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Generation of *in vitro* porcine monocyte-derived dendritic cells (MoDcs) by new modified protocol

การสร้างเดนไดรติกเซลล์ในห้องปฏิบัติการจากโมโนซัยต์ของสุกรโดยวิธีดัดแปรแบบใหม่

โดย

ผู้ช่วยศาสตราจารย์ทันตแพทย์หญิง ดร. พัชรี ฤทธิ์ประจักษ์

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ABSTRACT

Generation of *in vitro* porcine monocyte-derived dendritic cells (MoDcs) by new modified protocol

Teerawut Nedumpun, Sanipa Suradhat, Patcharee Ritprajak

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The derivation of dendritic cells (DCs) *in vitro* is an alternative system to overcome the low frequency of primary DCs and the difficulty of isolation techniques for study of DC immunobiology. To date, conventional culture protocol of porcine monocyte-derived DCs (MoDCs) has been widely used. However, this protocol is sometimes not practical due to the requirement of substantial monocyte number from the blood sample, and the process often interferes with DC maturation. To improve the protocol for porcine MoDC generation, we altered the previous conventional protocol, based on the human MoDC and mouse bone-marrow derived DC (BM-DC) culture system, and compared phenotypic and functional features of MoDC derived from the modified protocol to the conventional protocol. The modified protocol consumed less amount of monocytes but generated higher $CD1^+$ cells with DC-like morphology and ability of maturation. In addition, MoDCs from the modified protocol exhibited increased antigen uptake capability and cytokine gene expression in response to LPS stimulation. Our findings indicate that the modified protocol is expedient and reliable for generating potent MoDCs that substitute for primary DCs. This will be a valuable platform for future research in antigen delivery, vaccine and immunotherapy in pigs, as well as relevant veterinary species.

บทคัดย่อ

การสร้างเดนไดรติกเซลล์ในห้องปฏิบัติการจากโมโนซัยต์ของสุกร โดยวิธีดัดแปรแบบใหม่

ธีรวุฒิ เนตรอำพันธ์, สันนิภา สุรทัตต์, พัชรี ฤทธิ์ประจักษ์

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การพัฒนาเดนไดรติกเซลล์ในห้องปฏิบัติการเพื่อใช้ศึกษาเดนไดรติกเซลล์เชิงภูมิคุ้มกันชีววิทยา เป็นระบบทางเลือกที่ใช้ก้าวข้ามปัญหาเรื่องเดนไดรติกเซลล์ปฐมภูมิที่มีจำนวนน้อยและความยากของเทคนิคในการแยกเซลล์ ปัจจุบันวิธีการเพาะเลี้ยงเดนไดรติกเซลล์จากโมโนซัยต์ของสุกรแบบดั้งเดิมนั้นนิยมใช้อย่างกว้างขวาง อย่างไรก็ตาม วิธีการเพาะเลี้ยงนี้บางครั้งไม่สามารถนำมาประยุกต์ใช้ได้เนื่องจากจำเป็นต้องใช้โมโนซัยต์ในปริมาณมากจากตัวอย่างเลือด และกระบวนการดังกล่าวมักรบกวนการเจริญเต็มที่ของเดนไดรติกเซลล์ เพื่อปรับปรุงวิธีการสร้างไดรติกเซลล์จากโมโนซัยต์ของสุกร ผู้วิจัยได้ปรับเปลี่ยนวิธีการเพาะเลี้ยงแบบดั้งเดิม โดยยึดระบบการเพาะเลี้ยงไดรติกเซลล์จากโมโนซัยต์ของมนุษย์ และการเพาะเลี้ยงไดรติกเซลล์จากเซลล์ไขกระดูกของหนูเมาส์เป็นแนวทาง จากนั้นทำการเปรียบเทียบฟีโนไทป์ และการทำงานของไดรติกเซลล์ที่สร้างจากโมโนซัยต์ของสุกรด้วยวิธีดัดแปรกับวิธีดั้งเดิม พบว่าวิธีดัดแปรใช้ปริมาณโมโนซัยต์น้อยกว่าแต่สามารถสร้างเซลล์ที่มีการแสดงออกของ CD1 ได้จำนวนมาก โดยเซลล์เหล่านี้มีลักษณะคล้ายเดนไดรติกเซลล์และมีความสามารถในการเจริญเต็มที่ นอกจากนี้ไดรติกเซลล์ที่สร้างจากโมโนซัยต์ของสุกรด้วยวิธีดัดแปรสามารถจับกินแอนติเจนได้มากขึ้น และมีการแสดงออกของไซโตไคน์ยีนที่สูงขึ้นเมื่อถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์ จากผลการทดลองแสดงว่าวิธีดัดแปรนั้นสะดวกและเชื่อถือได้สำหรับการสร้างไดรติกเซลล์จากโมโนซัยต์ของสุกรที่มีศักยภาพเพื่อทดแทนเดนไดรติกเซลล์ปฐมภูมิ งานวิจัยนี้เป็นแนวทางที่มีคุณประโยชน์สำหรับการวิจัยในอนาคตทางด้านการนำส่งแอนติเจน วัคซีน และการรักษาเชิงวิทยาภูมิคุ้มกันในสุกร และสัตว์ชนิดอื่นๆ ที่เกี่ยวข้องกับงานทางสัตวแพทยศาสตร์

