# Functional characterization of bovine peripheral blood dendritic cells before and after parturition by a novel purification method

# Functional characterization of bovine peripheral blood dendritic cells before and after parturition by a novel purification method

（新規精製法による分娩前後における
ウシ末梢血樹状細胞の機能的特性解析）

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# Chapter 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). They express higher levels of major histocompatibility complex (MHC) class II and accessory molecules on their surface than other professional APCs. DCs also play an important role in the innate and adaptive immune response (Banchereau & Steinman, 1998). It was known that once a mature DC presented antigen to T cells, in the context of co-stimulatory molecules and cytokines, the net result was induction of T cell activation and proliferation. It also indicates that a mature DC phenotype is required for the induction and maintenance of tolerance (Joffre et al., 2009; Akbari et al., 2001). 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.

Peripheral blood DCs have been divided into two main subsets: conventional DC (cDC) and plasmacytoid DC (pDC). cDC are known to efficient naive T cell stimulators by presenting antigenic peptides to T cells in the context of MHC molecules. cDC also produce pro-inflammatory cytokines, which have potent downstream immune stimulatory function (Banchereau & Steinman, 1998). pDC have been shown to produce large amounts of type I interferons (IFN) that limit virus spread, enhance antigen presentation, and increase cytotoxic function (Siegal et al., 1999; Megjugorac et al., 2004; Fitzgeraldbocarsly, 1993).

Some studies have investigated the phenotype and of bovine peripheral blood DCs (Miyazawa et al., 2006; Gibson et al., 2012; Renjifo et al., 1997; Reid et al., 2011). In these studies, enrichment protocols were utilized to deplete non-DCs. While the DCs population is enriched, a major limitation of this approach is the difficulty of entirely depleting other cell types, thus reducing the overall purity of the DC yield. Consequently, careful interpretation should be exercised when attributing DC immuno-phenotype and functions to DC enriched populations.

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 is a C-type lectin receptor (CLR) that can function as an endocytic receptor (Jiang et al., 1995). And 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). 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). Cytotoxic T Lymphocyte-associated Antigen-4 (CTLA-4) interacts with B7 molecules to inhibit T cell activation and induce T cell anergy by competitive antagonism of CD28:B7 mediated co-stimulation. However, the complete absence of CTLA-4 results in unrestricted activation of T cells (Carreno et al., 2000; Carter & Carreno, 2003).

Naive T cell activation requires signals from the co-stimulatory molecules and cytokines provided by the mature DCs (Greenwald et al., 2005). Depending on the type of infection and antigen presented, T helper (Th) cells will develop into immune phenotypes that are characterized by their transcription factors, cytokine profiles, and effector functions. Upon encountering foreign antigens and costimulatory molecules presented by DCs, naive $CD4^+$ T cells can differentiate into Th1, Th2, Th17 and Regulatory T (Treg) cells. These differentiation programs are mainly shaped by cytokines produced by DCs and are characterized by the expression of lineage-specific transcription factors and production of signature cytokines. IL-12 is important for the differentiation of Th1 cells with an important function in host defense against intracellular pathogens. $CD8\alpha^+$ DCs have been shown to drive the development of Th1-type immune response in vivo in an IL-12 and IFN-$\gamma$ depending manner, whereas $CD8\alpha^-$ DCs induce Th2-type responses which play a crucial role in anti-parasite and humoral immunity with IL-4 and IL-10 (Coquerelle & Moser, 2010). TGF-$\beta$ together with IL-6 and IL-23 instruct naive T cells to develop into Th17 cells that mediate immunity against fungi and extracellular bacteria. In the absence of inflammatory cytokines, TGF-$\beta$ promotes naive T cells to differentiate into Foxp3-expressing Treg cells for the maintenance of immune tolerance (Huang et al., 2012).

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). 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-$\gamma$ occurring after parturition and interleukin (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). Treg as 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, TNF-$\gamma$, 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.

In recent years, *Bacillus subtilis* C-3102 strain (BS) has been widely used in livestock as feed probiotics additives (Hatanaka et al., 2012; He et al., 2013; Jeong & Kim, 2014; Kritas et al., 2015; Michiels et al., 2016), but there were few reports studying on dairy cattle. In our laboratory, the proportion of $CD172a^{+}/CD11c^{+}$ DCs in bovine peripheral blood decreased in mastitis, and that the feeding of *Bacillus subtilis* C-3102 strain (BS) prevented the cattle from mastitis (Urakawa et al. 2013). It was thought that there was a relationship between the DCs and mastitis. DCs present in the bovine mammary gland are hypothesized to play an important role in defense against invading pathogens (Maxymiv et al., 2012). However, the mechanism of mastitis prevention by DCs has not been described clearly in dairy cattle feeding a diet with *Bacillus subtilis* probiotics.

Bovine DCs function also has not been characterized during the parturition despite their important role in both orchestrating innate and adaptive immune activation and inducing tolerance. The object of the study is to analyze the phenotypic and functional characterization of bovine peripheral blood DCs before and after parturition by BS-feeding comparing the control group. Before this, as the population of DCs is less than 5% in bovine peripheral blood mononuclear cells (PBMC), it is needed to isolate highly purified DCs

subpopulations in sufficient numbers. Therefore, a novel method of two-step Magnetic-activated cell sorting (MACS) for bovine peripheral DCs must be established. After the purification, the expressions of surface markers (MHC II, CD205, CD40, CD80 and CD86) on DCs were determined using flow cytometry, and the expressions of a number of cytokines (IL-12a, IL-12b, IFN-$\gamma$, IL-4, IL-10, TGF-$\beta$ and IL-6) were analyzed by qRT-PCR. This study provides the evidence for immune regulation of bovine DC populations before and after parturition.

# Chapter 2

# Purification and surface marker analysis of bovine peripheral blood dendritic cells

## 2.1 Introduction

It is well known that once a mature DC presented antigen to T cells, in the context of co-stimulatory molecules and cytokines, the net result was induction of T cell activation and proliferation. Recent evidence indicates that a mature DC phenotype is also required for the induction and maintenance of tolerance(Joffre et al., 2009; Akbari et al., 2001). 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). A few studies have investigated the phenotype and of bovine peripheral blood DCs (Miyazawa et al., 2006; Gibson et al., 2012; Renjifo et al., 1997; Reid et al., 2011). In these studies, enrichment protocols were utilized to deplete non-DCs.

The object of this chapter is to analyze the phenotypic and functional characterization of bovine peripheral blood DCs before parturition. As the population of DCs is less than 5% in bovine PBMC, it is needed to isolate highly purified DCs subpopulations in sufficient numbers. Therefore, a novel method of two-step MACS for bovine peripheral DCs must be established. After the purification, the expressions of surface markers (MHC II, CD205, CD40, CD80 and CD86) on DCs were determined using flow cytometry.

## 2.2 Materials and Methods

### 2.2.1 Animals

Sixteen Holstein Friesian cows, 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.

### 2.2.2 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). Peripheral blood mononuclear cells (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 ℃. 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 ℃.

### 2.2.3 Purification of peripheral blood dendritic cells

The anti-bovine antibodies in this study were purchased from WSU (Pullman WA, AL, 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 dendritic cells were negatively selected using Auto MACS magnetic columns (Miltenyi Biotec). After negative selection, $CD3^-$/$sIgM^-$/$CD14^-$/$Granulocytes^-$ cells were incubated with anti-bovine CD172a antibodies (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.

2.2.4 Flow cytometry

In order to detect bovine dendritic cells, 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 (diluted 1/200) 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 mouse anti-bovine CD3, sIgM, CD14 or Granulocytes antibody for confirming 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 fluorescent antibodies were selected as controls.

2.2.5 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 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 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 then 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.

### 2.2.6 Statistical Analysis

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

## 2.3 Results

### 2.3.1 Purification of bovine peripheral blood dendritic cells

Figure 1 showed the purification process of bovine peripheral blood DC. The expressions of the surface molecules such as CD172a, CD11c, and MHC class II, such as specific markers of DCs, 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, it was necessary 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 dendritic cells, 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, the DCs was purified $CD172a^{+}/CD11c^{+}$ cells from the negative-selected cells. The positive selection with CD172a antibody revealed that the purity of $CD172a^{+}/CD11c^{+}$

dendritic cells was 84.8%, and that they also expressed MHC class II strongly.

2.3.2 Photographs of bovine peripheral blood dendritic cells

Peripheral blood dendritic cells after the negative and positive selections were stained with mouse 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, $CD172a^{-}/CD11c^{-}$/MHC class $II^{-}$ non-DC cells 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.

2.3.3 Phenotypic analysis of bovine peripheral blood $CD172a^{+}/CD11c^{+}$ dendritic cells 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 $CD172a^{+}/CD11c^{+}$ dendritic cells 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 the parturition, there were strong correlations between the percentage of CD86 and the percentages of CD80 or CD205 on $CD172a^{+}/CD11c^{+}$ DCs (Fig.3 B).

## 2.4 Discussion

In this study, a novel purification method for bovine peripheral blood DCs was established. The phenotype of the DCs was also characterized. 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-collected 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, it was 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. 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 (Fig.3). The majority of the DCs expressed the molecules of MHC class II and CD40. It has been reported that CD205 can lead to tolerance in the steady-state immunity after DC maturation (Bonifaz et al., 2002). It is well known that CD205 has been expressed on many dendritic cells in the T cell areas of lymphoid tissues(Gliddon et al., 2004). Therefore, a part of bovine peripheral blood DCs before parturition might have differentiated into activated DCs with high CD205.

In this study, before the parturition there were strong correlations in $CD172a^{+}/CD11c^{+}$ DCs between the CD86 expression and the expressions of CD80, as well as CD205. Therefore, the 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.

## 2.5 Summary

The results in this chapter suggest:

1) A novel method for the purification of DCs from PBMC was established by using two-step MACS, and the $CD172a^+/CD11c^+$ DCs with high expression of MHC class II and CD40 were purified at 84.8% purity.

2) Cell surface markers as MHC class II and CD40 were expressed on almost $CD172a^+/CD11c^+$ DCs, and the individual differences existed in the expressions of CD205, CD80 and CD86 molecules.

3) Before parturition, there were strong correlations between the percentage of CD86 and the percentages of CD80 and CD205 on DCs. It suggested 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.

