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Phenotypic and functional analysis of bovine peripheral blood dendritic cells before parturition by a novel purification method

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Abstract

Dendritic cells (DCs) are specialized antigen presenting cells specializing in antigen uptake and processing, and play an important role in the innate and adaptive immune response. A subset of bovine peripheral blood DCs was identified as CD172a$^+$/CD11c$^+$/MHC (major histocompatibility complex) class II$^+$ cells. Although DCs are identified at 0.1%–0.7% of peripheral blood mononuclear cells (PBMC), the phenotype and function of DCs remain poorly understood with regard to maintaining tolerance during the pregnancy. All cattle used in this study were 1 month before parturition. We have established a novel method for the purification of DCs from PBMC using magnetic-activated cell sorting, and purified the CD172a$^+$/CD11c$^+$ DCs, with high expression of MHC class II and CD40, at 84.8% purity. There were individual differences in the expressions of CD205 and co-stimulatory molecules CD80 and CD86 on DCs. There were positive correlations between expression of cytokine and co-stimulatory molecules in DCs, and the DCs maintained their immune tolerance, evidenced by their low expressions of the co-stimulatory molecules and cytokine production. These results suggest that before parturition a half of DCs may be immature and tend to maintain tolerance based on the low cytokine production, and the other DCs with high co-stimulatory molecules may already have the ability of modulating the T-cell linage.

KEYWORDS
cattle, cytokine, dendritic cell, phenotype, positive-selection

Tao Zhuang and Megumi Urakawa contributed equally to this work.

1 | INTRODUCTION

Dendritic cells (DCs) were first identified in the peripheral lymphoid organs of mice (Steinman & Cohn, 1973), specializing in antigen uptake and processing as an antigen-presenting cell (APC). DCs also play an important role in the innate and adaptive immune response (Banchereau & Steinman, 1998). The phenotypic and functional characterizations of peripheral blood DCs in humans have been described in several studies (MacDonald et al., 2002; Odoherty et al., 1994; Thomas, Davis, & Lipsky, 1993). However, the phenotype and function of peripheral blood DCs in cattle remain poorly understood.

A subset of bovine peripheral blood DCs was identified as $CD172a^+/CD11c^+$/MHC (major histocompatibility complex) class II^+ cells in the $CD3^-/B\text{-}B2^-/CD14^-$ population (Miyazawa et al., 2006) and expressed a CD205 molecule on the cell surface (Gonzalez-Cano, Arsic, Popowych, & Griebel, 2014). CD205, as an antigen-uptake receptor, was also expressed on DCs in lymphoid tissue (Gliddon, Hope, Brooke, & Howard, 2004). In addition, it has previously been reported that the surface molecules of CD40, CD80 and CD86 in DCs provided co-stimulate signals in T cell activation (Van-Gool, Vandenberghe, DeBoer, & Ceuppens, 1996).

In order to prevent fetal rejection caused by the recognition of paternal antigens, the maternal immune system has to be mobilized toward tolerance (Zenclussen, 2013). T helper (Th) cells play a central role in immune responses. However, the expression of Th1 and Th17-related genes is inhibited in bovine late gestation (Maeda, Ohtsuka, Tomioka, & Oikawa, 2013). The previous report showed the characterization of higher Th2/regulatory immunity by the increases of *IFN-γ* occurring after parturition and *IL-4* production before calving (Paibomesai, Hussey, Nino-Soto, & Mallard, 2013).

Among periparturient Jersey cows during the 2 weeks before and 2 weeks after parturition, the percentage of T cells with CD3, CD4 and gamma delta T-cell receptors reduced substantially in blood (Kimura, Goff, Kehrli, & Harp, 1999). During the periparturient period there is a decline in T-lymphocyte cell subsets, which parallels a reduction in functional capacities of blood lymphocytes (Kimura, Goff, Kehrli, Harp, & Nonnecke, 2002). Paternal T cells are aware of the presence of paternal antigens during pregnancy, where they acquire a transient state of tolerance specific for paternal antigens (Tafuri, Alferink, Moller, Hammerling, & Arnold, 1995). Regulatory T cells (Treg), the main function of which is to prevent autoimmunity, emerged as important players in regulating tolerance toward paternal and fetal antigens (Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995). Treg must first encounter antigens presented by antigen-presenting cells, as for example, DCs in an appropriate cytokine environment, to proliferate and function. In addition, DCs represent the first event leading to a protective adaptive immune response (Robertson, Mau, Tremellen, & Seamark, 1996), and contribute to the expansion of the peripheral Treg population (Schumacher et al., 2012). Immature DCs expressed a low level of MHC molecules and co-stimulatory molecules such as CD40, CD80 and CD86, and showed the reduced production of pro-inflammatory cytokines (*IL-12*, *TNFα*, *IL-6*) (Lutz & Schuler, 2002). These data are compatible with the hypothesis that declining T-cell populations may contribute to the immunosuppression reported for dairy cows at calving, and that DCs may regulate the population and functions of T cells during the days and weeks before and after parturition. However, the function for maintaining the tolerance during the pregnancy has not been clearly described in DCs in bovine blood. Previous works showed that in late gestation, the cows had a heightened susceptibility to persistent infections caused by mastitis and abortion-causing pathogens (Green, Green, Medley, Schukken, & Bradley, 2002; Williams et al., 2000). Therefore, we studied cattle which were 1 month before parturition.

