

# Galectin-8 Modulates Innate and Adaptive Immune Response Genes in Bovine Neutrophils

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## Abstract

Galectins (Gals) are a family of animal lectins that bind  $\beta$ -galactosides through a carbohydrate recognition domain. Galectin-8 is a tandem-repeat galectin, secreted intracellularly and extracellularly. It is associated with neutrophil migration and has been studied as a possible therapeutic to combat inflammation. The objective of this study was to evaluate the translational and the transcriptional effects of recombinant Galectin-8 (rGal-8) on cow neutrophils. Blood was collected aseptically from Holstein-Friesian cows (n=10) from the North Carolina A&T State University Dairy Unit. Neutrophils isolated were treated with rGal-8 (2 $\mu$ g), or PBS (control) and were incubated at 37°C, 5% CO<sub>2</sub> for 1 hour. Supernatant from treated neutrophils was evaluated for total protein concentration, and galectin-8 secretion using bovine Galectin-8 Enzyme Linked-Immuno-Sorbent Assay (ELISA) kit. Total RNA was extracted, reverse transcribed, and RT-qPCR was performed using the RT<sup>2</sup> Profiler Cow Innate & Adaptive Immune Responses Array with 84 genes. The Livak method was used to calculate transcript abundance and fold change (FC>2 considered significant). Total protein concentration increased (P=0.0361) after rGal-8 treatment compared to the untreated control. Galectin-8 secretion was not significantly different in control compared to treated group (P=0.5819). Out of the 84 genes, 81 genes were differentially expressed in response to rGal-8; 14 up-regulated, 5 down-regulated, 61 genes remained unchanged. Treatment with rGal8 induced the expression of IRF7. The top five up-regulated genes include FAS, CD40, CD86, IFNGR1, STAT1; down-regulated genes were TLR9, CD14, CCR6, TICAM1, and TLR1. Selected genes were probed to validate fold change; the levels of gene expression were comparable to data from RT<sup>2</sup> array. Exposure of bovine neutrophils to rGal-8 modified expression of immune response genes. The functional significance of the change needs further studies.

**Keywords:** Galectin-8, cow, neutrophils, immune

## 1. Introduction

The innate immune system, primarily leukocytes, serve as the first line of host defense and plays a crucial role in early recognition and pro-inflammatory response (Medzhitov & Janeway, 2000). Neutrophil are primary leukocytes characterized by a polymorphic-segmented nucleus, contain numerous cytoplasmic granules, and large stores of glycogen for energy (Paape, Bannerman, Zhao, & Lee, 2003). Numerous receptors are present on the neutrophil surface, some of these receptors detect chemoattractants that allow neutrophils to migrate toward areas of inflammation (Paape et al., 2003). Bovine neutrophils are comparable to neutrophils of other species in their functionality, activation mechanisms, diapedesis, and modulation of immune response (Bassel & Caswell, 2018). The balance between microbial killing and host tissue damage is linked to neutrophil biochemical composition which is species specific (Bassel & Caswell, 2018).

Galectin-8 is a mammalian lectin, made of two carbohydrate-recognition domains in the same polypeptide chain, joined by a link peptide (Hadari et al., 1995). Galectins have been shown to modulate the innate and adaptive immune system (Rabinovich & Gruppi, 2005; Vasta et al., 1994). Galectin expression and modulation in ruminants have been well- studied and reported previously (Eluka-Okoludoh et al., 2018; Ekwemalor et al., 2018; Asiamah et al., 2018; Osei et al., 2017, 2018; Mulakala et al., 2018). Galectin-8 acts as an extracellular matrix (ECM) protein that positively or negatively regulates cell adhesion, depending on the extracellular context and cell surface

counter-receptors like integrins (Troncoso et al., 2014). Galectin-8 involvement in the migration or activation of inflammatory cells suggests a role in the pathogenesis of inflammatory diseases (Tribulatti, Cattaneo, Hellman, Mucci, & Campetella, 2009). Galectin-8 modulates the neutrophil function via superoxide production (Nishi et al., 2003). The transcriptional and translational effects of Galectin-8 in bovine neutrophils is not well understood. The objective of this study was to evaluate the effect of recombinant Galectin-8 (rGal-8) on transcription and translation of genes associated with immunity in cow neutrophils.

