Communication

Epitope Mapping of Anti-Mouse ACKR4 Monoclonal Antibodies Developed by N-Terminal Peptide Immunization

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Posted Date: 5 May 2026

doi: 10.20944/preprints202605.0155.v1

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Epitope Mapping of Anti-Mouse ACKR4 Monoclonal Antibodies Developed by N-Terminal Peptide Immunization

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Abstract

Leukocyte migration is a fundamental process in both innate and adaptive immune responses. This process is tightly regulated by chemokines and their cognate receptors. The bioavailability of chemokines is further modulated by atypical chemokine receptors (ACKRs), a subset of chemokine receptor–like molecules that lack coupling to canonical G protein–mediated signaling pathways. Among these, ACKR4 regulates dendritic cell migration through ligand scavenging and has been implicated in tumor progression in murine models. We previously established anti-mouse ACKR4 (mACKR4) mAbs, A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3, by N-terminal peptide immunization. This study examined the binding epitopes of A$_4$Mabs. Alanine (or glycine) scanning within the N-terminal region (amino acids 2–19) was performed using flow cytometry and Western blotting. The results demonstrated that Tyr11, Tyr12, Glu14, Glu15, and Glu17 are critical for recognition by A$_4$Mab-1, while Tyr11, Tyr12, Tyr13, Glu15, and Asn16 are essential for recognition by A$_4$Mab-2 in flow cytometry and Western blotting. Furthermore, Glu14, Asn16, and Glu17 are essential for recognition by A$_4$Mab-3 in flow cytometry. These findings contribute to the understanding of mACKR4 recognition by A$_4$Mabs.

Keywords: mouse ACKR4; monoclonal antibody; epitope mapping; alanine scanning; flow cytometry

1. Introduction

Immune cell priming, effector responses, and memory formation are governed by chemokines and the spatially restricted expression of G protein–coupled receptors (GPCRs) [1]. Chemokine receptors constitute a major class of seven-transmembrane receptors and are categorized as canonical GPCRs and atypical chemokine receptors (ACKR1–ACKR4) [2,3]. Ligand binding to canonical chemokine receptors activates heterotrimeric G proteins [1,4]. In contrast, ACKRs, despite structural homology to GPCRs, do not couple to G protein signaling. Instead, they mediate β-arrestin–dependent internalization and degradation of chemokines, thereby functioning as scavenger receptors that regulate chemokine bioavailability [2,5]. Cryogenic-electron microscopy analyses of agonist-bound ACKR3 revealed a distinct chemokine-binding mechanism, and provided a structural basis for β-arrestin-biased receptor [6,7].

ACKR4 is expressed in T lymphocytes [8] and in stromal compartments [9,10] and binds CCL19, CCL20, CCL21, CCL22, and CCL25 [2]. Through ligand sequestration, ACKR4 modulates CCR7-, CCR6-, CCR4-, and CCR9-dependent migratory responses [2]. This activity establishes chemokine gradients that direct dendritic cell (DC) trafficking from peripheral tissues to draining lymph nodes [9,11,12]. ACKR4 is also expressed in a flow-dependent manner in afferent lymphatic collectors, where it removes CCL21 from the luminal surface and limits aberrant T-cell entry into inflamed

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dermal collectors. Consistent with this function, ACKR4 deficiency impairs T-cell migration to draining lymph nodes [13].

For functional analysis of ACKR4-expressing cells in mouse preclinical models, monoclonal antibodies (mAbs) against mouse ACKR4 (mACKR4) are required. Using immunization with the N-terminal mACKR4 peptide, we previously generated anti-mACKR4 mAbs, including A4Mab-1, A4Mab-2, and A4Mab-3 [14]. This study determined their specific binding epitopes using an alanine scanning strategy.

2. Materials and Methods

2.1. Plasmid Construction

The mACKR4 (Accession No.: NM_145700.2) cDNA with N-terminal MAP tag was described previously [14]. Alanine (or glycine)-substituted mutants of mACKR4 were generated using QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies Inc., Santa Clara, CA, USA). The PCR fragments containing the desired mutations were inserted into the pCAG-Ble vectors (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

2.2. Cell Line and Plasmid Transfection

The Alanine (or glycine)-substituted mutant plasmids were transfected into Chinese hamster ovary (CHO)-K1 cells (American Type Culture Collection, Manassas, VA, USA) using the Neon Transfection System (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Antibodies

A4Mab-1 (rat IgG2b, kappa), A4Mab-2 (rat IgG2b, kappa), and A4Mab-3 (rat IgG2b, kappa) were established as described previously [14].

