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Short communication

EFFECT OF *PASTEURELLA MULTOCIDA* SOLUBLE ANTIGEN STIMULATION ON THE *IN VITRO* RESPONSE OF PERIPHERAL BLOOD MONONUCLEAR CELLS OF HOLSTEIN CALVES

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The expressions of cytokines mRNA, including interleukin-4 (IL-4), interleukin-17A (IL-17A) and interferon-gamma (IFN-γ), their master regulatory transcription factors, and signal transducers and activator of transcription (STAT) stimulated in vitro with Pasteurella (P.) multocida soluble antigen were examined in peripheral blood mononuclear cells (PBMC) from Holstein calves. The healthy Holstein calves were divided into three groups; 2 weeks old (2W Group, N=8), 6 weeks old (6W Group, N=8), and 10 weeks old (10W Group, N=8). PBMC were stimulated in vitro by soluble antigen of P. multocida. There were significantly lower expressions of IFN-γ, IL-4, and STAT-6 mRNA of PBMC stimulated with P. multocida soluble antigen in the 2W Group compared to that in the 10W Group. Expression of IL-17A and IFN-γ in PBMC stimulated with P. multocida soluble antigen were significantly higher compared with the PBMC without stimulation in the 6W groups. The results of the present study demonstrated that 2W old calves had decreased cytokine expression of PBMC when in vitro stimulated with P. multocida soluble antigen in vitro.

Key words: calves, cytokine, immune function, *Pasteurella multocida*

INTRODUCTION

The immunologic competence of the neonate progresses rapidly after birth as the cells acquire maturity of immunological competence through antigenic experiences. During this period, the neonate mainly depends upon components of the innate or antigen-independent immune system [1, 2]. The overall performance of the immune system in the neonatal period lacks several important aspects. Neonates and young children typically present poor responses of immune cells to pathogens [3, 4]. As a result, young calves may be more susceptible than older calves to serious bacterial infections.

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Pasteurella (P.) multocida is one of the most common bacteria, which can cause high morbidity in young calves. *P. multocida* is considered as a part of the normal bacterial flora of the upper respiratory tract of most cattle, and it usually does not cause illness in healthy animals. Although inhaled bacteria such as *Pasteurella* are usually removed by the immune system, these bacteria can cause diseases by reaching the deeper portions of the respiratory tract when the animal's normal defense system is impaired. In the immunohistological and cytokine expression studies of the calves infected with *P. multocida,* increased numbers of $CD8^+$ blast T cells expressing MHC class-II in the bronchoalveolar lavage as well as increased expressions of Interleukin-8 (IL-8) in lung lymph nodes were observed [5].

Although all essential immune components are present in neonates at birth, many of the components are not fully functional until calves reach at least 2 to 4 weeks of age and may continue developing until puberty [6]. The numbers of peripheral $CD4^+$, $CD8^+$, $CD21^+$ and $CD2^+CD25^+$ cells are lower in neonates compared to adults, and these lymphocytes increase numbers gradually with the growth of animals [7]. The response of T cells to mitogen activation is slightly weaker at birth and remains constant throughout 4 weeks after the birth [7, 8]. These studies have provided the experimental evidence of age-related resistance to bacterial infection in calves. It has been shown the markedly highest interferon-gamma (IFN-γ) production of mediastinal lymph node cells occurred in the calves inoculated with *P. haemolytica* live vaccine [9]. However, the T cell reaction of peripheral blood mononuclear cells (PBMC) with *P. multocida* soluble antigens stimulation in young calves has not been clearly defined. Therefore, we analyzed the T cell cytokines mRNA expression in PBMC isolated from young Holstein calves by stimulating with *P. multocida in vitro*.