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CONTENT

INTRODUCTION

Dendritic cell (DC) is a unique innate immune cell capable of antigen processing and presentation. Signal transduction through DC activation in response to stimuli provides the foundation to develop adaptive immune response and direct the type of effector T lymphocytes (Merad et al., 2013; Pulendran et al., 2010). Furthermore, immune modulation properties of DC could contribute the great advantage towards the field of swine vaccinology and immunotherapy. Porcine DCs have been characterized and typified by the expression of the surface molecules, CD1, CD14, CD16, CD80/86, CD172 (SWC3) and MHC class II (Summerfield and McCullough, 2009). However, study of porcine DC biology is limited due to low frequency of DC population in blood circulation and tissues, and laborious DC isolation technique (Cooper et al., 1985; Summerfield and McCullough, 2009; Zhang et al., 2009). Therefore, *in vitro* monocyte-derived DC (MoDC) is an alternative tool to examine porcine DC biological properties and functions.

Two different *in vitro* culture procedures for generation of porcine DC from peripheral blood monocytes have been established. Fast protocol can generate porcine monocyte-derived DC (MoDCs), so-called fast MoDCs, within 48 hours but the phenotype and function of these DCs are partially developed (Wongyanin et al., 2012). Conventional protocol requires 7-9 days to induce MoDCs, but these DCs are more potent and favorable for investigating the interaction of swine pathogens and innate immunity (Lecours et al., 2011; Summerfield and McCullough, 2009).

However, there is the limitation of the conventional protocol which obligates to a large amount of peripheral blood mononuclear cells (PBMCs). In addition, this protocol includes harvesting and re-plating steps prior to *in vitro* stimulation which may possibly induce undesirable DC maturation by physical and pressure stimulation (Banchereau et al., 2000). The biological and functional properties of immature and mature DC are different. Immature DCs are capable of high antigen uptake but low T lymphocyte activation properties, while mature DCs loss their phagocytic ability but can mediate T lymphocyte activation and differentiation (Schnurr et al., 2000). Therefore, the stage of DC maturation is crucial for experimental interpretation.

The standard protocol for generating *in vitro* DCs in human and mouse are quite similar and have been well validated (Dewitte et al., 2014; Frindel et al., 1967; Inaba et al., 2009; Qu et al., 2014). Generation of human MoDCs and murine bone marrow-derived DCs (BM-DCs) requires much less DC precursors than the porcine MoDCs protocol. Furthermore, the harvesting and re-plating step used in the porcine system are generally excluded from human and mouse system. Due to aforementioned disadvantages of the current porcine MoDC generation protocol, our study thus aimed to improve a porcine MoDC culture technique by modifying the current conventional protocol, referred as the modified protocol, based on human MoDC and murine BM-DC culture procedures. This work will be beneficial for the study of immunobiology of porcine DC and mechanism of host-pathogen interaction. The validated MoDC will also be expedient for future research works in antigen delivery, vaccine and immunotherapy in pigs, as well as relevant species.

MATERIAL AND METHODS

Isolation of porcine peripheral blood mononuclear cells (PBMCs)

Porcine PBMCs were isolated from heparinized whole blood by density gradient centrifugation using Lymphosep® separation medium (Biowest, Kansas, Mo, USA) according to previously described protocol (Suradhat and Thanawongnuwech, 2003). PBMCs were further subjected to generation of porcine MoDC.

In vitro generation and stimulation of porcine MoDC

For the conventional protocol, 25 x 10^6 porcine PBMCs were plated in 1 ml of IMDM (GIBCO, Carlsbad, CA, USA) containing 5% fetal bovine serum or FBS (GIBCO) in a 6 well-plate (Corning-Costar, Corning, NY, USA) for 2 hours. Non-adherent cells were removed. Adhered cells, referred as monocytes, were subsequently cultured with 1 ml of RPMI complete medium, containing advanced RPMI (GIBCO), 10% FBS (GIBCO), 2 mM L-glutamine (GIBCO), antibiotic/antimycotic solution (GIBCO), 25 mM HEPES (GIBCO) and 50 μM β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), and supplemented with 10 ng/ml recombinant porcine IL-4 (R&D system, Minneapolis, MN, USA) and 25 ng/ml recombinant porcine GM-CSF (R&D system), referred as DC medium, for 7 days. Every 2 days, the culture medium was 50% replaced with fresh DC medium. At day 7, MoDCs were harvested and re-plated for 1 x 10^6 per ml DC medium in a 24 well-plate prior to DC activation experiment.