2.4. Flow Cytometry

Cells were collected 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, 2×10^5 cells were treated with 1 μg/mL of A4Mab-1, A4Mab-2, A4Mab-3, or an anti-MAP tag mAb (clone PMab-1 [15]) for 30 minutes at 4°C, followed by incubation with Alexa Fluor 488-conjugated anti-rat IgG (1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA). Fluorescence data (from a total of 5,000 cells per sample) were collected using the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan). Using FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA), single cells were gated based on side scatter versus forward scatter, and the fluorescence intensity was plotted.

2.5. Western Blotting

Cell lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.). Proteins (10 μg/lane) were electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% non-fat milk (Nacalai Tesque, Inc., Kyoto, Japan), PVDF membranes were incubated with 1 μg/mL of A4Mab-1, A4Mab-2, or PMab-1, followed by incubation with horseradish peroxidase-conjugated anti-rat IgG (1:10,000; Sigma-Aldrich Corp., St. Louis, MO). Finally, protein bands were detected with Pierce™ ECL Plus (Thermo Fisher Scientific, Inc.) or ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

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3. Results

3.1. Determination of A4Mab-1, A4Mab-2, and A4Mab-3 Epitopes by Flow Cytometry Using Alanine Scanning

Alanine scanning was performed on the N-terminal region (aa 2–19) of mACKR4. Eighteen mutants with alanine (or glycine) substitutions in mACKR4 with an N-terminal MAP tag were created (Figure 1). These mutant proteins were transiently expressed in CHO-K1 cells. Reactivity with A4Mab-1, A4Mab-2, and A4Mab-3 was assessed by flow cytometry. As shown in Figure 2A, A4Mab-1 did not react with the Y12A mutant. A4Mab-2 did not react with three mutants (Y12A, E15A, and N16A) (Figure 2B). Moreover, A4Mab-3 did not react with three mutants (E14A, N16A, and E17A) (Figure 2C). The cell surface expression of each mutant was confirmed with PMab-1, an anti-MAP tag mAb (Figure 2D).

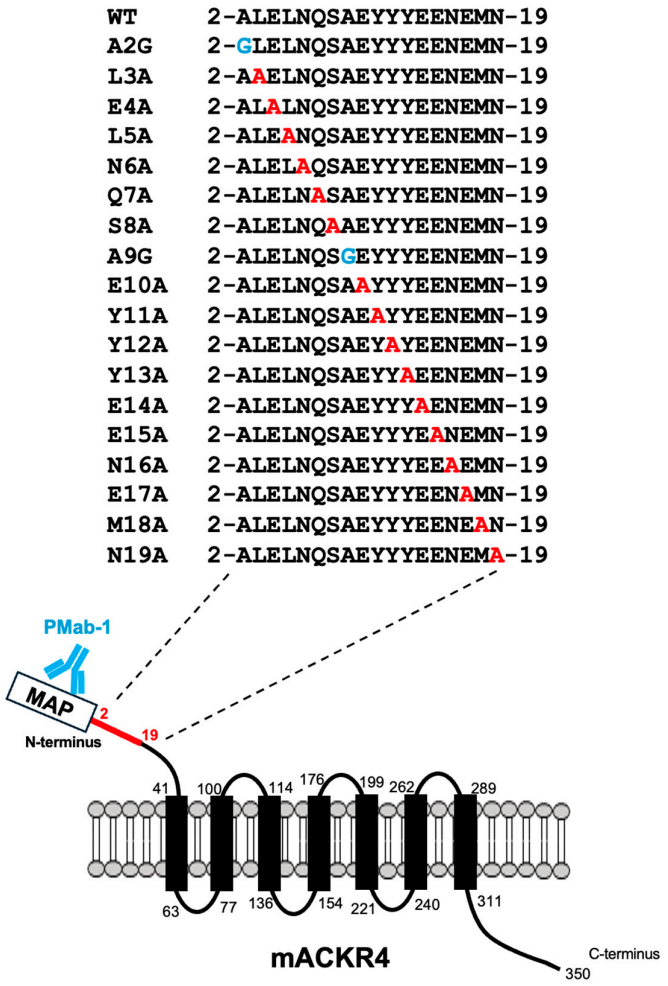

Figure 1. The illustration of alanine (or glycine)-substituted mutants of mACKR4. The N-terminal amino acids of mACKR4 were substituted with alanine (or glycine). PMab-1 recognizes the N-terminal MAP tag.

