Communication

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PMab-301: An Anti-Giraffe Podoplanin Monoclonal Antibody for Immunohistochemistry

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Abstract: Immunohistochemistry staining is an essential method in pathological diagnoses. Podoplanin (PDPN) is a specific maker of alveolar epithelium, lymphatic vessels, and glomeruli. In this study, we established a novel anti-giraffe PDPN (girPDPN) mAb, PMab-301, using the Cell-Based Immunization and Screening (CBIS) method. PMab-301 (mouse IgG_1, kappa) detected girPDPN in various applications, such as flow cytometry, western blot, and immunohistochemistry. PMab-301 specifically stained type-I alveolar cells using formalin-fixed paraffin-embedded giraffe lung tissues. Our findings suggest the potential usefulness of PMab-301 for the pathophysiological analyses of giraffe tissues.

Keywords: podoplanin; PDPN; giraffe; Immunohistochemistry

1. Introduction

Podoplanin (PDPN) is a mucin-type glycoprotein, [1] which plays an important role in the lymphatic system development [2]. PDPN interacts with C-type lectin-like receptor-2 (CLEC-2) which is an endogenous receptor of PDPN [3,4]. The PDPN-CLEC-2 interaction promotes the embryonic blood and lymphatic vessel separation [5]. PDPN is expressed in human cancers and is associated with malignant progression and metastasis via platelet aggregation [6–9]. PDPN is expressed in many cell types, including podocytes in glomerulus [1], pulmonary type I alveolar cells [10], and lymphatic endothelial cells [11]. In contrast, PDPN is not expressed in renal tubule cells, pulmonary type-II alveolar cells, and vascular endothelial cells [1]. Therefore, PDPN is a useful maker to distinguish these cells.

We have developed anti-PDPN mAbs for various species including tiger [12], cat [13], pig [14], horse [15], bovine [16], goat [17], sheep [18], alpaca [19], Tasmanian devil [20], bear [21], whale [22], California sea lion [23], golden hamster [24], and ferret [25] using the Cell-Based Immunization and Screening (CBIS) method [26–30]. The Cell-Based Immunization and Screening (CBIS) method is an effective method to develop mAbs for various applications, such as flow cytometry, western blot, and immunohistochemistry. Here, we established a novel anti-giraffe PDPN (girPDPN) mAb using the CBIS method.

2. Materials and Methods

2.1. Cell lines

P3X63Ag8U.1 (P3U1) and Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection (Manassas, VA). P3U1 and CHO-K1 were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), with 100 units/mL

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of penicillin, 0.25 μg/mL of amphotericin B, 100 μg/mL of streptomycin (Nacalai Tesque, Inc.), and 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc.). girPDPN coding sequences (cds) were obtained from the WGS database (SJXV01006036.1) by BLAST search using bovine PDPN cds as a query. The girPDPN sequence was registered in DNA Data Bank of Japan (DDBJ, accession number: LC780730). The synthesized DNA of girPDPN (Eurofins Genomics KK), in which the original signal sequence ($_{1}$-MWKVPVLFFILGSASFWVLAGA-$_{23}$) was deleted, was subsequently subcloned into a pCAGzeo_ssPA16 or ssMAP16 vector (IL2-signal sequence and PA16 tag or MAP16 tag added to N-terminus). The amino acid sequence of the tag system was as follows: PA16 tag, [31] sixteen amino acids (GLEGGVAMPGAEDDVV); MAP16 tag [32], sixteen amino acids (PGTGDGMVPPGIEDKI). The PA16 tag and the MAP16 tag can be detected by an anti-human PDPN mAb (clone NZ-1) and an anti-mouse PDPN mAb (clone PMab-1), respectively. Using a Neon transfection system (Thermo Fisher Scientific Inc., Waltham, MA), the girPDPN plasmid was transfected into CHO-K1 cells. Using a cell sorter (SH800; Sony Corp., Tokyo, Japan), stable transfectants were established and maintained in the medium, containing 500 μg/mL of Zeocin (InvivoGen, San Diego, CA).

All cells were grown in a humidified incubator at 37°C, in an atmosphere of 5% CO_2 and 95% air.

