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COMMUNICATION

Galectin-3 functions as an opsonin and enhances the macrophage clearance of apoptotic neutrophils

Anna Karlsson[2,3], Karin Christenson[2,3], Mustafa Matlak[3], Åse Björstad[3], Kelly L Brown[3], Esbjörn Telemo[3], Emma Salomonsson[4], Hakon Leffler[4], and Johan Bylund[1,3]

[3]Department of Rheumatology and Inflammation Research, University of Gothenburg; and [4]Section for Microbiology, Immunology, Glycobiology, Department of Laboratory Medicine, University of Lund, Sweden

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Galectin-3, a β-galactoside binding, endogenous lectin, takes part in various inflammatory events and is produced in substantial amounts at inflammatory foci. We investigated whether extracellular galectin-3 could participate in the phagocytic clearance of apoptotic neutrophils by macrophages, a process of crucial importance for termination of acute inflammation. Using human leukocytes, we show that exogenously added galectin-3 increased the uptake of apoptotic neutrophils by monocyte-derived macrophages (MDM). Both the proportion of MDM that engulfed apoptotic prey and the number of apoptotic neutrophils that each MDM engulfed were enhanced in the presence of galectin-3. The effect was lactose-inhibitable and required galectin-3 affinity for *N*-acetyllactosamine, a saccharide typically found on cell surface glycoproteins, since a mutant lacking this activity was without effect. The enhanced uptake relied on the presence of galectin-3 during the cellular interaction and was paralleled by lectin binding to apoptotic cells as well as MDM in a lactose-dependent manner. These findings suggest that galectin-3 functions as a bridging molecule between phagocyte and apoptotic prey, acting as an opsonin. The process of clearance, whereby apoptotic neutrophils are removed by macrophages, is crucial for the resolution of acute inflammation and our data imply that the increased levels of galectin-3 often found at inflammatory sites could potently affect this process.

Keywords: clearance/inflammation/mdm/phagocytosis

Introduction

The β-galactoside binding lectin galectin-3 has been shown to be involved in host defense, both as an endogenous modulator of inflammatory processes and as an anti-infective agent (Almkvist et al. 2002; Liu 2005; Kohatsu et al. 2006; Farnworth et al. 2008). Galectin-3 has been implicated in several stages of inflammation from the initiation phase (chemotaxis, microbial recognition) (Mey et al. 1996; Sano et al. 2000; John et al. 2002), through the elicitation of cellular effector functions (phagocytosis, oxygen radical production) (Yamaoka et al. 1995; Karlsson et al. 1998; Sano et al. 2003; Farnworth et al. 2008), to the final modulation of apoptotic cell death (Hsu and Liu 2004; Farnworth et al. 2008). Here, we extend these findings to include a role for extracellular galectin-3 also in the final clearance of apoptotic neutrophils by macrophages, a process of crucial importance for the termination of acute inflammation.

Neutrophils are short-lived cells and continuously undergo apoptosis within hours to a few days in the tissues. After apoptosis, these inactive cells are cleared by other (active) phagocytes, most often macrophages. Clearance is doubly anti-inflammatory in that it ensures safe removal of tissue-destructive enzymes and molecules (with which the neutrophils are filled) and in that the macrophages secrete anti-inflammatory cytokines upon phagocytosis of apoptotic bodies (Savill et al. 2002).

The major recognition mechanism in apoptotic clearance seems to involve phosphatidyl serine on the apoptotic cell, but the macrophage receptor interacting with this ligand is so far unknown, although several potential receptor candidates have been proposed (Hume 2008). Phagocytosis of microbes can be enhanced by opsonization, and the involvement of opsonins has been suggested also for the uptake of apoptotic bodies. Since high levels of galectin-3 can be present in extracellular fluids at inflammatory sites (Farnworth et al. 2008), we hypothesized that this lectin can bind to the surface of apoptotic neutrophils and facilitate the uptake of these cells by macrophages, i.e., function as an opsonin during apoptotic clearance.

