

# ChLpMab-23: Cancer-Specific Human–Mouse Chimeric Anti-Podoplanin Antibody Exhibits Antitumor Activity via Antibody-Dependent Cellular Cytotoxicity

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Podoplanin is expressed in many cancers, including oral cancers and brain tumors. The interaction between podoplanin and its receptor C-type lectin-like receptor 2 (CLEC-2) has been reported to be involved in cancer metastasis and tumor malignancy. We previously established many monoclonal antibodies (mAbs) against human podoplanin using the cancer-specific mAb (CasMab) technology. LpMab-23 (IgG<sub>1</sub>, kappa), one of the mouse anti-podoplanin mAbs, was shown to be a CasMab. However, we have not shown the usefulness of LpMab-23 for antibody therapy against podoplanin-expressing cancers. In this study, we first determined the minimum epitope of LpMab-23 and revealed that Gly54–Leu64 peptide, especially Gly54, Thr55, Ser56, Glu57, Asp58, Arg59, Tyr60, and Leu64 of podoplanin, is a critical epitope of LpMab-23. We further produced human–mouse chimeric LpMab-23 (chLpMab-23) and investigated whether chLpMab-23 exerts antibody-dependent cellular cytotoxicity (ADCC) and antitumor activity. In flow cytometry, chLpMab-23 showed high sensitivity against a podoplanin-expressing glioblastoma cell line, LN319, and an oral cancer cell line, HSC-2. chLpMab-23 also showed ADCC activity against podoplanin-expressing CHO cells (CHO/podoplanin). In xenograft models with HSC-2 and CHO/podoplanin, chLpMab-23 exerts antitumor activity using human natural killer cells, indicating that chLpMab-23 could be useful for antibody therapy against podoplanin-expressing cancers.

**Keywords:** podoplanin, monoclonal antibody, ADCC, CDC, antitumor activity

## Introduction

**P**ODOPLANIN IS EXPRESSED in many cancers, including oral cancers and brain tumors.<sup>(1–13)</sup> C-type lectin-like receptor 2 (CLEC-2) is an endogenous receptor of podoplanin and is involved in platelet aggregation.<sup>(14–22)</sup> Recently, podoplanin was reported to be a potential biomarker for identifying the presence of early infiltrative esophageal squamous cell carcinoma.<sup>(23)</sup> Previously, podoplanin has also been reported as a marker of tumor-initiating cells (TICs) in a human squamous cell carcinoma cell line, A431.<sup>(24)</sup> The same group recently showed that high clonal expansion capacity of podoplanin-positive TICs populations was the result

of reduced cell death caused by podoplanin-mediated signaling, indicating that podoplanin may be a therapeutic target in squamous cell carcinoma treatment.<sup>(25)</sup>

Transforming growth factor- $\beta$  (TGF- $\beta$ ) regulates many physiological events including tumorigenesis.<sup>(26)</sup> We previously found that TGF- $\beta$  induced podoplanin in human fibrosarcoma HT1080 cells and enhanced the platelet aggregating ability of HT1080.<sup>(27)</sup> TGF- $\beta$  type I receptor inhibitor (SB431542) and short hairpin Smad4 inhibited the podoplanin induction by TGF- $\beta$ , suggesting that TGF- $\beta$  is a physiological regulator of podoplanin in tumor cells. Recently, another group confirmed this phenomenon using *in vivo* analysis.<sup>(28)</sup> Podoplanin-mediated epithelial–mesenchymal transition (EMT)

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reportedly resulted in increased invasiveness and extravasation of tumor cells, and treatment of mice with a TGF- $\beta$ -neutralizing antibody inhibited podoplanin-mediated hematogenous metastasis *in vivo*, indicating that podoplanin promoted hematogenous metastasis in part by releasing TGF- $\beta$  from platelets that was essential for EMT of tumor cells.

Many anti-podoplanin monoclonal antibodies (mAbs) have been reported, and almost all of them, such as D2-40, 18H5, and NZ-1, react with the peptides of podoplanin.<sup>(19,29–35)</sup> We have used our original technology to produce anti-glycopeptide mAbs (GpMabs) against podoplanin.<sup>(36–40)</sup> One of these GpMabs, LpMab-2, was shown to be a cancer-specific mAb (CasMab).<sup>(36)</sup> LpMab-2 recognizes both an aberrant *O*-glycosylation and a Thr55–Leu64 peptide from podoplanin. Because LpMab-2 reacts with podoplanin-expressing cancer cells but not with normal cells, as shown by flow cytometry and immunohistochemistry, it is expected to be useful for molecular targeting therapy against podoplanin-expressing cancers.<sup>(41)</sup> Another anti-podoplanin CasMab is LpMab-23.<sup>(42)</sup>

In this study, we produced human–mouse chimeric LpMab-23 (chLpMab-23) and investigated whether chLpMab-23 exerts antibody-dependent cellular cytotoxicity (ADCC) and antitumor activity.

