

# Galectin-3 Attenuates Lipopolysaccharides-induced Inflammation in Adipocyte and Macrophage Co-culture System

Praveen Kumar Vemuri<sup>1</sup>, Bhavana Talluri<sup>1</sup>, V. B. S. C. Thunuguntla<sup>1</sup>, Charishma Cherukuri<sup>1</sup>, Anupama Mallampati<sup>2</sup>, Suryanarayana Veeravalli<sup>3</sup>, Vijaya Lakshmi Bodiga<sup>4</sup>

<sup>1</sup>Department of Biotechnology, Centre for Genomics and Proteomics, K L University, Vaddeswaram, Guntur, Andhra Pradesh, India, <sup>2</sup>Department of Biotechnology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India, <sup>3</sup>Department of Humanities & Basic Sciences, Aditya Engineering College, Surampalem, Kakinada, East Godavari, Andhra Pradesh, India, <sup>4</sup>Department of Molecular Biology, Institute of Genetics & Hospital for Genetic Diseases, Osmania University, Hyderabad, Telangana, India

## Abstract

**Introduction:** Galectin-3 (Gal-3) expression act as a potential novel therapeutic strategy in the treatment of a broad range of inflammatory diseases. **Aim:** This study aims to study the cross-talk between macrophage and adipocyte cells with Gal-3 upon lipopolysaccharides (LPSs) challenge. **Materials and Methods:** Cytokine secretion in 3T3-L1 adipocyte-RAW 264.7 macrophage cells were greatly enhanced on LPS stimulation compared to isolated cell culture system, suggesting the cross-talk between these cell types during LPS exposure. Gene and protein expression profile was analyzed by reverse transcription-polymerase chain reaction for understanding the inflammatory responses. **Results:** Decreased expression of interleukin 6 was confirmed as lowered suppressor of cytokine signaling 3 expressions downstream. A critical appraisal of the role of nuclear factor- $\kappa$ B (NF- $\kappa$ B) in LPS induced alteration of inflammatory phenotype was confirmed as decreased formation of tumor necrosis factor- $\alpha$  transcript using specific inhibitors of NF- $\kappa$ B, along with LPS treatment. Further, Gal-3 also inhibited nuclear p65 translocation, I $\kappa$ B- $\alpha$  degradation and induction of inducible nitric oxide synthase, in response to LPS. **Conclusion:** These results clearly suggest decreased oxidative burden and inflammation are significantly affected by Gal-3.

**Key words:** 3T3-L1 adipocyte, co-culture system, galectin-3, lipopolysaccharides, RAW 264.7 macrophage, suppressor of cytokine signaling 3

## INTRODUCTION

Galectin-3 (Gal-3), a 30 kDa molecule, appears to be altered with oligomerization through the self-assembly of the N-terminal regulatory domain<sup>[1]</sup> produced by macrophages and can also act in a cytokine-like fashion.<sup>[2]</sup> The provided signal can be used either by the macrophages themselves, to sustain Gal-3 dependent activation<sup>[3]</sup> or it can provoke various downstream effects in the surrounding cells.<sup>[4]</sup> Besides regulating inflammation and tissue remodeling through its roles in phagocytosis, pattern recognition, and signaling, an emerging view connects Gal-3 to intracellular sorting and trafficking of

glycoproteins.<sup>[5]</sup> Inflammation and its associated metabolic pathologies are the most common metabolic diseases, with increased cytokine production and acute-phase inflammatory signaling in the adipose tissue.<sup>[6]</sup> The presence of cyclic adenosine monophosphate responsive element and nuclear factor- $\kappa$ B (NF- $\kappa$ B)-like sites in the promoter region suggests

### Address for correspondence:

Praveen Kumar Vemuri, Department of Biotechnology, Centre for Genomics and Proteomics, K L University, Vaddeswaram, Guntur, Andhra Pradesh, India.  
E-mail: vemuripraveen@kluniversity.in

**Received:** 14-09-2016

**Revised:** 29-09-2016

**Accepted:** 05-10-2016

that Gal-3 expression could be regulated through metabolic signaling pathways involving the NF- $\kappa$ B transcription factor that is at the core of inflammatory pathways.<sup>[7]</sup> Based on the reports that co-existence of adipocytes and macrophages in the presence of lipopolysaccharide (LPS) further exacerbates obesity-associated inflammatory phenotype<sup>[8]</sup> as compared with culturing each cell type alone in the presence of LPS and earlier literature suggesting physical interactions between LPS and Gal-3.<sup>[9]</sup> The data presented in this work clearly reveal that Gal-3 treatment attenuate the LPS-primed cytokine production in co-culture systems. Therefore, it appears that Gal-3 plays a critical role in a variety of inflammatory disorders.

