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1 **Functional role of galectin-9 in directing human innate immune reactions**

2 **to Gram-negative bacteria and T cell apoptosis**

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21

22   **Abstract**

23   Galectin-9 is a member of the galectin family of proteins, which were first identified to

24   specifically bind to carbohydrates containing β-galactosides. Galectin-9 is conserved through

25   evolution and recent evidence demonstrated its involvement in innate immune reactions to

26   bacterial infections as well as the suppression of cytotoxic immune responses of T and natural

27   killer cells. However, the molecular mechanisms underlying such differential immunological

28   functions of galectin-9 remain largely unknown. In this work we confirmed that soluble

29   galectin-9 derived from macrophages binds to Gram-negative bacteria by interacting with

30   lipopolysaccharide (LPS), which forms their cell wall. This opsonisation effect most likely

31   interferes with the mobility of bacteria leading to their phagocytosis by innate immune cells.

32   Galectin-9-dependent opsonisation also promotes the innate immune reactions of

33   macrophages to these bacteria and significantly enhances the production of pro-inflammatory

34   cytokines – interleukin (IL) 6, IL-1β and tumour necrosis factor alpha (TNF-α). In contrast,

35   galectin-9 did not bind peptidoglycan (PGN), which forms the cell wall of Gram-positive

36   bacteria. Moreover, galectin-9 associated with cellular surfaces (studied in primary human

37   embryonic cells) was not involved in the interaction with bacteria or bacterial colonisation.

38   However, galectin-9 expressed on the surface of primary human embryonic cells, as well as

39   soluble forms of galectin-9, were able to target T lymphocytes and caused apoptosis in T cells

40   expressing granzyme B. Furthermore, "opsonisation" of T cells by galectin-9 led to the

41   translocation of phosphatidylserine onto the cell surface and subsequent phagocytosis by

42   macrophages through Tim-3, the receptor, which recognises both galectin-9 and

43   phosphatidylserine as ligands.

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Introduction

Galectin-9 is a member of the galectin family of proteins which were first identified to specifically bind to carbohydrates containing β-galactosides [1-5]. Galectins vary in their structural organisation and, so far, three different forms of galectin structure were discovered. Galectins can display dimeric, chimeric or tandem structures [1-3]. Galectin-9 has a tandem structure and contains two distinct carbohydrate recognition domains (CRDs) within one polypeptide [1-5]. The CRDs are fused together by a peptide linker. Galectin-9 may be present in three main isoforms characterised by the length of their linker peptide which can be long (49 amino acids), medium (27 amino acids) and short (15 amino acids) [1-5].

Galectins are conserved through evolution and have various intracellular and extracellular functions including both normal and pathophysiological processes [1, 2]. Galectin-9 is one of the most important galectins and is a major contributor to human immune reactions [6, 7], particularly because of its ability to suppress the cytotoxic activities of T and natural killer (NK) cells. In cytotoxic T cells galectin-9 acts through receptors such as Tim-3 (T cell immunoglobulin and mucin-containing protein 3) and VISTA (V-domain Ig-containing suppressor of T cell activation) [7]. Galectin-9 can induce leakage of granzyme B proteolytic enzyme from the intracellular granules of cytotoxic T cells thus leading to their programmed death [7]. In NK cells, galectin-9 acts mainly through Tim-3 and impairs their cytotoxic activities [6]. As such, galectin-9 is used by cancer cells to escape immune surveillance and also by foetus cells where it protects the embryo against rejection by the mother's immune system [8]. Furthermore, galectin-9 was found to participate in neutrophil-mediated killing of Gram-negative bacteria by opsonisation, thus promoting their phagocytosis by neutrophils [9].

However, the actual biochemical role of galectin-9 in anti-bacterial immune defence and suppression of T cell functions remains to be comprehensively understood. Here we report that galectin-9 binds Gram-negative bacteria (*E. Coli XL-10 Gold*) by interacting with lipopolysaccharide (LPS), which is a crucial cell wall component. This opsonisation effect renders the bacteria less mobile thus facilitating their capture and phagocytosis by macrophages. Opsonisation also promotes the innate immune reactions of macrophages to Gram-negative bacteria and significantly enhances the production of pro-inflammatory cytokines – interleukin (IL) 6, IL-1β and tumour necrosis factor alpha (TNF-α). Galectin-9 was almost incapable of binding peptidoglycan (PGN), which forms the cell wall of Gram-positive bacteria. Galectin-9 associated with the cell surface (studied in primary human embryonic cells) was not involved in the interaction with bacteria or bacterial colonisation. However, cell-surface-based galectin-9 on human embryonic cells, as well as secreted galectin-9, targeted T lymphocytes and caused apoptosis in T cells expressing granzyme B. T cells "opsonised" by galectin-9 were phagocytosed by macrophages through Tim-3. Furthermore, galectin-9 induced the release of transforming growth factor beta type 1 (TGF-β) and high mobility group box 1 (HMGB1) from T cells. TGF-β induces the expression of galectin-9 in cancer and embryonic cells and HMGB1 enhances the ability of macrophages to phagocyte apoptotic T cells.

Taken together our results suggest that galectin-9 is capable of opsonising LPS-containing bacteria and T cells triggering their phagocytosis by macrophages. Moreover, galectin-9 provokes the activation of anti-bacterial innate immune reactions and, in the case of T cell suppression, indirectly enhances the phagocytic activity of macrophages.

Materials and Methods

Materials

RPMI-1640 cell culture medium, foetal bovine serum and supplements as well as basic laboratory chemicals were obtained from Sigma (Suffolk, UK). Microtitre plates for Enzyme-Linked Immunosorbent Assay (ELISA) were provided by Oxley Hughes Ltd (London, UK). Rabbit antibodies against VISTA (ab243891, BLR035F), galectin-9 (ab69630), granzyme B ab134933, EPR8260), CD3 (ab21703, SP7 and LPS (lipid A, ab8467, 26-5), as well as mouse antibody against Toll-like receptor 2 (TLR2, ab9100, TL2.1), were purchased from Abcam (Cambridge, UK). Antibody against actin (66009-I-Ig) was purchased from and Proteintech (Manchester, UK). Goat anti-mouse (925-32210 and 926-68070) and anti-rabbit (926-3211 and 926-68071) fluorescence dye-labelled antibodies were obtained from Li-COR (Lincoln, Nebraska USA). ELISA-based assay kits/antibodies for the detection of galectin-9 (DY2045), Tim-3 (DY2365), VISTA (DY7126), IL-6 (DY206), IL-1β (DY201) and TNF-α (DY210) were purchased from Bio-Techne (R&D Systems, Abingdon, UK). Anti-Tim-3 mouse monoclonal antibodies (detection (3B1) and neutralising (4BS)) were generated by Dr Luca Varani and were used in this work [7, 10]. Human recombinant VISTA protein was obtained from Sino Biological US Inc (Wayne, PA, USA). Human recombinant Ig-like V-type domain of Tim-3 (amino acid residues 22-124) was described before [7]. Annexin V/propidium iodide apoptosis assay kits were purchased from Invitrogen (Carlsbad, USA). All other chemicals purchased were of the highest grade of purity commercially available.