## Materials and Methods

### Cell lines

Chinese hamster ovary cell lines (CHO)-K1, LN229, and P3U1 were obtained from the American Type Culture Collection (Manassas, VA). HSC-2 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The LN319 cell line was provided by Prof. Kazuhiko Mishima (Saitama Medical University, Saitama, Japan).<sup>(43)</sup> CHO-S was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). Core fucose-knockout CHO-S cells (PDIS-5) was established using CRISPR-Cas9 in our previous study.<sup>(41)</sup> CHO-K1 and LN229 cells were transfected with podoplanin plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA) according to the manufacturer's instructions.<sup>(36)</sup>

The CHO-K1, CHO-K1/podoplanin, and P3U1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing  $L$ -glutamine (Nacalai Tesque, Inc., Kyoto, Japan) and 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc.). HSC-2 cells were cultured in Eagle's minimal essential medium (Nacalai Tesque, Inc.) or Dulbecco's modified Eagle's medium (DMEM) containing  $L$ -glutamine and 10% heat-inactivated FBS. The LN319, LN229, and LN229/podoplanin cells were cultured in DMEM containing  $L$ -glutamine and 10% heat-inactivated FBS. Cells were kept at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. All media contained 100 U/mL of penicillin, 100  $\mu$ g/mL of streptomycin, and 25  $\mu$ g/mL of amphotericin B (Nacalai Tesque, Inc.).

### Antibodies

LpMab-23, a mouse anti-podoplanin mAb (IgG<sub>1</sub>, kappa), was developed as previously described.<sup>(42)</sup> Human IgG was purchased from Beckman Coulter, Inc. (Fullerton, CA) or Sigma-Aldrich Corp. (St. Louis, MO). To generate human–mouse chimeric anti-podoplanin (chLpMab-23), appropriate

$V_H$  and  $V_L$  cDNAs of mouse LpMab-23 and  $C_H$  and  $C_L$  of human IgG<sub>1</sub> were subcloned into pCAG-Ble and pCAG-Neo vectors (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively. Antibody expression vectors were transfected into CHO-K1 or PDIS-5<sup>(41)</sup> using the Lipofectamine LTX reagent (Thermo Fisher Scientific, Inc.). Stable transfectants of CHO-K1/chLpMab-23 or PDIS-5/chLpMab-23 cells were selected by cultivating the transfectants in a medium containing 0.5 mg/mL of both geneticin and zeocin (InvivoGen, San Diego, CA). CHO-K1/chLpMab-23 and PDIS-5/chLpMab-23 cells were cultivated in CHO-S-SFM II medium (Thermo Fisher Scientific, Inc.). ChLpMab-23 was purified using Protein G-Sepharose (GE Healthcare UK Ltd, Buckinghamshire, England).

### Production of point mutants

The amplified human podoplanin cDNA was subcloned into a pcDNA3 vector (Thermo Fisher Scientific, Inc.), and a FLAG epitope tag was added at the C-terminus. Amino acids in podoplanin were substituted with alanine using a Quik-Change Lightning site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA) with oligonucleotides containing the desired mutations. CHO-K1 or CHO-S cells were transfected with the plasmids using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc., Berkeley, CA). The point mutant cells of CHO-K1 were cultured in RPMI 1640 medium containing  $L$ -glutamine and 10% heat-inactivated FBS as described above. The point mutant cells of CHO-S were cultured in CHO-S-SFMII medium.

### Flow cytometry

The cell lines were harvested after brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin in phosphate-buffered saline (PBS), the cells were treated with primary mAbs for 30 min at 4°C, followed by treatment with fluorescein isothiocyanate (FITC)-conjugated goat antihuman or antimouse IgG (Thermo Fisher Scientific, Inc.). Fluorescence data were acquired using Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

### Determination of the binding affinity using flow cytometry

LN319 ( $2 \times 10^5$  cells) were resuspended with 100  $\mu$ L of serially diluted chLpMab-23 (0.02–50  $\mu$ g/mL) or NZ-12 (0.01–20  $\mu$ g/mL) followed by secondary antihuman IgG. Fluorescence data were collected using EC800. The dissociation constants ( $K_D$ ) were obtained by fitting the binding isotherms using the built-in one-site binding models in GraphPad PRISM 6 (GraphPad software, Inc., La Jolla, CA).

### Western blot analyses

Cell lysates (10  $\mu$ g) were boiled in SDS sample buffer (Nacalai Tesque, Inc.). The proteins were electrophoresed on 5%–20% polyacrylamide gels (Wako Pure Chemical Industries Ltd.) and were transferred onto a polyvinylidene difluoride membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), the membrane was incubated with chLpMab-23, NZ-12, or anti- $\beta$ -actin (clone AC-15; Sigma-Aldrich Corp.) and then with

peroxidase-conjugated antihuman IgG (Sigma-Aldrich Corp.) and was developed with the ImmunoStar LD Chemiluminescence Reagent (Wako Pure Chemical Industries Ltd.) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