## MATERIALS AND METHODS

### Co-culture of adipocytes and macrophages

RAW 264.7 and 3T3-L1 were co-cultured in a transwell system (Corning, USA) with a 0.5 mm porous membrane for separation of lower and upper chambers. Then,  $1 \times 10^4$  differentiated adipocyte cells were cultured in the lower chamber, whereas  $5 \times 10^5$  macrophage cells were cultured in the upper chamber.<sup>[10]</sup> The cells were treated with 1  $\mu$ g/ml of LPS (Sigma-Aldrich, USA) and/or 20  $\mu$ g/ml recombinant Gal-3 to compare the pro- and anti-inflammatory cytokine expression and alteration of signaling pathways.

### Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Co-culture cells were treated and total RNA was isolated using the RNeasy Tissue Mini Kit from Qiagen according to the manufacturer's instructions. DNase was used to remove any contaminating DNA using the DNA-free kit from Applied Biosystems. cDNA was then synthesized using the RT-PCR kit from Thermo Scientific according to the manufacturer's protocol. 50 ng of cDNA was used for PCR amplification and primers for the mouse genes studied were as follows: Suppressor of cytokine signaling 3 (Socs-3) forward primer 5'-CCCTGCACAGCCCTCCTTCTCAC-3' reverse primer, 5'-GCC CCA CCC AGC CCC ATA CC-3'. Tumor necrosis factor- $\alpha$  forward primer: AGCCCCAGTCTGTATCCTT Reverse primer: CTCCTTTGCAGAACTCAGG mouse glyceraldehyde 3-phosphate dehydrogenase primers were as follows: Forward primer, 5'-ATG TCA GAT CCA CAA CGG ATA CAT-3'; reverse primer, 5'-ACT CCC TCA AGA TTG TCA GCA AT-3'. LBP forward primer: ATG GGG GCC TTG GCC AGA GC; reverse primer: TGC AGT CCC TTG TCG GTG ATC; CD14 forward primer: GGAAGA CTT ATC GAC CAT GGA G; reverse primer: ACA AGG TTC TGG CGT GGT; TLR2 forward primer: GGC CAG CCA ATT ACC TGT GTG; reverse primer: AGG CGG ACA TCC TGA ACC T. The PCR reaction conditions are as follows: 94°C for 30 s, 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 5 min.<sup>[11]</sup>

### Western blot analysis

Cells ( $2 \times 10^6$ )/well were seeded into a six-well plate and treated or not with 1  $\mu$ g/ml LPS, with or without 20  $\mu$ M Gal-3<sup>[12]</sup> for 30 min before performing Western blotting. Primary antibodies, anti-phospho-p44/42-mitogen-activated protein kinase (MAPK), anti-phospho-p38-MAPK, anti-phospho-stress-activated protein kinases/jun amino-terminal kinases and anti-phospho-NF- $\kappa$ B p65 (Ser536; all from Cell Signaling Technology, Boston, MA, USA) were used at a dilution of 1:1000. Horseradish peroxidase-conjugated antirabbit secondary antibody (Sigma-Aldrich) was used in a 1:5000 dilutions. Bands were visualized with Enhanced Chemiluminescence (ECL - Pierce Chemical, Rockford, IL, USA) and quantified using NIH Image J software.<sup>[13]</sup>

### Statistical analysis

All data are presented as means  $\pm$  standard error of the mean. Statistical significance was determined by analysis of variance (one-way ANOVA and Bonferroni *post-hoc* test), independent sample *t*-test and where appropriate, Kruskal–Wallis and Mann–Whitney *U*-test. Relationships between variables were assessed using Pearson's correlation. Statistical significance was assumed at  $P < 0.05$ . Statistical analyses were performed using the SPSS 13.0.