Results and discussion

About 30–40% of the monocyte-derived macrophages (MDM; detected as CD14+) were associated with apoptotic neutrophils (detected as CFDA+) after 90 min co-incubation in medium (Figure 1A; Ctrl; upper-right quadrant). This association represents uptake of the apoptotic neutrophils by the MDM, since it was inhibited by cytochalasin B (Figure 1A), a well-known disruptor of actin polymerization that blocks phagocytosis but not attachment (Keller and Niggli 1995). The addition of exogenous galectin-3 significantly enhanced MDM association with apoptotic neutrophils (Figure 1A), again mainly due to uptake since it was inhibited by cytochalasin B (Figure 1B). The effect was evident at a galectin-3 concentrations of 50 μg/mL (Figure 1C) corresponding to levels reported from inflammatory sites in vivo (Farnworth et al. 2008), but not

[1]To whom correspondence should be addressed: Tel: +46-31-342-46-85; e-mail: Johan.Bylund@rheuma.gu.se
[2]These authors contributed equally to this work.

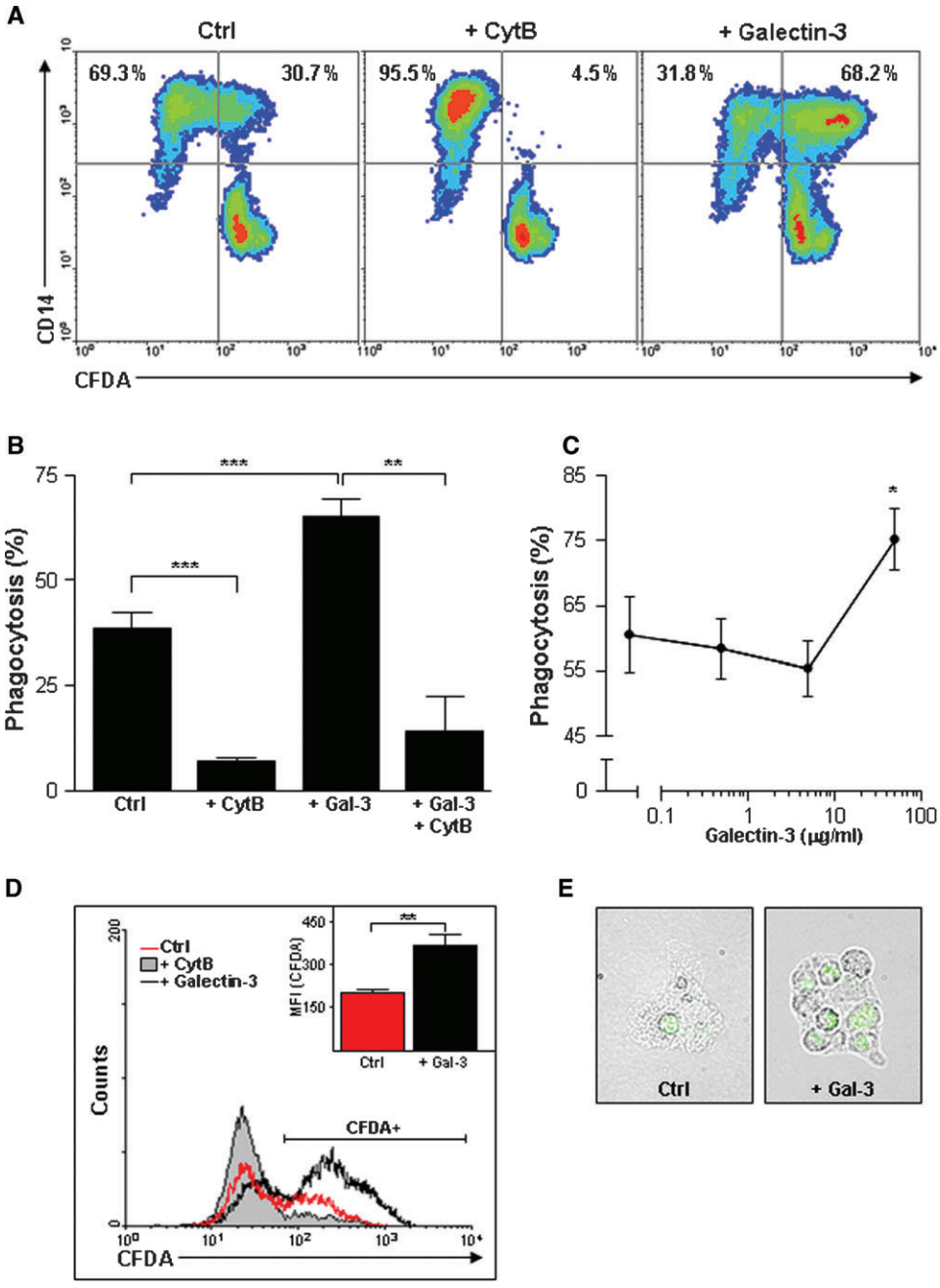

Fig. 1. Galectin-3 increases MDM phagocytosis of apoptotic neutrophils. After 90 min coculture of MDM (CD14+) and apoptotic neutrophils (CFDA+), the cells were analyzed by FACS and double-positive events (upper-right quadrant) were considered MDM having phagocytosed apoptotic neutrophils (**A**). Representative density plots (**A**; $n \geq 6$ independent experiments) are shown and the percentage of MDM found in CFDA− (upper-left) quadrant and the CFDA+ (upper-right) quadrant are indicated. The addition of the cytoskeletal disrupting