#### *Antibody-dependent cellular cytotoxicity*

Effector cells were prepared as previously described.<sup>(7)</sup> Human peripheral blood mononuclear cells (MNCs) were obtained from leukocytes, which were separated from the peripheral blood of healthy donors. The study with human subjects was approved by the Ethics Committee of the Tokushima University. ADCC was determined using the <sup>51</sup>Cr-release assay.<sup>(7)</sup> Target cells were incubated with 0.1  $\mu$ Ci of <sup>51</sup>Cr-sodium chromate at 37°C for 1 hour. After washing three times with 10% FBS-supplemented RPMI 1640, <sup>51</sup>Cr-labeled target cells were seeded in 96-well plates in triplicate. Human peripheral blood MNCs and chLpMab-23 or control human IgG were added to the cells. After 6 hours of incubation, <sup>51</sup>Cr released from cells into the supernatant (100  $\mu$ L) was measured using a gamma counter (PerkinElmer, Waltham, MA). The percentage of cytotoxicity was calculated using the following formula: % specific lysis =  $(E-S)/(M-S) \times 100$ , where *E* is the release in the test sample, *S* is the spontaneous release, and *M* is the maximum release.

#### *Antitumor activity of anti-podoplanin antibodies*

CHO/podoplanin cells and HSC-2 cells were trypsinized and washed with PBS. The cell density was adjusted with PBS to  $5.0 \times 10^7$  cells/mL (CHO/podoplanin) or  $1.0 \times 10^7$  cells/mL (HSC-2), and 100  $\mu$ L/animal of the cell suspension was subcutaneously inoculated into BALB/c nude mice. After 1 day, 100  $\mu$ L of 1 mg/mL of chLpMab-23 and human IgG were injected into the peritoneal cavity of mice. Additional antibodies were injected once a week for several weeks. Human natural killer (NK) cells ( $5.0 \times 10^5$  cells; Takara Bio, Inc., Shiga, Japan) were injected around the tumors several times. The tumor diameter was measured every 3–7 days and was calculated using the following formula: volume =  $W^2 \times L/2$ , where *W* is the short diameter and *L* is the long diameter. The mice were euthanized 21 (CHO/podoplanin) or 35 days (HSC-2) after cell implantation.

#### *Statistical analyses*

All data were expressed as means  $\pm$  SEMs. Student's *t*-test, Mann–Whitney *U*-test, one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons, and two-way ANOVA were performed as appropriate. *p*-Values <0.05 were considered to be significant. All statistical tests were two-sided.

## Results

#### *Epitope mapping of LpMab-23*

We performed flow cytometry using several point mutants of human podoplanin to determine the epitope of LpMab-23. Seventeen podoplanin point mutants against Val50–Thr66 were transiently expressed in CHO-K1 cells. As shown in Figure 1A, LpMab-23 did not detect G54A, T55A, S56A, E57A, D58A, R59A, Y60A, or L64A. In contrast, LpMab-7,<sup>(30)</sup> a positive control mAb against human podoplanin, recognized

all point mutants. These results indicate that the minimum epitope of LpMab-23 is Gly54–Leu64, which is very similar to that of LpMab-2 (Fig. 1B). Flow cytometry and western blot analyses using several glycan-deficient podoplanin cells revealed that the epitope of LpMab-23 is independent of glycans (data not shown), whereas the epitope of LpMab-2 includes *O*-glycan of Thr55/Ser56.<sup>(36)</sup> Here, we produced a human–mouse chimeric antibody from LpMab-23 and investigated whether chLpMab-23 possesses ADCC and antitumor activity.

