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Original Article

GALECTIN-3 IN REGULATION OF INFLAMMATORY RESPONSES MEDIATED BY LIPOPOLYSACCHARIDE IN MACROPHAGES AND ADIPOCYTES CULTURE SYSTEMS

PRAVEEN KUMAR VEMURI[1]*, BHAVANA TALLURI[1], SURYANARAYANA VEERAVALLI[2], VIJAYA LAKSHMI BODIGA[3]

[1]Centre for Genomics and Proteomics, Department of Biotechnology, K L University, Vaddeswaram, Guntur District, Andhra Pradesh, India, [2]Department of Humanities and Basic Sciences, Aditya Engineering College, Surampalem, Kakinada, East Godavari District, Andhra Pradesh, India, [3]Department of Molecular Biology, Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad, Telangana, India
Email: vemuripraveen@kluniversity.in

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ABSTRACT

Objective: The rationale of the present research work was to get insights of galectin-3 function in modulating inflammation in macrophages and adipocytes culture systems.

Methods: Recombinant galectin-3 was prepared, and anti-inflammatory effect of galectin-3 was studied in 3T3-L adipocytes and RAW264.7 macrophages stimulated with lipopolysaccharide followed by western blot analysis. Furthermore, we determined the galectin-3 effect on LPS-mediated ROS and NO generation in RAW macrophage cells by using DHE and mitochondrial membrane potential was measured by JC-1 respectively.

Results: Galectin-3 negatively regulates the exaggerated inflammatory response in the presence of lipopolysaccharide, by lowering cytokines in adipocytes and macrophages. Reduced oxidative stress was evident as the production of ROS and NO was diminished to a great extent by galectin-3 in lipopolysaccharide-treated macrophages. This was also confirmed by the ability of galectin-3 in the maintenance of mitochondrial membrane potential against lipopolysaccharide-induced massive membrane depolarization by galectin-3.

Conclusion: Based on the results obtained, it is rational to mention that galectin-3 exhibits significant anti-inflammatory and anti-oxidative effects in adipocyte and macrophage culture systems, when exposed to lipopolysaccharide.

Keywords: Galectin-3, Adipocytes, Macrophages, LPS, Glycobiology, Toll-like receptor

INTRODUCTION

Regardless of the broad explore in the territory of glycobiology concerning the structure and capacity of glycans, lectins, and glycosylation forms, numerous viewpoints are still left unexplored. Earlier studies have demonstrated that galectin family is involved in inflammatory activities; however, the role of galectin-3 remains to be elucidated that is of further interest because of its complexity. We investigate the hypothesis that galectin-3 induces proinflammatory effects, transcendently keeping a view on a diverse field of galectin family. Apart from responding to proinflammatory signals to exhibit a wide array of complement factors, growth factors, cytokines, and chemokines [1], adipose tissue also secretes proinflammatory molecules. Beutler recognized Toll-like receptor (TLR4) as the cellular mediator of lipopolysaccharide (LPS) response [2]. The discovery of tumor necrosis factor-α synthesis and secretion by adipocytes, as well as the description of TLR expression and LPS-elicited responses in adipocytes has initiated this relatively new field of basic science research [3]. Both invading macrophages and adipocytes in an adipose tissue can express Galectin-3 [4]. Several studies have demonstrated that MAPK pathways are associated with triggering of NF-κB and AP-1transcription factors, which is investigated as signaling cascade in LPS-mediated proinflammatory responses [5]. Carbohydrate-recognition domains are highly conserved, which bind β-galactose containing glycoconjugates by galectins [6]. Galectin-3 promotes the respiratory burst in neutrophils and monocytes [7,8], and this activity is dependent on the galectin property of the protein as it is inhabitable by lactose; it induces mediator release from mast cells [9] and downregulates interleukin-5 production from eosinophils [10]. Galectin-3 promotes the survival of B cells by inhibiting the differentiation into plasma cells, thus allowing the rise of a memory B cell [11]. Inflammatory responses to LPS result in the release of proinflammatory cytokines, Nitric Oxide (NO), Reactive Oxygen Species (ROS), and other cell mediators from monocytes and macrophages, which can cause fever, shock, organ failure, and death [12]. Mitochondria are involved, partially as a result of their membrane polarization/depolarization state, in LPS-induced ROS production, which can contribute to local inflammatory response, as well as to systemic tissue damage [13]. As a signaling loop, an inflated level of ROS activates MAPKs and inflammatory transcription factors [14]. Therefore, we have checked the role of galectin-3 in modulating the action of LPS on macrophages and/or adipocytes and thereby its effects on pro and anti-inflammatory cytokines using *in vitro* culture system.

