Natural and synthetic pathogen associated molecular patterns modulate galectin expression in cow blood

Emmanuel Kwaku Asiamah[1*], Kingsley Ekwemalor[2], Sarah Adjei-Fremah[3], Bertha Osei[4], Robert Newman[3] and Mulumebet Worku[2]

[1]Department of Agriculture- Animal Science, University of Arkansas at Pine Bluff, AR 71601, USA
[2]Department of Animal Sciences, North Carolina A&T State University, Greensboro, NC 27411, USA
[3]Department of Biology, North Carolina A&T State University, Greensboro, NC 27411, USA
[4]Functional and Chemical Genomics, Oklahoma Medical Research Foundation, OK 73104, USA

Abstract

Pathogen-associated Molecular Patterns (PAMPs) are highly conserved structural motifs that are recognized by Pathogen Recognition receptors (PRRs) to initiate immune responses. Infection by these pathogens and the immune response to PAMPS such as lipopolysaccharide (LPS), Peptidoglycan (PGN), bacterial oligodeoxynucleotides [CpG oligodeoxynucle-otides 2006 (CpG ODN2006) and CpG oligodeoxynucleotides 2216 (CpG ODN2216)], and viral RNA Polyinosinic-Polycy-tidylic Acid (Poly I:C), are associated with infectious and metabolic diseases in animals impacting health and production. It is established that PAMPs mediate the production of cytokines by binding to PRRs such as Toll-like receptors (TLR) on immune cells. Galectins (Gal) are carbohydrate-binding proteins that when expressed play essential roles in the resolution of infectious and metabolic diseases. Thus it is important to determine if the expression of galectin gene (LGALS) and Gal secretion in blood are affected by exposure to LPS and PGN, PolyI:C and bacterial CpG ODNs. LPS increased transcription of LGALS4 and 12 (2.5 and 2.02 folds respectively) and decreased secretion of Gal 4 ($p < 0.05$). PGN increased transcription of LGALS-1, -2, -3, -4, -7, and -12 (3.0, 2.3, 2.0, 4.1, 3.3, and 2.4 folds respectively) and secretion of Gal-8 and Gal-9 ($p < 0.05$). Poly I:C tended to increase the transcription of LGALS1, LGALS4, and LGALS8 (1.78, 1.88, and 1.73 folds respectively). Secretion of Gal-1, -3, -8 and nine were significantly increased in treated samples compared to control ($p < 0.05$). CpG ODN2006 did not cause any significant fold changes in LGALS transcription (FC < 2) but increased secretion of Gal-1, and-3 ($p < 0.05$) in plasma compared to control. Gal-4 was however reduced in plasma ($p < 0.05$). CpG ODN2216 increased transcription of LGALS1 and LGALS3 (3.8 and 1.6 folds respectively), but reduced LGALS2, LGALS4, LGALS7, and LGALS12 (−1.9, −2.0, −2.0 and; −2.7 folds respectively). Secretion of Gal-2 and -3 in plasma was increased compared to control ($p < 0.05$). Gal-4 secretion was reduced in plasma ($p < 0.05$). The results demonstrate that PAMPs differentially modulate galectin transcription and translation of galectins in cow blood.

Keywords: PAMPs, Galectins, Cow, Disease

Background

Cows are infected by numerous pathogens that affect their productivity and health. These pathogens include bacteria, protozoa, and viruses. Diseases caused by pathogens affect the profitability of rearing animals [1,2]. Pathogen-associated Molecular Patterns (PAMPs) are recognized by pathogen recognition receptors (PRRs) on host cells to trigger immune responses [3]. Lipopolysaccharide (LPS), peptidoglycan (PGN), double-stranded viral RNA (Poly I:C) and bacterial oligonucleotides (CpG ODNs) are associated with infectious and metabolic diseases in the cow [2,4–6]. PAMPs mediate the production of cytokines by primarily binding to PRRs, such as TLRs on immune cells to activate several signaling pathways [7–9]. There are 10 TLRs identified in cows that recognize PAMPs [10]. Previous studies have reported that the increase of LPS in the digestive tract of the cow phenomenon called subacute ruminal acidosis, and its translocation into the bloodstream, results in immune responses which if not controlled, causes serious health consequences [11]. Proper recognition and elimination is essential to animal health and production [12]. Aside from TLRs, other receptors and co-receptors play significant roles in recognition and pathogen elimination.

Numerous studies have shown that Gal, a family of proteins defined by their affinity for β-galactosides, participate in the regulation of both innate and adaptive immunity [13–15].

