

Communication

# C<sub>8</sub>Mab-21: A Novel Anti-Human CCR8 Monoclonal Antibody for Flow Cytometry

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**Abstract:** C-C motif chemokine receptor-8 (CCR8) belongs to class A of G protein-coupled receptors (GPCRs). CCR8 interacts with the specific chemokine ligand CCL1/I-309 in humans, which is produced by various cells, including tumor-associated macrophages and regulatory T cells (Treg). CCR8 is highly expressed on Treg and T-helper 2 (T<sub>H</sub>2) cells recruited to the inflammation site and is implicated in allergy, asthma, and cancer progression. The CCR8<sup>+</sup>Treg cells have been suggested to be an important regulator in the immunosuppressive tumor microenvironment (TME); therefore, it has been desired to develop sensitive monoclonal antibodies (mAbs) for CCR8. This study developed a specific mAb for human CCR8 (hCCR8), which is useful for flow cytometry by employing the Cell-Based Immunization and Screening (CBIS) method. The established anti-hCCR8 mAb, C<sub>8</sub>Mab-21, (mouse IgM, kappa) reacted with hCCR8-overexpressed Chinese hamster ovary-K1 (CHO/hCCR8) cells, TALL-1 (acute T lymphoblastic leukemia), CCRF-HSB2 (human T-lymphoblastic leukemia), and natural killer cells, which express endogenous hCCR8 by flow cytometry. Furthermore, EC<sub>50</sub> values of C<sub>8</sub>Mab-21 for CHO/hCCR8 and TALL-1 were determined as 6.5×10<sup>-8</sup> M and 2.0×10<sup>-8</sup> M, respectively. C<sub>8</sub>Mab-21, which was established by the CBIS method, could be a useful tool for analyzing the hCCR8-related biological response using flow cytometry.

**Keywords:** CCR8; CBIS method; monoclonal antibody; flow cytometry

## 1. Introduction

Targeting of immune checkpoint has become effective and powerful strategy for cancer therapy.[1] In particular, the development of antibody drugs targeting immune checkpoint molecules, such as programmed-cell death-1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), and PD-1 ligand 1 (PD-L1), has achieved remarkable therapeutic results.[2–4] PD-1 inhibits the excessive activation of conventional T cells by suppressing costimulatory signaling and renders them dysfunctional or exhausted.[5] PD-1 and CTLA-4 are also expressed in regulatory T cells (Treg), one of the immunosuppressors in the tumor microenvironment (TME). The inhibition could potentiate the activation and immunosuppressive function of Treg cells.[6]

Treg is defined as a CD4<sup>+</sup>T cell with CD25 and Foxp3 and maintains self-tolerance to prevent excessive immune responses and autoimmune diseases.[7] Treg suppresses the effector functions of T cells through the secretion of immunosuppressive cytokines, such as interleukin-10, transforming growth factor-β, and cytotoxic granzyme/perforin.[7–11] Intratumoral Treg suppresses antitumor T cell responses and thus resists the effects of immune checkpoint inhibitor therapy.[12,13] Antibodies against T cell immunoreceptors with Ig and ITIM domains (TIGIT), one of the immune checkpoint molecules, improve the effectiveness of PD-L1 antibodies by suppressing Treg.[14] Therefore, the development of immunotherapy targeting Treg is expected.[6,15]

Intratumoral Treg expresses a high level of C-C motif chemokine receptor-8 (CCR8). Additionally, the CCR8-expressing Treg has also increased the expression of CD25 and Foxp3 compared with CCR8-negative Treg.[16] Therefore, CCR8-expressing Treg is considered to have

potent immunosuppressive functions. The CCR8-expressing Treg is known to be correlated with poor prognosis in some cancer patients.[17,18] CCR8 is attractive as a target molecule for the next cancer immunotherapy.[19] Anti-CCR8 drugs, including S-531011,[20] IPG7236,[21] and SRF114[22] are undergoing clinical trials.