For the modified protocol, 5 x 10^6 PBMCs in 1 ml of IMDM containing 5% FBS were plated in a 24 well-plate. Non-adherent cells were removed, and the adhered cells were further cultured with DC medium for 7 days. Every 2 days, the culture medium

was 50% replaced with fresh DC medium. Then, the MoDCs were incubated with stimulus without harvesting and re-plating step.

MoDCs derived from both protocols were cultured in a 24 well-plate, and stimulated with 0.1 mg/ml LPS (Sigma aldrich, St. Louis, MO, USA) at 37 °C in a 5% CO_2 incubator for 24 hrs.

Percentage of adhered monocyte and number of adhered MoDCs

Following PBMC plating, the adhered cells were harvested to determine the cell number using Z2 coulter counter (Beckman Coulter, CA, USA). Percentage of the cells was calculated by number of the adhered cells/total number of PBMCs x 100. In addition, the 7-day cultured MoDCs were harvested and examined the cell number using Z2 coulter counter.

MoDC morphology

The morphology of cultured monocytes and MoDCs at day 7 were observed under the inverted bright field microscopy (Olympus), 100× stage objectives.

Flow cytometric analyses

MoDCs were collected and washed twice with 1 X PBS containing 0.1% sodium azide (NaN_3) and 1% FBS. The cells were then incubated with the primary antibodies for 30 minutes at 4°C. After washing twice, the cells were incubated with the secondary antibodies for 30 minutes at 4°C, and then washed twice. Finally, the cells were fixed with 2% formaldehyde in PBS. Stained MoDCs were analyzed by Beckman FC550 (Beckman Coulter), and FlowJo software.

Primary antibodies included anti-porcine CD1-PE (clone 76-7-4, SouthernBiotech, Birmingham, AL, USA), anti-human CD86-PEcy7 (clone IT2.2, BioLegend, San Diego, CA, USA), biotinylated anti-porcine SWC3 (clone 74-22-15,

SouthernBiotech), anti-porcine SLA-DR IgG2a,κ (clone 1053H2-18, SouthernBiotech) and isotype control antibody (clone MPC-11, BioLegend). Secondary antibodies included goat anti-mouse IgG2a,κ-FITC (Invitrogen, Carlsbad, CA, USA) and streptavidin-APC (Invitrogen, USA).

Phagocytosis assay

The phagocytosis assay was previously described (20). Briefly, MoDCs (5×10^5 cells/well) were incubated with FITC-conjugated E.coli (Molecular Probes, Invitrogen, USA) at various ratios. Then, the cells wereshaken at incubator at 37 °C for 10 minutes. After incubation, cold PBS was added to stop phagocytosis activity, and the cells were washed at 4 °C for 5 minutes for 4 times. MoDCs were resuspended in FACs buffer for flow cytometry analysis.

Quantitative real-time polymerase chained reaction (qRT-PCR)

After LPS stimulation, total mRNA was extracted from the MoDCs using UPzol™ (Biotechrabbit, Germany), and converted to cDNA by Supercript III First-Strand cDNA synthesis kit (Invitrogen). The qRT-PCR reaction was performed as previous described (Wongyanin et al., 2012). The specific primers are shown in the Table 1. The amplification reaction consisted of initial denaturing at 95 °C for 15 minutes, followed by 35 cycles of denaturing at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds. The final extension was performed at 72 °C for 5 minutes. The specificity of amplified product was verified by melting curve analysis. The Ct values of the target gene was normalized against the housekeeping gene; *GAPDH*. Differences in Ct values between the treatments were analyzed by the formula $2^{-(\Delta CP_{target\ gene}\ -\ \Delta CP_{gapdh})}$, using a Rotor-Gene Real-Time Analysis Software 6.0 (Corbett Research).

Table 1 Oligonucleotide sequences of primers for qRT-PCR

Gene	Protein	Forward primer	Reverse primer	Product size
IL1B	IL-1β	AAC-GTG-CAA-TGA-TGA-CTT-TG	CAC-TTC-TCT-CTT-CAA-GTC-CC	292 bp
IL6	IL-6	AGA-ACT-CAT-TAA-GTA-CAT-CCT-CG	AGA-TTG-GAA-GCA-TCC-GTC	180 bp
IL12A	IL-12p35	ATG-CCT-CAA-CCA-CTC-CCA-AA	GGC-AAC-TCT-CAT-TCG-TGG-CT	182 bp
IL12B	IL-12p40	CCC-TGG-AGA-AAT-GGT-GGT-CC	GGC-CAG-CAT-CTC-CAA-ACT-CT	137 bp
IFNA	IFN-α	CAC-CAC-AGC-TCT-TTC-CAT	CTG-CAT-CTT-CCT-TCT-GGA-AT	225 bp
IFNB	IFN-β	CTG-GAG-GAG-GAC-TCC-AT	GAG-TCT-GTC-TTG-CAG-GTT	268 bp
IFNG	IFN-γ	GAG-GTT-CCT-AAA-TGG-TAG-CTC	GTC-TGA-CTT-CTC-TTC-CGC	213 bp
GAPDH	GAPDH	AAG-TGG-ACA-TTG-TCG-CCA-TC	TCA-CAA-ACA-TGG-GGG-CAT-C	123 bp

Statistical analysis

The statistical analysis is performed by using Student's t-test.