In this study, we investigated the phenotypic and functional characterization of bovine peripheral blood DCs before parturition. As the population of DCs is less than 5% in bovine peripheral blood mononuclear cells (PBMC), there is a need to isolate highly purified DC subpopulations in sufficient numbers. Therefore, we have established a novel method of two-step magnetic-activated cell sorting (MACS) for bovine peripheral DCs, and were able to obtain DCs at a purity of more than 85% from PBMC. After the purification, we determined the expressions of surface markers (MHC II, CD205, CD40, CD80 and CD86) on DCs using flow cytometry and analyzed the expression of a number of cytokines (*IL-12a*, *IL-4*, *IFN-γ*, and *IL-6*). This study provides the evidence for immune regulation of bovine DC populations before parturition.

2 | MATERIALS AND METHODS

2.1 | Animals

Sixteen Holstein Friesian cows (average age at 5.2 ± 2.2 years, calving number at 2.3 ± 1.8), housed at the Miyagi Prefecture Animal Industry Experiment Station, were used in this study. All animal handing and experimental protocols were conducted in compliance with guidance approved by the Tohoku University Environmental and Safety Committee on Experimental Animal Care and Use, and the Environmental and Safety Committee on Miyagi prefecture animal industry experiment station. These animals were clinically healthy and kept in the same conditions.

2.2 | Blood sampling

Jugular venous blood (200 ml) was obtained from the cows at 1 month prior to parturition, into tubes containing sodium heparin, and was diluted 1:1 with phosphate-buffered saline (PBS). PBMC were separated from the buffy coat using Lympholyte®-H (1.077 g/ml; CEDARLANE, Burlington, Ontario, Canada) gradient centrifuged at 600 × *g* for 30 min at 18°C. PBMC were washed once with lysing buffer (Tris-HCl buffer containing 0.83% ammonium chloride) and twice with PBS at 450 × *g* each for 10 min at 4°C.

2.3 | Purification of peripheral blood DCs

The anti-bovine antibodies in this study were purchased from WSU (Pullman, WA, USA), Bio-Rad (Hercules, CA, USA), SouthernBiotech

(Birmingham, AL, USA), BD Biosciences (Franklin Lakes, NJ, USA) and Miltenyi Biotec (Bergisch Gladbach, Germany) (Table 1). For the sorting of $CD3^-/sIgM^-/CD14^-$/granulocytes$^-$ cells, PBMC were washed with PBS containing 0.2% bovine serum albumen (BSA), and incubated with the mixture of mouse anti-bovine CD3 (diluted 1/50), mouse anti-bovine sIgM (diluted 1/100), mouse anti-bovine CD14 (diluted 1/50), and mouse anti-bovine granulocytes (diluted 1/1000) antibodies for 30 min on ice, followed by the incubation with rat anti-mouse IgG1 Micro Beads and rat anti-mouse IgM Micro Beads for 30 min on ice, respectively. $CD3^-/sIgM^-/CD14^-$/granulocytes$^-$ cells containing DCs were negatively selected using Auto MACS magnetic columns (Miltenyi Biotec, Bergisch Gladbach, Germany). After negative selection, $CD3^-/sIgM^-/CD14^-$/granulocytes$^-$ cells were incubated with mouse anti-bovine CD172a antibody (diluted 1/200) and rat anti-mouse IgG1 Micro Beads for 30 min on ice, respectively. $CD172a^+$ cells were positively selected from $CD3^-/sIgM^-/CD14^-$/granulocytes$^-$ cells using Auto MACS magnetic columns.

2.4 | Flow cytometry

In order to detect bovine DCs, PBMC, $CD3^-/sIgM^-/CD14^-$/granulocytes$^-$ negative-selected cells in MACS step 1 (negative-selected cells) and $CD172a^+$ positive-selected cells in MACS step 2 (positive-selected cells) were stained with mouse anti-bovine CD172a antibody and co-stained with mouse anti-bovine CD11c (diluted 1/500) and MHC class II (diluted 1/250) antibodies. PBMC and negative-selected cells were incubated with anti-bovine CD3, sIgM, CD14 or granulocytes antibody in order to confirm the deletion of T cells, B cells, monocytes and granulocytes. Negative-selected cells were incubated with mouse anti-bovine MHC class II, CD40, CD205, CD80 or CD86 antibody, and treated with secondary fluorescent antibodies for 30 min on ice in the dark. After the treatment of secondary fluorescent antibodies as shown in Table 1, each cell was subjected to the flow cytometry analysis using the Accuri C6 flow cytometer (BD Biosciences) and the BD Accuri C6 software, Version 1.0.264.21 (BD Biosciences). In each experiment, cells incubated with isotype-matched antibodies and secondary fluorescent antibodies were selected as controls.

