

*Full Length Research Paper*

# Toll-like receptor-4 (TLR-4) expression on polymorphonuclear neutrophil leukocytes during perinatal period of dairy cow

Qun-Wei Cui<sup>1</sup>, Xuan Zhou<sup>1</sup>, Ning Guo<sup>1</sup>, Zhen Zhang<sup>1</sup>, Ya-Chun Wang<sup>2</sup> and Gen-Lin Wang<sup>1\*</sup>

<sup>1</sup>College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, P. R. China.

<sup>2</sup>College of Animal Science and Technology, China Agricultural University, Beijing 100193, P. R. China.

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To establish a foundation for further researches on the improvement of polymorphonuclear neutrophil leukocytes (PMN) functions in dairy cow during perinatal period, the counting of PMN, as well as the mRNA and protein expression of toll-like receptor-4 (TLR-4) on PMN was studied during this critical period. Blood samples were taken 21, 14 and 7 days, and at calving (0) day before expected calving time, and 7, 14 and 21 days after calving. The PMN changes were measured by automatic blood cell analyzer, and mRNA and protein expression of TLR-4 were analyzed by quantity real-time PCR (RT-PCR) and western blot. The results show that the quantity of neutrophil leukocytes reached the peak ( $3.12 \pm 0.26 \times 10^9$ ,  $p < 0.05$ ) at 0 day. The mRNA expression of TLR-4 was down-regulated from the -21 days before calving to the 14 day after calving ( $P < 0.01$ ). The protein of expression TLR-4 was lower from 7 to 14 days. The down-regulation of TLR-4 expression may be the major factor of PMN dysfunction of cows from 7 to 14 days after calving.

**Key words:** Cow, hematology, perinatal period, toll-like receptor-4 (TLR-4) pathway.

## INTRODUCTION

During the perinatal period, cows are more susceptible to intramammary and uterus infections by environmental pathogens like *Escherichia coli* due to the depression of non-specific or innate immunity (Burvenich et al., 2003; Williams et al., 2007). It is reported that hematological changes take place in dairy cows during periparturient period (Lee and Kehrl, 1998) and 25 to 60 days after calving (Nazifi et al., 2008). Dairy cows subjected to these changes may easily suffer from immune suppression and induce bacterial infections. Polymorphonuclear neutrophil leukocytes (PMN) acts as the first defense line against bacterial infections. Therefore, their reduction and dysfunction can affect the immune status (Kehrl and Harp, 2001) and then influence PMN recognition of pathogenic bacteria.

The discovery of toll-like receptors as conservative

molecules and their role in innate defense have aroused a new area of interest. Toll-like receptor-4 (TLR-4), one member of this family, has been implicated in the recognition of *E. coli* (Quesniaux et al., 2004; Yadav and Schorey, 2006; Weiss et al., 2007) by mediating cytokine production and stimulating host defense (Ferwerda et al., 2007). TLR-4 recognizes the conserved lipopolysaccharide (LPS) pattern of Gram-negative bacteria. TLR-4 may, therefore, play an important role in the innate immune status of cows during periods of risk of intramammary infection by Gram negative organisms (Miller et al., 2005). It has been reported that the actual number of TLR-4 molecules involved in recognition is important for initiation of signaling that leads to activation of the innate immune response (Triantafyllou and Triantafyllou, 2005), and then initiates an intracellular signaling cascade that induces an array of responses including the activation of the transcription factor, nuclear factor  $\kappa\beta$  or NF- $\kappa\beta$  (Chow et al., 1999).

When intramammary of dairy cows were infected by Gram negative organisms, PMN rapidly increase to clear

\*Corresponding author. E-mail: [genlinwang@gmail.com](mailto:genlinwang@gmail.com). Tel: +86-25-84395045. Fax: +86-25-84395314.