## 2. Materials and Methods

### 2.1 Sample Collection

Ten clinically healthy Holstein Friesian dairy cows from the North Carolina Agricultural and Technical State University (NC A&T) Dairy Unit were used for this study. This study was approved by the Institutional Animal Care and Use Committee. Whole blood samples (50mL) were collected from the jugular vein aseptically into tubes containing the anticoagulant citrate dextrose (ACD). Blood samples were processed within 2 hours of collection.

### 2.2 Neutrophil Isolation Analysis

Neutrophils were isolated from whole blood (50mL) using the modified Carlson and Kaneko method (Carlson & Kaneko, 1973). Whole blood was centrifuged on a swinging bucket rotor (Eppendorf Centrifuge 5810 R) at 1800 x g at 4°C for 20 minutes. Isolated total cell count and viability was assessed using TC-20 (Bio-Rad), White blood cell differential count was calculated as described previously (Worku et al., 2016; Adjei-Fremah et al., 2016, 2018; Ekwemalor et al., 2016, 2018).

### 2.3 Preparation of Treatments

Recombinant human galectin-8 (source: *E. coli*, Novo protein, Summit, NJ), was reconstituted in endotoxin-free ultra-pure water (Invitrogen). Neutrophils ( $10^6$  cells/ml, viable cells) were treated with 2µg/ml rGal-8 in PBS (PH 7.4), and untreated control samples were maintained in Phosphate buffer saline (PBS) served as control. All reagents used were endotoxin-free as tested with the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit following the manufacturer's protocol (GenScript, Piscataway, NJ) as described previously (Adjei-Fremah et al., 2016; Adjei-Fremah, 2017; Ekwemalor et al., 2018).

### 2.4 Isolation of Total RNA and cDNA Synthesis

Total RNA was isolated using Trizol according to the manufacturer's instructions (Sigma-Aldrich St. Louis, MO). RNA concentration and purity were assessed using a Nanodrop Spectrophotometer ND 1000 (Thermo Scientific Inc., Waltham, MA). RNA integrity purity was analyzed on an RNA 6000 Nano LabChip (Agilent Technologies, DE) following manufacturers protocol as described previously (Adjei-Fremah et al., 2016, 2018). Isolated total RNA (200ng/µl) was converted to complementary DNA (cDNA) from RNA using Retroscript kits (Ambion, Inc), following the manufacturer's instructions. Selected Primers used to validate RT<sup>2</sup> Profiler Cow Innate & Adaptive Immune Responses PCR Array (Table 1).

Table 1. Sequences of primers used for validation

Accession Number	Gene	Primer	Sequence 5'>3'	Product size (bp)
NM_174197	<i>TLR2</i>	Forward	<i>ACGCCTTTGTGCCTAC</i>	192
		Reverse	<i>CCGAAAGCACAAAGATGGTT</i>	
NM_173966	<i>TNFα</i>	Forward	<i>CTGCACTTCGGGGTAATCGG</i>	549
		Reverse	<i>CAGGGCGATGATCCCAAAGTA</i>	
NM_174088	<i>IL-10</i>	Forward	<i>TGCTGGATGACTTTAAGGGTTACC</i>	186
		Reverse	<i>TCATTTCCGACAAGGCTTGG</i>	
NM_174008	<i>CD14</i>	Forward	<i>GACGACGATTCCGTTGTGT</i>	600
		Reverse	<i>TGCTAGCGCTAGATATTGGA</i>	
AB098788	<i>RPLPO</i>	Forward	<i>CAACCCTGAAGTGCTTGACAT</i>	564
		Reverse	<i>AGGCAGATGGATCAGCCA</i>	
XR_003033789	<i>18S rRNA</i>	Forward	<i>TTCGATGGTAGTCGCTGTGC</i>	99
		Reverse	<i>TTGGATGTGGTAGCCGTTTCT</i>	

### 2.5 First Strand cDNA Synthesis and RT<sup>2</sup> Profiler Array Analysis

Purified Total RNA was reverse transcribed using First Strand cDNA Synthesis Kit for RT-qPCR (Qiagen), following manufacturer's instruction as described previously (Adjei-Fremah et al., 2017, 2018; Ekwemalor et al., 2016; Worku et al., 2016). Briefly, reaction mixture to eliminate genomic DNA, was incubated for 5 min at 42°C, then place immediately on ice. To reverse transcribe, 10µl reverse-transcription mix was added to 10µl genomic DNA elimination mix. Mixture was incubated at 42°C for exactly 15 min, then incubate at 95°C for 5 min. Ninety-one (91µl) RNase-free water was added to reaction.