2.2. Hybridomas

To develop mAbs against giraffe PDPN, we intraperitoneally immunized two BALB/c mice (CLEA Japan,Tokyo, Japan) with CHO/MAP16-girPDPN cells (1×10^8 cells/mouse) plus Imject Alum (Thermo Fisher Scientific, Inc.). The procedure included three additional injections every week (1×10^8 cells/mouse), which were followed by a final booster intraperitoneal injection (1×10^8 cells/mouse), two days before harvesting splenocytes. The harvested splenocytes were subsequently fused with P3U1 cells, using PEG1500 (Roche Diagnostics, Indianapolis, IN). For the hybridoma selection, cells were cultured in the RPMI-1640 medium with 10% FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B, 5 μg/mL of plasmocin, 5% Briclone (NICB, Dublin, Ireland), and hypoxanthine, aminopterin and thymidine (HAT; Thermo Fisher Scientific, Inc.). The supernatants were subsequently screened using flow cytometry using CHO-K1 and CHO/PA16-girPDPN.

2.3. Flow cytometric analysis

CHO/PA16-girPDPN and CHO-K1 cells were harvested after a brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA, Nacalai Tesque, Inc.). The cells were treated with 1 μg/mL NZ-1 or PMab-301 for 30 min at 4°C. The cells were treated with 2 μg/mL of anti-mouse IgG conjugated with Alexa Fluor 488 (Cell Signaling Technology, Inc., Danvers, MA). The fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corporation, Tokyo, Japan).

To determine the dissociation constant (K_D), PMab-301 was serially diluted from 10 μg/mL to 0.61 ng/mL. The geometric mean of fluorescence intensity of CHO/PA16-girPDPN at each concentration was calculated by FlowJo v10.8.1 (Becton, Dickinson & Company, Ashland, OR). The K_D was estimated by fitting saturation binding curves to the built-in; one-site binding models in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA).

2.4. Western blotting

Cell lysates were treated with sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.) at 95°C for 3 min. The proteins (10 μg) were separated on polyacrylamide gels (5%–20%, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and transferred to polyvinylidene difluoride membranes (Merck KGaA). After blocking with 4% skim milk (Nacalai Tesque, Inc.) in PBS-0.05% Tween 20, membranes were incubated with 5 μg/mL of PMab-301, 1 μg/mL of an anti-PA16 tag mAb (NZ-1), or 1 μg/mL of an anti-IDH1 mAb (RcMab-1). IDH1 is an internal control. Then, they were incubated again with peroxidase-conjugated anti-mouse immunoglobulins (for PMab-301; diluted

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1:1000; Agilent Technologies, Inc., Santa Clara, CA) or anti-rat immunoglobulins (for NZ-1 and RcMab-1; diluted 1:10000; Sigma-Aldrich Corporation, St. Louis, MO). Finally, protein bands were detected using ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

2.5. Immunohistochemical analyses

Giraffe lung tissue samples obtained from the laboratory of veterinary pathology, the University of Tokyo. The tissues were fixed in 10% neutral-buffered formalin and processed to make formalin-fixed paraffin-embedded (FFPE) tissue sections. To deparaffinize, rehydrate, and retrieve antigen, the sections were autoclaved in Deparaffinization/Antigen Retrieval Solution pH6 (low pH; Nichirei Biosciences, Tokyo, Japan) 121°C for 20 minutes. Then, sections were blocked using the Super Block T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), incubated with 5 μg/mL PMab-301 for 1 h at room temperature, and treated with the Envision + Kit for mouse (Agilent Technologies, Inc.) for 30min. Finally, Color was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 5 min, and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

3. Results

3.1. Establishment of a novel anti-girPDPN antibody

We immunized mice with girPDPN-overexpressing CHO-K1 (CHO/MAP16-grPDPN) cells (Figure 1A). To produce hybridomas, the splenocytes from these mice were fused with P3U1 cells using polyethylene glycol (Figure 1B). To select anti-girPDPN mAb-producing wells, the reactivities to CHO/PA16-girPDPN cells were observed by flow cytometry (Figure 1C). After limiting dilution, PMab-301 (mouse IgG_1, kappa) was finally established (Figure 1D).