agent cytochalasin B (CytB; 10 µg/mL) blocked phagocytosis, whereas the presence of galectin-3 (50 µg/mL) increased the proportion of phagocytosing MDM. Graphs ((**B**) mean +SEM, $n = 3$–7, and (**C**) mean ± SEM, $n = 5$) display the percentage of MDM found in the upper-right quadrant after indicated additions. To determine the phagocytic capacity of each phagocytosing MDM, the CD14+/CFDA+ populations were analyzed regarding CFDA fluorescence intensity ((**D**) representative histograms of CFDA fluorescence including all CD14+ events). The mean fluorescence intensity (MFI) of cells gated as indicated (CFDA+) is given in the inset (MFI+SEM, $n = 6$). The presence of galectin-3 significantly increased the level of CFDA MFI in phagocytosing MDM. Fluorescence microscopy (**E**) revealed multiple CFDA-labeled neutrophils ingested by single MDM in the presence of galectin-3. Asterisks denote statistically significant differences using paired Student's *t*-tests: $^{***} = P < 0.0001$, $^{**} = P < 0.005$.

at lower concentrations. In addition, the average level of CFDA staining per double positive particle was higher after incubation with galectin-3 (Figure 1D), suggesting that each MDM contained a higher number of apoptotic neutrophils, which was also seen using fluorescence microscopy (Figure 1E). Thus, galectin-3 appears to increase both the proportion of MDM that engulf apoptotic prey and the number of apoptotic neutrophils that each MDM engulfs.

The galectin-3-induced increase in phagocytosis was dependent on its carbohydrate binding activity, since the effect