CCR8 is one of the seven transmembrane-spanning G protein-coupled receptors (GPCRs). Human CCR8 is known to bind to five C-C chemokine ligands (CCLs): CCL1/I-309, CCL4, CCL16, CCL17, and CCL18.[23] CCR8 is upregulated in not only Treg, but also various cancers, including breast, non-small cell lung (NSCLC), bladder, and colorectal cancer.[17,18] CCR8 mediates bladder cancer cell migration, invasion, and epithelial-mesenchymal transition by interacting with CCL18.[24] CCR8 and its specific ligand CCL1/I-309 regulate the immune system, which mediates the progression of diseases such as cancers. CCR8-CCL1/I-309 axis promotes migration and inhibits apoptosis in Treg and lymphomas.[25,26] Therefore, CCR8-targeting antibodies will contribute to the elucidation of pathological mechanisms, diagnosis, and therapy.

Using the Cell-Based Immunization and Screening (CBIS) method, we previously developed numerous monoclonal antibodies (mAbs) against chemokine receptors, including mouse CCR3,[27,28] mouse CCR8,[29–31] human CCR9,[32] and mouse CXCR4.[33] In this study, we have successfully developed an anti-human CCR8 (hCCR8) mAb using the CBIS method, which is applicable to flow cytometry.

## 2. Materials and Methods

### 2.1. Preparation of Cell Lines

LN229, Chinese hamster ovary (CHO)-K1, and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). TALL-1 and CCRF-HSB2 cells were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). The human natural killer (NK) cells (donor lot. 4022602, purity > 70%) were purchased from Takara Bio (Shiga, Japan). pCMV6neo-myc-DDK vector with hCCR8 (Accession No.: NM\_005201) was purchased from OriGene Technologies, Inc. (Rockville, MD). The plasmid was transfected into the cell lines using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). Subsequently, LN229 and CHO-K1, which stably overexpressed hCCR8 with C-terminal myc-DDK tags (hereinafter described as LN229/hCCR8 and CHO/hCCR8, respectively) were established using a cell sorter (SH800; Sony Corp., Tokyo, Japan), following cultivation in a medium containing 0.5 mg/mL G418 (Nacalai Tesque, Inc., Kyoto, Japan).

### 2.2. Production of Hybridomas

For developing anti-hCCR8 mAbs, two female 6-week-old BALB/c mice were immunized intraperitoneally with  $1 \times 10^8$  cells of LN229/hCCR8. The immunogen was harvested after brief exposure to 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). We added Imject Alum Adjuvant (Thermo Fisher Scientific Inc.) as an adjuvant in the first immunization. Three additional injections of  $1 \times 10^8$  cells of LN229/hCCR8 were performed without an adjuvant every week. We performed a final booster immunization of  $1 \times 10^8$  cells of LN229/hCCR8 intraperitoneally two days before harvesting splenocytes from mice. We conducted cell-fusion of the harvested splenocytes with P3U1 cells using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN).

### 2.3. Flow Cytometric Analyses

CHO-K1 and CHO/hCCR8 cells were harvested after brief exposure to 1 mM EDTA. CHO-K1, CHO/hCCR8, TALL-1, and CCRF-HSB2 cells were washed with 0.1% bovine serum albumin in phosphate-buffered saline and treated with primary mAbs for 30 min at 4°C. Afterward, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000) following the collection of fluorescence data, using the SA3800 Cell Analyzer (Sony Corp.). The anti-human CD198 (CCR8) mAbs (clones S19017D and L263G8) were purchased from BioLegend (San Diego, CA). The Alexa

Fluor 488-conjugated anti-mouse IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

