ACTA VET. BRNO 2013, 82: 131–134; doi:10.2754/avb201382020131

Gene expression of T lymphocytes of gravid cows during preimplantation

Yousuke Maeda[1], Kana Yamamoto[1], Hiromichi Ohtsuka[1], Takaaki Ando[2], Michiko Tomioka[3], Masaaki Oikawa[1]

[1]Kitasato University, Faculty of Veterinary Medicine, Laboratory of Large Animal Internal Medicine, Japan
[2]Kagoshima University, School of Joint Faculty of Veterinary Medicine, Laboratory of Veterinary Theriogenology, Japan
[3]Kitasato University, Faculty of Veterinary Medicine, Laboratory of Large Animal Surgery, Japan

Received September 20, 2012
Accepted February 19, 2013

Abstract

An interaction between the conceptus and the immune system of animals is important during implantation. The aim of this study was to clarify the gene expression of T cell subsets in gravid cows during the preimplantation period. Peripheral blood from 14 Holstein dairy cows was taken 14 days after artificial insemination. Based on the gravidity, cows were divided into gravid (n = 8) and nongravid (n = 6) groups. Mononuclear cells from peripheral blood were stimulated with phytohaemagglutinin and then $CD4^+$, $CD8^+$, and $WC1^+$ γδ T cell subsets were isolated using magnetic cell sorting. The expression of interferon γ, interleukin 4, and progesterone induced blocking factor were determined using real-time PCR. The expression of interleukin 4 and progesterone induced blocking factor was significantly higher in $WC1^+$ γδ T cells from gravid cows. In addition, interleukin 4 expression in $WC1^+$ γδ T cells from gravid cows was significantly higher than that in $CD4^+$ and $CD8^+$ T cells. This study describes for the first time the important role of $WC1^+$ γδ T cells during the preimplantation period in cows.

CD4, CD8, IFN-γ, IL-4, PIBF, WC1

Interaction between the conceptus and the immune system of animals is important during implantation in cows (Hansen 1995). T cells play a central role in the immune response. T cell subsets, $CD4^+$ (helper), $CD8^+$ (cytotoxic) αβ T cells, and $WC1^+$ γδ T cells have different functions in the immune system (Tanaka et al. 2008). The gravidity requires maternal immunological adjustments. Previous studies have reported that the percentage of γδ T cells in peripheral blood of women in the first trimester of gravidity was higher compared to that of nonpregnant women (Polgar et al. 1999). Oliveira et al. (2008) reported that bovine gravidity at 33–34 days is associated with increase of $CD4^+CD25^+$ T cells, which could be analogous to the regulatory T (Treg) cells described as increasing during gravidity in mice and humans. However, functions of individual T cell subset during preimplantation period are unknown. One of the reasons causing early embryonic death is low progesterone production (Mehmet et al. 2011). Progesterone induced blocking factor (PIBF) is a molecule secreted by T cells in women during gravidity (Polgar et al. 1999). Progesterone induced blocking factor upregulates the levels of T-helper (Th)2 immunity (Druckmann et al. 2005). Th2 immunity induces interleukin (IL)-4 production and suppresses Th1 immunity that induces interferon (IFN)-γ production. Thus shifting immunity toward Th2 predominance is essential for successful gravidity in women (Saito 2000). Phytohaemagglutinin (PHA) is a mitogen which is generally used to activate T cell subsets (Quade and Roth 1999) and activated T cells express various cytokine mRNAs associated with T cell mediated immunity.

The aim of this study was to explain the gene expression of T cell subsets with PHA stimulation in gravid cows during the preimplantation period.

Address for correspondence:
Hiromichi Ohtsuka, PhD.
Laboratory of Large Animal Internal Medicine
Faculty of Veterinary Medicine, Kitasato University
35–1 Higashi 23 bancho, Towada, Aomori 034-8628, Japan

Phone: +81-17-623-4371
Fax: +81-17-623-8703
E-mail: otsuka@vmas.kitasato-u.ac.jp
http://actavet.vfu.cz/

Materials and Methods

Healthy Holstein dairy cows (n = 14) were artificially inseminated (AI) and examined for gravidity by rectal palpation 50–60 days later and divided into two groups: gravid (n = 8) and nongravid (n = 6) cows. They showed no significant differences in age, postpartum days, and body condition score (Table 1).

Table 1. Characteristic of gravid and nongravid Holstein dairy cows.