Cell lines and primary human cells/samples

Cell lines used in this work were purchased from the European Collection of Cell Cultures (Porton Down, UK). Cell lines were accompanied by identification test certificates and were

grown according to corresponding tissue culture collection protocols. *Escherichia coli* (*E. Coli*) XL10 Gold® bacteria were purchased from Stratagene Europe (Amsterdam, The Netherlands).

Blood plasma of healthy human donors was obtained from buffy coat blood (purchased from healthy donors undergoing routine blood donation) which was procured from the National Health Blood and Transfusion Service (NHSBT, UK) following ethical approval (REC reference: 16-SS-033). The procedure was completed as described previously [6, 7]. Primary human AML plasma samples were obtained from the sample bank of University Medical Centre Hamburg-Eppendorf (Ethik-Kommission der Ärztekammer Hamburg, reference: PV3469) and kindly provided by Professor Walter Fiedler and Dr Jasmin Wellbrock.

Placental tissues (CVS, chorionic villus sampling) and amniotic fluids were collected after obtaining informed written consent from pregnant women at the University Hospital Bern. Cells were prepared and cultured as described before [8, 11]. CVS was washed with PBS, treated with 270 U/ml of collagenase type 2 (Sigma, Buchs, Switzerland) for 50 min at 37° C, washed twice with PBS and cells were then re-suspended and cultured in CHANG medium (Irvine Scientific, Irvine, USA) according to the manufacturer's instructions. Amniotic fluid samples were centrifuged and cell pellets were then re-suspended in CHANG medium. The first medium change was performed after 5 days of incubation at 37° C. The medium was then changed every second day until the number of cells was sufficient.

Primary human T cells where isolated from PBMCs with a CD3 T cell negative isolation kit (Biolegend). 200.000 T cells per 200 µl were incubated with and without Gal-9 at a final concentration of 2.5 µg/ml in RPMI medium. After 16 h cells were stained with anti-CD4,

140 anti-CD-8, anti-CD3 and AnnexinV (Miltenyi Biotec) according to manufacturer's

141 recommendation and analysed on a MacsQuant 16 Analyzer (Miltenyi Biotec).

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143 **In-cell and on-cell Western analysis**

144 In order to detect phagocytosis of bacterial cells or Jurkat T cells by THP-1 macrophages, we

145 analysed these cells by employing the use of specific markers following coculturing of the

146 respective cells. We used a standard LI-COR in-cell Western assay (methanol was used as a

147 permeabilisation agent) [12] to detect bacterial LPS or T cell-associated CD3 in THP-1

148 macrophages. Rabbit anti-LPS (which recognises lipid A) and anti-CD3 antibodies were used

149 to detect specific targets and a goat anti-rabbit Li-Cor secondary antibody was employed for

150 visualisation purposes.

151 In order to characterise the presence of galectin-9 and VISTA on the surface of human

152 embryonic cells or Jurkat T cells (galectin-9 only) we used a standard Li-COR on-cell

153 Western assay [12] where the cells were not permeabilised thus measuring only the proteins

154 present on the cell surface.

155

156 **Preparation of bacterial cell extracts and measuring galectin-9 in cytoplasmic and cell**

157 **wall fractions**

158 *E. Coli XL10 Gold*® bacterial cells were collected and lysed as described before by

159 sonication on ice in a buffer containing 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 μM

160 $ZnCl_2$, 0.5% NP-40, 0.5 mM dithiothreitol and 1 mM phenyl-methyl-sulfonyl-fluoride.

161 Lysates were then centrifuged and both supernatant (cytoplasm extract) and pellet (containing

162 cell wall components) were subjected to further analysis. Lysates were used to detect

163 galectin-9 by Western blot analysis (see below). Cell wall pellets were incubated with

164 biotinylated antibodies against galectin-9, Tim-3 or VISTA for 2 h at room temperature with

165 constant agitation. Pellets were then precipitated by centrifugation (5 min at 13,000 rpm)

166 followed by three washes with TBST buffer and centrifugation after each wash. After this,

167 pellets were re-suspended in PBS containing HRP-labelled streptavidin and incubated for 1 h

168 at room temperature with constant agitation. This was followed by 3 washes (as described

169 above) and development by re-suspending in 6 mg/ml ortho-phenylendiamine (OPD) solution

170 containing hydrogen peroxide. After 5 min incubation at room temperature with constant

171 agitation in the darkness, equal amount of 10 % sulfuric acid solution was added to stop the

172 reaction. Mixtures were centrifuged for 5 min at 13,000 rpm, supernatants were transferred to

173 the wells of a 96-well plate and absorbances were measured at 492 nm.

174 We also measured galectin-9, Tim-3 and VISTA on the surface of bacterial cells using on-

175 cell ELISA. Bacterial pellets were incubated for 1 h at room temperature in PBS containing

176 antibodies against galectin-9, Tim-3 or VISTA for 2 h at room temperature with constant

177 agitation. Bacterial cells were then precipitated by centrifugation (5 min at 13,000 rpm)

178 followed by three washes with TBST buffer and centrifugation after each wash. After this,

179 pellets were re-suspended in PBS containing HRP-labelled streptavidin and incubated for 1 h

180 at room temperature with constant agitation. Visualisation was performed using OPD as

181 described above.

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183 **Measurement of IL-6, IL-1β, TNF-α, TGF-β and released HMGB1 concentrations**

184 Concentrations of secreted cytokines/growth factors (IL-6, IL-1β, TNF-α and TGF-β) were

185 measured by ELISA using Bio-techne kits according to the manufacturer's protocols.

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186 HMGB1 was measured using a MyBioSource ELISA assay kit according to the

187 manufacturer's protocol.

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189 **Assessment of binding of galectin-9 and associated proteins with LPS and PGN**

190 ELISA plates were coated with anti-LPS antibody and blocked with BSA. 1 µg/well

191 *Pseudomonas aeruginosa* (*P. aeruginosa*) LPS (Sigma) was immobilised on the plate for 2 h

192 followed by application of human blood plasma. Blood plasma was then washed away 5

193 times with TBST buffer and biotinylated antibodies against galectin-9, Tim-3 or VISTA were

194 added. Binding was visualised as described above.

195 In order to assess the interaction of PGN with galectin-9 we coated the ELISA plate with 5

196 µg/well *Staphylococcus aureus* (*S. aureus*) PGN and blocked with BSA. Human blood

197 plasma or 500 ng/well human recombinant galectin-9 (dissolved in PBS) were then applied

198 and incubated for 2 h. The presence of galectin-9 was then detected as described above. To

199 confirm that the plate was successfully coated with PGN, we incubated some of the wells

200 with 10 µl of THP-1 cell lysate (which contains TLR2 – a PGN receptor) followed by

201 extensive washing with TBST. TLR2 binding was measured using rabbit anti-TLR2 antibody

202 (1:500) and visualised using goat anti-rabbit HRP-labelled antibody (1:1000).

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208 **Western blot analysis**

209 Western blot analysis of galectin-9, VISTA, Tim-3 and granzyme B was performed as

210 described before [7]. Actin staining was used as a protein loading control.