#### *Production of chLpMab-23*

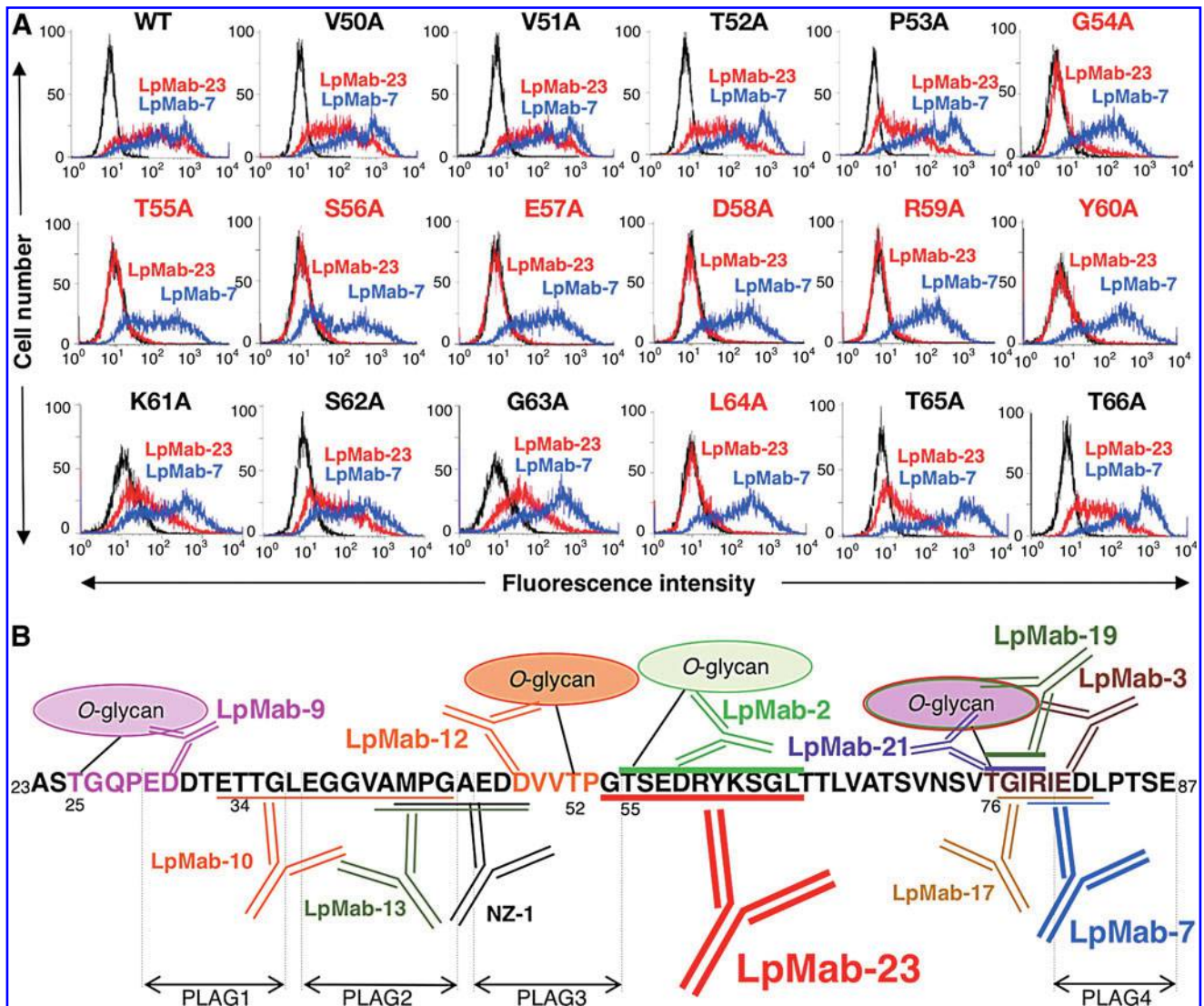
We first checked the sensitivity of chLpMab-23 using flow cytometry. As shown in Figure 2A, chLpMab-23 reaction against human glioblastoma cell line, LN319, was observed at a very low concentration of 10 ng/mL. A positive control NZ-12, the most sensitive chimeric anti-podoplanin antibody,<sup>(44)</sup> reacted with LN319 in the same way. Both antibodies reacted with LN319 in a dose-dependent manner. We next performed a kinetic analysis of the interaction of chLpMab-23 with LN319 using flow cytometry. As shown in Figure 2B,  $K_D$  of chLpMab-23 was determined to be  $1.2 \times 10^{-8}$  M. The  $K_D$  of NZ-12 was also determined to be  $1.2 \times 10^{-8}$  M. NZ-12 was derived from the rat anti-podoplanin mAb, NZ-1, which possesses the highest binding affinity<sup>(8)</sup>; this showed that chLpMab-23 also possesses high binding affinity against podoplanin. Western blot analysis showed that chLpMab-23 did not detect T55A and S56A (Fig. 2C), which is consistent with the results of flow cytometry using LpMab-23 (Fig. 1A). chLpMab-12,<sup>(30)</sup> a human–mouse chimeric antibody produced from another GpMab, LpMab-12 against human podoplanin, did not recognize T52A, which is consistent with our previous study.<sup>(37)</sup> chLpMab-7, a human–mouse chimeric antibody produced from LpMab-7 against human podoplanin,<sup>(45)</sup> and NZ-12 reacted with all point mutants. Because the podoplanin expression level in S56A mutant is comparatively low, the signals of chLpMab-12, chLpMab-7, and NZ-12 were lower than those of the other mutants. These results indicate that the epitope of chLpMab-23 was confirmed to include Thr55 and Ser56.

#### *ADCC of chLpMab-23 against podoplanin-expressing cells*

Next, we investigated whether chLpMab-23 possesses ADCC activity against podoplanin-expressing cells. chLpMab-23 reacted with CHO/podoplanin in a dose-dependent manner (Fig. 3A). A positive control NZ-12 showed a similar reaction. Western blot analysis showed that chLpMab-23 specifically detected podoplanin, which is expressed in LN229 and CHO-K1 (Fig. 3B). NZ-12 also helped detect podoplanin in the same way. As shown in Figure 3C, NZ-12 showed ADCC activity against CHO/podoplanin cells. In the same way, chLpMab-23 exerted ADCC against the CHO/podoplanin cells but not against CHO-K1 cells, indicating that ADCC against CHO/podoplanin is specific against podoplanin.

