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COMMUNICATION

C_8Mab-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. 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 cells recruited to the inflammation site and is implicated in allergy, asthma, and cancer progression. CCR8$^+$Treg cells have been suggested an important regulator in the immunosuppressive tumor microenvironment. Therefore, it has been proposed for use in the development of sensitive monoclonal antibodies targeting 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_8Mab-21; mouse IgM, kappa) demonstrated reactivity with hCCR8-overexpressed Chinese hamster ovary-K1 (CHO/hCCR8) cells, TALL-1 (human adult acute T-lymphoblastic leukemia), CCRF-HSB2 (human T-lymphoblastic leukemia), and natural killer cells expressing endogenous hCCR8, as confirmed by flow cytometry. Furthermore, EC_{50} values of C_8Mab-21 for CHO/hCCR8 and TALL-1 were determined as 6.5×10^{-8} M and 2.0×10^{-8} M, respectively. C_8Mab-21, established by the CBIS method, provides a useful tool for analyzing the hCCR8-related biological response using flow cytometry.

Keywords: CCR8; CBIS method; Monoclonal antibody; Flow cytometry

1. Introduction

Targeting immune checkpoint has become an effective and powerful strategy for cancer therapy.[1-4] 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.[5-7] PD-1 inhibits the excessive activation of conventional T cells by suppressing costimulatory signaling and renders them dysfunctional or exhausted.[8] PD-1 and CTLA-4 are also expressed in regulatory T cells (Treg), one of the immunosuppresses in the tumor microenvironment (TME).[9] Inhibition of

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these molecules could potentiate the activation and immunosuppressive function of Treg.[10-12]

Treg is defined as $CD4^+$T cell that expresses CD25 and FOXP3, playing a role in maintaining self-tolerance to prevent excessive immune responses and autoimmune diseases.[13,14] Treg suppresses the effector functions of T cells through the secretion of immunosuppressive cytokines, such as interleukin-10 (IL-10), transforming growth factor-β, and cytotoxic granzyme/perforin.[14-18] Intratumoral Treg suppresses antitumor T cell responses and thus resists the effects of immune checkpoint inhibitor therapy.[19,20] Antibodies against T cell immunoreceptors with Ig and ITIM domains (TIGIT), one of the immune checkpoint molecules, have shown to improve the effectiveness of PD-L1 antibodies by suppressing Treg.[21] Therefore, the development of immunotherapy targeting Treg is expected.[11,22]

Intratumoral Treg expresses high levels of C-C motif chemokine receptor-8 (CCR8).[4] In addition, CCR8-expressing Tregs exhibit increased expression of CD25 and FOXP3 compared to CCR8-negative Tregs,[23] indicating their potent immunosuppressive functions. The CCR8-expressing Treg is known to be correlated with poor prognosis in some cancer patients.[24,25] Thus, CCR8 is emerging as an attractive target for the next cancer immunotherapy.[26] Several anti-CCR8 drugs, including S-531011,[27] IPG7236,[28] and SRF114[29] are undergoing clinical trials.

CCR8 is one of the seven transmembrane-spanning G protein-coupled receptors.[4] Human CCR8 (hCCR8) is known to bind to five C-C chemokine ligands (CCLs): CCL1/I-309, CCL4, CCL16, CCL17, and CCL18.[30-32] CCR8 is upregulated not only in Treg but also in various cancers, including breast, non-small cell lung (NSCLC), bladder, and colorectal cancer.[24,25] In bladder cancer, CCR8 mediates cell migration, invasion, and epithelial-mesenchymal transition by interacting with CCL18.[33] In addition, CCR8 and its specific ligand CCL1/I-309 regulate the immune system, which mediates the progression of diseases such as cancers by promoting migration and inhibiting apoptosis in Treg and lymphomas.[34,35] Therefore, CCR8-targeting antibodies will contribute to the elucidation of pathological mechanisms, diagnosis, and therapy.[27,36]

Using the Cell-Based Immunization and Screening (CBIS) method, we previously developed numerous monoclonal antibodies (mAbs) against chemokine receptors, including mouse CCR3,[37] mouse CCR8,[38] human CCR9,[39] and mouse C-X-C chemokine receptor type 4 (CXCR4).[40] In this study, we successfully developed an anti-hCCR8 mAb using the CBIS method, which is applicable to flow cytometry.

