Title: 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 professional antigen presenting cell specialized 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 class II^+ cells. Although DCs are identified at 0.1-0.7% of PBMC, the phenotype and function of DCs remains poorly understood with regard to maintaining tolerance during the pregnancy. All cattle used in this study were one month before parturition. We have established a novel method for the purification of DCs from PBMC using MACS, 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: *dendritic cell; cattle; positive-selection; phenotype; cytokine*

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 the human have been described in several studies (Thomas et al., 1993; Odoherty et al., 1994; MacDonald et al., 2002). 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 class II^{+} cells in the $CD3^{-}$/B-$B2^{-}$/$CD14^{-}$ population (Miyazawa et al., 2006) and expressed a CD205 molecule on the cell surface (Gonzalez-Cano et al., 2014). CD205, as an antigen-uptake receptor, was also expressed on DCs in lymphoid tissue (Gliddon et al., 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 (VanGool et al., 1996).

In order to prevent the 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 gene was inhibited in bovine late gestation (Maeda et al., 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 et al., 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 et al., 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 et al., 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 et al., 1995). Regulatory T cells (Treg), the main function for which is to prevent autoimmunity, emerged as important players in regulating tolerance toward paternal and fetal antigens (Sakaguchi et al., 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 represented the first event leading to a protective adaptive immune response (Robertson et al., 1996), and contributed 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 the late gestation, the cows had a heightened susceptibility to persistent infections caused by mastitis and abortion-causing pathogens (Green et al., 2002; Williams et al., 2000). Therefore, we studied the cattle which were one 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 DCs 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.

Materials and Methods

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.

Blood sampling

Jugular venous blood (200 mL) was obtained from the cows at one month prior to parturition, into the 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.

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 0.5% 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.

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 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.

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 PerCP conjugated rat anti mouse-IgG1, PE conjugated goat anti mouse IgM and FITC 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 minutes. 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 400X with LSM software ZEN 2012, Version 8.0.0.273.

Quantitative real-time polymerase chain reaction (qRT-PCR) 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 the spectrophotometry at 260 nm. The reverse transcription and cDNA synthesis were described as below. In brief, 2 μg of total RNA

was mixed with 500 ng oligo $(DT)_{12-18}$ and 1 μL of 10 mM 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 h, and then at 70°C for 15 min.

One μL cDNA sample, 7 μL SYBR Green Premix Taq (Takara Bio Inc.), 1 μL of 5pM corresponding primer pair, and RNase-free water were added in a 20 μL final volume per well in 96-well plate. The primer sets of bovine cytokines were listed in Table 2 (Takara Bio Inc.). The transcripts using the bovine peripheral blood DCs cDNA were amplified with the Thermal Cycler Dice Real Time System Single (Takara Bio Inc.): 1 cycle at 95°C for 30 sec; 40 cycles at 95°C for 5 sec, 60°C for 30 sec, then 95°C for 15 sec, 60°C for 30 sec, and finally 95°C for 15 sec. From template DNA, SYBR green fluorescence was 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 target gene were presented as the relative expression level to the expression of house-keeping *GAPDH* gene.

Statistical Analysis

Values are reported as means ± SD. Statistical analyses 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 (*: $p<0.05$, **: $p<0.01$).

Results

Purification of bovine peripheral blood DCs

We tried to purify bovine blood DCs from PBMC. Fig.1 shows the purification process of bovine peripheral blood DC. The expression of the surface molecules such as CD172a, CD11c, and MHC class II, such as specific markers of DC, were assessed by three-color flow cytometry without any gate (Fig.1 A). Among the total PBMC, 14.8% $CD172a^{+}CD11c^{+}$ cells were present and almost expressed a MHC class II molecule. 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 monocyte/Mø. Therefore, we attempted to remove these cell populations from PBMC using each 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 (Fig.1 B). Therefore, $CD172a^{+}/CD11c^{+}$ cells in the negative-selected cells were considered as bovine peripheral blood DCs, which also expressed MHC class II molecule. However, the negative-selected cells contained a large amount of 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 expressed MHC class II strongly.

