



Cx₃Mab-4: A Novel Anti-Mouse CXCR3 Monoclonal Antibody for Flow Cytometry

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C-X-C motif chemokine receptor 3 (CXCR3, CD183) is a G-protein-coupled receptor for CXCL9, CXCL10, and CXCL11. CXCR3 induces chemotaxis of immune cells and promotes inflammation. Various mouse models have been developed to mimic the pathogenesis of diseases and used in the evaluation of therapeutics for these diseases. Although CXCR3 is an attractive target to suppress inflammation, anti-CXCR3 therapeutic agents have not been approved. In this study, we established a novel anti-mouse CXCR3 (mCXCR3) monoclonal antibody, Cx₃Mab-4 (rat IgG₁, kappa), using the Cell-Based Immunization and Screening method. Flow cytometric analysis demonstrated that Cx₃Mab-4 bound to mCXCR3-overexpressed Chinese hamster ovary-K1 (CHO/mCXCR3) cells, but did not react to parental CHO-K1 cells. The dissociation constant of Cx₃Mab-4 was determined as 1.3×10^{-9} M, indicating that Cx₃Mab-4 possesses a high affinity to mCXCR3-expressing cells. Cx₃Mab-4 could be useful for targeting CXCR3-expressing cells in preclinical mouse models.

Keywords: mouse CXCR3, monoclonal antibody, CBIS method

Introduction

C-X-C MOTIF CHEMOKINE RECEPTOR 3 (CXCR3, CD183) is a G-protein-coupled receptor expressed on T cells and natural killer cells.¹ CXCR3 signaling contributes to the accumulation of these cells to inflammation sites. Interferons (IFNs), cytokines secreted in inflammation sites, regulate CXCR3 signaling and expression. IFN- α , - β , and - γ elevate the expression of CXCL9, CXCL10, and CXCL11, which induce CXCR3-mediated chemotaxis in various types of cells including macrophages, dendritic cells (DCs), fibrocytes, and endothelial cells.^{2,3} Additionally, IFN- γ is essential for CXCR3 expression induced by T cell receptor stimulation in T cells.⁴ CXCR3 activation leads to actin polymerization and migration of T cells to inflammation sites through G-protein and phospholipase C-dependent pathways.⁵

CXCR3 is involved in inflammatory diseases.^{6,7} Dextran Sulfate Sodium (DSS)-induced colitis is a commonly used mouse model for inflammatory bowel disease (IBD).⁸ In CXCR3-deficient mice challenged with 2.5% DSS, body weight loss, a sign of the severity of colitis, was lower than that of wild type mice.⁹ The number of leukocytes in the

colon did not increase after the challenge. Moreover, the elevations of proinflammatory cytokines, such as IL-6, TNF- α , and IFN- γ by DSS-challenge reduced in CXCR3-deficient mice.⁹ These facts indicate that CXCR3 plays a pivotal role in the development of IBD.

To understand pathogenesis and develop therapeutics for inflammatory diseases, various mouse models have been reported.^{8,10-12} Although CXCR3 is an attractive target for anti-inflammatory therapies, few preclinical models using anti-mouse CXCR3 (mCXCR3) monoclonal antibodies (mAbs) have been developed. We have established mAbs against mouse CCR3¹³ and CCR8¹⁴ using the Cell-Based Immunization and Screening (CBIS) method. In this report, we developed a novel anti-mCXCR3 mAb using the CBIS method and evaluated its applications.

Materials and Methods

Antibodies, cell lines, and plasmids

An anti-mCXCR3 mAb (clone CXCR3-173) was purchased from BioLegend (San Diego, CA). Alexa Fluor 488-conjugated

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anti-rat IgG (4416) and anti-Armenian hamster IgG (ab173003) were purchased from Cell Signaling Technology, Inc., (Danvers, MA) and Abcam (Cambridge, UK), respectively.

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

The synthesized DNA (Eurofins Genomics KK) encoding mCXCR3 (Accession No.: NM_009910.3) was subcloned into a pCAGzeo_PAch vector (PA tag¹⁵ added to the C-terminus of mCXCR3). The pCAGzeo_mCXCR3-PA was transfected into LN229 and CHO-K1 cells using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). Clones stably expressing mCXCR3-PA were established using a cell sorter (SH800; Sony Corp., Tokyo, Japan) after culturing in a medium, containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA).

