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# Detection and chromatographic removal of lipopolysaccharide in preparations of multifunctional galectins

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ABSTRACT

The functional spectrum of human galectins is currently explored, with a wide range of activities being described. The role of galectin-3 as adhesin for bacteria is based on its strong binding to lipopolysaccharides (LPSs), which brings the possibility of such a contamination in galectin preparations to awareness. This assumption was verified in three independent functional assay systems using polymyxin B as inhibitor of LPS-dependent effects. Moreover, a commercial LPS quantification kit also revealed LPS in galectin preparations. Chromatography was effective in removing LPS, suggesting that such a technique needs to be applied to prevent assigning cellular responses to galectins rather than LPS.



The capacity of glycans for high-density information coding directs increasing attention to endogenous lectins as translators of sugar-encoded signals [1,2]. Their recombinant production has enabled convenient access to these tissue effectors, which is a key step to map their functional profiles. If, however, a role as adhesin for bacteria is among the functions of a galectin, the possibility of lipopolysaccharide (LPS) binding and LPS-triggered effects should be considered. This is indeed the case for at least one member of the galectin family, galectin (Gal)-3, which is a multifunctional regulator of cell adhesion, differentiation, growth, and migration [3,4].

Historically, this lectin was first shown to associate with LPS from *Klebsiella pneumoniae* via β-galactosides in the outer core and with the lipid A/inner core region of LPS from *Salmonella minnesota* R7, here via the non-lectin part of the protein [5]. The outer core section of *Pseudomonas aeruginosa* LPS, lipooligosaccharides from *Neisseria gonorrhoeae*, and the O-antigen of *Helicobacter pylori* also appear to target Gal-3, as do mycobacterial phosphatidylinositolmannans, mycolic acids and, of particular note, *Escherichia coli* LPS [6–10]. This collective evidence, flanked by the enormous functional potency of even minute quantities of LPS, calls for a rigorous analysis of LPS contamination in galectin preparations.

In this report, we use three independent functional assay methods and a commercial kit for LPS quantification. Routinely, the cationic inhibitor of LPS binding, polymyxin B, was tested in parallel. These experiments show LPS contamination at notable level in galectin-3 preparations. Due to the recent delineation of a lipid (farnesyl)-binding region in a galectin other than Gal-3 [11], we systematically tested other human galectins for LPS contaminations as well. Indeed, LPS was present in most of these preparations. Chromatography was effective in removing these contaminations. Our data suggest that functionally significant LPS contaminations in galectin preparations and show a method for their removal.

## Materials and methods

*Galectins and LPS.* The human galectins were purified after recombinant production, checked for purity and quaternary structure and subjected to activity assays as described in [12–15]. Human Gal-3 was exposed to collagenase D to obtain the proteolytically truncated (trGal-3) derivative with cleavages at the Tyr106/Gly107 and Glu229/Ile230 peptide bonds [16]. LPS from *E. coli* for the controls and polymyxin B (PMB) were purchased from Sigma–Aldrich (Schnelldorf, Germany).

*Chromatographic removal of LPS.* Solutions of galectins (at a concentration of 1 mg/ml in phosphate-buffered saline (PBS)) were mixed at a volume ratio of 2:1 with EndoTrap™ red agarose resin (Profos AG, Regensburg, Germany), equilibrated with buffer solution of 10 mM $Na_2HPO_4$, pH 7.4, containing 80 mM NaCl, the slurry

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was kept for 30 min at rt under gentle agitation and then centrifuged at 1200*g* for 5 min. Supernatants were collected in endotoxin-free tubes.

