping of C_6Mab-13 [48,49]. The REMAP method is useful for determining the conformational epitope, which could not be identified by alanine scanning. The REMAP method will be effective for more detailed epitope analysis of C_6Mab-13.

When CCL20 is secreted in tumor tissues, it attracts CCR6-expressing Treg cells [50], which are involved in tumor progression and poor prognosis in patients with hepatocellular carcinoma [51]. Using this biological response of CCR6, novel cancer treatment strategies using CCR6-expressing chimeric antigen receptor-T (CAR-T) cells were designed [52,53]. Also, removal of immunosuppressive cells, such as CCR6+ Treg cells, may enhance antitumor efficacy [54]. We demonstrated that C_6Mab-13 possesses high binding affinity against mCCR6, which is expressed in Chinese hamster ovary-K1 cells (K_D: 2.8×10^{-9} M by flow cytometric analysis) in the previous study [29]. Therefore, C_6Mab-13 is expected to be useful for antitumor evaluation by depletion of CCR6-expressing Treg cells in mouse models.

Author Contributions: T.T. and M.T. performed the experiments. M.K.K. and Y.K. designed the experiments. T.T. and H.S. analyzed the data. T.T., H.S. and Y.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All related data and methods are presented in this paper. Additional inquiries should be addressed to the corresponding authors.

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

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