#### *Antitumor activity of chLpMab-23*

To study the antitumor activity of chLpMab-23 on primary tumor growth *in vivo*, CHO/podoplanin cells were subcutaneously implanted into the flanks of nude mice. To upregulate the ADCC activity of chLpMab-23, PDIS-5 cells



**FIG. 1.** Epitope mapping of LpMab-23 using point mutants of human podoplanin in flow cytometry (A) Seventeen podoplanin point mutants were transiently expressed in CHO-K1 cells. Cells were treated with LpMab-23 (1  $\mu$ g/mL; red), LpMab-7 (1  $\mu$ g/mL; blue), or control phosphate-buffered saline (PBS) (black) for 30 min at 4°C, followed by treatment with anti-mouse IgG–Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. (B) Schematic illustration of the epitope of anti-podoplanin mAbs including LpMab-23 (red) and LpMab-7 (blue). mAbs, monoclonal antibodies.

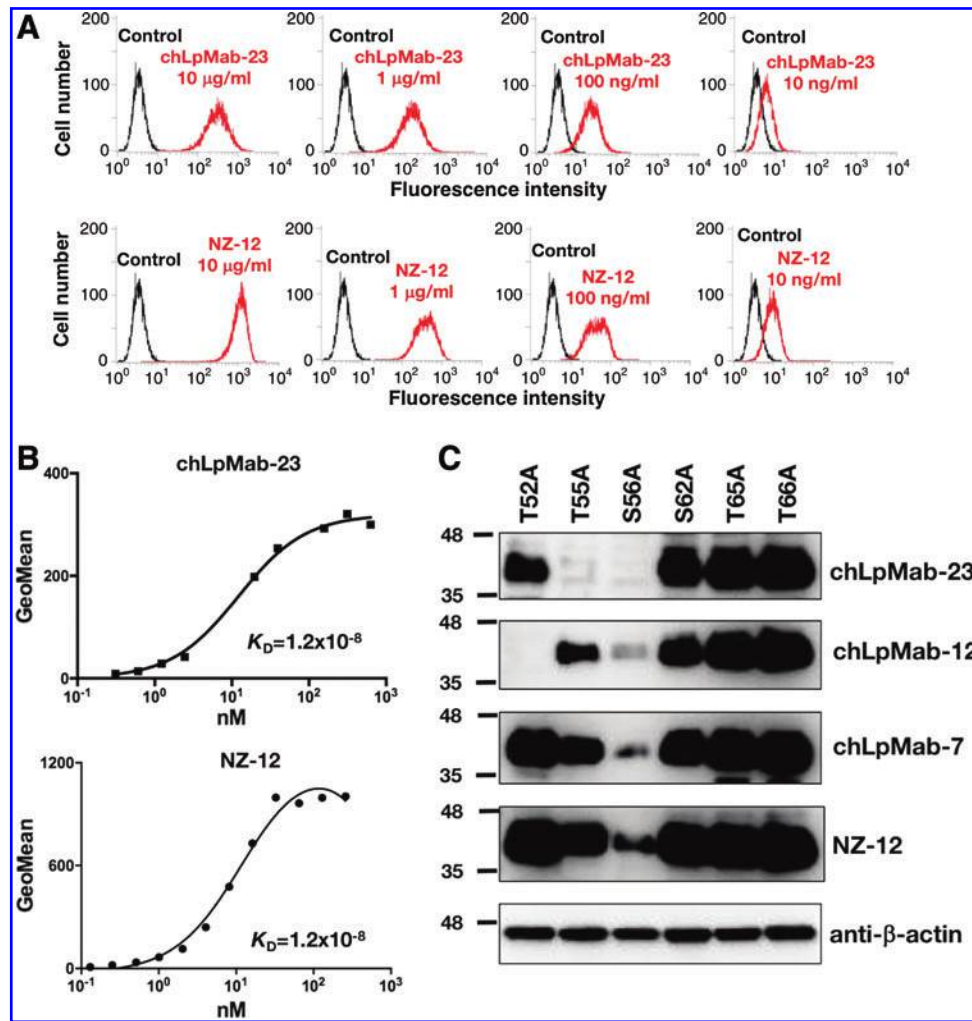
(FUT-8-knockout cell line) were used for producing chLpMab-23. Core fucose-negative-type chLpMab-23, which was produced using PDIS-5, and control human IgG were injected four times into the peritoneal cavity of mice, and human NK cells were injected twice around the tumors. Tumor formation was observed in mice from the control and treated groups. However, chLpMab-23 significantly reduced tumor development compared with control human IgG on day 21 (Fig. 3D).

