Communication

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Epitope Analysis of an Anti-Mouse CCR1 Monoclonal Antibody S15040E Using Flow Cytometry

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Abstract: The C-C motif chemokine receptor 1 (CCR1) is widely expressed in various immune cells and plays crucial roles in the maturation and migration of immune cells. CCR1 has been considered an attractive drug target for treating allergic and autoimmune diseases. An anti-mouse CCR1 (mCCR1) monoclonal antibody (clone S15040E) has been used in various in vivo studies to identify mCCR1-positive cells by flow cytometry. However, the binding epitope has not been determined. This study investigated the binding epitope of S15040E using flow cytometry. The mCCR1 extracellular domain-substituted mutant analysis showed that S15040E recognizes the extracellular loop 2 (ECL2, aa 172–197) of mCCR1. Next, alanine (or glycine) scanning was conducted in the ECL2 region. The results revealed that the Trp176, Phe178, Arg181, and Cys183 are essential amino acids for the recognition by S15040E. These results showed the involvement of the ECL2 of mCCR1 in the recognition by S15040E.

Key words: mouse CCR1, monoclonal antibody, epitope mapping, alanine scanning, flow cytometry

1. Introduction

Chemokine receptors belong to class A seven transmembrane (7TM) receptors and play an essential role in guiding leukocyte trafficking in immune surveillance and inflammatory response [1]. The cognate chemokines are named according to the sequence of the first two cysteines (CC, CXC, XC, or CX3C motif). The CC ligands (CCL1 to CCL28) are recognized by C-C chemokine receptor type 1 (CCR1) to CCR10 [2]. Upon ligand binding, chemokine receptors typically activate G protein pathways and recruit β-arrestins [3,4].

CCR1 mediates inflammatory responses and plays an essential role in the development of autoimmune diseases [1,5]. It has been considered an attractive drug target for treating allergic and autoimmune diseases [6]. Among the chemokine receptors, CCR1 possesses ligand promiscuity, which allows it to recognize at least nine human CC chemokines, including CCL3, CCL5, CCL7, CCL8, CCL13–16, and CCL23 [2,7,8].

For the development of therapeutic agents in the chemokine system, the structural understanding of the chemokine receptor activation is essential. Among the CCR family members, CCR2 and CCR5 have been characterized in both inactive and active states [9–12], while active-state of CCR8 and CCR6 and inactive-state of CCR7 and CCR9 structures were also determined [13–16]. Furthermore, the CCL15-CCR1 complex showed a crucial sequence for ligand binding distinct from many other chemokine–receptor complexes, providing new insights into the mode of chemokine recognition [13]. Moreover, the structures of CCR8 in complex with either the endogenous ligand CCL1 or the antagonistic monoclonal antibody (mAb) were solved, which provides the specific activation mechanism by CCL1 and inhibition by mAb [17]. Therefore, anti-chemokine receptor mAbs with defined epitopes are useful for the analysis of specific structures.

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An anti-mouse CCR1 (mCCR1) mAb (clone S15040E) has been used in various in vivo studies to identify mCCR1-positive cells using flow cytometry [18–20]. However, the binding epitope has not been determined. To determine the epitopes of 7TM proteins, we have faced difficulty using conventional methods such as enzyme-linked immunosorbent assay. This study investigated the binding epitope of S15040E using flow cytometry-based approaches.

2. Materials and Methods

2.1 Plasmid Construction

pCAG-Ble-mCCR1 and pCAG-Ble-mouse CCR5 (mCCR5) were generated as previously described [21,22]. Chimeric mutants including mCCR5 (mCCR1p2–34), mCCR5 (mCCR1p92–107), mCCR5 (mCCR1p172–197), and mCCR5 (mCCR1p265–281) were produced with a PA16 tag at their N-terminus using the HotStar HiFidelity polymerase kit (Qiagen Inc., Hilden, Germany). Alanine (or glycine) substitutions in mCCR1 were conducted using QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies Inc., Santa Clara, CA, USA).

2.2. Cell Lines

Chinese hamster ovary (CHO)-K1 cell was obtained from the America Type Culture Collection (ATCC, Manassas, VA, USA). The chimeric and the point mutant plasmids were transfected into CHO-K1 cells using the Neon Transfection System (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Antibodies

An anti-mCCR1 mAb (clone S15040E) was purchased from BioLegend (San Diego, CA, USA). NZ-1 (an anti-PA16 tag mAb) was described previously [23].