MATERIALS AND METHODS

Recombinant human galectin-3 preparation

Galectin-3 was produced via the *Pichia pastoris* and purified as previously described [15] and stored at 4 °C in phosphate buffer saline; pH7 containing 100 mM lactose. Before use, galectin-3 was further purified by nickel-based affinity chromatography to eliminate contaminating LPS. Western blot analysis was done to illuminate if the galectin-3 expression is controlled during the growth of yeast cells. Mass spectrometric fingerprinting was performed essentially of the purified p29 and identified it as galectin-3.

Cell culture

Adipocytes 3T3-L1 (86052701, Sigma) were coated in 48-well plates and cultured [16] as per manufacturer instructions. Cells were split at 70-80 % confluency, approx. 1:50 to 1:100 (2-4 × 10^4 cells/cm^2) and trypsinized using 0.25 % solution, with or without EDTA, at 37 °C and 5 % CO2. RAW 264.7 cells (85062803, Sigma) were seeded [17] in T-75C+culture flasks, suspended in DMEM+1% Non-Essential Amino Acids+2-5 mM Glutamine+5-10% FBS or DMEM+2-5 mM Glutamine+5-10% FBS as per manufacturer's instructions.

Determination of cytokine levels in the adipocyte and macrophage conditioned medium by ELISA

IL-6 levels were detected in differentiated 3T3-L1 [18], or RAW 264.7 cells [19], amicon ultra centricon tubes were used to

concentrate the cell supernatant. All ELISAs were performed according to the protocols of the manufacturer for detection of cytokine levels in the conditioned medium. Protein concentration in 3T3-L1 adipocytes or RAW 264.7 was determined by Bradford assay, using BSA and was expressed in pg or ng/ml.

Binding assay in response to LPS and galectin-3

ELISA plates (96-welled) were seeded overnight with LPS (100µg/ml) or with recombinant galectin-3 (10µg/ml) or PBS as a control in triplicates [20]. The plate was then washed and blocked with 10% FCS for one hour at 37 °C and different doses of recombinant galectin-3 were added. In some wells, the galectin-3 was incubated with IgG as control or with the galectin-3 antibody (Abcam) for 30 min before being added to the wells. Samples were then incubated for 2 h at 37 °C, washed thoroughly, and reinsulated with the biotinylated galectin-3 antibody (2-3 µg/ml). Different amounts of biotinylated LPS (Chondrex, Inc, USA) were added for one hour. ExtrAvidin peroxidase (Sigma) was then added for one hour, followed by TMB substrate and analyzed by the ELISA reader at 630 nm.

Western blot analysis

Cells (2×10^6)/well were seeded into a six-well plate and treated or not with 1 µg/ml LPS, with or without 20 µM galectin-3 for 30 min before performing western blotting [21]. Primary antibodies were used, anti-phospho-p38-MAPK, (Cell Signaling Technology, USA) with 1:1000 dilution and HRP-conjugated anti-rabbit secondary antibody (Sigma, USA) with 1:5000 dilution. Bands were visualized with ECL (Pierce Chemical, Rockford, IL, USA) and measured using Image J 1.49.

Superoxide measurement in macrophages

RAW 264.7 cells with 0.5 mg/ml zymosan is added to stimulate oxidative burst and suspended in six-well plates, and experiments were carried out for the cells. After serum starvation (1% FBS) for 16h, macrophages were treated with LPS (1µg/ml) and/or galectin-3 (20µg/ml) in 2 mL DMEM containing 1% FBS and 1% penicillin/streptomycin) for additional 24 hours. During the last 10 min of treatment, cells were incubated with 10 µM DHE and kept in an incubator at 37 °C with 5% CO2/95% air. Cells were visualized under an inverted fluorescent microscope (Leica) with an excitation filter of 535±25 nm and an emission filter of 610±25 nm. The images were acquired for an average of 1s using a spot digital camera and software [22].

