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Epitope Mapping of an Anti-Mouse CXCR6 Monoclonal Antibody (Cx_6Mab-1) Using the 2×Alanine Scanning Method

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The CXC chemokine receptor 6 (CXCR6) is a member of the G protein-coupled receptor family that is highly expressed in helper T type 1 cells, cytotoxic T lymphocytes (CTLs), and natural killer cells. CXCR6 plays critical roles in local expansion of effector-like CTLs in tumor microenvironment to potentiate the antitumor response. Therefore, the development of anti-CXCR6 monoclonal antibodies (mAbs) is essential to evaluate the immune microenvironment of tumors. Using N-terminal peptide immunization, we previously developed an anti-mouse CXCR6 (mCXCR6) mAb, Cx_6Mab-1 (rat IgG1, kappa) , which is useful for flow cytometry and western blotting. In this study, we determined the critical epitope of Cx_6Mab-1 by enzyme-linked immunosorbent assay (ELISA) using the 1×alanine scanning (1×Ala-scan) method or the 2×alanine scanning (2×Ala-scan) method. Although we first performed ELISA by 1×Ala-scan using one alanine-substituted peptides of mCXCR6 N-terminal domain (amino acids 1–20), we could not identify the Cx_6Mab-1 epitope. We next performed ELISA by 2×Ala-scan using two alanine (or glycine) residues-substituted peptides of mCXCR6 N-terminal domain, and found that Cx_6Mab-1 did not recognize S8A–A9G, A9G–L10A, L10A–Y11A, and G13A–H14A of the mCXCR6 N-terminal peptide. The results indicate that the binding epitope of Cx_6Mab-1 includes Ser8, Ala9, Leu10, Tyr11, Gly13, and His14 of mCXCR6. Therefore, we could demonstrate that the 2×Ala scan method is useful for determining the critical epitope of mAbs.

Keywords: mouse CXCR6, epitope mapping, monoclonal antibody, enzyme-linked immunosorbent assay, 2×Ala scanning

Introduction

The CXC chemokine receptor 6 (CXCR6) is primarily expressed in T cells, including cytotoxic T lymphocytes (CTLs), helper T type 1 (Th1) cells, natural killer (NK) cells, and NK T cells. Upon the ligand (CXCL16) binding to CXCR6, it stimulates intracellular signaling pathway and mediates various cellular functions, including the infiltration into target tissues.[1] CXCL16 is constitutively secreted by liver sinusoids, which plays critical roles in the maintenance of NK, NK T, and $CD8^+$ memory T cells and lymphocyte homeostasis in the liver.[2]

CXCL16-CXCR6 axis also plays complex roles in tumor microenvironment (TME).[3] It promotes tumor progression directly through enhancing survival, proliferation, and metastasis of tumor cells. Furthermore, it potentiates tumor-promoting M2 macrophages infiltration,[4] and stimulates the conversion from mesenchymal stem cells into cancer-associated fibroblasts.[5] In contrast, CXCR6 mediates the differentiation into effector-like CTLs. In TME, CXCL16 is secreted by $CCR7^+$ dendritic cells, which also *trans*-present the survival cytokine interleukin-15 (IL-15). CXCR6 expression and IL-15 *trans*-presentation to effector-like CTLs are critical for the survival and local expansion in TME to enhance their antitumor immune response.[6] These results indicate a critical function of CXCR6 for potentiating $CD8^+$ CTLs-mediated antitumor immune responses.

We have developed monoclonal antibodies (mAbs) against chemokine receptors, including anti-mouse CCR2,[7] mouse

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CCR3,[8–10] mouse CCR4,[11] mouse CCR8,[12–14] and human CCR9,[15] and also determined the binding epitope.[16] We also established an anti-mouse CXCR6 (mCXCR6) mAb Cx_6Mab-1 (rat IgG_1, kappa) by N-terminal peptide immunization.[17] To clarify further characteristics of Cx_6Mab-1, we performed epitope mapping by enzyme-linked immunosorbent assay (ELISA) using the 1×alanine scanning (1×Ala-scan) method or the 2×alanine scanning (2×Ala-scan) method.

