

Novel Monoclonal Antibody LpMab-17 Developed by CasMab Technology Distinguishes Human Podoplanin from Monkey Podoplanin

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Podoplanin (PDPN) is a type-I transmembrane sialoglycoprotein, which possesses a platelet aggregation-stimulating (PLAG) domain in its N-terminus. Among the three PLAG domains, *O*-glycan on Thr52 of PLAG3 is critical for the binding with C-type lectin-like receptor-2 (CLEC-2) and is essential for platelet-aggregating activity of PDPN. Although many anti-PDPN monoclonal antibodies (mAbs) have been established, almost all mAbs bind to PLAG domains. We recently established CasMab technology to produce mAbs against membranous proteins. Using CasMab technology, we produced a novel anti-PDPN mAb, LpMab-17, which binds to non-PLAG domains. LpMab-17 clearly detected endogenous PDPN of cancer cells and normal cells in Western-blot, flow cytometry, and immunohistochemistry. LpMab-17 recognized glycan-deficient PDPN in flow cytometry, indicating that the interaction between LpMab-17 and PDPN is independent of its glycosylation. The minimum epitope of LpMab-17 was identified as Gly77–Asp82 of PDPN using enzyme-linked immunosorbent assay. Of interest, LpMab-17 did not bind to monkey PDPN, whereas the homology is 94% between human PDPN and monkey PDPN, indicating that the epitope of LpMab-17 is unique compared with the other anti-PDPN mAbs. The combination of different epitope-possessing mAbs could be advantageous for the PDPN-targeting diagnosis or therapy.

Introduction

PODOPLANIN (PDPN) IS HIGHLY expressed not only in many cancers but also in normal cells such as lymphatic endothelial cells (LECs) and podocytes.^(1,2) PDPN is also called as “Aggrus” because PDPN possesses a platelet aggregation-inducing activity, which is associated with cancer metastasis.⁽³⁾ Another name is “T1 α ”; its expression is observed in lung type I alveolar cells.⁽⁴⁾ PDPN binds to C-type lectin-like receptor-2 (CLEC-2)^(5,6) and rearranges the actin cytoskeleton in dendritic cells to promote efficient motility along stromal surfaces.⁽⁷⁾ The physical elasticity of lymph nodes is maintained by PDPN of stromal fibroblastic

reticular cells.⁽⁸⁾ The signaling from CLEC-2 to PDPN controls the contractility of fibroblastic reticular cells and lymph node microarchitecture.⁽⁹⁾ PDPN is known as a specific lymphatic endothelial marker,⁽¹⁰⁾ and PDPN–CLEC-2 interaction is critical for the embryonic blood–lymphatic vascular separation.⁽¹¹⁾ The development of ectopic lymphoid follicles is dependent on Th17-expressing PDPN.⁽¹²⁾

The expression of PDPN has been reported in many tumors, including oral cancer, lung cancer, esophageal cancer, malignant brain tumors, mesotheliomas, testicular tumors, and osteosarcoma.^(3,13–25) To study these complicated functions of PDPN, different epitope-possessing anti-PDPN monoclonal antibodies (mAbs) are necessary. Using conventional methods

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of antibody production, almost all anti-PDPN mAbs react with platelet aggregation-stimulating (PLAG) domain of human PDPN.^(18,24,26,27) Rabbit polyclonal antibodies, which were produced by Matsui et al., also recognized the PLAG domain because the PLAG domain was shown to be immunodominant antigenic sites.⁽²⁸⁾ We have further produced many mAbs against mouse, rat, and rabbit PDPNs.^(29–31)

Recently, we have established the CasMab technology to produce cancer-specific mAbs or antiglycopeptide mAbs. Using CasMab technology, we established different epitope-possessing anti-PDPN mAbs such as LpMab-2, LpMab-3, LpMab-7, LpMab-9, and LpMab-10.^(32–38) Interestingly, LpMab-2, LpMab-3, and LpMab-9 detect the site-specific *O*-glycosylation: LpMab-2 against Thr55/Ser56, LpMab-3