MATERIALS AND METHODS

Animals and sampling

The procedures used in the present study were performed in accordance with the principles and guidelines for animal use set by the Animal Experiment and Care Committee of Kitasato University, Towada, Japan. Twenty-four healthy female Holstein calves of 2, 6 and 10 weeks (W) of age, 8 calves for each age group, were used in this study. There was no clinical evidence of diseases in any of the calves during this study. Blood samples were collected once from each calf into heparinized tubes (Terumo, Tokyo, Japan) from the jugular vein.

Cell culture

Soluble *P. multocida* antigen was isolated from a calf with clinical signs of pneumonia as the stimulant for this study. The isolated and identified bacteria were cultured on blood agar for 12 hours at 37°C, and were harvested in sterile phosphate-buffered saline (PBS) by centrifugation at 15000 rpm for 10 min. After disrupting the cell wall of bacteria by freezing at −80°C, the bacteria suspension was centrifuged in order to separate the pellet of intact bacteria and debris. The resulting supernatant was filtered to obtain the soluble antigens. The antigen for use in proliferation assays was prepared as previously described [10].

PBMC were isolated by density gradient centrifugation, and 1×10^{6} PBMC in RPMI 1640 (Invitrogen, Tokyo, Japan) medium, supplemented with 2-ME and 10% heat-inactivated fetal calf serum (FCS; Cansera International Inc., Rexdale, Canada) were placed in a 24-well plate (Greiner Bio-One, Tokyo, Japan) for cytokines mRNA, and incubated with 1 μg/mL of soluble *P. multocida* antigen.

Real-time PCR

After 12 hours incubation at 37°C, PBMC were resuspended in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to analyze the cytokines mRNA expression of PBMC in the presence of soluble antigen. Analysis of cytokine mRNA levels was performed by a real-time PCR as described previously [11]. Two micrograms of total RNA from each sample were used for the synthesis of the first-strand of cDNA using oligo-dT primers (Invitrogen, Carlsbad, CA, USA) and superscript II reverse transcripts (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems, CA, USA) on an ABI prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The following genes were selected: β-actin, IFN-γ, IL-4, IL-17A, T-box transcription factor 21 (T-bet), GATA binding protein 3 (GATA-3), RAR-related orphan receptor C (RORC), STAT1, STAT3 and STAT6. Primers were designed using the publicly available web-based Primer3 program and are listed in Table 1. The Ct values define the threshold cycle of PCR, at which amplified products were detected. Results are expressed as Ct values, where Ct is the difference in threshold cycles for a target. β-actin was used as an internal control.

Table 1. Primers used for real-time PCR expression analysis

Gene	Accession Number	Product Length	Primer Designation	Sequence (5'-3')
IL-4	NM_173921	117	Forward	GCCCCAAAGAACACAACTGA
			Reverse	GAGATTCCTGTCAAGTCCGC
IL-17A	NM_001008412	83	Forward	TGTCTACAGTGAACTGGAAGGAAC
			Reverse	CCACCAGACTCAGAAGCAGTAG
IFN-γ	NM_174086	108	Forward	TCAAATTCCGGTGGATGATCT
			Reverse	CTTCTCTTCCGCTTTCTGAGG
GATA-3	NM_001076804	128	Forward	AGCACAGGCCGGGAGTGTGTGAACT
			Reverse	AGGGGCCGGTTCTGTCCGTTCATCT
RORγt	NM_001083451	145	Forward	ACAGCCCTCGTCCTCATCAATGCC
			Reverse	TGGGTGGCAGCTTTGCCAGGATA
T-bet	NM_001192140	132	Forward	CATGCCAGGGAACCGCCAGTATGT
			Reverse	ATCTGGGTCGCATTGTTCCAAGCCC
STAT1	NM_001077900.1	102	Forward	CCCCAGAGCCTATGGAACTTG
			Reverse	CATGCCACTCTTCTGTGTTCA
STAT3	XM_010816228.1	90	Forward	CCGTACCTGAAGACCAAGTTC
			Reverse	TGAATCTAAAGTGCGGGGG
STAT6	NM_001012673.1	118	Forward	CAATGGACAGTCTGGAGCCCT
			Reverse	CTGGGGATTGTTTCCACAGAGG