**Table 1.** Primers used in RT-PCR assay.

Gene	Serial number	5'-3'	Temperature (°C)
TLR-4	NM174198	CAAATGCCCTACTCAACCTCT TAAACCAGCCAGACCTTGAATACA	61
$\beta$ -actin	AY141970	TCCAGCC TTCCTTCCTGGGCAT GGACAGCACCGTGTGGCGTAGA	61

the invading bacteria in mammary gland. Interestingly, LPS has been described to enhance the activity and functional life span of PMN by respectively enhancing its oxidative burst activity (Francois et al., 2005; Remer et al., 2003) and inhibiting PMN apoptosis (Sabroe et al., 2005). As previously described, LPS is recognized by TLR-4, so there exist a possibility that the TLR-4 signaling pathway is involved in enhancing the PMN activity. Furthermore, recent researches have proved that TLR-4 regulates the expression of CD11b/CD18 integrin in PMN in response to LPS, and CD11b/CD18 adhesion molecules contribute to PMN diapedesis across the bovine blood-milk barrier (Smits et al., 2000). So, the down-regulation of TLR-4 might cause the dysfunction of PMN during the periparturient period.

In this study, we investigated the change rule of PMN, by RT-PCR and western blot methods to analyze the key gene of TLR-4 expression on PMN during periparturient period, thus established a foundation for further researches on the improvement of PMN functions.

## MATERIALS AND METHODS

In this study, we chose 70 Chinese Holstein dairy cattle (4 to 6 years old) and parity from 2 to 4 were evaluated, then the diseased and not pregnant ones were removed. The remaining 56 individuals were used for data statistics. Cows were reared in a free-stall barn and brought into maternity pens one week before calving. The blood samples were taken by jugular vein puncture into tubes containing acid citrate dextrose as anticoagulant. Blood sample of each animal was taken -21, -14 and -7 days before expected calving; (0) day at calving; and 7, 14 and 21 days after calving. All procedures of the experiment were carried out in accordance with the guidelines of regulation for the administration affairs of experimental animals in China.

### Examination and sampling

Total neutrophils (NEU) count was measured using an automatic blood cell analyzer (Weishikang, Shanghai, PR China) within 2 h after sample collection.

### Real-time PCR

Bovine PMN were isolated from blood samples by differential centrifugation using established procedures (Carlson GP and Kaneko JJ, 1973), cells were resuspended using TRIzol reagent (Invitrogen, Shanghai, PR China). The RNA samples were treated

with DNaseI to remove contaminating genomic DNA prior to reverse transcription. 2 mg total RNA from each sample was used for synthesizing the first strand cDNA with oligo-dT primers (Invitrogen, Shanghai, PR China) and superscript II reverse transcripts (Invitrogen, Shanghai, PR China) according to the manufacturer's protocols.

RT-PCR was performed using SYBR Green Master Mix (Rox, Beijing, PR China) on an ABI prism 7300 Sequence Detector (Applied Biosystems, PR China). The target DNA sequences were specifically amplified with the previously designed primers (Table 1).  $\beta$ -actin was selected as the control gene because its amplification efficiency was the same as that of all test genes and the abundance of its mRNA in bovine blood neutrophils does not change through the peripartum period (Weber et al., 2001; Madsen et al., 2002). The comparative threshold cycle number ( $2^{-\Delta\Delta C_t}$ ) method was used and the  $C_t$  values define the threshold cycle of PCR, at which amplified products were detected.

### TLR-4 expression detection by Western blot

According to previous method, PMN were isolated from each cows, and then the protein was extracted from PMN (Monfardini et al., 2002). The protein was incubated with TLR-4 antibody (1:1,500, Boster, BA1717) and  $\beta$ -actin antibody (1:500, Boster, BA0410), then incubated with the corresponding secondary antibody (1:15,000). The signal was detected with ECL Plus (Hangzhou, PR China) as described by the manufacturer. Protein gray scale was detected by Kodak Image Analysis System. The values were defined as the ratio of the gray scale of TLR-4 protein and  $\beta$ -actin protein at the corresponding time points.

### Statistical analysis

The neutrophils were analyzed by SPSS 17.0. Values shown in the text are least square means  $\pm$  standard error of mean (SE) unless otherwise stated.

Statistical comparisons of RT-PCR and protein results between groups were done by Student's t-test assuming equal variance and values of  $p < 0.05$  were regarded as significant. The mean values and standard errors of the clinical and laboratory data were calculated.

