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Article

Determination of the Binding Epitope of an Anti-mouse CCR9 Monoclonal Antibody (C9Mab-24) using the 1 × alanine and 2 × alanine-substitution Method

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Abstract: C-C chemokine receptor 9 (CCR9) is a receptor for C-C-chemokine ligand 25 (CCL25). CCR9 is crucial in the chemotaxis of immune cells and inflammatory responses. Moreover, CCR9 is highly expressed in tumors including several solid tumors and T-cell acute lymphoblastic leukemia. Several preclinical studies have shown that anti-CCR9 monoclonal antibodies (mAbs) exert antitumor activity. Therefore, CCR9 is an attractive target for tumor therapy. In this study, we conducted the epitope mapping of an anti-mouse CCR9 (mCCR9) mAb, C9Mab-24 (rat IgG_{2a}, kappa), using a 1 × alanine (1 × Ala) and 2 × alanine (2 × Ala)-substitution method via enzyme-linked immunosorbent assay. We first performed the 1 × Ala-substitution method using one alanine-substituted peptides of the mCCR9 N-terminus (amino acids 1-19). C9Mab-24 did not recognize two peptides (F14A and F17A), indicating that Phe14 and Phe17 are critical for C9Mab-24-binding to mCCR9. Furthermore, we conducted the 2 × Ala-substitution method using two consecutive alanine-substituted peptides of the mCCR9 N-terminus, and showed that C9Mab-24 did not react with four peptides (M13A–F14A, F14A–D15A, D16A–F17A, and F17A–S18A), indicating that ^{13}MFDDFS^{18} is involved in C9Mab-24-binding to mCCR9. Overall, combining, the 1 × Ala or 2 × Ala scanning methods could be useful for understanding for target-antibody interaction.

Keywords: mouse CCR9, monoclonal antibody, epitope mapping, alanine scanning, enzyme-linked immunosorbent assay

1. Introduction

C-C chemokine receptor 9 (CCR9) is a member of G-protein coupled receptors with seven transmembrane domains and four extracellular regions. Previous studies have shown that CCR9 is expressed on the surface of immature T lymphocytes and intestinal cells [1,2]. The C-C chemokine ligand 25 (CCL25), the only ligand for CCR9 [3,4], is mainly expressed in the thymus and intestinal epithelium [3,5-7]. CCL25 induces a chemotaxis of immature T cells into the thymus for their maturation [8]. Studies have demonstrated that CCR9 and CCL25 play important roles in inflammatory diseases, such as asthma [9], inflammatory bowel disease [10-12], and hepatitis [13,14]. Moreover, CCR9 is highly expressed in malignancies such as lung cancer [15], breast cancer [16,17], ovarian cancer [18],

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melanoma [19,20], and T-cell acute lymphoblastic leukemia (T-ALL) [21]. CCR9 is expressed in more than 70% cases of T-ALL. However, it is expressed in a small subset of normal T cells [21]. The interaction of CCR9 and CCL25 activates the phosphatidylinositide 3-kinase (PI3K)/Akt signaling pathway, which involves in the proliferation and survival of tumor cells [18,22,23].

Several anti-CCR9 mAbs have been developed for therapeutic uses. Anti-human CCR9 mAbs (clones 91R and 92R) exhibited the cytotoxicity against CCR9-positive tumors via antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. These antibodies suppressed T-ALL proliferation in mouse xenograft models [24,25]. Moreover, Maciocia *et al.* developed a CCR9 specific mAb by gene-gun vaccination of rats with a plasmid encoding human CCR9. They further produced the chimeric antigen receptor (CAR)-T cells, and showed a potent antitumor effect in cell lines and patient derived xenograft mouse models of T-ALL [21]. Therefore, CCR9 is considered as an attractive target for tumor therapy [5,26].

Using the N-terminal peptide immunization, we developed anti-mouse chemokine receptor mAbs against CCR2 [27], CCR3 [28], CCR4 [29], CCR6 [30], and CXCR6 [31]. Furthermore, we developed an anti-mCCR9 mAb, C$_9$Mab-24 (rat IgG$_{2a}$, kappa), via peptide immunization of the mCCR9 N-terminus (amino acids 1-19) [32]. In this study, we determined the binding epitope of C$_9$Mab-24 using two different alanine scanning strategies via enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. ELISA