2. Materials and methods

2.1. Cell lines

LN229, Chinese hamster ovary (CHO)-K1, and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). TALL-1 and CCRF-HSB2 cells were obtained from the Japanese Collection of Research Bioresources 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, USA). The plasmid was transfected into cell lines using the Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, LN229 and CHO-K1 stably overexpressing 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).

CHO-K1, P3U1, CHO/hCCR8, TALL-1, and CCRF-HSB2 cells were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc., Kyoto, Japan). LN229 and LN229/hCCR8 were cultured in a Dulbecco's Modified Eagle Medium (DMEM, Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc., Kyoto, Japan). All cells were cultured in a humidified incubator at 37°C with 5% CO_2 and 95% air.

2.2. Antibodies

The anti-human CD198 (CCR8) mAb (clones S19017D and L263G8) were purchased from BioLegend (San Diego, CA, USA). The Alexa Fluor 488-conjugated anti-mouse Immunoglobulin (Ig)G was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.3. Hybridoma production

For developing anti-hCCR8 mAbs, two female 6-week-old BALB/c mice were immunized intraperitoneally with 1×10^8 cells of LN229/hCCR8. The immunogen was harvested after brief exposure to 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc., Kyoto, Japan). Imject

Alum (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used as an adjuvant for the first immunization. Subsequently, three additional weekly injections of 1×10^8 cells of LN229/hCCR8 were administered without an adjuvant. A final booster immunization with 1×10^8 cells of LN229/hCCR8 was given intraperitoneally 2 days before harvesting splenocytes from the mice. Harvested splenocytes were then fused with P3U1 cells using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN, USA).

Hybridomas were cultured in RPMI-1640 medium supplemented as shown above and additional supplements included hypoxanthine, aminopterin, and thymidine (HAT; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 5% Briclone (NICB, Dublin, Ireland), and 5 μg/mL of Plasmocin (InvivoGen, San Diego, CA, USA). The hybridoma supernatants were screened by flow cytometry using CHO/hCCR8 and parental CHO-K1 cells. The cultured supernatant of C_8Mab-21-producing hybridomas was filtrated and purified using Capto L (Cytiva, Tokyo, Japan).

2.4. Flow cytometry

CHO-K1 and CHO/hCCR8 cells were harvested after brief exposure to 1 mM EDTA. Subsequently, 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. The cells were then treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000) following the collection of fluorescence data, using the SA3800 Cell Analyzer and SA3800 software ver. 2.05 (Sony Corp, Tokyo, Japan).

2.5. Determination of the EC_{50} by flow cytometry

CHO/hCCR8 and TALL-1 were suspended in 100 μL of serially diluted C_8Mab-21 (100 μg/mL – 0.006 μg/mL), S19017D (10 μg/mL – 0.0006 μg/mL for CHO/hCCR8; 10 μg/mL, 2.5 μg/mL – 0.0006 μg/mL for TALL-1), or L263G8 (10 μg/mL – 0.0006 μg/mL for CHO/hCCR8; 0.625 μg/mL – 0.0006 μg/mL for TALL-1). Alexa Fluor 488-conjugated anti-mouse IgG (1:200) was then added. Fluorescence data were subsequently collected using the BD FACSLyric (BD Biosciences, Franklin Lakes, NJ, USA), and EC_{50} values were calculated by fitting the binding isotherms into the built-in one-site binding model in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Establishment of anti-hCCR8 mAbs by the CBIS method

The CBIS method was employed using hCCR8-overexpressing cells to develop anti-hCCR8 mAbs. 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-non-reactive supernatants of hybridomas. From 956 wells, only one (0.10%) yielded CHO/hCCR8-reactive supernatant. Finally, we established the clone C_8Mab-21 (mouse IgM, kappa) by limiting dilution and additional screening.

