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Characterization of monoclonal antibodies against porcine pulmonary alveolar macrophages of gnotobiotic miniature swine☆

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ABSTRACT

There is no sufficient porcine specific antibody available to investigate the ontogeny and development of porcine pulmonary alveolar macrophage (PAM). Therefore, several mouse anti-porcine macrophage mAbs have been produced and characterized in this study. First, the monoclonal antibody PM18-7, an IgG1, kappa isotype, bound to 91% of PAM, 6% of monocytes, and 2% of granulocytes indicating PM18-7 was found to be PAM specific. Monocyte derived macrophages could not be induced to express the PM18-7 antigen by culture. PM18-7 was immunoprecipitated with a molecule of 260 kDa under non-reducing conditions and with that of 130 kDa under reducing conditions in SDS-PAGE. Second, the monoclonal antibody PM3-15, an IgG1, kappa isotype, bound to 92% of PAM, 86% of monocytes, and 3% of granulocytes indicating PM3-15 was mononuclear phagocyte specific. PM3-15 was immunoprecipitated with a molecule of 245 kDa under non-reducing conditions and those of 150, 95 kDa under reducing conditions in SDS-PAGE. Third, the monoclonal antibody PM16-6, an IgM isotype, bound to 82% of PAM, 89% of monocytes, and 82% of granulocytes indicating PM16-6 recognizes those cells of myeloid lineage such as macrophages, monocytes and granulocyte. The antigen immunoprecipitated by PM16-6 was 120 kDa under non-reducing conditions and was 75, 25 kDa under reducing conditions. Finally, the antigen bound to PM18-7 was identified as ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1, CD203a), PM3-15 was figured out as integrin alpha M beta 2 precursor (ITGaM, CD11b) and PM16-6 was identified as Thimet oligopeptidase (THOP-1) by the LC-MS/MS protein sequencing method.

1. Introduction

The swine is an excellent animal model for examination of the ontogeny and function as well as migration [1] of cells in the immune system since large numbers of cells can be obtained from a single animal [2]. Additionally, gnotobiotic miniature swine has immunological similarities to humans. Also, macrophages are one of the most powerful antigen presenting cells from innate to adaptive immunity [3]. Unfortunately, few mAbs reactive with swine lymphocytes, monocytes, and macrophages are available [4,5]. Moreover, a recent study of xenotransplantation has focused on the use of gnotobiotic miniature swine as an organ donor [6] as well as immunological study of the innate immune system for human [7]. However, the knowledge about the porcine innate immune system was not enough understood. Therefore, to find out the innate immune system of porcine, innate immune cells such as macrophages, monocytes, granulocytes, dendritic cells and NK cells need to be first determined [8,9]. Among these cells, macrophages play a key role in innate immune regulation [10]. In addition, the development of hybridoma technology [11–13] has permitted the production of numerous monoclonal antibodies (mAbs) reactive to a variety of cell surface molecules. The mAbs reactive to porcine

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macrophages would be useful tools to investigate the ontogeny and development of the porcine innate immune system and immune regulation. Furthermore, studies on porcine innate immune regulation has been getting easier because mAbs could have the functions as activation or inhibition [14].

The mAbs reactive to porcine macrophage was reported at International Swine CD Workshops for the first time [15]. However, it is not clearly identified at the moment and so far, there is no additional study carried out.

Therefore, we report here the development and characterization of porcine macrophage specific mAbs. These mAbs will be useful tools for future studies of macrophage development and differentiation [16].

2. Materials and methods

2.1. Animals

Minnesota miniature swine at the animal facilities of the University of Health Sciences/The Chicago Medical School were used. At present, the gnotobiotic miniature swine from the Chicago Medical School has been moved to Konkuk University, Seoul, South Korea [17].

For the lung lavage to get pulmonary alveolar macrophage (PAM), the sow sacrificed for producing germfree miniature swine was used. Sacrifice was conducted by CO_2 under the provision of Institutional Animal Care and Use Committees (IACUC) of Konkuk University, Seoul, Korea.

2.2. Preparation of pulmonary alveolar macrophage (PAM)

PAM was obtained by bronchial lavage [18]. Briefly, 2 L of phosphate-buffered saline (PBS) was injected intratracheally and re-collected. The lavage fluid was withdrawn, and centrifuged for 10 min at 1000 × g. The pelleted cells were washed three times in Hank's balanced salt solution (HBSS), resuspended in complete medium (RPMI-1640 medium supplemented with 25 mM Hepes, 2 mM L-Glutamine, 100 U penicillin/ml, 100 ug streptomycin/ml, and 10% heat inactivated FBS) and counted. Over 90~95% purified PAM has been collected by this method.

2.3. Preparation of peripheral blood mononuclear cells (PBMC)

Mononuclear cells from peripheral blood were prepared by the Ficoll–Paque PLUS (GE healthcare, Germany) density gradient centrifugation method of Boyum [19]. Mononuclear cells were resuspended in complete medium and incubated for 1 h at 37 °C and 5% humidified CO2. Peripheral blood lymphocytes (PBL) were decanted with the non-adherent layer containing monocytes, and were placed on ice for 5 min. The monocytes were then gently scraped from the plate with a rubber policeman and resuspended in complete media.