RESULTS

MoDC generation by modified protocol was able to increase the number of adherent monocytes, but still maintained dendritic cell-like morphology

Generation of MoDCs by the conventional and the modified culture procedure differed in 2 steps, which are initial plating and re-plating (Table 2).

Table 2 Comparison of conventional and modified protocol

Culture procedure	Conventional	Modified
Number of initial PBMCs per well	25 x 10^6 cells	5 x 10^6 cells
Culture ware	6-well plate	24-well plate
Volume of culture medium	1 ml	1 ml
Cytokines	25 ng/ml GM-CSF 10 ng/ml IL-4	25 ng/ml GM-CSF 10 ng/ml IL-4
Harvesting step	Yes	No
Re-plating step	Yes	No

At the initial step, PBMC number in the modified protocol was 5-fold less than the conventional protocol; however, the percentage of adherent monocytes in the modified protocol was obviously greater than those in the conventional protocol (Figure 1A). During the culture period, the cells from both procedures retained attachment approximately 70-80% (Figure 1B). The purpose of re-plating step in the conventional protocol is to manipulate the individual number of monocytes. Notwithstanding, we have concerned with the maturation of MoDCs prior to stimulation which could affect the accuracy and reliability of the downstream experiment. The re-plating step thus was excluded from our modified protocol, and the number of differentiated MoDC in individual well was confirmed. After 7-day culture, the number of MoDC generated from modified protocol was consistent

among the individual well (Figure 1C), suggesting that the re-plating step is dispensable for MoDC culture.

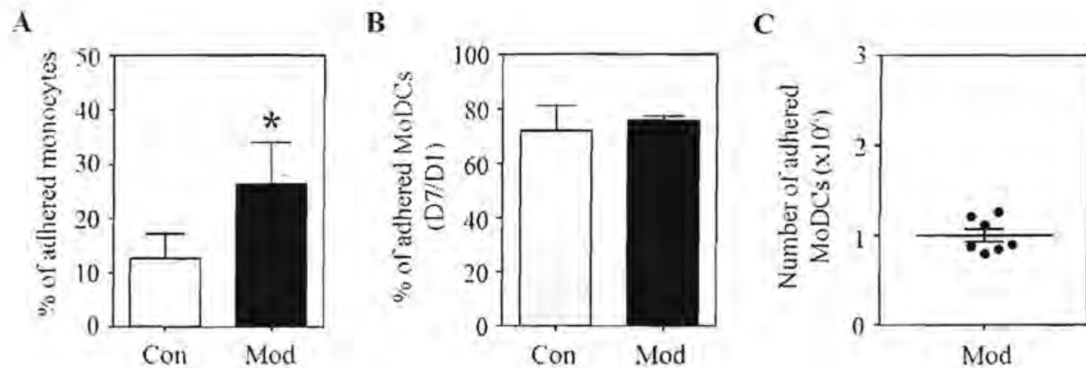

Figure 1 Percentage of adhered monocytes in different culture protocol. (A) The percentage of adhered monocyte prior to MoDC induction of conventional and modified protocol was determined by calculating the ratio of adhered monocytes per total PBMCs. (B) The percentage of MoDC on day 7 per day 1 after MoDC induction from different protocol. (C) Total number of 7-day cultured MoDC generated by modified protocol from 7 individual samples. $^{*}p<0.05$ comparing between MoDCs from both protocols. Con, conventional protocol; Mod, modified protocol.

We also observed the change in cell morphology at the end of culture period. All MoDCs elongated and transformed to dendritic cell-like morphology (Figure 2B-C and 2E-F), which are absent in monocytes (Figure 2A, 2D). In addition, MoDCs from the modified protocol (Figure 2C and 2F) were resembled to those from the conventional protocol (Figure 2B and 2E) both in size and morphology.