We further investigated whether chLpMab-23 also inhibited the tumor growth of an oral cancer cell line, HSC-2. chLpMab-23 reacted with HSC-2 in a dose-dependent manner (Fig. 4A). NZ-12 also reacted with HSC-2 in a dose-dependent manner. The reaction of NZ-12 is much more sensitive compared with that of chLpMab-23. For *in vivo* analysis, we used normal-type chLpMab-23, which was produced in CHO-K1 cells. HSC-2 cells were subcutaneously

implanted into the flanks of nude mice. chLpMab-23 and control human IgG were injected seven times into the peritoneal cavity of mice, and human NK cells were injected three times around the tumors. As shown in Figure 4B, tumor formation was observed in mice from the control and treated groups. chLpMab-23 significantly reduced tumor development of HSC-2 compared with control human IgG on day 35 (Fig. 4C). These results indicate that administration of chLpMab-23 with the NK cells inhibited the primary tumor growth *in vivo*.

## Discussion

In a previous study, through an immunohistochemical analysis, we showed that LpMab-23 reacted with tumor cells of human oral cancer but not with normal cells such as lymphatic endothelial cells (LECs).<sup>(42)</sup> Furthermore, flow

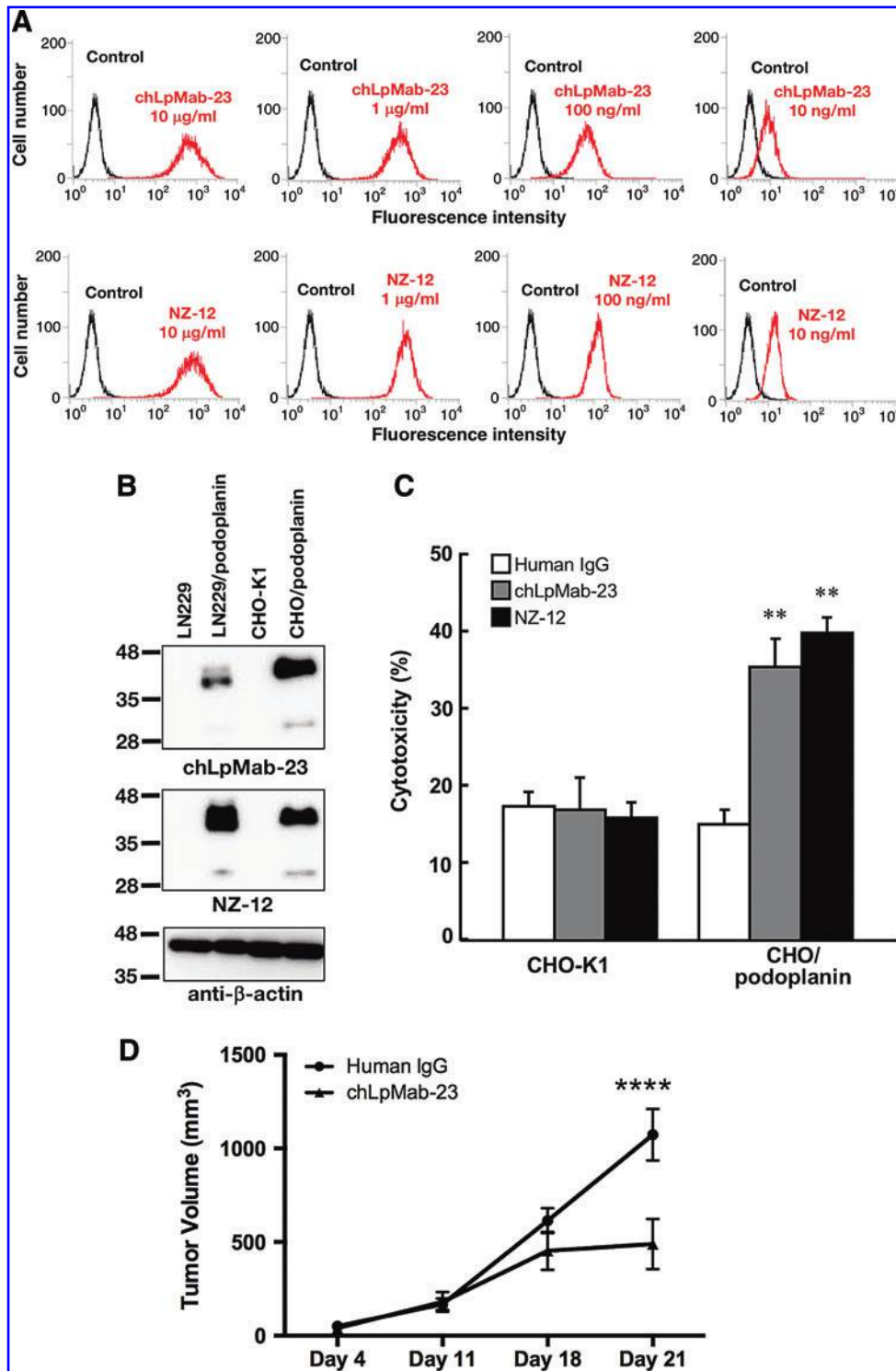


**FIG. 2.** Characterization of chLpMab-23 (A) LN319 cells were treated with chLpMab-23 or NZ-12 (10 µg/mL–10 ng/mL, red) and PBS (black) for 30 min at 4°C, followed by treatment with antihuman IgG–FITC. Fluorescence data were collected using Cell Analyzer EC800. (B) Determination of the binding affinity of chLpMab-23 or NZ-12 using flow cytometry. (C) Six podoplanin point mutants were stably expressed in CHO-S cells. Western blot analysis with chLpMab-23, chLpMab-12, chLpMab-7, or NZ-12. The membrane was incubated with 10 µg/mL of primary antibodies and then with peroxidase-conjugated antihuman IgG; the membrane was detected using a Sayaca-Imager. AC-15 (anti-β-actin) was used as internal control. FITC, fluorescein isothiocyanate.