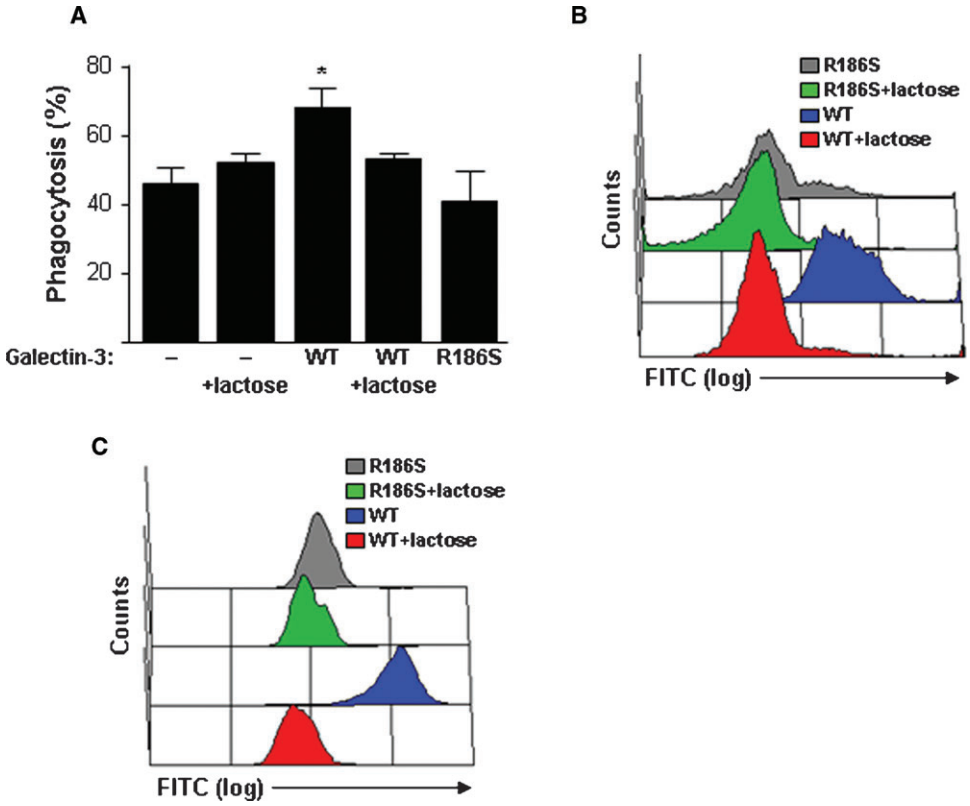

Fig. 2. Importance of the carbohydrate recognition domain (CRD) for the galectin-3-induced increase in clearance and for cell binding. Panel (**A**) shows phagocytosis of apoptotic neutrophils by MDM as measured by FACS and as described in the legend to Figure 1. The proportions of phagocytosing MDM when incubated with or without wild-type galectin-3 (50 μg/mL) ± lactose (5 mM) or galectin-3 R186S (50 μg/mL) are shown (mean + SEM, $n = 3$–5). Lactose blocked the galectin-3-enhanced phagocytosis and the galectin-3 R186S mutant was without effect. The asterisk denotes a statistically significant difference compared to phagocytosis in the control system (without additions) using one-way ANOVA: $^{*} = P < 0.05$. The panels show binding of FITC-labeled wild-type galectin-3 (20 μg/mL) or galectin-3 R186S mutant (20 μg/mL) to apoptotic neutrophils (**B**) or MDM (**C**) in the presence or absence of lactose (5 mM; representative histograms, $n = 2$–3). The wild-type galectin-3 bound both apoptotic neutrophils (**B**) and MDM (**C**) in a lactose sensitive manner, and the galectin-3 R186S mutant displayed considerably less binding.

was completely blocked by the competitive inhibitor lactose (Figure 2A). Moreover, galectin-3 that was mutated in the carbohydrate recognition domain (CRD; R186S) did not affect the MDM phagocytosis of apoptotic neutrophils (Figure 2A). This mutant has lost the affinity for *N*-acetyl-lactosamine (LacNAc) and thus for glycoproteins with *N*-linked glycans where LacNAc is typically found (Cumpstey et al. 2007; Cederfur et al. 2008). These data, hence, suggest that the galectin-3 effect is dependent on binding to *N*-linked glycans of glycoproteins. The basal level of phagocytosis (in the absence of added galectin-3) was not affected by lactose (Figure 2A), indicating that the endogenous amount of extracellular galectin-3 secreted in our in vitro system is too low to affect the clearance process.

The results reported above may be due to preactivation of the MDM by galectin-3, increasing their phagocytic potential. To test this possibility, we preincubated the MDM with galectin-3 (50 μg/ml) after which the cells were washed with lactose-containing PBS followed by PBS and then used in the clearance assay. This treatment had no effect on the MDM uptake capacity, i.e., the control system (44.5 ± 14.6% uptake, $n = 3$) and the galectin-3 treated, washed cells (41.4 ± 9.3% uptake, $n = 3$) did not differ significantly ($P > 0.45$). Hence, galectin-3 had to be physically present during the cellular interaction to induce its enhancing effect.

We next investigated the direct binding of galectin-3 to apoptotic neutrophils and found that the cells readily bound a substantial amount of FITC-labeled galectin-3 and that this binding was markedly decreased in the presence of lactose (Figure 2B). Further, the galectin-3 R186S mutant showed much reduced binding, similar to wild-type galectin-3 in the presence of lactose. FITC-labeled wild-type galectin-3 bound also to MDM in a lactose-inhibitable manner, whereas the R186S mutant displayed much lower binding (Figure 2C). This implies that galectin-3, by the use of the CRD region, crosslinks the active phagocyte (MDM) to the prey (the apoptotic neutrophil) and by this facilitates phagocytosis.