*TNF-α release assay*. Human CD14-positive monocytes were suspended in RPMI 1640 medium at $10^6$ cells/ml with aliquots at 50 μl/well. Following a 2-h incubation step at 37 °C/5% $CO_2$ to let cells adhere to the substratum, the cell layer was carefully washed with PBS to remove non-adherent lymphocytes and then cultured in RPMI 1640 medium supplemented with 10% autologous serum, 5 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-csf, Behringwerke, Marburg, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 mM L-glutamine (Gibco-Invitrogen, Karlsruhe, Germany) for 8 days at 37 °C/5% $CO_2$ to obtain monocyte-derived macrophages. At days 0, 3, and 5 200 μl of this medium supplemented with autologous serum and rhGm-csf was added to the cells. These cells were used for assays at 50,000 cells/ml and a galectin concentration of 10 μg/ml or stepwisely increasing concentrations up to 40 μg/ml. Controls were run in the presence of 100 mM lactose or sucrose and of 30 μg/ml PMB. Supernatants were collected after a period of 16 h, and the concentration of TNF-α was determined by ELISA using a suitable pair of monoclonal antibodies and control cytokine for the standard curve from BD Pharmingen (Heidelberg, Germany).

*NF-κB activation assay*. Cells of the murine macrophage-like Raw 264.7 line stably transfected with the pB2xluc plasmid (harboring the NF-κB-driven luciferase gene as reporter) were cultured in complete DMEM containing 300 μg/ml neomycin. Cells were seeded at a density of $10^5$ cells/well in 0.5 ml aliquots into 48-well plates in serum-free medium, kept overnight 37 °C/5% $CO_2$ and then exposed to galectins at a concentration of 10 μg/ml. After 8 h cells were carefully washed, then frozen, thawed, and exposed to lysis buffer (Promega, Mannheim, Germany), the resulting suspension was then cleared and tested for luciferase activity with appropriate substrate (Promega) and a luminometer (Berthold Technologies, Bad Wildbad, Germany).

*Culture of dendritic cells (DC) from peripheral blood*. Human CD14-positive monocytes were treated with IL-4 (100 U/ml, Miltenyi Biotech, Bergisch Gladbach, Germany) and rhGM-csf (500 U/ml) for 5 days at 37 °C/5% $CO_2$. Treatment of immature DC with 100 ng/ml LPS or galectins for 24 h followed, DC maturation was assessed by flow cytometric analysis of the markers CD86 (Southern Biotec, Birmingham, Alabama, USA) and CD54 (BD Pharmingen).

*LPS quantification*. Solutions containing galectins (at 10 μg/ml) were processed with the reagents of the chromogenic Limulus Amebocyte Lysate (LAL) test (Cambrex QCL 1000; Cambrex Bio Science, Walkersville, MD, USA). Absorbance of the samples was determined spectrophotometrically at 405 nm and the concentration of endotoxin calculated from a standard curve.

*Statistical analysis*. Data sets were routinely processed by the Student's *t*-test. All results are presented as mean ± standard deviation (SD) with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$. All experiments were done in triplicates.

## Results

### *Induction of TNF-α secretion by Gal-3*

As a first diagnostic assay to check for LPS contamination of Gal-3 preparations we tested human monocyte-derived macrophages for TNF-α secretion. The level of their spontaneous TNF-α production was low, whereas treatment with 100 ng/ml LPS lead to a strong and significant increase of TNF-α production (Fig. 1A). Full-length and trGal-3 at 10 μg/ml led to significant increase of TNF-α secretion as well. Having processed the two Gal-3 preparations chromatographically with resin as an endotoxin trap, the endotoxin-depleted protein fractions were tested in parallel with mock-treated fractions. Interestingly, virtually no TNF-α production could be detected in the endotoxin-depleted protein fractions (Fig. 1A). Moreover PMB, an inhibitor of LPS binding to cells,

