Communication

Development of specific Anti-Mouse Atypical Chemokine Receptor 4 Monoclonal Antibodies

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Abstract: Leukocyte migration is an essential function of innate and adaptive immune responses. Chemokines and their receptors control the migration system. The abundance of chemokines is controlled by atypical chemokine receptors (ACKRs), chemokine receptor-like molecules that do not couple to the G protein signaling pathways. Among them, ACKR4 regulates dendritic cell migration by controlling the ligands and is involved in tumor development in mouse models. Because no anti-mACKR4 mAb for flow cytometry has been reported, this study aimed to develop a novel monoclonal antibody (mAb) for mouse ACKR4 (mACKR4). Among the established anti-mACKR4 mAbs, A$_4$Mab-1 (rat IgG$_{2b}$, kappa), A$_4$Mab-2 (rat IgG$_{2b}$, kappa), and A$_4$Mab-3 (rat IgG$_{2b}$, kappa) recognized mACKR4-overexpressed Chinese hamster ovary-K1 (CHO/mACKR4) by flow cytometry. The dissociation constant (K_D) values of A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3 for CHO/mACKR4 were determined as 3.5×10^{-9} M, 6.0×10^{-9} M, and 1.6×10^{-9} M, respectively. Furthermore, A$_4$Mab-1 and A$_4$Mab-2 could detect mACKR4 by western blotting. These results indicated that A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3 help to detect mACKR4 by flow cytometry and western blotting and obtain the proof of concept in preclinical models.

Keywords: mouse ACKR4; monoclonal antibody; peptide immunization; flow cytometry; western blotting

1. Introduction

The immune cell priming, memory responses, and effector functions are controlled by chemokines and the restricted expression of the G protein-coupled receptors (GPCRs) [1]. The chemokine receptors are the most prominent family of receptors, which possess a seven-transmembrane domain. They can be categorized into a larger subgroup of G protein-coupled receptors and a smaller subgroup of atypical chemokine receptors (ACKR1 to ACKR4) [2,3]. Additional candidates designated as CCRL2 (ACKR5), PITPNM3 (ACKR6), GPR182, and CXCR3-B are identified, but they are required for further functional characterization [2].

Upon a chemokine binding to its GPCR, the G protein subunits are usually activated [4]. The ACKRs are homologous to GPCRs. However, ACKRs fail in inducing the classical signaling through G protein [2]. Instead, the signaling is transduced to β-arrestin-dependent internalization of receptor and ligand, which leads to degradation of chemokine [2]. Therefore, ACKRs function as a chemokine scavenger receptor that mediates chemokines' rapid internalization and degradation.

ACKR4 has been described as expressed in not only T lymphocytes [5], but also stromal cells [6,7]. ACKR4 binds to the chemokines, including CCL19, CCL20, CCL21, CCL22, and CCL25. ACKR4 regulates migratory responses driven through CCR7 (activated by CCL19 and CCL21), CCR6 (activated by CCL20), CCR4 (activated by CCL22), and CCR9 (activated by CCL25) [2]. Therefore, ACKR4 controls the bioavailability of the abovementioned chemokines by creating a chemokine gradient, which facilitates the directional migration of dendritic cells (DCs) from the non-lymphatic tissue to the draining lymph node [6–9].

ACKR4 expression is reduced in human colorectal cancer (CRC) compared with normal colon epithelial cells [10]. The downregulation of ACKR4 in CRC is associated with a weak antitumor immune response [10]. Loss of ACKR4 in mouse colorectal cancer cells impairs the DC migration to the tumor-draining lymph nodes, which leads to the reduced number of tumor-specific T-cells and resistance to immune checkpoint blockades [10]. In the MMTV-PyMT transgenic mouse model, which recapitulates the step-wise progression of human breast cancer [12], the loss of mACKR4 led to an increase in intratumor $CD8^+$ T cells and $CD103^+$ DCs and inhibited tumor development [11]. These findings promise further study of the targeting of ACKR4 to improve the efficacy of immunotherapy. Therefore, a specific monoclonal antibody (mAb) against mouse ACKR4 (mACKR4) is essential to identify and target the ACKR4-expressing cells in the preclinical tumor models.

