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COMMUNICATION

Ea_8Mab-9: A novel monoclonal antibody against erythropoietin-producing hepatocellular receptor A8 for flow cytometry

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Abstract

Erythropoietin-producing hepatocellular receptor A8 (EphA8) is a type I transmembrane protein that belongs to the largest erythropoietin-producing hepatocellular (Eph) family among receptor tyrosine kinases. By binding to its membrane-bound ephrin-A or ephrin-B ligands on adjacent cells, Eph receptors form complexes and mediate bidirectional signaling activities, triggering cell-cell adhesion and repulsion. Increased expression of EphA8 correlates with poor prognosis in some types of cancer. Therefore, developing sensitive monoclonal antibodies (mAbs) for EphA8 has been desired for treatment, diagnosis, and further basic research. In particular, there are no anti-EphA8 mAbs that can be used for flow cytometry. A novel, specific, and sensitive anti-human EphA8 mAb, which applies to flow cytometry, clone Ea_8Mab-9 (mouse immunoglobulin G_1, kappa), was established using the Cell-Based Immunization and Screening method. Ea_8Mab-9 reacted with EphA8-overexpressed Chinese hamster ovary-K1 cells (CHO/EphA8) and EphA8-overexpressed LN229 glioblastoma cells (LN229/EphA8) in flow cytometry. Notably, Ea_8Mab-9 did not recognize other members of the Eph receptor family. Furthermore, Ea_8Mab-9 demonstrated a high binding affinity for CHO/EphA8 and LN229/EphA8, with dissociation constants of 1.3×10^{-9} M and 1.6×10^{-9} M, respectively. The reaction of Ea_8Mab-9 with CHO/EphA8 was completely blocked by a recombinant EphA8 protein. Ea_8Mab-9 could be useful for analyzing the EphA8-related biological responses using flow cytometry, owing to its high affinity and specificity.

Keywords: EphA8; Cell-Based Immunization and Screening method; Monoclonal antibody; Flow cytometry

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1. Introduction

Erythropoietin-producing hepatocellular (Eph) receptors are the most prominent family of receptor tyrosine kinases (RTKs) and regulate tissue homeostasis, including cell proliferation and migration, tissue remodeling, angiogenesis, axon guidance, and synaptic plasticity in the nervous system.[1-3] Eph receptors are classified into EphA and EphB subfamilies according to the sequence homology and binding mode to membrane-bound ephrin ligands. The EphA subfamily includes nine members, such as EphA1

to EphA8 and EphA10. The EphB subfamily consists of five members, such as EphB1 to EphB4 and EphB6. Eight ephrin ligands have been identified, including glycosylphosphatidylinositol-anchored ephrin A1 to A5 and transmembrane ephrin B1 to B3. Following the binding to receptors, forward signaling is activated on the receptor side, and reverse signaling is generated on the ligand side, controlling various biological homeostasis.[3] Eph receptors play critical roles in the nervous system (EphA3, EphA4, EphA5, EphB1, EphB2, and EphB3),[4,5] cardiovascular system (EphB4),[6] immune system (EphA1, EphA2, EphA3, EphA4, EphA7, EphA10, EphB1, EphB2, EphB4, and EphB6),[7] and gastrointestinal system (EphB2 and EphB3).[8]

EphA8 complementary DNA (cDNA) was first isolated from a rat brain cDNA library and named eek (eph- and elk-related kinase, EEK) in 1991.[9] Another group cloned the mouse EphA8 molecule in 1997.[10] EphA8 is one of the members of the RTK family, and its regulatory mechanism is thought to be based on tyrosine kinase (TK) activity. Phosphorylation of Tyr-615 in the EphA8 juxtamembrane domain mediates a strong association with the SH2 domain of Fyn, a member of the Src TKs.[11] Phosphorylation of Tyr-838 in the EphA8 kinase domain modulates Fyn binding to Tyr-615, resulting in attenuation of cell adhesion through cellular cytoskeletal modifications.[11] Interestingly, TK activity-independent functions of EphA8 are also emerging. Ephrin A5-induced EphA8-integrin interaction is promoted by phosphatidylinositol 3-kinase in a TK-independent manner.[12] Similar to representative growth factor receptors such as epidermal growth factor receptor, Eph receptors play a role in cell proliferation primarily through forward signaling.[13,14] In addition to regulating cell-cell attachment and cell motility, EphA8 is involved in organ development and axon growth.[15,16] EphA8 induces caspase-dependent apoptotic cell death of ephrin A5-expressing neural epithelial cells during early brain development.[15] Loss of EphA8 disrupts axon guidance during mammalian nervous system development.[16] Furthermore, EphA8 facilitates neurite outgrowth by sustaining mitogen-activated protein kinase activity in neuronal cells.[17]

EphA8 expression has also been reported to be associated with cancer.[2,18-21] EphA8 upregulation is observed in various cancers, including oral tongue squamous cell carcinoma (OTSCC),[22] ovarian cancer,[23] gastric cancer,[24] and breast cancer.[25] EphA8 and ephrin A5 contribute to the invasiveness of stem cells isolated from MDA-MB-231, a triple-negative invasive breast cancer cell line.[26] In contrast, tumor suppressor functions of EphA8 have also been proposed. Reducing expression of miR-10a, a promoter of cancer invasion, leads to increased EphA8 expression and suppression of cancer progression in colorectal cancer and glioma.[27,28] Further research is necessary to clarify the role of EphA8 in either promoting or suppressing cancer-related functions.