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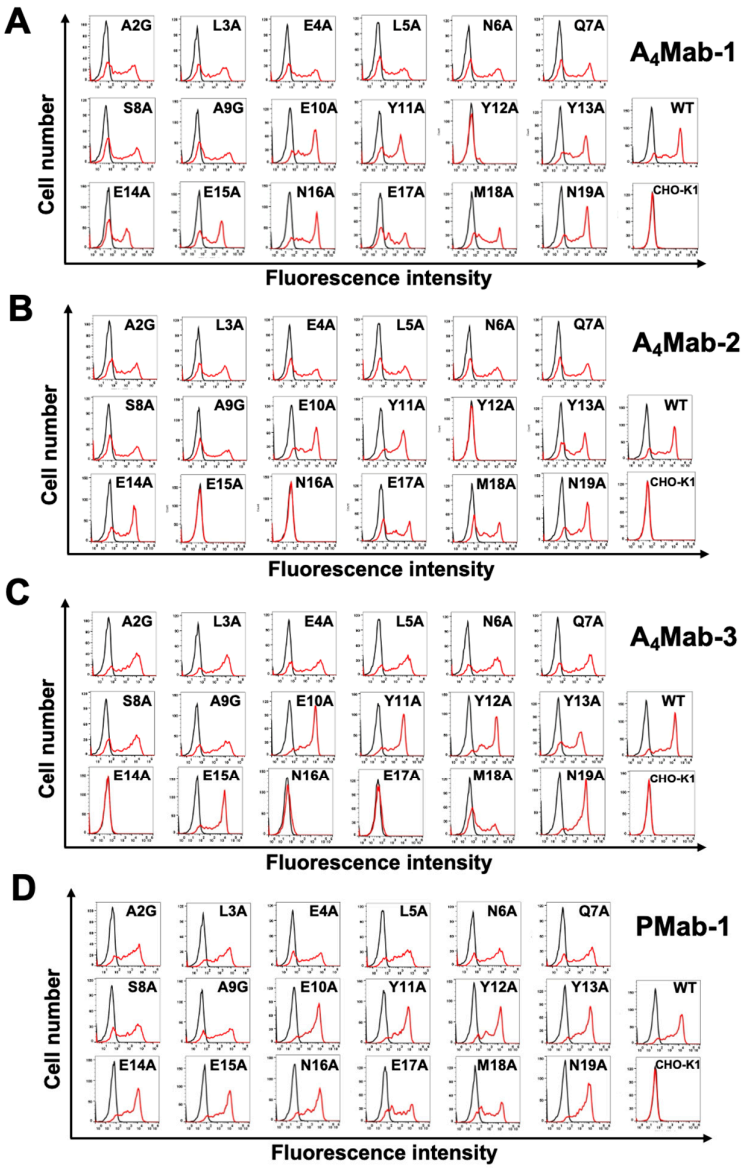

Figure 2. Determination of A4Mab-1, A4Mab-2, and A4Mab-3 epitopes by flow cytometry using alanine scanning. CHO-K1 cells transiently expressing mACKR4 mutants and wild-type (WT) were treated with A4Mab-1 (1 μg/mL, A), A4Mab-2 (1 μg/mL, B), A4Mab-3 (1 μg/mL, C), PMab-1 (1 μg/mL, D), or blocking buffer for 30 minutes at 4℃. The cells were then incubated with Alexa Fluor 488-conjugated anti-rat IgG. Red lines indicate cells treated with the primary mAbs, while black lines show cells treated with the blocking buffer.

3.2. Determination of the A4Mab-1 and A4Mab-2 Epitopes Using Western Blotting

A4Mab-1 and A4Mab-2 are suitable for western blotting. Therefore, mutant lysates were prepared and analyzed by western blotting [14]. As shown in Figure 3A, A4Mab-1 detected about 48 kDa major bands, which completely disappeared in the lysates of five mutants (Y11A, Y12A, E14A, E15A, and E17A). Furthermore, A4Mab-2 also detected 48 kDa major bands, which completely disappeared in the lysates of five mutants (Y11A, Y12A, Y13A, E15A, and N16A). (Figure 3B). PMab-1 was used to confirm the expressions (Figure 3C).

Figure 4 summarizes the results and illustrates the epitope of A4Mab-1, A4Mab-2, and A4Mab-3 in the N-terminal region of mACKR4.