We have employed the Cell-Based Immunization and Screening (CBIS) method and developed anti-mouse chemokine receptor mAbs against CCR1 (clone C_1Mab-6) [12], CCR3 (clones C_3Mab-2, C_3Mab-3, and C_3Mab-4) [13–15], CCR5 (clone C_5Mab-2) [16], CCR8 (clones C_8Mab-1, C_8Mab-2, and C_8Mab-3) [17–19], CXCR1 (clone Cx_1Mab-1) [20], CXCR3 (clone Cx_3Mab-4) [21], and CXCR4 (clone Cx_4Mab-1) [22]. Furthermore, we established anti-mouse chemokine receptor mAbs against CCR2 (clone C_2Mab-6) [23], CCR3 (clones C_3Mab-6 and C_3Mab-7) [24], CCR4 (clone C_4Mab-1) [25], CCR5 (clones C_5Mab-4 and C_5Mab-8) [26], CCR9 (clone C_9Mab-24) [27], and CXCR6 (clone Cx_6Mab-1) [28] using the N-terminal peptide immunization. Because no anti-mACKR4 mAb was commercially available, we aimed to develop novel anti-mACKR4 mAbs.

2. Materials and Methods

2.1 Cell lines and Plasmids

P3X63Ag8U.1 (P3U1), LN229, and Chinese hamster ovary (CHO)-K1 cell lines were obtained from the American Type Culture Collection (Manassas, VA).

The synthesized mACKR4 (Accession No.: NM_145700.2) cDNA (Eurofins Genomics KK, Tokyo, Japan) was subsequently subcloned into pCAGzeo-nMAP vectors (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The MAP tag can be detected by an anti-mouse podoplanin mAb (clone PMab-1) [29]. The mACKR4 plasmids were transfected into CHO-K1 and LN229. Stable clones were sorted using a cell sorter (SH800; Sony Corp., Tokyo, Japan). These cells were cultured as described previously [28].

2.2. Peptides

The N-terminal extracellular region of mACKR4 ($_1$-MALELNQSAEYYYEENEMN-$_{19}$) plus C-terminal cysteine was synthesized by Eurofins Genomics KK. The keyhole limpet hemocyanin (KLH) was subsequently conjugated at the C-terminus.

2.3. Production of Hybridomas

The approval of animal experiments was obtained from the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001). A five-week-old Sprague-Dawley rat (CLEA Japan, Tokyo, Japan) was intraperitoneally immunized with 100 µg of the KLH-conjugated mACKR4 peptide (mACKR4-KLH) with Alhydrogel adjuvant 2% (InvivoGen). After three additional weekly immunizations (100 µg/rat) and final booster immunizations (100 µg/rat), hybridomas were produced and cultured in the medium containing hypoxanthine, aminopterin, and thymidine (HAT; Thermo Fisher Scientific Inc., Waltham, MA), 10% FBS, and 5% BriClone (NICB, Dublin, Ireland). The supernatants were screened using enzyme-linked immunosorbent assay (ELISA) using the mACKR4 peptide. The supernatants were further screened by flow cytometry using CHO-K1 and CHO/mACKR4.

2.4. Purification of Antibodies

The cultured supernatants of A_4Mab-1, A_4Mab-2, and A_4Mab-3 hybridomas were purified using 1 mL of Ab-Capcher (ProteNova, Kagawa, Japan). The mAbs were eluted with an IgG elution buffer

(Thermo Fisher Scientific Inc.), and replaced with phosphate-buffered saline (PBS) using Amicon Ultra (Merck KGaA, Darmstadt, Germany).

2.5. ELISA

The synthesized mACKR4 peptide (MALELNQSAEYYYEENEMNC) was immobilized on 96 well immunoplates (Thermo Fisher Scientific Inc.). Blocking was performed with 1% bovine serum albumin (BSA)-PBS containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc.). The plates were incubated with supernatants, followed by peroxidase-conjugated anti-rat IgG (Sigma-Aldrich Corp., St. Louis, MO). The peroxidase reactions were performed using ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.).

2.6. Flow Cytometric Analysis

Cells were harvested after brief exposure to 1 mM EDTA. The cells were washed with blocking buffer (0.1% BSA in PBS) and treated with 1, 0.1, and 0.01 µg/mL of A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3 for 30 min at 4°C. For peptide inhibition assay, A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3 (0.1 µg/mL) were pre-incubated with 10 µg/mL of mACKR4 peptide or dimethyl sulfoxide (DMSO) for 25 min at 4°C, and incubated with the cells for 30 min at 4°C. Alexa Fluor 488-conjugated anti-rat IgG was used as a secondary antibody. The SA3800 Cell Analyzer (Sony Corp.) was used for fluorescence data collection and analysis. The dissociation constant (K_D) was determined as described previously [26].

