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

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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: giraffe, podoplanin, PDPN, PMab-301

Introduction

Podoplanin (PDPN) is a mucin-type glycoprotein,[1] which plays an essential role in the development of the lymphatic system.[2] The interaction of PDPN and C-type lectin-like receptor-2 (CLEC-2), an endogenous receptor of PDPN,[3,4] facilitates the separation of embryonic blood and lymphatic vessels.[5] Human PDPN is expressed in many malignant tumors and is associated with malignant progression and cancer metastasis via platelet aggregation.[6–9] PDPN is expressed in many cell types, including podocytes,[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 cat,[12] tiger,[13] horse,[14] pig,[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 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.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). girPDPN coding sequences (cds) were obtained from the WGS database (SJXV01006036.1) by BLAST search using bovine PDPN cds as a query. The synthesized DNA of girPDPN (Eurofins Genomics KK), in which the original signal sequence ($_1$-MWKVPVLFFILGS ASFWVLAGA-$_{22}$) 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] 16 amino acids (GLEGGVAMPGAEDDVV); MAP16 tag,[32] 16 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.

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The girPDPN plasmid was transfected into CHO-K1 cells, using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were established through cell sorting using a cell sorter (SH800; Sony Corporation, Tokyo, Japan), after which cultivation in a medium, containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA) was conducted.

CHO-K1, girPDPN-overexpressed CHO-K1 (CHO/MAP16-girPDPN and CHO/PA16-girPDPN), and P3U1 cells were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). All cells were grown in a humidified incubator at 37°C, in an atmosphere of 5% CO_2 and 95% air.

Hybridomas

Two 5-week-old BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under specific pathogen-free conditions. All animal experiments were performed according to the relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. The Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001) approved animal experiments. The mice were monitored daily for health during the complete 4-week duration of the experiment. A reduction of more than 25% of the total body weight was defined as a humane endpoint. During the sacrifice, the mice were euthanized through cervical dislocation, after which deaths were verified through respiratory and cardiac arrest.

To develop mAbs against girPDPN, we intraperitoneally immunized two mice 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 was followed by a final booster intraperitoneal injection (1×10^8 cells/mouse), 2 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/PA16-girPDPN and CHO-K1.

Purification of mAbs

The cultured supernatants of PMab-301-producing hybridomas were filtrated with Steritop (0.22 μm; Merck KGaA, Darmstadt, Germany). The filtered supernatants were subsequently applied to 1 mL of Ab-Capcher ExTra (ProteNova, Inc., Kagawa, Japan). After washing with phosphate-buffer saline (PBS), bound antibodies were eluted with an IgG elution buffer (Thermo Fisher Scientific, Inc.), followed by immediate neutralization of eluates, using 1 M Tris-HCl (pH 8.0). Finally, the eluates were concentrated, after which PBS was replaced with the elution buffer using Amicon Ultra (Merck KGaA).

Flow cytometric analyses

CHO-K1 and CHO/PA16-girPDPN cells were harvested after a brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). The cells were subsequently washed with 0.1% bovine serum albumin (BSA) in PBS and treated with 1 μg/mL NZ-1 or PMab-301 for 30 minutes at 4°C. The cells were treated with 2 μg/mL of Alexa Fluor 488-conjugated anti-rat IgG (Cell Signaling Technology, Inc., Danvers, MA) for NZ-1 or Alexa Fluor 488-conjugated anti-mouse IgG (Cell Signaling Technology, Inc.) for PMab-301. The fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corporation).

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).

Western blotting

Cell lysates (10 μg) were boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.), after which proteins were separated on 5%–20% polyacrylamide gels (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 0.05% Tween 20-containing PBS, 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 horseradish peroxidase-conjugated anti-mouse immunoglobulins (for PMab-301; diluted 1:1,000; Agilent Technologies, Inc., Santa Clara, CA) or anti-rat immunoglobulins (for NZ-1 and RcMab-1; diluted 1:10,000; 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).

Immunohistochemical analyses

Giraffe lung tissue samples obtained from routine necropsies performed at the Laboratory of Veterinary Pathology, the University of Tokyo, fixed in 10% neutral-buffered formalin, were 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; No. 415281, 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 hour at room temperature, and treated with the EnVision + Kit for mouse (Agilent Technologies, Inc.) for 30 minutes at room temperature. Finally, Color was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 5 minutes, and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation). Hematoxylin and eosin (H&E) staining was performed using the serial sections.