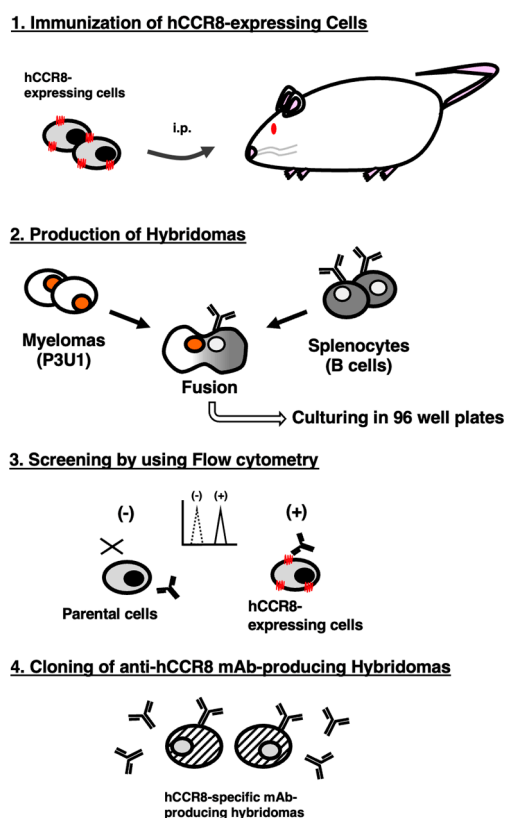
#### 2.4. Determination of the $EC_{50}$ by Flow Cytometry

CHO/hCCR8 and TALL-1 were suspended in 100  $\mu$ L serially diluted of C<sub>s</sub>Mab-21 (100  $\mu$ g/mL to 0.006  $\mu$ g/mL), S19017D (10  $\mu$ g/mL to 0.0006  $\mu$ g/mL for CHO/hCCR8, 10  $\mu$ g/mL, 2.5  $\mu$ g/mL to 0.0006  $\mu$ g/mL for TALL-1), or L263G8 (10  $\mu$ g/mL to 0.0006  $\mu$ g/mL for CHO/hCCR8, 0.625  $\mu$ g/mL to 0.0006  $\mu$ g/mL for TALL-1), after which Alexa Fluor 488-conjugated anti-mouse IgG (1:200) was added. Fluorescence data were subsequently collected, using the BD FACSLyric (BD Biosciences, Franklin Lakes, NJ), following the calculation of  $EC_{50}$  by fitting the binding isotherms into the built-in; one-site binding model in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

### 3. Results

#### 3.1. Establishment of Anti-hCCR8 mAbs by the CBIS Method

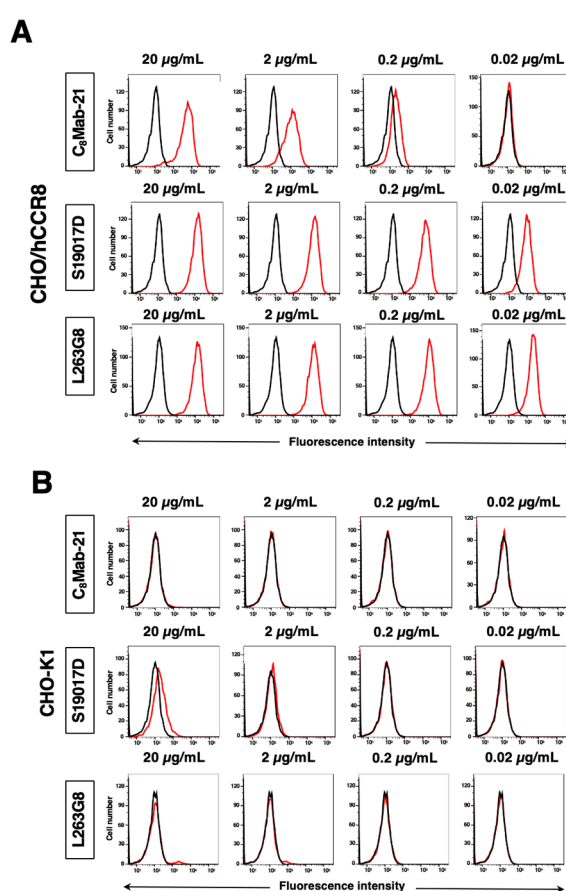
To develop anti-hCCR8 mAbs, we employed the CBIS method using hCCR8-overexpressed cells. Anti-hCCR8 mAbs-producing hybridoma screening was conducted using flow cytometry (Figure 1). Two mice were intraperitoneally immunized with LN229/hCCR8 weekly for a total of 5 times. Subsequently, hybridomas were seeded into 96-well plates, after which flow cytometric analysis was used to select CHO/hCCR8-reactive and CHO-K1-nonreactive supernatants of hybridomas. Afterward, we obtained CHO/hCCR8-reactive supernatant in only 1 of 956 wells (0.10%). We finally established clone C<sub>s</sub>Mab-21 (mouse IgM, kappa) by limiting dilution and additional screening.



