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Epitope Mapping of the Anti-Human CC Chemokine Receptor Type-2 Monoclonal Antibody (K036C2)

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CC chemokine receptor type-2 (CCR2) belongs to the G protein-coupled receptors superfamily, and is localized on cell surface of tumor cells and some immune cells, including monocytes and macrophages. CCR2 is a receptor for monocyte chemoattractant protein-1/C-C motif chemokine 2, and is involved in the progression of various diseases such as cancers. Therefore, the development of CCR2-targeted monoclonal antibody (mAb) is desired. Its characterization, including epitope of mAb, is very important for antibody applications. In this study, we investigated the critical epitope of K036C2, which is a commercially available anti-human CCR2 (hCCR2) mAb. We conducted enzyme-linked immunosorbent assay (ELISA) using three N-terminal peptides of hCCR2 and demonstrated that K036C2 recognizes 11–29 and 21–39 amino acids of hCCR2. We further performed ELISA using 20 peptides, which include alanine substitution of hCCR2. K036C2 lost the reaction to the alanine-substituted peptides of D25A, Y26A, D27A, G29A, and A30G. These results indicate that the critical binding epitope of K036C2 includes Asp25, Tyr26, Asp27, Gly29, and Ala30 of hCCR2.

Keywords: human CCR2, K036C2, epitope, monoclonal antibody, enzyme-linked immunosorbent assay

Introduction

CHEMOKINE RECEPTORS BELONG to the G protein-coupled receptor (GPCR) with seven transmembrane regions. Chemokines are a family of cytokines, and divided into four different subfamilies of XC, CC, CXC, and CX3C, depending on the number and position of N-terminus cysteine residues.[1,2] Chemokines orchestrate many cellular functions including immune responses.[3–5]

CC chemokine receptor type-2 (CCR2) is expressed in various cell types, including epithelial cells, macrophages, dendritic cells, and monocytes. CCR2 is involved in the regulation of migration and positioning of immune-related cells.[6–8] CCR2 is the primary receptor of C-C motif chemokine 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1). CCL2 and CCR2 play an important role in regulating the key immune regulators, including T lymphocytes, natural killer cells, stromal cells, and monocytes.[9,10] CCL2-CCR2 axis is correlated with many diseases such as immune disorders and cancer.[8,11,12]

CCR2-expressing cells are often involved in tissue damage at the site of inflammation.[13] Interferon-γ-regulated CCR2+ monocytes become a driver of lung damage during influenza A virus infection.[14] During viral and bacterial infection in respiratory organs, the recruitment of macrophages and neutrophils mediated by CCL2-CCR2 axis contribute to the innate immune responses.[15] High CCR2 levels in blood samples have been detected in patients with severe COVID-19.[16]

Furthermore, CCL2 expression has been reported to be upregulated in several tumors, such as inflammatory breast cancers, bladder cancers, and bone tumors.[9,17,18] High CCR2 expression has been confirmed in the invasive lesion of breast cancers and melanoma.[19] In sarcoma, CCR2 correlates with poor prognosis by regulating the infiltration of multiple immune and stromal cells in tumor microenvironment.[20,21] In cancer treatment, the blockade of CCR2 functions has been reported to enhance the effectiveness of immune checkpoint inhibitors, such as an anti-programmed-cell death-1 monoclonal antibodies (mAbs) in mouse models.[18]

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We have produced numerous mAbs against GPCRs, including mouse CCR2,[22] mouse CCR3,[23–25] mouse CCR4,[26] mouse CCR8,[27] and human CCR9,[28] and also determined the binding epitope.[29] In addition, we further determined the binding epitope of mAbs against numerous membrane proteins, including CD20,[30] CD44,[31,32] CD133,[33] and podoplanin.[34–36] In this study, we performed the epitope identification of anti-human CCR2 mAb (K036C2) by using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Enzyme-linked immunosorbent assay

The human CCR2 peptides (Accession No. NM_001123041), including three N-terminal peptides (Table 1) and 20 alanine-substituted mutants (Table 2), were synthesized by utilizing PEPscreen (Sigma-Aldrich Corp., St. Louis, MO). Each peptide was 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), wells were blocked with 1% bovine serum albumin-containing PBST for 30 minutes at 37°C. The plates were then incubated with K036C2 (BioLegend, San Diego, CA) (1 μg/mL), followed by a 1:2000 dilution of peroxidase-conjugated anti-mouse immunoglobulins (Agilent Technologies, Inc., Santa Clara, CA). Enzymatic reactions were performed using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc., Kyoto, Japan). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Results

Epitope determination of K036C2 using N-terminal hCCR2 peptides

To characterize the binding epitope of anti-hCCR2 mAb, K036C2, for hCCR2, we synthesized three N-terminal peptides: 1–19 amino acids (aa), 11–29 aa, and 21–39 aa (Table 1). The results of ELISA demonstrated that K036C2 reacted with 11–29 aa and 21–39 aa of hCCR2 (Fig. 1A). These results are summarized in Figure 1B.