# Figures and Tables

# Table 1 Antibodies used in this study

| Antibodies | Isotype | Clone | Supplier |
|---|---|---|---|
| CD3 | IgG1 | MM1A | WSU |
| surface IgM | IgG1 | IL-A30 | Bio-Rad |
| CD14 | IgG1 | CAM36A | WSU |
| Granulocytes | IgM | CH138A | WSU |
| CD172a | IgG1 | DH59B | WSU |
| CD11c | IgM | BAQ153A | WSU |
| MHC II | IgG2a | TH14B | WSU |
| CD205 | IgG2a | ILA53A | WSU |
| CD40 FITC | IgG1 | IL-A156 | Bio-Rad |
| CD80 FITC | IgG1 | IL-A159 | Bio-Rad |
| CD86 FITC | 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 |

# Figure 1

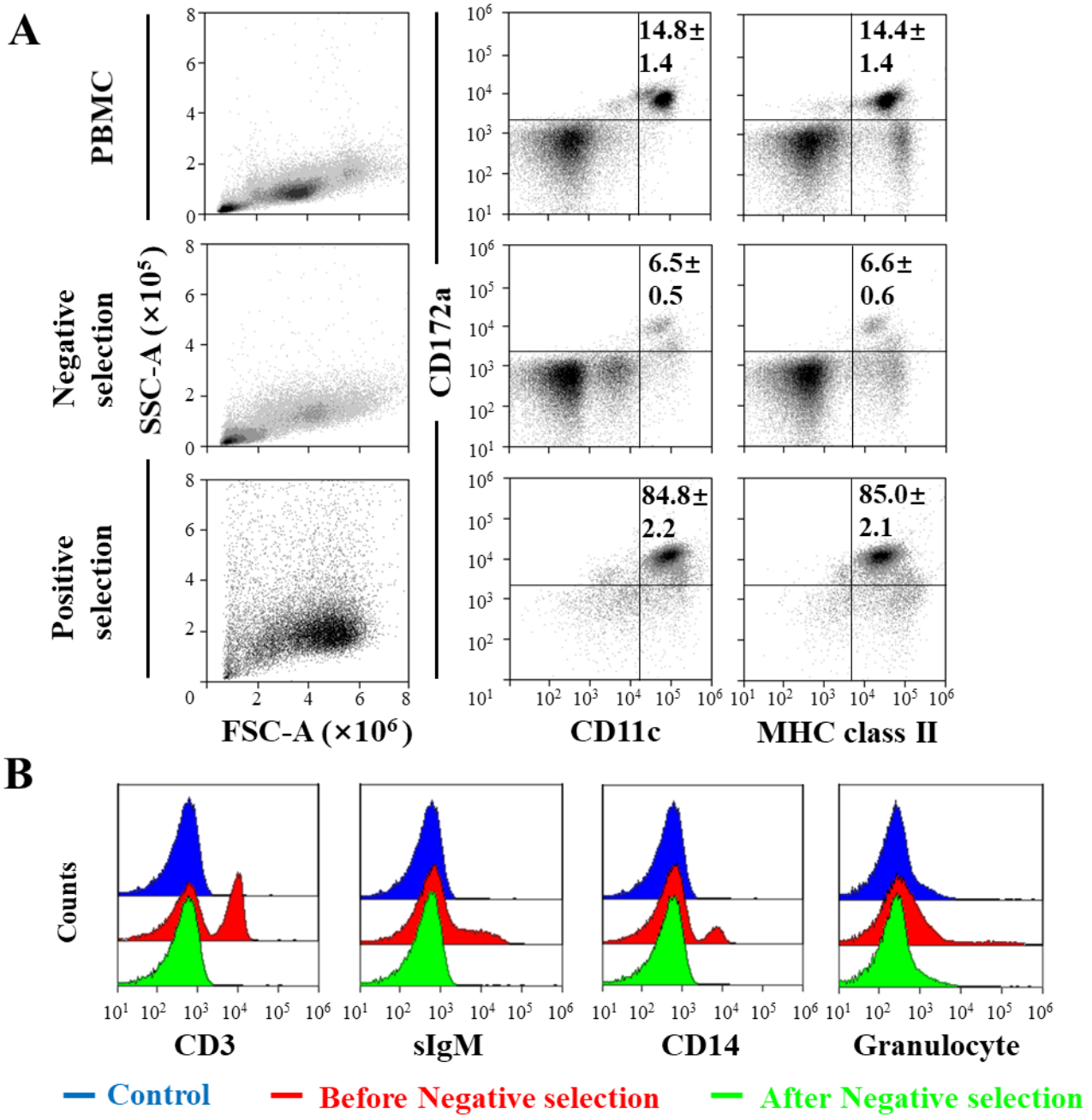
A
PBMC
Negative selection
Positive selection
SSC-A (×10⁵)
FSC-A (×10⁶)
CD172a
CD11c
MHC class II
14.8± 1.4
14.4± 1.4
6.5± 0.5
6.6± 0.6
84.8± 2.2
85.0± 2.1
B
Counts
CD3
sIgM
CD14
Granulocyte
Control
Before Negative selection
After Negative selection

**Fig.1 Purification of bovine peripheral blood dendritic cells.**

T cells, B cells, monocytes and granulocytes were removed from PBMC by the negative selection using 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 expressions 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.

# Figure 2

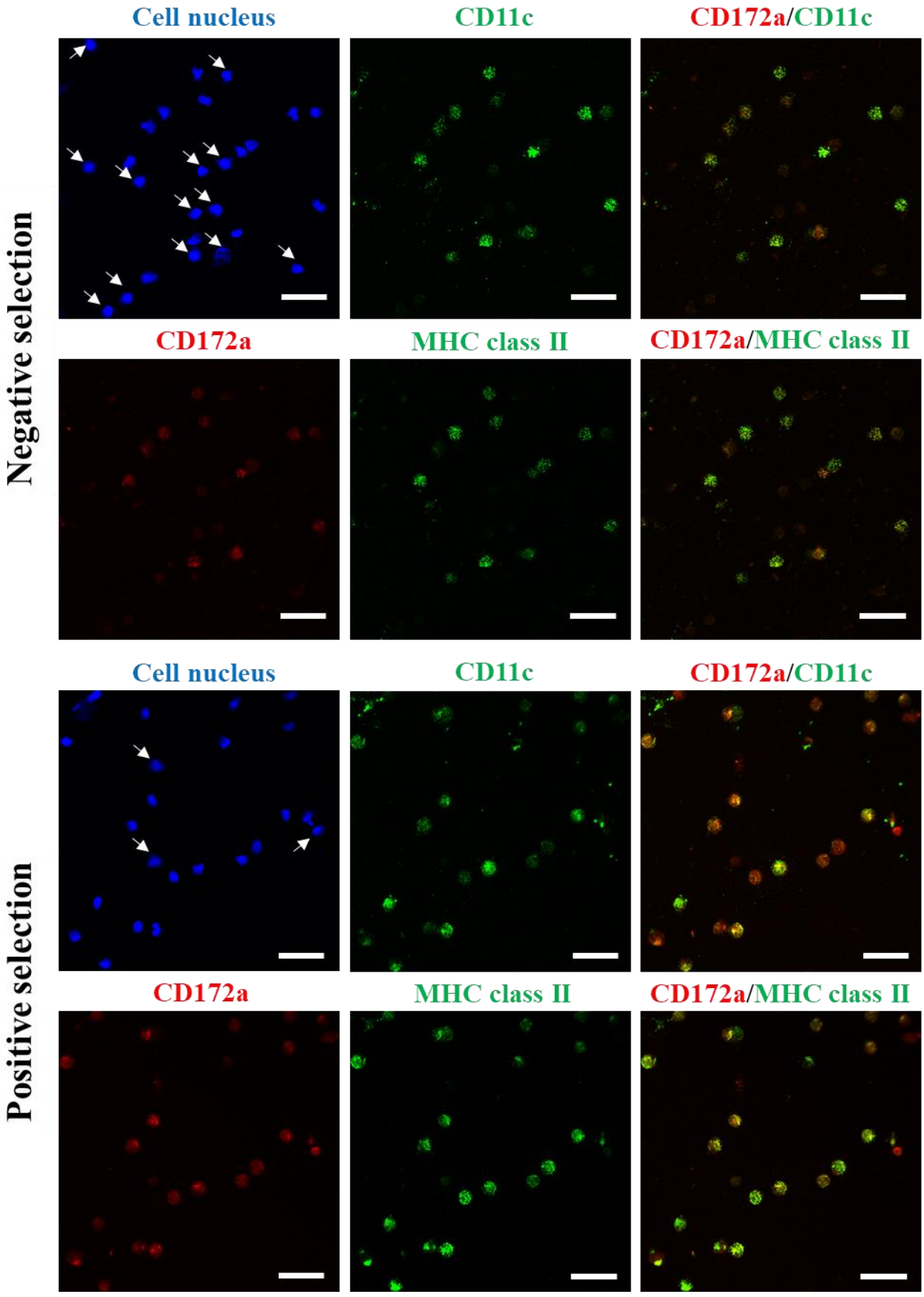
Negative selection
Cell nucleus
CD11c
CD172a/CD11c
CD172a
MHC class II
CD172a/MHC class II
Positive selection
Cell nucleus
CD11c
CD172a/CD11c
CD172a
MHC class II
CD172a/MHC class II

**Fig.2 Photographs of peripheral blood dendritic cells.**

Peripheral blood dendritic cells 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 showed the unstained cells by CD172a and CD11c. Bars: 50 μm.