Results and Discussion

Establishment of anti-girPDPN mAbs

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

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 (Fig. 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 fluorescence intensity 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 (Fig. 2B).

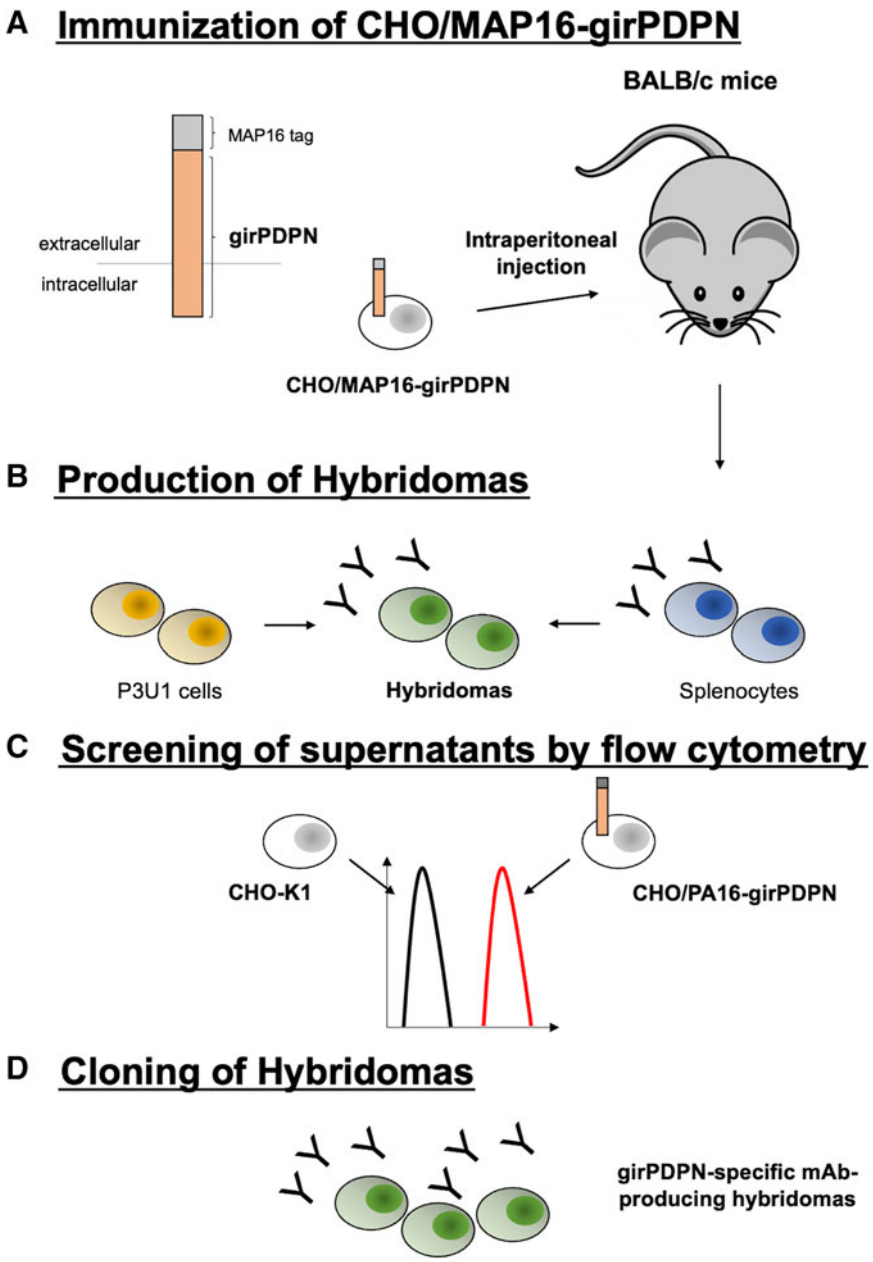

FIG. 1. The establishment of PMab-301 using the CBIS method. **(A)** MAP16-tagged girPDPN-overexpressing CHO-K1 (CHO/MAP16-girPDPN) cells were immunized into two BALB/c mice using an intraperitoneal injection. **(B)** The splenocytes were fused with P3U1 cells. **(C)** The culture supernatants were screened through flow cytometry to select anti-girPDPN mAb-producing hybridomas. **(D)** PMab-301 was established through limiting dilution and some additional screenings. CBIS, Cell-Based Immunization and Screening; CHO, Chinese hamster ovary; girPDPN, giraffe podoplanin.