Statistical analysis

Data are presented as median (25th, 75th percentiles). The Mann-Whitney U test was used to determine the level of significance of the data between with and without antigen of the same sample. Statistical analysis for each parameter among the three groups was performed using Kruskal-Wallis's test in order to determine the differences among the three age groups. Statistical significance was considered when $P < 0.05$. All analyses were performed using software Excel-Toukei 2015 (Social Survey Research Information, Tokyo, Japan).

RESULTS AND DISCUSSION

After 12 hours stimulation in the presence of *P. multocida* soluble antigen, mRNA of IL-4 and IFN-γ in the 2W Group were the lowest among the three groups and significantly lower levels were found. Although there was no difference in IL-17A and IFN-γ between with vs without antigen in the 2W Group, these cytokines mRNA levels increased significantly by *P. multocida* soluble antigen stimulation in the 6W Group (Figure 1). After activation T helper (Th) cells develop into different subsets, and the T cell subsets express different cytokines and mediate distinct effector functions during immune responses. The concentrations of IFN-γ and IL-4 in PBMC were markedly lower at birth and remained low even at 3 months of age in the previous study [12]. These cytokines are associated with an immune development in calves, and as a result, very young calves seem to be more susceptible than older calves to serious bacterial infection, as well as some viral and fungal infections. However, there were no significant differences in mRNA of T-bet, GATA-3 and RORC of PBMC among the three groups (Figure 2).

Significantly lower mRNA expression of STAT 1 without stimulation was obtained from the 2W Group compared with the 10W Group, and significantly lower mRNA expression of STAT6 with the *P. multocida* soluble antigen stimulation was obtained within the 2W Group compared with the 6 W or 10W Group. There were no significant differences in mRNA relative expression of STAT 1, 3 and 6, between antigen stimulated vs. unstimulated PBMS, within all three experimental groups (Figure 3). Signaling via receptors on leukocytes involves different Janus kinase-STAT pathways. STAT1 is involved in gene upregulation after stimulation by IFNs. STAT1 is a key mediator of IFN-γ stimulation; while STAT6 plays a major role in IL-4 stimulated signaling [13]. STAT1 or STAT6 phosphorylations in response to IFN-γ or IL-4 significantly decreased in monocytes from neonates compared to those from adults [14]. There is a possibility that the lower cytokine expressions in the PBMC of neonatal calves are due to a lower activation of the STAT pathway.

Despite the significantly low levels of IFN-γ and IL-4 of PBMC by *P. multocida* soluble antigen stimulation in the 2W Group compared to 10W Group, an expression of IL-17A in the 2W Group calves was not significantly different compared to other groups.