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) (Fig.2). After the negative selection, $CD172a^+/CD11c^+$ and $CD172a^+$/MHC class II^+ DCs were detected as a small population in the photographs. Indeed, there was a plenty of $CD172a^-/CD11c^-$/MHC class II^- non-DC cells indicated with arrows. However, this cell population indicated with arrows decreased after the 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 purify highly DCs from bovine blood.

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 (Fig.3 A). 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 (Fig.3 B).

As the purity of bovine peripheral blood DC was more than 85% after positive

249 selection, it became available for the examination of the expression of T cell-modulation
250 cytokines in DCs (Fig.4). There were significant correlations in bovine peripheral DCs
251 with the activated molecule of CD205 and the mRNA expressions of *IFN-γ* and *IL-6*. In
252 addition, there were significant correlations between the co-stimulatory molecule CD80
253 and the expressions of *IL-12a*, *IL-4*, and *IFN-γ*, and between CD86 and the expressions
254 of *IL-4*, *IFN-γ* and *IL-6*.

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 (Renjifo et al., 1997; Miyazawa et al., 2006; Gibson et al., 2012; Sei et al., 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 molecule were detected at 6.5% of the negative-selected cells. This cell fraction was revealed as DCs (Miyazawa et al., 2006; Gonzalez-Cano et al., 2014), 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 molecule at 84.8% purity, and also confirm the purified cells as DCs using the immunofluorescence photographs (Fig.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 et al., 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

279 are characterized by the increased expression of MHC II, CD11c, CD80/CD86 and the
280 decreased expression of CD14 and CD21 surface markers (Denis & Buddle, 2008).
281 CD80 and CD86 on DCs and interact with the CD28 (stimulatory) and CTLA-4
282 (inhibitory) receptors of the T cell. The absence of CD80 and CD86 results in lack of
283 co-stimulatory signal delivery to T cells and leads to clonal anergy and lack of proper T
284 cell response (Schwartz, 1990). The signaling molecule CD40 is required to induce
285 immunogenic DCs and for the induction of *IFNα* (Martin et al., 2003; Le Bon et al.,
286 2006).

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

300 DCs collect and process antigens for presentation to T cells, and differ in the
301 regulatory signals they transmit, directing T cells to different types of immune response
302 or to tolerance (Shortman & Liu, 2002; Steinman, 1991). The priming with DCs was

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

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Figure Legends

Fig.1 Purification of bovine peripheral blood DCs.

T cells, B cells, monocytes and granulocytes were removed from 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 the positive selection using MACS with anti-bovine CD172a antibody. The size (FSC), complexity (SSC), and expression of surface molecule CD172a, CD11c and 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.

Fig.2 Photographs of peripheral blood DCs.

Peripheral blood DCs after the negative selection and the positive selection were stained by CD172a (Red), CD11c (Green) and MHC class II (Green). All samples were counterstained with DAPI (Blue). Arrows show the unstained cells by CD172a and CD11c. Bars: 50 μm.

Fig.3 Phenotypic characterization of bovine peripheral blood CD172a$^+$CD11c$^+$ DCs before parturition.

After the negative selection, the surface expression of MHC class II, CD40, CD205, CD80 or CD86 on DCs was analyzed on CD172a$^+$CD11c$^+$ DCs (A). Figure B showed the correlations between the percentage of CD86 and the percentage of CD80 and

CD205 on DCs. **: $p<0.01$

Fig.4 Relationship between expression of cytokines and surface molecule positivity in bovine peripheral blood DCs before parturition.

The correlations between the expression of *IL-12a*, *IL-4*, *IFN-γ*, and *IL-6* and the percentages of surface molecules CD205, CD80 or CD86 were shown in DCs after the negative and positive selections. *: $p<0.05$, **: $p<0.01$