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212 **Granzyme B in-cell activity, caspase-3 activity, cell viability and Annexin V tests**

213 In-cell activity of granzyme B was measured as described before [7]. Briefly, living cells

214 were incubated with 150 µM Ac-IEPD-AFC (granzyme B substrate) for 1 h at 37°C in sterile

215 PBS. This did not affect the cell viability, as described below. Total cell fluorescence was

216 then measured in living cells using excitation and emission wavelengths recommended by the

217 Ac-IEPD-AFC manufacturer (Sigma). An equal number of cells, which were not exposed to

218 granzyme B substrate were used as a control.

219 Capsase-3 activity in cell lysates was measured using a colorimetric assay kit based on

220 cleavage of the substrate Ac-DEVD-pNA according to the manufacturer's (Bio-techne)

221 protocol. Cell viability was measured by MTS assay (Promega kit was used); measurements

222 were performed according to the manufacturer's protocol).

223 An annexin V test was performed [7] using an Invitrogen assay kit according to the

224 manufacturer's protocol.

225

226 **Statistical analysis**

227 Each experiment was performed at least three times and statistical analysis, when comparing

228 two events at a time, was performed using a two-tailed Student's t-test. Multiple comparisons

229 were conducted by ANOVA. Post-hoc Bonferroni correction was applied. Statistical

230 probabilities (p) were expressed as * when $p < 0.05$; and ** when $p < 0.01$.

Results

Galectin-9 opsonises Gram-negative bacteria *via* binding to LPS, triggering their phagocytosis and enhancing anti-bacterial innate immune reactions

Galectin-9 was found to be able to opsonise Gram-negative bacteria by direct interaction with them. We first investigated the reactions of galectin-9 with Gram-negative bacteria and with LPS (component of their cell wall) as well as the impact of these interactions on phagocytosis of target bacteria and innate immune reactions to them. We used THP-1 cells which were differentiated into macrophages by 24 h exposure to 100 nM PMA. Upon completion of differentiation, medium was then replaced (PMA and antibiotic free). 50 µl of *E. Coli XL10 Gold®* were added to the culture and incubated under normal cell culturing conditions for 16 h in the presence or absence of 10 mM lactose to block the sugar-binding activity of galectin-9 (Figure 1A). A concentration of 10 mM lactose was sufficient to block the sugar-binding activities of THP-1 cell-derived galectin-9 and neither affected cell viability (when measured by an MTS test) nor proliferation velocity (assessed by counting the cells). Bacterial cells were then washed away with sterile PBS and THP-1 cells were permeabilised with methanol, as outlined in Materials and methods, and the presence of LPS was detected using anti-LPS antibody (specific to lipid A) by in-cell Western (Figure 1 B). We found that LPS was highly present in THP-1 macrophages when co-cultured with bacteria and these levels were substantially attenuated by the presence of lactose in the culture medium (Figure 1 B). Importantly, co-incubation with bacteria provoked high levels of inflammatory cytokine release from THP-1 cells, where secretions of TNF-α, IL-1β and IL-6 were significantly upregulated (Figure 1C). The presence of lactose in the medium significantly reduced the levels of secreted cytokines (Figure 1C). Importantly, upon completion of co-incubation, we measured galectin-9, Tim-3 and VISTA levels by ELISA. In the presence of bacteria, the level of galectin-9 was 8.7 ± 1.1 ng galectin-9 per 10^6 THP-1 cells. Tim-3 and VISTA levels

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256 were 1.12 ± 0.2 and 0.91 ± 0.14 ng per 10^6 THP-1 cells, respectively. Bacteria washed away

257 from the co-culture were lysed and the cytoplasmic components then extracted and subjected

258 to Western blot analysis for presence of galectin-9. It was not detectable in bacterial

259 cytoplasm (Figure 1D left panel). Pellet containing bacterial cell wall was exposed to

260 biotinylated antibody against galectin-9 for 1 h. Then antibody was washed away with PBS 3

261 times by re-suspension followed by centrifugation. Pellet was exposed to HRP-labelled

262 streptavidin for 1 h followed by washing as described above and measurement of HRP as

263 outlined in Materials and methods. We found that cell wall pellet derived from bacterial cells

264 that were not co-cultured THP-1 cells did not contain galectin-9. In contrast, galectin-9 was

265 present in the pellets from bacterial cells co-cultured with THP-1 cells. The presence of

266 lactose reduced the amount of galectin-9 associated with bacteria (Figure 1D right panel). We

267 also assessed if Tim-3 and VISTA, which were found to associate with galectin-9 in T cells,

268 were attached to bacteria. We used the same approach as for galectin-9 (see above and Figure

269 1D right panel for schemes of the assays) and found that both Tim-3 and VISTA were indeed

270 associated with galectin-9 and that their presence, as with galectin-9, was reduced by lactose

271 (Figure 1D right panel). In order to confirm that Tim-3 and VISTA interact with galectin-9

272 and not directly with bacteria we exposed bacterial cells (*E. Coli XL10 Gold®)*, described

273 above, for 1 h to 0.1 μM human recombinant galectin-9, 0.1 μM human recombinant Tim-3

274 or 0.1 μM human recombinant VISTA. In addition, we exposed bacterial cells to a mixture of

275 0.1 μM galectin-9 and 0.1 μM Tim-3 or VISTA (see scheme of the experiment in

276 Supplementary figure 1). We found that Tim-3 and VISTA were associated with bacteria

277 only when co-incubated with galectin-9 and not on their own (Supplementary figure 2),

278 which provides further confirmation that Tim-3 and VISTA associate with galectin-9 and not

279 with bacteria. Finally, we sought to confirm that galectin-9 interacts with LPS. We coated the

280 ELISA plate with anti-LPS antibody (3 μg/well) and immobilised *P. aeruginosa* LPS on it (1

281 µg LPS per well), see Materials and methods for further details. We then loaded human blood

282 plasma obtained from healthy donors containing 520 pg/ml galectin-9, 790 pg/ml Tim-3 and

283 335 pg/ml VISTA with or without 30 mM lactose (this high lactose concentration was used

284 given the viscosity of human blood plasma and the presence of proteins other than galectin-9,

285 which can potentially interact with lactose). We then measured galectin-9 as well as Tim-3

286 and VISTA associated with LPS. We found that blood plasma galectin-9 was bound to the

287 LPS and associated with Tim-3 and VISTA (Figure 1E). The presence of lactose attenuated

288 the association of galectin-9 (and respectively Tim-3 and VISTA) with LPS (Figure 1E).

289 To confirm the observed effects with whole bacterial cells we incubated *E. Coli XL10 Gold®*

290 (50 µl stock) with 500 µl of blood plasma obtained from healthy donors containing 460 pg/ml

291 galectin-9, 410 pg/ml Tim-3 and 285 pg/ml VISTA for 1 h in the absence or presence of 30

292 mM lactose. We then precipitated bacteria and measured galectin-9, Tim-3 and VISTA

293 associated with them as outlined in Materials and methods. We found that galectin-9, as well

294 as Tim-3 and VISTA, were associated with bacteria (Figure 2) and this association was

295 significantly downregulated by the presence of lactose.