Materials and Methods

Peptides

The mCXCR6 (Accession No. NM_030712) peptide ($_1$-MDDGHQESALYDGHYEGDFW-$_{20}$) and one alanine (or glycine) residue-substituted peptides (Supplementary Table S1) and two alanine (or glycine) residues-substituted peptides (Table 1) were synthesized by utilizing PEPscreen (Sigma-Aldrich Corp., St. Louis, MO).

Enzyme-linked immunosorbent assay

Synthesized mCXCR6 peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham, MA) at a concentration of 1 μg/mL for 30 minutes at 37°C. 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-containing PBST for 30 minutes at 37°C. The plates were incubated with 1 μg/mL of Cx_6Mab-1, followed by a peroxidase-conjugated anti-rat immunoglobulins (1:10000 diluted; Sigma-Aldrich Corp.). Enzymatic reactions were performed 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).

Results

Epitope mapping of Cx_6Mab-1 with alanine-substituted mCXCR6 peptides

We previously established an anti-mCXCR6 mAb, Cx_6Mab-1 (rat IgG1, kappa), by immunizing rats with KLH-conjugated mCXCR6 N-terminal domain ($_1$-MDDGHQESALYDGHYEG DF-$_{19}$)+C-terminal cysteine residue.[17] To reveal the binding epitope of Cx_6Mab-1, we first synthesized one-alanine (or glycine)-substituted peptides of mCXCR6, which is called as 1×Ala-scan method (Supplementary Table S1). However, Cx_6Mab-1 reacted with all one-alanine (or glycine)-substituted peptides and wild-type (WT) (Supplementary Fig. S1).

We next synthesized mutant peptides that sequential two amino acids were substituted to two alanine (or glycine) residues, which is called as 2×Ala-scan method. For instance, a peptide (M1A–D2A) indicates the alanine substitution of first Met and second Asp of mCXCR6 peptide (Table 1). As shown in Figure 1A, Cx_6Mab-1 exhibited reaction with M1A–D2A, D2A–D3A, D3A–G4A, G4A–H5A, H5A–Q6A, Q6A–E7A, E7A–S8A, Y11A–D12A, D12A–G13A, H14A–Y15A, Y15A–E16A, E16A–G17A, G17A–D18A, D18A–F19A, F19A–W20A, and WT. In contrast, Cx_6Mab-1 did not react with S8A–A9G, A9G–L10A, L10A–Y11A, and G13A–H14A (Fig. 1), indicating that Ser8, Ala9, Leu10, Tyr11, Gly13, and His14 are included in the critical epitope of Cx_6Mab-1. The results are summarized in Table 1. Figure 1B shows the schematic illustration of mCXCR6 and the critical epitope of Cx_6Mab-1.

Discussion

A strategy, called alanine-scanning mutagenesis, was first used to identify specific residues in human growth hormone (hGH) that participate in the binding to the hGH receptor. Single alanine mutations were introduced at every residue within the regions that have been suggested in receptor

Table 1. Identification of the Cx_6Mab-1 Epitope Using 2×Alanine-Substituted mCXCR6 Peptides

Peptides	*Sequences*	*Cx_6Mab-1*
M1A–D2A	AADGHQESALYDGHYEGDFW	+++
D2A–D3A	MAAGHQESALYDGHYEGDFW	+++
D3A–G4A	MDAAHQESALYDGHYEGDFW	+++
G4A–H5A	MDDAAQESALYDGHYEGDFW	+++
H5A–Q6A	MDDGAAESALYDGHYEGDFW	+++
Q6A–E7A	MDDGHAASALYDGHYEGDFW	+++
E7A–S8A	MDDGHQAAALYDGHYEGDFW	+++
S8A–A9G	MDDGHQEAGLYDGHYEGDFW	–
A9G–L10A	MDDGHQESGAYDGHYEGDFW	–
L10A–Y11A	MDDGHQESAAADGHYEGDFW	–
Y11A–D12A	MDDGHQESALAAGHYEGDFW	+++
D12A–G13A	MDDGHQESALYAAHYEGDFW	+++
G13A–H14A	MDDGHQESALYDAAYEGDFW	–
H14A–Y15A	MDDGHQESALYDGAAEGDFW	+++
Y15A–E16A	MDDGHQESALYDGHAAGDFW	+++
E16A–G17A	MDDGHQESALYDGHYAADFW	+++
G17A–D18A	MDDGHQESALYDGHYEAAFW	+++
D18A–F19A	MDDGHQESALYDGHYEGAAW	+++
F19A–W20A	MDDGHQESALYDGHYEGDAA	+++

+++, OD655 ≧ 0.3;–, OD655 < 0.1.