Various monoclonal antibodies (mAbs) against human Eph receptors, including EphA2,[29] EphB2,[30] and EphB4,[31] have previously been developed by the Cell-Based Immunization and Screening (CBIS) method. This method preserves the native structure of membrane proteins during immunization and enables the efficient generation of antibodies that recognize modifications and/or three-dimensional structures of the extracellular domains of membrane proteins. Since flow cytometry is used for high-throughput screening in the CBIS method, mAbs suitable for this application are prioritized. However, anti-EphA8 mAbs suitable for flow cytometry are not yet available. Therefore, the establishment of anti-EphA8 mAbs is essential to support basic research and preclinical studies related to cancer therapy.

In this study, an anti-human EphA8 mAb (clone Ea_8Mab-9) suitable for flow cytometry was successfully established using the CBIS method.

2. Materials and methods

2.1. Cell lines and stable transfectants

LN229, Chinese hamster ovary (CHO)-K1, and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (USA). The cDNA encoding human EphA8 (Accession No. NM_020526; Catalog No.: RC220352) was purchased from OriGene Technologies Inc. (USA). The open reading frame of EphA8, excluding the signal sequence, was subcloned into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Japan) with either an interleukin 2-signal sequence and PA16 tag or a MAP16 tag at the N-terminus, using the in-fusion HD Cloning Kit (Takara Bio Inc., Japan). The resulting plasmid was transfected into the cell lines using the Neon Transfection System (Thermo Fisher Scientific Inc., USA). Subsequently, LN229 and CHO-K1 cells stably overexpressing EphA8 with a deletion of amino acids 1 – 27 and an N-terminal MAP16 tag (hereafter described as LN229/EphA8 and CHO/EphA8, respectively), as well as LN229 cells stably overexpressing EphA8 with the same deletion and an N-terminal PA16 tag (hereafter described as LN229/PA16-EphA8), were established using a cell sorter (SH800, Sony Corp., Japan).

cDNAs for various Eph receptors were obtained, including EphA1 (Catalog No.: RC213689, Accession No.: NM_005232), EphA4 (Catalog No.: RC211230,

Accession No.: NM_004438), EphA5 (Catalog No.: RC213206, Accession No.: NM_004439), EphA6 (Catalog No.: RC223510, Accession No.: NM_001080448), EphA7 (Catalog No.: RC226293, Accession No.: NM_004440), EphA10 (Catalog No.: RC218374, Accession No.: NM_001099439) EphB1 (Catalog No.: RC214301, Accession No.: NM_004441), EphB2 (Catalog No.: RC223882, Accession No.: NM_004442), EphB6 (Catalog No.: RC229404, Accession No.: NM_004445), were purchased from OriGene Technologies Inc. (USA). EphA2 (Catalog No.: HGY095959, Accession No.: NM_004431), EphA3 (Catalog No.: HGY053437, Accession No.: NM_005233), and EphB3 (Catalog No.: HGX039581, Accession No.: NM_004443) cDNAs were purchased from RIKEN DNA Bank (Japan).

EphA2 and EphB3 cDNAs were cloned into a pCAGzeo vector (FUJIFILM Wako Pure Chemical Corporation, Japan). EphB6 cDNA was cloned into a pCMV6 vector. EphA1 cDNA was cloned into a pCAGzeo-ssnPA vector. EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, and EphB1 cDNAs were cloned into a pCAGzeo_ssnPA16 vector.

The plasmids were also transfected into CHO-K1 cells, and stable transfectants were established by staining with specific antibodies: an anti-EphA2 mAb (clone SHM16; BioLegend, USA), an anti-EphB3 mAb (clone 647354; R & D Systems Inc., USA), an anti-EphB6 mAb (clone T49-25; BioLegend), and an anti-PA16 tag mAb (clone NZ-1 for EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA10, and EphB1), followed by sorting using the SH800 cell sorter. After sorting, cells were cultivated in a medium containing 0.5 mg/mL of Zeocin (InvivoGen, USA) or 0.5 mg/mL of G418. Eph receptors-overexpressed CHO-K1 (e.g., CHO/EphA1) clones were finally established. CHO/PA16-EphB4 cells were described previously.[31]

CHO-K1, P3U1, and CHO-K1 cells overexpressing each Eph receptor were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque Inc., Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc., USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque Inc., Japan). LN229 and LN229/EphA8 cells were cultured in Dulbecco's Modified Eagle Medium (Nacalai Tesque Inc., Japan) supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific Inc., USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque Inc., Japan). All cells were cultured in a humidified incubator at 37°C with 5% CO_2 and 95% air.