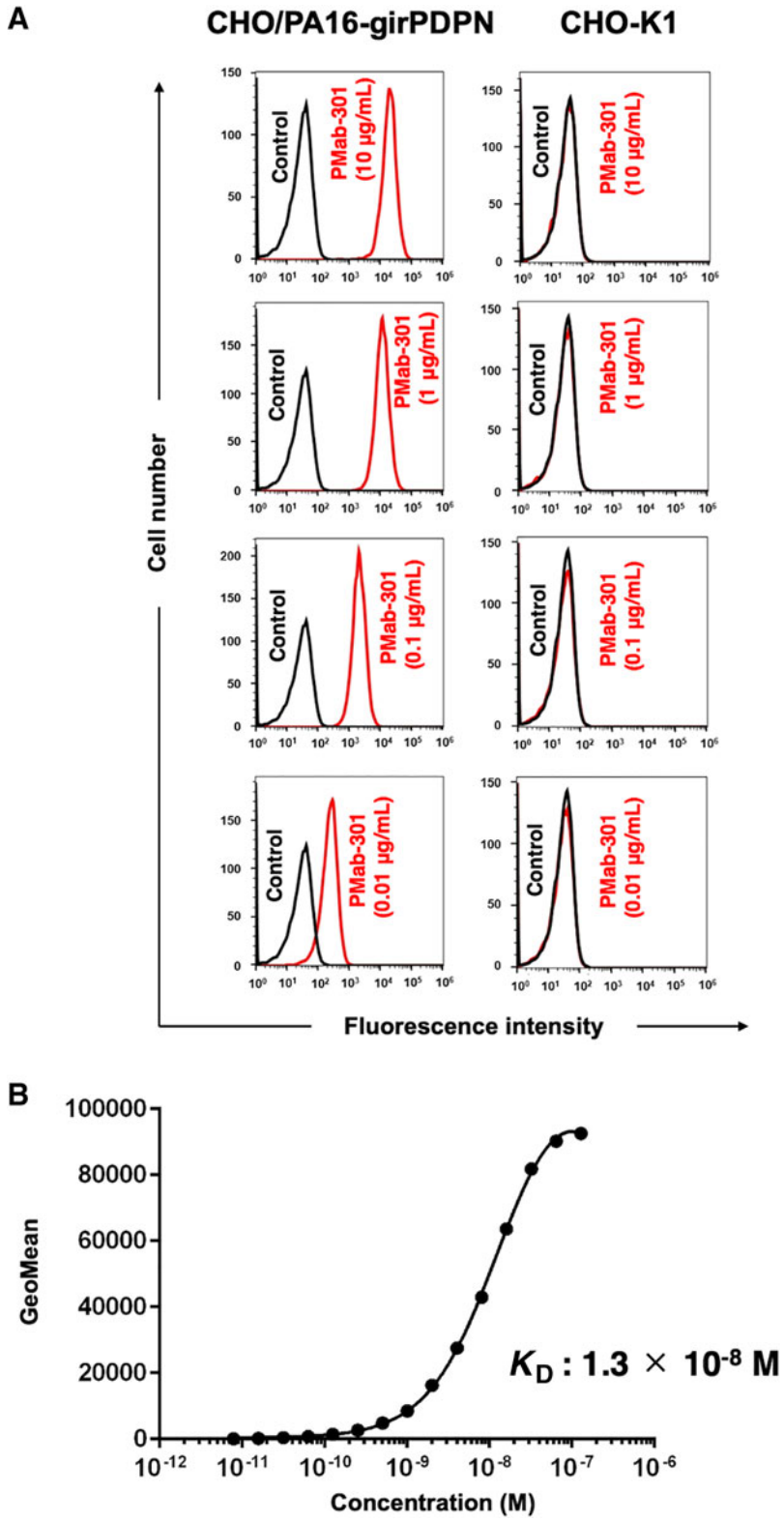

FIG. 2. Flow cytometry of girPDPN-overexpressing cells using PMab-301. **(A)** CHO/PA16-girPDPN and CHO-K1 cells were treated with 0.01–10 μg/mL of PMab-301 (red line) or blocking buffer (negative control, black line), followed by treatment with AlexaFluor488-conjugated anti-mouse IgG. **(B)** The binding affinity of PMab-301 was determined against CHO/PA16-girPDPN cells by flow cytometry. The dots show the geometric mean of fluorescence intensity of CHO/PA16-girPDPN at each concentration.