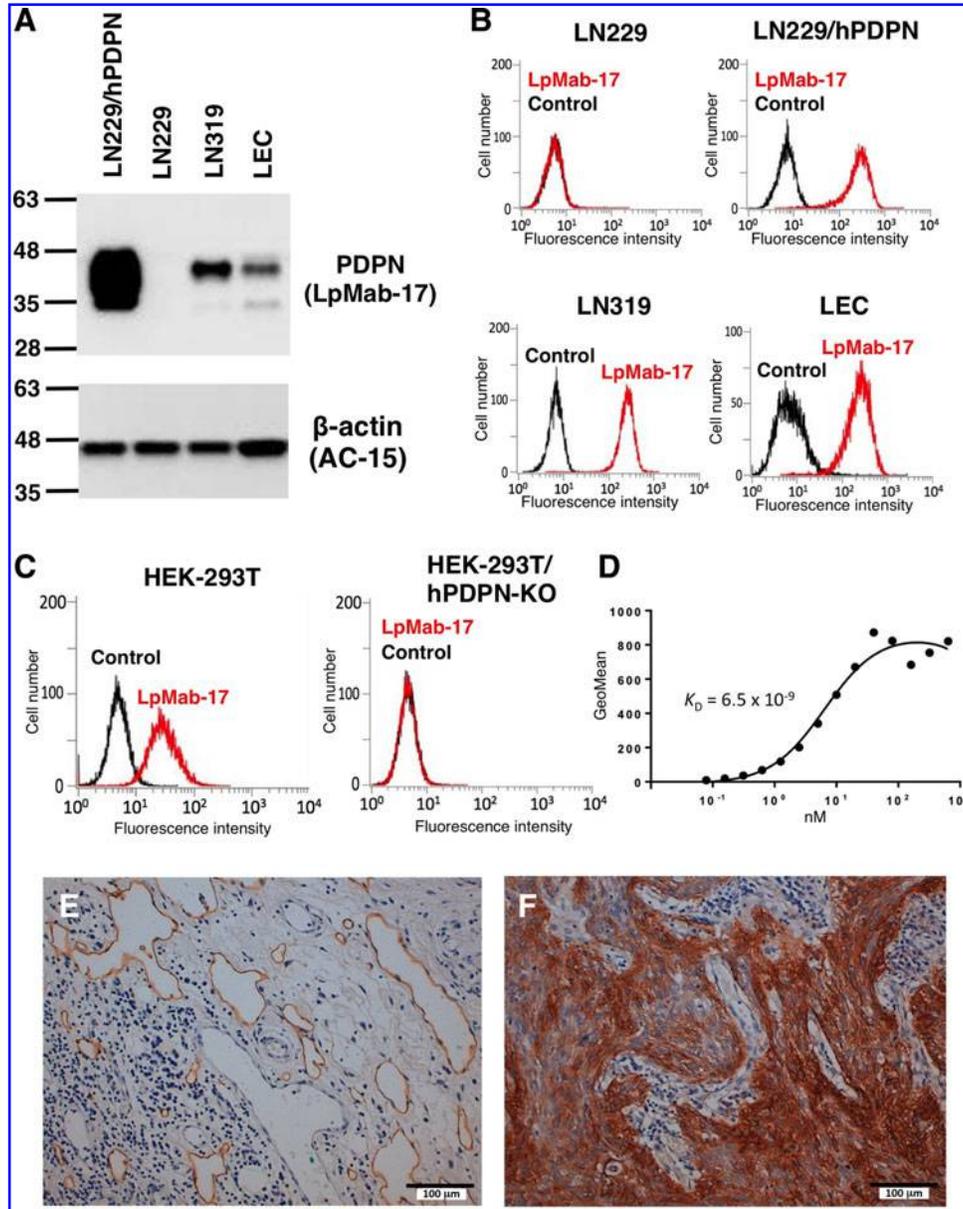


FIG. 1. Characterization of a novel anti-PDPN mAb, LpMab-17. (A) Western-blot analysis by LpMab-17. AC-15 (anti- β -actin) was used as internal control. Total cell lysates were electrophoresed on 5%–20% polyacrylamide gels, and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μ g/mL of LpMab-17 and then with peroxidase-conjugated antimouse IgG; the membrane was detected using a Sayaca-Imager. (B, C) Flow cytometric analysis by LpMab-17. (B) Four cell lines were treated with LpMab-17 (1 μ g/mL; red) or control PBS (black) for 30 minutes at 4°C, followed by treatment with antimouse IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. (C) HEK-293T and HEK-293T/hPDPN-KO cells were treated with LpMab-17 (1 μ g/mL; red) or control PBS (black) for 30 minutes at 4°C, followed by treatment with antimouse IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. (D) Determination of binding affinity of LpMab-17 using flow cytometry. (E, F) Immunohistochemical analysis by LpMab-17 against lymphatic endothelial cells (E) and cancer cells (F) of oral squamous cell carcinomas. Sections were incubated with 1 μ g/mL of LpMab-17, followed by Envision+ kit, and color was developed using DAB and counterstained with hematoxylin. Scale bar: 100 μ m. DAB, 3,3-diaminobenzidine tetrahydrochloride; KO, knock out; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PDPN, podoplanin; PVDF, polyvinylidene fluoride.

against Thr76, and LpMab-9 against Thr25. Here, we developed and characterized a novel anti-PDPN mAb, LpMab-17, which possesses a novel epitope of PDPN.

Materials and Methods

Cell lines, animals, and tissues

LN229, HEK-293T, Chinese hamster ovary (CHO)-K1, glycan-deficient CHO cell lines (Lec1, Lec2, and Lec8), COS-7, and P3U1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Human LECs were purchased from Cambrex (Walkersville, MD). The human glioblastoma cell line, LN319, was donated by Dr. Kazuhiko Mishima (Saitama Medical University, Saitama, Japan) and Dr. Webster K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA). LN229, CHO-K1, Lec1, Lec2, and Lec8 were transfected with human *PDPN* plasmids (LN229/hPDPN, CHO/hPDPN, Lec1/hPDPN, Lec2/hPDPN, and Lec8/hPDPN) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA) according to the manufacturer's instructions.