## RESULTS

### Socs-3 mRNA expression in co-culture system

Gal-3 *per se* failed to show any effect and was always comparable to controls. These data clearly suggest that cytokine secretion is enhanced in the presence of LPS (1  $\mu$ g/ml) and this increase is alleviated by Gal-3 pre-treatment of co-culture, suggesting an anti-inflammatory effect. Interleukin 6 (IL-6) is a strong inducer of insulin resistance via its ability to upregulate Socs-3 gene expression.<sup>[14]</sup> We compared Socs-3 gene expression in co-culture system challenged with LPS and co-treated with Gal-3. The mRNA expression analysis indicates that Gal-3 was effective in inhibiting Socs-3 expression [Figure 1]. These results suggest that down-regulation of IL-6 brought about by Gal-3 during LPS challenge further signals reduces the synthesis of Socs-3, which is a critical component of the inflammasome.

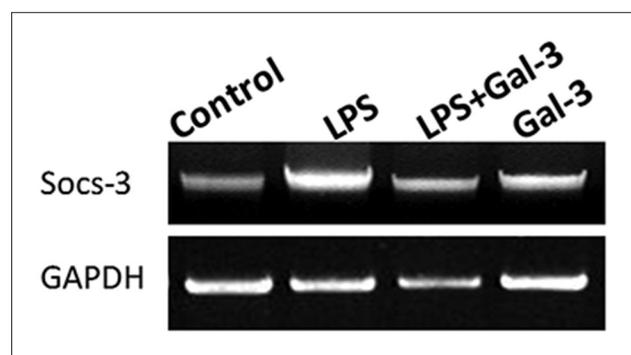
### Effect of LPS and Gal-3 on NF- $\kappa$ B and inducible nitric oxide synthase (iNOS) activity

To confirm these data, we have performed Western blot analysis of p65 and I $\kappa$ B. Results showed Gal-3 downregulates NF- $\kappa$ B activation by LPS in co-culture system [Figure 2]. Whereas there was a clear induction of p65 nuclear translocation and I $\kappa$ B- $\alpha$  degradation in response to LPS, Gal-3 attenuated this

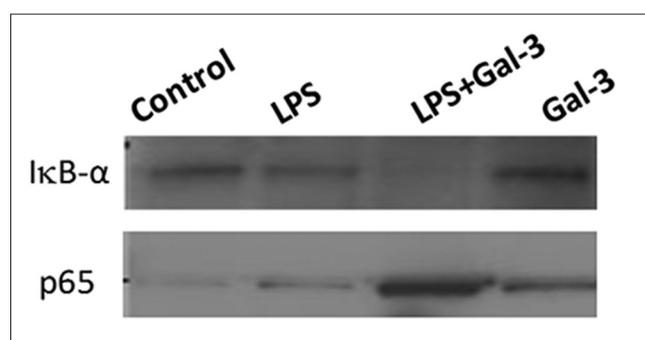
response. Strikingly, the ability of LPS to induce the cytokine production correlated well with NF- $\kappa$ B activation and Gal-3 repression correlated with lowered cytokine production. We have also followed induction and expression of iNOS as a surrogate downstream marker of NF- $\kappa$ B activity in response to LPS treatment in the co-culture system. LPS increases the production of superoxide and nitric oxide, by increased activation of nicotinamide adenine dinucleotide phosphate oxidase and iNOS, resulting in enhanced oxidative stress. LPS showed a significant increase in the expression of iNOS that was repressed to a large extent in the presence of Gal-3 [Figure 3]. These findings indicate NF- $\kappa$ B signaling as a critical effector of LPS-induced inflammation in adipocytes.<sup>[15]</sup>

### Cytokine secretion in co-culture system are exaggerated upon LPS stimulation, but alleviated by Gal-3 treatment

To define the role of Gal-3 as a pathogen pattern recognition receptor and the immunological significance related to the interplay between Gal-3 and LPS in a mixed culture system



**Figure 1:** Suppressor of cytokine signaling 3 mRNA expression in adipocytes-macrophage treated with lipopolysaccharide and/or galectin-3