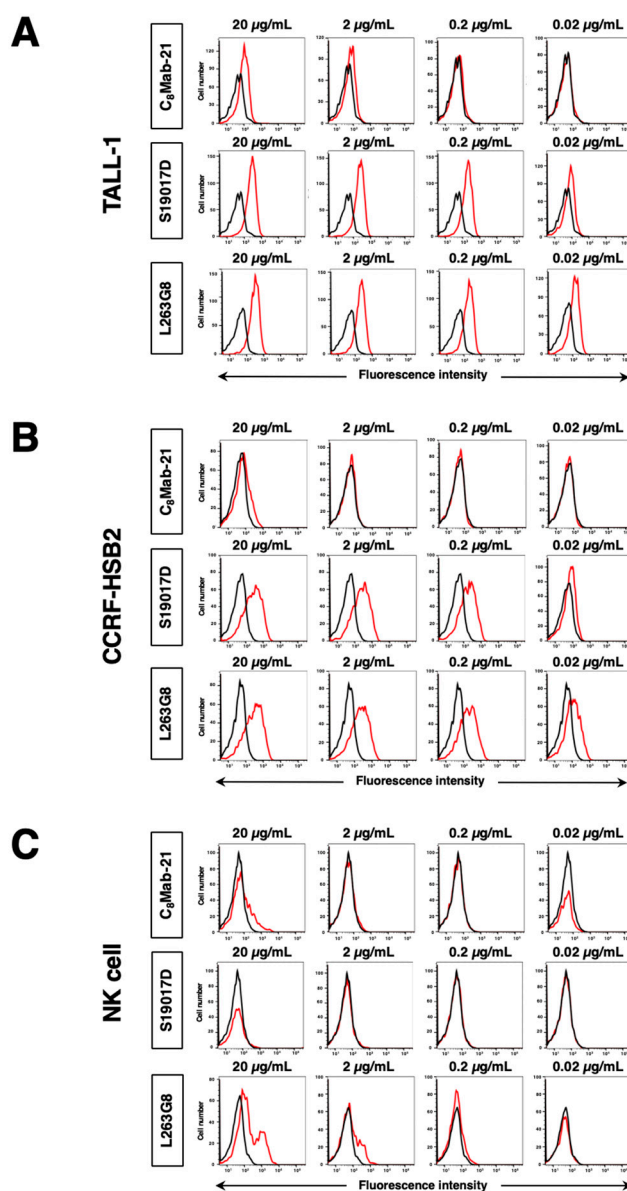
**Figure 1. A schematic procedure of anti-hCCR8 mAbs production.** The procedure of the Cell-Based Immunization and Screening (CBIS) method for antibody development. **(A)** LN229/hCCR8 cells were immunized into two mice using intraperitoneal injection. **(B)** The spleen cells from mice were fused with P3U1 myeloma cells. **(C)** The culture supernatants of hybridoma were screened by flow cytometry using CHO-K1 and CHO/hCCR8. **(D)** After limiting dilution of hybridomas and additional analysis, C<sub>s</sub>Mab-21 was finally established.