## RESULTS

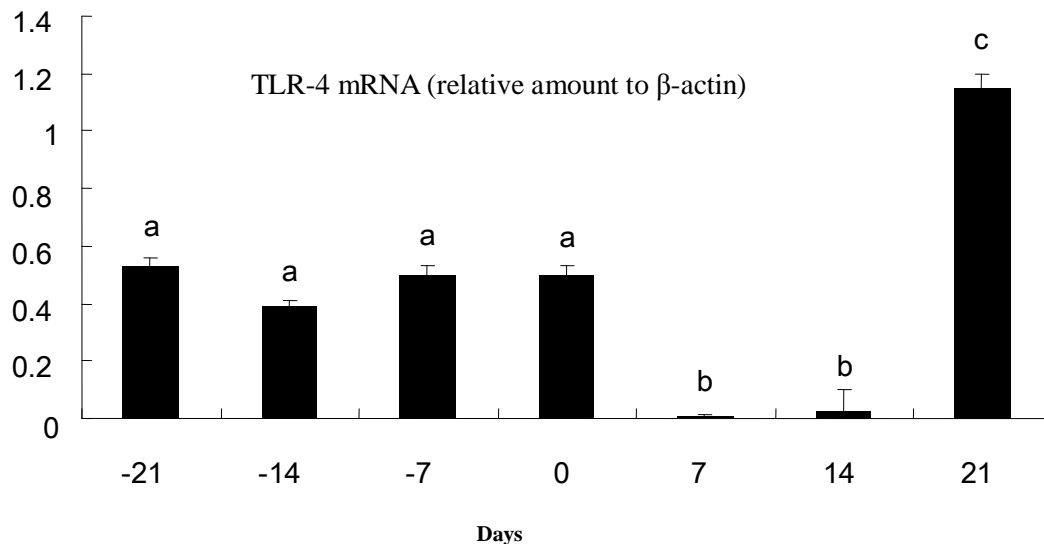
### PMN of dairy cows in peripheral blood

The results indicate that the quantity of NEU reached the peak ( $3.12 \pm 0.26 \times 10^9$ ,  $p < 0.05$ ) at 0 day, and then sharply decreased to  $2.56 \pm 0.24 \times 10^9$  at 7 days. The quantity of NEU increased gradually from 7 to 21 days after calving, but no significant difference is shown (Table 2).

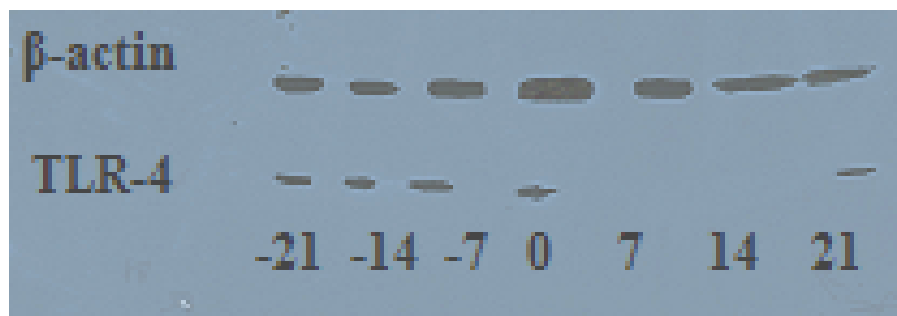
**Table 2.** PMN parameters of dairy cows in perinatal period.

Value	-21 Day	-14 Day	-7 Day	0 Day	7 Day	14 Day	21 Day
NEU 10 <sup>9</sup> /l	2.35±0.23 <sup>a</sup>	2.76±0.23 <sup>b</sup>	2.28±0.22 <sup>a</sup>	3.12±0.26 <sup>c</sup>	2.56±0.24 <sup>b</sup>	2.67±0.25 <sup>b</sup>	2.71±0.23 <sup>b</sup>

Values with different superscripts in each row are significantly different ( $p < 0.05$ ).



**Figure 1.** The mRNA expression of TLR-4 in perinatal period. Means not sharing common uppercase letter differ significantly ( $p < 0.01$ ). Means not sharing common lowercase letter differ significantly ( $p < 0.05$ ).



**Figure 2.** TLR-4 and  $\beta$ -actin protein expression of PMN during perinatal period.

### The mRNA expression of TLR-4

The mRNA expression of TLR-4 in different days in perinatal period is shown in Figure 1. The TLR-4 mRNA expression has no significant difference before calving, but the expression was down-regulated from 7 to 14 days and reduced to the minimum on the 7th day, and then sharply reached the peak at 21 days ( $p < 0.05$ ).