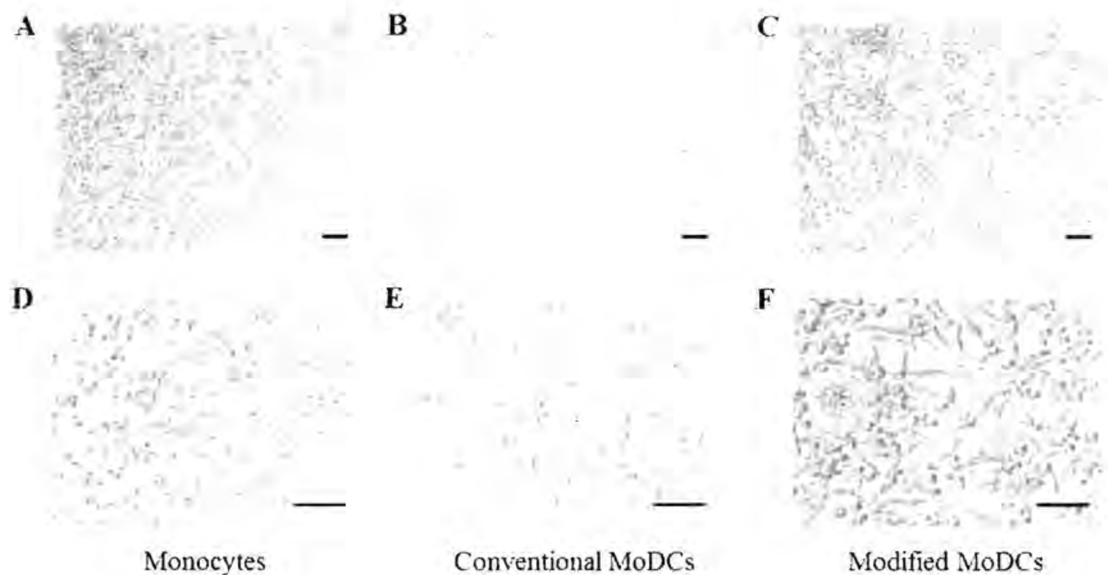

Figure 2 Cellular morphology of monocytes and MoDCs. (A, D) Porcine peripheral blood monocytes were cultured in non-DC medium and MoDCs generated from (B, E) conventional and (C, F) modified protocol were cultured in DC medium. At day 7, the cell morphology was observed under the bright field microscope. Scale bares, 50 μm.

MoDCs generated from the modified protocol highly expressed dendritic cell marker, CD1 molecules

To characterize MoDC phenotype, surface expressions of CD1, SLA-DR and SWC3 (CD172) markers were determined (Figure 3). CD1 is not detectable in monocytes, but it is upregulated when monocytes differentiated into DCs (Summerfield and McCullough, 2009). Notably, MoDC from the modified protocol highly expressed CD1 molecules while MoDCs from the conventional protocol expressed CD1 at the comparable level to monocytes (Figure 3A and 3B, left panel). Furthermore, $CD1^{+}$ population was markedly present in MoDCs from the modified

protocol, but was moderately found in MoDCs obtained from the conventional protocol (Figure 3C, left panel).

The SLA-DR marker obviously separated monocytes into 2 subpopulations of $SLA\text{-}DR^-$ and $SLA\text{-}DR^+$ cells. In the MoDCs, $SLA\text{-}DR^-$ population was decreased, and replaced by $SLA\text{-}DR^+$ cells (Figure 3A, middle panel). The obtained MoDCs from both protocols showed upregulated expression of SLA-DR and the elevated percentage of $SLA\text{-}DR^+$ population when compared to the monocytes. However, there was no difference in the SLA-DR expression level and $SLA\text{-}DR^+$ cells between the 2 protocols (Figure 3B, middle panel).

Two distinct subpopulations of the $SWC3^+$ cells, $SWC3^{lo}$ and $SWC3^{hi}$, were detected in the monocytes, but homogeneous $SWC3^{hi}$ population was found in all MoDCs (Figure 3A, right panel). Furthermore, the expression level of SWC3 and the percentage of $SWC3^+$ cells were significantly higher in the MoDCs, while there was no difference between the MoDCs obtained from both protocols (Figure 3B and 3C, right panel). Taken together, the results demonstrated that modified culture procedure was more efficient to generate porcine MoDCs.