**Figure 3**

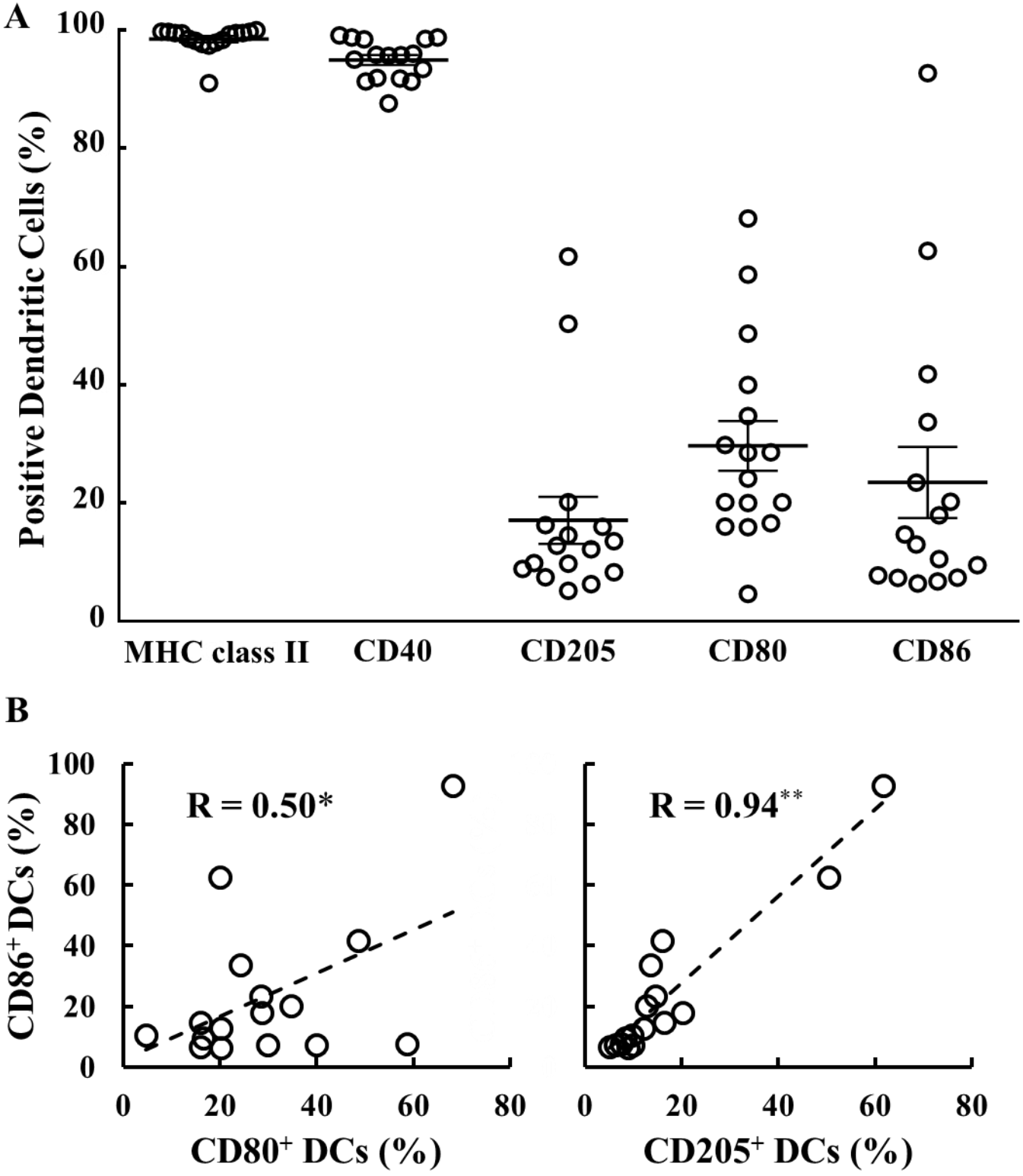
A
Positive Dendritic Cells (%)
100
80
60
40
20
0
MHC class II
CD40
CD205
CD80
CD86
B
CD86+ DCs (%)
R = 0.50*
R = 0.94**
CD80+ DCs (%)
CD205+ DCs (%)
0
20
40
60
80

**Fig.3 Phenotypic characterization of bovine peripheral blood CD172a$^{+}$/CD11c$^{+}$ dendritic cells before parturition.**

After the negative selection, the surface expressions of MHC class II, CD40, CD205, CD80 or CD86 on DCs were analyzed on CD172a$^{+}$/CD11c$^{+}$ dendritic cells (A). Figure B showed the correlation between the percentage of CD86 and the percentage of CD80 or CD205 on DCs. *: $p<0.05$, **: $p<0.01$

# Chapter 3

# Effect of *Bacillus subtilis* C-3102 stain as feed probiotics additives on surface markers of bovine peripheral blood dendritic cells before and after parturition

## 3.1 Introduction

In our laboratory, the proportion of $CD172a^+/CD11c^+$ DCs in bovine peripheral blood decreased in mastitis, so it was thought that there was a relationship between the DCs and mastitis. Besides, the feeding of *Bacillus subtilis* C-3102 strain (BS) prevented the cattle from mastitis (Urakawa et al. 2013), but the reason was not clarified.

Bovine peripheral blood DCs were identified as a lineage negative of $CD172a^+/CD11c^+$/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). The phenotype of the DCs in bovine peripheral blood has not been described before and after parturition.

In this chapter, cattle were fed with or without BS probiotics for two months before and after parturition. After the MACS negative selection, the proportion of the $CD172a^+/CD11c^+$ DCs in the peripheral blood and the expressions of surface markers such as MHC Class II, CD205, CD40, CD80 and CD86 on DCs were analyzed by flow cytometry.

## 3.2 Materials and Methods

### 3.2.1 Animals

Twenty Holstein cows, 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.

### 3.2.2 *Bacillus subtilis* feeding

The cows were divided into the control-feeding group with ten cows and the *Bacillus subtilis* (BS)-feeding group with the other ten cows. The study applied *Bacillus subtilis* C-3102 stain ($1.5\times10^8$ CFU/g). The BS-feeding group started the feeding one month prior to the parturition and ended one month past the parturition. The cows were individually fed twice a day in the morning and evening, each with 20 g. Water was fed ad libitum.

### 3.2.3 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, CA) 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.

### 3.2.4 Purification of peripheral blood dendritic cells

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 2). 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).

### 3.2.5 Flow cytometry

In order to detect bovine DCs, negative-selected cells were stained with mouse anti-bovine CD172a (diluted 1/50) and CD11c (diluted 1/500) antibodies. These cells were also incubated with mouse anti-bovine MHC class II, CD40, CD205, CD80 and CD86 antibodies, and treated with

secondary fluorescent antibodies for 30 min on ice in the dark. After the treatment of secondary fluorescent antibodies in Table 2, 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 fluorescent antibodies were selected as controls.

### 3.2.6 Statistical Analyses

Values are reported as means ± SD. Statistical analyses were performed using software GraphPad 6.00 program (GraphPad software Inc., CA, USA). Differences between results were tested with a paired, two-tailed, Student's t-tests. The correlation between two parameters was analyzed by Pearson correlation coefficient test (*: $p<0.05$, **: $p<0.01$).

## 3.3 Results

### 3.3.1 Proportion of bovine peripheral blood CD172a$^+$/CD11c$^+$ dendritic cells before and after parturition

Holstein cows were fed with or without probiotics (*Bacillus subtilis* C-3102 strain) from one month before parturition to one month after parturition. The peripheral bloods were obtained from the cattle at one month before and after parturition, respectively. After the MACS negative selection, the percentages of the CD172a$^+$/CD11c$^+$ DCs in the peripheral blood were determined by the flow cytometry.

As shown in Figure 4, the data had been transferred into the increase rate. The increase rate of bovine peripheral blood CD172a$^+$/CD11c$^+$ DCs did not change in the control-feeding group before and after parturition. However, the BS-feeding significantly increased ($p$<0.01) the proportion of CD172a$^+$/CD11c$^+$ DCs after parturition.

### 3.3.2 Comparison of surface molecules expressed on bovine peripheral blood CD172a$^+$/CD11c$^+$ dendritic cells before and after parturition

After the MACS negative selection, bovine peripheral blood DCs were determined with the anti-bovine CD172a and CD11c antibodies on a three-color flow cytometry. The rest surface molecules were analyzed with the corresponding antibodies. Meanwhile, as shown in Figure 5, no differences of the proportion of MHC class II, CD40, CD205 and CD86 positive cells before and after parturition were detected in both the control- and BS-feeding

groups. However, the proportion of CD80 positive cells of DCs remained unchanged in the control-feeding group throughout the parturition while that in the BS-feeding group significantly increased ($p<0.05$) after parturition (Fig.5). Therefore, *Bacillus subtilis* feeding significantly increased the proportion of DCs and the expression of the surface co-stimulatory molecule CD80 compared to that in the control-feeding group.

Based on the percentages of CD205, CD86, and CD80 expressions, the correlations among the surface molecules before and after parturition were shown in Figure 6. As previously elaborated in chapter 2 (Fig.3 B), before the parturition, there were strong correlations between the percentage of CD86 and the percentages of CD80 and CD205 on DCs. After the parturition, the strong correlations remained in the control-feeding group. However, in the BS-feeding group, although the strong correlation between CD80 and CD86 was found, the correlation between CD86 and CD205 disappeared (Fig.6).

## 3.4 Discussion

In this chapter, the unique profiles in bovine blood DCs were identified at specific stages of gestation and lactation that suggest greatest alterations to these cell populations occurs over the late gestation. Bovine blood nonclassical monocytes (ncM) were identified as $CD172a^{+}/CD14^{-}/CD16^{+}$ cells (Hussen et al., 2013), and the $CD172a^{+}/CD11c^{+}/CD14^{-}$ DCs in this study could be considered as a population of these cells, and the proportion of ncM in bovine blood also increased from the late dry period in late gestation to the post-calving period (Pomeroy et al., 2016). As shown in Figure 4, the BS-feeding significantly increased ($p<0.01$) the proportion of $CD172a^{+}/CD11c^{+}$ DCs after parturition. It was found that the proportion of $CD172a^{+}/CD11c^{+}$ DCs in bovine peripheral blood decreased in mastitis, and the feeding of *Bacillus subtilis* C-3102 strain prevented the cattle from mastitis (Urakawa et al., 2013). Therefore, it was considered that the BS-feeding could prevented from mastitis by rising the proportion of $CD172a^{+}/CD11c^{+}$ DCs in bovine peripheral blood.

As shown in Figure 5, no differences of the proportions of MHC class II, CD40, CD205 and CD86 positive cells were detected in both groups before and after parturition. MHC class II and CD40 were expressed on the DC surface at a high expression in the two groups, while the CD205 and CD86 expressions were lower than them. However, the proportion of CD80 positive cells of DCs remained unchanged in the control-feeding group throughout the parturition while that in the BS-feeding group significantly increased

($p<0.05$) after parturition (Fig.5). 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). As CD80 high-positive DCs well induced IL-12a, there might be an autocrine effect of IL-12a on DCs maturation in the BS-feeding group (Fig. 5).

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 and Sallusto, 2001) and co-stimulatory molecules CD80 and CD86 (Mellman and 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 and Buddle, 2008). $CD205^{+}$ DCs can also induce tolerance if they are not activated but induce T cell activation following activation through CD40 which up-regulates CD86 (Bonifaz et al., 2002). However, in the BS-feeding group, although the strong correlation between CD80 and CD86 was found, the correlation between CD86 and CD205 disappeared (Fig.6). These data indicated that the DCs in BS-feeding group could be activated DCs with the high CD80 expression after parturition.