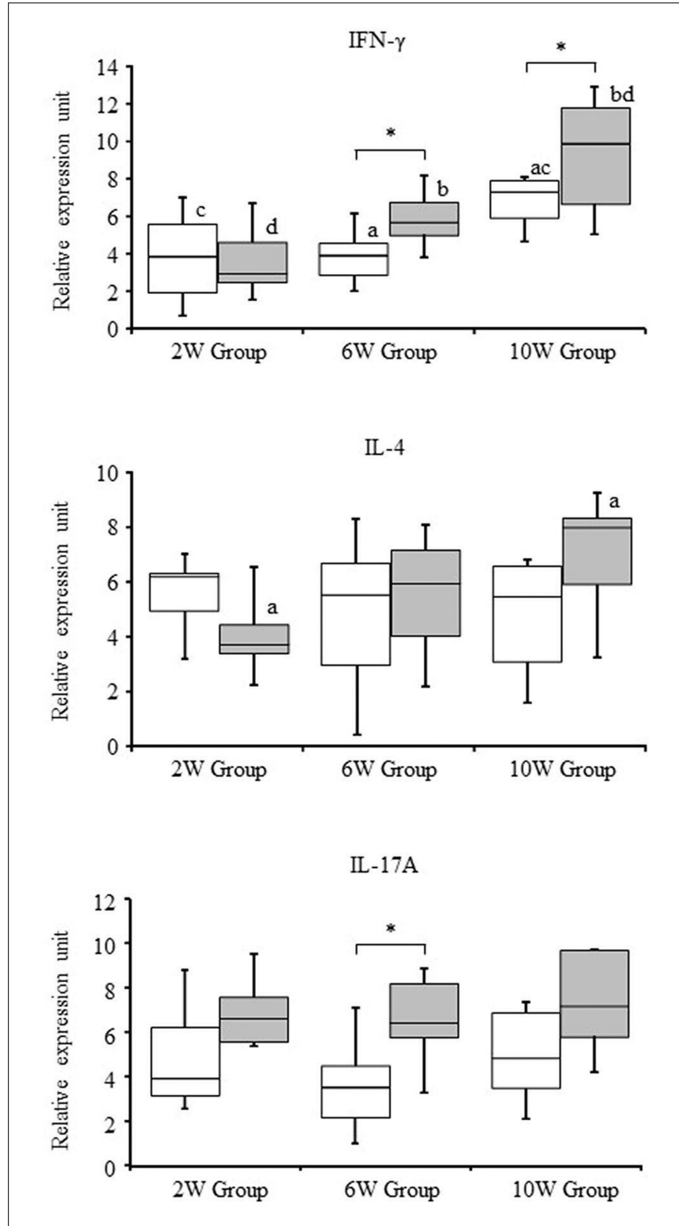

Figure 1. Expressions of cytokine mRNA in PBMCs with soluble *P. multocida* antigen (gray bars) or without antigen (empty bars). Box = 25th and 75th percentiles; bar = min and max values, and the line within box means median values. Asterisks indicate the time point where significant differences between with antigen and without antigen was observed (P<0.05). Same letters indicate significant differences of the same mRNA among the three age groups (P<0.05). The lower responses of PBMC with antigen stimulation were detected at higher frequencies in the 2 weeks old calves.