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 demonstrated 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 absent in lysates of CHO-K1 cells. These data indicate that PMab-301 specifically detects girPDPN in western blot.

PMab-301 recognized girPDPN in immunohistochemistry

Finally, we investigated whether PMab-301 can be used for immunohistochemical analyses using 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.

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 (Fig. 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.

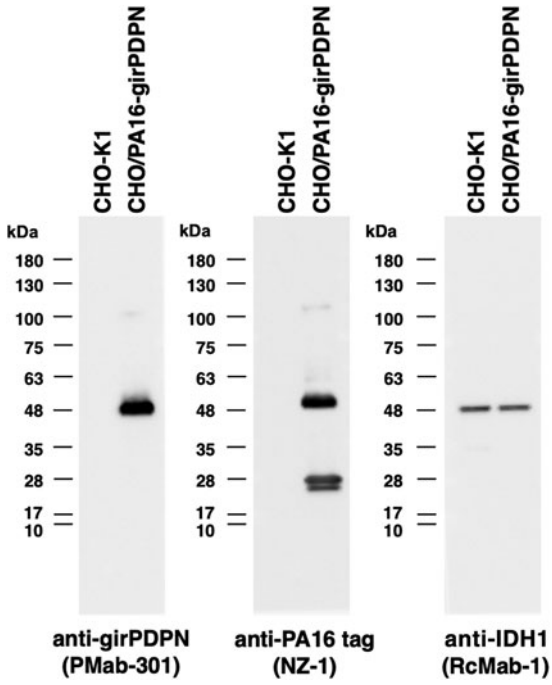

FIG. 3. Western blot with PMab-301. Cell lysates (10 μg) of CHO-K1 and CHO/PA16-girPDPN cells were electrophoresed, after which proteins were transferred to PVDF membranes. After blocking, membranes were subsequently incubated with PMab-301, anti-PA16 tag mAb (NZ-1), or anti-IDH1 mAb (RcMab-1). RcMab-1 was used to detect an internal control. They were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulins (for PMab-301) or anti-rat immunoglobulins (for NZ-1 and RcMab-1).

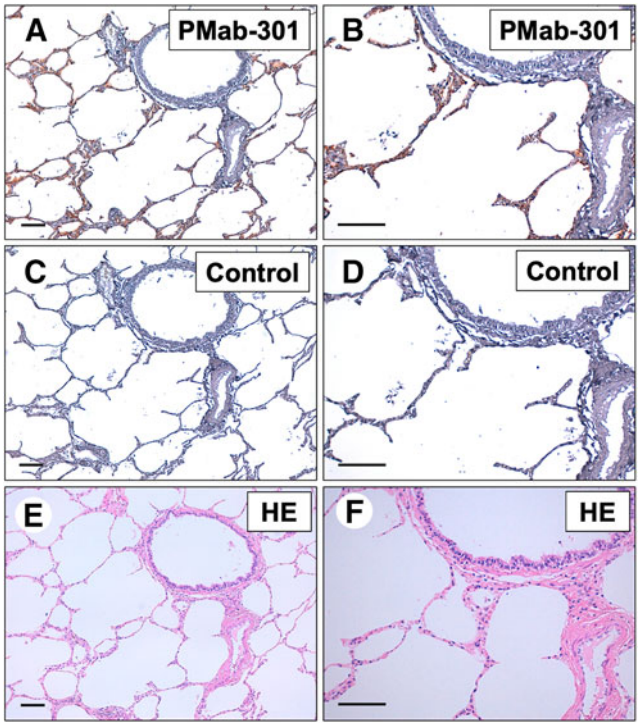

FIG. 4. Immunohistochemical staining of giraffe lung with PMab-301. Histological sections of the giraffe lung were autoclaved in a citrate buffer and incubated with 5 μg/mL of PMab-301 **(A, B)** or a blocking buffer **(C, D)**, followed by the EnVision+Kit. **(E, F)** H&E staining was performed. Scale bars = 100 μm. H&E, hematoxylin and eosin.

Authors' Contributions

T.O., T.T., H.S., G.L., T. Nakamura, M.Y., and S.H. performed the experiments. K.U., T. Nakagawa, M.K.K., and Y.K. designed the experiments. H.S. and M.K.K. analyzed the data. T.O. and Y.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Author Disclosure Statement

The authors have no conflict of interest.

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