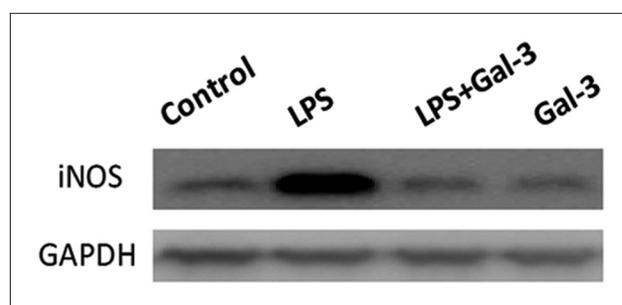


**Figure 2:** Effect of lipopolysaccharide (LPS) and galectin-3 (Gal-3) on nuclear factor (NF- $\kappa$ B) subunit activity. The activity of NF- $\kappa$ B is influenced by the degradation of I $\kappa$ B subunit. LPS and Gal-3 induced I $\kappa$ B- $\alpha$  degradation (top) and nuclear p65 translocation (bottom) in adipocytes. Treatment was performed with LPS (1  $\mu$ g/ml) and/or Gal-3 (20  $\mu$ g/ml) for the indicated amounts of time, and total cellular I $\kappa$ B levels and nuclear p65 levels were determined by Western blot analysis

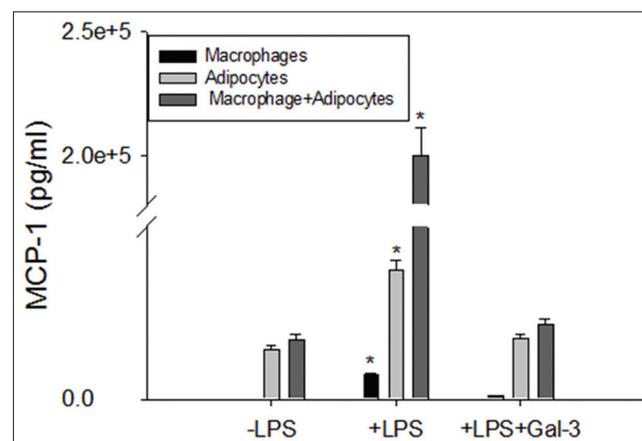
consisting of adipocytes and macrophages,<sup>[16]</sup> we tested the effects of 1  $\mu$ g/ml *Escherichia coli* LPS on the production of cytokines viz. Monocyte chemoattractant protein 1 [Figure 4], RANTES [Figure 5], and CXCL1/KC [Figure 6]. As expected, protein expressions of all these molecules were markedly upregulated in adipocyte-macrophage co-cultures stimulated with endotoxin as compared with co-cultures without endotoxin stimulation or with each cell type alone ( $P \leq 0.05$ ), except for CXCL1/KC, where adipocyte-macrophage co-culture showed lower concentration compared with adipocytes alone.

### Effect of LPS and Gal-3 on LPS signaling mediator expression in co-culture system

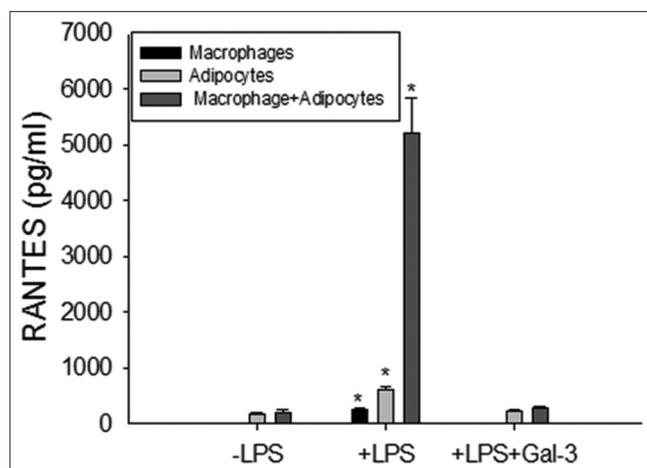
It is well known that LPS signaling requires co-effector molecules such as LPS-binding proteins and/or CD14 for



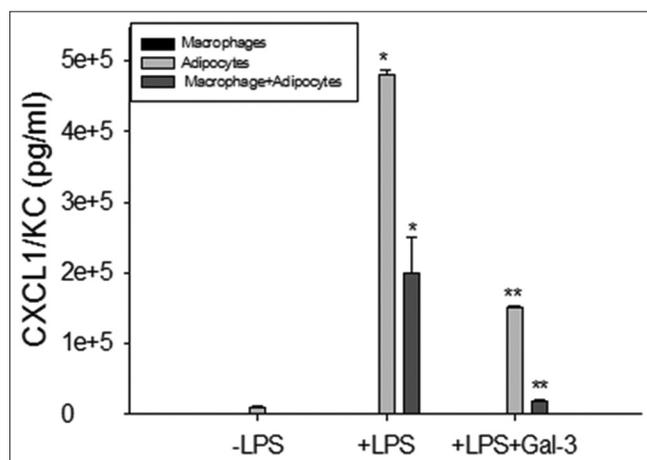
**Figure 3:** Galectin-3 (Gal-3) inhibits lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression in 3T3L1 adipocytes. 3T3L1 adipocytes were treated with LPS (1  $\mu$ M) alone or in combination with Gal-3 (20  $\mu$ M) for 24 h. Total protein was isolated, and 30  $\mu$ g of protein was loaded onto 12% SDS-PAGE and subjected to Western blotting, using a specific antibody for iNOS as described in Materials and Methods. Images shown are representative images from three independent experiments