Tables

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

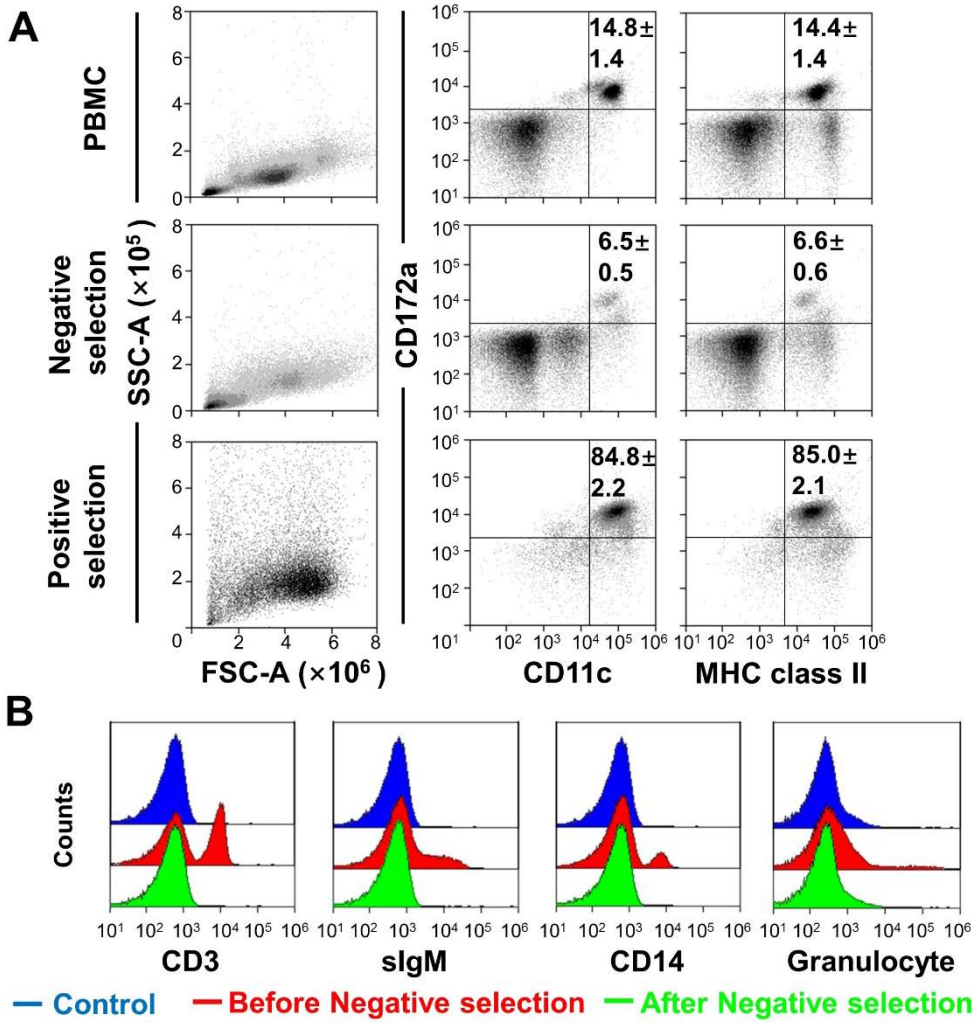
502 **Table 2 Primer information for quantitative real-time PCR in this study**

Primer		Sequence	Size (bp)
IL-12a	FW[a]	GGCAGCTATTGCTGAGCTGATG	136
	RV[b]	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	

503 [a] Forward primer.