2.5 | Immunocytochemical staining

Negative- and positive-selected cells were stained with mouse anti-bovine CD172a antibody and co-stained with mouse anti-bovine CD11c and MHC class II antibodies, and then stained with peridinin chlorophyll protein complex conjugated rat anti-mouse IgG1, Phycoerythrin (PE) conjugated goat anti-mouse IgM and fluorescein isothiocyanate conjugated goat anti-mouse IgG2a fluorescent antibodies (Table 1). Cells were then centrifuged onto glass slides (Cytospin 2 Thermo Shandon, Pittsburgh, PA, USA) at 600 × *g* for 5 min. After air drying for 5 min, cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature in the dark, and were washed three times with PBS. Slide images were viewed using a Laser Scanning Microscope 700 (Carl Zeiss, Jena, German), and photographed at 400× with LSM software ZEN 2012, Version 8.0.0.273.

TABLE 1 Antibodies used in this study

Antibodies	Specificity	Isotype	Clone	Supplier
CD3	Pan T cells	IgG1	MM1A	WSU
Surface IgM	Pan B cells	IgG1	IL-A30	Bio-Rad
CD14	Mø, monocytes	IgG1	CAM36A	WSU
Granulocytes	Granulocytes	IgM	CH138A	WSU
CD172a	Mø, monocytes, DCs	IgG1	DH59B	WSU
CD11c	Mø, monocytes, DCs T cell subset, B cell subset	IgM	BAQ153A	WSU
MHC II	MHC class II	IgG2a	TH14B	WSU
CD205	Mø, DCs	IgG2a	ILA53A	WSU
CD40 FITC	Co-stimulatory molecule	IgG1	IL-A156	Bio-Rad
CD80 FITC	Co-stimulatory molecule	IgG1	IL-A159	Bio-Rad
CD86 FITC	Co-stimulatory molecule	IgG1	IL-A190	Bio-Rad
Control		Mouse IgG1	COLIS69A	WSU
Control		Mouse IgM	COLIS52A2	WSU
Control		Mouse IgG2a	COLIS205C	WSU
FITC IgG2a-secondary ab		Goat anti-mouse		SouthernBiotech
PE IgM-secondary ab		Goat anti-mouse		SouthernBiotech
PerCP IgG1-secondary ab		Rat anti-mouse		BD Biosciences
IgG1 Micro Beads ab		Rat anti-mouse		Miltenyi Biotec
IgM Micro Beads ab		Rat anti-mouse		Miltenyi Biotec

TABLE 2 Primer information for quantitative real-time PCR in this study

Primer		Sequence	Size (bp)
IL-12a	FW	GGCAGCTATTGCTGAGCTGATG	136
	RV	ACGAATTCTGAAGGCGTGAAG	
IFN-γ	FW	CATAACACAGGAGCTACCGATTTCA	197
	RV	CCCTTAGCTACATCTGGGCTACTTG	
IL-4	FW	CTTAGGCGTATCTACAGGAGCCACA	112
	RV	TCGTCTTGGCTTCATTCACAGAAC	
IL-6	FW	ATGCTTCCAATCTGGGTTCAATC	98
	RV	ATGCTTCCAATCTGGGTTCAATC	
GAPDH	FW	GATGGTGAAGGTCGGAGTGAAC	100
	RV	GTCATTGATGGCGACGATGT	

FW, forward primer; RV, reverse primer

2.6 | Quantitative real-time polymerase chain reaction (qPCR) analysis

After the negative and positive selections, the purified bovine peripheral blood DCs were stored at −80°C. Total RNA was extracted from them using ISOGEN II reagent (Takara Bio Inc., Siga, Japan) following the manufacturer's instructions, and its concentration was determined by spectrophotometry at 260 nm. The reverse transcription and complementary DNA (cDNA) synthesis are described below. In brief, 2 μg of total RNA was mixed with 500 ng oligo $(DT)_{12-18}$ and 1 μl of 10 mmol/L deoxynucleotide triphosphates (dNTPs) (Invitrogen, Carlsbad, CA, USA). The mixture was heated to 65°C for 10 min in order to prepare for cDNA synthesis. Then the first-strand cDNA was incubated with 200 units of Superscript RT III, 0.1M DTT and 5 × First-Strand Buffer (Invitrogen) at 50°C for 1 hr, and then at 70°C for 15 min.