Measurement of ΔΨm in macrophages

ΔΨm was measured using the mitochondrial-specific dual-fluorescence probe, JC-1 (Molecular Probes, USA). RAW 264.7 cells were seeded to glass coverslips, treated as indicated, washed twice in ice-cold PBS, and loaded with JC-1 for 15 min at 37 °C. When excited at 488 nm, the dye emits green fluorescence when the mitochondria are depolarized and red for normal ΔΨm. Images of the cells were taken with a Leica fluorescent microscope equipped with CCD camera and epifluorescent illumination. The same microscopic field was imaged in both the red and green channel, and then the images were merged. In control experiments, we did not observe considerable bleed-through between the red and green channels [23].

Determination of nitrite concentration in macrophages

RAW 264.7 cells were cultured in 5% CO2 at 37 °C in DMEM with 10% FCS and L-glutamine (Sigma-Aldrich). Cells were coated at 2×10^4 or 10^5 cells/well in a 96-well plate or 2 × 106 cells/well into a six-well plate. Nitrite concentrations were determined [24] using the Griess reagent-based photometric assay in 96-well plates after 24h incubation of 10^5 cells/well with the indicated concentrations of LPS and galectin-3.

RESULTS

Release of cytokines into adipocyte and macrophage medium in challenge to LPS with galectin-3

ELISA was carried out to assay the release of various cytokines into the adipocyte and macrophage medium accordingly to LPS and galectin-3. IL-6 levels (fig. 1) in the adipocyte culture medium after 24h were 8~fold higher upon LPS challenge than in the control group ($P<0.05$). Interestingly, the IL-6 concentrations were lower in the LPS+galectin-3 group compared with LPS ($P<0.05$). A similar trend of higher cytokine concentrations with LPS treatment was observed with TNF-α (fig. 2), IL-8 (fig. 3) and IL-10 (fig. 4), whereas galectin-3 was effective in alleviating the rise in cytokine release to a significant extent. Most importantly, galectin-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, and this increase is alleviated by galectin-3 pretreatment of adipocytes, suggesting an anti-inflammatory effect. Similar experiments were conducted to assess the production of TNF-α, IL-6 and IL-1β in response to LPS and galectin-3 in RAW264.7 macrophage cells. Incubation of RAW 264.7 cells with 1.0 µg/ml LPS caused a marked increase in TNF-α (fig. 5), IL-6 (fig. 6) and IL-1β (fig. 7) secretion, where simultaneous incubation of cells with increasing concentrations of galectin-3 (5, 10 and 20 µg/ml) dose dependently decreased the release of these cytokines triggered by LPS into the incubation medium. The role of the p38 MAPK pathway in modulating the secretion of TNF-α in response to LPS and galectin-3 has been determined by western blotting (fig. 8). Interestingly, galectin-3 treatment along with LPS significantly attenuated the phosphorylation evoked by LPS. We also determined galectin-3's effect on LPS-induced ROS and NO production in RAW macrophage cells by using DHE and a Griess reagent based assay respectively. We followed the ROS and NO production in RAW cells after LPS challenge for 24 h, which was found not to have a considerable cytotoxic effect. LPS significantly increased the formation of ROS (fig. 9 and 10) and NO (fig. 11) that was reduced by galectin-3. This finding is consistent with the notion that inhibiting the production of the highly reactive radicals during the early phase of the inflammatory response attenuates inflammatory damage. At 20 µg/ml concentration, galectin-3 diminished LPS-induced ROS and nitrite accumulation in the cells to below and close to the control levels respectively. Excessive intracellular ROS can be generated by mitochondria, and mitochondrial oxidative stress and dysfunction result in the collapse of mitochondrial membrane potential ($\Delta\psi_m$). We investigated the mitochondrial membrane potential using a cell-permeable, voltage-sensitive fluorescent mitochondrial dye, JC-1, which emits green fluorescence when the mitochondria are depolarized and red for normal $\Delta\psi_m$ when excited at 488 nm. Microscopic analysis and monitoring of the red/green ratio revealed that LPS induced substantial mitochondrial depolarization. Galectin-3 diminished this effect of LPS on $\Delta\psi_m$. Massive depolarization of mitochondria by LPS was indicated by faint green fluorescence. However, $\Delta\psi_m$ was preserved by 20 µg/ml galectin-3, as indicated by the appearance of red fluorescence emitted by JC-1 aggregates in the mitochondria (fig. 12). Protection of mitochondria against LPS-induced massive membrane depolarization by galectin-3 indicates the importance of mitochondrial integrity in an inflammatory response of macrophages.