Epitope determination of K036C2 using alanine-substituted hCCR2 peptides

We further synthesized 20 different alanine-substituted hCCR2 peptides (Table 2). The results of ELISA demonstrated that K036C2 reacted with point mutants, such as T21A, T22A, F23A, F24A, Y28A, P31A, S32A, H33A,

Table 1. Identification of the K036C2 Epitope Using N-Terminal Human CC Chemokine Receptor Type-2 Peptides

Peptides	*Sequences*	*K036C2*
1–19	MLSTSRSRFIRNTNESGEE	–
11–29	RNTNESGEEVTTFFDYDYG	+++
21–39	TTFFDYDYGAPSHKFDVKQ	+++

+++, OD655 ≧ 0.3; –, OD655 < 0.1.

Table 2. Identification of the K036C2 Epitope Using Alanine-Substituted Human CC Chemokine Receptor Type-2 Peptides

Peptides	*Sequences*	*K036C2*
T21A	ATFFDYDYGAPSHKFDVKQI	+++
T22A	TAFFDYDYGAPSHKFDVKQI	+++
F23A	TTAFDYDYGAPSHKFDVKQI	+++
F24A	TTFADYDYGAPSHKFDVKQI	+++
D25A	TTFFAYDYGAPSHKFDVKQI	–
Y26A	TTFFDADYGAPSHKFDVKQI	–
D27A	TTFFDYAYGAPSHKFDVKQI	–
Y28A	TTFFDYDAGAPSHKFDVKQI	+++
G29A	TTFFDYDYAAPSHKFDVKQI	–
A30G	TTFFDYDYGGPSHKFDVKQI	–
P31A	TTFFDYDYGAASHKFDVKQI	+
S32A	TTFFDYDYGAPAHKFDVKQI	+++
H33A	TTFFDYDYGAPSAKFDVKQI	+++
K34A	TTFFDYDYGAPSHAFDVKQI	+++
F35A	TTFFDYDYGAPSHKADVKQI	+++
D36A	TTFFDYDYGAPSHKFAVKQI	+++
V37A	TTFFDYDYGAPSHKFDAKQI	+++
K38A	TTFFDYDYGAPSHKFDVAQI	+++
Q39A	TTFFDYDYGAPSHKFDVKAI	+++
I40A	TTFFDYDYGAPSHKFDVKQA	+++

+++, OD655 ≧ 0.3; +, 0.1 ≦ OD655 < 0.2; –, OD655 < 0.1.

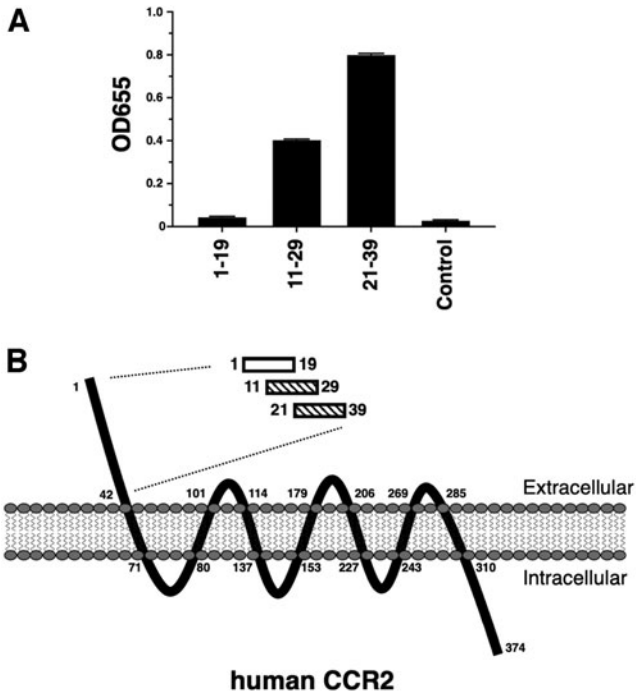

FIG. 1. Determination of the K036C2 epitope for hCCR2 by ELISA using N-terminal peptides. **(A)** N-terminal synthesized peptides of hCCR2 were immobilized on immunoplates. The plates were incubated with K036C2 (1 μg/mL) followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Schematic illustration of hCCR2 and the K036C2 epitope. ELISA, enzyme-linked immunosorbent assay; hCCR2, human CC chemokine receptor type-2.

K34A, F35A, D36A, V37A, K38A, Q39A, and I40A as well as the 21–40 aa wild-type sequence (positive control) (Fig. 2A). In contrast, K036C2 did not bind to alanine-substituted hCCR2 peptides, such as D25A, Y26A, D27A, G29A, and A30G (Fig. 2A), indicating that Asp25, Tyr26, Asp27, Gly29, and Ala30 were determined to be the critical aa, which are included in the K036C2 epitope. The results are summarized in Figure 2B.