The mCCR9 (accession no.: NP_001160097) peptides, such as wild type (WT), 19 of 1 × alanine (1 × Ala)-substituted peptides (Table 1), and 18 of 2 × alanine (2 × Ala)-substituted peptides (Table 2), were synthesized using PEPscreen (Sigma-Aldrich Corp., St. Louis, MO). Each peptide was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham) at a concentration of 1 μg/mL for 30 minutes at 37℃. As a negative control, no peptide was immobilized on the immunoplates. After washing with phosphate-buffered saline containing 0.05% Tween20 (PBST), the wells were blocked with 1% bovine serum albumin containing PBST for 30 minutes at 37℃. The plates were then incubated with C$_9$Mab-24 (1 μg/mL), followed by a 1:20000 dilution of peroxidase-conjugated anti-rat immunoglobulins (Sigma-Aldrich Corp.). Enzymatic reactions were performed using an ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc., Japan). Optical density was detected at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

3. Results

3.1. Epitope determination using 1 × Ala-substituted mCCR9 peptides.

We synthesized 19 different 1 × Ala-substituted mCCR9 peptides (Table 1). According to the ELISA results, C$_9$Mab-24 reacted to 1 × Ala-substituted peptides, such as M1A, M2A, P3A, T4A, E5A, L6A, T7A, S8A, L9A, I10A, P11A, G12A, M13A, D15A, D16A, S18A, and Y19A, as well as WT (positive control) (Fig. 1A). In contrast, C$_9$Mab-24 did not react with 1 × Ala-substituted peptides, such as F14A and F17A as well as a negative control (Fig. 1A). These results indicate that Phe14 and Phe17 of mCCR9 are the critical residues for the C$_9$Mab-24 binding to mCCR9. Figure 1B summarizes the results.

Table 1. Identification of the C$_9$Mab-24 epitope using 1×Ala-*substituted* mCCR9 peptides.

Peptides	*Sequences*	*C$_9$Mab-24*
WT	MMPTELTSLIPGMFDDFSY	++
M1A	AMPTELTSLIPGMFDDFSY	++
M2A	MAPTELTSLIPGMFDDFSY	++

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P3A	MMATELTSLIPGMFDDFSY	++
T4A	MMPAELTSLIPGMFDDFSY	++
E5A	MMPTALTSLIPGMFDDFSY	++
L6A	MMPTEATSLIPGMFDDFSY	++
T7A	MMPTELASLIPGMFDDFSY	++
S8A	MMPTELTALIPGMFDDFSY	++
L9A	MMPTELTSAIPGMFDDFSY	++
I10A	MMPTELTSLAPGMFDDFSY	++
P11A	MMPTELTSLIAGMFDDFSY	++
G12A	MMPTELTSLIPAMFDDFSY	++
M13A	MMPTELTSLIPGAFDDFSY	++
F14A	MMPTELTSLIPGMADDFSY	–
D15A	MMPTELTSLIPGMFADFSY	++
D16A	MMPTELTSLIPGMFDAFSY	++
F17A	MMPTELTSLIPGMFDDASY	–
S18A	MMPTELTSLIPGMFDDFAY	++
Y19A	MMPTELTSLIPGMFDDFSA	++