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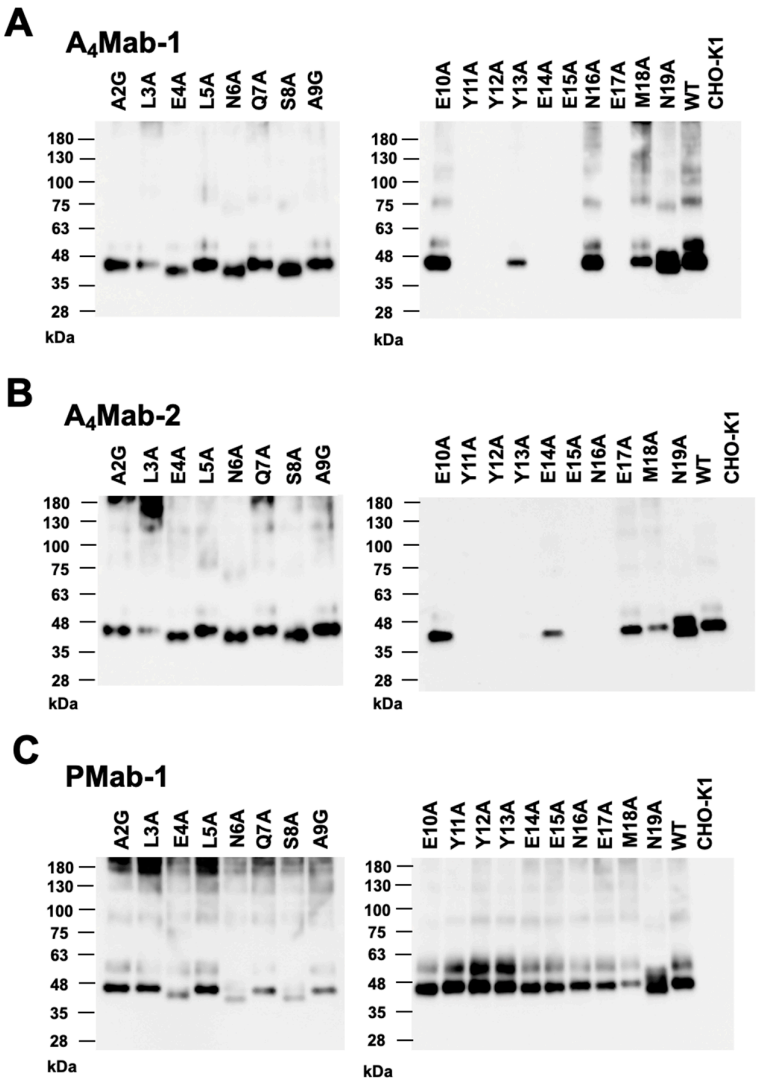

Figure 3. Determination of A4Mab-1 and A4Mab-2 epitopes by western blotting. The cell lysate of CHO-K1 transiently expressed mACKR4 mutants and wild-type (WT) was electrophoresed on 5%–20% polyacrylamide gels and transferred onto PVDF membranes. The membranes were treated with A4Mab-1 (1 μg/mL, A), A4Mab-2 (1 μg/mL, B), and PMab-1 (1 μg/mL, C). Then, the membranes were treated with horseradish peroxidase-conjugated anti-rat IgG. Protein bands were detected with Pierce™ ECL Plus or ImmunoStar LD.

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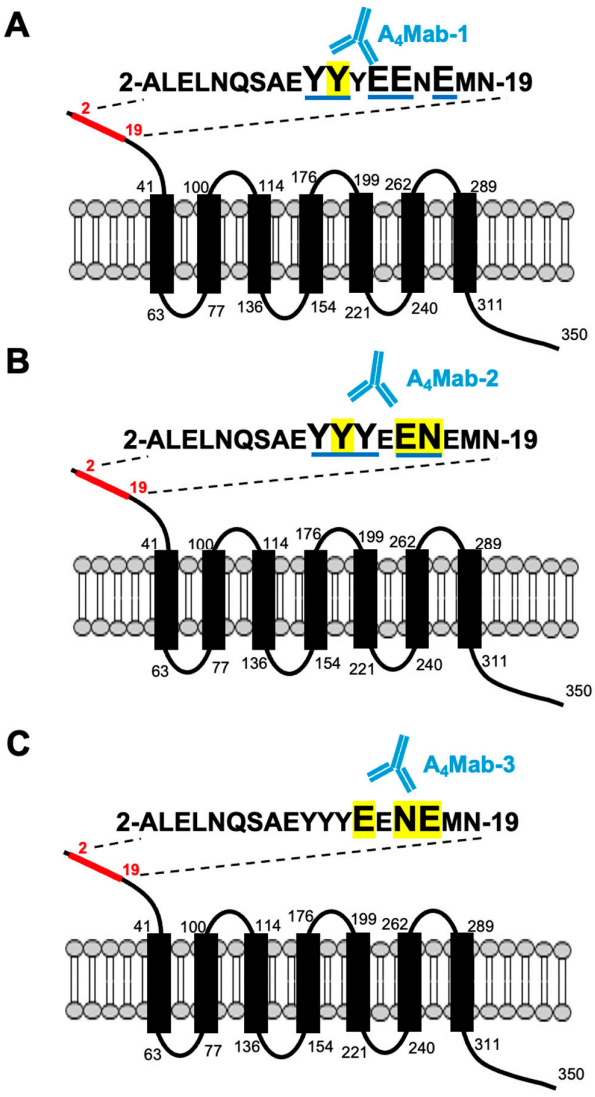