2.4. Preparation of polymorph nuclear cells (PMN)

Granulocytes were recovered by dextran sedimentation. Briefly, 20 ml of heparinized whole blood was mixed with 4 ml of dextran (2% Dextran 500 and 4% Dextran 70, Pharmacia), and then the mixture was allowed to stand at room temperature for 1 h. The upper layer containing the plasma-dextran-leukocyte mixture was removed and washed twice with balanced salt solution (BSS). Contaminating red blood cells were lysed by red blood cell lysis buffer (Sigma™, St. Louis, MO).

2.5. Preparation of single cell suspensions

Single cell suspensions were obtained from the liver, mesenteric lymph nodes, spleen, and thymus, all of which were removed aseptically from gnotobiotic miniature swine.

Cells were teased from the, tissue and placed to allow debris allowed to settle. Cells in the supernatant fluid were withdrawn, centrifuged, and washed 3 times in HBSS, then resuspended in complete medium. Bone marrow cells were obtained by removing the ribs or femurs from swine. The bones were flushed using a syringe with 30 ml of BSS and 18 gauge needles. Single cell suspensions were washed 3 times in HBSS and resuspended in complete medium.

2.6. Monoclonal antibodies

Spleen cells from mice immunized with PAM were fused with P3X63-Ag8-653 murine myeloma cells [11]. Hybridomas producing anti-macrophage antibodies were assayed for reactivity to PAM by ELISA. Hybridomas producing anti-macrophage antibodies were cloned several times in limiting dilution by microscopic observation to assure a single clone. The selected clones were expanded for culture supernatant and for ascites fluid production in pristane-primed balb/c mice.

2.7. Flow cytometric analysis and fluorescent microscopy

Macrophages, monocytes, or granulocytes resuspended in 100 ul of complete media at 4×10^6 cells/ml were incubated with 100 ul of monoclonal antibody (mAb) supernatant for 30 min at 4 °C. The cells were then washed twice with PBS-BSA-Azide and incubated for an additional 30 min at 4 °C with 50 ul of a 1:1000 diluted FITC conjugated goat anti-mouse IgG (Tago, Inc., Burlingame, CA, R&D systems, USA, and Santacruz Korea, Seoul). The cells were washed two more times. 2nd controls consisted of cells incubated with FITC conjugated goat anti-mouse IgG (H + L) only. The mAbs 76-7-4 (mAb to porcine anti-B cell) and 74-22-15 (porcine anti-phagocytes) were purchased from American Type Culture Collection (ATCC) and were used as negative and positive controls in some experiments. HB-140 (anti-porcine CD1a), HB-142 (anti-porcine macrophages, granulocytes), HB-143 (anti-porcine $CD4^+$ T cell), and HB-147 (anti-porcine cytotoxic T cell) were purchased from ATCC and were used as comparison of reactivity in some experiments. The analysis was based on 5000 cells using the MDADS program (Coulter). The flow cytometric analysis and calculation of mean of flouresence intensity (MFI) were conducted by WinMDI (version 2.09).

2.8. Immunoprecipitation and SDS-PAGE

Macrophages washed 3 times with cold PBS were lysed with 1 ml of 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, and 0.5% NP-40, at pH 7.95, resuspended and placed on ice for 30 min, and then centrifuged for 15 min, at 4 °C, at 12000 × g. The cell lysate was precleared by adding 20 ul of mAb MOPC ascites (ATCC) and 20 ul of Protein A & G (sc-2003, Santacruz) and was rotated overnight at 4 °C. 100 ul of the precleared lysate was rotated with 100 ul of mAb culture supernatant for 1 h at room temperature. 20 ul of Protein A & G agarose bead was added and the mixture was rotated for an additional 1 h. The products of immunoprecipitation were washed 5 times, and then 30 ul of sample buffer containing 2% SDS was added. For reducing conditions, the sample buffer also contained 5% 2-mercaptoehanol. The samples were analyzed SDS-PAGE under non-reducing and reducing conditions using a 10%~4% acrylamide gel. Prestained 10~245 kDa standards

(PM007-0500, Genedirx™) were used. Photographs of acrylamide gels, stained with coommassie blue solution, were taken by the DIA program of LAS-3000 machine (Fujifilm™, Boston, MA.).