2.2. Antibodies

The Alexa Fluor 488-conjugated anti-mouse immunoglobulin g (IgG) secondary antibody was purchased from Cell Signaling Technology Inc. (USA).

2.3. Development of hybridomas

To develop anti-EphA8 mAbs, two 6-week-old female BALB/cAJcl mice purchased from CLEA Japan (Japan) were immunized intraperitoneally with 1×10^8 LN229/PA16-EphA8 cells. The immunogen was harvested after a brief exposure to 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque Inc., Japan). For the initial immunization, Alhydrogel adjuvant 2% (InvivoGen, USA) was added. Three additional injections of 1×10^8 LN229/PA16-EphA8 cells were performed without an adjuvant addition every week. A final booster immunization was performed with 1×10^8 LN229/PA16-EphA8 cells intraperitoneally 2 days before harvesting splenocytes. Splenocytes from the immunized mice were fused with P3U1 myeloma cells using polyethylene glycol 1,500 (PEG1,500; Roche Diagnostics, USA) under warmed conditions.

Hybridomas were cultured in RPMI-1640 medium supplemented as shown above, with additional supplementation of hypoxanthine, aminopterin, and thymidine (HAT) (HAT; Thermo Fisher Scientific Inc., USA), 5% BriClone (NICB, Ireland), and 5 μg/mL of plasmocin (InvivoGen, USA). The hybridoma supernatants were screened by flow cytometry using CHO/EphA8 and parental CHO-K1 cells. The culture supernatant from Ea_8Mab-9-producing hybridomas was filtrated and purified using Ab-Capcher Extra (ProteNova, Japan).

2.4. Flow cytometric analysis

CHO-K1, CHO/EphA1, CHO/EphA2, CHO/EphA4, CHO/EphA6, CHO/EphA7, CHO/EphA8, CHO/EphB1, CHO/EphB6, LN229, and LN229/EphA8 cells were harvested after brief exposure to 1 mM EDTA (Nacalai Tesque Inc., Japan). CHO/EphA3, CHO/EphA5, CHO/EphA10, CHO/EphB2, CHO/EphB3, and CHO/EphB4 cells were harvested after brief exposure to 0.25% trypsin and 1 mM EDTA (Nacalai Tesque Inc., Japan). All cells were then washed with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), treated with the primary mAb for 30 min at 4°C, and subsequently treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000). Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp., Japan).

2.5. Determination of K_D by flow cytometry

CHO/EphA8 and LN229/EphA8 were suspended in 100 µL of serially diluted Ea_8Mab-9 (10 µg/mL to 0.0006 µg/mL), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG (1:200). Fluorescence data were subsequently collected using the BD FACSLyric system (BD Biosciences, USA). The dissociation constant (K_D) was calculated by fitting the binding isotherms into the built-in one-site binding model in GraphPad PRISM 6 (GraphPad Software Inc., USA).

2.6. Determination of K_D by enzyme-linked immunosorbent assay (ELISA)

The recombinant EphA8-Fc (recEphA8) (Sino Biological Inc., China) was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc., USA) at 10 µg/mL for 30 min at 37°C. After washing with PBS containing 0.05% Tween20 (PBST; Nacalai Tesque Inc., Japan), wells were blocked with 1% BSA in PBST for 30 min at 37°C. The plates were then incubated at serially diluted Ea_8Mab-9 (10 µg/mL to 0.0006 µg/mL), followed by treatment with peroxidase-conjugated anti-mouse IgG_1 (1:2000; SouthernBiotech, USA). Finally, enzymatic reactions were conducted using the ELISA POD substrate TMB kit (Nacalai Tesque Inc., Japan). The K_D value was determined as described above.

3. Results

3.1. Development of anti-EphA8 mAbs using the CBIS method

Currently, polyclonal antibodies against EphA8 for flow cytometry are commercially available. However, they are insufficient for therapeutic applications. Therefore, the establishment of mAbs targeting EphA8 is essential to develop various mAb-based therapeutic modalities. To develop anti-EphA8 mAbs for flow cytometry, the CBIS method was employed using EphA8-overexpressed cells. Hybridoma that produced anti-EphA8 mAbs were screened by flow cytometry (Figure 1). Two female BALB/cAJcl mice were intraperitoneally immunized with LN229/PA16-EphA8 cells once weekly for 5 weeks. Subsequently, hybridomas were seeded into 96-well plates, and the culture supernatants were screened to identify those that specifically reacted with CHO/EphA8 cells but not with parental CHO-K1 cells. Several highly CHO/EphA8-reactive supernatants of hybridomas were obtained. The most sensitive clone, Ea_8Mab-9 (mouse IgG_1, kappa), was ultimately established through limiting dilution and additional analysis.