RT<sup>2</sup> Profiler Cow Innate & Adaptive Immune Responses PCR Array containing primers for 84 tested and 5 housekeeping genes, and controls for RT reactions. Total volume of cDNA synthesized from First Strand Synthesis was used for preparation of reaction mixture. To each of 96 well-plates, 25µl reaction mixture comprised of 2x RT<sup>2</sup> SYBR Green Mastermix, cDNA synthesis reaction and RNase-free water (Qiagen) was added. Array plate was processed in CFX-96 (Bio-Rad), thermal cycling condition recommended by manufacturer (Qiagen), 10min 95°C, 15s 95°C, 1min 60°C repeat for 40 cycles.

Gene expression was normalized to housekeeping genes TYK2, ACTB, GAPDH, HPRT1, and TBP to determine fold change between treated and control samples using 2<sup>-ΔΔCt</sup> method (Livak & Schmittgen, 2001).

### 2.6 Total Protein Concentration

Total protein concentration was measured in supernatant after treatment with galectin-8, in individual and pooled samples using Pierce BCA Assay kit (Rockford, IL), following manufacturer's instructions as described previously (Obanla et al., 2016; Adjei-Fremah et al., 2015; Hamilton et al., 2017; Karlton-Senaye et al., 2018).

### 2.7 Quantification of Galectin-8 Secretion

Bovine Galectin-8 Enzyme Linked-Immuno-Sorbent Assay (ELISA) kit (MyBiosource, Inc., San Diego, CA), was used to measure quantification of Galectin-8 Concentration in supernatant of treated and control samples. Details described elsewhere (Asiamah et al., 2018), briefly, 50µL of each standard and sample, and 100µl HRP conjugate was added to appropriate wells. Wells were covered and incubated for an hour at 37°C. The solution was discarded, and plate washed four times with 1X Wash Buffer. Fifty microliters (50)µl of chromogen A and chromogen B substrate was added to each well and incubated for 15 minutes at 37°C, after which stop solution was added. Absorbance was measured at 450 nm using microplate reader (Bio Tek).

### 2.8 Statistical Analysis

One-way Analysis of variance (ANOVA) was performed on all variables using the statistical analysis software package SAS version 9.3 (SAS Institute, Cary, NC) to evaluate differences between treatment and control groups. P-value of <0.05 was considered to be significant.

## 3. Results

### 3.1 Effect of rGalectin-8 Treatment on Neutrophil Count and on Cow Innate & Adaptive Immune Response

Treatment of bovine neutrophils with exogenous Galectin-8 had no effect on the neutrophil count and viability (Table 2). There was no significant difference ( $p>0.05$ ) in the neutrophil cell count between treated and untreated groups. Individual animal variation was observed in the percentage of neutrophils. The average purity of neutrophil preparations was 94% or higher, as identified by stained smears.

Table 2. Analysis of bovine neutrophils count average percent viability following galectin-8 treated and control

Parameter	Control	rGal-8 treated	P-value
Total cell count (cells/ml)	7.10 x 10 <sup>6</sup> ±	6.19 x 10 <sup>6</sup> ±	NS
Total viable cells (cells/ml)	1.07 x 10 <sup>6</sup>	1.12 x 10 <sup>6</sup> ±	NS
Neutrophils (%)	94 ± 96	95±96	NS

Note. rGal-8 = recombinant Galectin-8; NS =  $P < 0.05$ .

RT<sup>2</sup> Profiler™ PCR Array Cow Innate & Adaptive Immune Responses was observed at the level of mRNA transcription in recombinant Galectin-8 treated bovine neutrophils. The genes on the array consist of Innate and Adaptive immunity genes, Pattern Recognition Receptors (PRRs), Cytokines, Th1 and Th2 markers and Treg markers. Out of the 84 genes on the array, 81 genes were detected, 80 in the control group (Figure 1) and 81 in the treated group. Four genes were not detected in the control group IL18, IRAK1, MAPK1, and IRF7. Treatment with

rGalectin-8 induced the expression of IRF7 in bovine neutrophils, this gene was not expressed in the control group. Treatment with rGalectin-8 differentially affected the expression of 19 genes; 13 genes were up-regulated, 5 genes were down-regulated, and 61 genes remained unchanged or not significantly expressed. The up-regulated genes were FAS, CD40, CD86, IFNGR1, STAT1, CXCL8, TLR2, CD80, IL10, TNF, NLRP3, NFKB1, IFNG (Table 3). Down-regulated genes were TLR9, CD14, CCR6, TICAM1, TLR1 (Table 4). The highest fold changes were observed with cell surface receptor genes and cytokines.