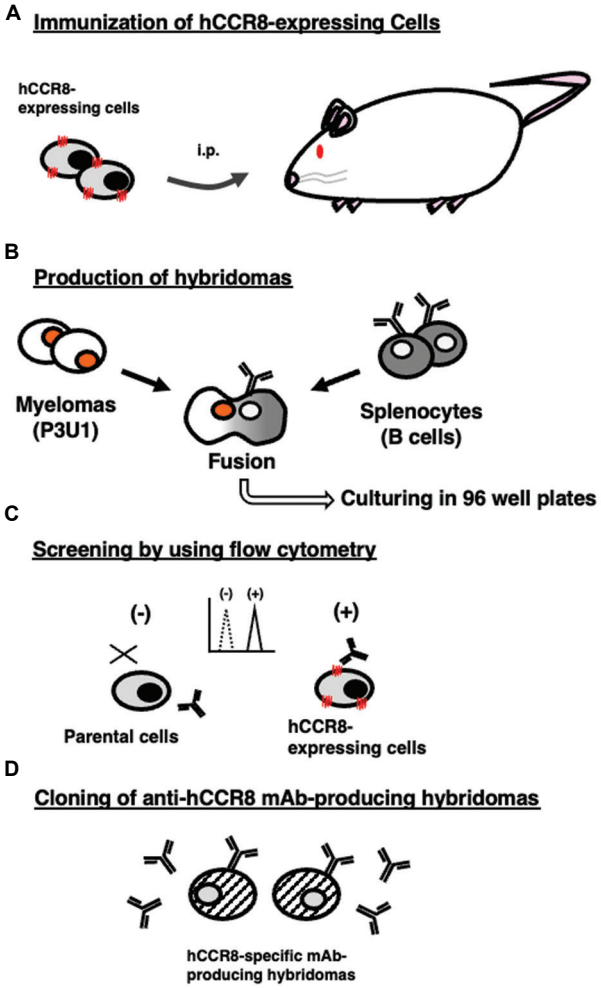

Figure 1. A schematic procedure of anti-hCCR8 monoclonal antibodies production. The procedure of the CBIS method for antibody development. LN229/hCCR8 cells were immunized into two mice via intraperitoneal injection (A). Spleen cells harvested from mice were fused with P3U1 myeloma cells (B). The culture supernatants of hybridoma were screened by flow cytometry using CHO-K1 and CHO/hCCR8 (C). After limiting dilution of hybridomas and additional analysis, the C_8Mab-21 clone was finally established (D).
Abbreviations: i.p.: Intraperitoneal; CHO: Chinese hamster ovary; CBIS: Cell-based immunization and screening; hCCR8: Human C-C motif chemokine receptor-8.

3.2. Flow cytometric analysis

Flow cytometric analysis was conducted using purified C_8Mab-21 (Figure S1) and commercially available anti-human CD198 (CCR8) mAbs (clone S19017D and L263G8) against CHO-K1, CHO/hCCR8, TALL-1, CCRF-HSB2, and NK cells. The results showed that C_8Mab-21, S19017D, and L263G8 recognized CHO/hCCR8 in a dose-dependent manner (Figure 2A). Neither C_8Mab-21 nor L263G8 reacted with parental CHO-K1 cells, even at a concentration of 20 μg/mL. However, S19017D showed slight reactivity with CHO-K1 cells at concentrations of 20 μg/mL and 2 μg/mL (Figure 2B). For endogenously hCCR8-expressing cells, C_8Mab-21 recognized TALL-1, CCRF-HSB2, and NK cells at concentrations of 2 μg/mL and 20 μg/mL (Figure 3). S19017D reacted with TALL-1 and CCRF-HSB2 in a dose-dependent manner, even at a concentration of 0.02 μg/mL, but did not recognize NK cells, even at a concentration of 20 μg/mL (Figure 3). L263G8 reacted with TALL-1 and CCRF-HSB2 at concentration as low as 0.02 μg/mL, and with NK cells at concentrations of 2 μg/mL or higher (Figure 3). Thus, C_8Mab-21 could detect both exogenously and endogenously expressed hCCR8 in its native conformation using flow cytometry.