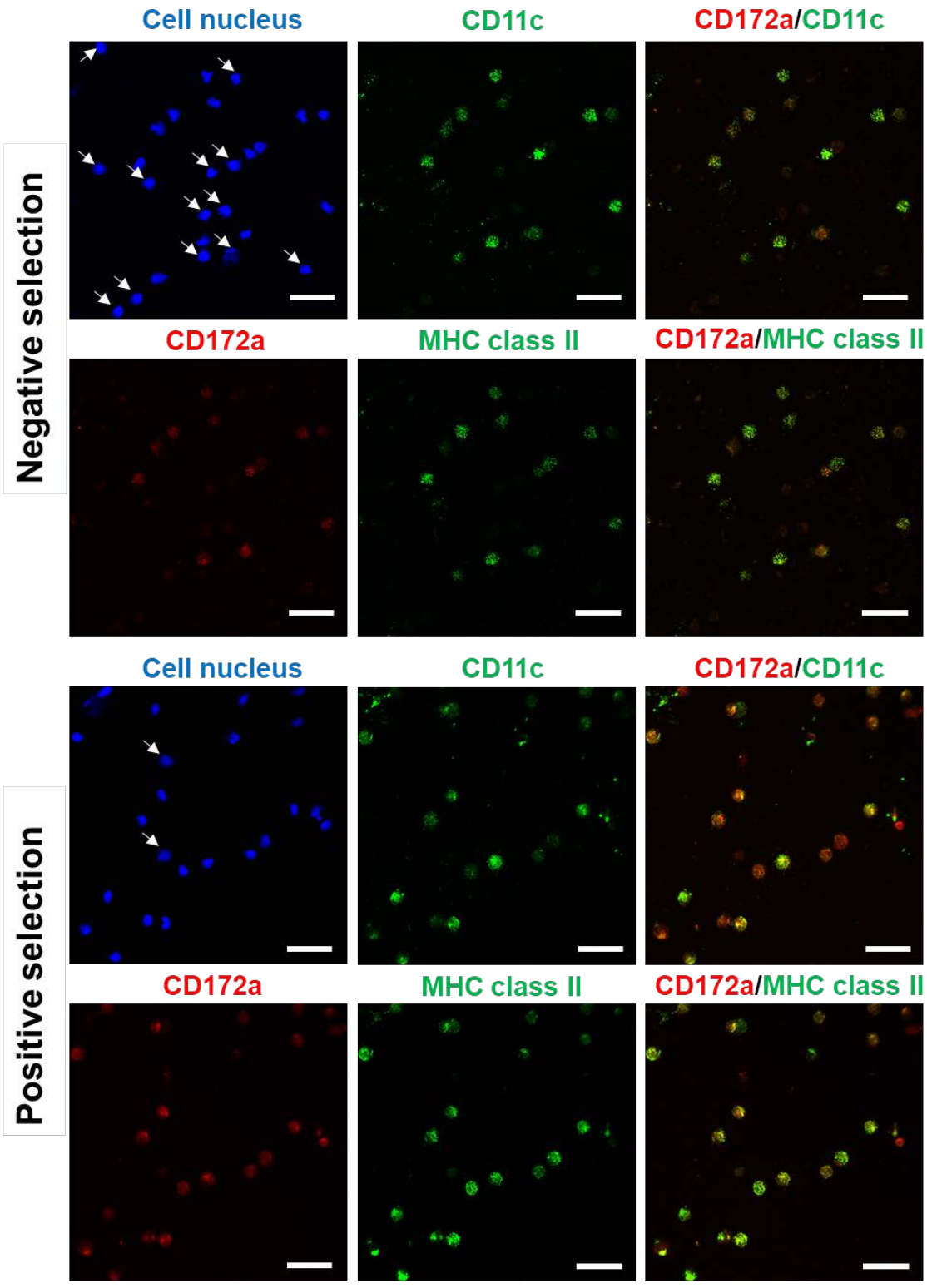
504 [b] Reverse primer.