LN229 and mCXCR3-PA-overexpressed LN229 (LN229/mCXCR3-PA) cells were cultured in Dulbecco's Modified Eagle Medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 μ g/mL streptomycin, 100 U/mL of penicillin, and 0.25 μ g/mL amphotericin B (Nacalai Tesque, Inc.). CHO-K1 and mCXCR3-PA-overexpressed CHO-K1 (CHO/mCXCR3-PA) cells were maintained in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc.), supplemented with 10% FBS, 100 μ g/mL of streptomycin, 100 U/mL of penicillin, and 0.25 μ g/mL of amphotericin B. All cells were grown in a humidified incubator at 37°C, in an atmosphere of 5% CO₂ and 95% air.

Production of hybridomas

A five-week-old Sprague–Dawley rat was purchased from CLEA Japan (Tokyo, Japan). The animal was housed under specific pathogen-free conditions. All animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

To develop mAbs against mCXCR3, we intraperitoneally immunized one rat with CHO/mCXCR3-PA cells (1×10^9 cells) plus Alhydrogel adjuvant 2% (InvivoGen). After four additional injections every week (1×10^9 cells), the splenocytes were harvested. The splenocytes were fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). Hybridomas were selected by cultivation in the RPMI-1640 medium with 10% FBS, 100 μ g/mL of streptomycin, 100 U/mL of penicillin, 0.25 μ g/mL of amphotericin B, 5 μ g/mL of Plasmocin, 5% Briclone (NICB, Dublin, Ireland), and hypoxanthine/aminopterin/thymidine (HAT; Thermo Fisher Scientific, Inc.). The supernatants were screened using flow cytometry using LN229/mCXCR3-PA.

Purification of mAbs

The cultured supernatants of anti-mCXCR3 mAbs-producing hybridomas were filtrated with Steritop (0.22 μ m; Merck KGaA, Darmstadt, Germany). After filtration, the supernatants were applied to 1 mL of Ab-Capcher ExTra (ProteNova Co., Ltd., Kagawa, Japan). Bound Cx₃Mab-4 was eluted with an IgG elution buffer (Thermo Fisher Scientific, Inc.), followed by immediate neutralization of eluates, using

1 M Tris-HCl (pH 8.0). The buffer of eluates was replaced with phosphate-buffered saline (PBS) using Amicon Ultra (Merck KGaA).

Flow cytometry

CHO-K1 and CHO/mCXCR3-PA cells were harvested after a brief exposure to 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). The cells were washed with 0.1% bovine serum albumin in PBS and treated with anti-mCXCR3 mAbs for 30 min at 4°C. After washing, the cells were treated with Alexa Fluor 488-conjugated secondary antibodies. Flow cytometric analysis was performed using the SA3800 Cell Analyzer (Sony Corp.).

To determine the dissociation constant (K_D), anti-mCXCR3 mAbs were diluted serially from 100 μ g/mL to 6 ng/mL. The geometric mean of fluorescence intensities of CHO/mCXCR3-PA at each concentration was calculated by FlowJo v10.8.1 (BD Biosciences, Franklin Lakes, NJ). The K_D was estimated by fitting saturation binding curves to the built-in, one-site binding models in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

Results

Development of anti-mCXCR3 mAbs

We developed anti-mCXCR3 mAbs using the CBIS method. The CBIS method is composed of four steps (Fig. 1). First, a rat was immunized with CHO/mCXCR3-PA cells (Fig. 1A). The splenocytes were collected and fused with P3U1 cells (Fig. 1B). The reactivities to LN229/mCXCR3-PA cells of each supernatant were observed by flow cytometry (Fig. 1C). Finally, Cx₃Mab-4 (rat IgG₁, kappa) was developed (Fig. 1D).

Flow cytometry

To check the specificity of Cx₃Mab-4 and CXCR3-173 (a commercially available anti-mCXCR3 mAb), we performed flow cytometry against CHO/mCXCR3-PA and CHO-K1. Both Cx₃Mab-4 and CXCR3-173 reacted to CHO/mCXCR3-PA cells in a dose-dependent manner (Fig. 2A). In contrast, Cx₃Mab-4 and CXCR3-173 did not bind to CHO-K1 cells even at 10 μ g/mL (Fig. 2B). No difference was observed between two anti-mCXCR3 mAbs.

Determination of dissociation constant of anti-mCXCR3 mAbs against CHO/mCXCR3-PA cells

We determined the apparent dissociation constant (K_D) of Cx₃Mab-4 and CXCR3-173 against mCXCR3 by flow cytometry. The geometric mean of the fluorescence intensity of CHO/mCXCR3-PA at each concentration of Cx₃Mab-4 and CXCR3-173 was plotted. By fitting one-site binding models, both K_D values of Cx₃Mab-4 and CXCR3-173 for CHO/mCXCR3-PA were determined as 1.3×10^{-9} M (Fig. 3), indicating that both Cx₃Mab-4 and CXCR3-173 possess high affinity for CHO/mCXCR3-PA cells.