296 Finally, we sought to confirm that galectin-9 can bind only LPS and not peptidoglycan (PGN),

297 which forms the cell wall of Gram-positive bacteria. For this purpose, we coated the plate

298 with 5 µg/well PGN and applied human blood plasma obtained from healthy donors

299 containing 560 pg/ml galectin-9. For comparison, we applied 500 ng per well of human

300 recombinant galectin-9 (this is approximately 20% of the amount of PGN used to coat the

301 wells of the plate). This high amount was applied alone to assess the possibility of chemical

302 interactions between the two substances – PGN and galectin-9. To confirm the successful

303 immobilisation of PGN on the ELISA plate surface, we loaded cell lysate of THP-1 cells

304 containing TLR2 (PGN receptor [13]) and then measured its presence by ELISA (see

305 Materials and methods for details). We found that PGN did not bind galectin-9 from blood

plasma (Figure 3) but traces of interactions were detectable with recombinant galectin-9 (the concentration here was 1000 times higher than in blood plasma). TLR2 was clearly interacting with PGN, suggesting that it was successfully immobilised on the ELISA plate. These results indicate that galectin-9 at physiological concentration does not interact with PGN and thus, in line with previous observations, opsonises only Gram-negative bacteria which contain LPS. Opsonisation of Gram-negative bacteria with galectin-9 enhances innate immune reactions to these bacteria and their phagocytosis by macrophages.

Cell surface-based galectin-9 in human embryonic cells protects them against cytotoxic T cell attack but is not involved in bacterial colonisation

Recently, we reported that human embryonic cells express high levels of galectin-9 at the early stages of pregnancy [8]. We sought to confirm whether embryonic galectin-9 can suppress the cytotoxic activity of T cells. We compared the levels of galectin-9, Tim-3 and VISTA in embryonic cells obtained during the chorion stage (13-14 weeks of pregnancy) and amnion stage (ca 20 weeks). As expected, all of the proteins were expressed at higher levels in the earlier pregnancy stage (Figure 4 A-C). We asked whether Tim-3 or VISTA, or both proteins, act as traffickers/carriers of galectin-9 in order to translocate it onto the surface. We prepared ELISA formats coating the plate with mouse or rabbit anti-galectin-9 antibody to capture galectin-9 from the cell lysates of embryonic cells obtained at chorion stage (which express high levels of galectin-9). We confirmed successful capturing of galectin-9 by detecting it using rabbit anti-galectin-9 antibody (mouse antibody was used to capture galectin-9 in this case) and visualised the interaction using goat anti-rabbit fluorescently-labelled secondary antibody (Figure 4 C). We detected Tim-3 and VISTA associated with galectin-9. We found that both proteins were detectable but the signal obtained with Tim-3

330 was much more intense suggesting that Tim-3 is likely to act as carrier/trafficker for galectin-

331 9 in embryonic cells and VISTA possibly associates with the complex. Using on-cell Western,

332 we measured galectin-9 and VISTA on the surface of embryonic cells and found that they

333 were both present and when merging the fluorescence – yellow fluorescence was also

334 detectable suggesting that galectin-9 and VISTA could possibly be located close to each other

335 on the cell surface. Galectin-9 and VISTA could thus associate when interacting with T cells,

336 as we have recently reported for acute myeloid leukaemia cells [7]. To verify this we co-

337 cultured primary human embryonic cells with Jurkat T cells, which were pre-treated for 24 h

338 with 100 nM PMA [7] in order to activate granzyme B production (Figure 4E). PMA treated

339 Jurkat T cells expressed granzyme B, Tim-3 and VISTA (Figure 4 E). Medium was then

340 replaced with PMA-free medium and cells were co-cultured with embryonic cells for 16 h

341 with or without pre-treatment with galectin-9 or/and VISTA neutralising antibodies. We

342 found that presence of antibodies in the co-culture reduced intracellular activation (most

343 likely caused by leakage) of granzyme B as well as caspase 3 activity and increased the

344 viability of Jurkat T cells (Figure 4F).

345 We sought to understand if cell surface-based galectin-9 in human embryonic cells can be

346 involved in the colonisation of Gram-negative bacteria. We co-cultured embryonic cells

347 (chorion stage) with 50 µl stock of *E. Coli XL10 Gold*® for 16 h in antibiotic-free medium

348 allowing bacteria to form colonies on the monolayer of embryonic cells (Figure 5A). Then

349 we removed the medium containing bacteria and added THP-1 monocytes (10^6 cells per dish

350 containing 3 ml of culture medium) and incubated for 16 h in antibiotic-free medium under

351 normal cell culture conditions in the absence or presence of 10 mM lactose. We then

352 measured IL-6, IL-1β and TNF-α in cell culture medium (Figure 5). We found background

353 levels of all three cytokines in the co-culture of embryonic cells with THP-1 cells, which

354 were not exposed to bacteria. However, cytokine levels were significantly upregulated in the

355 presence of bacteria and were not reduced in the co-cultures by lactose (Figure 5B). These

356 results suggest that cell surface-based galectin-9 in human embryonic cells is not involved in

357 bacterial colonisation and does not influence the association of bacteria with embryonic cells

358 and thus does not determine the innate immune response to bacteria infecting embryonic cells.

359 However, galectin-9 is involved in suppressing the cytotoxic activities of T cells, thus

360 protecting embryonic cells against cytotoxic immune attack.

361

Secreted galectin-9 "opsonises" T cells and provokes their phagocytosis by macrophages

363 Given the results presented above, and the current knowledge on galectin-9-triggered

364 suppression and even apoptosis of T cells, we asked whether T lymphocytes opsonised by

365 galectin-9 can be phagocytosed by macrophages. For this purpose, we used Jurkat T cells

366 activated with 100 nM PMA for 24 h. These cells were then exposed to 2.5 µg/ml galectin-9

367 in PMA free medium (Figure 6A). This concentration of galectin-9 was used based on our

368 previous observations. Importantly, recombinant galectin-9, in terms of inducing biological

369 responses, is about 250-500 times less active than myeloid cell-derived protein [7]. After

370 exposure to galectin-9 we characterised the presence of phosphatidylserine (PS, known as an

371 "eat me signal" for macrophages) on the cell surface using annexin V staining, cell viability,

372 as well as the release of TGF-β (known to be released by dying T cells [14]) and HMGB1

373 (released by damaged, stressed or dying cells). We found that cell viability measured by MTS

374 test was not significantly affected (although some of the cells were apoptotic) despite the

375 significant increase in annexin V staining, indicating increased surface-based PS levels

376 (Figure 6B). Secreted levels of TGF-β and HMGB1 were significantly upregulated in cells

377 treated with galectin-9. These cells were co-cultured for 3 h with THP-1 macrophages

378 (differentiated for 24 h by exposure to 100 nM PMA). Some of the macrophages were pre-

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379 stimulated for 1 h with 1 µg/ml HMGB1 to assess the possibility of phagocytic activity of

380 macrophages being enhanced by HMGB1. We then permeabilised THP-1 cells with methanol

381 and assessed presence of the T cell marker CD3 in THP-1 cells by in-cell Western. We found

382 that galectin-9-treated Jurkat T cells were phagocytosed at significantly higher levels

383 compared to cells which were not pre-exposed to galectin-9 (Figure 6C, top panel). HMGB1

384 significantly enhanced the ability of macrophages to phagocytose T cells opsonised with

385 galectin-9. Since, in addition to galectin-9, Jurkat T cells had high amounts of PS on their

386 surface, we asked whether macrophage surface-based Tim-3 is involved in the phagocytosis

387 of T cells as both galectin-9 and PS are Tim-3 ligands. We co-cultured PMA-differentiated

388 THP-1 cells with PMA-activated galectin-9 pre-treated Jurkat T cells (as described above)

389 with or without 1 h pre-exposure of macrophages to 2 µg/ml Tim-3 neutralising antibody. We

390 observed that neutralisation of Tim-3 reduced phagocytosis of T cells (Figure 6C bottom

391 panel).