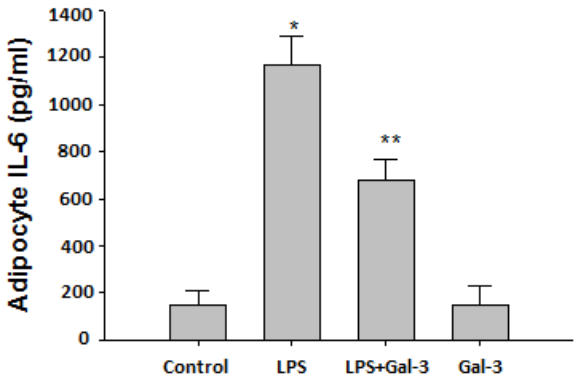

Fig. 1: Adipocytes treated with LPS (1µg/ml), LPS+galectin-3 (20µg/ml), galectin-3. n = 6 for all groups and the release of IL-6 in the culture medium is analyzed. *p<0.05 in relation to control, **p<0.05 in relation to LPS

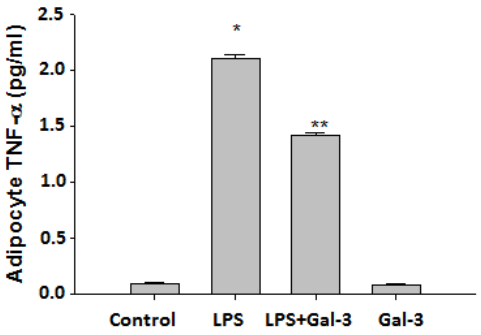

Fig. 2: Adipocytes treated with LPS (1µg/ml), LPS+galectin-3 (20µg/ml), galectin-3. n = 6 for all groups and the release of TNF-α release in the culture medium is analyzed. *p<0.05 in relation to control, **p<0.05 in relation to LPS

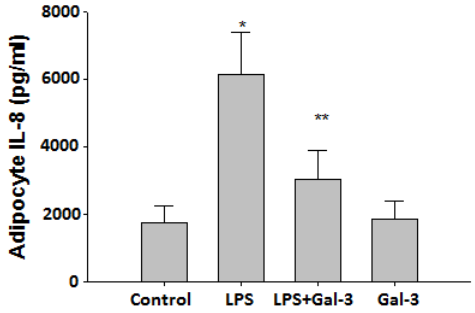

Fig. 3: Adipocytes treated with LPS (1µg/ml), LPS+galectin-3 (20µg/ml), galectin-3. n = 6 for all groups and the release of IL-8 in the culture medium is analyzed. *p<0.05 in relation to control, **p<0.05 in relation to LPS

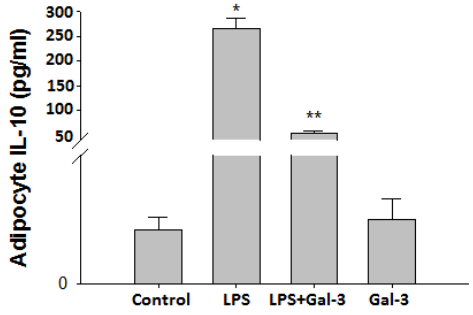

Fig. 4: Adipocytes treated with LPS (1µg/ml), LPS+galectin-3 (20µg/ml), galectin-3. n = 6 for all groups and the release of IL-10 in the culture medium is analyzed. *p<0.05 in relation to control, **p<0.05 in relation to LPS

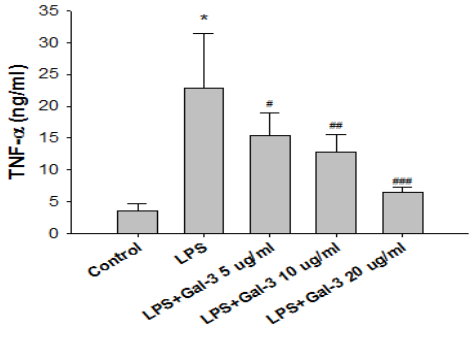

Fig. 5: Dose-dependent decrease in LPS-induced TNF-α production from macrophages by addition of galectin-3

