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Development of a Novel Anti-Mouse CCR4 Monoclonal Antibody (C_4Mab-1) by N-Terminal Peptide Immunization

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The CC chemokine receptor type-4 (CCR4) belongs to the G-protein-coupled receptor superfamily, expressed on the cell surface of T cells and its malignancy. Two CCR4 ligands (CCL17 and CCL22) bind to CCR4 that mediate physiological and pathological functions of T cell immune responses. Anti-CCR4 monoclonal antibody (mAb) mogamulizumab is approved for adult T cell leukemia/lymphoma and cutaneous T cell lymphomas. In addition, mogamulizumab can deplete regulatory T cells, implying the application to solid tumors as an immunomodulator. Therefore, the development of sensitive mAbs for CCR4 has been desired for basic research, diagnosis, and therapy. In this study, a specific, and sensitive anti-mouse CCR4 (mCCR4) mAb, C_4Mab-1 (rat IgG_1, kappa), was established using N-terminal peptide immunization. C_4Mab-1 reacted with mCCR4-overexpressed Chinese hamster ovary (CHO)-K1 cells, P388 (mouse lymphoid neoplasm), and J774-1 (mouse macrophage-like) cells in flow cytometry. Kinetic analyses using flow cytometry showed that K_Ds of C_4Mab-1 for CHO/mCCR4, P388, and J774-1 cells were 4.2×10^{-9} M, 5.4×10^{-7} M, and 1.1×10^{-6} M, respectively. C_4Mab-1 could be a valuable tool for elucidating mCCR4-related biological responses.

Keywords: CCR4, monoclonal antibody, flow cytometry

Introduction

CC chemokine receptor 4 (CCR4) is a chemokine receptor for two CC chemokine ligands, CCL17 and CCL22. CCR4 is primarily expressed in T cells, including helper T type 2 (Th2), regulatory T (Treg), and Th17-cells.[1] Upon the chemokines binding to CCR4, the chemokines can trigger intracellular signaling of CCR4 expressing cells, which mediates various cellular functions, including the infiltration into target tissues.[2]

In addition, elevated serum levels of CCL17 and CCL22 have been reported in patients with atopic dermatitis and correlate with disease severity and treatment response.[3–5] Accordingly, the serum CCL17 test was approved by the Japanese Pharmaceutical and Medical Devices Agency and is now regarded as an excellent biomarker for disease severity and therapeutic response in the disease.[6]

In addition, CCR4 is strongly expressed in T cell malignancies, including adult T cell leukemia/lymphoma (ATLL)[7] and cutaneous T cell lymphomas (CTCLs).[8] Therefore, CCR4 has been regarded as a possible therapeutic target for these malignancies. Mogamulizumab (Poteligeo) is a defucosylated humanized monoclonal antibody (mAb) targeting CCR4. Mogamulizumab binds to Fcγ receptor IIIa on natural killer cells and exerts potent antibody-dependent cellular cytotoxicity (ADCC) against the $CCR4^+$ cells.[9,10] Mogamulizumab was first approved in Japan for ATLL therapy.[11,12]

Clinical studies of mogamulizumab in CTCLs were conducted. Mogamulizumab efficiently decreased levels of $CCR4^+$ malignant T cells and $CCR4^+$ Tregs in CTCL patients.[13,14] Mogamulizumab significantly improved the progression-free survival and overall response rate compared with vorinostat, a histone deacetylase inhibitor approved for treating CTCLs.[15,16] From these results, the US Food and Drug Administration approved mogamulizumab in 2018 for treating adult patients with relapsed or refractory mycosis fungoides (MF) and Sézary syndrome (SS), which are the most common types of CTCLs.[17]

In solid tumors, Treg cells play a critical role in suppressing tumor immunity.[18] Treg cells abundantly infiltrate into tumor tissues, associated with poor prognosis in cancer patients. Removal of Treg cells is thought to increase the

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antitumor immune responses and potentiate the efficacy of immune checkpoint inhibitors. Since mogamulizumab efficiently depletes Treg cells, clinical trials in advanced solid tumors are currently conducted singly or combined with immune checkpoint inhibitors.[19,20]