392 To confirm the physiological relevance of this effect we co-cultured THP-1 macrophages (24

393 h PMA differentiation was applied) with PMA-activated Jurkat T cells which were first

394 cultured for 16 h in the presence of 10 % human blood plasma obtained either from healthy

395 donors (contained 370 pg/ml galectin-9) or from AML patients (contained 8200 pg/ml

396 galectin-9). In co-cultures where Jurkat T cells were pre-treated with AML patient plasma,

397 the level of phagocytosis was significantly higher, while no significant changes were

398 observed in phagocytosis of cells pre-treated with healthy donor blood plasma. Neutralisation

399 of Tim-3 downregulated phagocytosis of Jurkat T cells pre-treated with blood plasma from

400 AML patients (Figure 6D). Exposure of Jurkat T cells to blood plasma of AML patients

401 significantly increased galectin-9 levels on their surface (Figure 6E) confirming an

402 opsonisation effect.

17

403   We then sought to confirm that opsonisation of primary human T cells with galectin-9 leads

404   to the appearance of PS on their surface. CD4 and CD8-positive primary human T cells were

405   treated with 2.5 µg/ml galectin-9 for 16 h followed by measurement of PS levels using

406   annexin V staining. We found that, in both cell types, PS levels were significantly

407   upregulated with higher level of upregulation observed in CD8-positive T cells (Figure 7).

408   The differences in effects are most likely determined by granzyme B levels in both types of T

409   cells (which are higher in CD8-positive cells).

410   Taken together our results suggest that galectin-9 affects T cells, causing their phagocytosis

411   by macrophages.

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Discussion

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425 Galectin-9 is known to contribute to immunosuppressive functions in the malignant tumour

426 microenvironment by impairing the anti-cancer activities of cytotoxic lymphoid cells and

427 thus allowing cancer cells to escape immune attack [7]. However, the exact role of galectin-9

428 in normal human immune reactions remains to be understood.

429 Here we confirmed that the secreted form of galectin-9, normally produced by macrophages

430 and other cells of myeloid lineage, is capable of opsonising Gram-negative bacteria. The

431 effect takes place through the interaction of galectin-9 with LPS present on the cell wall of

432 these bacteria (Figures 1 and 2). Galectin-9 most likely interacts with sugar components of

433 LPS since the binding is strongly inhibited by lactose, but occurs when lipid A is occupied by

434 interaction with antibody. Furthermore, during opsonisation of Gram-negative bacteria, the

435 galectin-9 binding partners, Tim-3 and VISTA, form multiprotein associations in a way

436 similar to the one recently reported for T cells [7]. These interactions most likely render the

437 bacteria less mobile. As such, they can be more easily captured by macrophages and

438 phagocytosed. Opsonisation also increases the number of bacteria interacting with innate

439 immune cells and thus enhancing their cytokine production (IL-6, IL-1β and TNF-α). In

440 contrast, PGN, which forms the cell wall of Gram-positive bacteria [13], is poorly recognised

441 by galectin-9 and, as such, galectin-9 cannot be involved in the opsonisation of Gram-

442 positive bacteria (Figure 3), which is in line with previous observations [9].

443 Interestingly, galectin-9 is highly expressed in human embryonic cells especially at the early

444 stages of pregnancy (Figure 4). When present on the cell surface it protects embryonic cells

445 against the cytotoxic activity of T cells by stimulating the upregulation of intracellular

446 granzyme B activity and caspase 3 in attacking T cells, which then undergo apoptosis (Figure

447 4). This takes place in the way similar to the one reported for AML cells, which secrete

448 galectin-9 to impair cytotoxic activities of lymphoid cells [6, 7].

449 However, surface-based galectin-9 in embryonic cells is not involved in the interactions of

450 Gram-negative bacteria infecting embryonic cells. When infecting human cells, bacteria

451 normally use their pili and bind various substances on the host cell surface [17]. Pili form a

452 "first class" of organelles involved in the binding of bacteria to host cells [17]. For example,

453 *E. Coli* pili can use the adhesion factor PapG to interact with glycosphingolipids on the

454 kidney epithelium. Another type of pili, called "Type I pili", binds D-mannosylated receptors

455 (e. g. uroplakins in the bladder) [17-20]. From our results, it is clear that cell surface-based

456 galectin-9 does not appear to be involved in adhesion/colonisation of Gram-negative bacteria

457 on the host cell surface (Figure 5).

458 In sharp contrast, soluble galectin-9, known to impair cytotoxic activities of T and NK cells

459 [6, 7, 21], opsonised T cells. This effect leads to activation of granzyme B in T cells

460 expressing this enzyme (e. g. cytotoxic T cells) [7] and can induce apoptosis of T cells and

461 causes the release of TGF-β and HMGB1 (Figure 6B). Dying T cells are known to release

462 high levels of TGF-β [14], which can upregulate expression of galectin-9 in cancer cells [7,

463 22] and possibly also in malignant tumour-associated macrophages (or placental

464 macrophages involved in protection of the embryo). Galectin-9-dependent opsonisation of T

465 cells leads to the appearance of PS on their surface (Figure 6B and Figure 7). This is the

466 process which is most likely triggered by scramblases of types TMEM16F and Xk-related

467 protein 8 (Xkr8) [23-26]. TMEM16F is also a calcium-dependent scramblase [23-26] and, as

468 such, is most likely involved in the translocation of PS onto the T cells surface since galectin-

469 9 induces intracellular calcium mobilisation in T cells of all types [27]. Xkr8 is a caspase-3-

470 dependent scramblase and can be activated by caspase-3 [23, 25, 26], the activity of which is

471 significantly upregulated in cytotoxic T cells in a granzyme-B-dependent manner [7].

472   HMGB1, as a ligand of Toll-like receptors 2 and 4 [15, 16], activates macrophages and their

473   ability to phagocytose target cells. Tim-3 present on macrophage cell surfaces is involved in

474   phagocytosis of T cells affected by galectin-9 (Figure 6), which have two Tim-3 ligands

475   present on their surface, galectin-9 and PS (known as an "eat me" signal [28]). This discovery

476   explains the phenomenon of host T cells being phagocytosed by tumour-associated

477   macrophages or placental macrophages.

478   Our results demonstrated another reason why LPS induces TGF-β production (the effect

479   which has recently been reported [29]). While LPS directly induces innate immune reactions

480   [13], the upregulation of TGF-β secretion triggers the production of the opsonising protein

481   galectin-9 [8], which significantly enhances innate immune reactions to bacteria (Figure 1).

482   Interestingly, other galectins (-4 and -8) with tandem structure and galectin-3 (a chimeric type

483   of galectin) were recently reported to interact with bacterial LPS [30]. Further investigations

484   would have to unravel the role of these galectins in the opsonisation of bacteria and T cells in

485   human immune responses.