One μl cDNA sample, 7 μl SYBR Green Premix Taq (Takara Bio Inc.), 1 μ of 5pM corresponding primer pair, and RNase-free water were added in a 20 μl final volume per well in a 96-well plate. The primer sets of bovine cytokines are listed in Table 2 (Takara Bio Inc.). The transcripts using the bovine peripheral blood DC cDNA were amplified with the Thermal Cycler Dice Real Time System Single (Takara Bio Inc.): one cycle at 95°C for 30 s; 40 cycles at 95°C for 5 s, 60°C for 30 s, then 95°C for 15 s, 60°C for 30 s and finally 95°C for 15 s. From template DNA, SYBR green fluorescence was

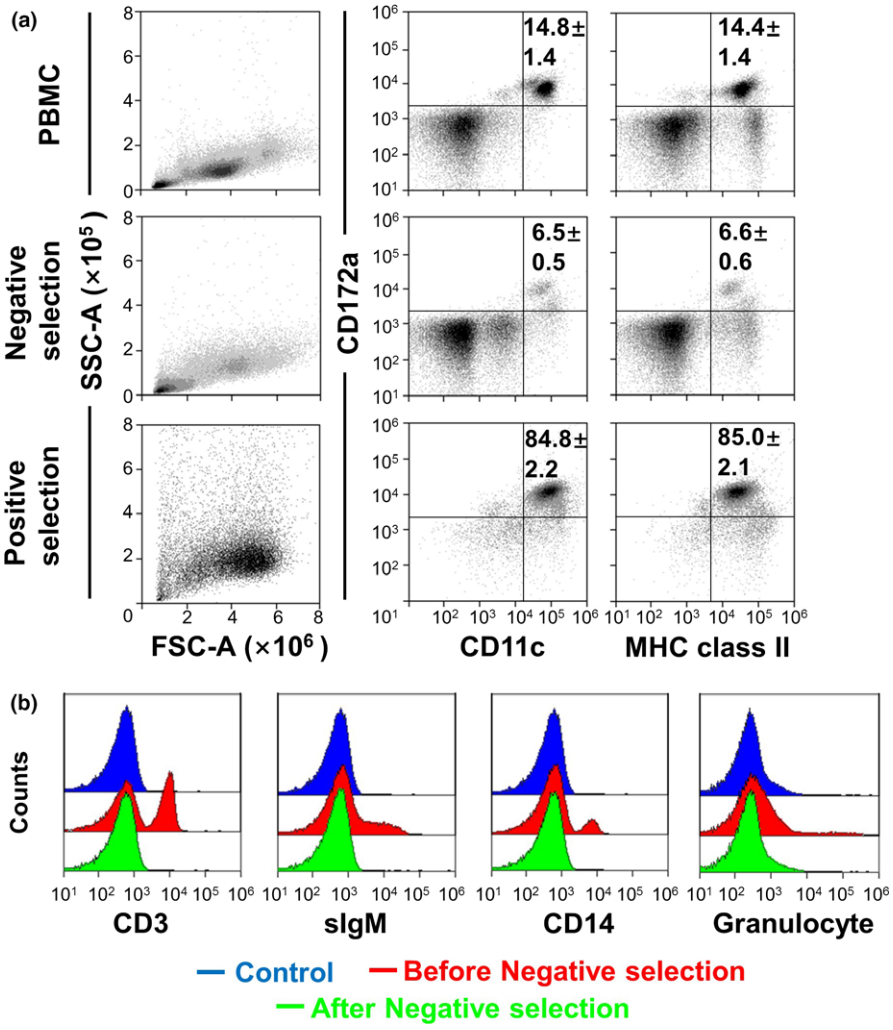

FIGURE 1 Purification of bovine peripheral blood dendritic cells (DCs). T cells, B cells, monocytes and granulocytes were removed from peripheral blood mononuclear cells (PBMC) by the negative selection using magnetic-activated cell sorting (MACS) with anti-bovine CD3, sIgM, CD14 and granulocytes antibodies. After the negative selection, peripheral DCs were purified from the negative-selected cells by positive selection using MACS with anti-bovine CD172a antibody. The size (forward scatter, FSC), complexity (side scatter, SSC), and expression of surface molecule CD172a, CD11c and major histocompatibility complex (MHC) class II were analyzed on PBMC, the negative-selected cells and the purified DC by flow cytometry (a). The flow cytometry histograms show the expression of CD3, sIgM, CD14 or granulocytes in PBMC and the negative-selected cells (b). Data are representative from six independent experiments

detected for the calculation of copy numbers. The specificity and the integrity of PCR product were confirmed by the dissociation curve analysis. *GAPDH*-specific primers were used as the internal controls, and the reactions without template were used as negative control experiments. The results of the target gene are presented as the relative expression level to the expression of house-keeping *GAPDH* gene.