2.4. Flow Cytometry

Cells were harvested after brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin in phosphate-buffered saline, cells were treated with S15040E (1 μg/mL) or NZ-1 (1 μg/mL) for 30 min at 4°C and subsequently with Alexa Fluor 488-conjugated anti-rat IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA, USA). Fluorescence data were obtained using the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

3. Results

3.1. Determination of the Epitope of an Anti-mCCR1 mAb, S15040E, by Flow Cytometry Using Chimeric Proteins

An anti-mCCR1 mAb (clone S15040E) is applicable for flow cytometry. To investigate the binding epitope of S15040E, we focused on four extracellular regions of mCCR1, including the N-terminal region (aa 2–34), extracellular loop 1 (ECL1; a 92–107), ECL2 (aa 172–197), and ECL3 (aa 265–281). The four extracellular regions of mCCR1 were substituted into the corresponding regions of mCCR5, which possesses a similar structure to mCCR1. As shown in Figure 1, plasmids encoding mCCR5 (mCCR1p2–34), mCCR5 (mCCR1p92–107), mCCR5 (mCCR1p172–197), and mCCR5 (mCCR1p265–281) were generated. The chimeric proteins were transiently expressed on CHO-K1 cells, and the reactivities to S15040E were analyzed using flow cytometry (Figure 2A). S15040E reacted with mCCR5 (mCCR1p172–197), but not with other chimeric proteins (Figure 2A). The cell surface expression of each mutant was confirmed by an anti-PA16 tag mAb, NZ-1 (Figure 2B). These results indicated that S15040E recognizes the ECL2 of mCCR1.

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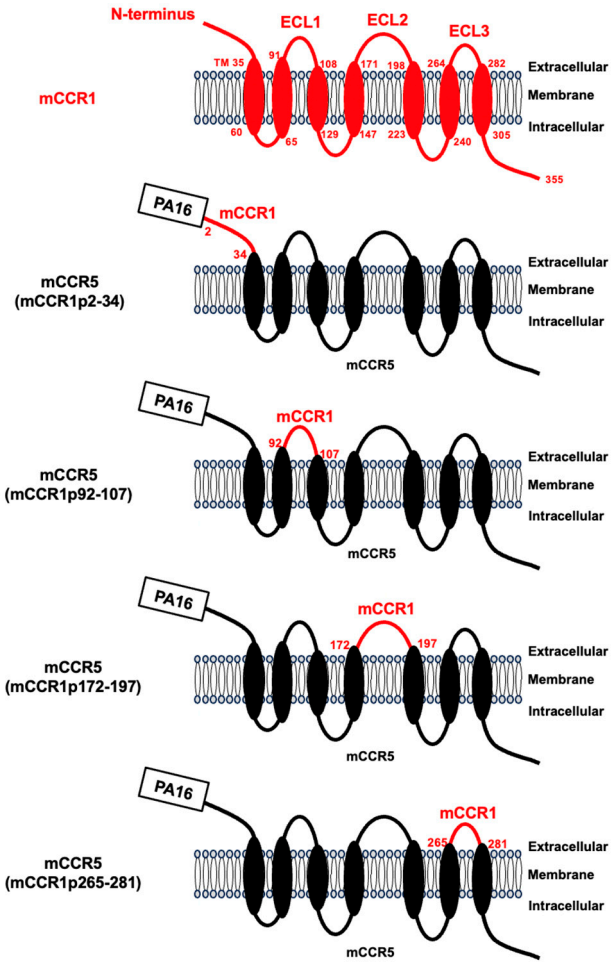

Figure 1. Schematic illustration of chimeric proteins. The four extracellular regions of mCCR1, including the N-terminal region (residues 2–34), ECL1 (residues 92–107), ECL2 (residues 172–197), and ECL3 (residues 265–281) were substituted into the corresponding regions of mCCR5. ECL, extracellular loop.