### 3.2. Flow Cytometric Analysis

Flow cytometric analysis was conducted using purified CsMab-21 (Supplementary Figure S1) and commercially available anti-human CCR8 (CCR8) mAbs (clone S19017D and L263G8) against CHO-K1, CHO/hCCR8, TALL-1, CCRF-HSB2, and NK cells. Results showed that CsMab-21, S19017D, and L263G8 recognize CHO/hCCR8 dose-dependently (Figure 2A). CsMab-21, and L263G8 did not react with parental CHO-K1 cells even at 20  $\mu\text{g/mL}$  of mAbs. S19017D slightly reacted to CHO-K1 cells at 20  $\mu\text{g/mL}$  and also 2  $\mu\text{g/mL}$  (Figure 2B). Regarding endogenously hCCR8-expressing cells in Figure 3, CsMab-21 recognized TALL-1, CCRF-HSB2, and NK cells at a concentration of 2  $\mu\text{g/mL}$  or 20  $\mu\text{g/mL}$  (Figure 3). S19017D reacted to TALL-1 and CCRF-HSB2 dose-dependently even at a concentration of 0.02  $\mu\text{g/mL}$ . However, NK cells were not recognized by S19017D even at a concentration of 20  $\mu\text{g/mL}$  (Figure 3). L263G8 reacted to TALL-1 and CCRF-HSB2 even at 0.02  $\mu\text{g/mL}$ , and also NK cells at a concentration of 2  $\mu\text{g/mL}$  or higher of mAb (Figure 3). To conclude, CsMab-21 could detect exogenously and endogenously expressed hCCR8 in native conformation using flow cytometry.



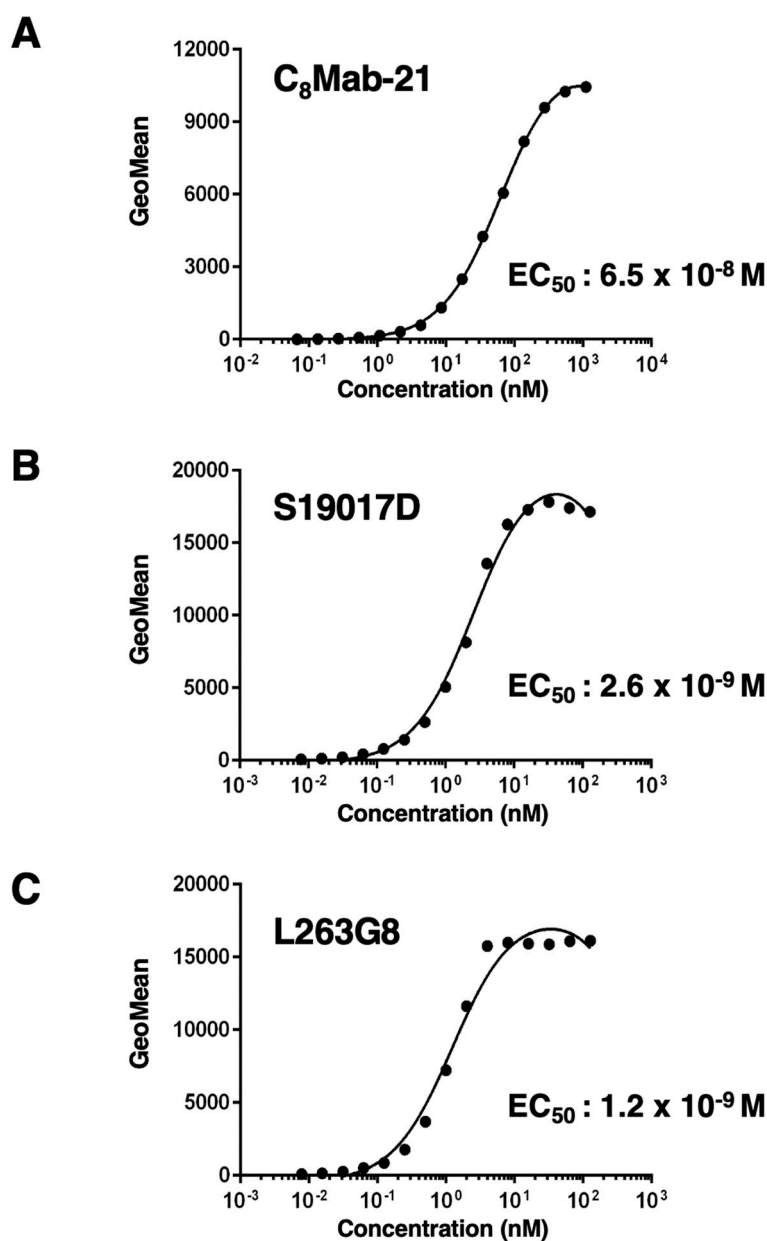
**Figure 2. Flow cytometric analysis of anti-hCCR8 mAbs against CHO/hCCR8 and CHO-K1.** CHO/hCCR8 (A) and CHO-K1 cells (B) were treated with 0.02–20  $\mu\text{g/mL}$  of CsMab-21, S19017D, and L263G8 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. Black line, control (no primary antibody treatment).