Some galectins have been studied to bind microorganism-specific glycans, or host-like glycans on microorganisms to initiate immune responses [16]. Galectin mediated immune response could either lead to clearance of microorganism or favor establishment of the pathogens. Galectins (Gal) act as PRRs that orchestrate immune responses according to the level of pathogenicity of the invading microorganisms [17]. Some members of the galectin family are also known alarmins for sepsis, a condition caused by an overwhelming immune response to microbial infection [18].

The most significant advancement in animal health in the past three decades has been the paradigm shift from treatment of clinical illness to disease prevention [19]. Overuse of antibiotics in animal production has raised food safety and public health concerns in addition to its reduced effectiveness and resistance in animals [20]. As such, numerous studies are emerging to better understand the molecular mechanisms leading to infectious diseases to design more effective management practices to reduce cow health disorders [7,8]. An increased understanding of the genetics underlying the immune response mechanism of cows during infections may offer opportunities for alternative control strategies.

Because galectins are involved in immune responses and the outcome of microbial infections, it was essential to evaluate whether its expression in cows is affected by pathogen-associated molecular patterns (PAMP) stimulation in whole blood. Understanding galectin expression in response to PAMP stimulation will aid in drug design aimed at galectins as targets. This study aimed to evaluate the effect of stimulation with known PAMPs (natural and synthetic) on galectin gene expression in cow blood.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee of North Carolina A&T State University approved all protocols for animals handling. Five multiparous Holstein Friesian periparturient cows (N = 5) from North Carolina A&T State Dairy farm were used. None of the animals showed any signs of disease or received medications during the 4-week period before blood sampling.

Blood sampling

Complete details of blood sampling have been reported elsewhere [21]. Briefly, Whole blood (10 mL) was collected aseptically from the jugular vein of the animals into vacutainer tubes containing 1mL of the anti-coagulant Acid Citrate Dextrose. The tubes were gently mixed and placed on ice immediately after collection. The samples were transported to the Laboratory for Animal Genomic Diversity and Biotechnology at North Carolina A&T State University for further analysis.

Stimulation of whole blood

One mL of blood from the cows was each incubated with 10 μg/mL each of either *Escherichia coli* derived LPS (Sigma-Aldrich, St. Louis, MO), *Staphylococcus aureus*-derived PGN (Sigma-Aldrich), CpG ODN (2216) class A (Invivogen, San Diego CA), CpG ODN (2006) class B (Invivogen) and 12.5 μg/mL of poly I:C (Invivogen) individually to assess the expression of *LGALS*. Samples treated with phosphate buffer saline (PBS) served as control. Samples were incubated at 37℃, with 85% humidity and 5% CO_2 for 30 minutes. At the end of the incubation period, the tubes were spun down at 1,700 × g at 4℃ for 10 minutes. Supernatants were collected and stored at −80℃ to measure secreted galectins concentration. Trizol (1 mL) was added to cell pellets and stored for RNA isolation (Ambion®, Thermo Fisher Scientific Inc, Waltham, MA).

Total RNA isolation and cDNA synthesis

Total RNA was isolated using Trizol according to the manufacturer's instructions (Sigma-Aldrich St. Louis, MO). The appropriate precautions were used to avoid RNase contamination throughout the entire procedure [9]. The RNA concentration (ng/μL) and purity (260/280) were assessed using a Nanodrop Spectrophotometer

ND 1000 (Thermo Scientific Inc., Waltham, MA). Total RNA was pipetted into an RNA 6000 Nano LabChip® (Agilent Technologies, Santa Clara, CA) and RNA integrity was determined using Bioanalyzer following manufacturer's protocol (Agilent). Complimentary DNA (cDNA) synthesis was performed with 500 ng/μL RNA (purity 260/280 = 1.8, RIN = 7). Retroscript kits (Ambion®) were used to synthesize cDNA for real-time Polymerase chain reaction. cDNA synthesis was performed in an MWG AG Biotech Primus 96 Plus Industrial Lab Thermal Cycler (MWG Biotech Huntsville, AL). Total RNA (1 μg) was heated to 85℃ for 3 minutes and placed on ice, centrifuged briefly, and returned to ice. The remaining RETROscript® components were added: 10X RT (PCR) buffer (2 μL), dNTP mix (4 μL), RNase inhibitor (1 μL), and MMLV-RT (1 μL), for a final volume of 20 μL. The components were mixed gently, centrifuged briefly and incubated at 42–44℃ for 1 hour, followed by an incubation step at 92℃ for 10 minutes to inactivate the Reverse Transcriptase (RT). Polymerase chain reaction was performed by assembling the following components: RT reaction buffer (5 μL), 10X PCR buffer (5 μL), dNTP (2.5 μL), Nuclease-free water (37.5 μL), PCR primers (provided in kit) (2.5 μL), and thermostable DNA Polymerase (1–2 units (U)). The PCR cycle was: Step 1) Initial denaturation, 94℃ for 2 minutes; Step 2) Amplification, 94℃ for 30 seconds (denaturation), 60℃ (annealing) for 30 seconds, 72℃ (extension) for 1 minute; Step 3) to Step 2 for 30 cycles; and Step 4) Final Extension, 72℃ for 5 minutes.