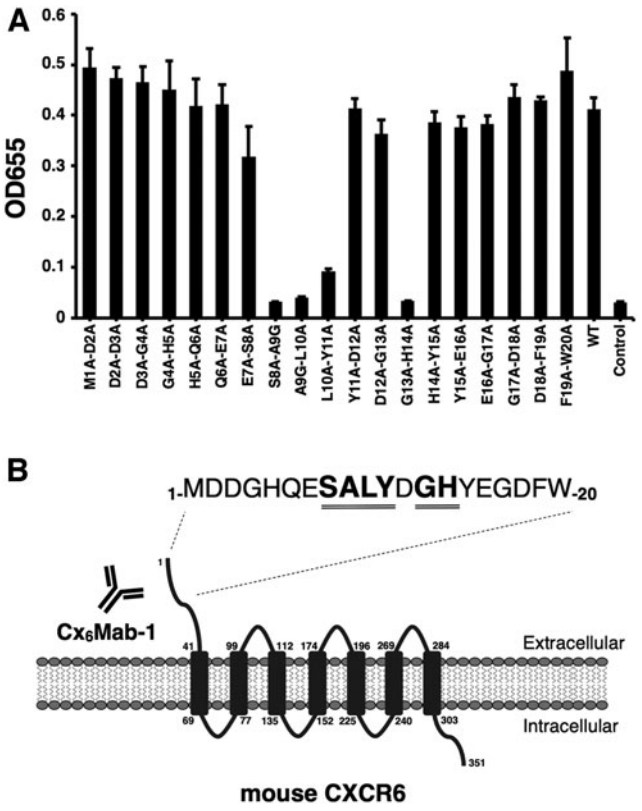

FIG. 1. Determination of the Cx_6Mab-1 epitope of mCXCR6 by ELISA using 2×Ala-scan method. **(A)** The 2×alanine-substituted mCXCR6 peptides were immobilized on immunoplates. The plates were incubated with Cx_6Mab-1 (1 μg/mL), followed by peroxidase-conjugated anti-rat immunoglobulins. **(B)** Schematic illustration of mCXCR6 and the Cx_6Mab-1 epitope. The Cx_6Mab-1 epitope involves Ser8, Ala9, Leu10, Tyr11, Gly13, and His14 of mCXCR6. ELISA, enzyme-linked immunosorbent assay; 2×Ala-scan, 2×alanine scanning; mCXCR6, mouse CXC chemokine receptor 6.

recognition.[18] The strategy was also applied to antibody–antigen interaction to analyze the functional epitope of hGH important for the binding to 21 different anti-hGH mAbs using ELISA.[19] In this study, we could not identify the epitope of Cx_6Mab-1 by 1×Ala-scan (Supplementary Fig. S1). However, the introduction of sequential amino acids substitution (2×Ala-scan) could determine the epitope of Cx_6Mab-1 (Fig. 1). This 2×Ala-scan method could be another option to determine the epitope of mAbs.

The N-terminus of chemokine receptors is known to be the ligand-binding domain.[20] The 3D structure of CXCR6 and its ligand CXCL16 has not been solved. In contrast, a molecular dynamics simulation of CXCR6-CXCL16 was reported. According to the simulation, acidic residues of human CXCR6, including E8, D9, and D17, interact with basic residues of human CXCL16.[21] In mCXCR6, these amino acids are thought to be conserved in and out of Cx_6Mab-1 epitope. Therefore, further investigation is required to evaluate the agonistic or antagonistic effects of Cx_6Mab-1 through the investigation of intracellular signaling pathways and cellular responses.[3,22]

CXCR6 plays a critical function for sustaining antitumor immune responses of $CD8^+$ CTLs through the conversion of stem-like memory cells into effector-like CTLs in TME.[6] In tumor immune therapy, the infiltration of CTLs into tumor is important factors to predict the efficacy.[23,24] Cx_6Mab-1 could be useful for investigating the expression of mCXCR6 in tumor infiltrating CTLs and use of combination therapy with immune checkpoint inhibitors in mice experiments.

Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1
Supplementary Table S1

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