**Figure 3.** Flow cytometric analysis of anti-hCCR8 mAbs against endogenously hCCR8-expressing cells. TALL-1 (A), CCR8-HSB2 (B), and NK cells (C) were treated with 0.02–20 µg/mL of CsMab-21, S19017D, and L263G8 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. Black line, control (no primary antibody treatment).

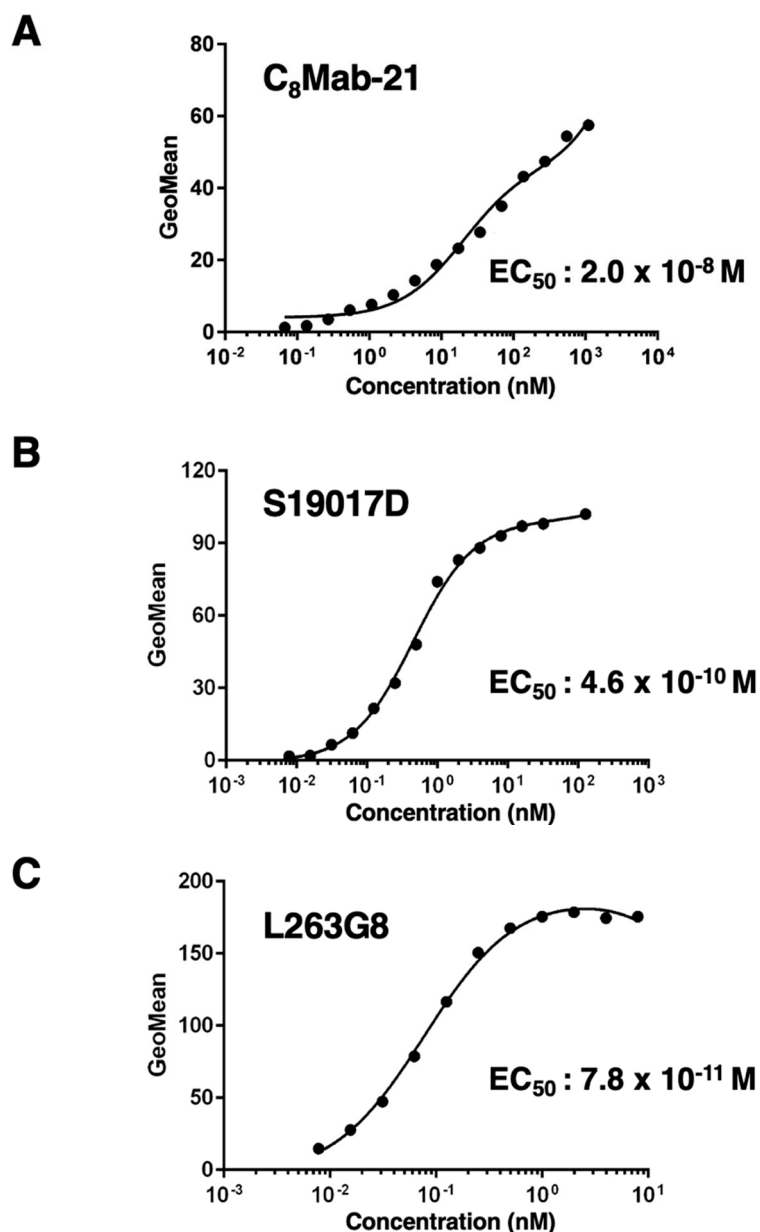
### 3.3. Titration of CsMab-21 and Anti-CCR-8 Control Antibodies on hCCR8 Overexpressed and Endogenously hCCR8-Expressing Cell Lines