Gene expression profiling

With the use of Primer-3 online tool (http://bioinfo.ut.ee/primer3-0.4.0/), Forward and reverse primers for cow galectin genes *LGALS1, 2, 3, 4, 7, 8, 9, 12,* and *15* were designed commercially. Based on published sequences, cow specific Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and β actin were purchased from MWG, Biotech Huntsville AL. β-actin and GAPDH were used as internal controls and for normalization.

Real-time qPCR

Real-time qPCR was performed in the CFX Connect real-time system (Bio-rad Laboratories, Hercules, CA). The qPCR reaction mixture consisted of template, primer (μL), intercalating dye SYBR Green, DH_2O, 200 ng of cDNA, 1 μL (100 μM) of forward primer, 1 μL (100 μM) of reverse primer, 10 μL of SYBR Green and DH_2O to the volume of 20 μL.

All samples were carried out in triplicate 20-μL reactions in 96-well plates, and a negative control with no cDNA template were included in every run. The run program used were: Step 1) 95℃ for 15 seconds for denaturing, Step 2) 60℃ for 30 seconds for primer annealing, and 72℃ for elongation. Step 3) to Step 2 for

30 cycles; and Step 4) Final Extension, 72℃ for 5 minutes. Specificity of the amplicon products was confirmed by visual inspection of melting curves. Real-time PCR data was analyzed using the Livak's method [22]. The housekeeping genes (GAPDH and β actin) and samples from far-off/PBS treated cows were used to determine the ΔΔCt [22]. Fold change in transcript abundance was calculated using the Livak method. Where

ΔCt = (Target genes treat – GAPDH/ β actin treat)
      – ΔCt (Target genes PBS – GAPDH/ β actin PBS).
Fold change = 2 (−ΔΔCt).

Evaluation of galectin secretion

Commercial bovine specific galectin ELISA kits were purchased and used to determine the concentrations of secreted Gal-1 (catalog no. MBS2882620, detection range; 0.31 ng/mL & 20.0 ng/mL), Gal-2 (catalog no. MBS033680 detection range; 0.625 ng/mL & 20 ng/mL), Gal-3 (catalog no. MBS017323 detection range; 0.156 ng/mL & 10 ng/mL), Gal-4 (catalog no. MBS028694, detection range; 0.25 ng/mL & 8 ng/mL), Gal-8 (catalog no. MBS041856 detection range; 0.5 ng/mL & 16 ng/mL), Gal-9 (catalog no. MBS033074 detection range; 0.625 ng/mL & 20 ng/mL), Gal-12 (catalog no. MBS032400, detection range; 31.2 pg/mL & 1,000 pg/mL) in plasma according to the manufacturer's instructions (My BioSource®)) [23]. A microplate reader was used to measure the absorbance at 450 nm (BioTek Instruments, Inc., Winooski, VT). Galectin concentration was then determined using a standard curve. The sensitivity was 0.1 ng/mL for all assays. Both intraassay CV (%) and inter-assay CV (%) for all assays was less than 15%. [CV (%) = SD/mean ×100].

Statistical analysis

Real-time PCR data were analyzed using Livak's method [22]. Housekeeping genes (GAPDH and β actin) and samples from PBS treated samples were used to determine the ΔΔCt, as described above. The Proc GLM procedure in SAS 9.4 (SAS Institute Inc, Cary NC) was used to analyze data obtained for total galectins concentrations. Each sample was assayed in triplicates. The PDIFF statement in SAS (SAS Institute Inc. Cary, NC) was used to compare all least square means. Significant differences were declared at $p < 0.05$. PROC corr was used to do a correlation analysis between *LGALS* and Gal concentrations in plasma.