Therefore, in this chapter, BS-feeding significantly increased the proportion of DCs and the expression of the surface co-stimulatory molecule CD80 and it suggested that the BS-feeding has induced the differentiation of immature DCs into activated DCs.

## 3.5 Summary

The results in this chapter indicated:

1) *Bacillus subtilis* feeding increased the proportion of bovine peripheral blood dendritic cells after parturition.

2) *Bacillus subtilis* feeding significantly increased the proportion of CD80 positive cells of dendritic cells, which is believed to induce the differentiation of immature DCs into activated DCs compared to that in the control-feeding group after parturition.

# Figures and Tables

# Table 2 Antibodies used in this study

| Antibodies | Isotype | Clone | Supplier |
|---|---|---|---|
| CD3 | IgG1 | MM1A | WSU |
| surface IgM | IgG1 | IL-A30 | Bio-Rad |
| CD14 | IgG1 | CAM36A | WSU |
| Granulocytes | IgM | CH138A | WSU |
| CD172a | IgG2b | CC149 | Bio-Rad |
| CD11c | IgM | BAQ153A | WSU |
| MHC II | IgG2a | TH14B | WSU |
| CD205 | IgG2a | ILA53A | WSU |
| CD40 FITC | IgG1 | IL-A156 | Bio-Rad |
| CD80 FITC | IgG1 | IL-A159 | Bio-Rad |
| CD86 FITC | IgG1 | IL-A190 | Bio-Rad |
| Control | Mouse IgG1 | COLIS69A | WSU |
| Control | Mouse IgM | COLIS52A2 | WSU |
| Control | Mouse IgG2a | COLIS205C | WSU |
| Control | Mouse IgG2b | COLIS169A | WSU |
| FITC IgG2a-secondary ab | Goat anti Mouse | | SouthernBiotech |
| PE IgG2b-secondary ab | Goat anti Mouse | | SouthernBiotech |
| Alexa Flour 647 IgM-secondary ab | Goat anti Mouse | | SouthernBiotech |
| IgG1 Micro Beads ab | Rat anti Mouse | | Miltenyi Biotec |
| IgM Micro Beads ab | Rat anti Mouse | | Miltenyi Biotec |

# Figure 4

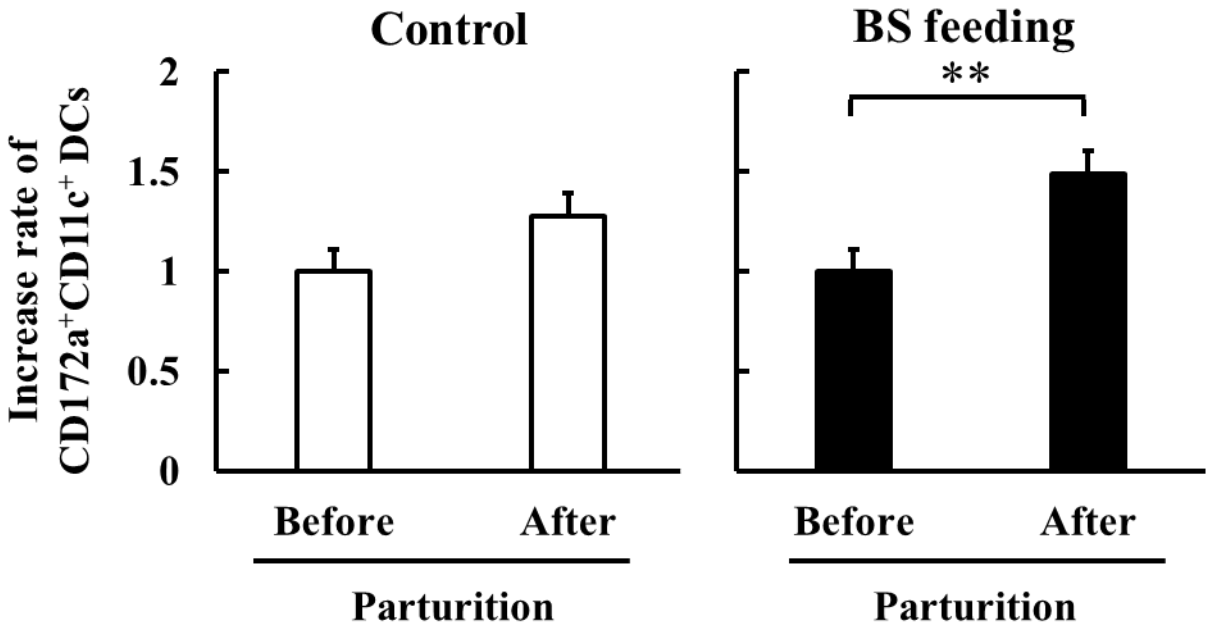


**Fig.4 Increase rate of bovine peripheral blood CD172a$^+$/CD11c$^+$ dendritic cells before and after parturition.**

Holstein cows were fed with or without probiotics (*Bacillus subtilis* C-3102 strain) from a month before parturition to a month after parturition. The peripheral bloods were obtained at a month before and after parturition. The percentages of CD172a$^+$/CD11c$^+$ dendritic cells in the negative-selected cells were determined using flow cytometry. **: $p<0.01$

**Figure 5**

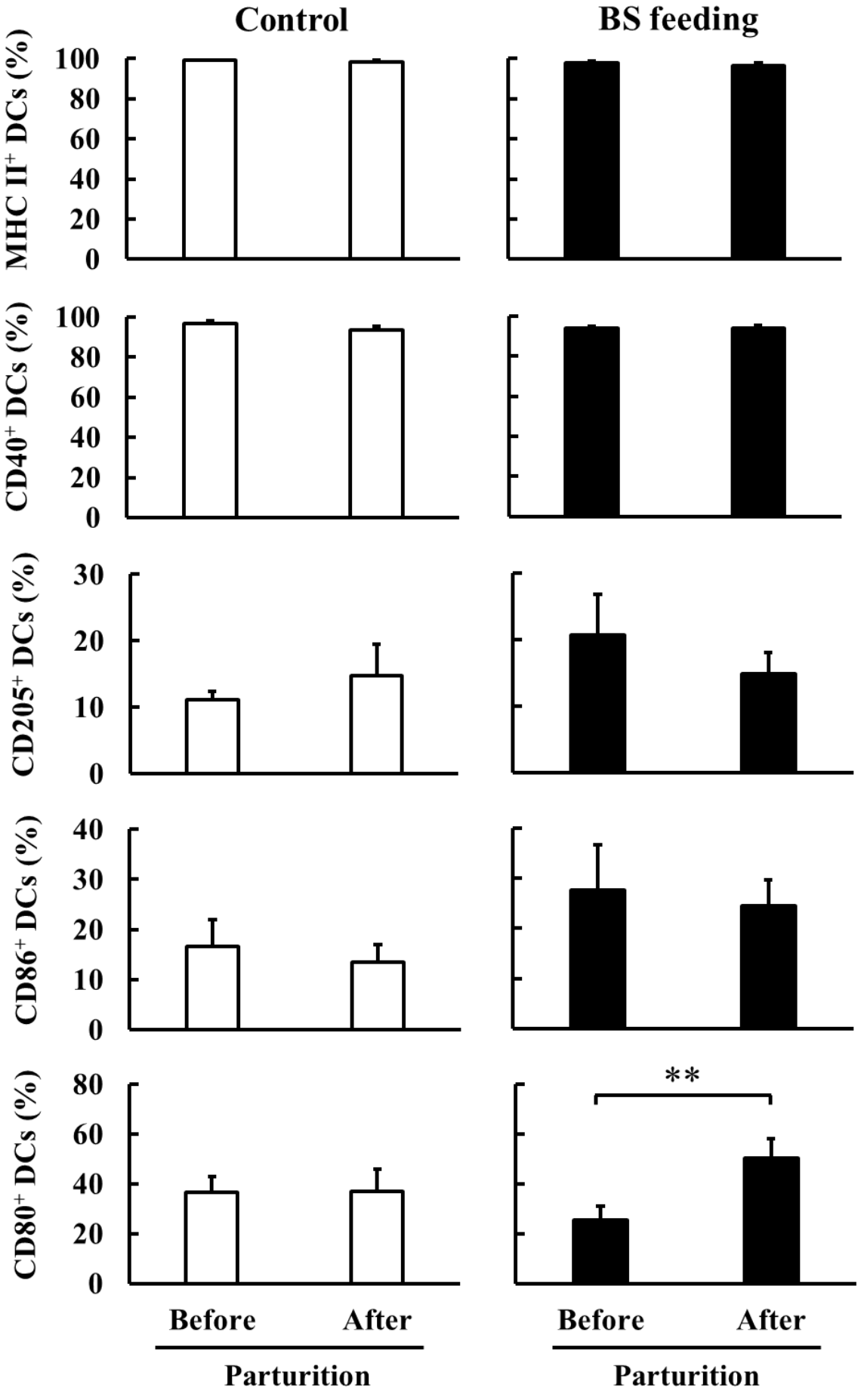
Control
BS feeding
MHC II+ DCs (%)
CD40+ DCs (%)
CD205+ DCs (%)
CD86+ DCs (%)
CD80+ DCs (%)
100
80
60
40
20
0
30
10
**
Before
After
Parturition

**Fig.5 Comparison of surface molecules expressed on bovine peripheral blood dendritic cells before and after parturition.**

Holstein cows were fed with or without probiotics (*Bacillus subtilis* C-3102 strain) from a month before parturition to a month after parturition. The peripheral bloods were obtained at a month before and after parturition. After the negative selectin, the expression percentage of MHC class II, CD205, CD40, CD86 and CD80 were observed in the $CD172a^{+}/CD11c^{+}$ dendritic cells before and after parturition by flow cytometry. **: $p<0.01$