The titration of CsMab-21, S19017D, and L263G8 was assessed with exogenously hCCR8-expressed CHO/hCCR8 using flow cytometry. Results showed that the EC<sub>50</sub> values of CsMab-21, S19017D, and L263G8 for CHO/hCCR8 are  $6.5 \times 10^{-8}$  M,  $2.6 \times 10^{-9}$  M, and  $1.2 \times 10^{-9}$  M, respectively (Figure 4). These results indicate that CsMab-21 possesses a moderate affinity for exogenously overexpressed hCCR8 in CHO-K1 cells.



**Figure 4.** The analysis of the binding affinity of anti-hCCR8 mAbs for CHO/hCCR8. CHO/hCCR8 cells were suspended in 100  $\mu\text{L}$  of serially diluted C<sub>8</sub>Mab-21 (100  $\mu\text{g}/\text{mL}$  to 0.006  $\mu\text{g}/\text{mL}$ ) (A), S19017D (10  $\mu\text{g}/\text{mL}$  to 0.0006  $\mu\text{g}/\text{mL}$ ) (B), or L263G8 (10  $\mu\text{g}/\text{mL}$  to 0.0006  $\mu\text{g}/\text{mL}$ ) (C). Then, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were subsequently collected using the BD FACSLyric, following the calculation of  $EC_{50}$  by GraphPad PRISM 6.

The titration of C<sub>8</sub>Mab-21, S19017D, and L263G8 was analyzed with endogenously hCCR8-expressing TALL-1 using flow cytometry. Results indicated that the  $EC_{50}$  values of C<sub>8</sub>Mab-21, S19017D, and L263G8 for TALL-1 are  $2.0 \times 10^{-8} \text{ M}$ ,  $4.6 \times 10^{-10} \text{ M}$ , and  $7.8 \times 10^{-11} \text{ M}$ , respectively (Figure 5). These results showed that C<sub>8</sub>Mab-21 possesses a moderate affinity for endogenously expressing hCCR8 in TALL-1 leukemia cells.



**Figure 5.** The analysis of the binding affinity of anti-hCCR8 mAbs for TALL-1. TALL-1 cells were suspended in 100  $\mu$ L of serially diluted C<sub>8</sub>Mab-21 (100  $\mu$ g/mL to 0.006  $\mu$ g/mL) (A), S19017D (10  $\mu$ g/mL, 2.5  $\mu$ g/mL to 0.0006  $\mu$ g/mL) (B), or L263G8 (0.625  $\mu$ g/mL to 0.0006  $\mu$ g/mL) (C). Then, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were subsequently collected using the BD FACSLyric, following the calculation of the EC<sub>50</sub> by GraphPad PRISM 6.