Variable	Gravid cows	Nongravid cows	*P*-value
Number	8	6	
Age (years)	3.2 ± 0.4	2.7 ± 0.3	0.377
Postpartum days (days)	122.0 ± 14.6	149.5 ± 21.1	0.289
BCS	2.94 ± 0.06	2.74 ± 0.10	0.100

Data are shown as mean ± SEM, BCS – body condition score

Peripheral blood samples were collected from the tail vein into heparin tubes from each animal 14 days after AI and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphosepar I: Immuno-Biological Laboratories, Gunma, Japan). Approximately 5×10^6 PBMCs/ml/well in RPMI 1640 medium (Invitrogen, Tokyo, Japan) supplemented with 10% foetal calf serum (Cansera International, Rexdale, Canada) were placed in 48-well plates. Cells were stimulated with 5 µg/ml of phytohaemagglutinin (PHA; Sigma-Aldrich, St. Louis, MO, USA) in triplicates and incubated for 12 h at 37 °C. After incubation, PHA-activated PBMCs in one of tripricate wells were first incubated for 60 min at 4 °C either with bovine anti-CD4 (CACT138A, IgG1), anti-CD8 (BAT82A, IgG1), or anti-WC1 (CACTB32A, IgG1) monoclonal antibody (VMRD, Pullman, WA, USA). After incubation, the cells bound either with anti-CD4, anti-CD8, and anti-WC1 mAbs were incubated with rat anti-mouse IgG1 antibodies conjugated with magnetic beads (Miltenyi Biotec, Auburn, CA, USA) for 20 min at 4 °C. The magnetic bead-labelled cells were positively separated using a mini MACS MS^+ column (Miltenyi Biotec) in accordance with manufacturer's instructions.

Total RNA from each sample was collected using TRIzol reagent (Invitrogen) and used for the synthesis of first-strand cDNA using oligo-dT primers and SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Real-time RT-PCR was performed with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) on a StepOnePlus machine (Applied Biosystems). The primers for β-actin, IFN-γ, and IL-4 were used as previously described (Riollet et al. 2001). The primers for PIBF were designed using Primer-Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) as follows, PIBF: forward (5'-CCATAGGGCAAGGCTAGACAC-3') and reverse (5'-CGGCTTCAACTTCATCGTCA-3'). The final quantification was carried out using the comparative CT method as previously described (Livak et al. 2001). Expression levels were normalized to endogenous β-actin levels and results were reported as the n-fold difference relative to mRNA expression in the sample with the lowest expression as previously described (Fumuso et al. 2003; Howarth et al. 2005).

Data were expressed as mean ± SEM. All statistical analyses were performed using software Ekuseru-Toukei 2010 (Social Survey Research Information, Tokyo, Japan). Data screening was performed with histograms and Chi-square test and mRNA expressions were log transformed for further statistical analysis due to the skewed distribution. The Student's *t*-test was used for comparisons of data from gravid and nongravid groups. Comparison of mean expression levels among $CD4^+$, $CD8^+$, and $WC1^+$ γδ T cells was done by two-way ANOVA followed by Tukey's test. A *P*-value less than 0.05 was considered significant.

Results

Table 2 represents the expression of IFN-γ, IL-4, and PIBF in $CD4^+$, $CD8^+$, and $WC1^+$ γδ T cells in gravid and nongravid groups of cows. In case of IFN-γ expression, significant difference between gravid and nongravid groups was proven. Expression of IL-4 in $WC1^+$ γδ T cells from gravid cows was significantly higher compared to the nongravid group ($P < 0.01$). In addition, IL-4 expression in $WC1^+$ γδ T cells from gravid cows was significantly higher than that in $CD4^+$ and $CD8^+$ T cells ($P < 0.05$). Regarding PIBF expression, $WC1^+$ γδ T cells from gravid cows showed significantly higher expression compared with the nongravid group ($P < 0.05$).

Table 2. The mRNA expression of IFN-γ, IL-4, and progesterone induced blocking factor in $CD4^+$, $CD8^+$, and $WC1^+$ γδ T cells isolated from phytohemagglutinin-stimulated peripheral blood mononuclear cells in gravid and nongravid cows 14 days after artificial insemination.

Gene for	T cell subsets	Gravid cows	Nongravid cows	
IFN-γ/β-actin	$CD4^+$	4.06 ± 0.67	3.57 ± 0.50	
	$CD8^+$	3.48 ± 0.42	3.57 ± 0.19	
	$WC1^+$	3.93 ± 0.44	3.17 ± 0.31	
IL-4/β-actin	$CD4^+$	2.62 ± 0.31[a]	2.40 ± 0.18	
	$CD8^+$	2.73 ± 0.18[a]	2.08 ± 0.34	
	$WC1^+$	3.45 ± 0.22[b]	2.04 ± 0.31	**
PIBF/β-actin	$CD4^+$	12.39 ± 0.94	12.27 ± 0.95	
	$CD8^+$	10.69 ± 1.14	11.10 ± 0.48	
	$WC1^+$	12.70 ± 1.08	9.47 ± 0.89	*

Data are shown as mean ± SEM. IFN-γ – interferon-γ, IL-4 – interleukin-4, PIBF – progesterone induced blocking factor. $^*P < 0.05$, $^{**}P < 0.01$ - significant difference in expression between gravid and nongravid cows; [a, b] - a significant difference among T cell subsets ($P < 0.05$).