486   Taken together, our results strongly suggest that galectin-9 is involved in the opsonisation of

487   Gram-negative bacteria thus promoting anti-bacterial immune defence, including innate

488   immune reactions and phagocytosis. Opsonisation of T cells by galectin-9 allows it to protect

489   embryos against cytotoxic attack by the mother's immune system and recruit placental

490   macrophages to phagocytose/remove T cells which potentially pose a threat to the developing

491   embryo. Unfortunately, this phenomenon is also successfully used to protect malignant

492   tumours against cytotoxic T cells and in recruiting tumour-associated macrophages to

493   participate in the suppression of anti-cancer T cell function. Furthermore, galectin-9 also

494   induces T cells to produce TGF-β and HMGB1 which contribute further to an

495   immunosuppressive milieu. Both factors can either directly (TGF-β) or indirectly (HMGB1,

496 through TLR4-mediated TGF-β expression) induce galectin-9 expression in cancer cells and

497 macrophages [8, 16]. Interestingly, recent evidence demonstrated that intracellular galectin-9

498 expressed by T cells enhances proximal T cell receptor signalling [31], thus further

499 biochemical studies may help to understand the mechanisms of regulation of galectin-9

500 expression in T cells, especially those infiltrated into malignant tumours.

501 Taken together, our results have shown that secreted and cell surface-associated galectin-9

502 plays crucial role both in anti-bacterial immune defence and in the suppression of cytotoxic

503 cell function during embryo development and malignant tumour progression.

512 **Conflicts of interest**

513 The authors have no conflicts of interest to declare

514 **Author contributions**

515 SS, NHM, IMY and BFG performed majority of the experiments and analysed data. NA,

516 EFK and SB completed the work with primary embryonic cells. VVS designed the study,

517 planned all the experiments together with EFK, analysed the data. VVS, BFG and EFK wrote

518 the manuscript.

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Figure legends

Figure 1. Opsonisation of Gram-negative bacteria with galectin-9 occurs *via* LPS binding, triggering phagocytosis of bacterial cells and innate immune cytokine secretion. THP-1 macrophages (obtained by PMA differentiation of monocytes) were co-cultured with *E. Coli XL10 Gold®* for 16 h in the absence or presence of 10 mM lactose (**A**). Phagocytosis of bacterial cells was then assessed using in-cell Western (**B**). Concentrations of TNF-α, IL-1β and IL-6 were measured in cell culture medium by ELISA (**C**). Bacterial cells were lysed and galectin-9 was measured in cytoplasmic extracts by Western blot (**D, left panel**). Cell wall-containing pellet was subjected to measurement of galectin-9, Tim-3 and VISTA as outlined in Materials and methods (**D, right panel**). Binding of galectin-9 to LPS and the association of Tim-3 and VISTA with the complex was performed by an ELISA-based method as outlined in Materials and methods (**E**). Images are from one experiment representative of five which gave similar results. Quantitative data represent mean values ± SEM of five independent experiments. * - p < 0.05 and ** - p < 0.01 *vs* control.

Figure 2. Galectin-9 from human blood plasma opsonises Gram-negative bacteria. *E. Coli XL10 Gold®* cells were incubated in human blood plasma obtained from healthy donors in the absence or presence of 30 mM lactose. Galectin-9 on the surface of bacteria and its association with Tim-3 and VISTA was detected as outlined in Materials and methods. Images are from one experiment representative of five which gave similar results. Quantitative data represent mean values ± SEM of five independent experiments. ** - p < 0.01 *vs* control.

662 **Figure 3. Galectin-9 from blood plasma does not bind PGN.** PGN from *S. aureus* was

663 immobilised on an ELISA plate and exposed to human recombinant galectin-9 (500 ng/well),

664 human blood plasma obtained from healthy donors or THP-1 cell lysate containing TLR2

665 (PGN receptor) to confirm successful immobilisation of PGN on the plate surface. Images are

666 from one experiment representative of five which gave similar results. Quantitative data

667 represent mean values ± SEM of five independent experiments. ** - $p < 0.01$ *vs* control.

668

669 **Figure 4. Galectin-9 and VISTA play a crucial role in suppressing the cytotoxic**

670 **activities of T cells on human embryonic cells.** Primary human embryonic cells were

671 cultured as described in Materials and methods. Levels of galectin-9, VISTA **(A)** and Tim-3

672 **(B)** were measured by Western blot analysis in cells obtained either from 7 patients at

673 chorion stage (weeks 13-14) or 7 patients at amnion stage (ca. week 20). Association of

674 galectin-9 with Tim-3 and VISTA was analysed as described in the text and as shown in

675 Supplementary figure 3 **(C)**. The presence of galectin-9 and VISTA on the cell surface was

676 analysed using on-cell Western **(D)**. Cells used for this analysis are also shown on the top of

677 the panel D. Embryonic cells (chorion stage) were then co-cultured for 16 h with Jurkat T

678 cells, which were pre-activated for 24 h with PMA to induce the expression of granzyme B.

679 PMA-activated cells expressed both Tim-3 and VISTA **(E)**. Jurkat T cells were then collected

680 and subjected to measurement of in-cell granzyme B activity, caspase-3 activity in cell

681 lysates and cell viability assay **(F)**. Images are from one experiment representative of seven

682 which gave similar results. Quantitative data represent mean values ± SEM of seven

683 independent experiments. * - $p < 0.05$ and ** - $p < 0.01$ *vs* control.

684

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Figure 5. Galectin-9 is not involved in colonisation of Gram-negative bacteria on embryonic cells. Primary human embryonic cells (chorion stage) were co-incubated with *E. Coli XL10 Gold®* cells for 16 h in the absence or presence of 10 mM lactose. Unbound bacteria were then removed and THP-1 cells (monocytes) were added. The innate immune response to these bacteria was measured by detecting the amounts of IL-6, IL-1β and TNF-α release using ELISA. Images are from one experiment representative of four which gave similar results. Quantitative data represent mean values ± SEM of four independent experiments. * - p < 0.05 and ** - p < 0.01 *vs* control.

Figure 6. Galectin-9 opsonises T cells and triggers their phagocytosis by macrophages. PMA-activated Jurkat T cells were exposed to 2.5 μg/ml human recombinant galectin-9 for 16 h followed by co-culturing for 3 h with PMA-differentiated THP-1 macrophages. PC – phosphatidylcholine, SM – sphingomyelin, PS – phosphatidylserine **(A)**. Cell viability, PS (annexin V staining), TGF-β and HMGB1 releases were measured as outlined in Materials and methods **(B)**. Phagocytosis of the T cells was measured in THP-1 cells with or without 1 h pre-activation with HMGB1 **(C, top panel)** or with or without neutralising Tim-3 **(C, bottom panel)**. PMA-activated Jurkat T cells were first cultured for 16 h in culture medium containing 10 % of blood plasma obtained from healthy human donors or AML patients. This was followed by co-culturing of these cells with THP-1 macrophages for 3 h. Phagocytosis of Jurkat T cells was then analysed using in-cell Western. Cells exposed to blood plasma obtained from AML patients were co-cultured with THP-1 cells with or without 1 h pre-exposure of macrophages to Tim-3 neutralising antibody **(D)**. Given the increased levels of T cell phagocytosis following their exposure to blood plasma obtained from AML patients, these cells were subjected to measurement of galectin-9 on their surface by on-cell Western **(E)**. Images are from one experiment representative of five which gave similar results.