⁽³²⁾ HEK-293T/hPDPN-knock out cells (HEK-293T/hPDPN-KO; PDIS-2) were produced by transfecting CRISPR/Cas plasmids (Target ID: HS0000333287), which target *PDPN* (Sigma-Aldrich Corp., St. Louis, MO), using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc., Philadelphia, PA). CHO-K1, Lec1, Lec2, Lec8, and P3U1 were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), and LN229, LN319, and COS-7 were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. L-Proline (0.04 mg/mL) was added for Lec1, Lec2, and Lec8. LEC was cultured in endothelial cell medium EGM-2MV supplemented with 5% FBS (Cambrex). Female BALB/c mice (4 weeks old) were purchased from CLEA

Japan (Tokyo, Japan). Animals were housed under pathogen-free conditions. The Animal Care and Use Committee of Tohoku University approved the animal experiments described herein. This study examined two oral cancer patients who underwent surgery at Sendai Medical Center. Informed consent for obtaining samples and for subsequent data analysis was obtained from patients or the patient's guardian.

Hybridoma production

BALB/c mice were immunized by intraperitoneal (i.p.) injection of 1×10^8 LN229/hPDPN cells together with Imject Alum (Thermo Fisher Scientific, Inc.). After several additional immunizations, a booster injection was given i.p. 2 days before spleen cells were harvested. The spleen cells were fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The hybridomas were grown in RPMI medium with hypoxanthine, aminopterin, and thymidine selection medium supplement (Thermo Fisher Scientific, Inc.). The culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA) for binding to recombinant human PDPN purified from LN229/hPDPN cells.

Enzyme-linked immunosorbent assay

Seven peptides of human PDPN⁽²⁶⁾ and 20 synthetic peptides, which include a point mutation among 75–94 amino acids of human PDPN,⁽³⁴⁾ were synthesized previously. Peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at 1 µg/mL for 30 minutes. After blocking with 1% bovine serum albumin in 0.05% Tween20/phosphate-buffered saline (PBS; Nacalai Tesque, Inc.), the plates were incubated with culture supernatant or purified mAbs (1 µg/mL) followed by 1:1000 diluted peroxidase-conjugated antimouse IgG or antirat IgG (Dako, Agilent Technologies, Inc., Santa Clara, CA). The enzymatic reaction was conducted with a 1-Step Ultra TMB-