# Figure 6

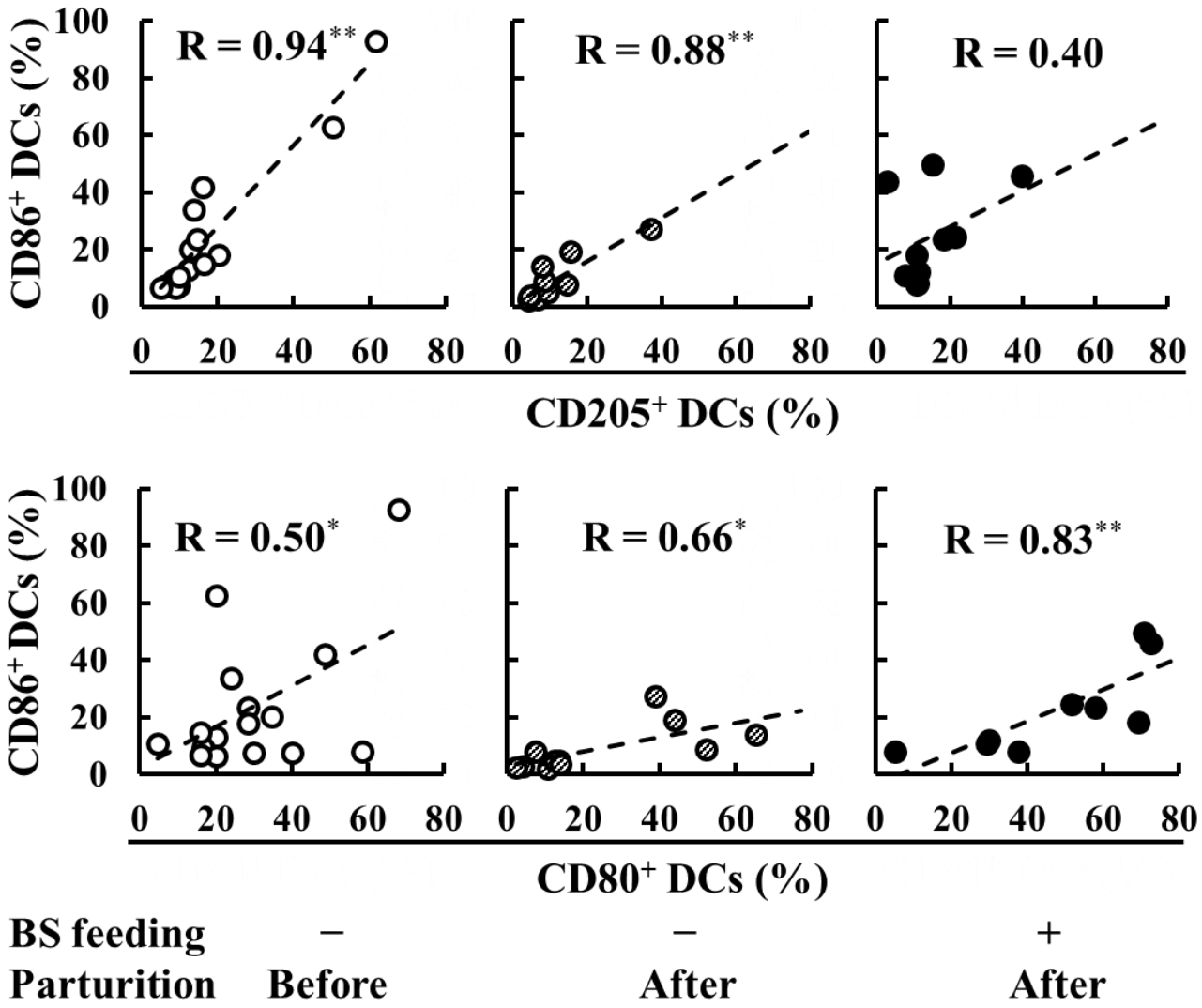
R = 0.94**
R = 0.88**
R = 0.40
R = 0.50*
R = 0.66*
R = 0.83**
CD86+ DCs (%)
CD205+ DCs (%)
CD80+ DCs (%)
0
20
40
60
80
100
BS feeding − − +
Parturition Before After After

**Fig.6 Relationship between CD86 expressions and the expression of CD80 or CD205 in bovine peripheral blood CD172a$^+$/CD11c$^+$ dendritic cells before and after parturition.**

After the negative selection, the expression of CD205, CD80 or CD86 was analyzed on bovine peripheral blood CD172a$^+$/CD11c$^+$ dendritic cells (Figure 5). Using these data, the correlations between the percentage of CD86 and the percentage of CD80 or CD205 in DCs were showed before and after parturition. *: $p<0.05$, **: $p<0.01$

# Chapter 4

# Effect of *Bacillus subtilis* C-3102 stain as feed probiotics additives on cytokine gene expression and its correlation with surface markers of bovine peripheral blood dendritic cells before and after parturition

## 4.1 Introduction

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). Once a mature DC presented antigen to T cells, in the context of co-stimulatory molecules and cytokines, the net result was induction of T cell activation and proliferation.

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, TNF-ɤ, IL-6) (Lutz & Schuler, 2002). Previous works 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.

In this chapter, cattle were fed with or without BS probiotics for two months before and after parturition. After the purification, the expression of surface marker CD80 on DCs was analyzed by flow cytometry, and the gene expressions of cytokines such as IL-12a, IL-12b, IFN-$\gamma$, IL-4, IL-10, TGF-$\beta$ and IL-6 of DCs were analyzed by qRT-PCR.

## 4.2 Materials and Methods

### 4.2.1 Animals

Twenty Holstein cows, 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.

### 4.2.2 *Bacillus subtilis* feeding

The cows were divided into the control-feeding group with ten cows and the *Bacillus subtilis* (BS)-feeding group with the other ten cows. The study applied *Bacillus subtilis* C-3102 stain ($1.5\times10^8$ CFU/g). The BS-feeding group started the feeding one month prior to the parturition and ended one month past the parturition. The cows were individually fed twice a day in the morning and evening, each with 20 g. Water was fed ad libitum.

### 4.2.3 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, CA) gradient centrifuged at 600 g for 30 min at 18°C. PBMC was 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.

4.2.4 Purification of peripheral blood dendritic cells

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 3). 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. $CD3^-$/$sIgM^-$/$CD14^-$/$Granuloytes^-$ cells containing dendritic cells were negatively selected using Auto MACS magnetic columns (Miltenyi Biotec). 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 (Miltenyi Biotec).

4.2.5 Flow cytometry

In order to detect bovine DCs, negative-selected cells were stained with mouse anti-bovine CD172a (diluted 1/500) and CD11c (diluted 1/500) antibodies. These cells were incubated with mouse anti-bovine CD80 antibody, and then treated with secondary fluorescent antibodies for 30 min on ice in the dark. After the treatment of secondary fluorescent antibodies in Table 3, 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 fluorescent antibodies were selected as controls.

4.2.6 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). 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, Carlsbad, CA) at 50°C for 1 hour, and then at 70°C for

15 min.

One μL cDNA sample, 7 μL SYBR Green Premix Taq (Takara Bio Inc., Japan), 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 4 (Takara Bio Inc., Japan). The transcripts using the bovine peripheral blood DCs cDNA were amplified with the Thermal Cycler Dice Real Time System Single (Takara Bio Inc., Japan): 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.

### 4.2.7 Statistical Analyses

Values are reported as means ± SD. Statistical analyses were performed using software GraphPad 6.00 program (GraphPad software Inc. California USA). Differences between results were tested with a paired, two-tailed, Student's t-tests. The correlation between two parameters was analyzed by Pearson correlation coefficient test (*: $p<0.05$, **: $p<0.01$).

## 4.3 Results

### 4.3.1 Expressions of cytokines in bovine peripheral blood dendritic cells before and after parturition

Holstein cows were fed with or without probiotics (*Bacillus subtilis* C-3102 strain) from a month before parturition to a month after parturition. The peripheral bloods were respectively taken at one month before and after parturition. After the negative and positive selections, the expressions of cytokines in DCs were analyzed by qRT-PCR, and the results were expressed relative to GAPDH.

In the both groups, the gene expressions of IL-12a and IL-12b in DCs presented no differences before and after parturition in the both groups. The gene expression of IFN-$\gamma$ in the control-feeding group after parturition was significantly ($p<0.01$) higher than that before the parturition (Fig.7), but no difference with that in the BS-feeding group after parturition. Among the three groups, no differences in the gene expressions of IL-4 and IL-10 of DCs were detected (Fig.8). The gene expression of IL-6 did not vary after parturition. However, the gene expression of TGF-$\beta$ in the control-feeding group after parturition was significantly ($p<0.01$) higher compared to that one month prior to the parturition. But the gene expression of TGF-$\beta$ in BS-feeding group did not increase after parturition, which was significantly ($p<0.01$) lower than that in the control-feeding group (Fig.9).

In the control-feeding group, the gene expressions of IFN-$\gamma$ and TGF-$\beta$ significantly increased after parturition.

4.3.2 Relationships between expressions of cytokines and CD80 in bovine peripheral blood dendritic cells before and after parturition.

The expression of co-stimulatory molecule CD80 as a signal of activating T cells was added in this chapter. Based on the expressions of cytokines in DCs, the correlations between cytokine expressions and surface marker expression in DCs were analyzed and shown in Figure 10. There was a strong correlation ($p<0.01$) between IL-12a gene expression and CD80 positivity one month before parturition. However, after parturition, this correlation disappeared in the control-feeding group while remained in the BS-feeding group. This research also discovered the correlations ($p<0.01$ and $p<0.05$) between IFN-$\gamma$ and IL-4 gene expressions with CD80 positivity one month before parturition, which disappeared after parturition in either the control-feeding group or the BS-feeding group. Before parturition, TGF-$\beta$ and IL-6 gene expressions were neither found correlated with CD80 positivity, or in the control-feeding group after parturition. However, after parturition, a strong negative correlation ($p<0.05$) between TGF-$\beta$ with CD80 in the BS-feeding group was observed, whilst a strong positive correlation ($p<0.01$) between IL-6 with CD80 was identified.

Therefore, the BS feeding led to a negative correlation between TGF-$\beta$ and CD80, also making IL-12a and IL-6 strongly and positively correlated with CD80 positivity.

## 4.4 Discussion

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).

Interleukin 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). The previous report showed the characterization of higher Th2/regulatory immunity by the increases of IFN-$\gamma$ occurring after parturition and IL-4 production before calving (Paibomesai et al., 2013). While in the both groups, the gene expressions of IL-12a and IL-12b in DCs presented no differences before and after parturition (Fig.7), but after parturition, there was a strong correlation ($p<0.01$) between IL-12a gene expression and CD80 positivity in the BS-feeding group (Fig.10).