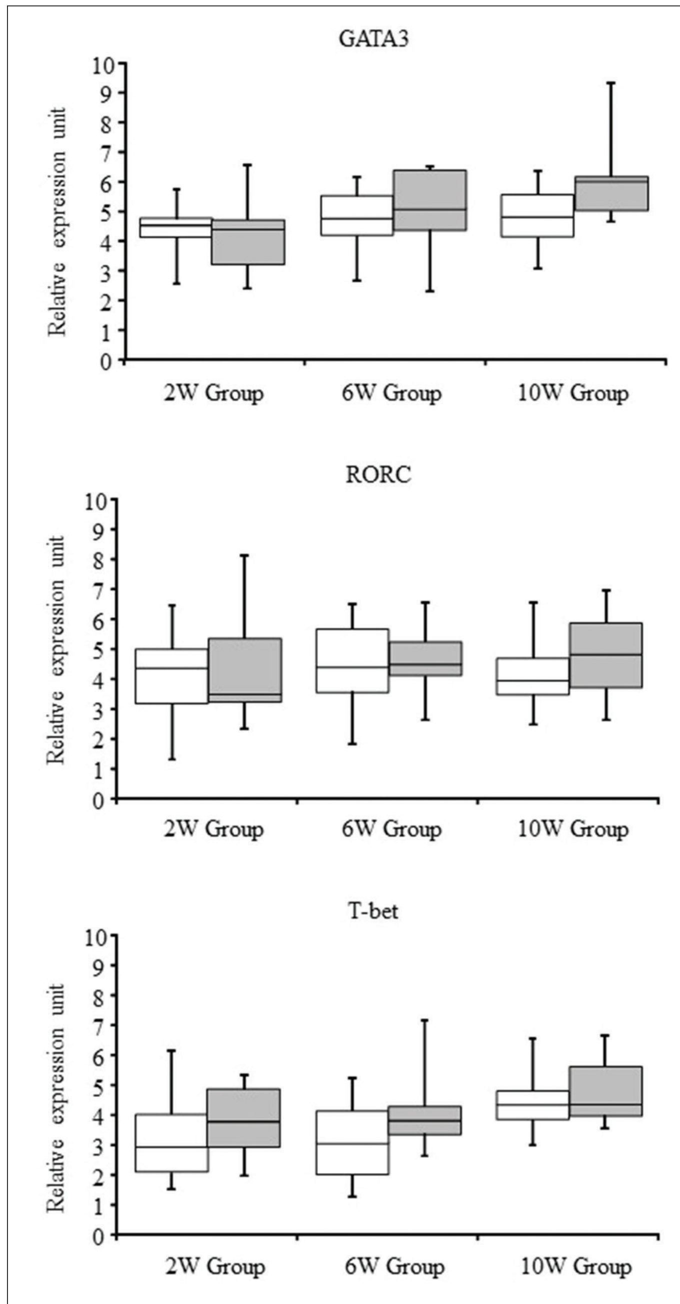

Figure 2. Expressions of cytokine master regulator transcription factor mRNA in PBMCs with soluble *P. multocida* antigen (gray bars) or without antigen (empty bars). Box = 25th and 75th percentiles; bar = min and max values, and the line within box means median values.