2.7. Western Blotting

Sodium dodecyl sulfate-treated cell lysates from LN229 and LN229/mACKR4 were separated on polyacrylamide gels. The proteins were transferred onto polyvinylidene difluoride membranes (Merck KGaA). The membranes were incubated with 1 µg/mL of A$_4$Mab-1, A$_4$Mab-2, PMab-1, or AC-15 (an anti-β-actin mAb; Sigma-Aldrich Corp.). For peptide blocking assay, 1 µg/mL of A$_4$Mab-1 and A$_4$Mab-2 were pre-incubated with 1 µg/mL of mACKR4 peptide or dimethyl sulfoxide (DMSO) for 15 min and incubated with the membranes. Peroxidase-conjugated anti-rat IgG or anti-mouse IgG (Agilent Technologies Inc., Santa Clara, CA) were used as secondary antibodies. The signals were detected using ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) and a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

3. Results

3.1. Development of Anti-mACKR4 mAbs Using N-Terminal Peptide Immunization

To develop anti-mACKR4 mAbs, one rat was immunized with mACKR4-KLH (Figure 1A). The splenocytes were fused with P3U1 myeloma cells and seeded into 96-well plates. Then, positive wells for the naked mACKR4 peptide were selected using ELISA. Then, CHO/mACKR4-reactive and CHO-K1-non-reactive supernatants were selected using flow cytometry (Figure 1B). After the cloning by limiting dilution and additional screenings, anti-mACKR4 mAbs, A$_4$Mab-1 (rat IgG$_{2b}$, kappa), A$_4$Mab-2 (rat IgG$_{2b}$, kappa), and A$_4$Mab-3 (rat IgG$_{2b}$, kappa) were finally established (Figure 1C).
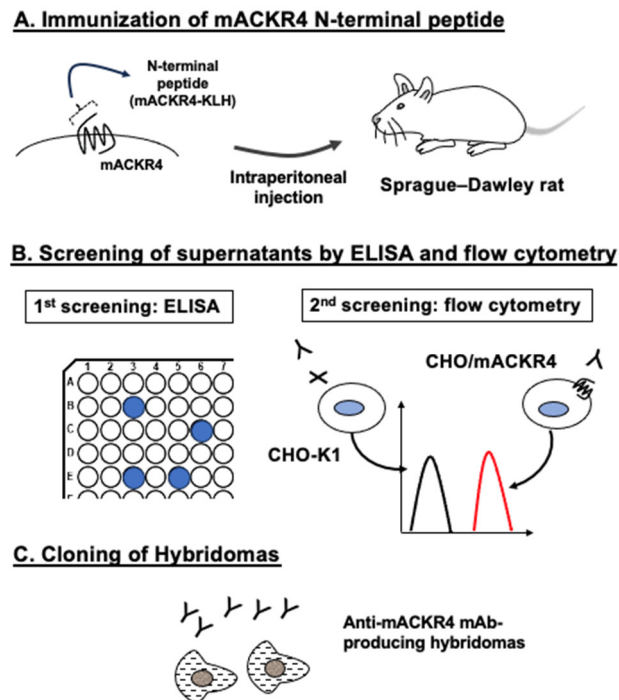
Figure 1. The production of anti-mACKR4 mAbs. (**A**) mACKR4 N-terminal peptide conjugated with KLH (mACKR4-KLH) was immunized into a Sprague–Dawley rat. The spleen cells were fused with P3U1 cells. (**B**) To select anti-mACKR4 mAb-producing hybridomas, the supernatants were screened by ELISA and flow cytometry using CHO-K1 and CHO/mACKR4 cells. (**C**) After limiting dilution, anti-mACKR4 mAbs, A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3 were finally established. ELISA, enzyme-linked immunosorbent assay.

3.2. Flow Cytometric Analysis Using A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3

We conducted flow cytometry using three anti-mACKR4 mAbs: A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3 against CHO/mACKR4 and CHO-K1 cells. A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3 recognized CHO/mACKR4 cells dose-dependently at 1, 0.1, and 0.01 µg/mL (Figure 2A). A$_4$Mab-3 exhibited a superior reactivity against CHO/mACKR4 cells compared to A$_4$Mab-1 and A$_4$Mab-2 (Figure 2A). Parental CHO-K1 cells were not recognized by any mAbs even at 1 µg/mL (Figure 2B). The superior reactivity of A$_4$Mab-3 was also observed in LN229/mACKR4 cells (supplementary Figure 1).