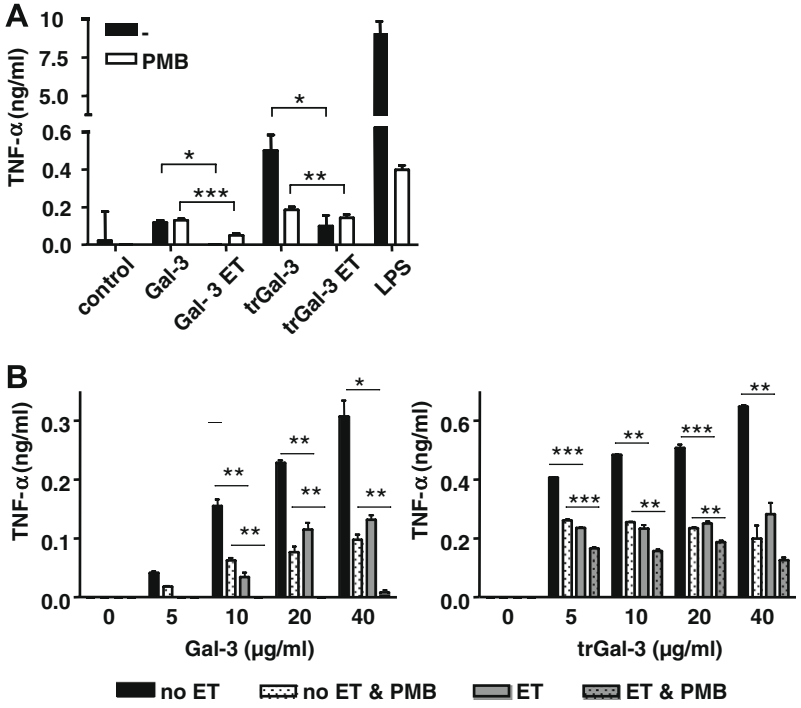


**Fig. 1.** (A) Effect of presence of LPS (100 ng/ml), full-length Gal-3 (10 μg/ml), and trGal-3 (10 μg/ml) on extent of secretion of TNF from human monocyte-derived macrophages. (B) Effect of a concentration range of Gal-3 and trGal-3 on the TNF secretion. The effects of exposure of lectin to the endotoxin-trapping resin (ET) and of presence of polymyxin B (PMB) were tested in parallel. Data are presented as means ± SD, $n = 3$; $^{*}p < 0.05$, $^{***}p < 0.005$.

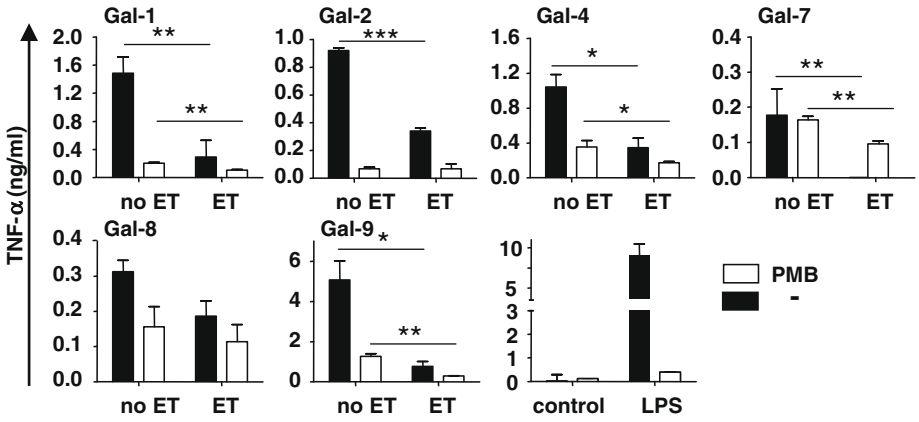


**Fig. 2.** Extent of secretion of TNF in the absence and presence of human galectins-1, -2, -4, -7, -8, and -9 (10 μg/ml), and the effects of lectin exposure as in Fig. 1. Data are presented as means ± SD, $n = 3$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

similarly reduced the TNF-α-inducing activity of trGal-3 (Fig. 1A). Full-length and trGal-3 induced a dose-dependent increase of TNF-α secretion among a concentration range of 5–40 μg/ml, when LPS had not been depleted (Fig. 1B).