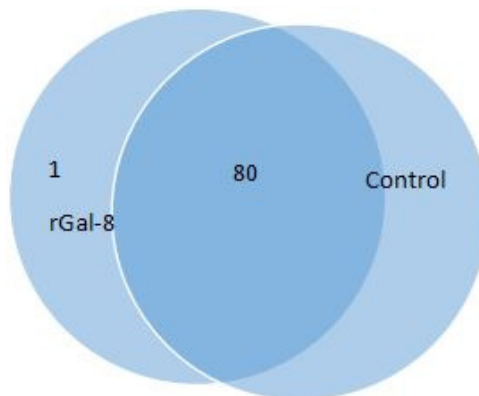


Figure 1. Venn diagram showing summary of 84 genes on the Innate & Adaptive Immune Responses RT<sup>2</sup> Profiler

PCR Array in response to treatment with rGalectin-8. Eighty genes were detected in the Galectin-8 treated and control groups, one gene was induced by Galectin-8, three genes were not expressed in both groups.

Table 3. Up-regulated Cow Innate & Adaptive Immune Responses PCR Array in response to rGalectin-8 treatment

Gene	Fold Change	Gene Function
<i>FAS</i>	4.66	Plays a central role in the physiological regulation of programmed cell death, adaptive immunity
<i>CD40</i>	3.77	Co-stimulates T-cell proliferation and cytokine production, innate immunity
<i>CD86</i>	3.59	Encodes a type I membrane protein that is a member of the immunoglobulin superfamily, Adaptive immunity
<i>IFNGR1</i>	3.58	Plays an essential role in the IFN-gamma pathway that is required for the cellular response to infectious agents, Adaptive immunity
<i>STAT1</i>	3.47	Transcription factor that can induce a set of IFN- $\gamma$ -regulated genes, Innate immunity
<i>CXCL8</i>	3.34	Neutrophil activation, released from several cell types in response to an inflammatory stimulus, Adaptive immunity - cytokine
<i>TLR2</i>	3.15	PRR, recognizes PAMPs, mediates the response to bacterial lipoproteins, innate immunity
<i>CD80</i>	3.07	Costimulatory molecule known for its role in T-cell activation, Adaptive immunity
<i>IL10</i>	3.05	Prevents inflammatory and autoimmune diseases, Adaptive immunity
<i>TNF</i>	2.74	Pro-inflammatory cytokine, Adaptive immunity
<i>NLRP3</i>	2.53	PRR, recognizes PAMPs, plays a crucial role in inflammation, Innate immunity
<i>NFKB1</i>	2.33	Activated by various stimuli such as cytokines, bacteria or viruses, Innate immunity
<i>IFNG</i>	2.0	Antiviral activity, and immunoregulatory functions, Adaptive Immunity

Table 4. Down-regulated Cow Innate & Adaptive Immune Responses PCR Array in response to rGalectin-8 treatment

Gene	Fold regulation	Gene Function
<i>TLR9</i>	-2.73	PRR, alerts the immune system of viral and bacterial PAMPs, Innate immunity
<i>CD14</i>	-2.53	Mediates the response to bacterial LPS, Innate immunity
<i>CCR6</i>	-2.31	Chemotaxis of dendritic cells, immune responses to microbes, Th17 marker
<i>TICAM1</i>	-1.96	Involved in alerting against invading pathogens, Innate immunity
<i>TLR1</i>	-1.94	PRR recognize pathogen-associated molecular patterns (PAMPs), Innate immunity

Note. Gene functions are from GeneCards<sup>®</sup> ([www.genecards.org](http://www.genecards.org))

Following RT<sup>2</sup> profiler array data analysis a set of genes was chosen for validation by RT-qPCR based on fold change and known effects of Galectin-8. Correlation between the RT<sup>2</sup> profiler array and RT-qPCR results was performed, and the statistical significance of the correlation determined. The Livak's method was used to calculate transcript abundance. The selected genes were: TLR2, IL-10, TNF and CD14, primer sequence listed (Table 1). Bovine primers were designed using NCBI and primer 3 tool. Our results show up-regulation in expression of TLR2, IL-10 and TNF, and down-regulation in CD14 (Table 5). RPLPO and 18S rRNA were used as housekeeping genes to normalize expression.