Results

Effect of PAMPs on LGALS transcription

LPS increased transcription of *LGALS4* and *LGALS12* (2.5 and 2.02 folds respectively) (Fig. 1A). PGN increased transcription of *LGALS-1, -2, -3, -4, -7,* and *-12* (3.0, 2.3, 2.0, 4.1, 3.3, 2.4
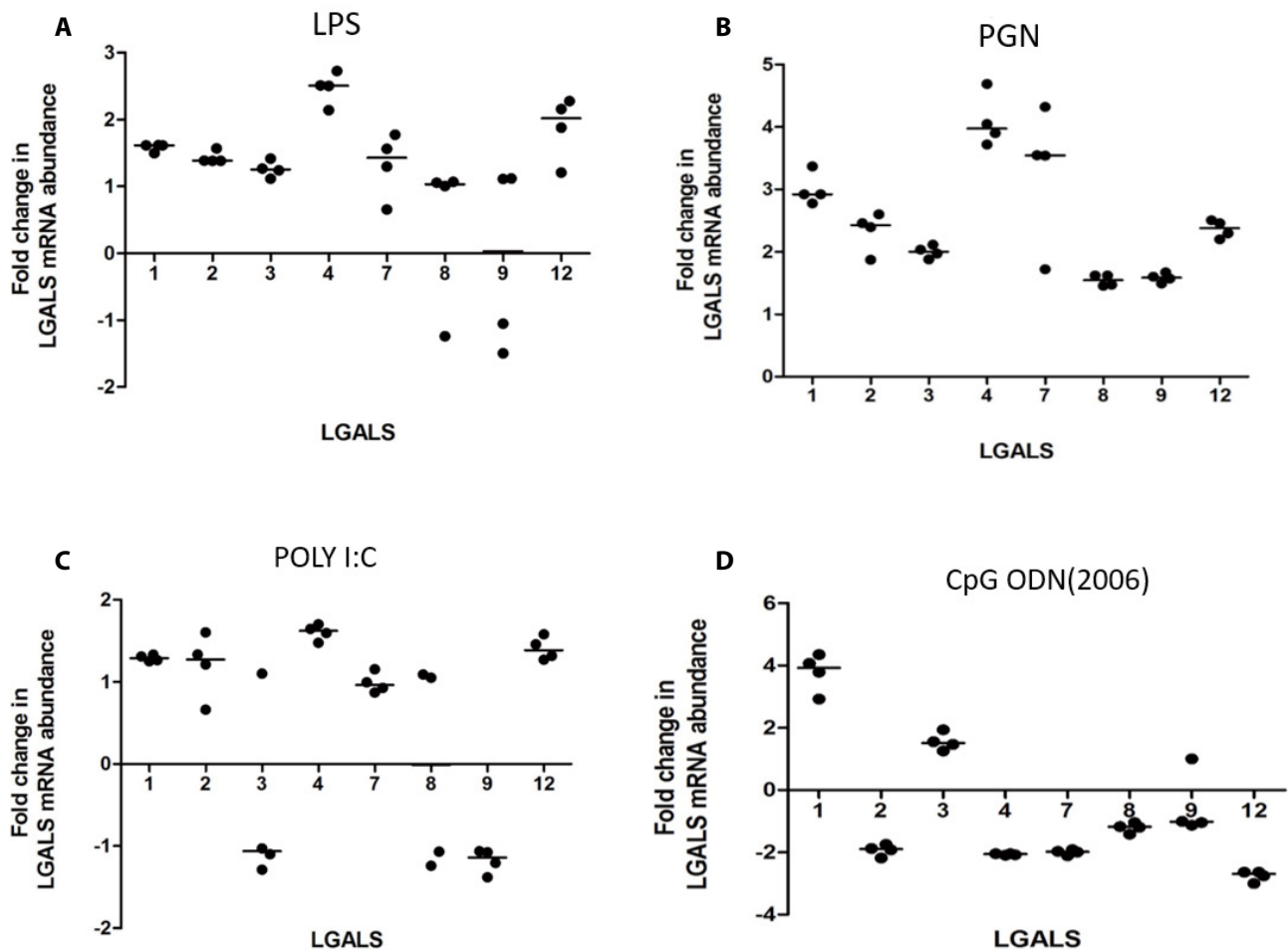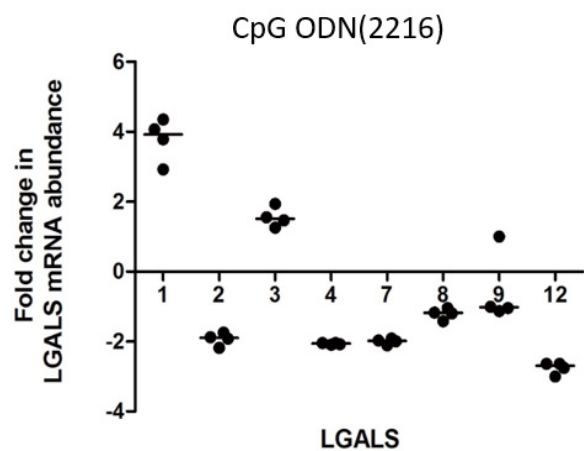
Fig. 1. **Fold changes in mRNA expression of *LGALS-1, -2, -3-,4, -7, -8, -9,* and *-12* after stimulation of whole blood with LPS (A), PGN (B), *POLY I: C* (C), CpG ODN 2006 (D).** Error lines represent the ± standard deviation of the mean.