2.9. Protein sequencing by MALDI-TOF

The bands of proteins detected by immunoprecipitation and SDS-PAGE were collected with staying in the 10% of acrylamide gel. Then, bounded peptides were eluted from acrylamide gel with 100 ul of 50% ACN, 20 ul of supernatant of each sample were mixed with 40 ul of alpha-cyano-4-hydroxycinnamic acid matrix solution (CHCA 6.2 mg/ml, MeOH:ACH:H_2O = 50:40:10) and 2 ul of this mixture were added on a GroundSteel MTP 384 target plate (Bruker Daltonics). All procedures were automated and carried out using a robot (ClinProt Robot, Bruker Daltonics). 2 ul of the eluted sample not used for MALDI-TOF analysis were collected and stored at −80 °C for liquid chromatography-electrospray ionization (LC-ESI) MS/MS characterization of protein/peptides peaks of interest. Mass spectra in a mass range from 1000 to 10,000 Da were acquired using a Reflex IV MALDI-TOF MS instrument (Bruker Daltonics) in positive linear mode as previously reported.

3. Results

3.1. Immunoglobulin class determination

The mAbs to PAM were produced and immunoglobulin heavy and light chain types of the three mAbs were determined. All mAbs had kappa light chains. The mAb PM16-6 contained a mu heavy chain and therefore was an IgM molecule. PM3-15 and PM18-7 mAbs had gamma 1 heavy chains, and thus, they were of the IgG1 subclass (Table 1).

3.2. Complement dependent cytotoxicity

The ability of PM3-15, PM16-6 and PM18-7 to fix complement was determined by using a complement dependent cytotoxicity assay. Over Ninety percents of PAM lysed by complement and PM16-6, consistent with its IgM isotype, and with the known ability of the IgM isotype to efficiently bind complements. In contrast, when PAM was reacted with PM3-15 or PM18-7, the percentage of lysis was much lower, ranging from 23 to 27% (Table 2). It has been shown that antibodies of the IgG1 isotype have little or no ability to fix complement. Therefore, the complement fixing ability of these other mAb is consistent with their IgG1 subclass.

3.3. Tissue distribution of Epitopes Recognized by mAbs

An ELISA assay was used to determine the reactivity of each anti-macrophage mAb with single cell suspensions from different tissues. Based on these results, three mAbs were selected for further characterization (Table 3). Thus, PM3-15, PM16-6 and PM18-7 displayed strong (++++) binding to immunizing PAM and moderate (++) binding to adherent newborn bone marrow cells and adherent newborn liver cells, but did not bind to lymphocytes, red blood cells, thymocytes, or mouse J774 cells. In addition, PM3-15 and PM16-6 showed a positive reaction with monocytes. The mAb PM3-15 also demonstrated a reactivity pattern with lymph node macrophages, which was not seen with PM16-6 or PM18-7.

Table 1
Isotyping of Anti-PAM MAbs.

Monoclonal antibody	Source	Isotype
PM18-7	SUPERNATANT	IgG1, K
PM3-15	SUPERNATANT	IgG1, K
PM16-6	SUPERNATANT	IgM, K

Isotype was determined by ouchterlony double diffusion. The source was the hybridoma culture supernatant at the normal conditions.

Table 2
Ability of mAbs to fix complement in a complement dependent cytotoxicity assay.

mAb	Cytotoxicity (%)
18-7H7D2	27.9 ± 5.4
3-15A2A8	23.5 ± 7.8
16-6E8A6	88.0 ± 2.9

50 ul of macrophage at 2 × 10^6 cells/ml were incubated with 100 ul of mAb culture supernatant for 30 min before addition of complement. After 1 h, the incubation cells were examined for viability.

Table 3
Tissue distributions of epitopes recognized by mAbs.

mAb (clone)	PAM	PBMO	PBL	Spleen	MLN	Newborn B.M	Newborn LIVER	THY.	Mouse (J774)
PM18-7 (H7D2)	++++	−	−	−	−	++	++	−	−
PM3-15 (A2A8)	++++	+++	−	−	+++	++	++	−	−
PM16-6 (E8A6)	++++	++	−	−	−	++	++	−	−

Antibody reactivity determined by optical density in the ELISA assay is indicated using an arbitrary scale from (−) negative to (++++) strongly positive. Data is the mean obtained from 5 experiments.

3.4. Reaction of mouse anti-porcine macrophage mAbs with human and mouse macrophage lineage cells

The mAbs reactive with human or mouse cells are generally not reactive with swine cells. It was not known whether mAbs reactive with swine cells would react with human or mouse cells. Therefore, cross-reactivity was assayed. Human primary cultured monocytes and human (U937) and mouse (WEHI-164) cell lines were reacted with each mAb and then were examined for the percentage of fluorescent cells. PM3-15, PM16-6, and PM18-7 did not bind to any cells tested (Fig. 1), it means that the mAbs were specific to swine macrophages/monocytes.

3.5. Distribution of PM3-15, PM16-6 and PM18-7 antigens on porcine phagocytes

The reaction of PM18-7, PM3-15 and PM16-6 with PAM, Peripheral blood monocyte (PBMO), and granulocytes was analyzed by flow cytometry. Each mAbs showed a different pattern of binding to these 3 kinds of phagocytes (Fig. 2).