We next performed a peptide-blocking assay. As shown in Figure 3, A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3 reacted with the CHO/mACKR4. The mACKR4 peptide wholly neutralized these reactions (Figure 3).
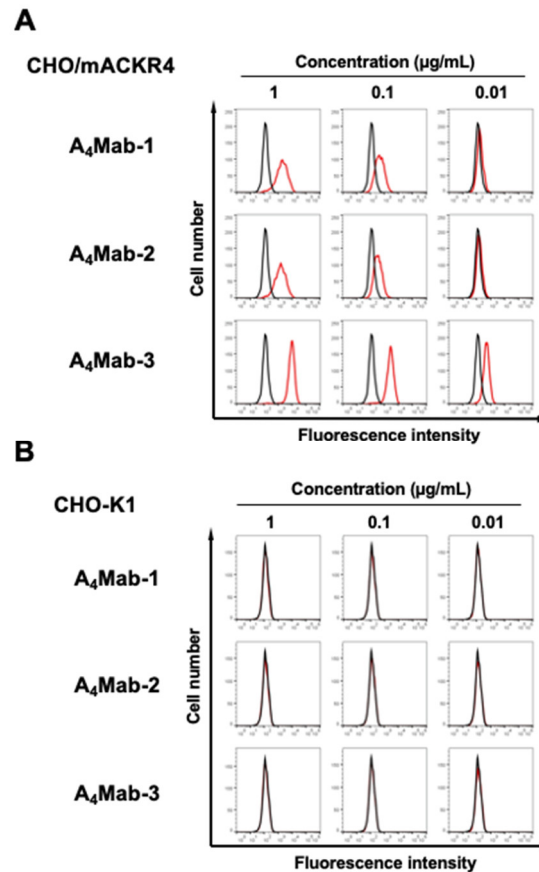
Figure 2. Flow cytometry of mACKR4-overexpressed CHO-K1 cells using anti-mACKR4 mAbs. CHO/mACKR4 (**A**) and CHO-K1 (**B**) cells were treated with 0.01–1 μg/mL of A₄Mab-1, A₄Mab-2, or A₄Mab-3 (red line). The mAbs-treated cells were further incubated with anti-rat IgG conjugated with Alexa Fluor 488. The black line represents the negative control (blocking buffer).
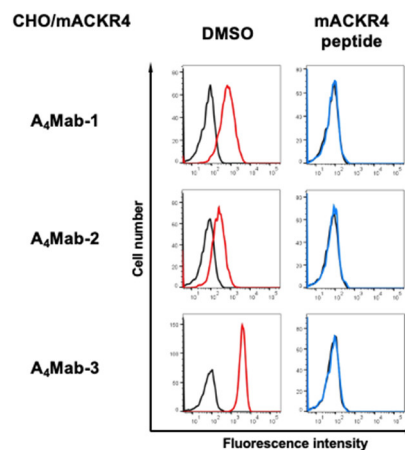

Figure 3. A peptide-blocking assay using A₄Mab-1, A₄Mab-2, and A₄Mab-3 with mACKR4 peptide. A₄Mab-1, A₄Mab-2, or A₄Mab-3 (0.1 μg/mL) plus mACKR4 peptide (10 μg/ml, blue line) or control (1% DMSO in blocking buffer, red line) were reacted with CHO/mACKR4 for 30 min at 4°C, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. The black line represents the negative control (blocking buffer). DMSO, dimethyl sulfoxide.