folds respectively) (Fig. 1b). Poly I:C increased the transcription of *LGALS1, LGALS4,* and *LGALS8* (1.78, 1.88, and 1.73 folds respectively) (Fig. 1c). CpG oligodeoxynucleotides 2006 (CpG ODN2006) did not cause any significant fold changes in *LGALS* transcription (FC < 2) (Fig. 1d). CpG oligodeoxynucleotides 2216 (CpG ODN2216) increased transcription of *LGALS1* and *LGALS3* (3.8 and 1.6 respectively), but reduced *LGALS2, LGALS4, LGALS 7,* and *LGALS12* (–1.9, –2.0, –2.0 and –2.7) (Fig. 2).

Effects of PAMPs on galectin secretion

Poly I:C and CpG ODN2006 increased plasma secretion of Gal-1 compared to control (PBS) (p = 0.10 and p = 0.0125 respectively) (Fig. 3a). CpG ODN 2216 increased Gal-2 concentration in plasma compared to control (PBS) (p = 0.0459) (Fig. 3b). CpG ODN 2216 and CpG ODN 2006 increased Gal-3 compared to control (PBS) (p = 0.013 and p = 0.0195 respectively) (Fig. 3c). LPS, CpG ODN2216, and CpG ODN2006 reduced Gal-4 concentrations in plasma compared to control (PBS) (p = 0.04, p = 0.0005 and p