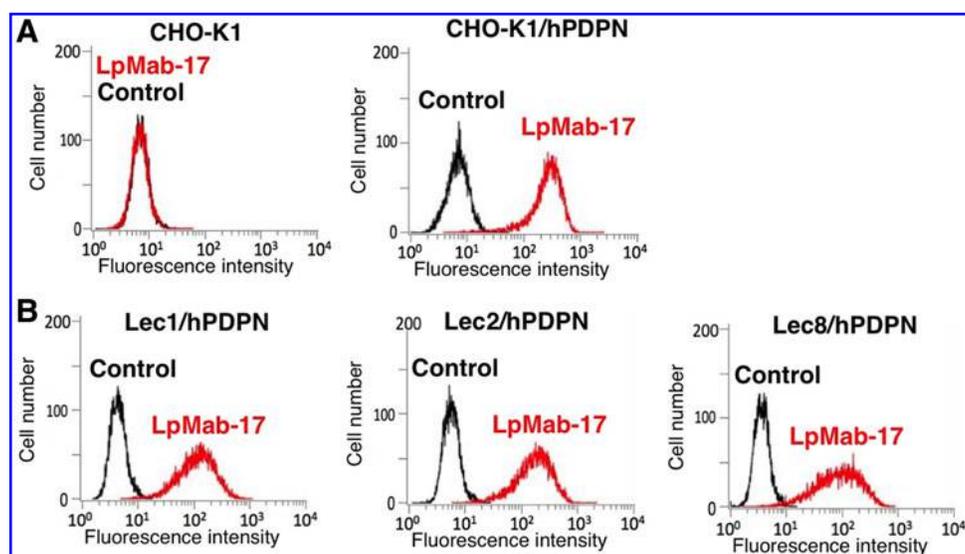


FIG. 2. Flow cytometric analysis by LpMab-17. (A) CHO-K1 and CHO-K1/hPDPN cells were treated with LpMab-17 (1 µg/mL; red) or control PBS (black) for 30 minutes at 4°C, followed by treatment with antimouse IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. (B) Glycan-deficient PDPN-expressing cells (Lec1/hPDPN, Lec2/hPDPN, Lec8/hPDPN) were analyzed in the same way. CHO, Chinese hamster ovary.

ELISA (Thermo Fisher Scientific, Inc.). The optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc.).

Western-blot analyses

Cell lysates (10 µg) were boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.). The proteins were electrophoresed on 5%–20% polyacrylamide gels (Wako Pure Chemical Industries Ltd., Osaka, Japan) and were transferred onto a polyvinylidene fluoride (PVDF) membrane (EMD Millipore Corp., Billerica, MA). After blocking with 4% skimmed milk (Nacalai Tesque, Inc.), the membrane was incubated with LpMab-17 or anti-β-actin (clone AC-15; Sigma-Aldrich Corp.) and then with peroxidase-conjugated antimouse IgG (1:1000 diluted; Dako, Agilent Technologies, Inc.), and developed with the ImmunoStar LD Chemiluminescence Reagent (Wako Pure Chemical Industries Ltd.) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Flow cytometry

Cell lines were harvested by brief exposure to 0.25% Trypsin/1 mM EDTA (Nacalai Tesque, Inc.).⁽⁶⁾ After washing with PBS, the cells were treated with LpMab-17 or LpMab-7 (1 µg/mL) for 30 minutes at 4°C, followed by treatment with Oregon Green 488 goat antimouse IgG (Thermo Fisher Scientific, Inc.). Fluorescence data were collected using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

Determination of the binding affinity using flow cytometry

LN319 (2×10^5 cells) were resuspended with 100 µL of serially diluted LpMab-17 (0.0061–50 µg/mL) followed by secondary antimouse IgG (Thermo Fisher Scientific, Inc.). Fluorescence data were collected using a cell analyzer (EC800; Sony Corp.). 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).

Immunohistochemical analyses

Four-micrometer-thick histologic sections were deparaffinized in xylene and rehydrated. Without antigen retrieval procedure, sections were incubated with 1 µg/mL of LpMab-17 for 1 hour at room temperature followed by treatment with Envision+ kit (Dako, Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dako, Agilent Technologies, Inc.) for 5 minutes, and then the sections were counterstained with hematoxylin (Wako Pure Chemical Industries Ltd.).