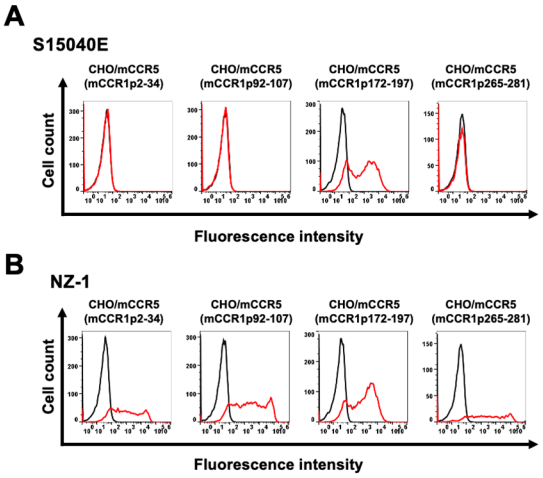

Figure 2. Determination of the epitope of an anti-mCCR1 mAb, S15040E, by flow cytometry using chimeric proteins. CHO-K1 transiently expressed the chimeric proteins were treated with S15040E (1 μg/mL, A), an anti-PA16 tag mAb, NZ-1 (1 μg/mL, B), or blocking buffer for 30 min at 4°C, followed by the addition of Alexa Fluor 488-conjugated anti-rat IgG. Red lines show the cells with S15040E or NZ-1 treatment, and black lines show cells treated with a blocking buffer as a negative control.

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3.2. Determination of the S15040E Epitope by Flow Cytometry Using Alanine Scanning

Next, alanine scanning was conducted in the ECL2 of mCCR1. Twenty-six alanine (or glycine) substituted mutants of mCCR1 were constructed (Figure 3), and the mutant proteins were transiently expressed on CHO-K1 cells. The reactivity against S15040E was assessed using flow cytometry. As shown in Figure 4A, S15040E did not react with four mutants (W176A, F178A, R181A, and C183A). In contrast, S15040E reacted with the other twenty-two mutants. The cell surface expression of each mutant was confirmed by NZ-1 (Figure 4B). These results showed that Trp176, Phe178, Arg181, and Cys183 of mCCR1 are important for S15040E binding.

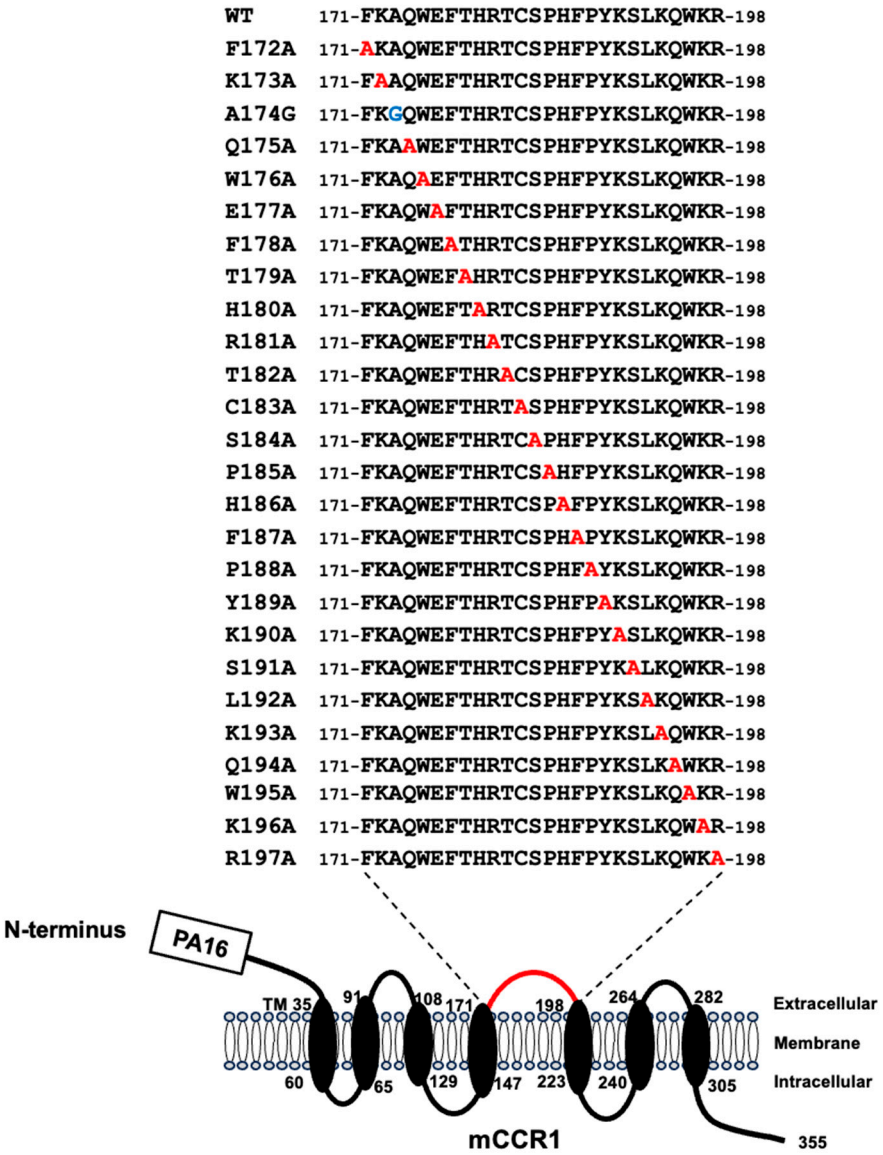

Figure 3. The illustration of alanine (or glycine) substituted mutants of mCCR1.