In order to investigate whether galectin-3 binding to the surface was sufficient to increase phagocytosis, we performed experiments using freshly prepared, viable neutrophils as prey. Viable neutrophils also bind galectin-3 in a lactose-dependent manner (Karlsson et al. 1998), but the presence of extracellular galectin-3 (50 μg/mL) did not affect the uptake of viable neutrophils by MDM (data not shown).

The presented data extend into a human setting the findings that galectin-$3^{-/-}$ murine macrophages display impaired phagocytic capacity regarding apoptotic neutrophils (Sano et al. 2003; Farnworth et al. 2008). Our data also imply that the role galectin-3 plays is most probably an extracellular one,

functioning as a bridging molecule between the macrophage and neutrophil, i.e., playing the role of an opsonin. It is, however, clear that galectin-3 on the surface of a target cell is not sufficient to mediate phagocytosis and the minor uptake of viable neutrophils was not enhanced by the presence of the lectin; additional "eat me" signals are likely required for phagocytosis to occur. Extracellular galectin-3 concentrations can be very high in an inflammatory setting (Farnworth et al. 2008), considerably higher than the 50 μg/mL required for a significant effect in our in vitro system. Thus, high levels of extracellular galectin-3 in inflamed tissues may play an important role for the clearance of apoptotic neutrophils and subsequently for the resolution of acute inflammation.

Material and methods

Culturing of cells

Monocytes isolated from buffy coats from healthy donors as described (De Almeida et al. 2003) were diluted in RPMI-1640 with 10% fetal calf serum and 1% PEST to a concentration of 5×10^5 cells/mL and cultured in TC plates in the presence of M-CSF (R&D Systems, Minneapolis, MN; 15 ng/mL). On day 7, MDM were detached using PBS with 0.02% EDTA, counted, and allowed to reattach in culture medium in 24-well plates (3×10^5 cells/well) overnight. Neutrophils were isolated from buffy coats as previously described (Böyum 1968), CFDA-labeled (Invitrogen; Eugene, OR) according to manufacturer's instructions, resuspended to 5×10^6 cells/mL in RPMI-1640 with fetal calf serum (10%), and incubated in the presence of an anti-Fas antibody (eBioscience, San Diego, CA; 10 μg/mL) at 37°C in 5% CO_2. After 18 h, the cells were washed and diluted in RPMI-1640 without serum. Viable neutrophils were used directly after CFDA labeling and washing.

Clearance assay

The attached MDM were incubated with indicated supplements (legend to figures) in RPMI-1640 without serum for 10 min prior to the addition of apoptotic neutrophils (∼80% apoptotic as determined by annexin V staining; neutrophil/MDM ratio 4/1). After coincubation (90 min), the cells were detached, washed, fixed in PFA (2%, 4°C, 10 min), stained for CD14 (60 min; anti-CD14-PE/Cy7; eBioscience), analyzed by FACS (FACSort, Becton Dickinson, Mountain View, CA) and in certain instances by fluorescence microscopy. Data were analyzed using WinMDI 2.8 and results are presented as the percentage of the CD14 positive events that were also positive for CFDA.

Galectin-3 binding to apoptotic neutrophils and MDM

Apoptotic neutrophils or MDM were incubated in a 10 mM HEPES buffer containing 140 mM NaCl and 2.5 mM $CaCl_2$ on ice with FITC-labeled galectin-3 (Patnaik et al. 2006) or FITC-labeled galectin-3 mutant R186S (Cumpstey et al. 2007; Cederfur et al. 2008) in the presence or absence of lactose (5 mM) for 40 min on ice after which the samples were analyzed by FACS.

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Conflict of interest statement

None declared.

Abbreviations

CRD, carbohydrate recognition domain; LacNAc, *N*-acetyllactosamine; MDM, monocyte-derived macrophages; MFI, mean fluorescence intensity.

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