IL-12 induces IFN-$\gamma$ production in NK cells, T cells, and macrophages, and provides the necessary signal to direct Th1 differentiation following T cell activation (O'Garra & Murphy, 2009). The secretion of IL-12, IFN-$\gamma$ and IL-4 from DCs induced the development of T lymphocytes (Debecker et al., 1994), and the expression of CD80 in DCs increased after parturition which was shown in chapter 3 (Fig.5). Therefore, it was considered that DCs in BS-feeding group would rise the secretion of IL-12a after parturition, and there might be an autocrine effect of IL-12a on DCs maturation. A previous study indicates that bovine DCs in late gestation have reduced Th1-promoting

cytokine production compared with regulatory cytokine production (Pomeroy et al., 2015), the DCs may induce the tolerance before parturition and lead to Th1-promoting after parturition in the BS-feeding group. It also showed that cattle with the low CD80 expression would be a high IL-12a production in the next lactation period because of the positive correlation between CD80 and IL-12a. The BS-feeding induced the differentiation of immature DCs into activated DCs earlier after parturition than the control-feeding group.

Among the three groups, no differences in the gene expressions of IL-4 and IL-10 of DCs were detected (Fig.8). In the control-feeding group, the gene expressions of IFN-$\gamma$ and TGF-$\beta$ significantly increased after parturition (Fig.9).

Cytotoxic T Lymphocyte-associated Antigen-4 (CTLA-4) is an essential negative regulator of T cell immune responses. CTLA-4 also shares two ligands (CD80 and CD86) with a stimulatory receptor, CD28. It revealed a mechanism of immune regulation whereby CTLA-4 acts as an effect or molecule to inhibit CD28 co-stimulation by the cell-extrinsic depletion of ligands, accounting for many of the known features of the CD28-CTLA-4 system (Qureshi et al., 2011). Natural Treg cells may critically require CTLA-4 to suppress immune responses by affecting the potency of antigen-presenting cells to activate other T cells (Wing et al., 2008). Treg-specific CTLA-4 deficiency impairs in vivo and in vitro suppressive function of Tregs-in particular, Treg-mediated down-regulation of CD80 and CD86 expression on DCs (Maeda et al., 2014; Onishi et al., 2008).

The priming with DCs was strictly dependent on CD80／CD86, and CD80 was well known to induce naive T cells to become IL-4 producers (Debecker et al., 1994). The signaling molecule CD40 is required to induce immunogenic DCs and for the induction of IFNα (Martin et al., 2003, Le Bon et al., 2006). 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). Therefore, the DCs in control-feeding group were considered induced the tolerance as the high production of TGF-*β* and the negative correlation between CD80 and TGF-*β*. But the expression of TGF-*β* in BS-feeding group did not increase after parturition, which was significantly lower than that in the control-feeding group (Fig.9).

Interleukin 6 is the dominant factor to initiate the transcriptional program of Th17 cells (Heink et al., 2017). Th17 cells are differentiated by IL-6 in the presence of TGF-*β* which induces differentiation of Treg cells (Noack & Miossec, 2014). When IL-6 proinflammatory cytokine is added to naive $CD4^+$ T cells with TGF-*β*, Th17 cell differentiation is promoted more than with TGF-*β* alone due to reduced expression of Foxp3 which inhibits RORγt expression (Song et al., 2014; Keswani et al., 2016). In this chapter, the positive correlation between CD80 and IL-6 and the negative correlation of CD80 and TGF-*β* indicated that the DCs would induce the naive T cell into Th17 cells also with a lower expression of TGF-*β* (Fig.8) in the BS-feeding group. It also showed that cattle with the low CD80 expression would be a high IL-6 production in the next lactation period as the same as IL-12a.

Therefore, the DCs in the control-feeding group with the high expression

of TGF-$\beta$ would induce the tolerance, while the DCs in the BS-feeding group may already have the ability of modulating the T-cell linage involved in the removal of bacteria by the high expressions of CD80 and the cytokines of IL-12a and IL-6.

## 4.5 Summary

In this chapter the results demonstrate:

1) In the control-feeding group, gene expressions of IFN-$\gamma$ and TGF-$\beta$ of bovine peripheral blood dendritic cells significantly increased after the parturition. The DCs in the control-feeding group may lead the immunity towards tolerance.

2) In *Bacillus subtilis*-feeding group, the increase of TGF-$\beta$ gene expression was not observed after the parturition, but a strong negative correlation between TGF-$\beta$ and the expression of the co-stimulatory factor CD80 molecule was detected.

3) In *Bacillus subtilis*-feeding group, it was verified that expressions of IL-12a and IL-6 were strongly and positively correlated with CD80 positivity after parturition. These data indicate that DCs of the BS-feeding group may have the ability to lead naive T cells to cell-mediated immunity involved in the removal of bacteria.

# Figures and Tables

# Table 3 Antibodies used in this study

| Antibodies | Isotype | Clone | Supplier |
|---|---|---|---|
| CD3 | IgG1 | MM1A | WSU |
| surface IgM | IgG1 | IL-A30 | Bio-Rad |
| CD14 | IgG1 | CAM36A | WSU |
| Granulocytes | IgM | CH138A | WSU |
| CD11c | IgM | BAQ153A | WSU |
| CD172a | IgG1 | DH59B | WSU |
| CD172a | IgG2b | CC149 | Bio-Rad |
| CD80 FITC | IgG1 | IL-A159 | Bio-Rad |
| Control | Mouse IgG1 | COLIS69A | WSU |
| Control | Mouse IgM | COLIS52A2 | WSU |
| Control | Mouse IgG2a | COLIS205C | WSU |
| Control | Mouse IgG2b | COLIS169A | WSU |
| FITC IgG2a-secondary ab | Goat anti Mouse | | SouthernBiotech |
| PE IgG2b-secondary ab | Goat anti Mouse | | SouthernBiotech |
| Alexa Flour 647 IgM-secondary ab | Goat anti Mouse | | SouthernBiotech |
| IgG1 Micro Beads ab | Rat anti Mouse | | Miltenyi Biotec |
| IgM Micro Beads ab | Rat anti Mouse | | Miltenyi Biotec |

# Table 4 Primer information for quantitative real-time PCR in this study

| Primer | | Sequence | Size (bp) |
|---|---|---|---|
| IL-12a | FW[a] | GGCAGCTATTGCTGAGCTGATG | 136 |
| | RV[b] | ACGAATTCTGAAGGCGTGAAG | |
| IL-12b | FW | ATTCTCGGCAGGTGGAGGTC | 164 |
| | RV | GGCATCCTTGTGGCATGTG | |
| IFN-γ | FW | CATAACACAGGAGCTACCGATTTCA | 197 |
| | RV | CCCTTAGCTACATCTGGGCTACTTG | |
| IL-4 | FW | CTTAGGCGTATCTACAGGAGCCACA | 112 |
| | RV | TCGTCTTGGCTTCATTCACAGAAC | |
| IL-10 | FW | AGCAGCTGTATCCACTTGCCAAC | 133 |
| | RV | CCAGCAGAGACTGGGTCAACAGTA | |
| IL-6 | FW | ATGCTTCCAATCTGGGTTCAATC | 98 |
| | RV | ATGCTTCCAATCTGGGTTCAATC | |
| TGF-β | FW | CGAGCCCTGGACACCAACTA | 137 |
| | RV | AGGCAGAAATTGGCGTGGTA | |
| GAPDH | FW | GATGGTGAAGGTCGGAGTGAAC | 100 |
| | RV | GTCATTGATGGCGACGATGT | |

[a] Forward primer.

[b] Reverse primer.

**Figure 7**

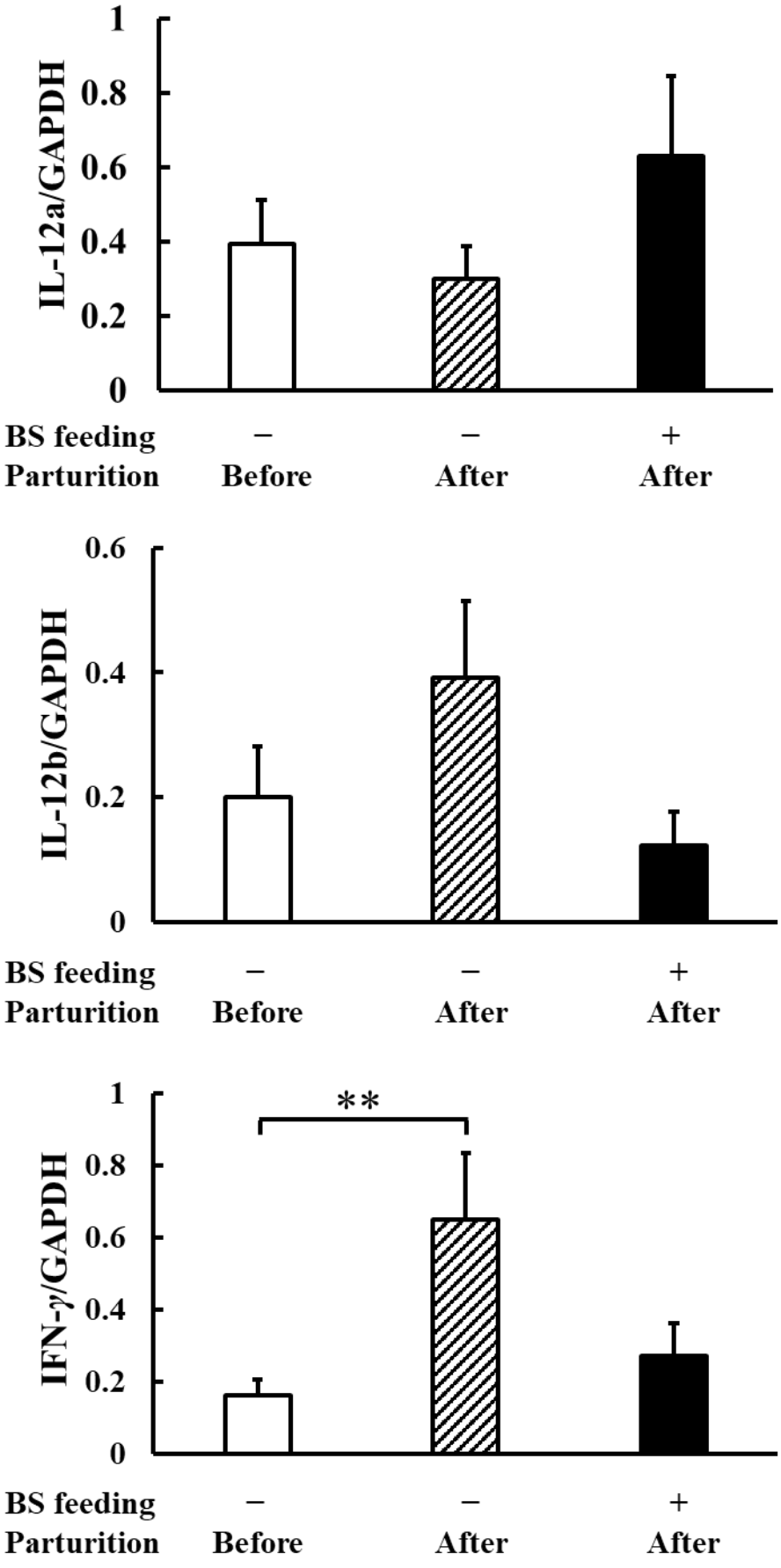
IL-12a/GAPDH
1
0.8
0.6
0.4
0.2
0
BS feeding − − +
Parturition Before After After
IL-12b/GAPDH
0.6
0.4
0.2
0
BS feeding − − +
Parturition Before After After
IFN-γ/GAPDH
**
1
0.8
0.6
0.4
0.2
0
BS feeding − − +
Parturition Before After After