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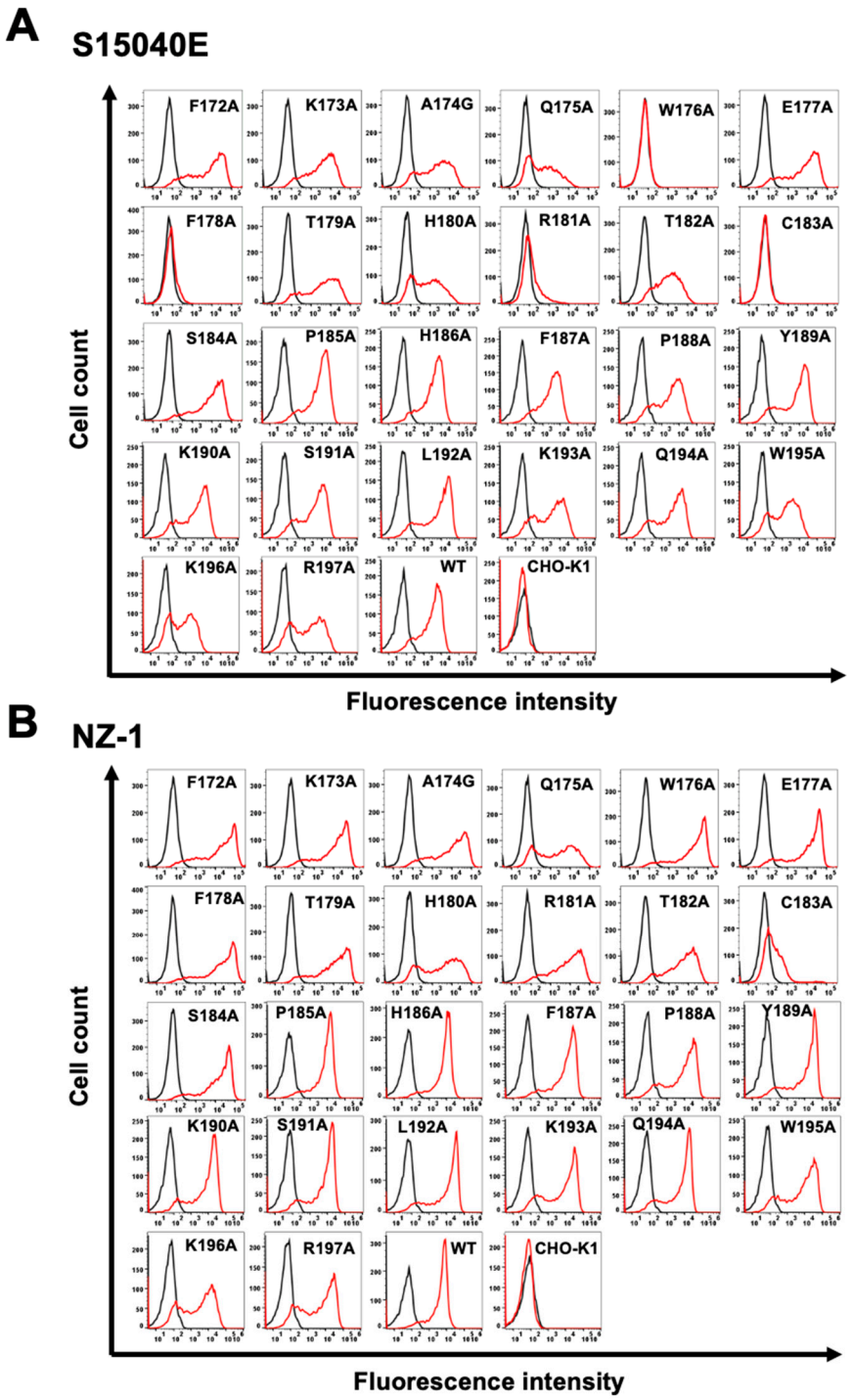

Figure 4. Determination of the S15040E epitope by flow cytometry using alanine scanning. CHO-K1 transiently expressed the mutant proteins and wild-type mCCR1 (WT) were treated with S15040E (1 μg/mL, A), NZ-1 (1 μg/mL, B), or blocking buffer for 30 min at 4°C. Then, cells were treated with Alexa Fluor 488-conjugated anti-rat IgG. Red lines show the cells with S15040E or NZ-1 treatment, and black lines show cells treated with a blocking buffer as a negative control.