Figure 4. The schematic illustration of A4Mab-1, A4Mab-2, and A4Mab-3 epitopes. (A) Tyr12 of mACKR4 is essential for recognition by A4Mab-1 in flow cytometry (yellow), and Tyr11, Tyr12, Glu14, Glu15, and Glu17 are essential for the recognition by A4Mab-1 in western blotting (underlined). (B) Tyr12, Glu15, and Asn16 of mACKR4 are essential for recognition by A4Mab-2 in flow cytometry (yellow), and Tyr11, Tyr12, Tyr13, Glu15, and Asn16 are essential for the recognition by A4Mab-2 in western blotting (underlined). (C) Glu14, Asn16, and Glu17 of mACKR4 are essential for the recognition by A4Mab-3 in flow cytometry (yellow).

4. Discussion

This study conducted the epitope mapping of anti-mACKR4 mAbs (A4Mab-1, A4Mab-2, and A4Mab-3) by alanine scanning. Results revealed that Tyr12 is essential for recognition by A4Mab-1 in flow cytometry (Figure 2A), while Tyr11, Tyr12, Glu14, Glu15, and Glu17 are crucial in western blotting (Figure 3A). In A4Mab-2, Tyr12, Glu15, and Asn16 are vital in flow cytometry (Figure 2B), and Tyr11, Tyr12, Tyr 13, Glu15, and Asn16 are important in western blotting (Figure 3B). Additionally, Glu14, Asn16, and Glu17 are essential for recognition by A4Mab-3 in flow cytometry (Figure 2C). The recognition mode by A4Mab-1 and A4Mab-2 may be different between native structure in flow cytometry (Figure 2) and denatured structure in western blotting (Figure 3). These results would contribute to the understanding of mAb-epitope interaction.

ACKR4 has been established as a critical regulator of dendritic cell (DC) trafficking through modulation of CCR7-dependent pathways, via regulation of the abundance of CCR7 ligands, CCL19

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and CCL21 [16]. The N-terminal region of CCR7 contains sulfated tyrosine residues that are essential for high-affinity binding to these ligands [17,18]. Although ACKR4 also binds to these ligands, the requirement for tyrosine sulfation in the ligand binding has not been investigated. A4Mabs were established by immunization with the N-terminal 1-19 peptide without any modification, including sulfation [14]. The epitope of A4Mab-1 and A4Mab-2 includes a Tyr triplet (Figure 4), suggesting that A4Mab-1 and A4Mab-2 recognize non-sulfated tyrosine residues.

Unlike canonical GPCRs, ACKR4 does not elicit classical G protein–mediated signaling; instead, it functions as a scavenging receptor that facilitates chemokine clearance [19,20]. Mechanistically, ACKR4 is coupled to the endocytic machinery via β-arrestin. Upon ligand engagement, ACKRs undergo internalization into endosomal compartments, followed by lysosomal degradation of the bound chemokine [5]. Although the N-terminal region of ACKR4 has not been definitively characterized as a ligand-binding interface, it remains of interest to determine the relationship between A4Mab epitopes and the neutralizing or receptor internalization effects. These activities would be expected to attenuate mACKR4 function, potentially leading to an accumulation of its cognate chemokines.

Author Contributions: Conceptualization, M.K.K and Y.K.; investigation, M.H., T.T., M.Y., T.N; writing—original draft preparation, H.S.; writing—review and editing, Y.K.; project administration, Y.K.; funding acquisition, Y.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported in part by Japan Agency for Medical Research and Development (AMED) under Grant Numbers: JP26am0521010 (to Y.K.), JP26ama121008 (to Y.K.), JP25ama221153 (to Y.K.), JP25ama221339 (to Y.K.), and JP25bm1123027 (to Y.K.), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) grant no. 25K10553 (to Y.K.).

Data Availability Statement: The data presented in this study are available in the article.

Conflicts of Interest: The authors declare no conflict of interest.

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