++, OD655≧0.3; –, OD655<0.1

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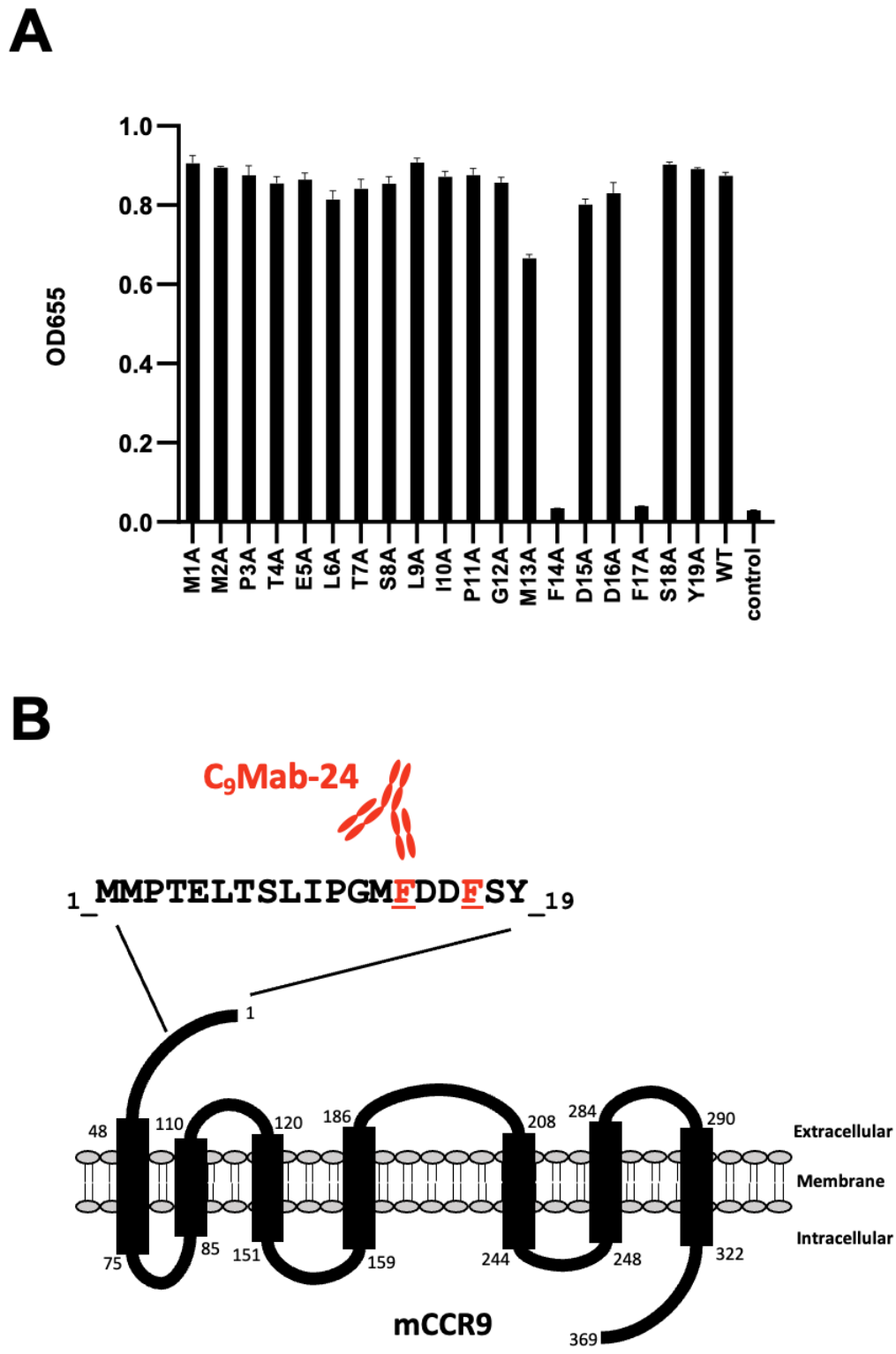

Figure 1. Determination of the C9Mab-24 epitope for mCCR9 by ELISA using 1×Ala-substituted peptides. (A) Synthesized 1 × Ala-substituted peptides of mCCR9 were immobilized on immunoplates. The plates were incubated with C9Mab-24 (1 μg/mL), followed by peroxidase-conjugated anti-rat immunoglobulins. (B) Schematic illustration of mCCR9 and the critical amino acids for C9Mab-24 epitope.

3.2. Epitope determination using 2 × Ala-substituted mCCR9 peptides

The result of 1 × Ala-substitution showed that Phe14 and Phe17 of mCCR9 are the most critical for the C9Mab-24-mCCR9 interaction, but did not show that only Phe14 and

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Phe17 of mCCR9 are enough for the C9Mab-24 binding to mCCR9. Therefore, we further investigated the C9Mab-24 epitope using 2×Ala-substituted mCCR9 peptides, as described previously [33].

We synthesized 18 different 2×Ala-substituted mCCR9 peptides (Table 2). According to the ELISA results, C9Mab-24 reacted to 2×Ala-substituted peptides, such as M1A–M2A, M2A–P3A, P3A–T4A, T4A–E5A, E5A–L6A, L6A–T7A, T7A–S8A, S8A–L9A, L9A–I10A, I10A–P11A, P11A–G12A, G12A–M13A, and S18A–Y19A as well as WT (positive control) (Fig. 2A). In contrast, C9Mab-24 did not react with 2 × Ala-substituted peptides, such as M13A–F14A, F14A–D15A, D16A–F17A, and F17A–S18A along with the negative control (Fig. 2A). Moreover, the reactivity of C9Mab-24 with D15A–D16A was weaker than that with WT (Fig. 2A). These results indicate that 13-MFDDFS-18 is involved in C9Mab-24-binding to mCCR9. Figure 2B summarizes the results.