Results

Production of a novel anti-PDPN mAb LpMab-17

In this study, we employed the CasMab technology. In brief, we immunized mice with LN229/hPDPN cells to develop novel anti-PDPN mAbs. Using ELISA, the culture supernatants were screened for binding to recombinant human PDPN purified from LN229/hPDPN cells. After the limiting dilution, LpMab-17 (mouse IgG₁, kappa) was es-

TABLE 1. DETERMINATION OF NZ-1 AND LpMAB-17 EPITOPES BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Mutants	LpMab-17	LpMab-7	NZ-1
hpp23–36	–	–	–
hpp29–47	–	–	–
hpp38–51	–	–	+++
hpp49–68	–	–	–
hpp69–88	+++	+++	–
hpp89–108	–	–	–
hpp109–128	–	–	–

+++; OD450 ≥ 2.0; –, OD450 < 0.1; hpp, human podoplanin peptide.

tablished. LpMab-17 reacted with LN229/hPDPN, not with LN229, a PDPN-negative cell in Western-blot analysis (Fig. 1A) and flow cytometry (Fig. 1B). LpMab-17 also recognized endogenous PDPN, which is expressed in a glioblastoma cell line LN319 and a LEC in Western-blot analysis (Fig. 1A) and flow cytometry (Fig. 1B). LN229, which does not express PDPN, was not detected by LpMab-17, indicating that LpMab-17 does not recognize nonrelated antigens (Fig. 1A, B). The LpMab-17 reaction against HEK-293T was lost by knocking out of human PDPN using CRISPR/Cas (Fig. 1C).

We next performed a kinetic analysis of the interaction of LpMab-17 with LN319 using flow cytometry. As shown in Figure 1D, K_D was determined to be 6.5×10^{-9} M, indicating that LpMab-17 possesses high affinity against human PDPN. We further investigated whether LpMab-17 is useful for immunohistochemical analyses. LpMab-17 stained LECs (Fig. 1E) and oral squamous cell carcinoma cells (Fig. 1F) in a membranous/cytoplasmic staining pattern, demonstrating that LpMab-17 is very useful in immunohistochemical analysis.

TABLE 2. DETERMINATION OF LpMAB-17 EPITOPE BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Mutation	Sequence	LpMab-17	LpMab-7
Wild type	VTGIRIEDLPTSESTVHAQE	++	+++
V75A	ATGIRIEDLPTSESTVHAQE	+++	+++
T76A	VAGIRIEDLPTSESTVHAQE	+++	+++
G77A	VTAIRIEDLPTSESTVHAQE	–	+++
I78A	VTGARIEDLPTSESTVHAQE	–	+++
R79A	VTGIAIEDLPTSESTVHAQE	±	–
I80A	VTGIRAEDLPTSESTVHAQE	–	–
E81A	VTGIRIADLPTSESTVHAQE	–	–
D82A	VTGIRIEALPTSESTVHAQE	–	–
L83A	VTGIRIEDAPTSESTVHAQE	+	–
P84A	VTGIRIEDLATSESTVHAQE	+	+++
T85A	VTGIRIEDLPASESTVHAQE	++	+++
S86A	VTGIRIEDLPTAESTVHAQE	+	+++
E87A	VTGIRIEDLPTSASTVHAQE	++	+++
S88A	VTGIRIEDLPTSEATVHAQE	+	+++
T89A	VTGIRIEDLPTSES AVHAQE	+	+++
V90A	VTGIRIEDLPTSESTAHAE	++	+++
H91A	VTGIRIEDLPTSESTVAAQE	+	+++
Q93A	VTGIRIEDLPTSESTVHAAE	++	+++
E94A	VTGIRIEDLPTSESTVHAQA	+	+++

+++; OD450 ≥ 2.0; ++, 1.0 ≤ OD450 < 2.0; +, 0.2 ≤ OD450 < 1.0; ±, 0.1 < OD450 < 0.2; –, OD450 < 0.1.

We also performed flow cytometric analysis using LpMab-17 against several glycan-deficient PDPN transfectants such as Lec1/hPDPN (*N*-glycan deficient), Lec2/hPDPN (sialic acid deficient), and Lec8/hPDPN (*O*-glycan deficient). LpMab-17 reacted with CHO-K1/hPDPN, not with CHO-K1 (Fig. 2A). Furthermore, all transfectants of glycan-deficient PDPN were detected by LpMab-17 (Fig. 2B), showing that the epitope of LpMab-17 is independent of glycan.