3.3. Titration of anti-CCR-8 mAbs on hCCR8-overexpressed and endogenously hCCR8-expressing cell lines

The titration of C_8Mab-21, S19017D, and L263G8 was assessed with exogenously hCCR8-expressed CHO/hCCR8 using flow cytometry. The results showed that the EC_{50} values of C_8Mab-21, S19017D, and L263G8 for CHO/hCCR8 are 6.5×10^{-8} M, 2.6×10^{-9} M, and 1.2×10^{-9} M, respectively (Figure 4). The histogram of C_8Mab-21 is shown in Figure S2. These results indicate that C_8Mab-21 possesses a moderate affinity for CHO/hCCR8 cells.

The titration of C_8Mab-21, S19017D, and L263G8 was analyzed with endogenously hCCR8-expressing TALL-1 using flow cytometry. The EC_{50} values of C_8Mab-21, S19017D, and L263G8 for TALL-1 are 2.0×10^{-8} M, 4.6×10^{-10} M, and 7.8×10^{-11} M, respectively (Figure 5). These results showed that C_8Mab-21 possesses a moderate affinity for endogenously expressed hCCR8 in TALL-1 leukemia cells.

Further investigation was conducted to explore other applications, such as immunohistochemistry. However, hCCR8 could not be detected by immunohistochemistry using cell blocks of CHO/hCCR8 (Figure S3).

4. Discussion

Chemokine receptors are focused as targets for many diseases, including inflammatory disorders and cancers.[41,42] The receptors transmit signals to intracellular molecules regarding extracellular conditions and govern broad cellular dynamics, such as proliferation, homeostasis, migration, and motility of the cells.[41,43] Although the therapeutic drugs including mAbs have been developed, significant challenges remain. These challenges include the complexity of the structure, the small area of epitope regions, and the difficulty in purifying these protein as immunogens.[42,44]

Unlike protein purifications, the preparation of antigens using the CBIS method is relatively simple. Furthermore, the CBIS method allows for the retention of the antigens' structure, including modifications such as glycosylation and folding. We have successfully developed multiple mAbs using the CBIS method, targeting human epidermal growth factor receptor 1 (HER1; EGFR),[45] HER3,[46] trophoblast cell surface antigen 2,[47] CD44,[48] and podoplanin.[49] Furthermore, some of the mAbs developed by the CBIS method exhibited cancer specificity by recognizing unique cancer-specific epitopes.[50,51] Therefore, the CBIS method is one of the efficient and useful tactics for generating diverse antibodies targeting membrane proteins. Further investigations are required to determine the epitope of C_8Mab-21.

In the immunosuppressive TME, $CD8^+$T cells are exhausted along with the induction of $CCR8^+$Treg. The infiltration of $CCR8^+$Treg has been shown to associate with high thymocyte selection associated high mobility group box (TOX), an exhaustion marker in $CD8^+$T cells, in some types of cancer patients.[52,53] Elimination of $CCR8^+$Treg using antibodies is expected to advance the treatment of these cancer patients. Targeting CCR8 may offer more specific antitumor activity than other approaches aimed at Treg removal.[53,54] In mice, $CCR8^+$T cell depletion therapy using anti-CCR8 mAbs induces tumor-specific immune responses without triggering autoimmune responses or immune reactions in the TME.[36] Since C_8Mab-21 recognizes cell surface hCCR8, we plan to investigate its potential function against Treg, such as detection and interfering effects, in future studies. Furthermore, antibody-dependent cellular cytotoxicity (ADCC) activity and complement-dependent cytotoxicity have previously been enhanced by modifying isotypes and defucosylating mAbs.[55,56] C_8Mab-21 is a mouse IgM, which lacks ADCC activity. Although the crosslinking property of IgM is lost, it will be converted into a mouse IgG_{2a} version to evaluate the effect of antitumor activities in xenograft models. We successfully cloned and determined the complementarity-determining regions of C_8Mab-21 (Figure S4).