Discussion

The CXCR3 ligands, CXCL9, CXCL10, and CXCL11, are abundantly expressed in the intestinal mucosa of IBD

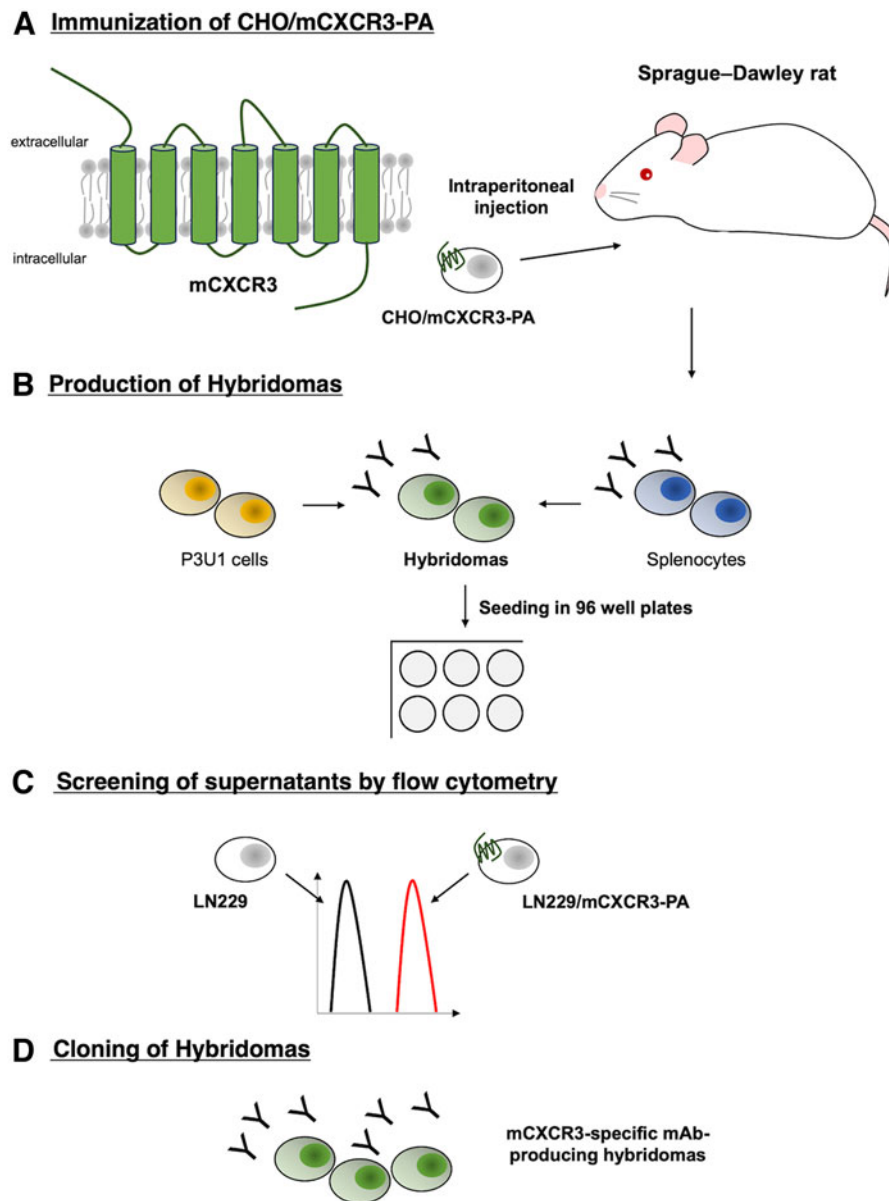


FIG. 1. The workflow of development of Cx₃Mab-4 by CBIS method. **(A)** CHO/mCXCR3-PA cells were injected intraperitoneally into a Sprague–Dawley rat. **(B)** After five immunizations, the splenocytes from the rat were fused with P3U1 cells. Hybridomas were seeded in 96-well plates. **(C)** The culture supernatants of each well were screened through flow cytometry to distinguish anti-mCXCR3 mAb-producing hybridomas. **(D)** Cx₃Mab-4 was established through limiting dilution and some additional screenings. CBIS, Cell-Based Immunization and Screening; mAb, monoclonal antibody; mCXCR3, mouse CXCR3.