Epitope mapping using ELISA

To investigate whether LpMab-17 reacts with synthetic peptides, we performed ELISA using several synthetic pep-

tides of human PDPN (Table 1). A rat anti-PDPN mAb NZ-1 reacted with PLAG2/3 domain that corresponds to 38–51 amino acids of human PDPN (hpp38-51). In contrast, LpMab-17 and LpMab-7 strongly recognized non-PLAG domain that corresponds to 69–88 amino acids of human PDPN (hpp69-88). We further performed ELISA using 20 peptides that corresponds to 75–94 amino acids of human PDPN to determine the LpMab-17 epitope in detail (Table 2). The results showed that LpMab-17 reaction was lost in point mutations of 77–82 amino acids. LpMab-17 did not react with COS-7, whereas LpMab-7 did (Fig. 3A). Sequence comparison between human PDPN and monkey PDPN revealed that the epitope of LpMab-17 is 77-GIRIED₈₂, whereas the

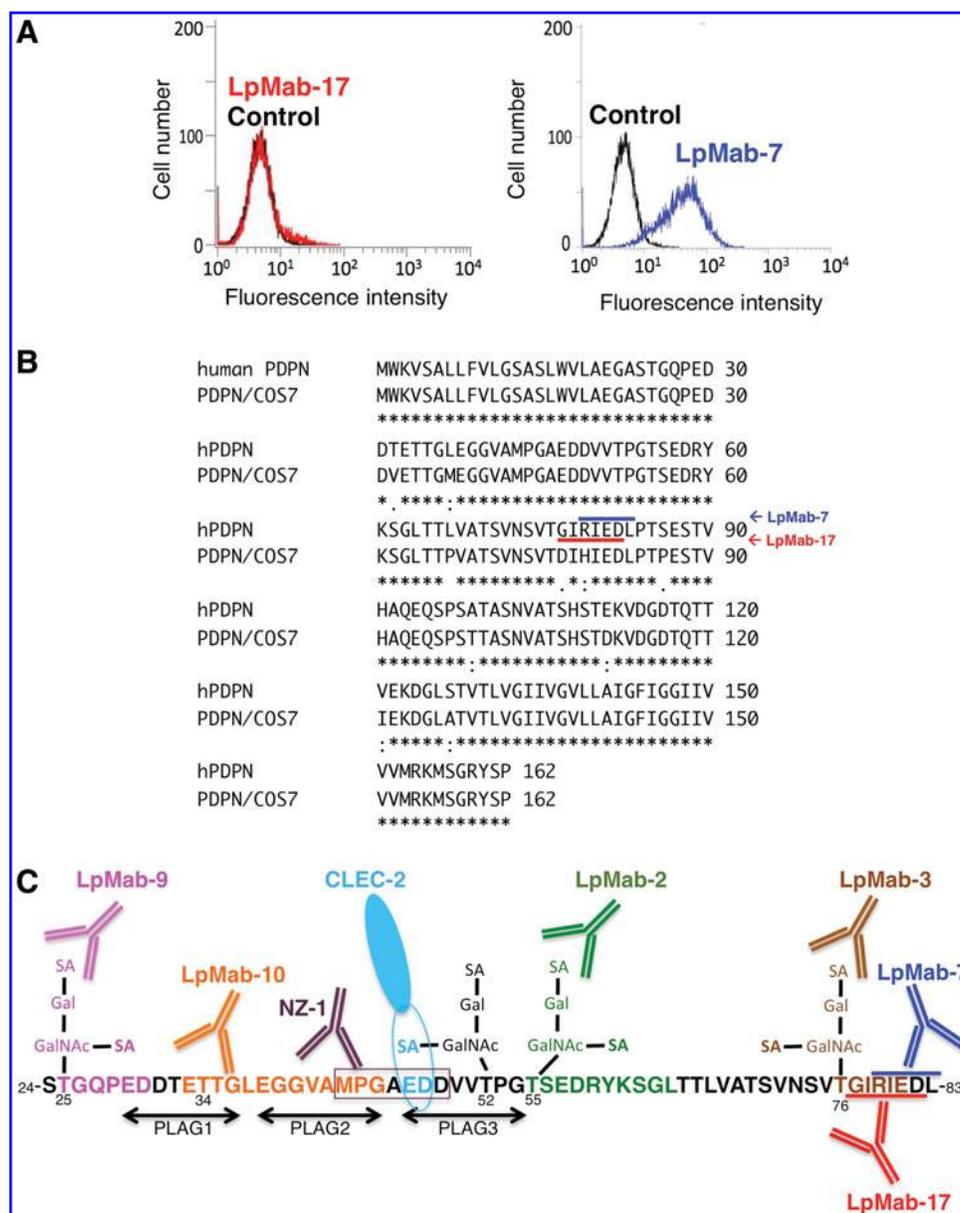


FIG. 3. The epitope difference between LpMab-17 and LpMab-7. **(A)** Flow cytometric analysis. COS-7 cells were treated with LpMab-17 (1 μ g/mL; red), LpMab-7 (1 μ g/mL; blue), or control PBS (black) for 30 minutes at 4°C, followed by treatment with antimouse IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. **(B)** The sequence comparison between human PDPN and monkey PDPN. **(C)** Comparison of epitopes of anti-PDPN mAbs.

corresponding sequence of monkey PDPN is $_{77}$ -DIHIED- $_{82}$; the amino acids consistency is only 67% (Fig. 3B).

Discussion

We recently established the CasMab technology, which can produce cancer-specific mAb, although one protein in cancer cells and normal cells possesses the same amid acid sequence.⁽³²⁾ Using the CasMab technology, we are able to try to develop antibody drugs against many targets, which were excluded from antibody-drug candidates. The post-translational difference such as glycans should be utilized to produce cancer-specific mAbs.

In this study, we used LN229/hPDPN cells, not purified recombinant proteins, for immunization to develop novel anti-PDPN mAbs. LpMab-17 recognized not only exogenous PDPN but also endogenous PDPN, which is expressed in a glioblastoma cell line LN319 and a LEC in Western-blot analysis and flow cytometry. LpMab-17 also detected many other PDPN-expressing cancer cell lines such as glioblastoma, lung squamous cell carcinoma, oral squamous cell carcinoma, and malignant mesothelioma (data not shown).