Interestingly, a correlation between cancer-associated fibroblasts (CAFs) and CCR8 has been found from

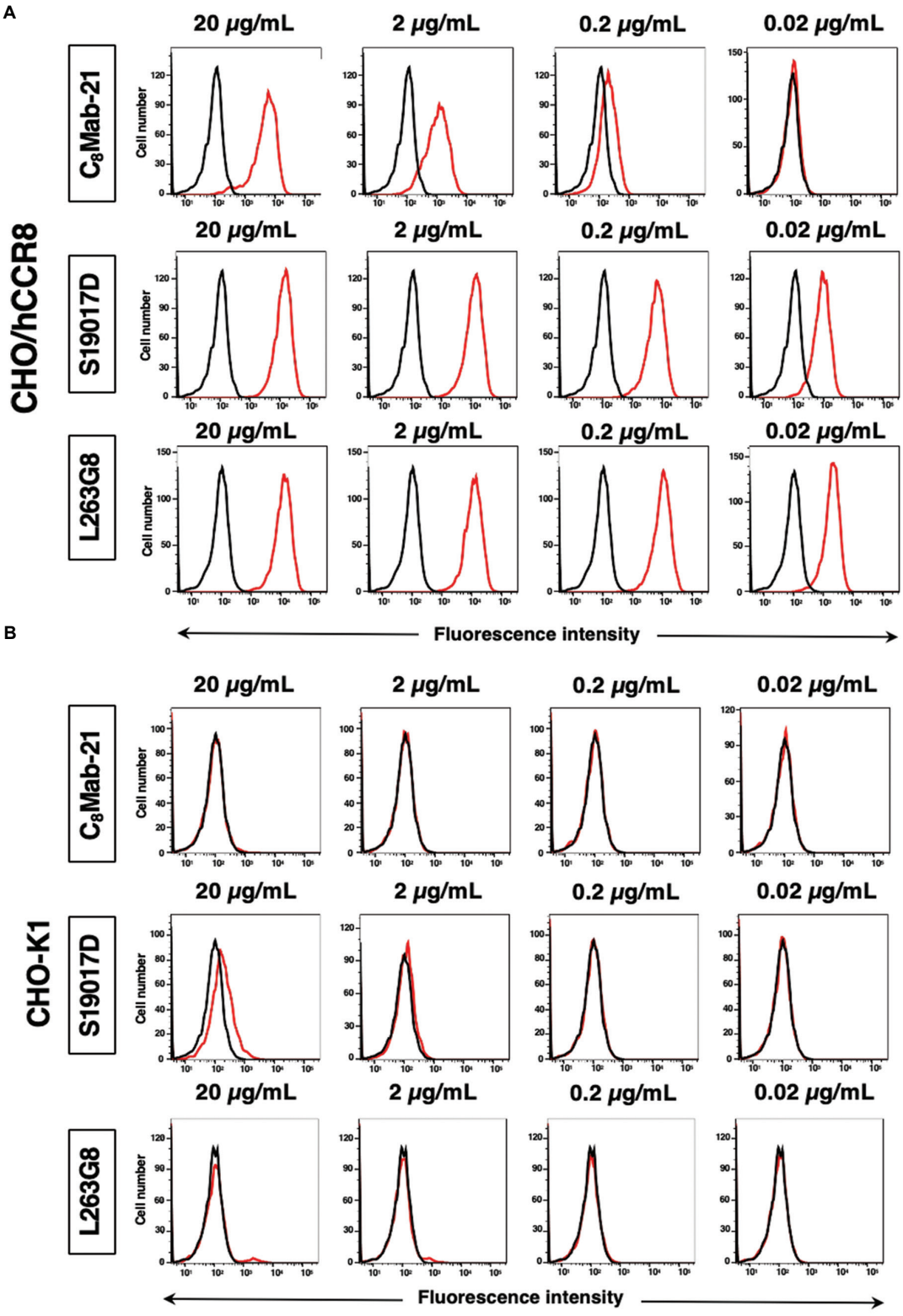

Figure 2. Flow cytometric analysis of anti-hCCR8 monoclonal antibodies against CHO/hCCR8 and CHO-K1. CHO/hCCR8 (A) and CHO-K1 (B) cells were treated with 0.02 – 20 μg/mL of C_8Mab-21, S19017D, and L263G8 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse Immunoglobulin G. Fluorescence data were collected using the SA3800 Cell Analyzer. The black line represents the control group (no primary antibody treatment).