3.3. Determination of the Binding Affinity of A₄Mab-1, A₄Mab-2, and A₄Mab-3 Using Flow Cytometry

To determine the K_D values of A₄Mab-1, A₄Mab-2, and A₄Mab-3 against CHO/mACKR4, we conducted flow cytometry, and the geometric mean of the fluorescence intensity was plotted versus the concentration. The K_D values of A₄Mab-1, A₄Mab-2, and A₄Mab-3 for CHO/mACKR4 were determined as 3.5×10^{-9} M, 6.0×10^{-9} M, and 1.6×10^{-9} M, respectively. (Figure 4). These results indicate that A₄Mab-3 possesses the most superior affinity to CHO/mACKR4.
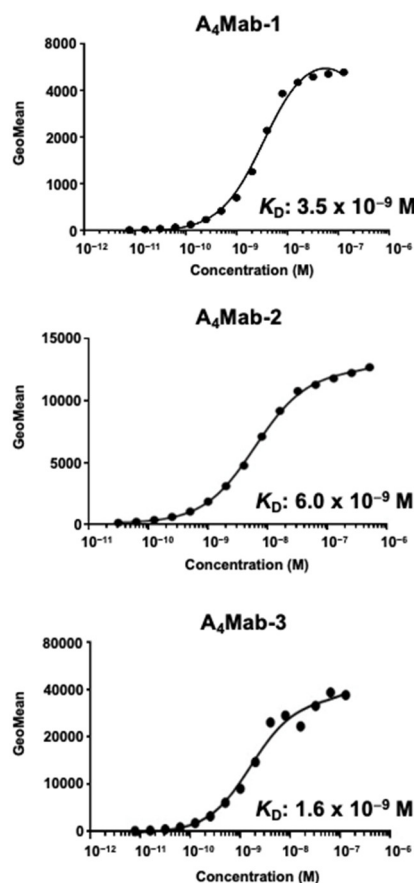

Figure 4. The binding affinity of anti-mACKR4 mAbs. CHO/mACKR4 cells were suspended in serially diluted A₄Mab-1, A₄Mab-2, or A₄Mab-3. The cells were treated with anti-rat IgG conjugated with Alexa Fluor 488. The fluorescence data were subsequently collected using the SA3800 Cell Analyzer, followed by the calculation of the K_D using GraphPad PRISM 6.

3.4. Detection of mACKR4 Using A₄Mab-1, A₄Mab-2, and A₄Mab-3 by Western Blotting

Western blotting was performed to assess the reactivity of A₄Mab-1, A₄Mab-2, and A₄Mab-3. Lysates of LN229 and LN229/mACKR4 cells were probed. A₄Mab-1 (Figure 5A) and A₄Mab-2 (Figure 5B) detected mACKR4 as a ~50-kDa band. In contrast, A₄Mab-1 and A₄Mab-2 did not show any bands from the lysates of LN229 cells. A₄Mab-2 exhibited a superior reactivity against LN229/mACKR4 cell lysate compared to A₄Mab-1 (Figure 5A and B, the same exposure time). In contrast, A₄Mab-3 could not detect any bands in the LN229/mACKR4 cell lysate (data not shown). A similar molecular weight band was also detected by PMab-1 (Figure 5C). An anti-β-actin was used as an internal control (Figure 5D). We also performed the peptide blocking experiment. We found that the 50-kDa band detected by A₄Mab-1 and A₄Mab-2 was blocked in the presence of mACKR4 peptide (Figure 5E and 5F, respectively). These results suggest that A₄Mab-1 and A₄Mab-2 are useful for detecting mACKR4 by western blotting.
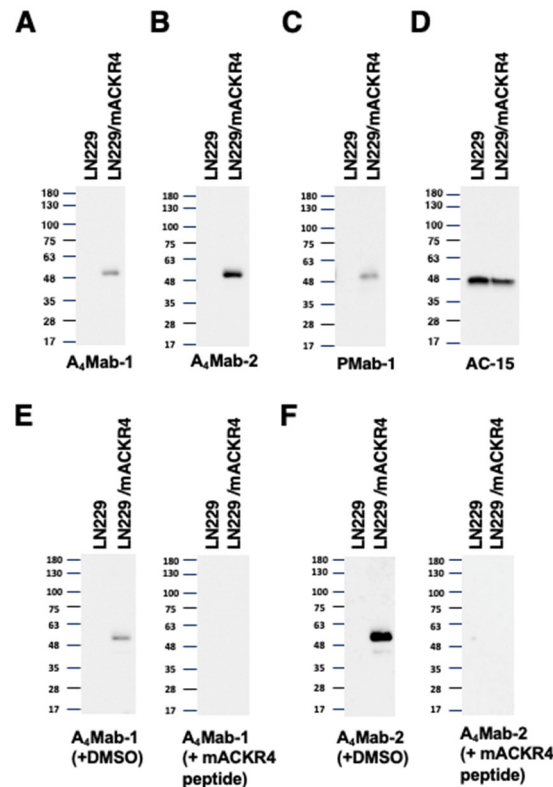
Figure 5. Western blotting using anti-mACKR4 mAbs. The lysates of LN229 and LN229/mACKR4 cells were electrophoresed and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with 1 μg/mL of A4Mab-1 (A), 1 μg/mL of A4Mab-2 (B), 1 μg/mL of PMab-1 (an anti-MAP tag mAb) (C), or 1 μg/mL of AC-15 (an anti-β-actin mAb) (D). The membranes were subsequently incubated with peroxidase-conjugated anti-rat IgG (for A4Mab-1, A4Mab-2, and PMab-1) or anti-mouse IgG (for AC-15). Note that the exposure time of A4Mab-1 and A4Mab-2 blotting was the same. (E) The membranes were incubated with 1 μg/mL of A4Mab-1 plus the mACKR4 peptide (1 μg/mL) or DMSO. (F) The membranes were incubated with 1 μg/mL of A4Mab-2 plus the mACKR4peptide (1 μg/mL) or DMSO. They were subsequently incubated with peroxidase-conjugated anti-rat IgG.