3.2. Flow cytometric analysis

Flow cytometric analysis was conducted using Ea_8Mab-9 against CHO-K1, CHO/EphA8, LN229, and LN229/EphA8 cells. Results indicated that Ea_8Mab-9 bound to CHO/EphA8 (Figure 2A, upper) and LN229/EphA8 (Figure 2B, upper) in a dose-dependent manner. In contrast, no binding was observed to parental CHO-K1 (Figure 2A, lower) or LN229 (Figure 2B, lower) cells, even at the highest tested concentration of 10 µg/mL. These findings indicate that Ea_8Mab-9 specifically reacts to EphA8 in flow cytometric applications.

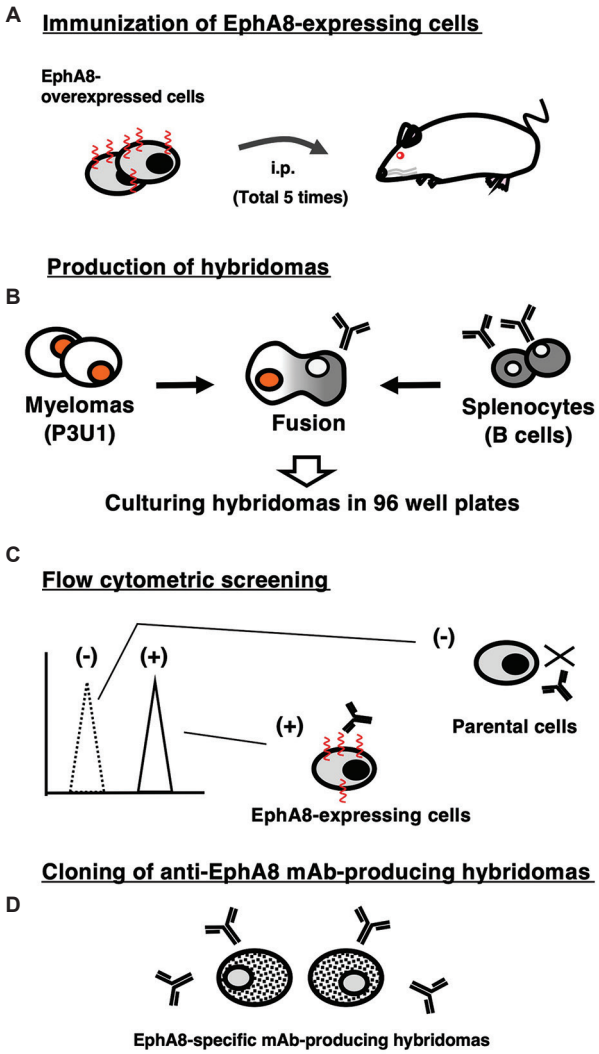

Figure 1. A schematic diagram of anti-EphA8 mAb development using the CBIS method. (A) LN229/PA16-EphA8 cells were immunized into two mice by intraperitoneal injection. (B) The spleen cells from immunized mice were fused with P3U1 myeloma cells using PEG1,500. (C) The culture supernatants of hybridoma were screened by flow cytometry using CHO-K1 and CHO/EphA8 cells to select EphA8-specific mAb-producing hybridomas. (D) After limiting the dilution of hybridomas to obtain the single clone and additional screening, the mAb clone Ea_8Mab-9 (mouse IgG_1, kappa) was finally established. Abbreviations: CBIS: Cell-Based Immunization and Screening; CHO: Chinese hamster ovary; i.p.: Intraperitoneal; mAb: Monoclonal antibody; EphA8: Erythropoietin-producing hepatocellular receptor A8; IgG: immunoglobulin g; PEG1,500: Polyethylene glycol 1,500.