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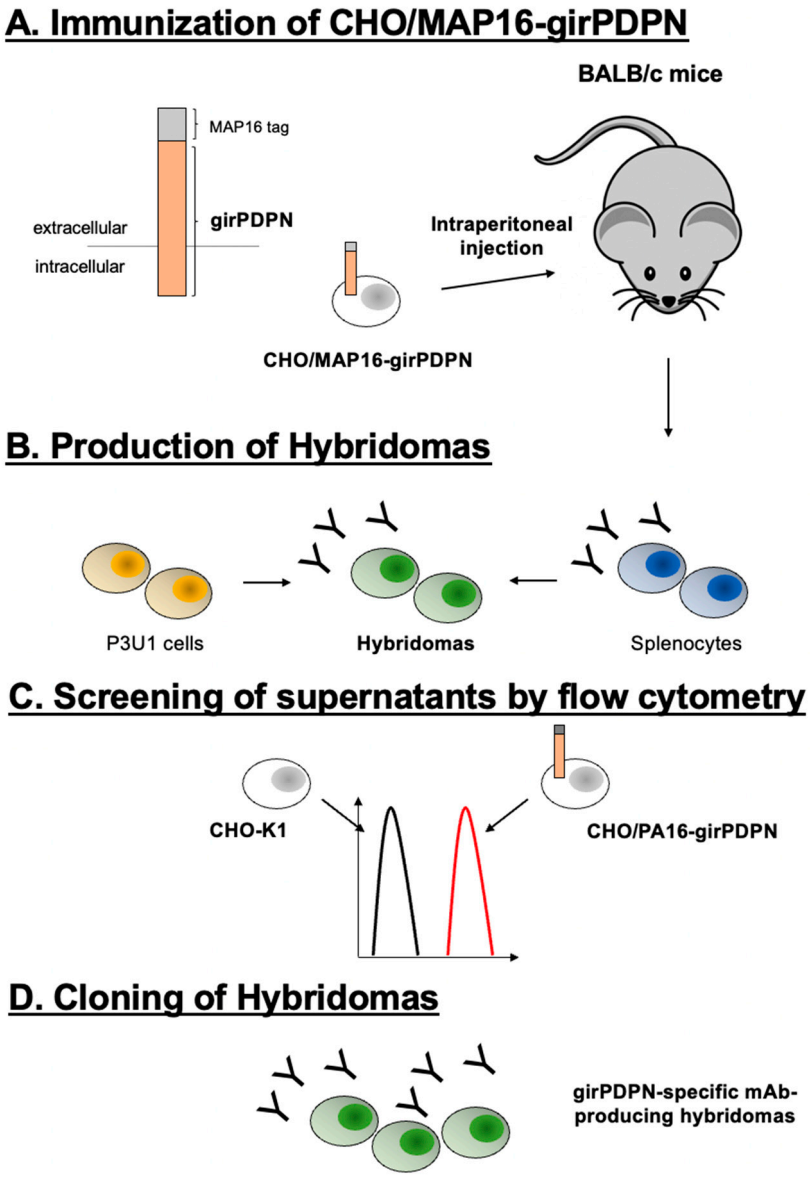

Figure 1. The establishment of PMab-301 using the CBIS method. (A) MAP16-tagged girPDPN-overexpressing CHO-K1 (CHO/MAP16-girPDPN) cells were immunized into two BALB/c mice. (B) The splenocytes were fused with P3U1 cells. (C) The culture supernatants were screened using flow cytometry to select anti-girPDPN mAb-producing hybridomas. (D) PMab-301 was established through limiting dilution and some additional screenings.

3.2. PMab-301 reacted girPDPN-overexpressing CHO-K1 in flow cytometry

We checked the reactivity of PMab-301 to girPDPN by flow cytometry. PMab-301 reacted to CHO/PA16-girPDPN cells in a dose-dependent manner, but did not react with CHO-K1 cells (Figure 2A). To determine the K_D of PMab-301 against CHO/PA16-girPDPN cells, we conducted kinetic analysis by flow cytometry. The geometric mean of the data was plotted versus the concentration of PMab-301. The K_D value of PMab-301 for girPDPN was determined as 1.3×10^{-8} M (Figure 2B).