Using the cell-based immunization and screening (CBIS) method, many mAbs have been developed against membrane proteins, such as anti-CD19,[21] anti-CD20,[22,23] anti-CD44,[24] anti-CD133,[25] anti-EpCAM,[26,27] anti-HER3,[28] anti-KLRG1,[29] anti-TIGIT,[30] anti-TROP2,[31,32] anti-programmed cell death ligand 1,[33] and anti-podoplanin[34–44] mAbs. CBIS method includes immunizing antigen-overexpressing cells and the high-throughput hybridoma screening using flow cytometry. Anti-chemokine receptors mAbs, including anti-mouse CCR3,[45] anti-mouse CCR8,[46] and anti-human CCR9[47] mAbs, were also successfully developed using the CBIS method. This study developed anti-mouse CCR4 (mCCR4) mAbs using N-terminal peptide immunization.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). P388 (mouse lymphoid neoplasma) and J774-1 (mouse macrophage-like) cells were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Miyagi, Japan). Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding mCCR4 (accession no. NM_009916) was subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

mCCR4 plasmid was transfected into CHO-K1 cells using the Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were developed through cell sorting using a cell sorter (SH800; Sony Corp., Tokyo, Japan) and cultivation in a medium containing 0.5 mg/mL Zeocin (InvivoGen, San Diego, CA).

CHO-K1, P3U1, mCCR4-overexpressed CHO-K1 (CHO/mCCR4), P388, and J774-1 were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100-μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). Cells were grown in a humidified incubator at 37°C with an atmosphere of 5% CO_2 and 95% air.

Antibodies

The anti-mCCR4 mAb (clone 2G12) was bought from BioLegend (San Diego, CA). The secondary Alexa Fluor 488-conjugated anti-rat IgG and anti-Armenian hamster IgG was bought from Cell Signaling Technology, Inc. (Danvers, MA).

Hybridoma production

A 6-week-old female Sprague–Dawley (SD) rat was bought from CLEA, Japan (Tokyo, Japan). The animal was housed under specific pathogen-free conditions. Then, animal experiments were performed, following relevant guidelines and regulations to reduce animal suffering and distress in the laboratory. The Animal Care and Use Committee of Tohoku University approved the animal experiments (Permit no. 2019NiA-001). Rat health was monitored daily during the entire 4-week duration of the experiment, and a decline of >25% of the total body weight was denoted as a humane endpoint. Subsequently, the rat was euthanized through cervical dislocation, after which respiratory and cardiac arrest was used to verify death.

The mCCR4 peptide immunization method was used to develop mAbs against mCCR4. In brief, one SD rat was immunized with the keyhole limpet hemocyanin-conjugated mCCR4 peptide (100 μg): $_1$-MNATEVTDTTQDETVYNSY-$_{19}$ + C-terminal cysteine. The administration was performed through the intraperitoneal route with an Imject Alum (Thermo Fisher Scientific, Inc.).

The procedure involved three additional immunization procedures (100 μg), followed by a final booster intraperitoneal injection (100 μg) 2 days before its spleen cells were harvested. The harvested spleen cells were subsequently fused with P3U1 cells using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN). Then, hybridomas were cultured in an RPMI 1640 medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). Cultured supernatants were finally screened by enzyme-linked immunosorbent assay (ELISA) and flow cytometric analysis.

ELISA

The synthesized mCCR4 peptide: $_1$-MNATEVTDTTQ DETVYNSY-$_{19}$ + C-terminal cysteine was immobilized on Nunc Maxisorp 96-well Immuno plates (Thermo Fisher Scientific, Inc.) 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.), wells were blocked with 1% bovine serum albumin (BSA)-containing PBST for 30 minutes at 37°C.