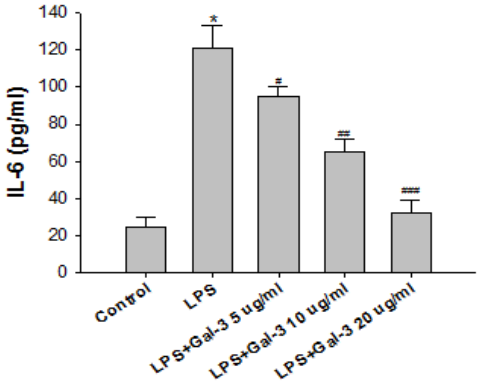

Fig. 6: Dose-dependent decrease in LPS-induced IL-6 production from macrophages by addition of galectin-3

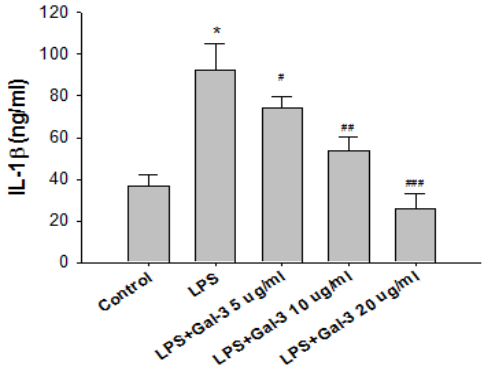

Fig. 7: Dose-dependent decrease in LPS-induced IL-1β production from macrophages by addition of galectin-3

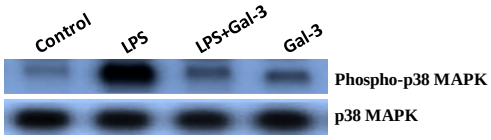

Fig. 8: Galectin-3 significantly attenuates p38 MAPK phosphorylation induced by LPS in adipocytes. Proteins (50 µg per lane) were separated by SDS-PAGE and analyzed by Western blotting using an anti-phospho-p38 MAP Kinase protein antibody (Thr180/Tyr182). Loading equality was controlled using an antibody against the unphosphorylated isoform of p38. The data represent a typical result from three independent experiments

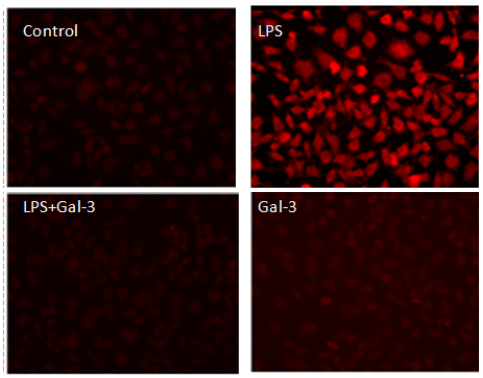

Fig. 9: Detection of superoxide production in macrophages by microscopy using Di hydroethidium (DHE) dye. Macrophage cells were treated or not for 30 min with 1µg/ml LPS together with or without 20 µg/ml galectin-3, stained with 10 µg/ml DHE and then assayed by fluorescent microscopy

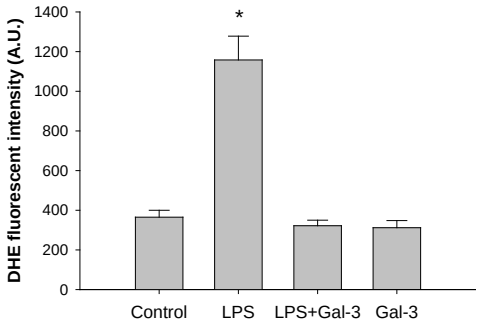

Fig. 10: Quantitative analysis of DHE fluorescence in macrophages upon treatment with LPS and/or galectin-3

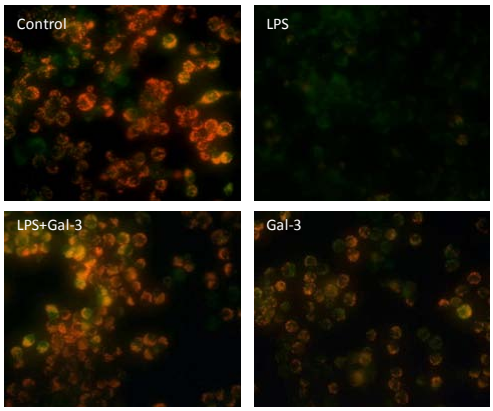

Fig. 11: Detection of mitochondrial membrane potential by microscopy using JC-1 dye: RAW 264.7 cells were treated or not for 30 min with 1 µg/ml LPS together with or without 20 µg/ml. Galectin-3, stained with JC-1 and then assayed by fluorescent microscopy. Fluorescence emission shift from red (≈590 nm) to green (≈529 nm), representing mitochondrial membrane depolarization, was monitored at 488 nm excitation. Representative images acquired 30 min after the LPS challenge, are presented. Green and red fluorescent images were taken of the same microscopic field and merged images of three independent experiments are presented