The mAb PM18-7 was 91% bound to PAM, yet showed little or no binding to PBMO or granulocytes. The expression of the antigen recognized by PM18-7 on PAM showed a bright fluorescence intensity, suggesting that the majority of PAM expressed this molecule on the cell surface at a high density. Thus, it can be affirmed that PM18-7 recognizes a PAM specific antigen. PM3-15 was 92% bound to PAM, 86% to PBMO, but did not bind to granulocytes (3.5%). PM3-15 showed bright fluorescence intensity on PAM and had a moderate to bright fluorescence intensity on PBMO. Thus, a high density of antigens reactive to PM3-15 was absent on the cell surface of most PAM and less abundantly present on the cell

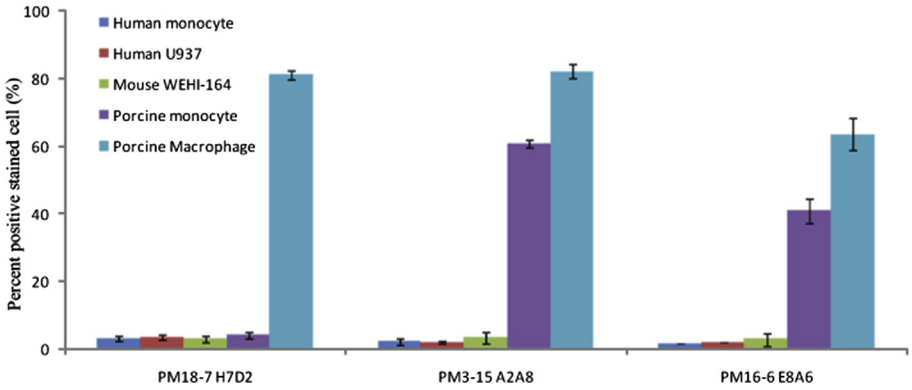

Fig. 1. Reactions of mAbs with human or mouse monocyte/macrophage cell lines. Porcine monocyte was obtained from attached population of PBMC. Cells were examined by FACS Calibur machine (BD™, New Jersey, USA).

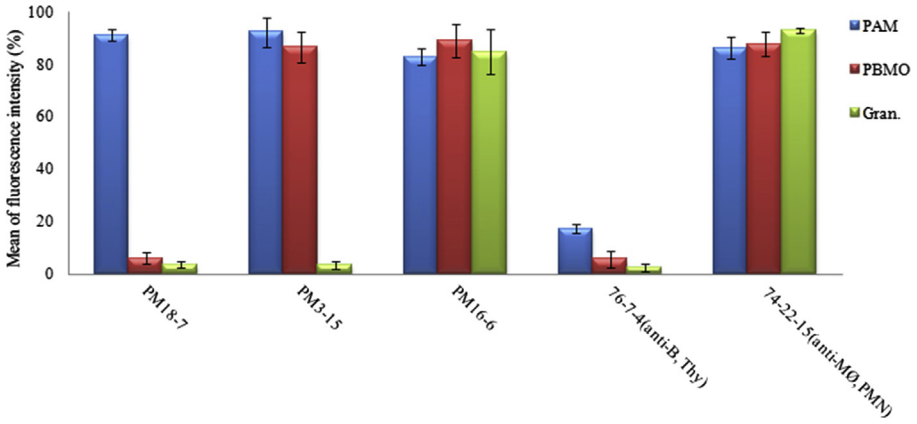

Fig. 2. Flow cytometric analysis was conducted by each mAb to each myeloid lineage cell, macrophage (PAM), monocyte (PBMO) and granulocyte (PMN). 76-7-4 mAb was used as negative control and mAb 74-22-15 was used as positive control. The y-axis indicated that percentage of stained cell by mAbs comparable to negative control (only 2nd antibody stained control).