= 0.0273 respectively (Fig. 4a). PGN and Poly I:C increased Gal-

Fig. 2. **Fold changes in mRNA expression of *LGALS-1, -2, -3-,4, -7, -8, -9,* and *-12* after stimulation of whole blood with CpG ODN (2216) class A (N = 5).** Error lines represent the ± standard deviation of the mean.
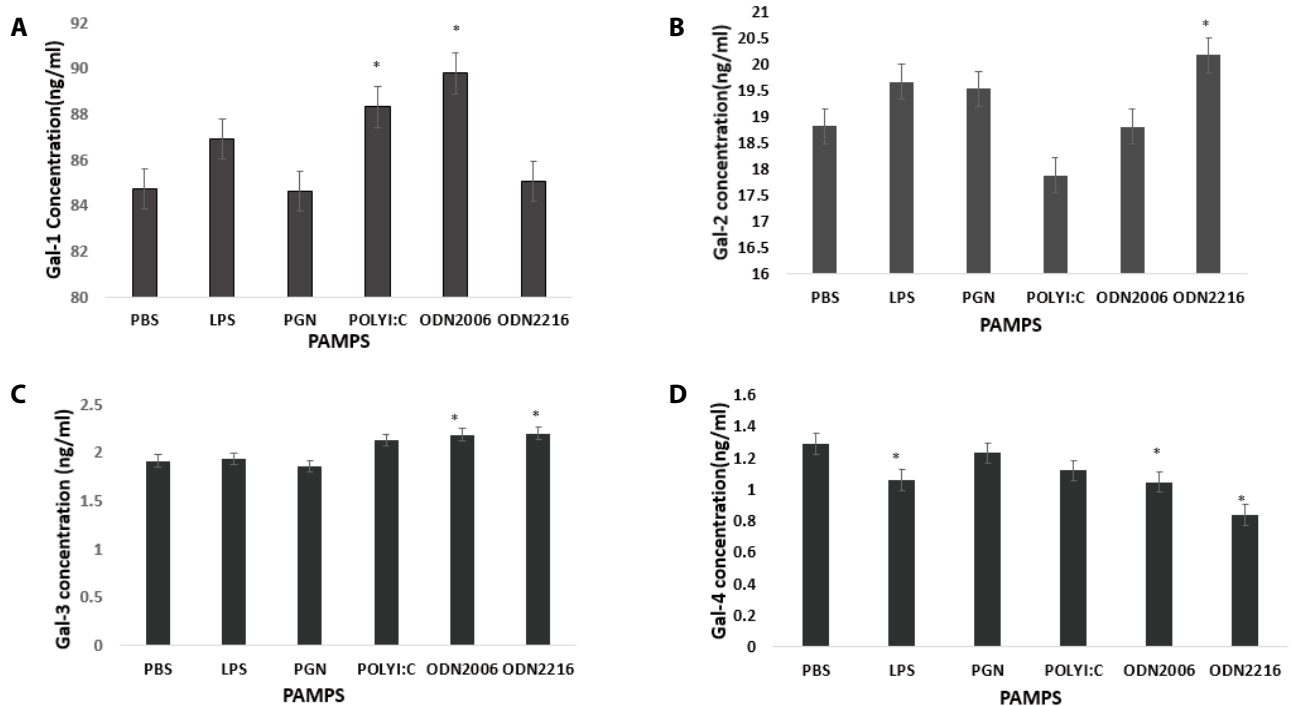
Fig. 3. Effects of stimulating cow blood from multiparous Holstein with Lipopolysaccharide (LPS), Peptidoglycan (PGN), Polyinosinic-Polycytidylic Acid (Poly I:C), CpG ODN (2216) class A, CpG ODN (2006) class B on plasma Gal-1 (A), Gal-2 (B), Gal-3 (C), and Gal-4 (D) (N = 5). Error lines represent the ± standard deviation of the mean. *significantly different from the control (PBS) at $p < 0.05$.
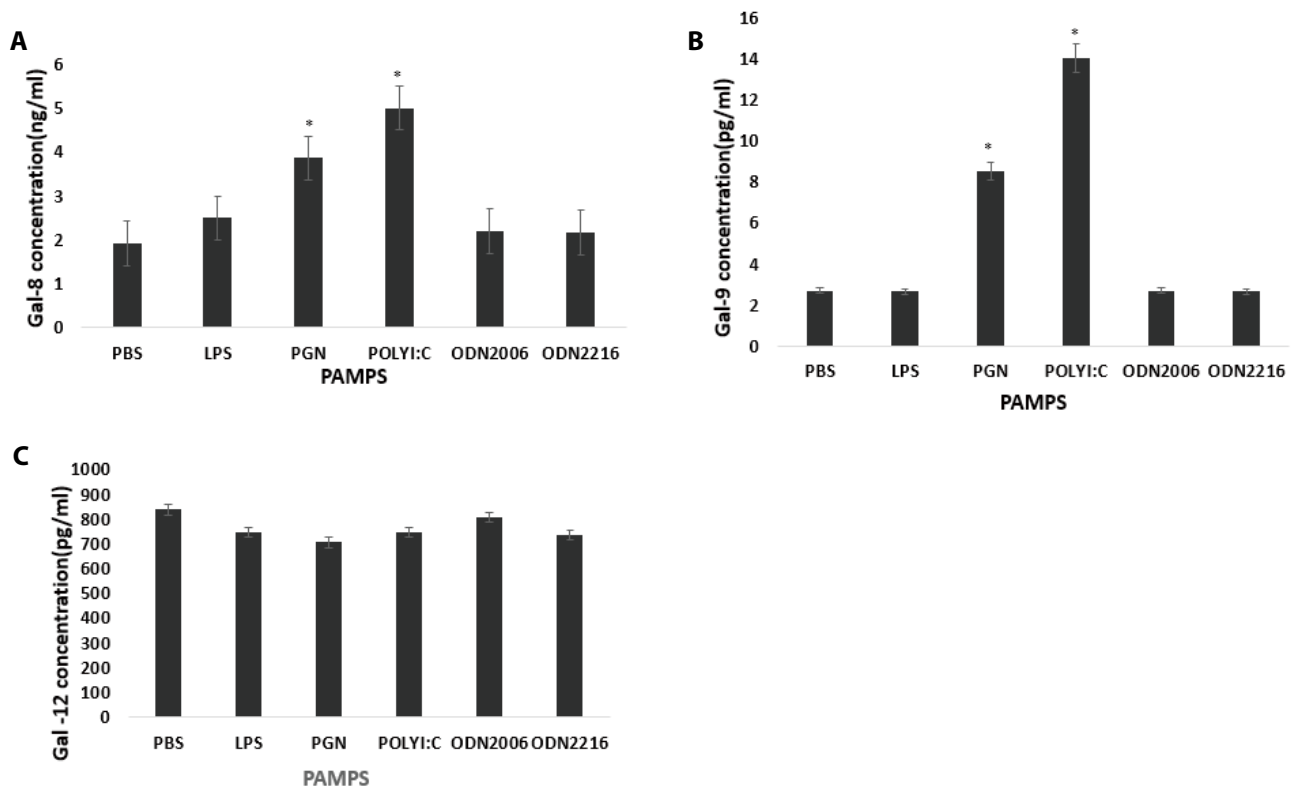
Fig. 4. Effects of stimulating cow blood from multiparous Holstein with Lipopolysaccharide (LPS), Peptidoglycan (PGN), Polyinosinic-Polycytidylic Acid (Poly I:C), CpG ODN (2216) class A, CpG ODN (2006) class B on plasma Gal-8 (A), Gal-9 (B) and Gal-12 (C) (N = 5). Error lines represent the ± standard deviation of the mean. *significantly different from the control (PBS) at $p < 0.05$.