Table 5. Validation of RT<sup>2</sup> array results using Real Time-qPCR

Gene	RT <sup>2</sup> Array Fold change	RT-qPCR Fold change
TLR2	3.15	2.00
IL-10	3.05	5.10
TNF	2.74	14.22
CD14	-2.53	-2.77

### 3.2 Effect of Galectin-8 on Total Protein Concentration

Total protein concentration was analyzed in treatment and control neutrophils. There was a significant difference in protein content of control compared to treated neutrophils  $P = 0.036$  (Figure 2) in pooled and individual animal samples. There was variation in the total protein concentration level among the different animals used. Consequently, rGal-8 increased the total protein concentration in bovine neutrophils.

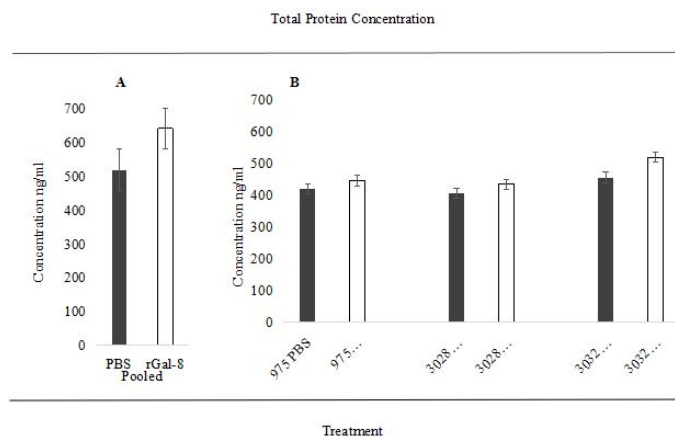


Figure 2. Total protein concentration in recombinant galectin-8 treated and control neutrophils ( $P = 0.036$ )

**A.** Total protein concentration in treated supernatant of pooled neutrophils ( $n=10$ ). **B.** Total protein concentration in neutrophils of individual animals ( $n=3$ , Individual animal identification 975, 3028, 3032).

### 3.3 Effect of rGalectin-8 Treatment on Galectin-8 Secretion

Levels of secreted galectin-8 were analyzed in both treatment and control groups. After treatment, the supernatant was used to detect levels of secreted galectin-8. Galectin-8 is secreted in bovine neutrophils. There was no difference in the amount of secreted Galectin-8 ( $P = 0.58$ ) in treated and control groups (Figure 3).

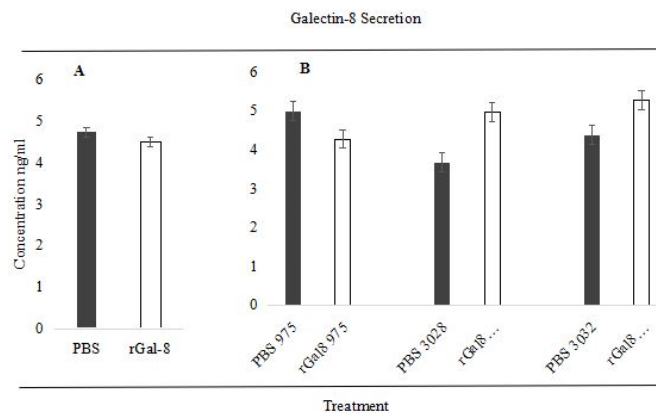


Figure 3. Secretion of Galectin-8 in bovine neutrophils following treatment with recombinant Galectin-8

Bovine specific Galectin-8 Enzyme-Linked Immunosorbent Assay (ELISA) on treated and control group ( $P = 0.36$ ). **A.** Galectin-8 secretion in pooled neutrophils ( $n=10$ ), **B.** Galectin-8 secretion in individual animals ( $n=3$ , Individual animal identification 975, 3028, 3032). PBS=Phosphate Buffer Saline served as control.