2.7 | Statistical analysis

Values are reported as means ± SD. Statistical analysis were performed using the software GraphPad 6.00 program (GraphPad software Inc., La Jolla, CA, USA). The correlation between two parameters was analyzed by Pearson correlation coefficient test (*$p < 0.05$, **$p < 0.01$).

3 | RESULTS

3.1 | Purification of bovine peripheral blood DCs

We tried to purify bovine blood DCs from PBMC. Figure 1 shows the purification process of bovine peripheral blood DC. The expression of the surface molecules such as CD172a, CD11c and MHC class II, specific markers of DC, were assessed by three-color flow cytometry without any gate (Figure 1a). Among the total PBMC, 14.8% of $CD172a^+CD11c^+$ cells were present and almost expressed MHC class II molecules. However, it is well known that CD11c is highly expressed on monocytes, macrophages (Mø) and natural killer (NK) cells, and that $CD172a^+/CD11c^+$ cells possibly include a subset of T cells, B cells, NK cells and monocytes/Mø. Therefore, we attempted to remove these cell populations from PBMC using each

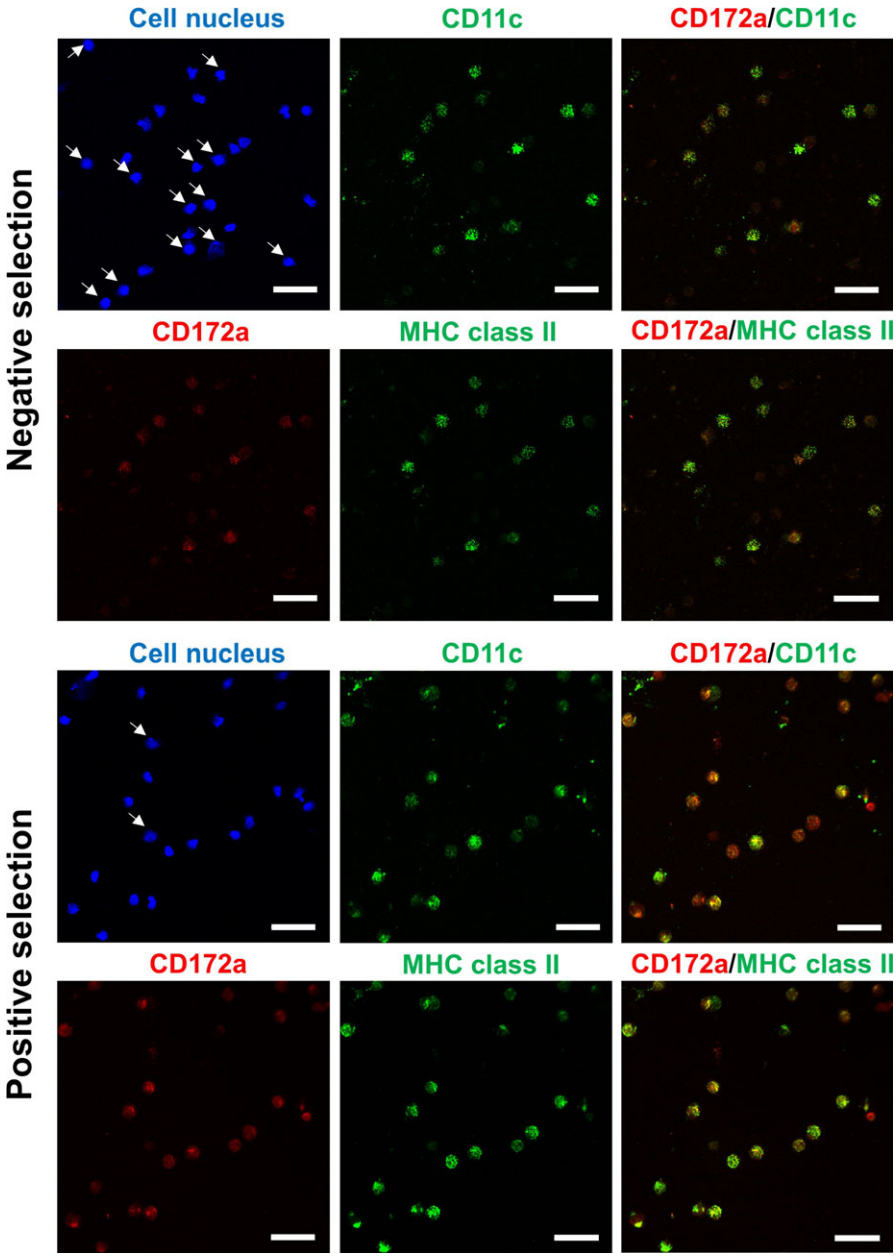

FIGURE 2 Photographs of peripheral blood dendritic cells (DCs). Peripheral blood DCs after the negative selection and the positive selection were stained by CD172a (red), CD11c (green) and major histocompatibility complex (MHC) class II (green). All samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Arrows show the unstained cells by CD172a and CD11c. Bars: 50 μm