**Figure 4:** Monocyte chemoattractant protein 1 expression analysis in 3T3-L1 adipocytes co-cultured with RAW 264.7 macrophages with or without lipopolysaccharide (LPS) stimulation and also tested the effect of recombinant galectin-3 in alleviating the inflammatory cytokine production in response to LPS

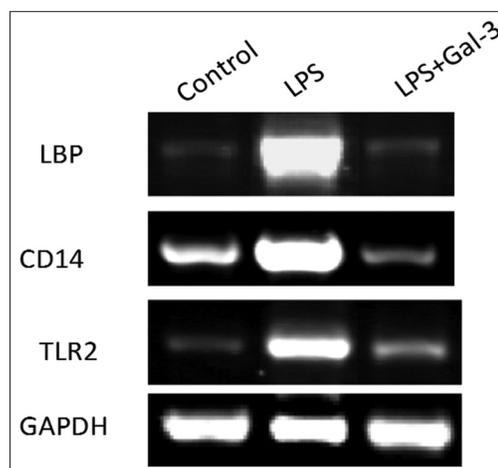


**Figure 5:** Plot representing the levels of RANTES in macrophages, adipocytes, and mixed cultures after 24 h of stimulation with lipopolysaccharide (LPS). RANTES is barely detectable in the culture supernatants of macrophages without LPS stimulation but secreted in detectable amounts from adipocyte culture and mixed culture with macrophages. LPS stimulus (1 ng/ml) significantly increased the RANTES production in macrophages and more so in mixed cultures than in adipocytes alone and this effect is almost nullified/normalized when cells were co-treated with LPS and galectin-3



**Figure 6:** CXCL1/KC production from macrophage alone culture, adipocyte alone culture and adipocyte-macrophage co-cultures stimulated with lipopolysaccharide and exposed to galectin-3

transducing signals into the cells via toll-like receptor 4 (TLR4).<sup>[17]</sup> In the present study, we observed elevated mRNA expression encoding LBP, CD14 and TLR2 in adipocytes co-cultured with macrophages in the presence of LPS than the cells without LPS (16.6 times higher at 24 h after LPS stimulation for LBP; 7.7 times higher expression for CD14; 3.3 times higher expression for TLR2). The expression of effector molecules of LPS signaling mentioned above was dramatically down-regulated in the presence of Gal-3 in the co-cultured cells [Figure 7].



**Figure 7:** mRNA expression analysis of lipopolysaccharide-binding protein, CD14, and TLR2 in macrophages treated with lipopolysaccharide and/or galectin-3

## DISCUSSION

In this direction, using isolated cultures of adipocytes, macrophages, and cocultures, we present the first comprehensive description of changes in LPS-induced inflammatory cytokine production, and consequent anti-inflammatory effects of Gal-3 in 3T3-L1 adipocytes, RAW 264.7 macrophages, and cocultures stimulated with LPS. In co-culture system, the cytokine secretion in 3T3-L1 adipocyte-RAW 264.7 macrophage cells were greatly enhanced on LPS stimulation compared to isolated cell culture system, suggesting the cross-talk between these cell types during LPS exposure. Gal-3 efficiently repressed the heightened cytokine secretion in this mixed culture system, through downregulation of LBP, TLR2, and CD14 molecules. Most importantly, the physical interactions between Gal-3 and LPS and reduced bioavailability of the endotoxin were revealed using binding assays. Based on the results obtained, it is logical to mention that Gal-3 exhibits significant anti-inflammatory and antioxidative effects in adipocyte and macrophage culture systems when exposed to LPS. These results provide convincing evidence in favor of the effective function of Gal-3 in modulating sepsis and endotoxic activity.