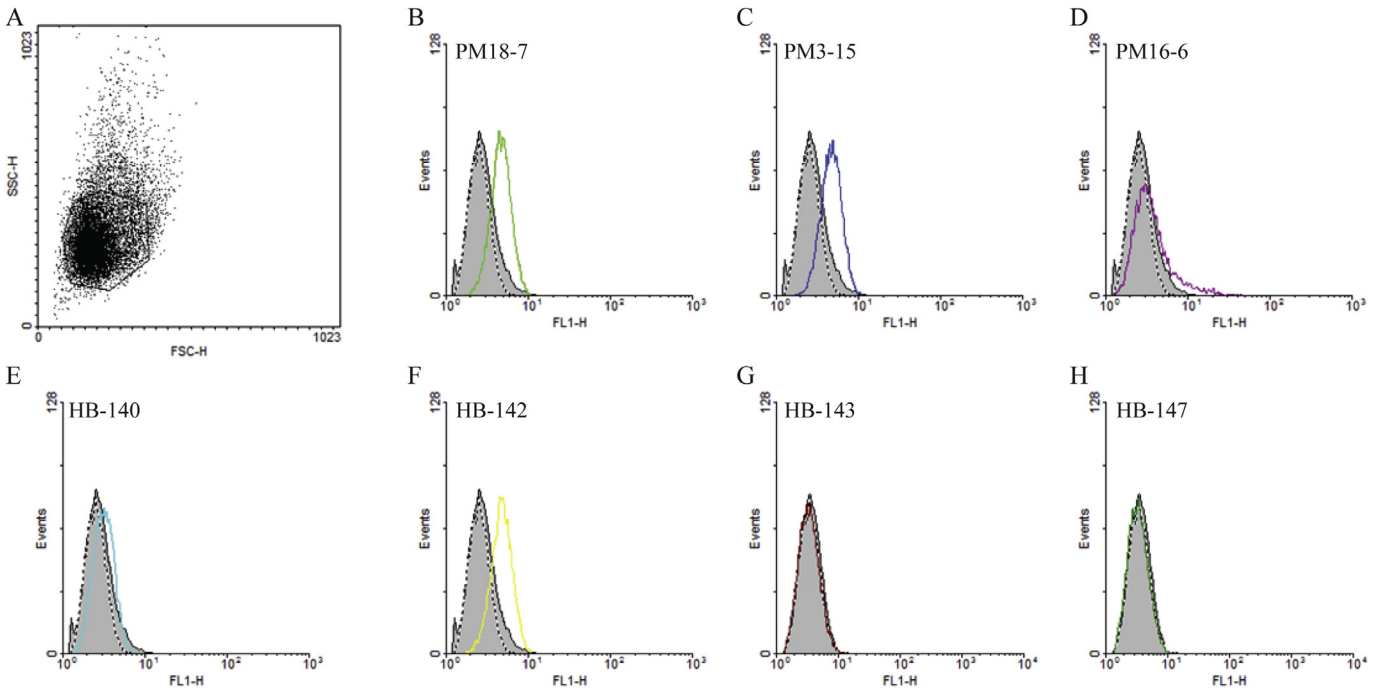

Fig. 3. The mAbs were compared with comparisons by FACS analysis. FACS plots (A) gated on live cluster represents PAM and mAbs, PM18-7 (B), PM3-15 (C), PM16-6 (D) were compared with being commercially sold mAbs, anti-porcine CD1a (E), anti-porcine macrophages and granulocytes (F), anti-porcine cytotoxic T lymphocyte (G), anti-porcine CD4+ T lymphocyte (H).

surface of PBMO. The antigen recognized by PM3-15 was absent on the cell surface of granulocytes. Thus, the results show that PM3-15 recognizes mononuclear phagocyte specific antigens. PM16-6 was 82% bound to PAM, 89% to PBMO, and 84% to granulocytes. The fluorescence intensity binding pattern of PM16-6 was moderate to bright on PAM and dull to moderate on PBMO and granulocytes.

These results indicate that most PAM, PBMO, and granulocytes had an antigen recognized by PM16-6 present on their cell surface, but at different densities. The antigen was present at a higher density on PAM than on PBMO or granulocytes. Thus, it can be inferred that PM16-6 recognizes phagocyte specific antigens.

3.6. The comparisons of each mAbs to commercial antibodies to PAM

To figure out the affinity of each mAbs to porcine PAM, the commercial mAbs used for comparison group, such as HB-140 (anti-porcine CD1a), HB-142 (anti-porcine macrophages, granulocytes), HB-143 (anti-porcine CD4+ T cells), and HB-147 (anti-porcine cytotoxic T cells). Moreover, all these mAbs were used as the positive controls of hybridoma culture supernatant. At the results, mAb, PM18-7 and PM3-15 bound to porcine PAM as strong as HB-142 mAb known as anti-porcine macrophages and granulocytes. However, the mAb PM16-6 shows us respectively positive tendency to the PAM but weak due to its mu heavy chain (Fig. 3).

3.7. The recognition of porcine PBMC population by each mAbs

The ability of cell type separation was determined by each mAbs against to porcine periphery blood mononuclear cell (PBMC). As known well, PBMC had various cell types such as lymphocytes included B and T cell, myeloid cell contained monocyte/macrophage, granulocyte and NK cells. According to the result (Fig. 4), mAb PM18-7 recognized over 14% of PBMC, might be monocyte-macrophage population. The mAb PM3-15 recognized over 13% of PBMC, might be monocyte or same population of those PM18-7 and mAb PM16-6 recognize over 10% of PBMC, thought to be granulocytes. In this figure, the affinity of each mAbs comparison to commercial antibodies was confirmed by FACS analysis, and it can be used for immune assay without efforts.