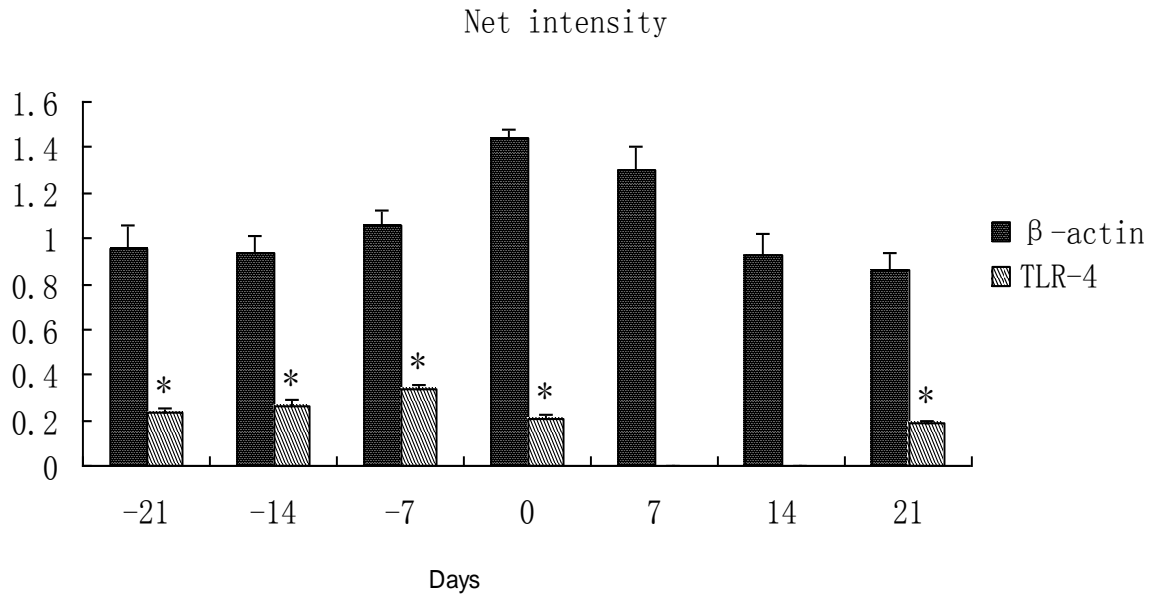
### The protein expression of TLR-4

The protein expression and net intensity of TLR-4 in

different days in perinatal period are shown in Figures 2 and 3. There was no significant difference from -21 to 0 days, but the expression of protein is lower from 7 d to 14 days ( $p < 0.05$ ).

### DISCUSSION

The well-documented immunosuppression of PMN around the time of parturition occurs through impairment of chemotaxis, superoxide production, myeloperoxidase activity and phagocytic capabilities (Cai et al., 1994; Detilleux et al., 1995; Lee and Kehrl, 1998). It leads to



**Figure 3.** Net intensity of TLR-4 and  $\beta$ -actin protein expression of PMN during perinatal period (\* $p < 0.05$ ).

the reduction of recognition and phagocytosis of pathogenic bacteria, thus the variation of PMN quantity may result in its earlier mentioned functions.

TLR-4 recognizes the conserved LPS pattern of Gram-negative bacteria. It has been reported that the actual number of TLR-4 molecules involved in recognition play a critical role in the signal initiation that leads to activation of the innate immune response (Triantafilou and Triantafilou, 2005). As mentioned earlier, we believe that the TLR-4 expression level might directly affect PMN discrimination of Gram-negative bacteria during perinatal period. Our study shows that the PMN quantity reached the peak at 0 day, and then sharply decreased at 7 days, but the quantity was still more than that of 7 and 21 days before excepted calving which may be caused by its hormone variation (Hoedemaker et al., 1992). Our study results on NEU listed in Table 2 are similar to that of Meglia et al. (2005). But as shown in Figure 1, it can be concluded that the mRNA expression down-regulation of TLR-4 on cows might occur within the 14 days after calving. The results of our western blot analysis showed similar change rule as mRNA expression. The down-regulation of TLR-4 might regulate the expression of CD11b/CD18 integrin and reduce the cytokine production in PMN. Thus, lead to the declined ability for pathogenic bacteria discrimination during this period.

## Conclusions

The results show that the quantity of PMN has limited effects on immune response during the perinatal period. However, the down-regulation of TLR-4 expression may be the major factor of PMN dysfunction of cows from 7 to

14 days after calving.

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