Plates were then incubated with culture supernatants, followed by peroxidase-conjugated anti-rat immunoglobulins (1:2000 diluted; Agilent Technologies, Inc., Santa Clara, CA). Afterward, enzymatic reactions were performed using the ELISA POD substrate TMB kit (Nacalai Tesque, Inc.), followed by the optical density measurement at 655-nm, using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Flow cytometry

CHO-K1 and CHO/mCCR4 cells were harvested after brief exposure to 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). CHO-K1, CHO/mCCR4, P388, and J774-1 cells were washed using 0.1% BSA in PBS and treated with primary mAbs for 30 minutes at 4°C. Afterward, cells were treated using Alexa Fluor 488-conjugated anti-rat IgG or anti-Armenian hamster IgG (1:1000) also, followed by the collection of fluorescence data, using the EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Determination of the binding affinity by flow cytometry

CHO-K1, CHO/mCCR4, P388, and J774-1 cells were suspended in 100 μL serially diluted anti-mCCR4 mAbs,

after which Alexa Fluor 488-conjugated anti-rat IgG anti-Armenian hamster IgG (1:200) was added. Fluorescence data were subsequently obtained, using the BD FACSLyric (BD Biosciences, Franklin Lakes, NJ), followed by the calculation of the dissociation constant (K_D) by fitting the binding isotherms into the built-in one-site binding model in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

Results

Establishment of anti-mCCR4 mAbs

To develop anti-mCCR4 mAbs, the mCCR4 peptide immunization method was employed. Hybridoma screening was performed using ELISA and flow cytometry (Fig. 1). A rat was immunized with the CCR4 synthetic peptide. Subsequently, hybridomas were seeded into 96-well plates, after which ELISA was used to obtain positive wells for mCCR4 peptides, followed by selecting CHO/mCCR4-reactive and CHO-K1-nonreactive supernatants using flow cytometry. Finally, C_4Mab-1 (rat IgG_1, kappa) was established after limiting dilution.

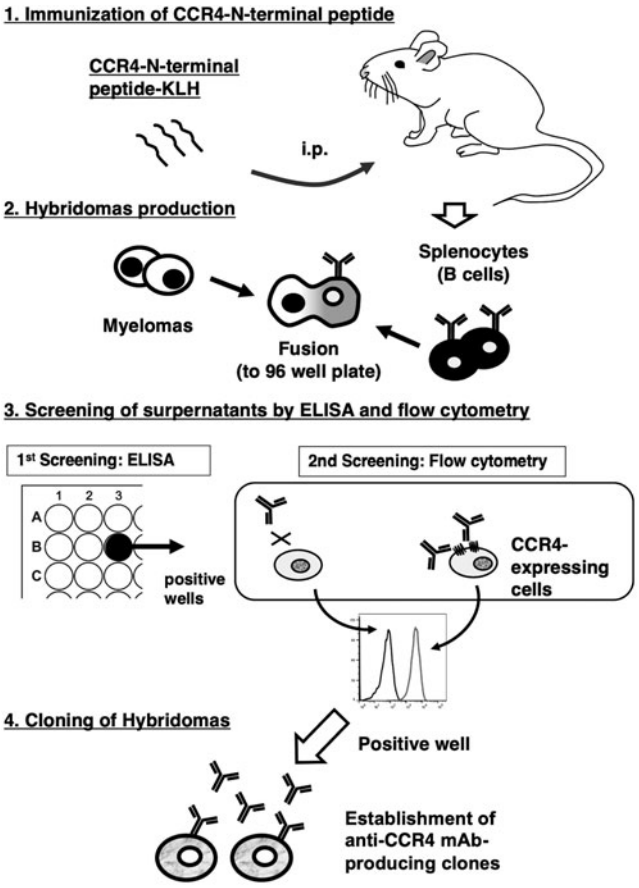

FIG. 1. A schematic procedure of anti-mCCR4 mAbs production. The rat was intraperitoneally immunized with the mCCR4 peptide. The screening was then conducted by ELISA, using the mCCR4 peptide, flow cytometry using parental cells and mCCR4-overexpressed CHO-K1 cells. CHO-K1, Chinese hamster ovary-K1; ELISA, enzyme-linked immunosorbent assay; mAbs, monoclonal antibodies; mCCR4, mouse CCR4.