4. Discussion

In this study, we developed novel anti-mACKR4 mAbs (A4Mab-1, A4Mab-2, and A4Mab-3) using the N-terminal peptide immunization and showed the usefulness for flow cytometry (Figures 2−4) and western blotting (Figure 5) to detect mACKR4. Because no anti-mACKR4 mAb for flow cytometry has been reported, these mAbs could be the first ones for detecting mouse ACKR4 in flow cytometry.

ACKR4 has emerged as an essential regulator of DC migration via CCR7, as ACKR4 binds to the CCR7 ligands, CCL19 and CCL21 [30]. ACKR4 dose not induce classical GPCR signaling and instead leads to the degradation of chemokine [31,32]. ACKRs are linked to endocytic machinery via β-arrestin. Upon chemokine ligation to ACKRs, the complex is generally internalized to endosomal machinery and subsequent chemokine degradation by lysosome [33]. A4Mab-1, A4Mab-2, and A4Mab-3 recognize the N-terminal region of mACKR4 with different K_D values from 6.0×10^{-9} M to 1.6×10^{-9} M (Figure 4). Although the ACKR4 N-terminus has not been determined as a ligand-binding region, it would be interesting to investigate whether these mAbs possess the neutralizing activity to the ligands or promote the internalization of mACKR4. These functions are expected to inhibit the mACKR4 activity, which may increase the number of mACKR4 ligands.

A4Mab-1, A4Mab-2, and A4Mab-3 are applicable for flow cytometry (Figures 2−4), and A4Mab-1, A4Mab-2, but not A4Mab-3 are useful for western blotting (Figure 5). These results suggest that

A$_4$Mab-3 possesses the different epitope from A$_4$Mab-1 and A$_4$Mab-2. Previously, we determined the epitope of Cx$_6$Mab-1 (an anti-mouse CXCR6 mAb) using 1× and 2× alanine scanning methods [34]. In future studies, we should evaluate the epitopes of A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3. The identification of epitopes would contribute to understanding the properties of mAbs.

Immune checkpoint blockades have developed for cancer treatments [35]. The extent of intratumor CD8$^+$ cell infiltration is correlated with improved responsiveness of anti-PD-1/PD-L1 therapy in melanoma [36] and improved outcomes in colorectal cancer [37]. A study investigated the roles of host mACKR4 in the development of mammary tumor by crossing MMTV-PyMT transgenic mice with the mACKR4-knockout strain [11]. The mammary tumor development was delayed in the mACKR4-knockout compared to control mACKR4-sufficient transgenic mice [11]. Mechanistically, the loss of mACKR4 leads to increased intra-tumor CCL21 levels and elevated numbers of CD103$^+$ DCs and CD8$^+$ cells within tumors. However, the contribution of mACKR4-expressing host cells to tumor microenvironment (TME) has not been investigated. Therefore, A$_4$Mab-1, A$_4$Mab-2, and A$_4$Mab-3 would help identify the host cells that contribute to the formation of immunosuppressive TME. Furthermore, these mAbs could contribute to the preclinical studies for the depletion of mACKR4-expressing cells to enhance responsiveness to immune checkpoint blockade or T-cell co-stimulation.

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Yukinari Kato: Conceptualization, Funding acquisition, Project administration, Writing – review and editing. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The animal study protocol was approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001) for studies involving animals.

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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