**Fig.7 Expressions of cytokine IL-12a, IL-12b and IFN-γ in bovine peripheral blood dendritic cells before and after parturition.**

Holstein cows were fed with or without probiotics (*Bacillus subtilis* C-3102 strain) from a month before parturition to a month after parturition. The peripheral bloods were obtained from Holstein cows at a month before and after parturition. After the negative and positive selections, the expressions of IL-12a, IL-12b and IFN-γ cytokines in dendritic cells were analyzed by qRT-PCR, and the results were expressed relative to GAPDH. **: $p<0.01$

# Figure 8

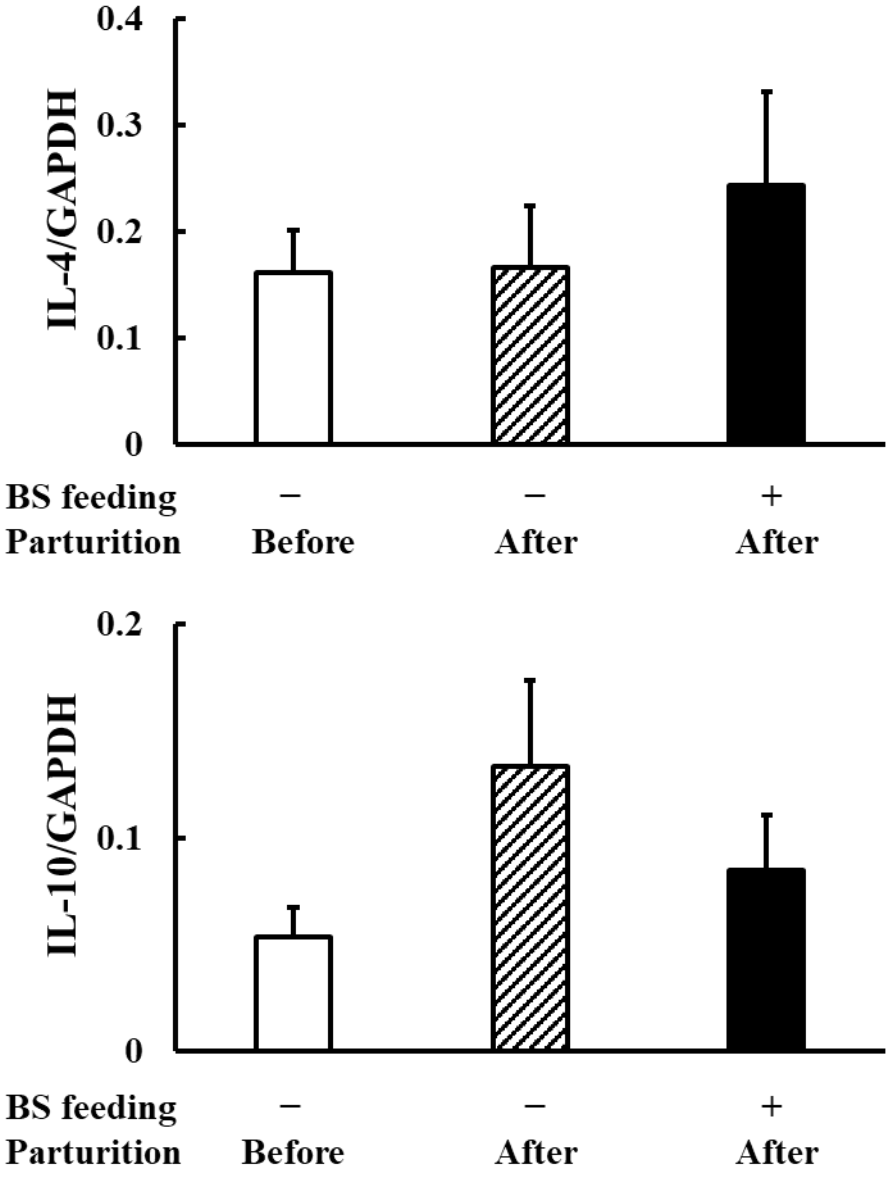
0.4
0.3
0.2
0.1
0
IL-4/GAPDH
BS feeding
Parturition
−
Before
−
After
+
After
0.2
0.1
0
IL-10/GAPDH
BS feeding
Parturition
−
Before
−
After
+
After

**Fig.8 Expressions of cytokine IL-4 and IL-10 in bovine peripheral blood dendritic cells before and after Parturition.**

Holstein cows were fed with or without probiotics (*Bacillus subtilis* C-3102 strain) from a month before parturition to a month after parturition. The peripheral bloods were obtained from Holstein cows at a month before and after parturition. After the negative and positive selections, the expressions of IL-4 and IL-10 cytokines in dendritic cells were analyzed by qRT-PCR, and the results were expressed relative to GAPDH.

# Figure 9

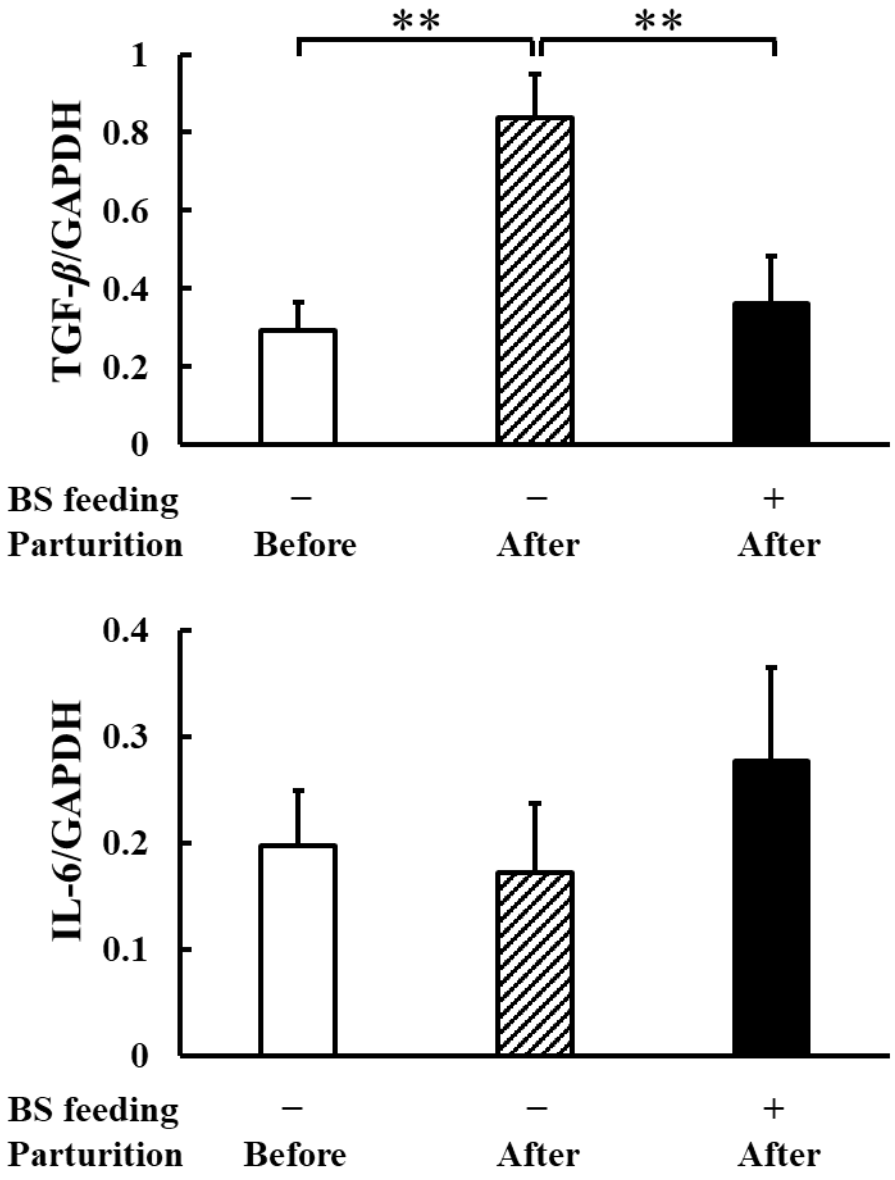
**
**
TGF-β/GAPDH
1
0.8
0.6
0.4
0.2
0
BS feeding
Parturition
−
Before
−
After
+
After
IL-6/GAPDH
0.4
0.3
0.2
0.1
0
BS feeding
Parturition
−
Before
−
After
+
After

**Fig.9 Expressions of cytokine TGF-$\beta$ and IL-6 in dendritic cells from bovine peripheral blood before and after Parturition.**

Holstein cows were fed with or without probiotics (*Bacillus subtilis* C-3102 strain) from a month before parturition to a month after parturition. The peripheral bloods were obtained from Holstein cows at a month before and after parturition. After the negative and positive selections, the expressions of TGF-$\beta$ and IL-6 cytokines in dendritic cells was analyzed by qRT-PCR, and the results were expressed relative to GAPDH. **: $p<0.01$