#### 4. Discussion

Galectins have been studied for their multifunctional roles. Galectin-8 has been reported to be expressed in a variety of tissues. Previous studies conducted by our research group reported the expression of Galectin-8 in bovine peripheral blood (Asiamah et al., 2017, 2018). Our current study investigated the effects of recombinant Galectin-8 on secretion and expression of innate and adaptive immune response genes in bovine neutrophils.

Our RT<sup>2</sup> Profiler™ PCR Array Cow Innate & Adaptive Immune Responses result shows modulation in expression of neutrophils treated with exogenous Galectin-8. Dairy bovine neutrophils genes were expressed in the five control housekeeping genes ACTB, GAPDH, HPRT1, TBP, and YWHAZ. Three genes were not expressed at all in the control and treatment group, IL-18, MAPK1, and IRAK1. Treatment with rGal-8 induced the expression of Interferon regulatory factor 7 (IRF7). IRF is a member of the interferon regulatory transcription factor (IRF) family and plays a critical role in the innate immune response against DNA and RNA viruses (GeneCards, www.genecards.org). Furthermore, IRF7 is a crucial regulator of type I interferons (IFNs) against pathogenic infections, activation triggers signaling cascades from pathogen recognition receptors (PRRs) (Ning, Pagano, & Barber, 2011). IFNG and IFNGR1 have increased fold change in response to Galectin-8 stimulation. IFN function with receptors, one of the key receptors is the cargo receptor NDP52 which detects ubiquitinated structures on bacteria (MacMicking, 2012). Galectin-8 has been shown to utilize the cargo receptor NDP52 to recruit autophagy to engulf lysosomal bacteria (Thurston et al., 2012). IRF7 activation in the presence of exogenous Galectin-8 demonstrates that Galectin-8 modulates the IFN-IRF7 system.

Previous studies have reported the expression of Toll-Like Receptors in cow neutrophils (Worku et al., 2016; Adjei-Fremah et al., 2017, 2018). In our study treatment with recombinant Galectin-8 increased the expression of TLR2, and down-regulated the expression of TLR9. Toll-Like Receptor stimulation induced multiple signaling pathways. Some of these pathways include nuclear factor- $\kappa$ B (NF- $\kappa$ B), mitogen-activated protein kinases (MAPKs) and type-I interferon (IFN) response (Akira et al., 2006). Treatment with recombinant Galectin-8 activated NF- $\kappa$ B1 and increased the expression of IL-10, CXCL8 and TNF cytokines. Previous studies have reported increase in expression of pro-inflammatory cytokines in human endothelial cells when treated with Galectin-8 (Cattaneo et al., 2014). The CD14 glycoprotein and receptor was found to be down regulated with treatment of Galectin-8. The interaction between recombinant Galectin-8 and TLRs triggers the expression of several cytokines and may activate the innate immune system through the NF- $\kappa$ B pathway.

Gene expression analysis on the array showed Galectin-8 increased the expression of FAS, a member of the TNF-receptor superfamily. Our results are consistent with previous research indicating that Jurkat T cells treated with galectin-8 had an increase in FAS expression (Norambuena et al., 2009). Furthermore, Norambuena et al. (2009) showed that Galectin-8 exerted proapoptotic effects via activation of ERK1/2 in Jurkat T cells through FAS ligand. FAS is a part of the TNF superfamily, our result showed increased TNF expression in the presence of Galectin-8 is consistent with previous findings. Treatment with Galectin-8 increased the fold change in bovine neutrophils: analogous to our findings, mice splenocytes with exogenous galectins-8 induced the expression of CD86 (Carabelli et al., 2017).

Validation results using IL-10, TNF, TLR2 and CD14, showed comparable results to the array. When these primers were tested on individual animals, there were varying expressions of each gene (data not shown). Striking evidence has demonstrated that galectin-8 can stimulate dendritic cells that secrete pro-inflammatory cytokines and stimulate antigen specific T-cells (Brinchmann et al., 2018; Carabelli et al., 2017).

In conclusion, this study provided preliminary evidence that exogenous recombinant Galectin-8 is involved in modulation of the innate and adaptive immunity in bovine neutrophils. Binding of recombinant Galectin-8 to TLRs through the NF- $\kappa$ B pathway may be important in bovine immune system. Further definition of the interaction between genes and pathways need further study.

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### Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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