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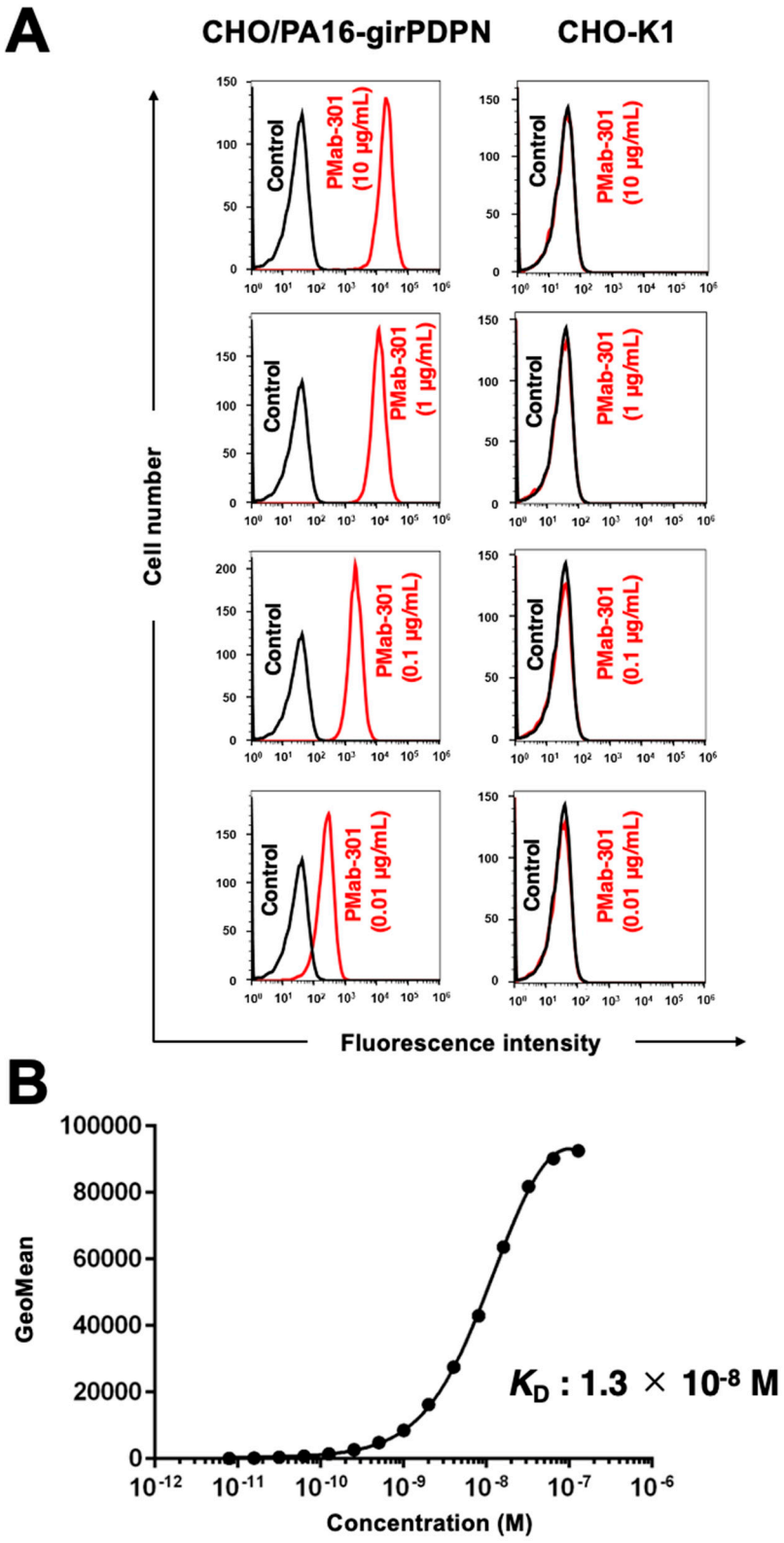

Figure 2. Flow cytometry of girPDPN-overexpressing cells using PMab-301. (A) CHO/PA16-girPDPN and CHO-K1 cells were treated with 0.01–10 μg/mL of PMab-301 (redline) or blocking buffer (negative control, blackline), followed by treatment with AlexaFluor488-conjugated anti-mouse IgG. (B) The binding affinity of PMab-301 was determined against CHO/PA16-girPDPN cells by flow cytometry.

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3.3. girPDPN was detected by western blot by PMab-301

We examined whether PMab-301 is applicable for western blot. Due to glycosylation, PDPN has been detected as about a 48 kDa-band [18,19,33]. As shown in Figure 3, PMab-301 and NZ-1 detected the 48-kDa band of girPDPN in lysates from CHO/PA16-girPDPN cells, whereas this band was not detected in lysates CHO-K1 cells. These data indicate that PMab-301 specifically detects girPDPN in western blot.