*Indication for LPS contamination of human galectins by functional assays*

After gaining first evidence for a contamination of Gal-3 preparations by LPS, it is reasonable to assume that the carbohydrate recognition domain of other members of this lectin family may also associate with LPS. Therefore, we performed similar experiments with aliquots of cell batches and various human galectins to address this question. Examining preparations of human homo-dimeric Gal-1, -2, and -7 as well as the tandem-repeat-type Gal-4, -8, and -9 revealed obvious differences in the capacity to induce TNF-α secretion (Fig. 2). Gal-9 yielded a strong signal, whereas Gal-7 and -8 did not. Exposure of galectins to the LPS-binding resin reduced the observed activity similarly as it had been observed with Gal-3. Treatment with PMB decreased the TNF-α response to a comparable extent as with resin treatment (Fig. 2). The presence of the haptenic inhibitor lactose further decreased the activity of Gal-1 and -4, a control for blocking sugar-dependent galectin binding (not shown).

In order to extend this evidence for LPS contamination in galectin preparations, we next performed systematic assays in the murine macrophage-like Raw 264.7 line stably transfected with a vector containing the luciferase gene, its expression driven by NF-κB activation. This system sensitively detected LPS and spontaneous signal generation reached a constant value of around 2 RLU/s. In this type of assay, all tested galectin preparations were active. Exposure to the LPS-binding resin again led to signal reduction, as did PMB (Fig. 3). In addition, we used another functionally relevant test system for LPS effects, which is maturation of dendritic cells. In line with the two other assay systems, chromatographic treatment and PMB led to reduction in dendritic cell marker appearance (not shown). These data suggested that preparations of human recombinant galectins contain active LPS.

*Quantitative LPS determination*

Based on the functional experiments we next attempted to perform a quantification of LPS content by standard *Limulus* amebocyte lysate assay. Mock controls ascertained the absence of LPS in the buffer. Titrations for Gal-3 and trGal-3 ascertained the expected concentration dependence (Fig. 4A). Assays with galectins at 10 μg/ml revealed the presence of LPS and the reduction

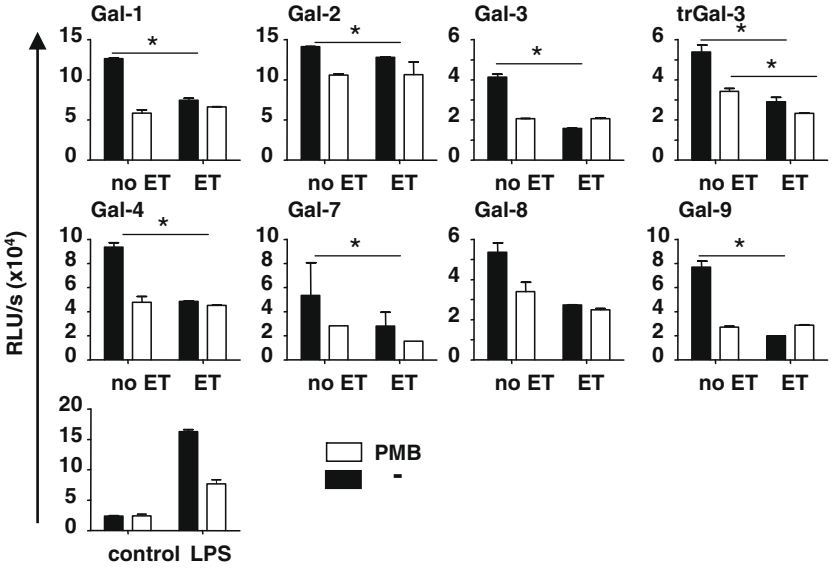


**Fig. 3.** Extent of NF-κB activation in cells of the murine macrophage-like Raw 264.7 line engineered for luciferase-dependent response quantification by human galectins (at 10 μg/ml) and the effects of lectin exposure as in Fig. 1. Data are presented as means ± SD, $n = 3$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