Table 2. Identification of the C9Mab-24 epitope using 2 × Ala-*substituted* mCCR9 peptides.

Peptides	*Sequences*	*C9Mab-24*
WT	MMPTELTSLIPGMFDDFSY	++
M1A–M2A	AAPTELTSLIPGMFDDFSY	++
M2A–P3A	MAATELTSLIPGMFDDFSY	++
P3A–T4A	MMAAELTSLIPGMFDDFSY	++
T4A–E5A	MMPAALTSLIPGMFDDFSY	++
E5A–L6A	MMPTAATSLIPGMFDDFSY	++
L6A–T7A	MMPTEAASLIPGMFDDFSY	++
T7A–S8A	MMPTELAALIPGMFDDFSY	++
S8A–L9A	MMPTELTAAIPGMFDDFSY	++
L9A–I10A	MMPTELTSAAPGMFDDFSY	++
I10A–P11A	MMPTELTSLAAGMFDDFSY	++
P11A–G12A	MMPTELTSLIAAMFDDFSY	++
G12A–M13A	MMPTELTSLIPAAFDDFSY	++
M13A–F14A	MMPTELTSLIPGAADDFSY	–
F14A–D15A	MMPTELTSLIPGMAADFSY	–
D15A–D16A	MMPTELTSLIPGMFAAFSY	++
D16A–F17A	MMPTELTSLIPGMFDAASY	–
F17A–S18A	MMPTELTSLIPGMFDDAAY	–
S18A–Y19A	MMPTELTSLIPGMFDDFAA	++

++, OD655≧0.3; –, OD655<0.1

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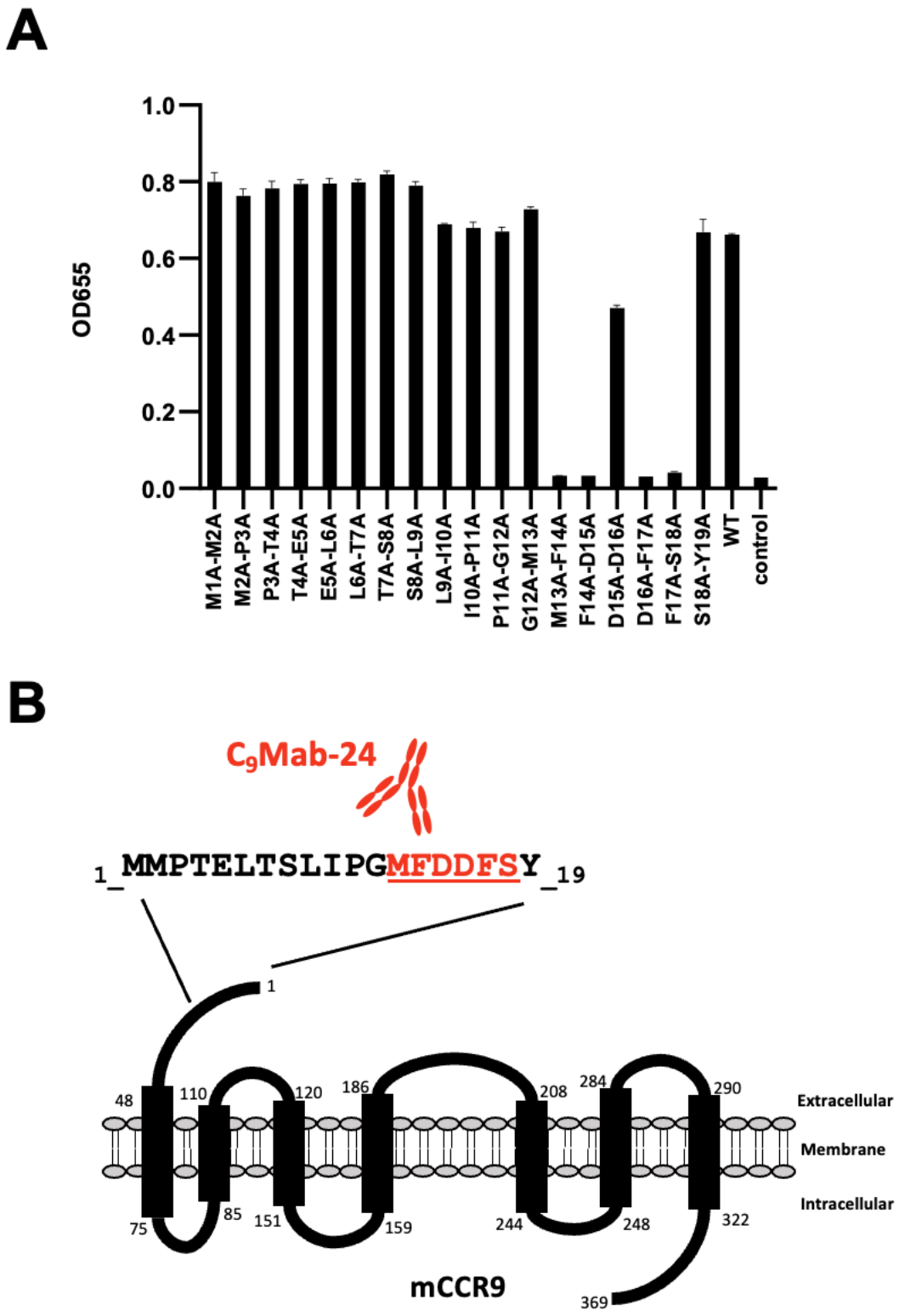