specific monoclonal antibody. After the negative selection, $CD172a^+/CD11c^+$ cells were found to represent about 6.5% of the negative-collected cells and also expressed MHC class II on the cell surface. The negative selection using MACS removed T cells ($CD3^+$), B cells (surface IgM^+), monocytes ($CD14^+$) and granulocytes from PBMC, and these populations in negative-selected cells disappeared (Figure 1b). Therefore, $CD172a^+/CD11c^+$ cells in the negative-selected cells were considered as bovine peripheral blood DCs, which also expressed MHC class II molecules. However, the negative-selected cells contained a large population of $CD172a^-/CD11c^-$ non-DC cells. Next, we tried to purify $CD172a^+/CD11c^+$ cells from the negative-selected cells. The positive selection with CD172a antibody revealed that the purity of $CD172a^+/CD11c^+$ DCs was 84.8%, and that they also strongly expressed MHC class II.

3.2 | Photographs of peripheral blood DCs

Peripheral blood DCs after the negative and positive selections were stained with anti-bovine CD172a (red), CD11c (green) and MHC class II (green) antibodies. All samples were counterstained with DAPI (blue) (Figure 2). After the negative selection, $CD172a^+/CD11c^+$ and $CD172a^+$/MHC class II^+ DCs were detected as a small population in the photographs. Indeed, there were plenty of $CD172a^-/CD11c^-$/MHC class II^- non-DC cells which are indicated with arrows. However, this cell population indicated with arrows decreased after positive selection with anti-CD172a antibody. Almost all the positive-selected cells expressed CD172a, CD11c and MHC class II, which were considered as the bovine peripheral blood DCs. These data suggest that the two-step MACS method can highly purify DCs from bovine blood.

3.3 | Phenotypic analysis and cytokine expression of bovine peripheral blood $CD172a^+/CD11c^+$ DCs before parturition

Next, the surface expression of MHC class II, CD40, CD205, CD80 or CD86 was analyzed on $CD172a^+/CD11c^+$ cells after the negative selection (Figure 3a). The results demonstrated that almost all the $CD172a^+/CD11c^+$ DCs expressed the molecules of MHC class II (98.48 ± 0.54%) and CD40 (94.98 ± 0.88%). However, there were individual differences in the expression of CD205, CD80 or CD86 in the $CD172a^+/CD11c^+$ DCs. The percentages of CD205, CD80 and CD86 positive cells were 17.08 ± 3.97, 29.68 ± 4.23, and 23.50 ± 6.02 of $CD172a^+/CD11c^+$ DCs, respectively. Before parturition, there were significant correlations between the percentage of CD86 and the percentages of CD80 or CD205 on $CD172a^+/CD11c^+$ DCs (Figure 3b).

As the purity of bovine peripheral blood DC was more than 85% after positive selection, it became available for the examination of the expression of T cell-modulation cytokines in DCs (Figure 4). There were significant correlations in bovine peripheral DCs with the activated molecule of CD205 and the messenger RNA expressions of *IFN-γ* and *IL-6*. In addition, there were significant correlations between the co-stimulatory molecule CD80 and the expressions of *IL-12a*, *IL-4* and *IFN-γ*, and between CD86 and the expressions of *IL-4*, *IFN-γ* and *IL-6*.

4 | DISCUSSION

In this study, we have established a novel purification method for bovine peripheral blood DCs. We have also characterized the phenotype and function of the DCs. A previous study revealed that DCs were identified at 0.1%–0.7% of PBMC (Renjifo et al., 1997). Because of the low percentage of DCs in the PBMC, it was necessary to deplete the non-DC from bovine PBMC (Gibson, Miah, Griebel, Brownlie, & Werling, 2012; Miyazawa et al., 2006; Renjifo et al., 1997; Sei, Ochoa, Bishop, Barlow, & Golde, 2014). In this study, T cells, B cells, monocytes and granulocytes were depleted from PBMC by negative selection. However, $CD172a^+/CD11c^+$ cells with MHC class II molecules were detected at 6.5% of the negative-selected cells. This cell fraction was revealed as DCs (Gonzalez-Cano et al., 2014; Miyazawa et al., 2006); however, it was very difficult to investigate the functional and the genetic analysis of bovine blood DCs using it. Using positive selection with anti-bovine CD172a antibody and immunomagnetic microbeads, we were able to purify the $CD172a^+/CD11c^+$ DCs with MHC class II molecules at 84.8% purity,