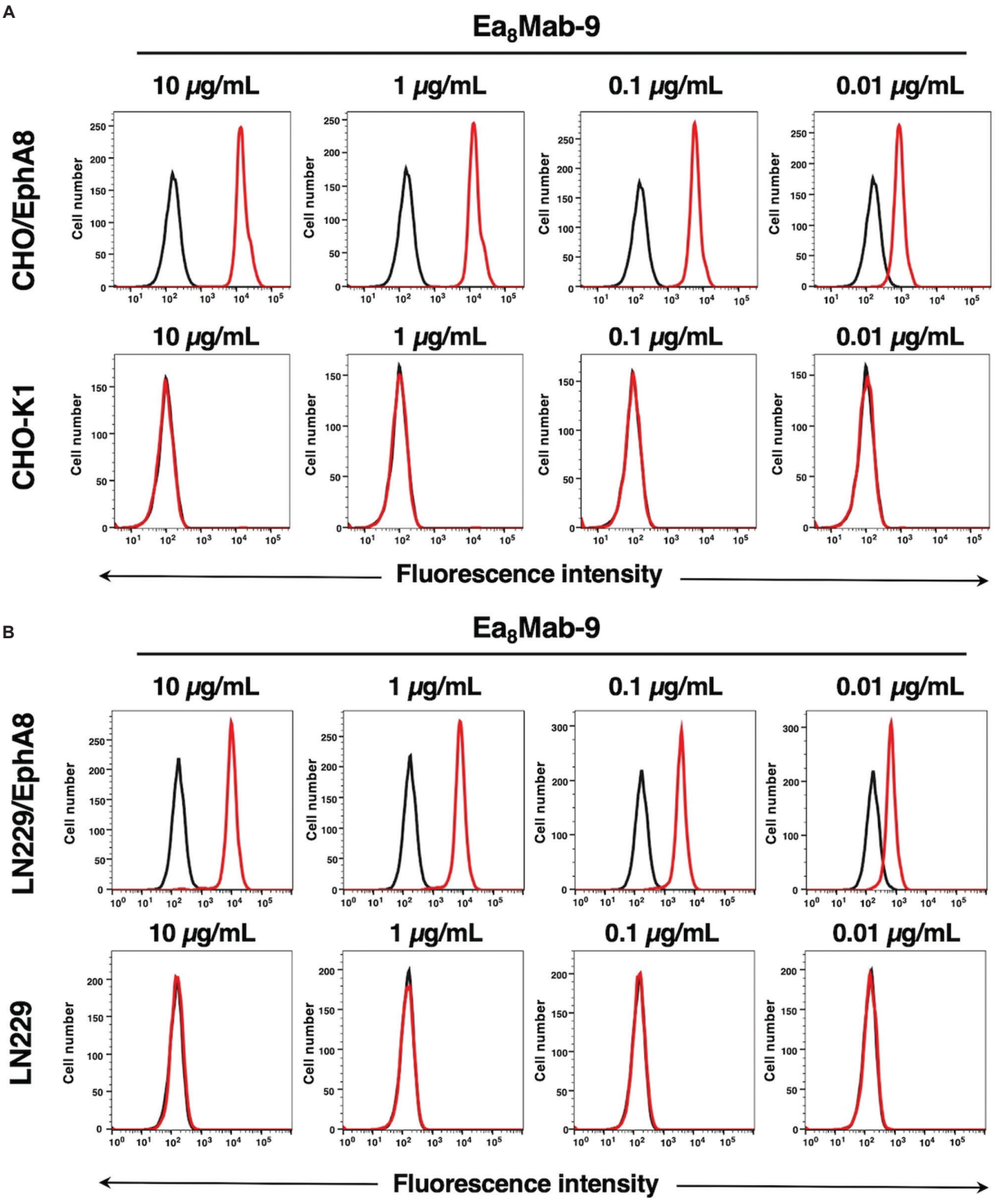

Figure 2. Flow cytometric analysis of Ea_8Mab-9 in EphA8 receptor-expressed CHO-K1 and LN229 cells. (A) CHO/EphA8 (upper panels) and CHO-K1 (lower panels) cells were treated with 0.01 – 10 μg/mL of Ea_8Mab-9 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. The black line represents the negative control (no primary antibody treatment). (B) LN229/EphA8 (upper panels) and LN229 (lower panels) cells were treated with 0.01 – 10 μg/mL of Ea_8Mab-9 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. The black line indicates the negative control (no primary antibody treatment).
Abbreviations: CHO: Chinese hamster ovary; EphA8: Erythropoietin-producing hepatocellular receptor A8; IgG: immunoglobulin g.

3.3. Specificity of Ea_8Mab-9 to Eph receptor-expressed CHO-K1 cells

CHO-K1 cells overexpressing all Eph receptors, including EphA1 to A8, EphA10, EphB1 to B4, and EphB6, were established. Using these fourteen cell lines, the specificity of Ea_8Mab-9 was evaluated. As shown in Figure 3, Ea_8Mab-9 recognized CHO/EphA8 cells but did not bind to any other Eph receptor-expressed CHO-K1 cells. These results confirm the high specificity of Ea_8Mab-9 for EphA8 among the Eph receptor family.

3.4. Determination of the binding affinity of Ea_8Mab-9 by flow cytometry

The binding affinity of Ea_8Mab-9 was assessed with exogenously EphA8-expressed CHO/EphA8 and LN229/EphA8 cells using flow cytometry. Results showed that the K_D values of Ea_8Mab-9 were 1.3×10^{-9} M for CHO/EphA8 cells and 1.6×10^{-9} M for LN229/EphA8 cells (Figure 4). The reproducibility of the binding data was confirmed (Figure A1). These results demonstrate that Ea_8Mab-9 possesses a high affinity for EphA8.