#### 4. Discussion

GPCRs, including chemokine receptors, are focused as targets for many diseases, including inflammatory disorders and cancers.[34,35] GPCRs transmit signals to intracellular molecules about extracellular conditions and govern broad cell dynamics, such as proliferation, homeostasis, migration, and motility of the cells.[34,35] Therapeutic drugs, including mAbs targeting GPCRs, have been developed to date; however, the complexity of the structure, the small area of epitope regions, and the difficulty of purifying the protein as an immunogen pose high hurdles.[36,37]

Unlike protein purifications, the preparation of antigens is not complicated in the CBIS method. Furthermore, it is possible to retain the antigen structure and modification such as glycosylation and folding using the CBIS method. We have successfully developed multiple mAbs by the CBIS method against human epidermal growth factor receptor 1 (HER1; EGFR),[38] HER3,[39] trophoblast cell

surface antigen 2,[40] epithelial cell adhesion molecule,[41] CD44,[42–44] and podoplanin.[45] Furthermore, some of the developed mAbs by the CBIS method exhibited cancer specificity by recognizing unique cancer-specific epitopes.[46–51] Therefore, the CBIS method is an efficient and useful tactic for generating diverse antibodies targeting membrane proteins.

In the immunosuppressive TME, CD8<sup>+</sup>T cells are exhausted along with the induction of CCR8<sup>+</sup>Treg. CCR8<sup>+</sup>Treg infiltration is confirmed to be associated with high TOX, an exhaustion marker of T cells, expression in CD8<sup>+</sup>T cells in some types of cancer patients.[52,53] Elimination of CCR8<sup>+</sup>Treg using antibodies is expected to advance the treatment of these cancer patients. Targeting CCR8 might be more specific in antitumor activity than other approaches aimed at Treg removal.[53,54] In mice, CCR8<sup>+</sup>T cell depletion therapy using anti-CCR8 mAbs induces tumor-specific immune responses without triggering autoimmune responses or immune reactions in TME.[55] Since C<sub>s</sub>Mab-21 could recognize cell surface hCCR8, we plan to investigate the function of C<sub>s</sub>Mab-21 against Treg, such as detection and interfering effects in future studies. Furthermore, we have previously enhanced antibody-dependent cellular cytotoxicity (ADCC) activity and complement-dependent cytotoxicity (CDC) by modifying isotypes and defucosylation in mAbs.[56,57] C<sub>s</sub>Mab-21 is mouse IgM, which has no ADCC activity, Although the property of IgM such as crosslinking activity is lost, it will be converted into a mouse IgG<sub>2a</sub> version to evaluate the effect of antitumor activities in xenograft models. We successfully cloned the cDNA of V<sub>H</sub> and V<sub>L</sub> region of C<sub>s</sub>Mab-21 (Supplementary Figure S2).

Interestingly, a correlation between cancer-associated fibroblasts (CAFs) and CCR8 has been found from the results of omics analysis.[22] CCR8 is suggested to be involved in the pathogenesis of various cancer types. CAFs are one of the tumor suppressor factors, the same as Treg, that are known to interfere with the function of tumor immune cells by promoting fibrosis and constructing an extracellular matrix in the TME.[58,59] CAFs with a myofibroblastic-like phenotype transfer large amounts of proteins to the surrounding endothelial cells through matrix-bound vesicles, which might contribute to cancer progression and treatment resistance.[60,61] Recently, the phenotypes of CAF have been reported to be associated with better or worse outcomes in NSCLC patients.[62] Although further functional analysis of CCR8-expressing CAFs is required, targeting CCR8 might suppress Treg and CAFs, and lead to synergistic antitumor immunotherapy results. In that case, it is well worth evaluating the impact of C<sub>s</sub>Mab-21 on CAFs as well in future studies.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

**Authorship confirmation/contribution statement:** T.T. and G.L. performed the experiments. M.K.K. and Y.K. designed the experiments. T.T. analyzed the data. T.T., H.S., and Y.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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**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.

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