Abbreviations: CHO: Chinese hamster ovary; hCCR8: Human C-C motif chemokine receptor-8.

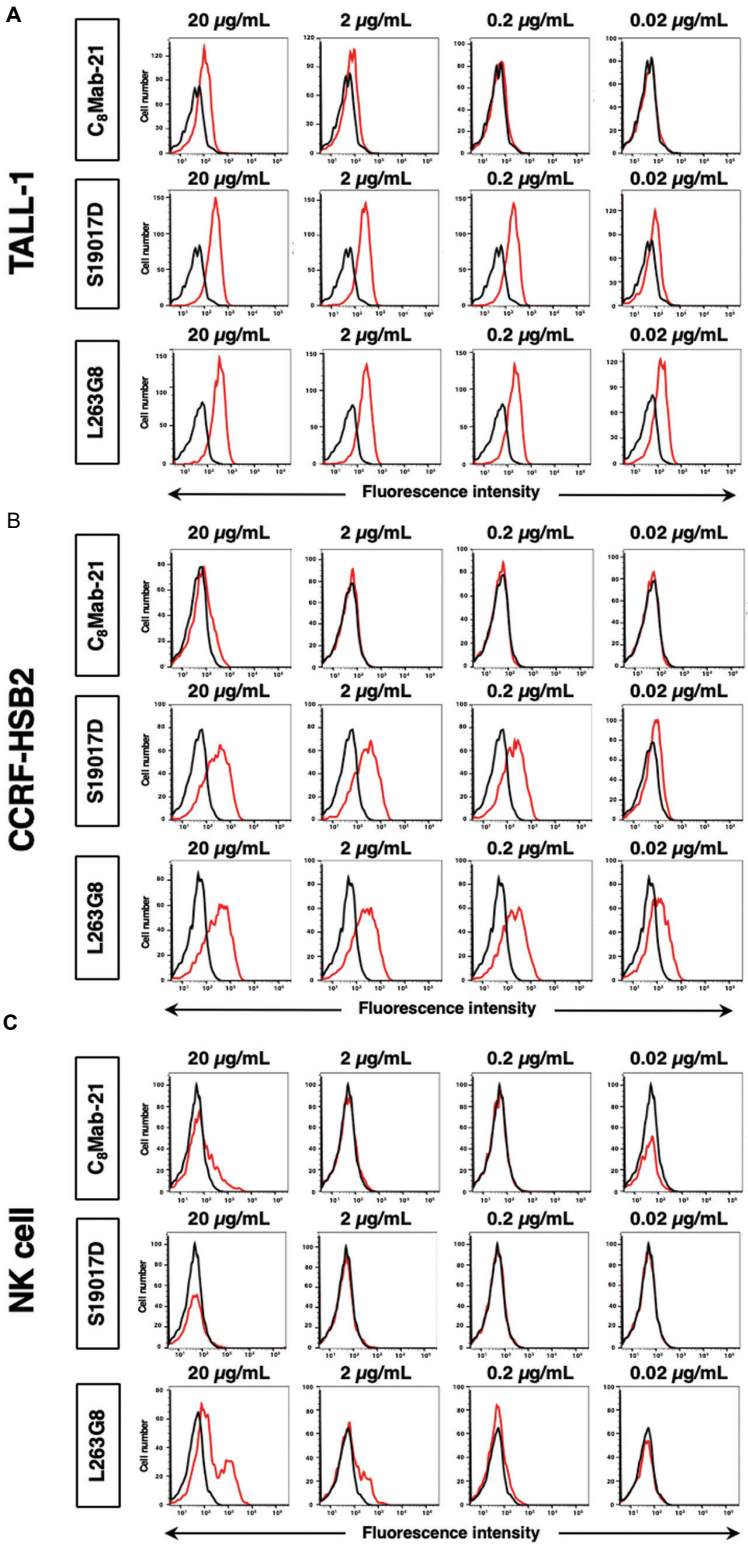

Figure 3. Flow cytometric analysis of anti-hCCR8 monoclonal antibodies against endogenously hCCR8-expressing cells. TALL-1 (A), CCRF-HSB2 (B), and NK cells (C) were treated with 0.02 – 20 μg/mL of C_8Mab-21, S19017D, and L263G8 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse Immunoglobulin G. Fluorescence data were collected using the SA3800 Cell Analyzer. The black line represents the control group (no primary antibody treatment).

Abbreviations: NK cells: Natural killer cells; hCCR8: Human C-C motif chemokine receptor-8.