710   Quantitative data represent mean values ± SEM of five independent experiments. * - p < 0.05

711   and ** - p < 0.01 *vs* control.

712

713   **Figure 7. Exposure of primary human T cells to galectin-9 upregulates PS translocation**

714   **onto the cell surface.** CD4- and CD8-positive T cells isolated from blood of healthy human

715   donors were exposed to 2.5 µg/ml human recombinant galectin-9 for 16 h followed by PS

716   detection of their surface using annexin V staining. Quantitative data represent mean values ±

717   SEM of eleven independent experiments. ** - p < 0.01 *vs* control.

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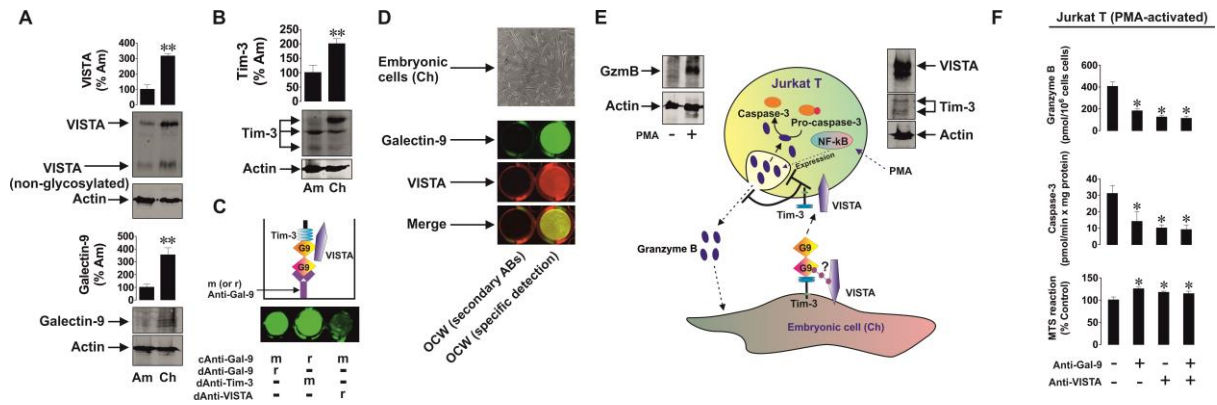