# Figure 10

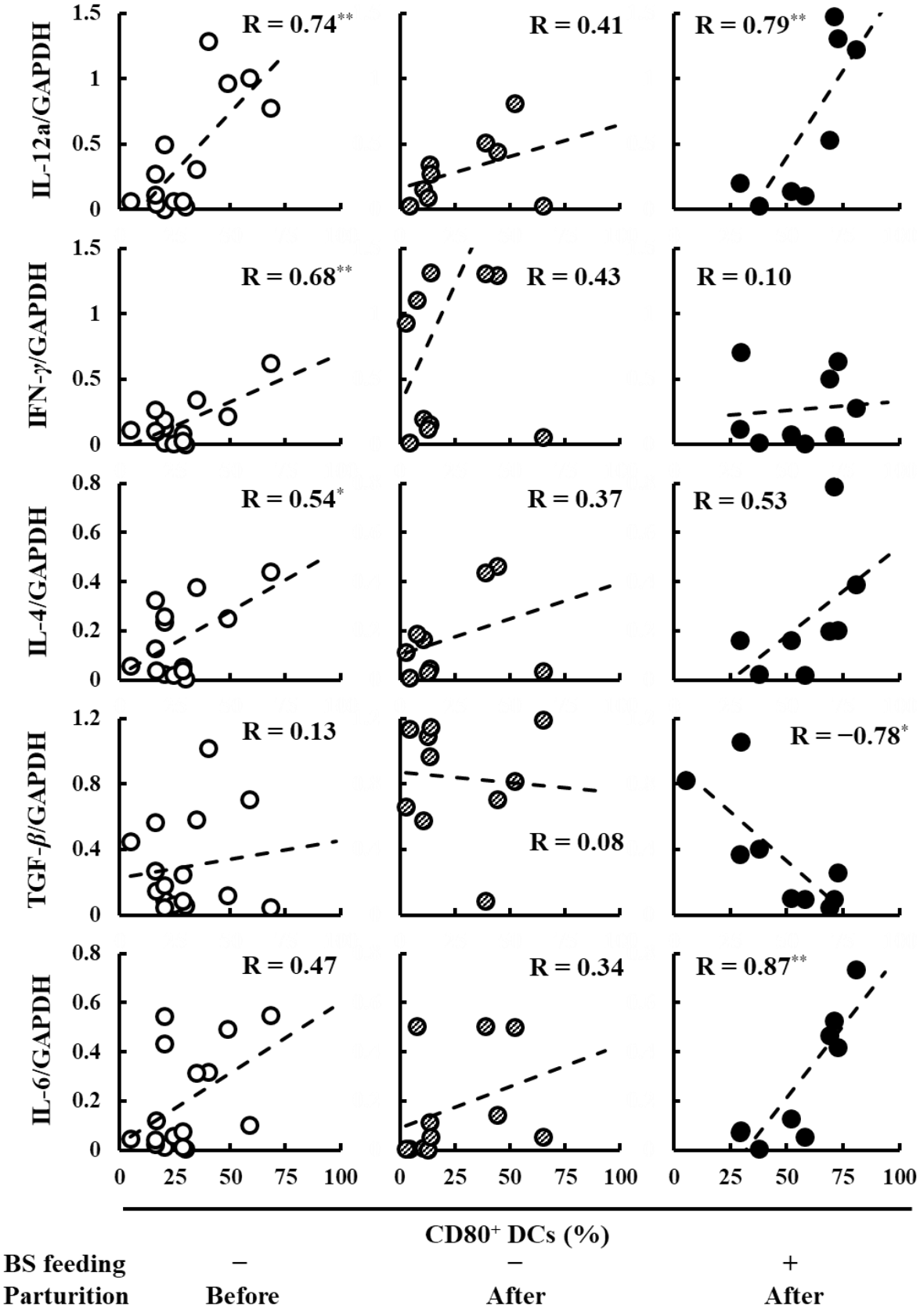
IL-12a/GAPDH
IFN-γ/GAPDH
IL-4/GAPDH
TGF-β/GAPDH
IL-6/GAPDH
R = 0.74**
R = 0.41
R = 0.79**
R = 0.68**
R = 0.43
R = 0.10
R = 0.54*
R = 0.37
R = 0.53
R = 0.13
R = 0.08
R = −0.78*
R = 0.47
R = 0.34
R = 0.87**
0 25 50 75 100
CD80+ DCs (%)
BS feeding − − +
Parturition Before After After

**Fig.10 Relationship between expressions of cytokines and CD80 in bovine peripheral blood dendritic cells before and after parturition.**

The correlations between the expressions of IL-12a, IFN-$\gamma$, IL-4, TGF-$\beta$ and IL-6 and the percentages of co-stimulatory molecule CD80 were shown in dendritic cells after the negative and positive selection. *: $p<0.05$, **: $p<0.01$

# Chapter 5

# Conclusion

A novel method for the purification of the $CD172a^+/CD11c^+$ DCs with high expression of MHC class II at 84.8% purity from bovine PBMC by using MACS was successfully established in this study. The individual differences existed in the expressions of CD205, CD80 and CD86 molecules on DCs. The BS-feeding significantly increased the proportion of $CD172a^+/CD11c^+$ DCs and CD80 positive DCs in the peripheral blood one month after parturition compared with that before parturition. The TGF-$\beta$ expression of DCs in the control-feeding group after parturition was significantly higher than that before parturition. In DCs of the BS-feeding group after parturition, there was a strong negative correlation between the expression of TGF-$\beta$ and the co-stimulatory factor CD80 molecule expression, and there were also positive correlations between the expressions of IL-12a and IL-6 and the CD80 expression.

These results suggested that the BS-feeding prevented the cattle from mastitis by increasing the proportion of DCs in bovine peripheral blood, and that the *Bacillus subtilis* C-3102 strain changed the surface markers and the cytokine production in DCs. These data also indicate that DCs of the BS-feeding group may have the ability to lead naive T cells to cell-mediated immunity involved in the removal of bacteria (Fig.11).

# Figures and Tables

# Figure 11

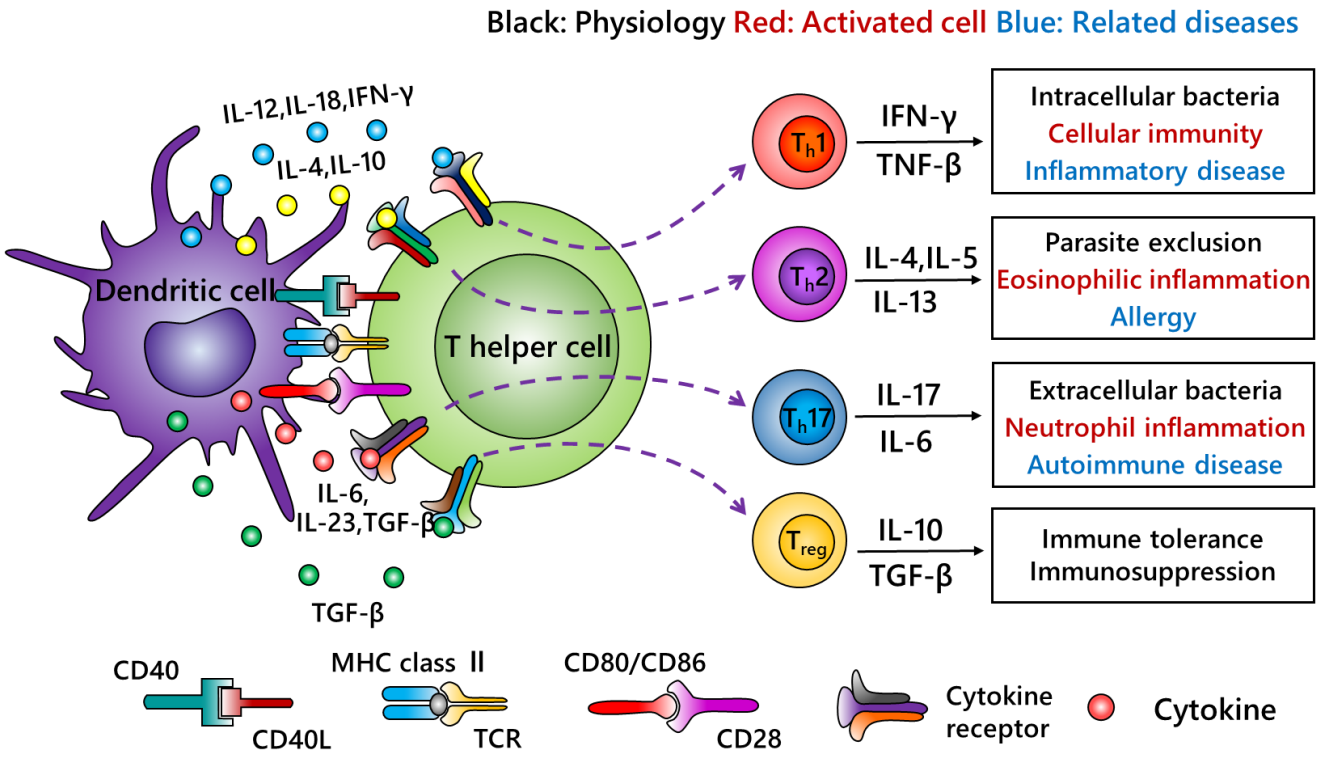


**Fig.11 Differentiation induction and function of T helper cells**

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.

# Acknowledgements

This dissertation is the work of joint efforts from many people, and here I would like to express my deepest gratitude to them.

First and foremost, I would like to express my heartfelt thanks to Prof. Hisashi ASO, my academic supervisor, from whom I have learned so much, and whose inspiring instruction and patience as well as the careful modification to my dissertation have proved invaluable. Then to Prof. Masaaki TOYOMIZU, Kentaro TANEMURA, Assoc. Prof. Tomonori NOCHI, and Assoc. Prof. Hitoshi SHIRAKAWA for their advice and guidance, which have greatly contributed to the completion of this dissertation.

I would like to express my thanks to Mrs. Megumi URAKAWA, for her instruction to carry out my research task, and for her practical advice to overcome difficulties concerning experiments.

Also, I would like to express my thanks to all members in Laboratory of Functional Morphology for providing a supportive and friendly atmosphere to study here. I really enjoy wonderful studying times with you and keep memories forever.

I also would like to thank to everyone who I met here, they made my life enriched and interesting in Japan.

Without everyone’s support, this dissertation can’t be accomplished. Thus, this paper is rather a common possession than a private work.

Finally, I should thank my family and my friends for their love and friendship.

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