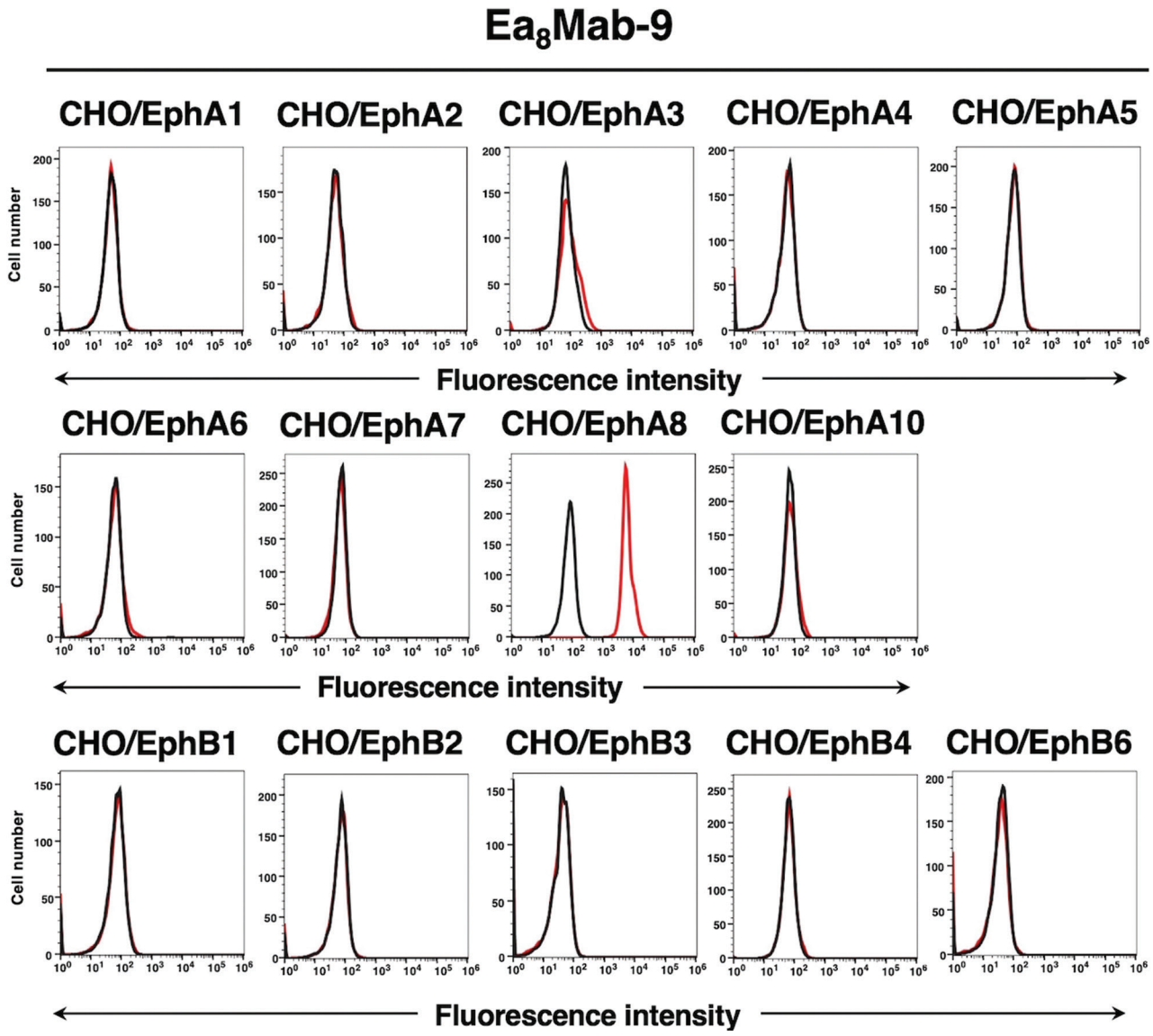

Figure 3. Flow cytometry of Ea_8Mab-9 in Eph receptor-expressed CHO-K1 cells. CHO-K1 cells transfected to express each of the fourteen Eph receptors were treated with 10 μg/mL of Ea_8Mab-9 (red line) or control blocking buffer (black line), followed by the treatment with Alexa Fluor 488-conjugated anti-mouse IgG.
Abbreviations: CHO: Chinese hamster ovary; EphA8: Erythropoietin-producing hepatocellular receptor A8; IgG: immunoglobulin g.