Discussion

CCR2 is a seven-transmembrane receptor with four extracellular regions (Fig. 2B). N-terminal domains of some GPCRs, such as CCR2, CCR3, CCR5, and CXCR1, have been determined as their ligand-binding sites.[2] We previously developed an anti-hCCR2 mAb (clone C_2Mab-9) by using N-terminal peptide immunization method and clarified the epitope of C_2Mab-9 as Phe23, Phe24, Asp25, and Tyr26.[37] We identified the critical epitope of K036C2 as Asp25, Tyr26, Asp27, Gly29, and Ala30 using ELISA in this study; therefore, the C_2Mab-9 epitope was shown to be different from that of K036C2 (Fig. 2).

We have previously developed various novel epitope mapping system, named RIEDL insertion for epitope mapping (REMAP)[38,39] and histidine-tag insertion for epitope mapping (HisMAP) method.[30] These methods are effective in determining linear and structural epitopes. Therefore, we will try to determine the binding epitope of K036C2 using REMAP and HisMAP methods in the future study.

The several amino acids in the N-terminal region of CCR2B (predominant isoform of CCR2) were reported to show pivotal roles for CCL2-triggered cell migration and lamellipodium formation,[40] indicating that anti-hCCR2 mAbs targeting N-terminal region might be advantageous for the functional study about the CCL2-CCR2 axis. It is expected that information on epitopes will be helpful for anti-GPCR mAb drugs that have high development hurdles.[41]

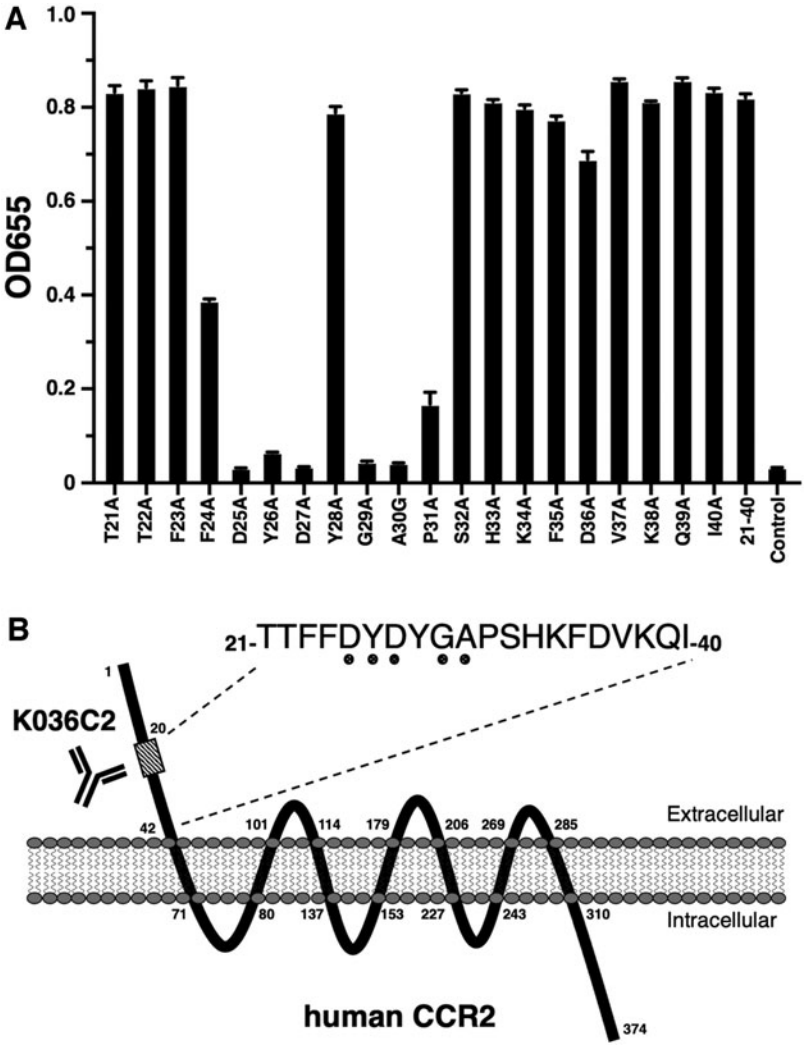

FIG. 2. Determination of the K036C2 epitope for hCCR2 by ELISA using alanine-substituted peptides of hCCR2. **(A)** Synthesized peptides of hCCR2 were immobilized on immunoplates. The plates were incubated with K036C2 (1 μg/mL), followed by peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Schematic illustration of hCCR2 and the K036C2 epitope. The K036C2 epitope of hCCR2 involves Asp25, Tyr26, Asp27, Gly29, and Ala30.

Author Disclosure Statement

No competing financial interests exist.

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