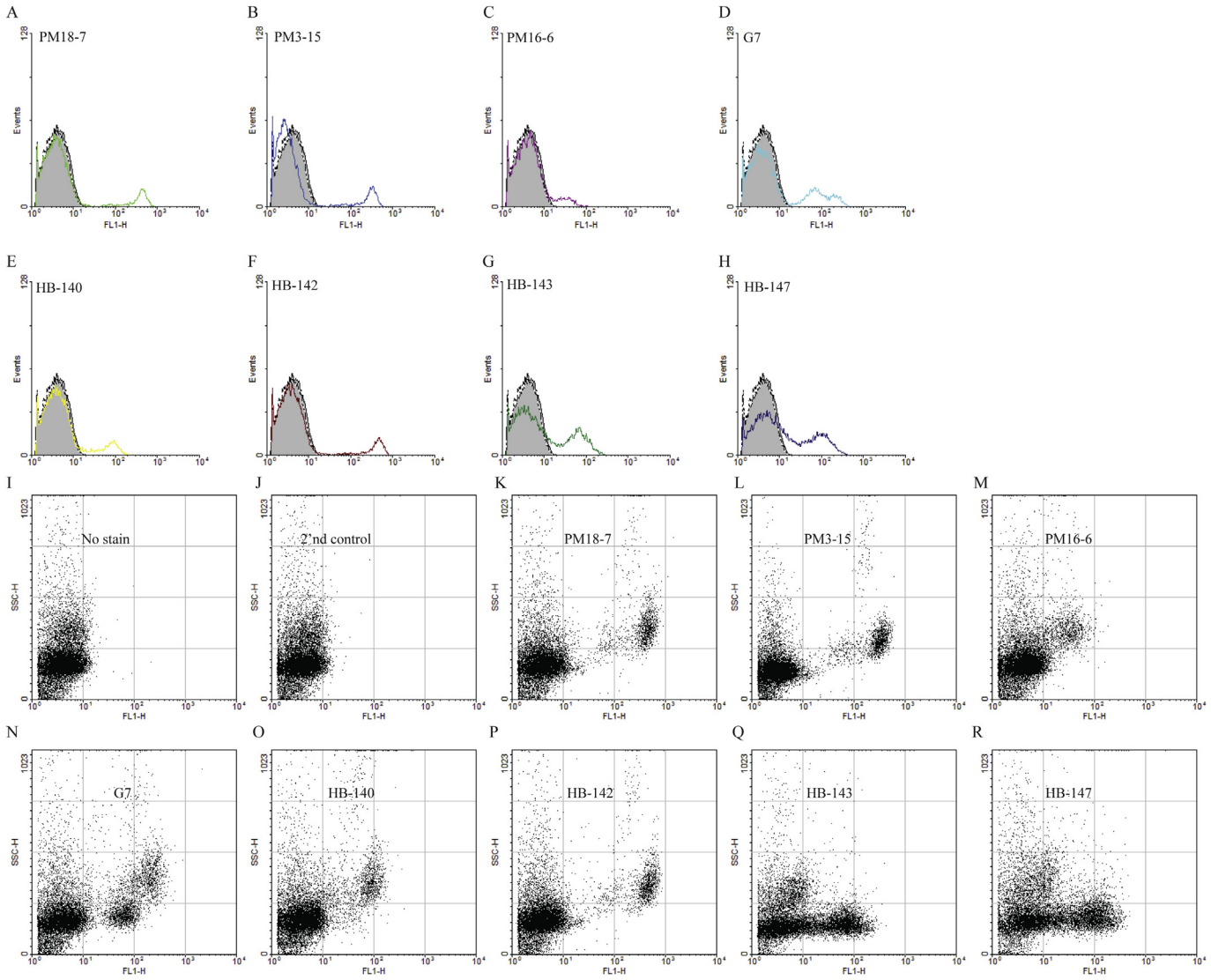

Fig. 4. The reactivity of mAbs to porcine PBMC was confirmed by FACS analysis. Each mAbs were recognized each population in the PBMC (A ~ H). Dot plots were indicated antibodies and SSC as X, Y axis (I ~ R).

3.8. Identification of antigens localization bound to each mAb to PAM

The mAbs recognize distinct antigens on the myeloid lineage cells. In the present study localization of antigen was identified by immunocytochemistry methods. Antigens bound to PM18-7, PM3-15 were observed on the surface of PAM and 3d4/2, antigens for PM16-6 were observed both on the surface and in the cytoplasm (Fig. 5). These data indicated that these fabricated mAbs in this study could be used for application of immunocytochemistry and for the ontogeny of myeloid lineage differentiation.

3.9. Apparent molecular weight of antigens recognized by mAb

Whole cell lysate of PAM were precleared with MOPC supernatant and protein L agarose beads. Then, Immunoprecipitation with each respective mAbs followed by SDS-PAGE was performed to determine the molecular weight and amino acid sequences of the cell surface antigens recognized by PM18-7, PM3-15, and PM16-6.