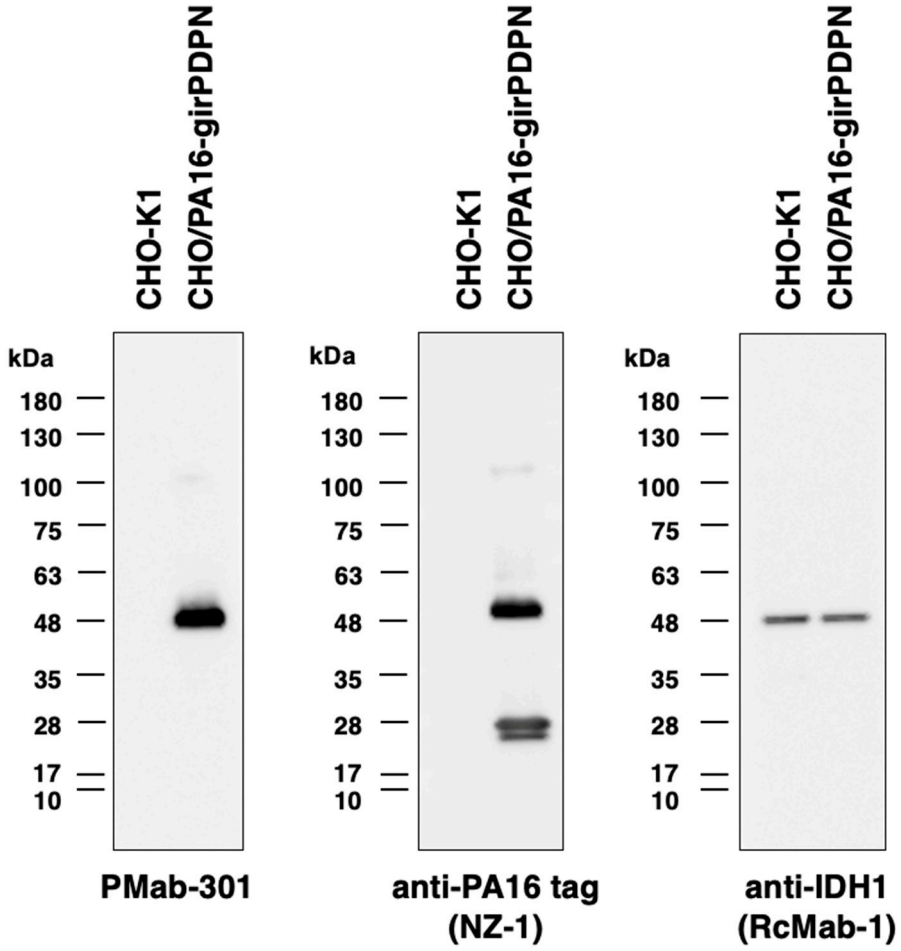

Figure 3. **Western blot with PMab-301**. Cell lysates (10 μg) of CHO/PA16-girPDPN and CHO-K1 cells were electrophoresed and transferred to PVDF membranes. After blocking, membranes were incubated with PMab-301, anti-PA16 tag mAb (NZ-1), or anti-IDH1 mAb (RcMab-1). RcMab-1 was used to detect an internal control.

3.4. PMab-301 recognized girPDPN in immunohistochemistry

Finally, we investigated whether PMab-301 can be used for immunohistochemical analysis using formalin-fixed paraffin-embedded (FFPE) giraffe lung tissue sections because PDPN is known to be expressed on pulmonary type I alveolar cells of various species [10]. As shown in Figure 4A and 4B, PMab-301 strongly stained alveolar epithelial cells, but did not react with vascular endothelial cells, indicating that PMab-301 is useful for detecting girPDPN in immunohistochemistry.