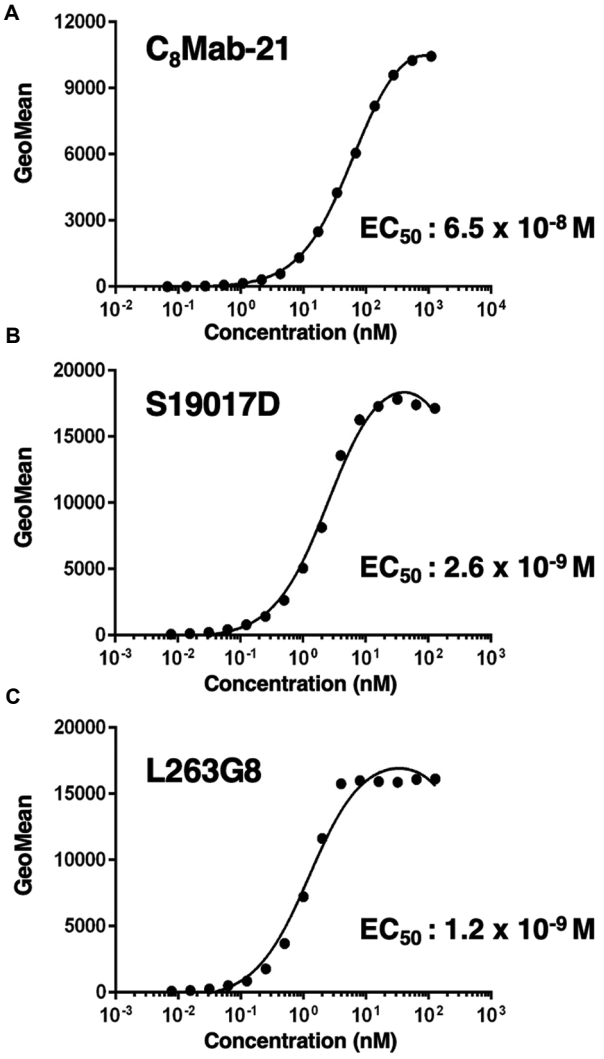

Figure 4. The analysis of the binding affinity of anti-hCCR8 monoclonal antibodies for CHO/hCCR8. CHO/hCCR8 cells were suspended in 100 µL of serially diluted C_8Mab-21 (100 µg/mL – 0.006 µg/mL) (A), S19017D (10 µg/mL – 0.0006 µg/mL) (B), or L263G8 (10 µg/mL – 0.0006 µg/mL) (C). The cells were then treated with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G. Fluorescence data were subsequently collected using the BD FACSLyric, and the EC_{50} values were calculated by GraphPad PRISM 6.
Abbreviations: GeoMean: Geometric mean; hCCR8: Human C-C motif chemokine receptor-8.