505 **Figure 1**

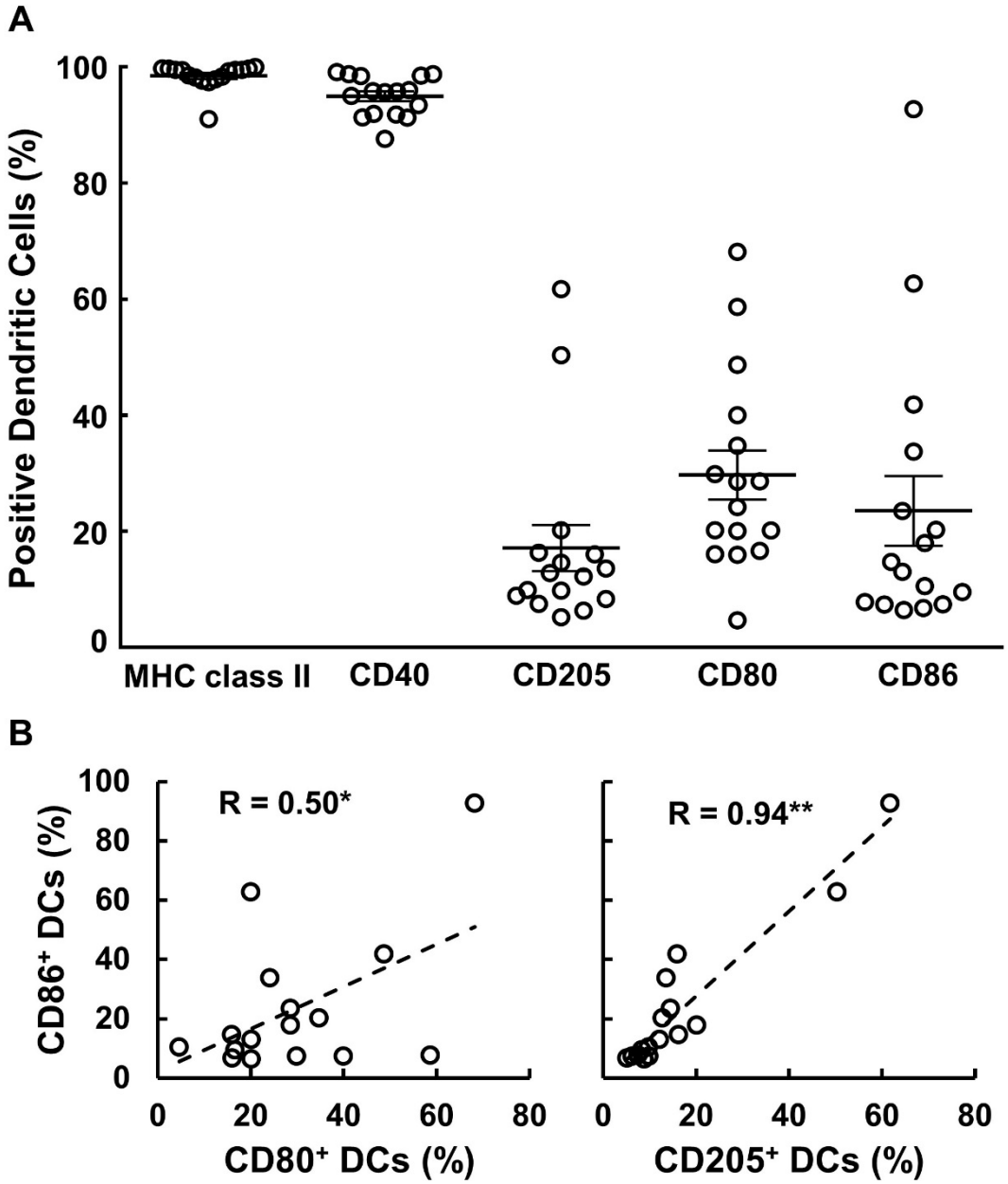


506

Figure 2

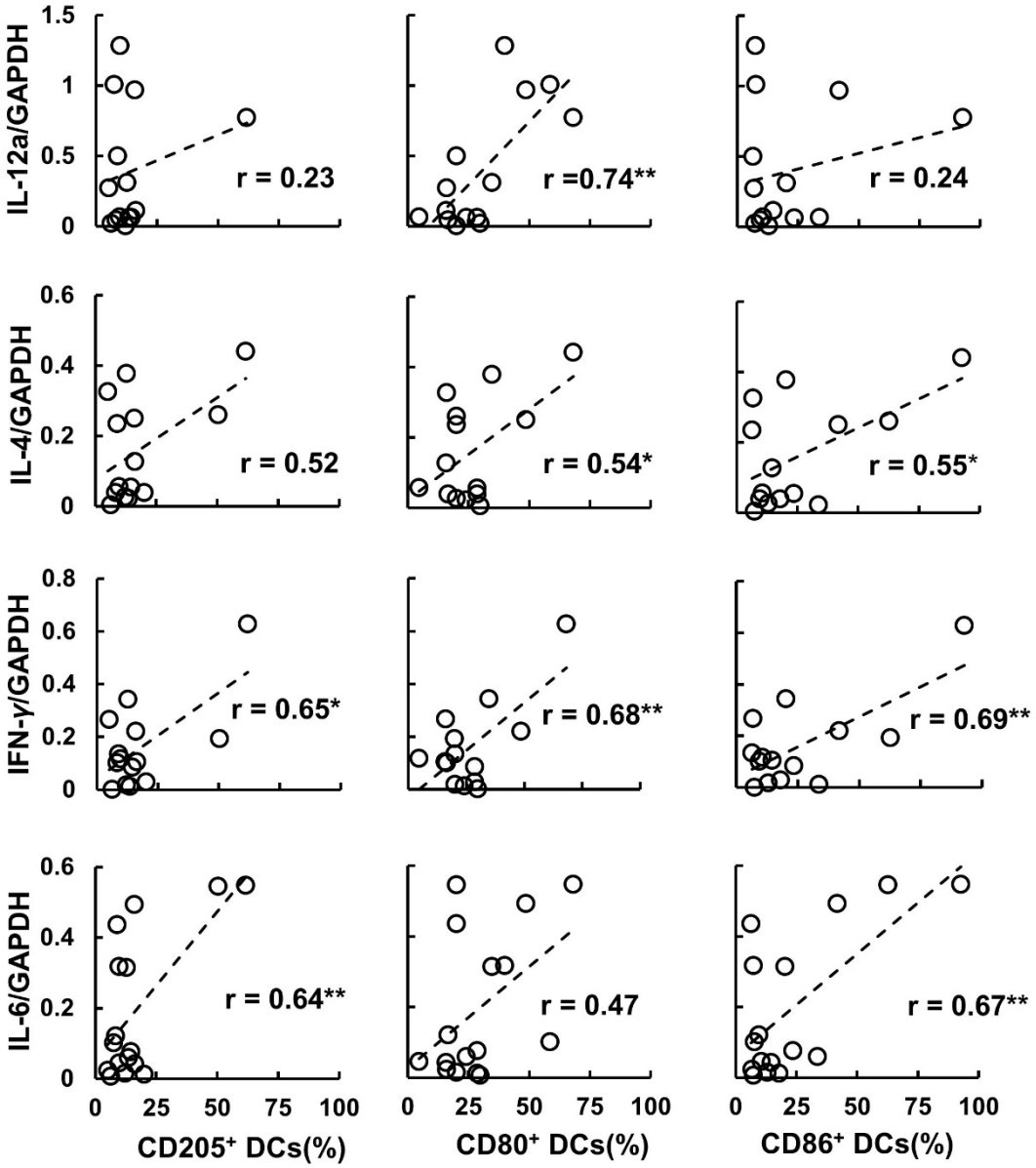

Cell nucleus
CD11c
CD172a/CD11c
Negative selection
CD172a
MHC class II
CD172a/MHC class II
Cell nucleus
CD11c
CD172a/CD11c
Positive selection
CD172a
MHC class II
CD172a/MHC class II

509 **Figure 3**



510

511 **Figure 4**



512

513 和文抄録

514

515 新規精製法によるウシ末梢血樹状細胞の分娩前における表現型と機能の解析

516

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529

530 樹状細胞（DC）は抗原提示細胞であり、自然免疫および適応免疫応答において
531 重要な役割を果たす。ウシ末梢血樹状細胞は、CD172a^{+}/CD11c^{+}/MHC class II^{+}を
532 発現するが、末梢血単核球中に 0.1～0.7%しか存在しないために、妊娠中の免
533 疫寛容性維持に関する表現型および機能は未だ解明されていないのが現状であ
534 る。本研究では、磁気細胞分離装置（MACS）を用いてウシ末梢血単核球から樹

535 状細胞の精製を試み、純度 84.8%の MHC class II および CD40 を高発現する
536 CD172a^{+}/CD11c^{+}樹状細胞を精製する新規法を確立した。分娩前末梢血中樹状細胞
537 では、抗原取込みに関わる CD205 および共刺激分子 CD80 と CD86 の発現には多
538 様性があったが、共刺激分子の発現強度とサイトカイン発現には正の相関があ
539 ることを発見した。また、共刺激分子およびサイトカインの発現が低い樹状細
540 胞も存在して免疫寛容の維持に関わると考えられた。本研究成果によって、分
541 娩前のウシ末梢血中では、低サイトカイン産生能の未成熟な樹状細胞と、高い
542 共刺激分子発現して T 細胞を調節する能力を持つ成熟した樹状細胞が混在する
543 ことにより、免疫寛容性を維持していることが示唆された。