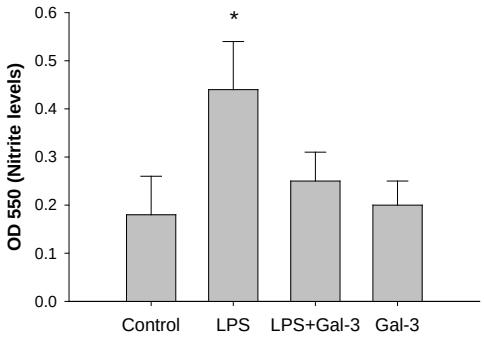

Fig. 12: NO levels in macrophages measured as nitrite after treatment with LPS and/or galectin-3

DISCUSSION

Obesity is associated with a state of chronic and low-grade inflammation. Various studies have provided clear evidence that obese adipose tissue is characterized by increased infiltration of macrophages [25], suggesting that there is crosstalk between macrophages and adipocytes [26]. It was, therefore, important to elucidate the changes in proinflammatory signals during crosstalk and to evaluate natural endogenous compounds that can lower the proinflammatory signals and understand the signaling mechanisms associated with the changes.

In this direction, using isolated cultures of adipocytes and macrophages, we present the comprehensive description of changes in LPS-induced inflammatory cytokine production and consequent anti-inflammatory effects of Galectin-3 in 3T3-L1 adipocytes and RAW264.7 macrophages stimulated with LPS [27]. The adipocytes treated with LPS showed an enormous response in the production of inflammatory cytokines that included IL-6, TNF-α, IL-8 and IL-10 [28], which was prevented to a significant extent by 20 µM Galectin-3. Adipose tissue has been postulated to be a primary tissue producing TNF-α and IL-6 in obese subjects, although the exact cell types responsible for the production of each cytokine remain unknown. In the present study, LPS acting through TLR-4 dramatically changed the expression profile of cytokines in 3T3-L1 adipocytes. The LPS-activated macrophages are known to interact with mature adipocyte, resulting in further exacerbation of the inflammatory cascade. The anti-inflammatory effect of Galectin-3 was also evidenced as a decrease in Socs-3 expression (not shown here), which is inducible by IL-6. Down-regulation of IL-6 by Galectin-3 has a direct effect on the suppression of cytokine signaling. This clearly suggests that the bioavailability of LPS is regulated by co-incubation of cells with Galectin-3, which thus negatively affects the TLR4 expression. Marked increase in phospho-p38, MAPK, JNK and ERK often reported to increase with LPS was confirmed in the present study and Galectin-3 was able to repress efficiently the activation of these signaling pathways, thereby rendering the cells to produce lower levels of inflammatory cytokines [29]. Reduced levels of inflammatory cytokines were also confirmed as levels of NO were found to decrease with Galectin-3, indicating lowered oxidative burden in adipocytes. Taken together, Galectin-3 exhibited potential anti-inflammatory effects both in adipocytes and macrophages, when treated with LPS.

CONCLUSION

The conclusion of this research work is to characterize the functional capacity of galectin-3 to modulate the LPS signaling in adipocytes, to investigate the mechanisms of LPS-induced cytokine and chemokine release, and to detect potential differences in response to LPS in the presence of galectin-3. It's significant where, galectin-3 secreted in situ by macrophages or adipocytes, may render inflammation particularly sensitive to LPS. LPS concentrations as low as 1 µg/ml, galectin-3 had no effect on LPS-pretreated macrophages. However, galectin-3 pre-incubated with LPS enhanced the ability of these low LPS concentrations to activate immature, unprimed macrophages. Based on the results obtained, it is logical to mention that galectin-3 exhibits significant anti-inflammatory and anti-oxidative effects in adipocyte and macrophage culture systems, when exposed to LPS. These results provide convincing evidence in favor of the effective function of galectin-3 in modulating sepsis and endotoxic activity. Prospective work might include expanding our *in vivo* studies depleting various macrophage populations in the wild type mouse and reconstituting them with wild-type or galectin-3-/-macrophages.

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COMPETING INTERESTS

The authors have declared that no competing interests exist

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