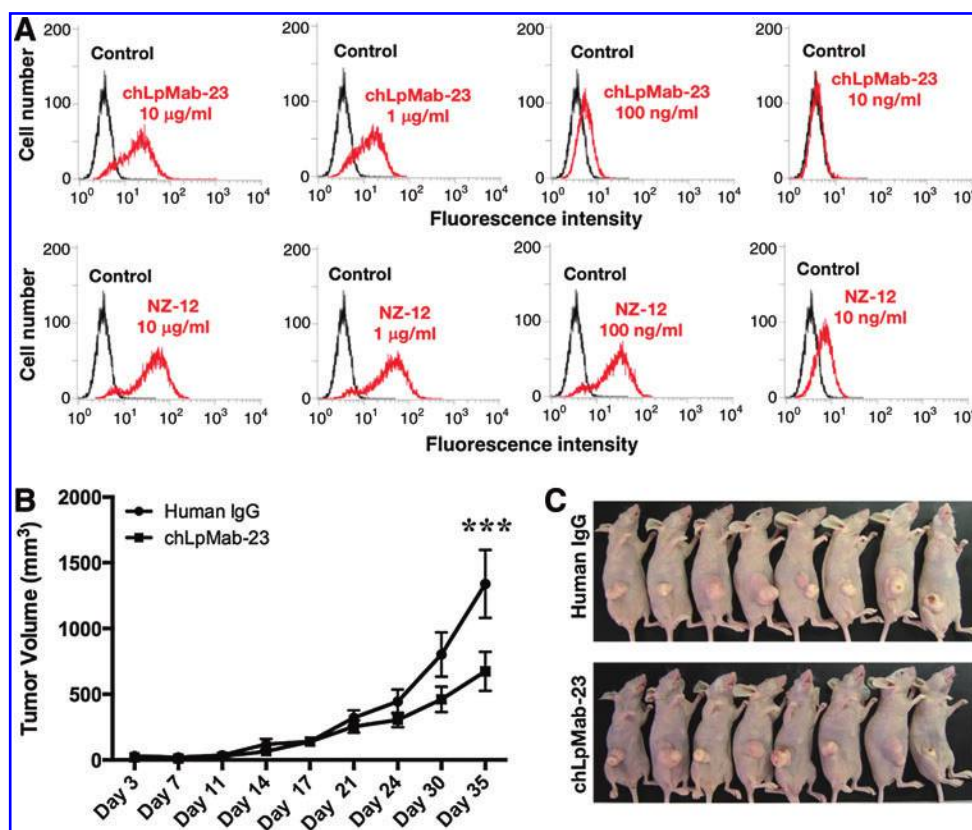
cytometric analysis revealed that LpMab-23 reacted with podoplanin-expressing cancer cell lines but showed little reaction with normal cells including LECs and HEK-293T. Although the epitope of another CasMab, LpMab-2, was determined to be Thr55–Leu64 peptide from podoplanin, the epitope of LpMab-23 has not been clarified. In this study, we performed flow cytometry using several point mutants of human podoplanin and revealed that Gly54–Leu64 peptide from podoplanin is the LpMab-23 epitope (Fig. 1A). Furthermore, eight amino acids namely Gly54, Thr55, Ser56, Glu57, Asp58, Arg59, Tyr60, and Leu64 of podoplanin are important for LpMab-23. In contrast, seven amino acids namely Thr55, Ser56, Glu57, Asp58, Arg59, Tyr60, and Leu64 of podoplanin are important for LpMab-2,<sup>(36)</sup> indicating that epitopes of the two different CasMabs LpMab-2 and LpMab-23 are very similar (Fig. 1B).

We had not shown that LpMab-23 possesses ADCC against podoplanin-expressing cancer cells because LpMab-

23 is a mouse IgG<sub>1</sub> subclass, which does not exert ADCC. In this study, we developed a human–mouse chimeric antibody, chLpMab-23, from a mouse mAb, LpMab-23. chLpMab-23 reacted with LN319, CHO/podoplanin, and HSC-2 in a dose-dependent manner (Figs. 2A, 3A, and 4A). chLpMab-23 showed ADCC against the CHO/podoplanin cells (Fig. 3C) but not against the CHO-K1 cells, indicating that ADCC against CHO/podoplanin is specific against podoplanin. chLpMab-23 also showed ADCC against the LN319 glioblastoma cell line (data not shown). However, chLpMab-23 did not exert ADCC against malignant mesothelioma cell line NCI-H226 or lung cancer cell line PC-10 (data not shown) because podoplanin expression in those cell lines may be comparatively low. Furthermore, we investigated the complement-dependent cytotoxicity (CDC) of chLpMab-23 against several podoplanin-expressing cancer cells. However, chLpMab-23 showed no CDC against podoplanin-expressing cancer cells (data not shown). In our previous