LpMab-17 is very useful in immunohistochemical analysis. Importantly, LpMab-17 does not need antigen retrieval procedure to detect human PDPN in immunohistochemical analysis, although LpMab-2 and LpMab-3, which were also produced by CasMab technology, need antigen retrieval procedure.^(32,35) Furthermore, LpMab-9 is not useful for immunohistochemistry, whereas it is very sensitive in flow cytometry or Western-blot analysis,⁽³⁶⁾ indicating that the CasMab technology is not always helpful to produce mAbs for immunohistochemical analysis. The antigen retrieval procedure often causes nonspecific staining; therefore, LpMab-17 is advantageous for not only reducing costs and saving time but also showing specific immunohistochemical staining against human PDPN.

Because human and monkey PDPN shows 94% homology, almost all anti-PDPN mAbs react with PDPN of a monkey renal epithelial cell, COS-7. LpMab-17 did not detect mouse, rat, rabbit, dog, and bovine PDPNs (data not shown); therefore, LpMab-17 is an absolutely specific antihuman PDPN mAb. As shown in Figure 3C, LpMab-17 epitope is independent of previously established anti-PDPN mAbs such as LpMab-2, LpMab-3, LpMab-7, LpMab-9, LpMab-10, and NZ-1. The epitope of D2-40, the most prevalent anti-PDPN mAb, is also different from that of LpMab-17.⁽²⁶⁾ The epitopes of LpMab-17 and LpMab-7 are very similar; however, the critical difference has been observed in immunohistochemical analysis against one respiratory disease (data not shown). The combination of different epitope-possessing mAbs could be advantageous for the PDPN-targeting diagnosis or therapy; therefore, we should further characterize the activity of LpMab-17 and compare the difference among anti-PDPN mAbs.

In conclusion, LpMab-17 could be advantageous for investigating the expression and function of PDPN in cancers and normal tissues. Further different epitope-possessing anti-PDPN mAbs should be established as powerful tools for uncovering the function of PDPN in the future.

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Author Disclosure Statement

No competing financial interests exist.

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