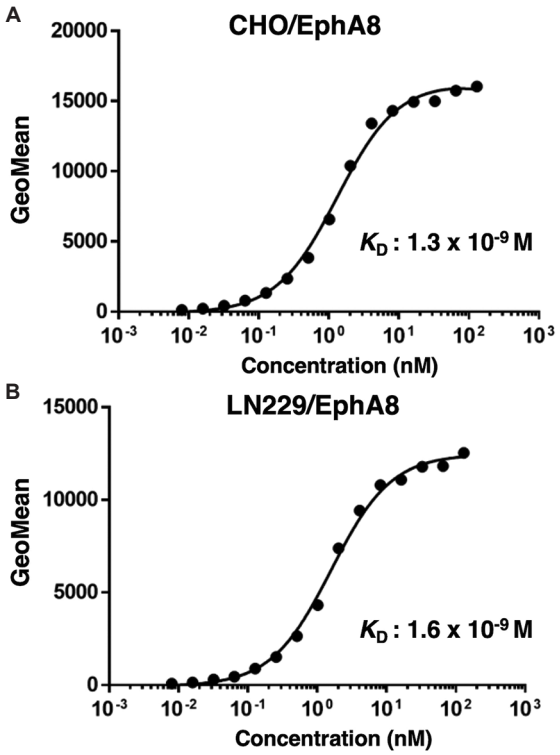

Figure 4. Determination of the binding affinity of Ea_8Mab-9 by flow cytometry. (A) CHO/EphA8 and (B) LN229/EphA8 cells were incubated in 100 µL of serially diluted Ea_8Mab-9 (10 µg/mL to 0.0006 µg/mL). Cells were then treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the BD FACSLyric system, and K_D was calculated using GraphPad PRISM 6 software.
Abbreviations: CHO: Chinese hamster ovary; GeoMean: Geometric mean; K_D: Dissociation constant; EphA8: Erythropoietin-producing hepatocellular receptor A8.

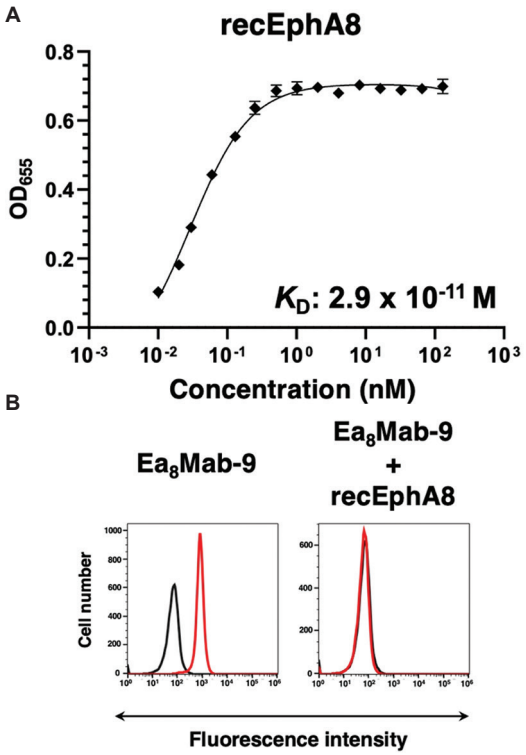

Figure 5. Determination of the binding affinity of Ea_8Mab-9 by ELISA and blocking assay. (A) Recombinant EphA8 (recEphA8) was immobilized on immunoplates and incubated with the serially diluted Ea_8Mab-9, followed by detection with peroxidase-conjugated anti-mouse immunoglobulins (n = 3). Enzymatic reactions were conducted, and the optical density at 655 nm (OD_{655}) was measured. Data are presented as mean ± SD. The K_D value was determined as described above. (B) Blocking assay using recEphA8. CHO/EphA8 cells were treated with control blocking buffer, Ea_8Mab-9 (0.01 µg/mL), or Ea_8Mab-9 (0.01 µg/mL) preincubated with recEphA8 (3 µg/mL) for 30 min at 4°C, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. The black line represents the negative control (blocking buffer).
Abbreviations: CHO: Chinese hamster ovary; ELISA: Enzyme-linked immunosorbent assay; K_D: Dissociation constant; OD: Optical density; SD: Standard deviation; EphA8: Erythropoietin-producing hepatocellular receptor A8.

3.5. Determination of the binding affinity of Ea_8Mab-9 using ELISA

The binding affinity of Ea_8Mab-9 against recEphA8 was evaluated using ELISA. As shown in Figure 5A, the K_D value of Ea_8Mab-9 was 2.9 × 10^{-11} M. Furthermore, the recEphA8 competitively inhibited the binding of Ea_8Mab-9 in flow cytometry (Figure 5B). These results also confirmed the high affinity and specificity of Ea_8Mab-9 for EphA8.

4. Discussion

In this study, an anti-EphA8 mAb, Ea_8Mab-9, was first developed and showed high affinity and specificity in flow cytometry (Figures 2-4) and ELISA (Figure 5). Additional anti-EphA8 mAb clones for flow cytometry were also established (http://www.med-tohoku-antibody.com/topics/001_paper_antibody_PDIS.htm#EphA8). Ea_8Mab-9 and these clones are expected to facilitate the elucidation of EphA8 functions in various research fields. In particular, no commercially available anti-EphA8 antibodies are suitable for flow cytometry. Ea_8Mab-9 is expected to lead to a more detailed elucidation of the role of EphA8 in cancer, including the tumor microenvironment and neuronal research. Furthermore, previous efforts have enhanced antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity activities by switching isotypes and eliminating fucosylation in mAbs.[32] Since Ea_8Mab-9 is a mouse IgG_1 subclass, which lacks ADCC activity, a mouse IgG_{2a} version will be generated to examine antitumor efficacy in tumor xenograft models in future studies.