Flow cytometry

Flow cytometry was performed using C_4Mab-1 against CHO-K1, CHO/mCCR4, P388, and J774-1. Results indicated that C_4Mab-1 dose-dependently recognized CHO/mCCR4, but not CHO-K1 (Fig. 2A). Another anti-mCCR4 mAb (clone 2G12 from BioLegend) also dose-dependently recognized CHO/mCCR4, but not CHO-K1 (Fig. 2B). In addition, C_4Mab-1 and 2G12 reacted with CHO/mCCR4, even at 0.01-μg/mL concentration (Fig. 2A, B). C_4Mab-1 and 2G12 also reacted dose-dependently with endogenous mCCR4-expressing P388 and J774-1 (Fig. 2A, B).

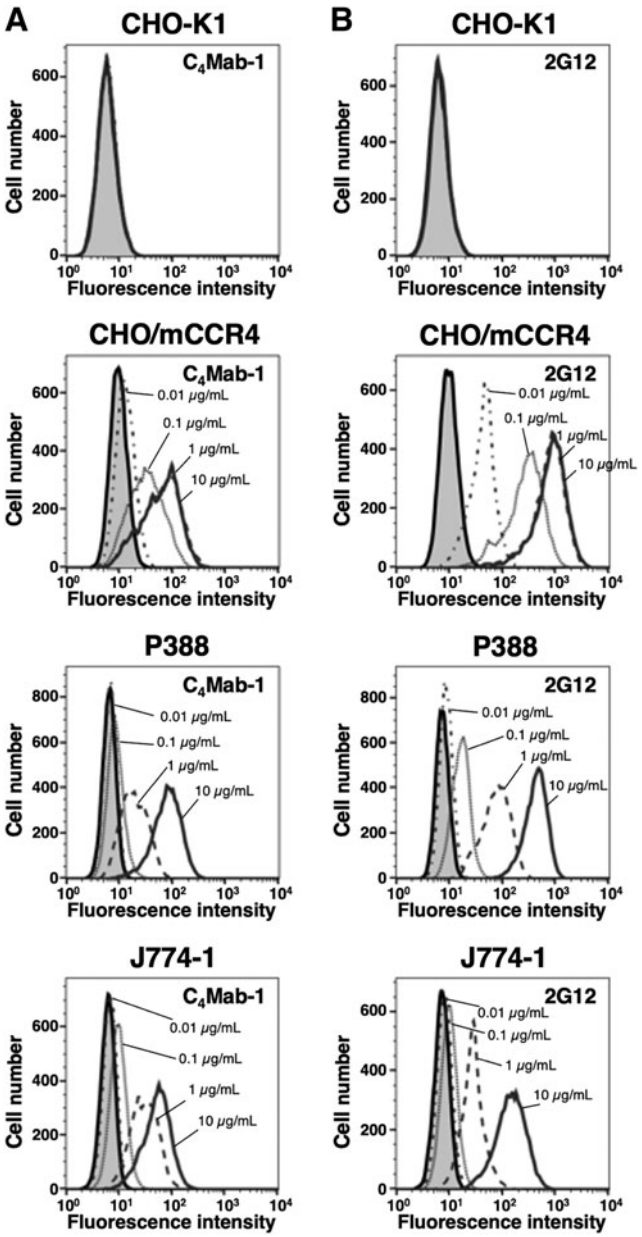

FIG. 2. Flow cytometry using anti-mCCR4 mAbs. CHO-K1, CHO/mCCR4, P388, and J774-1 were treated with 0.01–10 μg/mL of C_4Mab-1 **(A)** and 2G12 **(B)**, followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG or anti-Armenian hamster IgG.