Figure 2. Determination of the C9Mab-24 epitope for mCCR9 by ELISA using 2×Ala-substituted peptides. (A) Synthesized 2 × Ala-substituted peptides of mCCR9 were immobilized on immunoplates. The plates were incubated with C9Mab-24 (1 μg/mL), followed by peroxidase-conjugated anti-rat immunoglobulins. (B) Schematic illustration of mCCR9 and the C9Mab-24 epitope region.

4. Discussion

We have established various anti-chemokine receptor mAbs against mouse CCR3 [34,35], mouse CCR8 [36-38], and human CCR9 (hCCR9) (clone C9Mab-1) [39] using the Cell-Based Immunization and Screening (CBIS) method. We also identified the epitope of

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C9Mab-1 in the N-terminal region of hCCR9 [40]. Then, we immunized the peptide of the region, and established a clone, C9Mab-11, which possesses a binding affinity comparable to C9Mab-1 [41]. C9Mab-1 possesses a wider epitope (10-IPAMA-14 and 16-DY-17) [40] than that of C9Mab-11 (11-PAMA-14) (manuscript submitted). In this study, we identified the epitope of the anti-mCCR9 mAb, C9Mab-24 which was established by the N-terminal peptide immunization of mCCR9 [32]. Using 2 × Ala and 1 × Ala-substitution methods, we found that 13-MFDDFS-18 is involved in C9Mab-24-binding to mCCR9, and phenylalanines at 14 and 17 are critical among the amino acids (Fig. 1 and Fig. 2). The combination of 2 × Ala and 1 × Ala-substitution methods could contribute the determination of region and center of mAb epitope.

The N-terminal region of CCR9 is important for the CCL25 interaction, and anti-hCCR9 mAbs (91R and 92R) recognized the N-terminus, and inhibited the binding to CCL25 [3,25]. The epitopes of 91R and 92R are located within 11-16 amino acids of hCCR9 [24,25], which is almost identical to the epitopes of C9Mab-1 and C9Mab-11. C9Mab-24 also binds to the N-terminal region of mCCR9 (13-MFDDFS-18). Therefore, the neutralizing activity of C9Mab-24 should be investigated in the future.

The therapeutic success for refractory childhood leukemia relies on the development of CAR-T targeting B-cell–specific antigen CD19 for B-cell acute lymphoblastic leukemia [42]. Although the therapy targets common B cell antigen, the background of the success is the ability to tolerate B-cell aplasia. Compared to B-cell aplasia, T-cell aplasia exhibits intolerable immunosuppression. Therefore, a major hurdle for the development of CAR-T cell therapy for T-ALL is the inability to find T-ALL antigens that are not expressed in normal T-cells [43]. To overcome this problem, CCR9 is expected for cell surface antigens unique to T-ALL cells. The CARCCR9, a CAR-T with a single-chain variable fragment (scFv) for hCCR9, demonstrated the cytotoxicity against CCR9-positive but not CCR9-negative T-ALL cells [21]. Although the epitope of the scFv has not been reported, the investigation of a suitable epitope to exert the CAR-T-mediated cytotoxicity is thought to be important for the future therapeutic applications of our anti-CCR9 mAbs.

Author Contributions: HK, TT, and TA performed the experiments. MKK and YK designed the experiments. HK, TA, HS, and YK analyzed the data. HK, TA, HS, and YK wrote the manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

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