According to the result of immunoprecipitation and MALDI-TOF, PM18-7 which precipitated one band with a molecular weight of 260 kDa under non-reducing conditions and that of 130 kDa under reducing conditions was observed (Fig. 6A). PM3-15 precipitated a

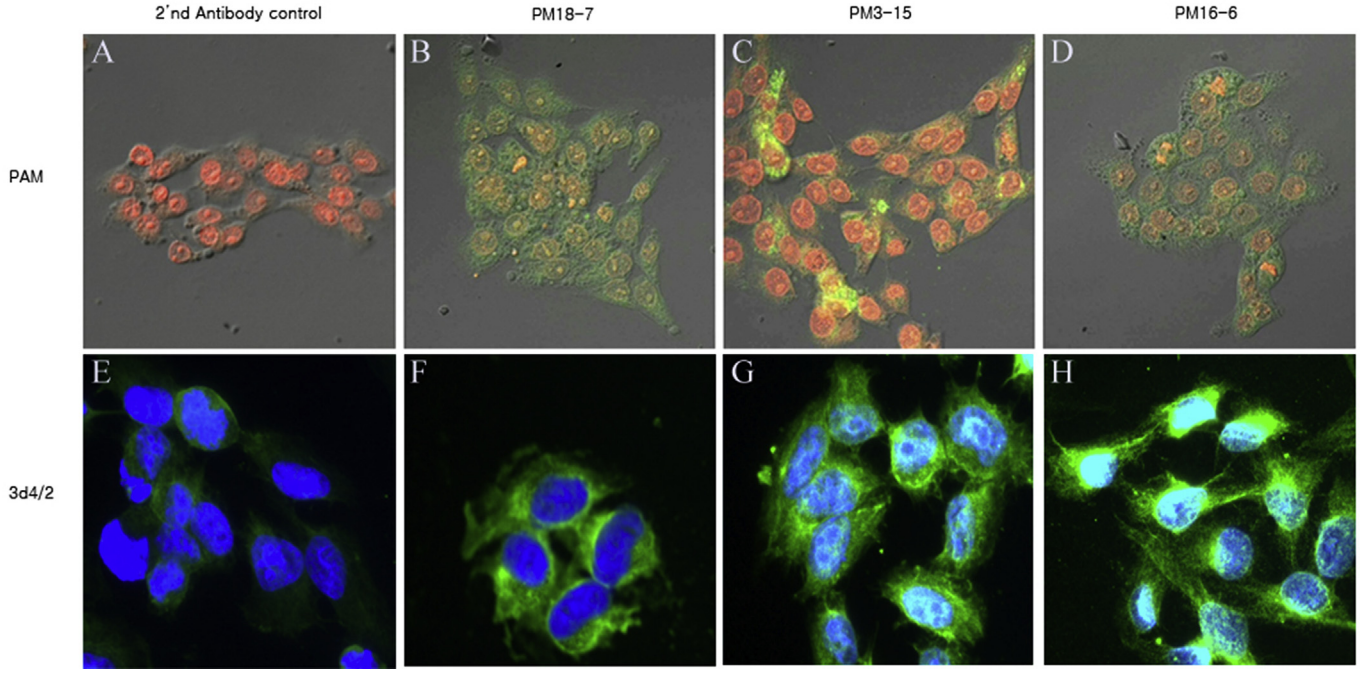

Fig. 5. Immunocytochemistry was conducted by each mAb to PAM and 3d4/2 (PAM cell line). A, E; only secondary antibody (goat anti-mouse Ig (H + L)) was treated to PAM and 3d4/2, B, F; PM18-7 was used as primary antibody, C, G; PM3-15 was used as primary antibody, D, H; PM16-6 was used as primary antibody.