patients.^{16–18} IBD is characterized by chronic, uncontrolled inflammation in the intestinal mucosa.¹⁹ The chemokines induce cellular trafficking and enhance inflammation through binding to CXCR3 expressed on proinflammatory lymphocytes. The inhibition of infiltration has been shown to attenuate inflammation.¹⁹ Eldelumab (MDX-1100, BMS-936557) is a fully human IgG₁ mAb to CXCL10 and have been developed for IBD treatment. Unfortunately, in the phase II study, the eldelumab exposure-remission was not sufficient in patients with IBD.^{20,21} Since not only CXCL10 but also CXCL9 and CXCL11 are elevated in IBD, anti-CXCR3 therapy is also expected for IBD treatment. In this

study, we developed a novel anti-mCXCR3 mAb, Cx₃Mab-4 which is useful for flow cytometry (Figs. 2 and 3). To apply preclinical mouse models of IBD, we should determine the epitope of Cx₃Mab-4 and investigate the neutralizing effect against the ligands. We have already determined the epitopes of anti-mouse chemokine receptor mAbs against CCR2 (C₂Mab-6),²² CCR3 (C₃Mab-3, 4, 6, and 7),^{23,24} CCR6 (C₆Mab-13),²⁵ CCR9 (C₉Mab-24),²⁶ and CXCR6 (Cx₆Mab-1).^{27,28}

Infiltration of regulatory T (Treg) cells into solid tumors represents a barrier to cancer immunotherapy.²⁹ Chemokine receptors including CXCR3 play a critical role in Treg cells