8 secretion compared to control (PBS) (p = 0.001 and p <.0001 respectively) (Fig. 4a). PGN and Poly I:C increased Gal-9 secretion compared to control (PBS) (p = 0.001 and p <.0001 respectively) (Fig. 4b). PAMPs did not affect plasma concentration of Gal-12 (p > 0.05) (Fig. 4c).

Analysis of correlation between galectin transcription and secretion

LGALS1 correlated negatively with Gal-1 (p = 0.02) and correlated positively with Gal-2 (p = 0.03). *LGALS2* correlated positively with Gal-4 (p =< 0.0001) and Gal-3 (p = 0.0009). *LGALS3* correlated negatively with Gal-1 (p = 0.006) and Gal-3 (p = 0.05). *LGALS4* correlated positively with Gal-4 (p =< 0.0001) and negatively with Gal-3. *LGALS8* correlated positively with Gal-8 (p =< 0.0001), Gal-9 (p =< 0.0001) and Gal-4 (p = 0.0002), and negatively with Gal-2 (p =< 0.0002). *LGALS9* correlated positively with Gal-9 (p = 0.007), Gal-8 (p = 0.0006) and Gal-4 (p = 0.002), and negatively with Gal-3.

LGALS12 correlated positively with Gal-4 (p =< 0.0001) and negatively with Gal3 (p = 0.0073).

Discussion

In the past few years, galectins have been shown to participate in the regulation of both innate and adaptive immunity [13,24]. Furthermore, there is research that supports the ability of Gals to recognize microbial pathogens like viruses, bacteria and protozoan parasites [15] directly. The ability of Gals to function as PRRs in the immune defense against invading microbes makes them indispensable components in the innate immune response [16]. To test whether microbial infection affects galectin expression in cow blood, we treated blood with natural bacteria cell wall components (LPS and PGN), synthetic bacterial DNA adjuvants (CpGODN2006 and CpGODN2216) and viral RNA (Poly I:C). Treatment of cells with PAMPs mimic microbial infection and regulates expression of various genes [25]. The current study showed that PAMP modulates *LGALS* gene transcription as well as Gal secretion differentially in cow blood. Both transcription and secretion depended on the type of stimulant used.

Bacterial LPS and PGN designated PAMPs, are recognized by TLR-4 and TLR-2 respectively [26]. The binding of these PAMPs to their receptors is characterized by transcription of genes involved in immune responses and secretion of cytokines [8,9,27]. In the present study, LPS stimulation increased the transcription of *LGALS4* and *LGALS12* in cow blood (Fig. 1a). Gal-4 has been studied to be only expressed in inflamed cells and therefore has a more restricted distribution in normal cells [28]. Gal-4 also promotes resolution of inflammatory diseases, therefore, making it an

important player in immune responses [29]. Gal-12, on the other hand, is preferentially expressed in adipose tissues and has also been studied to be associated with impaired metabolic conditions [30]. Both Gal-4 and Gal-12 exert regulatory functions in immune cells and have strong potential as biomarkers. This data suggests a possible role of Gal-4 and -12 in the recognition and resolution of gram-negative bacteria in cows. The relationship between LPS and the transcription of these galectins need to be explored.

The gram-positive bacterial cell wall, PGN also increased transcription of *LGALS4* and *LGALS12* as well as all galectins tested. The differential *LGALS* transcription and secretion in response to LPS and PGN observed in the study suggest distinct immunological activities in response to these PAMPs. This also accentuates the fact that both gram-positive and gram-negative are recognized by different receptors to elicit different immunological responses. Thus, either LPS or PGN can be used as adjuvants to induce the production of different galectins.

Recently, galectins have been observed to interact directly with the β-galactosides on the surface of viruses thereby participating in antiviral defense, via the activation of the innate and adaptive immune responses [31,32]. Previous research has shown that viral infection has modulatory effects on galectin expression and function [33]. In this study, galectin expression in blood in response to viral challenge assessed by a PolyI:C challenge. Poly I:C is a viral PAMP that promotes cellular recognition of RNA viruses by binding to TLR3 to induce proinflammatory, as well as regulating and cytokine responses [34]. In this study, Poly I:C increased the transcription of *LGALS1, LGALS4,* and *LGALS8* with a subsequent secretion of Gal-1, -3, -8 and 9 in plasma. In a previous study, secretion of several galectins, including Gal-1 and -3 were increased in virus-infected macrophages [35]. Gonzalez et al. (2005) has also postulated that induced Gal-1 secretion after a viral infection is beneficial for the virus due to its anti-inflammatory functions [31]. On the contrary, Gal-3 has opposing functions since it recruits inflammatory cells to the site of infection. It is possible that the balance between the secreted extracellular Gal-1 and -3 in the current may regulate the direction of the inflammatory response in cows. It is also noteworthy that Gal-8, which has been known to be involved in phagocytosis [36] is transcribed and secreted upon viral PAMP stimulation. The current study suggests that these galectins may also be involved in recognition of viral PAMPs and promote the removal of these viral pathogens through phagocytosis, or prevent viruses from entering the host cell.