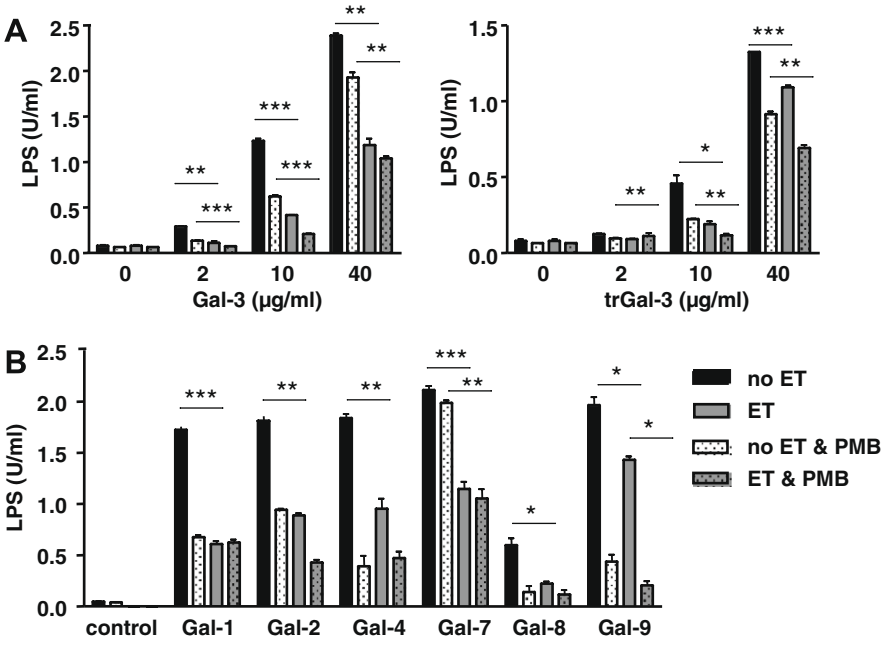


**Fig. 4.** Determination of the LPS associated to (A) full-length Gal-3 and trGal-3 at different concentrations and to (B) human galectins-1, -2, -4, -7, -8, and -9 (at 10 μg/ml) by the chromogenic *Limulus* amebocyte lysate assay and the effect of lectin exposure to the endotoxin-trapping resin (ET). Data are presented as means ± SD, $n = 3$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

of its concentration after chromatographic processing (Fig. 4B). An influence of the lectin activity on the assay was excluded by controls in the presence of 100 mM haptenic inhibitor lactose, flanked by experiments with 100 mM sucrose to exclude an effect of increase in osmolarity. The detection of LPS is embedded into the general pattern recognition process responding to several pathogen-related danger molecules, which consecutively leads to inflammatory responses of the host. The contribution to pattern recognition of LPS may vary in dependence of the inflammatory pathway(s) involved.

## Discussion

The reported affinity of Gal-3 to LPS can engender problems concerning the purity of protein from recombinant production. To address this issue and to extend it to preparations of other human galectins, we systematically applied three different functional test systems, sensitive for LPS detection as well as a quantitative LPS detection assay. In detail, we examined TNF-α secretion, NF-κB activation and maturation of dendritic cells. Invariably, positive responses were measured and they could be reduced by PMB. False-negative results due to the presence of this inhibitor of LPS binding are unlikely, because a plant lectin is known to induce TNF-α secretion in a PMB-sensitive and carbohydrate-dependent manner [17]. The case for LPS contamination was reinforced by application of a LPS-trapping resin with a $K_D$ of $5 \times 10^{-8}$ M, at the same time providing a means for decontamination. LPS presence might especially be problematic in those cases, where rather high concentrations of galectin are required, warranting to exclude LPS presence in controls. In summary, our results signify potential for substantial LPS contamination in galectin preparations and report a convenient way to remove them.

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