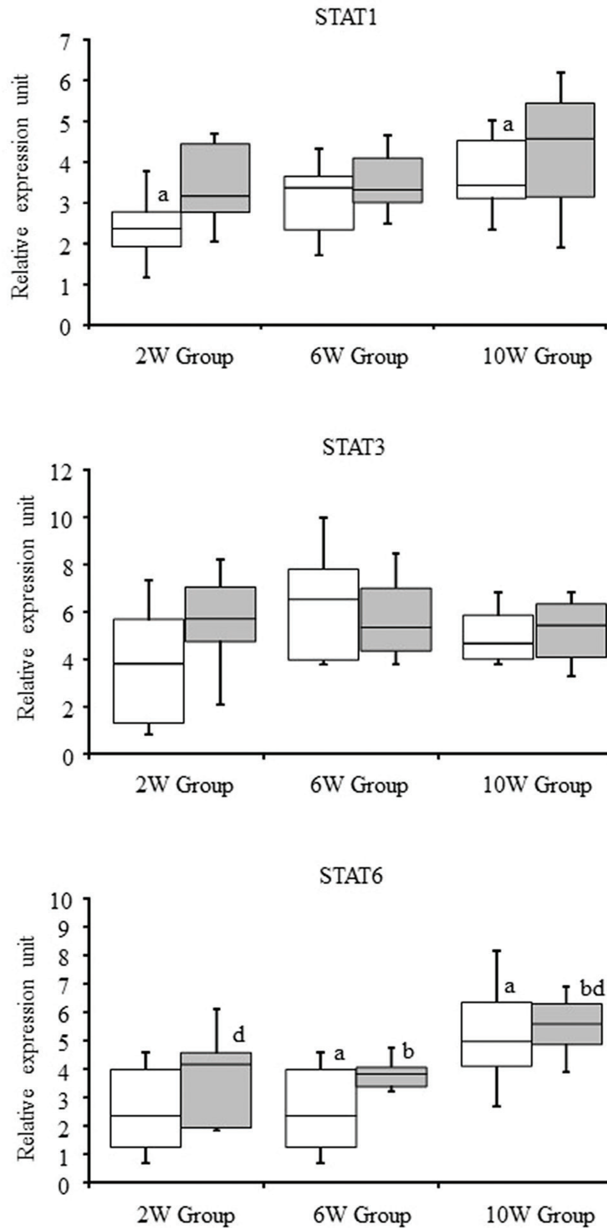

Figure 3. Expressions of Signal Transducers and Activators of Transcription mRNA in PBMCs with soluble *P. multocida* antigen (gray bars) or without antigen (empty bars). Box = 25th and 75th percentiles; bar = min and max values, and the line within box means median values. Asterisks indicate the time point where significant differences between with antigen and without antigen was observed (P<0.05). Same letters indicate significant differences of the same mRNA among the three age groups (P<0.05). The lower responses of PBMC with antigen stimulation were detected at higher frequencies in the 2 weeks old calves.

Th17 cells are characterized by the production of IL-17A, and can recruit neutrophils and confer host protection against extracellular bacteria and fungi at epithelial barriers [15, 16]. Although the fetal immune system develops in a sterile and protected environment and therefore lacks antigenic experience, soon after birth, the newborn is exposed to bacteria, viruses, fungi, and parasites and must immediately defend itself. These microbes may induce the production of IL-17A in neonates. IFN-γ inhibits the production of IL-17A in a STAT1-dependent, T-bet-independent manner [17]. In the 2W Group, expressions of IFN-γ with *P. multocida* soluble antigen stimulation were significantly lower than those in other older groups, thus it is speculated that these insensitive Th1-type immune reaction may enhance expression of IL-17A mRNA of Th17 cells in young calves.

In conclusion, there appears to be multiple pathways for Th1 and Th2 PBMCs insufficiently respond to stimuli in young calves. Therefore, it is possible that neonatal calves are prone to an insufficient immune response and have an increased risk of serious infections. Decreased STAT phosphorylation and activation may represent developmental immaturity and may contribute to the unique susceptibility of neonates to infections by *P. multocida*. The ideal immune conditions are still unclear in the neonatal calves. Further studies are needed in this field.

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Authors' contributions

MI and YM carried out the cell culture and immune factors PCR analysis. TT made the *P. multocida* soluble antigen. HO participated in the design of the study, performed the statistical analysis, conceived of the study, drafted the manuscript, and participated in its design and coordination. MT helped to perform the statistical analysis and draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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EFEKTI STIMULACIJE SOLUBILNIM ANTIGENOM *PASTEURELLA MULTOCIDA*, NA *IN VITRO* ODGOVOR MONONUKLEARNIH ĆELIJA PERIFERNE KRVI KRAVA RASE HOLŠTAJN

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Ispitivan je uticaj *in vitro* stimulacije sa solubilnim antigenom *Pasteurella multocida* (*P. multocida*) na ekspresiju iRNK citokina, uključujući interleukin-4 (IL-4), interleukin-17A (IL-17A) i interferon-gama (IFN-γ), na njihove značajne faktore regulacije transkripcije, prenosa signala i aktivatora transkripcije (STAT) kod mononuklearnih ćelija periferne krvi (PBMC), kod krava rase Holštajn. Životinje su podeljene po grupama i to: telad stara 2 nedelje (2W grupa, 8 teladi), 6 nedelja (6W, 8 teladi) i jedinke stare 10 nedelja (W10, 8 teladi). PBMC su stimulisani in vitro solubilnim antigenima *P. multocida*. Uočena je značajno manja ekspresija IFN-γ, IL-4 i STAT-6 iRNK kod PBMC stimulisanih sa *P. multocida* solubilnim antigenom kod životinja 2W grupe u poređenju sa rezultatima grupe 10W. Ekspresija IL17A i IFN-γ u PBMC stimulisanim sa solubilnim antigenom *P. multocida* bila je značajno veća u poređenju sa PBMC , bez stimulacije u grupi 6W. Rezultati ispitivanja ukazuju da telad stara 2 nedelje (grupa 2W) ima smanjenu ekspresiju citokina ukoliko se PBMC in vitro stimulišu sa solubilnim *P. multocida* antigenom.