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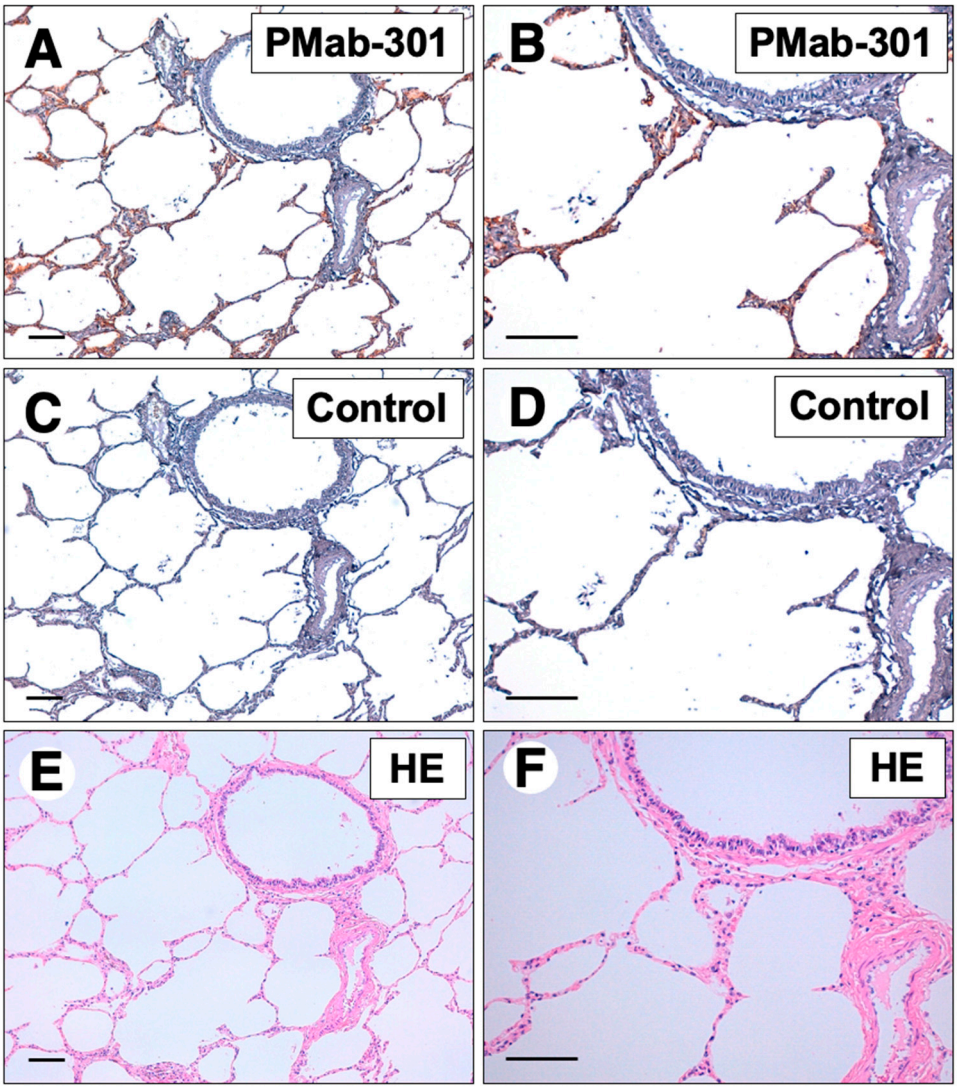

Figure 4. Immunohistochemical staining of giraffe lung with PMab-301. Immunohistochemical analysis for the giraffe lung. The sections of the giraffe lung were incubated with 5 μg/mL of PMab-301 (A, B) or a blocking buffer (C, D), followed by the EnVision+Kit. (E, F) Hematoxylin and eosin (HE) staining was performed. Scale bars = 100 μm.

4. Discussion

We successfully established PMab-301 against girPDPN by the CBIS method and assessed the application of PMab-301. PMab-301 could recognize girPDPN in flow cytometry, western blot, and immunohistochemistry, indicating that PMab-301 can be used in various applications to detect girPDPN.

Several studies have reported that many giraffes died from unknown causes [34–36]. Immunohistochemistry is a commonly used method in pathological examination to find out the cause of death. In this study, PMab-301 could stain alveolar epithelium cells, but did not react with vascular endothelial cells in giraffe lung tissues (Figure 4). These data indicate that PMab-301 could bind to girPDPN specifically in immunohistochemistry of giraffe tissue. Because PDPN is a maker of not only alveolar epithelium but also lymphatic endothelial cells in various animals [16–19], PMab-301 will be a helpful antibody to distinguish lymphatic endothelial cells from vascular endothelial cells in giraffe tissues.

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