## CONCLUSION

The present results point to a role of Gal-3 in modulating the role of adipose tissue in innate immunity and modifying the response of endotoxin (LPS) as TLR4 ligand in adipocytes. In this way, Gal-3 may have a defensive part in infection/inflammation with metabolic alterations ordinarily seen in inflammatory response syndrome/sepsis, for example, insulin resistance, hyperglycemia, and hypertriglyceridemia. Future prospects may involve expanding our *in vitro* studies to fully investigate the role of Gal-3 on adipocyte and macrophage activation and function.

## REFERENCES

1. Liu FT. Molecular biology of IgE-binding protein, IgE-binding factors, and IgE receptors. *Crit Rev Immunol* 1990;10:289-306.
2. MacKinnon AC, Farnworth SL, Hodgkinson PS, Henderson NC, Atkinson KM, Leffler H, *et al.* Regulation of alternative macrophage activation by galectin-3. *J Immunol* 2008;180:2650-8.
3. Nishi Y, Sano H, Kawashima T, Okada T, Kuroda T, Kikkawa K, *et al.* Role of galectin-3 in human pulmonary fibrosis. *Allergol Int* 2007;56:57-65.
4. Jeon SB, Yoon HJ, Chang CY, Koh HS, Jeon SH, Park EJ. Galectin-3 exerts cytokine-like regulatory actions through the JAK-STAT pathway. *J Immunol* 2010;185:7037-46.
5. Delacour D, Koch A, Jacob R. The role of galectins in protein trafficking. *Traffic* 2009;10:1405-13.
6. Dunic J, Lauc G, Flögel M. Expression of galectin-3 in cells exposed to stress-roles of jun and NF-kappaB. *Cell Physiol Biochem* 2000;10:149-58.
7. Kim K, Mayer EP, Nachtigal M. Galectin-3 expression in macrophages is signaled by Ras/MAP kinase pathway and up-regulated by modified lipoproteins. *Biochim Biophys Acta* 2003;1641:13-23.
8. Catalán V, Rodríguez A, Becerril S, Sáinz N, Gómez-Ambrosi J, Frühbeck G. Adipopharmacology of inflammation and insulin resistance. *Biomed Rev* 2006;17:43-51.
9. Dabelic S, Supraha S, Dunic J. Galectin-3 in macrophage-like cells exposed to immunomodulatory drugs. *Biochim Biophys Acta* 2006;1760:701-9.
10. Yamashita A, Soga Y, Iwamoto Y, Yoshizawa S, Iwata H, Kokeguchi S, *et al.* Macrophage-adipocyte interaction: Marked interleukin-6 production by lipopolysaccharide. *Obesity (Silver Spring)* 2007;15:2549-52.
11. Bodiga VL, Inapurapu S, Vemuri PK, Kudle MR, Bodiga S. Intracellular zinc status influences cisplatin-induced endothelial permeability through modulation of PKC $\alpha$ , NF- $\kappa$ B and ICAM-1 expression. *Eur J Pharmacol* 2016;791:355-68.
12. Vemuri PK, Veeravalli S. Expression, purification and characterization of human recombinant galectin 3 in *Pichia pastoris*. *Iran J Biotech* 2014;12:3-8.
13. Souktani R, Pons S, Guegan C, Bouhidel O, Bruneval P, Zini R, *et al.* Cardioprotection against myocardial infarction with PTD-BIR3/RING, a XIAP mimicking protein. *J Mol Cell Cardiol* 2009;46:713-8.
14. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 2003;374:1-20.
15. Vemuri PK, Talluri B, Veeravalli S, Bodiga VL. Galectin-3 in regulation of inflammatory responses mediated by lipopolysaccharide in macrophages and adipocytes culture systems. *Int J Pharm Pharm Sci* 2016;8:356-60.
16. Hagemann T, Robinson SC, Schulz M, Trümper L, Balkwill FR, Binder C. Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF- $\alpha$  dependent up-regulation of matrix metalloproteases. *Carcinogenesis* 2004;25:1543-9.
17. Henderson NC, Mackinnon AC, Farnworth SL, Kipari T, Haslett C, Iredale JP, *et al.* Galectin-3 expression and secretion links macrophages to the promotion of renal fibrosis. *Am J Pathol* 2008;172:288-98.

**Source of Support:** Nil. **Conflict of Interest:** None declared.