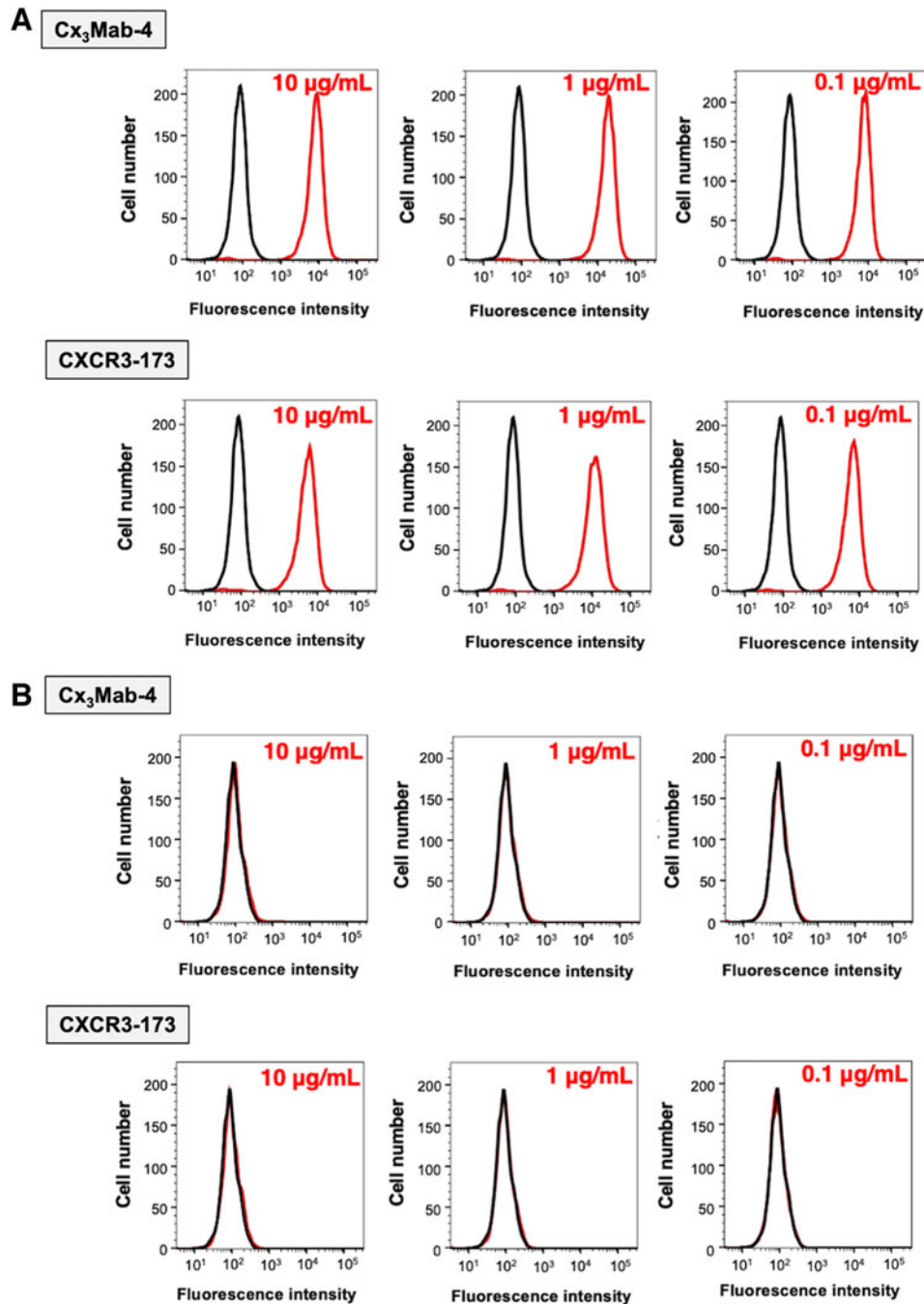


FIG. 2. Flow cytometry of mCXCR3-overexpressed cells using Cx₃Mab-4. CHO/mCXCR3-PA (A) and CHO-K1 (B) cells were treated with 0.1–10 µg/mL of Cx₃Mab-4 or CXCR3-173, followed by treatment with Alexa Fluor 488-conjugated secondary antibodies. The red lines show the cells treated with each mAbs. The black lines show the cells treated with blocking buffer and Alexa Fluor 488-conjugated secondary antibodies (negative control). CHO, Chinese hamster ovary.

recruitment into inflamed tumor tissue, and are important therapeutic targets.³⁰ CXCR3⁺ Treg cells increase in multiple tumor models and are activated through interaction with DCs.³⁰ CXCR3 ablation in Treg cells disrupted the DC-Treg cells interaction and increased DC-CD8⁺ T cell interactions, which resulted in increased CD8⁺ T cell priming and activation in tumors.³⁰ These results show that CXCR3 is critical for Treg cells accumulation and immune suppression in tumors. Therefore, depletion of CXCR3⁺ Treg cells in tumors is

expected to potentiate antitumor immunity. In our previous studies, we changed the isotype of mAbs into mouse IgG_{2a} to retain antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).³¹ Since the subclass of Cx₃Mab-4 is rat IgG₁, it does not possess ADCC and CDC. Therefore, in further studies, the subclass of Cx₃Mab-4 will be converted into mouse IgG_{2a} to evaluate the effect of depletion of mCXCR3⁺ Treg cells in preclinical mouse models.

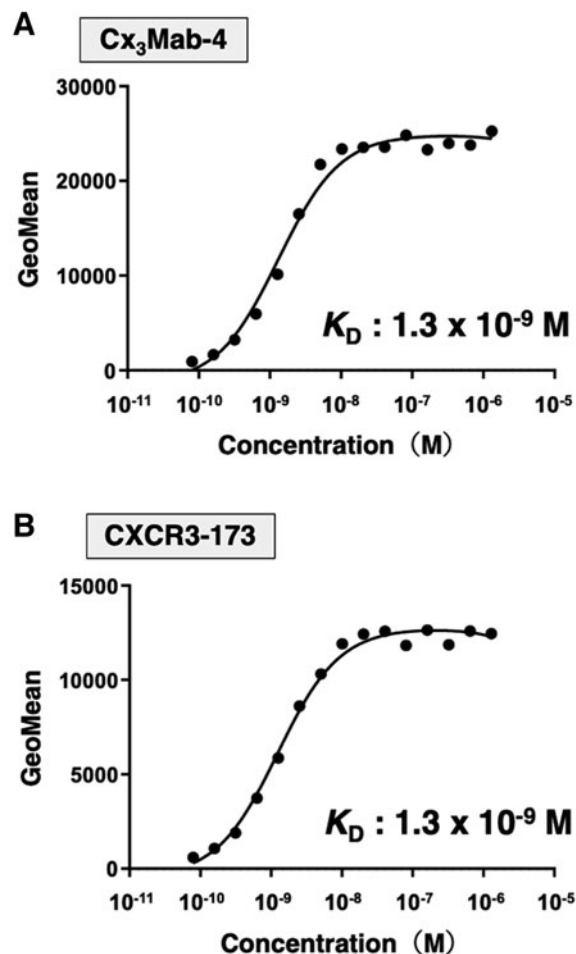


FIG. 3. Determination of dissociation constant of Cx₃Mab-4 against mCXCR3-overexpressed cells. The binding affinity of Cx₃Mab-4 (**A**) and CXCR3-173 (**B**) against CHO/mCXCR3-PA cells was determined by flow cytometry. The dots show the geometric mean of fluorescence intensity of CHO/mCXCR3-PA at each concentration. The solid lines are the fitting curve calculated by GraphPad PRISM 8.

Authors' Contributions

T.O., Y.I., and T.T. performed the experiments. M.K.K. and Y.K. designed the experiments. H.S. and M.K.K. analyzed the data. T.O. and Y.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Author Disclosure Statement

No competing financial interests exist.

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