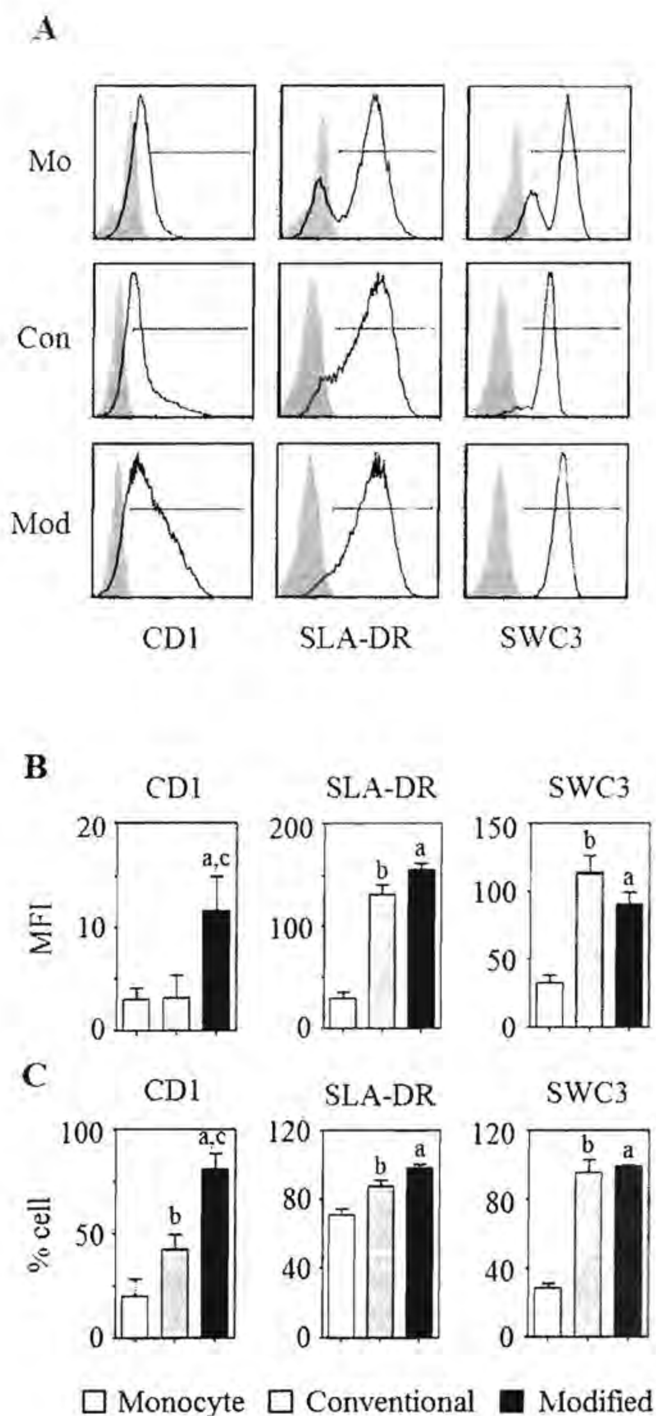

Figure 3 Comparative analysis of monocytes, and MoDC phenotype. Monocytes and immature MoDCs generated from modified and conventional protocol were harvested at day 7. The cells were stained against the surface markers, CD1, SWC3 and SLA-DR, and analyzed by flow cytometry. (A) Histogram demonstrated the

expression level of DC marker (thick line) and isotype control staining (gray shed). Data are representative of three independent experiments. (B) Mean fluorescent intensity (MFI) and (C) percentage of positive cells of CD1, SWC3 and SLA-DR in monocytes, MoDCs from conventional and modified protocol were determined; n = 3-4 pigs; [a] $p<0.05$ when compared MoDCs from modified protocol to monocytes (); [b] $p<0.05$ when compared MoDCs from conventional protocol to monocytes; [c] $p<0.05$ when compared MoDC from modified protocol to those of conventional protocol.

MoDCs generated from modified protocol were capable of undergoing maturation

According to the efficient dendritic cell differentiation obtained from the modified protocol, we next questioned whether these MoDCs were able to undergo maturation. To evaluate the maturation stage, the cultured MoDCs were stimulated with LPS and the expression of maturation markers, SLA-DR, CD80 and CD86 were determined (Figure 4). From flow cytometric analysis, all MoDCs altered their phenotype upon LPS stimulation by upregulating expression of SLA-DR and CD86, but not CD80 molecules (Figure 4A). MoDCs from the modified protocol possessed the frequency and levels of maturation markers similar to those from the conventional protocol (Figure 4B and 4C). The data suggested that our modified culture procedure was capable of generating the potent *in vitro* MoDCs.