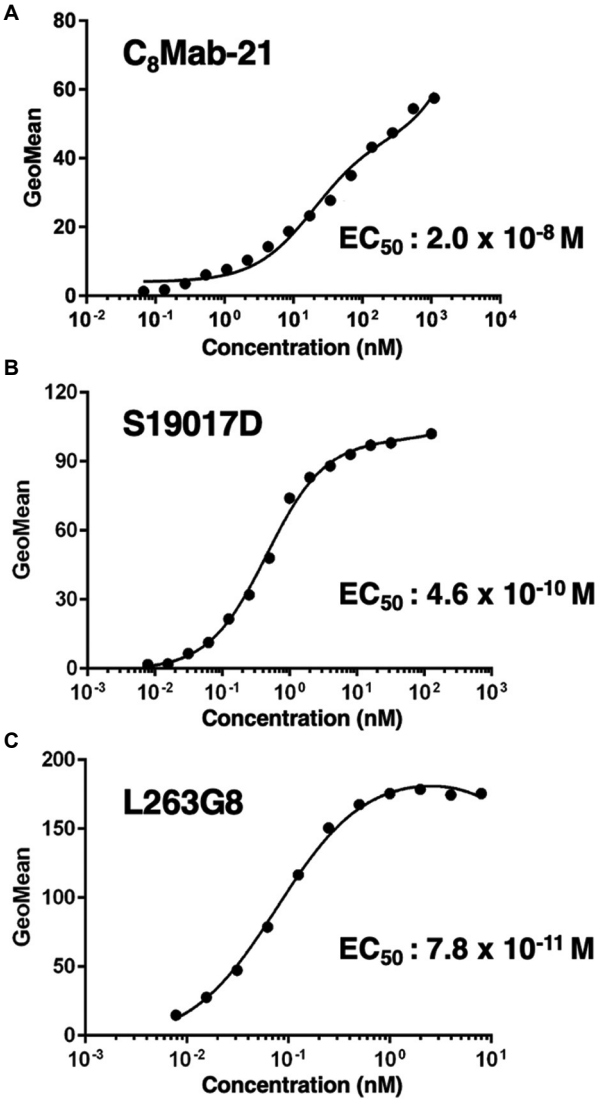

Figure 5. The analysis of the binding affinity of anti-hCCR8 monoclonal antibodies for TALL-1. TALL-1 cells were suspended in 100 µL of serially diluted C_8Mab-21 (100 µg/mL – 0.006 µg/mL) (A), S19017D (10 µg/mL, 2.5 µg/mL – 0.0006 µg/mL) (B), or L263G8 (0.625 µg/mL – 0.0006 µg/mL) (C). Then, cells were treated with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G. Fluorescence data were subsequently collected using the BD FACSLyric, following the calculation of the EC_{50} by GraphPad PRISM 6.
Abbreviations: GeoMean: Geometric mean; hCCR8: Human C-C motif chemokine receptor-8.

the results of omics analysis.[29] CCR8 is suggested to be involved in the pathogenesis of various cancer types.[24,25] CAFs, similar to Tregs, are one of the tumor-suppressive factors known to interfere with the function of tumor immune cells by promoting fibrosis and constructing the extracellular matrix in the TME.[1,57] CAFs with a myofibroblastic-like phenotype transfer large amounts of proteins to the surrounding endothelial cells through matrix-bound vesicles, which may contribute to cancer progression and treatment resistance.[58,59] Recently, the phenotypes of CAF have been reported to associate with either better or worse outcomes in NSCLC patients.[60] Although further functional analysis of CCR8-expressing CAFs is required, targeting CCR8 could suppress Treg and CAFs, and lead to synergistic antitumor immunotherapy results. Therefore, it is well worth evaluating the impact of C_8Mab-21 on CAFs.

5. Conclusion

C_8Mab-21, established by the CBIS method, is a useful tool for analyzing the hCCR8-positive cells by flow cytometry.

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Conflict of interest

The authors declare no conflicts of interest.

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Writing – review & editing: Hiroyuki Suzuki, Yukinari Kato

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

Consent for publication

Not applicable.

Availability of data

The data from this study are available in the article.

Further disclosure

The paper has been uploaded to a preprint server (DOI: 10.20944/preprints202403.1166.v1).

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