Discussion

In the present study, both IL-4 and PIBF expressions were significantly higher in $WC1^+$ γδ T cells from gravid cows compared to nongravid cows. Bovine γδ T cells have a suppressive effect on αβ T cell responses (Rhodes et al. 2001) and $WC1^+$ γδ T cells act as immune regulatory cells (Hoek et al. 2008). Progesterone induced blocking factor is secreted predominantly by γδ T cells in women (Polgar et al. 1999). Therefore, $WC1^+$ γδ T cells are involved in conception through PIBF and Th2-associated cytokine production. On the other hand, there were no differences in IL-4 and PIBF expressed by $CD4^+$ and $CD8^+$ T cells. However, it may be necessary to investigate other Th1 and Th2 cytokines in $CD4^+$ and $CD8^+$ T cells to determine their association with gravidity (Ng et al. 2002).

In our study, $WC1^+$ γδ T cells from gravid cows displayed significantly higher IL-4 expression after PHA stimulation compared to $CD4^+$ and $CD8^+$ T cells. This suggests that activated $WC1^+$ γδ T cells are more capable of producing Th2 cytokines than $CD4^+$ and $CD8^+$ T cells. Alterations in Th1/Th2 balance affect gravidity and Th2 immunity plays an important role in successful pregnancies (Saito 2000). Therefore, activated $WC1^+$ γδ T cells may be a more critical factor for embryonic implantation than $CD4^+$ and $CD8^+$ T cells via increasing Th2 cytokines during the preimplantation period. In conclusion, the present study demonstrated that activated $WC1^+$γδ T cells from gravid cows released Th2 cytokine and PIBF in gravid cows 14 days after AI. Thus, successful implantation may be associated with $WC1^+$γδ T cells during the preimplantation period.

Acknowledgments

This study was supported in part by a Grant-in-Aid for General Scientific Research (23580448) of Japan.

References

Ataman MB, Erdem H, Bülbül B, Ümütlü S, Çolak M 2011 The effect of buserelin injection 12 days after insemination on selected reproductive characteristics in cows. Acta Vet Brno **80**: 171-177

Druckmann R, Druckmann MA 2005: Progesterone and the immunology of pregnancy. J Steroid Biochem Mol Biol **97**: 389-396

Fumuso E, Giguère S, Wade J, Rogan D, Videla-Dorna I, Bowden RA. 2003: Endometrial IL-1beta, IL-6 and

TNF-alpha, mRNA expression in mares resistant or susceptible to post-breeding endometritis. Effects of estrous cycle, artificial insemination and immunomodulation. Vet Immunol Immunopathol **96**: 31-41

Hansen PJ 1995: Interactions between the immune system and the ruminant conceptus. J Reprod Fertil Suppl **49**: 69-82

Hoek A, Rutten VP, Kool J, Arkesteijn GJ, Bouwstra RJ, Van Rhijn I, Koets AP 2009: Subpopulations of bovine $WC1^{+}$ γδ T cells rather than $CD4^{+}CD25^{high}$ $Foxp3^{+}$ T cells act as immune regulatory cells *ex vivo*. Vet Res **40**: 6

Howarth PH, Babu KS, Arshad HS, Lau L, Buckley M, McConnell W, Beckett P, Al Ali M, Chauhan A, Wilson SJ, Reynolds A, Davies DE, Holgate ST. 2005: Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. Thorax **60**: 1012-1018

Livak KJ, Schmittgen TD 2001: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**: 402-408

Ng SC, Gilman-Sachs A, Thaker P, Beaman KD, Beer AE, Kwak-Kim J 2002: Expression of intracellular Th1 and Th2 cytokines in women with recurrent spontaneous abortion, implantation failures after IVF/ET or normal pregnancy. Am J Reprod Immunol **48**: 77-86

Oliveira LJ, Hansen PJ 2008: Deviations in populations of peripheral blood mononuclear cells and endometrial macrophages in the cow during pregnancy. Reproduction **136**: 481-490

Polgar B, Barakonyi A, Xynos I, Szekeres-Bartho J 1999: The role of gamma/delta T cell receptor positive cells in pregnancy. Am J Reprod Immunol **41**: 239-244

Quade MJ, Roth JA 1999: Dual-color flow cytometric analysis of phenotype, activation marker expression, and proliferation of mitogen-stimulated bovine lymphocyte subsets. Vet Immunol Immunopathol **67**: 33-45

Riollet C, Rainard P, Poutrel B 2001: Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. J Dairy Sci **84**: 1077-1084

Rhodes SG, Hewinson RG, Vordermeier HM 2001: Antigen recognition and immunomodulation by gamma delta T cells in bovine tuberculosis. J Immunol **166**: 5604-5610

Saito S 2000: Cytokine network at the feto-maternal interface. J Reprod Immunol **47**: 87-103

Tanaka S, Nagai Y, Noguchi Y, Miyake M, Watanabe K, Ohwada S, Aso H, Yamaguchi T 2008: Differential cytokine expression in T cell subsets from bovine peripheral blood. Anim Sci J **79**: 597-604