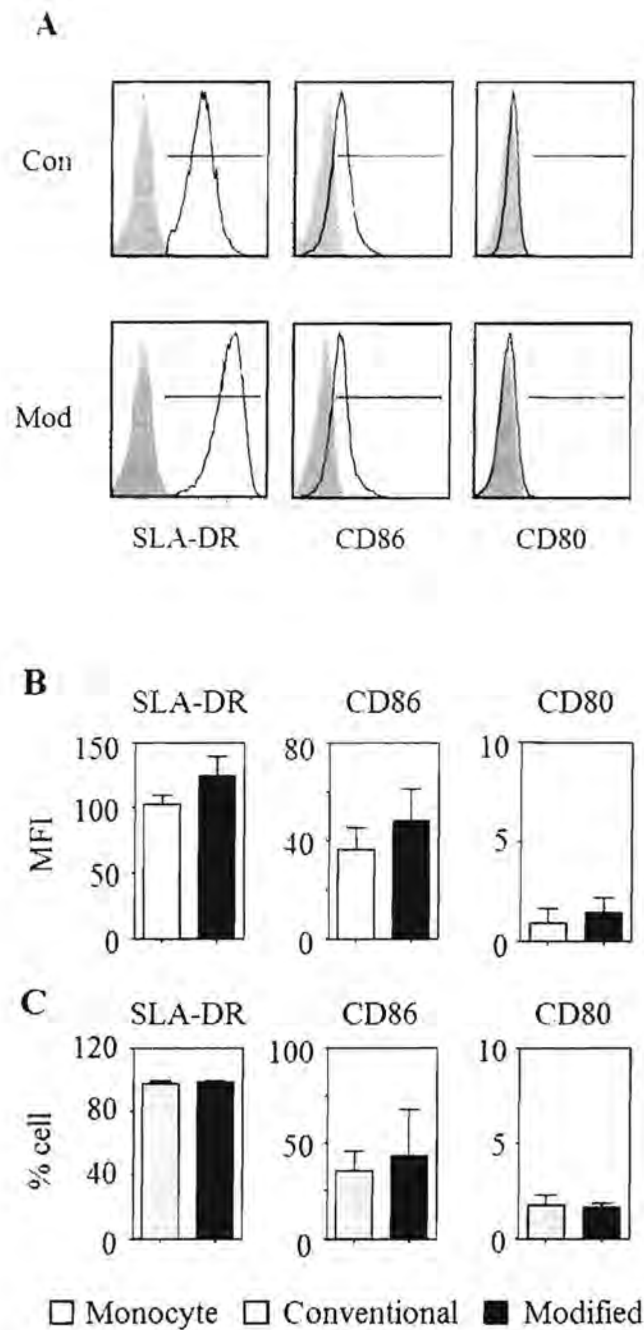

Figure 4 Maturation stage of MoDC. MoDCs generated from conventional and modified protocol were stimulated with 1 μg/ml of LPS for 24 hrs. Then, the cells were stained for the activation markers, SLA-DR, CD80 and CD86, and analyzed by flow cytometry. (A) Histogram demonstrated the expression level of activation marker (thick line) and isotype control staining (grey shed). Data are representative of

three experiments. (B) Mean fluorescent intensity (MFI) and (C) percentage of positive cells of SLA-DR, CD80 and CD86 in monocytes, MoDCs from conventional and modified protocol were determined; n = 3-4 pigs.

The modified protocol improved functional properties of MoDCs

Generation of porcine MoDCs is not only beneficial for the study of porcine DC biology, but also essential for *in vitro* tests in clinical application. *In vitro* generated DCs, therefore, must represent *in vivo* DCs in both cellular phenotype and functional properties. Accordingly, MoDC function was investigated by determining the mRNA expression of pro-inflammatory cytokines (Figure 5) and antigen uptake capability in response to stimulus (Figure 6). Upon LPS stimulation, *IL1B*, *IL6* and *IFNB* mRNA, encoding IL-1β, IL-6 and IFN-β, respectively, were induced in MoDCs generated from the modified protocol comparable to the cells from the conventional protocol. It should be noted that the expression level of *IFNG* gene, encoding IFN-γ, was significantly upregulated in MoDCs obtained from the modified protocol (Figure 5).

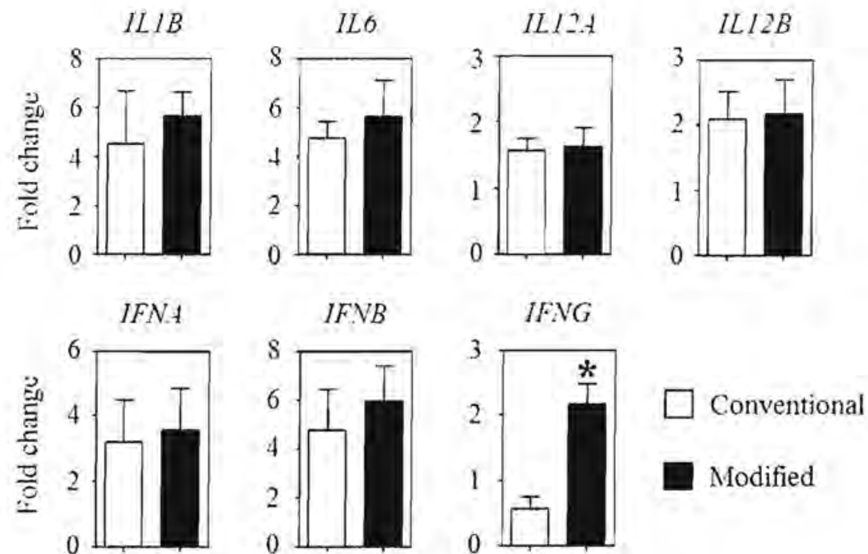

Figure 5 The mRNA expression of proinflammatory cytokines in activated MoDCs. MoDCs generated from conventional and modified protocol were stimulated with 1 μg/ml LPS for 24 hrs. The mRNA expression of *IL1B*, *IL6*, *IL12A*, *IL-12B*, *IFNA*, *IFNB* and *IFNG* were evaluated by quantitative real-time PCR. The relative mRNA expression was shown as fold induction over the negative control. $^{*}p<0.05$ when compared MoDC from modified protocol to those of conventional protocol; n = 3 pigs.

To assess phagocytosis activity, FITC-labeled *E.coli* was used as antigen, and flow cytometric analysis of FITC positive MoDCs was performed. Comparing between the two culture protocols, MoDCs derived by the modified protocol displayed higher efficacy of *E.coli* engulfment (Figure 6).