EphA8 regulates brain development and neural outgrowth in normal tissues,[15,17] and its overexpression

has been reported in cancers.[22,23,25] EphA8 TK-dependent activation appears to be essential for its function. For example, Tyr-phosphorylated EphA8 regulates cell-cell adhesion by interacting with Fyn.[11] In breast cancer, EphA8 suppresses cell apoptosis via Akt activation and correlates with poor prognosis.[25] In addition, EphA8 expression may mediate the resistance to paclitaxel treatment.[25] In gastric cancer, EphA8 promotes malignancy through Akt signaling and interaction with a disintegrin and metalloproteinase domain-containing protein 10.[24] In OTSCC patients, EphA8 expression significantly correlates with tumor, node, metastasis stage, but not with other risk factors such as age, gender, drinking, and smoking history.[22] Since EphA8 has been reported to cooperate with stem cells,[26] further evaluation of its relationship with other membrane protein markers such as CD44 and CD133 may be valuable.[33,34] To analyze the population of EphA8-expressing cancer cells, detecting naïve EphA8 on these cells is necessary. Because Ea_8Mab-9 recognizes EphA8 with high affinity, it will be helpful for diagnosis and experiments using flow cytometry. However, the suitability of Ea_8Mab-9 for immunofluorescence or immunohistochemistry has not yet been investigated. If unsuitable for these applications, identifying EphA8-expressing cells in tissue samples may be challenging.

Eph receptors have been widely studied in the context of cancer and are gaining attention as therapeutic targets.[2,35] Clinical trials have been conducted for various modalities, including compounds, antibody drugs, and chimeric antigen receptor (CAR)-T cells targeting Eph receptors and ephrin ligands such as EphA2, EphA3, EphA5, EphB4, ephrin A4, and ephrin B2.[2,21] However, there are currently no drugs approved specifically for Eph receptors or ephrin ligands. Given that mAbs against HER2,[36] podoplanin,[37] and podocalyxin[38] established using the CBIS method have revealed applicability in CAR-T therapy, it is worthwhile to investigate the potential application of Ea_8Mab-9 for CAR-T therapy targeting EphA8-positive tumors.

5. Conclusion

Ea_8Mab-9, established using the CBIS method, may serve as a valuable tool for analyzing EphA8-related biological responses by flow cytometry, owing to its high affinity and specificity.

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Conflict of interest

The authors declare that they have no conflict of interest

Author contributions

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Methodology: Mika K. Kaneko
Writing – original draft: Tomohiro Tanaka
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 of this study are available in the article.

Further disclosure

The paper has been uploaded to a preprint server (DOI: 10.20944/preprints202412.1044.v1).

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Appendix

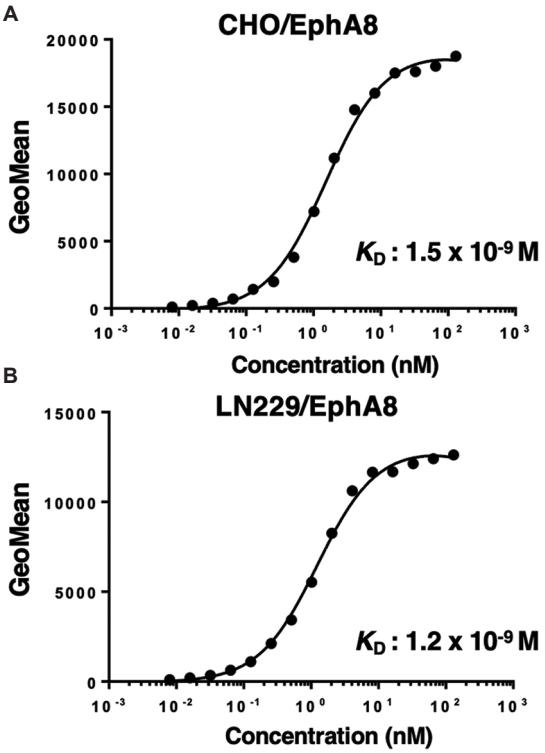

Figure A1. Reproducibility of the binding affinity of Ea_8Mab-9 against EphA8-expressed cells. (A) CHO/EphA8 and (B) LN229/EphA8 cells were incubated in 100 μL of serially diluted Ea_8Mab-9 (10 μg/mL to 0.0006 μg/mL). Cells were then treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the BD FACSLyric system, and K_D was calculated using GraphPad PRISM 6 software.
Abbreviations: CHO: Chinese hamster ovary; GeoMean: Geometric mean; K_D: Dissociation constant; EphA8: Erythropoietin-producing hepatocellular receptor A8