4. Discussion

This study demonstrated the flow cytometry-based epitope mapping of an anti-mouse CCR1 mAb (S15040E) using the chimeric proteins (Figs. 1 and 2). Furthermore, we determined that the Trp176, Phe178, Arg181, and Cys183 in ECL2 are essential for the recognition by S15040E in alanine scanning (Figures 3 and 4). We previously determined the epitope of an anti-mouse CCR8 mAb,

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C8Mab-2 [24]. Our strategy for epitope identification would contribute to the understanding of mAb-epitope interaction.

The 7TM receptors have conserved disulfide bridge between transmembrane helix 3 (TM3) and ECL2 [25]. The Cys183 is well conserved and sole cysteine in ECL2, which forms a disulfide bridge with TM3. We previously confirmed that S15040E could not detect mCCR1 in western blotting [21]. Therefore, S15040E recognizes the conformational epitope, which depends on the disulfide bridge between TM3 and ECL2. Figure 5 summarizes the epitope of S15040E.

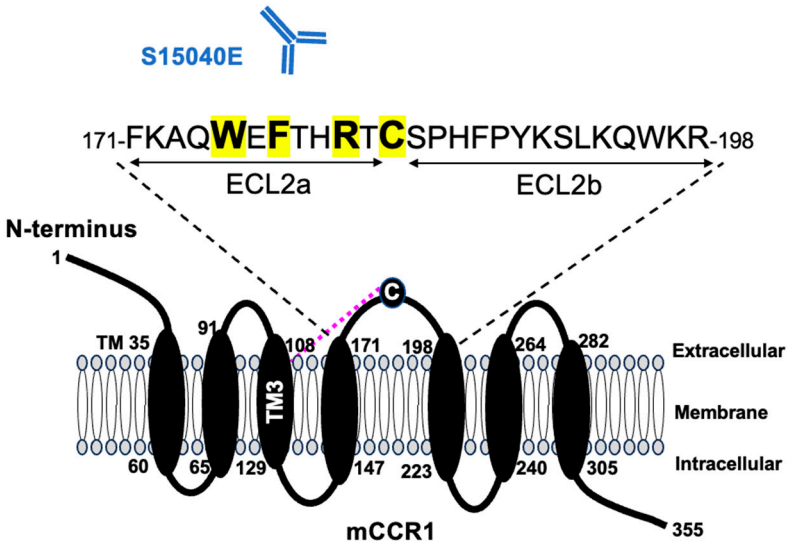

Figure 5. The schematic illustration of the S15040E epitope. Trp176, Phe178, Arg181, and Cys183 are essential amino acids for the recognition by S15040E. The dotted magenta line represents the disulfide bridge between TM3 and ECL2. TM3, transmembrane helix 3; ECL, extracellular loop.

ECL2 is essential for interaction with chemokines and is the largest region covering the activation-associated receptor binding pocket. ECL2 is divided into two parts before and after the disulfide bridge (ECL2a and ECL2b, respectively). Both parts involve the chemokine signaling selectivity and pharmacological activity [26–29]. The Trp176, Phe178, and Arg181 are in the ECL2a of mCCR1. In human CCR1, the ECL2a is essential for the recognition of CCL15 [13]. Therefore, S15040E may possess the neutralization activity to the ECL2a-bound ligands.

G protein-coupled receptors can transduce intracellular signaling through G proteins and β-arrestins. "Balanced" agonists or antagonists can activate or inhibit these signaling pathways. In contrast, specific pathways can be selectively triggered in a "biased" response. The biased responses can arise from biased ligands or biased receptors, all of which can drive preferential activation of either G protein- or β-arrestin-mediated pathways [30]. CCR1 is known to be a biased receptor that can selectively activate G proteins or β-arrestin pathways by diverse CCL15 isoforms [13,31]. Further structural analysis of the S15040E-mCCR1 complex may provide new insights into the mechanism of biased response and the development of therapeutic drugs.

Author Contributions: Ayaka Okada: Investigation; **Hiroyuki Suzuki:** Writing – original draft; **Tomohiro Tanaka:** Investigation, Funding acquisition; **Mika K. Kaneko:** Conceptualization; **Yukinari Kato:** Conceptualization, Funding acquisition, Project administration, Writing – review and editing; All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

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 conflict of interest involving this article.

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