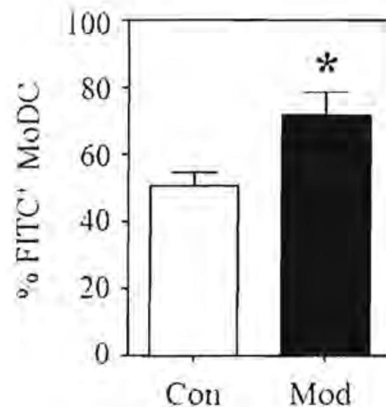

Figure 6 Antigen uptake capability of MoDCs. MoDCs generated from conventional and modified protocol were cultured with FITC-labeled *E. coli*. The percentage of FITC positive cells, represented MoDCs engulfed *E. coli*, were analyzed by flow cytometry. * $p<0.05$ when compared MoDC from modified protocol to those of conventional protocol; n = 5 pigs. Con, conventional protocol; Mod, modified protocol.

DISCUSSION

Acquisition and isolation of the substantial number of primary porcine DCs is the major obstacle in favor of *in vitro* MoDC culture. It has been demonstrated that phenotypes and properties of *in vitro* DC generated from blood monocytes ultimately resembled tissue-infiltrating MoDCs (Carrasco et al., 2001).

To generate potent porcine DCs in culture system, we modified the previous porcine MoDC culture protocol based on human MoDC and murine BM-DC culture procedures (Dewitte et al., 2014; Inaba et al., 2009; Lecours et al., 2011; Qu et al., 2014; Summerfield and McCullough, 2009). Comparing with the original protocol, the modified protocol required less PBMCs for generating the equal amount of MoDCs

(Figure 1A-B). It is possible that the less number of PBMCs in the cultured system improved the ratio of the cells to contact surface area on the culture plate leading to enhanced monocyte adherence. Harvesting and re-plating of MoDCs in the conventional protocol aim to control the accuracy in the cell numbers, however, these steps may induce premature DC as the consequence of mechanical stimulation (Craig et al., 2009) leading to the reduced phagocytosis ability and cytokine production in response to stimuli (Mellman and Steinman, 2001). Accordingly, we excluded the above steps from our modified protocol, and found that the frequency of MoDCs was quite consistent among the individual samples (Figure 1C). The morphology of porcine MoDCs has been well characterized by cytoplasmic projection around the cell surface (Guzylack-Piriou et al., 2006; Lecours et al., 2011; Lin et al., 2012). The obtained MoDCs from the modified protocol exhibited morphological changes and expressed DC markers, CD1, SWC3 and SLA-DR, similar to those generated by the conventional protocol, concordant with previous reports (Chamorro et al., 2005; McCullough et al., 1997; Summerfield and McCullough, 2009).

CD1 molecule has been recognized as a lipid presentation molecule restrictedly expressed on the DC population (Summerfield and McCullough, 2009). The increased $CD1^+$ cells and enhanced phagocytic activities in MoDCs obtained from the modified protocol (Figure 3) indicated the more efficient generation of porcine MoDC, compared to the original protocol. As the downstream signals and the optimal doses of GM-CSF and IL-4 are mandatory for MoDC differentiation *in vitro* (Heystek et al., 2000; Menges et al., 2005), it is possible that the less number of cultured monocytes per well in the modified protocol allowed better utilization of the supplements, leading to the higher quantity and quality of the generated porcine MoDCs. In addition, the omitted

mechanical stimulation in the modified protocol could benefit to the more efficient DC maturation process.

Of interest, MoDCs generated by the modified protocol displayed the high level of *IFNG* expression upon maturation (Figure 5). IFN-γ is not only important for Th1 polarization but also essential for driving DC maturation and endowing with the effector properties of DCs (Frasca et al., 2008; Han et al., 2009; He et al., 2007; Pan et al., 2004). DCs produce high level of IFN-γ in response to stimuli, especially LPS (Fedele et al., 2008; Pan et al., 2004). IFNGR (interferon gamma receptor) deficient BM-DCs exhibited the decreased maturation marker, CD86, reduced the production of pro-inflammatory cytokines, IL-1β and IL-12, and impaired function to activate alloreactive T cells (Pan et al., 2004). In addition, exogenous IFN-γ enhanced DC maturation and functions (Frasca et al., 2008; Han et al., 2009; Moretto et al., 2007). IFN-γ producing DCs potentially induced the antigen-specific cytotoxic T cell responses both *in vitro* and *in vivo* (He et al., 2007; Lemoine et al., 2010; Moretto et al., 2007). IFN-γ thus acts as an autocrine mediator for DC maturation, and exert T cell effector functions. It is likely that our modified protocol improve MoDCs maturation and functions via the induction of IFN-γ, which is useful for both in vitro tests and clinical application.

CONCLUSION

The development of MoDC in the culture system provides the great advantages to study DC immunobiology, especially in the aspect of host-pathogen interaction. Here, we proposed the protocol that required less number of porcine PBMCs and yielded good quantity and quality of *in vitro* porcine MoDC generation.

The modified protocol MoDCs has potential for numerous downstream applications and will be a valuable platform for future research in antigen delivery, vaccine and immunotherapy in pigs, as well as relevant veterinary species.

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