**FIG. 3.** ADCC and antitumor effects of chLpMab-23 against CHO/podoplanin (A) CHO/podoplanin cells were treated with chLpMab-23 or NZ-12 (10 µg/mL–10 ng/mL, red) and PBS (black) for 30 min at 4°C, followed by treatment with antihuman IgG–FITC. Fluorescence data were collected using Cell Analyzer EC800. (B) Western blot analysis with chLpMab-23 or NZ-12. The membrane was incubated with 10 µg/mL of chLpMab-23 and then with peroxidase-conjugated antihuman IgG; the membrane was detected using a Sayaca-Imager. AC-15 (anti-β-actin) was used as internal control. (C) ADCC of chLpMab-23 or NZ-12 against CHO and CHO/podoplanin using human MNCs was evaluated using a 6-hour <sup>51</sup>Cr-release assay in the presence of 10 µg/mL of antibodies with an E/T ratio of 100. \*\**p* < 0.01 with Student's *t*-test. (D) Antitumor effects of chLpMab-23 on tumor development of CHO/podoplanin xenograft. CHO/podoplanin cells ( $5 \times 10^6$  cells/100 µL) were subcutaneously inoculated into BALB/c nude mice. After 1 day, 100 µg of core fucose-negative chLpMab-23 or control human IgG was injected into the peritoneal cavity of the mice. The antibodies were further injected three times (days 4, 11, and 18; control group: *n* = 6; chLpMab-23 group: *n* = 6). NK cells were injected around the tumor on days 4 and 11. The tumor diameter was measured at 3–4-day intervals and was calculated using the following formula: tumor volume =  $W^2 \times L/2$ , where *W* = short diameter and *L* = long diameter. \*\*\*\**p* < 0.001 with two-way ANOVA. ADCC, antibody-dependent cellular cytotoxicity; ANOVA, analysis of variance; MNCs, mononuclear cells; NK, natural killer.



**FIG. 4.** Antitumor effects of chLpMab-23 on tumor development of HSC-2 xenograft, (A) HSC-2 cells were treated with chLpMab-23 or NZ-12 (10 µg/mL–10 ng/mL, red) and PBS (black) for 30 min at 4°C, followed by treatment with antihuman IgG–FITC. Fluorescence data were collected using Cell Analyzer EC800. (B) HSC-2 cells ( $1 \times 10^6$  cells/100 µL) were subcutaneously inoculated into BALB/c nude mice. After 1 day, 100 µg of chLpMab-23 or control human IgG was injected into the peritoneal cavity of the mice. The antibodies were further injected six times (days 10, 14, 18, 22, 26, and 30; control group:  $n=8$ ; chLpMab-23 group:  $n=8$ ). NK cells were injected around the tumor on days 10, 18, and 26. The tumor diameter was measured at 3–4 day intervals and was calculated using the following formula: tumor volume =  $W^2 \times L/2$ , where  $W$  = short diameter and  $L$  = long diameter. \*\*\* $p < 0.005$  with two-way ANOVA. (C) Comparison of the tumor size (day 35).

study, we showed that ADCC of anti-podoplanin mAbs is much more important for antitumor activity.<sup>(7,44)</sup> Therefore, we moved on to the investigation of antitumor activity using chLpMab-23.

To study the antitumor activity of chLpMab-23 on the primary tumor growth of CHO/podoplanin cells *in vivo*, core fucose-negative-type chLpMab-23 was used because core fucose-negative mAbs show higher ADCC activity.<sup>(46)</sup> chLpMab-23 significantly reduced tumor development of CHO/podoplanin on day 21 (Fig. 3D). We previously used normal-type (core fucose-positive-type) chLpMab-23 for the same xenograft model of CHO/podoplanin cells; however, chLpMab-23 did not reduce tumor development of CHO/podoplanin xenograft. We further showed that normal-type chLpMab-23 significantly reduced tumor development of HSC-2 (Fig. 4B). The different results for the CHO/podoplanin and HSC-2 xenograft models may depend on the growth speed of each xenograft or the sensitivity of human NK cells. We did not compare the antitumor activity of core fucose-negative-type and normal-type chLpMab-23 in the HSC-2 xenograft model. In the near future, we should determine the best format of chLpMab-23 for antibody therapy against podoplanin-expressing tumors.

Taken together, chLpMab-23 could be useful for antibody therapy against podoplanin-expressing cancers. The Cas-Mabs and chLpMab-23 developed by us could be applied to novel antitumor reagents to provide strict specificity against tumor cells.

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#### Author Disclosure Statement

No competing financial interests exist.

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