Studies have indicated that oligodeoxynucleotides (CpG ODNs) containing unmethylated CpG dinucleotides are potent activators of both innate and adaptive immunity [37]. CpG ODNs are recognized by the Toll-like receptor 9 (TLR9) [38]. The TLR9 signaling pathway involves mitogen-activated protein kinases, and

NF-kB-inducing kinase-IKK- Inhibitor of KappaB Kinase (IkB) pathways [39]. Both CpG ODN 2216 and CpG ODN 2006 have been studied to show distinct functional profiles depending on the type of CD8+ cells they are exposed to. CpG ODN 2216 induces high amounts of interferon alpha in plasmacytoid dendritic cells whereas CpG ODN2006 only induces small amounts of IFN alpha [40]. Furthermore, CpG ODN2006 has also been studied to be a weak activator of natural killer cells compared to CpG ODN2216 [41]. This proves that both CpG ODN motifs show distinct immunologic activities. In the present study, the 2 types of CpG ODN adjuvants also affected *LGALS* transcription and secretion distinctively in cow blood. Although no fold changes were observed in *LGALS* transcription (FC < 2), CpG ODN2006 treatment increased secretion of Gal-1 and Gal3 in plasma (Fig. 1d). This increase supports the proinflammatory roles of CpG ODNs since Gal-1 and Gal-3 are proinflammatory in pathogenic conditions. CpG ODN2216, however, increased Gal-2, a pro-apoptotic galectin and Gal-3, a proinflammatory galectin. This also points to a balance in immune response upon PAMP recognition. Improper orchestration of the immune response to microbial infections may lead to sepsis, a condition caused by an overwhelming immune responses [7].

It was also important to note from this study that although LPS and the CpG ODNs motifs are both of bacteria origin, LPS did not affect galectin secretion in plasma. This could mean that Gal secretion in cow blood is increased in response to bacteria DNA compared to its cell wall components. The concentration of LPS (10 µg) used as well as the length of exposure could also cause variation in transcription and translation in blood [42]. Also, the fact that transcription of *LGALS4* was reduced in blood treated the CpG ODN motifs is noteworthy since both PAMPs are synthetic adjuvants. This points to a negative regulatory effect of these PAMPs on *LGALS4*. The results of this study have implications for vaccination and use of synthetic PAMPs in cows and immunomodulation.

Conclusion

The results demonstrate that PAMPs differentially modulate transcription of mRNA and secretion of Gal in cow blood. Elucidation of the relationship between PAMPS and galectin expression may help to define their roles in infectious diseases as well as aid in drug design for the dairy industry.

Competing interests

No potential conflict of interest relevant to this article was reported.

Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

Conceptualization: Worku M.
Data curation: Worku M, Asiamah EK.
Formal analysis: Worku M, Asiamah EK, Adjei-Fremah S, Ekwemalor K, Osei B.
Methodology: Worku M, Asiamah EK, Adjei-Fremah S, Ekwemalor K, Osei B.
Software: Asiamah EK, Adjei-Fremah S.
Validation: Newman R.
Investigation: Asiamah Ek, Worku M.
Writing - original draft: Worku M, Asiamah EK.
Writing - review & editing: Worku M, Asiamah EK, Adjei-Fremah S, Ekwemalor K, Osei B, Newman R.

Ethics approval and consent to participate

All protocols for handling of animals was approved by the Institutional Animal Care and Use Committee (IACUC) of North Carolina A&T State University.

ORCID

Emmanuel K Asiamah   https://orcid.org/0000-0001-8142-6211
Kingsley Ekwemalor   https://orcid.org/0000-0003-2073-2623
Sarah Adjei-Fremah   https://orcid.org/0000-0001-6198-7097
Bertha Osei   https://orcid.org/0000-0001-5162-3388
Robert Newman   https://orcid.org/0000-0002-3865-0266
Mulumebet Worku   https://orcid.org/0000-0003-3663-4745

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