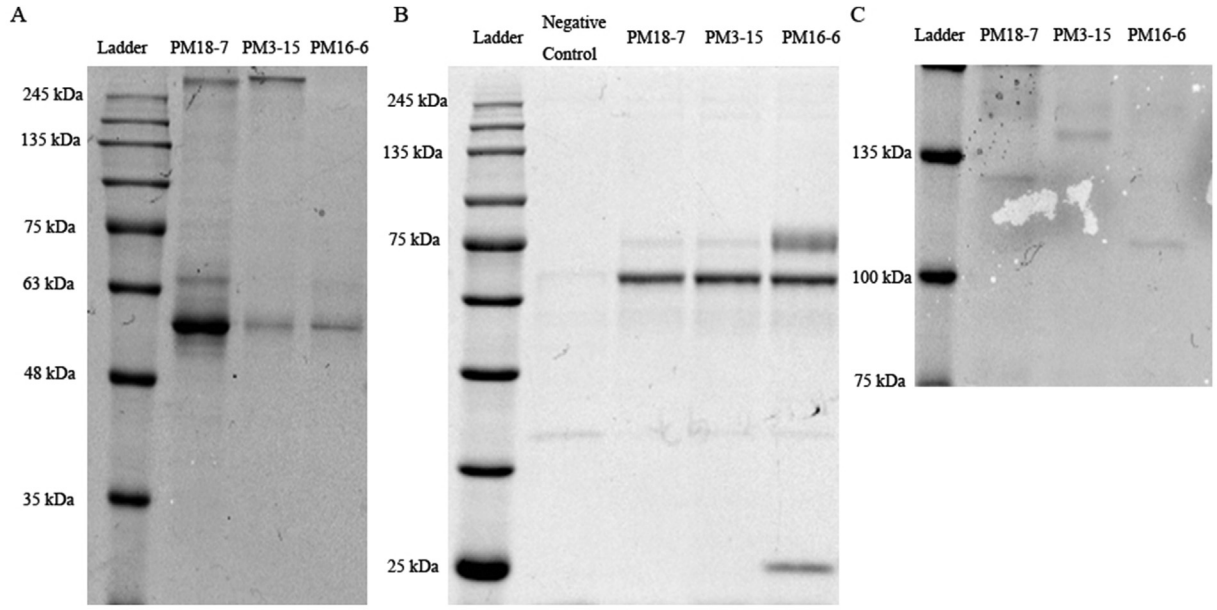

Fig. 6. Molecular weight of antigens detected by mAb immunoprecipitation of PAM whole cell lysate was identified under non-reducing and reducing conditions with SDS-PAGE. Immunoprecipitates of mAbs under non-reducing (A) and reducing (B) conditions were indicated antigens bound to each mAbs. Immunoprecipitates of PM18-7 and PM3-15 under reducing conditions (C) were figured out.

Table 4
Antigens identification by LC/MS-MS protein sequencing analysis.

Sample	Protein description	Size (kDa)	Mascot[a] score	Queries matched
PM18-7 H7D2	Ecto-nucleotide pyrophosphatase/ phosphodiesterase 1 (ENPP1/CD203)	130/ 130	80	49
PM3-15 A2A8	Integrin alpha M isoform 2 precursor (CD11b)	150/95	99	30
PM16-6 E8A6	Thimet Oligopeptidase 1 (THOP-1)	75/25	42[b]	19

[a] Protein score is −10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 54 are significant.
[b] The reason why score is below than 54 is too small amount of input mass list.

band under non-reducing conditions, molecular weights of 245 kDa and under reducing conditions, molecular weights of 150, 95 kDa were observed. PM16-6 precipitated a band under non-reducing conditions, molecular weights of 125 kDa and reducing conditions, molecular weights of 75, 25 kDa were observed (Fig. 6B). Using these bands, MALDI-TOF protein sequencing was conducted and then, the sequence analysis was carried out by mascot software program. As a result (Table 4), the peptides obtained in five more experiments is that the antigens recognized by PM18-7 was identified as ectonucleotide pyrophosphatase type 1 (ENPP1, CD203a), PM3-15 was figured out as complement receptor type 3 (CD11b/CD18) and PM16-6 was investigated as thimet oligopeptidase type 1 (THOP-1). It was highly trustworthy value by mascot score.

4. Discussion

These studies document the production and characterization of three mAbs which react with surface membrane molecules present on the porcine alveolar macrophage. 1) PM18-7 was found to immunoprecipitated a 130 kDa dimeric molecule that was present on virtually all PAM and not on monocytes or granulocytes. 2) PM3-15 immunoprecipitated 245 kDa molecule under non-reducing conditions and 150, 95 kDa under reducing conditions and reacted with PAM, splenic macrophages, mesenteric lymph node macrophage, and monocytes. 3) PM16-6 immunoprecipitated 125 kDa molecule under non-reducing conditions and 75, 25 kDa molecules under reducing conditions and reacted with PAM, monocytes and poly-morpho-nuclear cell called as granulocytes.

To further characterize the ontogeny of porcine PAM and to identify surface antigens that appear during development, mAbs reactive with porcine macrophages were needed. The mAbs made against mouse or human macrophages which are readily available are not reactive with porcine macrophage and thus not useful for porcine macrophage studies. Therefore, hybridoma producing mAbs reactive with PAM and nonreactive with RBC, peripheral blood lymphocytes and thymocytes were selected to obtain mAbs recognizing epitopes specific to macrophages and to eliminate mAbs recognizing epitopes common to other cell types. Many of the hybridoma supernatants originally reactive to PAM only were nonreactive upon retesting, but the three clones that were selected for further characterization (PM18-7, PM3-15 and PM16-6) were produced by hybridoma that were stable and continued to secrete mAbs.

Finally the antigens bound to each mAbs and specifically exist on the macrophages were identified as CD203a, CD11b/CD18 and THOP-1. Moreover, the epitopes recognized by each mAbs in these molecules will be figured out. We can suggest that those mAbs characterized in this study were recognized distinguished molecules exist on the myeloid cells, even though using these mAbs, we can make the distinction respective myeloid cells. Also we might guess that those have potential capacity to be functional antibodies due to the antigens were located on the surface of the target cells. If it happened, these mAbs will be useful tools for ontogeny of macrophage and myeloid lineage cells also it can be used as functional antibodies in the veterinarian molecular and cellular immunology fields. In addition, the molecule CD11b as target of PM3-15 had been used for monocyte/macrophage marker in other species [20] as well as porcine [21]. However, CD203a and THOP-1 as target of PM18-7 and PM16-6 has not been reported as markers for macrophage and granulocyte in other species, thus we have to dig it out about these markers for using myeloid cell ontogeny and development.

Conflict of interest

Authors declare no conflict of interest including any financial, personal or other relationships with other people or organization that could inappropriately influence, or be perceived to influence the work.

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Transparency document

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