Determination of the binding affinity of C_4Mab-1

The binding affinity of C_4Mab-1 was analyzed with CHO/mCCR4, P388, and J774-1, using flow cytometry. Results indicated that the K_D of C_4Mab-1 for CHO/mCCR4, P388, and J774-1 was 4.2×10^{-9} M, 5.4×10^{-7} M, and 1.1×10^{-6} M, respectively (Fig. 3A). In contrast, the K_D of 2G12 for CHO/mCCR4, P388, and J774-1 was 3.6×10^{-9} M, 1.2×10^{-6} M, and 1.4×10^{-6} M, respectively (Fig. 3B). These results, therefore, show that C_4Mab-1 has a high affinity for CHO/mCCR4 and a moderate affinity for P388 and J774-1 cells.

Discussion

Chemokine receptors, such as CCR4, are a family of the seven-transmembrane domain G protein-coupled receptors (GPCRs). The generation of anti-GPCR mAbs is difficult because of the problems in protein folding, small epitope regions, and purification of functional antigens.[48] Therefore, there were attempts to develop different anti-GPCR mAbs, including anti-mouse CCR3,[45] anti-mouse CCR8,[46] and anti-human CCR9[47] mAbs, using the CBIS method.

In this study, a novel anti-mCCR4 mAb (C_4Mab-1) through peptide immunization was developed. The K_Ds of C_4Mab-1 for CHO/mCCR4, P388, and J774-1 cells were 4.2×10^{-9} M, 5.4×10^{-7} M, and 1.1×10^{-6} M, respectively, showing that C_4Mab-1 possessed a high to moderate affinity against mCCR4-expressing cells. The epitope of mogamulizumab was shown at positions 12–29 of the N-terminal residues (DESIYSNYYLYESIPKPC)