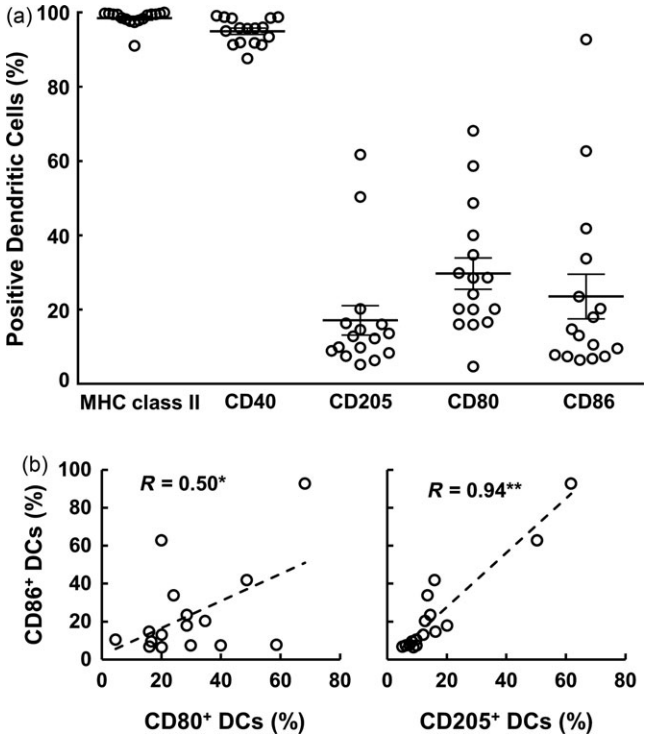

FIGURE 3 Phenotypic characterization of bovine peripheral blood $CD172a^+CD11c^+$ dendritic cells (DCs) before parturition. After the negative selection, the surface expression of major histocompatibility complex (MHC) class II, CD40, CD205, CD80 or CD86 on DCs was analyzed on $CD172a^+CD11c^+$ DCs (a). The correlations between the percentage of CD86 and the percentages of CD80 and CD205 on DCs (b). $*p < 0.05$, $**p < 0.01$

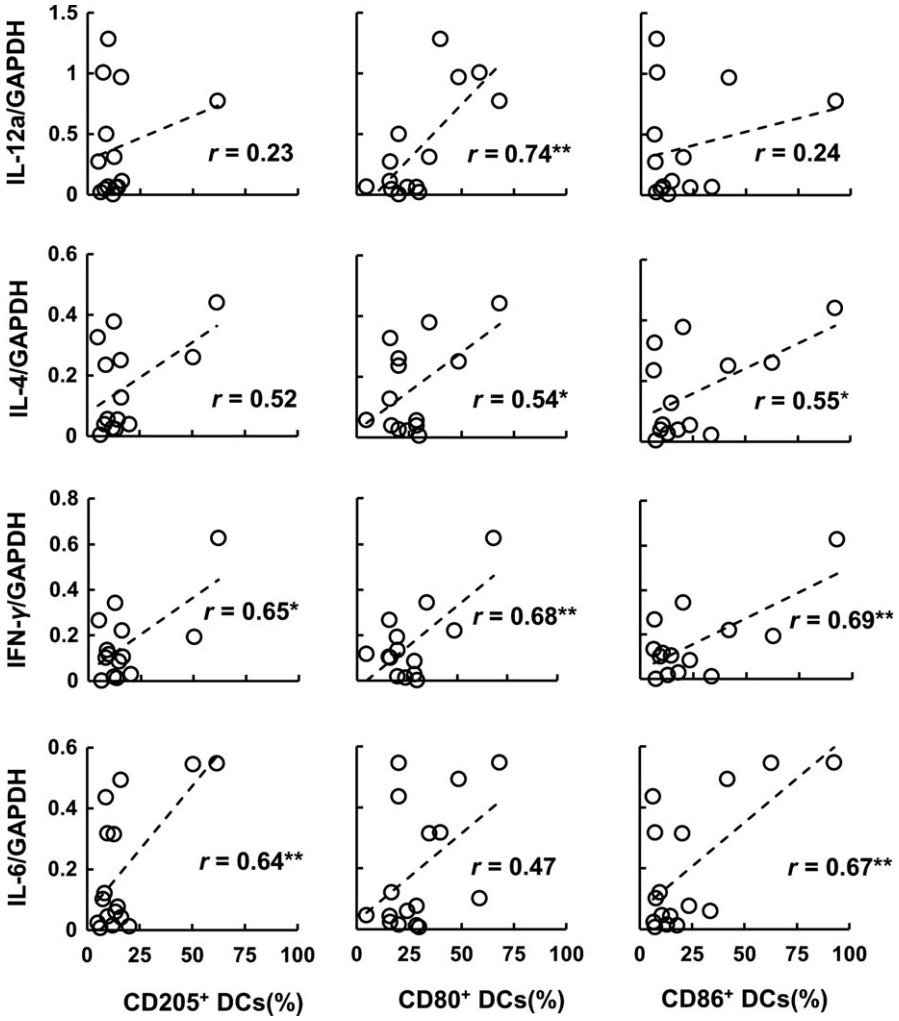

FIGURE 4 Relationship between expression of cytokines and surface molecule positivity in bovine peripheral blood dendritic cells (DCs) before parturition. The correlations between the expressions of *IL-12a*, *IL-4*, *IFN-γ* and *IL-6* and the percentages of surface molecules CD205, CD80 or CD86 are shown in DCs after the negative and positive selections. $*p < 0.05$, $**p < 0.01$

and also confirm the purified cells as DCs using the immunofluorescence photographs (Figure 2).