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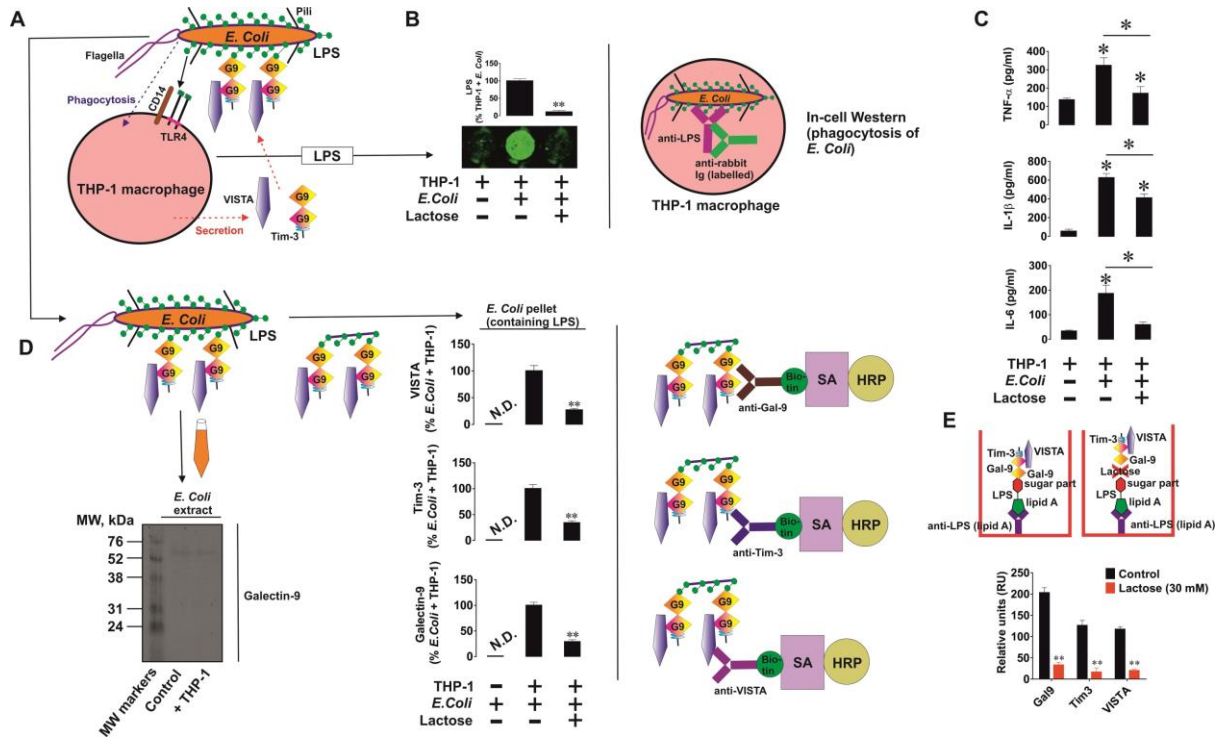
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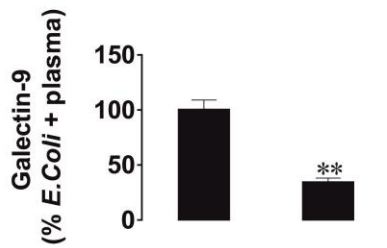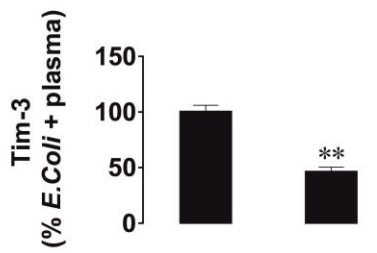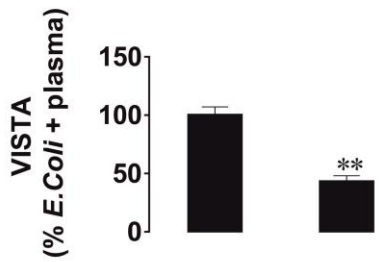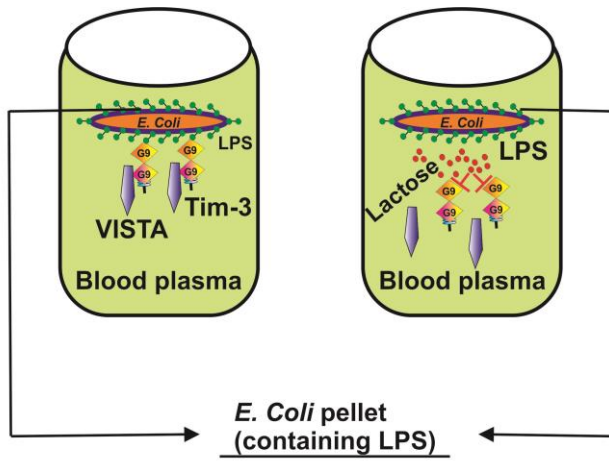
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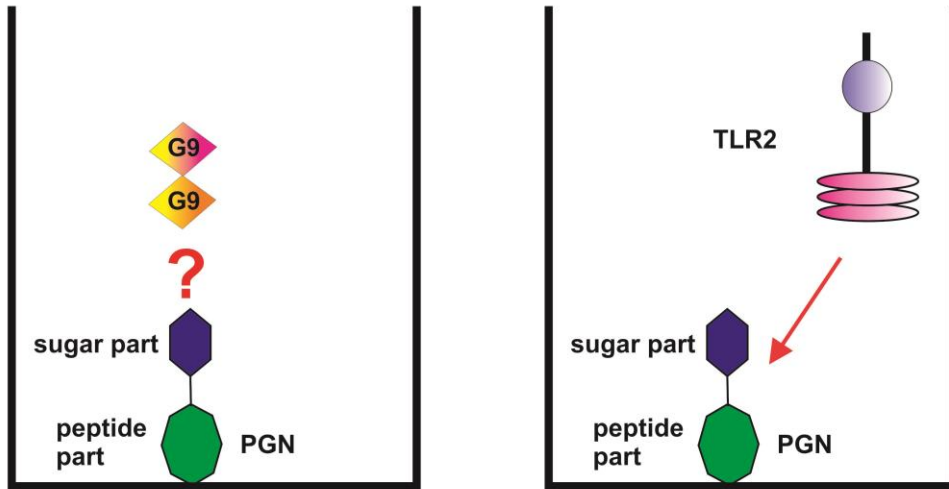
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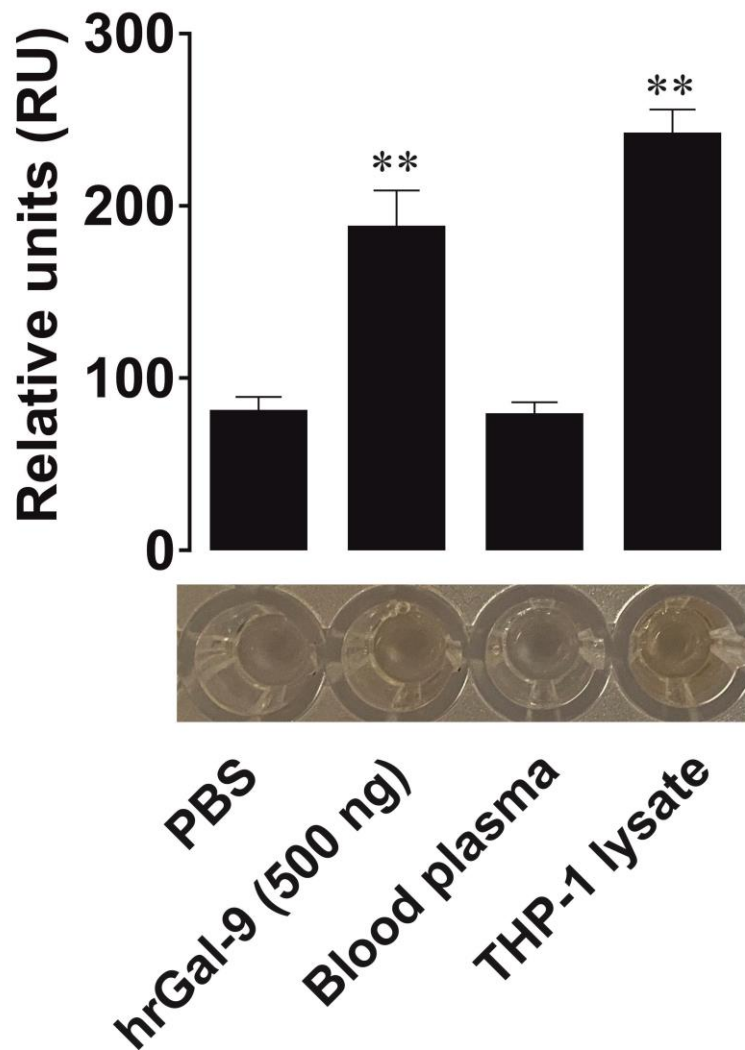
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739 Figure 5
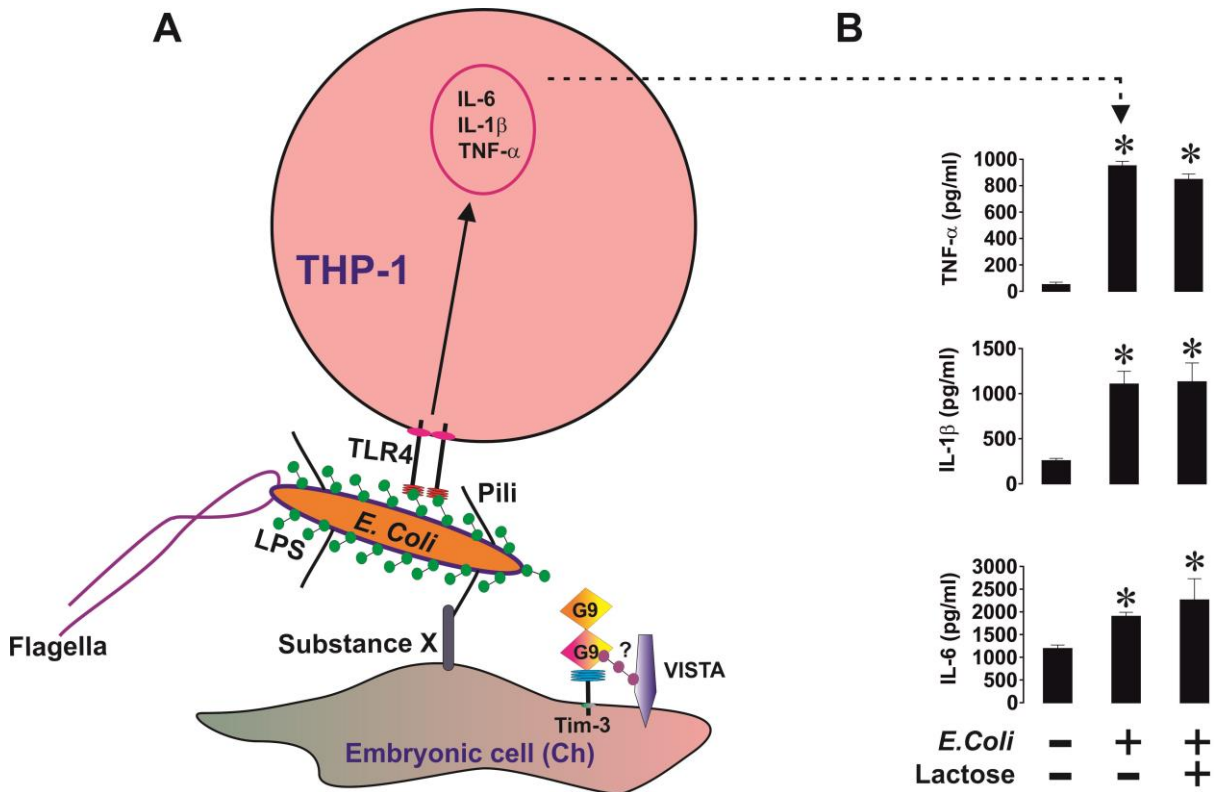
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741 Figure 6

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743   Figure 7