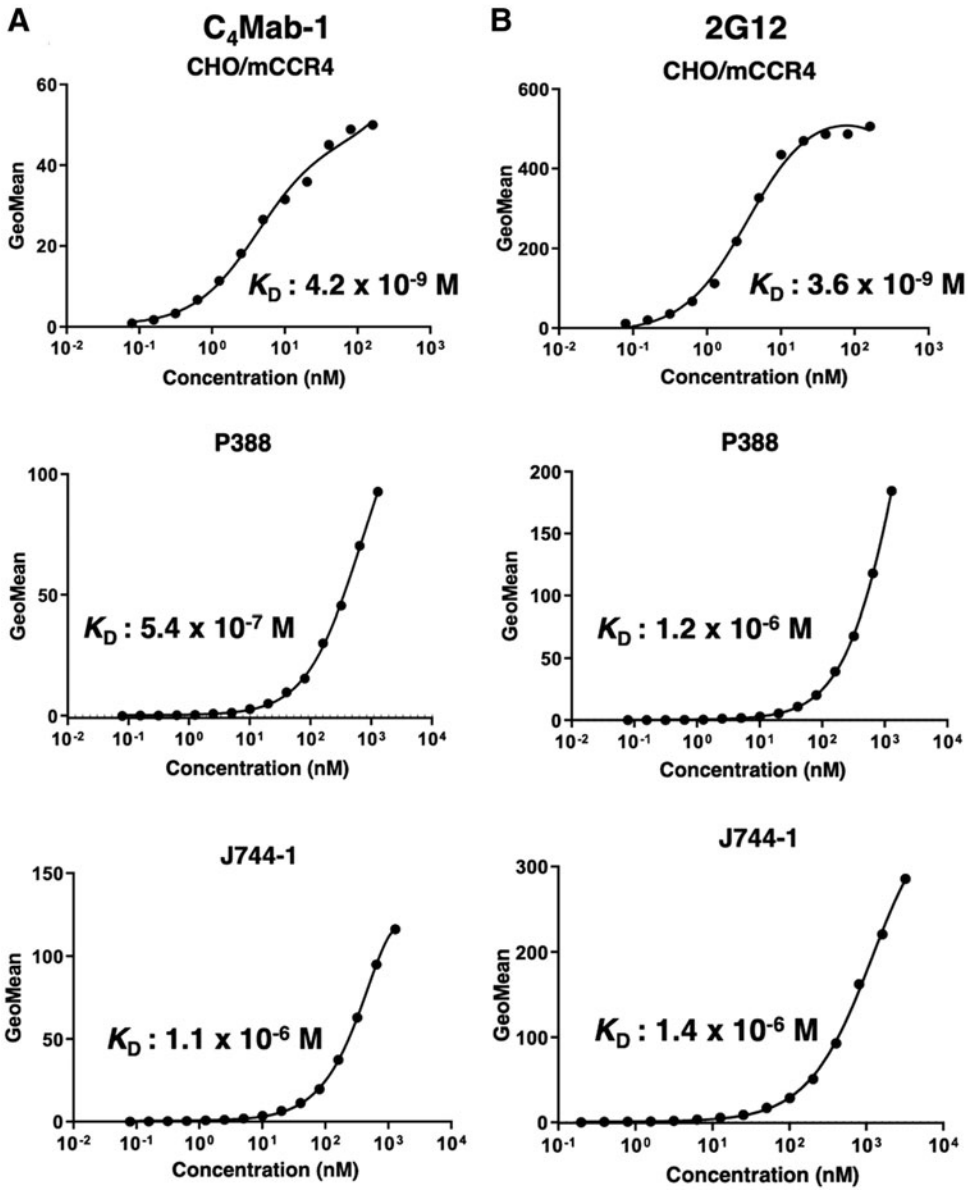

FIG. 3. The determination of the binding affinity of C_4Mab-1. CHO/mCCR4, P388, and J774-1 cells were suspended in 100 μL serially diluted C_4Mab-1 **(A)** and 2G12 **(B)**. The concentration of each mAb is as follows: C_4Mab-1 for CHO/mCCR4, 12.5–0.006 μg/mL; C_4Mab-1 for P388, 100–0.006 μg/mL; C_4Mab-1 for J774-1, 100–0.006 μg/mL; 2G12 for CHO/mCCR4, 12.5–0.006 μg/mL; 2G12 for P388, 100–0.006 μg/mL; 2G12 for J774-1, 250–0.02 μg/mL. Then, cells were treated with Alexa Fluor 488-conjugated anti-rat IgG or anti-Armenian hamster IgG.

of human CCR4.[49] Therefore, in humans and mice, N-terminal CCR4 is an essential region to generate high affinity mAbs.

CTCLs are a heterogeneous group of non-Hodgkin skin lymphomas obtained from skin-homing T cells. The skin-homing effector/memory T cells express cutaneous lymphocyte antigen (CLA) and CCR4.[5] In contrast, malignant T cells of MF/SS are characterized as $CLA^+CCR4^+CD4^+$ T cells, while skin lesions express high levels of CCR4 ligands, CCL17 and CCL22.[50] Therefore, the neutralizing activity of C_4Mab-1 is also important to suppress the T cell homing to target lesions. Further investigation is needed to evaluate the neutralizing activity of C_4Mab-1 for CCR4-CCL17/CCL22-mediated homing and chemoattraction.

Foxp3-expressing Treg cells are indispensable for preventing autoimmunity. Treg cells also abundantly infiltrate into tumor tissues and suppress antitumor immunity. Therefore, the removal of Treg cells improves antitumor immunity but may also trigger autoimmunity.[18] Therefore, an essential point in developing Treg-depleting cancer immunotherapy is how to specifically deplete Treg cells infiltrating into tumors without affecting tumor-reactive effector T cells, while suppressing autoimmunity.

These marker proteins include T cell accessory molecules, including CD25, CTLA-4, GITR, 4-1BB, OX-40, LAG3, TIGIT, CCR8, and CCR4.[18] In our previous studies, we changed the isotype of mAbs into mouse IgG_{2a} to retain ADCC and complement-dependent cytotoxicity (CDC), which caused high antitumor activities in mouse xenograft models.[51–53] Since the subclass of C_4Mab-1 was rat IgG_1, it did not possess ADCC and CDC. Therefore, in further studies, the subclass of C_4Mab-1 will be converted into mouse IgG_{2a} to evaluate the effect of depletion of $CCR4^+$ Treg cells in cancer immunotherapy.

Author Disclosure Statement

No competing financial interests exist.

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