DCs are specialized antigen-presenting cells that regulate both immunity and tolerance. DCs in the periphery play a key role in induction of T cell immunity, as well as tolerance. DCs are phenotypically and functionally heterogeneous, and further classified into several subsets depending on distinct marker expression and their location. Co-stimulatory molecules were necessary to the T-cell responses and were up-regulated during DC activation (Cools, Ponsaerts, Van Tendeloo, & Berneman, 2007). The program of maturation of DCs brings about the up-regulation of MHC II (Lanzavecchia & Sallusto, 2001) and co-stimulatory molecules CD80 and CD86 (Mellman & Steinman, 2001). Bovine DCs are characterized by the increased expression of MHC II, CD11c, CD80/CD86 and the decreased expression of CD14 and CD21 surface markers (Denis & Buddle, 2008). CD80 and CD86 on DCs interact with the CD28 (stimulatory) and CTLA-4 (inhibitory) receptors of the T cells. The absence of CD80 and CD86 results in lack of co-stimulatory signal delivery to T cells and leads to clonal anergy and lack of proper T cell response (Schwartz, 1990). The signaling molecule CD40 is required to induce immunogenic DCs and for the induction of *IFNα* (Le Bon et al., 2006; Martin, O'Sullivan, Low, & Thomas, 2003).

The purified DCs from peripheral blood not only expressed CD172a, CD11c and MHC class II on the surface, but also expressed CD40, CD205, CD80 and CD86 (Figure 3). The majority of the DCs expressed the molecules of MHC class II and CD40. It is well known that CD205 has been expressed on many DCs in the T cell areas of lymphoid tissues (Gliddon et al., 2004). It has been reported that CD205 can lead to tolerance in the steady-state immunity after DC maturation (Bonifaz et al., 2002). Therefore, a part of bovine peripheral blood DCs before parturition might have been differentiated into activated DCs with high CD205. In this study, before parturition there were strong correlations in $CD172a^+/CD11c^+$ DCs between the CD86 expression and the expressions of CD80, as well as CD205. Therefore, our phenotype analysis of DCs revealed that there were both immature DCs and activated DCs in the peripheral blood, and that the peripheral blood DCs might have the potential of regulation for T cell lineage.

DCs collect and process antigens for presentation to T cells, and differ in the regulatory signals they transmit, directing T cells to different types of immune response or to tolerance (Shortman & Liu, 2002; Steinman, 1991). The priming with DCs was strictly dependent on CD80 / CD86, and CD86 was well known to induce naive T cells to become *IL-4* producers (Debecker et al., 1994). DCs may

determine the specificity, the amplitude and the character (Th1 / Th2) of the immune response. Therefore, we also investigated the cytokine production of the DCs and the correlations between expression of cytokine and co-stimulatory molecules. As the secretion of *IL-2*, *IFN-γ* and *IL-4* from DCs induced the development of T lymphocytes (Debecker et al., 1994), there were great positive correlations between CD80/CD86 positivity and the expressions of *IL-6*, *IFN-γ* and *IL-4* (Figure 4). *IL-12* from DCs appeared as a potent and obligatory inducer of Th1 priming (De Becker et al., 1998). In addition, *IL-12* is produced by DCs and is able to increase their stimulatory capacity of DCs (Kelleher & Knight, 1998). As CD80 high-positive DCs well induced *IL-12a*, there might be an autocrine effect of *IL-12a* on DC maturation (Figure 4). In contrast, a half of cattle in this study showed low expressions of CD205, CD80 and CD86 with low expressions of *IL-12a*, *IL-4*, *IFN-γ* and *IL-6*. A previous study indicates that bovine DCs in late gestation have reduced Th1-promoting cytokine production compared with regulatory cytokine production (Pomeroy, Sipka, Klaessig, & Schukken, 2015). Therefore, a half of bovine peripheral DCs before parturition may be immature and tend to maintain tolerance based on the low cytokine production. In addition, the other DCs with high CD205 and CD80/CD86 may already have the ability of modulating the T-cell linage. Our purification method in this study was considered as a useful tool to identify the capacity of DCs for activating T cells in vitro. Further research should explore into the similar phenotype DCs in bovines after parturition during the lactation period.

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